

AAV Gene Therapy in Large Animal Models

1. In Vivo Selection of Randomly Integrated rAAV Vectors

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Recombinant adeno associated viruses (rAAVs) are a powerful tool for gene delivery. While historically thought to be non-integrating, it is now known that rAAVs can randomly integrate into the genome at low frequencies. Compared to episomal expression, these integrated vectors have the advantage of persisting through cell divisions. However, these integration events are typically too infrequent to result in therapeutic levels of expression of the delivered transgene. Our lab has previously demonstrated a strategy to expand a small population of hepatocytes that have the desired gene edit to therapeutic levels. This strategy utilizes a drug selectable cassette consisting of a Cytochrome P450 reductase (*Cypor*) targeted shRNA. *Cypor* is an obligate cofactor of Cytochrome P450 enzymes and is necessary for the metabolism of the drug acetaminophen to its hepatotoxic byproduct. In the absence of *Cypor*, acetaminophen cannot be metabolized, and hepatocytes that have lost *Cypor* are protected against high doses of acetaminophen. Due to the regenerative nature of hepatocytes, these acetaminophen-protected cells can expand to therapeutic levels. When the selectable shRNA is linked in cis with a therapeutic transgene, this method can selectively expand hepatocytes that have a stably integrated transgene. We hypothesized that this selection method could be used to expand rare random rAAV integration events to therapeutic levels. We delivered rAAV containing a U6-driven shRNA targeting *Cypor* and a CAG promoter driving human Factor IX (hFIX) into wildtype neonatal and adult mice. To expand the population of hepatocytes with the integrated rAAV, the mice were placed on an acetaminophen containing diet. Blood hFIX concentration increased over time up to 50-fold, readily reaching therapeutic levels. This demonstrates the expansion of hepatocytes expressing integrated copies of transgene. Immunofluorescent staining for *Cypor* confirmed the clonal expansion of *Cypor*-negative hepatocytes to 20% of the liver. Thus, we conclude that the random integration of conventional rAAV gene therapy vectors in combination with a selection cassette and regimen can be utilized to expand small populations of hepatocytes bearing chromosomal rAAV integrations to therapeutic levels.

2. Bicistronic AAV Gene Therapy for Tay-Sachs and Sandhoff Diseases in the Sheep Model of Tay-Sachs

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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 the enzyme Hexosaminidase A (HexA). Hex A deficiency results in GM2 ganglioside storage and subsequent neuronal death. Here we report on the preclinical therapeutic efficacy of a bicistronic AAV9 vector in TSD sheep after intravenous (IV, n=5) or cerebrospinal fluid injection by combined bilateral ventricular injection, cisternal magna and lumbar intrathecal injection (CSF, 1E14 vg n=5 long term; n=4 short term end point at 5 months). Sheep treated IV survived to 18±5 months and CSF treated sheep are ongoing with the oldest now more than 4 years old (untreated TSD sheep survive ~ 9±0.5 months). Both IV and CSF treated TSD sheep exhibited marked attenuation of neurologic disease as well as normal cognition as measured by maze testing. GM2 ganglioside and HexA levels in CSF normalized by 1 month after CSF administration and remained at normal range. CSF GM2 in the IV cohort were intermediate of TSD and normal sheep. No increase in Hex A levels in serum was noted in either the IV or CSF cohorts. Magnetic resonance spectroscopy (MRS) showed normalization of markers of neuronal health, myelination, and metabolism in the CSF treated cohort and partial improvement in the IV cohort. Diffusion tensor imaging (DTI) demonstrated the decrease in microstructural integrity and increase in water diffusion through white matter of TSD thalamus. In line with MRS findings, CSF and IV treatment normalized the abnormalities of white matter structure. The CSF treated cohort had serum anti AAV9 neutralizing antibodies (Nab) > 1:10 and 1:20 at the time of treatment that increased to a maximum of 1:160. CSF treatment resulted in at or above normal Hex A levels (3X-20X) throughout the brain and spinal cord. IV treatment resulted in Hex A activity in spinal cord at ~ 70% and 100% of normal level in cervical and lumbar sections, ~ 20% in cerebellum and brainstem and low levels in other brain regions (up to 10%). CSF treatment normalized GM2 levels in brain and spinal cord, with the exception of the thalamus and temporal cortex where GM2 levels decreased by 50%. IV treatment lowered GM2 levels to ~30% of TSD in cervical and lumbar spinal cord, while GM2 levels in brain remained at untreated TSD levels. Vector biodistribution in CNS is in line with Hex A expression. Partial normalization in peripheral nerve was noted in both the IV and CSF cohorts. QPCR detected up to 10X higher copy number for IV cohort in autonomic nerves (vagus, sympathetic chain

and vago-sympathetic trunk), but was not reflected by Hex A levels. CSF treatment, increased Hex A levels to 10% normal in muscle and ~7% normal in liver. IV treatment normalized Hex A levels in muscle and resulted in 10% normal Hex A activity in heart and spleen and ~4% normal in liver. The increase in Hex A was sufficient to decrease GM2 levels to below 50% TSD level in heart, muscle, and liver in both cohorts. Change in Hex A levels for both cohorts were not sufficient to decrease GM2 levels in spleen, lung and kidney as compared to TSD. These data show promise for a minimally invasive treatment for TSD and SD using this bicistronic vector construct, which provided the basis for submission of a pre-IND application.

3. Vagus Nerve Delivery of AAV9 to Treat Autonomic Nervous System Dysfunction in Giant Axonal Neuropathy

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Giant axonal neuropathy (GAN) is a rare pediatric disorder caused by autosomal recessive loss-of-function mutations in the *GAN* gene. A Phase I study (NCT02362438) is underway to test the safety of lumbar-injected, intrathecal (IT) delivered scAAV9/JeT-GAN to treat the most severe aspects of GAN. While GAN is primarily described as a progressive motor and sensory neuropathy, pathology is apparent throughout the autonomic nervous system (ANS) and patients frequently present with enteric and autonomic dysfunction. The distribution of IT-delivered AAV9 to peripheral ganglia is limited, so we hypothesized that if AAV9/GAN is also delivered via the vagus nerve (VN) then critical ANS neurons will be transduced, resulting in a greater amelioration of ANS dysfunction as compared to IT delivery alone. We tested this approach in a GAN rat model that we previously developed. Adult GAN rats received a single IT or a combination IT and vagus nerve injection of AAV9/GAN or vehicle and were monitored for up to 20 months post-injection. Rats were implanted with telemetry devices to record blood pressure, heart rate, respiration and body temperature under basal conditions and following ANS stimulation. At baseline, there was not a detectable difference in autonomic function between WT and GAN KI rats. However, upon challenge of the autonomic nervous system with pilocarpine, GAN KI rats had abnormal blood pressure, heart rates and respiration as compared to WT rats. The effect of pilocarpine on body temperature was normal in the KI rats. IT only delivery of AAV9/GAN improved respiration and blood pressure in GAN KI rats. Combined IT+VN delivery of AAV9/GAN showed greater efficacy than IT alone with normalization of respiration, blood pressure and heart rate responses as compared to control WT rats. Histological analysis of tissues also showed an unexpected additive benefit of combined IT+VN delivery in preventing nerve fiber loss within the dorsal columns of the spinal cord as compared to IT alone treatment. These results support that a simultaneous dual-route administration could be proposed for future patients. To our knowledge, VN injection of AAV has not been utilized in large animals or humans, so we tested the feasibility of this approach in dogs. Adult hounds received a direct vagus nerve injection of AAV9/

CBh-GFP and were observed for 3 weeks post-injection. Vocalization, weight, eating and fecal output were monitored pre and post-surgery. Overall, the injections were well-tolerated. Post-mortem analysis showed that in agreement with our previous studies in rats, there was efficient targeting of vagal neurons of the nodose ganglia, dorsal motor nucleus, visceromotor neurons and fibers of the nucleus ambiguus, and viscerosensory neurons and fibers of the nucleus tractus solitarius. Together these results support that direct VN delivery of AAV9 can efficiently transduce neurons critical for ANS function in large animals. Previously, we found that rats pre-immunized with AAV9 had efficient GFP transduction in vagal nerve fibers and neurons when they received a second dose of AAV9 via the vagus nerve, albeit to a reduced level as compared to naïve rats. Histological analysis showed an excellent safety profile without evidence of neuroinflammation or significant chronic inflammatory infiltrates. It remains to be determined in a large animal if direct vagal nerve injection could allow for redosing of AAV9 to target the ANS in subjects that previously received an AAV9 gene therapy. Additional studies are warranted to translate this approach into clinical testing for patients with GAN. Results from these studies are immediately applicable to GAN but may extend to treating a wider variety of neurological diseases.

4. Assessment of Gene Therapy Treatment in the Pompe Disease Canine Model

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Pompe disease is a recessive lysosomal storage disease caused by a deficiency in the lysosomal enzyme acid α -glucosidase (GAA). A naturally occurring nonsense mutation in the Finnish Lapphund canines have been found to have many similarities to infantile onset Pompe disease (IOPD) in humans based on the pathophysiology in skeletal muscle, smooth muscle, motor neurons and cardiac function. The only approved treatment for Pompe disease in humans is enzyme replacement therapy (ERT) that improves striated muscle function yet does not cross the blood brain barrier and requires life-long treatment. Additionally, patients with no-endogenous activity also require immune management to prevent anti-drug antibodies. We have studied the long-term effects of systemic gene therapy using recombinant adeno-associated virus serotype 9 (rAAV9) to replace the human GAA (hGAA) gene in 6 Pompe disease affected canines. Concomitant administration of a second AAV vector to promote hGAA tolerance in target hepatocytes was given to select canines. We followed the disease progression with baseline physical exams, total health blood panel through IDEXX (including chem 27 with IDEXX SDMA[®], IDEXX CBC-select[®], canine Cardiopet[®] proBNP, total T4, canine troponin I), anti-capsid and anti-transgene antibody levels in peripheral blood, electrocardiogram (ECG), echocardiogram and photographic documentation prior to dosing. Canines were started on an immunomodulation regime of prednisone (1mg/kg) and sirolimus (1mg/m²) administered orally once a day for 7 days prior to dosing and

continued post dosing for up to 3 months. On the day of dosing, prior to gene therapy delivery, a dose of diphenhydramine (1mg/kg) is given subcutaneously (SQ) followed by anti-thymocyte globulin (rabbit) (3mg/kg) intravenously (IV) and then Solu-Medrol (1mg/kg) IV. Two unaffected canines and one affected canine served as controls. One affected canine was treated at 8 months old with rAAV9-Des-hGAA at 2×10^{13} vg/kg IV to study the impact of established disease pathology reversibility of the phenotype. One affected canine was treated at 4 weeks old with rAAV9-Des-hGAA at 3×10^{12} vg/kg intrathecally (IT) to evaluate the option of CNS treatment only. The remaining 3 canines were treated at 3-4 weeks old with rAAV9-Des-hGAA at 3×10^{13} - 1×10^{14} vg/kg and rAAV9-LSPcoGAA at 1×10^{12} - 1×10^{13} vg/kg IV simultaneously. Post dosing, we monitored anti-capsid antibody levels and vector genome circulation levels at 1 hour, 24 hours, 3, 7, and 14 days post dosing. Daily adverse event assessments and weight monitoring were performed with monthly physical exams, total health blood panel through IDEXX, anti-capsid and anti-transgene antibodies, circulating vector genomes, ECG, echocardiogram, and photographic documentation. Every 3 months gait mat analysis (ProtoKinetics®) and a hindlimb muscle biopsy is performed. Gene therapy treatment at 3-4 weeks old demonstrated significant improvement to skeletal muscle development, prevention of cardiomyopathy and prolongs the expected lifespan of Pompe affected canines. Sustained correction of GAA activity was achieved for over 9 months in all systemically treated animals. These results support the potential use of a dual vector administration to establish tolerance to GAA which is critical in IOPD. Additional studies are underway using pre-treatment with a canine specific anti-CD20 antibody to block anti-capsid antibody responses and allow subsequent dosing to maintain GAA level in the growing musculature over the life span.

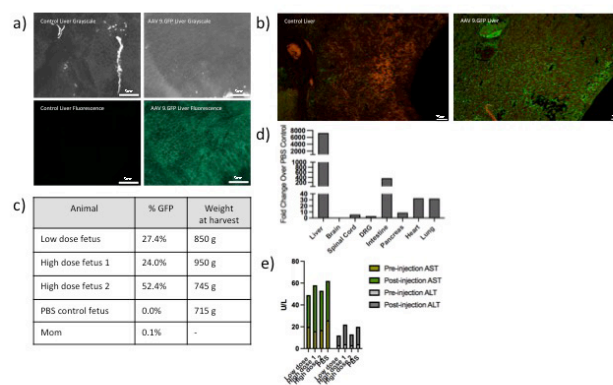
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6. The Porcine Model for In Utero Gene Therapy

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Introduction In utero gene therapy and gene editing technology (IUGT) offer the potential to treat genetic diseases before the onset of pathology and take advantage of the fetal properties of small size, access to progenitor cell populations, and an immature immune system that are barriers to postnatal therapies. Although proof-of-principle and toxicity studies have demonstrated the safety and feasibility of IUGT in small animal models, studies in large animal models are less prevalent. The nonhuman primate (NHP) model provides obvious advantages but is limited by cost, availability, husbandry, and a limited number of fetuses per pregnancy. The pig is a large animal model with more similarity to humans than small animals in terms of anatomy, development, and immunology, and has the added benefit of a shorter gestation and multiparity over NHPs, facilitating faster evaluation of multiple variables. Furthermore, there are an increasing number of pig models to recapitulate human genetic diseases, highlighting the relevance of this preclinical model. As such, we initiated studies to

determine the feasibility and safety of AAV mediated IUGT in the fetal pig model. **Methods** An adeno-associated virus serotype 9 (AAV9) designed to carry the green fluorescent protein (GFP) transgene under a CAG promoter (AAV9.GFP) was intravenously administered to 104 day gestation (term = 115 days) Yucatan mini-pigs. Specifically, after a maternal laparotomy and exposure of the uterus, a uterine incision was made and the umbilical vessels identified. The umbilical vein was cannulated and the viral vector was delivered in a total of 10 milliliters. The hysterotomy was closed in two layers to obtain a water-tight seal and the uterus returned to the maternal abdomen, which was closed prior to recovery from anesthesia. Fetuses were injected with either phosphate buffered saline (PBS) (n=1), low dose AAV9.GFP (4.5×10^{13} vg/kg; n=1) or high dose AAV9.GFP (9×10^{13} vg/kg; n=2). One week after injection, the sow was euthanized and fetuses were harvested for analyses. **Results** At the time of harvest, the sow was healthy and all fetuses were viable with a strong umbilical arterial pulse. Fetal weights were within normal limits for gestational age (715-950g). The livers of all fetuses that had been injected with AAV9.GFP demonstrated strong GFP fluorescence on stereomicroscopy (Figure 1a) and histology (Figure 1b). On flow cytometry, 24.0-52.4% of non-hematopoietic liver cells were GFP positive, while there were no GFP positive cells in the livers of the PBS injected fetus or mom (0.0-0.1%) (Figure 1c). Quantitative RT-qPCR for GFP mRNA demonstrated expression in multiple organs including low level expression in the heart, lung, and intestine, and a 7,000 fold higher expression in the liver of a high dose recipient compared to the PBS injected control (Figure 1d). Pre- and post-injection liver function tests showed a rise in aspartate aminotransferase (AST) and alanine transaminase (ALT) in all fetal pigs including the PBS injected control (Figure 1e). **Conclusion** This study validates that cannulation and injection of the umbilical vein for IUGT in fetal pigs is feasible, safe, and does not result in preterm labor at short-term time points. This supports the porcine model as a worthwhile large animal model for future testing and optimization of pre-clinical *in utero* gene therapies.



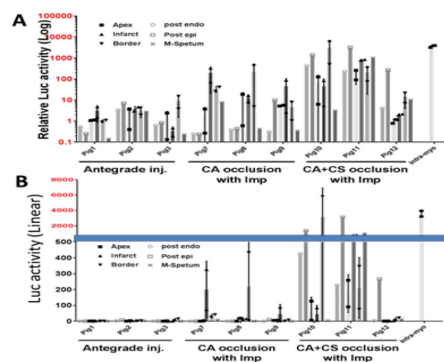
7. Temporary Mechanical Support Improves Cardiac AAV Gene Transfer Efficacy in a Pig HF Model

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Purpose: Gene therapy using adeno-associated virus (AAV) is increasingly promising as a potential breakthrough therapy for heart failure (HF) as evidenced in animal studies. However, challenges remain in achieving successful gene transduction for human heart. Using a catheter-based approach, we hypothesized that compared to conventional delivery by slow continuous infusion, AAV exposure to the heart can be prolonged using temporary coronary balloon occlusions aided by a mechanical cardiac support device for hemodynamic maintenance. We predicted that this novel technique would lead to increased gene expression in the heart in a pig model of HF. **Methods:** Yorkshire pigs received AAV-6 encoding luciferase gene (5.0×10^{13} vg) by antegrade injection into the coronary artery one week after myocardial infarction (MI). Pigs were allocated to one of the following groups: conventional delivery ($n=3$), delivery with coronary artery occlusion (CAO, $n=3$), and delivery with coronary artery and coronary sinus occlusions (CAO + CSO, $n=3$). A catheter-based cardiac support device was inserted to preserve hemodynamics during coronary balloon occlusion. Four weeks later, tissues were harvested from various heart regions and other organs to assess luciferase expression. Two other animals underwent MI creation and were injected one week later with 20 nm gold nanoparticles (the size of AAV) by either CAO + CSO or conventional delivery with mechanical support in order to visualize distribution by electron microscopy (EM). Infarct and remote regions from explanted hearts were further processed and analyzed for the presence of injected gold nanoparticles between delivery methods. **Results:** Catheter-based support offered safe vector delivery during 1-1.5 minutes of CA and CS occlusions in preventing hemodynamic instability and arrhythmia. CAO delivery enhanced luciferase expression up to 20-fold globally in the heart, but not in non-cardiac tissues, compared to conventional delivery. CAO + CSO delivery further increased luciferase expression, also noted by increased vector genome in the tissues detected by PCR. At infarct border and remote regions, CAO + CSO delivery led to a more than 300-fold increase. EM data demonstrated a greater presence of gold nanoparticles after CAO + CSO delivery in contrast to conventional delivery. The majority of gold nanoparticles seen were at the interface of the vessel lumen and endothelium, with some observed within vesicles in remote areas in the CAO + CSO delivery group, as well as occasional incidence in infarct area. **Conclusion:** Coronary artery and sinus occlusion with catheter-based cardiac support dramatically improved AAV gene expression in the heart without compromising hemodynamics or increasing off-target expression. Furthermore, this delivery approach leads to higher vector uptake into cardiac tissue. Higher detection of gold nanoparticles beyond the arterial lumen with this technique suggests greater opportunity for AAV transduction by optimizing the time of vector exposure to tissue. This method offers clinically applicable and efficient cardiac gene delivery as a new therapeutic option for patients with heart failure.



Luciferase expression in pig heart tissues following catheter-based injection with AAV-6 encoding luciferase gene in 3 delivery groups: conventional delivery (Antegrade inj.), delivery with coronary artery occlusion with mechanical cardiac support device (CA occlusion with Imp), and delivery with coronary artery and coronary sinus occlusions with mechanical cardiac support device (CA+CS occlusion with Imp) in contrast to intra-myocardial delivery (Intra-myo).

Biology of Gene Edited Cells

8. Double Strand Break Free Genome Editing to Target Hematopoietic Stem and Progenitor Cells: Therapeutic Applicability in Fanconi Anemia

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CRISPR/Cas9 genome editing tools have demonstrated their efficacy to specifically control gene editing outcomes, with a promising influence in the treatment of many genetic disorders, including hematological diseases. The recent description of Base (BE) and Prime editing (PE) that enable the correction of many small insertions, as well as small deletions and point conversions avoiding the use of donor templates and the generation of double strand breaks (DSB) in the cell genome, have become an attractive option in Fanconi anemia (FA). To implement BE and PE strategies in clinically relevant cell types, we aimed at optimizing specific base substitutions using adenine BE in healthy donor cord blood and mobilized peripheral blood CD34⁺ cells. *In vitro* studies showed up to 85% specific A to G conversion in hematopoietic progenitor cells (HPCs). To investigate the efficacy of BE in more primitive hematopoietic stem cells (HSCs), BE-CD34⁺ cells were transplanted into immunodeficient NSG mice. Results in primary and secondary recipients showed the efficacy of BE in HSCs capable of repopulating these animals. In subsequent studies we aimed at applying BE for the correction of HSPCs from FA patients. In this disease we estimate that around 40% of FA mutations could be potentially targeted by this strategy. In particular, we pursued the correction of HSPCs harboring the most frequent mutation described in FA patients from Spain, c.295 C - T, which results in a stop codon in

exon 4 of *FANCA*. So far, *in vitro* data has shown efficient phenotypic correction in these cells thanks to therapeutic base conversion. To expand the application of gene editing to other frequent mutations from FA patients, we focused on PE, a more versatile strategy that can install all types of targeted DNA base pair substitutions, small insertions and small deletions in the absence of a donor template. First, PE3 and the recently described PE5 systems were tested in healthy donor K562 cells. In these cells, up to 42% and 38% point conversion were respectively determined at day 5 post-nucleofection. Also, we designed a PE approach for the therapeutic correction of two prevalent *FANCA* mutations, c.295 C - T and c.3788_3790del in HSPCs from FA patients. In this case, 6.5% and 36% of intended correction were respectively observed 5 days after nucleofection. Moreover, analyses performed 30 days after nucleofection revealed a marked proliferative advantage of PE as compared to not edited FA cells. Additionally, an evident correction of the FA phenotype was revealed by acquisition of mitomycin C resistance in these cells. Altogether, our results show that precise gene editing may constitute an alternative new therapeutic approach for the future treatment of the bone marrow failure in FA patients carrying specific mutations.

9. Cellular Senescence and Inflammatory Programs Are Unintended Consequences of CRISPR-Cas9 Gene Editing in Hematopoietic Stem and Progenitor Cells

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Gene editing (GE) in hematopoietic stem and progenitor cells (HSPC) is a revolutionary site-specific gene correction strategy for a plethora of immune-hematological diseases. Despite the rapid development of advanced GE-based therapies, a few challenges remain to be faced to improve GE efficiency and the reduced repopulating potential upon transplantation. We previously showed that the combination of nuclease-induced Double Strand Break with DNA repair template for Homology Directed Repair (HDR) delivered by AAV6 caused cumulative activation of the p53-mediated DNA Damage Response (DDR) pathway constraining HSPC proliferation and yield, suggesting that DDR-related cellular programs may inadvertently contribute to HSPC dysfunctions upon gene editing. Protracted DDR signaling has been causatively linked to the establishment of cellular senescence, a condition in which cells, despite being still alive, are unable to further proliferate. By integrating transcriptional analysis (up to the single cell level) with innovative imaging-based cellular assays we reported induction of cellular senescence markers (p16 and Senescence-Associated β -Galactosidase) and pro-inflammatory programs across edited HSPC subtypes and *in vivo* in the human graft. Consistently, we found open chromatin at promoters of several senescence-gene categories and inflammatory genes of the *IL1* axis (an upstream mediator of DDR-dependent inflammation) and NF- κ B pathway (a key regulator of inflammatory genes upon several stressors) especially in HDR-edited cells. Moreover, transcriptional activation of

inflammatory cytokines in edited HSPC was dependent on the apical DDR kinase ATM and partly mitigated by transient administration of a p53 dominant negative form (GSE56). In this context, temporary inhibition of *IL1* and NF- κ B pathways at the time of GE resulted in increased clonogenicity of edited HSPC *in vitro* and ameliorated long-term hematopoietic reconstitution in xenotransplanted mice. We also reported a decrease in senescence markers in both cord blood and mobilized peripheral blood-derived HSPC upon GE. By a barcoding-based strategy, we performed *in vivo* clonal tracking of HDR-edited HSPC revealing that *IL1* inhibition improved polyclonal reconstitution and better preserved self-renewal and multi-potency of individual edited HSPC. Our findings define senescence and inflammatory programs as long-term consequences of CRISPR-Cas9 engineered human HSPC and pave the way for the development of novel strategies based on senescence modulation and anti-inflammatory molecules to overcome adverse cellular responses for efficient HSPC-based clinical applications.

10. The Choice of Template Delivery Mitigates the Genotoxic Risk and Adverse Impact of Editing in Human Hematopoietic Stem Cells

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Gene editing by homology-directed repair (HDR) remains the most versatile and best performing strategy for targeted integration of a gene-sized therapeutic sequence. Delivery of the large DNA template for such application might be challenging in cell types relevant for gene therapy, such as hematopoietic stem/progenitor cells (HSPCs). So far, recombinant adeno-associated virus (AAV) serotype 6 is the most efficient repair template for HDR gene editing in HSPCs. However, we previously reported that AAV transduction triggers a robust p53-mediated DNA damage response (DDR) thus lowering long-term-repopulating (LT)-HSCs engraftment and clonal complexity in hematochimeric mice. Here, we showed that this response is entirely due to the nucleic acid content and not to capsid proteins of the AAV vector. Moreover, we uncovered an unexpected load and prolonged persistence of AAV DNA, which trigger a sustained DDR, upon recruiting the Mre11-Rad50-Nbs1 (MRN) complex on the AAV viral inverted terminal repeats (ITRs), independently from transgene cassette, AAV purification process or targeted locus. Furthermore, sequencing of the nuclease target site in human xenografts revealed that a detectable fraction of edited cells in the long-term graft carried on-target trapping of short AAV DNA fragments (median = 0.5%), predominantly made of transcription-competent ITR (\approx 70%) and possibly originating from AAV contaminants or MRN-mediated degradation of the AAV vector genome. Genome-wide molecular analysis also showed ITR prevalence (median 0.1-0.2 copies/cell)

in all hematopoietic lineages and secondary recipients. Retrieval of AAV integration sites (IS) by an unbiased bioinformatic pipeline (RAAVIoli) showed preference for the nuclease target site, despite detectable integrations at predicted nuclease off-targets and random sites. These findings aggravate the genotoxic burden and adverse impact of AAV-based editing by limiting the intended editing outcome at the target site and potentially altering endogenous transcription at IS. To mitigate these potential issues, we optimized and refurbish HDR editing in human HSPCs based on Integrase-Defective Lentiviral Vector (IDLV) delivery, as it may provide several advantages over AAV. Whereas both DNA delivery templates showed similar propensity for non-homologous end joining (NHEJ)-mediated trapping of the full-length genome, IDLV transduction resulted in a lower and less persistent vector DNA load, which triggered less prolonged DDR. This allowed for better preservation of clonogenic capacity and more efficient HDR editing of long-term repopulating HSPCs with our newly optimized IDLV-based editing protocol. Because insertions of viral DNA fragments at the target site was instead much less frequent with IDLV compared to AAV, and its long terminal repeats (LTRs) are transcriptionally silent, the choice of IDLV as repair template significantly mitigates the overall toxicity burden of HDR editing and should facilitate clinical translation of HSPC gene therapy.

11. Assessing Stealth and Sensed Base Editing in Human Hematopoietic Stem/Progenitor Cells

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Engineered nucleases, such as CRISPR/Cas9, enable gene disruption and site-specific targeted integration in human hematopoietic stem and progenitor cells (HSPCs) for therapeutic purposes. Yet, we showed that Cas9-induced DNA double-strand breaks (DSBs) trigger a p53-dependent DNA damage response, which correlates with the specificity of the nuclease, and is further aggravated by the viral delivery of a DNA template suitable for homology-direct repair (HDR)-mediated targeted integration, ultimately decreasing HSPC repopulation potential and clonality. Moreover, DSB repair may cause unintended deletions and chromosomal rearrangements. Adenine (A-) and cytosine (C-) base editing (BE) hold the promise for precise editing by site-specific conversion of single nucleotides, while bypassing the requirement for DNA DSBs and HDR engagement. Despite promising results have been achieved in human HSPCs, a comprehensive characterization of efficiency, tolerability and genotoxicity of CBE and ABE is lacking and will be required to instruct the rationale towards safe and effective clinical translation of this approach. Here, we performed side-by-side comparison of these gene editing platforms in human HSPCs by electroporating late-generation base editors CBE4max or ABE8.20m-, or Cas9 nuclease, as mRNAs and independently targeting different loci using sgRNAs compatible with all editing platforms. Common outcome for all editors was the lack of surface protein expression and/or installation of the intended edit, measured by flow cytometry and

next-generation sequencing, respectively. ABE8.20m showed higher efficiency than CBE4max and Cas9 nuclease, reaching up to 90% allele editing, which was consistent across HSPC subpopulations, comprising the most primitive compartment endowed with long term repopulation potential, source (e.g., cord blood- and mobilized peripheral blood HSPCs) and targeted loci. Cas9-, but not CBE4max- and ABE8.20m-, treated HSPCs showed lower in-vitro clonogenic capacity compared to mock electroporated cells, indicating milder impact of BE vs. Cas9-editing on HSPCs. Correspondingly, Cas9-edited HSPCs displayed the lowest repopulation potential in xenotransplanted mice, despite all treatments maintained long-term multilineage engraftment. However, CBE4max 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 BE trigger p53 pathway activation, albeit to lower extent as compared to Cas9 treatment, likely due to the conversion of nickase-induced DNA single-strand breaks to DSB. Additionally, both BE, but not Cas9, upregulated the expression of interferon-stimulated genes, which could indicate innate cellular sensing of their longer encoding mRNAs. Indeed, engineering of the BE mRNA construct allowed to decrease the effective mRNA dose and abolished activation of interferon response for both BEs, without aggravating p53 activation. On-going studies are aimed to investigate genome integrity and clonal dynamics upon transplantation of base-edited HSPCs with the goal to further build confidence on their prospective clinical translation.

12. DNA Barcode as a Useful Tool to Study Hematopoietic Stem Cell Fate in Gene Editing Strategies

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Gene editing strategies using a combination of a rAAV6 donor and RNP to modify hematopoietic stem and progenitor cells (HSPCs) through homology directed repair (HDR) have shown great promise for the treatment of hematologic diseases. However, due to homogeneous modification of the cell genome in HDR gene editing, clonality studies of edited HSPCs cannot be carried out. DNA barcoding offers a powerful approach for labeling individual cells to allow their characterization, their growth and their differentiation pattern. Here, we describe an AAV-barcoding strategy to track the clonal outputs of gene edited human CD34⁺ cells after transplantation into immunodeficient mice. We first developed a rAAV6-barcode library including a semi-random 65bp sequence oligonucleotide carrying the required sequences to knock-in a codon optimized version of the Erythroid Pyruvate Kinase cDNA (coRPK) in the *PKLR* locus. Theoretically this system allows the tracking of up to 10⁴ different clones. We used this rAAV6-barcode

library to optimize HSPC gene editing conditions, addressing both gene editing efficacy and clonality of modified HSPCs. Two different culture media (StemSpan™ SFEM II or GMP SCGM) in combination or not with StemRegenin 1 (SR-1) were used. The rAAV6-barcode showed a high *in vitro* targeting efficiency, with up to 60% targeted hematopoietic progenitors in all tested condition. When this edited HSPC was transplanted into immunodeficient mice gene editing efficiencies were slightly reduced, dropping to 40% gene editing in human hematopoietic cells 3 months post-transplant when cells were cultured in StemSpan™ SFEM II medium. Analysis of clonal output *in vivo* showed up to 2000 individually marked HSPCs in each mouse, with 9 to 12 unique HSPCs clones per mouse responsible for 90% of the human hematopoietic engraftment. Importantly, there was no evidence of progressive clonal dominance in any of the conditions tested. Overall, the DNA barcode methodology has been demonstrated to be a powerful approach to study hematopoietic fate and behavior of gene edited HSPCs and cultured conditions for the optimization of the gene editing technology to make it clinically applicable.

13. High Frequency of AAV Integration at Double Strand Breaks Induced in Preclinical Model of Gene Therapy and in Edited Long-Term Engrafted HSPCs

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Recombinant Adeno Associated Viral (rAAV) vectors have been exploited in gene therapy (GT) applications for the treatment of several genetic disorders. Despite rAAVs have been considered episomal vectors, it has been shown that fragmented or full-length AAV DNA can integrate within the genome of host cells leading to hepatocellular carcinoma and clonal expansion events in some preclinical models. Yet, AAV integrations have been reported at the nuclease target site in which they are used in *in-vivo* gene editing (GE) studies. Hence, a deeper assessment of AAV integration events in GT and GE is required. Here we analyzed the genomic distribution of rAAV integrations in T-cells and liver tissues of immune-deficient ZAP70 knock-out mice that were intra-thymically injected with a rAAV expressing the therapeutic transgene. In this model, the long-term transgene expression in functional and proliferating T lymphocytes suggested for the presence of AAV integrated copies. Moreover, we investigated the distribution of AAV IS in two clinically relevant GE contexts based on human hematopoietic stem cells (HSPCs) edited at the AAVS1 safe harbor locus or at the intron of a disease causing gene and engrafted long-term in immune-deficient mice. As repair templates, rAAVs encoding for eGFP under the human PGK promoter or the promoter-less sequence of the therapeutic gene were adopted. Because different portions of the rAAV genome may be involved in the integration process, several PCR primers were designed and combined with a DNA-sonication based PCR protocol to amplify all possible vector/

cellular genome junctions. Amplicons were then analyzed by a novel bioinformatics tool allowing the identification and characterization of rAAV integration sites (IS). By this approach, we retrieved 640 AAV IS from ZAP70-treated mice and Inverted Terminal Repeats (ITR) were mainly involved in these events. Similarly to other studies, IS in liver were found throughout the genome of host cells (N=197) with a bias for micro-homology regions, palindromes and telomeres. Differently, more than 80% of the IS derived from T-cells (N=443) clustered within T cell receptor genes (TCR) were enriched in Rag Signal Sequences and were represented by multiple cellular genomes. Analyzing the DNA collected from bone marrow and spleen of 29 mice transplanted with edited HSPCs, we retrieved 182 AAV IS. The two editing sites were the preferential loci of IS accounting for 21% and 34% of derived IS. As before, ITRs were involved in more than 90% of the IS at the on-target sites. AAV IS were also found in predicted off-target sites and at the cellular PGK promoter. Overall, these data showed that AAV ITRs have an intrinsic and strong tendency of being captured at DNA damage sites by non-homologous end joining mechanisms. The intra-thymic delivery of rAAV can be considered as a “naturally occurring GE procedure”, since double strand breaks produced by RAG complexes in TCR genes during T cell maturation promoted AAV integration and conferred a long-term therapeutic benefit to treated mice. On the other hand, the presence of ITR fragments as inadvertent consequence of HDR protocols in engrafted HSPCs raises concerns regarding the use of AAV in GE applications. Hence, IS studies provide insights on the genomic integrity of transduced cells even in AAV-based GT and GE applications and may suggest for improvements in the choice and design of the vector used for a more effective and safer clinical translation.

14. Base Editing of a γ -Globin *cis*-Regulatory Element in Human Hematopoietic Stem Cells for Reactivation of Therapeutic Fetal Hemoglobin

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β -thalassemia and sickle cell disease (SCD) 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 (HPFH). HPFH mutations upstream of the TSS of the γ -globin (*HBG*) genes either disrupt the binding site (BS) of the fetal Hb (HbF) repressor LRF or generate a *de novo* BS for the KLF1 activator. To reactivate γ -globin expression, nuclease-based approaches have been explored. However, nucleases generate double-strand breaks (DSBs), raising safety concerns. Base editing (BE) allows the introduction of point mutations (C>T by

cytidine base editors, CBEs; A>G by adenine base editors or ABEs) without generating DSBs. We have previously reported that BE allowed the introduction of HPFH mutations in the *HBG* promoters, which disrupt the LRF BS (via CBE) or generate the KLF1 BS (via ABE). This led to therapeutically relevant HbF levels in red cells differentiated from edited SCD or β -thalassemia HSPCs, and rescued the pathological phenotypes. Importantly, higher HbF reactivation levels were observed in BE- than Cas9-treated samples. Here, we evaluated the safety of these approaches and their efficacy *in vivo*. First, we performed RNA-seq in control and edited HSPCs to assess the impact of BE on the global gene expression profile. Overall, BE led to fewer differentially expressed genes (DEG) than Cas9 nuclease. As expected, Cas9 nuclease-treated samples showed DEG associated with DNA damage response and apoptosis. Surprisingly, genes involved in apoptosis and HSC biology were dysregulated in CBE-treated samples, while only globin genes were differentially expressed in ABE-treated cells. To assess BE RNA off-target activity in HSPCs, we further analyzed RNA-seq data. A similar number of C>T or A>G variants were observed in control and edited samples, demonstrating no increase in RNA deamination due to CBE or ABE. To evaluate the DNA off-target activity of BE, we performed whole exome sequencing analysis. For each donor, 99.9% of single nucleotide variants were shared between untreated and edited samples, indicating no detectable gRNA-dependent or -independent off-target activity in exons. Low to undetectable gRNA-dependent DNA off-target activity at coding and non-coding regions was also observed by GUIDE-seq and NGS sequencing. Finally, to evaluate the BE efficacy in repopulating HSCs, we transplanted edited HSPCs in immunodeficient mice. We observed no differences in the engraftment and differentiation potential of edited and control HSCs, as measured by the frequency of human CD45⁺ cells and the analysis of lineage-specific markers. A lower BE efficiency was observed *in vivo* using CBE compared to ABE, suggesting either some toxicity induced by CBEs or their low efficiency in *bona fide* HSCs. Importantly, ABE efficiency reached levels of ~50%, and a clonal analysis showed that ~70% of HSCs harbored at least 1 edited *HBG* promoter, which was sufficient to reactivate HbF. In conclusion, we provided sufficient proof that BE of a γ -globin *cis*-regulatory element is safe and efficient in repopulating HSCs and reactivate HbF in their erythroid progeny, thus enabling the clinical development of base-edited HSPCs for the therapy of β -hemoglobinopathies.

Oligonucleotide Therapeutics

15. Development of an AIMER for the Treatment of Alpha-1 Antitrypsin Deficiency

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Introduction: Alpha-1 antitrypsin deficiency (AATD) is an inherited genetic disorder that is most commonly caused by a G-to-A point mutation in the *SERPINA1* gene (*PiZ* allele). The resulting E342K mutation leads to misfolding and aggregation of mutant Z-AAT protein in hepatocytes resulting in a lack of functional wild-type AAT (M-AAT) in circulation. This leads to progressive lung injury, liver injury, or both and culminates in end-stage pulmonary and liver disease. Current standard of care is weekly intravenous AAT protein augmentation therapy, which addresses only the lung manifestation. While there are multiple approaches in development, these address only a subset of patient needs (*e.g.*, silencing approaches to reduce liver aggregates) and do not restore M-AAT (*e.g.*, small-molecule approaches). We aim to 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 stereopure design as well as phosphoryl-guanidine (PN) backbone chemistry, and they recruit endogenous adenosine deaminase acting on RNA (ADAR) enzymes. This approach is expected to retain physiological regulation of M-AAT while protecting the lungs and decreasing Z-AAT protein aggregation in liver. **Methods:** We evaluated AIMers conjugated to *N*-Acetylgalactosamine (GalNAc-AIMers), to facilitate delivery to hepatocytes, in primary human hepatocytes and *in vivo* studies, using a mouse model for AATD that expresses both the human *PiZ* allele and human ADAR1. We administered GalNAc-AIMers by subcutaneous injection. We determined the percentage of edited *SERPINA1* mRNA with Sanger sequencing, quantified restored serum M-AAT protein by mass spectrometry, determined amount of total AAT protein in the media (*in vitro*) or serum (*in vivo*) with ELISA, and confirmed functionality of restored M-AAT protein in a neutrophil elastase inhibition assay. We applied PAS-D (Periodic acid Schiff-Diastase) staining and immunohistochemistry against polymeric and total AAT protein and quantified the positive signal using an image analysis platform, HALO, to evaluate liver Z-AAT polymers. **Results:** GalNAc-AIMers support dose-dependent *PiZ* mRNA base editing and AAT secretion *in vitro*. In the mouse model, these molecules

direct significant PiZ editing in hepatocytes and durably increase serum AAT protein levels. With an initial 3-dose loading regimen, followed by bi-weekly single dosing, serum AAT levels are maintained at concentrations >11 μM , the expected therapeutic threshold. These serum concentrations increase over time to ~19 μM , corresponding to a 4.9-fold higher concentration than in controls. Secreted AAT protein has the expected wild-type amino acid sequence and inhibits neutrophil elastase. Over time, hepatocytes in treated animals also show a clear decrease in Z-AAT polymers. **Conclusion:** AATD is a chronic disease of the liver and lung with high unmet need. Repeat dosing with GalNAc-AIMers restores expression and function of serum M-AAT protein as well as clearance of Z-AAT protein aggregates from liver in a mouse model for AATD. These findings highlight the therapeutic potential for GalNAc-AIMers as a treatment of both liver and lung manifestations of AATD.

16. STK-002, an Antisense Oligonucleotide (ASO) for the Treatment of Autosomal Dominant Optic Atrophy (ADOA), Localizes to Retinal Ganglion Cells (RGC) and Upregulates OPA-1 Protein Expression After Intravitreal Administration to Non-Human Primates (NHP)

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ADOA is the most common inherited optic neuropathy, starting in the first decade of life and resulting in severe and progressive visual decline due to loss of RGCs. Most patients harbor loss-of-function mutations in the *OPA1* gene that lead to haploinsufficiency. Reduced *OPA1* protein levels result in impaired mitochondrial function in RGCs leading to cell death. Currently, there is no treatment for patients with ADOA. Targeted Augmentation of Nuclear Gene Output (TANGO) ASOs such as STK-002 reduce the inclusion of a non-productive, alternatively spliced exon in *OPA1*, and leverage the wild-type allele to increase productive *OPA1* mRNA and protein. We previously demonstrated that TANGO ASOs can increase *OPA1* protein levels in human cell lines, rabbit retina, and ADOA patient fibroblasts. In this study, we evaluated ASO localization and *OPA1* protein levels in the retina following intravitreal administration of STK-002 to NHPs. Cynomolgus monkeys (N=22) received bilateral intravitreal injections of vehicle or STK-002. Eyes were collected at 4 or 8 weeks after injection. Retinas were isolated for molecular analyses and whole globes were prepared for histology. Retinal *OPA1* mRNA and protein were measured using qPCR (Taqman) and enzyme-linked immunosorbent assay (ELISA), respectively. A hybridization ELISA (HELISA) method was used to quantitate STK-002 levels in retina. Whole globes were sent for custom assay development and detection of STK-002 by miRNAscope™ in situ hybridization (ISH), and detection of *OPA1* protein by immunofluorescence (IF). Retinal exposure of STK-002 increased in a dose-dependent manner and remained high at the last timepoint evaluated (Week 8). STK-002 also dose-dependently increased protein levels at Week 4, ranging from 31 to 47% compared to vehicle, and levels were maintained at Week 8. ISH and IF analysis demonstrated

that both STK-002 and *OPA1* protein levels increased in RGCs, the target cells for ADOA. STK-002 produced a dose-dependent and persistent increase in *OPA1* protein expression in the retinas of NHPs. ASO-induced increase in *OPA1* protein levels in RGCs represents a potentially disease-modifying therapy for patients with ADOA.

17. Repeat Dosing with DYNE-101 is Well Tolerated and Leads to a Sustained Reduction of *DMPK* RNA Expression in Key Muscles for DM1 Pathology in hTfR1/DMSXL Mice and NHPs

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Myotonic dystrophy type 1 (DM1) is a severe neuromuscular disease caused by the expansion of CUG triplets in the 3'-untranslated region of the dystrophin myotonia protein kinase (*DMPK*) RNA. The expanded CUG repeats form hairpin-loop structures that sequester the MBNL splicing regulators into the nucleus and inhibit their function, leading to a spliceopathy that drives the clinical manifestations of DM1. DYNE-101 is being developed for the treatment of DM1 and consists of an antigen-binding fragment (Fab) that binds specifically to transferrin receptor 1 (TfR1), conjugated to a gapmer antisense oligonucleotide (ASO) designed to target the *DMPK* RNA. Of note, both the Fab and ASO recognize their respective targets in humans and cynomolgus monkeys. Previous work conducted in hTfR1/DMSXL mice, a novel model that expresses human TfR1 (hTfR1) and a toxic human mutant *DMPK* transcript with more than 1,000 CUG repeats, revealed that DYNE-101 reduces the levels of toxic human *DMPK* RNA in nuclei from cardiac and skeletal muscle, consequently improving multiple DM1 splicing defects. To determine the impact of prolonged repeat dosing of DYNE-101 on toxic human *DMPK* expression in cardiac and skeletal muscle, hTfR1/DMSXL mice were administered with 4 monthly doses of DYNE-101, or vehicle. We demonstrated a robust reduction of mutant human *DMPK* RNA in the heart, diaphragm, gastrocnemius, and tibialis anterior. Importantly, low and infrequent administration of DYNE-101 was sufficient to achieve strong pharmacological activity in hTfR1/DMSXL mice. To establish the translatability of these observations to primates, wild-type (WT) cynomolgus monkeys were treated with 2 monthly doses of DYNE-101, or vehicle. We observed substantial suppression of WT *DMPK* expression up to 70% in the heart, diaphragm, gastrocnemius, tibialis anterior, masseter, esophagus, and duodenum. Taken together, these data demonstrate that administration of DYNE-101 to rodents and non-human primates (NHPs) effectively reduces *DMPK* expression in different muscle types affected by DM1 pathology. A subsequent 13-week GLP toxicology study demonstrated that repeated administrations of DYNE-101 were well-tolerated in NHPs. In conclusion, these data support advancement of DYNE-101 into the clinic for the treatment of DM1.

18. SNCA Reduction for the Treatment of Synucleinopathies

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Long-term neuroprotective therapy is of considerable interest for the treatment of neurodegenerative synucleinopathies like Parkinson's disease or multiple system atrophy (MSA). These diseases are characterized by the toxic accumulation of alpha-synuclein protein in the brain with associated neurodegeneration. Multiple lines of evidence strongly implicate alpha-synuclein as a causal factor in neurodegeneration, and reduction of total alpha-synuclein has been well demonstrated to be neuroprotective in preclinical models of synucleinopathy. RNA interference (RNAi) mediated by artificial miRNAs has shown increasing promise as a therapeutic modality to reduce levels of target mRNA and protein. Adeno-associated viruses (AAV) are well-validated gene delivery vectors with the ability to express therapeutic constructs for years in the brain. Therefore AAV-mediated RNAi represents a promising approach for the long-term reduction of SNCA and treatment of synucleinopathy. Artificial miRNAs (amiRNA) were designed to target alpha-synuclein mRNA (SNCA). In vitro screening demonstrated robust target knockdown in human cells. Reduction of SNCA in differentiated human dopaminergic cells (LUHMES) did not cause detectable toxicity and was neuroprotective against rotenone-induced cell death supporting the therapeutic concept of total SNCA lowering. AAV-RNAi-mediated reduction of SNCA was evaluated in the brains of wild type mice and was maintained for up to 6 months post-injection without detectable behavioral effects. PoC efficacy studies in a transgenic model of multiple system atrophy and a pre-formed fibril (PFF)-model of synucleinopathy are ongoing to provide additional in vivo validation of this approach. Our results support the continued development of SNCA lowering therapeutics as a safe and efficacious approach for treating patients with synucleinopathies.

19. Alpha-Synuclein Lowering and Rescue of Motor Phenotype by miRNA-Based AAV Gene Therapy in *In Vivo* Parkinson's Disease Models

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Parkinson's disease (PD) is a progressively debilitating neurodegenerative disease with an increasing prevalence with age. Only symptomatic

therapies are available which do not tackle the underlying disease mechanism. One of the underlying causes of PD is aggregation of alpha-synuclein protein (α -syn), encoded by the SNCA (Synuclein Alpha) gene. Aggregated α -syn is one of the main components of Lewy Bodies (LBs), a key neuropathological hallmark in PD. Typically, LB pathology originates in brainstem and extends to midbrain and cortical regions with disease progression, in parallel with neurodegeneration of nigro-striatal dopaminergic circuits and other neurotransmitter systems. Our hypothesis for a disease modifying therapy is that lowering α -syn protein levels in relevant brain regions may reduce aggregation and degeneration, ultimately halting disease progression. We are developing an adeno-associated virus (AAV) gene therapy to deliver SNCA-targeting miRNAs (miSNCA) engineered to decrease α -syn mRNA and protein levels. Several miSNCA candidates were designed, targeting all SNCA splicing variants. After initial screening with luciferase-based assays, selected AAV-miSNCA candidates were assessed by small RNA sequencing. The miSNCA backbone of choice avoids off-target effects by not having the passenger strand after expression and processing in the cell. The lead miSNCA candidates were all correctly processed and expressed within endogenous miRNA levels. We then tested efficacy in both *in vitro* and *in vivo* model systems, achieving a dose-dependent lowering of α -syn mRNA and protein. Finally, the potential phenotypic improvement by miSNCA was evaluated in a *C. elegans* model of PD and also in a α -syn rat model with AAV1/2-induced overexpression of A53T- α -synuclein. Lead miSNCA candidates decreased α -syn mRNA and protein levels and rescued the motor phenotype in the *C. elegans* PD model. Results in the α -syn rat model supported the previous findings and showed target engagement at both mRNA and protein levels. Moreover, dopamine metabolite deficiencies and motor deficits in α -syn rats were corrected by AAV-miSNCA. These results support the potential therapeutic value of α -syn RNAi-based gene therapy for disease modification in PD.

20. Discovery of Translation Initiation Elements Enabled by a Parallel Arrayed Screen of Full-Length Viral UTRs in Synthetic Circular RNA

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Linear messenger RNA (mRNA) has emerged as a validated modality for vaccines and is being investigated for a spectrum of other therapeutic applications. We are developing a new class of synthetic, protein-coding circular RNA (oRNATM) that has advantages over mRNA including ease of production, high levels of protein expression, and enhanced stability. Lacking the cap structure and termini of mRNA, oRNAs require a sequence-based element, such as a viral internal ribosome entry site (IRES), to initiate translation. Tens of thousands of unique UTRs containing putative IRESs are known to exist, often found in the 5' untranslated region (UTR) of viruses in the *Picornaviridae* and *Flaviviridae* families; yet only a handful of

these sequences have been evaluated for activity. Here, we report the discovery of hundreds of potent oRNA-active IRESs using FoRCE™ (Formulated oRNA Cell-based Expression): a robust high-throughput platform that enables parallel arrayed synthesis, purification, lipid nanoparticle (LNP) formulation, and cell-based screening of oRNAs. Importantly, FoRCE maximizes therapeutic translatability by 1) measuring IRES activity in circular oRNA instead of DNA plasmid, 2) incorporating LNP formulation into the process, 3) using primary cells instead of cancer cell lines, and 4) exploring full-length UTRs up to 1000nt instead of short oligo-length fragments. We applied FoRCE to almost 3,000 unique oRNAs containing UTRs extracted from viral genomes and discovered hundreds of IRESs that drive translation from synthetic oRNA in primary human T cells, hepatocytes, and myotubes. Many of these IRESs exhibited far greater activity than those commonly used by the broader scientific community such as the EMCV IRES. Although some IRESs maintained a high level of expression across all cell types tested, others showed a strong preference for a specific cell type, suggesting a novel means for selectively tuning tissue expression. This evaluation of viral IRES activity, the most comprehensive to date, represents a major advance in our ability to control and maximize cap-independent translation. Continued exploration of both natural and synthetic translation initiation elements will further actualize the therapeutic potential of oRNA.

21. Tumor-Targeted miRNA Agent for Pediatric Glioblastoma

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Despite intensive efforts in surgical and medical management, the prognosis of pediatric glioblastoma (pGBM) is unsatisfactory. The large datasets analyses revealed significant differences between pGBM and normal brain tissues at the molecular levels such as miRNA expression. In particular, miRNA20 is reportedly elevated in pGBM and functions as an oncogenic miRNA. As such, targeting specific oncogenic miRNA in pGBM is an attractive approach to develop new miRNA-based agents for pGBM. To diminish levels of oncogenic miRNA in pGBM by using antisense-miRNA is a promising approach, however, i) transport to the brain by crossing the blood-brain barrier (BBB), and ii) tumor-targeted delivery of anti-miRNAs are currently major challenges for advancement into the clinic. In this study, we seek to overcome current limitations by using a cell penetrating-peptide, p28 as a carrier molecule. We have demonstrated that the redox protein azurin, secreted by an opportunistic pathogen *Pseudomonas aeruginosa*, preferentially enters human cancer cells and induces apoptosis. We have further identified that a fragment of azurin, p28, can cross the BBB and preferentially enters human pGBM cells, SJ-GBM2 and CHLA-200. In addition, p28 has no apparent toxicity or immunogenicity in a clinical trial of pediatric CNS patients. Thus, p28 is a potentially ideal carrier for the pGBM targeted delivery. Here, we created a unique agent by covalently linking p28 and antisense-miRNA20 (namely AmiR20-p28). AmiR20-p28 exposure showed clear dose-dependent anti-proliferative/apoptotic effects on pGBM cell lines, but not on human astrocytes used as a non-malignant control. In contrast, antisense-miRNA20 alone without p28 conjugation showed very little effect at any concentration tested in these cancer cells. Such apoptotic

effects induced by AmiR20-p28 were associated with a significant reduction of endogenous miRNA20 levels in pGBM. Further, the p53 and ERK signaling pathways were altered by AmiR20-p28, suggesting that AmiR20-p28 can regulate multiple pathways simultaneously. In an orthotopic xenograft model, AmiR20-p28 significantly inhibited the SJ-GBM2 tumor growth over the course of seven weeks tail vein *i.v.* treatment. The PCR analyses to detect AmiR20-p28 also showed the preferential accumulation of AmiR20-p28 at the given tumor in the mouse brain. Based on these findings, the development of p28 as a carrier will provide a novel strategy for tumor-targeted delivery of miRNAs. It can potentially overcome the current limitations of developing miRNA-based agents and provide a positive impact on the treatment outcomes (*e.g.*, morbidity, quality of life) for pGBM.

Cardiovascular and Pulmonary Diseases

22. Development of Passive Immunoprophylaxis Against SARS-CoV-2 Using Elderly and Immunodeficient Mice Models

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Vaccines for COVID-19 are now a crucial public health need but the degree of protection provided by conventional vaccinations for individuals with weakened/damaged immune systems is unclear. The use of viral vectors to express neutralizing monoclonal antibodies (mAbs) in the lung is an alternative approach that does not depend on the immune response of individuals. We have shown that this strategy, termed vector-mediated immuno-prophylaxis (VIP), can result in the sustained production of neutralising mAbs against infectious diseases such as Influenza (Tan Thorax 2020;75:1112) and RSV (Antepowicz Sci Reports 2021; 11;15694). We have developed a third-generation, self-inactivating recombinant Simian Immunodeficiency Virus (rSIV) pseudotyped with the Fusion and Haemagglutinin-Neuraminidase surface glycoproteins from Sendai Virus (rSIV.F/HN) for efficient transduction of the murine lung (Alton Thorax 2016;20:8406). To investigate rSIV.F/HN as a vector to prevent SARS-CoV-2 infection, we used the prototype anti-SARS-CoV-2 RBD mAb NC0321. This mAb can efficiently neutralise a range of SARS-CoV-2 variants, including Alpha, Beta, Delta and Eta, with an IC₅₀ of 1.3, 27.63, 3.014 and 2.041 ug/ml, respectively. Both prophylactic and therapeutic strategies were assessed. Injection of purified NC0321 mAb (10 mg/kg, intraperitoneal) can protect against 1E5 focus forming units (FFU)/ml of SARS-CoV-2 infection in mice transduced with Adenovirus expressing human ACE2 (Ad5-hACE2). To evaluate the VIP strategy *in vivo* using NC0321 as an exemplar, we constructed a single open reading frame of NC0321 IgG1 cDNA in rSIV.F/HN vector. We previously showed that VIP for COVID-19 (COVIP), in which NC0321 IgG1 was expressed in the proximal airway after a single *in vivo* intranasal dose (I.N. 5E8 Transducing Unit (TU)/ml) conferred

protection against a SARS-CoV-2 mimic in a mouse model expressing hACE2 (Du Thorax 2022; accepted). Here, we extend our studies to compare levels of protection observed in healthy young mice (5-8w), with healthy older mice (7-8m), as well as mice with severe immunodeficiency (NOD.Cg -Prkdc^{IL-2R-gamma null}/SzJ, SCID, 6m). We administered a single I.N. dose of rSIV.F/HN expressing NC0321 (5E8 TU/ml) to each group of mice (young, elderly, and SCID mice of mixed gender; n=6/group). After 28 days we found that the young mice achieved NC0321 mAb in serum (~3 µg/ml) and ELF (~10 µg/ml) comparable to old and SCID mice (ns p>0.999). Moreover, comparable serum and ELF IgG expression were observed in all groups. Importantly, strong protection was observed in all groups (95.3%, 83.7% and 93.1% protection against mouse-adapted SARS-CoV-2 surrogate challenge for the young, elderly, and SCID groups respectively. **** p<0.0001). Finally, we showed immunoprophylaxis from a single I.N. dose of rSIV.F/HN.NC0321 afforded both short-term (28 days, ~1.5 log at day 1, **** p<0.0001; 1 log reduction at day 3, * p=0.0226), and long-term (> 6m, ~2 logs at day 1, *** p=0.0006; ~1 log reduction at day 3, ** p=0.0077) prevention of authentic SARS-CoV-2 replication in Ad5-hACE2 transduced mouse lungs. In summary, these data demonstrate that a single vaccination of rSIV.F/HN expressing an anti-SARS-CoV-2 mAb can provide similar high protection in mice of older age or with a weakened immune system, compared to young, healthy mice. Combining these findings with our earlier studies, we believe that this rSIV.F/HN vector delivery of mAb by direct lung inhalation could be useful, especially for individuals who cannot gain effective immunity from existing vaccines and could potentially prevent clinical sequelae for COVID-19 and other respiratory infections.

23. F/HN Pseudotyped Lentiviral Vector Uses Alpha 2,3 Sialylated N-Acetylglucosamine to Efficiently Transduce Human Airway Cells

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New pseudotypes for Lentiviral vectors (LV) are being developed to improve efficiency of targeting in the human lung. The F/HN pseudotype (Alton et al, 2017) mediates efficient transduction of murine airway *in vivo* without the need for adjuncts that disrupt the airway epithelia. This pseudotype is based on the fusion (F) and attachment hemagglutinin-neuraminidase (HN) glycoproteins of a murine respirovirus (Sendai). Glycoproteins such as HN can agglutinate red blood cells by binding sialylated glycans abundant on the cell surface. The availability of different subtypes of sialylated glycans on airway epithelium varies between species and can affect the targeting specificity and efficiency of the pseudotype. We investigated the sialylated glycans used by F/HN and determined the implications for transducing human airway. Firstly, we used lectin binding studies to show that the human airway contains an abundance of α2,6 and α2,3 linked sialylated glycans, although the α2,3 sialylated glycans were of a specific human subtype (N-Acetylglucosamine). This is in contrast with murine airways, which lack α2,6 linkages but are abundant in

both α2,3 subtypes. This highlights a particular problem in using the mouse lung model to predict transduction of human airway when using sialic acid as receptors. Next, we used lectin binding in three types of air-liquid interface (ALI) cultures derived from primary human airway epithelial cells to show that the ALI cultures shared a similar availability of sialylated glycans (specifically α2,6 and the human α2,3 subtype) with the human airways. To investigate the use of these receptors for F/HN transduction, we generated recombinant Human Immunodeficiency Virus vectors expressing EGFP (LV.F/HN). All three types of ALI culture were transduced by LV.F/HN, but ALI cultures with the greatest abundance of the human α2,3 subtype were most efficiently transduced (up to 52% area EGFP; n=4 donors, n=2 replicates). We co-localised EGFP expression with lectin binding and cell-type specific antibodies and showed LV.F/HN transduction of basal, ciliated, goblet and club cells, all expressing human α2,3 subtype. In separate studies, ALI cultures were treated with either Sialidase S (to specifically cleave α2,3) or Sialidase A (to cleave both α2,3 and α2,6) prior to transduction with LV.F/HN. Both pre-treatments resulted in significantly reduced transduction (p=0.0002; Sialidase A, P=0.003; Sialidase S) indicating the F/HN use of human α2,3 for transduction. To assess the relative efficiency in using these receptors, we compared LV.F/HN transduction with another hemagglutinin pseudotype: HA Rostock, based on avian influenza, is similarly successful at transducing the murine airway *in vivo* (McKay et al, 2006). We showed that transduction of human ALI cultures by LV.F/HN was significantly more efficient (up to 300-fold) than transduction with LV.HA Rostock (n=2 types, n=2-4 donors per type, n=2 replicates). We also compared LV.F/HN and LV.HA Rostock for their affinity to bind soluble sialylated glycans and inhibit hemagglutination. As expected, both pseudotypes showed a greater affinity to bind human α2,3 than α2,6. Interestingly, LV.F/HN required 16-fold less of the human α2,3 subtype glycan (LSTd) to inhibit hemagglutination. This suggests that LV.F/HN has a strong affinity to bind the human α2,3 glycans abundant on ALI cultures and human airway. Altogether these results highlight the importance of accurately modelling the receptors present on target cells. In addition, the prevalence of α2,3 sialylated N-Acetylglucosamine on human airways, and its efficient use for transduction by the F/HN pseudotype, supports its potential in human airway gene therapy applications.

24. Extended Results from First-in-Human Clinical Trial of RP-A501 (AAV9:LAMP2B) Gene Therapy Treatment for Danon Disease

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Background: Danon disease (DD) is an X-linked disorder caused by mutations in the *LAMP2* gene which is essential for autophagy. DD is characterized by severe cardiomyopathy with median survival for male patients (pts) to approximately 19 years (y) of age. In males, DD natural history is characterized by progressively worsening

cardiac abnormalities including wall thickening culminating in severe ventricular hypertrophy, impaired left ventricular global longitudinal strain (GLS), increased brain natriuretic peptides (BNPs) and propensity for arrhythmias; abnormalities frequently manifest in childhood followed by end-stage heart failure during late adolescence. Treatment options are limited. **Methods:** This open-label Phase 1 study evaluates the safety and efficacy of a single IV infusion of RP-A501 (AAV9.LAMP2B) at doses 6.7×10^{13} GC/kg (low dose) and 1.1×10^{14} GC/kg (high dose) in male DD pts in two age groups: ≥ 15 y and 8-14 y. Eligibility criteria include a DD diagnosis with LAMP2 mutation and cardiac involvement. **Results:** All adolescent and adult patients who were treated (≥ 15 y; N=3 pts at 6.7×10^{13} GC/kg, N=2 pts at 1.1×10^{14} GC/kg) with observed immunosuppressive regimen compliance (N=4) had evidence of cardiac LAMP2B expression which ranged from 67-100% vs. normal control by immunohistochemistry (IHC). Adverse events were manageable with transient immunosuppression. Reversible platelet decreases and transaminase elevations returned to baseline within 2 months. Steroid induced myopathy which was considered severe in 2 patients resolved with steroid taper. One patient with decreased LVEF treated at 1.1×10^{14} GC/kg had complement-mediated thrombocytopenia and acute kidney injury requiring transient hemodialysis with full recovery. This patient later required heart transplantation due to Danon disease progression. All other patients have had stable or improved NYHA Class, 6 MWT and reported increases in physical activity. BNPs were reduced by 57- 81% from baseline; echocardiograms indicate trends towards decreased wall thickness. Treatment has been initiated and well-tolerated in the pediatric cohort (n=1; 8-14y at 6.7×10^{13} GC/kg) with an enhanced immunosuppressive regimen intended to minimize potential for complement-mediated events and steroid exposure. **Conclusions:** This ongoing first-in-human trial demonstrates that RP-A501 gene therapy for DD was generally well-tolerated and confers cardiac LAMP2B gene expression. Patients exhibited improvements in multiple cardiac and clinical parameters. Initial pediatric safety results will be presented at the meeting.

25. Amphiphilic Peptides Deliver Adenine Base Editor RNPs to Rhesus Monkey Airway Epithelial Cells *In Vivo*

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The development of safe and effective gene editing strategies for cystic fibrosis and other lung diseases is challenged by the complex barrier properties of airway epithelial cells. To overcome this limitation, we utilized novel amphiphilic shuttle peptides to deliver base editor ribonucleoprotein (RNP) into the airways to edit airway epithelial cells of rhesus monkeys. We previously reported that non-covalently combining shuttle peptides in solution with protein cargoes (CRISPR-

Cas nucleases, GFP) facilitates rapid protein delivery into human and mouse airway epithelia. Using primary air liquid interface cultures of rhesus tracheal epithelial cells, we demonstrated that the shuttle peptides S10 and S315 delivered Cas9-ABE8e RNP and achieved editing at the CCR5 safe harbor locus (editing efficiency 3-9%). To investigate the delivery efficiency *in vivo*, the Cas9-ABE8e RNP and shuttle peptides S10 or S315 were aerosolized into the trachea of sedated young rhesus monkeys during tidal breathing. Seven days later, tissues were obtained and dissected, and airway epithelia collected from the trachea, mainstem, and segmental bronchi using cytology brushes. DNA was extracted from epithelial cells and subjected to high-throughput sequencing. Using the S315 shuttle peptide and Cas9-ABE8e, we achieved a mean editing efficiency of 2.8% at the CCR5 locus in airway epithelial cells (range 0.02 - 5.3%) depending on the anatomic region sampled. To visualize the biodistribution of the RNPs within the respiratory tract and in specific cell types, we delivered a Cy5-fluorescent peptide fused to a nuclear localization signal (NLS-Cy5) using the S10 peptide. The lungs were obtained 1 and 2 hours post-delivery, fixed, and examined by microscopy. Epifluorescence and confocal microscopy documented an effective intra-nuclear delivery of NLS-Cy5 into epithelial cells throughout the respiratory tract, including large, medium, and small airways, and alveolar regions. Ongoing analyses will identify the NLS-Cy5-positive epithelial cell types using co-localization with fluorescently-labeled antibodies. Of significant interest is the capacity of this method to target regional airway progenitor cell types. In summary, using a rhesus monkey model, following a single delivery of adenine base editor RNPs to the airways in a clinically relevant manner we achieved up to 5.3% editing efficiency of the CCR5 locus in airway epithelia, a level considered therapeutically relevant in cystic fibrosis.

26. Gene Therapy Induced Cholesterol Catabolism to Treat Atherosclerosis and NASH

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Repair Biotechnologies, Inc., Syracuse, NY

Excessive and abnormal accumulation of cholesterol has emerged as a critical determinant in the pathogenesis of cardiometabolic diseases. Humans cannot degrade excess cholesterol and are therefore prone to developing life-threatening forms of these diseases. We have developed the Cholesterol Degrading Platform (CDP), an engineered and optimized human protein fusion complex that confers on cells the ability to safely degrade excess cholesterol into a non-toxic, soluble catabolite that can be readily eliminated. We believe that targeted CDP gene and cell therapy can be a novel approach to treating and reversing cardiometabolic disease. Atherosclerosis is a chronic maladaptive inflammatory response triggered by the retention of cholesterol-rich low-density lipoproteins (LDL-C) within the walls of arteries. Circulating monocytes enter the arterial wall, differentiate into macrophages, and ingest LDL-C to prevent its further accumulation. Evidence points to the inability of macrophages to degrade excess cholesterol as a critical determinant in atherosclerosis progression. ApoE-null mice were implanted with a subcutaneous osmotic minipump to allow continuous angiotensin II release (0.7mg/kg/day, 4 weeks) and placed on an atherogenic diet for 4 weeks. Mice were randomly assigned into two groups and intravenously administered

one dose of either AAV-Empty or AAV-CDP (1013 vg/kg, iv tail vein n=7/group). Both groups were placed on normal chow for 4 weeks until euthanasia. Aortic roots were sectioned and stained for plaque lipids with Oil Red O. Compared to the control AAV-Empty treated group, AAV-CDP was well-tolerated and resulted in a significant 48% reduction in plaque lipids ($P < 0.05$). Mice did not exhibit weight loss or evidence of toxicity. Excess accumulation of cholesterol in non-alcoholic fatty liver disease (NAFLD) liver is believed to be a trigger in the onset and progression of non-alcoholic steatohepatitis (NASH), a condition where hepatic steatosis coupled with sustained and maladaptive inflammation lead to progressive liver cell injury and fibrosis. ApoE-null mice were fed a high fat diet for 8 weeks to develop NASH. We found that treatment with AAV-CDP (vs AAV-Empty, 1013 vg/kg iv tail vein; n=12/group) resulted in a 34% reduction in liver macrosteatosis ($P < 0.01$) and a 42% reduction in α -smooth muscle actin (SMA) staining ($P < 0.01$), suggesting reduced hepatic cell injury, hepatic stellate cell activation, and liver fibrosis. Separately, using the MSNASH/Pco) mouse model of NASH, we found that weekly intravenous administrations of 1 mg/kg of LNP encapsulated CDP mRNA (n=11 LNP-mRNA-CDP, n=11 LNP-empty control) resulted in a 17% improvement in blood glucose tolerance ($P < 0.01$) over the course of 8 weeks. However, after 12 weeks of treatment, liver toxicity, and loss of improvement was observed, possibly a result of mRNA immunogenicity. In conclusion, our data provide initial evidence that systemic administration of CDP can be safe and well-tolerated, and potentially implemented as an efficient treatment for atherosclerosis and NASH. We are now testing the efficacy of CDP-expressing iPSC-derived anti-inflammatory M2 macrophage cell therapy in animal models of atherosclerosis.

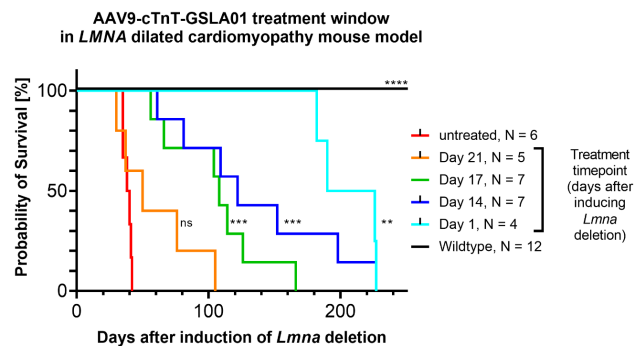
27. AAV Gene Therapy Using a Genetic Suppressor Treats LMNA Dilated Cardiomyopathy in a *Lmna* Mouse Model Following a Decline in Ejection Fraction

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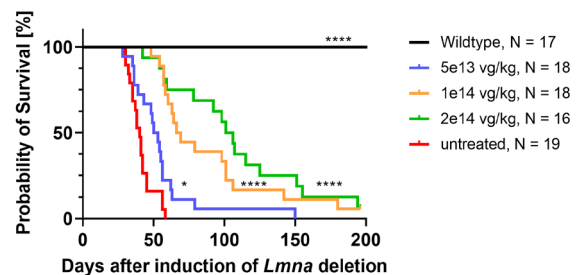
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Autosomal dominant, gain-of-function mutations in the *LMNA* gene encoding the nuclear envelope protein lamin A/C result in dilated cardiomyopathy (DCM), with a prevalence of as high as 1/12500. In previous studies, we established a mouse model for *LMNA* DCM where inducible cardiac deletion of *Lmna* resulted in ventricular dilation, reduced ejection fraction, fibrosis and mortality within 5-6 weeks. Using AAV9 and the cTnT (cardiac Troponin T) promoter to express a genetic suppressor of *Lmna* mutations, GSLA01, in cardiomyocytes *in vivo*, we prevented development of cardiac pathology, extending the lifespan of the mice to over one year after induction of the *Lmna* mutation. The phenotypic correction is the result of the GSLA01 protein disrupting the nuclear envelope-localized LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. The *Lmna* DCM mice exhibit a relatively normal cardiac ejection fraction 14 days after *Lmna* gene deletion in the heart, but this declines significantly at 21 days post-deletion. To determine whether our AAV9-cTnT-GSLA01 vector can be

used to treat rather than prevent *LMNA* DCM, we treated mice 1, 14, 17 and 21 days after deletion of *Lmna* in mouse cardiomyocytes *in vivo*. The lifespan of *Lmna* DCM mice was increased by 177 % to 108 days 17 days after induction of the *Lmna* mutation ($P = 0.0002$), with earlier treatment resulting in longer survival. Dose response experiments delivering 5×10^{13} , 1×10^{14} and 2×10^{14} vg/kg of AAV9-cTnT-GSLA01 vector in mice 17 days after inducing the *Lmna* mutation resulted in statistically significant ($P < 0.05$) increases in median lifespans to 51.5, 67.5 and 103.5 days respectively, whereas untreated mice survived only 40 days. The dose-efficacy response and lifespan extension following treatment of mice with reduced ejection fraction suggest that our genetic suppressor approach is a promising one for treating *LMNA* DCM patients using AAV gene therapy.



AAV9-cTnT-GSLA01 dose response for *LMNA* DCM mouse model treated 17 days after induction of *Lmna* mutation



28. A First-in-Human Phase 1 Clinical Gene Therapy Trial for the Treatment of Heart Failure Using a Novel Re-Engineered Adeno-Associated Vector

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Congestive heart failure (HF) remains a progressive disease with a critical need for innovative therapies to reverse the course of ventricular dysfunction. Recent advances in understanding the molecular basis of

myocardial dysfunction, together with the evolution of increasingly efficient gene transfer technology, have placed HF within reach of gene-based therapies. One of the key abnormalities in HF is abnormal intracellular Ca^{2+} handling and increased protein phosphatase activity. We have developed a novel Adeno-Associated Vector, AAV2i8, that is cardiotropic and de-targets the liver to deliver a constitutively active protein phosphatase 1 inhibitor 1 (I-1c) - AAV2i8.I-1c. We initiated a First-in-Human dose escalation gene therapy trial to evaluate the safety and feasibility of a single antegrade coronary artery infusion of three dose levels of AAV2i8.I-1c in patients with non-ischemic cardiomyopathy and New York Heart Association Class III symptoms of HF. The trial has enrolled three patients in cohort 1 and five patients in cohort 2. Twelve month follow-up data are available for all three participants in cohort 1. The safety profile was excellent with no AEs related to the investigational product over 12 months' observation. Efficacy measurements included changes in NYHA classification, VO_2 max assessed by cardiopulmonary exercise testing, six-minute walk test, left ventricular ejection fraction, and left ventricular regional wall motion. Compared to baseline, all three patients showed clinically meaningful improvements in endpoints such as LVEF and NYHA at month 12 (see table 1). The 12-month follow-up of cohort 2 is ongoing. Table 1: summary of results for the 12 months follow up of cohort 1. Quantifiable measures of biological activity across a number of parameters, important for assessing HF status, could be detected in patients in cohort 1 in this cardiac gene therapy trial. We will report preliminary safety and efficacy data on patients who have completed 12 month follow-up.

	BASELINE			Difference at 12 months from baseline		
	1	2	3	1	2	3
Subject	1	2	3	1	2	3
NYHA	3	3	3	-1	-2	-1
LVEF (%)	37.5	29	34	+10.5	+22	+6
MLWHFQ	81		51	-43		-12
6MWT (m)	314	381	24.6	+51.75	+38.1	+235.4
p VO_2 max (ml/kg/mim)	12.3	14.3	7.9	+0.9	+1.5	+5.1
NT-proBNP(pg/ml)	225	232	165	+552	-38	-60

Musculo-skeletal Diseases

29. Muscle-Specific Tyrosine Kinase Chimeric Autoantibody Receptor T Cells (MuSK-CAART): A Precision Cellular Immunotherapy for Antigen-Specific B Cell Depletion in MuSK Myasthenia Gravis

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Muscle-specific tyrosine kinase (MuSK) myasthenia gravis is an autoimmune disease that causes life-threatening muscle weakness due to MuSK autoantibodies that disrupt acetylcholine receptor clustering and neuromuscular junction signaling. MuSK myasthenia gravis therapy with steroids and the anti-CD20 monoclonal antibody rituximab aims to suppress or eliminate antibody-producing B cells, although disease often relapses due to incomplete B cell depletion and even transient B cell depletion risks serious infections. The ideal therapy would eliminate only the autoantibody-producing B cells while preserving healthy B cells to protect from infection. Chimeric antigen receptor (CAR) T cells have transformed cancer treatment by re-programming T cells to eradicate B cell cancers, raising hope that precision medicine cures can be extended to other B cell-mediated diseases. By expressing the MuSK autoantigen as the extracellular targeting domain of a chimeric autoantibody receptor (CAAR) with 4-1BB/CD3 ζ cytoplasmic signaling domains, we genetically engineered human MuSK CAAR T cells (MuSK-CAART) to selectively kill anti-MuSK B cells expressing a surface-bound autoantibody (anti-MuSK B cell receptor). Expression of the MuSK CAAR in primary human T cells directs T cell cytotoxicity toward B cells targeting a broad range of physiologic MuSK epitopes. MuSK-CAART demonstrated comparable activity as anti-CD19 CAR T cells in eliminating anti-MuSK B cells in immunodeficient NSG mice xenografted with anti-MuSK Nalm6 cells, in the presence or absence of soluble anti-MuSK antibodies. In an immunocompetent experimental autoimmune myasthenia gravis model, treatment with murine anti-CD19 CAR T cells depleted B cells and decreased both serum anti-MuSK IgG and total IgG levels, whereas treatment with murine T cells expressing a human MuSK CAAR induced comparable decreases in anti-MuSK IgG titers without decreasing total B cells or IgG levels, reflecting MuSK-specific B cell depletion. Toxicology studies in the NSG xenograft model and screens of a high-throughput human membrane proteome array and primary human cells did not identify MuSK-CAART off-target cytotoxic interactions. These data in part formed the basis of an investigational new drug application for MuSK-CAART for the treatment of MuSK autoantibody-positive myasthenia gravis, which has been allowed to proceed by the FDA.

30. Novel Single AAV Vector Treatment for Congenital Muscular Dystrophy Type 1A (MDC1A) Using CRISPR-GNDM® Technology

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Traditional AAV gene replacement therapy is challenging for genes with large coding sequences due to AAV's packaging size limitations (under 4.7kb). While this could be addressed with CRISPR-mediated activation (CRISPRa) on a modifier gene, present CRISPRa systems are also notably beyond the packaging capacity of a single AAV vector. Additionally, specificity of CRISPRa can present as a challenge as off-target gene perturbation is often observed. In effort to overcome these obstacles, we developed our CRISPR-GNDM® (Guide Nucleotide-Directed Modulation) technology, which includes an engineered Cas9 variant fused with compact transcription activator and sequence specific guide RNA cassette, all at a reduced size to be packaged into a single AAV vector and applied it to develop a therapy for MDC1A disease. MDC1A is a severe, early onset form of congenital muscular dystrophy (CMD) involving muscle weakness, loss of ambulation, and respiratory failure. Majority of patients do not survive past the second to third decade of their life, noninvasive ventilatory (NIV) support is required in all patients, and quality of life is diminished. There is currently no effective treatment or cure. MDC1A is characterized by mutations in *LAMA2* which causes functional impairment of the laminin $\alpha 2$ subunit, a critical component of the heterotrimeric extracellular matrix proteins laminin-211/221. Laminin $\alpha 1$ subunit encoded by the *Lama1* gene has been shown to compensate the loss of *Lama2* in mouse. As *LAMA1* coding sequence is too large (over 9kb) to package into a single AAV vector and it is not actively transcribed in muscle tissues, it serves as an ideal target for our CRISPR-GNDM® technology. We first evaluated efficacy of AAV9-CRISPR-GNDM®-mLAMA1 in neonatal DyW mice via systemic delivery. DyW is a well characterized *Lama2* knockout mouse strain with severe muscular dystrophy phenotypes. While untreated homozygous animals typically die within 1-3 months, GNDM® treatment extended their survival in a dose-dependent manner to more than 8 months. In these mice, using immunofluorescent staining, we observed robust LAMA1 protein expression in the basement membranes of skeletal muscle fibers. H&E staining showed less histopathology compared to untreated animals. Treated animal also showed over 60% reduced serum CK levels, a circulating marker of muscle degeneration. Additionally, we evaluated long-term efficacy of AAV9-CRISPR-GNDM® in C57BL6 animals and observed sustained GNDM® expression and/or *Lama1* mRNA upregulation in muscle tissues up to 2 years. Towards developing human CRISPR-GNDM® vector, we screened over 400 human guide RNAs in human primary skeletal muscle cells, through which we identified a few human guide RNA sequences that strongly upregulate *LAMA1*. Using whole transcriptome analyses, we found that CRISPR-GNDM® demonstrated much higher transcription modulation specificity than CRISPRa. We also optimized the size of our vector by further engineering GNDM® to have a compact activation moiety to achieve efficient bioprocessing of AAV with high therapeutic potency. In summary, our proprietary CRISPR-GNDM® technology offers a

single vector treatment for MDC1A with high potency and specificity, and can be customized and applied to other genetic disease caused by haploinsufficiency.

31. Regeneration of Articular Cartilage, Suppression of Synovial Inflammation, and Alleviation of Joint Pain After Intra-articular Injection of ICM-203 in Canine OA Model

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While inflammation in osteoarthritis (OA) needs to be controlled to attenuate OA pathogenesis, maintenance of proper dynamics of chondrocyte physiology would also be critical to suppress OA. Due to the complicated etiology of OA, significant unmet medical needs exist for a disease-modifying OA drug (DMOAD). Nkx3.2 was initially identified as a pro-chondrogenic factor promoting cartilage development. Our previous studies have revealed that reduced Nkx3.2 expression can be tightly linked with OA and compensation of Nkx3.2 expression can suppress OA in mouse models. Nkx3.2-D2, a truncated form of Nkx3.2 encoding a.a. 123-320, was identified to be resistant to OA-induced protein degradation leaving its OA suppression activity intact. In this study, ICM-203, an AAV vector encoding Nkx3.2-D2, was investigated for its DMOAD activity in a beagle OA model. Modulation of OA pathogenesis was examined by using intra-articular (IA) injection of ICM-203 in a surgery-induced beagle OA model. Combined knee joint surgery, including anterior cruciate ligament transection and partial medial meniscectomy, was employed to thirty-two male beagle dogs to induce OA. Prior to dosing, radiography was analyzed to confirm the baseline OA conditions. Then, a single administration of ICM-203 (Low, Mid, High), along with placebo, was performed in the OA-induced knee joint (n=8 per group). Longitudinal observation for cartilage regeneration and synovitis were examined by MRI. In addition, various functional assessments including manual joint palpation, gait analysis, and static weight bearing (SWB) was performed for 26 weeks. Pre-dose analyses of radiography revealed that beagle dogs' operated knee joint condition was comparable to KL grade 2 to 3 in human patients. After ICM-203 administration, cartilage regeneration was evaluated by longitudinal MRI analyses. Semi-quantitative MOAKS revealed pronounced linear improvements in cartilage surface area and full thickness cartilage damage in ICM-203 high-dosed group. Furthermore, cartilage conditions were assessed by quantitative MRI as well. Centro-medial femur (cMF) cartilage was thickened by 9.6% for ICM-203 high-dosed group at week-26 post-dose, and the average thickness for the entire cMF cartilage was increased by 0.04 mm. In addition, IA injection of ICM-203 decreased the areas with full cartilage defect in cMF; the average recovery of

denuded area (dAB) in placebo and ICM-203 high-dose group was 0.14 and 26.6%, respectively. Consistently, significantly accelerated recovery in Hoffa and Effusion synovitis was observed for ICM-203-dosed group. Finally, IA administration of ICM-203 was effective in restoring knee joint function by alleviating pain as evidenced by manual palpation, gait analysis, and SWB analyses. All ICM-203 dosed group showed continuous improvement in pain up to week-26, whereas the placebo treated group remained with sustained pain. IA delivery of ICM-203 was found to be capable of regenerating functional cartilage, inhibiting synovial inflammation, and restoring normal joint functions. Considering these pre-clinical observations, first in human trial using Nkx3.2-based AAV gene therapy could potentially show function/pain and structural benefits for human OA patients.

32. Lentiviral Vector-Based Gene Therapy for Type II Collagen Disorders

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Background- Type II collagen disorders are skeletal dysplasia resulting from mutations in *COL2A1*. Clinical presentations range from perinatal lethal disease (Achondrogenesis type II or hypochondrogenesis) to disproportionate short stature (Spondyloepiphyseal dysplasia congenita or SEDc), or normal stature children with severe myopia, retinal detachment and early onset inflammatory arthritis (Stickler syndrome). SEDc is the most common severe non-lethal type II collagen disorder, defined by disproportionate short stature, progressive scoliosis/kyphosis, joint degeneration and pain, myopia and various non-skeletal features. No disease-modifying treatment is available. **Methods-** We have developed a VSVG-pseudotyped third-generation self-inactivating lentiviral vector (INS-101) for *in vivo* systemic use to restore normal cartilage growth in bone growth plates and prevent skeletal deformities related to the most severe clinical complications. As a proof-of-concept, we tested INS-101 in a spontaneous mouse model of SEDc (B6(Cg)-*Col2a1*^{SEDc}/J or “SEDc mice”), which recapitulates the key histologic, radiographic and clinical characteristics of pediatric SEDc patients. INS-101 cassette includes a codon-optimized wild-type *COL2A1* cDNA under the control of the hybrid promoter HB13a, which was specifically developed to support high and sustained expression in chondrocyte lineage, but no activity in hematolymphoid cells. **Results-** Following repeat systemic injections of INS-101 at days 1, 7 and 15 in SEDc mice, survival was improved from 55 to 86% at doses as low as 1E6 Transducing Unit per kg (TU/kg) in the treated SEDc mice compared to untreated controls. Results from SEDc mice treated at doses ranging from 1E7 to 1E9TU/kg suggested a dose-dependent and long-term correction of histologic, radiographic and clinical anomalies, associated with: 1) high expression of *COL2A1* in the chondrocytes of bone growth plates, 2) restoration of columnar architecture and chondrocyte differentiation in growth plates, 3) radiographic correction of sternum, hip, cranio-cervical and long bone measurements, 4) restoration of the thoracic cage volume and correction of the cranio-cervical junction instability and vertebrae maturation, and 5) prevention of respiratory

insufficiency, spine deformities and locomotor dysfunction. An increase in total body length was observed, although INS-101 treatment did not fully restore the body length of wild-type mice. No adverse event was observed in over 400 SEDc and wild-type mice treated. To assess the activity of INS-101 in patient-derived specific SEDc mutations, induced Pluripotent Stem Cells (iPSCs) were generated from SEDc, Stickler and healthy control subjects. We tested INS-101 in iPSC-derived human mesenchymal stem cells and chondrocytes for *in vitro* activity and coverage of several mutations in long-term 3D chondrocyte cell culture systems. Preliminary results suggest that INS-101 treatment *in vitro* restores the production of the extracellular matrix and accelerates the growth and differentiation of subset proliferative and hypertrophic chondrocytes. **Conclusions-** Overall, *in vitro* and *in vivo* proof-of-concept studies suggest that systemic delivery of INS-101 can efficiently target chondrocytes of the skeletal bone growth plates and prevent the development of severe complications related to type II collagen disorders. Further development is ongoing to delineate the efficacy of INS-101 in the SEDc and Stickler mouse models, and its biodistribution and safety profile in relevant large animal models.

33. Multicenter AAV Gene Therapy Studies for SMARD1/CMT2S Establish Safety and Efficacy in Multiple Animal Models and Pave the Way for Initiation of a Phase I/II Clinical Trial

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Mutations in the immunoglobulin mu DNA binding protein 2 (IGHMBP2) gene lead to a spectrum of rare, autosomal recessive diseases characterized by degeneration of α -motoneurons and ganglion cells. Currently, there is no evidence of a close genotype-phenotype correlation, as even siblings carrying the same mutation can present a broad spectrum of clinical features, ranging from distal muscle weakness with fatal respiratory distress / failure (Spinal muscular atrophy with respiratory distress type 1 - SMARD1) to sensory and milder motor neuropathies with lesser respiratory involvement (Charcot Marie Tooth Disease 2S - CMT2S). Currently, only symptomatic treatments are available for SMARD1/CMT2S, which have no effect on disease progression. Thus, there is an urgent need to develop an effective therapy for SMARD1/CMT2S. We conducted multicenter blinded, dose-ranging IND-enabling safety and efficacy studies evaluating Adeno-Associated virus Serotype 9 (AAV9)-mediated delivery of a functional IGHMBP2 coding sequence. The therapy was tested in three different mouse models comprising the whole SMARD1/CMT2S disease spectrum. We demonstrated that

a single, intracerebroventricular injection of ssAAV9.IGHMBP2 in EM3, nmd-2] and EM5 mice at post-natal day 1 resulted in widespread expression of IGHMBP2 with improvement in functional measures in a dose dependent manner. All three mouse models showed a marked and dose-dependent response to the treatment leading to improvement of body weight, strength, neuromuscular junction innervation and survival. In addition, we also saw an increase in CMAP and MUNE, potentially clinically relevant biomarkers indicating improved muscle innervation. Importantly, ssAAV9.IGHMBP2 had no adverse effects when administered in wild type mice, showing dose-dependent levels of transgene expression in brain and spinal cord. To further translate this approach to the clinic, we administered ssAAV9.IGHMBP2 intrathecally to six cynomolgus macaques aged 1-6 years. ssAAV9.IGHMBP2 treatment was safe and well tolerated in all non-human primates with widespread transgene expression throughout the central nervous system up to 6 months post-injection. Importantly, no significant pathology was observed throughout peripheral organs as well as brain, spinal cord and dorsal root ganglia. Collectively, these studies resulted in FDA approval of the first in human phase I/II clinical trial for intrathecal administration of ssAAV9.IGHMBP2 in SMARD1/CMT2S patients.

34. AAV-CRISPR-Cas13 Gene Therapy for FSHD: DUX4 Gene Silencing Efficacy and Immune Responses to Cas13b Protein

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The Ohio State University, Columbus, OH, USA. Facioscapulohumeral Muscular Dystrophy (FSHD) is among the most prevalent muscular dystrophies, ranging from 1 in 8,333 to 1 in 20,000. Currently, no treatment exists that alters the course of FSHD, and therapy development remains an unmet need in the field. Abnormal reactivation of the embryonic *DUX4* gene in skeletal muscle has emerged as an underlying cause of muscle weakness and wasting in FSHD. *DUX4* silencing is the most direct method to develop an effective FSHD therapy. Toward this goal, we developed an AAV6-CRISPR-Cas13 strategy to silence toxic *DUX4* expression in muscles of a FSHD mouse model. CRISPR-Cas13 is a programmable RNA-targeting system that can be guided to silence target mRNAs in mammalian cells and may allow long-term silencing of target genes while applying the precision of the CRISPR system. Since Cas13 only targets and cleaves RNA instead of DNA, it will avoid potential risks of permanent off-target genome editing that could arise with DNA-targeting systems. Moreover, potentially hundreds of *DUX4* copies exist in the human genome, making DNA editing more challenging. Therefore, we hypothesized that CRISPR-Cas13 could be a powerful method for silencing *DUX4* at the mRNA level. Here, we demonstrate that intramuscular delivery (5e10 total particles) of an AAV6 vector encoding a PspCas13b enzyme and *DUX4*-targeting guide RNAs reduced *DUX4* mRNA by >50% ($P < 0.003$) and improved histopathological outcomes in treated FSHD mice. We further investigated the safety of RNA targeting using the

CRISPR-Cas13 system. To investigate possible off-target effects, we performed RNA-seq analysis of treated versus control or untreated human myoblasts and also examined potential collateral RNA cleavage activity (i.e. non-specific cleavage of co-expressed mRNAs) using a dual reporter system. Although we did not detect collateral cleavage, our RNA-sequencing results suggested some guide RNAs, but not others, could induce potential off-target gene expression changes. We are currently exploring mechanisms to explain these differential off-target effects. To address whether PspCas13b can activate a mammalian host immune response, we injected wild-type mouse muscles with various doses of AAV-Cas13b and investigated immune cell infiltration in injected muscles as well as pro-inflammatory cytokine profiles. We find evidence of an immune response against PspCas13b in mice, including CD8⁺, CD3⁺, and NK T-cell, but not B-cell, infiltration in injected muscles. Importantly, transient immunosuppression reduced immune responses to Cas13b in treated animals. In conclusion, our data support that Cas13b can target and reduce *DUX4* expression in FSHD muscles, but minimizing cellular immune response might be necessary in order to translate AAV-Cas13 therapy to the clinic. **Keywords:** Facioscapulohumeral Muscular Dystrophy, FSHD, *DUX4*, D4Z4, CRISPR-Cas13, gene silencing, RNA targeting therapy, Adeno-associated virus vectors, AAV, Muscle

35. Development of Dual AAV-Mediated RNAi and Protein Expression Therapy for Myotonic Dystrophy

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INTRODUCTION. Gain-of-function, dominant genetic disease requires a therapy that eliminates or inactivates a mutant cell component. Recombinant AAV-delivered, RNAi technology has been used to target RNA for gene silencing through mRNA reduction or translation inhibition. We are developing systemic rAAV-RNAi as a therapy for dominant genetic disease of muscle. Our focus includes myotonic dystrophy type 1 (DM1), a common (1 in 2100) debilitating multi-systemic adult muscular dystrophy caused by a DNA microsatellite repeat. Transcription of the DNA repeat results in production of an expanded repeat-containing RNA that is retained in muscle cell nuclei. The expanded repeat containing RNA affects splicing of a subset of unrelated cellular RNAs primarily through the sequestration of the muscle-blind like protein 1 (MBNL1), a key splicing factor. **BACKGROUND.** Our previous efforts to develop AAV-RNAi therapy for myotonic dystrophy type 1 (DM1) targeted the human α -skeletal actin mRNA (*ACTA1* or HSA) which carries an expanded toxic repeat in the widely used HSA^{LR} mouse model of DM1. The HSA transgene acts as a skeletal muscle restricted platform for expressing the expanded CTG repeat in a similar 3' UTR genetic disease context as the dystrophin myotonia protein kinase gene (*DMPK*) gene in individuals with DM1. We have previously shown that it is possible to decrease disease burden with systemic HSA RNAi expression in the HSA^{LR} mouse by reducing the amount of toxic HSA repeat mRNA found in muscle nuclei to improve hallmarks of disease, including defects in cellular transcript splicing and myotonia. **CURRENT WORK.** To further develop an rAAV-RNAi approach for

DM1, we seek to safely integrate therapeutic RNAi hairpin expression into the normal cellular RNAi pathway, balancing the level of RNAi hairpin expression with efficacy. Our current approach, incorporating muscle restricted RNA polymerase II (Pol2) promoter mediated expression, reduces miRNA expression relative to the previous Pol3 promoter expressed miRNAs *in vivo* and *in vitro*. To increase the therapeutic potential of vector delivered Pol2 promoter expressed miRNAs, which typically express at lower levels than Pol3 promoters, we began exploring the possibility of co-expressing our engineered miRNAs with MBNL1 as a combinatorial gene therapy. Studies have suggested that supplemental MBNL1 expression to counteract its deficiency in splicing activities could be a beneficial approach for a DM1 therapy; however, conflicting data showing MBNL1 expression in muscle could be harmful warranted a deeper understanding of the effects of MBNL1 expression in muscle. To limit the potential for MBNL1 toxicity in the heart, we systemically administered 7.5e12 vg of 1) AAV6-CK8-MBNL1 (n=4), or 2) a cardiac low MBNL1 expressing version of AAV6-CK8-MBNL1 (n=7), or 3) a promoter-less AAV6-FLuciferase (negative control, n=7) to WT B6/J mice at 4 weeks of age. We found that AAV6-CK8-MBNL1 is cardiotoxic, by histology and echocardiogram, and led to the death of all treated mice within 5 weeks of administration. Conversely, we observed that our cardiac CK8-MBNL1 low expressing vector resulted in 100% survival 8 weeks post injection. Skeletal muscle histological examination of these mice showed features comparable to the control mice for all groups. These results indicate that MBNL1 expression in skeletal muscle, but not cardiac tissue, is feasible. Our goal is to combine controlled expression of MBNL1 with *DMPK* targeted RNAi to develop a combinatorial therapy for DM1.

Immune Responses to AAV Vectors

36. Characterizing AAV-Mediated Immune Responses in a Mouse Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene, which encodes dystrophin, and is amenable to gene-editing and gene replacement therapies. Serious adverse events have been reported in patients dosed with AAV-microdystrophin during Phase I/II clinical trials, likely due to immune responses against the AAV capsid. In this study, we comprehensively characterize AAV-induced immune responses *in vivo* in order to gain insights on AAV-immune interactions. We systemically dosed and re-dosed dystrophic mice with AAV9 at 1.16E14vg/kg carrying either micro-dystrophin, Cas9, or Cas9 with a frameshift mutation (Cas9-FS), which disrupts Cas9 protein expression and production. Peripheral blood mononuclear cells (PBMCs) were collected at baseline and after the first and second administrations of AAV (five hours, two weeks and four weeks post

dosing). First, we investigated TLR-induced cytokine responses using Luminex since AAV genomes contain unmethylated CpG motifs that have been shown to activate the TLR9:MyD88 pathway. We calculated the risk potential for TLR9 activation for each transgene: Cas9 ($K_{TLR9}=20$), Cas9-FS vectors ($K_{TLR9}=20$) and micro-dystrophin ($K_{TLR9}=13$). All three vectors are above the speculated threshold ($K_{TLR9}>10$) to activate TLR9. Accordingly, Luminex assays revealed that all three vectors significantly increased TNF α , IP-10, MCP-1, and MIP-1 β following the second dose of AAV at the five-hour time point, strongly suggestive of TLR9:MyD88 activation. In addition, we probed for complement levels, as consumption of C3 and C4 were reported in ongoing DMD clinical trials. In our study, we observed significant consumption of C3 and C4 across all three vectors after the second dose at the five-hour time point, suggesting activation of the classical complement pathway, which relies on antibody production. Our preliminary findings support this hypothesis, as we observed peak anti-AAV IgG responses after the second dose as well as cross-reactivity against AAV2 and AAV8. Finally, we performed single cell RNA-sequencing to analyze immune cell phenotypes and our initial findings suggest pronounced activation of all main immune cell types following the second administration of AAV. Ongoing analysis and validation studies are needed to identify critical immune cell types that could be targeted to improve the safety and efficacy of AAV-based therapies.

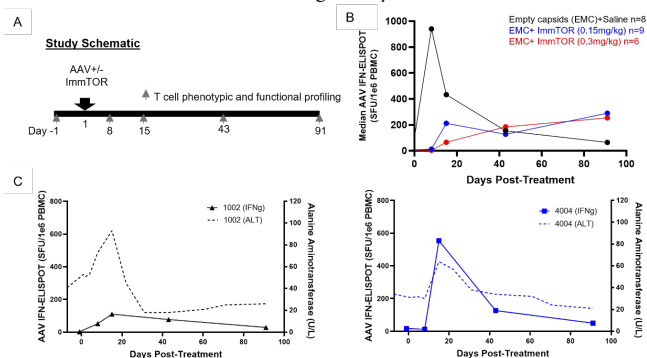
37. Functional Assessment of T Cell Responses to AAV8 Empty Capsids in Healthy Volunteers

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Gene therapy using recombinant adeno-associated virus (AAV) is a promising treatment for both monogenic and complex acquired diseases. However, clinical trials with systemically delivered AAV have demonstrated that T-cell responses directed against the capsid may impact the safety and efficacy of gene transfer. Specifically, capsid-specific CD8+ T cells are induced and associated with elevated liver transaminase levels in a dose dependent manner. Antigen presentation also activates CD4+ T-helper cells, which facilitate humoral and cell-mediated immune responses. Thus, there is a need to characterize the kinetics and polyfunctional profile of CD4+ and CD8+ T cells after AAV gene therapy and evaluate the potential of immune modulators to inhibit the AAV-specific T-cell response. We conducted an experimental medicine trial in 23 healthy volunteers in which subjects were administered 2e12 vp/kg of a novel formulation of AAV8-empty capsids alone (n=8) or in conjunction with tolerogenic ImmTOR nanoparticles, which encapsulate rapamycin (n=15). Empty capsids (intact viral capsid devoid of DNA) were manufactured by double transfection of rep/cap and helper plasmids and purified by ultracentrifugation and chromatography. The results suggest combination treatment was safe and tolerated in healthy volunteers with no serious

adverse events. We performed a multiparametric analysis of the cell-mediated immune response in blood from all individuals before and after dosing (Fig A). The activation, differentiation and functional profile of the T-cell response was evaluated using IFN γ ELISpot, flow cytometry. Dosing with AAV8 empty capsids activated and induced proliferation of CD4 $^{+}$ and CD8 $^{+}$ T cells. Responding T cells produced a robust, capsid serotype cross-reactive IFN γ response detected as early as seven days after dosing (Fig B). When AAV8 empty capsids were co-administered with ImmTOR, there was a significant delay in the induction of the AAV8 specific T-cell response. Administration of AAV8 empty capsid \pm -ImmTOR induced distinct populations of capsid-reactive immune cells with unique profiles of cytokine expression associated with antibody production and immune cell activation. Four subjects showed asymptomatic increases in serum transaminases, with the peak T-cell response coinciding with peak blood alanine amino-transferase (ALT) levels in 3 of 5 subjects (Fig C). A fifth subject, treated with AAV8 capsid alone, showed high elevation of ALT at the end of the 90-day observation. Interestingly, neither the magnitude of the T-cell response, cytokine profile, HLA type, nor in silico-simulated MHC-peptide binding affinity was predictive of elevations in liver transaminases. We believe our results show that empty AAV capsids can be used as a model antigen to assess the effects of immune modulators in development, and that immune-monitoring protocols based on IFN γ ELISPOT should be supplemented with other measures of T-cell function to better assess the contribution of specific cellular subsets to AAV immunogenicity.



38. Characterization of the Innate Immune Response to AAV in Human Blood and the Central Role of Complement

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Adeno-associated virus (AAV) is emerging as a promising vector for gene therapy. It has been previously shown that complement components can bind directly to AAV and promote vector uptake into innate immune cells and that this uptake is associated with increased cytokine secretion in vitro (Zaiss et al. 2008). Additionally, complement receptors were shown to promote anti-AAV humoral response in gene transfer mouse model (Zaiss et al. 2008). While in clinic AAV has shown good safety profile and low immunogenicity, some clinical trials have observed complement activation following intravenous

infusion of AAV. Therefore, we sought to further characterize the innate immune response to AAV in human whole blood. First, we studied the innate immune reaction to AAV (Spark100 and Spark 200) vectors in human whole blood with different titers of AAV neutralizing antibodies (NAb). Using cell surface and intracellular staining for capsid particles at various timepoints, we found that neutrophils, monocyte-related dendritic cells (moDC - HLA-DR+CD11c+CD14 $^{+}$), and monocytes were the most numerous cells that take up AAV in blood. The conventional dendritic cells (cDC - HLA-DR+CD11c+CD14 $^{-}$) were the most activated cell subset upon AAV uptake as evidenced by a 2.4-fold increase of costimulatory molecule (CD86) expression. Presence of AAV NABs in blood enhanced uptake of AAV vector into neutrophils and other cell subsets. AAV NABs enhanced anti-AAV pro-inflammatory cytokine response in blood, in particular interleukin 6 (IL-6) secretion. High NAB titers and high doses of AAV were both required for significant complement activation in blood measured by C3a ELISA assay and C3 fragment deposition on neutrophils. Next, to understand the importance of the complement activation in mediating the immune response to AAV, we studied effects of the investigational complement C3 inhibitor, APL-9, developed by Apellis. APL-9 is a derivative of compstatin, which binds to C3 and C3b and blocks C3 activation. In a whole blood assay, AAV activated complement only in donors with high titers ($\geq 1:100$) of AAV NABs. APL-9 was highly effective at suppressing this activation and it significantly reduced basal levels of C3a in seronegative samples and samples with low NAB titers ($\leq 1:10$). Blocking complement C3 activation significantly reduced vector internalization into monocytes, moDC and cDC and decreased levels of co-stimulatory CD86 molecule. These results suggest that complement plays a major role in driving phagocytosis of AAV. APL-9 treatment also reduced cytokine and chemokine secretion in all donors, regardless of whether they exhibited overt complement activation in response to AAV. This suggests that suppressing even a low level of background complement activation reduces the immune response to AAV. In conclusion, both anti-AAV NAB and complement are important mediators of anti-AAV immunity and complement pathway inhibition reduces multiple readouts of immune response to AAV vectors. These findings warrant further investigation of the complement inhibitors in AAV-based gene transfer. **Disclaimer:** This communication reflects the views of the authors and neither the IMI nor the European Union, EFPIA or any other partners are liable for any use that may be made of the information contained herein.

39. Deimmunized Micro-Dystrophin Vectors Blunt Patient Immunity *In Vitro* & Restore Cardiac Functional Deficits *In Vivo* in *mdx*^{4cv} DMD Mice

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Duchenne muscular dystrophy (DMD) is caused by mutations within the dystrophin gene. A clinically identified hurdle for DMD gene

therapy relates to patient immune system recognition of dystrophin as a foreign protein, potentially resulting in destruction of expressing myofibers and/or cardiomyocytes. In fact, within the past year several serious adverse events have been reported in DMD patients treated with AAV-microdystrophin (μ Dys) due to an immune response against dystrophin. We have been developing an approach to avoid or minimize presentation of dystrophin to T-cells via Rosetta-based computational protein deimmunization & redesign protocols with integration of experimental immunogenic epitope data, MHC epitope prediction tools, and host genomic data. Our initial tests aimed to establish whether μ Dys can be redesigned to deimmunize immunodominant epitope(s) within the actin-binding domain (exons 6-8) that displayed T-cell reactivity in the first AAV- μ Dys human clinical trial (Mendell et al; NCT00428935). Importantly, the algorithm used in our studies predicted T-cell responses to the same epitopes, independent of their detection in this clinical trial. We used Rosetta to develop 3 redesigns of the first epitope (P17, exon-6), 5 redesigns of the second epitope (P19, exon-7), and 10 redesigns of the third epitope (P23 in exon-8). While some redesigned proteins displayed reduced stability, several of the redesigns appeared as stable and functional as the original μ Dys in both myogenic cultures and in skeletal muscles of mdx4cv mice following AAV gene therapy. We also evaluated cardiac function of the two leading P17/P19 redesigns by infusing redesigned (R) AAV6-CK8- μ Dys vectors into mdx4cv mice at 2 weeks of age and evaluating them via cardiac magnetic resonance (CMR) and late gadolinium-enhanced (LGE) MR imaging at \sim 1.5 years of age. Ejection fractions (EF) of mdx4cv mice treated with redesigned AAV- μ Dys returned to wildtype levels (wildtype mice = $73.63 \pm 3.33\%$; untreated mdx4cv = $59.07 \pm 3.54\%$; R-P17 treated = $72.24 \pm 6.22\%$; R-P19 treated = $70.66 \pm 5.91\%$). Furthermore, we quantified the fibrosis via the collagen fraction (extracellular volume, ECV) using T1 weighted Gd contrast. Treatment with a combinatory redesigned AAV- μ Dys consisting of P17 redesign#2 + P19 redesign#2 decreased ECV compared to untreated mdx4cv myocardium (0.04 ± 0.03 vs 0.07 ± 0.02) to be closer to wildtype control animals (0.03 ± 0.007). Finally and most importantly, some redesigns for the first two epitopes (P17 and P19) displayed loss of immune recognition when assayed via interferon- γ ELISpot against T-cell lines derived from the patients who developed an immune response against μ Dys in the Mendell et al. clinical trial. Unfortunately, such cells were not available to test the P23 redesigns. These studies demonstrate that multiple dystrophin T cell epitopes can be deimmunized and redesigned without loss of function in both skeletal muscles and the heart. Multiple versions can be developed in parallel for expression, functional & immunological studies. Taken together, our approach could greatly facilitate the clinical application of safer and more efficacious gene therapies for DMD and other muscle disorders.

40. Pretreatment with IVIG Reduces Peripheral Transduction of AAV9 Delivered to the CNS

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Direct administration of AAV vectors to the central nervous system (CNS) has the potential to limit off-target effects in the peripheral nervous system and peripheral organs. However, preclinical studies have shown that AAV9, administered directly to the CNS in nonhuman primates, is present in peripheral organs, especially the liver (Meyer 2015; *Mole Ther*). Additionally, a recent meta-analysis suggests that dorsal root ganglia (DRG) pathology results across a variety of AAV serotypes and transgenes, and following both direct cerebrospinal fluid (CSF) and IV administration (Hordeaux 2020; *HGT*). We evaluated the potential of IV immunoglobulin G (IVIG), a widely available therapy containing antibodies against a broad variety of antigens, including AAV capsids, to limit biodistribution of AAV9 to peripheral organs and DRG following administration to the CSF. Four groups of 5 C57BL/6 mice were administered AAV9 capsid carrying CAG.GFP by either IV (1 group) or intracerebroventricular (ICV) injection (3 groups). Two of the ICV groups were pretreated with IVIG (blood concentration 10 mg/ml) either 24h or 2h prior to the AAV9 vector administration. Across peripheral tissues there was no difference in vector genomes (by droplet digital polymerase chain reaction [ddPCR]) at 14d (necropsy) between animals administered AAV9.CAG.GFP by IV or ICV with no IVIG pretreatment, confirming CNS vector escape. Analyses by immunohistochemistry in liver, kidney, and heart suggest IVIG pretreatment may limit transduction and protein expression. In liver, IVIG pretreatment at both 24h and 2h appeared to decrease AAV9 transduction, and there were minimal GFP+ cells in the IVIG pretreated groups. In kidney we saw significant reduction in transduction and no GFP+ cells in either pretreatment group. In heart, transduction was reduced with 24h IVIG pretreatment and appeared reduced with 2h pretreatment, with no GFP+ cells in either IVIG pretreated group. There was an increase in vector genome copies in the spleens of animals pretreated with IVIG 2h prior to AAV9 administration, but no increase in GFP expression, suggesting that the antibody-bound vector was phagocytosed by splenic cells. In DRG (combined cervical, thoracic, lumbar), there was no significant difference in vector genome transduction or protein expression across groups. These findings suggest that IVIG may reduce AAV9 transduction and protein expression in peripheral organs following direct CNS administration in C57BL/6 mice. Confirmation of these findings may suggest an available means to limit off-target effects in peripheral organs following AAV administration to the CSF.

41. Differential T Cell Immune Responses to Deamidated Adeno-Associated Virus Vector

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Despite the high safety profile demonstrated in clinical trials, the immunogenicity of adeno-associated virus (AAV)-mediated gene therapy remains a major hurdle. Specifically, T cell-mediated immune responses to AAV vectors are related to loss of efficacy and potential liver toxicities. As post-translational modifications in T cell epitopes have the potential to affect immune reactions, the cellular immune responses to peptides derived from spontaneously deamidated AAV were investigated. Here, we report that highly deamidated sites in

AAV9 contain CD4 T cell epitopes with a Th1 cytokine pattern in multiple human donors with diverse HLA backgrounds. Furthermore, some PBMC samples demonstrated differential T cell activation to deamidated or non-deamidated epitopes. Also, *in vitro* and *in silico* HLA binding assays showed differential binding to the deamidated or non-deamidated peptides in some HLA alleles. This study provides critical attributes to vector-immune mediated responses, as AAV deamidation can impact the immunogenicity, safety, and efficacy of AAV-mediated gene therapy in some patients.

42. Interplay Between Plasmacytoid Dendritic Cells and Kupffer Cells in IL-1R1-MyD88 Driven Cellular Immune Responses to Hepatic AAV Gene Transfer

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Immune responses to the viral vector and encoded therapeutic protein can adversely affect the safety and efficacy of gene therapy. Even though adeno associated virus (AAV) vectors are known for their comparatively low immunogenicity, immune responses remain major hurdles to the success of AAV-based gene therapies. Toll like receptor (TLR) 2, 9 and their downstream adaptor molecule: myeloid differentiation primary response 88 (MyD88) have been described as primary innate sensors to the AAV capsid and genome, respectively. We previously demonstrated that TLR9-MyD88 mediated sensing of the AAV genome by plasmacytoid dendritic cells (pDCs) and crosstalk with conventional dendritic cells (cDCs) is essential for priming of capsid-specific CD8⁺ T cells. In contrast, cellular responses to the transgene product in the liver is dependent on IL-1R1-MyD88 signaling, and both cytokines IL-1 α and IL-1 β contribute to these inflammasome independent responses. We have also shown that activation of transgene specific responses take place in the liver and that cross presenting CD11c⁺XCR1⁺ dendritic cells are critical for MHC I antigen presentation. In the present study, we sought to identify the cell types that are critical for IL-1 (α/β) cytokine production and signaling in response to the AAV encoded transgene. We injected C57BL/6-WT mice with hepatotropic AAV8 serotype encoding ovalbumin (AAV8-OVA). Five mice each on days 3, 7, 10 and 14 post injection (PI) were euthanized, and intra-hepatic lymphocytes and splenocytes were analyzed for expression of IL-1R by flow cytometry. Intra-hepatic lymphocytes and splenocytes from naïve mice were used to obtain baseline IL-1R expression. Immunohistochemistry was performed on liver sections to identify cells that produced IL-1 (α/β) cytokine. In comparison to naïve mice, significantly higher number of IL-1R expressing pDCs were observed on day 3 PI in the liver ($p < 0.0001$) and on day 3 and 7 PI in spleen ($p < 0.0001$). Although increased pDC frequencies were observed in both the liver and spleen, intensity of IL-1R expression was higher on hepatic pDCs (peaking on day 3 PI). Significantly increased numbers of IL-1R expressing cDCs were also observed in liver ($p = 0.0023$) and spleen ($p < 0.0001$) on day 3 PI. However, the intensity of IL-1R on cDCs from AAV injected mice was comparable to that of naïve mice. Similarly, increased numbers of IL-1R expressing Kupffer (F4/80⁺) cells were observed on day 3 and

7 PI in liver ($p < 0.0001$ and 0.0021 respectively) and on day 3 PI in spleen ($p < 0.0001$). Similar to liver pDCs, intensity of IL-1R expression on F4/80⁺ liver cells was higher (peaking on day 3 PI) as compared to F4/80⁺ cells from spleen. Immunohistochemical analysis of liver sections from AAV injected mice revealed that PDCA1⁺B220⁺ cells (pDCs) were the chief IL-1 (α/β) cytokine producing cells. Co-staining of IL-1 α and other cellular markers such as F4/80, CD4 and CD8 showed IL-1 producing pDCs in proximity to F4/80⁺, CD4⁺ and CD8⁺ cells. These results suggest an interaction between pDCs and Kupffer cells that might be critical for transgene specific cellular responses. Furthermore, these results indicate that, similar to capsid-specific CD8⁺ T cell responses, pDCs are also central to the activation of cellular responses against the AAV encoded transgene product.

Vector Manufacturing and Engineering 1: Deciphering AAV Vector Genomes

43. Direct ITR-To-ITR Nanopore Sequencing of Plasmid and Vector Preparations and Their Implications for *In Vivo* Gene-Editing Vector Performance

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Many gene therapy concepts and platforms to treat human diseases are moving from *in vitro* and *in vivo* pre-clinical testing to clinical trials. However, establishing vector potencies remains difficult, especially for CRISPR/Cas9 gene therapy platforms that promote gene editing. Specifically, observed differences in indel efficiencies between tissue culture experiments with Cas9 expression plasmids and animal models treated with vectored Cas9 components are not completely understood. Therefore, means of evaluating these inconsistencies are needed. Using third-generation sequencing, we recently demonstrated that certain recombinant adeno-associated virus (rAAV) vector designs carrying sgRNA cassettes can cause heterogeneity in packaged rAAV particles. These high-throughput sequencing platforms have enabled the characterization of mutations, contaminants, truncations, and heterogeneity in vector preparations that can impact editing efficiency. In our recent studies, we screened different dual-guide rAAV-Cas9 vector designs intended to target two regions upstream and downstream of a mutated gene for segmental deletion *in vivo*, and found that one vector design exhibited lowered indel efficiencies. Using long-read sequencing with the Oxford Nanopore Technologies MinION platform, we captured vector genomes and their parental plasmids from inverted terminal repeat (ITR)-to-ITR. Surprisingly, we found that 20% of the full-length vector genomes contained truncations that contained only a single-guide Cas9 (i.e. single indel target region). Importantly, we found that this truncated form was present in 3% of the parental vector plasmid preparation (likely a contaminant during cloning steps) that was not identified by conventional restriction digests and Sanger sequencing. While the importance of parental plasmid

quality is paramount in vector production, the link between plasmid quality and vector heterogeneity to *in vivo* efficiency has not been described previously. In this specific case study, we provide evidence of a low-level contaminant that was preferentially packaged to yield higher proportions within the vector population. As a result, the outcome provides a mechanistic explanation for the compromised indel frequencies observed *in vivo* among the two editing targets. Although the proportion of single-guide versus dual-guide vector population does not directly correspond to the specific indel efficiency at the two target regions, our analysis shows the devastating effect of vector plasmid impurities on vector heterogeneity and potential complications for vector potency. Our results emphasize the need for revamped quality control workflows for both vector plasmids and produced vectors to improve the efficiency of rAAV-based gene therapies. *Co-corresponding authors

44. Physico- and Biochemical Characterization of Linear Covalently Closed dbDNA™ for rAAV Manufacturing

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DNA used for the generation of recombinant Adeno-Associated Virus (rAAV) is a key starting material for successful vector production. Doggybone™ (dbDNA™) is a newly developed technology of DNA, which is synthetically manufactured to produce double stranded linear DNA molecules with covalently closed ends. The molecule is industrially manufactured from small quantities of a precursor plasmid and a specific oligonucleotide. The process entails a series of enzymatic reactions using Phi29 polymerase for rolling cycle amplification, restriction enzymes to break up the backbone, protelomerase TelN from *E. coli* N15 phage for covalent closure of both ends, and exonucleases treatment to remove residual DNA. In addition, clearing of enzymes and residuals of the manufacturing process is achieved using special chromatographic and tangential flow filtration steps. dbDNA™ offers many advantages over plasmid for the manufacturing of rAAV, key among them are quality of the material, purity, fast manufacturing turnaround time, and lower amounts of DNA required for transfection. Besides, dbDNA™ technology can address challenges in DNA manufacture since it provides scalability, the ability to amplify accurately complex sequences like AAV-ITRs and safety, as it does not carry bacterial sequences. To closely monitor the manufacturing process and further characterize the final dbDNA™ product, a series of physico- and biochemical techniques were utilized. Manufacturing internal process controls (IPCs) and final product dbDNA™ from helper, capsid and transgene were analyzed by High Liquid Pressure Chromatography in a UPLC-DAD-TQD equipment with PDA detector at 260nm and Reverse Phase DNAPac RP (100 x 3mm, 4um) column. The chromatogram's profiles of final dbDNA™ product showed symmetric peaks with good resolution and over 95% purity. In all cases relative standard deviation between triplicates of main peak areas were less than 5%. In general, dbDNA™ of molecular weights from 4500 to 9500 bp elute at 4-5 minutes. Individual loading of each reaction component as well as dbDNA™ samples treated with DNase or Proteinase K confirmed the nature of the DNA peaks. This system was capable of separating dbDNA™, different DNA fragments,

residual specific oligonucleotide and Tris. However, enzymes and dNTPs/dNMPs resolved in the solvent front. The feasibility of separating dbDNA™ molecules by Capillary Electrophoresis Laser Induced Fluorescence (CE-LIF) was also studied using a SCIEX PA 800 Plus equipment and a modified dsDNA 1000 Kit. Successful separation was achieved for IPCs and final dbDNA™ product. Repeatability demonstrated migration time variation below 0.7% and purity variation below 1.5%. To further characterized the final dbDNA™ product, we set out to interrogate the Phi29 polymerase error rate during dbDNA™ manufacture using two high-throughput sequencing methods, NGS with the Illumina platform and Nanopore. Helper, capsid and ITR containing transgene dbDNA™ samples were compared to the precursor plasmid template used in the manufacturing process. No indels were observed in dbDNA™ molecules compared to the reference template sequence, which shows the high degree of fidelity of the Phi29, even during the amplification of regions of high complexity such as AAV's ITRs. Finally, Transmission electron cryomicroscopy (CryoTEM) analyses of the final product showed that sample distribution differs depending on the concentration and the thickness of the vitreous ice along the hole of the grid, with molecules at the edges forming big loops which could correspond to dbDNA™. Considering the synthetic origin, the controlled manufacturing process, and the high purity of the product, dbDNA™ is an attractive alternative starting material for rAAV manufacture.

45. Assessment and Comparison of Digital PCR Platforms for AAV Viral Genome Titration

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Adeno-associated virus (AAV) has emerged as the primary vector for *in vivo* gene therapy applications, and there is a need to establish and optimize methods for viral genome quantification of AAVs to support both preclinical and clinical studies. FDA recommendations specify that a titer assay used for clinical dosing should demonstrate a CV at $\leq 15\%$ and accuracy at $\pm 20\%$. Conventional quantitative PCR (qPCR) methods can fall short of these standards, while droplet digital PCR (ddPCR) eliminates the need for a standard curve and conducts an absolute count of viral genome copies. Hence, ddPCR is becoming the gold standard for viral genome quantification. Until recently, ddPCR required longer experiment time and had lower throughput compared to qPCR. However, multiple digital PCR platforms have been developed with varying degrees of automation, throughput and format including nanoplate partitions (replacing droplet-based technology). Here we describe a streamlined workflow that maintains good precision across analysts while reducing sample preparation time. Using this workflow, we evaluated 2 transgenes and 2 AAV serotypes across three digital PCR platforms including Qiagen QIAcuity nanoplate digital PCR (ndPCR), BioRad QX200™ AutoDG™ ddPCR and BioRad QX ONE integrated ddPCR. The sample preparation workflow produced accurate, precise, linear and specific results across both transgenes and serotypes. All platforms exhibited inter-assay and intra-assay precision within the FDA recommendation of 15% for all samples across 2 analysts. Comparable results were obtained between the QX200™ and QX ONE™ ddPCR. These results demonstrate that current QX200™ assays could be effectively bridged to the higher-throughput and fully

integrated QX ONE™ platform in a QC environment. The QIAcuity ndPCR also met precision requirements, however a systematic shift in quantification was observed when compared to the two ddPCR platforms. The QIAcuity ndPCR provides a precise alternative for antiquated qPCR approaches, as it is more affordable and faster than ddPCR technologies. The streamlined sample preparation workflow utilized in this study provided reliable data across multiple transgenes, serotypes and instruments.

46. rAAV Vector Breakpoints Determined Using Single-Molecule, Modified Base Sequencing

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Recombinant adeno-associated virus (rAAV) is an important gene therapy vector due to its low immunogenicity and long track record for use in humans. As with all gene therapy systems, the quality of the material delivered is critical. The presence of empty or partially full capsids is one factor that affects rAAV quality. When rAAV is delivered to cells or analyzed in vitro after capsid disruption, the mixed ssDNA hybridizes to complementary partners, forming heteroduplex double stranded DNA molecules. Standard next generation sequencing (NGS) library preparation methods result in extension of DNA at pre-existing breakpoints, making their identification difficult. When there is no knowledge of breakpoint location, it is challenging to assess vector quality or to fix any sequence-dependent issues. We have developed a NGS library preparation method that allows us to distinguish pre-existing DNA from any DNA added during NGS library preparation. Modified nucleotides are included in the library preparation so that any new DNA includes stretches of modified bases while the pre-existing ssDNA consists of natural, unmodified bases. Using the Sequel II system, the modified bases can be distinguished from unmodified bases, enabling breakpoint identification at high resolution. This method was used on a particular rAAV that was known to break during packaging, a phenomenon that can occur in AAV vectors of various serotypes, allowing us to precisely map the sites that were fracturing and measure the packaged lengths of rAAV ssDNA molecules. These rAAV breakpoint data have allowed us to design better therapeutic vectors and generate more precise quality control data. Partially filled capsids have been an ongoing FDA concern and the new technology provides information that standard methods do not. This use of modified bases for localizing DNA breaks enables improved vector designs and provides better quality metrics for AAV vectors, resulting in higher quality gene therapy vectors.

47. Positioning Short-Hairpin Stopper Sequences Outside of the ITRs Reduces Encapsidation of Non-Vector DNA in rAAV Preparations

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Adeno-associated virus (AAV) vectors have emerged as one of the most attractive gene transfer systems for gene therapy. Nevertheless, AAV manufacturing still faces many challenges, especially for large-scale clinical grade vectors. Among the issues for standard production schemes are the presence of vector impurities. These undesirable impurities come in the form of empty particles, particles with truncated vector genomes, and non-vector DNA in AAV vector preparations. Non-vector DNAs originate from replication-competent (pseudo-wild type) AAVs, host genomes, and prokaryotic DNA sequences from reverse-packaged plasmid backbones. Reverse-packaged DNAs, including antibiotic resistance genes and bacterial origins of replication, are the major non-vector DNA present in preparations. These elements should be eliminated, since they pose risks by potentially evoking immune responses and other untoward effects in patients. Our previous study revealed that nucleotide sequences that form short hairpins hinder AAV genome replication by redirecting polymerase activity during AAV vector manufacturing. We therefore hypothesized that placing short-hairpin DNA sequences (shDNA) flanking outside of the ITRs as “stoppers” can reduce the packaging of non-vector DNAs by suppressing replication read-through beyond the ITR or abolish reverse packaging events. We incorporated shDNAs outside of the ITRs in self-complementary (scAAV) or single-stranded AAV (ssAAV) vector plasmids. We then packaged the scAAV vector with AAV.rh32.33, while the ssAAV vector was packaged with AAV8. Vector purification was performed by two rounds of CsCl gradient ultracentrifugation or by iodixanol gradient, respectively. Digital droplet PCR analysis of the scAAV.rh32.33 vectors showed that the stopper sequence reduced reverse packaging by more than two-fold (from 10.2% reverse-packaged genomes without stopper, to 4.3% with stopper). We also analyzed the vector genomes extracted from purified scAAV.rh32.33 and ssAAV8 vectors by AAVGP-Seq developed by our lab. This sequencing analyses confirmed that the stopper sequences indeed reduced reverse packaged genomes in both scAAV.rh32.33 and ssAAV8 vectors by two-fold. In summary, incorporation of shDNAs beyond the ITRs can reduce the frequency of reverse packaging in both sc- and ssAAV vectors independent of vector purification methods and serotypes. Optimization of the shDNA may further reduce the encapsulation of non-vector DNA in rAAV preparations. (*Co-corresponding authors)

48. Characterization of Residual DNA in rAAV Products Made in the Baculovirus/Sf9 Platform

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Recombinant Adeno-associated virus (rAAV) vector preparations contain large amounts of residual DNA in comparison to other protein-based biologicals. Most of the residual DNA is inside AAV capsids, which results in copurification with the rAAV product. Here,

we describe some of the characteristics of residual DNA in rAAV vectors made in the baculovirus/Sf9 platform as analyzed by southern blot and digital droplet PCR (ddPCR). The main findings are: 1) All or most of the residual DNA present in purified rAAV preparations is encapsidated; 2) Residual DNA fragments cover a wide range of sizes from approximately 4,700 nucleotides to less than 1,000 nucleotides; 3) Residual DNA is present inside AAV capsids as single-stranded DNA (ssDNA); 4) Encapsidation of residual DNA is Rep-dependent; 5) Residual DNA derived from the baculovirus regions adjacent to the ITRs is over-represented and corresponds mostly to the strand with 3' end pointing towards the ITR. Baculovirus and host cell genome sequences are present in residual DNA, but at different ratios than in the cell substrate. Estimation of the total amount of baculovirus and host cell residual DNA requires understanding the relative abundance of different regions, which can be achieved by ddPCR quantification of multiple markers (high resolution) or by next-generation sequencing (higher resolution).

49. NGS Based Evaluation of AAV Genome Integrity for Improved Production and Function

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Adeno-associated virus has become a popular tool for gene therapy due to its ability to safely and efficiently deliver genetic elements to multiple tissue types *in vivo*. Genetic elements with a total size within ~4.7 kilobases (kb) can be assembled between two ITRs, packaged into AAV particles, and be used to deliver expression cassettes, ultimately in the form of episomal DNA circles that do not integrate into the host genome, to animal and human tissues. Production of complete AAV particles can be challenging and result in a heterogeneous mixture of capsids with complete and incomplete genomes, which can increase the amount of virus needed to achieve the desired GOI expression *in vivo*. This increase can make manufacturing AAV difficult, affect potency, and result in increased toxicity due to larger dosing requirements. Taken together, achievement of reliable methods for evaluation of integrity of AAV genome packaging remains a challenge. Here we sought to develop a system to 1) identify the truncated genomes that result from incomplete AAV genome packaging, 2) identify genetic elements that might compromise the full AAV genome packaging, and 3) augment the AAV genome to provide optimal functionality and packaging of genetic elements. To this end, we have established a pipeline where AAV genomes are extracted, subjected to size analysis by agarose gel electrophoresis, and analyzed by multiple Next-Generation Sequencing (NGS) platforms. Consolidation of these data allows a more complete picture of the packaged AAV genome and provides the groundwork for studies aimed at augmenting AAV genomes for manufacturing and robust gene expression. For this pipeline generation we manufactured AAV particles containing AAV genomes of varying promoters and organization of components. These genomes also contained different combinations of promoter and transcription termination signals in an effort to analyze the influences of size and sequence of the AAV genome. AAV genomes were first extracted and resolved on an alkaline

agarose gel. AAV particles packaging constructs closer to the packaging limit formed full-length ssAAV genomes, but many also contained truncated genomes. Given this variation we sought to characterize the AAV genomes by NGS to gain a more mechanistic understanding of AAV packaging at the sequence level. AAV genomes can be sequenced by various techniques, each with advantages and pitfalls. For these analyses we chose to analyze the AAV genomes using a combination of Illumina and Nanopore sequencing, thus combining short- and long- read sequencing approaches. Illumina sequencing uncovered that there is preference for reads near the 5' ITR and promoter regions when using longer, more complex promoters. Further analysis of the reads mapping to these regions also uncovered likely hairpin formation at these sites. Indeed, these analyses provided a nucleotide resolution of alternative AAV truncation sites and provided a framework to test for problematic sequences and/or position of elements in AAV genomes. To test possible mechanisms of AAV replication termination during packaging, we are testing alternative designs of AAV genomes. One approach has been to rearrange the genetic elements to test if position elements influence the AAV termination. Alternatively, sequences have been removed or augmented to reduce the capacity for hairpin formation. This data can be utilized to enhance titer and potency to select optimal construction design. Taken together, we have developed a pipeline for the analysis of AAV genomes following AAV packaging that will guide future clinical AAV therapeutics.

Gene and Cell Therapy Trials in Progress

50. High Anti-Sickling Potency of a Gamma Globin in the Phase 1/2 MOMENTUM Study of ARU-1801 Gene Therapy and Reduced Intensity Conditioning for Sickle Cell Disease

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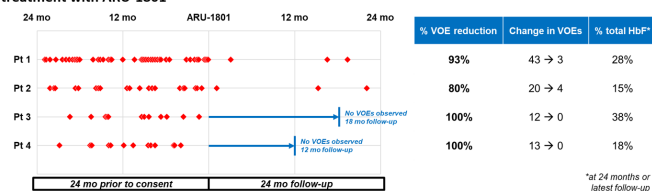
Introduction: Sickle cell disease (SCD) is a genetic red blood cell (RBC) disorder that causes hemolytic anemia, painful vaso-occlusive crises, and life-threatening complications. Myeloablative allogeneic hematopoietic stem cell transplant (allo-HSCT) remains the only curative therapy for SCD, but has several limitations, including lack of donors, conditioning-related toxicities, and risk of graft-versus-host disease (GVHD). ARU-1801 is an autologous lentiviral gene therapy that utilizes reduced-intensity conditioning (RIC), designed to address these limitations and widen access for SCD patients. Updated data on an ongoing Phase 1/2 study (NCT02186418) are presented here. **Methods:** Adults (18-45 years old) with severe SCD (as defined by recurrent vaso-occlusive events [VOE] and acute chest syndrome) were screened for eligibility. Prior to infusion of ARU-1801, all patients

received a single intravenous dose of RIC melphalan (140 mg/m²). Endpoints included measures of safety, engraftment, vector copy number (VCN), hemoglobin, and frequency of VOs. Patients were weaned off transfusions 3-6 months after drug product (DP) infusion. Outcomes are reported at 24 months or latest follow-up for each patient.

Results: As of 1 Jan 2022, four patients (mean age [range], 26 [19-34] years old) have been treated and followed for ≥12 months post-transplant. Transient neutropenia and thrombocytopenia lasted a median of 7 days. There have been no other serious adverse events related to chemotherapy or ARU-1801 to date. Marked improvements in SCD manifestations include 80-93% reduction in annualized VOs in the first two patients and complete absence of VOs (100% reduction) in the next two patients to date. Preclinical studies suggest HbF^{G16D} may have superior anti-sickling potency compared to wild-type HbF. Clinically, patients in this study expressed a mean 42% HbF^{G16D} per DP VCN, which appears more efficient at expressing anti-sickling globin (ASG) than other lentiviral vectors used for gene therapy, suggesting ARU-1801 may reach effective levels of HbF at relatively lower VCN. Combined, efficacy at low VCN and RIC may increase the safety profile of ARU-1801 over other SCD gene therapies. Furthermore, ARU-1801 appears to be a more potent ASG. At screening, Patient 4 had recurrent VOs despite 16% HbF and 20% F cells; but, at 12 months following ARU-1801 gene therapy, had complete absence of VOs with 18% HbF (15% HbF^{G16D}) and 68% F-cells. Furthermore, Patient 4 showed a similar level of remarkable improvement in RBC sickling kinetics by oxygen gradient ektacytometry (Lorrca®) as seen in Patient 3 with 38% HbF.

Conclusion: ARU-1801 delivers a potent anti-sickling HbF^{G16D} with RIC, making it a promising gene therapy alternative to therapies that require myeloablative conditioning and offering amelioration of SCD symptoms without the toxicities and resources associated with full myeloablation.

Figure 1. Reduction in VOs from 24 months prior to consent to 24 months (or latest follow-up) after treatment with ARU-1801



51. Safety and Outcomes of Intravenous scAAV9.U7-ACCA for the Treatment of Duchenne Muscular Dystrophy Caused by Exon 2 Duplications

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Duplication of exon 2 in the *DMD* gene is the most common duplication causing Duchenne muscular dystrophy and is a promising candidate for exon skipping therapy as skipping the single exon copy should result in full length dystrophin expression. We previously reported the early safety and efficacy of the therapeutic exon skipping viral 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. Enrollment is now complete with three subjects (9 years, 13.7 years and 7 months) receiving a dose of 3.0x10¹³ vg/kg intravenously without incident - no SAEs and no biochemical measures of toxicity. Through 18 months, Subject 1 continues to do well. His creatine kinase level (CK) remains improved (4, 237 IU; 31% of baseline); his 100M % predicted walk time was stable from baseline; his NSAA has dropped from 25 to 23 due to increasing heel cord tightness and his time to climb 4 stairs has increased from 2.17s to 2.4s. Pulmonary and cardiac function remain normal. Through 12 months, subject 2 reports clinical stability and his CK remains improved (1,458 IU; 29% of baseline); his 100M % predicted walk time was slightly increased (28.3 to 25%); his NSAA dropped from 22 to 20 and his time to climb 4 stairs was stable at 4.1s. Pulmonary and cardiac function remain normal. Subject 3 was dosed prior to symptom onset at 7 months of age. Through 60 days he is doing well. His CK has dropped significantly (1,163 IU; 9% of baseline) and he is achieving developmental milestones on time. Muscle biopsy of the first two subjects through 12 and 6 months respectively shows expression of apparent full-length dystrophin protein, quantified by Western blot at levels of ~6% in the younger (9.0 years) subject and BLOQ/~1-2% in the older (13.7 years) subject, possibly reflecting differences in myofiber transfection due to differing degrees of dystrophic skeletal muscle changes. These findings represent the continued therapeutic expression of apparent full-length dystrophin in subject 1, continued safety through up to 18 months post gene transfer, and the first evidence of safety for early systemic viral vector delivery in an infant with DMD.

52. RGX-121 Gene Therapy for the Treatment of Severe 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 in tissues. Severe 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. In this phase 1/2, first-in-human, multicenter, open-label, dose escalation trial (NCT03566043), participants with severe MPS II ages 4 months to 5 years receive one image-guided RGX-121 injection to the CNS with follow-up for safety, tolerability, and efficacy for 104 weeks. Assessments include CSF, plasma and urine biomarkers; cognition, language, and motor neurodevelopmental scales; and imaging. Nine participants have been enrolled in 3 dose cohorts (1.3×10^{10} , 6.5×10^{10} , and 2.0×10^{11} genome copies/gram brain mass) as of April 25, 2021. As of April 25, 2021, 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 two years. Heparan sulfate CSF levels, which are elevated in neuronopathic MPS II disease, showed consistent reductions, and interim neurodevelopmental testing demonstrated ongoing skill acquisition in multiple domains. Plasma I2S enzyme expression and total urine GAGs suggested emerging evidence of systemic RGX-121 activity. Updated interim results from this clinical trial will be presented. RGX-121 has the potential to provide sustained CNS clinical outcomes and additional systemic effects in MPS II patients.

53. Up to 10.5 Years of Follow-Up in 17 Subjects Treated with Hematopoietic Stem and Progenitor Cell Lentiviral Gene Therapy for Wiskott-Aldrich Syndrome

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Background: Wiskott-Aldrich Syndrome (WAS) is a rare, X-linked, life-threatening primary immunodeficiency and platelet disorder caused by WAS protein (WASP)-encoding gene mutations. WAS is characterized by recurrent or severe infections, microthrombocytopenia, eczema, and an increased risk of autoimmunity and malignancy. WAS can be treated with allogeneic hematopoietic stem cell transplantation (HSCT); however, a suitable donor is not always available and associated complications may include graft-versus-host disease, rejection, and toxicity from myeloablative conditioning. Also, HSCT carries higher risks in children ≥ 5 years of age (Moratto, 2011; Burroughs, 2020). Gene therapy (GT) is currently being studied as an alternative treatment option. OTL-103 is an investigational autologous hematopoietic stem and progenitor cell (HSPC) GT composed of CD34⁺ HSPCs transduced *ex vivo* with a self-inactivating lentiviral vector encoding human WAS cDNA under the control of an endogenous human WAS promoter. Here we report updated safety and efficacy results of 17 male subjects treated with OTL-103 as part of a Phase I/II trial or Expanded Access Program (EAP) with up to 10.5 years of follow-up. **Methods:** All subjects received rituximab and reduced-intensity conditioning with busulfan and fludarabine pre-GT. Patient demographics and characteristics are included in the table. **Results:** At time of analysis, median follow-up was 8.4 years for Trial (range: 5.2 - 10.5) and 3.3 years for EAP (range: 0.4 - 4.9). All subjects were alive except one EAP subject who died 4.5 months post-GT due to deterioration of a pre-existing neurological condition considered unrelated to OTL-103 by Investigator. To date, there have been no reports of OTL-103-related adverse events (AEs), serious AEs, or signs of insertional mutagenesis or replication-competent lentivirus. Sustained engraftment of genetically modified HSPCs resulted in WASP expression restoration in lymphocytes and platelets. A reduction in severe infection rate was observed and all evaluable subjects stopped immunoglobulin replacement therapy and prophylactic antimicrobials, suggesting immune reconstitution and T-cell function normalization. A reduction in bleeding rate and severity was observed with no severe events >6 months post-GT. All evaluable subjects achieved platelet transfusion independence and median platelet counts improved from baseline (see Table). Eczema resolved in all evaluable subjects. Similar clinical benefit was seen in subjects ≥ 5 years of age.

Conclusions: This safety and efficacy analysis of subjects treated with OTL-103 demonstrates the potential of GT as an effective treatment for patients with WAS, including in those ≥ 5 years.

Table

Study	NCT01515462 (Trial) (n=8)	EAP (n=9)
Median age at GT, years (range)	2.2 (1.1–12.4)	3.8 (1.4–35.1)
Subjects ≥ 5 years of age at GT, n (%)	3 (37.5)	4* (44.4)
HSPC source	BM (%)	5 (62.5)
	mPB (%)	2 (25)
	Both (%)	1 (12.5)
Severe infection events per PYO (12 months pre-GT) (95% CI)	2.1 (1.2–3.4)	1.4 (0.8–2.5)
Severe infection events per PYO (>6 months post-GT**) (95% CI)	0.1 (0.1–0.3)	0.1 (0.0–0.4)
Median time to IgRT cessation, days post-GT (range)	577 (307–1843)	159 (91–503)
Moderate and severe bleeding events per PYO (12 months pre-GT) (95% CI)	3.4 (2.2–4.9)	1.9 (1.1–3.0)
Moderate and severe bleeding events per PYO (>6 months post-GT**) (95% CI)	0.1 (0.0–0.2)	0.1 (0.0–0.4)
Median time to PLT transfusion independence, days post-GT (range)	85.5 (15–261)	20 (9–68)
Median PLT count at baseline, $\times 10^9/L$ (range)	11.5 (5.5–21.0)	21.3 (8.0–142.0)
Median PLT count post-GT, $\times 10^9/L$ (range)	67.5 (32.0–84.0) (n=4, Year 8)	54.5 (30.5–227.0) (n=4, Year 3)

BM = bone marrow; CI = confidence interval; EAP = Expanded Access Program; GT = gene therapy; HSPC = hematopoietic stem and progenitor cell; IgRT = immunoglobulin replacement therapy; mPB = mobilized peripheral blood; PLT = platelet; PYO, person-years of observation.

*2 subjects were adults and splenectomized

**events occurring more than 6 months after OTL-103 infusion (up to last follow-up)

54. Anti-GD2 CAR NKT Cells Are Safe and Produce Antitumor Responses in Patients with Relapsed/Refractory Neuroblastoma

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Background: T cells expressing chimeric antigen receptors (CARs) remain largely ineffective in solid tumors. Va24-invariant natural killer T cells (NKTs) are an innate-like T cell subset with natural antitumor properties. NKTs expressing a GD2-CAR and IL-15 showed potent antitumor activity in neuroblastoma (NB) mouse models, justifying evaluation of CAR-NKTs in NB patients. **Methods:** Our phase 1 clinical trial aims to assess safety, persistence, and efficacy of autologous CAR-NKTs in patients with relapsed/refractory NB (NCT03294954) using 3+3 dose-escalation schema. Twelve patients have been infused to date on four dose levels (DL; CAR+ NKTs/m²): DL1 (3×10^6), DL2 (1×10^7), DL3 (3×10^7), and DL4 (1×10^8) following lymphodepletion. Antitumor responses were evaluated using 3D imaging and quantified by changes in Curie scores. CAR-NKT frequency in peripheral blood was measured by flow cytometry and qPCR. To estimate *in vivo* CAR-NKT dose, we calculated area under the curve (AUC) for CAR-NKT frequency in peripheral blood over a four-week period post-infusion. **Results:** Median age was 7.6 years (range 2–12 and patient-derived NKTs were expanded to produce $\geq 10^9$ CAR-NKTs with 93.1% median NKT purity (range 74.1–97.2%) and 60.1% median CAR expression (range 20.2–87.7%). No dose limiting toxicities or grade 2+ toxicities related to CAR-NKTs were observed. CAR-NKTs expanded in all patients, peaking two-to-four weeks post-infusion. Five patients progressed, four had stable disease (SD), two had a partial response (PR), and one achieved complete response (CR, Fig 1). Patients were divided into non-responders (7) and responders

(5; including 3 PR/CR and 2 SD, 1 with 30% Curie score reduction and 1 with clearance of bone marrow metastases). Responders had higher CAR-NKT AUC than non-responders (911 vs 261.3, $p=0.032$, Fig 2A), CD62L+ NKT frequency in infused products correlated with AUC ($R^2=0.61$, $p=0.003$, Fig 2B) and was higher in responders than non-responders (71% vs 35.3%, $p=0.002$, Fig 2C). Transcription factor LEF1 was the top overexpressed gene in CD62L+ vs CD62L-NKTs; loss-of-function experiments showed that LEF1 is required for central memory differentiation in NKTs. NKTs co-expressing a GD2-CAR and LEF1 resisted exhaustion during *in vitro* serial tumor challenge and had superior antitumor activity in a xenogeneic NB model vs control GD2-CAR NKTs. **Conclusions:** CAR-NKTs are safe and can expand post-transfer, producing objective responses in NB patients. CAR-NKT antitumor activity is associated with *in vivo* rate of expansion and CD62L+ frequency in infused products. LEF1 maintains central memory-like differentiation in CD62L+ NKTs, and transgenic overexpression of LEF1 helps CAR-NKTs resist exhaustion and boosts therapeutic efficacy in a NB model.

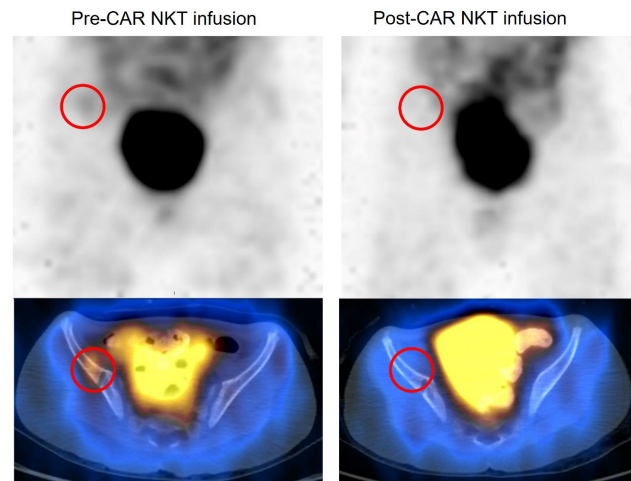


Figure 1. Partial response in patient #12: Pre- and post-GD2-CAR NKT infusion images from MIBG scans. Planar images (top row) and fused SPECT/CT images (bottom row) visualize pelvic bone metastasis (red circle) visible prior to infusion (left column) and complete resolution after infusion (right column). MIBG avid areas mid-image correspond to bladder with MIBG-containing urine.

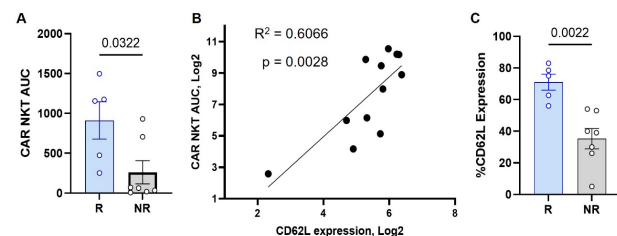


Figure 2. GD2-CAR NKT expansion and antitumor activity in patients. A) CAR-NKT expansion represented by area under the curve (AUC) in responders and non-responders. B) Correlation of CD62L+ CAR-NKT frequency in infusion products with CAR-NKT expansion using regression analysis. C) CD62L+ CAR-NKT frequency in indicated patient groups.

55. Exploratory Immuno-Safety Profile of EDIT-101, a First-in-Human *In Vivo* CRISPR Gene Editing Therapy for *CEP290*-Related Retinal Degeneration

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Mutations in the *CEP290* gene cause severe early onset retinal degeneration, often with severe visual impairment from early childhood. The most common mutation in *CEP290* is c.2991+1655A>G located within intron 26 (*CEP290-IVS26*). To treat retinal degeneration due to this mutation, we developed EDIT-101, an adeno-associated virus type 5 (AAV5) encoding *Staphylococcus aureus* Cas9 (SaCas9) expressed under the photoreceptor-specific *GRK1* promoter and two guide RNAs designed to excise this disease-causing mutation. Safety and tolerability of EDIT-101 is being evaluated in the ongoing BRILLIANCE (NCT03872479) trial, an open-label, Phase 1/2 single ascending dose study enrolling adults and children. Key exploratory safety endpoints include assessing humoral and cell-mediated immunogenicity generated towards the AAV5 capsid and the expressed SaCas9 component of EDIT-101. To understand the immuno-safety profile of EDIT-101 in treated subjects we developed and validated three assays. First, an IFN- γ Enzyme Linked Immuno Spot (ELISpot) assay to detect cell-mediated immune responses towards the AAV5 capsid and SaCas9 from isolated peripheral blood mononuclear cells. Second, an electrochemiluminescence immunoassay to detect binding antibodies (BABs) to AAV5 and SaCas9 in serum. Finally, a cell-based virus neutralization assay to detect AAV5 neutralizing antibodies (nAbs) in subject plasma. These validated assays were used to test the available baseline and post-treatment samples from subjects in the adult low (2 subjects, 6×10^{11} vg/mL), medium (5 subjects, 1.1×10^{12} vg/mL), and high (1 subject, 3×10^{12} vg/mL) dose cohorts in the BRILLIANCE trial. The one subject in the high dose cohort had detectable pre-existing SaCas9 cell-mediated immunity. No subjects developed consistent anti-SaCas9 cell-mediated responses post-dosing. There were no detectable BABs against SaCas9 in any of the cohorts pre- or post-dosing. Pre-existing cell-mediated AAV5 immune responses were detected in two subjects in the mid-dose cohort and one subject each within the low-dose and high dose cohorts. These responses continued to be detected post-treatment. AAV5 naïve patients did not develop consistent cell-mediated responses post-treatment. The presence of BABs to AAV5 correlated with detection of AAV5 nAbs in all subjects. Only one of eight subjects had detectable pre-existing AAV5 nAbs. After dosing, four subjects developed AAV5 nAb with titers plateauing around week six. The nAb titers in some subjects began to decline by three months post-treatment. The anti-AAV5 antibodies detected in either pre- or post-treated subject samples had no correlation with detected cell-mediated AAV5 responses. We report development of immunogenicity assays to evaluate humoral and cell-mediated responses to AAV5 and SaCas9 in multiple cohorts

dosed with EDIT-101. Despite ubiquitous presence of *S. aureus* as a pathogenic and commensal bacterium in humans, sub-retinal injection of EDIT-101 did not induce an immune response directed towards SaCas9. No subjects developed consistent cell-mediated responses to SaCas9 or AAV5, but multiple had pre-existing immunity that persisted after treatment. Pre-existing humoral or cell-mediated immunity in subjects did not lead to immune-related adverse events, which is encouraging for subsequent treatment of the contralateral eye. Our data suggests that EDIT-101 has a favorable immunogenic profile and no adverse events have been attributable to immunogenic responses in subjects treated in the BRILLIANCE trial.

56. Hematopoietic Stem Cell Gene Therapy for Cystinosis: Updated Results from a Phase 1/2 Clinical Trial

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Cystinosis is a lysosomal storage disorder characterized by cystine accumulation within the lysosomes of all organs and 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, eye pathology that can lead to blindness, but also impairment of the heart, thyroid, skeletal muscle, pancreas, and central nervous system, eventually causing premature death in early adulthood. Cysteamine, the FDA-approved drug to treat cystinosis, delays, but does not stop, the progression of the disease. Here we report the results from the phase 1/2 open-label clinical trial (NCT03897361) evaluating the safety and efficacy of CTNS-RD-04, consisting of autologous CD34⁺ cells transduced with a lentiviral vector (LV) carrying the *CTNS* cDNA encoding for cystinosin (CCL-EFS-CTNS-WPRE) in cystinosis. Peripheral blood CD34⁺ HSPCs are collected via apheresis after mobilization with granulocyte colony stimulating factor 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 cysteamine and cysteamine eye drops are withdrawn prior to infusion. Four patients (ages 20 to 46 years) have been treated with CTNS-RD-04 so far with follow-up ranging from 1-month to 27 months. CTNS-RD-04 cell doses ranged from 3.63×10^6 to 9.59×10^6 CD34⁺ cells/kg with VCNs ranging from 0.59 to 2.1 copies/dg. No adverse events related to the drug product and no serious adverse events have been reported to date. In the three infused patients with follow-up ranging from 12-months to 26 months, polyclonal hematopoietic reconstitution occurred in all infused patients. Peripheral blood VCN at 12 months post-gene therapy ranged between 1.03 to 2.59. White blood cell cystine was decreased in these three treated patients as well as tissue cystine crystals in the skin and rectal mucosa. The four patients are no longer taking oral cysteamine. Patient 2 has elected to restart eyedrop cysteamine after a year post-infusion. Updated data will be presented for the four patients infused to date.

AAV Preclinical CNS Gene Therapy

57. Selection of Clinical Doses for SBT101, an AAV9-hABCD1 Vector for the Treatment of Adrenomyeloneuropathy

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X-linked adrenoleukodystrophy (ALD) is an inherited neurodegenerative disease caused by pathogenic variants in the *ABCD1* gene which encodes a peroxisomal transporter for very long-chain fatty acids (VLCFA). Pathogenic variants lead to the accumulation of VLCFA in plasma and tissues, including adrenal glands, testes, and the central nervous system. Adrenomyeloneuropathy (AMN), the adult-onset form of ALD, is characterized by a slowly progressive spinal cord disease leading to loss of mobility, incontinence, and debilitating pain. AMN is the most frequent clinical manifestation of ALD affecting virtually all adult males and >80% of females. So far there is no therapy available for AMN, leaving the patients with progressive neurodegeneration and lifelong disability. We are developing SBT101, an adeno-associated virus serotype 9 (AAV9)-based gene therapy capable of delivering a functional copy of the human *ABCD1* (*hABCD1*) gene, as a treatment for AMN. Selection of doses for a planned Phase 1/2 study was made based on safety, efficacy, and biodistribution data from preclinical studies in rodents and non-human primates (NHPs). In an 8week dose-finding study, there was a dosedependent increase in *hABCD1* protein levels in the spinal cords of all *Abcd1*^{-/-} mice following intrathecal (IT) administration of SBT101, and mice receiving 2.0E10 vector genomes/animal (vg/an) had a statistically significant improvement in mtDNA compared to untreated littermate controls. In a second mouse study there was an increased grip strength at 7-8 months post administration of SBT101 to *Abcd1*^{-/-}/*Abcd2*^{-/-} mice at 3.3E10 and 3.3E11 vg/an when compared to control mice administered AAV9-Null. The increased grip strength equated that of wild-type mice and occurred together with a dose dependent decrease of VLCFA compared to *Abcd1*^{-/-}/*Abcd2*^{-/-} animals administered AAV9Null. The lowest dose of 3.3E10 vg/an produced both a functional improvement and reduction in VLCFA. Taken together, the minimum effective dose in mice was considered to be 2.0E10 vg/an, translating to a human dose of 7.5E13 vg/person, based on relative volumes of CSF between species. In biodistribution studies in NHPs, IT infusions of 1.1E13 and 3.4E13 vg/an of rAAV9-CBA-GFP-WPRE in cynomolgus monkeys resulted in expression of the reporter gene in 25% to 100% of spinal cord and dorsal root ganglia (DRG) neurons as measured by immunohistochemistry. Safety of SBT101 was demonstrated at both similar and higher doses in NHPs scaled from mouse doses. One-time IT administration up to

doses of 7.6E13 vg/an (highest dose tested, corresponding to human doses up to 8.8E14 vg/person) was well tolerated by NHPs. Based on efficacy, biodistribution, and safety data and taking into consideration differences in CSF volumes across species, a first in human dose range of SBT101 was calculated for patients to be 1.0E14 and 3.0E14 vg/person. This range falls within doses that have been shown to be associated with activity and efficacy in mice. These levels also translate into doses that efficiently transduce the spinal cord and DRG neurons in NHPs at >25% and have shown safety in both species with a safety margin for the high dose of approximately 2.75fold.

58. 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 treated at different neuropathologic stages, and meaningful *ARSA* expression and biodistribution in non-human primates (NHPs). We demonstrate that AAV.SAN002-hARSA mediated gene replacement in MLD mice (*Arsa*^{-/-}) at late (13mo), early (6mo), and pre (2mo) neuropathologic stages resulted in the reversal of MLD-associated pathology. In contrast to vehicle treated MLD mice, AAV.SAN002-hARSA treated mice demonstrated 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 exhibited prominent *hARSA* expression, secretion, and uptake by non-transduced cells (cross-correction) leading to improved myelination in the corpus callosum, recovery in brain oligodendrocyte cell numbers (snRNAseq), and normalization of plasma Nf-L levels. We also report a NHP study evaluating *hARSA* expression and biodistribution. Here, juvenile cynomolgus monkeys received AAV.

SAN002-hARSA at a low and high doses. Broad and dose-dependent supra-physiological sulfatase activity was noted in NHP brain. AAV.SAN002-hARSA treatment also resulted in significantly increased sulfatase activity in the spinal cord. Furthermore, hARSA in situ hybridization and immunohistochemistry confirmed broad biodistribution and robust transgene expression in NHP brain and spinal cord. Our results indicate that AAV.SAN002-hARSA mediated gene replacement is a viable approach to achieve broad and therapeutic levels of ARSA in the CNS and PNS.

59. Three Examples of Long-Term AIDS-Virus Suppression Using AAV-Delivered Monoclonal Antibodies

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Background: Long-term delivery of anti-HIV monoclonal antibodies using adeno-associated virus (AAV) holds promise for the treatment of HIV infection. We have previously reported monkey rh2438 in which a single administration of AAVs encoding a combination of potent and broadly neutralizing antibodies during the chronic phase of infection resulted in an abrupt decline in plasma viremia which remained below the limit of detection for 38 successive measurements over a 3-year period. The field has nicknamed this monkey “the Miami monkey” analogous to “the Berlin patient”, a person that was cured of his HIV infection. This monkey never received antiviral drugs at any time and therefore appears to have been functionally cured. **Methods:** Indian-origin rhesus macaques housed at the Wisconsin National Primate Research Center were used for our studies. Monkeys received recombinant AAV vectors expressing full length IgG1 versions of the monoclonal antibodies. Rhesus monkeys were infected with SHIV-AD8 for months before receiving AAV expressing constant-region *rhesusized* versions of select anti-HIV monoclonal antibodies. Antibody and anti-drug antibody (ADA) levels were measured by ELISA. **Results:** Here we report that monkey rh2438 continues to be suppressed for over 6 years and continues to express high levels of antibodies 10-1074 and 3BNC117 in serum (approx. 50-150 µg/ml). Monkey rh2438 generated little or no ADA responses to these antibodies. We have two additional macaques, r14097 and r14121, which also received AAVs coding for a cocktail of neutralizing anti-HIV antibodies during the chronic phase of infection and have shown suppressed viral loads for over 3 years. Monkey r14097 showed sustained delivery of antibodies PGT128 (approx. 20 µg/ml) and N6 (5-10 µg/ml) and a late rise of antibodies 35O22 (to >5 µg/ml) and PGT145 (to approx. 1 µg/ml). Monkey r14121 showed sustained delivery of antibody PGT128 (approx. 10 µg/ml) and a late rise of antibody N6 (to approx. 2 µg/ml). **Conclusions:** Our data show that durable, continuous antibody expression can be

achieved with AAV (in the absence of strong ADA responses) and support the potential for lifelong suppression of viral loads with the AAV-antibody approach.

60. AAV-Mediated Delivery of Anti-HIV Antibodies to the CNS

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Background: Intramuscular inoculation of recombinant adeno-associated virus (AAV) vectors encoding potent and broadly neutralizing antibodies has the potential to strongly and durably suppress HIV replication in infected individuals. However, circulating antibodies do not efficiently reach the CNS. Additionally, AAV delivery can be hampered by the generation of anti-drug antibody (ADA) responses to the delivered antibody which can severely limit levels and efficacy. We performed intrathecal delivery of AAV to test whether the immunoprivileged status of the CNS can prevent the generation of ADAs. **Methods:** Three SHIV-infected rhesus macaques received intrathecal inoculation of recombinant AAV9 vectors with CMV-promoter driven expression of anti-HIV antibodies 3BNC117 and 10-1074 at week 8 post-infection. Antibody levels and ADAs were measured by ELISA in cerebrospinal fluid (CSF) and serum. **Results:** Both 3BNC117 and 10-1074 were detected in serum of all three monkeys in the initial weeks following AAV-mediated delivery. 3BNC117 was detected in the CSF of all three monkeys in the initial weeks following AAV-mediated delivery. 10-1074 was also detected in the CSF of two of the three monkeys in the initial weeks following the AAV-mediated delivery. Two of the three monkeys had early, strong ADA responses to both of the antibodies and this correlated with rapid declines in the levels of both antibodies in both serum and CSF to below the limit of detection by 4-6 weeks post AAV administration. One of the monkeys had delayed ADA responses to both antibodies and this correlated with delayed declines in the levels of both antibodies in both serum and CSF. **Conclusions:** To the best of our knowledge, this was the first time that AAV-mediated delivery of antibodies in the CNS has been attempted. While the ADA problem was not solved in our experimental conditions, our data show AAV-delivered antibodies were detectable in CSF with the delivery method used. Eradicating or minimizing ADA responses is crucial to make the AAV-delivery of antibodies a consistent and reliable approach against HIV.

61. Antibody Gene Therapy for Rabies Encephalitis

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Rabies kills more than 60,000 people annually, mostly children in 68 countries and there is no reliable treatment to save them. Rabies causes a rapidly progressive encephalitis that is almost universally fatal once infection is established. Recombinant adeno associated viral (AAV) vectors that cross the blood brain barrier are being used to treat human neurodegenerative diseases and can deliver antibodies to the nervous system, providing an opportunity to treat neuropathogenic infections such as rabies. An AAV9 vector expressing a broadly neutralizing human anti-rabies antibody against rabies G glycoprotein (AAV-RAB) was evaluated in mice and cats for rabies neutralizing antibody production following a single intravenous administration as a possible modality for human antibody gene therapy for rabies. Female C57BL/6J mice were treated intravenously (IV) with doses ranging from 1×10^{10} to 1×10^{13} vg/kg for a dose escalation study and 6×10^{13} vg/kg for a longitudinal study. Two weeks after treatment, animals treated with 1×10^{11} , 1×10^{12} , and 6×10^{13} vg/kg had serum rabies neutralizing titers well above the protective level of 0.5 IU/mL, ranging from 54.8 to $>1,519$ IU/mL. Neutralizing antibodies above protective titers persisted in sera and brain for at least 60 days following treatment. Sera of mice dosed with 1×10^{10} vg/kg were negative for neutralizing antibodies. Antibody was expressed broadly in neurons and brain endothelial cells as shown by immunohistochemistry (Fig 1). Cats were treated via IV administration of either 2.0×10^{12} or 1.0×10^{13} vg/kg AAV-RAB ($n=3$ each) at approximately 4 months of age. Both low dose and high dose cohorts expressed therapeutic neutralizing antibodies in the sera two weeks following treatment. Animals treated with the high dose displayed neutralizing antibodies in the CSF 1 month post treatment. One animal in the high dose cohort show persistent expression in both sera and CSF at 17 months following treatment. These preclinical studies support the use of antibody gene therapy for rabies encephalitis.

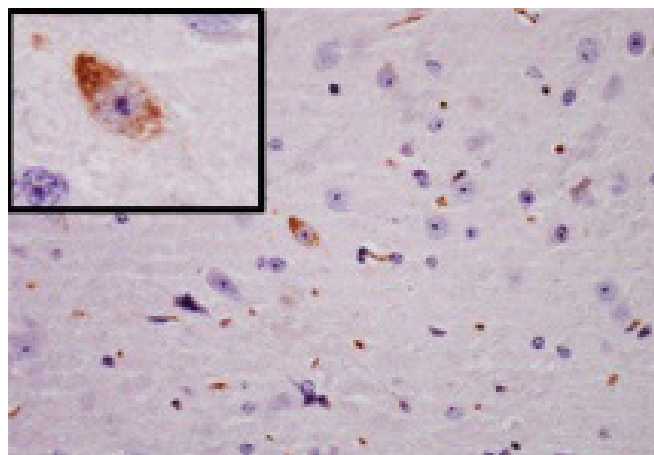


Figure 1. Immunohistochemistry of mouse brain. Rabies antibody expression in neurons and glia (brown stain) after systemic injection of AAV-RAB. Biotin-SP-conjugated AffiniPure Goat Anti-Human IgG, Fcγ Fragment Specific (Jackson ImmunoResearch). (Magnification 100X, inset 214X).

62. Development of an Intrathecal AAV9/AP4M1 Gene Therapy for Hereditary Spastic Paraplegia 50 (SPG50)

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Hereditary spastic paraplegia type 50 (SPG50) is an ultrarare childhood-onset neurological disease caused by biallelic loss-of-function variants in *AP4M1*, encoding the Mu subunit of the adaptor protein complex 4 (AP-4). The AP-4 is an obligatory heterotetrameric complex that plays an important role in vesicle-mediated protein transport. Clinically, SPG50 is characterized by childhood-onset progressive spastic paraplegia, global developmental delay and later intellectual disability, microcephaly, foot deformities, and epilepsy. At present, there is no specific treatment for SPG50. To develop an AAV9/*AP4M1* gene therapy for SPG50, preclinical efficacy and safety studies were conducted. Proof-of-concept *in vitro* studies by Dr. Ebrahimi-Fakhari's group showed that transduction of patient-derived fibroblasts with AAV2/*AP4M1* resulted in phenotypic rescue including restored ATG9A trafficking and increased AP4E1 levels, and a reduction in cell survival at the highest MOI of 1×10^5 . Similar results were obtained in independent *in vitro* studies by Dr. Bonifacino's group using two additional fibroblast cell lines from two SPG50 sibling patients, where 49% and 77% of the fibroblasts from patient 1 and 2, respectively were rescued with AAV2/*AP4M1*. Importantly, these fibroblasts did not show any sign of toxicity following infection. In an ongoing *in vivo* efficacy study, *Ap4m1* knockout (KO) mice were administered intrathecally (IT) with high (5E11 vg), mid (2.5E11 vg), or low (1.25E11

vg) doses of AAV9/AP4M1 at postnatal day p7-10 (pre-symptomatic cohorts) or p90 (early-symptomatic cohorts), which led to clear age and dose effects with early intervention and high dose achieving the best therapeutic benefits. For instance, the high dose of AAV9/AP4M1 injected IT at p7-10 resulted in: 1) significantly higher levels of AP4M1 mRNA in all brain regions assessed; 2) minimal immune responses to AAV9 or AP4M1 peptides; 3) minimal organ toxicity; 4) significant improvement of impaired behavior on Hindlimb Claspings and Elevated Plus Maze tests; 5) minimal adverse effects on body weight or survival; and 6) no neurological abnormalities, indicating no apparent adverse effects. In parallel, a non-GLP one-year toxicity study in C57BL/6J wild type (WT) mice, a GLP three-month toxicity study in Sprague Dawley WT rats, and a GLP three-month toxicity study in WT Non-Human Primates (NHP) were conducted to further evaluate the safety of AAV9/AP4M1 administration. All three studies indicated AAV9/AP4M1 had an acceptable safety profile up to a target human dose of 1E15 vg. Some adverse effects were noted at a higher dose, such as increased excitability, increased activity, and decreased body weight in the rat GLP study at 12 weeks post administration at the highest dose, and neuronal degeneration in the lumbar dorsal root ganglion (DRG) at higher doses with no recovery. In the NHP non-GLP study, decreased nerve conduction occurred in the sural nerve at the 1.68E14 vg dose, corresponding to a human dose of 2E15 vg. Interestingly, a similar occurrence/severity of DRG toxicity was noticed in both rat and NHP toxicity studies, which extends our knowledge for the field and supports the use of rat model to monitor DRG toxicity in future studies. Taken together, these preclinical results identified an acceptably safe and efficacious dose of AAV9/AP4M1 corresponding to 1E15 total vg delivered IT. An investigational Phase I intrathecal gene transfer trial using 1E15 vg of AAV9/AP4M1 to treat SPG50 was approved by Health Canada.

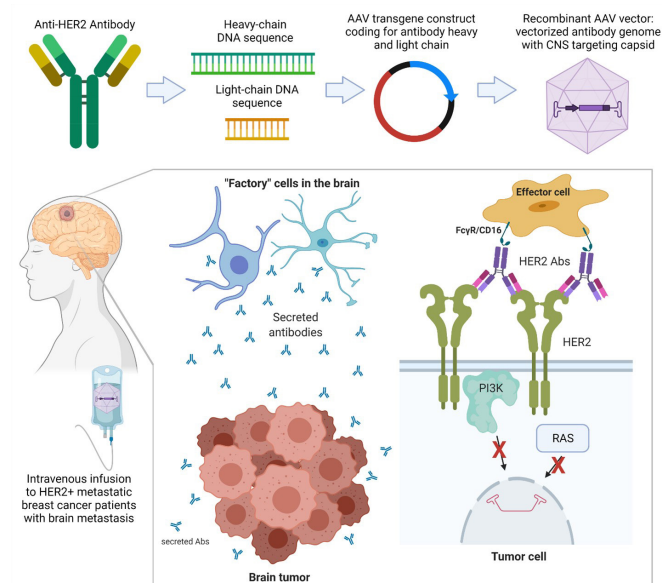
63. CNS Penetrant AAV Vectors Encoding HER2 Antibodies Reduce Tumor Burden in Models of Breast Cancer Brain Metastasis

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Amplification of the growth factor receptor HER2 occurs in ~20% of breast cancer patients. HER2-directed antibody (Ab) therapy, while efficacious against peripheral tumors, has limited central nervous system exposure (CNS). Along with improving early detection and screening for tumors, current anti-HER2 therapies confer prolonged control of metastatic disease in the periphery. As a result, CNS tumors that emerge in 30-50% of metastatic HER2+ breast cancer patients drive mortality and necessitate improved treatments. Our strategy utilizes CNS-targeted AAV vectors to deliver broad brain expression of a vectorized HER2 Ab. The design aims to achieve efficacious brain tumor exposure by creating “factory cells” to secrete antibody into the interstitial fluid (ISF) and cerebrospinal fluid (CSF). The therapy benefits patients by being a single, intravenous administration utilizing capsids that readily cross the blood-brain barrier. To that end, we designed a HER2 Ab that inhibited HER2+ breast cancer cell growth in vitro. Engineering also led to a profound increase in Ab-dependent CD16A receptor stimulation relative to trastuzumab, a standard of care for HER2+ tumors. The in vitro data suggests that the antibody blocks HER2-mediated signaling and can trigger immune cell mediated killing

of tumors. After encoding the HER2 Ab and comparator Abs into AAV transgenes, AAV vectors utilizing CNS-targeted capsids with selective brain cell tropism relative to AAV9 were generated. Blood-brain barrier penetrant AAV vectors were administered intravenously to mice orthotopically engrafted with HER2+ cancer cells. In both prophylactic AAV administration and a treatment model where AAV was dosed post-engraftment, the vectorized Ab significantly reduced tumor burden. Additionally, Ab levels in CSF, serum, and brain parenchyma exceeded the in vitro IC50 for cell growth inhibition. To understand target engagement and the mechanism behind observed tumor suppression, we employed single-cell RNA-Seq to characterize immune responses to these vectorized Abs. Upon dissociation of brains from tumor engrafted mice that underwent intravenous AAV treatment, we performed transcriptomic profiling of CD45+ immune cells. Our results indicate a pronounced innate immune response to the HER2 Ab that increased tumor-proximal natural killer cells and proliferating microglia. Response to the gene therapy also included dendritic cells that are mediators of adaptive immune activation. Therefore, the adaptive immune system could augment the efficacy of the therapy when non-exhausted, tumor-associated T lymphocytes are present. In conclusion, we have utilized intravenous administration of AAV vectors engineered to deliver transgene expression broadly in the CNS. Our animal data indicate that vectorized HER2 antibody gene transfer can potentially address the unmet needs of patients afflicted with brain metastases. **Figure 1:** AAV-mediated gene therapy to deliver vectorized antibodies to the CNS of metastatic breast cancer patients. Illustration depicts HER2 antibodies, with heavy and light chains vectorized into a transgene construct, packaged into AAV capsids engineered to efficiently cross the blood-brain barrier and selectively transduce brain cells. Transduced cells become factories and secrete HER2 antibodies. Antibodies bind to the overexpressed HER2 receptors on metastatic tumors, inhibit pro-growth signaling, and induce tumor cell killing by immune-cell cells via Fc-mediated interactions.



Gene Editing in Blood and Immune Disorders

64. Two is Better Than One: CRISPR/Cas9 Based Gene Editing with FOXP3 Isoforms for IPEX Therapy

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Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is caused by mutations in the forkhead box protein 3 (*FOXP3*) gene, a critical transcription factor required for T regulatory cells (Tregs). Patients present with severe early onset autoimmunity that can be fatal within the first year of life. As a monogenic immune disease with limited treatment options, IPEX is an ideal candidate for a gene therapy approach whereby patient hematopoietic stem cells are gene edited for autologous transplant. In healthy Treg and activated effector T cells (Teffs), *FOXP3* is expressed in 2 predominant isoforms: the full length (*FOXP3^{FL}*) and exon 2 deleted (*FOXP3 Δ 2*) isoform. We developed a CRISPR/Cas9 approach combined with AAV delivery of a donor template to restore *FOXP3^{FL}* expression at the endogenous locus, permitting regulated expression of wild-type *FOXP3^{FL}* irrespective of downstream mutations. We demonstrated precise editing of T cells from IPEX patients and healthy donors. *FOXP3^{FL}* edited Tregs showed restoration of *FOXP3* expression and functional suppression. Additionally, we demonstrated that gene edited HSPCs can be transplanted into NSG-SGM3 mice for multilineage reconstitution. However, we found that the *FOXP3* expression and suppressive capacity of *FOXP3^{FL}* or *FOXP3 Δ 2* edited Tregs is towards the lower end of the range of unmodified cells. HSPCs edited with *FOXP3^{FL}* also gave rise to less Tregs in vivo. In order to delineate the role of isoforms in human Tregs, we induced the expression of each isoform on *FOXP3* knockout CD4⁺ T cells by lentiviral gene transfer. When compared to *FOXP3^{FL}* or *FOXP3 Δ 2* alone, or double transduction of the same isoform, co-expression of *FOXP3^{FL}* and *FOXP3 Δ 2* induced the highest overall *FOXP3* protein expression. This condition, in turn, led to optimal acquisition of Treg-like cell phenotypes including downregulation of cytokines, such as IL-17, and increased suppressive function. Together with literature showing that each of the isoforms has independent functions, our data suggests that the expression of both isoforms may be essential for controlling *FOXP3* expression and fine tuning the development of functional Treg and Teff cells. Therefore, we designed and tested a new knock-in construct, confirming that it allows alternative splicing of both isoforms in edited T cells and preserves the endogenous *FOXP3* isoform ratio. To our knowledge this is the first CRISPR/Cas9 construct that maintains an alternative splice event and could instruct other editing therapies where gene isoforms confer independent functions.

65. Preclinical Safety and Feasibility Study of a CRISPR/Cas9 Gene Editing Platform to Treat Wiskott Aldrich Syndrome

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Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disease caused by mutations in the *WAS* gene resulting in the development of microtrombocytopenia, eczema and increased susceptibility to infections, tumors and autoimmune disorders. For those children who are not eligible for an allogeneic stem cell transplantation, lentiviral gene therapy has proved effective, although full immune and platelet reconstitution as well as physiological *WAS* protein expression is not always achieved in treated patients. We have recently concluded a proof-of concept study aiming at correcting the genetic defects in patient-derived *WAS* HSPCs by gene knock-in into the *WAS* locus using CRISPR/Cas9 and AAV6, and showed the ability of the gene editing strategy to correct the *WAS* defects both in vitro and in vivo. We have now progressed this gene editing-based therapy to preclinical studies to assess its feasibility at the clinical scale. By ex-vivo gene editing of healthy donor HSPCs, we have demonstrated successful optimisation of medium and large-scale cell manipulation protocols using clinical grade reagents and reproducible rates of HSPC gene targeting, viability and colony forming efficiency. Large-scale manufactured cells were able to engraft and give rise to mature hematopoietic cells in xenotransplantation studies with no major toxicity observed. Moreover, a comprehensive in vitro and in vivo assessment of potential off-targeting and chromosomal instability caused by the gene editing procedure confirmed the safety of the platform. Overall, data coming from this study provide specificity, toxicity and efficacy evidence supportive of continued development of the platform and will pave the way for the translation of CRISPR/Cas9 gene editing into the next generation of therapeutic tools for *WAS* and for diseases that require gene correction at the HSPC level.

66. Development of a Beta-Globin Gene Replacement Strategy as a Therapeutic Approach for β -Thalassemia

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β -thalassemia is a genetic disorder characterized by reduced production of β -globin, a protein that forms functional, oxygen-carrying hemoglobin with α -globin (HbA, $\alpha_2\beta_2$). In its most severe form, β -thalassemia is caused by mutations in both alleles of the β -globin gene. These patients fail to produce functional β -globin, resulting in severe anemia. More than 300 β -thalassemia mutations are known; most are small nucleotide insertions, substitutions, or deletions within or directly adjacent to the β -globin (*HBB*) gene. A gene editing platform using homology directed repair (HDR) that replaces the *HBB* gene to achieve a normal or β -thalassemia trait phenotype with HbA expression similar to healthy individuals is an ideal strategy. The challenge is achieving high levels of gene replacement that result in high HbA expression.

We designed a homologous DNA donor that can replace a mutated *HBB* gene with a functional *HBB* gene and overcomes the two main challenges with gene replacement at this locus. First, to avoid sequence homology of the insert with the endogenous locus, we used a diverged *HBB* coding sequence. Second, because introns are required for high *HBB* expression, we hypothesized that incorporation of non-homologous introns would result in endogenous HbA production. Using a high-efficiency gene editing platform that combines: 1) a high fidelity Cas9 precomplexed with chemically modified guide RNAs, 2) a DNA template delivered by AAV6, and 3) CD34+ hematopoietic stem and progenitor cell culture optimization, we developed an *HBB* gene replacement strategy. To identify DNA templates resulting in high *HBB* expression, we screened 39 versions of T2A-EGFP-tagged *HBB* coding sequences containing various heterologous introns and polyadenylation signals. Different DNA donors were knocked into primary CD34+ hematopoietic stem and progenitor cells (HSPCs, $n = 3$ healthy donors), differentiated into red blood cells (RBCs) *in vitro*, then evaluated for EGFP mean fluorescence intensity (MFI) by flow cytometry, as a surrogate for beta-globin expression. Adding heterologous introns to the *HBB* coding sequence significantly increased MFI by three-fold ($p=0.0008$). To evaluate if the MFI positively correlated with HbA expression, the T2A-EGFP sequence was removed from the DNA donors and HbA expression was measured by HPLC in HSPC-derived RBCs. MFI and HbA output were positively correlated, and the top DNA donors resulted in HbA expression on par with endogenous levels. Using this gene replacement strategy, we achieved HDR frequencies of up to 40% in CD34+ HSPCs. To increase gene replacement frequencies, the top DNA donors were further optimized by truncating the introns to create a smaller insertion cassette that resulted in higher HDR and maintained high levels of HbA expression. Using HSPCs from patients with sickle cell disease as a therapeutically relevant model, we investigated if this DNA donor can replace the non-functional *HBB* gene that produces HbS. Gene replacement of the sickle allele using the optimized DNA donor restored HbA expression as measured by HPLC comparable to an HDR gene correction strategy that corrects the HbS point mutation. Future experiments are investigating *in vitro* and *in vivo* stem cell repopulation capacity of HSPCs targeted for *HBB* gene replacement. In summary, we developed a precise *HBB* gene replacement strategy that is highly efficient and results in high HbA production, offering a potential differentiated approach for treating β -thalassemia.

67. Adenine Base Editor-Mediated Correction of Three Prevalent and Severe β -Thalassemia Mutations

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β -thalassemia is a highly prevalent monogenic recessive disease caused by mutations affecting the synthesis of the adult hemoglobin β -chains. Point mutations in the β -globin gene (*HBB*) locus reduce (β^+) or

abolish (β^0) the production of β -globin chains. The imbalance between α - and β -globin production leads to the precipitation of uncoupled α -globins, which causes erythroid cell death, ineffective erythropoiesis and severe anemia. The most severe form of the disease, β -thalassemia major, is typically associated with a β^0/β^0 genotype. Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an attractive therapeutic option. However, current gene therapy strategies based on the use of lentiviral vectors or CRISPR/Cas9 nuclease are not equally effective in all the patients and/or raise safety concerns. The two β^0 mutations CD39 (CAG>TAG) and IVS2-1 (G>A) and the β^+ mutation IVS1-110 (G>A) are among the most common and severe β -thalassemia mutations in the Mediterranean area and Middle East. Here, we exploited the capacity of adenine base editors (ABEs) in combination with specific single guide RNAs (gRNAs) to convert A>G and correct these mutations in HSPCs from β -thalassemia patients. First, we screened different combinations of ABEs/gRNAs in T cells from β -thalassemia patients harboring these mutations. The absence of the canonical *Sp*yCas9 NGG PAM close to the target bases prompted us to test a variety of non-NGG Cas9 variants in combination with a highly processive deaminase (ABE8e). In particular, we generated and tested the novel ABE8e-SpRY base editor harboring the near-PAMless SpRY-Cas9. We selected the best performing gRNAs that were used in combination with ABE8e-SpRY or ABE8e-NRCH to revert CD39, IVS2-1 and IVS1-110 mutations with a gene correction efficiency in T cells of 100%, 53% and 86%, respectively. We then tested selected ABEs/gRNAs combinations in HSPCs from β -thalassemia patients and achieved correction efficiencies of up to ~90%. Control and edited β -thalassemic HSPCs were differentiated towards the erythroid lineage to evaluate globin and hemoglobin production. Red blood cells derived from edited HSPCs exhibited high β -globin levels and improvement of the α /non- α globin ratios. The delayed erythroid differentiation typically observed in β -thalassemic cell cultures was corrected by our treatment. Indeed, along the differentiation, we observed an increased enucleation rate (measured as frequency of DRAQ5⁻ cells) compared to unedited β -thalassemic controls, reaching the levels observed in healthy donor cells. Furthermore, the size of enucleated cells (typically reduced in β -thalassemic cells) was normalized. Finally, measurement of Annexin⁺ cells by flow cytometry showed a substantially reduced apoptotic rate in edited β -thalassemic samples compared to untreated controls. In conclusion, we developed three efficient base editing approaches to revert highly prevalent and severe β -thalassemia mutations and correct the β -thalassemic cell phenotype. Validation of these results in HSPCs *in vivo* and genotoxicity studies will provide sufficient proof of efficacy and safety to enable the clinical development of base-edited HSCs for gene therapy of β -thalassemia.

68. Improvement of *PKLR*-gene Editing in Human Hematopoietic Stem and Progenitor Cells Towards its Clinical Application for Pyruvate Kinase Deficiency

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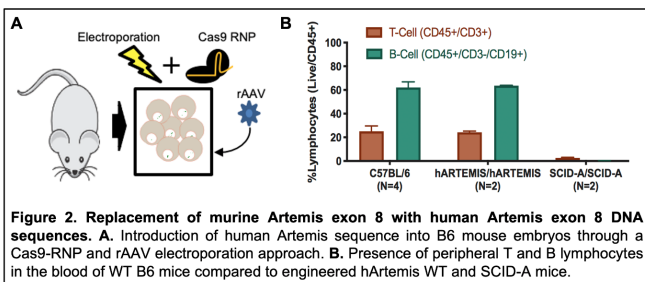
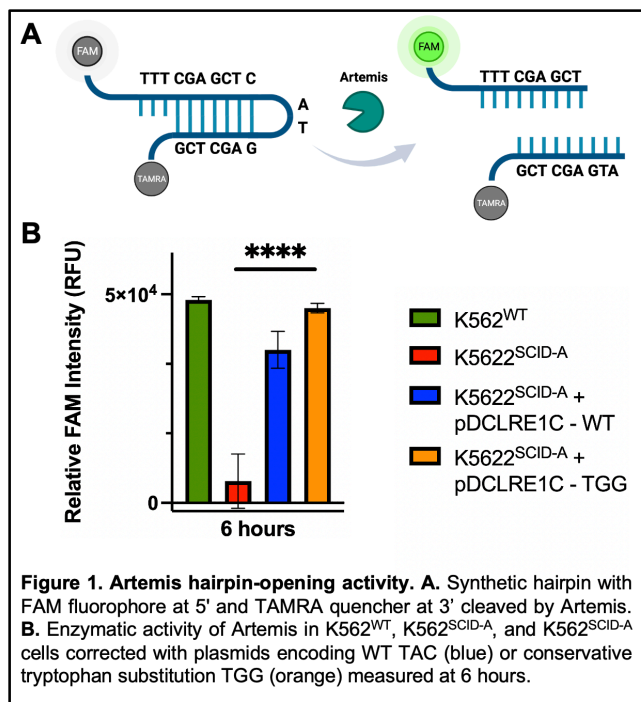
Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in the *PKLR* gene. PKD-erythroid cells suffer from an energy imbalanced cause by a reduction of the erythroid pyruvate kinase (PK) protein activity. This disease is associated with reticulocytosis, splenomegaly and iron overload, and may be life-threatening in severely affected patients. Autologous Hematopoietic Stem Cell Transplantation (HSCT) of genetically corrected cells using lentiviral vectors has demonstrated a durable and curative therapeutic option (NCT#04105166). With that in mind, we developed a knock-in gene editing strategy at the genomic starting site of the *PKLR* gene by combining RNP electroporation and two different recombinant adeno-associated viral vector (rAAV6) donors to deliver either a TurboGFP expression cassette or a promoter-less therapeutic codon optimized RPK cDNA (coRPK), flanked by specific homologous arms. We were able to obtain a stable integration in up to 40% of colony forming units (CFUs) generated from healthy donor cord-blood hematopoietic stem and progenitor cells (HSPCs, CB-CD34⁺ cells) in the absence of toxicity. These gene edited CB-CD34⁺ cells engrafted efficiently in both primary and secondary NSG mice, although clinically relevant correction remained a challenge. Different alternatives for modifying our original gene editing protocol were explored in order to reach therapeutic levels in long-term HSPCs. Firstly, we assessed the addition of different compounds either involved in AAV transduction (Bleomycin, Bortezomib, Teniposide, Daunorubicin and Polyvinyl alcohol), in the homology repair pathway (BRCA1 and MRE11A proteins) or in the maintenance of the stem compartment (SR1) during HSPC gene editing process. Although some compounds were promising and showed a moderate increase in gene editing percentage, the best results were obtained when culture timing and cell density were adjusted. 48h-expansion of pre-stimulation period and reduction of cell density during the editing process doubled HSPC gene editing frequency, reaches up to 70% HDR in CFUs. Edited HSPCs under this optimized protocol were infused into immunodeficient mice and the specific integration was detected in up to 25% of the human cells three months after transplant. These results confirm the feasibility of reaching therapeutic levels of *PKLR* gene editing in human HSPCs and therefore its future potential clinical application for the treatment of PKD.

69. Site-Specific Editing Methods to Reverse Severe Combined Immunodeficiency (SCID) in Athabaskan-Speaking Native Populations

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Severe combined immunodeficiency of Athabaskan-speaking Natives (SCID-A) is caused by the absence of Artemis protein due to a premature stop codon (pmSTOP) in exon 8 of the *DCLRE1C* gene. Artemis plays a key role in the canonical non-homologous end joining NHEJ (c-NHEJ) DNA repair pathway as well as V(D)J recombination. Therefore, SCID-A patients have hypersensitivity to DNA damaging agents like ionizing radiation and alkylating agents, along with a T-B⁺NK⁺ lymphocyte population, leading to a high incidence of severe infections. *Ex vivo* lentiviral complementation of Artemis in SCID-A hematopoietic stem cells (HSCs) is currently in clinical trials (NCT03538899); however, this approach carries the risk of insertional mutagenesis and can result in suboptimal gene expression due to the semi-random integration pattern of lentiviruses and the necessity of driving transgene expression with an exogenous promoter. Here we show progress towards a superior approach of site-directed genetic repair of Artemis *in situ*, correcting the pmSTOP using homology-directed repair (HDR) or adenine base editor (ABE). For induction of HDR-mediated correction, we used Cas9 nickase to avoid genotoxic double-strand breaks (DSBs) and delivered a template for DNA repair using recombinant adeno-associated virus (rAAV), achieving >50% correction of the pmSTOP codon that leads to Artemis deficiency in a model K562^{SCID-A} cell line. For ABE-mediated conversion, we targeted the mutation using ABE8e-NG, achieving >40% restoration of the reading frame by converting the pmSTOP to tryptophan (TAA→TGG). This conservative amino acid substitution resulted in the rescue of functional Artemis protein as demonstrated by enzymatic hairpin-opening activity (**Figure 1**). To create *in vivo* models to validate our proposed therapeutic approaches, we engineered a pre-clinical mouse model with a humanized *DCLRE1C* Exon 8 with either wild-type (WT) or SCID-A sequences using Cas9-ribonucleoprotein (RNP)/rAAV embryo electroporation (**Figure 2A**), which recapitulates the immunophenotype of SCID-A, as shown by the presence/absence of circulating T and B lymphocytes in **Figure 2B**. Here we show *in vitro* proof of concept of two superior approaches for correcting *ARTEMIS* in its endogenous genetic context. Moreover, we have developed a robust mouse model that recapitulates the human SCID-A disease phenotype and will be used to test our proposed therapies. Our work demonstrates the broader potential of these site-specific approaches for the treatment of other primary immunodeficiencies and genetic diseases.



70. Development of a Double shmiR Lentivirus Effectively Targeting Both BCL11A and ZNF410 for Enhanced Induction of Fetal Hemoglobin to Treat β -Hemoglobinopathies

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A promising treatment for β -hemoglobinopathies is the de-repression of fetal hemoglobin (HbF). Decreasing the expression of γ -globin repressor BCL11A results in a coordinated increase in γ -globin expression and reduction in β -globin expression. Here, we aim to improve lentivirus vectors (LVV) containing a single BCL11A shmiR (SS) currently in clinical trials (Esrick et al. NEJM, 2021) to further increase γ -globin induction. We developed a novel LVV expressing two shmiRs simultaneously targeting BCL11A and the independent γ -globin repressor, ZNF410. Healthy human HSC-derived erythroid cells transduced with the double shmiR (DS) LVV showed up to 80% reduction of both BCL11A and ZNF410 proteins with a consistent

and significant ($p < 0.05$) additional 10% HbF induction compared to targeting BCL11A alone. Erythrocytes differentiated from SCID HSCs transduced with the DS LVV expressed higher therapeutic levels of HbF and demonstrated significantly reduced *in vitro* sickling phenotype ($p < 0.001$) compared to the SS BCL11A LVV. Erythrocytes differentiated from DS LVV transduced HSCs from β -thalassemia major patients increased γ -globin expression to more than 60% and demonstrated restored globin chain balance and reduced microcytosis. Erythroid cells derived from the bone marrow of immunodeficient mice engrafted with DS LVV gene-modified cells also showed simultaneous target gene knockdown and significantly higher γ -globin and HbF levels compared with SS BCL11A ($p < 0.05$). Normalized to VCN in erythroid cells, γ -globin expression was also significantly higher in DS transduced cells compared with BCL11A shmiR. Finally, in Berkeley SCD mice reconstituted with DS-transduced cells the average levels of γ -globin in erythroid cells from BM were 1.7%, 18.5%, and 25.4% for NT group, SS, and DS, respectively. This increase in γ -globin was associated with a statistically larger reduction in peripheral blood hemolysis markers compared with the SS vector (see Table). As an additional marker of erythroid stress, average spleen mass of SS LVV and DS LVV group were also decreased (Table). In summary, these results indicate that the DS LVV targeting BCL11A and ZNF410 can enhance HbF induction which could potentially improve the efficacy of current approaches of treating β -hemoglobinopathies and could be used as a model to simultaneously and efficiently target multiple gene products.

Table. Hematologic parameters and spleen mass in Berkeley-SCD mouse model.

	Erythroid precursor cells (%)	Reticulocytes (%)	Sickled cells (%)	Spleen mass (g)
NT (non-targeting)	35.0 \pm 6.9	34.0 \pm 4.3	50.4 \pm 6.7	0.53 \pm 0.06
SS (single shmiR)	8.8 \pm 1.8	12.6 \pm 3.8	14.8 \pm 4.2	0.25 \pm 0.06
DS (double shmiR)	6.2 \pm 1.8	7.9 \pm 4.1	10.0 \pm 4.3	0.18 \pm 0.04
P value (SS vs DS)	0.0068	0.0187	0.0255	0.0123

Synthetic Nanoparticle-Based Gene Transfer

71. Ministring DNA: A Durable and Safe Nonviral Delivery Platform

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Ministring DNA (msDNA) is a novel mini linear DNA vector with closed ends, without bacterial plasmid sequences, which can be a safe, redosable, and effective non-viral gene delivery vector. First, we screened precursor plasmids containing different cis-regulatory

sequences and 2 proprietary supersequences (SSeq), with nuclear localization signals, to establish a highly efficient, ubiquitous expression cassette template that will be incorporated into the msDNA. All plasmids carried both secreted luciferase (NanoLuc) and cytoplasmic eGFP reporter genes driven by the CMV promoter (pSNLuc), the CMV promoter with WPRE (2xSSeq pGSNLuc-WPRE), or the CAG promoter with chimeric intron (2xSSeq pCAGLuc), or the CAG promoter with chimeric intron and WPRE (2xSSeq pCAGLuc-WPRE). Groups of mice were administered with 50 µg of respective plasmids via hydrodynamic injection. The plasma of the treated mice was collected on day 1, 7, 14 and every two weeks afterwards to examine the luciferase gene expression in treated mice. On day 1 post-vector administration, all mice exhibited high levels of luciferase expression (10^8 - 10^9 RLU per mg of plasma protein). On day-7, the 2xSSeq pCAGLuc and the 2xSSeq pCAGLuc-WPRE treated mice produced 10^7 - 10^8 RLU/mg of plasma protein, but the pGSNLuc-WPRE treated mice yielded lower luciferase levels ($\sim 10^6$ RLU/mg protein). After 8-weeks post-vector administration, all mice exhibited low levels of luciferase expression (around 10^5 RLU/mg protein). Due to the rapid drop of luciferase levels, it is suspected that humoral or cell-mediated immune responses may be induced in the plasmid treated mice. To test dose response of plasmid DNA following nonviral gene delivery in animal models, the mice were administered with 5 µg plasmids instead of 50 µg plasmids using hydrodynamic injection. The mice treated with 2xSSeq pCAGLuc and the 2xSSeq pCAGLuc-WPRE showed sustained high luciferase expression levels (10^7 - 10^8 RLU/mg protein) more than 8 weeks post-vector administration, which were 100-fold higher than the conventional plasmid pSNLuc with no SSeq). Based on these results, the expression cassette in the precursor plasmid 2xSSeq pCAGLuc-WPRE was selected as the template for generating msDNA-CAG-W. msDNA containing the CMV promoter with WPRE (msDNA-CMV-W) was also generated and used as control. Groups of mice received hydrodynamic injection of 5 µg msDNA-CMV-W, msDNA-CAG-W and pSNLuc as benchmark control respectively. Similarly to plasmid treated mice, the msDNA-CAG-W treated mice produced sustained high levels of luciferase expression (10^7 - 10^8 RLU/mg protein) more than 8 weeks post-vector administration, whereas the luciferase expression in msDNA-CMV-W treated mice dropped to low levels ($\sim 10^6$ RLU/mg protein) in less than one month. The rapid drop of luciferase expression in msDNA-CMV-W treated mice was likely due to silencing of the CMV promoter in hepatocytes. Most importantly, mice treated with msDNAs produced more than fivefold higher gene expression levels compared with those generated from mice treated with the corresponding plasmids. In addition, luciferase gene expression was confirmed via whole body live imaging by IVIS. In conclusion, nonviral delivery with msDNA-CAG-W in mice was highly efficient and the resulting gene expression was stable for more than two months. The novel msDNA platform can be applied to change the paradigm of nonviral gene therapy to provide a safe, titratable, and redosable genetic medicine to cure disorders.

72. Mechanisms of siRNA Delivery by Cyclic Amphipathic Peptides

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Acute respiratory distress syndrome (ARDS) affects over 200,000 Americans each year (excluding those affected by COVID-19) and has a 30-40% mortality rate with no current pharmacological interventions. Moreover, it is estimated that 90% of covid deaths are due to ARDS. Additionally patients who survive may never fully recover normal lung function. In ARDS, the alveolar-capillary barrier separating airspace and blood weakens or is destroyed, resulting in edema, inflammation, and ultimately death. Targeted inhibition of inflammatory signaling to preserve barrier function may improve survival and help retain lung function. siRNA therapeutics against key inflammatory genes offer a potential treatment. However, delivery of siRNA and other RNAs remains a major challenge in the lung and other organs. We have identified several cyclic amphipathic peptides (CAPs) that can complex siRNA and promote delivery in cultured cells and in the lungs of mice. CAPs are a subset of cell penetrating peptides that can cross the cell membrane via endogenous cellular endocytic mechanisms. In order to understand the mechanisms of action and delivery of these peptide-RNA complexes, we have characterized their mechanism(s) of cell uptake. Using pharmacological and genetic knockdown approaches, we have studied the uptake of a number of different CAPs in cultured lung epithelial cells. We initially found that two peptides supported high level delivery in the lung: cyclic (WR)4 and cyclic (FKFE)2 and have created a series of peptides with amino acid substitutions to evaluate structure-activity relationships. We find that these different peptides utilize either clathrin-dependent endocytosis (CDE), caveolae, or macropinocytosis for their uptake. Each pathway results in uptake however, the membrane interaction and vesicles involved have different characteristics in terms of cargo delivery to desired locations in the cell. The specific pathway used for CAP-siRNA uptake is influenced by peptide design with differences in uptake and efficiency of siRNA cargo delivery. By imaging and quantifying the delivery of fluorescently labeled siRNA by different CAPs to A549 cells *in vitro* we show that cellular delivery is dependent on the presence and “strength” of a positively charged amino acid (Arg>Lys>His) and amino acids capable of pi-pi interaction (e.g. Trp or Phe). Comparisons of functional siRNA delivery between different CAPs was shown by delivering occludin siRNA then relative quantitation of knockdown by Western Blotting. Future work will focus on *in vivo* therapeutic potential by delivery of siRNA to downregulate pro-inflammatory factors such as mTOR and study of tissue and cell localization by CAP.

73. In Vivo Spatially Targeted Nonviral Optical Delivery of Genes in Mice to NHPs

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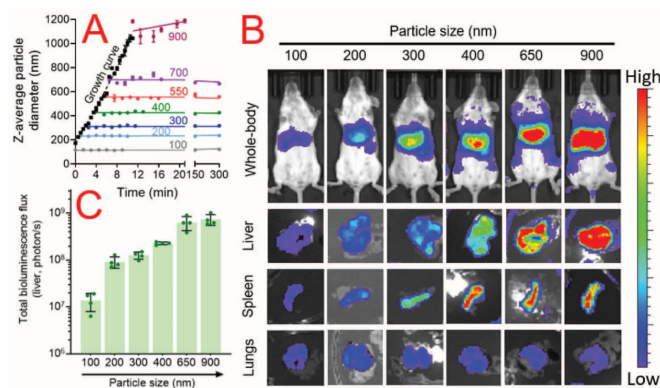
Introduction: Cell-specific opsin-based sensitization of neurons to light via optogenetics has allowed modulation of neural circuitry in-vivo for probing function and understanding of disease. Neurodegeneration diseases such as geographic atrophies (GA) in dry age-related macular degeneration (dry-AMD) would require spatially targeted delivery of opsin-encoding gene into cells in the targeted atrophic region(s) without interfering in the functioning of surrounding intact retina. Traditional methods for gene delivery require viral transduction, which suffer from lack of spatial localization, unexpected inflammatory responses, and immunological reactions. Here, we report use of an optical coherence tomography (OCT) guided, spatially targeted, near-infrared laser microirradiation platform for successful delivery of genes encoding for ambient-light activatable Multi-Characteristic opsin (MCO) to retinal cells in small and large animal models. **Methods:** Wild type and different retinal-degenerated (*rd1*, *rd10*, and laser-induced GA) mice models obtained from Jackson labs were used for OCT-image guided spatially targeted optical gene delivery. We also tested the non-viral laser delivery method in wild type as well as MNU-injected (model for photoreceptor degeneration) rats. MCO-plasmids were intravitreally injected, followed by OCT imaging and selection of target region(s) to achieve spatially localized laser gene transfection in retinal cells. For spatially-localized transfection, we utilized OCT-guided ultrafast near infrared (NIR) laser, or continuous wave (CW) NIR laser in conjunction with gold nanorods (GNRs). Efficacy was evaluated by measurement of fluorescence reporter expression and electrophysiological response to light activation. The toxicity was evaluated by OCT, intra-ocular pressure (IOP), Electroretinogram (ERG), immunoreaction and biodistribution. For clinical translation, we developed OCT-guided laser system to image and deliver therapeutic genes in a spatially targeted manner to retina in non-human primates (African green monkey). **Results:** Our rodent studies provided proof-of-concept for highly localized optical gene delivery in an efficient manner without causing toxicity (measured by no loss of cell viability in caspase/TUNEL assays, no increase in IOP or decrease in ERG of wildtype animals and OCT retinal thickness). Targeted optical delivery of MCO into GA lesion led to enhanced light-evoked electrophysiological response. We also demonstrated that OCT-guided optical gene delivery is an efficient non-viral gene delivery method to transfect NHP retina as shown by fundus imaging of the reporter (mCherry) fluorescence. In addition, functional improvement (measured by redERG) was detected, which was attributed to MCO's broadband activation spectrum. No structural and functional changes in NHPs have been observed from fundus/OCT imaging and IOP measurements. **Discussion and Conclusion:** The advantages of the optical delivery method with OCT guidance include: (i) real time imaging guidance to pathological areas of interest, (ii) monitoring of gene delivery to target region of interest, (iii) opportunity to re-dose the same atrophic area(s) with better opsin-encoding genes and newly-developed GA areas in subjects without immune rejection, and (iv) ability to deliver large size genes (that cannot be packaged in safe AAV vectors). Advancement of laser based targeted gene delivery will enable reliable delivery of therapeutic molecules including CRISPR/Cas9 gene editing agents, into targeted neurons to restore function in neurodegenerative diseases.

74. Particle Size Engineering to Enhance mRNA Delivery Efficiency via Biodegradable Carriers In Vivo

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Introduction. The tremendous success of lipid-based mRNA delivery systems for vaccination has provided the impetus for the development of a diverse range of carriers that can offer distinct tropisms desirable for a variety of therapeutic targets, especially those outside of the liver and the muscle. Poly(β -amino) ester (PBAE), as a biodegradable polymeric carrier, could deliver multiple types of nucleic acid cargos and access different organs and tissues, although the delivery efficiency can be further improved. In this study, we developed a new particle size control method that exponentially increases the payload capacity of mRNA/PBAE nanoparticles through a supra-molecular assembly technique. The assembled particles had a size in sub-micron range that yielded markedly higher transfection efficiency comparing with the nanoparticles prepared with the conventional method. **Methods.** The poly(β -amino ester) was synthesized using Michael addition reactions, with the structure optimized for mRNA delivery. To formulate the nanoparticles, mRNA in sodium acetate buffer at pH 5.0 and polymer ethanol solution were mixed at a volume ratio of 3:1 through the flash nano-complexation (FNC) technique. This step generated "building block" nanoparticles with a Z-average size of 100 nm. A supramolecular assembly strategy was previously developed for plasmid DNA/polycation nanoparticles and was applied to these nanoparticles. Briefly, an assembly buffer with optimized ionic strength and pH was added to remove the repelling forces on nanoparticle surfaces. Along the path of the induced size growth, reversal of the solution conditions re-stabilized the particles at desired sizes (**Fig. 1A**). **Results.** Upon mixing with the assembly buffer, the size of mRNA/PBAE particles grew in a predictable manner, with a constant rate of roughly 50 nm (diameter) per minute (**Fig. 1A**). This kinetics-based method generated highly uniform particles in a size range of 100 to 1000 nm that could stay stable for at least 5 hours. Upon i.v. injection of particles at varied sizes loaded with luciferase mRNA, the transfection efficiencies in the liver and spleen were found to be size-dependent. The larger the particle size, the higher the transfection efficiency (**Fig. 1B**). The transfection efficiency level obtained from 650-nm mRNA/PBAE particles were comparable to that from a benchmark mRNA lipid nanoparticle formulation (DLin-MC3-DMA and DSPC-based, data not shown). This level was not attainable by PBAE particles prepared using the conventional methods in previous reports that were generally below 200 nm in size. Particularly in the liver, the transfection efficiency with the 900-nm particles was 60-fold higher than that using the 100-nm particles (**Fig. 1C**). Besides, larger particles prepared with the supra-assembly process resulted in more durable mRNA expression: the overall luciferase expression level in mice treated with the 900-nm particles at 48-h post-injection was comparable to that in mice treated with the 100-nm particles at 12-h post-injection. **Conclusion.** The supra-molecular mRNA assembly of PBAE/mRNA nanoparticles markedly increased the transfection efficiency in the liver and spleen, matching the level achievable using the mRNA lipid nanoparticles. These sub-micron sized PBAE/mRNA particles have great potential as an effective gene delivery system for gene medicine.



75. Delivery of CRISPR/Cas9 mRNA LNPs to Repair a Small Deletion in FVIII Gene in Hemophilia A Mice

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Hemophilia A (HemA) is a bleeding disorder resulting from a deficiency of the X-linked factor VIII (FVIII) gene. Currently, treatment of repeated infusions with FVIII protein for HemA patients is costly and inconvenient. Gene therapy represents a very promising alternative treatment method. The combination of lipid nanoparticle (LNP) technology targeting specific organs and CRISPR/Cas9 technique allow *in vivo* gene correction of the mutant FVIII and regain the expression of FVIII. We aimed at optimizing *in vivo* delivery efficiency of mRNA encapsulated LNPs and FVIII gene-editing tools, respectively. Since FVIII protein is mainly and naturally made in liver sinusoidal endothelial cells (LSECs), we investigated various formulations of LNPs that can target LSECs. First, Luciferase mRNA (Luc) was encapsulated in MC3-based LNPs and its transfection efficiency was examined in HepG2 and HUVEC cells as liver cell models. Luciferase expression can be detected in both cell lines, indicating mRNA LNP can successfully transfect both hepatocytes and endothelial cells *in vitro*. Next, we delivered Luc LNPs and GFP LNPs into mice, respectively. The *in vivo* transfection was evaluated using *in vivo* imaging systems (IVIS) and immunofluorescent staining by Luciferase mRNA LNPs and GFP mRNA LNPs, respectively. Results suggested that this LNP formulation mainly targets hepatocytes *in vivo*. LNPs were further improved by different formulations and enhancement of LSEC-targeting efficiency was observed by immunofluorescent staining. Next, we evaluated the efficacy of different sgRNAs for *in vivo* gene editing of mutant FVIII. An immunodeficient hemophilia A (NSG HA) mice that contained premature stop codon in exon 1 of FVIII were used as the HemA mouse model. Two different sgRNAs that can edit wild-type FVIII sequence (mF8sgRNA) or mutant FVIII exon 1 sequence (NSGHAsgRNA) were designed, respectively. For a start, the Cas9/sgRNA plasmids were hydrodynamically injected into NSG HA mice and the FVIII expression was examined by aPTT assay. Both FVIII-targeting sgRNAs can promote the recovery of FVIII expression in NSG HA mice at least one month after treatment, suggesting successful gene editing of mutant FVIII. Finally, a combination of gene editing and LNP technology was performed. Encapsulated Cas9 mRNA and sgRNA LNPs were delivered into NSGHA mice intravenously. Expression of endogenous FVIII

was confirmed by aPTT assay and edited mutant FVIII was verified by DNA sequencing and online CRISPR analysis tools, respectively. Our data showed our LNP formulations can deliver mRNA into both hepatocytes and endothelial cells *in vivo*. Furthermore, combined with CRISPR/Cas9 technology, we have corrected the mutated FVIII gene and regained the FVIII activity in NSG HA mice. This gene-editing protocol has the potential to persistently regain therapeutic levels of FVIII gene expression for hemophilia A treatment.

76. Building a Genetic Medicine Platform for DNA-Encoded Antibody Therapeutics

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Within the field of genetic medicine, gene-based antibody therapy is a hot commodity. DNA-based delivery aims to administer the antibody-encoding nucleotides using a non-viral plasmid DNA (pDNA) vector. Such genetic shortcut allows for prolonged antibody production *in vivo*, and addresses some of the bottlenecks of conventional antibody protein therapeutics. This can allow for a broader antibody accessibility and e.g. facilitate more effective cocktails. We previously presented preclinical proof of concept for various DNA-encoded full-length monoclonal antibodies (mAbs) and nanobodies, delivered to muscle or tumor. To advance our DNA platform, we evaluated additional examples of product development. First, we focused on COVID-19. Using B cell mining in a convalescent patient, we identified a panel of human mAbs that potently neutralized the main SARS-CoV-2 variants of concern. The lead mAb 3B8 also retains its activity against omicron. In hamsters, intramuscular electroporation of DNA-encoded 3B8 resulted in median mAb serum levels up to 90 µg/ml ten days after pDNA delivery, and protected animals against SARS-CoV-2 infection. Second, we focused on immunotherapy by delivering a cocktail of DNA-encoded immunomodulators directly into the tumor. In a murine cancer model, intratumoral electroporation of plasmids encoding IL-12 and an anti-PD-1 and anti-CTLA-4 antibody significantly delayed tumor growth compared to IL-12 alone and the combination of anti-PD-1 and anti-CTLA-4 antibodies. The triple combination also enabled significant abscopal effects, which was not the case for the other treatments. To assess translational feasibility, we previously reported on intramuscular DNA-based antibody delivery in 40-70 kg sheep, a clinically relevant model. Here, we further aligned and affirmed the electroporation setup to clinical practice. In groups of eight sheep each, escalating doses of pDNA (1 and 4 mg) gave serum mAb levels up to the µg/ml range, without inducing anti-drug-antibodies during the six week follow-up. While the currently attained mAb titers are effective in various rodent disease models, we explored further improvements. *In vitro*,

mAb levels consistently improved with decreasing sizes of plasmid backbone. *In vivo*, following intramuscular pDNA electrotransfer in mice, the correlation was less consistent. While the largest conventional plasmid (10.2 kb) gave the lowest mAb levels, a regular conventional plasmid (8.6 kb) demonstrated similar levels as a minimal plasmid (6.8 kb). Thus, while improving biosafety, a reduction in size beyond a standard conventional plasmid backbone did not improve mAb levels *in vivo*. Cassette modifications, such as swapping antibody chain order or use of two versus a single encoding plasmid, significantly increased antibody expression *in vitro*, but failed to translate *in vivo*. Conversely, a significant improvement *in vivo* but not *in vitro* was found with a set of muscle-specific promoters compared to the ubiquitous CAG promoter. Overall, despite the limited translation between *in vitro* and *in vivo*, improvements in potency and biosafety were identified. In conclusion, our new findings contribute to a broadly and clinically applicable genetic medicine platform for DNA-based antibody therapeutics.

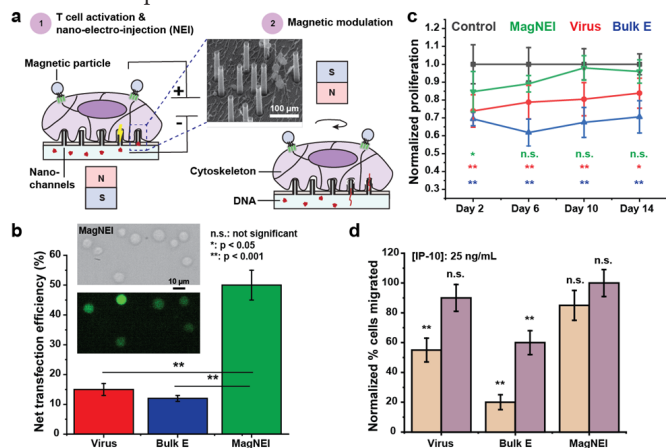
77. Nano-Structures for Efficient and Transgene-Free Immune Cell Transfection

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Background: Chimeric antigen receptor T cell (CAR-T) therapy is a promising treatment using genetically reprogrammed T cells for cancer immunotherapy. However, for genetic engineering of T cells to work, biomolecules like DNA must first be delivered successfully into the nuclei of cells, a process known as transfection. Despite several decades of research, it remains a challenge to transfect T cells with high efficiency while preserving critical biological polyfunctionalities. Gold standard viruses and bulk electroporation which are clinically used to transfect T cells offer low net transfection efficiency. They can also induce adverse immune responses and aberrant cytokine productions, and delay cell proliferation, leading to delayed, less efficacious, and more costly treatments. Emerging methods such as nanoparticles suffer from poorly controllable intracellular release of cargo while microfluidics run into high operating costs from use of biomolecule concentrates in continuous flow. **Methods:** Here, we describe the magnetic nano-electro-injection (MagNEI) platform, an improved technology for T cell immunoeengineering (Fig. 1a, the insert is a scanning electron image of nanochannels). During MagNEI transfection, localized electric fields transiently open pores on the membranes of cells magnetically stabilized onto hollow nanochannels. The electric fields then electrophoretically inject DNA into T cells. Once DNA enters the cells, magnetic forces are applied *via* FDA-approved Dynabeads to transport them into the nuclei. **Results:** MagNEI provided 50% net transfection efficiency for long-term, stable expression of GFP *i.e.* 3-4 folds better than gold standard AAVs and Lonza bulk electroporation (Fig. 1b). While viruses and Lonza bulk electroporation adversely reduced T cell proliferation by 20-30% (Fig. 1c) and T cell migration towards chemoattractant IP-10 by as much as 80% (Fig. 1d), MagNEI did not. Unlike viruses and Lonza bulk electroporation, MagNEI did not cause significant increase in IL-6 cytokine productions or changes in gene expression relative to untreated control T cells (data not shown). **Conclusions:** Our results demonstrate the technical and biological superiority of MagNEI as a transfection method - higher efficiency with minimal perturbations to critical biological attributes including

cytokine production, cell proliferation, trafficking, and gene expression. We envision that the use of MagNEI platform can overcome transfection difficulties in laboratories and clinics for genetic engineering of sensitive, primary immune cells to advance immunobiology and cancer immunotherapies.



Tools and Approaches for Inborn Errors of Metabolism

78. Nuclease-Free Promoterless Genome Editing for Wilson Disease

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Wilson Disease (WD, OMIM #277900) is a life-threatening rare autosomal recessive disorder of copper homeostasis, caused by mutations in *ATP7B* copper transporter, and characterized by toxic copper accumulation resulting in severe and progressive liver and brain diseases. WD represents an attractive target for liver-directed gene therapy. However, classic gene replacement strategies hold limitations associated to adeno-associated viral vector (AAV) cargo capacity, decline of therapeutic effect due to transgene dilution and genotoxic risk, particularly in children. We applied a nuclease-free genome editing approach, based on AAV-mediated targeted integration of a promoterless *ATP7B* cDNA into the albumin locus. To this aim we generated an AAV2/8 vector bearing a codon-optimized human mini-*ATP7B* cDNA flanked by two mouse *Alb* homology arms and preceded by a sequence encoding for a 2A peptide derived from porcine teschovirus-1 (AAV-*Alb*-miniATP7B). Intra-venous injection of AAV-*Alb*-miniATP7B at the dose of 2.3×10^{13} gc/kg in *Atp7b*^{-/-} pups and adult mice resulted in a complete rescue of survival. At sacrifice, these mice showed extensive liver repopulation by genome edited hepatocytes, associated to an amelioration of liver injury and rescue of serum ceruloplasmin oxidase activity. Furthermore, we combined promoterless nuclease-free genome editing with the administration of D-penicillamine, a copper chelator currently used for the therapy

of WD. *Atp7b*^{-/-} mice treated with D-penicillamine and AAV-AlbminiATP7B showed a significant improvement of liver pathology and reduction of copper storage compared to *Atp7b*^{-/-} mice administered with chelation therapy alone. In summary these results indicate that promoterless nuclease-free genome editing provide a significant and sustained therapeutic benefit in WD and may represent a safer alternative to classic gene replacement strategies.

79. Systemic Gene Therapy Using the Novel Adeno-Associated Viral Vector 44.9

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Background: Here we explore a novel capsid, AAV44.9, isolated from a laboratory stock of a simian adenovirus, as a candidate vector for systemic gene therapy. AAV44.9 vectors have previously been established to transduce acinar cells in the salivary glands and mediate highly efficient cone and rod transduction in macaques. However, the close phylogenetic relationship between AAVrh8R and AAV44.9 suggested that AAV44.9 vectors could be a potent in systemic human gene therapy applications, and led us to assess the biodistribution of AAV44.9 reporter vectors in mice, conduct proof of concept studies in mouse models of the lethal metabolic disorder methylmalonic acidemia (MMA), and finally, survey neutralizing antibody (NAb) titers in serum collected from both healthy adult blood donors as well as a large cohort of patients with MMA studied as part of a dedicated natural history study at the NIH (clinicaltrial ID: NCT00078078). **Methods:** To assess biodistribution of AAV44.9 following systemic delivery, adult Tomato floxed-GFP mice were treated by tail vein injection (dose 1e12 GC/mouse) with AAV44.9 carrying either Cre-recombinase or a luciferase reporter, followed by subsequent detection of mTomato and GFP using confocal microscopy (liver, heart) or the measurement of luminosity. The therapeutic efficacy of AAV44.9 gene delivery was examined in two murine models of MMA, representing neonatal lethal (*Mmut*^{-/-}) and juvenile phenotypes (*Mmut*^{-/-} MCK-*Mmut*⁺). An AAV44.9 CBA-Mmut vector was administered by retroorbital injection at doses of 2e11vc/pup and 7e12vc/kg in the respective models. Survival, weight gain, metabolic correction, transduction (ddPCR), and transgene expression (qPCR and Western blotting) were measured in the treated MMA mice and compared to the respective untreated mutants and littermate controls. Neutralizing antibody assays were performed by incubating serum samples, diluted 1:16 then six further 1:2 dilutions, with AAV44.9, AAV9, and/or AAV2 luciferase reporters, followed by infection of COS cells, and the subsequent measurement of luminescence. A titer 1:32 reflected the presence of NAb. **Results:** Confocal microscopy confirmed close to 100% red to green fluorescent transition in the liver and heart 4 weeks after AAV44.9 Cre treatment, and independently, mice treated with the related AAV44.9 luciferase cassette had reporter signal overwhelmingly localized in the region of the heart and liver. AAV44.9 CBA-Mmut treatment rescued *Mmut*^{-/-} mice from lethality, lowered disease related metabolites, increased growth in *Mmut*^{-/-} MCK-*Mmut*⁺ mice, enabled efficient transduction of the liver and heart, and resulted in robust hepatic expression of MMUT.

AAV44.9 seropositivity measured in serum from a cohort of healthy volunteers (n = 19, mean age 47+/-11 years old) was 11% as compared to AAV2 (36%) at a titer of 1:32. Similarly, the seroprevalence of anti-AAV44.9 antibodies at a titer of 1:32 in the entire MMA cohort, all with biallelic lesions in the *MMUT* gene (n=48), was 12% and comparable to that of AAV9 seroprevalence, which was 9%. **Conclusions:** In summary, we establish that AAV44.9 has pronounced hepato-cardiac tropism, is highly efficacious in the treatment of clinically severe MMA mouse models, and exhibits a low prevalence of pre-existing NAb in humans, both in healthy adult blood donors and patients with MMA. Vectors based on AAV44.9 are therefore promising for the treatment of MMA and, by extension, related metabolic disorders, such as propionic acidemia.

80. Single, Systemic Administration of BEAM-301 Mitigates Fasting Hypoglycemia and Restores Metabolic Function in a Transgenic Mouse Model of Glycogen Storage Disease Type Ia

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Glycogen storage disease type Ia (GSD-Ia) is an inborn error of metabolism caused by mutations in the *G6PC* gene that inactivate glucose-6-phosphatase- α (G6Pase- α or G6PC), a key enzyme in regulating interprandial euglycemia. GSD-Ia patients exhibit impaired glucose homeostasis and life-threatening fasting hypoglycemia. The most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients is G6PC-p.R83C containing a single G to A transition mutation in the *G6PC* gene. Adenine base editors (ABEs) enable the programmable conversion of A•T to G•C in genomic DNA. Here, we evaluated the efficacy of a novel ABE to correct the G6PC-p.R83C mutation in a transgenic mouse model of GSD-Ia, homozygous for human G6PC-p.R83C (huR83C) and deficient of G6Pase- α activity. Given the high rate of neonatal lethality in huR83C mice, subjects were treated shortly after birth with BEAM-301, a lipid nanoparticle (LNP) containing guide RNA and mRNA encoding ABE. BEAM-301-treated mice grew normally to at least 35 weeks of age without hypoglycemia-induced seizures and exhibited base-editing efficiencies of up to ~60% in total liver extracts. Notably, single digit percentage base-editing rates were sufficient to restore physiologically relevant levels of hepatic G6Pase- α activity, normalize serum metabolites, and most importantly prevent hypoglycemia during a 24h fast. A preliminary assessment of guide-dependent off-targets in primary human hepatocytes and guide-independent DNA deamination in immortalized cells indicated a low risk profile for the ABE and guide RNA used in these proof-of-

concept studies. In summary, these data demonstrate clinically-relevant correction of G6PC-p.R83C and support further development of BEAM-301 as a potential one-time treatment for GSD-1a.

81. CRISPR-Mediated Insertion of a Targeted GAA Transgene into Hepatocytes Provides Effective, Long-Lasting Gene Therapy in Neonate and Adult Pompe Disease Mice

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Pompe disease is a lysosomal disease characterized by progressive skeletal and cardiac myopathy due to loss of lysosomal alpha glucosidase (GAA) and accumulation of lysosomal glycogen. AAV-based gene therapies using the hepatocytes as an *in vivo* depot for production of GAA are in clinical trials. However, these technologies utilize AAV episomes to express GAA, which will be lost as hepatocytes divide due to liver growth or damage. Thus, liver-directed episomal gene therapy is not appropriate for treating the significant pediatric population of Pompe patients. Furthermore, GAA has poor tropism to muscle tissues, which necessitates high levels of secreted GAA to mediate uptake in muscles. We have previously shown that enzyme replacement therapy using a targeted GAA, where GAA uptake into muscles is aided by an antibody fragment, improves delivery to muscle tissues and corrects lysosomal glycogen storage in Pompe mice (Baik *et al.*, *Mol Ther* 2021). Here, we present a CRISPR-mediated insertion approach for Pompe disease, where a transgene is inserted into the first intron of the albumin locus to express targeted GAA under control of the endogenous albumin promoter. The insertion template DNA 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. We show that this insertion approach and liver-directed episomal AAV gene therapy correct Pompe phenotypes in **adult** mice. In addition, insertion into **neonatal mice** results in stable expression throughout a 15 month study, whereas expression from episomal AAV decreases over time. This neonatal insertion treatment leads to glycogen normalization in muscles and improved muscle strength 3 months and 15 months post dosing. Promisingly, 2-3 year old cynomolgus monkeys treated with CRISPR-mediated insertion express the targeted GAA transgene. This technology may be an alternative approach to treat Pompe disease in both late and infantile onset forms of the disease.

82. A Novel Human Liver Chimeric Mouse Model Lacking the Murine AAVR Gene for Validation of AAV Gene Therapy Vectors

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Adeno-Associated Virus (AAV) gene therapy has been extensively studied in preclinical animal models prior to clinical translation. However, AAV biology can display markedly significant differences between humans and experimental animal models in regards to tissue tropism, cellular uptake, entry into the nucleus, uncoating, second strand synthesis of the recombinant vector genome, transcript levels and eventually, transgene expression. Human liver chimeric mice helped to generate new insight into AAV biology and liver gene therapy applications in general. However, one of the main drawback of such models is that most of the clinically used AAVs display enhanced tropism for murine hepatocytes over human cells. As we have previously shown by injecting several AAV serotypes, most of the murine part of the chimeric liver is significantly transduced with AAVs, while only a low percentage of human hepatocytes express the AAV transgene. This limits the utility of conventional human liver chimeric mouse models and makes it difficult to model AAV vector biology and therapeutic efficacy that is relevant to a human setting. Recently, a canonical AAV receptor gene (*AAVR*) mediating entry of the most commonly used serotypes in gene therapy was identified. Here, by deleting early exons of the murine *AAVR*, we generated a novel TIRFA (*Transgene free Il2rg^{-/-}/Rag2^{-/-}/AAVR^{-/-}*) mouse strain, which can be highly repopulated with primary human hepatocytes expressing the cognate human *AAVR*. When injecting highly humanized TIRFA mice with AAV9 or AAV8 vectors carrying a tdTomato transgene driven by a ubiquitous promoter, only human hepatocytes were transduced while the rest of the murine liver and remaining organs were negative for transgene expression. Results from ongoing studies focused on vector biodistribution and efficiency of human hepatocyte transduction will be presented. The TIRFA mice can also be repopulated with hepatocytes from human patients with metabolic liver disorders, demonstrating its potential for human AAV gene therapy in a better humanized setting.

83. Lentiviral-Based Genetic Correction of IL10RB-Defect to Treat Very Early Onset-Inflammatory Bowel Disease

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Mutations in the IL-10 gene or its receptor chains lead to life-threatening Very Early Onset-Inflammatory Bowel Disease (VEO-IBD) usually within the first months of life. In these cases, allogeneic stem cell transplant is the only curative option, leading to prolonged disease remission and survival. Gene therapy represents an attractive therapeutic alternative, through autologous transplantation of genetically corrected hematopoietic stem and progenitor cells (HSPCs). We developed a lentiviral vector-based gene therapy approach for congenital defects of the murine IL-10 receptor beta chain (*mll10rb*). Genetic correction of *mll10rb* knock-out (KO) lineage negative (Lin⁻) by lentiviral vectors restores responsiveness to IL-10 *in vitro* as measured by phosphorylation of STAT3 in differentiated macrophages. Transplantation of gene-corrected Lin⁻ cells with expression of *mll10rb* gene under the constitutive PGK promoter leads to amelioration of IBD in *mll10rb*^{-/-} mice. Mice treated with gene therapy or with wild-type (WT) cells recovered their body weight within 20-60 days post-transplant, while mice treated with mock vector transduced *mll10rb* KO cells progressively lost weight. This was accompanied by a corresponding reduction of peripheral blood neutrophils from 56 to 40% in mock and gene therapy-treated animals, indicating normalization of myelopoiesis in response to reduced colitis-associated systemic inflammation in this mouse model. Analyses 60-80 days post-transplantation revealed lengthening of the colon after gene therapy by 20% relative to the mock group, colon histopathological score improvement, and increase of anti-inflammatory macrophages at the expense of the inflammatory counterpart reduced by 2.7x in the lamina propria. However, we observed a progressive decrease of gene marking over time in peripheral blood, from 90% *in vitro* to 60% 8 weeks post-transplantation. This effect was confirmed in competitive transplantation assays using mock vector control versus the therapeutic PGK-mll10rb. Gene marking was relatively preserved in the immature HSC/MPP1 fraction, but a pronounced 2-fold reduction of gene marking was seen in committed progenitor subpopulations of the bone marrow and downstream mature cell lineages. This finding is consistent with the hypothesis that IL-10 signaling mediates an antiproliferative response in progenitor cells, complementing the anti-inflammatory function it has in immune effector cells. We next investigated whether the competitive disadvantage of gene-corrected HSPC could be bypassed by expressing *mll10rb* under the myeloid-specific promoter CD68. In a competitive setting, we found that the CD68-mll10rb vector led to stable gene marking. Interestingly, myeloid-specific expression was sufficient to mediate clinical IBD improvement comparable to animals receiving PGK-based gene therapy and, most importantly, I10rb-WT Lin⁻ cells in terms of weight gain, and colon histopathology.

In conclusion, we demonstrated the therapeutic efficacy of a lentiviral-based HSPC gene therapy approach in a relevant mouse model of VEO-IBD, comparing both constitutive and myeloid-specific promoters and demonstrating that myeloid-specificity might be superior to achieve correction of the clinical features.

84. Ex Vivo Editing of Hematopoietic Stem Cells for Erythroid Expression of Therapeutic Proteins In Vivo for LAL-D Therapy

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Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive disorder caused by mutations in the *LIPA* gene. LAL is involved in lysosomal hydrolysis of cholesterol ester and triglyceride and its deficiency triggers fats accumulation in organs and tissues leading to multi-organs failure. The most severe form of LAL-D (Wolman disease, WD; $\leq 5\%$ LAL activity) affects $\sim 1/300,000$ live births and, if not treated, results in premature death within the first year of life due to malnutrition and hepatosplenomegaly. 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 risk of developing neutralizing antibodies that can decrease treatment efficacy. In order to develop a curative treatment for LAL-D, we assess hematopoietic stem cells (HSCs) based gene therapy (GT) for erythroid-specific expression of LAL into bloodstream. Using CRISPR-Cas9, we integrated LAL cDNA under the control of the endogenous α -globin promoter to redirect a fraction of the striking globin synthesis capacity of erythroid cells ($\sim 7.2\text{gr/day}$) for robust therapeutic LAL expression by the erythroid lineage (Pavani, et al, Nat. Commun. 2020). Secreted LAL enzyme retained enzymatic activity and cross-corrected the lipid accumulation in WD patients' fibroblasts. In addition, we transplanted modified HSCs into NSG immunodeficient mice and confirmed their homing, engraftment and multilineage differentiation, with the notable exception of erythroid cells due to model limitations. To achieve *in vivo* erythroid differentiation and protein expression, we moved to a mouse model humanized for the α -globin locus. We confirmed the presence of the human HBA locus via DNA sequencing (nanopore) and protein expression (HPLC). Then, we optimized the editing and transduction of murine (m)HSCs and we are currently analysing the level of LAL expression in the blood of mice transplanted with edited mHSCs. To further increase LAL expression and secretion, we engineered LIPA cDNA sequence by changing its signal peptide sequence and optimizing the codon usage. The optimized sequence leads to an 8-fold increase in enzymatic activity in the medium of erythroid-differentiated HSPCs. To investigate the therapeutic potential of this strategy, we take advantage of a recent LAL-D mouse model. We first confirmed the loss of LAL protein and activity leading to a cholesterol and triglyceride accumulation in liver, and we are currently transplanting this LAL-D mouse with edited humanized mHSCs to evaluate *in vivo* the therapeutic benefit of our HSC based platform.

Overall, using two different transgenic mouse models and engineering LAL cDNA, we are assessing the potential of a CRISPR based HSC gene therapy platform for erythroid mediated protein expression for ERT.

CAR-T Cells and Beyond

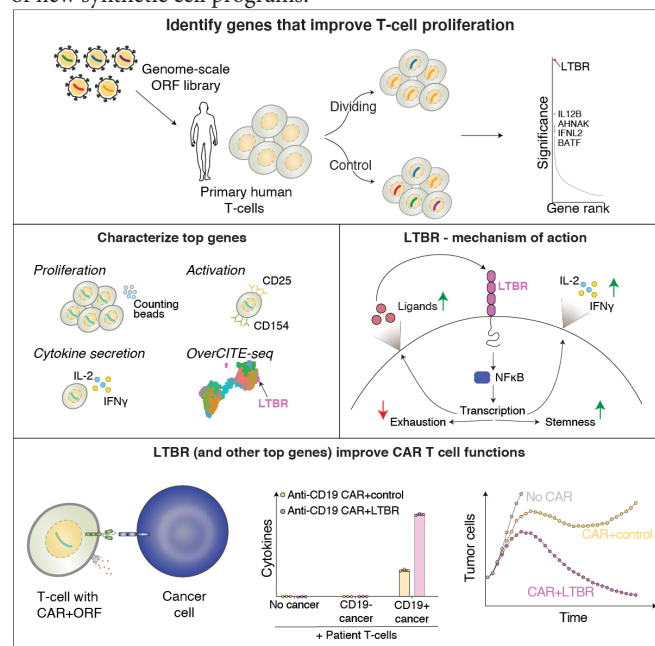
85. A Genome-Scale Screen for Synthetic Drivers of T Cell Proliferation

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The engineering of patient T-cells for adoptive cell therapies has revolutionized the treatment of several cancer types. However, further improvements are needed to increase durability and response rate. While CRISPR-based loss-of-function screens have shown promise for high-throughput identification of genes that modulate T-cell response, these methods have been limited thus far to negative regulators of T-cell functions, and raise safety concerns due to the permanent nature of genome modification. Here we identify positive T-cell regulators via overexpression of ~12,000 barcoded human open reading frames (ORFs). Using this genome-scale ORF screen, we find modulator genes that may not normally be expressed by T-cells. The top-ranked genes increased primary human T-cell proliferation, activation, and secretion of key cytokines. In addition, we developed a single-cell genomics method for high-throughput quantification of the transcriptome and surface proteome in ORF-engineered T-cells (OverCITE-seq). Using OverCITE-seq, we discovered that overexpression of the top-ranked ORF, lymphotoxin beta receptor (LTBR), in primary T-cells results in induction of a distinct transcriptional program. Interestingly, LTBR is not naturally expressed by lymphocytes but is present in myeloid and stromal cells, playing an important role in formation of tertiary lymphoid structures. Following up on the transcriptomic results, we showed that LTBR overexpression results in an increase in T-cell stemness and effector functions, as well as resistance to apoptosis and exhaustion in chronic stimulation settings. This remodeling of T-cell programs was accompanied by extensive changes in chromatin accessibility, particularly at sites enriched for NF-kappaB binding motifs. Using mutagenesis and epistasis approaches, we demonstrated that LTBR constitutively activates both the canonical and the non-canonical NF-kappaB pathways via ligand short-circuiting and tonic signaling - but only the canonical pathway activation contributes to the observed phenotype. Expression of several top-ranked genes, including LTBR, improved antigen-specific chimeric antigen receptor (CAR) T-cell responses in healthy donors and cancer patients *ex vivo*. This effect was observed regardless of the costimulatory domain used in the CAR (CD28 or 4-1BB) or the targeting moiety (specific for either

CD19, a target in B-cell cancers, or mesothelin, a target in a range of solid tumors, including pancreatic ductal adenocarcinoma). Finally, the top-ranked genes discovered in alpha beta T-cells also improved antigen-specific responses of gamma delta T-cells, highlighting the potential for cancer-agnostic therapies. Our results provide several strategies for improving next generation T-cell therapies via induction of new synthetic cell programs.



86. CD45RA Expressing PBMCs Effect the Outgrowth of Epstein-Barr Virus Antigen Specific T-cells

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Introduction: In our clinical trial NCT01555892 using Epstein-Barr Virus (EBV) specific T-cells (EBVSTs) for the treatment of EBV+ lymphoma, we were unable to successfully manufacture EBVSTs from ~25% of patients. Failures resulted from lack of antigen specificity and/or failure to grow, likely because memory EBV Ag-specific T-cells are eliminated or rendered dysfunctional in the immunosuppressive lymphoma microenvironment. Since many EBVSTs from patients proliferate, but lack antigen specificity, we sought to reduce competitive bystander outgrowth by depleting the CD45RA+ fraction of PBMCs that contains naïve T-cells, T-regs and NK-cells prior to initiation. EBVSTs derived from CD45RA depleted (RAD)-PBMCs (RAD-EBVSTs) showed increased EBV Ag-specificity, enhanced proliferation, polyfunctionality, and cytotoxicity compared to EBVSTs derived from whole (W)-PBMCs (W-EBVSTs). RAD-EBVSTs also demonstrated more rapid EBV+ tumor clearance in NSG mice. **Most**

notably, CD45RA depletion enabled the outgrowth of EBVSTs from previously unresponsive lymphoma patient PBMCs. This unexpected finding suggested that CD45RA+ cells inhibit the reactivation and expansion of EBV Ag specific T-cells. Hence, **we hypothesized that CD45RA+ cells exert inhibitory effects on the outgrowth of stimulated memory EBV specific T-cells** and here we sought to understand the source and mechanism of this inhibition. **Methods:** To pinpoint the CD45RA+ subset that inhibited EBVSTs, we separated CD45RA+ PBMCs into CD3+ /CD3- cells, CD3- CD56+ NK cells, CD3+ CCR7+ naïve T_N-cells, CD3+ CCR7- terminally differentiated T_{EMRAS} and CD3+ CCR7+ CD95+ (putative) T_{SCMs}, and added them individually to CD45RA depleted (RAD)-PBMCs, then expanded EBVSTs for 16 days by stimulation with peptide libraries and cytokines. We then evaluated their fold expansion and antigen specificity using g-IFN ELISpot assays. To elucidate the effects of CD45RA+ PBMCs on clonal expansion, we sequenced TCR Vβ CDR regions of W- and RAD-EBVSTs and evaluated their clonal composition. **Results:** Initial add-back experiments suggested that the CD3+ fraction of CD45RA+ PBMCs contained the inhibitory activity, while NK cells and myeloid cells had no effect. Further fractionation of CD3+ CD45RA+ PBMCs suggested potentially T_N cells but not T-regs, T_{EMRAS} or T_{SCMs} inhibited the reactivation and clonal expansion of Ag-specific memory T-cells. Further experiments are however, required to confirm the effects of T_N cells. TCR-β chain sequencing revealed a distinct clonal repertoire in RAD-EBVSTs with dominant clonotypes not present in the 50 most abundant clonotypes in W-EBVSTs, despite their presence in W-PBMCs. W-EBVSTs showed greater clonal similarity to that of W-PBMCs and EBVSTs derived from CD45RA+ PBMCs that lacked apparent EBV specificity. **Conclusion:** The less selective clonal expansion of W-EBVSTs associated with reduced antigen specificity suggests that CD45RA+ cells inhibited the outgrowth of antigen-stimulated memory T-cells, which would otherwise grow in RAD-PBMCs. Such inhibitory effects could represent a physiological strategy to prevent the oligoclonal outgrowth of cross-reactive memory T-cells in response to infections with new pathogens. Further experiments are required to elucidate the mechanisms underlying the effects of CD45RA+ cells on Ag-specific T-cells. RAD-EBVSTs are currently under evaluation in clinical trials NCT01555892 and NCT04288726 for the treatment of lymphoma.

87. IFNγ Impedes Antigen-Specific Proliferation of CAR-T with a CD28, but Not 4-1BB, Costimulatory Domain

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Background: Chimeric antigen receptor T cells (CAR-T) have yielded impressive responses in patients with hematologic malignancies but can be unsuccessful if the engineered T cells are incapable of mounting an adequate response or persisting long-term. CAR-T constructs containing CD28 costimulatory domains demonstrate heightened antigen sensitivity and cytotoxic activity compared to 4-1BB but have

elevated expression of immune checkpoint proteins and reduced persistence in the clinic. A recent report showed that interferon-gamma (IFNγ), a pro-inflammatory cytokine commonly used as a potency assay for CAR-T cells, is not required for clearance of leukemia or lymphoma by CD19-targeting CAR T cells bearing 4-1BB (BBζ) or CD28 (28ζ) costimulatory domains. However, loss of IFNγ yielded significantly greater antigen-specific proliferation of 28ζ, but not BBζ, CAR T cells. Paradoxically, previous reports suggest that IFNγ is a driver of natural T cell proliferation, but the effect of this cytokine on CAR-T expansion has yet to be studied. Given the differential effect on CAR-T proliferation in the absence of IFNγ, we sought to determine the mechanism by which IFNγ impedes the expansion of 28ζ CAR T cells. **Results:** Pharmacologic blockade or genetic knockout of IFNγ using CRISPR/Cas9 editing was used to generate IFNγ-deplete CAR-T as confirmed by ELISA and flow cytometry. IFNγ knockout CAR-T (IFNγKO) were combined with Nalm6 leukemia cells in the presence or absence of donor-matched macrophages capable of amplifying the IFNγ signal. As expected, 28ζ IFNγKO CAR-T cultures had significantly greater proliferation in response to antigen than control 28ζ cells while BBζ CAR-T expanded similarly regardless of IFNγ status. This phenomenon was not solely caused by the reduced expression of immune checkpoint proteins as CTLA-4, Lag3, PD-1, and Tim-3 were reduced on both BBζ and 28ζ IFNγKO CAR-T. Transcriptional analysis of 28ζ IFNγKO CAR T cells showed upregulation of genes involved in T cell survival and expansion (*BATF*, *BCL2L1*), memory (*OPA1*, *LEF1*), lipid synthesis (*FASN*), fatty acid oxidation (*SLC25A20*) and protection from oxidative stress (*PYCR2*), and downregulation of genes involved in glycolysis (*SLC2A1*, *NEK2*, *ALDOC*, *PDK1*, *PGK1*, *LDHA*), immune checkpoint interactions (*CTLA4*) and T cell anergy and dysfunction (*NFIL3*, *NR3C1*, *SLAMF6*) compared to 28ζ control CAR-T. These changes were 28ζ-dependent as they were not observed in cultures given BBζ IFNγKO or BBζ control CAR-T. **Conclusions:** Collectively, these data suggest that IFNγ hinders the antigen-specific proliferation of 28ζ CAR-T through a combination of glycolytic skewing, inhibitory immune checkpoints, and T cell anergy. These findings highlight an important consequence of IFNγ production that can be targeted to improve 28ζ CAR-T proliferation and persistence in the clinic.

88. Naturally Occurring CD7- T Cells Mark a Functional Effector and Persistent CAR T Cell Population

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Background Expanding the availability of CAR T cells for T-cell leukemias has been challenging due to an increased overlap in antigen expression between blasts and healthy T cells. We previously reported

on using a two-step magnetic bead manufacturing process to select naturally occurring CD7⁻ T cells. We have successfully expressed a CD7-CAR on these cells and have targeted T-ALL with robust antitumor activity. However, other investigators have described CD7⁻ T cells as dysfunctional. Here we compare functional differences between CD7⁻ and bulk T cells in a CD19-CAR T cell model. **Methods and Results** We generated CD19-CAR^{Bulk} and CD19-CAR^{CD7⁻} T cells using a second-generation retroviral vector. We found no significant difference in CD19-CAR expression on bulk and CD7⁻ T cells (68.97 ± 12.20% vs. 73.75 ± 8.27%, N=10). Immunophenotypic characterization highlighted a predominance of CD4⁺ memory subsets in CD7⁻ T cells when compared to bulk T cells (p<0.001). In serial stimulation assays, CAR T cells were exposed to fresh BV173 cells every 72 hours. CD19-CAR^{Bulk} T cells had antitumor activity for 4-13 stimulations while CD19-CAR^{CD7⁻} T cells killed target cells for 7-14 stimulations (N=3). We also measured cytokine secretion by multiplex analysis. CD19-CAR^{CD7⁻} T cells maintained high levels of secretion (>1,000 pg/mL) of GM-CSF to stimulation 9 and IFN-γ through stimulation 11, whereas GM-CSF and IFN-γ concentrations decreased between stimulations 5-7 in CD19-CAR^{Bulk} T cells. We performed TCR sequencing to determine T cell clonality and show comparable clonal focusing between CD19-CAR^{Bulk} and CD19-CAR^{CD7⁻} T cells. To test anti-tumor activity in an *in vivo* BV173 model, mice received a single iv dose of 3x10⁶ T cells 7 days after tumor injection. CD19-CAR^{Bulk} and CD19-CAR^{CD7⁻} T cells significantly reduced tumor burden compared to tumor-only and non-transduced T cell controls. However, CD19-CAR^{CD7⁻} T cells had significantly greater survival and persistence compared to CD19-CAR^{Bulk} T cells (p<0.001). To gain insight into the behavior of CAR^{CD7⁻} T cells in patients, we mined publicly available single-cell gene expression data from pre- and post-infusion samples from an institutional Phase I study of CD19-CAR T cells for refractory/relapsed B-ALL (NCT03573700). Unsupervised clustering of 184,791 CD19-CAR positive T cells led to the identification of 21 transcriptional clusters functionally annotated based on key expression markers. Clusters with the overall lowest relative CD7 expression and the fewest cells with any expression of CD7 encompassed either dysfunctional or CD4⁺ effector subsets. In addition, we identified distinctly enriched CD7^{lo} populations within post-infusion CD8⁺ functional effector clusters. Corresponding flow cytometric analysis supported an enrichment of CD7-negative T-cell subsets in pre- and post-infusion samples. **Conclusion** Here we demonstrate CAR^{CD7⁻} T cells derived from naturally occurring CD7⁻ T cells have robust antitumor activity, as judged by their ability to expand, persist, and elicit potent antitumor activity against CD19⁺ B-ALL. In addition, CD7^{lo} populations identified in clinical CD19-CAR T cell post-infusion samples were either dysfunctional or highly effector subsets, reconciling findings in our work and in previous literature. In summary, CD7⁻ T cells expressing CAR molecules are promising and warrant further exploration.

89. Tim-4-Chimeric Engulfment Receptor (CER) T Cell Therapy Elicits Phosphatidylserine-Dependent Cytotoxic and Antigen-Presenting Cell-Like Function and Synergizes with Approved BTK Inhibitors for the Treatment of Hematologic Malignancies

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Background: Tumor microenvironments largely suppress immune responses, in part due to dysfunction of antigen presentation. While activated conventional αβ T cells are nominally capable of processing and displaying antigens, they are limited in antigen-presenting capacity due to inefficient antigen capture. To overcome this, we developed Tim-4-CERs, novel synthetic antigen capture receptors designed to facilitate antigen-presenting cell (APC)-like function and impart target-dependent cytotoxic responses. We fused the phosphatidylserine (PS) receptor Tim-4, which plays a central role in cross-presentation in subsets of dendritic cells, with innate signaling domains to drive tumor antigen uptake and enhance APC-like function. Using CER T cells engineered with Tim-4 fused with a toll/interleukin-1 (TIR) domain and the T cell-derived signaling domains CD28 and CD3z, we tested for APC-like and cytotoxic responses against Mantle Cell Lymphoma (MCL) cell lines. **Methods:** We engineered novel Tim-4 CER constructs designed to enhance target-dependent phagocytosis and cytotoxicity. Using an engineered TMEM30a phospholipid flippase knockout JeKo-1 lymphoma cell line with constitutive PS exposure (JeKo-1 PS⁺), we evaluated tumor fragment uptake, cytotoxicity, cytokine secretion (GrB, IFN-g, and TNF-a), and APC-like activity *in vitro*. JeKo-1 PS⁺ cells were labeled with the pH-sensitive pHrodoTM dye to quantify the uptake of tumor cells. CER-T cells were tested for antigen-display capacity by evaluating autologous HPV E7 TCR T cell activation and proliferation following CER-T co-culture with JeKo PS⁺ cells and HPV-derived peptides. Finally, CER T cell anti-tumor activity was examined in xenografted MCL models. Statistical analysis was performed using 2-way ANOVA. **Results:** We engineered CER-1234 and CER-1236 T cells that readily express Tim-4 on the cell surface and differ in their orientation of the TIR signaling domain relative to the T-cell derived signaling domains CD28 and CD3z. Both CER T cells showed increased uptake of JeKo-1 PS⁺ tumor cell fragments (p < 0.001) upon co-culture *in vitro*. Internalization of pHrodo⁺JeKo-1 fragments was dependent on PS binding, and treatment with Cytochalasin D, an inhibitor of actin polymerization, or Bafilomycin A, a lysosome inhibitor, blocked tumor cell fragment uptake (p < 0.0001 and p < 0.0001). In BTKi-primed REC-1 cells, CER-1234 internalized fragments were localized to lysosomal compartments. Upon *in vitro* co-culture with JeKo-1 PS⁺ cells, both CER T cell constructs displayed cytotoxic function, with CER-1234 eliminating 90% of targets at 96 hrs at low effector:target (E:T) ratios. BTKi treatment in combination with CER-1234 exhibited > 90% cytotoxicity, with production of Th1 cytokines (GrB, IFN-g, and TNF-a). Lastly, CER T cells were evaluated for APC-like activity *in vitro*. HPV E7 TCR T cells showed a significant increase in proliferation (p < 0.05)

at 72h relative to controls, indicating Tim-4 CER T cells are capable of processing, displaying, and triggering proliferation of antigen-specific T cells. *In vivo*, CER T cells reduced tumor burden relative to controls in xenograft models of MCL, with no overt morbidity observed.

Conclusion: Tim-4 CER T cells are a novel approach to overcome antigen presentation defects in tumor microenvironments by enhancing antigen acquisition, APC-like function, and cytotoxic responses. CER T cells exhibit these combined functions *in vitro*, synergize with BTK inhibition, and mediate significant anti-tumor effects *in vivo* against a clinically relevant model of mantle cell lymphoma.

90. Peptide-scFv Bispecific CAR-T Cells Targeting Acute Myeloid Leukemia

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Background Target antigen selection for acute myeloid leukemia (AML) immunotherapy is challenging due to significant overlap between normal hematopoietic progenitor cells (HPCs) and blasts. We have demonstrated AML blasts express cell-surface GRP78 and CD123. To bypass immune escape mechanisms, the goal of this project was now to design a bispecific CAR and evaluate T cells expressing these CARs in preclinical models.

Methods and Results Bispecific CARs that rely on using two signaling chain variants (scFVs) as antigen binding domains, often do not fold properly due to scFv/scFv interaction. We therefore took advantage of a peptide that recognizes GRP78 and a CD123-specific scFv for our bispecific antigen binding domain, and designed four bispecific CARs with different linkers: short (G4S)3, long (*mutIgG4*), 2 rigid (B2m, GPcPcPc) [G4S-, *mutIgG4*-, B2m-, GPc-CAR T cells). Bispecific CAR T cells were generated by retroviral transduction and > 85% of T cells expressed CARs for all constructs (N=4). All bispecific CAR T cell populations recognized and killed GRP78⁺/CD123⁺ tumor cells (KG1a, MOLM13) as evidenced by sustained antitumor activity and cytokine secretion compared to controls (p<0.01, N=4). However, only (G4S)3-, *mutIgG4*-, B2m-CAR T cells recognized GRP78⁺/CD123- targets. We first evaluated the anti-AML activity of G4S-, *mutIgG4*-, and B2m-CAR T cells *in vivo* against AML cells that express both target antigens (MOLM13); mono-specific CAR T cells served as a control. A single infusion of 3x10⁶ CAR T cells had potent anti-AML activity, resulting in a significant survival advantage (p<0.001, N=5-15). Leukemia recurred in all mice treated with monospecific-CAR T cells on average by day 40 and *mutIgG4*- by day 35, while G4S- and B2m-CAR T cells induced complete responses in 7/15 and 6/15 treated mice, respectively. Extramedullary disease (chloromas) was the main mechanisms of relapse, and chemokine expression analysis of chloromas and chemokine receptor expression on CAR T cells are in progress to provide further mechanistic insights. Based on these studies, we selected one of the top performing bispecific CARs (G4S-CAR) for further evaluation. To demonstrate that the GRP78-specific peptide and the CD123-specific scFv do not interfere with binding of their respective antigen, we analyzed immune synapse

formation of G4S-CAR and monospecific CAR T cells against KG1a and GRP78⁺/CD123⁺ KG1a^{CD123.KO} cells. We observed no difference in synapse formation as judged by lysosome or F-actin recruitment to the synapse within 30 minutes of activation, indicating that the (G4S)3 linker enables both antigen binding domains to interact with their respective target antigen. Finally, we evaluated mono- and G4S-CAR T cells in GRP78⁺/CD123⁺ KG1a and KG1a^{CD123.KO} xenograft models. In both models, there was a significant reduction in tumor burden and survival benefit in mice treated with G4S-CAR T cells compared to tumor only (p<0.01), demonstrating that (G4S)3-linker preserved the antigen recognition of bispecific CARs to GRP78 and CD123 also *in vivo*.

Conclusions We demonstrate here that it is feasible to target two antigens on AML cells with a peptide-scFv bispecific CAR design, and that these bispecific CAR T cells not only prevent immune escape, but also have improved antitumor activity compared to their mono-specific counterparts when both antigens were expressed. This has been rarely observed for scFv-scFv-based bispecific CARs, in which normally one of the scFvs dominate. Thus, peptide-scFv-based CARs present a promising bispecific CAR design to prevent immune escape for a broad range of malignancies.

91. CD28-Based B7-H3 CAR T Cells Have Superior Anti-glioma Efficacy in an Immune Competent Glioma Model While Suppressive Macrophages at Tumor Edges Are Associated with Therapeutic Resistance

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Introduction. We and others have demonstrated that B7-H3-CAR T cells have potent antitumor responses in xenograft models for brain tumors. However, their effect is limited in immune-competent mouse models indicating a suppressive role of the tumor immune microenvironment (TIME) on CAR T cell therapy. Thus in this study, we set to optimize B7-H3 CAR structure and examine effects of the TIME on CAR design using the GL261 immunocompetent glioma model. **Methods and Results.** We generated a library of second-generation murine B7-H3-specific CARs with different transmembrane (CD8, CD28), costimulatory (CD28, 4-1BB), and activation (ζ , *mut* ζ) domains. We then compared the cytolytic activity, expansion, and anti-tumor activity of T cells expressing these CARs. Results showed that T cells expressing B7-H3 CARs with CD28 transmembrane/costimulatory domains have superior efficacy compared to all other CARs we tested *in vitro*. Yet, providing optimized costimulation and signaling (CD28.*mut* ζ) did not induce superior anti-glioma efficacy of B7-H3 CAR T-cells *in vivo*. Thus, we next investigated whether incorporating 4-1BB signaling into CD28-based CAR T cells by expressing 4-1BBL on their cell surface enhances the therapeutic efficacy and persistence of B7-H3 CAR T-cells. We found that in repeat stimulation assays, surface expression of 4-1BBL enhanced expansion of B7-H3 CAR T-cells at least 300-fold more than T-cells with CD28 or 4-1BB costimulatory domains alone. Additionally, 4-1BBL expression

significantly enhanced the sequential killing capacity compared to CD28- or 41BB-based B7-H3 CAR T-cells. However, transgenic expression of 4-1BBL in CD28-based CARs was not associated with further enhanced anti-glioma efficacy of B7-H3 CAR T-cells *in vivo*. To understand the contribution of the glioma TIME to this therapeutic failure, we next investigated the immune composition of the TIME suppressing CAR T cell functions. Tumor samples were collected from non-responder (NR) mice at the endpoint and used for spatial transcriptomic analysis. High dimensional flow cytometry was also used to validate our findings. These analyses revealed that i) infused CAR T cells were not detectable in NRs at the endpoint; ii) lymphoid populations represented a very small fraction of immune infiltrates (15-20%) compared to myeloid cells (45-60%); iii) monocyte-derived macrophages (MDM) formed a tight ring surrounding the tumor edges while microglia-derived macrophages (MiDM) were dispersed around and within the tumor; iv) MDMs expressed predominantly suppressive protumorigenic signatures while MiDMs expressed inflammatory anti-tumor signatures; and (v) recruitment and spatial localization of described immune populations were only observed in NR mice treated with functional CARs and not in mice treated with control CARs. **Conclusions.** To our knowledge, this is the first study to comprehensively evaluate the functions of distinct CAR domains in an immune-competent brain tumor model. Results demonstrate that CD28-based CARs have the best overall performance while transgenic expression of 4-1BBL has the potential to further enhance their persistence and anti-glioma efficacy. Our TIME-based studies show that suppressive myeloid cells are recruited into brain tumors upon CAR T cell treatment. Studies evaluating the contributions of specific myeloid populations to the limited therapeutic responses and potential inhibition of CAR T cell function are ongoing.

Cancer - Oncolytic Viruses

92. The Potential of Oncolytic Virotherapy in Synovial Sarcoma

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Background: Synovial sarcoma (SS) is a rare and aggressive mesenchymal malignancy that disproportionately affects young adults. Patients with disseminated SS have a median survival of 1 year with the presently available systemic therapies. Little to no responses to immune checkpoint inhibitor-based regimens have been seen in SS patients in clinical trials to date. We have engineered and reported the enhanced anti-tumor effect of measles virus encoding *Helicobacter pylori* neutrophil-activating protein (MV-s-NAP) as a potent immunostimulatory transgene. In this study, we evaluated the infiltrating T-cell immune status in SS tissue samples and tested the oncolytic MV-s-NAP efficacy against SS in a xenograft model. **Methods:** T-cell analysis was run on samples from SS patients treated at our institution under an institutional review board approved protocol. We performed T-cell receptor (TCR) sequencing (Adaptive

Biotechnologies) on archived formalin-fixed, paraffin-embedded (FFPE) specimens to evaluate the relative differences in the infiltrating T-cell fraction repertoire between SS and undifferentiated pleomorphic sarcoma (UPS). We determined the *in vitro* anti-tumor activity of the oncolytic MV-s-NAP against validated monophasic (Fuji, HSSY-II) and biphasic (SYO-1, Yamato-SS) SS cell lines. Subsequently, *in vivo* therapeutic effect was tested in the athymic mouse Syo-1 flank-tumor model following repeated intratumor (i.t.) injection of MV-s-NAP. **Results:** We analyzed 46 patient samples total. Nine patients had UPS. There were 37 SS patient samples (including 9 biphasic and 28 monophasic SS, in 8 of which paired primary and metastatic lesions were available). Patients with SS significantly lower infiltrating T-cell number, lower clonality and higher TCR diversity than those with UPS. There was no difference in the T-cell repertoire between monophasic and biphasic as well as primary and metastatic SS patient samples. MV-s-NAP demonstrated dose dependent killing in all 4 SS cell lines. As typified by our SYO-1 line, infection at multiplicity of infection (MOI) =1 MV-s-NAP induced strong cytopathic effect at 48-72 hrs. of infection with less than 25% viable cells by 120 hrs.). In addition, treatment with MV-s-NAP significantly increased the release of danger associated molecular patterns and cell surface expression of programmed death ligand 1 and major histocompatibility complex I and II in all SS lines as compared with untreated controls. MV-s-NAP showed significant anti-tumor effect in the SYO-1 SS xenograft model with improved median survival of 26 days for the treated mice vs. 19 days for the control group (p <0.0001). **Conclusion:** Oncolytic virotherapy with a MV encoding bacteria-derived NAP as a potent TLR agonist could provide an alternative viro-immunotherapy approach in the treatment of recurrent or disseminated SS. Both *in vitro* and *in vivo* results warrant further exploration on the mechanisms of direct oncolytic MV effect and potential NAP-mediated immunophenotype alteration of the SS tumor microenvironment as a key step in clinical translation to SS patient treatment.

93. Oncolytic Adenovirus Expressing IFN-alpha with Chemoradiation (GEM+nab-PTX) Synergistically Inhibits Pancreatic Cancer Cell Growth In Vitro and In Vivo

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Objectives: In the United States, pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer related death and 5-year survival of PDAC remains lower than 10%. IFN-Alpha (IFN) has multiple functions, including antitumor effect, immunomodulation and sensitizing cancers to radiation. Clinical trials of adjuvant therapy combined with IFN, 5-FU, CDDP, and radiation improved the 5-year survival of patients with PDAC by 35%. However, this also showed the disadvantages of IFN including the high drop-out rate due to the systemic toxicity of IFN as well as insufficient delivery of IFN to the tumor. To improve efficacy and tolerability of IFN therapy, we have developed an oncolytic adenovirus expressing IFN (OAd-IFN). Based on these findings, we evaluated OAd-IFN in combination with chemoradiation. **Methods:** In all experiments, we used HP1 cells, one of the hamster

pancreatic cancer cell line. OAd-IFN has RGD fiber modification and overexpresses Adenoviral Death Protein. Hamster IFN- α gene was placed in the Adenovirus E3 region. We used GEM+nab-PTX, which are common used combination chemotherapy for patients with PDAC. In vitro experiment, we assessed the colony formation assay, the gold standard method to analyze radiosensitivity of cancer cells. Cells were infected with OAd-IFN (0.0625, 0.3 and 0.625 VP/cell) and on the following day, cells were irradiated (0.625, 1.25 and 2.5 Gy). After that chemo drugs are added to media. After 14 days, colony number were counted. Calculation of the Combination Index (CI) to determine synergism ($CI < 1$), antagonism ($CI > 1$), or additive effect ($CI = 1$) between virus, chemotherapy, and radiation was performed. To evaluate the effect of OAd-IFN in the hamster syngeneic tumor, HP1 cells were injected subcutaneously into the backs of the Syrian hamsters. When tumor volume reached to 200 mm³, we performed intratumoral viral injection (OAd-IFN: 2.5x10⁹ PFU). 3 days after virus administration, a clinically feasible fraction of 8 Gy was given. We also intraperitoneally injected GEM (20mg/kg) + nab-PTX (2mg/kg) twice weekly. At the end of this experiment, the hamsters were sacrificed. Results: Triple-therapy regimen (OAd-IFN + Chemo + Radiation) showed remarkable inhibition of colony formation and CI analysis showed that combination therapy of OAd-IFN and chemoradiation were synergistic ($CI < 1$). In vivo experiment, the average tumor volumes of the control, chemoradiation (CR), OAd-IFN only (V), triple-therapy regimen (CRV) were 2027 \pm 1296, 908 \pm 849, 767 \pm 593 and 202 \pm 161 mm³, respectively (Figure 1). A significant tumor growth suppression was observed in CRV group without any serious side effect. Conclusion: In the colony formation assay, OAd-IFN potentiated the inhibition of radiation and chemotherapy (GEM+nab-PTX). Triple-therapy regimen (OAd-IFN + Chemo + Radiation) showed remarkable inhibition in the hamster syngeneic sub-cu tumor model. Combination therapy of OAd-IFN and chemoradiation might be applicable as a novel PDAC therapy

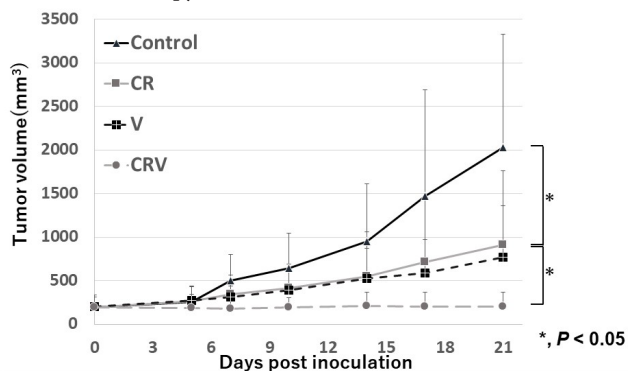


Figure 1. Triple-therapy regimen (OAd-IFN + GEM + nab-PTX + Radiation) showed remarkable inhibition in the hamster syngeneic sub-cu tumor model. The average tumor volumes of the control, chemoradiation (CR), OAd-IFN only (V), triple-therapy regimen (CRV) were 2027 \pm 1296, 908 \pm 849, 767 \pm 593 and 202 \pm 161 mm³, respectively at Day 21. A significant tumor growth suppression was observed in CRV group. Data are presented as mean \pm SD. * $P < 0.05$

94. Oncolytic HSV-1 rQNestin34.5v2 Sensitizes IDH1-Mutated Glioma to Immunotherapy

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Diffuse infiltrating *low-grade gliomas* include oligodendrogliomas and astrocytomas and account for approximately 20% of all primary brain tumors. Again of function mutation in isocitrate dehydrogenase 1 (mIDH1) gene has been frequently reported in low-grade gliomas. Low grade mIDH1 gliomas are classified into 2 molecular subgroups: 1p/19q codeletion/TERT-promoter mutations in oligodendroglioma or inactivating mutations in α -thalassemia/mental retardation syndrome X-linked (ATRX) and tumor suppressor protein 53 (TP53) in astrocytoma. Glioma patients with IDH1 mutant status exhibit a more favorable prognosis compared to patients with IDH1 wild-type tumors. Oncolytic herpes simplex virus 1 (oHSV-1) is emerging as a promising approach to promote anti-tumor responses. In this study, we aimed to assess whether expression of IDH1 by glioma cells favors the anti-tumor response to HSV-1 oncolytic virotherapy and to identify molecular mechanisms that contribute to treatment effect. A novel oHSV-1 (rQNestin34.5v2) currently in clinical trial for recurrent glioblastoma (ClinicalTrials.gov: NCT03152318) was employed, that maintains a copy of the HSV-1-ICP34.5 gene, which is responsible for HSV-1 neurovirulence under transcriptional control of the tumor-specific promoter nestin to drive robust tumor-selective replication. The anti-tumor activity of this HSV-1 was evaluated in C57BL/6 mice harboring orthotopic glioma expressing mutant and wild type IDH1 with loss of ATRX and TP53. HSV-1 rQNestin34.5v2 infected wild type and mIDH1 murine glioma cells in vitro triggering cytotoxicity and release of proinflammatory chemokines in infected cells. In vitro screens for upregulation of immune checkpoint molecules following infection identified CD155/TIGIT, L-selectin/LAG-3 and PD-L1/PD-1 inhibitory axes as promising targets for combination therapies. Mice harboring mIDH1 gliomas exhibited a less aggressive phenotype and significantly longer survival period compared to those with IDH1 wild type gliomas. These data demonstrate that mouse gliomas expressing IDH1 resemble human glioma cells harboring IDH1 and are suitable models to study translational immunotherapies for low grade gliomas. Experiments to address the therapeutic and immunostimulatory potential of intratumoral HSV-1 administration in primary and recurrent IDH1-mutant gliomas are ongoing.

95. Characterization of the Cancer-Targeted Oncolytic Adenoviruses with Fiber-Knob Modification

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Oncolytic adenovirus (OAd) is one of the promising anti-cancer agents under development. Oncolytic adenovirus (OAd) has high transduction efficiency *in vitro* and *in vivo* mediated by specific protein-to-protein binding and the exponential replication causing oncolysis. However, many cancer cells are resistant to wild-type Ad infection due to the low expression of adenovirus primary receptor (coxsackie-adenovirus receptor, CAR). In order to overcome this issue, several infectivity-enhanced oncolytic adenoviruses (OAds) with modified fibers has been developed (e.g. RGD, Ad5/3). These vectors confer augmented anti-tumor effect in CAR-negative cancer cells. However, these OAds still lack binding selectivity for cancer cells and are at risk for causing various adverse effects by increasing distribution to normal organs (e.g. innate immune response, hepatotoxicity) with systemic administration. Therefore, precise targeting of oncolytic virus at the level of infection (transductional targeting) is needed to achieve *in vivo* therapeutic benefits and safety for systemic treatment of cancer. To generate the cancer-targeting OAd, we have previously developed a novel system to identify the cancer-specific binding OAd by Ad-based library screening approach. To generate Ad library, we replaced a 21 base pair random sequence (7 amino acids) in the AB-loop of the fiber-knob region that is responsible for the binding affinity to the Ad receptor, CAR. To date, we have identified several cancer-targeted OAds by using this screening system. Among them, the Mesothelin-targeted OAd (MSLN-OAd), and the CD133-targeted OAd (CD133-OAd) showed strong anti-tumor effect with systemic injection in human tumor xenograft models. Here, we characterized these two fiber-modified cancer-targeted OAds (MSLN-OAd and CD133-OAd) toward systemic application. Since the adenovirus type 5 wild-type vector (Ad5-WT) has a liver tropism after intravenous injection, we assessed *in vivo* viral distribution of our fiber-modified cancer-targeted OAds after i.v. injection. We measured the virus copy number in major organs (lung, liver, spleen, kidney, intestine, and tumor) after i.v. injection of the viruses. The MSLN-OAd and CD133-OAd vectors show reduced liver targeting compared to untargeted OAd (Ad5-WT). On the other hand, the viral copy number of MSLN-OAd and CD133-OAd in the tumor was significantly higher than Ad5-WT. These results suggest that systemic injection of the tumor targeted-OAd showed significantly lower liver sequestration and better tumor accumulation. Next, we tested the effect of preexisting immunity to the fiber-modified OAd in human serum because neutralizing antibodies (nAbs) are one potential obstacle in oncolytic virotherapy. In a neutralization assay, the MSLN-OAd and CD133-OAd was more resistant to human serum, compared to Ad5-WT. These data suggest that OAd with a targeting motif in the AB-loop allow a degree of escape from pre-existing Abs. Interestingly, when we check the hemagglutination profile of the MSLN-OAd and CD133-OAd, the fiber-modified virus abolishes hemagglutination of human red blood cells. This profile change is thought to be due to the replacement of the AB-loop CAR binding domains to the cancer-targeted motif. These results suggest that the fiber-modification can be

one of the most effective ways to generate retargeting OAds to alternate sites *in vivo*, such as tumor site. The fiber-modified OAds also has a greater potential to facilitate systemic therapy.

96. Optimizing NIS-Expression for Oncolytic Adenovirus-Based Radiotherapy and Imaging of Breast Cancer

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Breast cancer is the most frequently diagnosed cancer in women worldwide with more than 2 million new cases in 2020. Treatment of breast cancer involves the combination of surgery, radiotherapy, chemotherapy, hormonal therapy, or biological therapies delivered in diverse sequences. The therapeutic use of viruses for cancer treatment has been revived these last two decades. Oncolytic adenoviruses (OAd) are engineered to selectively replicate in cancer cells leading to the destruction of the targeted cancer tissue. Thus, OAd can be a valuable tool to ameliorate immunogenicity and tumor suppression. Our laboratory has engineered an adenovirus encoding the sodium/iodide symporter (NIS), a native protein that mediates cellular uptake of iodine. NIS gene was inserted into the E3 region to allow continual gene expression as the virus replicates. Infected tumor cells can be synergistically treated with the intrinsic viral oncolysis and radiotherapy with radioactive iodine. This allows the detection and monitoring of metastases with high sensitivity by radiotracers. Our OAd-NIS also include a chimeric Ad5/3 fiber to improve its infectivity, the Cox2 promoter to drive tumor-selective replication and the deletion of the Adenovirus Death Protein (ADP). The initial purpose of ADP deletion was to prevent potential deleterious effects on the membrane localization and functionality of NIS, by lowering the rate of infection. Interestingly, the usage of six human breast cancer cell lines from different molecular classifications, reflecting breast cancer heterogeneity, allowed us to demonstrate that ADP deletion did not drastically affect the cytolytic activity of OAd-NIS in all the assessed cell lines. More importantly, while ADP deletion had a limited impact on the spread and replication of NIS-expressing vectors in breast cancer cells, it was found to significantly improve NIS expression in these cells. The same trend was observed in our previous studies carried out with pancreatic cancer cells *in vitro* and *in vivo* models including Patient-Derived Xenografts (PDX). We hypothesized that ADP expression could not affect the expression of NIS by tumor cells but impaired its anchorage to the cytoplasmic membrane. To verify this hypothesis, we employed an antibody targeting the extracellular domain of NIS with and without permeabilization conditions. We determined that NIS-membrane exportation was indeed not affected by ADP expression. However, ADP expression seemed to block the general expression of NIS in tumor cells. Furthermore, our pilot *in vivo* monotherapy study demonstrated that Ad5/3-COX2-ADP deleted-NIS can reduce tumor growth of xenograft of MDA-MB-231 cells into mice. Therefore, we have optimized our adenoviral vector for the therapeutic delivery of NIS to human breast cancer cells and will pursue its evaluation for the monitoring and radiotherapy of tumors. Our next step will aim for the use of a radiotracer for PET/CT imaging to visualized radioiodine

uptake by MDA-MB-231 tumor xenograft cells into mice infected by our OAd expressing NIS. We will also evaluate the combination therapy of our OAd-NIS vectors with radiotherapy using ¹³¹I.

97. Tumor Regression of Oncolytic Adenovirus-Treated Melanoma Rely on the Gut Microbiome

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In the last decade, cancer immunotherapy has achieved tremendous results, however the outcome of immune checkpoint inhibitors (ICIs) therapy has many genetic and environmental sources of variability. Several studies demonstrated the influence of gut microbiome on immunotherapy outcomes and in particular commensal *Bifidobacterium* was identified as a positive regulator of antitumor immunity *in vivo*, promoting anti-PD-L1 efficacy. Recently, novel strategies of active immunotherapy, based on oncolytic viruses (OVs) are achieving preclinical and clinical success, with a relevant contribution to the treatment of several types of tumors. We hypothesized that modulation of gut microbiota could also affect OVs therapeutic efficacy and, therefore, decided to investigate whether the effect of OVs on tumor progression could be altered by manipulation of the intestinal microbial community. To address this question, we pre-treated a group of C57BL/6J mice with an oral administration of vancomycin and, subsequently, inoculated subcutaneously syngeneic B16.OVA melanoma cells to the groups. We then treated both groups with an intratumorally injection of oncolytic adenovirus Ad5-CpG and we observed that its antitumoral effect was extremely reduced in mice pre-treated with vancomycin, showing a faster tumor progression and less tumor-infiltrating lymphocytes (TILs), compared to the control group. To confirm that alteration of the intestinal microbiota was involved in this phenomenon, we performed the rescue of the group pre-treated with vancomycin, by cohousing with a Ad5-CpG-treated control group. Successively, we combined the adjuvant activity of virus with a probiotic containing *Bifidobacterium spp.* that increased the response to adenoviral therapy, reducing tumor-infiltrating T-cells and stimulating an enrichment of bacterial genera belonging to Firmicutes phylum. Using a bioinformatic tool Homologous Evaluation Xenopeptides (HEX) we identified specific *Bifidobacterium*-derived peptides highly similar to melanoma epitopes. As confirmed by IFN- γ ELISPOT assay, these peptides are able to trigger a robust CTL-response. Our data indicate that gut microbiota affects the immune responses elicited by oncolytic adenovirus Ad-CpG and *Bifidobacterium* vaccination optimized its antitumor activity in melanoma, because of a possible mechanism of molecular mimicry between *Bifidobacterium*-derived peptides and melanoma epitopes.

98. Oncolytic Adenovirus with Hyaluronidase Activity That Evades Neutralizing Antibodies and Allows Re-Administration: VCN-11

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VCN-11 is a novel oncolytic adenovirus genetically modified to express hyaluronidase (PH20) and display an albumin-binding domain (ABD) on the hexon. ABD allows the virus to self-coat with albumin when entering the bloodstream and evade neutralizing antibodies (NABs). Here, we validate the VCN-11's mechanism of action, characterize its toxicity and study its antitumor activity with pre-existing immunity. VCN-11 evaded NABs *in vitro* and efficiently targeted tumors after intravenous administration in the presence of high levels of NABs, whereas the control virus without ABD was neutralized. Intravenous administration of VCN-11 induced lower Nab levels than the administration of Ad5 in immunocompetent mice and hamsters. When re-administered in hamsters, VCN-11 was able to reach all tumors after systemic administration, even in the presence of high anti-VCN-11 Nabs. Antitumor efficacy of VCN-11 was demonstrated in several tumor models in the presence of NABs against wild type Ad5 and also in animals with pre-existing immunity against ABD capsid, demonstrating its effectiveness after re-administration. A low toxicity profile was observed in athymic nude mice and Syrian hamsters and no major toxicities were observed after administration of fractionated high doses (up to 1.2×10^{11} vp/mouse and 7.5×10^{11} vp/hamster) and when readministered. Fractionated intravenous administrations improved circulation kinetics and tumor targeting. Similar hepatic toxicity was observed after single and repeated virus administration. These features suggest that VCN-11 is a safe and promising candidate to treat tumors in the presence of pre-existing immunity against adenovirus and using a multi-dosing strategy for therapy.

Cell-Based Cancer Immunotherapies I

99. Endowing Universal CAR T-cell with Immune-Evasive Properties Using TALEN-Gene Editing

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Universal CAR T-cell therapies are poised to revolutionize cancer treatment and to improve patient outcomes. However, realizing these advantages in an allogeneic setting requires universal CAR T-cells that can kill target tumor cells, avoid depletion by the host immune system, and proliferate without attacking host tissues. Here, we describe the development of a novel immune-evasive CAR T-cells scaffold that evades NK cell and alloresponsive T-cell attacks and imparts efficient antitumor activity *in vitro* and *in vivo*. This novel immune-evasive CAR T-cells ($\Delta\text{TRAC}_{\text{CAR}}\Delta\text{B2M}_{\text{HLAE}}$), were developed using a combination of TALEN-mediated gene editing and AAV-dependent gene insertion. $\Delta\text{TRAC}_{\text{CAR}}\Delta\text{B2M}_{\text{HLAE}}$ is devoid of TCR $\alpha\beta$ and HLA Class I expression and endowed with an engineered surface-exposed HLA-E. These three features could enable CAR T-cells to prevent GvH reaction and to evade the cytolytic activities from alloresponsive T-cells and NK cells. We confirmed that HLA-ABC(-) $\Delta\text{TRAC}_{\text{CAR}}\Delta\text{B2M}_{\text{HLAE}}$ overcame alloresponsive T-cell attack, and we demonstrated their ability to evade NK cells attack by the significant enrichment of HLA-ABC(-) HLA-E(+) $\Delta\text{TRAC}_{\text{CAR}}\Delta\text{B2M}_{\text{HLAE}}$ in the presence of primary NK cells from healthy donors and Acute myeloid leukemia (AML) patients. We further confirm by mass cytometry (CyTOF) that NK cells from healthy donors, AML patients and Acute lymphocytic leukemia (ALL) patients display similar phenotypic and fitness characteristics, whatever the stage of their disease. Thus, we anticipate that expression of HLA-E is likely to protect $\Delta\text{TRAC}_{\text{CAR}}\Delta\text{B2M}_{\text{HLAE}}$ from NK cells in most AML and ALL patients. We showed that $\Delta\text{TRAC}_{\text{CAR}}\Delta\text{B2M}_{\text{HLAE}}$ T-cells exhibit antitumor activity *in vitro* and *in vivo*, demonstrating that their hypoimmunogenic features do not affect their cytolytic functions. This new cellular scaffold could enable the broad use of universal CAR T-cells in allogeneic settings and holds great promise for clinical applications.

100. Engineering Stealth CAR T Cells to Evade the Host Immune Responses

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Background: Currently 4 of the 5 FDA-approved CAR T cell products contain the FMC63-based αCD19 single chain variable fragment (scFv) antigen-recognition domain originating from a murine antibody. Clinical trials have indicated that treatment with FMC63-based autologous CAR T cells elicits an immunological T cell response in the patient against the murine scFv-fractions of the CAR. The problem of CAR T cell rejection is even greater when using allogeneic immune effector cell products. In this study, we aimed to use a one-shot approach to prevent cellular immunity rejection of engineered T cells by simultaneously reducing the surface expression of human leukocyte antigens via expression of viral inhibitors of transporter associated with antigen processing (TAP) in combination with shRNA targeting class II major histocompatibility complex (MHC) transactivator (CIITA) in CAR T cells

Results: Expression of the small viral TAP inhibitors (TAPi) Herpes Simplex virus (HSV) ICP47, Human Cytomegalovirus (HCMV) US6 or Epstein-Barr (EBV) BNLF2a in primary T cells resulted in reduced HLA class I expression on its cell surface. This reduced expression of HLA class I on the cell surface successfully prevented presentation of the NLV-peptide originating from the HCMV pp65 protein and allogeneic responses in a mixed lymphocyte reaction (MLR), whilst enough HLA I remained on the cell surface to limit susceptibility to lysis by NK cells. Importantly, expression of these small viral sequences did not elicit immune responses in donors with a cellular immunity against the respective virus. Next, we developed shRNA targeting CIITA to reduce HLA class II from the T cell surface. Expression of shRNA targeting CIITA in primary T cells resulted in reduced HLA class II expression, and resulted in a reduced allogeneic response as measured by MLR. Combined expression of both viral TAPi and shRNA targeting CIITA reduced both HLA class I and II expression, and mitigated allogeneic responses in an MLR assay. Next we combined our stealth technology with FMC63-based αCD19 CARs in a single lentiviral vector. No differences in tumor clearance were observed between αCD19 CAR T cells and Stealth αCD19 CAR T in *in vitro* killing assays against the acute lymphoblastic leukemia line NALM6 and Mantle cell lymphoma line JeKo-1, and in *in vivo* mouse models utilizing the NALM6 line. Furthermore, ELISpot assays with PBMC from patients who had received the FMC63-based αCD19 CAR T cell (axi-cel or tisa-cel) products indicated active cellular immune responses against autologous αCD19 CAR T. Moreover, an increased T cell response against autologous αCD19 CAR T cells was observed when the patient had received multiple αCD19 CAR T cells infusions. Importantly, no cellular immune response was observed when these patient PBMC were assayed against autologous Stealth αCD19 CAR T cells.

Conclusion: Collectively, this data indicates that expression of the small EBV TAPi sequence in combination with an shRNA targeting CIITA effectively reduced HLA expression and prevented allogeneic response in primary T cells, whilst limiting killing by NK cells. Incorporating these HLA-reducing sequences in FMC63-based αCD19 CAR T cells did not impair their ability to clear tumor *in vitro* and *in vivo*. These stealth αCD19 CAR T cells were able to prevent the immune-activation of T cells from patients pre-exposed to αCD19 CAR T cells. Our data suggest a potential advantage of using stealth CAR T cells to increase persistence and reduce rejection, including in cases of multiple infusions, which could apply to both autologous and allogeneic adoptive cell therapy settings.

101. Tumor-Directed, Myeloid Cell-Based Cytokine Gene Delivery Unleashes CAR T Cells in the Immunosuppressive Glioblastoma Microenvironment to Control Tumor Growth

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Background: The immunosuppressive tumor microenvironment (TME) of solid tumors represents a major hurdle for effective chimeric antigen receptor (CAR) T-cell therapies. While immunostimulatory cytokines can counteract immune suppression, their systemic administration entails high risks of toxicity and counter-regulatory responses. We have developed a gene and cell therapy to selectively deliver cytokines to the TME, while sparing unaffected tissues from exposure. In this strategy, currently in phase I/IIa clinical trial for glioblastoma (GBM) patients, hematopoietic stem cells (HSCs) are modified to target interferon (IFN)- α expression in the TIE2-expressing monocyte (TEM) progeny. Upon HSC transplantation, TEMs recruited by tumors release IFN- α and reprogram the TME inducing anti-tumor immunity and inhibiting growth of several tumor models. Here we investigated whether TEM-based IFN- α or interleukin (IL)-12 delivery may rescue CAR T-cell activity in the non-permissive GBM TME and safely synergize to thwart tumor growth. **Methods:** We first evaluated the expression of candidate targets at transcriptome and protein levels in a new murine GBM model (mGB2), which closely mimics the human disease. mGB2 cells express the immune checkpoint B7H3, sharing high human-mouse (h/m) homology. We generated both B7H3-redirection (h/m cross-reactive) and control mouse CAR T cells at high efficiency by optimizing a lentiviral-based protocol and administered them via systemic or intratumoral route in syngeneic mice, previously transplanted with engineered HSCs for TEM-mediated IFN- α or mock delivery, and orthotopically injected with mGB2. Tumor growth was assessed by magnetic resonance imaging; quantitative, immunophenotypic and transcriptomic analyses were performed on peripheral blood and tumor infiltrate; efficacy and tolerability of the treatments were monitored in survival studies. To demonstrate the portability of our platform to other cytokines, we also delivered IL-12, whose use is currently limited by systemic toxicities, in the same setting. **Results:** CAR_{B7H3} T cells were found at lower levels than control cells in the peripheral blood of both mock and IFN- α mice, while displaying a more activated phenotype in the latter ones. Conversely, CAR_{B7H3} T cells were enriched at the tumor site compared to control CAR T cells, suggesting a cognate antigen-driven homing and/or local expansion of the former. IFN- α delivery reprogrammed the TME leading to reduced exhaustion and increased activation of tumor-infiltrating CAR_{B7H3} T cells. While IFN- α gene therapy limited tumor growth *per se*, CAR_{B7H3} T cells alone had minor or no effect. Nonetheless, marked inhibition of tumor growth and prolonged survival of mice were achieved in the combination group, in absence of any overt toxicity. This therapeutic effect was further potentiated by intratumoral

injection of CAR T cells. Safety and efficacy were also observed by delivering IL-12 through TEMs in combination with CAR_{B7H3} T cells. **Conclusions:** IFN- α -driven TME reprogramming overcame some key limitations of CAR T-cell therapy targeting a clinically relevant tumor antigen in an immunocompetent mouse model of GBM, rescuing T cell effector function and synergizing in achieving tumor inhibition while preserving full tolerability of the treatment. TEM-based gene therapy is also portable to other cytokine payloads to specifically act on tumor-infiltrating CAR T cells while avoiding systemic toxicities.

102. Activation Regulated Gene Circuit for Controlling Payload Expression in Cell Therapies

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Background: The efficacy of immune cell therapies in solid tumors is hampered by poor persistence and limited effector functions in the immunosuppressive tumor microenvironment. Pro-inflammatory cytokines, such as IL-12, have been shown to enhance CAR-T cell activity and stimulate the innate immune antitumor response. However, IL-12 has a narrow therapeutic window and requires tight regulation to avoid triggering severe toxicity in humans. Previously, an activation responsive promoter containing binding sites for the NFAT transcription factor was used to control production of IL-12 by CAR-T cells, but had toxic basal expression levels in unactivated cells. A novel cell activation responsive promoter would provide a desirable expression profile, where expression would occur only after the immune cells reach their target and are triggered by the presence of tumor cells, resulting in more precise local production of therapeutic cytokines, which in turn may enhance antitumor efficacy and safety in patients. **Methods:** To discover tightly regulated activation responsive promoters in primary immune cells, two strategies were implemented. The first was native promoter screening in which we identified 20 genes upregulated in response to T cell activation and derived promoters from the native DNA sequences upstream of those genes. Each promoter was used to control expression of a fluorescent reporter and was transduced into primary T cells. T cells were activated with CD3/CD28 Dynabeads to induce T cell activation and promoter activity was measured by flow cytometry. The second strategy was a 15,000 member synthetic promoter library containing transcription factor binding sites and chromosomal regions associated with immune cell activation. This library was transduced into NK cells at MOI 1 and the cells were activated with K562 feeder cells. The NK cells were then sorted by FACS for high and low reporter expression in the activated and unactivated states. DNA was isolated from the sorted cells and submitted for NGS. Hits were identified that were specific to the high expression, activated NK cells. **Results:** In T cells, the NFAT promoter has high basal expression (25% the strength of a constitutive promoter). The NFAT promoter responds to T cell activation with a 5 fold induction in expression. Using the native promoter screening approach we identified 2 promoters with high specificity for the activated state (no basal expression and 4-6 fold induction upon activation). One of these promoters was then engineered by removing bioinformatically informed regions to

identify sequences that impact activation responsiveness. This led to the discovery of promoters with enhanced activation response. We also identified regions of the promoter that could be removed without impacting performance, thereby decreasing promoter size by 40% from 2000 bp to 1221 bp. This final promoter had zero basal activity and an impressive 20 fold induction in response to T cell activation. In NK cells, the NFAT promoter has low basal activity but also low induction upon activation. Using the pooled library approach we identified 19 promoters that could be specific to NK activation. We screened each clonally and found that 4 promoters showed no basal activity and high activation responsiveness (5-20 fold induction of reporter upon activation). These promoters are also compact: 250 bp, which enables their incorporation into complex gene circuits with size constraints. We developed two successful strategies for promoter discovery which generated several promising promoters that can tightly regulate gene expression based on activation state of primary immune cells. These promoters could enable gene circuits that regulate the expression of potent immune effectors, allowing for safely armed CAR immune cell therapies.

103. Preclinical Development of Safe and Effective T Cell Receptors Specific for Mutant KRAS G12V and G12D Peptides

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Adoptive T cell therapy (ACT) for solid tumors still requires further refinement, improvement, and mechanistic understanding to become a reproducibly effective treatment. T cell receptor (TCR)-engineered T cells are an attractive choice for solid tumor ACT because they can recognize peptides from both surface and intracellular proteins, including oncogenic drivers. An ideal TCR peptide target would be: 1) derived from a tumor-specific protein that is essential for tumor growth, 2) immunogenic, and 3) stably expressed in the context of a common HLA allele. KRAS is the most frequently mutated gene in human cancers and is a regulator of cell growth and proliferation. Hotspot mutations in KRAS codon 12 are present in ~30% of colon cancers and ~80% of pancreatic cancers, and are associated with poor survival, with KRAS substitutions G12V and G12D being most prevalent. Recent evidence suggests that mutant KRAS (mKRAS) is immunogenic and targetable, as adoptive transfer of mKRAS-specific tumor infiltrating lymphocytes led to a durable clinical response in a patient with colorectal cancer. We therefore hypothesized that TCR-engineered T cells specific for mKRAS G12V and G12D would be beneficial in treating KRAS-mutant solid tumors. To test this hypothesis, we used our high-throughput *in vitro* TCR discovery platform to isolate candidate TCRs. Briefly, we stimulated CD8⁺ T cells from healthy donors with mKRAS peptides

predicted to favorably bind to HLA-A*11:01, identified the highest avidity T cells by stringent fluorescence-based cell sorting, and then sequenced, synthesized and functionally compared the isolated TCRs. We expressed the TCRs in primary CD8⁺ T cells and tested the cells for specificity (response to peptide-loaded presenting cells and tumor cells endogenously expressing the antigen) and cytotoxicity (*in vitro* tumor cell killing assays), and selected for further study the candidates that exhibited the best functional avidity and tumor-killing capacity. We determined TCR recognition motifs by evaluating responses to peptides with alanine substituted at each position one residue at a time. We then searched the human proteome for proteins that contained the TCR peptide motif and determined if the T cells could recognize those peptides. We also evaluated potential alloreactivity to >50 lymphoblastoid cell lines with different HLA MHC class I haplotypes. These experiments revealed a favorable safety profile, without detected cross-reactivity or alloreactivity. Since effective anti-tumor immunity involves a coordinated CD4⁺ and CD8⁺ T cell response, we engineered both CD4⁺ and CD8⁺ T cells to express our most promising TCR candidate and the CD8 α β co-receptor to enhance signal transduction and peptide/MHC binding. Transduced CD4⁺ T cells were functional and provided help to CD8⁺ T cells, supporting tumor elimination in an *in vitro* model of repeated exposure to tumor. In summary, we report a TCR gene therapy approach targeting mutant KRAS peptides G12V and G12D with a coordinated CD4⁺ and CD8⁺ T cell response, and a promising efficacy and safety profile. Our work to date supports the clinical development of these novel products for treating KRAS-mutant solid tumors.

104. Disruption of H3K9me3-Mediated Gene Silencing Augments CAR T Cell Functional Persistence

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CD19-specific chimeric antigen receptors (CARs) that incorporate CD28 and CD3 ζ signaling motifs (1928z) induce remarkable T cell responses in patients with refractory B cell malignancies. 1928z CAR T cells rapidly acquire potent effector functions but display limited persistence. It would thus be beneficial to tune the effector function of 28z CAR T cells to extend their functional persistence in patients without compromising their anti-tumor function, particularly for adapting CAR T cell therapy to solid tumors. T cell differentiation and dysfunction under chronic stimulation are accompanied by global changes in transcriptional profiles and epigenetic states. These changes are in part mediated by key transcriptional and epigenetic factors, including the histone methyl transferase, *SUV39H1*. In a murine model of *Listeria monocytogenes* infection, Pace et al. showed that loss of *SUV39H1* regulates the transition between memory and effector states in OT-1 transgenic CD8⁺ T cells. In the present study, we examine the impact of disrupting *SUV39H1* in human 1928z CAR T cells, hypothesizing that *SUV39H1* edited, retrovirally transduced (Rv-1928z) T cells will limit the induction of T

cell dysfunction and enhance the functional persistence of CAR T cells. To assess the impact of *SUV39H1* on human Rv-1928z CAR T cells, we treated immune deficient mice bearing the human ALL cell line, NALM6, with limiting doses of *SUV39H1*-edited CAR T cells. *SUV39H1* editing (*SUV39H1^{ed}*) significantly enhanced the anti-tumor efficacy of Rv-1928z CAR T cells relative to their unedited counterparts, with 9/10 NALM6 bearing mice treated with *SUV39H1^{ed}* Rv-1928z CAR T cells surviving over the duration of observation (90 days) as compared to 1/12 mice treated with WT Rv-1928z CAR T cells. This enhanced tumor control in *SUV39H1^{ed}* Rv-1928z CAR T cells was associated with greater initial CAR T cell expansion and enhanced long-term CAR T cell persistence (> day 50). Single-cell transcriptional profiling revealed greater clonal diversity and prolonged maintenance of a memory phenotype of *SUV39H1^{ed}* Rv-1928z CAR T cells compared to WT Rv-1928z CAR T cells. To assess whether the persisting *SUV39H1^{ed}* Rv-1928z CAR T cells can mount an effective effector response upon tumor rechallenge, we modified our NALM6 model such that post primary tumor clearance (day 17), mice were rechallenged by tumor 5 times over 70 days. *SUV39H1^{ed}* Rv-1928z CAR T cells outperformed WT Rv-1928z CAR T cells in eliminating NALM6 upon rechallenge. Paired genome accessibility (ATACseq) and transcriptional analysis revealed epigenetic changes associated with *SUV39H1* loss in Rv-1928z CAR T cells, potentially accounting for continued expression of memory-associated transcription factors that suppress the expression of inhibitory receptors and curtail T cell dysfunction. In summary, we find that loss of *SUV39H1* in human Rv-1928z CAR T cells improves their anti-tumor efficacy by altering their epigenome to enhance their functional persistence, demonstrating the potential of combining CAR and epigenome engineering.

105. Mechanisms Regulating the Resistance of Normal T-cells to CD5 CAR-Mediated Cytotoxicity

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T-cell fratricide, or self-elimination, is a major factor limiting the development of CAR T-cell therapies for T-lineage malignancies. One notable exception is a CD5-specific CAR which produces minimal fratricide despite uniformly high expression of CD5 on human T-cells. We found CD5 protein is rapidly degraded in T cells after transduction with the CD5 CAR thus enabling these CAR T-cells to evade self-elimination. In our ongoing Phase I clinical trial (NCT03081910), CD5 CAR T-cells produce robust anti-tumor activity in patients with recalcitrant T-cell malignancies but spare a population of normal T-cells, thereby avoiding complete T-cell aplasia. The mechanism of normal T-cell resistance to CD5-directed CAR T-cell cytotoxicity remains unknown. Flow cytometric analysis showed the expansion of CD5 CAR T-cells in peripheral blood of patients with T-cell malignancies coincides with the loss of detectable CD5 expression on normal circulating T-cells. After CD5 CAR T-cell numbers subside, CD5 is re-expressed

on peripheral T-cells suggesting the loss of protein is transient and triggered by interactions with CD5 CAR T-cells leading to temporary protection against fratricide. To investigate this possibility, we replicated clinical findings by co-culturing T-cells with autologous CD5 CAR T-cells. We found normal T-cells downregulate CD5 *in trans* upon contact with CD5 CAR T-cells resulting in the emergence of a T-cell subset that resists CD5 CAR T-cell cytotoxicity. Using engineered variants of CD5, we showed internalization and complete degradation of CD5 protein in target cells upon contact with CD5 CAR T-cells by flow cytometry, western blot, and live-imaging fluorescence microscopy. CD5 loss was triggered by physical ligation by the CAR and did not require CAR signaling. Notably, removal of CD5 CAR T-cells restored freshly synthesized surface CD5 expression on T-cells within 16-24hr, mirroring clinical findings. Next, we investigated whether transient loss of CD5 contributes to the resistance of normal T-cells to CD5 CAR T-cell cytotoxicity. Because conventional methods of static cell culture do not accurately recapitulate the dynamic environment in blood vessels, we established an alternative method of measuring cell cytotoxicity where CAR T-cells and target cells are continuously agitated in conditions that approximate shear stress in large blood and lymphatic vessels. We found that the transient loss of CD5 on normal T-cells resulted in a significant protection from CD5 CAR-mediated cytotoxicity in both short term (T=6hr, mean cytotoxicity 12.06% vs 60.06% in control, p<0.001) and longer-term (T=20hr, mean cytotoxicity 51.4% vs. 86.6% in control, p<0.0001) only in continuously agitating conditions approximating shear stress in large blood and lymphatic vessels, but not static cultures with minimal external stress. This suggests the temporary downmodulation of CD5 on normal T-cells upon exposure to CD5 CAR T-cells in a dynamic environment in blood vessels destabilizes cytotoxic CAR-mediated immune synapse, contributing to the resistance of normal T-cells to CD5-directed cytolysis. These findings highlight a mechanism by which target cells can temporarily evade CAR T-cell cytotoxicity by internalizing and degrading target antigen. Our clinical and experimental data support the contribution of this process to the resistance of normal T-cells to CD5-directed cytotoxicity thus limiting the off-tumor toxicity of CD5 CAR T-cells. Tumor cells may employ a similar mechanism to resist CAR T-cell activity. Understanding how normal and malignant T-cells evade CAR-mediated cytotoxicity will inform the design of next-generation cell therapies for patients with T-cell malignancies.

Hematopoietic Stem Cell Gene Therapy

106. Inhibition of P38-MAPK Counteracts Culture Stress Induced by *Ex Vivo* Expansion of Hematopoietic Stem and Progenitor Cells (HSPCs) for Efficient Genetic Engineering

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Prolonged time in culture is still a prerequisite to reach sufficient levels of gene correction by gene transfer or homology-driven repair (HDR) for HSPC-based clinical applications. We recently discovered that gene-editing by co-delivery of nuclease-induced DNA double strand break (DSB) and corrective DNA templates via AAV6 in cultured HSPCs triggers a strong p53-mediated DNA damage response (DDR) affecting their functionality post-transplant. Instead, despite similar DDR activation, we now report only a transient cell cycle delay and a higher clonogenic potential when HSPCs were edited anticipating time in culture, suggesting that prolonged time in culture makes edited HSPCs to exceed a threshold of tolerable DDR with adverse cellular outcomes. Indeed, although current culture conditions have been tailored over the past decade to ensure high gene-transfer and gene editing efficiencies while preserving the long-term repopulating capacity of HSPCs upon *ex vivo* gene manipulation, we hypothesize that activated stem cells, when exiting from their quiescent status, accumulate elevated levels of physical DNA damage, ultimately resulting in stem cell dysfunctions. By combining comet assay for physical DNA damage evaluation, measurement of ROS, imaging of double and single strand break nuclear markers (γ H2AX and pRPA respectively) and Break labelling *in situ* and sequencing (by BLISS), we reported aberrant mitogenic ROS signalling and increased levels of DNA replication stress in activated human HSPCs from cord blood and mobilized peripheral blood. Interestingly, we identified as a key mediator of the observed culture stress the activation of the p38-MAPK and found that its chemical inhibition diminished ROS thus counteracting uncontrolled proliferation of HSPCs that ultimately leads to DNA damage accumulation. More importantly, edited HSPCs treated with p38i prior to genetic engineering maintained high editing efficiency and displayed a significant enhancement in clonogenic potential and *in vitro* single cell differentiation output and higher engraftment and repopulation capacity upon transplantation in immunocompromised mice. By *in vivo* clonal tracking of edited cells, we discovered that pre-treatment with p38i led to a higher clonal output compared to control genetically modified cells in terms of number of both indels (HSPC clones that underwent NHEJ repair) and BARs (HSPC clones that repaired by HDR), pointing to the preservation of the more rare HSC subset responsible for long-term hematopoietic reconstitution of genetic engineered cells. Similar observations were made when human HSPCs were corrected by means of gene transfer with lentiviral vectors.

Altogether, our findings identified a novel molecular axis in activated human HSPCs linking HSPC activation with physical DNA damage, p38 activation and premature exhaustion of HSPCs upon genetic engineering. Because in clinical settings the oligoclonal composition of the human graft observed upon transplantation of gene edited cells may delay hematopoietic recovery after conditioning restricting the size, their presence long-term and over all the safety of the engineered cell graft we reason that pharmacological inhibition of p38 may be exploited to ameliorate the yield and the quality of engineered cells to further advance HSPC-based gene and cell therapies.

107. 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 engineered hematopoietic stem/progenitor cells (HSPCs) has shown curative potential in murine models and patients affected by monogenic neurodegenerative diseases when treated in very early disease stages. The engrafted cells can act not only as vehicles for therapeutics to the brain, but also as modulators of neuroinflammation, possibly extending the use of HSPC gene therapy to a broader spectrum of neurodegenerative diseases. Nonetheless, the impact of this approach is affected by the slow pace of central nervous system (CNS) engraftment and differentiation of the engineered HSPCs into microglia-like cells (MLCs) as compared to the rapid progression of neurodegeneration of most targeted disorders. To foster this process, we focused on CX3CR1 (CX), a microglia chemokine receptor that regulates microglial recruitment to sites of neuroinflammation and microglia ontogeny. Firstly, we observed that transplantation of CX haploinsufficient (CX-haplo) HSPCs resulted in a more robust engraftment and generation of MLCs as compared to wild type (WT) HSPCs. Specifically, in competitive transplantation settings, CX-haplo HSPCs prevailed over WT in the repopulation of hematopoietic organs (60% vs 40%) and brain (80% vs 20%) both at short and long term timepoints post-transplant. Moreover, a branching analysis performed on brain-engrafted MLCs revealed a greater extent of ramifications and complexity of CX-haplo vs WT cells, 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 a single cell resolution on FACS-sorted MLCs from competitively transplanted mice. Gene ontology analysis on signature genes revealed significant upregulation of a signal transduction pathway in a cluster enriched with CX-haplo cells, that may account for a greater activity in cytoskeleton rearrangement, cell motility and cell cycle progression explaining the quantitative and qualitative advantage observed. Next, to develop a therapeutic strategy combining the improved features of CX-haplo HSPC transplantation with a microglia specific transgene expression, we designed a CRISPR-based gene addition strategy. We first validated

the use of a promoterless, splice trapping cassette encoding for a fluorescent reporter to be inserted into CX intron in a CX expressing cell line, showing that targeted insertion allows regulated transgene expression and CX knock-out only in HDR edited cells, while NHEJ-indels did not impacted CX expression. We then used an established editing protocol based on Cas9/gRNA electroporation and AAV6 transduction to deliver the cassette in human CD34+ HSPCs (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 recipient mice, showing higher transgene expression on human MLCs that reconstituted the brain compared to hematopoietic organs, as per physiologic CX expression. Interestingly, expression of the transgene driven by CX promoter in engrafted human MLCs was stronger than the expression driven by a conventional PGK promoter. In conclusion, we identified and validated *CX3CR1* as a key target to enhance the ability of HSPCs to generate a microglia-like progeny upon transplantation and allow specific and robust transgene expression in the CNS.

108. Ex Vivo Lentiviral-Mediated Gene Therapy for Patients with Fanconi Anemia [Group A]: Updated Results from Global RP-L102 Clinical Trials

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Background: Fanconi anemia (FA) is a rare inherited disorder of defective deoxyribonucleic acid (DNA) repair leading to 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 BMF; however, its efficacy is limited by human leukocyte antigen (HLA)-matched sibling donor availability and transplant-related toxicities including an increased solid tumor risk. *Ex vivo* lentiviral mediated gene therapy utilizing autologous FA-A CD34+ enriched hematopoietic stem and progenitor cells (HSPCs) relies upon the proliferative advantage of gene-corrected FA HSPCs, enabling engraftment without antecedent conditioning, as demonstrated in pre-clinical studies and the FANCOLEN-I clinical trial ("Process A" manufacturing). We report results from ongoing global RP-L102 studies using "Process B" manufacturing optimizations including transduction enhancers, commercial grade vector, and modified cell processing, and enrollment of patients (pts) with more limited BMF. **Methods:** Pts with mutations in *FANCA*, age ≥ 1 year with no HLA-matched sibling donor and at least 30 CD34+ cells/ μ L in bone marrow (BM) are eligible. Mobilized peripheral blood (PB)

mononuclear cells are collected via leukapheresis. CD34+ cells are enriched, transduced with a lentiviral vector encoding for the *FANCA* gene, and infused without cryopreservation or conditioning. Pts are followed for 3 years post-infusion for safety and efficacy evaluations including increasing PB and BM vector copy number (VCN), development of mitomycin-C (MMC) resistance in BM colony forming cells (CFCs), and stabilization/correction of cytopenias. **Results:** As of December 2021, 11 pts age 2 to 6 years have received RP-L102. Sustained engraftment has been demonstrated in 6 of 8 enrolled pts with ≥ 12 months of follow up as indicated by PB VCN. Concomitant increases in BM CFC MMC resistance ($\geq 10\%$ of baseline) have been observed, with concurrent blood count stabilization in at least 5 pts. The RP-L102 safety profile has been highly favorable; 1 pt had a transient serious Grade 2 RP-L102 infusion-related reaction which resolved without sequelae. **Conclusions:** Engraftment and proliferative advantage of transduced HSPCs has been confirmed in 6 of 8 evaluable (≥ 1 year of follow-up) pts in the absence of antecedent conditioning. Increases of $\geq 10\%$ BM CFC MMC resistance relative to baseline were identified in each of these 6 patients at ≥ 12 months post-infusion (at 1 timepoint or more) with concomitant blood count stabilization identified subsequent to the development of MMC-resistance in at least 5 pts. Additional clinical updates will be presented including initial correlations between BM CFC MMC-resistance, genetic correction and hematologic stabilization.

109. Preclinical Evidences Towards Lentiviral Gene Therapy for *RPS19*-Diamond Blackfan Anemia Patients

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Allogenic hematopoietic stem cell transplantation (HSCT) currently represents the only curative treatment for the bone marrow failure (BMF) of patients with Diamond Blackfan Anemia (DBA). However, the mortality and morbidity associated to HSCT demand new therapies able to restore hematological signs of DBA and improve the quality of life of these patients. Mutations in 20 ribosomal genes, plus 4 "DBA-like" genes account for 70-80% of DBA patients. However 25% of mutations have been identified to occur in ribosomal protein (RP) gene *RPS19*. During last years, autologous ex vivo lentiviral mediated gene therapy (GT) has become a true alternative for the treatment of different hematopoietic diseases. In this study we have focused in the development of a clinically applicable efficient and safe lentiviral mediated ex vivo gene therapy approach to correct *RPS19*-haploinsufficient DBA patient-derived primary hematopoietic

stem cells with the final intention to treat these patients in a Phase I/II GT clinical trial in the next future. Initial gene therapy studies were conducted in K562 cells in which *RPS19* was down-regulated with a sh-*RPS19* LV. In these studies, we observed that transduction with two different therapeutic LVs (*PGK.CoRPS19.Wpre** and *EF1 α (s).CoRPS19.Wpre*-LVs*) restored the expression of *RPS19* and corrected the ribosomal biogenesis defects characteristic of sh-*RPS19* transduced cells. The therapeutic efficacy of *PGK.CoRPS19.Wpre** LV was confirmed then in primary CD34⁺ cells from *RPS19*-deficient patients. Transduction of these CD34⁺ cells with the therapeutic LV significantly increased the number of BFU-E colonies. Moreover, the therapeutic LV reverted the red blood cell differentiation defect characteristic of DBA CD34⁺ cells, increasing the output of CD71⁺/CD235⁺ mature erythroid cells both in vitro and in vivo. Remarkably, CD34⁺ DBA cells transduced with the therapeutic LV were also capable of repopulating the hematopoiesis of NSG mice. In preliminary studies, no evident changes in the repopulating function of healthy donors (HD) CD34⁺ cells were observed after transduction with the therapeutic vector, revealing the safety associated with the ectopic expression of *RPS19*. These studies were also confirmed by the health status of transplanted immunodeficient recipients and by the polyclonal integrational pattern of the therapeutic proviruses. Taken together, the preclinical studies conducted in this work support that the lentiviral-mediated gene therapy of *RPS19*-deficient DBA patients should constitute an efficient and safe approach for the treatment of DBA patients.

110. A Mouse Model of Severe Alpha-Thalassemia with Abnormal Iron Metabolism, Erythropoiesis and Coagulation Can Be Rescued by a Novel Gene Therapy Approach

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Clinical presentation of α -thalassemia (AT) varies from an asymptomatic condition (one inactivated α -globin gene), mild to severe (deletion or mutation of 2, 3 or 4 copies of the α -globin genes, leading to formation of β -globin tetramers known as HbH), to Hb Bart's Hydrops Fetalis (complete absence of active α -globin genes). Recent estimates indicate that ~70,000 infants are born yearly with severe AT. In these patients, a blood transfusion independent state is achievable through allogeneic bone marrow transplantation. New therapies are desperately needed. We aim to develop a novel adult mouse model of AT and a gene therapy approach for this disease. We developed a novel adult mouse model of severe AT to study erythropoiesis, iron metabolism, coagulation, and for use as a test bed for a gene therapy approach for this disease. We generated adult animals that do not produce α -globin chains (α -KO) through transplantation of homozygous fetal liver cells (isolated at E14.5) into WT recipient mice. The α -KO animals demonstrate a severe phenotype, paradoxically showing elevated hematocrit, high reticulocyte count, and high

numbers of red blood cells (RBC). These RBC expressed only β -globin chains, showed aberrant morphology (Fig. 1A) and aggregation of β -globin tetramers on RBC membranes. Due to the inability of these RBC to deliver oxygen, animals succumb to the disease, displaying splenomegaly and other organ pathologies, including vaso-occlusive events, associated with neutrophil infiltration, fibrinogen staining, von Willebrand factor release, and platelet recruitment and activation. These animals also show iron deposition in the liver and kidney, in agreement with very low levels of hepcidin expression in the liver, and elevated erythropoietin (EPO) in the kidney. We screened multiple therapeutic lentiviral vectors using murine erythroleukemia cells and human umbilical cord derived erythroid progenitor (HUDEP) cells, modified by knocking out all the human α -globin genes. Since the regulatory sequences necessary for expressing α -globin at high levels are too large to be included in lentiviral vectors, we identified ALS20 α I, a vector in which the α -globin gene is under the control of the β -globin promoter and its locus control region, as the most efficient vector. One copy of ALS20 α I produces exogenous α -globin at a level comparable to that produced by one endogenous α -globin gene, suggesting that a low VCN could result in dramatic therapeutic benefits. Use of ALS20 α I resulted in correction of the disease phenotype in a dose-dependent manner in α -KO mice. At VCN>1 we observe phenotypic normalization, including HPLC profile (Fig. 1B), Hb, hepcidin and EPO levels, and RBC morphology (Fig. 1A). Additional data in CD34 cells isolated from patients with both deletional and non-deletional HbH disease also indicated that low integrations of ALS20 α I can significantly improve β/α -globin mRNA ratio (β/α R) and reduce HbH. In conclusion, we generated an adult mouse model of lethal α -thalassemia and a vector, ALS20 α I, that successfully improves α -globin levels in mice and patient cells.

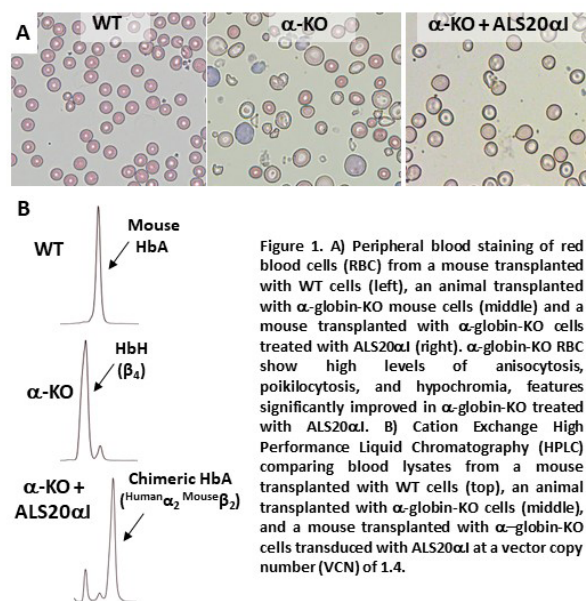


Figure 1. A) Peripheral blood staining of red blood cells (RBC) from a mouse transplanted with WT cells (left), an animal transplanted with α -globin-KO mouse cells (middle) and a mouse transplanted with α -globin-KO cells treated with ALS20 α I (right). α -globin-KO RBC show high levels of anisocytosis, poikilocytosis, and hypochromia, features significantly improved in α -globin-KO treated with ALS20 α I. B) Cation Exchange High Performance Liquid Chromatography (HPLC) comparing blood lysates from a mouse transplanted with WT cells (top), an animal transplanted with α -globin-KO cells (middle), and a mouse transplanted with α -globin-KO cells transduced with ALS20 α I at a vector copy number (VCN) of 1.4.

111. Treating Sickle Cell Disease with Lentiviral Vectors Combining an Anti-Sickling β^{AS3} -Globin Gene with *BCL11A* and *ZNF410* MicroRNA Adapted Short Hairpin RNAs

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Gene therapy is an emerging strategy that has become a promising clinical approach for ameliorating beta-hemoglobinopathies. For sickle cell disease (SCD), multiple strategies have been employed from introducing anti-sickling beta-globin variants, correcting the E6V mutation in beta-globin through *in situ* gene editing, and upregulating fetal hemoglobin (HbF) production through silencing or disruption of *BCL11A*, a fetal globin repressor. While current clinical gene therapy strategies focus on single mechanisms to mitigate the SCD phenotype, we have developed a gene therapy approach that utilizes a lentiviral vector (LVV) of reduced size with locus control regions cores expressing an anti-sickling beta-globin gene (UV+EC1, Morgan Mol Ther, 2020) and a *BCL11A* microRNA adapted short hairpin RNA (shRNA^{mir}) to induce fetal globin expression (Brendel JCI, 2017, Esrick NEJM, 2021) (UV+EC1+SS). We also developed a novel LVV expressing two shRNA^{mir}s simultaneously targeting *BCL11A* and the independent γ -globin repressor, *ZNF410* (termed double shRNA^{mir}, DS). We optimized and selected the location to incorporate the *BCL11A* shRNA^{mir} in the UV+EC1 vector by assessing titer, β^{AS3} -globin, and fetal-globin expression (UV+EC1+SS). We inserted the double shRNA^{mir} targeting *BCL11A* and *ZNF410* into the UV+EC1 vector to engineer the novel vector, UV+EC1+DS. These combination vectors are significantly reduced in size, compared to many beta-globin lentiviral vectors in clinical trials, and have increased titer and gene transfer to CD34+ HSPCs, which can lead to reduced costs of goods per patient treated. These vectors express β^{AS3} -globin and induce fetal-globin expression 5-6 fold over background in transduced human CD34+ cells differentiated *in vitro* to erythroid cells and increase total anti-sickling globin induction around 10 fold (Table). Erythrocytes differentiated from SCD patients HSCs transduced with UV+EC1+SS express therapeutic levels of anti-sickling globin of 47%, which is significantly higher than UV+EC1. The DS vector induced more γ -globin than the SS vector with a total anti-sickling globin of 57%, which is 2.5-fold over UV+EC1 (see Table). SCD derived erythroid cells transduced with UV+EC1+SS or UV+EC1+DS demonstrated a significantly reduced *in vitro* sickling phenotype (see Table). This data highlights the potential of these combination vectors to ameliorate SCD by two distinct mechanisms while also being advantageous for production and transduction.

Globin expression and quantification of sickled cells in erythrocytes differentiated from SCD HSPCs					
	AS3 globin (%)	γ -globin (%)	% Anti-sickling globin	Sickled cells (%)	VCN (c/dg)
SCD	0.02 ± 0.03	5.8 ± 1.9	5.8 ± 1.9	58.8 ± 5.0	0.028 ± 0.02
UV+EC1	17.5 ± 1.9	5.6 ± 1.9	23.1 ± 3.8	40.1 ± 12.5	1.3 ± 0.5
UV+EC1+SS	19.1 ± 2.7	27.8 ± 2.5	46.9 ± 0.6	19.8 ± 1.9	1.8 ± 0.5
UV+EC1+DS	23.8 ± 7.3	33.7 ± 1.8	57.5 ± 4.6	14.63 ± 3.4	1.9 ± 0.3
P value (UV+EC1 vs UV+EC1 SS)	0.9934	0.0004	0.0009	0.0272	0.5485
P value (UV+EC1 SS vs UV+EC1 DS)	0.6334	0.0152	0.0177	0.0875	0.9999

112. Transplantation without Myeloablation: Novel Conditioning Enables Robust Repopulation of Macrophage/Microglia Niches by Bone Marrow-Derived Cells

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Hematopoietic stem cell transplantation (HSCT) has been used for decades to treat multi-systemic diseases including several lysosomal and peroxisomal disorders. The therapeutic effect of HSCT for these diseases is mediated by bone marrow (BM)-derived cells that migrate to non-hematopoietic compartments as tissue-resident myeloid cells where they become a source of healthy cells and can cross-correct protein deficiencies. The engraftment and migration of BM-derived cells is partly influenced by the conditioning regimen, a pre-transplant treatment required to deplete host stem cells. However, despite its great promise, HSCT's risk-benefit assessment is often seen unfavorably. Its morbidity is largely due to the need for pre-transplantation conditioning that can have severe toxicities. Furthermore, HSCT's benefit is limited by the low efficiency of penetration of BM-derived cells in the central nervous system (CNS) and other non-hematopoietic tissues. Given the important therapeutic implications of HSCT for non-hematological diseases, we set out to establish conditioning regimens that act more specifically on the myeloid cell niches. To this aim, we exploited the transient inhibition of the Colony-stimulating factor 1 receptor (CSF1R) which is specifically expressed in CNS microglia and tissue-resident myeloid cells, and whose genetic depletion results in robust CNS repopulation by BM-derived cells. We evaluated the effect of the CSF1R inhibitor PLX3397 (CSF1Ri) alone or in combination with irradiation or Busulfan in conditioning regimens prior to BM transplant (BMT) in C57BL/6 mice. Enhanced green fluorescent protein-positive (EGFP+) bone marrow cells, derived from CAG:GFP/GFP mice were used to quantify donor-derived cells in recipient mice. We found that CSF1Ri combined with either irradiation or Busulfan dramatically increased the homing of BM-derived cells in the CNS, leading to superior engraftment of GFP+ microglia-like cells.

Quantification of GFP+CD45+CD11b+ cells in the brain by flow cytometry showed that they represented the 6±2.4% (Busulfan), 33±2.3% (irradiation), 90±7% (CSF1Ri+irradiation) and 89±5% (CSF1Ri+Busulfan) of host microglia. A safer regimen combining CSF1Ri with non-myeloablative Busulfan (50 mg/kg) also provided superior engraftment of donor-derived cells in the CNS and other non-hematopoietic tissues compared to Busulfan-myeloablation alone. The addition of CSF1Ri did not impact BM engraftment (%GFP+/CD45+: 90±2.8% Busulfan, 92±0.6% irradiation, 92±0.8% CSF1Ri+Irradiation, 92±1% (CSF1Ri+Busulfan) or lineage reconstitution. Furthermore, comprehensive neurobehavioral phenotyping confirmed no added toxicities of the CSF1Ri+Busulfan regimen as compared to Busulfan alone. In-depth characterization of endogenous and donor-derived CD45+CD11b+ cells from controls and transplanted brains by single-cell RNA-seq analyses showed a non-proinflammatory gene expression profile, supporting the therapeutic application of this novel conditioning regimen prior to HSCT for the treatment of CNS diseases. The CSF1Ri PLX3397 (Pexidartinib) is an attractive candidate as a CNS conditioning drug as it received FDA approval in 2019 for the treatment of diffuse-type tenosynovial giant cell tumors, has good CNS permeability and a favorable safety profile. The availability of more effective and specific conditioning regimens would make HSCT safer for the diseases for which HSCT is standard of care, de-risk it for diseases with a theoretical benefit, and ultimately establish a new treatment option for neurological and non-neurological diseases.

Vector Manufacturing and Engineering 2: Next Generation Methods

113. Stable BaEVRless Producer Cell Line for the Production and In Vivo Application of Alpha-Retroviral Particles

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In vivo hematopoietic stem cell (HSC) gene therapy has several potential advantages over *ex vivo* gene therapy, such as reduced cost, less need for individual GMP cell manipulation and, ideally, no need for toxic chemo-conditioning. However, the commonly used pseudotype for viral delivery, namely VSVg, is not suitable for *in vivo* application due to serum sensitivity and poor ability to target quiescent HSCs. In contrast, the baboon endogenous retrovirus (BaEV) glycoprotein and its derivative BaEVRless, which bind to the neutral amino acid transporters ASCT1 and ASCT2 for cell entry, efficiently transduce resting HSCs and are serum resistant. The aim of this study is to generate a stable BaEVRless producer cell line for the production and *in vivo*

application of alpha-retroviral particles. Alpha-retroviral particles can be stably produced long-term, but BaEVRless is highly fusogenic and as such not ideal for a stable producer cell line. To avoid superinfection and syncytia formation during virus production, we first used CRISPR-Cas9 to knockout the entry receptors ASCT1 and ASCT2 in HEK293T cells. We found due to low ASCT1 expression in HEK293T cells that knockout (KO) of ASCT2 is sufficient to eliminate superinfection and cell fusion. Abrogation of syncytia formation reduced the toxicity of concentrated viral supernatants as indicated by a 3-fold reduction of apoptotic cells after transduction of K562 cells. Reduced cell death and sequestration of viral particles resulted in a 2-fold increase in viral titers (400-fold concentrated, ~5 x 10⁸ infectious particles/mL). For *in vivo* application, CD47 was overexpressed on ASCT2 KO cells to reduce non-specific macrophage-mediated uptake of viral particles. Transduction rates of macrophages were reduced by 5-10% using CD47 overexpressing (OE) viral particles, while gene transfer into lymphoid cell lines was not affected. A BaEVRless expression cassette including a puromycin resistance gene was stably integrated into ASCT2 KO CD47 OE cells. The overall viral titer of selected BaEVRless cells was 1-log lower compared to the parental cell line. To increase the titer, clones were picked and screened for a high-titer producing clone. The selected clone achieved similar titers compared to the parental cell line and contains four copies of the BaEVRless expression cassette. The alpha-Gag/Pol expression plasmid was then stably integrated to generate the ASCT2 KO/CD47 OE/BaEVRless/alpha-Gag/Pol producer cell line. This resulting cell line thus only requires transient transfection of the therapeutic vector for virus production and achieves similar viral titers as the parental cell lines in the range of 10⁸ to 10⁹ infectious particles/mL. Alpha-retroviral particles generated from producer clones were applied to transduce HSCs *in vivo* in a humanized mouse model. Initial experiments achieved *in vivo* transduction rates of up to 2% GFP-positive cells. Gene marking was observed in human myeloid and lymphoid lineages as well as in bone marrow hCD34⁺ cells. Consistent with the species specificity, no gene marking was observed in murine cells. In summary, we engineered a stable alpha-retroviral producer cell line to produce high-titer and high-quality BaEVRless-pseudotyped alpha-retroviral particles that overexpress CD47 for *in vivo* gene therapy application.

114. Generation and Characterization of Rep Expressing AAV Packaging Cell Lines

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Complexity and cost of large-scale recombinant adeno-associated virus (rAAV) production still present a serious obstacle in commercialization of rAAV gene therapies. Improvements in rAAV vector manufacturing are achievable using packaging or producer cell lines, which in the case of rAAVs are difficult to generate due to the toxicity of AAV Rep proteins

required for production. The AAV *REP* gene encodes four Rep proteins (Rep-78, -68, -52, and 40) which have multiple overlapping functions and roles in AAV production. We have previously demonstrated transient transfection rAAV production with low amounts of plasmid encoding Rep cDNA. Only two Rep proteins (Rep68 and Rep40) were sufficient to obtain rAAV titers comparable to standard triple plasmid transfection production with the endogenous *REP* gene. With this in mind, we created expression cassettes regulated with two inducible gene expression systems (cumate and coumermycin). Two different expression cassettes were created, a tightly regulated, but weaker cassette for expression of *REP68* and a stronger less stringent cassette for *REP40* expression. We produced lentivirus encoding the expression cassettes and transduced suspension and serum-free media adapted HEK 293SF cells to generate packaging cell pools. We were able to obtain multiple stable cell lines and characterize them with regard to titer, stability, *REP* copy number and its expression level. Titers obtained in suspension rAAV production using Rep packaging cells are also comparable to triple plasmid transfection rAAV2 production. In addition to producing rAAV2, we were able to produce the chimeric rAAV-DJ serotype with similar infectious AAV particle titers. The packaging cell lines were stable in culture for at least 20 passages (7 weeks) without showing decrease in titers, Rep expression levels and loss of integrated expression cassettes. We assessed the quality of rAAVs produced by the packaging cell lines by ddPCR, total particle ELISA and transmission electron microscopy. Currently, adenoviral helper genes are being integrated in the Rep expressing clones to generate packaging cell lines that would only require the single-plasmid transfection carrying gene of interest.

115. Synthetic Biology Approach to Nucleic Acid Clearance in Lentiviral Vector Production

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To improve lentiviral vectors (LVV) purity for cell and gene therapy applications manufacturers' currently remove residual nucleic acids using a DNase treatment step with endonucleases such as Benzonase. This is a prohibitively expensive but necessary step in the manufacturing process as regulators require concentrations of less than 10 ng/dose at sizes less than 200 base pairs in the final product. As an alternative we adapted adherent HEK293T cells to serum-free culture and then engineered them to secrete nuclease enzymes to remove nucleic acid impurities from LVV supernatant. Three nuclease expression cassettes were designed by fusing the N-terminus of the *Staphylococcus aureus* nuclease B (nucB) open reading frame to: the native *S. aureus* nuclease signal peptide, the mammalian murine Igk chain leader sequence, and a novel virus-encoded transport protein. The Tet repressor system was selected to regulate expression of nucB to reduce unwanted cell cytotoxicity and any negative effects on lentivirus production from potential intracellular nuclease expression and constitutive nuclease secretion. All three nuclease expression cassettes demonstrated levels of activity equivalent to or better than Benzonase at 250 units in growth medium harvested from engineered cell lines 24 hours post

tetracycline induction. When treating 1.5 µg 1 kb DNA ladder (500 base pairs to 10 kilobase pairs), 2-hour incubations at 37°C with 10 µL of growth media reduced DNA ladder to non-visible sizes on 1% agarose gel. To verify that a nuclease secreting cell line can support LVV production, a LVV encoding for green fluorescent protein was prepared by transient transfection. Nuclease activity was demonstrated in fractions collected at each step of the LVV production process and had no measurable effect on infectious titre of the clarified supernatant when used to transduce both HEK293T and AGF-T cells under serum-free conditions. This provides a holistic approach to remove residual nucleic acids early in the process stream and improve the purity of the final product, whilst avoiding the addition of exogenous nuclease and its associated costs at scale. This may also potentially reduce any deficiencies of downstream processing attributed to the viscosity of residual nucleic acids.

116. Modification and Optimization of an AAV Purification Process to Accommodate Increased Upstream Yield and Reduce Manufacturing Bottlenecks

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As AAV continues to be a prominent modality in gene therapy, more and more resources are being invested into increasing the productivity of AAV manufacturing. Optimization of bioreactors, transfection reagents, and transfection conditions has led to 10 to 25-fold increases in the crude harvest titer from transient transfection processes developed at LogicBio. Downstream purification strategies initially designed around lower-yielding upstream production must be reevaluated to better accommodate the large increases in vector. This work highlights what was done to modify downstream purification steps to handle a 10-fold titer increase seen during the implementation of a next-generation upstream process. Initially, process modeling was used to predict the impact of upstream changes and identify potential bottlenecks and areas of improvement. Small-scale experiments were used to justify a new clarification train, the removal of two concentration and buffer exchange steps, and sizing adjustments to the affinity chromatography column. Additionally, extensive development work was done to replace a legacy density gradient empty/full enrichment process with a chromatography based polishing step that produces material of comparable quality in a more manufacturing friendly manner. Initial scalability of the downstream process was evaluated using multiple lots of material produced in a 50L STR and purification equipment appropriately sized to handle the harvest pool. In-process analytics were performed on intermediate process samples to compare step by step performance to what was predicted by the bench scale work. The new process successfully reduced the downstream manufacturing timeline from 2-weeks to four days and combined with the improvements in upstream, showed a 10-fold productivity increase compared to the legacy process. Finally, a proof of concept "platform evaluation" was performed

on a gene therapy product that used a new therapeutic cassette packaged in an identical capsid to investigate the translatability of the manufacturing process across similar programs.

117. Adenovirus Purification Method Using Scalable System with Single Use Anion Exchange Fiber Chromatography Capsule

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Easy and efficient production of viral vectors is a key issue for clinical application of viral vectors. Large batch adenovirus (Ad) production still requires further improvement on scalability and process economics. Currently, small batch Ad vectors in the laboratory scale have been purified mainly by double CsCl ultracentrifugation, while large batches are purified by anion exchange resin chromatography. However, both processes require centrifugation steps for removal of cell debris and processing of large volume with ultracentrifuge has many challenges. It is therefore difficult to establish a closed in-line purification system. 3M™ Harvest RC™ and 3M™ Emphaze™ AEX Hybrid Purifier are the next generation single-use harvest and clarification devices for biopharmaceutical processing of biologics. These devices utilize fibrous anion exchange (AEX) chromatography to efficiently separate the cells, cell debris and soluble contaminant (host cell proteins and DNA) from the target biotherapeutic product with precision quaternary ammonium (Q) functionalized polypropylene fiber, combined with a 0.2 μm membrane. They have already been used by therapeutic antibody manufacturers and have provided scalable and predictable clarification in a broad range of manufacturing scales. 3M™ Harvest RC is designed direct clarification of cell culture fluid in a single stage, while 3M™ Emphaze™ AEX Hybrid Purifier is designed for polishing by removing insoluble contaminants after primary clarification. In this project, in order to improve clinical batch production processes for Ad, we aimed at 1) elimination of low speed centrifugation for cell debris removal, 2) removal of large genomic DNA, 3) concentrating Ad for further purification, and 4) elimination of ultracentrifugation by AEX fiber chromatography. First, we applied producer cells with virus after 3 times of freeze and thaw to a Harvest RC capsule after adding 50mM HEPES (pH 7.4) and 300mM NaCl (flowthrough process), as well as with 50mM HEPES (pH 7.4) and 100mM NaCl for binding followed by PBS with 50mM HEPES (pH 7.4) and 300mM NaCl (bind and elute process). The eluates were analyzed by CsCl gradient ultracentrifugation. In both cases, the adenovirus was recovered without loss, and neither protein nor chromosomal DNA band was observed after ultracentrifugation. Next, we performed bind and elute experiments with an Emphaze capsule. The CVL prepared by regular centrifugation procedure was applied to Emphaze capsule after adding 50mM HEPES (pH 7.4) and 100mM NaCl for binding, followed by elution with PBS with 50mM HEPES (pH 7.4) and 100, 140, 180, 220, 260, and 300mM NaCl (5ml each). The eluates were analyzed by banding pattern after ultracentrifugation. There was no virus elution up to 180mM NaCl solution (conductivity 31.3 mS/cm). Addition of 200mM NaCl (35.1mS/cm) showed large quantity elution of the virus, and it was followed by smaller quantity elution with 260 and 300mM NaCl. These results show that cleanup of debris and

cell with Harvest RC was possible without centrifugation of CVL, and Harvest RC also removed large chromosomal DNA. The polishing processes with Emphaze enabled bind and elute, through which the concentrating the virus was possible. Also this step seemed to provide sufficient clarification to replace the ultracentrifugation process. Further optimization of buffers as well as capsule housing design may enable improvement of bind and elute patterns, which may permit total elimination of ultracentrifugation steps from the virus purification process. Considering the compatibility of the devices for in-line system as well as unparalleled scalability (the largest capsule permits processing of CHO cell in 2000L tank), these devices may revolutionize the large clinical batch adenovirus purification processes.

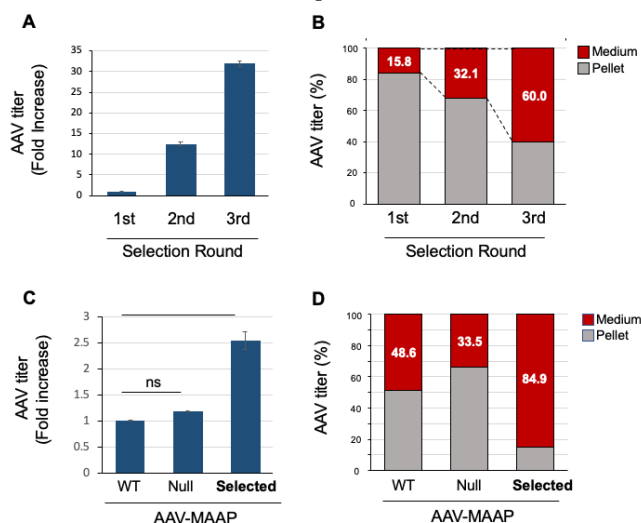
118. Membrane-Associated Accessory Protein Variants Improve Adeno-Associated Virus Production in HEK293 Cells

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Gene therapies delivered by adeno-associated virus (AAV) have shown significant clinical success in recent years. However, manufacturing quantities of good manufacturing practice (GMP)-grade AAV necessary to achieve current and projected dosing requirements presents a significant hurdle to expanding AAV-based gene therapies. The natural genome of AAV contains ~4.7 kb of ssDNA that encodes up to ten known proteins in a highly overlapped fashion. Among these proteins, the most recently discovered is the membrane-associated accessory protein (MAAP). MAAP is encoded by an alternative open reading frame (ORF) in the AAV cap gene that is found in all presently reported natural serotypes. Recently, evidence has emerged supporting a functional role of MAAP in AAV egress. We hypothesized that directed evolution, the iterative process of sequence diversification and selection of functional gene variants, could be utilized to identify MAAP variants that confer increased AAV secretion during packaging. To test this hypothesis, we generated a library of over 1E6 MAAP variants, which we subjected to five rounds of packaging into an AAV2 capsid for which MAAP expression was inactivated without altering the VP1 ORF (AAV2-MAAP-null). Among each iterative packaging round, we observed a progressive increase in both overall titer and ratio of secreted vector genomes conferred by the bulk selected MAAP library population. Next-generation sequencing uncovered common mutational features that were enriched up to over 10,000-fold on the amino acid level, including truncation mutants. Individual MAAP variants were isolated and systematically tested for effect on recombinant AAV2-MAAP-null packaging in HEK293 cells. Individual MAAP variants tested so far conferred an increase in overall AAV2 packaging titer of up to three-fold higher than levels conferred by wildtype MAAP2. We predict that this work may be applicable to increasing per-cell AAV output in industrial settings, potentially reducing global costs and increasing functional vector recovery in downstream manufacturing processes.

Figure Information: **A)** Head-to-head comparison of total vector genome titer (obtained by qPCR) of the MAAP library bulk population after one, two, or three rounds of AAV2-MAAP-null packaging selection relative to the titer after the first round. **B)** Ratio of secreted vector genomes for samples described in A. **C)** Head-to-head comparison of relative total vector genome titer (obtained by qPCR) when packaged into AAV2-MAAP-null containing wild type (WT) MAAP2, no MAAP, or the selected MAAP library bulk population. **D)** Ratio of secreted AAV for samples in C.



of our proprietary animal component-free growing suspension cells to stably express all the components essential for rAAV production as well as the transgene flanked by the ITRs. As a first step, clonal Alpha cell lines were generated harboring Tet-inducible expression of the Rep genes and the adenoviral helper functions E2A, E4orf6, and VA RNA. Lastly, the capsid genes and the transgene flanked by the ITRs have been stably integrated into the Alpha cell lines, generating an inducible rAAV stable helper virus-free ELEVECTA producer cell line. Production using the stable ELEVECTA system is initiated by induction with doxycycline, a straightforward and efficient procedure to generate high AAV vector quantities in a suspension system. The true power of our production system was revealed through upstream process optimization for our first proof of concept AAV8-GFP producer cell line. An attractive advantage of a stable AAV producer cell is the practicability of using manufacturing process intensification. We applied ATF perfusion technology to implement an upstream intensified large-scale cell culture process for rAAV production. The perfusion-based production process led to cell-specific yield (VG/cell) 8-fold higher when compared to the reference process in batch mode. Remarkably, this ATF perfusion process resulted in very high titers (E15 vg/L) and a high percentage of full particles (35-40%). The productivity improvement observed with upstream process intensification is a noteworthy success of the ELEVECTA platform, confirming the achievement of high quantities of AAV vector using a stable producer cell system for AAV manufacturing.

119. AAV Manufacturing with Stable Helper-Virus Free ELEVECTA® Producer Cells for Industrial Scale Vector Production

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Over the past decade, gene therapy applications have revolutionized the landscape of clinical medicine for inherited and acquired human diseases. In particular, recombinant adeno-associated virus (AAV) has been used extensively as a vehicle for transgene delivery in clinical studies, with several rAAV-based therapies recently gaining regulatory approval. The growing interest in rAAV-based gene therapy underscored a critical need for scalable high-yield production and demand for groundbreaking manufacturing approaches. To bridge the production gap in gene therapy, CEVEC combined its expertise in cell line development technologies and in-depth understanding of the rAAV basic biology to generate an innovative stable helper-virus free rAAV production platform based on ELEVECTA stable producer cells. Development of the ELEVECTA platform involved genetic engineering

AAV Vectors - Virology and Vectorology I

120. Aberrant Resolution Sites Lead to Backbone DNA Contamination of rAAV Vectors

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Recombinant adeno-associated virus (rAAV) vectors carry a cassette of interest retaining only the two inverted terminal repeats (ITRs) from the viral genome. Conventional rAAV production methods use plasmid DNA to provide the vector genome and the AAV/Adenovirus proteins required for replication and packaging. Contamination of rAAV vectors for gene therapy is an emerging issue in the field. rAAV vector DNA contamination mainly originates from the vector plasmid backbone. The mechanism driving the contamination phenomenon has yet to be elucidated. The ITRs are prone to mutation and deletion by bacterial hosts that are used to amplify the DNA for vector production. Here we systemically investigated how single ITR-containing DNA species affect rAAV vector production and lead to contamination. Interestingly, various forms of DNA with a single ITR were able to rescue and replicate efficiently. Their presence in the rAAV production system significantly competes for trans factors required for rAAV rescue, replication, and packaging. Although the replication of single ITR contaminants in triple plasmid AAV production system is independent of its size, the packaging of these species is affected by size. These results show that

there exist various aberrant resolution sites on rAAV vector plasmid backbone, thereby leading to truncated DNA generation. These single ITR-containing DNA species are a negative factor that contributes to impurities in purified rAAV vectors, reduces rAAV vector yield and quality, and potentially affects long term rAAV performance.

121. Directed Evolution of AAV for Retinal Gene Therapy

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Gene therapy is already a therapeutic option in the retinal degenerative disease field. Currently the only FDA approved gene therapy is for a rare childhood disease and relies on subretinal injections of naturally occurring Adeno-associated viral vector (AAV) carrying the therapeutic gene. To develop gene therapies applicable to a larger number of patients, AAVs that can target the retina more broadly and surgically less complex routes are necessary. An ideal route of injection is intravitreal injection. Intravitreal injections are easy to perform and put the virus in contact with the entire retinal surface. However, efficient gene delivery to the retina following intravitreal injection requires a vector able to cross the vitreous humor and inner limiting membrane. Unfortunately, none of the natural serotypes cross these barriers sufficiently to provide pan-retinal gene delivery with a well-tolerated dose. In an effort to find AAV variants capable of such tasks we used directed evolution, an approach that mimics evolution and rapidly selects optimised molecules for a specific task. In prior efforts, directed evolution was used to generate AAV variants that can infect the primate retina. Variants such as NHP26 and R100 have been optimized in the non-human primate retina and show efficient gene delivery in the foveal and peripheral regions throughout retinal layers. Since gene delivery has species specific attributes to date it is unknown how these variants will behave in a human retina, particularly in the macular region where the inner limiting membrane is the thickest. To develop an AAV variant for these specific needs, we used directed evolution to select AAV variants based on their ability to infect the macular region of human retinal explants. We then characterized the selected variant on the most adequate ex vivo and in vivo model systems available. Finally, in an effort to understand the effect of the vitreous on infectivity of our variant, we also tested its infectivity on macaque retinal explants, a tissue without the vitreous. Our data support the possibility to develop specifically tailored AAVs for human retinal gene therapy and provide new analysis methods based on next generation sequencing to track the directed evolution process.

122. Engineering AAV Capsid Variants to Overcome Pre-Existing Immunity and Improve Gene Delivery to Human Liver

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Gene therapy vectors based on adeno-associated virus (AAV) offer robust and long-term gene expression. Through numerous clinical trials they have emerged as the vector of choice for a wide variety of disease targets. However, continued challenges remain regarding immunogenicity and toxicity at high vector doses. In recent years, there has been a concerted effort in the field to identify and develop novel capsid variants to improve efficacy. Indeed, a small number of capsid variants with improved tropism for human liver cells have emerged from such studies, including our first-generation capsid, AAVS3. This report focuses on the development and characterization of capsid variants arising from directed evolution on primary human hepatocytes in the presence of highly AAV-neutralizing human plasma. In this approach, 23 AAV capsid sequences were shuffled, a replication competent AAV library (diversity $\sim 7 \times 10^5$) was produced, and five rounds of evolution on primary human hepatocytes *in vitro* were performed. Highly neutralizing plasma was incorporated during the final round to select capsids that evade existing anti-AAV antibodies. Using long-read sequencing, the most abundant variants (26) were selected to be characterized through a pipeline designed to assess functional transduction, seroprevalence, and manufacturability. The variants displayed a broad range of transduction profiles compared to AAVS3; from reduced transduction to greater than 4-fold improved transduction efficiency in primary hepatocytes. Work is ongoing to characterize lead candidates in a humanized liver model relative to wild-type serotypes. In addition, the neutralization profiles of the variants were assessed. Variants were ranked based on resistance to neutralization by intravenous immunoglobulin (IVIG) and a human plasma pool. Subsequently, the seroprevalence of a lead candidate was established against 96 human plasma samples in a high throughput transduction inhibition assay. Increased ability to overcome pre-existing immunity was inversely correlated to the ability to transduce liver cells. Our capsid engineering process provides the opportunity to explore capsid sequence space, and in conjunction with functional data, to accelerate the evolution and rational design of AAV capsids that will advance gene therapy. Through this approach, we have identified novel capsid variants with promising combinations of desirable characteristics such as improved liver tropism and potency, as well as reduced immunogenicity.

123. Bioengineered Hybrid Rep 2/6 Improves Encapsulation of Single-Stranded Expression Cassette into AAV6 Vectors

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Introduction: Recombinant Adeno-associated viruses (rAAVs) are one of the most widely used vector systems for gene therapy applications

and the number of clinical trials increases year by year. With more than thousand clinical trials ongoing, gene therapy is a growing part of biopharmaceutical research. The current challenge remains unresolved for sufficient vector production with high purity and guaranteed safety to satisfy increasing demand in pre-clinical testing on the large animal models and human clinical trials. AAV is a gutless vector without viral genes or *wild-type* viral protein expression, leading to a low cytotoxic reaction by transduced cells. However, regardless of AAV production methods, around 50% of generated capsids are lacking fully encapsulated therapeutic genome called “empty capsids” and are considered one of the main byproducts of AAV production. Thus, full vs. empty ratio improvement can help to enhance AAV treatment efficiency and reduce the risk of initiating cellular immune response toward AAV transduced cells and eventually lead to inactivation of AAV-mediated therapeutics. Hypothesis: Historically, AAV vectors were produced with matching pairs of AAV serotype 2 Rep and ITRs with only variables of capsid from different serotype of choice. We hypothesize that molecular bioengineering of Rep proteins may generate improved ratios of fully encapsulated vs. empty capsid, reducing the amount of non-active vectors. Approach: In common plasmid-based production systems expression of the Rep gene is driven by two endogenous promoters, p5 and p19, generating four splice variant proteins, Rep 78 and Rep 68, Rep 52 and Rep 40, what are responsible for ITR contained expression cassette replication and packaging. The expression of Cap gene, downstream of Rep, is driven by p40 promoter in C-terminus part of Rep and produces the three structural proteins for the capsid. Thus, we generated the hybrid Rep 2/6 gene which combined amino acids 1 to 92 of N-terminus part of Rep 2 containing DNA binding domain and C-terminus part of Rep 6 which matches corresponding downstream Cap 6 gene. We next compared the packaging of single-stranded expression cassette contained firefly luciferase - GFP fusion flanked with ITR 2 by hybrid Rep2/6 was with full Rep2 and Rep 6. Results: Our preliminary results obtained by WB analysis on packaging cell line transfected for AAV preparation indicate that there is clear reduced expression of Rep78/68 by Rep6 and that expression is restored in Rep2/6 hybrid to the level produced by the Rep2 gene. On the another hand, all three Rep genes produced similar levels and ratio of Rep52/40 and VP proteins. Consequentially, Rep6 failed to generate high titer of DNA encapsulated viral particles confirmed by qPCR with common promoter region primers. However, Rep2/6 hybrid generated AAV titer similar to Rep2. Encapsulation of DNA was evaluated by TEM imaging. Hybrid Rep2/6 improved full/empty ratio compared to Rep2 suggesting that C-terminus part of Rep6 encapsulates DNA more efficiently into AAV6 capsids than Rep2. AAV-mediated luciferase activity in vitro on K562 cells and in vivo on C57Bl6/J mice normalized to viral titer was similar for production with all three Rep constructs. Conclusions and future direction: Manipulation with Rep gene can be used to improve empty/full ratio without compromising viral titer and activity. The future direction of our studies include understanding the precise mechanism of improved packaging by hybrid Rep2/6 and evaluation of difference in AAV associated immunogenicity toward AAV6 capsid encapsulated by different Rep genes. Acknowledgements: This project is supported by NIH R01HL131586 and FDA R01FD007483 funding.

124. Programmable Adeno-Associated Viral Vectors for Cell Specific Targeting

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Adeno-associated virus (AAV) are nowadays the most utilized viral vectors for in vivo gene transfer. Their safety and efficacy continue to be extensively studied in clinical trials. Nevertheless, the wide tropism that characterizes these vectors prevents their use to specifically target a certain type of cells or organs. Several groups included ours have developed novel AAV capsids through DNA shuffling or peptide display techniques to make vectors capable to improve the transduction of specific tissues. Here, we developed an AAV vector (Nε-AAV) displaying a single unnatural amino acid substitution on the capsid surface for post-production vector engineering through click chemistry. First, we rationally screened a panel of different AAV serotypes and regions amenable for the insertion of the unnatural amino acid. Next, we identified the AAVs that would tolerate the unnatural amino acid substitution on the capsid without disrupting their packaging efficiency. As a proof-of-concept, we functionalized the Nε-AAVs through the conjugation with a widely characterized DNA aptamer (AS1411) or a folic acid moiety (FA). In vitro specific uptake of the AS1411-AAVs via aptamer targeting was assessed by using a specific antidote against the AS1411. On the other hand, the specific uptake of FA-AAVs was evaluated co-culturing the cells with an excess of folic-acid or employing an antibody directed against the folic acid receptor. AS1411-AAV showed up to 9-fold increase in transduction compared to the non-conjugated counterpart in MCF-7 and A549 cells, two different cell lines commonly used for this type of aptamer. FA-AAV displayed specific transduction in cell lines that overexpress the folic acid receptor with an average 4-fold increase in transduction compared to the non-conjugated vector. Studies in mice are underway to evaluate whether the FA-AAV and AS1411-AAV might specifically transduce cancer cells in a xenograft animal model. Overall, the high versatility of these novel Nε-AAVs might pave the way to tailoring gene therapy vectors toward specific type of cells both in ex vivo or in vivo approaches.

125. Methylation of CpG Dinucleotides in AAV Vector Plasmids Reduces TLR9 Immune Activation

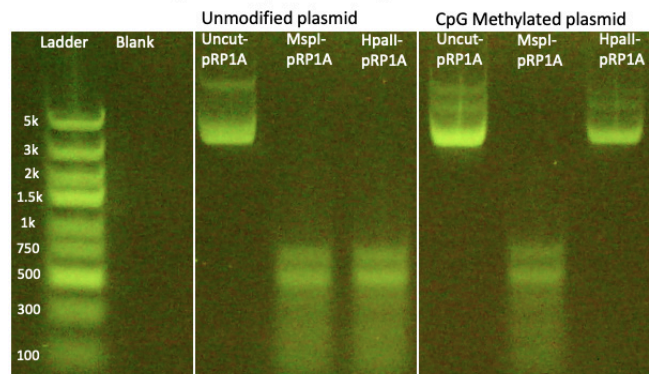
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Capsid-specific Cytotoxic T Lymphocytes (CTL) triggered through toll-like receptor (TLR9) signaling can reduce AAV delivered transgene expression. Unmethylated CpG (Me⁻ CpG) dinucleotides are ligands for TLR9, and dimerization of TLR9 receptors triggers pro-inflammatory cytokines that potentiate capsid specific CTLs. AAV genomes are hypomethylated at CpG. Previously, we performed HEK triple transfection with BrDU-labeled plasmids. We observed a bimodal density distribution of subsequent AAV particles with 50% at a lower density suggesting eukaryotic origin, but unexpectedly 50% were detected at a higher density suggesting BrDU-labeled plasmid DNA was directly packaged into AAV particles. Thus, any strategy to increase

CpG methylation must provide methyltransferase to AAV genomes originating in both prokaryotic cells, predicted to be unmethylated at CpGs, as well as eukaryotic cells predicted to be hypomethylated. We are investigating vector genome methylation to decrease CTL immune responses to AAV gene therapy. Our initial study focuses on the methylation of AAV vector plasmids. *We hypothesize that methylated CpG in AAV vector plasmids will reduce TLR9 activation.* Our initial experiments confirmed that adjuvant CpG containing oligodeoxynucleotide (ODN) sequences stimulated TLR9-specific immune signaling in an HEK reporter cell line, and significantly reduced after CpG methylation. CpG containing AAV vector plasmids stimulated a dose dependent increase in signaling from TLR9 reporter cells. We next incubated AAV vector plasmids with a methyltransferase, MSsSI and verified methylation by comparing MSsSI treated and untreated plasmids after digestion with methylation insensitive and sensitive restriction enzyme isoschizomers MspI and HpaII, respectively (Figure 1). We then interrogated differential transgene expression (GFP) on HeLa cells, and TLR9 immune signaling in an HEK reporter cell line. Here, we report that transgene expression is apparent after transfection by methylated plasmid, although levels are significantly reduced compared to those from unmodified plasmid. Moreover, we show that TLR9 activation was significantly decreased ($p=0.012$) in reporter cells transfected by methylated plasmid, compared to unmethylated controls. The observed decrease in transgene expression may be due to promoter methylation, or open reading frame methylation, or both. Methylation dependent promoter inactivation could be prevented with the use of a methylation independent promoter. Open reading frame CpG methylation dependent decreases in expression is a possibility, and is being characterized. Understanding the effect CpG methylation in the ORF in the vector plasmids has on expression efficiency is a key pre-requisite to our planned experiments to methylate CpG dinucleotides in the expression cassette of AAV vectors. TLR9 recognition of unmethylated CpGs is the first step in a signal transduction pathway that eventually leads to CTL responses that can eliminate therapeutic transgene expression. Here, we present preliminary data supporting that AAV vector genome methylation may be a viable strategy to decrease the CTL response to systemically administered AAV. We previously reported that nearly half of AAV vector genomes are directly rescued from vector plasmids. Therefore, these studies are a necessary first step toward the production of AAV vectors that avoid TLR9 recognition.

Figure 1: Unmodified plasmid pRP1A was susceptible to digestion using CpG methylation sensitive RE HpaII, but resistant after CpG methylation using MSsSI



126. A Novel Engineered AAV2 Capsid Variant CereAAV™ for Efficient In Vivo Gene Transduction into Mouse Brain Neurons and Microvascular Endothelial Cells

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Introduction: Recombinant adeno-associated virus vectors (AAVs) have been widely used as vehicles to deliver gene of interest for research tool as well as for therapeutic purpose. Recently, Zolgensma® for spinal muscular atrophy, was approved by the US Food and Drug Administration (FDA) and many pre-clinical and clinical studies targeting brain diseases have been performed using AAV2 and AAV9 serotypes via local and systemic administration. In particular, AAV9 have been commonly used for gene delivery into brain including central nervous system (CNS) and several AAV9 derivatives such as AAV-PHPB, have been reported to transduce CNS efficiently. It has been reported that AAV2 variant, designated AAV-BR1 efficiently transduced the Blood Brain Barrier (BBB) -associated endothelial cells by systemic administration. However, there is still no AAV2 variant is reported to transduce CNS across the BBB efficiently. To overcome shortcoming of AAV2 property, we addressed to develop a novel brain-targeting AAV vectors based on AAV2 serotype. **Method:** To date, peptide library screening approach by inserting short peptide exposed on viral particle surface, have been established as AAV capsid engineering method for tissue-specific transduction. In this study, we generated an AAV2 random peptide library and then injected into Balb/c mice intravenously. After 3 weeks, AAV genomic DNA was extracted from mouse brain and recovered by PCR amplification followed by re-cloning as a new library. After 3 rounds of those directed evolution, we identified several clones as brain targeted AAV2 vector candidates. **Result and Discussion:** To evaluate peptide mediate-transduction of engineered AAV2 mutant vector (AAV2mt) into mouse brain, we generated three candidate AAV2mt harboring AcGFP gene under the control of CAG promoter and then injected these vectors intravenously at 1×10^{12} vector genome/mouse into Balb/c mice. After 4 weeks, we analyzed AcGFP expression in brain by

immunohistochemistry (IHC) and AAV vector genome distribution was quantified by qPCR in several organs. Significant AcGFP expression mediated by AAV2mt were detected in whole brain and the expression level and coverage were much higher than wild-type AAV2 and AAV9. To confirm these results, we injected the AAV2mt into mouse again and then determined the gene transfer target by IHC. AAV2mt showed stronger AcGFP expression in the whole brain than AAV2 and AAV9 reproducibly, and the significant higher expression were detected in hippocampus (CA2 region) and thalamus than in cerebral cortex, striatum, and cerebellum. In addition, AAV2mt was efficiently transduced into neurons and microvascular endothelial cells in the mouse brain, but not astrocyte, oligodendrocyte and microglia. The amount of AAV2mt vector genomes in brain was more than 250-folds and 50-folds compared to wild-type AAV2 and AAV9, respectively. On the other hand, the AAV2mt vector genomes in liver was 300-folds less than wild-type AAV2. These results suggested that the AAV2mt named CereAAV™ efficiently transduced brain and strongly de-targeted from the liver. Now, we are trying to analyze the gene transduction capability of this CereAAV™ in non-human primate model, such as marmoset and reveal the mechanism of efficient gene transfer in brain, especially in neurons. This AAV2 mutant, CereAAV™, may have a benefit to use as a transduction tool for research use, but also clinical studies against brain diseases, due to efficient gene delivery to whole brain and de-targeting from liver by intravenously administration.

127. Applications of Light Scattering for Adeno-Associated Virus (AAV) Production and Characterization

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Light scattering methods can be employed to assess the mass, size and other critical quality attributes for AAV material testing and characterization. When coupled with size-exclusion chromatography (SEC), ultraviolet (UV) absorbance and differential refractive index (dRI) detectors, SEC-MALS has the potential for execution as an informative and efficient multi-attribute method (MAM). This session will cover how the Gene Therapy group at Resilience is utilizing multi-angle light scattering (MALS) to help drive process development decisions for AAV production and purification. Recent findings from SEC-MALS analysis will be compared with data generated by alternative light scattering methods, specifically dynamic light scattering (DLS) and mass photometry, with an aim to pinpoint specific process steps where each method may generate the greatest value.

128. Capsid Selection Strategy for the Development of Gene Therapies Based on Structural and Functional Analyses of a Panel of AAVHSCs

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Capsid selection, when developing a gene therapy, is a decisive step to increase success in the clinic. The capsid specific tissue, cellular pathway and cell-type tropism among different species and routes of administration all play critical roles in this strategy. First identifying the cell/tissue/organ or organ systems that need to be corrected based on the clinical presentation of the target disease and then overlapping this need with a capsid's characteristics can give rise to the best comprehensive outcome for the affected individual. We leverage AAVHSCs, a panel of 15 naturally occurring adeno-associated virus (AAVs) isolated from healthy human hematopoietic stem cells (HSC) which differ from Clade F member AAV9 and each other by one to four amino acids. The naturally occurring variations in the AAVHSCs are located in different functional regions of the capsid, including but not limited to, hypervariable regions, phospholipase domain, basic regions and glycan binding sites, which render each capsid with a unique set of features. By understanding the relationship of how each variation, based on structural and functional mapping, contributes to the unique characteristics of each capsid, we identify key insights to help guide our AAVHSC program capsid selection strategy across rare metabolic and neurological diseases. These capsid specific features are not limited to tropism, but also include overall immune responses, cellular or glycan binding and transduction kinetics. Herein we present, *in vivo* murine and non-human primate data, as well as *in vitro* data sets illustrating our AAVHSC capsid selection strategy for therapeutic applications. *In vivo*, we execute this strategy by comparing cellular vector genomes (vg), biodistribution, transgene output, translatable biomarkers and/or behavioral analyses across our intravenous programs or studies. Additional disease-specific evaluations, such as accessing the blood circulation or cerebrospinal fluid via secretion or crossing successfully into the nervous system via its wide range of blood-barriers, are included when necessary to guide the dose-selection rationale and achieve the most appropriate systemic distribution. *In vitro*, information on how the unique and naturally occurring capsid variations influence detailed functions such as trafficking, second strand synthesis or vg translation is also being utilized to further guide capsid selection. This vast amount of information and understanding of the AAVHSCs has been utilized in selecting capsids for therapies targeting lysosomal storage disorders. An example exemplifying this rationale is our gene therapy approach to Hunter Syndrome, which required not only widespread distribution to key tissues such as lung and kidney, but also benefited from a capsid targeting the choroid plexus for secretion and cross-correction in the central nervous system. These approaches in mind along with our panel of 15 naturally

occurring AAVHSCs allows Homology Medicines to embrace a capsid selection rather than a “one-capsid-fits-all” limitation to best develop disease specific therapeutics.

129. Directed Evolution of an AAV5 Capsid Library Identifies a Variant with Enhanced Transduction in Non-Human Primate and Rodent Brain Following Systemic Administration

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Recent efforts aiming at engineered Adeno-Associated Virus (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 applied our proprietary RNA-driven TRACER evolution method to AAV5, a highly divergent AAV serotype with a low prevalence of preexisting neutralizing antibodies in the general population. In order to increase the likelihood of identifying capsids with cross-reactivity between primates and rodents, the library was screened in parallel in non-human primates (NHP) and in rats. Following two rounds of biopanning, we identified a variant, VCAP-100, that showed a marked improvement in brain transduction in both NHPs and rats (60- and 40-fold, respectively) relative to AAV5. The VCAP-100 capsid was tested in cynomolgus macaques by intravenous dosing of 5×10^{13} viral genomes per kg with a self-complementary transgene. Relative to AAV9 used as a benchmark, VCAP-100 allowed 20-fold higher brain transduction and 5-fold higher spinal cord transduction. Transduction was widespread throughout multiple brain regions, but was noticeably high in the caudate, putamen, geniculate nucleus, thalamus, hippocampus, cerebellar cortex and deep cerebellar nuclei. Transduction was detected in cells showing either neuronal or glial morphology, suggesting a broad cell tropism. Transgene expression from the VCAP-100 capsid was similar to AAV9 in the liver and the heart but was 50% lower in the dorsal root ganglia (DRG). Consistently, whereas mild histopathology findings were observed in the lumbar DRGs of NHPs injected with AAV9, no microscopic findings were noted in the animals dosed with the VCAP-100 capsid. Our data indicate that the VCAP-100 capsid presents a combination of favorable attributes including high CNS transduction from the intravenous route in NHPs, cross-species translatability, a favorable immunological profile and an improved safety profile, possibly caused by reduced DRG transduction.

130. Engineering AAV Capsids with CNS-Targeted Biodistribution from Massively Diverse Libraries Using Machine Learning

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Targeted biodistribution of AAV remains a critical challenge for human gene therapies. CNS-targeted therapies currently use wild type AAV9, which is limited by high transduction of the liver and the dorsal-root-ganglia (DRG). Off-target AAV transduction limits therapeutic efficacy and increases toxicity, leading to great interest in capsid engineering to improve biodistribution. Previous capsid engineering strategies have generally been limited to libraries of relatively low diversity (thousands to millions of capsids), probing only a small portion of the vast possible space of capsid design; and employed positive-only selection, which fails to select against off-target tissue tropism. The AAVidTM platform leverages massive diversity capsid libraries and direct-to-non-human primate (NHP) biological selection for high-resolution mapping of the AAV mutational space to determine features affecting capsid assembly and tissue tropism. We have generated two distinct AAVidTM libraries: a combinatorial variant library with mutations in the sialic acid binding pocket on the AAV5 backbone (AAV5id) and a dual loop extension library on an AAV9 backbone (AAV9id). The total estimated viral diversity input in NHP was >1 billion for AAV5id and ~500M for AAV9id. We screened each library in four animals (two males, two females) and recovered >30 million unique AAVidTM capsid sequences from over 50 distinct tissues. To analyze this primary dataset, we developed a novel computational pipeline that accounts for sparse sampling and clusters similar sequence variants, enabling us to identify high-confidence capsid variants for a tissue of interest (positive selection) while de-targeting others (negative selection) for advancement into a functional secondary screen. We present data from applying the AAVidTM platform for identification of CNS-targeting capsid variants following intravenous administration. From our AAV5id primary screening dataset, we selected ~3000 capsid variants that were only observed in the CNS across multiple brain regions in multiple animals, as well as CNS-specific variants with similar primary amino acid compositions. We applied supervised machine learning to identify variant amino acid sequences with the highest probability of CNS-targeting/peripheral-detargeting (top 1.1% likelihood). Using the outputs of these models, we also expanded our search space to include synthetic, machine learning-generated CNS-targeting variants (top 0.03% likelihood). In total, we advanced 5000 capsid variants and controls into an NHP secondary screen that profiles both DNA and RNA to measure functional, cell-type-specific transduction within the target tissue. The AAVidTM platform significantly advances the magnitude of novel capsid screening power and computational analysis to identify next-generation AAV capsids with tissue- and cell type-specific biodistribution.

131. Investigation of AAV-Like Capsid Candidates for Functional Gene Delivery

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Adeno-associated viruses (AAVs) are non-pathogenic parvoviruses that are currently the foremost gene therapy vectors. As gene therapy is increasingly employed to treat a variety of indications, AAV capsids serve a critical role in delivering therapeutic nucleic acids. However, frequent natural AAV infection in humans causes high rates of pre-existing antibodies against AAV in clinical populations, and this seropositivity can exclude patients from AAV gene therapy. Further, effective delivery of larger transgenes in AAV vectors remains a major challenge to successful gene therapy, due to the restrictive 4.7 kb packaging capacity of AAVs. In particular, this packaging capacity presents a challenge for delivery of gene editing cassettes, which often extend beyond 6kb even before including optimal regulatory elements. To overcome these limitations, we investigate non-AAV capsids sharing structural features of AAV virions to elucidate novel gene therapy vectors. Amongst our capsid candidates are isolates from Densovirus (DNV), a subfamily of parvoviruses which include isolates with genomes extending beyond 6kb. Although DNV naturally infect invertebrates, we find that capsid proteins from many DNV isolates, including *Galleria mellonella* densovirus and *Penaeus monodon* hepadensovirus, are effectively expressed in mammalian cells when codon optimized and controlled by mammalian regulatory elements. Using transmission electron microscopy, we show that DNVs produced from mammalian cells can assemble capsid structures with diameters of approximately 25 nm, as would be expected in their natural host. We explore genome packaging strategies using both the natural genome replication machinery of our capsid candidates, as well as synthetic genome packaging machinery to encapsidate an exogenous transgene cassette. We aim to engineer the tropism of these novel vectors in hopes of generating an immune-evading AAV alternative for delivery of genome editors.

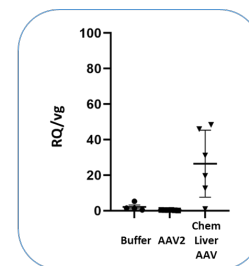
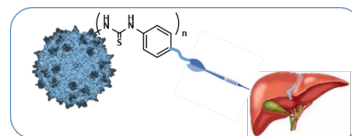
132. Chemically-Engineered AAV Vectors: How Chemistry Improves AAV Liver Gene Delivery

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Results from gene therapy clinical trials for hemophilia demonstrate the potential of the adeno-associated virus (AAV) vector platform to target the liver. However, to achieve therapeutic transgene expression, high vector doses are often required which raises safety concerns. To address this issue and increase the therapeutic index of AAV vectors, we developed a strategy of covalent grafting of chemical organic molecules on the surface of AAV capsids. In order to target asialoglycoprotein receptors (ASGPR) expressed on the surface of hepatocytes, a family of GalNAc ligands associated with a phenylisothiocyanate coupling

function was synthesized and conjugated to AAV2 vectors to generate chemically-engineered AAV vectors (ChemLiver-AAV2)¹. After validation of the chemical coupling using a panel of analytical assays, we evaluated the *in vivo* efficiency of ChemLiver-AAV2 for liver cell transduction. Mice were injected with either unmodified AAV2 or ChemLiver-AAV2 vectors both carrying an eGFP reporter gene expression cassette. One month post injection, ratios between eGFP mRNA levels (RQ) and vector copy numbers (vg/dg, vector genomes per diploid genome) analyzed in liver resulted in a value of 0.37 ± 0.1 vs 30 ± 15 respectively with non-modified AAV2 and ChemLiver-AAV2. Said differently, one copy of ChemLiver-AAV2 vector expressed up to 80 times more eGFP mRNA than one copy of AAV2. Furthermore, ChemLiver-AAV2 vectors showed a moderate reduced interactions with pre-existing capsid neutralizing factors when compared to AAV2 vectors, which, if confirmed, would be of interest in individuals with preexisting humoral anti-AAV immunity. Taken together, our findings reveal the great potential of creating a liver specific engineered AAV platform *via* chemical coupling to improve AAV-mediated liver gene therapy. 1. Mével *et al.*, *Chemical Science*, **2020**, *11*, 1122. Mével *et al.*, Patent WO2017EP64089 20170609, **2017**. Mével *et al.*, Patent WO2020EP69554 20200710, **2019**.



133. RBSeq: A Novel Bayesian Approach for More Comprehensive Characterization of Variant Performance in High-Throughput Capsid Screening

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Adeno-associated virus (AAV) vectors have become a promising gene delivery modality in treating a wide variety of diseases. However, due to the high complexity of the AAV sequence space, we are still searching for the ideal capsids that would satisfy multiple optimization objectives such as tropism, safety, and production fitness. Using next-generation sequencing (NGS) technologies, we can more efficiently explore the large AAV capsid sequence space through screening of high diversity libraries, allowing for comparison across many AAV capsids in a single experiment. However, analyses of capsid screening datasets often employ simple output/input ratios or co-opt differential expression methods to estimate capsid functionality. These approaches can suffer from several drawbacks, including (1) unreliable and unstable estimation due to a preponderance of low or zero-counts in high-diversity screens, and inability to (2) accurately quantify uncertainty in enrichment estimates and (3) directly

compare capsid enrichment results across screening experiments. To address these concerns, we propose RBSeq, a novel empirical Bayes beta-binomial model, where shared beta priors estimated from the data induce borrowing across capsids, creating a stabilizing effect on fold-change estimates for low-count variants. Beta-binomial conjugacy enables RBSeq to efficiently simulate directly from log fold-change posterior distributions even for very large screens. This allows for a complete characterization of the uncertainty in enrichment estimates, as well as increased flexibility in downstream analysis pipelines, where fold-change point estimates as well as measures of variability can be used to inform machine learning models to predict capsid efficiency from amino acid sequence. Combination of RBSeq with spike-in experimental designs additionally facilitates full posterior inference on enrichment of experimental capsids relative to well-characterized variants, allowing results to be compared more directly across experiments. We show that RBSeq, relative to existing approaches, exhibits comparable or superior statistical properties in simulated datasets and increased estimation stability in real capsid screening experiments. Additionally, we show how to leverage full posterior inference in RBSeq to enhance and increase performance of downstream machine learning analysis pipelines.

134. AAV Hijacks Cellular Secretion Machinery for Efficient Viral Egress

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Cellular egress of wild type Adeno-Associated Viruses (AAV) likely plays a role in viral dissemination and spread. Recombinant AAV vectors belonging to different clades are known to be secreted into cell culture media during production with variable efficiency. We recently demonstrated that the membrane-associated accessory protein (MAAP), expressed from a frameshifted open reading frame in the AAV *cap* gene, is an AAV viral egress factor. However, the cellular mechanisms exploited by MAAP to stimulate viral egress remain unknown. In order to elucidate this process, we employed a BioID proximity-based proteomics approach to identify host cellular proteins that participate in viral egress and secretion. Following MAAP8-BioID2 expression during rAAV8 MAAP Δ production, we found that MAAP was able to differentially biotinylate host proteins when compared to the 13x-BioID2 biotin ligase alone. Proteomic analysis of biotinylated MAAP interacting proteins revealed nearly 400 candidates that were enriched (\log_2 fold change greater than 1.5) in MAAP8 expressing cells over control. The main predicted biological functions of these host genes as determined by GO term analysis are regulation of protein localization to the plasma membrane, exocytic processing and vesicle docking. These interactors were of particular interest as they further suggest a role for MAAP in regulating AAV virion release by co-opting the cellular exosomal biogenesis machinery. Results from ongoing studies further exploring the relative contributions of these cellular host factors to AAV egress will be presented. Taken together, our findings provide a roadmap for manipulating AAV egress mechanisms to improve manufacturing processes.

135. GRE Modification of AAV D-Sequence Does Not Enhance Baseline Transduction Efficiency in Self-Complementary AAV2 or 3

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Self-complementary Adeno-associated Virus (scAAV) vectors are currently being investigated to treat genetic diseases and have a trans-gene expression advantage over typical single-stranded AAV (ssAAV). This is believed to be due to the ability to bypass second-strand synthesis. Previous work has suggested that glucocorticoids may modulate scAAV vector transduction *in vitro*, but this same effect is not observed in ssAAV vectors. It was previously hypothesized based on sequence alignment that the AAV D-sequence may harbor a partial glucocorticoid response element (GRE) which may lead to transcription regulation in its self-complementary form. As previously reported, analysis by EMSA showed partial binding of glucocorticoid receptor (GR) to double-stranded AAV2 & 3 D-sequence. It was then investigated if modification of the D-sequence to share more similarity with the human GRE would enhance vector transduction efficiency through enhanced transcription activation. Different huGRE-D-sequence vectors were then generated and packaged to investigate their effect on transduction efficiency *in vitro*. Both scAAV2 (pCBA-EGFP) & scAAV3 (pTTR-EGFP) vectors were packaged with either wild-type (wt) D-sequence or GRE-modified D-sequences. No significant differences in PEI-transfection efficiency were found between these packaging plasmids. Southern blot analysis of rescue replication showed no inhibition of rescue replication from these GRE D-sequence modified packaging plasmids compared to equivalent wt-D-sequence packaging plasmids. These GRE-D-sequence AAV vectors and their equivalent wt-D-sequence vectors were used to transduce a hepatocellular carcinoma cell line (Huh7) at 1,000 & 5,000 MOI. GFP activity was compared via fluorescent microscopy and spectrophotometric methods. Of all the GRE modifications investigated in AAV2 & AAV3, none were able to exceed transduction efficiency of their respective wt-D-sequence vectors, including in the presence of glucocorticoids. In fact, GRE-modified vectors had a slightly lower overall transgene expression efficiency compared to their respective wt-D-sequence controls at 1,000 MOI (AAV2-CBAp $p < 0.001$; AAV3-TTRp $p < 0.05$) and at 5,000 MOI for AAV2 (AAV2-CBAp $p < 0.05$; AAV3-TTRp $NS p > 0.05$). While glucocorticoid treatment may have a modest effect on transgene expression *in vitro*, these results suggest the use of GRE-D-sequence modified vectors is not an effective method for improving AAV transduction efficiency.

136. ITRs from AAV Serotypes Differentially Stimulate Innate Immune Signaling through TLR9

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Every year, nearly eight million children are born with serious genetic disorders, and over three million die before age five¹. While two AAV gene therapies have been FDA approved, many candidates have been hindered by safety and/or efficacy challenges. **To fully realize the**

potential of AAV therapies, factors contributing to efficacy-limiting immune responses must be better understood and addressed.

Cytotoxic T Lymphocyte (CTL) responses to AAV destroy transduced cells displaying AAV antigens and eliminate therapeutic transgene expression. The CTL response is initiated when Toll-Like Receptor 9 (TLR9) encounters unmethylated CpG dinucleotide pathogen associated molecular patterns. The CpGs in AAV vector genomes are 99% unmethylated, likely due to replication kinetics in production cell-lines. **Several strategies are being tested, in our lab and others, to reduce TLR9 signaling and CTL immune responses to AAV vector genomes.** At minimum, AAV vector genomes are comprised of a promoter-driven transgene flanked by Inverted Terminal Repeat (ITR) sequences. ITRs are essential for transgene packaging and are the only required viral sequences in AAV vector particles. For historical reasons, the ITRs employed in most AAV vectors are derived from AAV serotype 2 (AAV2). Little is known about the relative immunogenicity of ITR sequences derived from other AAVs. **We hypothesize that ITRs with lower CpG frequency will initiate less signaling through TLR9.** We compared ITR sequences from 11 AAV genomes available in NCBI: serotypes 1-8, avian, bat and bovine AAV. Six ITR sequences, including AAV2, have 16 CpGs (frequencies ~11%); AAV1 and AAV3 have 14 CpGs (9.8%); AAV8 has 12 (8.3%) and AAV4 has 10 (6.8%). Intriguingly, the Bat ITR has only 4 CpGs (a frequency of 2.8%). We synthesized these AAV^{ITR} sequences, as well as a CpG-free^{ITR}, and compared TLR9 activation in HEK cells expressing exogenous hTLR9 (HEKTLR9) with downstream promoters driving a Secreted Enzymatic Alkaline Phosphatase (SEAP) reporter gene. TLR9 signaling leads to SEAP secretion and substrate cleavage and is measured by absorbance. We found significant differences in absorbance after ITR exposure in HEKTLR9, but not in parental cells lacking TLR9. Surprisingly, CpG frequency did not correlate with TLR9 activation. TpCpG motifs found in the REP protein binding element of the ITRs are potent TLR9 agonists, and we are currently investigating their impact. AAV3^{ITR} was the strongest TLR9 activator, with 200% the signal of AAV2^{ITR} (p=0.0004). In contrast, AAV8^{ITR} was least stimulatory, activating TLR9 48% less than AAV2^{ITR} (p=0.01) and insignificantly less than the CpG free^{ITR}. Non-denaturing agarose electrophoresis showed banding patterns consistent with base paired ITR secondary structures, which migrate faster than polyT unpaired controls. Intriguingly, AAV8^{ITR} and BatAAV^{ITR} ran slower than all other AAV^{ITR} hinting at secondary structure differences, although these differences did not correlate with TLR9 activation. We also tested if methylation of CpGs would decrease TLR9 activation and observed a decrease in TLR9 activation to levels equal to CpG-free^{ITR}. Migration of methylated ITRs was indistinguishable from unmethylated counterparts after electrophoresis, suggesting no change in secondary structure. This study demonstrates the potential for decreasing AAV immunogenicity through alternative ITR design.

137. Characterizing the Amino Acid Distribution of a 7-mer Peptide Insertion at Variable Regions IV, V and VIII on the AAV Capsid

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Insertion of short, random peptides into the adeno-associated virus (AAV) capsid variable regions is a commonly used strategy to produce high-diversity AAV libraries for directed evolution. However, not all amino acid insertions are well-tolerated in the context of the AAV capsid, and incorporation of certain amino acids, particularly bulky residues, in a peptide insertion may negatively impact capsid production fitness. Here, we generated libraries containing 7-mer peptide insertions at variable regions (VRs) IV, V and VIII on an AAV capsid. For the library constructs, the Cap gene was placed between the AAV inverted terminal repeats (ITRs) such that each capsid produced would be packaged with its own originating genome. Following library production in HEK293T cells, PCR was used to recover the peptide-encoding amplicons from the produced AAV library for deep sequencing. We compared DNA and AAV library amplicon sequencing data to establish a capsid fitness map, where amino acids at each insertion position were evaluated based on their enrichment in the produced AAV library compared to the DNA library. Toward this aim, a fitness score for every amino acid at each position in the peptide sequence was calculated. Across all peptide insertion positions, smaller residues (e.g., glycine, serine, asparagine) were generally better tolerated compared to bulkier residues. We observed depletion of bulky amino acids such as arginine, tyrosine and tryptophan across the three tested variable regions at most positions (tryptophan fitness score at VR-IV: -0.41 to -0.55, VR-V: -0.16 to -0.44, VR-VIII: -0.28 to -0.65). From this analysis, we determine that properties such as amino acid size, hydropathy, charge and other factors may substantially affect production fitness of the resulting variant when inserted into the AAV capsid at the VR-IV, VR-V and VR-VIII insertion sites. Delineating these relationships may facilitate data-driven design of peptide insertion sequences that are less likely to impact AAV yields.

138. AAV.Ghost: Novel Stealth Capsid for Improved Central Nervous System Gene Delivery and Neutralizing Antibody Evasion

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In vivo selection of AAV9-based peptide display libraries has yielded new variants with improved gene transfer to various tissues including the central nervous system (CNS) and more recently skeletal muscle. Most libraries have been generated by insertion of a randomized library of seven amino acids (20⁷ or 1.28x10⁹ possible variants) inserted in the loop on variable region VIII (VR-VIII) after amino acid 589 in the VP1 gene. Various strategies have been employed to identify capsids that successfully navigate the black box of biological mechanisms necessary to transduce cells in the tissue of interest. We employed an expression-based strategy to enrich for functional capsids using a human synapsin-1 promoter-driven EGFP transgene cassette with the AAV p40 promoter and AAV9 cap-library in the 3'UTR. AAV capsid libraries using transgene expression for *in vivo* selection are compatible with selection in mice and other research animal species. We generated

the AAV9 capsid library by insertion of the 7-mer randomized library after position 589 of AAV9 VP1 using degenerate primers. NGS analysis of plasmid and viral libraries showed largely uniform peptide representation. Our goal in this study was to select an AAV9 variant that transduces the CNS more efficiently than AAV9 with lower sensitivity to pre-existing NABs. The viral library was incubated with AAV9 NAB-positive non-human primate (NHP) serum (1/5 dilution) pre-injection, and then 2E12 vg injected systemically into 6-8 week-old C57BL/6J male mice via the tail vein. Mice were sacrificed four weeks post-injection and total RNA isolated from brain and library sequences amplified by RT-PCR followed by NGS analysis of peptide frequency. AAV.Ghost (AAV.G) showed the highest frequency in the CNS and skeletal muscle compared to other variants, but among the lowest frequencies in liver. The sensitivity of AAV.G to antibody neutralization was assessed in HEK293T cells using both human IVIG, and AAV9 NAB-positive NHP serum. AAV.G resisted neutralization by IVIG to 2-fold higher concentrations than AAV9, while NAB-positive NHP serum had no effect in its transduction efficiency. The transduction profile of AAV.G was compared to AAV9 in C57BL/6J male mice injected systemically (1E12 vg) and intrathecally (IT; 1E11 vg). Mice were sacrificed at 4 weeks post injection and total DNA extracted from liver, brain and sciatic nerve for vector genome copy analysis by real-time quantitative PCR. The brain transduction efficiency of AAV.G was 10- and 70-fold higher than AAV9 when injected IT or systemically, respectively. No difference was apparent in sciatic nerve gene transfer efficiency. Liver transduction was considerably lower for AAV.G compared to AAV9. Additional studies are ongoing in mice and NHPs to determine whether the robust properties of AAV.G are conserved across species and thus determine its potential as a clinically relevant variant.

139. Profiling Promoters and Scanning for Enhancers to Refine Gene Modulation via AAV Gene Therapy

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Since the advent of Adeno-associated virus (AAV) vectors as a biotherapy, there has been a continuous effort to fine-tune its efficacy-this includes the design of therapeutic transgenes, which is constrained by the 4.7Kb packaging capacity of AAVs. Here we discuss strategies in refining the recombinant AAV cargo in the context of a rare disease called Dravet syndrome (DS). We will i) profile the cell-type-specific expression potency of commonly used promoters in the CNS and ii) use genomic libraries to identify enhancers with the goal of engineering a transgene cassette that can modulate gene expression in a highly targeted, cell-type-specific manner. DS is a severe epileptic encephalopathy caused by de novo mutations in the *SCN1A* gene. This results in haploinsufficiency for the $\alpha 1$ subunit of the voltage-gated sodium channels, which are localized to GABAergic interneurons. To treat haploinsufficiencies, one may deliver a transgene that increases the expression of the normal allele with the consideration that non-specific upregulation could have catastrophic consequences. For DS, this entails identifying transcriptional activators that will boost

endogenous *SCN1A* expression specifically in GABAergic interneurons. Selectively targeting a neuronal subtype will require a highly specific promoter. Studies have shown promoters like the hybrid CMV/chicken β -actin promoter (CAG), methyl CpG-binding protein 2 (MeCP2), ubiquitin C promoter (UBC), and synapsin-1 (Syn-1) to be broadly expressed in neurons. Yet, little is known about their functionality in neuronal subtypes. Here we are characterizing the potency of CAG, UBC, MeCP2, and Syn-1, at the single-cell level in different neuronal populations. AAV-PHP.eB vectors encoding NLS-eGFP driven by each of these promoters were delivered intravenously to 6-8week old C57BL/6J mice at a dose of 3e11 vector genomes. Brain tissue was collected 4 weeks post AAV delivery. From these samples, we will isolate GFP+ve nuclei, build single nuclei libraries with 10x Genomics, and perform snRNAseq on them. Such in-depth analysis will not only elucidate promoter functionality at the single-cell level but will also give us critical information that will drive a more rational selection of 'ubiquitous' promoters for CNS gene therapy. For our targeted gene therapy, we also plan to develop small, artificial regulatory elements by combining minimal promoters with enhancers from our genes of interest. Unlike promoters, enhancers are often located far away from their cognate genes. Thus, one must scan enormous genomic tracts to find potential enhancers. For DS, we have designed large-scale libraries that will probe genomic regions around *SCN1A*, *GAD1*, *GAD2* to identify putative GABAergic neuron and *SCN1A* specific enhancers. Our libraries consist of ~92,000, 130nt-long DNA fragments that are tiled across a 2Mb region around our candidate genes. These libraries were cloned downstream of a minimal *SCN1A* promoter in AAV-PHP.eB vectors encoding NLS-eGFP. Next, systemic delivery of the AAV libraries in mice, followed by snRNAseq on GFP+ve nuclei, should enable us to find enhancers that provide various levels of *SCN1A* expression restricted to GABAergic neurons. Additionally, existing ATACseq data from mouse and human brains will also be analyzed for candidate enhancer elements in *SCN1A*, *GAD1*, *GAD2* promoters. Information from the latter might complement our broad empirical approach and further refine the selection of enhancers. In conclusion, we hope to demonstrate that these methodologies will not only help us design a transgene cassette that can upregulate endogenous *SCN1A* transcription but these approaches can also be used to systematically find additional regulatory elements for other diseases.

140. Determining the Impact of rAAV Genome Size on Vector Production and Quality

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Recombinant Adeno-Associated Virus (rAAV) vectors have proven to be a compelling therapeutic for the treatment of debilitating genetic

diseases. The wild-type virus is a small non-enveloped dependovirus with a single stranded DNA genome of approximately 4.7kb. One of the most important features of the virus are the inverted terminal repeats (ITRs), which are positioned at both ends of the viral genome. The ITRs are the only viral sequence needed for rAAV genome replication and long term episomal formation. rAAV therapeutics are constructed by replacing the wild-type genomic DNA in-between the ITRs with a transgene cassette, containing a promoter, gene of interest, and other expression related components. The overall productivity, quality, and developability of rAAV therapeutics has been shown to be influenced directly by the design of the transgene cassette. Previously, studies have shown that the transgene cassette size can heavily influence quality characteristics that are crucial for successful gene therapies, including genome packaging and potency. To better understand the impact of genome size on vector quality and analytical characterization capabilities, we set out to create a set of ITR plasmids encoding different sized genomes expressing the same reporter gene. In this work we present results collected from three ITR plasmids encoding rAAV genomes that encompass the extremes of possible sizes, including undersized (1.37kb), close to wild-type (4.59kb), and oversized (5.67kb) vectors. The ITR plasmids were designed to have identical components (ITRs, promoter, reporter, polyadenylation signal, backbone) and only differ by the amount of stuffer sequence included to reach the specific genome sizes. After producing the three vectors using triple transfection, the purified rAAV were put through a panel of characterization assays ranging from biophysical (analytical ultracentrifugation (AUC), capillary isoelectric focusing (cIEF), size exclusion chromatography coupled to multiangle light scattering (SEC-MALS)), genomic (next generation sequencing (NGS)), and expression based analyses. Through these assays, specific trends were observed with each genome size, showing an impact on productivity and quality. This work helps further the understanding of extreme rAAV genome sizes on product quality and expand the knowledge of how these changes in quality can impact analytical characterization capabilities.

141. Ly6a Receptor Affinity Impacts the Blood-Brain Barrier Penetration and Tissue Targeting Properties of AAV9-PHP.B Vectors After Systemic Delivery

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AAV9-PHP.B, a gene therapy vector based on adeno-associated virus 9 (AAV9), crosses the blood-brain barrier (BBB) in C57BL/6 mice with unprecedented efficiency. AAV9-PHP.B achieves this brain transduction phenotype through its interactions with Ly6a, a cellular receptor expressed on the surface of murine brain microvascular endothelial cells. Here we explore how the intrinsic affinity of the PHP.B insert peptide for Ly6a impacts receptor engagement and BBB crossing. First, we developed a series of PHP.B peptide mutations that modulate the intrinsic affinity of AAV9-insert vectors for Ly6a. Surprisingly, AAV9-PHP.B insert variants with both increased and decreased receptor affinity localized to the brain with equal efficiency. However, BBB crossing and brain transduction was attenuated for variants with both weaker *and* tighter Ly6a binding, indicating the

original PHP.B peptide was near an affinity optimum for BBB function defined by more than simple localization to the BBB. Furthermore, while AAV9-PHP.B's enhanced transduction properties are specific for the central nervous system (CNS), Ly6a receptor expression can be detected in the endothelial layers of organs outside the CNS. We are presently investigating how receptor affinity impacts localization to, and successful transduction of, these peripheral tissues. We discuss the role of vector/receptor affinity in the targeted design of vectors to overcome delivery obstacles in humans.

142. Strong Universal Micro Promoters for Recombinant Adeno-Associated Viral (rAAV) Vectors

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Recombinant AAVs (rAAVs) have become a promising gene therapy tool. AAVs are considered safe for clinical trials due to low immunogenicity and low toxicity, compared to other viral vectors. A variety of cells and tissues can be targeted by rAAVs using various serotypes and recombinant capsids. Despite the progress in the development of new recombinant capsids, application of rAAV for some diseases remains challenging due to the packaging limit for the cargo DNAs. rAAVs can package up to 4.7 kb DNA including two inverted terminal repeats. Thus, rAAV allows a maximum of only ~ 4.3 kb DNA for gene expression cassette, which includes a promoter, a transgene, and a poly(A) transcription termination signal. The size of universally expressed promoters generally used in rAAV vectors range from 0.5 kb (CMV) - 1 kb (CAG), or larger. This is an impediment for delivery of large genes and DNA and RNA editing machinery such as CRISPR/Cas systems. Here we report two strong universal promoters that are only 84 bp (micro promoter μ P-84) and 135 bp (μ P-110) long for rAAV-based gene expression. We tested the activity of these promoters using a fluorescent reporter gene mRFP. Both micro promoters expressed high levels of mRFP in various cells and tissues *in vitro* and *in vivo*, comparable to the CAG promoter. Here we show the rAAV delivered micro promoter activity in cultured cells from the three different germ-layer lineages. In addition, *in vivo* expression was shown in multiple mouse tissues including liver, muscle, and brain in mice, as well as in human hepatocytes and in human pancreatic islets. The activity of the micro promoters is universal in all cells and tissues tested so far. Hence, our novel micro promoters will be valuable tools for rAAV gene therapy and enable the delivery of previously 'unpackable' large cargo DNAs.

AAV Vectors - Preclinical and Proof-of-concept Studies I

143. Production & Characterization of Adeno-Associated Viruses (AAV) Engineered with a Single Domain Antibody Against AXL into VP1

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The use of adeno-associated viruses (AAV) as vectors for gene therapy has increased in recent years due to their safety and the *in vivo* long-term expression of transgenes. Many common AAV serotypes, such as well characterized AAV2, are known for their wide tropism. However, for the delivery of a therapeutic payload such as suicide genes for cancer therapy, it is necessary to retarget those vectors to specific cells to further increase the safety of the treatment. To address this issue, we incorporated a single-domain antibody (sdAb) into the capsid of AAV2 to enable it to specifically bind to an ovarian cancer antigen (AXL) expressed on the cell surface. The sdAbs are getting popular due to their small size (~ 15 kDa), high stability, specificity and their ability to bind with high affinity to their antigen; making them interesting alternatives to conventional antibodies and allowing their incorporation into various proteins with minimal disruption of the native protein functionality. In this project, we have successfully produced a recombinant AAV-sdAb against AXL by transient transfection of HEK 293SF-3F6 cells in suspension culture and purified it by ultracentrifugation using Iodixanol step-gradients. Using two types of linker sequences, short: 5' (GGGGS)₃, 3' (GGGGS) and long (Mol. Ther. Methods Clin. Dev, 15: 211-220; 2019): 5' (GGGGS)₅, 3' (GGGGA), we incorporated the sdAb sequence into VP1 capsid protein of AAV. Production of infectious AAV particles expressing GFP as a reporter proved to be difficult when using the short linker sequence, while productions using longer linker sequences led to higher titers approaching those obtained with wild type AAV2 capsid (three times lower for genome copies) by standard triple plasmid transfection. HEK 293SF-3F6 cells were used for transduction optimization purposes which will be followed by *in vitro* model using the SKOV-3 ovarian cancer cell line that expresses AXL receptor on the cell surface to study the retargeting efficiency.

144. Lessons Learned: Using Convection-Enhanced Delivery to Infuse Gene Therapy Products to the Nonhuman Primate Cerebellum

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This compilation aims to demonstrate the feasibility and tolerability in targeting the nonhuman primate cerebellum to deliver gene therapy products using real-time MRI-guidance. Using convection-enhanced delivery (CED), all animals received a single, unilateral infusion of AAV5-GFP into the cerebellar parenchyma. Animals were monitored daily for signs of neurological impairment and weight loss until necropsy, which occurred 4-6 weeks post-infusion. Our observations revealed a 4-9% loss in body weight, with no untoward side effects post-infusion. Histological and immunohistochemical analyses were used to determine cerebellar expression of the GFP transgene, as well as tissue response to cannula insertion. Notably, when optimizing targeting of the cerebellum, we denoted challenges and corrections to maximize coverage and transgene transport distally and avoid leakage outside of the cerebellum. Even after performing focal intra-parenchymal infusions with little-to-no signs of leakage, GFP reporter detection was not without challenges. This further emphasizes the usefulness and practicality of performing these infusions under real-time MRI-guidance, allowing visualization of vector transport and infusion patterns unique to the cerebellum. Taken together, these data offer considerations when designing *in vivo* animal studies translatable to relevant clinical applications.

145. Quantification of DNA Contamination within Recombinant Adeno Associated Virus Preps Correlates with CD34+ Cell Potential

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Due to promising results in clinical trials, recombinant Adeno-Associated Virus (rAAV) vectors have been used to treat genetic diseases by gene therapy. One frequent application of rAAV is to provide donor sequences for gene editing in hematopoietic stem and progenitor cells (HSPC). We and others have reported that while rAAV may mediate efficient donor delivery, it has adverse effects on HSPC function, including reduced formation of progenitor colonies and impaired engraftment in xenograft models. Contaminating DNA may come from producer cells, or helper and packaging plasmids, and has the potential to elicit direct toxicity to HSPC via TLR activation. We characterized significant differences in contaminating DNA among rAAV preparations from different sources, and correlated findings with differences in HSPC clonogenic potential, acute cellular responses, cell cycle status, and apoptosis. We selected 6 previously used rAAV preps from two different manufacturers, treated preps with and without an endonuclease to allow for quantification of contaminating DNA inside (endonuclease-resistant) versus outside of the viral capsid. Following this, total DNA was isolated and analyzed by Illumina next generation sequencing (NGS). Knowing the sequences of plasmids used to create full rAAV viral preps, we were able to map each NGS read to individual contaminants and quantify levels of each present in a given prep. In parallel, CD34+ HSPC were edited and transduced with rAAV treated with and without endonuclease followed by colony forming unit (CFU) assay to measure clonogenic potential and RT-qPCR analysis of a panel of genes associated with cell cycle, acute immune response, and apoptosis. Significant differences in DNA contaminant levels were

observed among all 6 preps. Specifically, preps from manufacturer #1 had significantly less contaminating DNA with 80-95% of DNA within preps mapping to the rAAV genome. These values increased minimally to 92-95% with endonuclease treatment, indicating very low levels of extra-virion contaminating DNA. From manufacturer #2's preps, only 45-69% of all DNA was rAAV genome; upon endonuclease treatment, this number increased to 84-88%. A significant inverse correlation between the amount of contaminating DNA and HSPC clonogenic potential was observed following transduction of CD34+ HSPC with two preps from both manufacturer #1 and #2, with and without endonuclease treatment, as quantified by CFU assay ($p < .02$). CD34+ cells had significantly increased clonogenic potential when transduced by preps from manufacturer #2 treated with endonuclease compared to untreated preps, and comparable to that of cells treated with preps from manufacturer #1 ($p < .05$). Additionally, RT-qPCR analysis demonstrated significant increased expression of genes involved in acute immune responses, apoptosis, and cell cycle arrest for preps with high levels of contaminating DNA, which were decreased upon endonuclease treatment to levels similar to manufacturer #1. This study characterizes DNA contaminants within rAAV preps and correlates the extent of extra-virion DNA with effects on HSPC function upon transduction. While it was observed that DNA contamination plays a significant role in HSPC viability and clonogenic potential; endonuclease treatment of preps reduces toxicity and preserves function of these cells. Additionally, we measured variations in cell cycle, apoptosis, and acute immune response within HSPC, and found variations based on the amounts of contaminating DNA. This study identifies an important quality control parameter for rAAV manufacture for applications of HSPC editing by providing a relevant quality measurement.

146. Immune Response to AAV Vector Capsid and Assessment of Eligibility for AAV Mediated Gene Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a fatal genetic disorder caused by mutations in the dystrophin gene. During the past five years, gene therapy for NMD has rapidly accelerated, with many clinical studies underway and up to 40 product approval anticipated by 2025. Despite advances in AAV gene therapy, the field still faces a crucial unresolved challenge of the host immune response against vector capsid proteins. Exaggerated immune responses raise concerns regarding the safety and durability of the therapeutic effect. Preclinical data from our lab suggests that before therapeutic dosing (pre-existing immunity), natural exposure to AAV can lead to severe infusion reactions and a diminished therapeutic effect. Based on these limitations, current AAV-mediated gene therapy trials must utilize a single direction and exclude subjects with pre-existing AAV immunity. We designed an optimal construct that will lead to microdystrophin expression in skeletal, cardiac, and CNS tissues. We are testing this optimal construct with different immunosuppression regimens and immunomodulation in mdx mice with three capsids for optimal microdystrophin expression. We are developing and optimizing alternative immune modulation approaches to prevent immune responses against the AAV capsid,

manage pre-existing immunity to AAV in mdx mice, and then confirm our results in a canine model. The strategy mentioned above will help us overcome the strain of excluding patients with pre-existing immunity.

147. A High-Throughput Barcode Screening Identifies AAV-KP1 as a Capsid That Efficiently Transduces Pancreatic Islets in Non-Human Primates Following Retrograde Pancreatic Duct Injection

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The successful in-vivo reprogramming of non-beta cells into insulin-producing beta-like cells in rodent models by delivering key transcription factors has yielded encouraging results for the eventual success of viral vector-mediated gene therapy for type 1 diabetes (T1D). AAV vectors are the most prominent viral vectors for in vivo gene therapy in clinical settings; however, clinically relevant effective and safe approaches have yet to be established for AAV vector-mediated gene delivery to target cells in the pancreas. At the ASGCT2021 meeting, we reported successful establishment of real-time image-guided retrograde pancreatic duct (PD) injection of viral agents into non-human primates (NHPs). The procedure was conducted safely, with only showing a self-limited elevation of serum amylase and lipase that did not result in clinical symptoms. When this method was employed to deliver an AAV9 vector to the NHP pancreas, AAV9 predominantly transduced acinar cells and occasionally islet cells, including insulin-producing beta cells. Here, we report the result of a high-throughput Barcode-Seq screening for AAV capsids that efficiently transduce pancreatic cells following PD delivery in NHPs and show that AAV-KP1 outperforms other AAV capsids and transduces NHP pancreatic islet cells substantially better than AAV9. In this study, we produced a DNA/RNA-barcoded AAV library containing 45 different AAV capsids (major AAV serotypes and various capsid mutants) and injected three rhesus macaques via the PD route at a dose of 8.5 to 9.8×10^{12} vector genome (vg). We harvested tissues, including pancreas, 6 weeks post-injection, enzymatically digested the pancreas, and flow-sorted endocrine cells, duct cells and other cells using cell type-specific monoclonal antibodies. The AAV Barcode-Seq analysis revealed that the AAV-KP1 vector can efficiently transduce islet cells more than an order of magnitude better than AAV9. To validate the results obtained by the AAV Barcode-Seq analysis, we infused 8.4×10^{12} to 1.4×10^{13} vg of AAVKP1-CAG-tdTomato vector into the PD in four rhesus macaques, and assessed pancreatic transduction 4 weeks post-injection. Preliminary immunofluorescence microscopic analysis revealed that the AAV-KP1 vector preferentially transduces islet cells. Importantly, the relative vector genome DNA copy numbers of the AAV-KP1 vector in non-target organs, including the liver, were found to be substantially lower than those of AAV9, the serotype that effectively transduces the liver following PD injection. These observations indicate that retrograde PD injection of AAV-KP1 vector is a promising approach for AAV vector-mediated gene delivery to

pancreatic islets with minimal off-target effects. Further studies are warranted to support clinical translation of AAV-KP1 vector-mediated gene therapy for T1D.

148. Bioengineered AAV Vectors with Increased Skeletal Muscle Potency and Specificity for Systemic Gene Therapy

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Bioengineering of adeno-associated viral (AAV) vectors is a pivotal strategy for developing next-generation gene therapies for human disorders. In the present study, we assessed the efficacy of two novel AAV vectors, AAVMYO2 and AAVMYO3, which are composed of hybrid capsids and a small VP1 peptide insertion, in a mouse model of myotubular myopathy, a fatal pediatric disease affecting skeletal muscles that is caused by loss-of-function mutations in the *MTM1* gene. Intravenous administration of either AAVMYO2- or AAVMYO3-*MTM1* vectors at 2×10^{13} vg/kg in *Mtm1*-KO mice prolonged their lifespan and resulted in increased muscle mass and strength, and in liver transduction detargeting. In addition, we show efficient large-scale production and purification of AAVMYO2 and AAVMYO3, and data on their seroprevalence in a cohort of human sera. Our findings indicate that these novel vectors are potent in rescuing the phenotype of myotubular myopathy in mice, and hold great potential for gene therapy of other inherited disorders affecting skeletal muscles.

149. GT005, an AAV Gene Therapy Targeting the Alternative Pathway of the Complement System for the Treatment of Geographic Atrophy

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Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in the industrialised world and affects ~40 million people globally. Geographic atrophy (GA) is an advanced form of AMD that leads to irreversible loss of visual function through development of atrophic lesions of the outer retina, including loss of photoreceptors, retinal pigment epithelium (RPE) and the underlying choriocapillaris. Evidence has emerged implicating chronic local inflammation and activation of the complement system (CS) in

AMD pathogenesis. The genetic link between AMD and the CS was established by genome-wide association studies (GWAS) when highly associated genetic variants were discovered in specific complement proteins (complement factor (CF) B, H, I, and C3) of the alternative pathway (AP). While different therapeutic approaches are being investigated to reduce the rate of disease progression, there are currently no approved treatments for GA. Here, we explore supplementation of human CFI (hCFI) from an AAV vector as a gene therapy approach for AMD. First, the sequences of either wild type (WT) and codon-optimised (co) hCFI were subcloned into an AAV2 vector (GT005), and *in vitro* transduction of both vectors showed high expression of hCFI protein. Functional expression of hCFI was further confirmed by C3b cleavage assay. Subretinal injection of GT005 expressing either WT or co hCFI in mice led to detectable levels of secreted hCFI in ocular fluids and a concomitant reduction of C3b in retinal tissues. In addition, immunostaining of the mouse retina demonstrated predominant localisation of hCFI in the RPE and inner/outer segment of photoreceptors. However, no differences in expression were detected between the two CFI sequences, and hence the WT hCFI sequence was selected for progression owing to it being the native sequence. Furthermore, GT005 expressing mouse and human CFI showed a statistically significant reduction of CNV leakage area in a dose-dependent manner at Day 7 in the mouse laser-induced choroidal neovascularisation (CNV) model of AMD. Collectively, the non-clinical data provided here demonstrate the therapeutic potential of rAAV2-mediated expression of hCFI in slowing disease progression of GA by limiting complement-mediated inflammation in the posterior segment of the eye. More importantly, these data presented have paved the way for the evaluation of GT005 in Phase I and II clinical studies for GA secondary to AMD.

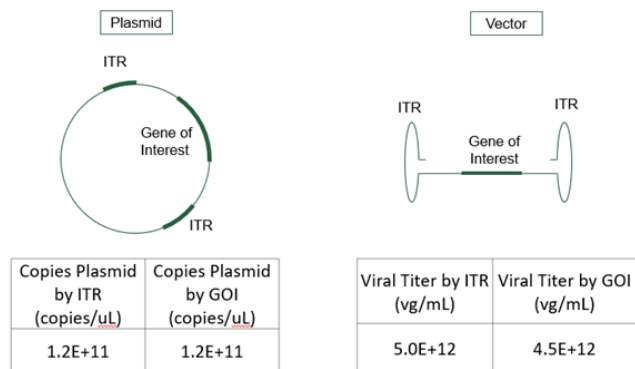
150. Viral Genome Titer Testing Strategy Using Multiplex for the Gene of Interest and ITR

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The viral genome titer method by PCR is the standard method to quantify AAV in order to predict precise doses in preclinical and clinical studies. Digital droplet PCR (ddPCR) represents a relatively new approach to determining genomic titer as compared to conventional real time quantitative PCR (rt-qPCR). ddPCR is preferable over qPCR due to its benefits of absolute quantification without use of a standard. Additionally, ddPCR allows for quantification of multiple targets within the same well, allowing for a direct comparison of two different targets. During early development of a gene therapy, there are often many constructs with different sequences that need to be evaluated while identifying a lead construct. While sequences unique to the gene of interest are preferable for titer determination, targeting the gene of interest is usually not viable prior to construct selection due to the differences between the sequences of the genes of interest. The use of a universal sequence for all constructs being considered allows for consistent dosing between constructs and between early preclinical studies. As the program advances towards final construct selection, titer determination via the gene of interest is important to bridge between nonclinical and clinical studies using multiple lots of vector. We propose a strategy of utilizing ddPCR duplexing of the universal sequence and the gene of interest in order to maintain

comparability to studies prior to construct selection to studies after construct selection. A case study will be presented where multiple candidates were evaluated in early preclinical studies with titer being assessed only by ITR. For subsequent preclinical studies, candidate selection had occurred allowing for titer to be determined using duplex of ITR and gene of interest targets. The performance of the methods was assessed through the evaluation of plasmids and synthetic DNA fragments as well as representative vector (Figure 1).



Duplex quantification of plasmid DNA verified the accuracy of both ITR and the gene of interest targets. Vector analysis for viral titer by duplex ddPCR provides a robust data set without increasing the timeframe for analysis or the number of samples analyzed. This strategy allows for comparisons between early preclinical titers with universal targets and later stage development/clinical titers with gene of interest targets.

151. A Breakthrough GLP AAV-ITR Sanger Sequencing Solution for New Drug Development

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Adeno-associated virus (AAV) is one of the most widely used vehicles for gene therapies given its high efficiency and safety in humans. One critical step for rAAV characterization is the sequencing of rAAV vectors which is challenging due to insufficient resolution of the inverted terminal repeat (ITR) regions using standard methods. To overcome these limitations, Azenta has developed a novel, proprietary Sanger ITR sequencing method that improves sequencing signals and extends read lengths through the entire ITR regions. Here, we provide an overview of our GLP-compliant AAV-ITR sanger sequencing process, and the improvements generated by our newly developed ITR sequencing method. Our results demonstrate that sequencing the AAV-ITR region is integral to identifying different types of mutations within the ITR and providing insight into sample clonality that was previously undetectable by traditional means. Our proprietary, GLP-compliant methods for complete AAV-ITR analysis enable an accurate and rapid solution in advance of FDA IND and BLA filings.

152. rAAV-Mediated TCAP Gene Replacement Therapy for LGMDR7 (LGMD2G)

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Limb girdle muscular dystrophy R7 (LGMDR7 or LGMD2G) is a relatively rare autosomal recessive muscular dystrophy caused by mutations in the *TCAP* gene, encoding telethonin or TCAP, a sarcomeric protein found exclusively in striated and cardiac muscle. Loss of functional TCAP leads to progressive proximal limb weakness, muscle atrophy, calf hypertrophy and cardiomyopathy in both males and females worldwide. We developed rAAV-mediated TCAP constructs, the expression of which results in amelioration of telethoninopathy phenotype and prevents disease progression. Here, we report the pilot efficacy data following treatment of 1.5-month-old humanized TCAPKI-null (hTCAPKI) mice with several TCAP vectors under different promoters. hTCAPKI males received a single intravenous infusion of vectors. The age matched hTCAPKI males injected with diluent and untreated C57Bl/6 males served as controls. Treated mice were sacrificed one month post injection for assessment of mouse TCAP (mTCAP) and human TCAP (hTCAP) transcripts by RT-PCR, and evaluation of TCAP protein expression by immunofluorescent (IF) analysis and western blot (WB). The RT-PCR results revealed the presence of both mTCAP and hTCAP transcripts in rAAV.TCAP-treated mice, while diluent-treated hTCAPKI and untreated C57Bl/6 animals showed the mTCAP transcript only as expected. The WB data confirmed the restoration of TCAP expression in rAAV.TCAP-treated mice showing up to 350% protein in triceps. IF staining revealed proper co-localization of TCAP protein with Titin in the Z-discs of sarcomeres in the skeletal muscle of rAAV.TCAP-treated mice. These results suggest that rAAV-mediated TCAP replacement is a potential therapy for LGMDR7 (LGMD2G). Our future studies will test the efficacy and potential toxicity of the developed vectors.

153. Abstract Withdrawn

154. Assessment of AAVrh.10 Tropism for Human Cardiomyocytes

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AAVrh.10, a clade E nonhuman primate serotype, is tropic for cardiomyocytes *in vivo* as assessed by systemic delivery in murine and non-human primate models. To help understand the mechanism of AAVrh.10 interaction with cardiomyocytes, we established an *in vitro* model using AAVrh.10 vectors to deliver reporter genes to AC16 and T0539, immortalized human ventricular cardiomyocyte cell lines. AAVrh.10 demonstrated dose-dependent expression of the β -galactosidase transgene 72 hr following treatment of AC16 and T0539 cells with 2×10^3 to 2×10^6 genome copies (gc) per cell. In contrast, at the

same doses A549 cells exhibited at least 10-fold lower levels of transgene expression compared to AC16 and T0539 cells. We hypothesized that the robust reporter transgene expression in cardiomyocytes resulted, at least in part, from interaction of AAVrh.10 with the AAVR receptor. To test this hypothesis, AC16, T0539 and A549 cells were evaluated for expression of AAVR and anti-AAVR-mediated inhibition of gene transfer by AAVrh.10. Western blot analysis demonstrated expression of AAVR in T0539 > AC16 > A549 cells. Comparison of AAVrh.10-mediated β -galactosidase transgene expression to AAVR expression in T0539, AC16, and A549 cells demonstrated a close relationship ($r^2=0.89$, $p<0.0005$). To further evaluate the importance of AAVR in cardiomyocyte AAVrh.10-mediated gene transfer to cardiomyocytes, AAVrh.10 mediated β -galactosidase expression was assessed as a function of treatment with AAVR blocking antibody or an equal amount of irrelevant mouse IgG at a viral dose of 2×10^4 gc per cell. The AAVR blocking antibody reduced viral gene expression in AC16 cells by $86.7 \pm 0.6\%$ ($p<0.005$) and in T0539 cells by $72.1 \pm 9.9\%$ ($p<0.005$). The same level of blocking antibody had no effect in gene expression in A549 cells ($p>0.6$). In conclusion, AAVR significantly contributes to AAVrh.10 *in vitro* infection of human cardiomyocyte cell lines and likely plays an important role in AAVrh.10-mediated gene delivery to the heart *in vivo*.

155. Biodistribution and Safety Comparison of AAV9 and AAV9PHP.B in Three Mouse Strains and Cynomolgus Macaques

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The field of gene therapy and especially the use of recombinant adeno-associated viruses (AAVs) continues to rapidly expand. To support development of potential AAV-based therapies, researchers leverage a variety of animal models ranging from mice to nonhuman primates (NHPs) to support pivotal pharmacology and toxicology studies. While use of these diverse species is critical for demonstrating safety and efficacy, few studies have directly compared biodistribution and safety between species. To help clarify this gap in knowledge, we generated AAV9 and AAV9PHP.B virus particles containing a CBA-tdTomato reporter transgene and performed intravenous administration at 5×10^{13} vg/kg in six-week-old C57Bl/6, BALB/C, and 129/J mice as well as in juvenile cynomolgus macaques with necropsies at four-weeks post dosing. Additionally, some of the NHPs also received prophylactic glucocorticoids beginning one day prior to dosing and continuing weekly throughout the study. In mice the treatments were well tolerated and biodistribution profiles were similar to previous reports with highest delivery to the liver and heart. The neurotropic properties of AAV9PHP.B were also limited to C57Bl/6 and 129/J mice as previously reported. In the NHPs, biodistribution profiles were similar between PHP.B and AAV9 and confirm recent reports that the neurotropic properties observed in some mouse strains are not extended to old world primates. Prophylactic glucocorticoids had minimal impact on biodistribution profiles. All dosed NHPs experienced acute elevation in transaminase levels with the most pronounced elevations occurring in the AAV9PHP.B group. Prophylactic glucocorticoids did not prevent or reduce this elevation. While all animals dosed with AAV9 recovered from this acute elevation in liver enzymes, the AAV9PHP.B-dosed

animals became clinically ill, with one animal dying on day 7, and the remainder of the cohort requiring treatment with glucocorticoids. Histologic evaluation of the CNS showed signs of necrosis and neuronal degeneration in the brain, spinal cord, and dorsal root ganglia of all animals. This observation aligns with previous findings that CNS toxicity is a common feature of fluorescent reporter transgenes. These data highlight the importance for testing biodistribution and safety in multiple species during drug development.

156. Exon Skipping for the Second Calponin Homology Domain of Dystrophin Using AAV.U7snRNA - In Vitro Intramuscular Studies Using a Novel Murine Model of Duchenne Muscular Dystrophy

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DMD, one of the largest gene encodes the protein dystrophin, which plays an essential role in muscle stabilization. Dystrophin connects the actin to the sarcolemma through its two actin binding domains (ABD1 & 2). Calponin homology (CH) domains, CH1 and CH2 forms ABD1 and encoded by exons 2-5 or 6-8 respectively. Out of frame DMD gene mutations cause dystrophin absence and leads into severe DMD phenotype while in frame mutations leads into truncated but functional dystrophin causing the milder Becker muscular dystrophy (BMD). Exon skipping approach converts DMD into milder BMD by restoring the reading frame uses antisense oligonucleotides (AONs). Canine models of DMD shows that skipping of exons 6-8 (CH2 domain) produce a highly functional dystrophin. This strategy of skipping of exons 6-8 would be a promising treatment option to treat 4% of DMD patients. Traditional exon skipping using antisense oligonucleotide (AON) has some limitations like stability and tropism into major affected tissues. We used AAV.U7 antisense delivery to overcome these limitations. U7 small nuclear RNA carrying antisense sequence improves the stability and adeno-associated virus (AAV) increases tropism. In this study we evaluated exon skipping, dystrophin expression & force restoration efficiency of our new AAV.U7.ex6-8 constructs using a novel humanized mice model for DMD, that we generated. We evaluated our lead candidate *in vitro* using transdifferentiated fibro myotubes and *in vivo*, Intramuscular (IM) studies using this novel humanized DMD mice model which we created using CRISPR/Cas9. This mouse carries a point mutation on hDMD exon 7. Exon skipping by RT-PCR, proteins restoration by immunostaining & muscle function using force transducer were performed. This new mouse colony referred as Δ CH2 was established and mice were evaluated for the DMD phenotype. *In vitro*, AAV1.U7.ex6-8 transduced wild type cells shows efficient exon skipping. 3-months post IM, AAV1.U7.ex6-8 induced efficient exon skipping, resulting in around 50-70% of truncated dystrophin expression and significant increase in muscle eccentric force. In conclusion, AAV1.U7.ex6-8 induced efficient DMD CH2 (exons 6-8) domain skipping, resulting in dystrophin production and muscle strength improvement. This AAV.U7 based vector represent a promising candidate for ~4% of DMD patients.

157. Preclinical Gene Therapy with AAV9/SLC6A1 in a Mouse Model of SLC6A1 Related Disorder

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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). SLC6A1 Related Disorder, a haploinsufficiency neurodevelopmental disorder, is caused by mutations in the SLC6A1 gene. Patients present with rare forms of infantile encephalopathy, developmental delay with cognitive impairment, movement disorders and features of autism spectrum disorder. 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 universal promoter (P1), the other is a neuronal-specific promoter (P2)) and conducted preclinical gene therapy studies using SLC6A1 knockout (KO) mice, a mouse model of SLC6A1. The two AAV9/SLC6A1 vectors were tested separately via lumbar intrathecal (IT) administration or intracerebroventricular (ICV) administration at different developmental ages. IT administration of either construct at postnatal day (PND) 7-10 or PND 28-35 in wild type C57BL/6 mice, using doses of 7.5×10^{11} or 7.0×10^{11} vector genomes (vg) (P1 and P2 constructs, respectively), did not lead to any adverse effects up to a year post-injection. Efficacy was evaluated in SLC6A1 KO mice as neonates by ICV injection (3×10^{11} vg per mouse) or at PND 7-10 or PND 28-35 by IT injection (7.5×10^{11} or 7.0×10^{11} vg for P1 and P2 constructs, respectively). Mice were assessed in a behavioral battery consisting of rotarod performance, open field, fear conditioning, nest building, hindlimb clasping and telemetry recording of EEG. The homozygous KO mice treated at PND 7-10 had moderate improvement on the nest building (P1 construct) or hindlimb clasping (P2 construct) behavioral endpoints, with a trend toward improved rotarod function, but treatment at PND 28-35 had no apparent treatment benefit. Homozygous KO mice treated as neonates by ICV injection with either construct showed significantly higher levels of human SLC6A1 mRNA in brain cortex region than IT administration, and significantly reduced seizures assessed by EEG. The P2 construct treatment in neonatal mice also ameliorated the fear conditioning, nest building, rotarod performance and hindlimb clasping phenotypes in homozygous KO mice. While the heterozygous KO mice showed minimal behavioral phenotypes, their abnormal EEG patterns were normalized following neonatal ICV administration of either vector design. While the treatment was tolerated well following IT administration at PND 7-10 or PND 28-35, some adverse effects (including death) were noted with both constructs after neonatal ICV treatment. Treatment with the P1 design had a higher death rate with more adverse effects. Taken together, safety concerns with neonatal administration of AAV9/SLC6A1 were noted, but this approach was also associated with strong treatment benefits. These results warrant further detailed dose exploration of AAV9/SLC6A1.

158. Precise Editing of the Human MECP2 Gene with AAVHSC Vectors

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Rett Syndrome (RTT) is a genetic neurodevelopmental disorder observed almost exclusively in females, and is caused by heterozygous, de novo mutations in the MECP2 gene, located on chromosome Xq28. Males born with the MECP2 mutations rarely survive due to the presence of a single X-chromosome. Females with Rett Syndrome display random X-chromosome inactivation and are mosaic for MeCP2 expression in all tissues. MeCP2 is a 486-amino acid methyl CpG-binding nuclear protein that functions as a methylation reader and regulates expression of thousands of genes through chromatin compaction at methylated sites and interaction with transcriptional regulators. MeCP2 is universally expressed, but the highest levels are observed in neurons. The MeCP2 protein contains a methyl binding domain (MBD) which binds to DNA, and a transcriptional repressor domain (TRD). Over 300 distinct mutations in the MECP2 gene have been reported in patients with Rett Syndrome, with almost all occurring within exons 3 and 4, and mapping to the MBD and C-terminus of TRD. Missense mutations account for approximately 70% of Rett Syndrome cases. Rett Syndrome is a direct result of the loss of MeCP2 function. However, overexpression of MeCP2 also results in a severe neurodevelopmental disorder, demonstrating the critical importance of MeCP2 gene dosage. Thus, MeCP2 gene dosage presents a "Goldilocks problem" and poses a significant challenge to traditional gene therapy approaches. To overcome these challenges, we explored the ability of the nonpathogenic, replication-defective stem cell-derived adeno-associated virus (AAVHSC) to carry out high-fidelity, precise, homologous recombination-based seamless genome editing to correct pathogenic mutations associated with Rett Syndrome. Importantly, our editing strategy preserves all regulatory elements associated with the MECP2 gene. The AAVHSC editing vector was designed to correct mutations in exons 3 and 4 of the MECP2 gene and to insert a promoterless Venus open reading frame immediately downstream of the coding sequence of exon 4. The MECP2 editing vector was packaged in AAVHSC7 and AAVHSC15 and was used to transduce cells derived from Rett Syndrome patients bearing mutations in exons 3 and 4. Venus expression was evaluated by flow cytometry and confirmed expression from the chromosomal MeCP2 promoter. Targeted integration analyses followed by Sanger sequencing confirmed successful editing of the MECP2 gene. No ITR sequences or insertion or deletion mutations were detected in the edited genomes. Our results demonstrate that AAVHSC editing vectors successfully edit the human MECP2 gene and may be used to correct genomic mutations that cause Rett syndrome.

159. Process Development for Recombinant Adeno-Associated Virus Production Using a Design of Experiments Model

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Background and novelty Viral vectors are used for gene transfer to specific tissue or to induce cell type modifications. Recombinant adeno-associated virus (rAAV) is one of the most promising vectors for gene therapies. The cell and gene therapy industry strives to establish efficient large-scale production of viral vectors with current good manufacturing practice (cGMP). To achieve the high amounts necessary for clinical use of rAAV, companies have moved away from production using adherent HEK293 cells to more scalable technologies using suspension cell culture. Transient transfection protocols are also being optimized for larger scales and efficiency in suspension. **Experimental approach** We have utilized a design of experiments (DoE) methodology to optimize rAAV production in HEK293T suspension cells. An efficient and scalable cell culture process for rAAV production was established for the rAAV2 serotype and verified for the rAAV5 serotype. First, we adapted HEK293T cells to a new animal origin-free cell culture medium and evaluated cell growth by population doubling time (PDT), morphology, aggregates, and viability. A DoE approach allowed us to evaluate the impact of plasmid concentration, cell densities, and harvest time. Here, we describe how to optimize the rAAV transient transfection, which forms the basis for a scalable rAAV production process in single-use bioreactors. **Results and discussion** To verify the robustness of our process, we have produced verification batches of rAAV5 at 10 L scale using Xcellerex™ XDR-10 and ReadyToProcess WAVE™ 25 bioreactors. The success criteria for rAAV5 production were met with the transfection protocol we developed. Our approach could be used in future work for scalable rAAV production — from small-scale shake flasks to large-scale, single-use bioreactor systems.

160. SP-101, a Novel Adeno-Associated Virus Vector, Functionally Corrects Human Airway Epithelia from Donors with Cystic Fibrosis at Low Multiplicity of Infection in the Presence of Doxorubicin

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Cystic fibrosis (CF) is the most common lethal monogenic disease among Caucasians. CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein that results in impaired chloride conductance across epithelia lining the airway, sweat glands, pancreas, and intestine. Although CFTR dysfunction affects multiple organs, lung damage caused by progressive infection and inflammation is the main cause for morbidity and mortality in CF patients. SP-101 (AAV2.5T-SP183-hCFTRΔR) is a novel recombinant adeno-associated virus (AAV) gene therapy vector being investigated as an inhalation treatment for people with cystic fibrosis (CF) in a mutation agnostic manner. SP-101 is composed of a novel capsid optimized for efficient apical transduction of human airway epithelial cultures (HAE) and encodes a promoter/enhancer that drives expression of a human CFTR minigene (hCFTRΔR). Doxorubicin is a small molecule known to enhance mRNA expression from a variety of AAV vectors. To understand the impact of doxorubicin on SP-101 expression, the relationship between SP-101 vector copy number (VCN), hCFTRΔR mRNA expression, and functional correction of CF

HAE was investigated in presence or absence of doxorubicin. SP-101 was applied to the apical surfaces of CF-HAE with Class I and Class II mutations cultured at the air-liquid interface and doxorubicin to the basal side. At 7 days post-transduction, SP-101 restored CFTR-mediated chloride conductance, measured via Ussing chamber assay, at multiplicity of infection (MOI) as low as 500 in the presence of doxorubicin. Functional correction of CF HAE increased with increasing MOI and correlated with increasing VCN (measured by ddPCR) and hCFTRΔR mRNA expression (measured by RT-qPCR). Increasing chloride conductance and hCFTRΔR mRNA expression in CF HAE also increased with increasing doxorubicin concentration. Even low MOI of SP-101 in the presence of low concentrations of doxorubicin were able to partially restore CFTR-mediated chloride conductance. Despite a significant impact on hCFTRΔR mRNA and chloride conductance, doxorubicin did not alter VCN of SP-101 in a dose-dependent manner. In summary, the apical tropism of the AAV2.5T capsid for human airway, combined with the enhanced mRNA expression afforded by doxorubicin, show promise for a novel CF treatment strategy and strongly support continued development.

161. Selection of NXL-001, a Clinical Candidate to Treat Ischemic Cortical Stroke Through AAV-Mediated Astrocyte-to-Neuron Conversion

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Loss of neurons is the hallmark of neurodegenerative disease and CNS injury and there is currently no approved therapy to replace or regenerate these cells. The prevailing dogma is that once neurons are gone, they are gone. Our work challenges this notion, with a platform of neuro-regeneration driven by the direct *in vivo* conversion of astrocytes into functional neurons. Among our most advanced programs is NXL-001, a gene therapy product that uses AAV9 to deliver the transcription factor NeuroD1 to astrocytes via intracranial injection for the treatment of ischemic cortical stroke. Stroke is a leading cause of morbidity worldwide with no treatment options following immediate intervention. We now present the series of datasets that culminated in the selection of our clinical candidate, currently being evaluated in pivotal IND-enabling studies. Initial studies used research vectors that specifically expressed NeuroD1 in astrocytes using the astrocyte-specific GFAP promoter and the Cre-FLEX expression system. This method demonstrated robust *in vivo* conversion of astrocytes to neurons in mouse and rat models of stroke. Newly generated neurons expressed appropriate markers of cortical glutamatergic neurons and exhibited appropriate electrophysiological profiles. Crucially, motor deficits in rodent models of stroke were reversed, demonstrating that the newly formed neurons had integrated into existing neural networks. This approach has since been extended to non-human primates with the demonstration of efficient *in vivo* conversion of astrocytes to functional cortical neurons in a monkey model of stroke. Due to the promising results obtained with research vectors in our initial studies, we have begun the process to translate this technology

into clinical use. We describe the optimization and selection of our clinical candidate vector for this program and provide an update on our ongoing and planned studies to advance NXL-001 to the clinic. We anticipate IND submission in early 2023.

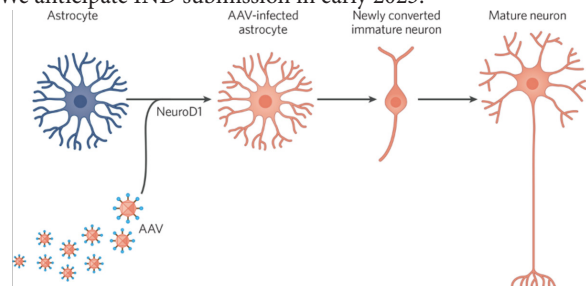


Figure: Astrocyte-to-neuron conversion to treat ischemic cortical stroke

162. Preclinical Pharmacology and Toxicology of Intrathecally Infused AAV9-hABCD1 (SBT101), a Gene Therapy Candidate for AMN, in Non-Human Primates

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OBJECTIVE: To evaluate the safety and biodistribution of SBT101, an AAV9 based gene therapy vector capable of delivering a functional copy of the human ABCD1 (*hABCD1*) gene into the intrathecal space in non-human primates (NHP). **BACKGROUND:** Adrenomyeloneuropathy (AMN) is a form of X-linked Adrenoleukodystrophy (ALD) caused by mutations in the gene encoding the ATP-Binding Cassette sub-family D member 1 (*ABCD1*). AMN is characterized by a slowly progressive dying-back axonopathy affecting sensory ascending and motor descending spinal cord tracts that manifests in aging patients, with 100% penetrance in men leading to loss of mobility. So far there are no therapies available for AMN/ALD, leaving the patients with progressive neurodegeneration and lifelong disability. Recombinant AAV9 vector-mediated gene therapy is showing great promise as a potential treatment for AMN, as these vectors deliver functional copies of the complementary deoxyribonucleic acid (cDNA) gene to cells, yielding rapid and robust transgene expression. We are developing SBT101, an AAV9-based gene therapy vector capable of delivering a functional copy of the human ABCD1 (*hABCD1*) gene, for use as a treatment for AMN. The safety and biodistribution of SBT101 was evaluated by delivery into the intrathecal space in NHPs in a dose ranging study. **DESIGN/METHODS:** Preclinical studies to assess the clinical chemistry, neurological and cardiac function, biodistribution, safety, and immune responses of SBT101 were performed in naïve cynomolgus monkeys with low pre-existing neutralizing antibodies (NAb) to AAV9. Animals were administered a single dose of SBT101 at concentrations of 1.5E13, 3.5E13, and 7.6E13 vector genomes (vg)/animal via intrathecal-Lumbar (IT-L) infusion over a 6-hour period. Animals were monitored for 3- or 6- months post administration to assess the reversibility or persistence of any effects. The administered doses were selected based on prior histopathological/functional studies after administration of SBT101 to mice and further extrapolation to NHPs based on the volume of cerebrospinal fluid (CSF). Formulation

buffer-administered NHPs were used as controls. **RESULTS:** Intrathecal delivery of SBT101 at all doses tested (up to 7.6E13 vg/animal) was well tolerated and no adverse events were noted. No SBT101-related effects on clinical, ophthalmological, neurobehavioral, or cardiac observations were noted. SBT101-related effects on clinical pathology test results were transient, observed on Days 7 and/or 28, and consisted of dose-dependent mild to moderate increased alanine aminotransferase and aspartate aminotransferase activity which returned to normal levels without any further intervention. Biodistribution studies indicated a dose dependent presence of AAV vector genomes and *hABCD1* transcript in Central Nervous System (CNS) tissues. Histopathological evaluations showed a slight to moderate increase in the incidence of axonal degeneration and presence of mononuclear infiltrates in CNS and peripheral tissue of SBT101 administered NHPs compared to controls at 3 months, which were not dose dependent, and found to be reduced or unremarkable by 6 months. High titers of serum NABs and antibodies against AAV9 were observed at the end of the study and were dose dependent. Titers of NABs and antibodies against AAV9 were marginal in CSF. **CONCLUSIONS:** Intrathecal delivery of SBT101, up to 7.6E13 vg/animal, was well tolerated in cynomolgus monkeys at doses predicted to be potentially clinically relevant in AMN patients. The safety profile and biodistribution of SBT101 further enables our path to the clinic for the investigation of AMN treatment.

163. AAV Gene Immunotherapy Induced Tolerance is Not Inhibited by Generalized Immune Suppression: A Beneficial Outcome for Patients with MS

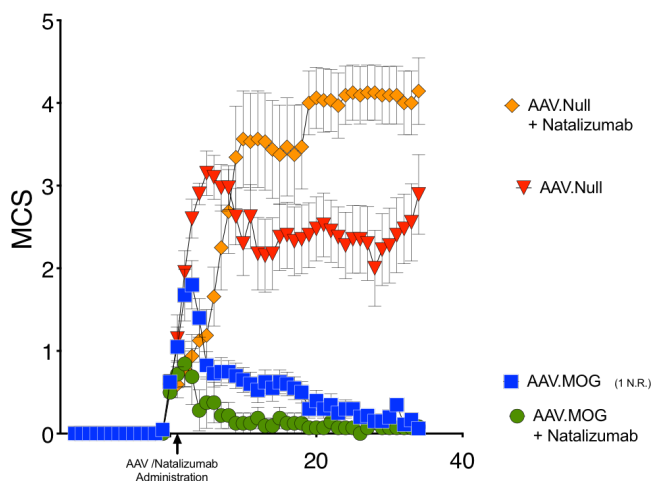
Addelynn Sagadevan^{1,2}, Kevin G. Senior¹, Geoffrey D. Keeler¹, Cristina Gaddie¹, Daniel Min¹, Brad E. Hoffman^{1,2}

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Multiple Sclerosis (MS) is a severely debilitating disease that affects nearly one million people in the US alone. While there are no known cures for MS, the FDA has approved several disease-modifying therapies (DMTs) based on generalized immune suppression that are designed to control disease inflammation and reduce exacerbations. One such DMT is natalizumab (Tysabri), a monoclonal antibody that acts as a $\alpha 4$ -integrin antagonist to reduce leukocyte infiltration into the CNS, thus reducing relapse rates in MS patients. However, as with all DMTs, natalizumab is not a permanent solution due to its suboptimal efficacy in patients over time and potential severe adverse side effects such as progressive multifocal leukoencephalopathy (PML). To provide a better long-term solution for MS, our lab has developed a pre-clinical Adeno-associated virus (AAV) gene immunotherapy that targets multiple neuro-proteins known to play a role in MS. Administration of this vector has shown to not only prevent the autoimmune disease, but can also reverse clinical symptoms in experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Importantly, as this gene immunotherapy moves into clinical trials, it is vital to determine if a patient's current standard of care using the immune suppressive natalizumab would interfere with the ability of our AAV gene immunotherapy to induce antigen specific Tregs and re-establish immunological tolerance. To test this, EAE was induced with the neuro-

protein myelin oligodendrocyte glycoprotein epitope (MOG₃₅₋₅₅) in 9-week-old female C57BL/6 mice. As mice developed clinical disease (tail paresis) AAV.MOG vector was administered. Concomitantly mice started a natalizumab treatment regimen administered every 48 hours for a total of 5 injections. Control mice received either natalizumab/AAV.Null or AAV.MOG/vehicle.

Natalizumab (α -alpha-4 integrin)



Overall, the results revealed that natalizumab administration did not interfere with our gene immunotherapy's ability to prevent/reverse clinical MS-like disease. Mice that received vector and natalizumab remained disease free (see figure). In contrast, mice treated with natalizumab, and null vector quickly experienced a significant rebound upon natalizumab withdraw, a side-effect known to occur in patients. In conclusion, it is evident that natalizumab does not have a negatively impact the ability of our gene immunotherapy to restore tolerance and ameliorate disease. Our gene immunotherapy displays promising clinical relevance for MS patients currently on natalizumab as we have demonstrated the ability of our gene therapy to curb disease progression of EAE and provide protection from severe relapses when natalizumab is withdrawn. These results, alongside our previous data observed with concomitant use of fingolimod and prednisolone, suggest that this novel approach has significant therapeutic potential for patients with MS.

164. Genome-Wide and Directed Integration Assays Identify and Quantify rAAV *In Vivo* Gene Editing Sites in Mice with Humanized Livers

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A variety of delivery methods and mechanisms have been used for gene editing across many systems. For all approaches, it is necessary to determine whether the desired DNA changes have been made as well as to determine whether any unintended changes have been introduced simultaneously. Because these changes may be different in each cell,

and sequencing all cells is not practical, selective evaluation of changes must be carried out. In assessing such changes, recombinant adeno-associated virus (rAAV) has the advantage of acting via homologous recombination (HR), but it is complicated by the presence of high levels of episomes that must be distinguished from integration events. Studies of viral integration generally include the use of sequences on both sides of the integration junction for specific amplification. Typically, primers directed at the virus being studied are combined with primers complementary to adaptors ligated non-specifically to genomic DNA. The high degree of sample processing can lead to a variety of artifacts and this is a particular problem when trying to identify rare potential off-target integrations where false positives can obscure real integration signals. At high doses, episomal AAV may be present at thousands of copies per cell and thus easily amplified or ligated inadvertently. Thus, false positive signals arising from high episomal background may occur more frequently than actual integration events. To overcome the high episomal background observed with rAAV, we have modified previous protocols to minimize the impact of competing vector genomes and focus only on rAAV that has integrated into the human genome. In addition, we have incorporated long-read sequencing to ensure that relevant viral and human genomic sequences are truly present on the same molecule and not inadvertently linked. Molecules spanning the integration junction include over 1 kb from the rAAV vector as well as sometimes many kb of human genomic sequence adjacent to the integration site. Positive control cell lines have been generated to establish the limit of detection in our assay. Genomic DNA from cell lines is spiked at known concentrations into genomic DNA purified from edited human hepatocytes engrafted in mice. The known integration frequency in the cell lines allows the frequency of real events to be estimated. rAAV with a codon-optimized *PAH* gene and homology arms targeted for insertion into human *PAH* was used as a model for examining both on- and off-target integration. On-target HR-mediated integration into the desired locus is found to be dose-dependent. There is no evidence of integration into any other location above the limit of detection, supporting the precision of this gene editing approach.

165. Biodistribution and Activity of a Novel AAV-hFXN Expression Vector in the MCK Mouse Model of Friedreich's Ataxia

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Background and objective: Friedreich's ataxia (FA) is a mitochondrial disease with a heterogeneous presentation that commonly features spinocerebellar ataxia and sensory neuropathy, hypertrophic cardiomyopathy, and increased incidence of diabetes mellitus. FA is an autosomal recessive ataxia in which trinucleotide repeat expansion of the *FXN* gene results in deficient expression of frataxin protein. Frataxin deficiency results in impaired iron homeostasis and dysregulation of mitochondrial respiration and oxidative stress, which

ultimately contribute to the clinical presentation of FA. As FA is a monogenic disease resulting from insufficient gene product, *FXN* gene replacement therapy is a promising therapeutic strategy for FA. We are developing an adeno-associated virus (AAV)-based approach to deliver the *FXN* gene to tissues affected in FA. The goal of this approach is to restore functional levels of frataxin in a safe manner while preventing FA progression. **Methods:** We engineered an expression cassette containing the human *FXN* sequence under the control of a synthetic Desmin promoter and a modified *FXN* 5' UTR. This construct was packaged in AAV serotype 7 for intravenous administration into 6-week-old MCK mice, which lack frataxin in cardiac and skeletal muscle. Four weeks post-administration, cardiac function in treated and untreated animals was assessed via MRI. Subsequently, animals were harvested for biochemical and histological examination. **Results:** Intravenous delivery of AAV7-*FXN* resulted in broad biodistribution to cardiac tissue, recovery of cardiac function, and tolerability in the MCK mouse. Administration of the target dose resulted in near-physiological frataxin expression in the heart, and this correlated with marked improvements in cardiac ejection fraction. Furthermore, our candidate therapeutic was well-tolerated up to 5-fold our target dose in MCK and non-transgenic mice. No gross histopathological findings were observed in heart or off-target tissues. **Discussion and Conclusion:** These results and ongoing studies strongly support further development of this promising therapeutic candidate.

166. Structure-Guided Evolution of a Novel, Neurotropic AAV Variant, STRV5 for Direct CNS Gene Transfer

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StrideBio, RTP, NC

Over the past decade the development of novel AAV variants to tackle challenges posed by neurological disorders has received significant attention and efforts with the goal of traversing the blood-brain barrier (BBB) following intravenous dosing. However, efforts to achieve improved spread in different regions of the brain and cell type-specific tropism have lagged behind. Here, we evolve a novel AAV variant, STRV5 following multiple rounds of evolution through different routes of administration in non-human primates (NHPs). Specifically, direct cisterna magna (ICM) injection yielded variants enriched in 12 different CNS regions. The lead capsid, STRV5 packaging different ss and sc reporter transgene cassettes was characterized in mouse and NHP models following different direct CNS dosing routes. First, bilateral injection directly into cerebral ventricles (ICV) in neonatal P1 mice, shows similar vector genome copy numbers (VCN) to AAV9 with predominantly neuronal transduction from rostral to caudal regions. The highest VCN was observed in the rostral region with a gradual decrease toward the caudal region (~1 to 2-log lower). Dual route of administration evaluating different doses and volumes, bilateral ICV and cisterna magna (ICM) in P5 mice shows increased VCN in caudal as well as deeper brain regions, when compared to bilateral ICV alone. We then evaluated two routes of administration independently, intracerebroventricular (ICV) and cisterna magna (ICM) in NHPs. These studies revealed that STRV5 spreads as efficiently as AAV9 upon ICM injection compared to the ICV route. Although, VCN in different brain regions were generally comparable, quantification of protein expression by ELISA shows a 5-fold increase in the premotor cortex

and 10-fold increase in the cerebellum for STRV5 compared to AAV9 vectors. Moreover, using dual immunohistochemistry for cellular marker, NeuN, and mCherry antibodies, we show a significant shift in the number of positive cells for NeuN colocalized with mCherry expression in the premotor cortex, hippocampus, and thalamus. Initial quantification using Zeiss imaging software shows ~26% of NeuN positive cells in case of STRV5 and additional analysis for different brain regions compared to AAV9 is ongoing. Together, our data highlights a novel, neurotropic AAV candidate with potential to display improved safety and CNS transgene expression for gene therapy of neurological disorders.

167. Immunohistochemistry to Identify Cell Type and AAV Transfection

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Neural directed engineered AAV vectors should produce an appropriate amount of transgene in specific cell subtypes. Immunohistochemistry (IHC) can identify cell type and transgene expression. Combining markers for cell type and transgene expression into one image is technically challenging. We demonstrate an IHC panel capable of meeting these needs. We use image analysis algorithms to identify cell type and AAV transfection. Multiplex IHC panels are useful for identifying engineered AAV vector tropism targeting and transgene expression in preclinical models.

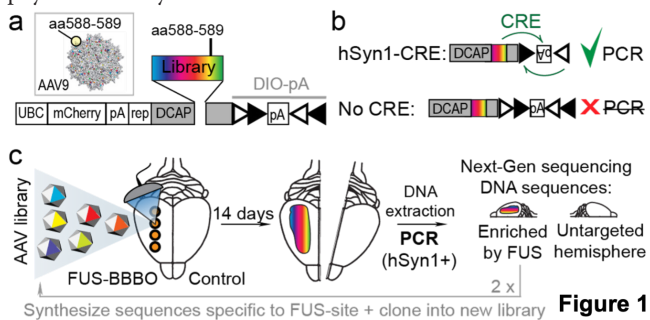
168. Engineering Viral Vectors for Acoustically Targeted Gene Delivery

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Introduction Targeted gene delivery to the brain is a critical tool for neuroscience research and has significant potential to treat human disease. However, the site-specific delivery of common gene vectors such as adeno-associated viruses (AAVs) is typically performed via invasive injections, limiting their scope of research and clinical applications. Alternatively, focused ultrasound blood-brain-barrier opening (FUS-BBBO), enables the noninvasive site-specific entry of AAVs into the brain from systemic circulation. However, when used in conjunction with natural AAV serotypes, this approach has limited transduction efficiency, requires ultrasound parameters close to tissue damage limits. Here, we use high throughput *in vivo* selection to engineer new AAV vectors specifically designed for local neuronal transduction at the site of FUS-BBBO. **Methods** To optimize new AAV variant for FUS-BBBO mediated delivery we employed a viral vector directed evolution method based on *in vivo* Cre recombination. In this approach, a library of AAVs with mutated capsid proteins (Fig. 1a) is injected intravenously into mouse. When a particular AAV variant transduces a cell expressing Cre, its viral genome is modified and becomes detectable by a Cre-dependent PCR (Fig. 1b). We generated a library of viral vectors with engineered capsids based on AAV9, which has high neuronal transduction but does not readily cross the BBB (Fig. 1a). We then used FUS-BBBO to deliver the AAV library (Fig. 1c) to one hemisphere of hSyn1-Cre mice that express Cre in neurons. Later, the DNA was extracted from both hemispheres (Fig. 1c) and sequenced

using next-generation sequencing (NGS). The variants with sequences detected uniquely in the targeted hemisphere were then subjected to a second round of screening to quantify their relative performance. As a result of this screen, we identified 5 engineered vector candidates (AAV.FUS.1 - 5) most enriched in the FUS-targeted hemisphere and evaluated their transduction efficiency and tissue tropism. **Results and Conclusions** Histological analysis revealed higher efficiency of transduction in the brain for all 5 candidates (Fig. 2a, b; up to 130% improvement over AAV9). At the same time, each serotype transduced the liver less strongly (Fig. 2c, d; up to 6.8-fold reduction compared to AAV9). Liver is naturally transduced by many AAVs, including AAV9, and represents an undesirable off-target site. The top AAV.FUS candidate (AAV.FUS.3) showed 12.1-fold improvement in overall tissue specificity (Fig. 2e). Moreover, all 5 candidates show improved neuronal tropism. Upon FUS-BBBO gene delivery, AAV.FUS.3 has a 69.8% likelihood of transducing a neuron, compared to 44.7% for AAV9 (Fig. 2f, g, h). Overall, this study shows that the molecular engineering of AAV capsids can lead to improved ultrasound-mediated gene delivery to the brain. Our screen yielded AAV.FUS.3, the first viral vector expressly engineered to work in conjunction with a specific physical delivery method.

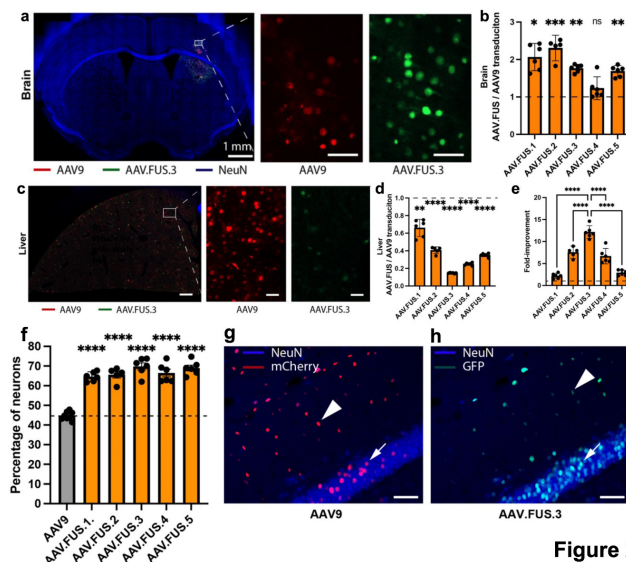


169. Prescreening of NHP's for Neutralizing Antibodies Against Adeno Associated Virus Serotype-2 and 9 (AAV2 and AAV9)

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Adeno-associated viruses (AAVs) are harmless viruses ubiquitous in nature which are critical components in the development of gene therapies. After the first FDA approval in December 2017, AAV vectors have emerged as a leading delivery method for gene therapies across a range of therapeutic indications and dosing routes. Most of over 200 gene therapy trials employing viral vectors are based on AAV vectors. A big challenge in these studies is immunogenicity as pre-existing immunity may interfere with vector distribution and consequently alter transduction and transgene expression both in humans and NHPs. Specifically, the presence of AAV neutralizing antibodies (NAb) in patients and research animals can undermine the potential efficacy of the drug being tested which biases the outcome of the study. Out of 13 serotypes, AAV2 and AAV9 are the best studied and most commonly used in gene therapies. Due to high AAV prevalence, routine ELISA antibody tests, which detect total antibody levels, may miss other neutralizing factors present in the subject are not useful. More commonly, cell based AAV NAb screening and titer assays are utilized for routine prescreening of NHPs prior to selection for gene therapy studies. Multi-tech multi-day qualification studies were performed to assess diagnostic sensitivity, diagnostic specificity, reproducibility and ruggedness of the in vitro AAV2 and AAV9 NAb assays for screening and determining the titer of NAb in test sera. Cells incubated with eight known positive and eight known negative samples were infected with AAV2-CAG-Luciferase or AAV9-CAG-Luciferase providing a luminescent readout of infection. In addition, to assess AAV2 and AAV9 titer assays, four samples with high titer were serially diluted 2-fold from up to 1/5,120 and measured by two techs. Results from the qualification of the AAV2 and AAV9 NAb assays show a high sensitivity, specificity and reproducibility. Overall, diagnostic sensitivity and specificity of the AAV2 and AAV9 NAb assays were found to be > 95% and >99%, respectively. Analytical specificity (selectivity) of the screening assays was also very high with no or low cross-reactivity observed by other AAV serotype antibodies including those against AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9 serotypes. In conclusion, we have validated cell based AAV2 and AAV9 NAb assays which are highly sensitive and specific for routine screening for the presence of neutralizing antibodies in NHPs.



170. Novel AAV Variants Targeting Schwann Cells

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Introduction Cell-targeted gene therapy remains a challenge in order to achieve higher efficacy and less off target toxic effects in gene replacement applications. In order to develop advanced gene therapy tools for more efficient transduction of Schwann cells in the peripheral nervous system (PNS) to treat Charcot-Marie-Tooth (CMT) inherited demyelinating neuropathies, there is a need for improved viral vectors. **Methods** Previous work in the lab of Dr. Gray generated shuffled capsid AAV variants for enhanced nervous system gene transfer (US patent publication 10532110B2). In order to study the efficacy of a set of these AAV variants to transduce Schwann cells we delivered by lumbar intrathecal injection five variants and AAV9 as a control, all utilizing the CBh promoter driving EGFP expression. We studied the biodistribution and expression of the six vectors and compared their efficacy to transduce Schwann cells in lumbar roots, sciatic and femoral nerves six weeks post-injection. **Results** Biodistribution analysis showed that most of the capsids had similar biodistribution in the PNS and CNS tissues while AAV9 showed higher transduction in the liver compared to the variant capsids. EGFP was detected in all tissues examined. In the spinal cord, EGFP was expressed mainly in oligodendrocytes and astrocytes in all the vectors examined, as indicated by double staining with different cell markers. In contrast, in the PNS and especially in lumbar roots, most of the variants were expressed in the axons, while two of the variants were expressed both in axons and in Schwann cells. In teased fibers we observed that in two variants EGFP was expressed in the Cajal bands of Schwann cells besides the axons. **Conclusion** This study provides evidence for the efficacy of novel shuffled capsid AAV variants to transduce Schwann cells. Although two of the variants were expressed at higher levels in Schwann cells, axonal expression remains prominent when using a ubiquitous promoter, and further studies are using a Schwann cell-specific promoter will be useful to evaluate expression rates and levels in Schwann cells in particular.

172. Single Dose Viral Vector Gene Immunotherapy to Treat Food Allergy

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Introduction: Food allergy (FA) is a health problem with an increased incidence. Sensitization to food allergens in humans occurs through different routes such as the gut, respiratory tract or even the skin. FA results from an abnormal response in gut immune tolerance. Activated dendritic cells present food antigens to Th2 cells that provide critical stimulation and signaling for B cell activation and immunoglobulin E

(IgE) class switching. Immunotherapies for patients with food allergy require lengthy protocols with repeated allergen dosing and patients can relapse following discontinuation of treatment. Therefore, we hypothesized that we could induce allergen specific regulatory T cells using a single dose of adeno-associated vectors (AAV) liver gene therapy to treat allergen sensitization and reduce the frequency of severe allergic reactions. **Methods:** 6-week-old heterozygous flaky tail (FT^{+/+}) female mice were sensitized by epicutaneous administration (8 doses) of *Alternaria alternata* protein extract followed by OVA protein. Later, mice were injected with 10¹¹ vg of AAV8-OVA (either with a liver-specific (TTR) or ubiquitous promoter (EF1α)) or an AAV8 vector expressing FIX protein as control, or 10¹² vg of AAV8-TTR-OVA. Then, mice were challenged by intraperitoneal OVA injection and the core body temperature, and symptom score (SS) were recorded every 15 minutes. One week after the challenge, cervical lymph nodes and splenocytes were collected for B and T cell flow cytometry analysis. Mice were bled at different timepoints to measure OVA-specific IgE and IgG antibodies and OVA expression. **Results:** Hypothermia was only significantly reduced in EF1α treated mice (-2°C ± 2.61) compared with TTR-OVA and FIX control groups (-4.93°C ± 3.02 and -6.52°C ± 0.78 respectively). Moreover, the SS severity was also significantly reduced in mice treated with the ubiquitous promoter (1.6 ± 1.34, 2.5 ± 1.22, and 4 for EF1α, TTR-OVA and FIX groups, respectively). IgG1 OVA-specific antibodies were slightly increased in EF1α treated mice however, no significant differences were observed in IgG1 and IgE titers between groups. B cell analysis by flow cytometry showed that AAV-OVA treatment resulted in a significant increase in germinal center B cells, plasmablasts, plasma cells and decrease in IgG⁺ memory B cells compared with the FIX control group. Analysis of T cells from spleen and cervical lymph nodes showed a significant increase in total and OVA-specific FoxP3⁺ T effector memory cells (Treg and follicular regulatory T cells (TFR)) after in vitro stimulation. Finally, we observed a significant increase in IL-10 and decrease in IL-13 cytokine production in supernatants from OVA-stimulated cells from AAV8-OVA treated mice. Next, we investigated if the symptoms difference observed between both AAV8-OVA vectors was dependent on the promoter or differences in plasma OVA levels (124.4 ± 38.55 and 534 ± 314.7 ng OVA/ml for TTR-OVA and EF1α respectively). To test this, we injected FT^{+/+} female mice with a higher dose of TTR-OVA AAV8 (10¹² vg) and repeated the same procedure detailed above. Mice injected with 10¹² vg of TTR-OVA AAV showed a comparable reduction in hypothermia (-1.34°C ± 2.38) and severity of SS (0.8 ± 1.09) compared with mice injected with 10¹¹ vg of EF1α. **Conclusions:** A single dose of an AAV8-OVA vector can safely treat FA in a skin sensitized FA model. Treatment success was in part mediated by an increase in OVA-specific FoxP3⁺ CD4 T cells (Treg and TFR) and increase in the regulatory cytokine IL-10 and a reduction in the IgE promoting cytokine IL-13. Finally, treating established allergy required a higher threshold of OVA expression.

173. Preclinical Development of a Gene Therapy for Gamma Sarcoglycanopathy

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Sarcoglycanopathies are rare autosomal limb girdle muscular dystrophies caused by mutations in one of the genes coding for sarcoglycans. Sarcoglycans form a tetrameric subcomplex part of the dystrophin-associated glycoprotein complex that protects sarcolemma against muscle contraction-induced damages. Absence of one of the sarcoglycan at the plasma membrane reduces the stability of the whole complex and perturbs muscle fiber membrane integrity. As of today, there is no curative treatment for any of the sarcoglycanopathies. A first clinical trial to evaluate the safety of a recombinant AAV2/1 vector expressing gamma-sarcoglycan using an intramuscular route of administration showed limited expression of the transgene but good tolerance of the approach. Considering the low efficiency of the serotype 1 to transduce muscle fibers, we switched to serotype 8 and undertook a series of experiments in mice. First, we evaluated the expression profile of three muscle promoters in an AAV8 expressing the human SGCG coding sequence to select the most adequate promoter to be used in a systemic mode of administration. We obtained an expression profile with an adequate balance for SGCG expression in skeletal muscle, heart and liver correlating with the ratio of the endogenous SGCG expression. Using an AAV2/8 expressing gamma-sarcoglycan under the control of the defined muscle-specific promoter, we observed a dose-related efficiency with a nearly complete restoration of SGCG expression, histological appearance, biomarker level and whole-body force. In addition, our data suggest that a minimal expression threshold level must be achieved for an effective protection of the transduced muscle. Following this proof-of-principle, we undertook a GLP-biodistribution and toxicology study up to 20 fold the dose chosen as the initial clinical dose. No drug-related mortality or sign of toxicity were observed during the course of the study. In parallel, a GMP production process was successfully established. Altogether, these data support the translation of this approach into a first-in-man clinical trial. This trial will be sponsored by Atamyo Therapeutics.

Gene Targeting and Gene Correction I

175. Workflow Optimization for Large Scale Genome Editing of Hematopoietic Stem/Progenitor Cells (HSPCs)

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Hematopoietic stem cell gene therapy (HSC GT) has generated significant interest in recent years to treat genetic blood cell disorders. The use of allogeneic hematopoietic stem cells (HSCs) has become clinical standard in this field yet it bears considerable risks for the patient for potential immunologic complications. Autologous HSC GT is a promising approach that potentially avoid these limitations and thus could be safer for the patients [1,2]. In this work, we describe a workflow optimization for the genome engineering of hematopoietic stem/progenitor cells. We have used the CTS Rotea Counterflow Centrifugation System developed by our team for the CD34+ cell pre-isolation leukocyte enrichment from mobilized leukopak [3]. CD34 tagged microbeads and column-based method was used for the positive selection of HSPCs. We have optimized a culture condition for

the CD34+ cells using the culture media developed by Sei et al. which supports superior expansion of human HSCs [4]. A flow panel has been designed for the phenotyping of HSCs/progenitor cells to identify the CD34+, CD38-, CD45- and CD90+ population. Initially, CD34+ cells were transfected with GFP plasmids using Neon Transfection system. In order to optimize the electroporation condition for the HSPCs, we have electroporated CD34+ cells with GFP Plasmids using a set of different electroporation parameters by changing the voltages, pulse widths and pulse numbers. CRISPR/Cas9 technique have been used for the genetic engineering of the HSPCs. We have optimized a set of sgRNAs to obtain the best knock-out efficiency targeting B2M gene in CD34+ cells. Optimal EP condition was further evaluated using the best performing sgRNA. We have designed and synthesized a double stranded donor DNA for the GFP Knock-In at the B2M gene knocked-out site of the CD34+ cells. We have obtained some promising data from our workflow optimization which could potentially lead to a large-scale genetic modification of CD34+ cells using CTS Xenon Electroporation System. There are already numerous evidences that autologous HSC GT could be a promising strategy for the future [2, 5]. Despite all the recent progress, the clinical application of this HSC gene therapy remains extremely challenging. CRISPR/cas9 based autologous HSC GT will probably require considerable optimization and will require further safety evaluations. Despite all the challenges ahead, we strongly deem that, autologous HSC GT will facilitate future advancements in treating genetic blood disorders [2,5].

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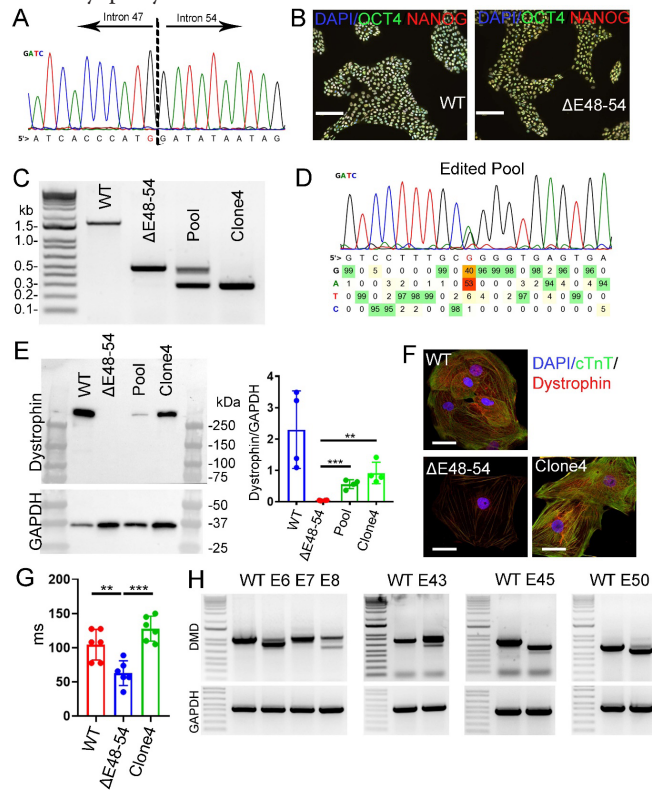
176. Correction of DMD in Human iPSC-Derived Cardiomyocytes by Base Editing-Induced Exon Skipping

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Duchenne muscular dystrophy (DMD) is the most common X-linked genetic muscle disease, caused by mutations in the *DMD* gene. Almost all DMD patients over 18 years of age show clinical symptoms of cardiomyopathy and over 90% of them eventually die from heart failure. Previously we showed that adenine base editing (ABE) can efficiently correct a nonsense point mutation in a mouse model of DMD. Here we further explored the feasibility of base editing-mediated exon skipping as a therapeutic strategy for DMD using cardiac cultures derived from human induced pluripotent stem cell (hiPSC). We first generated a DMD hiPSC line with a large deletion spanning exon 48

through 54 ($\Delta E48-54$) using CRISPR/Cas9 gene editing as about 70% DMD patients are caused by deletion mutations in *DMD* (Fig.1A). Cas9-gene editing did not affect the stemness as assayed for the expression of OCT4 and NANOG by immunofluorescence staining (Fig.1B). Dystrophin expression was disrupted as examined by RT-PCR (Fig.1C), Western blot (Fig.1E) and immunofluorescence staining (Fig.1F) in cardiomyocytes derived from the DMD hiPSC. Transfection of ABE and a guide RNA (gRNA) targeting the splice acceptor enabled efficient exon 55 skipping (Fig.1C), restored dystrophin expression (Fig.1 E,F) and improved the contraction amplitude in DMD hiPSC-derived cardiomyocytes (iCMs) (Fig.1G). The editing efficiency was ~40% as measured by Sanger sequencing data (Fig.1D). Moreover, we designed gRNAs to target the splice sites of exons 6, 8, 43, 45 and 50, and demonstrated that they can also induce exon skipping in WT iCMs (Fig.1H). These results highlight the great promise of ABE-mediated exon skipping as a novel therapeutic approach for DMD cardiomyopathy.

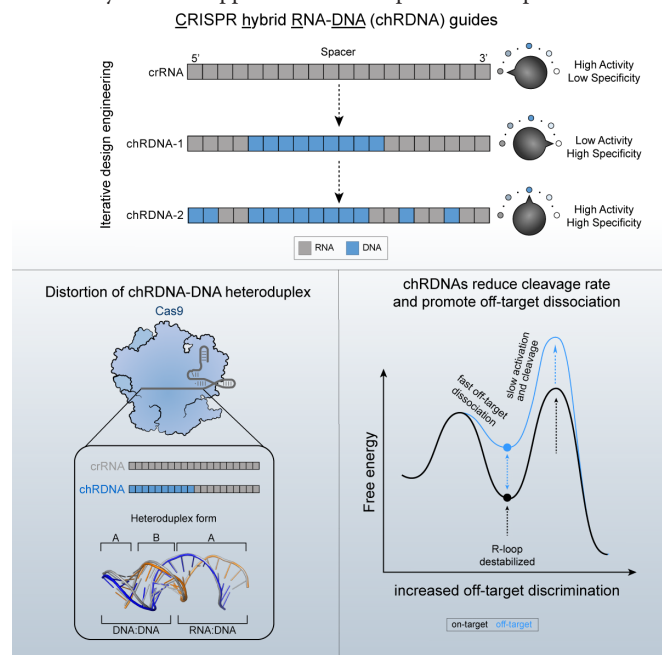


177. Conformational Control of Cas Endonucleases by CRISPR Hybrid RNA-DNA Guides Mitigates Off-Target Activity in T Cells

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CRISPR-based genome editing of primary human T cells has the potential to revolutionize disease-modifying therapies. However, substantial improvements in CRISPR-Cas9 specificity are needed to minimize its off-target activity in cells. A number of “high-fidelity” Cas9 variants have been engineered in an attempt to address specificity improvements, but these enzymes often are constrained by efficiency-specificity trade-offs, limiting their therapeutic application. Here we show that CRISPR hybrid RNA-DNA (chRDNA) guides composed of both RNA and DNA nucleotides are a highly effective approach to increase Cas9 specificity while preserving on-target editing activity. Across multiple genomic targets in therapeutically relevant primary human T cells, we show that 2'-deoxynucleotide (dnt) positioning affects guide activity in a sequence-dependent manner, and we leveraged this observation to engineer chRDNA guides with minimal to no detectable off-target activities. To gain mechanistic insight into chRDNA activity, we performed structural analysis of Cas9-chRDNA complexes, revealing that chRDNA guides adopt distorted helical conformations upon target hybridization to disfavor engagement of off-target sequences. Strikingly, through iterative engineering of dnt number and position in the chRDNA design, we fine-tuned specificity to discriminate between sequences that differ by only a single nucleotide, which was not achieved using “high-fidelity” Cas9 variants. From the combined understanding of Cas9 chRDNA design principles and mechanistic studies, we designed chRDNA guides for use with the Cas12a CRISPR system. Much like Cas9 chRDNAs, tailored Cas12a chRDNA guides supported robust Cas12a-mediated genome editing in primary human T cells without detectable off-target editing. This observation demonstrates the portability of the chRDNA platform across CRISPR systems and exhibits its advantages in terms of flexibility compared to traditional protein engineering approaches. Together, these results demonstrate that chRDNAs enable highly efficient and precise genome editing, paving the way for their utilization across CRISPR systems for application in therapeutic development.



178. Comprehensive Evaluation for Off-Target Editing of In Vivo Base Editing Medicines Targeting the PCSK9 Gene

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In vivo gene editing medicines that permanently modify DNA targets in the liver offer a potential for durable, even lifetime therapeutic benefit. Turning off PCSK9 production by the liver through a single base pair change in hepatocytes could permanently reduce low-density lipoprotein cholesterol and treat atherosclerotic cardiovascular disease, the leading cause of death worldwide. A key aspect of safety for in vivo base editing is the potential for unintended editing outcomes at the target site, unintended editing at non-target sites elsewhere in the genome, or for the creation of chromosomal structural variants. Such unintended editing outcomes need to be evaluated in the target organ, i.e. the liver, and in other cellular contexts guided by the biodistribution and pharmacokinetics of the lipid nanoparticle delivery system. We present a comprehensive approach to evaluate potential off-target editing for our lead program VERVE-101 targeting PCSK9. We applied bioinformatics analyses as well as two in vitro experimental techniques - a homology-based ONE-seq method and a homology-independent ABE-digenome-seq whole genome sequencing method - to prioritize ~3000 candidate off-target sites. We developed a hybrid capture sequencing panel to test whether off-target editing occurred at any of these 3000 sites. Evaluation in multiple primary human cell types (hepatocytes, splenic endothelial cells, and adrenal cells) revealed precise base editing at the on-target site, no evidence for bystander edits around the on-target site, no evidence of off-target base editing at non-target sites at relevant pharmacologic doses in hepatocytes, and no evidence of structural variant creation with base editing. In particular, analysis with two different structural variation detection techniques, a targeted locus-specific technique and an unbiased genome-wide high-resolution chromosomal imaging analysis technique, showed no evidence of translocations in primary human hepatocytes treated with VERVE-101; in contrast, primary human hepatocytes treated with CRISPR-Cas9 nuclease resulted in detectable translocation events above 0.25% at multiple sites. Comprehensive assessment for off-target editing reveals VERVE-101 to be a precise base editing therapy.

179. shRNA-Mediated Gene Therapy for the Treatment of Prader-Willi Syndrome

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Prader-Willi Syndrome (PWS) is a severe neurodevelopmental disorder caused by the loss of function of a series of PWS-related genes on human chromosome 15, including SNORD116 and SNRPN, resulting in weak muscle tone (hypotonia), feeding difficulties, poor growth, and delayed development. PWS genes are regulated through genetic imprinting whereby the paternal allele is expressed and the maternal allele is silenced. Previous studies demonstrated that maternal silencing of PWS genes is induced with euchromatic histone lysine methyltransferase 2 (EHMT2). Furthermore, small molecule targeting

of EHMT2 elevated maternal expression of SNORD116 and SNRPN and improved survival in a PWS mouse model. In this study, we tested the hypothesis that shRNA-induced inhibition of EHMT2 could induce maternal PWS gene expression. First, we screened siRNA candidates targeting EHMT2 in PWS patient-derived induced pluripotent stem cell (iPSCs) line. We discovered several candidates which reduced EHMT2 while concomitantly increasing SNORD116 transcript and SNRPN transcript and protein levels. Top siRNA candidates were converted into shRNA oligonucleotides and cloned into a self-complementary construct plasmid. Ongoing studies are underway to transfect these top shRNA plasmid candidates into PWS iPSC-derived neurons. This study provides the initial proof-of-concept of using an adeno-associated viral (AAV) vector design to express a candidate shRNA to treat PWS. Conceptually, an AAV gene therapy approach may provide a one-time dosing regimen for permanent maternal PWS gene expression. This work was funded by Taysha Gene Therapies, Inc.

180. AAV-Mediated Expression of α Klotho Isoforms Rescues Relevant Aging Hallmarks in SAMP8 Mice

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Senescence represents a stage in life associated with elevated incidence of morbidity and increased risk of mortality due to the accumulation of molecular alterations and tissue dysfunction, promoting a decrease in the organism's protective systems. Thus, aging presents molecular and biological hallmarks, which include chronic inflammation, epigenetic alterations, neuronal dysfunction and worsening of physical status. In this context, we explored the AAV9-mediated expression of the two main isoforms of the aging-protective factor Klotho (KL) as a strategy to prevent these general age-related features using the senescence-accelerated mouse prone 8 (SAMP8) model. Both secreted and transmembrane KL isoforms improved cognitive performance, physical state parameters and different molecular variables associated with aging. Epigenetic landscape was recovered for the analyzed global markers DNA methylation (5-mC), hydroxymethylation (5-hmC), as well as restoration occurred in the acetylation levels of H3 and H4. Gene expression of pro- and anti-inflammatory mediators in central nervous system such as TNF- α and IL-10, respectively, had improved levels, which were comparable to the senescence-accelerated-mouse resistant 1 (SAMR1) healthy control. Additionally, this improvement in neuroinflammation was supported by changes in the histological markers Iba1, GFAP and SA β -gal. Furthermore, bone tissue structural variables, especially altered during senescence, recovered in SAMP8 mice to SAMR1 control values after treatment with both KL isoforms. This work presents evidence of the beneficial pleiotropic role of Klotho as an antiaging therapy as well as new specific functions of the KL isoforms for the epigenetic regulation and aged bone structure alteration in an aging mouse model.

181. Development of a Gene Editing Therapeutic Approach for the Treatment of *RPL5*-Deficient Diamond-Blackfan Anemia Patients

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Diamond-Blackfan Anemia (DBA) is an inherited bone marrow failure (IBMF) syndrome mainly characterized by red cell aplasia, congenital abnormalities and increased risk of cancer. Mutations in more than 20 genes have been associated with DBA, being *RPS19* the most frequently mutated (25%), followed by *RPL5* (11%). A lentiviral gene therapy approach for the treatment of *RPS19*-deficient is under development in our laboratory. Additionally, here we propose a homologous recombination-mediated gene editing strategy for the correction of *RPL5* mutations, since this protein might require a tight endogenous regulation, due to its direct interaction with MDM2, P53-master regulator. With this purpose, we have developed an experimental approach based on the CRISPR/Cas9 system and adeno-associated viral vector 6 (AAV6) which harbors a codon optimized version of the *RPL5* gene. Designed sgRNAs delivered as a ribonucleoprotein allowed us to achieve around 90% INDELS both in healthy donor (HD) cord blood CD34⁺ cells and also in BM CD34⁺ cells from *RPL5*-deficient patients. CD34⁺ cells were transduced with the *CoRPL5*-AAV6, with the aim of optimizing the infectivity and efficacy of HR-mediated gene editing in these cells using different multiplicities of infection (MOIs). The qPCR detection of the *CoRPL5* sequence was indicative of the presence of this sequence in 38.89% and 17.86% of HD CD34⁺ cells transduced at MOIs of 10⁴ and 3x10³ viral particles per cell, respectively. Consistent with these results, the efficacy of homologous recombination was 31% and 14% in these samples, respectively. Similar studies are currently being conducted in lymphoblastic cell lines from *RPL5*-deficient patients to verify the phenotypic correction of these cells.

182. An Enhanced TcBuster™ (TcB-M™) Transposase Has Been Developed for Highly Efficient and Robust Delivery of Therapeutic Cargo for Both RUO and Clinical Applications

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Rapid development of genome engineering tools has driven a number of immune- and stem cell therapies in clinical trials with the goal of generating autologous and allogeneic therapeutics. A majority 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, manufacture delays, and is highly cost prohibitive. We have developed a non-viral transposase-based editing platform to overcome current viral limitations, which

allows for rapid cell manufacture, and reduced cell manufacturing cost. TcBuster is found in the red flour beetle and is a member of the hAT family of transposases. Using directed evolution, we engineered a hyperactive mutant (TcBuster-M™) that has improved integration rates using less plasmid DNA transposon. Since TcB-M™ is not constrained by cargo size, we are able to design large multicistronic plasmids for robust delivery of multiple proteins into various cell types, including primary T- and NK- cells, mesenchymal stem cells, and induced pluripotent stem cells. Additionally, TcB-M™ can be easily combined with endonucleases, such as CRISPR reagents, to generate combinatorial knock-out/over-expression edited cell products. The improved TcB-M™ has resulted in cargo integration rates of over 60% in primary T-cells, without sacrificing cell growth or clonal dominance concerns. Finally, we have conducted direct comparison against lentiviral engineered CAR-Ts, demonstrating TcB-M™ engineered CAR-Ts had higher integration percentage, comparable copy number per genome, and safer genome insertion loci. Overall, TcB-M™ is a proven non-viral gene editing technology to deliver large or difficult therapeutic cargos in variety of cell types. Thus, reducing many of the viral mediated editing hurdles, allowing faster generation of crucial therapeutics to market.

183. R-Loops as Determinant of AAV Integration by Homologous Recombination

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Genome editing approaches based on DNA homologous recombination (HR) have been extensively studied. However, most of these strategies utilize nucleases that generate DNA breaks to increase the rate of HR. Adeno-associated viral (AAV) vectors naturally stimulate HR, and we have optimized a promoterless nuclease-free approach (AAV-HR) for hepatic genome editing by exploiting the induction of HR upon AAV transduction in mice (Barzel et al., *Nature*, 2015). Specifically, murine *Albumin* homology arms to integrate a gene of interest (GOI) at the end of the locus in a non-disruptive way, resulting in a chimeric mRNA that produces both the albumin and GOI protein. Genome-editing is important for the treatment of pediatric subjects where the liver is growing and AAV expression would eventually be lost without genomic integration. Nevertheless, the percentage of edited cells, upon AAV-HR gene transfer, has been shown to be low at 0.4%-1%. We recently found that the inhibition of the Fanconi Anemia complementation group M (FANCM) significantly improves AAV-HR up to 4-fold in hematopoietic stem cells (De Alencastro et al., *Molecular Therapy*, 2020). Furthermore, FANCM has been reported to play a fundamental role in resolving RNA:DNA hybrid structures (R-loops) in the genome, and its inhibition significantly enhance the production of r-loops. Here, we report a preliminary r-loops characterization in vitro, using murine hepatoma cell line (HEPA1-6), and in vivo. The genetic inhibition of FANCM and SRSF1, another gene involved in r-loop formation, results in a significant improvement of AAV-HR up to 4.5-fold compared to cells treated with a scramble siRNA. Moreover, the use of a small molecule, reported to increase r-loops, can enhance AAV-HR by 15-fold in vitro. In a preliminary in vivo study, the use of this small molecule shows an increase in AAV-HR up to 2.5-fold in the liver. Finally, we are currently carrying out DNA/RNA immune precipitation

sequencing (DRIP-seq) experiments to map r-loops in vivo to find a direct correlation between AAV-HR and r-loops formation. The finding that genome region enriched in r-loops might influence the design of the homology arms that will ultimately allow for enhanced nuclease-mediated and nuclease-free AAV-HR making it amenable to treating a large group of diseases.

184. CRISPR/Cas9 Allele-Specific Design to Inactivate A Dominant Negative Mutation in COL6A1 Causing Ullrich Muscular Dystrophy

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Ullrich muscular dystrophy (UCMD) is a congenital disorder caused by defects in collagen VI genes (COL6A1, COL6A2 and COL6A3). Collagen VI is a multimeric essential component of the extracellular matrix and dominant negative mutations are frequently implicated in UCMD pathogenesis (50-75% of UCMD patients). CRISPR/Cas9 approach aimed at selectively knocking out mutated COL6A alleles could represent a therapeutic intervention. The genome editing strategy was assessed in primary fibroblasts from a UCMD patient carrying a heterozygous dominant mutation in the COL6A1 gene. This mutation is responsible for a marked reduction of collagen VI protein, thereby even a mild genomic correction should increase protein production and secretion in CRISPR-treated fibroblasts, ameliorating the disease phenotype. To specifically knock out the mutated allele, we electroporated patient's fibroblasts with ribonucleoprotein (RNP) carrying the high-fidelity SpCas9 and the mutation-specific gRNA. The indels frequency was scored by TIDE analysis and Sanger sequencing of the targeted genomic region. We also predicted possible genome wide off-targets by COSMID webtool. The top ranked off-target sites showed undetectable frequency of indels by TIDE analysis. Patient's fibroblasts were analyzed for functional collagen VI expression. RT-PCR demonstrated the decrease of mutated transcript respect to the untreated cells. Immunofluorescence analysis showed recovery of collagen VI secretion and, finally, ultrastructural analysis by Rotary-shadowing electron microscopy demonstrated the restoration of collagen VI microfibrillar network in the gene edited fibroblasts. In conclusion, these results support the application of CRISPR/Cas9-mediated genome-editing approach to knock out COL6A mutated alleles and rescue UCMD phenotype in patient-derived primary fibroblasts.

185. Novel Ultra-Small Type V CRISPR Nucleases with Expanded PAM Diversity

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Class 2 single-effector Cas proteins, such as Cas12a and Cas9, are revolutionizing genome editing through their programmable nature and robust endonuclease activity. However, their application remains limited due to their large size, which complicates in vivo delivery. To overcome these limitations, we employed genomics and biochemical approaches to discover next generation Cas effectors with enhanced capabilities. We mined through our multi-terabase metagenomic database for genes possessing a RuvC-like domain encoded near a CRISPR locus, and fine-tuned our search to only include Type V nucleases under 900 amino acids in length. Our search uncovered thousands of ultra-small Type V effectors from bacteria, viruses and phages, which we then grouped into families based on phylogenetic analysis. We identified diverse families composed of highly-novel sequences with predicted catalytic and binding domains critical for function. One large group of interest for the current study includes hundreds of Cas proteins that are less than 550 amino acids in length and is composed of novel families. We selected representatives from each family, predicted tracrRNA sequences based on RNA-sequencing and covariance models, designed single guide RNAs, and demonstrated cleavage activity for many members. Unlike most Type V effectors, biochemical analysis uncovered diverse PAM sequences that will allow these effectors to edit a variety of genetic targets. The compact nature and broad targetability of these systems should enable a broad range of genome engineering applications.

186. A Sickie HUDEP2 Cell Model with GFP Tagged Beta-Globin Gene for Analyzing Unintended Phenotypic Consequences of CRISPR/Cas9 Gene-Editing for Treating Sickie Cell Disease

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Sickle cell anemia (SCA), a β -hemoglobinopathy, is one of the most prevalent and severe inherited monogenic disorders. It is caused by a point mutation in the beta-globin gene (*HBB*), causing the replacement of glutamic acid by valine at the sixth residue. The resultant protein is hemoglobin S (HbS). CRISPR/Cas9 based gene correction approach for treating SCA relies on correcting the pathogenic point mutation in *HBB* via the homology-directed repair (HDR) pathway by delivering CRISPR/Cas9 along with a corrective donor template. However, CRISPR/Cas9 induced double-stranded breaks (DSBs) can result in insertions and deletions (indels) of large sizes. Indels that preserve the reading frame may yield proteins with altered sequences that may be functional, partially functional, truncated, or pathogenic. Larger unintended gene disruptions also raise a concern of possible *HBB* gene knockout or down-regulation, and the risk for known pathogenic phenotypes such as β -thalassemia major or minor, all of these need to

be further investigated. We aim to develop a cell model to assess these potential downstream consequences of *HBB* gene modifications. Sicklet Human Umbilical Cord Derived Erythroid Progenitor (S-HUDEP2) is an immortalized CD34⁺ hematopoietic stem and progenitor cell with homozygous sickle mutation. We delivered HiFi SpCas9/gRNA targeting the C- terminus of *HBB* and double-stranded DNA donor with self-cleaving peptide, eGFP, and β -globin polyA signal flanking homology arms into S-HUDEP2 for simultaneous expression of β -globin and eGFP under the *HBB* promoter. We created GFP⁺ single-cell clones using Fluorescent-Activated Cell Sorting (FACS) followed by clonal expansion. We confirmed successful biallelic precise in-frame targeted integration in established clones by in and out PCR followed by Sanger sequencing and digital droplet PCR-based GFP copy number variation (CNV) assay. To test the cell model, we delivered HiFi SpCas9/sgRNA as ribonucleoprotein (RNP) via electroporation using the two sgRNAs targeting the *HBB* currently in clinical and preclinical studies, R-02 and R-66S sgRNAs, respectively. We confirmed efficient indel frequency via Inference of CRISPR Edits (ICE) analysis. Edited clones were erythroid differentiated *in vitro* to monitor variations in eGFP fluorescent intensity based on *HBB* promoter activity. Using FACS, we observed an increase in GFP signal correlating with the expected increase in *HBB* expression and hemoglobin A production throughout erythroid differentiation. We found an apparent downward shift in the GFP signal in R-02 and R-66S RNP edited cells compared to mock-treated cells. In R-02 RNP-edited cells, we identified three distinct populations based on GFP intensity: GFP⁺, GFP^{low}, and GFP^{high}. Edited cells were bulk sorted into three sub-populations, and the indel frequencies in each subpopulation were quantified by ICE. We found minimal frameshift indels in the GFP^{high} population and a clear correlation between loss of GFP expression and indel rate. We observed a similar trend with R-66S edited cells but with distinct GFP signal and indels distribution based on the unique indel signature of the sgRNA. In summary, we have successfully established an S-HUDEP2 clonal cell model for analyzing *HBB* promoter activity and *HBB* frameshift genotype by GFP expression. Our cell model provides a useful tool for identifying gene modifications induced by CRISPR/Cas9 at *HBB* to evaluate the efficiency and safety of gene-editing based therapy for β -hemoglobinopathy.

187. Comprehensive Analysis of Prime Editing with CRISPResso2

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Prime editing is a highly versatile genome editing technology that significantly expands the ability to create precise changes to DNA. However, no standardized analysis strategy exists to quantify the efficiency and purity of editing products. This problem is compounded in that unintended editing outcomes (e.g. scaffold incorporation) are not accurately quantified using analysis approaches that have been used for other genome editing technologies. To address this point, we have extended our tool CRISPResso2 to rapidly and reproducibly characterize the editing outcomes of prime editors. Inputs for CRISPResso2 prime editing analysis is minimal, requiring only the prime editing guide RNA (pegRNA) spacer sequence (which specifies

the initial nick location) and pegRNA extension sequence (which encodes the desired edit). Based on these two input parameters, CRISPResso2 quantifies unintended edits that could result at the nicking site (with a narrow window), as well as at the 3' flap ligation site (with a wide window). Optionally, users may 1) customize analysis by modifying each window size, 2) specify a nicking sgRNA (ngRNA) sequence targeting the non-edited strand to quantify mutations at this site, or 3) specify the pegRNA scaffold sequence to quantify the rate of unintended scaffold incorporation. These extended capabilities make it possible to analyze simple or complex prime editing experiments with high reproducibility. We anticipate that our improvements will increase reproducibility and accuracy, and create a standard for the analysis of prime editing experiments moving forward.

188. Unraveling the Mechanism of Precise AAV-Based Genome Editing

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Genome editing has the potential to cure genetic diseases by correcting pathogenic mutations and reverting mutant sequences back to wild type. However, most current editing platforms require prior nuclease-mediated genomic breaks which can result in double stranded DNA breaks at off-target genomic sites. Both off- and on-target double stranded DNA breaks are then primarily repaired via the error-prone non-homologous end joining (NHEJ) DNA repair pathway. In contrast, the AAV-based genome editing platform does not require the use of exogenous nucleases, thus obviating off-target mutagenesis. We and others have previously shown that AAV editing requires the presence of AAV inverted terminal repeats (ITR), a single-stranded editing genome, homology to the target genomic loci, and the presence of BRCA2, an essential mediator of HR. AAV editing is seamless and precise. No insertion or deletion mutations or AAV ITRs have been observed at editing sites. These observations suggest that AAV utilizes the high-fidelity homologous recombination (HR) process for editing. The HR DNA repair pathway has evolved to accurately correct mutations and maintain genomic integrity. As a result, AAV editing is precise, with no on-target mutations. However, little is known about the mechanisms involved. In order to delineate the mechanism of AAV-mediated homologous recombination, we hypothesized that single stranded AAV editing genomes, which are bounded by the self-complementary palindromic AAV ITRs form unique structures which are recognized by the proteins of the HR complex and ultimately leads to the onset of DNA repair by HR. This hypothesis was tested using AAVHSC editing vectors designed to insert a promoterless Venus expression cassette in intron 1 of the human PPP1R12C gene on chromosome 19. Primary human fibroblasts were transduced with AAVHSC7 and AAVHSC15 editing vectors. Cells were harvested at different time points and intranuclear AAV vector genomes were analyzed for binding to cellular proteins. Chromatin immunoprecipitation analyses revealed that proteins of the HR complex, including BRCA2, RAD51, PALB2 and RPA, interacted with and bound to the AAV editing genomes. Further mapping analysis indicated that binding of these proteins occurred near the ITRs. Studies to further define the interaction of AAV editing genomes with cellular DNA repair proteins is ongoing. Overall, our results demonstrate that the AAVHSC editing genomes

specifically and preferentially recruit cellular proteins involved in HR-based DNA repair and begin to provide basis for the mechanism of accurate AAV-based genome editing.

189. A Versatile, Non-Viral Gene Editing Method for Directing Specificity and Enhancing Function of T Cells

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We have developed a robust non-viral method to efficiently manufacture T cells expressing a neoepitope-specific T cell receptor (neoTCR) from the endogenous locus while abolishing the expression of endogenous TCR. We have also developed a method to express a large transgene along with the neoTCR on the same RNA transcript, both expressed by the native T cell receptor promoter. Both methods allow the generation of several billion T cells expressing patient-specific, tumor-targeting neoTCRs with high efficiency. The diversity of tumor types and challenges that can be presented to the immune system suggest that engineered immune cells may be more effective against a subset of tumors when manufactured with additional modifications. To this end, we have developed multiple non-viral gene editing methods to disrupt genes, express additional transgenes, and knockdown RNA transcript via shRNA. In some instances, the presence of a gene product in the engineered therapeutic cell may inhibit the ability of the cell to either proliferate, kill, or signal within an immunosuppressive tumor microenvironment. Here, we demonstrate the generation of a T cell product expressing a neoTCR, without the endogenous TCR, and with the additional deletion of the TGFBR2 gene. This additional modification generates a cell that can both recognize a patient-specific tumor and is resistant to the immunosuppressive signaling of transforming growth factor beta (TGF- β). For some gene targets, the reduction of transcript may be favored to mitigate any negative effects of complete elimination. Accordingly, we have designed a shRNA expression construct that can efficiently knock down transcript levels of a chosen gene target in cells expressing the neoTCR transgene without the generation of any additional double-strand breaks. The use of this system allows the generation of enhanced T cells both recognizing the patient-specific tumor and depending on the targeted transcript, resistant to immunosuppression, increased persistence, and/or improved functional avidity. The expression of additional gene products can also be utilized to enhance the function of an engineered therapeutic cell therapy. Here, we demonstrate a non-viral knock-in strategy for expressing additional gene products under the control of a variety of promoters allowing the expression at various levels as well as at different cell states. Our strategy generates a product which expresses the neoTCR and the additional transgene product in the same cell using homology directed templates far exceeding the size limitations of AAV. Here, we successfully validate the expression of intracellular, secreted, and membrane-bound protein products

as well as products expressed only during the activated T cell state. In addition to the expression of a neoTCR and the elimination of the endogenous TCR, we show that our non-viral precision genome engineering technology is highly versatile with the abilities to knockout, knock down, and knock in additional genes, greatly expanding the applicability of our T cell drug product.

190. Engineered Novel Type-V Nuclease System for Efficient CRISPR Editing Delivered by AAV

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A lack of efficient delivery modalities has limited the use of CRISPR RNA-guided systems in vivo. LNP-based modalities have enabled delivery of CRISPR-Cas9 to the liver, but there are limitations of LNPs to access other organ systems, such as the CNS. Here we present a novel engineered Type V-I CRISPR system (Cas12i), ABR-001, which utilizes a tracr-less guide RNA. We conducted specificity studies using both unbiased methods and in silico prediction coupled with targeted NGS sequencing. Both methods revealed off-target profiles that were favorable to those of SpCas9. At <1100aa in length, ABR-001 is small enough to be packaged into an AAV along with its guide and full-size regulatory elements with remaining capacity to add additional functionalization. To assess the potential of ABR-001 for AAV delivery, we constructed an AAV vector expressing the ABR-001 effector under a CMV promoter and gRNA under a U6 promoter. Transduction of HEK293T cells resulted in robust editing levels >80%. The robust activity, high specificity, and compact size establishes ABR-001 as a versatile genome editing platform with broad capabilities for both ex vivo and in vivo gene therapy.

191. Bimodular CRISPR/Cas-lentiviral Vector for Modeling Diseases

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An aberrant level of gene expression has been implicated in the pathogenesis of a spectrum of diseases including those of neurological manifestations. The genetic animal models currently employed in the research focused on elucidating mechanisms of neurodegenerative diseases (NDDs) and pathologies have serious limitations in their ability to fully reconstitute the reliable and predictive phenotypes associated with the diseases. Furthermore, the current modeling of NDDs is time- and budget- prohibitive. To address these limitations, we developed an all-in-one bimodal lentiviral vector (LV) equipped with CRISPR/dCas9 system for achieving a reliable and effective perturbation in gene expression in inducible mode. The developed system harbors (i) an overexpression module for disease modeling; (ii) a cis-acting regulatory sequence controlling gene expression; and (iii) epigenome-editing gRNA/dCas9-effector system, expressed inducibly, which is capable of silencing the overexpression module via targeted binding to the regulatory element. As such, the cis-acting sequence could be tested and screened for its involvement in gene regulation. Furthermore, a possible rescue of the disease phenotypes could support the development of new therapeutics based on

epigenome-editing approach. In this study, we tested several inducible systems, including tetracycline- and 4-hydroxytamoxifen (4-OHT)-regulated. Furthermore, to achieve an efficient gene repression, we have improved and optimized dCas9- effector system. We demonstrated that the all-in-one vector could be efficiently packaged into lentiviral vector particles and consequently used for *in vitro* and *in vivo* disease modeling. Importantly, using this system, we were able to trigger Parkinson's disease (PD) phenotypes, by overexpressing alpha-synuclein protein encoded by *SNCA* gene. Furthermore, targeted, and inducible downregulation of *SNCA* expression allowed for rescuing PD pathologies. The study has established and validated the relevance and potential of cis-acting element located within Intron 1 of the *SNCA* gene, as a novel target for epigenetic-based therapeutic approach for PD.

192. Improved Methods for Large HDR Knock-Ins Using Alt-R™ HDR Donor Blocks and Alt-R HDR Enhancer V2

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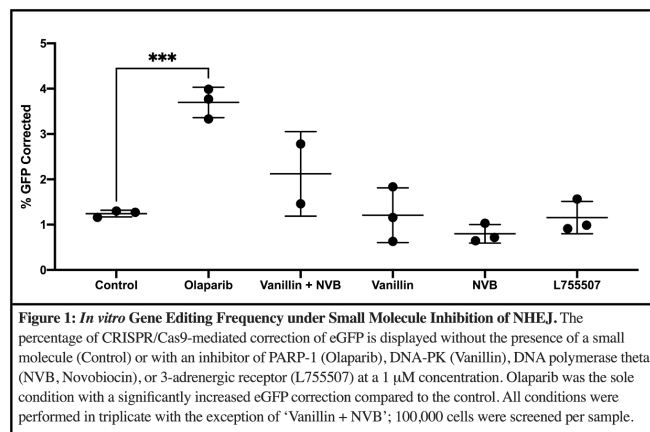
CRISPR-based homology-directed repair (HDR) is an invaluable tool to facilitate specific mutations in a genomic region of interest. While many methods have been reported for improving HDR efficiency, achieving precise changes via HDR remains a challenge particularly for large knock-ins. HDR repair outcomes are most efficient with single-stranded DNA (ssDNA) templates when small insertions, deletions, or SNP changes are desired edits. For these applications, synthetic oligonucleotides (ssODN) have been studied and optimized with modifications for enhanced efficacy in HDR. Larger insertions can be generated via HDR using enzymatically generated ssDNA or double-stranded DNA (dsDNA) donor templates. Here, we present work demonstrating that improved HDR efficiency for large insertions can be obtained when dsDNA donor templates include novel end-modifications. These modifications improve the frequency of HDR and reduce homology-independent (blunt) insertion events that can occur at both on- and off-target CRISPR edits relative to unmodified dsDNA. The use of Alt-R modified dsDNA improved the ratio of HDR:Blunt repair events 4.3-fold on average relative to unmodified dsDNA templates for short inserts and reduced blunt insertion of large templates 4.6-fold at a mock off-target site. We demonstrate further improvement to HDR rates when using Alt-R HDR Enhancer V2, a small molecule that increases the rate of HDR in varied cell types including iPSCs and primary human T-cells. Together the use of Alt-R modified repair templates and the Alt-R HDR Enhancer V2 improved HDR rates up to 5- to 10-fold across knock-in experiments. Finally, we present investigations into design considerations for large dsDNA HDR templates including homology arm length and the placement of blocking mutations to disrupt Cas9 re-cleavage.

193. PARP-1 Inhibition Increases Successful Gene Correction *In Vitro*

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The success of gene correction following a nuclease-induced targeted double-strand break (DSB) is dependent upon conditions favoring homology-directed recombination involving a provided repair template rather than simple healing of the DSB via non-homologous end joining (NHEJ). Our lab has previously demonstrated partial correction of blood phenylalanine concentrations in neonatal *Pah^{enu2/enu2}* mice, a model of human phenylketonuria (PKU) caused by phenylalanine hydroxylase (PAH) deficiency, following AAV-facilitated CRISPR/Cas9-mediated *Pah* gene correction. Achieving a physiologically relevant frequency of *Pah* gene correction required coadministration of vanillin, an inhibitor of the NHEJ repair pathway, suggesting that successful *in vivo* gene correction relies upon modulation of gene repair in favor of homologous recombination (HR). Based upon these results, we hypothesized that more potent inhibition of the NHEJ pathway would further facilitate HR, increase gene correction frequency, and improve the therapeutic efficacy of this approach. In order to identify compounds that promote HR, a selection of known small molecule NHEJ inhibitors were evaluated *in vitro* for their ability to facilitate gene correction. To evaluate correction efficiency, a HEK-293T cell line harboring an eGFP sequence with a premature stop codon integrated into the AAVS1 locus was used. Confluent cultures were co-transfected with two plasmids: the first expressing SpCas9 and a validated guide RNA that directs targeted nuclease activity to a site near AAVS1. The second plasmid carried an eGFP repair template with an intact open reading frame. Thus, the cells expressed functional eGFP only after HR-mediated gene correction. In addition to plasmid transfection, the cells were treated with the selected NHEJ inhibitors. 72 hours after treatment, cells were harvested and sorted by eGFP fluorescence using flow cytometry. Among several different NHEJ inhibitors, our results indicated that Olaparib, an inhibitor of Poly (ADP-ribose) polymerase 1 (PARP-1) and 2, is capable of increasing the baseline gene editing frequency nearly four-fold relative to vanillin. While prior published work has indicated that inhibiting PARP-1 leads to hyper-resection of free DNA strands and increased HR frequency, other studies conversely suggest that PARP-1 activity is required for HR. To help determine the exact cause of Olaparib's success as a gene editing enhancer, we will use our *in vitro* assay to screen other small molecule PARP inhibitors with different specificities to PARP-1 and varying tendencies to 'trap' PARP on the sites of DSBs. Based upon these preliminary results, future studies will evaluate the potential of Olaparib to improve gene correction *in vivo* with our previously validated AAV/Cas9 gene editing treatment for PKU. We will evaluate Olaparib's possible additive increase to our gene editing frequency by measuring blood Phe, PAH activity, and sequencing data. If the increased *in vivo* editing frequency reflects what we have observed *in vitro*, our results would validate a widely applicable strategy for increasing gene correction of pathogenic mutations.



194. Addition of Mismatches in Guide RNA Design Increased Allele Selectivity in a Gene Editing Strategy to Suppress a Dominant-Negative Single Nucleotide *COL6A1* Variant

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Allele-specific inactivation of dominant negatively acting variants via gene editing is a rational approach to treat dominant genetic conditions in which haploinsufficiency is tolerated. Achieving allele-specific targeting of pathogenic variants defined by only a single nucleotide change remains challenging. We first designed a guide RNAs (gRNA) to specifically target CRISPR/Cas9 a single-nucleotide, dominant-negative missense variant in the *COL6A1* gene (c.868G>A; G290R) that leads to collagen VI dysfunction in the extracellular matrix and is associated with a severe form of muscular dystrophy. We nucleofected primary fibroblasts derived from four patients and one control subject with a spCas9-GFP-expressing plasmid that contained the allele-specific gRNA, without providing a repair template. Using a deep-sequencing method, we found that a single frameshifting cytosine deletion accounted for a majority of edits (27.6% of all reads), and that this edit effectively suppressed expression. While editing activity was nearly 2-fold greater at the variant allele, complete allele selectivity was not attained (frequency of reads with indels: 21.2% vs 38.4%, normal vs variant allele respectively). Next, we introduced additional single mismatches in the gRNA sequence to decrease activity on the wild-type allele (2 resulting mismatches) while maintaining activity on the pathogenic allele (only 1 mismatch). In three of these designs, allele selectivity was increased, but on-target activity was decreased (in the optimal gRNA, frequency of reads with indels: 1.0% vs 19.7%, normal vs variant allele respectively). We are currently quantifying collagen VI matrix deposition, in cell cultures, following gene editing with these various gRNAs. Our preliminary data suggest that even without complete allele selectivity, the subset of cells that were effectively corrected by this gene editing approach were sufficient to improve the cellular phenotype. This study strengthens the potential of gene editing to treat dominant disorders, but also underscores the challenge in balancing activity and allele selectivity with gRNAs.

195. Chromatin Modulation Improves CRISPR Gene Editing in Human Pluripotent Stem Cells

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Genome-edited human induced pluripotent stem cells (iPSCs) have broad applications in disease modeling, drug discovery, and regenerative medicine. Despite the development of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system, the gene editing process can be inefficient and take several weeks to months to generate edited iPSC clones. Because chromatin compaction limits the Cas9 protein access to the target DNA, we developed a strategy to improve the speed and efficiency of the iPSC gene editing process via application of histone modifier, Trichostatin A (TSA; Class I and II histone deacetylase inhibitor or HDAC inhibitor). iPSCs were treated with varying amounts of TSA for 24 hours prior to lipofection of CRISPR-Cas9 ribonucleoproteins to target 10 different sites in the genome. Genomic DNA was extracted from the cells after 6 days and subjected to single cell ATAC-Seq and deep sequencing to characterize DNA accessibility as well as on- and off-target genome editing. TSA changed DNA accessibility within single cells and increased gene-editing efficiency of iPSCs by ~3.5 fold in a dose-dependent manner. A moderate concentration of TSA produced the highest genome editing efficiency for several targets across the genome, while excessive TSA concentration lowered efficiency, in part due to toxicity. Moreover, the TSA increased gene-editing efficiencies independent of the gRNA sequence. We observed no increased off-target effects upon TSA treatment, and the resultant iPSC edited lines retained pluripotency and genomic integrity. We also developed an *in situ* imaging-based pipeline to quantify the TSA-induced change in chromatin compaction of iPSCs to rapidly enable the identification of iPSCs that are amenable to gene editing. Overall, the combination of TSA and Cas9 ribonucleoproteins can be used to generate edited iPSCs efficiently for applications in cell/gene therapy and regenerative medicine.

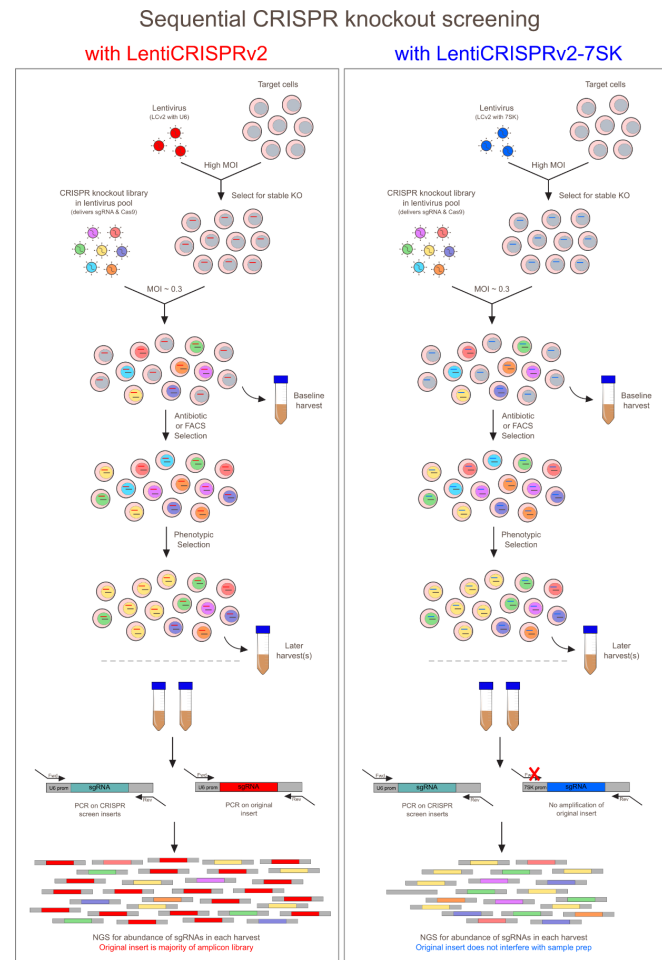
196. A Novel Lenti CRISPR-Based System for Sequential CRISPR/Cas9 Knockout Screening

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Genome-wide CRISPR/Cas9 screens are a popular high-throughput method used to investigate a wealth of biological questions in a single assay format. However, there are many biological questions with two or more components that cannot be readily approached with a single target CRISPR screen. Indeed, tools have been developed to investigate components in a "many vs many" format, such as combinatorial CRISPR/Cas9 screens to identify synthetic lethal gene pairs. Our goal is to develop a method to screen in a "one vs many" format, where one candidate of interest is perturbed against an existing genome-wide library. Here, we describe an easy-to-use system for sequential CRISPR/Cas9 knockout screening that avoids the cumbersome cloning and intensified effort of other dual CRISPR targeting systems. Nearly all human CRISPR/Cas9 knockout libraries are built using the same

lentiviral vector, LentiCRISPRv2, or its derivatives. This vector uses the U6 promoter to drive expression of the sgRNA, which is the standard promoter to drive nearly all sgRNA expression in other vectors. While the LentiCRISPRv2 system works well, it cannot be used twice in the same cell during a CRISPR/Cas9 knockout screen because the integrated inserts of both sgRNA cassettes will be amplified during sample preparation for next-generation sequencing, causing dilution of the resulting reads, which worsens with higher multiplicity of infection (MOI). Our system uses a novel LentiCRISPRv2 vector with a modified 7SK promoter to drive sgRNA expression without amplification in the library preparation process. This vector, called LentiCRISPRv2-7SK, was designed to be compatible with the cloning strategy of LentiCRISPRv2, but also, we show is undetectable by the common sequencing primers used to amplify the sgRNA inserts of a CRISPR screen. When used to edit the non-essential locus *AAVS1* in cells, LentiCRISPRv2-7SK gave similar patterns of indels compared to the original LentiCRISPRv2. We also tested LentiCRISPRv2-7SK functionally for its ability to evict Epstein-Barr Virus (EBV), a DNA tumor virus latently maintained as circular genomes in the nucleus of infected cells. A HEK293 cell line containing a recombinant form of the B95.8 strain of EBV was evicted equally efficiently when using either LentiCRISPRv2-7SK or LentiCRISPRv2 containing sgRNAs directed against viral maintenance elements. Ubiquitous use of the U6 promoter to drive sgRNA expression in the majority of vectors prevented the use of these vectors for sequential CRISPR screening. We have developed a novel 7SK promoter which we employ to drive expression of additional sgRNAs to a target of interest. This new addition to the LentiCRISPRv2 toolbox makes it possible to perform a “one vs many” CRISPR knockout screen. Such a screen could be performed on cells generated with LentiCRISPRv2-7SK to have a null mutation in one gene, then any of the available CRISPR knockout libraries using LentiCRISPRv2 could be used to screen the cell line without compromising the purity of the CRISPR inserts harvested for analysis. Similarly, this process could be reversed such that a CRISPR knockout screen is performed with a pre-existing LentiCRISPRv2 library, then the remaining cells after screening could be used to perturb an individual gene of interest. In summary, the sequential approach described here for “one vs many” screening represents a powerful tool that may help to examine questions across multiple biological systems.



197. Baculovirus-Mediated Gene Editing in Hematopoietic Cells Requires Inhibition of Cellular Innate Immune Pathways

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Gene editing using CRISPR-Cas9 can provide a curative approach for subjects with inherited blood disorders. Integration of a complete therapeutic open reading frame allows single-construct correction of a given genetic defect regardless of the underlying mutation. Large therapeutic genes flanked by sufficient homologous DNA sequences for effective homology-directed recombination (HDR) often exceed the cargo capacity of recombinant AAV6 (rAAV6)-based vectors commonly used for gene editing human hematopoietic stem and progenitor cells (HSPCs). In contrast, baculovirus (BV) vectors are capable of packaging of inserts of at least 38 kb. Additionally, BVs are non-replicating and non-integrating in mammalian cells and have not been associated with disease in humans. Although well characterized as a gene transfer vector, the potential of BV for the delivery of large donor DNA and gene editing tools remains largely unexplored in human HSPCs. To test the potential of BV as a gene delivery vector for hematopoietic cells, we constructed a VSV-G pseudotyped vector with a copGFP

reporter flanked by 4kb homology arms (HAs), sequences homologous to the *ITGB2* gene. Loss-of-function mutations within *ITGB2*, which encodes the $\beta 2$ integrin subunit CD18, are associated with leukocyte adhesion deficiency type 1 (LAD-1). To evaluate vector functionality, we transduced 293A cells, K562 hematopoietic cells and primary human HSPCs. We observed that copGFP expression was robust in 293A cells, approximately 7-fold lower in K562 cells and mostly undetectable in CD34+ HSPCs (Fig. A). We uncovered an innate immune block to BV in K562 cells and HSPCs mediated by the cGAS-STING DNA sensing pathway (Fig. B). This blockade could be overcome in part by a brief pre-treatment with H-151, a potent small molecule inhibitor of STING, in combination with zVAD and Nec-1, compounds which inhibit apoptotic and necrotic cell death, respectively. We next evaluated the ability of electroporated (EP) sgRNA/Cas9 ribonucleoprotein (RNP) complexes to direct the integration of a BV-packaged copGFP reporter at the *ITGB2* locus in K562 cells. Treated cells were cultured for 35 days, over which time copGFP expression in cells transduced with BV without RNP decreased to <1% by flow cytometry. In contrast, copGFP expression in cells treated with BV+RNP stabilized, with an average of ~12% copGFP+ cells up to 35 days (Fig. C). Stable targeted integration was confirmed by in-out PCR (Fig. D) and ddPCR of genomic DNA (Fig. E). To facilitate HDR-based genome engineering in an “all-in-one” approach, we assembled donor DNA with 4kb HAs and HiFiCas9/sgRNA (~22 kb in total), in a single BV vector. We transduced K562 cells as described and observed Cas9 activity by T7EI assay (Fig. F) and expression by Western blot (Fig. G). K562 cells stably expressed copGFP over the course of 35 days with approximately 11% copGFP+ cells, similar to the EP approach (Fig. H). Studies are ongoing to evaluate this strategy in human CD34+ HSPCs. Overall, we demonstrate the potential of a baculovirus approach to overcome the limited packaging capacity of current gene transfer vectors in hematopoietic cells. Our results also lay the groundwork for future studies characterizing vector-host interactions and innate immunity in human CD34+ HSPCs.

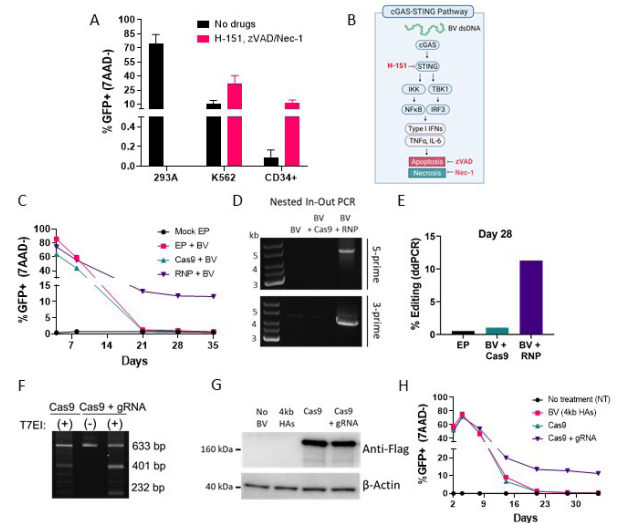


Figure. (A) Percentage of viable copGFP+ (7AAD-) 293A, K562, and human CD34+ HSPCs 24 hours after transduction with VSV-G-pseudotyped baculovirus (BV) containing copGFP flanked by 4kb homology arms (HA) to the *ITGB2* locus with or without small molecules, BV MOI = 25, n=3. (B) cGAS-STING innate immune pathway is activated by BV dsDNA. Small molecule inhibitors including H-151, zVAD, and Nec-1 can be used to inhibit the innate immune response and cell death, improving gene expression in hematopoietic cells. (C) Percentage of viable copGFP+ (7AAD-) K562 cells over the course of 35 days after editing with electroporated sgRNA/Cas9 and transduced VSV-G, copGFP/4kb HA BV, n=1. (D) Nested in-out PCR of genomic DNA isolated from K562 cells (Day 28). Primers are in the genomic DNA of *ITGB2* outside of the 4kb HA region and inside of the integrated copGFP donor. 5-prime PCR product size = ~4.5 kb, 3-prime PCR product size = ~4.2 kb. (E) Percentage of gene editing (HDR) estimated by ddPCR 28 days after electroporation and BV transduction, n=1. (F) T7EI assay indicating Cas9 activity in K562 cells edited with an “all-in-one” BV containing donor DNA/4kb HAs and HiFiCas9/sgRNA. (G) Western blot of flag-tagged Cas9 protein in K562 cells treated without BV, with 4kb HA containing BV (control), HiFiCas9 containing BV, or “all-in-one” BV. (H) Percentage of viable copGFP+ (7AAD-) K562 cells over the course of 35 days after “all-in-one” BV transduction with approximately 11% of cells copGFP positive on day 35, n=1.

198. AAVrh10-Based Gene Therapy for the Treatment of Frontotemporal Dementia Caused by *GRN* Mutations

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Frontotemporal lobar degeneration is one of the most common causes of early onset dementia and currently there are no disease-modifying therapies available. In up to 10% of frontotemporal dementia (FTD) patients, the disease is caused by dominantly inherited loss-of-function mutations in the granulin (*GRN*) gene (*GRN*-FTD), marked by > 50% reduction of progranulin (PGRN) in patients. Progranulin is a highly conserved secreted protein, primary expressed in neurons and microglia in central nervous system (CNS), and is believed to play critical roles in multiple cellular processes including lysosomal function, neuronal growth, and inflammation. Thus, adeno-associated virus (AAV) gene therapy to booster the levels of progranulin may represent a promising strategy to treat *GRN*-FTD. In this study, progranulin is expressed in CNS utilizing an AAV vector encoding a human *GRN* gene driven by a neuron-specific promoter packaged in AAVrh10 capsid and directly delivered to cerebrospinal fluid (CSF) via intracisternal magna (ICM) injection. The results show that a single ICM injection of AAVrh10-*GRN* results in a fair amount CNS-specific expression of human progranulin (hPGRN) in mouse models and

non-human primates. Using genetic mouse models of progranulin deficiency, we show that AAVrh10-GRN restores the phenotypes associated with PGRN deficiency including lysosomal pathology and lipofuscinosis. In NHP, ICM administration of AAVrh10-GRN resulted in a dose-dependent and sustained expression of human progranulin in CSF and achieved levels above the physiological CSF progranulin level in normal humans and didn't show any vector-associated adverse effects, demonstrating an appreciable safety profile. Collectively, these preclinical results support the feasibility of augmenting progranulin expression via AAV-GRN gene therapy for treating FTD caused by GRN mutations and suggest that our approach may provide an effective and safe treatment.

199. High-Efficiency, Large-Scale Manufacture of CCR5-Knockout Hematopoietic Stem Cell Products Based on mRNA Transfection in the GMP-Compliant CliniMACS Prodigy

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Background. Cases of functional HIV cure in patients who underwent allogeneic hematopoietic stem cell (HSC) transplantation from donors harboring homozygous CCR5 Δ 32 mutation sparked interest in development of curative treatment strategies based on CCR5 knockout. We previously introduced a GMP-compliant protocol for CCR5-Uco-TALEN mediated CCR5 gene editing in primary human HSC with breakthrough efficiency. Clinical translation of such approach requires upscaling of the manufacturing process to clinically relevant cell numbers in accordance with GMP accompanied by careful safety analysis. The aim of this study was to develop a clinical-scale protocol for highly efficient CCR5 knockout in HSC based on closed automated CliniMACS Prodigy platform using mRNA encoding CCR5-Uco-hetTALEN [Schwarze et al 2021] and to perform deep off-target analysis as well as in-vivo assessment of engraftment of CCR5^{ko} CD34+ hematopoietic stem cells. **Methods.** Apheresis products with mobilized CD34+ cells from 3 healthy donors were used for manufacturing. Automated HSC engineering was performed in the CliniMACS Prodigy and CliniMACS Electroporator using the customized cell engineering protocol with codon-optimized CCR5-Uco-hetTALEN mRNA produced by Trilink (USA). CCR5 gene editing events such as short indels and long homology-mediated repair events as well as off-target activity at CCR2 homolog were estimated by previously described digital droplet PCR (ddPCR) assay [Mock et al., 2015; Schwarze et al., 2021]. Top-10 potential off-targets were identified in silico and then amplicon NGS was performed by Microsynth (Switzerland). Unbiased TEG-seq [Tang et al., 2018] approach was used to predict additional potential off-target sites. Analysis of the structure and recovery dynamics of various populations of human immune cells was carried out after transplantation of edited cells (1

$\times 10^6$ HSC per mouse) after long-term (20 weeks) follow-up in the peripheral blood, bone marrow and spleen of NSG mice (The Jackson Laboratory). **Results.** Using the developed clinical-scale protocol, we confirmed the high on-target activity rate of CCR5-Uco-hetTALEN at CCR5 locus (up to 93.7%) by both ddPCR and NGS approaches. According to NGS of Top-10 in-silico predicted potential off-targets, pronounced off-target activity (2.53-5.12%) was seen in the CCR2 locus, only. Fifteen additional potential off-target sites were revealed by TEG-seq. Except of CCR2 gene, all potential off-targets located in introns or non-coding regions of the genome. After the transplantation of edited cells all animals of control (N=10) and experimental (N=10) groups were alive at the end of follow-up with no significant difference of body weights between them ($p > 0.05$). Flow cytometry data showed engraftment and multilineal recovery of lymphoid and myeloid hematopoietic lineages with no significant difference between groups. **Conclusion.** In summary, we have developed a reproducible clinical-scale GMP-compliant protocol for CCR5 knockout in primary human hematopoietic stem cells reproducibly facilitating high CCR5 gene editing efficiency. We have demonstrated a favorable safety profile for further product development.

200. Mitochondrial Genome Editing of Primary Cells Through the FusX TALE Base Editor (FusXTBE)

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Mitochondria are the principal generators of cellular ATP produced by oxidative phosphorylation (OXPHOS) and play critical roles in cell metabolism, organ homeostasis, apoptosis, and aging. The human mitochondrial DNA (mtDNA) is 16.5 kb circular molecule that contains 37 genes comprising 13 protein coding genes, 22 tRNAs, and 2 rRNAs. The mutation rate of mammalian mtDNA is significantly higher than the mutation rate observed for nuclear DNA, due to the lower fidelity of DNA polymerase and the ROS-saturated environment present within the mitochondrion. T-to-C or A-to-G point mutations in the mitochondrial genome have been associated with diverse forms of human diseases like mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke (MELAS-syndrome: associated mutation-A3243G), Leber hereditary optic neuropathy (LHON: associated mutation-T14484C), Epilepsy: associated mutation-T616C, and Myoclonic epilepsy with ragged-red fibers (MERRF: associated mutation-A8344G) etc. (Taylor et al., 2005, Skladal et al., 2003, Thorburn et al., 2004). Tools for introducing specific base changes in mitochondria has been hindered by the challenge of transporting RNAs into mitochondria, including guide RNAs that are required to program CRISPR-associated proteins. Although adenosine and cytidine deaminases are important for precision genome editing by enabling base editing in the nucleus, their adaptability in the mitochondria remain largely unexplored until recently. With the advent of the discovery of a novel bacterial cytidine deaminase protein capable of working on double-stranded DNA, DddA_{tox} has enabled the establishment of several mitochondrial base editing platforms. Utilizing the DddA_{tox} molecule, recently our group developed a next generation FusX TALE Base Editor (FusXTBE) that can induce high efficiency and precise editing of TC to TT (or GA to AA) in mtDNA in human cells *in*

vitro and zebrafish *in vivo*. The FusX system is a one-step rapid TALE assembly system that has been used to make an array of functional TALENs for nuclear gene editing. We observed that mRNA delivery of FusXTBE yielded a near completion editing of TC-to-TT in several loci of zebrafish. mRNA delivery provides rapid expression, avoids the requirement for DNA nuclear translocation, and achieves high levels of transfection in primary cells. In addition, mRNA delivery provides no risk of integration into the genomic DNA, adjustable gene expression and easier modulation of the immune system. Here we expand the FusXTBE technology to enhance base editing in human primary cells as potential models for downstream therapeutic considerations. Single dose delivery of FusXTBE demonstrates enhanced editing efficiency in primary fibroblast cells and activated T cells, respectively. These results establish a potential platform to revert pathogenically linked heteroplasmy sequences in mtDNA to wild-type from patient-derived cell lines. We continue to optimize the core editing activity and to enhance the targeting flexibility of the FusXTBE system. We have ongoing strategies in place for *in vivo* delivery options (viral and non-viral) with the goal of potential use in human therapeutics targeting patients with mtDNA-based mitochondrial disease.

201. CRISPR Screening Technologies to Identify and Dissect Gene Regulatory Regions That Control Complex Cell Phenotypes

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Introduction: Gene regulatory elements are critical for coordinating complex cell phenotypes as well as the cellular response to environmental stimuli. Consequently, these non-coding regions of the genome are promising targets for genome and epigenome editing therapeutics. In fact, early genome editing clinical trials have focused on a distal erythroid enhancer that controls BCL11A expression. This highly successful approach was facilitated by dissection of gene regulatory elements via CRISPR-Cas9 saturation mutagenesis. From this success, it is clear that precise identification of gene regulatory target regions is crucial for realizing the full potential of gene therapy and gene editing therapeutics. Here, we use next-generation genome engineering and screening technologies to dissect complex gene regulatory mechanisms, particularly in the context of cell-environment interactions. Mechanical cues resulting from cellular environments can have substantial effects on chromatin structure and epigenetic marks. These physical cues lead to changes in gene expression through largely undetermined mechanisms. To investigate these mechanisms, we utilize ATAC-seq combined with CRISPRi to screen for force sensitive enhancers. We further characterize these enhancer regions using CRISPR-Cas9 saturation mutagenesis screening. Using these methods, we identify key transcription factor motifs responsible for the mechanically controlled expression of MYH9. These findings could be used for targeted treatment of disease states where abnormal mechanical environments exacerbate disease progression, such as cancer and fibrosis, and more broadly can be applied to identify non-coding mediators of complex cell biology. **Methods and Results:** Fibroblast cells were cultured on polyacrylamide gels of differing stiffness. Following overnight culture, genome-wide ATAC-seq profiles were generated and used to determine force-sensitive nucleosome depleted regions (NDRs). From this genome-wide analysis, regions of

differentially accessible chromatin were found near MYH9, a canonical force-sensitive gene. Next, we sought to identify enhancers regulating the MYH9 gene using CRISPRi screening. We generated primary human fibroblasts expressing dCas9-KRAB and a library of gRNAs targeted to all NDRs within 440kb of the MYH9 gene. We discovered a region consisting of 3 enhancers located in MYH9 intron 3 (Mint3). The CRISPRi and ATAC-seq data were combined to determine that one of three enhancers located in the Mint3 region was a force-sensitive enhancer responsible for controlling MYH9 expression. To analyze the enhancer at higher resolution, we transduced fibroblasts with a vector expressing Cas9 and a targeted library of 64 gRNAs tiling across the Mint3 mechano-enhancer region and sorted cells into high and low myosin expressing bins. We sequenced the indels across the Mint3 enhancer in the sorted cell populations to determine which transcription factor binding motifs located in the enhancer region are critical for gene regulation. **Conclusion:** Here, we show differential ATAC-seq and CRISPR-based epigenetic screens can be used to identify context-specific enhancers. Additionally, we use Cas9 saturation mutagenesis screening to predict important transcription factor binding sites responsible for enhancer function. This method for high resolution dissection of critical enhancers could lead to the identification of specific DNA changes that would provide therapeutic effect for a range of applications.

202. Development of Purity Analysis Methods for Synthetic Prime Editing Guide RNAs (pegRNA) for Use in Prime Editing Research Applications

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Prime Editing (PE) is a gene editing strategy that can perform targeted, small insertions, deletions, and substitutions without introducing double-stranded breaks in the genome. Prime editing utilizes a novel dCas9-reverse transcriptase (RT) fusion protein and a guide RNA (pegRNA) that targets the complex to the correct location in the genome. The pegRNA also enables RT priming and serves as a site-specific DNA repair template. In some applications, synthetic RNA oligonucleotides are favored because they can be characterized as discrete gRNA molecules for use in genome editing. However, the length requirement of pegRNAs (120-200 nt) as well as potential secondary structures due to complementary or partial complementary sequences present analytical quality control challenges. These secondary structures can lead to retention times for RNA oligos that do not resolve in a size-dependent manner on capillary electrophoresis. Therefore, there's a need for the development of purity analysis methods to overcome the formation of energetically favorable secondary structures of these chemically synthesized RNA molecules. The use of common denaturants in analytical analysis cannot entirely disrupt the hydrogen bonds of these molecules nor maintain the denatured state, resulting in inaccurate determination of the purity of the chemically synthesized pegRNA. Here, we describe a method that uses capillary gel electrophoresis laserinduced fluorescence method (CGE-LIF),

which can offer high resolution and provide purity quantification of pegRNAs. We also study the effect of different purity levels on prime editing in functional studies using K562 cells.

203. CasPlus: Enhancement of Predictable and Template-Free Gene Editing by the Association of Cas with DNA Polymerase

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Introduction Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated proteins (Cas)-based genome editing has emerged as one of the most powerful tools for sequence-specific gene editing. However, common CRISPR-mediated gene editing strategies often require homology directed repair (HDR) mediated knock-ins, a method which can be inefficient or infeasible such as in the post-mitotic cells of the skeletal muscle, heart and central nervous system. Recently multiple groups demonstrated that SpCas9-mediated template-free editing also generates of undesirable large deletions extending over many kilobases. Hence, there remains an ongoing and unmet need for improved compositions and methods for precisely generating indels for a variety of purposes. In vivo editing efficacy and safety need to be evaluated and refined before being applied therapeutically in human patients. Duchenne muscular dystrophy (DMD) is associated with degeneration of cardiac and skeletal muscle caused by diverse mutations in the X-linked dystrophin gene (DMD). Dilated cardiomyopathy (DCM) is one of the most common lethal features of DMD. There is no curative treatment. Many challenges remain unaddressed. First and foremost, the current SpCas9-generated double-strand breaks repaired in a template-free manner led to imprecise insertion/deletion (indel) mutations in target genes and the repair products of in vivo genome editing are generally heterogeneous in their outcome and dismissed as random by nature. This project is pertinent to this need. **Methods** Our team systematically screened the factors in the DNA repair pathway and found that multiple enzymes promoting precise and template-free DNA repair, significantly increases the propensity for precise insertion through c-NHEJ (non-homologous end joining). Here we describe **CasPlus**, a template-free genome editing platform that precisely insert 1~3 bps into a targeted DNA site using engineered SpCas9 fused to an DNA polymerase. The CasPlus genome editing platform was developed with the objective of improving editing efficiency by controlling insertions and deletions through this repair mechanism and relying less on the cell's intrinsic DNA repair processes such as HDR and MMEJ (microhomology-mediated end joining). **Results** In this proof-of-concept study, our CasPlus system efficiently edit DMD out-of-frame mutation (del exon 52) via precise one base pair insertion in exon 51 or exon 53 with fewer byproducts than regular Cas9 system in human cardiomyocytes derived from patients' iPSCs. **Conclusions** CasPlus-mediated correction for DMD represents a safe and clinically feasible approach to permanently eliminate the genetic cause of this disease and restore cardiac and skeletal muscle structure and function. In principle, this approach could be applied to many genes harboring disease-causing indel mutations, such as *CFTR* delta F508 mutation. This approach bypasses the technical hurdles associated with other methods that have been tried over the past decade. Moreover, through

CasPlus editing, it will be possible to induce precise one to three base pairs insertion to reframing exons and to rescue the DMD mutations. Based on our preliminary results and the rapid pace of this field, we feel this is an opportune time to launch this new approach to permanently correct abnormalities associated with disease-causing indel mutations.

204. Dissecting the Differences in DNA Repair Pathway Choice of Cas12a

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Cas12a is a newly emerging genome editing tool that recognizes T-rich protospacer adjacent motif (PAM) regions and generates double-strand breaks (DSBs) with staggered ends. Previous studies demonstrated the limitations of Cas9 systems with a lower target site-specificity and difficulty efficiently editing GC-poor regions due to a preference for G-rich (PAM) sequences. Cas12a provides distinctive advantages for therapeutic purposes with low off-target effects and high editing efficiency within AT-rich target sites when compared to Cas9. Here, we evaluate the gene editing efficiency of Cas12a when utilizing homology-directed repair (HDR), non-homologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ) repair templates at the CD40L locus. Because HDR is limited to the S/G2 phases of the cell cycle, the majority of cells at the time of editing are not competent for HDR. To overcome this challenge, we utilize the NHEJ-based homology-independent targeted integration (HITI) and the MMEJ-based precise integration into target chromosomes (PITCh) because these repair pathways actively occur in proliferative as well as quiescent cells. We have tested guides for wild-type Cas12a (wtCas12a) in the K562 as well as Jurkat cell lines and noted highly efficient allelic disruption rates up to 91%. However, when transitioning to primary HSPCs, the majority of guides resulted in little to no allelic disruption. We then adopted the engineered variant, Cas12a-Ultra, which achieved robust allelic disruption for numerous gRNAs in PBSCs, reaching 71% targeted gene disruption. We have designed and packaged three different CD40L specific donors which will be used to evaluate repair through discrete DNA repair pathways in cell lines and primary HSPC.

205. Streamlined Detection of CRISPR Editing Using the rhAmpSeq™ CRISPR Analysis System

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Next generation sequencing (NGS) is the gold-standard method for characterizing the efficiency and specificity of genome editing with CRISPR systems. As the field progresses, CRISPR is being used as a screening tool and for therapeutics development. As a result, robust and accurate high-throughput genome editing analysis methods are needed. We have developed the rhAmpSeq CRISPR system, which includes straight-forward primer design, library preparation, and

editing analysis for single target interrogation associated with CRISPR screening projects. Additionally, the rhAmpSeq CRISPR system facilitates highly multiplexed, targeted NGS library preparation for simultaneous characterization of on- and off-target editing, used for lead target characterization. Here, we highlight a streamlined workflow using rhAmpSeq CRISPR for high-throughput genotyping of 95 edited genes involved in DNA repair and compare the editing analysis to other available pipelines. Additionally, we highlight the workflow for quantifying off-target editing levels of the lead gRNA targets. To ensure broad accessibility, we developed a web interface hosting our NGS analysis software solution, CRISPAItRations, which uses cloud-based resources to enable high-throughput batch analyses. Our results highlight the ability for the rhAmpSeq system to support high-throughput CRISPR screening analysis and quantification of off-target editing at 1000's of genomic loci (predicted or empirically defined to be of risk) in one multiplexed PCR. Last, we present the sensitivity and specificity afforded by coupling these tools with an in-depth evaluation of 20 genomic targets containing indel frequencies ranging from 0.05 - 3.5%. When applying the specific requirements our investigation suggests sensitivity and specificity in the evaluation of CRISPR editing can approach 95% confidence.

206. Optimizing Nonviral CRISPR Epigenome Editing in Human Mesenchymal Stem Cells

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Introduction: Human mesenchymal stem cell (hMSC) applications could be advanced by safe and efficient gene delivery. Nonviral systems, which usually deliver plasmid DNA (pDNA) complexed with cationic reagents, are safer and more flexible than immunogenic and mutagenic viral vectors, but are less efficient, especially in hMSCs. In this work, we optimize a transfection protocol to enable, for the first time nonvirally in hMSCs, CRISPR epigenome editing to upregulate expression of target genes with dCas9-p300 and promoter-targeting guide RNAs (gRNAs), a technology first demonstrated in the easily transfected HEK293T cells [1]. **Methods:** HEK293Ts or adipose hMSCs (hAMSCs) were seeded in 96-well plates, primed with DEX, and transfected with three commonly used reagents (Turbofect™, Lipofectamine 3000™, or 25 kDa branched polyethylenimine), complexed with pDNA encoding for reporter proteins to optimize pDNA and reagent dose. In subsequent experiments using optimized doses, pDNA encoding dCas9-EGFP or dCas9-p300 was co-delivered with a gRNA targeting the IL1RN promoter [1], either from a separate pDNA which also encoded for the dsRed fluorescent reporter, or as synthetic RNA oligos. Fluorescent reporter expression was quantified with imaging after 48 hrs, and target IL1RN mRNA upregulation was quantified by qRT-PCR. **Results:** Optimized EGFP transfection of HEK293Ts and hMSCs with pDNA (120 ng/well) complexed with Turbofect at a 1:3 ratio (µg:µL), resulted in about 70% and 20% transfection efficiency, respectively, and was significantly higher than with optimized LF3K and PEI complexes. Using these optimized doses, dCas9-EGFP transfection efficiency was about 85% and 10% in HEK293Ts and hAMSCs, respectively (**Fig 1A**), while transfection efficiency of the co-delivered gRNA pDNA reporter (dsRed) was about 65% and 5% in HEK293Ts and hAMSCs, respectively (**Fig 1B**). Consistent with seminal work [1], dCas9-p300 co-delivered with gRNA pDNA to HEK293Ts resulted in IL1RN mRNA

upregulation of over 3000-fold, relative to controls with no gRNA delivery. In comparison to HEK293Ts, identically treated hAMSCs displayed little to no IL1RN mRNA upregulation over controls, presumably due to low transfection efficiency. To achieve higher gRNA delivery than observed when delivered on separate pDNA in hAMSCs, we used synthetic gRNA oligos co-delivered with dCas9-p300 pDNA, which achieved up to 50-fold target gene IL1RN mRNA upregulation over controls in some hAMSC donors, but synthetic gRNA toxicity also resulted in significantly decreased cell viability.

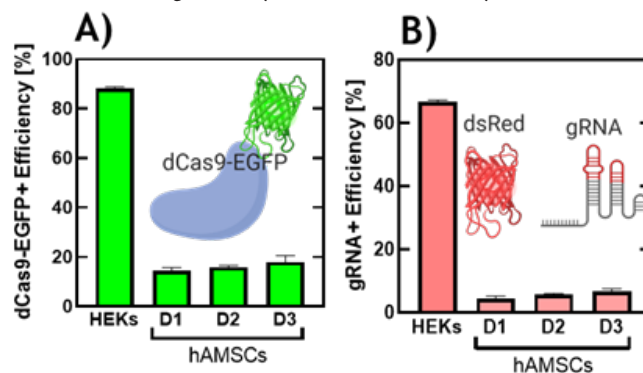


Figure 1: A) dCas9-EGFP transfection efficiency in HEK293T cells (HEKs) and hAMSCs derived from multiple donors (denoted as D#) after optimized co-delivery of pDNA expressing dCas9-EGFP (60 ng/well) and pDNA expressing dsRed reporter with gRNA (60 ng/well) B) Corresponding gRNA transfection efficiency from experiment described in A. Figure created with BioRender.com **Conclusion:** These results demonstrate the significant challenge of translating technologies first described in easily transfected cell lines to therapeutically relevant primary cells using safe gene delivery methods. Future work aims to increase transfection efficiency of CRISPR systems to hMSCs, in part by delivering both gRNA and dCas9 within the same pDNA. **References:** [1] Hilton et al, *Nat Biotechnol*, 2015, 33, 510.

207. In-Vitro Base Editing More Efficiently Targets and Mitigates Pathogenicity Associated with Free Sialic Acid Storage Disease R39C Variant Than Canonical Homology-Directed Repair

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Sialic acid storage diseases (SASDs) are a subset of lysosomal storage disorders resulting from pathogenic mutations in Solute Carrier Family 17 Member 5 (*SLC17A5*) gene. This gene is responsible for the production of **sialin**, a trans-membrane protein that facilitates the transport of free sialic acid out of the lysosome. *SLC17A5* variants generate a non-functional or partially functional sialin protein, resulting in the accumulation of free sialic acid within the lysosome. Dependent on the specific form of Sialic acid storage disease, affected individuals exhibit an array of symptoms from hypotonia, severe delay in normal development, and gradual neurological issues, among other complications. Currently, there are no available therapeutic interventions for sialic acid storage diseases, and treatment is directed towards managing symptoms and fostering a supportive quality of life

through care for patients. With the advent of CRISPR technology came a novel therapeutic approach for genomic modification. Yet, classical genome engineering utilizing CRISPR-Cas9 homology directed repair (HDR) has demonstrated its limitations with efficacy and efficiency of correction. Despite this, CRISPR-Cas9 continues to evolve as an essential instrument in the molecular toolbox, particularly with the development of base editing. **CRISPR-Cas9 base editing** allows for precise genomic modification of single base pairs without double stranded breaks through base excision repair. To take advantage of CRISPR-Cas9 base editing, our group utilizes this technology to perform genomic correction of a predominantly Finnish variant of *SLC17A5*, **c.115C>T (R39C)**, and attempt to improve editing efficiency and decrease indel formation when compared to CRISPR-Cas9 HDR. Future studies will aim at further enhancing the editing specificity and efficiency using base editing alternatives or through the potential use of prime editing systems.

208. Bacterial Retrons Enable Precise Gene Editing in Human Cells

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Retrons are bacterial genetic elements involved in anti-phage defense. They have the unique ability to reverse transcribe RNA into multicopy single-stranded DNA (msDNA) that remains covalently linked to their template RNA. Retrons coupled with CRISPR-Cas9 in yeast have been shown to improve the efficiency of precise genome editing via homology-directed repair (HDR). In human cells, HDR editing efficiency has been limited by challenges associated with delivering extracellular donor DNA encoding the desired mutation. In this study, we tested the ability of retons to produce msDNA as donor DNA and facilitate HDR by tethering msDNA to guide RNA in HEK293T and K562 cells. Through heterologous reconstitution of retons from multiple bacterial species with the CRISPR-Cas9 system, we demonstrated HDR rates of up to 11.4%. Overall, our findings represent the first step in extending retron-based precise gene editing to human cells.

209. All-in-One Adenoviral Vectors with CRISPR-Cas9 Precisely and Robustly Target R34G Mutation of NFE2L2/NRF2 Gene- Implicating Their Potential Applications In Vivo

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We develop a CRISPR/Cas9 gene editing system to disrupt gene functioning in solid tumors to resume the sensitivity to chemotherapy. Nuclear factor erythroid 2-related factor 2 (NRF2) is a master transcriptional regulator encoded by the NFE2L2 gene that activates antioxidant response element (ARE) genes in response to cellular stress. In some types of lung cancer, the tumor specific R34G mutation in NFE2L2 drives constitutive NRF2 activation leading to chemoresistance. In this study, we systematically compared the AAV

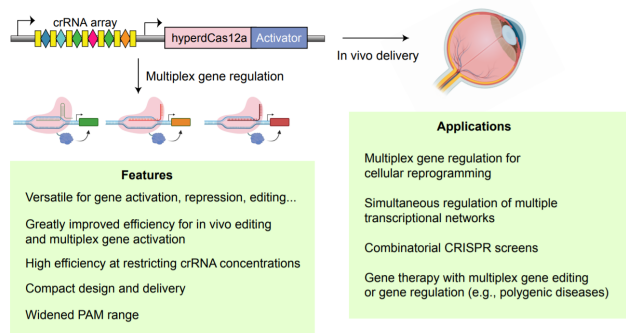
and adenoviral vector as vehicles to deliver CRISPR/Cas9 to edit the R34G mutation in cell lines derived from H1703. We identified AAV6 as the most effective serotype to transduce H1703 derived lines in vitro among many other serotypes. However, due to its size limitation, we cannot embed both gRNA and spCas9 in one vector. Even spCas9 alone almost maximizes the AAV capacity leaving little room for a full conventional promoter, like CMV, or EF1a, etc. We had to compromise to choose a mini-CMV or a core EF1a promoter to drive spCas9 in AAV shuttle vectors. EF1a Core promoter was superior in driving spCas9 expression in vitro as identified by western blotting although to a lesser degree compared to that of a full promoter. We prepared another U6 driven gRNA (R34G) in AAV6 separately, and co-infected H1703 derived lines with dual spCas9 AAV6 vectors. With the increment of MOI to 5×10^6 GC per cell, the maximum gene editing was about 12%. Alternatively, self-complementary AAV to express 3 copies of gRNAs with U6, H1, and 7SK promoters was constructed. When transducing the cells with dual AAV6 vectors, or adenoviral vector with a full CMV promoter-driven spCas9 in the presence of sc-AAV6-U6H17SK-R34G vector, the highest gene editing was up to 30% using AAV6 vectors (MOI up to 2×10^6 GC per cell); while when combined with adenoviral vector at MOI of 50 PFU/cell, the 72% of gene editing could be achieved readily. The data demonstrated that adenoviral vector was potent to express the highest levels of spCas9 to support effective gene editing. We further compared the potential between adenoviral vector and AAV6 in re-constituting the spCAS9 in cells by applying enough gRNA through LNP mediated transfection, consistently with the previous observation that adenoviral vector was superior in driving spCas9 expression at levels to match up the maximum R34G gRNA to achieve up to 95% of gene editing. Since the capacity of adenoviral vector was up to 8 Kb, we built up adenoviral vectors with a full CMV driven spCas9 in conjunction with 1 to 3 copies of gRNAs driven by U6 or U6, H1, 7SK promoters respectively. We also constructed two additional adenoviral vectors with one expressing only 3 copies of gRNA, and the other expressing spCas9. Unlike AAV6 vectors, 1 copy of gRNA in an all-in-one adenoviral vector was sufficient to edit the genome at a level like that of 3 copies of gRNA, and the dual vectors with gRNA or spCas9 separately did not show any advantage compared to that of an all-in-one vector. In addition, we built up additional vector sets where spCAS9 was switched to espCas9 and fused to 2 copies of NLS at both N- and C-terminal driven by a full CAG promoter. These adenoviral vectors with gRNA and espCas9 worked as efficiently as previous vector sets. To better understand the function of each polymerase III promoter, a serial of vectors where R34G gRNA was driven by U6, H7, or 7SK respectively was prepared and is under investigation. We anticipate one simple formulation to comprise spCas9-gRNA in an all-in-one adenoviral vector for ex vivo and in vivo application for cancer management.

210. Multiplexed Genome Regulation In Vivo with Hyper-Efficient Cas12a

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Multiplexed modulation of endogenous genes is crucial for sophisticated gene therapy and cell engineering. CRISPR-Cas12a systems enable versatile multiple genomic loci targeting by processing numerous crRNAs from a single transcript, however, their low efficiency has hindered applications *in vivo*. Through structure-guided protein engineering, we develop a hyper-efficient LbCas12a variant, termed hyperCas12a, with significantly enhanced efficacy for gene activation, particularly at low crRNA conditions. We demonstrate that hyperdCas12a has minimal off-target effects compared to the wildtype system and exhibits enhanced activity for gene editing and repression. Delivery of the hyperdCas12a-activator and a single crRNA array simultaneously activating endogenous *Oct4*, *Sox2*, and *Klf4* genes in the retina of postnatal mice alters the differentiation of retinal progenitor cells. The hyperCas12a system offers a versatile *in vivo* tool for a broad range of gene modulation and gene therapy applications.



211. CRISPR/Cas Knockout of miR-21 Reduces Glioma Growth

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Non-coding RNAs, including microRNAs, support the progression of glioma. miR-21 is a small, non-coding transcript, involved in regulating gene expression in multiple cellular pathways, including the regulation of proliferation. High expression of miR-21 has been shown to be a major driver of glioma growth. Manipulating the expression of microRNAs is a novel strategy in the development of therapeutics in cancer. In this study we aimed to target miR-21. Using CRISPR genome editing technology, we disrupted the miR-21 coding sequences in glioma cells. Depletion of this miRNA resulted in the upregulation of many downstream miR-21 target mRNAs involved in proliferation. Phenotypically, CRISPR-edited glioma cells showed reduced migration, invasion and proliferation *in vitro*. In immunocompetent mouse models, miR-21 knockout tumors showed reduced growth resulting in an increased overall survival. In summary, we show that by knocking out a key miRNA in glioma, these cells have decreased proliferation capacity both *in vitro* and *in vivo*. Overall, we identified miR-21 as a potential target for CRISPR-based therapeutics in glioma.

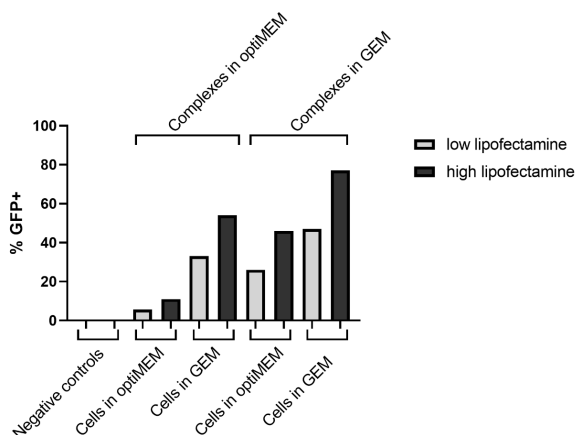
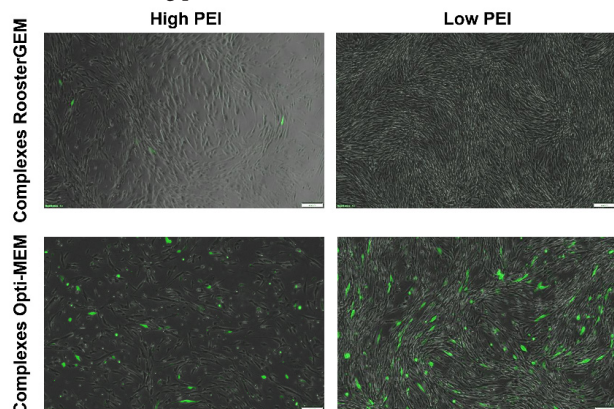
Oligonucleotide Therapeutics I

212. Optimization of Transient Transfection of Mesenchymal Stromal Cells with Plasmid DNA or Messenger RNA

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RoosterBio, Frederick, MD

Mesenchymal stromal cells (MSCs) are increasingly investigated in preclinical and clinical trials due to their immunomodulatory and regenerative functions. The transient transfection of MSCs with genetic material is an invaluable technique that can yield new mechanistic insights into MSC function and improve the effectiveness of MSC-based therapies. For example, transient transfection of MSCs with plasmid DNA (pDNA) has been demonstrated to improve MSC homing to a target injury site or increase MSC survival *in vivo*, thereby increasing rate of wound closure or neovascularization, respectively. Although viral-based methods historically yield highest transfection efficiencies, they also carry safety risks that may not suit all applications. Our objective was to produce an optimal protocol for the transient transfection of human MSCs (hMSCs) with pDNA or messenger RNA (mRNA) using non-viral means. hMSCs were transfected with pDNA or mRNA encoding GFP using Lipofectamine 3000. pDNA was additionally complexed with polyethylenimine (PEI) for transfection. The transfection complex medium, cell medium, concentrations of pDNA/mRNA and transfection reagent, cell plating density, and transfection duration were systematically altered to determine optimal conditions. hMSCs were imaged via fluorescence microscopy and analyzed using ImageJ software, or evaluated via flow cytometry to determine transfection efficiency. pDNA-transfected hMSCs displayed the most GFP-positive cells when pDNA complexes were formed with PEI in Opti-MEM, reaching transfection efficiencies of 30% (Fig 1). Culturing cells in RoosterNourish yielded the highest pDNA transfection efficiency compared to several other media. hMSCs could be transfected with pDNA using Lipofectamine 3000, but PEI is recommended due to its availability as a GMP-compliant reagent. hMSCs in RoosterGEM or

Opti-MEM had increased mRNA transfection efficiency compared to hMSCs in other media. Additionally, mRNA transfected MSCs showed the highest transfection efficiency when mRNA complexes were formed in RoosterGEM (~80%), followed by Opti-MEM (~55%, Fig 2). hMSCs transfected with either mRNA or pDNA began fluorescing by 24 hours post-transfection and maintained similar fluorescence levels up to day 5. In summary, we have developed optimized protocols for the transient transfection of hMSCs with pDNA or mRNA, and these protocols will serve those seeking to transfect MSCs early or late in the manufacturing process.



213. A Novel Method for Assessing Full-Length Sequence Integrity of RNA Therapeutics

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RNA therapeutics is a rapidly expanding area of gene therapy. RNA can be used to introduce exogenous copies of new transcripts, where it can be translated into a protein that can work as an enzyme or antigen or regulate signaling pathways. Confirmation of an intact full-length mRNA including exact length of the polyA tail is critical to ensure appropriate RNA stability and translation. Existing polyA

tail length assays have limitations when detecting the length of mixed populations and intermediates and can be prone to introducing artifacts that prevent accurate and precise quantitation. Compared to existing Quality Control (QC) methods, next-generation sequencing (NGS) offers an effective high-throughput approach for monitoring RNA therapeutics quality and purity. Using a novel approach, full-length manufactured RNA molecules are sequenced using PacBio long-read technology. This novel approach involves 3' adapter ligation and template switching during reverse transcription to keep the full length polyA tail intact. Following amplification and adapter ligation, the final library is then sequenced using PacBio Single-Molecule, Real-Time (SMRT) long-read sequencing technology. This method allows for full-length sequencing of products up to and beyond 10kb without limitations or bias due to repetitive sequence or C/G content. Crucially, this new method preserved the entire length of the polyA tail enabling sensitive and specific counting of the polyA tail length. Here we describe our novel approach and present final QC results using this methodology. In addition to sequence fidelity, this NGS approach offers read-depth sufficient to identify and confirm overall sample purity, including intermediate and alternative products present within the sample. With this approach, the entire molecule, including complete polyA tail is sequenced end-to-end to ensure complete fidelity of the manufactured RNA therapeutic.

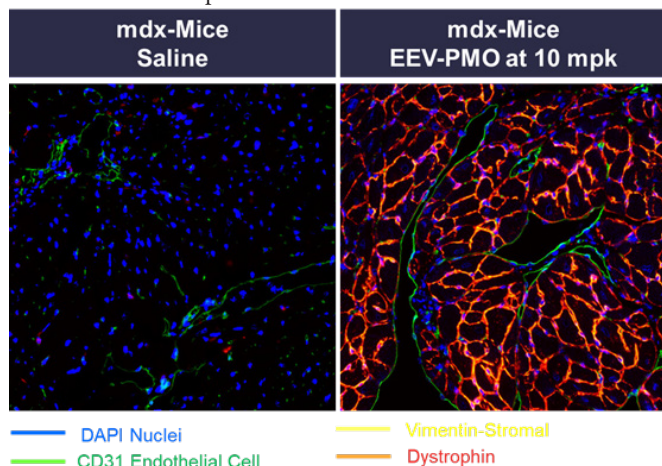
214. Development of Endosomal Escape Vehicles to Enhance the Intracellular Delivery of Oligonucleotides

Xiang Li, Patrick G. Dougherty, Mahboubeh Kheirabadi, Ajay Kumar, Amy Hicks, Mark Wysk, Kimberli J. Kamer, Nelsa L. Estrella, Xiulong Shen, Wenlong Lian, Nanjun Liu, Matthew Streeter, Suresh Peddigari, Chance Brandt, Christine R. Waters, Tyler Ironside, Meagan Mycroft, Roshni Mukundan, Vyoma Patel, Phallika Mon, Arianna Bonilla, Andy Stadheim, Mohanraj Dhanabal, Mahasweta Girgenrath, Natarajan Sethuraman, Ziqing Qian

Entrada Therapeutics, Boston, MA

Biological therapeutics exhibit high target specificity and potency but are limited in their ability to reach intracellular targets of interest. These limitations often necessitate high therapeutic doses and can be associated with less-than-optimal therapeutic activity. One promising solution for the intracellular delivery of biologics is through the use of cell-penetrating peptides (CPPs). However, canonical CPPs are limited by relatively low efficiencies of cellular uptake and endosomal escape, minimal proteolytic stability, and toxicity. To overcome these limitations, we have designed a family of proprietary cyclic CPPs that form the core of our Endosomal Escape Vehicle (EEV™) technology, which is capable of delivering covalently conjugated cargo across all tissue types. To demonstrate the utility of our platform, we employed our EEV technology for the delivery of splice-modulating oligonucleotides and evaluated the EEV-oligonucleotide conjugates in preclinical models of Duchenne muscular dystrophy (DMD). EEV-oligonucleotide conjugates demonstrated durable exon skipping and broad dystrophin protein expression in target tissues including skeletal

and cardiac muscles. These results suggest the significant therapeutic potential of our EEV-oligonucleotides for neuromuscular diseases, as well as the broader application of our EEV platform for the delivery of intracellular therapeutics.



215. Oligo-Protac Strategy for Cell-Selective and Targeted Degradation of Activated Stat3

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Oncogenic and/or tolerogenic transcription factors (TFs), such as STAT3 or NF- κ B, are enticing but pharmacologically challenging targets for cancer therapy. Decoy oligodeoxynucleotides (dODN), which comprise the TF specific DNA-binding sequence, are alternative, competitive inhibitors of TF transcriptional activity. Their development so far has been hindered by the lack of targeted delivery methods, limited stability, and rather modest efficacy of reversible target inhibition. We previously generated scavenger receptor (SR)/toll-like receptor 9 (TLR9)-mediated delivery strategy for nuclease-resistant STAT3 or NF- κ B dODNs to target myeloid cells, B lymphocytes and certain cancer cells *in vitro* as well as *in vivo*. Here, we adapted a proteolysis targeting chimera strategy to TF-specific CpG-STAT3dODN (C-STAT3dODN^{PROTAC}) to enable STAT3 protein degradation in cell-selective manner. We used computational modeling of the C-STAT3dODN with STAT3 protein dimers and ubiquitin ligase components in solution to design C-STAT3dODN^{PROTAC}. Phthalimide derivatives, such as thalidomide, were tethered to the 3' end of the decoy molecule without or with aliphatic linkers of different length. The optimal linker length proved consistent with the computational model. As verified by *in vitro* competition studies, the inhibitory effect of STAT3dODN^{PROTAC} was thalidomide- and STAT3dODN-specific. We then verified that STAT3dODN^{PROTAC} reduces protein levels of STAT3 in target immune cells through proteolysis as assessed using proteasome inhibitors such as epoxomicin. The inhibitory effect was also abolished by target immune cells lacking expression of cereblon (CRBN), a known E3 ubiquitin ligase component and recognized by thalidomide. Next, we generated CpG-STAT3dODN^{PROTAC} conjugates to enable

spontaneous oligo-PROTAC conjugate uptake by target cells such as primary myeloid cells, B cells, established dendritic cells (DC2.4) and B cell lymphoma (A20) but not by T cells. In DC2.4 cells with constitutive STAT3 activation, CpG-STAT3dODN^{PROTAC} dose-dependently reduced STAT3 levels (IC₅₀=250nM) within 2 days of treatment. Importantly, the inhibitory effect of C-STAT3dODN^{PROTAC} was specific to STAT3 without affecting levels of the related STAT1 or STAT5 proteins and resulted in improved immunostimulatory effects on mouse dendritic cells. To our knowledge, this is the first demonstration that the PROTAC strategy can be employed to decoy DNA-based targeting of undruggable TFs. Our initial results underscore the potential of using this strategy for cell-selective targeting of oncogenic and immunosuppressive STAT3 with potential application to cancer immunotherapy.

216. Impact of Phosphoryl Guanidine-Containing Backbone Linkages on Stereopure Antisense Oligonucleotides in the CNS

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Wave Life Sciences, Cambridge, MA

Attaining sufficient tissue exposure at the site of action to achieve the desired pharmacodynamic effect on a target is an important determinant for any drug discovery program, and this can be particularly challenging for oligonucleotides in deep tissues of the CNS. Herein, we report the stereopure synthesis and the impact of phosphoryl guanidine-containing backbone linkages (PN linkages) to oligonucleotides acting through an RNase H-mediated mechanism, using *Malat1* and *C9orf72* as benchmarks. We found that the incorporation of various types of PN-based moieties to the oligonucleotide backbone can increase potency *in vitro* under free-uptake conditions in cultured neurons ~10-fold compared with comparably modified stereopure phosphorothioate (PS) and phosphodiester (PO)-based molecules. One of these backbone types, called PN-1, also yielded profound pharmacological and activity benefits *in vivo* throughout the mouse CNS. These benefits include increased tissue exposure for the oligonucleotides, especially in deeper brain regions, and both an increase in percentage of mRNA knockdown observed and the durability of the knockdown. Given these benefits in preclinical models, the incorporation of PN linkages into stereopure oligonucleotides with chimeric backbone modifications has the potential to render regions of the brain beyond the spinal cord

more accessible to oligonucleotides and, consequently, may also expand the scope of neurological indications amenable to oligonucleotide therapeutics.

217. Treating “N-of-Few” Genetic Conditions with Antisense Oligonucleotides (ASOs): Current State and Ethical Challenges

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In 2018, the first individualized antisense oligonucleotide (ASO) therapeutic was created for a 6-year-old girl named Mila, who had a previously unknown variant of Batten Disease. The ASO, called Milasen, was developed in just over one year and effectively reduced some of the symptoms of Mila's condition. Although Mila passed away a few years later, her unique treatment experience marked the beginning of a paradigm shift in rare disease treatment. There are countless others like Mila who face devastating diagnoses with no treatment options and no market incentive for such to be developed, due to their ultra-rare, “n-of-1” or “n-of-few,” incidence. Individualized ASOs offer hope to these patients, however they bring with them a host of ethical challenges, some familiar and some novel. As these are innovative treatments, blurring the historically distinct line between treatment and research, these agents raise regulatory, research oversight, and clinical use questions. In addition, individualized ASOs are currently very expensive and resource intensive to develop, raising concerns about justice, both for the patients and families who desire such individualized treatments, as well as for healthcare systems which must now grapple with the projected costs and benefits of individualized treatments in the context of other demands. Indeed, at present, most individualized therapeutics are being funded by philanthropic efforts in lieu of industry or governmental funding. Other ethical considerations include evaluating the risk/benefit profile and actualizing informed consent for the use of novel interventions with minimal preclinical evidence and in desperate situations lacking any other therapeutic options. Finally, we touch on the question of how and to what degree should “n-of-few” interventions prioritize evidence generation and sharing of such findings. Recognizing the promise of individualized ASOs, this project aims to identify the relevant ethical concerns, foresee potential pitfalls, and help to guide this promising area of inquiry.

218. Development of Synthetic Stem-Loop RNA (sl-RNA) Fragment Derived from Sendai Virus Genome for Inducing Antitumor Immunities Combined with a Pyro-Drive Jet Injector

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In this study, we developed synthetic stem-loop (sl-) RNA fragment which induces strong antitumor immunities. The sl-RNA sequence is derived from Sendai virus DI (defective interfering) particle genome. Previous studies showed that inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) particles called HVJ-envelope

(HVJ-E) have multiple-anti-cancer activities. One of the activities is activation of anticancer immunities through inactivation of Treg (regulatory T cell), promotion of NK cells activation and generation of CTL against cancers. Another is the cancer selective apoptosis by induction of proapoptotic genes such as TRAIL and Noxa in various human cancer cells but not in normal cells. Most of these anti-cancer activities are conducted by RNA genome fragments of HVJ-E through RIG-I/MAVS (retinoic-acid inducible gene-I, mitochondrial antiviral signaling protein) signal pathway. Among various Sendai virus strains, Sendai virus Cantell strain showed the highest production of IFN- β in dendritic cells (DCs) and the strongest DC maturation and we found that DI particles of Cantell strain resulted in those immunostimulatory activities. The Cantell strain HVJ DI RNA has complementary termini (approximately 100nt) and exhibits the highest binding affinity to RIG-I. The DI RNA induces higher expression level of those apoptosis-related proteins such as Noxa and TRAIL and more cancer cell death than whole-genome RNA (approximately 15 kb) of complete Cantell strain without DI particles. Moreover, sin-sl-RNA (114nt) and sl-RNA-57 (57nt) have been developed as a third generation of synthetic RNA fragment which originated in Sendai virus DI particle genome. Sin-sl-RNA is a single fragment which has complementary base sequence (25nt) at both ends of fragment. The complementary base sequence helps to form double strand stem and single strand loop structure. In other words, sin-sl-RNA can form stem-loop structure by a single RNA fragment. On the other hand, sl-RNA-57 (57nt) is a half size of the sin-sl-RNA. Two sl-RNA-57 fragments are needed to be annealed to form the stem-loop structure. The sin-sl-RNA and sl-RNA-57 were injected to B16F10 (mouse melanoma) tumor by pyro-drive jet injector (PJI) three times. The PJI is a novel injector system capable of injection depth adjustment. The tumor growth was strongly suppressed in sin-sl-RNA and sl-RNA-57 injected group. Chemokine and cytokine array revealed that MCP-2, IP-10, RANTES and MIP-2 secretions were increased in the B16F10 tumor tissues after sl-RNA-57 injections. Additionally, macrophage infiltrations and macrophage polarization to M1 (antitumorogenic) were observed in the sl-RNA-57 injected B16F10 tumor sections by F4/80 and NOS-2 immunostaining. Originally HVJ-E was recognized as the main antitumor immunity inducing factor. Further investigations revealed that the core of antitumor immunity induction part might be the sl-RNA fragment derived from Sendai virus genome. These findings provide a novel nucleic acid medicine for the cancer treatment.

219. A Large-Scale Saturation Mutagenesis Screen Identifies Design Principles of siRNA Selectivity

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Most RNAi studies assume a perfect match between the target sequence and the guide strand. However, in reality, the guide tends to tolerate certain mismatches, resulting in significant hindrances to therapeutic development due to off-target effects, altered activity and poor discriminatory capacity to support allele-specific targeting (e.g., as is required for Huntington disease). On the flip side, we can harness this

phenomenon to our advantage by tuning the level of selectivity to a group of variants (isoforms). For instance, when designing an antiviral siRNA, a key property is the ability to neutralize a broad spectrum of strains by overcoming common genetic polymorphisms. We therefore wish to understand the effect of mismatches on RNAi silencing activity. Previous studies that tried to address this question mostly focused on a few specific siRNAs or generated a small number of data points, severely limiting the generalizability of their findings. Hence, the factors that shape siRNA selectivity, specificity, and potency are not well understood. Here, we harnessed a massively parallel siRNA screening platform to infer the design principles governing siRNA selectivity, specificity and potency, generating and analyzing tens of thousands of data points per experiment. Our platform uses an innovative synthetic biology approach, consisting of oligonucleotide sensors that harbor both an siRNA guide and a 50nt target site fused to a reporter gene. We evaluate siRNA efficacy by sorting the cells based on the quantitative signal of the reporter gene expression and then performing high-throughput sequencing. We used our platform to unravel the design principles of mismatch tolerance. First, to study the factors affecting siRNA specificity, we selected four highly potent siRNAs and performed an exhaustive saturation mutagenesis screen. To this end, we paired each of the four siRNAs against each and every mismatched target site harboring one or two SNPs at its 22nt target sequence for a total of 8,282 data points. Second, to study the factors affecting the interplay between siRNA selectivity and potency, we selected 2,060 siRNAs with potential clinical relevance. We synthesized multiple sensors for each siRNA, where each sensor paired an siRNA against either a perfectly matched target site or a mismatched target site harboring one or two SNPs at the 22nt target site, for a total of 29,052 data points. Third, to study how RNA conformation affects siRNA potency, we selected 318 siRNAs characterized in our lab for their activity profile and evaluated their sensitivity to SNPs that alter their target RNA conformation. To this end, for each siRNA we designed two sensors with the same guide and the same 22nt target site, but with different sequences flanking their target site. We used these datasets to train a machine learning model for predicting siRNA potency, selectivity and specificity. Our model takes as input the sequences of an siRNA guide and of the target RNA (including the 50nt region that flanks the target site), extracts relevant features such as thermodynamic stability and minimum free energy, and predicts silencing efficacy. Using this model, we were able to design siRNAs that are either highly tolerant or highly intolerant to specific types of mismatches, allowing us to modulate siRNA mismatch tolerance according to the desired clinical outcomes. We used our model to identify hyper-potent siRNAs that can target specific alleles while leaving alternative alleles unperturbed. In conclusion, our work infers the principles underlying siRNA selectivity and potency. These principles can guide the design of hyper potent allele-specific siRNAs as desperately needed therapeutics for dominant gain-of-function disorders.

220. Multimodal Immunotherapy for Malignant Glioma Using TLR9-Targeted STAT3 CpG-Oligodeoxynucleotides Based Immunotherapy Reprogramming the Glioma Microenvironment

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Malignant gliomas are rapidly fatal despite multimodal treatments including cellular immunotherapies. Therapeutic effects of radiation therapy (RT), used to treat nearly all MG patients, are only modest and short-lived. Glioma resistance to RT results from both intrinsic cancer cell properties and protective influences of the tumor microenvironment. We previously demonstrated that the release of danger signals from irradiated cells recruits Toll-like Receptor-9 (TLR9)⁺ myeloid cells with active STAT3 signaling which drive tumor vascularization and regrowth. Here, we describe development of TLR9-targeted STAT3 antisense oligonucleotide (CpG-STAT3ASO) with optimized stability and efficacy for glioma radio-immunotherapy. To augment the STAT3 knockdown in glioma cells, we utilized locked-nucleic acid (LNA) chemistry within ASO part of the molecule generating single-stranded and double-stranded CpG-STAT3ASO conjugates. All chemically-modified and fluorescently labeled CpG-STAT3ASO^{Cy3} variants were rapidly internalized by human and mouse glioma cells and myeloid cells *in vitro*. Compared to our benchmark 2'-O-methyl-modified oligonucleotides, the LNA-modified CpG-STAT3ASO^{LNA} 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 double-stranded and to lesser extent single-stranded CpG-STAT3ASO (1 mg/kg/q2w) inhibited tumor growth and extended survival of immunodeficient NSG mice. Since the combination of CpG-STAT3ASO with focal glioma irradiation did not significantly enhance the overall effect in NSG mice, we next tested CpG-STAT3ASO variants against syngeneic GL261 model in immunocompetent mice. Our results demonstrated that both single and double-stranded CpG-STAT3ASO^{LNA} (1 mg/kg) synergized with radiotherapy (15 Gy), however, repeated treatments with double-stranded variant proved to be better tolerated. To reveal changes in the glioma microenvironment after STAT3-inhibition/TLR9-activation, we analyzed transcriptomic changes in mouse glioma-associated CD11b⁺ myeloid cells from mice with inducible STAT3 deletion (Mx1^{Cre}/Stat3^{fllox/fllox}) treated using intracranial CpG ODN injection. Global transcriptome analysis using RNAseq indicated prominent IFN gene signature triggered by the combined treatment but not by STAT3 deletion or TLR9 triggering alone. These results were consistent with the maturation and activation of antigen-presenting cells, such as DCs, microglia and macrophages together with increased T cell infiltration into glioma, after the intracranial injections of CpG-STAT3ASO^{LNA} in immunocompetent mice. We believe that our results will pave way to clinical translation of CpG-STAT3ASO for radio-immunotherapy of malignant glioma.

221. Improved Immune Cell Expression with Circular RNA (oRNA) *In Vivo*

Kevin Kauffman, Junghoon Yang, Zifiso Nyoni, Emily Fitzgerald, Fraser Leslie, Meaghan Richardson, Wuliang Zhang, Tammy Tai, Dylan Hoots, Kristen Ott, Trent Stevens, Yessica Wiryawan, Corey Ciullo, Ian Langer, Rahul Vungutur, Ashley Wong, Akinola Emmanuel, Thomas Lee, David Soto, Akshi Thakkar, Magnolia Chinn, Ramya Elangovan, Renee Wright-Michaud, Nelson Chau, R. Alexander Wesselhoeft, Amy Becker, Allen Horhota, Thomas Barnes

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Approvals of *ex vivo* CAR-T immunotherapies like Yescarta, Kymriah, and Breyanzi illustrate the extraordinary promise in harnessing patients' own immune systems to treat certain cancers. However, *ex vivo* CAR-T therapies are challenged with preconditioning-associated toxicity, as well as complex, costly, and poorly scalable manufacturing processes tailored for each patient. Off-the-shelf protein-coding RNA therapeutics capable of *in situ* CAR expression offer an attractive alternative to *ex vivo* therapies, but they have been limited by effective delivery of RNA to immune cells and the inability to achieve meaningful protein expression from mRNA. To overcome these barriers, we are developing two key proprietary technologies: 1) protein-coding circular RNA (oRNATM), which results in enhanced protein expression compared to commercially-available modified linear mRNAs, and 2) effective delivery solutions based on proprietary lipid nanoparticles (LNPs) which are capable of transfecting immune cells in mice, rats, and NHPs *in vivo* as well as primary human cells *in vitro*. oRNA can be readily formulated into a variety of LNPs with suitable sizing, polydispersity, encapsulation efficiency, and RNA stability. Moreover, oRNA-LNPs demonstrate robust *in vitro* transfection of immortalized human T cell lines and primary human T cells at least as efficiently as mRNA-LNPs. Interestingly, when administered intravenously to mice, we discovered that LNPs containing luciferase-encoding oRNA have relatively higher expression in the immune cell-rich spleen than LNPs containing conventional modified mRNA. Furthermore, oRNA-LNPs result in significantly higher protein expression in splenic effector immune cell subsets relevant to CAR therapies (including T cells) compared to mRNA-LNPs. Together, these data suggest that our novel oRNA and LNP technologies favorably combine to maximize immune cell expression *in vivo* and support our plans to bring *in situ* CAR therapies to the clinic.

222. Self-Delivering RNAi Targeting BRD4 (PH-894) Improves the Phenotype of HER2-CAR-T Cells During Expansion

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Chimeric antigen receptor (CAR) T cell therapies can elicit remarkable responses toward treating hematological malignancies. However, the full therapeutic potential of CAR-T in treating solid tumors remains unrealized. Strategies under investigation to improve CAR-T

therapeutic efficacy include *ex vivo* intervention during manufacture to improve the quality and potency of the CAR-T product. The activation and expansion phases of a typical CAR-T manufacturing process with anti-CD3/CD28 antibody coated beads or similar are known to induce exhaustion and differentiation phenotypes, which have a negative impact on the potency of the final therapeutic product. Gene therapy approaches have shown promise in this area; however, obstacles remain including safety considerations deriving from the permanent deletion of T cell suppressive mechanisms, potential off-target impacts of gene editing the cell product, and technical challenges of low editing efficiencies and process integration. The INTASYLTM platform is a self-delivering RNAi technology that imparts small molecule-like properties to siRNAs, providing efficient delivery to target cells without need for specialized formulations or drug delivery systems, allowing seamless incorporation into CAR-T manufacturing protocols. INTASYL provides robust and highly specific on-target gene silencing. While durable, the effects of INTASYL-mediated silencing are transient, potentially mitigating safety considerations arising from permanent deletion of T cell suppressor mechanisms. Here we assessed the potential of the BRD4-targeted INTASYL PH-894 to improve the quality and potency of HER2-targeted CAR-T cells (HER2CART) in a 19-day model of CAR-T expansion. HER2CART cells were activated with plate-bound OKT3 antibody (10 ng) and anti-CD3/CD28 beads on Day 0 and Day 6 respectively and treated with PH-894 (2 μ M), a non-targeting control INTASYL (NTC; 2 μ M), or vehicle (PBS) on Day 0 and Day 5. PH-894 reduced the expansion-associated induction of BRD4 and BRD4-regulated MYC. Additionally, PH-894 mitigated the induction of inhibitory receptors and markers of T cell exhaustion, TIGIT and TIM3, on CD8⁺ HER2CART cells and secretion of expansion-induced immunosuppressive IL-10. PH-894 elicited durable repression of PD-1 on CD4⁺ HER2CART cells (through Day 19). Finally, PH-894 preserved putative T cell stem-cell memory (T_{scm} defined by CCR7⁺CD62L⁺CD95⁺CD45RA⁺) and central memory (T_{cm} defined by CCR7⁺CD62L⁺CD95⁺CD45RA⁻) CD8⁺ phenotypes associated with cell persistence, on HER2CART populations at Day 19 that were otherwise depleted by cell expansion without PH-894 use. These data provide proof-of-concept to suggest that silencing BRD4 with INTASYL PH-894 can be used to improve the phenotype of CAR-T during activation and expansion phases of their manufacturing process by reducing exhaustion, immunosuppression, and conferring a phenotype associated with cell persistence, thus serving to improve the quality of a final CAR-T cell product. Follow-up studies are ongoing on the use of PH-894 in T cell adoptive cell therapy.

223. Development of Conjugated siRNAs for Allele-Specific Silencing of a Dominant *Col6a3* Pathogenic Variant in Mouse Cultured Cells

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Collagen VI-related dystrophies (COL6-RD) are a spectrum of early onset congenital muscular dystrophies. *COL6A1*, *COL6A2* and

COL6A3 genes encode collagen $\alpha 1$, $\alpha 2$ and $\alpha 3$ (VI) chains of collagen VI. Dominant negatively acting pathogenic variants in those genes produce chains capable of incorporating into the hierarchical assembly of collagen VI and as a consequence, produce a dysfunctional collagen VI extracellular matrix. Inactivation of such dominantly acting alleles in the *COL6* genes is a valid strategy as haploinsufficiency is clinically tolerated. A common class of variants associated with severe COL6-RD results in dominant in-frame skipping of exon 16 in the *COL6A3* gene. Oligonucleotides, specifically small interfering RNAs (siRNAs), offer a promising avenue as molecular-based therapies to treat COL6-RD by effecting allele specific knockdown. Collagen VI in muscle is produced by interstitial fibroblastic cells, which need to be effectively targeted for this approach to work. It has been shown that maximal bioavailability of siRNAs can be achieved through chemical modifications and lipid conjugation of the siRNA molecule. Using cultured fibroblasts from a *COL6A3* Δ 16 mouse model carrying a heterozygous deletion of *Col6a3* exon 16, we tested eleven siRNAs modified with 2'-O-methyl, phosphorothioate linkages, and a 3' cholesterol tag for their efficacy and allele silencing specificity. We used reverse transcription PCR and digital PCR and found that two of the eleven siRNAs tested were most effective at knocking down the pathogenic *Col6a3* Δ 16 transcripts, while preserving expression from the normal allele. Next, we performed a dose-response experiment to determine the optimal siRNA concentration for maximal efficiency while maintaining allele specificity. Additional functional assays are ongoing. This study provides insight into the molecular therapeutic potential for RNA interference in the treatment of dominant collagen VI related dystrophies.

224. Identification of Cardiac-Specific Aptamer Ligands Using a Novel Whole-Organ SELEX Process with a Langendorff Heart

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Cardiovascular disease remains the number one cause of death in the US with the expectation that this situation will worsen over the next decade. Exacerbating this problem, is the steady decline in the number of cardiovascular drugs entering the clinical pipeline over the past twenty years. Furthermore, only 10% of FDA approved drugs are for treatment of cardiovascular disease and only a third of these cardiovascular drugs target a novel mechanism. To address this problem, we applied aptamer technology to the clinically relevant problem of generating cardiac-specific ligands. We hypothesized that the SELEX process would provide an "unbiased" approach to generate novel cardiac-specific aptamer ligands with the potential to lead to the discovery of new mechanistically based cardiovascular therapeutics. We performed a first-of-its-kind *ex vivo* organ-based aptamer selection using a Langendorff heart preparation, a process we term herein "Whole-Organ SELEX." By using a Langendorff heart preparation with intact murine hearts, cardiac-specific aptamers can be identified that bind ventricular cardiomyocytes within their native environment where cell-cell contact are preserved. Negative and positive selection pressure was applied during Whole-Organ SELEX by dissociating and separating the ventricular cardiomyocytes from all other cell types found within the heart (e.g. vascular cells, fibroblasts). We conducted nine rounds of Whole-Organ SELEX, alternating between

male and female hearts to avoid sex bias, and used at least two hearts per selection round to mitigate biological bias of a single heart. High-throughput sequencing and aptamer bioinformatics analysis revealed 77 unique sequence clusters of potential cardiac-specific aptamers. We perfused mouse hearts by Langendorff heart preparation with fluorescently labeled (Alexa Fluoro 647) cardiac aptamer or control aptamer. From these experiments, we observed significantly more fluorescence with the mouse hearts perfused with the cardiac aptamers as compared to control. A z-stack 3D reconstruction confirmed these results by showing fluorescence of the cardiac aptamer throughout the ventricular cardiomyocytes (Fig. 1). These results were repeated with shorter perfusion times and lower aptamer concentrations. To validate the specificity of the cardiac aptamer, we treated primary mouse aortic vascular smooth muscle cells (VSMCs) and vascular endothelial cells with the cardiac aptamer or the control aptamer. No significant difference or degree of fluorescence was observed between the cardiac aptamer or the control aptamer with the mouse aortic VSMCs or endothelial cells. These data collectively suggest that our innovative Whole-Organ SELEX process using a Langendorff heart preparation successfully generates cardiac specific aptamers. Since aptamers have utility as a therapeutic modality, a delivery tool, an imaging agent, and as a diagnostic; generation of cardiac specific aptamer ligands provides multiple new unexplored avenues for cardiovascular research and drug development. Furthermore, our innovative and novel Whole-Organ SELEX process using the Langendorff heart can be applied to models of heart failure and cardiac disease (e.g., dilated, hypertrophic, diabetic) to generate a diverse toolbox of cardiac specific aptamer ligands that target both normal healthy hearts and diseased hearts.

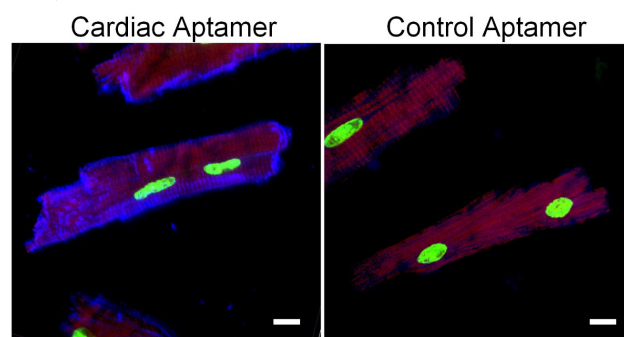


Fig. 1: Cardiac aptamer specificity for ventricular cardiomyocytes. **Aptamer**, nucleus (**Sytox Green**) and cytoplasm (**AF568-Ph**). Scale = 10 μ m

225. Antisense Treatment Improves Sarcolemma Repair in a Mouse Model of Facioscapulohumeral Muscular Dystrophy

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Background: Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common inherited muscular dystrophies with an incidence of 1:8,000 to 1:20,000. Studies showed that FSHD is caused by aberrant expression of double homeobox 4 (DUX4) due to epigenetic changes of the D4Z4 macrosatellite repeat region at chromosome

4q35. We have developed antisense oligonucleotides (AONs) targeting DUX4, which reduces DUX4 in FSHD models. Our recent study identified sarcolemma repair deficits in human myoblasts affected by FSHD. We hypothesize that reduction of DUX4 will correct the cellular deficits observed in the FSHD cells. **Objective:** To reduce the pathogenic DUX4 mRNA in affected muscles using the AONs and determine whether the treatment improves membrane repair deficits in muscles of the FLExDUX4 mouse model which expresses human DUX4. **Approach:** 2'-MOE gapmers were delivered by subcutaneous injections (s.c.) to the FLExDUX4 mice (n=6). FLExDUX4 mice without treatment (PBS only) and wildtype littermates receiving PBS were used as control. The reduction of DUX4 transcripts was determined by qRT-PCR. Sarcolemma repair capacity was evaluated by a laser injury assay. Change in fluorescence intensity of FM-1-43 dye entry ($\Delta F/F$) into the myofiber after laser injury were measured and used to determine the repair capacity. **Results:** The data show that membrane repair capacity is reduced as expected in the myofibers of the FLExDUX4 mice, which expresses DUX4 at a very low level. The treatment of 2'MOE antisense oligonucleotides (20mg/kg s.c.) improved sarcolemma repair of myofibers of the FLExDUX4 mice, which received the 2'MOE gapmers. The treatment also reduced the number of myofibers that were not able to repair and recover from the laser injury. **Conclusion:** Routine and repetitive sarcolemmal injury from daily muscle contraction can potentially be a central contributor to muscle pathology and FSHD disease progression, if these injuries cannot be effectively repaired. Our findings showed that the DUX4 expressing muscles exhibit sarcolemma repair deficits and the treatment of the 2'MOE antisense oligonucleotides improves the sarcolemma repair capacity after laser injury.

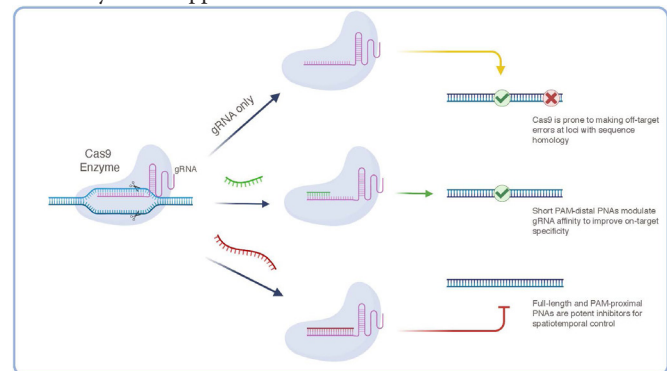
226. Antisense Peptide Nucleic Acids for Sequence-Specific CRISPR-CAS9 Modulation

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Despite the rapid and broad implementation of CRISPR-Cas9-based technologies, convenient tools to modulate dose, timing, and precision remain limited. While multiple technologies have been put forward to address these needs including anti-CRISPR protein and small molecule inhibitors, inducible Cas9 fusions, gRNA structural variants, and off-target shields, they feature notable drawbacks such as sequence and design restriction, modest potency, challenges of protein delivery or expression, and restricted functionalities. Overcoming many of these challenges and building on methods using synthetic peptide nucleic acids (PNAs) to bind RNA with unusually high affinity, we describe a novel class of antisense guide RNA (gRNA) spacer-targeted, or "antisense", PNAs as a tool to modulate Cas9 binding and activity in cells in a sequence-specific manner. In this study, we designed, chemically synthesized, and tested a series of PNA oligomers and demonstrate their use as powerful modifiers of Cas9 activity via sequence-specific antisense gRNA binding. We employ diverse methods *in vitro* and in three human cell lines to describe the effects of 60 rationally designed PNAs across 10 gRNA targets. We demonstrate that PNAs rapidly and efficiently target complexed gRNA spacer sequences at low stoichiometric doses and

without design restriction for sequence-selective Cas9 inhibition. We further show that short PAM-proximal antisense PNAs achieve potent cleavage inhibition at low doses (over 2,000-fold reduction), outperforming previously published anti-CRISPR approaches, and that PAM-distal PNAs modify gRNA affinity to improve on-target specificity. We go on to generate *in silico* and empiric models to describe the latter phenomenon and predict the effects of PNA binding on Cas9 affinity for a given target. Finally, we apply antisense PNAs for temporal manipulation of two dCas9-fusion systems. Broadly, these results present a novel rational approach to nucleoprotein engineering and describe a rapidly implementable antisense platform for CRISPR-Cas9 modulation to improve spatiotemporal versatility and safety across applications.



227. Short Hairpin RNA-Expressing Oncolytic Adenovirus-Mediated HER3 Inhibition Enhances Tumor Growth Inhibition and Apoptosis in Breast Cancer

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RNA interference has promising therapeutic potential due to its sequence-specific silencing of target gene expression. However, the absence of an efficient delivery system for siRNA is a major hurdle to its clinical application. Oncolytic viruses are attractive as a delivery platform of nucleic acid or protein-based therapeutics to the target sites, particularly tumors. Here, we applied adenovirus as carrier to deliver short hairpin RNA (shRNA) capable of silencing HER3, one of the members of the EGFR family. We demonstrated that both replication-incompetent and replication-competent adenovirus-mediated shRNA against HER3 (shHER3) delivery could induce the effective downregulation of HER3, resulting from inhibition of cell proliferation and enhancement of apoptosis *in vitro* as well as *in vivo*. Furthermore, the oncolytic adenovirus-mediated shHER3 delivery induces HER3 down-regulation along with suppression of HER2 by the E1A gene of replication-competent adenovirus, thereby inducing potent antitumor efficacy compared to replication-incompetent adenovirus. These findings suggest shHER3-expressing oncolytic Ad could induce combined efficacy in treatment targeting EGFR family.

228. Combined Overexpression of hATXN1L and Mutant ATXN1 Knockdown Rescues Motor Phenotypes and Disease-Related Gene Expression Changes in B05 Mice

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Spinocerebellar ataxia type I (SCA1) is an autosomal dominant neurodegenerative disease caused by a (CAG) expansion within the coding sequence of the *ATXN1* gene. The primary mechanism of disease in SCA1 is through toxic gain-of-function by polyglutamine-expanded protein and is compounded by concomitant loss of normal ATXN1 protein function. In prior work, we demonstrated the efficacy of virally expressed RNA interference (RNAi) molecules targeting *ATXN1* to prevent and reverse disease phenotypes in SCA1 mice. Furthermore, we showed that overexpression of the ATXN1 homolog, ATXN1-like (ATXN1L) improves disease readouts when delivered pre-symptomatically. Here we developed two dual expression transgenes, combining our ATXN1-targeting miRNA (miS1) with hATXN1L mRNA in single adeno-associated viruses (AAV), to measure their ability to reverse disease in B05 mice as assessed by behavioral and next generation sequencing assays. Mice treated with control vectors expressing human ATXN1L (hATXN1L) alone showed motor improvements and changes in gene expression that reflected increases in pro-development pathways and a decrease in angiogenic gene expression. When combined with miS1, motor improvements were complemented by robust normalization of disease allele-induced changes in gene expression. The observed additive effect by overexpression of ATXN1L with knockdown of mutant ATXN1 support further development of our dual expression transgenes for a more effective SCA1 therapy.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases I

229. Novel Approach to Elucidate the Biological Mechanism of Gene Therapies for Lysosomal Diseases Using Quantitative Pharmacology

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Background: There is a lack of analytical methods that can quantify the therapeutic efficacy of gene therapy over time in pre-clinical studies. According to the Food and Drug Administration, "the preclinical data generated for [cell and transgene therapy (CGT)] products may

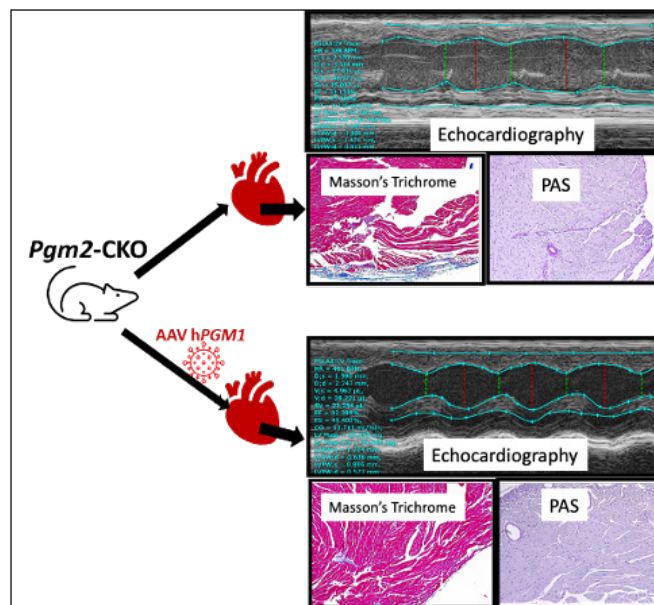
not always be as informative as for small molecule pharmaceuticals, particularly since it usually is not feasible to conduct traditional preclinical pharmacokinetic (PK) studies with CGT products" [Guidance on Considerations for the Design of Early-Phase Clinical Trials of CGT Products]. Adapting traditional pharmacokinetics for gene therapy would advance the translatability of preclinical studies. Such an approach could elucidate the biological mechanism of gene therapy with the rigorous, reproducible quantitative analysis needed in the drug approval process. **Objective:** The objective of this study was to investigate the mechanism of gene therapy encoding a novel fusion enzyme, iduronidase-LT, by reconciling quantitative pharmacokinetic methods with the biological process. In the lysosomal disease, mucopolysaccharidosis type I (MPS I), also called Hurler syndrome, homozygous *IDUA* mutations result in systemic iduronidase deficiency and life-threatening multi-organ manifestations, including cardiac valve disorders. Normal human iduronidase enzyme was compared to an experimental variant, iduronidase-LT, an iduronidase terminally modified with a 6-mer peptide. **Methods:** Seventy-one MPS I mice were hydrodynamically injected with a plasmid containing the liver-specific promoter, hAAT HCR-ApoE, and either iduronidase-LT or unmodified iduronidase. Catalytic activity was determined in the plasma and liver at timepoints ranging from 0.5 to 168 hours. Activity in other tissues was also measured, and physical levels of iduronidase in the liver were measured using ELISA. For the pharmacokinetic analysis, enzymatic activity-time in the liver and plasma were analyzed by non-compartmental methods (WinNonLin Phoenix) to measure area under the curve, $t_{1/2}$ and C_{max} using the linear trapezoidal rule. Pharmacokinetic parameters were recontextualized for gene therapy. **Results:** In the plasma, iduronidase-LT had a significantly two-fold higher area under the curve (AUC) than iduronidase ($p=0.003$). In traditional pharmacokinetics, the AUC represents the total drug exposure over time. Therefore, one interpretation is that the plasma is being exposed to 100% higher enzyme activity from iduronidase-LT than iduronidase. Moreover, iduronidase-LT had a significantly higher activity level than iduronidase in the heart ($p=0.02$). The iduronidase-LT's higher activity levels in the plasma and heart were not caused by a higher transgene expression in the liver. In the liver, both enzymes had similar physical levels over time. Both enzymes also had similar activity levels in the liver over time assessed by comparing their AUCs ($p=0.64$). Pharmacokinetic approaches were utilized to support or refute other potential mechanisms of iduronidase-LT. For example, a difference in the enzymes' distribution between the site of transgene expression, plasma, or the desired site of action. **Conclusion:** A novel method of elucidating the biological mechanism of gene therapy was developed using quantitative pharmacological methods. This approach does not require the use of multiple timepoints in tissues, preserving the time and cost needed for studies. Moreover, this approach could be readily applied to other gene therapies to deduce mechanisms, assess therapeutic potential, and address barriers such as dosing feasibility.

230. Pioneering AAV Gene Therapy for Congenital Disorders of Glycosylation (CDG): Multi-Faceted Correction of Disease Progression in a Mouse Model of PGM1-CDG

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Only 5% of patients in the rapidly growing group of more than 150 types of Congenital Disorders of Glycosylation (CDG) have a therapy. For patients with Phosphoglucomutase 1 deficiency, also known as PGM1-CDG, oral galactose therapy has shown some efficacy in the treatment of liver, coagulation, and endocrine disease, but it has no effect on the lethal dilated cardiomyopathy (DCM) and myopathy where complex metabolic background, including abnormal glycogen metabolism, may play a role in the pathophysiology. We recently constructed a cardiomyocyte-specific conditional *Pgm2* (mouse equivalent of human *PGM1*)-KO mouse model that recapitulates the DCM phenotype at the molecular, biochemical, histological, and functional levels. With this novel and critical research tool, we evaluated different experimental modalities with the ultimate goal to address the unmet medical needs for the patients. As human *PGM1* cDNA is ~1.4kb in length and almost all known patients have deleterious missense mutations, we launched a pilot study to examine the efficacy of AAV9-mediated *PGM1* gene replacement therapy in correcting cardiac dysfunctions in our mouse model. 4-week-old mice were injected with a single IV dose of 5×10^{11} AAV9 *hPGM1* vector genome or control. Two weeks after injection, cardiac functions were continuously monitored over a period of 5 months after the injections and before the mice were sacrificed for structural and biochemical evaluation. AAV9-mediated *PGM1* gene replacement prevented decline in cardiac functions and delayed the progression of the disease. Particularly, AAV9 *hPGM1* treated mice showed a normal distribution of PGM1 in heart, normalized the glycogen accumulation and significantly reduced fibrosis. The promising *in vivo* POC data warrant further optimization and safety studies, and support an AAV gene replacement therapy modality for patients with PGM1.



231. Improved Efficacy of Glucose Production in Later Age-Treated Gene Therapy for Murine Model of Glycogen Storage Disease Typela

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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 with metabolic disturbances including excessive accumulation of glycogen and triglycerides in the liver. Recently, a recombinant adeno-associated virus (rAAV)-mediated gene therapy had demonstrated its ability to restore hepatic G6Pase- α and 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. However, the long-term persistence of the gene therapy still needs more investigation since it is directly correlated to the efficacy of the gene therapy. In a previous study, it had been shown that the transduced rAAV vectors are gradually diluted out or particularly lost in the livers of young animals due to high rate of hepatocyte proliferation associated with liver growth. This result had been applied to the gene therapy targeting GSD-Ia mice. Additionally, we have found that GSD-Ia mice have a later onset of growth and do not reach their full bodyweight until approximately 9-10 weeks of age compared to control group. Therefore, in this study, given the liver growth rates have close correlation to body weight, we investigate the relationship between gene therapy efficacy and GSD-Ia specific age. **Methods:** The advantage of using GSD-Ia mice in gene therapy study is easy outcome measure by blood glucose level after the treatment. We first examined the efficacy of a rAAV8 vector expressing G6PC when administered in young (3-4 weeks) mice versus adult (12 weeks) mice by serial blood glucose level measurements. The efficacy of the

gene therapy in these conditions were finally evaluated after 12 weeks post gene therapy with biochemical analysis of the collected tissues.

Results: To overcome the extremely low survival rate of GSD-Ia mice prior to gene therapy, we set up a protocol to supplement the mice's water with 5% glucose and easy access to soft food at the bottom of the cage. This protocol greatly improved the survival rate of GSD-Ia mice and we were able to obtain later- aged GSD-Ia mice (over 10-12 weeks) for this study. We infused the mice with the rAAV8 vector at either 3-4 weeks or 12 weeks of age. Here we show that at 2 weeks after administering gene therapy, both groups show similar fasting glucose levels. However, at 6 weeks after gene therapy, the 12-week treated mice showed normoglycemia through the 24 hours of fasting period, whereas, the 3-week treated group had lower glucose levels.

Conclusion: Our results show that administering gene therapy in 3-4 week young mice lowers the efficacy of the treatment with aging, due to a high rate of hepatocyte proliferation and continued liver growth in the GSD-Ia mouse model. Growth rate guided determination of the gene therapy-favorable age enabled persistence of gene therapy efficacy against age in GSD-Ia mice. This study can provide a valuable insight for individual liver-targeting gene therapies.

232. Correction of Phenylketonuria by Drug-Mediated Expansion of Transplanted Hepatocytes In Vivo

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Hepatocyte transplantation is a promising approach for the treatment of genetic liver disorders. Hepatocytes delivered into the portal circulation can engraft in the liver and support hepatic function. However, typical engraftment rates are low and achieving a therapeutic cell replacement threshold is challenging. This problem could be overcome if it were possible to selectively expand the engrafted cells, as hepatocytes have the ability to proliferate in vivo. We have previously described a system to selectively expand gene-edited hepatocytes in vivo by targeting cytochrome P450 reductase (*Cypor*). *Cypor* is required for the metabolism of the drug acetaminophen (APAP) to its hepatotoxic byproduct. Therefore, hepatocytes deficient in *Cypor* are resistant to the hepatotoxic effects of APAP. Administration of high doses of APAP can expand a small population (< 1%) of *Cypor*-deficient hepatocytes to up to 40% of the liver. Here, we report the use of APAP-mediated expansion of transplanted hepatocytes to treat a mouse model of phenylketonuria (PKU). PKU is an inborn error of metabolism caused by a deficiency in the phenylalanine hydroxylase (*PAH*) gene, which is normally expressed in hepatocytes. The result is an inability to metabolize the amino acid phenylalanine. Hepatocyte transplantation has the potential to correct phenylketonuria, but complete correction by hepatocyte transplantation has not been reported. Wildtype mouse hepatocytes were treated in vitro with spCas9 ribonucleoprotein complexes to create a knockout of *Cypor* and then transplanted into *PAH*-deficient mice. Mice were subsequently treated with our established APAP selection protocols. Prior to APAP treatment, blood phenylalanine levels were > 1500 μ M, well above the healthy range. Blood phenylalanine levels in the APAP-treated mice dropped to within the physiological range, as low as 150 μ M. Upon

harvest, ~16% of hepatocytes contained insertion/deletion mutations in *Cypor*, corresponding to the *PAH*-expressing transplanted hepatocytes. Clonal expansion of *Cypor*-deficient hepatocytes was observed in the APAP-treated mice, and was not seen in transplanted animals that did not receive APAP. Together, these data indicate that selective expansion of transplanted *Cypor*-deficient hepatocytes is a viable therapeutic strategy to correct phenylketonuria.

233. AAVKP1 Shows a Peculiar Biological Phenotype Effective for In Vivo Localized Gene Delivery While Detargeting the Liver

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Local administration of adeno-associated virus (AAV) has the potential to enhance target organ transduction with a smaller vector dose while avoiding off-target gene delivery. This is critically important when AAV vector infection itself or subsequent transgene expression in non-target organs is detrimental and leads to toxic effects. It was once thought that local injection approaches, such as intramuscular or intrathecal injections confine AAV vector particles within the biological space where the vector is injected, therefore avoiding vector genome dissemination to remote organs. However, it is now widely appreciated that robust AAV capsid vectors such as those derived from AAV9 can efficiently transduce the liver and other organs following local vector delivery due to the inadvertent leakage into the systemic circulation. Here, we report that AAVKP1, a novel AAV capsid identified by directed evolution in human pancreatic islets in vitro, has a peculiar biological phenotype that allows effective hepatic gene delivery when injected systemically while detargeting the liver when delivered locally. This biological attribute is unique among other liver-tropic AAV capsids including AAV8 and AAV9. In mouse experiments, we comprehensively characterized transduction efficiencies of 47 AAV capsids in various organs following intravenous vector injection using AAV DNA/RNA Barcode-Seq. A set of AAV capsids including AAV8, AAVrh8, AAVrh10, AAVAnc80 and AAVKP1 transduced the liver effectively at levels equal to or higher than that of AAV9, demonstrating the liver-tropic nature of AAVKP1. To compare liver transduction efficiencies of various AAV capsids following local gene delivery, we took advantage of our ongoing project using rhesus macaques in which we deliver AAV vectors into the pancreatic duct (PD) and assessed off-target gene delivery to non-pancreatic organs. In this PD injection study, we performed duodenotomies to directly visualize the ampulla of Vater. A catheter was then inserted into the pancreatic duct using fluoroscopic imaging. AAV DNA/RNA-barcode library that covers more than 40 serotypes and mutants was injected into the PD locally and blood samples were collected at various time points up to 72hr after injection for pharmacokinetic profiling of each AAV capsid. The animals were euthanized 4 weeks after injection and multiple tissues were harvested for downstream analyses. The results showed that the blood vector concentrations after injection as an indicator of vector spillover are highly variable among AAV capsids, showing several

orders of magnitude higher and lower compared to AAV9. Among a set of the capsids that showed robust local transduction within the pancreas, we identified AAVKP1 as an AAV capsid whose blood concentrations are substantially lower than those of AAV9 at any time points. Consistent with its low blood concentrations, AAVKP1 transduced the liver 100 times less efficiently than AAV9 although both AAVKP1 and AAV9 are liver tropic capsids when intravenously administered. On the other hand, another liver tropic capsid, AAV8 showed several times higher blood concentrations and accordingly led to higher liver transduction than AAV9. These data demonstrate that the degree of vector spillover after local administration of AAV is capsid dependent and varies significantly among different AAV capsids. The vector pharmacokinetic profile is one of the important determinants of vector bio-distribution. Importantly, our study has shown that AAVKP1 is an attractive vehicle for effective local gene delivery while substantially limiting off-target vector spillover particularly in the liver.

234. Application of a Novel Murine Model of Mucopolysaccharidosis Type II for Developing Human Hematopoietic Stem Cell Gene Therapy

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Hematopoietic stem cell gene therapy (HSC-GT) has attracted interest as one of the promising therapies for lysosomal storage diseases including mucopolysaccharidosis type II (MPS II), which is caused by a deficiency of iduronate-2-sulfatase (IDS). *In vivo* characterization of gene-modified human CD34+ (hCD34+) cells before human trial is an important point for the efficient clinical development of HSC-GT. However, a model animal of MPS II, which is evaluable the therapeutic performance of human HSC, has not been established yet. In this study, we generated an immunodeficient NOG/MPS II mouse using CRISPR/Cas9 technology and analyzed the application of those mice in the characterization of gene-transduced hCD34+ cells *in vivo*. NOG/MPS II mice showed accumulation of glycosaminoglycans, the substrates of IDS, and decreased IDS activity in the whole tissues. Transplantation of gene-modified hCD34 cells with lentiviral vector carrying IDS gene, or untransduced cells into NOG/MPS II mice reconstituted the human white blood cells such as lymphoid cells and myeloid cells, respectively. However, significant improvement of biochemical abnormalities in several pathogenic tissues including the central nervous system was only observed in the mice of the HSC-GT group. Additionally, analysis of the integration sites in bone marrow- and spleen-derived cells of HSC-GT group mice showed a typical oligoclonal integration pattern of lentiviral vectors. Our results indicate the promising application of NOG/MPS II mice to the evaluation of therapeutic efficacy and safety of HSC-GT for MPS II using human cells.

235. Development 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 enzyme. The enzymatic deficiency causes lysosomal accumulation of undegraded metabolites and secondary mechanisms of cell damage. The rapid neurodegeneration and severity of GM1, requires a therapy providing a prompt and robust enzyme delivery to the central nervous system, possibly associated to 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 for other LSDs in multiple preclinical and pivotal clinical studies. The rationale is based on the replacement of patient's microglia with a metabolically-competent myeloid progeny of the transplanted HSPCs, which restore the metabolic defect and a normal scavenging function, rescuing the storage and mitigating tissue damage. To this aim, we developed multiple therapeutic lentiviral vectors (LVs) expressing a codon-optimized sequence of the human GLB1 gene, alone or in combination with an immunomodulatory molecule, i.e. the Metallothionein 1G (MT1G), potentially contributing to the down-regulation of neuroinflammation. Both LVs can be produced with a high titer and infectivity, and are able to deliver multiple gene copies safely and efficiently in human and murine HSPCs. LV-transduced cells express dose-dependent, supra-physiological levels of the therapeutic enzyme, which is correctly secreted and uptaken by target cells, as determined by enzyme-specific activity assays performed on total protein extracts and cell culture media. In patient-derived primary fibroblasts, transduction with the therapeutic LVs determines a stable genetic and metabolic correction, proven by the rescue of storage material as early as two weeks post-transduction with an average of ≥ 3 vector copies/cell. The co-expression of the therapeutic enzyme and the immunomodulatory MT1G has been determined by western blotting analysis performed on human cell lines and patient-derived cells. 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 the animal model of the disease.

236. Investigational Liver Gene Transfer of Alpha Galactosidase for the Treatment of Fabry Disease

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Fabry disease is a lysosomal storage disease caused by loss-of-function mutations in the GLA gene which codes for the soluble lysosomal enzyme alpha-galactosidase A (α -gal A, AGA). Loss of GLA expression results in significant accumulation of globotriaosylceramide (GL-3, GB3) in tissues resulting in multi-systemic pathology such as kidney failure, ventricular hypertrophy, and stroke. While the current standard of care, enzyme replacement therapy (ERT), increases survival and slows disease progression, ERT has significant drawbacks such as variable uptake to affected tissues, short circulating half-life, neutralizing antibody development to recombinant α -gal A, and challenges to treatment administration and patient burden. To address these challenges, we present the development of SPK-10002, an investigational liver-directed adeno-associated viral (AAV) gene therapy expressing secretable GLA with an optimized expression cassette and a highly hepatotropic capsid. Administration of SPK-10002 in GLA ko mice results in dose-dependent expression in the liver and dose-dependent secretion of the modified, active α -gal A into circulation. Utilizing immunostaining, we observe that the modified α -gal A is increased in relevant peripheral tissues, and tissue distribution of α -gal A corresponds with decreased intensity of GL-3 staining. We also observe a decrease in the vacuolization within dorsal root ganglia correlating with decrease in GB3 staining. As GL-3 is an FDA-approved clinical biomarker of drug efficacy, we measured GL-3 levels using LC/MS in GLA ko mice treated with SPK-10002 and observe decreased GL-3 accumulation in a dose and time-dependent manner in both kidney and heart. We also observe in serum and relevant tissues a dose-dependent decrease in globotriaosylsphingosine (lyso-GL-3), a clinical biomarker of Fabry disease and a suggested pathological molecule. These data demonstrate that consistent, low level circulating modified α -gal A (<100 ng/ml serum) is sufficient for significant reduction in GL-3 and lyso-GL-3. Additional *in vitro* and *in vivo* studies are ongoing to further characterize SPK-10002 and to determine the biochemical properties of the transgene-derived protein product following liver expression of secretable α -gal A. Overall, these data suggest that low-dose administration of SPK-10002 may be a promising therapeutic strategy for treatment of Fabry disease.

237. Genetic Barcoding for Identifying Transduction Disparities in Disease Models of Sanfilippo Syndrome Type-B

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Adeno-associated Virus (AAV) is currently a modality for delivery of transgenes for various research applications, including correction of disease processes. Many capsid serotypes, production methods and point-mutations for optimized AAV capsids have been proposed for various specific applications. In order to identify optimal vectors for possible correction of Sanfilippo Syndrome type-B (MPSIIIB) as well as for general mouse central nervous transduction, we performed a barcoded vector comparison. In this study, 21 different AAV capsid variations were each packaged with a unique 6-nucleotide (nt) sequence upstream of the poly-A region of a typical GFP transgene. These viruses were pooled together in equimolar proportions and injected into the central nervous system (CNS) of adolescent (P28) mouse models of MPSIIIB that were N-acetylglucosamidase (*NAGLU*) Knock-out (-/-). The same vector mix was injected in phenotypic controls (*NAGLU* +/+). Two different intracranial injection methods were also assessed for effect on biodistribution: intraparenchymal six-site (IC6) and Intra-cisterna magna (ICM). AAV biodistribution and relative gene-expression levels in central nervous system and peripheral organs were compared using PCR metabarcoding and subsequent next-generation sequencing (NGS) via genotype-barcode linked Illumina sequencing. Using our previously developed pipelines, we assessed the differences in vector dynamics in both a diseased state and phenotypically normal animal models. This provides insight to future AAV therapies of MPSIIIB, as well as general CNS disease processes, and provides an NGS-based framework for future vector screening in disease models.

238. Efficacy of Genome Editing in Infant Mice with Glycogen Storage Disease Type Ia

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Background: Glycogen storage disease type Ia (GSD Ia), the inherited deficiency of glucose-6-phosphatase (G6Pase), is associated with life-threatening hypoglycemia, as well as longer-term adverse effects including hepatocellular adenoma and carcinoma formation. Gene replacement therapy has been developed, although it fails to stably reverse G6Pase deficiency. In order to more stably treat GSD Ia, genome editing was developed with adeno-associated virus (AAV) vectors. The vectors were designed to stably integrate a therapeutic *G6PC* transgene, which was mediated by CRISPR/Cas9 cleavage of the *G6PC* locus and homology-directed recombination (HDR). A Donor vector containing the *G6PC* transgene flanked by homology to the *G6PC* locus was integrated at a double-

stranded break created by a CRISPR/Cas9 vector that contains *Streptococcus pyogenes* Cas9 and a small guide RNA targeting *G6PC*. Methods: These two vectors were administered to 10 day old *G6pc* $-/-$ mice with GSD Ia, and efficacy was evaluated in comparison with *G6pc* $-/-$ mice that received only the Donor vector that would not integrate in absence of the CRISPR. The vectors were administered at low (Donor, $2E+12$ vector genomes/kg; CRISPR $4E+11$ vector genomes/kg), or medium dosages (each vector 4-fold higher). Benefits of genome editing were evaluated at 6 and 12 weeks of age, by comparison of the Donor + CRISPR group to the Donor only group at each dose. Results: Improved glucose homeostasis was demonstrated by the following statistically significant differences. Mice receiving both Donor + CRISPR vectors had increased blood glucose concentrations during fasting (low dose; $p < 0.0001$) and decreased liver glycogen compared with mice receiving Donor vector only (medium dose; $p < 0.001$). In the glucose tolerance test, Donor vector administration improved blood glucose at baseline following 4 hours fasting (low dose; $p < 0.0001$) and at 120 minutes following glucose administration (low dose; $p < 0.01$). Significantly more copies of the *G6PC* transgene were detected in the liver for the CRISPR + Donor treated group than for the Donor treated mice (medium dose; $p < 0.05$). Additionally, the liver glycogen concentrations of the medium dose CRISPR + Donor treated mice was significantly lower than untreated *G6pc* $-/-$ controls ($p < 0.001$). After 4 weeks of treatment with both AAV vectors, the medium dose mice had liver glycogen concentrations in the range of wildtype mice. This study has been extended to include evaluation of the same groups at 14 weeks of age and evaluation of transgene integration. For the low dose at 14 weeks the liver glycogen was significantly decreased for both Donor and CRISPR + Donor treated groups, revealing stable liver transduction. Conclusions: We have demonstrated delivery of a CRISPR/Cas9 vector increases efficacy of our *G6PC* transgene over the transgene vector alone, even at low vector dosages. Infant GSD Ia mice receiving the CRISPR to stimulate permanent integration of the *G6PC* transgene had higher blood glucose, increased copies of the transgene, and lower glycogen in liver. The CRISPR treated mice also had hepatic glycogen content similar to wildtype mice. None of these benefits were attributable to CRISPR/Cas9 expression, which has no known effects on glucose metabolism. This data suggests combination therapy with CRISPR/Cas9 helps prevent the loss of therapeutic AAV transgenes in young mice, and justifies the further, long-term evaluation of genome editing in GSD Ia.

239. Optimization of Hydroxyacidoxidase 1(HAO1) Targeting ARCUS Nucleases for the Treatment of Primary Hyperoxaluria Type 1 (PH1)

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PH1 is a rare autosomal recessive disorder affecting an estimated 1 to 4 individuals in a million, with most patients diagnosed as children or young adults. PH1 is caused by a mutation in the alanine glyoxylate aminotransferase (AGXT) gene, which encodes a key metabolic enzyme responsible for converting glyoxylate to glycine in the liver. The

inability to metabolize glyoxylate to glycine leads to an overproduction of systemic oxalate, resulting in the formation of insoluble calcium oxalate crystals in the kidneys. These calcium oxalate crystals lead to kidney stone formation, kidney failure, and further effects in the liver, heart, and other organs. To determine if ARCUS gene editing could be utilized to reduce systemic oxalate levels in PH1 patients, ARCUS nucleases were designed to target and disrupt the HAO1 gene that encodes glycolate oxidase (GO), an upstream enzyme in the metabolic pathway responsible for the conversion of glycolate to glyoxylate. By inhibiting the formation of glyoxylate, oxalate production should be minimized. As a proof of concept, first generation HAO1 targeting ARCUS nucleases were characterized in non-human primates (NHPs). Here, a single infusion of AAVs carrying an HAO1 ARCUS nuclease gene resulted in greater than 30% editing at the HAO1 locus, greater than 95% knock-down of GO protein in NHP liver, and therapeutically meaningful increases in serum glycolate up to 80uM. Candidate HAO1 ARCUS nucleases were optimized to improve specificity while maintaining high levels of on-target activity, and were further characterized in vitro and in vivo. In NHP's optimized nucleases achieved greater than 50% editing at the HAO1 locus, 98% Knock-down of GO in the liver, with serum glycolate levels greater than 80um out to 92 days. These data demonstrate the ability of ARCUS nucleases to edit the HAO1 gene and impact the glyoxylate metabolic pathway at therapeutically meaningful levels after a single administration in NHPs.

240. KT-A112: A Novel AAV-hIns/AAV-hGck Mediated Gene Therapy Works Synergistically to Normalize Blood Glucose in STZ-Induced Diabetic Mice

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Type 1 diabetes (T1D) is a chronic autoimmune disorder characterized by profound insulin deficiency that results from the progressive destruction of pancreatic β -cells. After decades of active research, T1D still has no cure. Current therapies aim to normalize blood glucose with injections of exogenous insulin and lifestyle modifications to prevent potentially life-threatening complications. Despite important advancements, reports indicate that nearly 80% of T1D patients fail to achieve target treatment goals. Skeletal muscle is responsible for the disposal of approximately 70% of circulating glucose after a meal, but only approximately 20-25% during the fasted state. Therefore, a one-time intramuscular administration of adeno-associated virus (AAV) vector-mediated gene therapy co-expressing insulin (INS) and glucokinase (GCK) has the potential to improve the lives of patients with T1D by genetically engineering skeletal muscle to counteract hyperglycemia. Therapeutic efficacy has previously been demonstrated in diabetic mice and dogs (1-3). In preparation for the clinic, we have designed a novel, modified version of AAV-mediated

human insulin (AAV1-hINS) and human glucokinase (AAV1-hGCK) optimized to improve insulin trafficking and secretion and gene of interest (GOI) immunogenicity after co-administration via intramuscular administration. In this study, 4-wk old C57Bl/6J mice were administered either STZ (40 mg/kg) or buffer on five consecutive days. Frank diabetes developed in STZ mice over the next 4 weeks. Prior to AAV injection, body weight, fed glucose and circulating insulin were determined and the mice blocked and sorted into groups such that there were no differences within or between treatment groups. Intramuscular injections of a low-, mid-, or high-dose of AAV1-hINS/AAV1-hGCK were administered bilaterally in the quadriceps, gastrocnemius and tibialis anterior muscles. hINS:hGCK ratio was studied using a 1:1, 1:0.5, 1:2 and 1:4 ratio based on the mid-dose of hINS. Fed glucose and lactate were measured weekly; fasted glucose and circulating insulin levels were determined on wks 4 and 8. An OGTT was performed in fasted animals at wk 8, and HbA1c measured at the end of the study. Muscles were collected and mRNA expression, protein expression and enzymatic activity (GCK) of vector-mediated INS and GCK were determined. Treatment with a 1:1 mixture of AAV1-hINS and AAV1-hGCK produced significant, dose related improvement in glycemic control in the STZ induced diabetic animals. Fasted blood glucose was restored at or below the level of non-STZ controls. Glucose disposal measured by the AUC of an OGTT was improved. HbA1c was significantly reduced (greater than 2%) in all treatments. No significant changes in blood lactic acid production or episodes of hypoglycemia were observed. Overall, results from this study demonstrate that IM administration of AAV1-hINS/AAV1-hGCK effectively normalize hyperglycemia in an STZ-induced mouse model of T1D. Furthermore, this novel, investigational therapy has the potential to provide safe, durable maintenance of glycemic control in T1D without the need to employ additional therapeutic agents. References: 1. Mas et al. *Diabetes* 2006; 2. Callejas et al. *Diabetes* 2013; 3. Jaen et al. *Mol Ther Met & Clin Dev* 2017.

241. 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 treatment is only symptomatic. We designed an original gene therapy (GT) approach based on the autologous transplantation of hematopoietic stem/progenitor cells genetically modified by lentiviral (LV) gene transfer to restore the IDS expression. The GT 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. 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. 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 event. Based on these results in the animal model of the disease, we are currently assessing the GT strategy *in vitro* and *in vivo* in a toxicology and biodistribution study in humanized immunodeficient mice (NSG). The study employs human HSPCs transduced with large-scale produced Lentiviral vector (LVV) and two different manufacturing protocols intended for clinical use. The study will last 12-16 weeks post-transplantation. At the end of the study, mice will be sacrificed, and the organs collected and analyzed for human-cell engraftment and chimerism, biodistribution in target and non-target organs, vector insertion profiling and multilineage human hematopoietic reconstitution. The study will be instrumental to a rapid progression toward the clinical development of our GT strategy for MPS II.

242. Optimized Base Editing Reagents Yield More Potent Genetic Correction in a Mouse Model of Alpha-1 Antitrypsin Deficiency

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Alpha-1 antitrypsin is a neutrophil elastase inhibitor primarily expressed and secreted by hepatocytes. Autosomal codominant mutations in the gene encoding alpha-1 antitrypsin, *SERPINA1*, can result in alpha-1 antitrypsin deficiency (AATD), a rare disease that predominantly manifests as pulmonary emphysema due to progressive loss of elastin in the lung. The most common high-penetrance pathogenic *SERPINA1* variant, p.Glu342Lys, also referred to as the Z allele (PiZ), results in protein misfolding and aggregation, leading to liver fibrosis and cirrhosis in a subset of patients. The p.Glu342Lys variant is caused by a G-to-A single nucleotide polymorphism and is amenable to correction by an adenine base editor (ABE), a gene editing construct capable of performing RNA-programmable A-to-G base conversions in genomic DNA. Previous reports demonstrate correction of the p.Glu342Lys mutation within the livers of transgenic AATD mouse model (NSG-PiZ) treated with lipid nanoparticles formulated with mRNA encoding an ABE and guide RNA. In this study, improvements upon the gene editing reagents were achieved through protein engineering of the base editor itself, mRNA codon optimization, and chemical modification of the guide RNA. These combined improvements yielded a >2-fold increase in potency of resulting lipid nanoparticles and therapeutically relevant increases in circulating alpha-1 antitrypsin in NSG-PiZ mice treated at clinically relevant doses (<1mg/kg). Furthermore, similar efficacy and durability was observed in NSG-PiZ mice dosed at >37 weeks of age despite liver fibrosis analogous to what might be encountered in a clinical

setting. These results go beyond previous proof-of-concept studies in demonstrating both therapeutic relevance and technical feasibility of base editing as a potential treatment for alpha-1 antitrypsin deficiency.

243. Primary Human Hepatocytes, Genetically Engineered Ex Vivo to be Hyperfunctional, Can Produce Clinically-Relevant Levels of Therapeutic Factors In Vivo

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Background: Hepatocyte transplantation is a potential alternative curative therapy for many devastating liver diseases, including genetic diseases such as hemophilia B. However, two major obstacles have limited the widespread use of hepatocyte transplantation in the clinic: a shortage of high-quality hepatocytes and limited therapeutic efficacy of engrafted cells. In this study, we hypothesized that human hepatocytes could be engineered ex vivo to be hyperfunctional and that these engineered hepatocytes could be expanded in an in vivo bioreactor to generate high quality enhanced hepatocytes suitable for therapeutic applications in many liver disease indications. **Methods and Results:** Optimized protocols were developed to enable >90% transduction of human hepatocytes with lentiviral vectors while preserving high viability. Using human Factors VIII (FVIII) and IX (FIX) as examples, repeated studies demonstrated that ex vivo lentivirus (LV) transduced human hepatocytes overexpressed either human FVIII or FIX in vitro at 5-100x levels (dependent on experimental conditions) compared to non-transduced human hepatocytes. Next, to demonstrate both the in vivo potential of these engineered hepatocytes and expandability of the cells, hyperfunctional (FIX overexpressing) or luciferase-labeled (control) engineered cells were transplanted into the liver of an immune-deficient mouse model of hereditary tyrosinemia type 1 (FRG mouse). Engrafted human hepatocytes overexpressed human FIX at a level comparable to physiological levels of FIX in peripheral blood at four weeks after transplantation and until the end of the study (14 weeks). Engineered human hepatocytes proliferated and repopulated the FRG mouse, demonstrating the ability to expand the quantity of engineered cells in vivo >100-fold. Importantly, engineered hepatocytes expanded in vivo at comparable kinetics to non-edited cells, and thus rescued the HT1 mice at levels comparable to normal function hepatocytes. **Conclusion:** The data generated clearly demonstrate efficient genetic engineering of primary human hepatocytes to render them hyperfunctional, and their subsequent expansion. We expect that combining this engineering approach with our ongoing development of the FRG rat bioreactor for large-scale expansion of primary human hepatocytes will provide large quantities of high-quality enhanced hepatocytes, potentially allowing for a wider spectrum of liver disease patients to be treated.

244. Gene Therapy for Guanidinoacetate Methyltransferase Deficiency Restores Cerebral and Myocardial Creatine While Resolving Behavioral Abnormalities

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Introduction Creatine is an essential molecule in energy homeostasis which facilitates buffering high energy phosphates. It is also important for neurite growth cone migration, dendritic and axonal elongation, co-transmission on GABA postsynaptic receptors in the central nervous system, and neurotransmitter release. Guanidinoacetate N-methyltransferase (GAMT) in the liver produces creatine from S-adenosylmethionine and guanidinoacetate (GAA), and its deficiency causes a rare disorder that results in developmental delay, intellectual disability, muscular hypotonia, involuntary movements, ataxia, and autistic or self-aggressive behavior. Impaired creatine synthesis leads to elevated GAA levels and reduced creatine levels in plasma, urine, and tissues, which together are thought to underlie the phenotypes. Current treatments for GAMT deficiency, such as oral creatine administration, are minimally effective in some, seizure risk often remains, and can lead to nephrolithiasis. In this study, we developed a gene therapy approach to restore hepatic GAMT gene expression by utilizing an AAV to express human codon-optimized GAMT in murine hepatocytes to overcome the limitations of current therapy. **Methods/Results** We generated an AAV expressing human codon-optimized GAMT (hcoGAMT) transcript variant 1 under a liver-specific promoter serotyped to rh10. Through a dose escalation experiment in 8-week-old mice, we found that a dose of 1×10^{14} genome copies (GC)/kg achieved restoration of creatine and amelioration of GAA to levels nearly equivalent to those of wild type. Equal numbers of mice per gender and genotype (Gamt^{+/+}, Gamt^{-/-}, treated Gamt^{-/-}, n=8 per genotype group with 4 per gender) were analyzed and followed in a yearlong study. For the treated Gamt^{-/-} group, a dose of 1×10^{14} GC/kg was administered at 8-12 weeks of age. While all mice started with similar weights, AAV-treated Gamt^{-/-} mice showed substantial weight gain over 12 months, and whole-body microCT at 8 months of age showed marked improvement in adipose tissue quantity, when compared to untreated Gamt^{-/-} mice. Plasma and urine were collected every 3 months for examining creatine and GAA level through liquid chromatography/mass spectrometry. In AAV-treated Gamt^{-/-} mice, plasma and urinary creatine levels were normalized, while GAA levels were reduced, approaching wild type. GAA levels in peripheral tissues (brain, heart, muscle, kidney, and liver) were also significantly reduced while there was marked improvement in creatine levels for AAV-treated Gamt^{-/-} mice. Brain glucose uptake was measured by fluorodeoxyglucose (18[F]-FDG)-positron-emission tomography (PET). Gamt-deficient mice showed decreased glucose uptake, while AAV-treated Gamt^{-/-} mice had glucose uptake comparable to wild type. Behavioral testing was conducted at 8 months of age. The Barnes maze showed that Gamt^{-/-} mice had deficits in learning, and these were resolved with AAV-based gene therapy. Additionally, while untreated Gamt^{-/-} mice depended on non-hippocampal serial strategies, AAV-treated Gamt^{-/-} mice advanced to a hippocampal direct strategy, resembling the wild type. AAV-treated Gamt^{-/-} mice also demonstrated grip strength comparable to the wild

type, while untreated mice showed significantly reduced strength. **Conclusion** We developed a gene therapy for GAMT deficiency that resolves the majority of the biochemical and behavioral abnormalities found in the mouse model of this disorder. This successful application of AAV-based gene therapy to GAMT deficiency suggests a possible path forward for clinical development considerations.

245. The Natural History of Vitamin B12-Responsive Cobalamin A-Type Methylmalonic Acidemia (MMA) Provides Clinical Benchmarks for the Treatment of Severe Forms of MMA Caused by MMUT and MMAB Deficiencies

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Background and Rationale: Isolated methylmalonic acidemia (MMA) results from a group of autosomal recessive disorders, including enzyme deficiencies of methylmalonyl-CoA mutase (*mut*⁰ or *mut*) and defects in the cellular metabolism of the cofactor 5-deoxyadenosylcobalamin (*cblA*, *cblB*, or *cblD*). *cblA* is caused by pathogenic variants in *MMAA*, which encodes a GTPase that regulates 5-deoxyadenosylcobalamin binding to methylmalonyl-CoA mutase. The majority of individuals with *cblA* respond clinically and biochemically to vitamin B12 injections, and, therefore, the natural history may inform the outcomes expected by “converting” more severe non-vitamin B12 responsive forms of MMA, such as *mut* and *cblB* subtypes, into milder conditions following genomic therapies, including mRNA, genome editing, and/or conventional AAV gene addition. **Methods:** A cohort of 17 individuals with *cblA*, ages 2 to 50 years, were evaluated per protocol (clinicaltrials.gov ID: NCT00078078) and followed over a period of 17 years. Clinical outcomes and biomarkers were compared to 91 individuals with *mut* and 17 with *cblB* subtypes. **Results:** Two individuals were diagnosed prenatally, due to family history, and treated *in utero* via maternal cobalamin supplementation, while 15 others presented symptomatically at various ages, ranging from newborn to 17 years. Presenting symptoms included hyperammonemia, basal ganglia stroke, renal tubular acidosis, and chronic kidney disease (CKD) with metabolic crisis after a renal transplant. Six individuals experienced a basal ganglia stroke around the time of first metabolic crisis between 11 and 62 months of age. No metabolic strokes occurred in individuals compliant with B12 therapy. Serum methylmalonic acid levels ranged from 5–223 μM (reference <0.40 μM) in *cblA* compared to 15 μM to 12 mM (3–50 fold higher) in *mut* and *cblB*. Similar trends were observed in hepatic mitochondrial biomarkers FGF21. For FGF21, individuals with *cblA* had a mean of 451 ± 369 pg/ml, as opposed to 6,137 ± 8,066 pg/ml in *mut* and 3,005 ± 2,098 pg/ml in *cblB*. Within the *cblA* cohort, 3/17 (18%) individuals had CKD stage 3 by age 10 years, compared to 50% of *mut*⁰ and 63% of *cblB*. At 47 years of age, one individual with *cblA* taking 25 mg/day of intramuscular hydroxocobalamin had a glomerular filtration rate of 61 mL/min/1.73m², suggesting that renal disease is progressive even at high doses of vitamin B12 administration. Cardiac conduction abnormalities (n=6) included ventricular bigeminy

or ectopy, and one had mild left eccentric ventricular hypertrophy with associated electrocardiographic findings. Among 11 individuals with *cblA*, ten had a full-scale intelligence quotient (IQ) greater than 70. All adult subjects with *cblA* lived independently and many were employed. Two females had a total of three healthy children. **Conclusions:** Prenatal and pre-symptomatic treatment of *cblA* appears to lessen the disease burden, emphasizing the importance of newborn screening for MMA. Despite known complications of *cblA* including strokes with neurological sequelae, CKD, and dysrhythmias, individuals supplemented with vitamin B12 did not experience metabolic crises or basal ganglia injury, achieved normal IQs (except for one individual), and lived independently. The natural history of this milder form of MMA establishes clinical outcomes and biomarker trends (reduced serum methylmalonic acid and FGF21 levels along with improved 1-¹³C propionate oxidation) that could be used as benchmarks for new genomic treatments for the more severe *mut* and *cblB* disease subtypes.

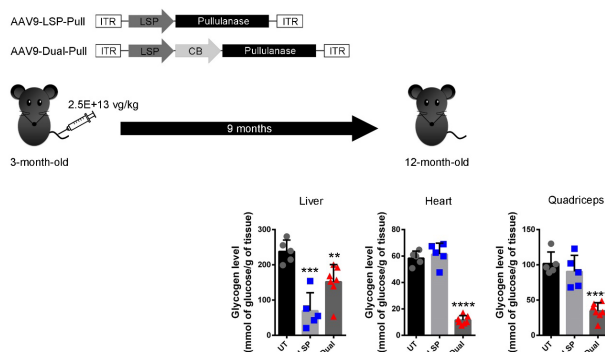
246. Long-Term Correction of Glycogen Storage Disease Type III by AAV-Mediated Gene Therapy Using a Dual Promoter to Express Bacterial Pullulanase

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Background: Deficiency of glycogen debranching enzyme in glycogen storage disease type IIIa (GSD IIIa) results in progressive accumulation of abnormal glycogen in the liver, heart, and skeletal muscles. Most patients have both liver and muscle involvement (GSD IIIa), while others have disease limited to the liver (GSD IIIb). Previously we reported that an AAV vector containing a dual promoter (Dual, combined a liver-specific promoter (LSP) and the ubiquitous CMV enhancer/chicken β-actin promoter) reduced Pullulanase-induced cellular immune responses in GSD IIIa mice and corrected disease phenotypes in the liver, heart and skeletal muscle after ten weeks of treatment. In this study, we evaluated the long-term efficacy of this gene therapy approach in GSD IIIa mice. **Methods:** Two Pullulanase-expressing AAV vectors, AAV-Dual-Pull and AAV-LSP-Pull, were packaged as AAV9 and intravenously injected into 3-month-old GSD IIIa mice at the same dose (2.5 × 10¹³ vg/kg). Age-matched untreated GSD IIIa mice (UT) were used as controls. Mice were monitored for up to nine months for functional improvements by treadmill, wire-hang, and grip strength tests. Blood samples were collected for analyzing plasma liver (ALT and AST) and muscle (CK) enzyme activities. At 12 months of age, mice were euthanized to collect tissues for analyses of AAV vector biodistribution, glycogen content, enzyme activity, protein expression, and histology. **Results:** During the nine months of treatment, the AAV9-Dual-Pull treated mice showed significantly better performances in the functional tests than the UT or AAV9-LSP-Pull treated mice. Plasma CK activity was significantly lowered in the AAV9-Dual-Pull treated mice but remained unchanged in mice treated with AAV9-LSP-Pull compared to UT; both AAV treatments decreased plasma ALT and AST activities. After nine months of treatment, liver size (liver/body-weight ratio) was significantly reduced in both AAV treatment groups compared with UT. AAV genome copy numbers (copies/diploid genome) of AAV9-Dual-Pull were higher than AAV9-

LSP-Pull in the heart (6.32 ± 5.01 vs. 1.47 ± 0.92) and quadriceps (2.09 ± 1.59 vs. 0.12 ± 0.05), but not significantly different (108.6 ± 118.7 vs. 56.0 ± 50.1). Pullulanase expression was increased highly in the heart, moderately in the quadriceps, and slightly in the liver of AAV9-Dual-Pull treated mice; AAV9-LSP-Pull treated mice had elevated the expression only in the liver. Consistent with the expression results, AAV9-Dual-Pull treatment reduced glycogen levels in the heart (-80%), quadriceps (-66%), and liver (-36%), while AAV9-LSP-Pull reduced glycogen content only in the liver (-71%) (**Figure 1**). PAS stain of tissue sections confirmed the glycogen content data. **Summary:** Persistent correction of disease phenotypes in the liver, heart, and skeletal muscle of GSD IIIa mice was achieved by the AAV9-Dual-Pull treatment. AAV9-LSP-Pull showed better efficacy than AAV9-Dual-Pull in the liver but did not affect other tissues, suggesting a favorable treatment for patients with GSD IIIb.



247. Low-Density Lipoprotein (LDL) Receptor Liver Gene Transfer with Lentiviral Vectors Normalizes Circulating LDL Cholesterol in a Mouse Model of Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is an autosomal dominant metabolic disease, characterized by high LDL cholesterol in the circulation, due to impairment in its clearance pathway by hepatocytes, mediated by LDL receptor (LDL-R). Affected patients rapidly progress to atherosclerosis, myocardial infarction, and death within the third decade of life, if left untreated. Current treatments with cholesterol-lowering drugs are only partially effective. Despite strategies targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) and angiopoietin-related protein 3 precursor (ANGPTL3) are showing promising results, they are, *per se*, unable to fully normalize cholesterol in homozygous patients. Liver gene therapy may represent a definitive cure, particularly if administered at an early stage of disease progression. Lentiviral vectors (LV) are attractive vehicles for liver-directed gene therapy since they integrate in the genome of target cells and may thus allow stable gene transfer following a single administration even in pediatric patients. We have developed vesicular stomatitis virus glycoprotein (VSV.G)-pseudotyped LV that achieve stable transgene expression in the liver and dose-dependent reconstitution of coagulation factors after

intravenous (i.v.) administration to small and large animals. We thus set out to apply our gene therapy strategy to FH. We first generated and produced LV encoding LDL-R under the control of a hepatocyte-specific expression cassette. We observed a considerable 10-fold drop in infectious titer, compared to other transgene-encoding LV, challenging *in vivo* use. Since LDL-R and its family members are the entry route of VSV.G LV, we hypothesized that the interaction between VSV.G and LDL-R during LV production and possibly the re-infection of producer cells were causing the observed outcome. We rescued the titer by changing LV pseudotype or abrogating transgene expression in producer cells, confirming the detrimental consequences of co-expression of VSV.G and LDL-R for LV production. We then assessed whether VSV.G LV liver gene transfer was feasible in the mouse model of FH, LDL-R knock out (KO), since these mice lack what is described as the main VSV.G receptor on hepatocytes. Surprisingly, we found that in LDL-R KO mice, hepatocyte gene transfer and expression of a reporter transgene were >2 fold higher than in control mice and the reasons for this outcome are still under investigation. We treated LDL-R KO adult mice with VSV.G LV encoding LDL-R i.v. and did not show phenotypic correction. Interestingly, when treating juvenile LDL-R KO mice with the same LV, we achieved stable and complete normalization of circulating LDL cholesterol for more than 6 months after gene therapy (N=20). We are currently challenging some of these mice with a high-cholesterol diet (HCD) for three months and cholesterol levels of gene therapy treated mice remain comparable to wild type mice at the last follow-up (1.5 months of HCD). Liver and aorta will be collected for end-point histopathologic analysis of steatosis and atherosclerosis. We are currently investigating the percentage of genetically corrected hepatocytes required for therapeutic efficacy. Enhancers of LV transduction may improve the therapeutic outcome in adult mice. Overall, we show here proof-of-concept of therapeutic efficacy in FH by liver-directed LV gene therapy and our results encourage further investigation towards clinical translation.

248. AAV Gene Therapy for Treating MPS IIIC: Facilitate by-Stander Effects by EV-mRNA Cargo

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Mucopolysaccharidosis (MPS) IIIC is a devastating neuropathic lysosomal storage disease, caused by autosomal recessive defects in heparan- α -glucosaminidase-N-acetyltransferase (HGSNAT) gene. No treatment is currently available for the disease. Recent success in AAV gene therapy offers great tool for treating neurogenetic diseases. However, for maximal therapeutic benefits, gene therapy for MPS IIIC will require high vector dose to transduce as many cells as possible, given that HGSNAT is a non-secretive, exclusive trans-lysosomal-membrane enzyme. Notably, all cells are known to continuously release extracellular vesicles (EVs) and communicate by exchanging large molecules via EV trafficking. In this study, we developed a novel single stranded (ss) AAV vector containing an EV-mRNA packaging zip code (ZC) signal linked to human HGSNAT cDNA. The rAAV-hHGSNAT^{ZC}

vector was tested *in vitro* in human MPS IIIC skin fibroblasts. As results, the rAAV-hHGSNAT^{ZC} mediated not only efficient expression of functional HGSNAT protein in MPS IIIC cells, but also the release of abundant EVs containing hHGSNAT mRNA to the media. Importantly, incubation with purified EVs from media of transduced cells resulted in the expression of functional HGSNAT protein and the clearance of GAG contents in non-transduced recipient MPS IIIC cells. In addition, human MPS IIIC cells excrete significantly more EVs than normal human cells, which were reduced to normal levels after incubation with the vector or the purified EVs containing hHGSNAT-mRNA, indicating the correction/cross-correction of broader disease aspects. This study demonstrates that the incorporation of the ZC signal sequence in rAAV-hHGSNAT vector mediates EV packaging of hHGSNAT-mRNA, which can be transported to recipient cells and translated into functional rHGSNAT protein there, thus providing by-stander effects and cross-correction of MPS IIIC pathology. Our data strongly support the great therapeutic potential of rAAV gene therapy with EV-mRNA-cargo facilitated by-stander effects for treating MPS IIIC, without having to transduce every single cell for optimal benefits. It may therefore reduce the burden of a high vector dose and the risk of potential toxicity for human application. Further, this technology may also be applicable to broad disease targets involving transcription factors and proteins that are not secreted.

249. Effects of AAV Serotype on Treatment of Feline GM1 Gangliosidosis by Cerebrospinal Fluid Delivery of AAV

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Feline GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency in lysosomal β -galactosidase (β -gal). GM1 in cats faithfully emulates the pathology of GM1 in humans, making cats an ideal animal model for this disease. Adeno-associated viral (AAV) gene therapy for GM1 aims to restore β -gal activity and minimize disease progression. Choosing the appropriate AAV serotype and delivery route is critical for GM1 therapy since the vector must display tropism for cells that can achieve therapeutic effect and reach the areas most affected. Intracranial injection of AAV into the thalamus and deep cerebellar nuclei resulted in a 7.5-fold increase in the lifespan of GM1 cats, but the procedure is invasive and requires a craniotomy for delivery to the brain parenchyma. Injection into the CSF via the cisterna magna (CM) is less invasive and could improve biodistribution to certain parts of the CNS and peripheral organs. This study compares the effects of AAV serotype on survival, β -gal distribution, viral distribution, and disease progression in GM1 cats following CM injection using serotypes AAV9 and AAVrh10. Each animal received 1.5×10^{13} vector genomes/kg body weight at 2.2 ± 0.3

months of age (symptom onset occurs at ~ 4 months). Enzyme assays with a synthetic fluorogenic substrate (4MU) were used to assess β -gal enzyme activity in various tissue samples in the CNS and peripheral organs. β -gal activity increased to 28-300% normal in the cerebellum and the spinal cord for both treatment cohorts; however, activity was lower in the cerebrum for both cohorts (untreated animals exhibit $\sim 3\%$ of normal β -gal activity in the brain and spinal cord). Both cohorts also displayed improved β -gal activity in peripheral tissues, such as the heart (2.5 ± 2.1 fold normal). 7T Magnetic resonance imaging documented delayed neurodegeneration in both cohorts, which correlated with delayed clinical symptoms such as inability to stand. These symptoms are reached at approximately 8 months in untreated animals, but were delayed by 2+ months in both treatment cohorts. Glycerophosphocholine and phosphocholine (GPC+PCh), an MRS biomarker that increases with loss of myelin integrity, showed no preservation in the GM1+AAVrh10 cohort, and partial preservation only in the cerebellum of the GM1+AAV9 CM cohort. NAA+NAAG (N-acetyl-aspartate+N-acetyl-aspartylglutamate) is a biomarker for neuronal health that decreases with age in untreated animals. Neither treatment group was able to preserve NAA+NAAG levels at endpoint, but NAA+NAAG levels declined at a slower rate when compared to the untreated group. Ultimately, untreated GM1 cats ($n=9$) survived 7.9 ± 0.4 months, GM1+AAV9 cats ($n=3$) survived 13.9 ± 2.3 months, and GM1+AAVrh10 cats ($n=3$) survived 11.9 ± 1.1 months. Statistical significance was found between the survival of untreated GM1 cats and GM1+AAV9 treated cats ($p=0.0009$) as well as untreated cats and GM1+AAVrh10 treated cats ($p=0.0002$) but there was no statistical difference between AAV9 and AAVrh10 treated cats ($p=0.2478$). This study demonstrates efficacy of AAV therapy after CM injection of either AAV9 or AAVrh10. Improvements in survival are anticipated to occur with optimization of dose and delivery protocols.

250. Using Directed Evolution to Overcome Therapeutic Deficiencies in Treating Lysosomal Storage Diseases

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The efficacy of gene therapies for lysosomal storage disorders can be impacted by optimization of many parameters such as vector serotype, promoter, codon usage, dose, and route of administration, while the primary amino acid sequence of the enzyme transgene itself is generally kept constant. Accordingly, the intrinsic properties of the enzyme may constrain the benefits of a therapeutic. Notably, many lysosomal enzymes are unstable in serum and poorly taken up by target cells, limiting cross-correction, or highly immunogenic, limiting long-term therapeutic outcomes. An enzyme transgene with a better expression and stability profile and lower immunogenicity may offer superior efficacy to patients at lower doses than gene therapies constrained by

limitations of the wild-type enzyme. Here, we provide case examples on the power of directed evolution in generating superior transgenes for potential gene therapy applications in Fabry and Pompe disease. Using our proprietary CodeEvolver® protein engineering platform, which entails high-throughput protein expression, high-throughput activity and cell-based assays, next-generation sequencing, and bioinformatics, we have identified α -galactosidase A (GLA) and α -glucosidase (GAA) variants with superior properties for addressing Fabry and Pompe diseases, respectively. GLA was optimized for improved lysosomal and serum stability and reduced predicted immunogenicity through 12 rounds of iterative directed evolution involving screening >12,000 variants. Up to 17 mutations in selected variants confer stability at both lysosomal and blood pH, stability to serum and elevated temperature, and up to ~20x increased activity in Fabry patient fibroblasts and GLA^{-/-} podocytes. To address the existing immunogenicity of GLA and the potential for introducing new immunogenic epitopes during directed evolution, we took a multi-pronged de-immunization approach focused on removing in silico-predicted major histocompatibility class (MHC) II epitopes and validating predictions with ex vivo MHC-associated peptide proteomics (MAPPs) and T cell proliferation assays. Finally, we show that the in vitro improvements translate to improved pharmacokinetics, biodistribution, and Gb3 substrate reduction in a mouse disease model. For the potential treatment of Pompe disease, we screened >19,000 GAA variants over nine rounds of iterative directed evolution. The resulting engineered GAA variants are substantially more stable than wild-type GAA, as evidenced by increases in melting temperature at lysosomal and neutral pH. Further, the engineered GAA variants retain >60% of their activity through 50 hours in plasma at 37°C, while wild-type GAA maintains <10% activity within 24 hours under these conditions. Pompe fibroblasts and GAA^{-/-} myoblasts treated with GAA variants have up to ~40x higher GAA activity in cell lysates compared to wild-type GAA. Collectively, our findings highlight the promise of sequence optimization to generate enzyme variants to inspire a new generation of gene therapies that may improve outcomes in patients.

251. Characterization of Transgenic Mouse Line with *Gaussia* Luciferase-Based Secreted ER Calcium-Monitoring Protein (SERCAMP)

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Introduction. Concentration of calcium within the endoplasmic reticulum (ER) is couple of orders of magnitude higher than in the cytosol. Maintenance of this gradient is important for proper ER functioning. When calcium levels in ER decrease in response to pathophysiological conditions, ER retention signal (ERS)-containing proteins are redistributed from the ER to the extracellular space in a process called exodosi. To readily detect ER exodosi in animal models of disease, we have developed a transgenic mouse containing a Cre-dependent, CAG promoter driven LoxP-STOP-LoxP *Gaussia* luciferase-based secreted ER calcium-monitoring protein (GLuc-SERCAMP) integrated into the genome at the ROSA26 locus. The goal

of this study was to characterize GLuc-SERCAMP reporter animals (mRosa26-LSL-CAG-GLuc-SERCAMP) in Albumin-Cre or Dopamine Transporter (DAT)-Cre background. **Methods.** Male and female animals with LSL-GLuc-SERCAMP, Albumin-Cre, DAT-Cre, LSL-GLuc-SERCAMP x Albumin-Cre, and LSL-GLuc-SERCAMP x DAT-Cre genotype were used. Immunohistochemistry (IHC) was performed to colocalize GLuc and Cre-reporter proteins as well as cell-specific markers. GLuc luminescence levels were measured from brain, skeletal muscle, liver, spleen, lungs, heart, kidney tissue and from blood and cerebral spinal fluid (CSF) samples. To induce ER calcium depletion, SERCA pump inhibitor, thapsigargin (males - 0.7 mg/kg, females 0.5 mg/kg), was injected i.p. or intracerebroventricularly via injection cannula after baseline plasma and CSF sample collection. **Results.** LSL-GLuc-SERCAMP crossed with Albumin-Cre or DAT-Cre mice showed strong colocalization of GLuc immunoreactivity in liver tissue and dopaminergic neurons in midbrain, respectively. Little or no staining was observed in other tissue or brain regions. GLuc luminescence in LSL-GLuc-SERCAMP x Albumin-Cre animals was detectable from liver tissue samples and plasma. Only low basal signal was seen in other tissue or genotypes. In SERCAMP x DAT-Cre animals, luminescence signal was significantly higher in samples from brain regions rich in dopamine neuros. ER calcium depletion with thapsigargin resulted in approximately two-fold increase in plasma luminescence compared to the baseline or vehicle measurements. **Conclusions.** This model provides Cre-dependent expression of GLuc-SERCAMP reporter protein for measurement of Ca-dependent ER exodosi with negligible expression in untargeted tissue. Luminescence associated with ER exodosi can be readily detected in bodily fluids from small cell populations in central nervous system or from whole organs.

252. Brain Single-Nuclei Sequencing and Lipidomics Reveal Reversible Cell Lineage and Pathway Changes in Myelin Producing Cells in Adult Pah^{enu2} Mice, a Model of Phenylketonuria

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Phenylketonuria (PKU) is a genetic defect caused by lack of liver enzyme phenylalanine hydroxylase (PAH). This deficiency results in elevated blood Phe levels that are toxic to the brain. We have previously confirmed brain myelin defects in adult PAH^{enu2} mice, a model of human PKU by MRI and myelin basic protein (MBP) staining and shown the partial reversibility of these changes with AAV-mPAH (encoding mouse PAH) treatment. To better understand the hyperphenylalaninemia mediated myelination defects in the brain, we performed targeted lipidomics and single nuclei transcriptomics of the corpus callosum (CC) of these mice. We measured the levels of cholesterol, a major component of myelin and other intermediate sterols of cholesterol biosynthesis in the CC (targeted to squalene to cholesterol). While there was no change in total cholesterol, a

significant reduction of sterol intermediates both in the Bloch (mainly in astrocytes) and Kandutsch-Russell (mainly in neurons) pathways were observed in the PAH^{enu2} mice compared to heterozygous mice (with normal blood Phe). These changes suggest potential compensatory mechanisms to maintain normal cholesterol synthesis in the brain. Single nuclei sequencing performed to evaluate the cell composition and cell-specific expression patterns revealed changes in the myelin producing cells (oligodendrocytes and astrocytes). In oligodendrocytes, the number of mature forms of oligodendrocyte 2 (mtOLG2) were reduced in PAH^{enu2} mice. The Ingenuity pathway analyses demonstrated an upregulation of EIF2 signaling, NRF2-mediated oxidative stress response and oxidative phosphorylation, all stress related pathways in mtOLGs and these were corrected by blood Phe normalization. In astrocytes, while the number of cells was not significantly changed, marked upregulation in LXR/RXR activation, a pathway involved in lipid metabolism and cholesterol regulation, was seen in all astrocyte subtypes. The neural stem cells (NSCs), a subtype of astrocytes, also showed upregulation in NRF2-mediated stress response, CREB signaling and energy metabolism pathways (Glycolysis and Gluconeogenesis). Analysis of altered genes highlighted increased expression of transthyretin (TTR), ApoE, Cst3 and Cd81. Particularly striking was the increased expression of ApoE in astrocytes and may represent compensatory mechanism to provide cholesterol transport to other cell types to maintain white matter function. In summary, our results demonstrated significant changes in the transcriptome and sterol profiles in the brains of PKU mice which are likely to contribute to white matter myelin deficits observed in the PKU brain pathology.

253. Neonatal Treatment of Murine GM1-Gangliosidosis Following Liver-Targeted Genome Editing with the PS Gene Editing System

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Introduction: GM1-gangliosidosis is a progressive and fatal neurological lysosomal disease caused by mutations in the gene encoding the lysosomal enzyme β -galactosidase (β -gal). Deficiencies in β -gal result in the progressive accumulation of GM1 and GA1 gangliosides within the lysosome, leading to chronic neuroinflammation and cell death. In the most severe infantile form, patients succumb to the disease by three years of age. Currently, there are no approved therapeutic options available to treat or prevent the onset of GM1-gangliosidosis. Previously, it has been demonstrated that AAV-mediated delivery of a CRISPR-Cas9 gene therapy, termed the PS Gene Editing System, results in therapeutic improvement in multiple lysosomal diseases, including mucopolysaccharidosis type I (MPS I) and Sandhoff disease. This approach allows for targeted integration of a lysosomal enzyme into the albumin locus, resulting in the high transgene expression and secretion of the mature enzyme out of modified hepatocytes.

Hypothesis: Utilizing this platform, it was hypothesized that insertion of a β -gal transgene into the albumin locus would provide permanent, high levels of secreted β -gal enzyme, which allowed for cross-correction

and prevention of the GM1-gangliosidosis disease pathology. **Materials & Methods:** AAV8 vectors delivering the PS Gene Editing System, encoding either a mouse-like (PS-mmGlb1) or human-like β -gal (PS-hsGLB1) cDNA, were administered intravenously into neonatal β -gal deficient mice (β -gal^{-/-}). β -gal enzyme activity was monitored monthly in plasma. At six months of age, mice were subjected to a battery of cognitive and motor behavioral tests, including the beam walk, accelerating rotarod, pole test, inverted screen, and Barnes maze. Between 8-10 months, mice were euthanized and analyses including enzyme activity, ganglioside quantification and brain pathology were conducted. **Results:** Over seven months, the average plasma β -gal enzyme activity of mice receiving the mid and high dose of the PS System was 1.8- and 9.6-fold of heterozygous levels in PS-hsGLB1 mice, and 3.2- and 90.7-fold in PS-mmGlb1 mice. Tissue analysis revealed a dose-dependent increase of β -gal enzyme activity, with a significant increase in the liver, heart, and spleen of mice receiving the high dose of PS-mmGlb1 and PS-hsGLB1. Importantly, a significant increase of enzyme activity was present in the brain of mice receiving the high dose of PS-mmGlb1. Ganglioside quantification using HPLC-MS/MS showed a decrease of GM1 in the heart and spleen of PS-mmGlb1 mice and a decrease of GA1 in the heart, liver, and spleen of both PS-mmGlb1 and PS-hsGLB1 mice. GA1 ganglioside in the hippocampus and GM1 in the cerebellum of mice receiving the high dose of PS-mmGlb1 were decreased. Comprehensive behavioral assessment utilizing the beam walk, rotarod, pole test, inverted screen, and Barnes maze revealed β -gal^{-/-} mice receiving the high-dose of PS-mmGlb1 had a significant improvement of cognitive and motor function, whereas high-dose PS-hsGLB1 mice had mild motor function improvement. **Conclusions:** These results demonstrate that the PS Gene Editing System can provide supraphysiologic levels of β -gal enzyme and treat murine GM1-gangliosidosis and supports the potential of this technology for treating lysosomal diseases.

254. Prime Editing Corrects the Gaa c.1826dupA Mutation in a C2C12 Mouse Myoblast Line of Infantile Onset Pompe Disease

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Pompe disease is a rare autosomal recessive disorder resulting from pathogenic mutations to the GAA gene, which encodes the lysosomal enzyme acid α -glucosidase (GAA). Loss or deficiency of GAA leads to the accumulation and storage of glycogen, skeletal muscle hypotonia, and hypertrophic cardiomyopathy. Though standard of care is enzyme replacement therapy, developing a strategy that fully attenuates clinical symptoms and overcomes the requirement for regular infusions is a critical next step. Animal and cell models that recapitulate disease pathology and harbor mutations relevant to Pompe disease provide a platform that supports the development of such therapeutic strategies including genome editing. Recent advances have extended CRISPR-based approaches to prime editing, which combines canonical CRISPR/Cas9 and a reverse transcriptase to precisely template desired edits, effectively correcting pathogenic variants. With this method, it is now

possible to correct small insertions, deletions, and both nucleotide transitions and transversions. Infantile-onset Pompe disease *Gaa* c.1826dupA C2C12 mouse myoblasts, generated via CRISPR/Cas9 homology directed repair, represent one such model for prime editing. Here, we describe the use of prime editing to target this *Gaa* pathogenic mutation in mouse myoblasts, and present molecular and biochemical data demonstrating its correction. *Gaa* c.1826dupA C2C12 myoblasts were nucleofected with plasmids containing the pegRNA, PE3b sgRNA, and PE2-Cas9 enzyme. The pegRNA, which contains the targeting guide sequence and the editing template, was optimized to promote maximal editing efficiency. PE3b, a second sgRNA targeting the non-edited strand was employed to increase editing. Treated cells demonstrated an increase in wild-type (c.1826A) *Gaa* sequence and showed significantly increased GAA activity when compared to unedited controls. Translation of these strategies to the c.1826dupA *Gaa* mouse model (*Gaa*^{em1Jhng/J}), also generated via CRISPR/Cas9 homology directed repair, will provide insight into the use of genome editing based therapeutics for infantile-onset Pompe disease and other monogenic genetic disorders.

255. Education Needs in Diagnosing Rare Diseases: A Clinician Survey

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Background: No individual clinician can be familiar with the >7,000 known rare diseases; however, recognizing when a patient may have a rare disease and appropriate referral is critical in shortening the pathway to diagnosis. Unfortunately, there continues to be a significant delay in diagnosis across rare diseases, which may lead to inappropriate or harmful testing or medication, and disease progression. **Methods:** A 14-question online survey to assess clinician knowledge, experience, and educational needs in rare disease was emailed to Medscape member physicians. It launched on March 31, 2021 and closed on August 2, 2021. **Results:** A total of 927 physicians specializing in 16 different therapeutic areas participated in the survey. Approximately 71% of physicians considered rare diseases on the order of 10- to 100-fold rarer compared with standard definitions in the EU (5 per 10,000) or US (<200,000). And 57% said they never or rarely (1-2x/year) see rare disease patients. Despite most rare diseases originating childhood, as many as 70% of pediatricians (n = 55) said they never or rarely see rare disease patients. Although 89% have been involved in a rare disease diagnosis, only 20% were mostly or very confident in making such a diagnosis. Barriers between US and EU physicians were similar, with 35-45% indicating sufficient knowledge regarding signs and symptoms that should trigger suspicion, sufficient time to investigate properly, availability of diagnostic guidelines, access to diagnostic tests, and knowing where to refer the patient were all barriers in their practice. When asked about what they consider most effective in rare disease education, case-based, text-based, and short-format education, given by world renowned clinicians were most popular. And a comprehensive online learning platform with current, organized education and resources was considered most valuable. **Conclusions:** This educational research study identifies gaps in

diagnosing rare diseases and supports the need to develop education for physicians on recognizing rare diseases earlier, shortening the diagnostic odyssey and enabling earlier referral and treatment.

256. Treatment of Glutaric Aciduria Type 1 (GA-1) by GCDH Gene Replacement Therapy via Intracerebroventricular AAV9 Delivery

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Glutaric aciduria type I (GA-1) is an autosomal recessive genetic disorder caused by the deficiency of glutaryl-CoA dehydrogenase (GCDH). GCDH contributes to amino acid metabolism and its deficiency results in cerebral accumulation of glutaric acid (GA), 3-hydroxy glutaric acid (3-OH-GA) and glutaconic acid that lead to selective striatal degeneration. Newborns with GCDH deficiency mutations may show macrocephaly and hypotonia. Without proper treatment, patients could die from acute encephalopathic crisis. Current treatments for GA-I patients consist of low-lysine diet in combination with oral supplementation of L-carnitine. Our laboratory has generated a mouse model of GCDH^{c.422_428del/c.422_428del} (GCDH^{-/-}) by CRISPR knockout. The GCDH^{-/-} mice showed significantly elevated GA in the plasma, liver and brain compared to the wild-type C57BL/6 mice. When challenged with high-protein diet for two days, approximately half of the GCDH^{-/-} mice would not survive the metabolic stress. To evaluate whether GCDH gene replacement therapy could be used to provide a sustained treatment for GA-1 patients, we prepared a rAAV9 carrying human GCDH expression cassette (rAAV9-hGCDH) and intracerebroventricularly injected it to the GCDH^{-/-} neonates for a proof-of-concept (PoC) study. The rAAV-treated mice were challenged with high-protein diet at 4 weeks old, then GCDH transgene protein expression, survival rate and GA concentration were analyzed. The rAAV treated GCDH^{-/-} mice showed significantly increased survival in a dose-dependent manner, consistent with the dose-dependent GCDH protein expression observed in the brain. In addition, the rAAV-treated GCDH^{-/-} mice showed significantly decreased brain GA concentration. To summarize, a GCDH deficient mouse model of GA-I was generated and high protein diet induced metabolic stress was used to evaluate and demonstrate the therapeutic effects of AAV-delivered GCDH gene replacement therapy. In light of the positive PoC in the GA-1 mouse model, an Investigator Initiated Trial (IIT) of the rAAV-hGCDH gene replacement therapy has been initiated with dosing of the first pediatric GA-1 patient completed and enrollment of additional patients ongoing.

257. In Vivo mRNA Therapy for Argininosuccinic Aciduria

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Background: Lipid nanoparticles (LNP) encapsulating mRNA are in phase I/II clinical trials for liver inherited metabolic diseases *e.g.* ornithine transcarbamylase deficiency, propionic and methylmalonic acidurias. Argininosuccinate lyase (ASL) converts argininosuccinic acid (ASA) into arginine and is integral to the liver-based urea cycle, which detoxifies neurotoxic ammonia. Inherited ASL deficiency causes argininosuccinic aciduria, which provokes hyperammonaemia, chronic liver disease and neurodisability. Standard of care aims to normalise ammonia with protein-restricted diet, ammonia-scavenger drugs and in severe cases liver transplantation. However limited efficacy of medical therapy and poor quality of life assert the need for novel therapies. **Methods:** We investigated *hASL* mRNA (ASL-LNP) versus Luciferase mRNA (Luc-LNP) containing LNP in ASL-deficient fibroblasts and in the knock-in *Asl^{Neo/Neo}* mouse, which recapitulates the human phenotype with early death and hyperammonaemia. **Results:** ASL-LNP increased ASL activity in ASL-deficient fibroblasts ($p < 0.01$). A single intravenous dose of 1mg/kg in juvenile *Asl^{Neo/Neo}* mice restored liver ASL activity to physiological levels at 24h post-injection ($p < 0.001$) with sustained increase up to 7 days post-injection. Ammoniaemia was normalised at 24h ($p < 0.05$) and reduced by 80% at 7 days post-injection ($p < 0.01$). Weekly intravenous administration from birth up to 7 weeks normalised the mouse phenotype with survival, growth, fur, ammoniaemia, plasma ASA and citrulline, orotate, liver ASL activity and C13 ureagenesis not significantly different from wild-type littermates. Repeated dosing did not increase markers of liver toxicity. A long-term experiment up to 16 weeks confirmed sustained efficacy. **Discussion:** Here, we show proof of concept of LNP.mRNA therapy in ASL deficiency *in vitro* and *in vivo* after systemic delivery. This approach could be of benefit for patients affected by argininosuccinic aciduria.

Frontotemporal Dementia (FTD, OMIM:607485) is the second most common dementia in people under the age of 65 after Alzheimer Disease and is devoid of any cure or specific approved treatment. The incidence of pathogenic loss-of-function mutations in the progranulin gene (GRN) accounts for approximately 5% of all familial FTD cases. 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/Progenitor Cell (HSPC) Gene Therapy (GT) based on the use of lentiviral vectors (LVs) for gene transfer could thus represent a valuable treatment option for GRN-FTD patients as it could generate a long-lasting source of GRN in their brains through its myeloid, microglia-like CNS progeny. To explore this novel treatment option and generate proof of concept preclinical data, we developed and extensively tested therapeutic LVs with novel myeloid/microglia-specific promoters able to safely deliver multiple copies of the human *GRN* cDNA in GRN knock-out (ko) cell models, inducing over-expression of the therapeutic protein which is then correctly secreted and taken up by target GRN knock-out cells. The most promising among these constructs have been tested *in vivo* in optimized transplant conditions enabling CNS-specific and selective engraftment of the transplanted cells and of their progeny. Thus far, we showed that this approach efficiently delivers human *GRN* to the brain of transplanted GRN-ko mice, with a newly designed promoter based on the HLA-DRA promoter sequence resulting in the most robust human *GRN* delivery potential. Cohorts of GRN-ko mice transplanted with this and reference vectors are currently being monitored to accumulate evidence supporting this potential therapeutic approach.

259. 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. Normal *ATXN1* alleles contain 6-42 CAG trinucleotide repeats with interspersed CAT nucleotides, while disease alleles have an uninterrupted CAG region with 39-100+ repeats. The mechanism of SCA1 pathogenesis is unknown; however, some features of the disease include neuronal degeneration and formation of toxic mutant ATXN1 (mATXN1) nuclear inclusions. 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. CRISPR Cas9 is a DNA editing tool used to induce knockout of the target gene. 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, while the second approach employs a dual guide system to delete the CAG repeat region. gRNAs were optimized *in vitro*, with each approach significantly reducing *ATXN1* expression. 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

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258. Development of an Ex Vivo Gene Therapy for Frontotemporal Dementia (FTD)

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reduced levels of mRNA and protein levels by 70-75% ($p < 0.001$) and 45-65% ($p \leq 0.03$), respectively. For testing *in vivo*, SCA1 mice, expressing mutant human *ATXN1*, were crossed to *spCas9* transgenic mice. Recombinant AAVs (rAAVs) expressing the optimized gRNAs from each strategy were delivered directly to the deep cerebellar nuclei of 5-week-old SCA1/*spCas9* mice for transduction of Purkinje cells. The exon-exon strategy reduced protein and mRNA levels by 55% ($p = 0.05$) and 50% ($p = 0.02$), respectively compared to saline injected controls. The dual guide strategy reduced *ATXN1* mRNA levels by 70-80% ($p < 0.001$). This shows that the dual guide strategy is more effective at inducing reduction of *ATXN1* levels *in vivo* and studies are in progress to assess the impact of both strategies on SCA1 mice phenotypes. In a third strategy, we assessed the utility of CasRx, an RNA specific nuclease which binds to target mRNA and cleaves the sequence complementary to the gRNA. CasRx guides designed to target *ATXN1* transcripts reduced *ATXN1* mRNA by 50% ($p < 0.001$) *in vitro*. gRNAs were then delivered via rAAV to the cerebellum of SCA1 mice and the treated cerebellum was collected and analyzed. *In vivo* CasRx resulted in a 10% *ATXN1* mRNA reduction ($p = 0.001$). Currently, we are designing and screening a multiple guide strategy to increase *ATXN1* knockdown. This work indicates several editing strategies to treat SCA1 in mice models with possible translation to human therapies.

260. Robust Suppression of Tau by CRISPR-GNDM® System for Treatment of Tauopathies

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Hyperphosphorylation and aggregation of Microtubule-associated protein Tau (MAPT) have been implicated in a number of pathologic conditions such as frontotemporal dementia and Alzheimer's Disease. Downregulation of MAPT in the central nervous system holds great promise towards a favorable clinical outcome for such diseases. Our proprietary CRISPR-based Guide Nucleotide Directed Modulation (CRISPR-GNDM®) system robustly upregulates or downregulates gene of interests with the help of activation or inhibition moieties which can all be delivered in a single AAV vector. In order to find the best guide RNA, 134 human guides and 96 mouse guides were screened on SK-N-AS human and Neuro-2 mouse neuroblastoma cell lines respectively. SK-N-AS cells transfected with the CRISPR-GNDM® plasmid DNA showed 90% suppression of tau expression. Similar levels of suppression (90%+) were also seen with mouse guides in Neuro-2a cells upon transfection with CRISPR-GNDM® plasmid with the inhibitory moiety. AAV9 encapsulating the CRISPR-GNDM® with the best mouse guide RNA was injected into adult mice via intracerebroventricular (ICV) route ($n = 4$, 3×10^{11} vg.) under the influence of hSyn promoter. As a control, AAV9-hSyn-GNDM with a non-targeting guide RNA was also injected via ICV route ($n = 4$, 3×10^{11} vg). After 3 weeks post-injection, RNAFISH assay showed robust CRISPR-GNDM® expression in certain parts of cortex and hippocampus. MAPT mRNA was drastically eliminated in the cells which are transduced with CRISPR-GNDM® with the therapeutic sgRNA, but not affected in the cells which were not transduced with CRISPR-GNDM® or transduced with CRISPR-GNDM® with non-targeting sgRNA. In order to achieve higher level of transduction efficiency, neonatal mice were injected with AAV9-

hSyn-GNDM containing therapeutic ($n = 4$, 9×10^{10} vg) and control ($n = 3$, 1.4×10^{11} vg) sgRNAs. Mice injected at postnatal day 2 (P2) via ICV resulted in 70% reduction in MAPT expression in cortex and hippocampus 3 weeks after injection compared to uninjected mice, determined by QPCR. The suppression of MAPT was maintained into adulthood to 8-weeks post injection, longest period tested, at 50% both in cortex and hippocampus even though the brain size significantly increased over the 8-week period ($n = 4$, 9×10^{10} vg). In order to test the effectiveness of a viral vector that can effectively pass through blood brain barrier, AAV.PHP.eB-hSyn-GNDM was injected via intravenous route (IV) to 8-week-old adult mice ($n = 3$, 1.9×10^{14} vg/kg). MAPT was suppressed to ~50% in the cortex and hippocampus of the adult mice 3 weeks after injection compared to the untouched mice, determined by QPCR. In conclusion, CRISPR-GNDM® is highly effective and gene-specific technology for downregulating disease-associated genes such as MAPT.

261. Can AAV-Mediated Lysosomal Enhancement Be a New Therapeutic Strategy for Niemann-Pick Type C Disease?

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Niemann-Pick type C disease is a rare and highly debilitating disease caused by deficiency of NPC1 or NPC2 protein leading to accumulation of cholesterol and sphingolipids in the lysosomes of affected cells which results in the broad CNS and visceral symptoms. The most severe manifestations include progressive psychomotor dysfunction leading to premature death with highly variable age of onset, severity, and life span among the patients. Despite many breakthroughs in the NPC research in the last decade, the unmet need remains very high with no approved disease-modifying therapies in the US. One of the established pathological mechanisms explaining impairment of lysosomal function in NPC relies on block of lysosomal Ca²⁺ release due to lipid-mediated inhibition of the lysosomal channel TRPML1 [1]. Activation of TRPML1 channel was shown to reduce excessive lipid accumulation in the NPC *in-vitro* models [1]. We have previously developed AAV vectors that overexpress human TRPML1 channel and showed efficacy of this approach to prevent and restore motor deficits in the TRPML1 KO mouse, a model of another lysosomal disorder mucopolysaccharidosis IV [2]. Here we set out to test whether lysosomal enhancement via AAV-mediated delivery of TRPML1 transgene was able to rescue disease phenotype in the NPC mice. To test this hypothesis, we used scAAV9-JeT-MCOLN1 vector in the well-characterized BALB/cNctr-Npc1m1N/J mouse model of NPC. To achieve broad distribution of the TRPML1 transgene in the CNS, we used intracerebroventricular (ICV) administration to neonatal mice. We detected dose-dependent overexpression of TRPML1 transgene in the NPC mouse brain at the levels that have been associated with the efficacious activity in TRPML1 KO model, and low but detectable expression of the transgene in the liver. We observed reduction of lysosomal aggregation in the brain of NPC-treated mice, as measured by expression of the lysosomal marker LAMP1, in response to the highest dose of scAAV9-JeT-

MCOLN1. Despite successful transgene expression in the brain in NPC mice, and reduction of lysosomal pathology, we observed no rescue in neuromotor phenotype, weight or lifespan of NPC mice, limiting applicability to CNS-targeted TRPML1 activation or lysosomal enhancement as potential treatment for NPC. References: 1. Shen, D., et al., Lipid storage disorders block lysosomal trafficking by inhibiting a TRP channel and lysosomal calcium release. *Nat Commun*, 2012. 3: p. 731. 2. De Rosa, S., et al., MCOLN1 gene therapy corrects neurologic dysfunction in the mouse model of mucopolidosis IV. *Human Molecular Genetics*, 2021.

262. Growing Clinical Gene Testing Considerations in the Era of RNA and Gene Therapy

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There has been exciting progress in the treatment of many diseases, including both cancer and hereditary disorders, using RNA and gene therapies. The Federal Drug Administration (FDA) has recently approved multiple RNA and gene therapies for neuromuscular diseases, and many are at clinical trial stages including several for other neurologic disorders, such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, frontotemporal dementia and spinocerebellar ataxia. These RNA and gene therapies function by replacing disease genes, modulating functional products at a gene level, as well as targeting specific mutations (or types of mutations) (table 1).

Date	Trade Name	Active Ingredient	Disease	Genetic mutation	Function on	Mechanism of action
2015-07-02	Orkambi	lumacaftor/ivacaftor	Cystic Fibrosis	F508del; 2 copies	Selected mutation	Chaperone
2016-09-19	Exondys 51	eteplirsen	Duchenne muscular dystrophy	DMD exon 51	Selected mutation	PMO-ASO
2016-12-23	Spinraza	nusinersen	Spinal muscular atrophy	SMN1 biallelic	Backup gene	ASO (SMN2)
2017-12-19	LUXTURN	voretigene nersarvecetyl	RPE65 - Retinal dystrophy	RPE65 biallelic	Gene	Gene therapy
2018-02-12	Syndeco	tezacaftor ivacaftor	Cystic Fibrosis	F508del; 2 or 1 copies	Selected mutation	Combination
2018-08-10	Galafold	migalastat	Fabry disease	GLA amendable mutation	Amendable mutation	Chaperone
2018-08-10	Onpatro	patisiram	Polyneuropathy hATTR	hATTR mutation	Gene	ASO
2018-10-05	Tegsedi	inotersen	Polyneuropathy hATTR	hATTR mutation	Gene	ASO
2019-05-03	Vyndaqel	tafamidis meglumine	Transferrin Amyloid	Wild type or hATTR	Gene	Transferrin stabilizer
2019-05-24	ZOLGENSMA	onasemnogene APOB-siRNA	Spinal muscular atrophy	SMN1 biallelic	Gene	Gene therapy
2019-10-21	Trikafta	tezacaftor/ivacaftor/tezacaftor	Cystic Fibrosis	F508del; 1 copies	Selected mutation	Combination
2019-12-12	Vyondys 53	golodirsen	Duchenne muscular dystrophy	DMD exon 53 skipping	Selected mutation	ASO
2020-08-07	Evrysdi	risdiplam	Spinal muscular atrophy	SMN1 biallelic	Backup gene	Splicing modifier (SMN2)
2020-08-12	Viltepso	viltolarsen	Duchenne muscular dystrophy	DMD exon 53 skipping	Selected mutation	ASO
2021-02-25	Amondys 45	casimersen	Duchenne muscular dystrophy	DMD exon 45 skipping	Selected mutation	ASO

Molecular-based therapeutics generally require a clinical genetic diagnosis to determine eligibility, and for mutation targeting drugs, only patients affected by “amenable” mutations (a term from chaperone therapy) will benefit. The rapid progress in RNA and gene therapy development and approval has resulted in challenges for clinicians and clinical diagnostic laboratories. We increasingly need clinical genetics testing to identify and molecularly characterize patients for clinical trial enrollment and access to approved drugs. Multiple pharmaceutical-sponsored genetic testing programs were launched to increase access. Early diagnosis is essential with a growing interest in presymptomatic diagnosis for best clinical outcome via presymptomatic treatment. State run newborn screening (NBS) programs provide opportunities for early diagnosis and intervention for all families regardless of socioeconomic status. Yet, NBS on spinal muscular atrophy (SMA) was introduced two years after first FDA approved drug by Recommended Uniform Screening Panel (RUSP) in 2018., and is not available to all states as of spring 2022. Although pharmaceutical-sponsored programs and newborn screening (NBS) have helped with both access and early diagnosis, these programs are not available for

several hereditary conditions that will benefit from molecular-based therapeutics. The availability of approved therapies also brings critical considerations for the clinical genetic testing laboratories to support treating specialty clinics. A central system resource to disseminate information regarding the availability of novel therapies, update clinical diagnostic algorithms, and further build evidence-based review and guidelines is needed to ensure patients receive appropriate care. In summary, RNA and gene therapy are in a race to the clinic. Joint efforts from medical and genetic communities are needed to increase genetic testing access and early diagnosis to unlock the utmost value.

263. Endogenous Human SMN1 Promoter-Driven Gene Replacement Improves the Efficacy and Safety of AAV9-Mediated Gene Therapy for Spinal Muscular Atrophy (SMA) in Mice

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Zolgensma[®] an FDA-approved scAAV9 ubiquitously expressing a human *SMN1* cDNA transgene under cytomegalovirus enhancer/chicken β -actin promoter (*CMVen/CB-hSMN1*), is a significant breakthrough for treating SMA. Nonetheless, a high dose of the vector is required, leading to liver damage and hematologic complications in most patients. Severe toxicities were also found in nonhuman primates and piglets with AAV9-like vectors expressing *hSMN1*. Here, we hypothesize that restoration of physiological levels of *hSMN1* expression in the appropriate cell types with the lowest effective dose of vector may be essential for the next generation SMA gene therapy. By optimizing the coding sequence of *hSMN1* (*co-hSMN1*) under *CMVen/CB* promoter, we increased *hSMN1* protein expression by ~8 fold over the published Zolgensma[®] construct in Neuro2a cells. However, when we treated postnatal day 1 SMNdelta7 mice with this potent vector rAAV9-*CMVen/CB-co-hSMN1* at 3.3 x10¹⁴ GCs/kg by facial vein administration, all treated animals (n = 6) died earlier than the untreated SMA mice. H&E staining of liver sections at day 8 revealed severe liver damage in both SMA mice and their healthy littermates treated with this vector, suggesting that the early lethality was likely to be associated with hepatic overexpression of *hSMN1*. In an attempt to achieve physiologically regulated *hSMN1* expression, we created a second generation (2nd gen) scAAV9 vector expressing *co-hSMN1* from an endogenous *hSMN1* promoter (i.e., scAAV9-SMN1p-*co-hSMN1*) and injected neonatal SMA mice with either 3.3x10¹⁴ GCs/kg (High dose, n = 9) or 1.1 x10¹⁴ GCs/kg (Low dose, n = 12). We also dosed another group of SMA mice with 3.3x10¹⁴ GCs/kg (high dose only, n = 13) of scAAV9-*CMVen/CB-hSMN1* harboring the same expression cassette as used in Zolgensma[®] as the benchmark vector. This side-by-side comparison study revealed that our novel 2nd gen vector has therapeutic potential overcoming the current SMA treatment limitations, which is supported by the following key findings. 1) All the mice injected with the high dose 2nd gen vector survived until the full 90-day study period compared with 60 days median survival from benchmark vector-treated mice. 2) The high dose 2nd gen vector-treated female mice gained more body weight than the benchmark vector

group starting from Day 25, while the low dose group showed similar body weights as the high dose benchmark vector. The average body weights of the treated male mice were equal in all three groups. 3) The high dose 2nd gen vector-treated mice have a faster righting response than benchmark vector-treated mice (Day 3-7 vs Day 7-13). In addition, the low dose group, also had an earlier righting response (Day 3-9) than the high dose benchmark vector group. 4) Rotarod tests of surviving mice at Days 30 and 80 showed comparable performances in all three groups. 5) At Day 60, 67% of mice treated with the benchmark vector developed ear necrosis, while none of the high dose 2nd gen vector-treated mice had ear necrosis at Day 90. In the low dose group, 33% of mice showed ear necrosis at Day 90. 6) The structures of neuromuscular junctions in the 2nd gen vector-treated mice were restored to that of wild-type mice, better than those of benchmark-treated SMA mice. 7) Less liver hSMN1 expression was detected from 2nd gen vector-treated healthy mice than the benchmark vector at Day 3 and Day 8, suggesting its potential to reduce hSMN1 expression associated liver toxicity. In summary, our novel hSMN1 AAV gene therapy vector consisting of the endogenous SMN1 promoter and codon-optimized hSMN1 has improved potency and safety profile as compared to the Zolgensma[®] vector, holding promise for clinical applications.

264. Therapeutic Effect of IL21 Blockage by Gene Therapy in Experimental Autoimmune Encephalomyelitis

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The pathogenic role of the interleukin 21 (IL21) in different autoimmune diseases, such as multiple sclerosis (MS), has been extensively studied. However, its pleiotropic nature makes it a cytokine that may exhibit different activity depending on the immunological stage of the disease. In this study, we developed a gene therapy strategy to block the interaction between IL21 and its receptor (IL21R) by using adeno-associated vectors (AAV) encoding a new soluble cytokine receptor (sIL21R) protein. We tested this strategy in a murine model of experimental autoimmune encephalomyelitis (EAE), obtaining different clinical effects depending on the time at which the treatment was applied. Although the administration of the treatment during the development of the immune response was counterproductive, the preventive administration of the therapeutic vectors showed a protective effect by reducing the number of animals that developed the disease, as well as an improvement at the histopathological level and a modification of the immunological profile of the animals treated with the AAV8.sIL21R. The beneficial effect of the treatment was also observed when inducing the expression of the therapeutic molecule once the first neurological signs were established in a therapeutic approach with a Doxycycline (Dox)-inducible expression system. All these clinical results highlight the pleiotropicity of this cytokine in the different clinical stages and its key role in the EAE immunopathogenesis.

265. Optimizing MRI-Guided Intra-Striatal AAV Dose Administration in Non-Human Primates

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Direct parenchymal infusion enables precision delivery of AAV vectors to deep subcortical structures, such as putamen and caudate, which are difficult to transduce by AAVs dosed via intra-CSF routes of administration. To ensure maximal coverage with minimum reflux or leakage, we conducted a study in non-human primates (NHP) to optimize intra-striatal dosing procedure. We report the evaluation of infusion volume, flow rate and cannula trajectories in three cynomolgus monkeys of approximately 2.5 years of age. The monkeys underwent bilateral intra-striatal convection-enhanced delivery (CED) using the Clearpoint system with real-time MRI guidance. Gadolinium was co-administered to visualize test article distribution in the brain with regular MRI scans taken during infusion. The animals were monitored for 10 days after dose administration and examined postmortem for tolerability of the dosing procedure. We were able to arrive at optimal dose volumes and flow rates that result in the dosing solution to distribute mostly within the targeted structures with minimal reflux or leakage. Coverage ranged from an estimated 26-38% and 30-56% in the caudate and putamen, respectively. Furthermore, the dosing procedure was well tolerated, and no animals showed adverse clinical signs or remarkable histopathology findings at injection sites in the brain beyond those attributable to the cannula penetration.

266. Evolved AAV Capsids for Gene Therapy of CLN2 Disease

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CLN2 disease is a childhood neurodegenerative disease due to deficiency of TPP1, a lysosomal enzyme amenable to cross-correction. Currently, enzyme replacement therapy (ERT) to the brain is the standard of care for patients, which requires treatment every two weeks directly to the CSF via a permanent Omayo reservoir. Gene therapy is an attractive alternative to ERT. Previously we showed that AAV-targeting of ependymal cells, which line the brain ventricles, allows for recombinant enzyme secretion into the CSF with dramatic extension of life span and clinical benefit in a canine model of CLN2 deficiency. For clinical translation of this approach, however, more efficient capsids are required for better transduction of the target cells. Here, we evolved AAV capsids for improved ependyma cell targeting *in vivo*. We identified peptide modified variants (PM-AAVs) that when delivered to mouse CSF provided levels of expression surpassing the current gold standard for mouse ependyma, AAV4. Our PM-AAVs resulted in up to 1000-fold increase in TPP1 levels in multiple brain areas, extended lifespan, and improved phenotypes at doses significantly lower than is required for AAV4, which could never achieve these levels of correction

even at the highest dose tested. Thus, capsid evolution can improve the translatability and applicability of AAVs for gene therapies for neurodegenerative diseases.

267. Temporal Phenotypic Changes in HD Models for Preclinical Studies

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Mouse models of Huntington's Disease (HD), is a late-onset lethal dominant genetic disorder due to a CAG repeat within exon 1 of Huntingtin (*Htt*), are routinely used to assess emerging therapies designed to treat symptoms or modify disease. HD models include fragment models, knock in models, and transgenic models that provide overlapping and distinct phenotypes to gauge therapeutic development. Additionally, the different HD mouse models present distinct onset, symptoms and disease progression. The objective of this study was to fully characterize 3 distinct model for use in gene therapy pre-clinical studies, culminating in a guidance document for sample sizes based on the study objective (e.g., disease modifying or symptom treatment). We assessed N171-82Q transgenic mice, zQ175 knock-in mice, and BACHD full-length mice in comprehensive behavior tests in early, mid- and late disease stages. Our results suggest the most effective behavioral tests and appropriate sample sizes to detect treatment efficacy in each model at varying ages. We also provide options for early detection of motor deficits while minimizing testing time and training. This information will inform researchers in the HD field as to which mouse model, tests and sample sizes can accurately and sensitively detect treatment efficacy in preclinical HD research.

268. Development of AAV Gene Therapy for ECHS1 Deficiency

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Short-chain enoyl-CoA hydratase (ECHS1) Deficiency is a rare and devastating pediatric disease that currently has no treatments that address the cause of disease. Caused by missense loss-of-function mutations in the ECHS1 gene, patients present with Leigh Syndrome-like symptoms that include severe developmental delays, regressions, encephalopathy, severe hypotonia, and white matter abnormalities. The cytoplasmic protein encoded by the ECHS1 gene, short-chain enoyl-CoA hydratase, is necessary for the oxidation of short-chain fatty acids and branched-chain amino acids within the mitochondrial matrix. As a monogenic, loss-of-function disorder, ECHS1 Deficiency is a viable candidate for gene replacement therapy using recombinant adeno-associated virus (rAAV) delivery. We developed two vectors with either a strong or weak ubiquitous promoter drive expression of a codon-optimized form of the human ECHS1 coding sequence. To confirm transgene expression and optimize dosing, our vectors were transfected into HEK293T cells. Quantitative PCR and western blotting confirmed a dose-dependent increase in ECHS1 mRNA and protein expression. Additionally, immunocytochemistry revealed co-localization of ECHS1

protein with mitochondrial protein HSP60, indicating the protein encoded by the transgene correctly translocates to the mitochondria. We've also begun the development of a second-generation vector. In an effort to achieve endogenous ECHS1 expression and regulation, we are exploring the use of the ECHS1 promoter in our vector. At 2.1kb, this promoter is too large for proper AAV packing, so we are assessing truncated forms of the ECHS1 promoter to identify the minimal sequence required to drive gene expression while maintaining some functional regulation. Moving forward, the weak-ubiquitous first-generation vector was used for virus production using a self-complementary AAV9 (sc-AAV9) vector, as this serotype is effective in transducing both neurons and glia along with peripheral organs. Virus was delivered at high and low doses to wildtype mice via intrathecal (IT) lumbar puncture at post-natal day 10 to study biodistribution of viral mRNA and protein at 8-weeks post-injection. Expression was found to be highest in the spinal cord, hind-brain regions, and liver, with moderate expression in other tissues. Long-term toxicology analyses in wild-type mice are ongoing to monitor for potential adverse effects from ECHS1 overexpression. To assess the therapeutic potential of our vector, we used novel ECHS1 transgenic mice, that similar to patients have very low levels of functional ECHS1 protein. Following IT delivery of our vector at P10, we found a significant increase in ECHS1 expression levels across tissues at 3-weeks post-injection. Furthermore, immunohistochemistry revealed vector expression in both neurons and glia throughout the brain, with transduction highest in the hindbrain. Long-term studies to assess functional improvements via disease biomarkers and behavior are still ongoing and results will be reported. Taken together, these preliminary studies support that IT delivery of sc-AAV9/ECHS1 may be a viable therapeutic approach for ECHS1 Deficiency patients.

269. Biodistribution and Comparative Performance of AAV-TT and AAV9 in the Nonhuman Primate Brain

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The adeno-associated viral vector capsid variant known as "true type" (AAV-TT) is a novel capsid with improved CNS tropism and retrograde transport through brain circuits. This retrograde spreading AAV-TT capsid enables neuronal transduction in multiple brain regions innervating the injected area (e.g. multiple targeting to be achieved with one single AAV-TT intraparenchymal deposit). Here we have injected a GFP-coding AAV-TT into the left post-commissural putamen of two adult juvenile *Macaca fascicularis*, and AAV9-GFP in two more animals for comparison. All animals were sacrificed four weeks post-AAV delivery. GFP+ neurons were consistently found throughout the layer V of the cerebral cortex (ipsi- and contralateral to the injected side), the caudal intralaminar nuclei and the substantia

nigra pars compacta. Within the cerebral cortex, cortical areas showing the highest neuronal transduction rates were the precentral gyrus, the superior frontal gyrus, the postcentral gyrus, the insular gyrus and the cingulate cortex. Although both AAV-TT-GFP and AAV9-GFP showed a similar pattern of retrograde transduction, AAV-TT capsid exhibited a slight enhanced transduction when compared to AAV9, as shown by its ability to transduce the dorsal raphe nucleus and the entorhinal cortex. Moreover, uptake of viral vectors through fibers of passage was only observed for AAV9 and not for AAV-TT capsid. These data represent a powerful ability of AAV-TT for transducing projection neurons in multiple brain areas located far away from the injection site, thus opening unprecedented possibilities when using retrograde spreading AAV-TT capsids for disease modeling purposes and for therapeutic interventions in the field of neurodegenerative disorders.

270. Transient Depletion of Pre-Existing Antibodies for Efficient AAV Gene Delivery Treating MPS IIIA in Mice

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As AAV gene therapy offers promise for treating diseases in humans, the high prevalence of pre-existing α AAV antibodies (Abs) in human populations poses a critical challenge in translation. To date, only individuals negative for α AAV-Abs are eligible for AAV gene therapy treatment. The IgG degrading enzyme of Streptococci (IdeS) has been demonstrated to specifically cleave IgG of humans, primates, sheep and rabbit (but not mouse) origin. Studies have shown effective transient IgG degradation by IdeS in animals and humans, with no detectable dose limiting toxicity. To address the challenge of pre-existing α AAV-Abs, we have developed a modified new IdeS protein product, Ab-cleaver (**Ab-C**). When incubated *in vitro*, **Ab-C** was shown to completely cleave human and rabbit IgGs within 6hr. For clinical relevance, we established a rabbitized α AAV9-Ab⁺ mouse model by an IV infusion of purified α AAV9-Ab⁺ rabbit IgG into MPS IIIA mice, resulting in serum levels of α AAV9-IgG at 1:6,400 and α AAV9 neutralizing Abs (α AAV9-NAbs) at 1:200. **Ab-C** Ab depletion was shown to be dose-dependent. An IV **Ab-C** infusion at the effective dose resulted in rapid IgG cleavage and clearance of pre-existing α AAV9-IgG and α AAV9-NAbs in rabbitized α AAV9-Abs⁺ MPS IIIA mice. Importantly, an IV injection of AAV9-hSGSH^{OP} vector at 24hr post **Ab-C** treatment led to effective transduction in α AAV9-Abs⁺ MPS IIIA mice, as in MPS IIIA mice negative for α AAV9-Abs. We believe that **Ab-C** offers a great tool to overcome the pre-existing α AAV-Abs for the translation of rAAV gene therapy to treat diseases in humans. This may broaden the patient eligibility and make rAAV gene therapy available to all patients in need of vector administration and re-administration.

271. Optogenetic Modulation of the Central Nervous System for Pain

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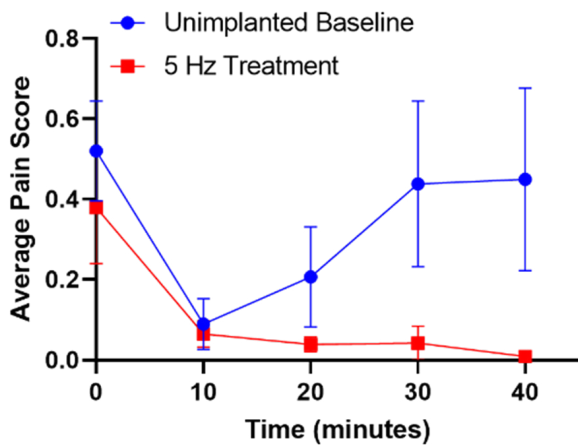
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Introduction: Pain is a common symptom of injury and disease which itself can be a pathological state. The nuanced nature of the pain experience is due to the many neurological structures involved in the pain experience. The Anterior Cingulate Cortex (ACC) is a neurological structure known to be involved in the affective aspects of pain. Here we present a combinatorial drug-device approach for cell-selective modulation of neuronal circuitry in ACC by red light for pain modulation.

Methods: We created a highly sensitive optogenetic actuator Multi-Characteristic Opsin (MCO), which can be specifically expressed in inhibitory (GABAergic) neurons by use of GAD promoter. We also developed a novel Bluetooth Optogenetic Pain Modulator (OPM) device to provide optogenetic stimulation with red (630 nm) light at various frequencies and intensities. We used formalin challenge and sciatic nerve constriction (SNC) as models of acute and chronic neuropathic pain respectively. We measured reflexive pain responses of mice hind paw during optogenetic stimulation and mechanical stimulation hypersensitivity in SNC models.

Results: Optogenetic stimulation of MCO-expressing inhibitory neurons of the ACC led to reduction of reflexive acute pain responses. Reflexive responses to pain were reduced significantly in the second (inflammatory) phase of responses to the presence of formalin. This reduction was specific to this phase even when optogenetic stimulation was initiated 20 minutes before formalin injection and persisted even if stimulation was terminated 20 minutes into the experiment. Taken together, these results suggest that MCO based optogenetic stimulation of the ACC causes changes in pain processing that are effective on the persistent inflammatory pain and the consequent analgesic effect lasts even after cessation of stimulation. Our results also confirm that optogenetic stimulation is effective in reducing chronic pain due to peripheral nerve injury. The threshold for mechanical allodynia measured in SNC mice increased with optogenetic stimulation.

Conclusion: Our results provide support for the viability of modulation of the Central nervous system for pain inhibition. The use of a central target structure allows the same modulation technique to be able to affect both inflammatory and neuropathic pain. The sensitivity of MCO and the programmable nature of the OPM allows a highly customizable cell-specific optogenetic stimulation for effective pain modulation with a low power burden. This technology has potential to manage a variety of difficult-to-treat pain syndromes.



Acute pain responses are reduced with continuous 5 Hz treatment. Formalin assay scores at 10 minute intervals with continuous 5Hz treatment (5 ms pulses at 630 nm) comparing baseline to treated experimental mice. Avg ± SEM. N=7 at baseline and 6 in treatment group.

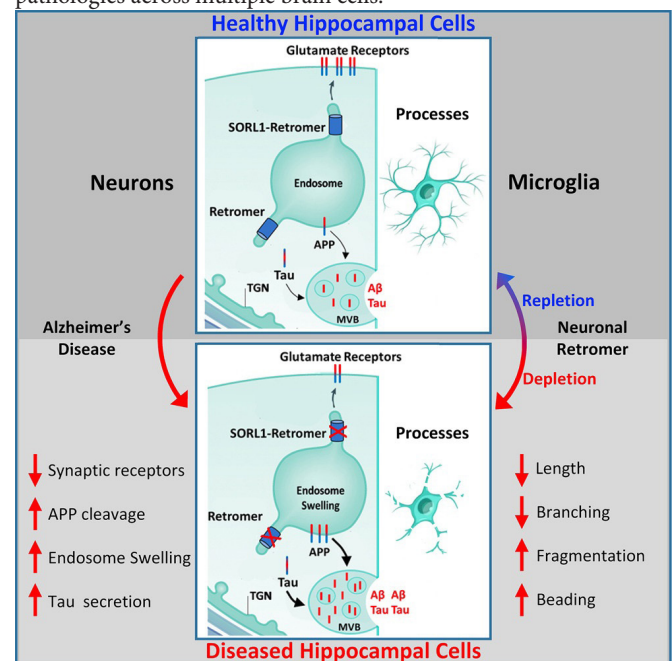
272. Alzheimer’s Associated Neuronal and Microglial Phenotypes are Normalized with Retromer Viral Vectors

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Introduction: The neuron’s early endosome, a central trafficking station of the endolysosomal network, has proven to be an organelle fundamental to Alzheimer’s disease (AD). A combination of genetic and functional studies have implicated endosomal trafficking as a pathogenic biological pathway in the disease. More specifically, recent studies have suggested that the endosome’s recycling routes might mediate AD’s pathophysiology. Endosomal recycling critically depends on retromer, a dedicated trafficking complex that is considered a “master conductor” of endosomal trafficking. Retromer core proteins are deficient in the hippocampal formation of patients with AD, and depleting VPS35, the core’s central protein, has emerged as the most reliable approach for disrupting retromer-dependent endosomal recycling. **Methods:** Attempts to model VPS35 depletion in the mammalian brain have been hindered because VPS35 knockout mice are embryonic lethal. To overcome this challenge, we have recently developed a mouse model in which VPS35 is genetically depleted selectively in hippocampal and forebrain neurons, allowing survival into adulthood. In parallel, we have optimized a VPS35 repletion strategy using AAV9-VPS35, overcoming the autoregulation observed for VPS35 expression, and showing that exogenous VPS35 can bind other retromer core proteins critical for retromer function. We then combined the VPS35 neuronal-selective knockout (VPS35 nsKO) mice with the AAV9-VPS35 protocol and completed an extensive series of

VPS35 depletion-repletion studies to strengthen the mechanistic link between retromer-dependent endosomal recycling and AD’s known cellular phenotypes. For neurons, we relied on prior studies that have associated retromer dysfunction with the pathogenic cleavage of the amyloid precursor protein (APP) and the loss of hippocampal-enriched glutamate receptors, which typify AD-associated amyloid and synaptic pathology. Besides neurons, glia are now considered a second brain cell type phenotypic of AD’s cellular pathologies. We therefore took advantage of our VPS35 depletion-repletion paradigm to ask whether the neuronal retromer will not only regulate AD-associated neuronal phenotypes but might also regulate glial phenotypes. **Results:** The VPS35 depletion-repletion studies strengthen the causal link between the neuronal retromer and AD-associated neuronal phenotypes, including the acceleration of amyloid precursor protein cleavage and the loss of synaptic glutamate receptors. Moreover, the studies show that the neuronal retromer can regulate astrocytic activation, and a distinct, dystrophic, microglia morphology, phenotypic of hippocampal microglia in AD. Finally, by crossing these mice with tau KO mice we show that the neuronal and, in part, the microglial responses to VPS35 depletion were found to occur independent of tau. **Conclusions:** Mechanistically, showing that disrupting retromer-dependent endosomal recycling can regulate both AD-associated neuronal and glial phenotypes strengthens and clarifies a causal link between this trafficking route and AD. Therapeutically, demonstrating that AD-associated neuronal and microglial phenotypes can be rescued by retromer viral vectors in adulthood implies that upregulating retromer function in patients might ameliorate AD-associated pathologies across multiple brain cells.



273. Using Genetically Modified Extracellular Vesicles as Non-Invasive Strategy to Evaluate Brain-Specific Cargo

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The lack of techniques to trace brain cell behavior *in vivo* hampers the ability to monitor status of cells in a living brain. Extracellular vesicles (EVs), nanosized membrane-surrounded vesicles, released by virtually all brain cells might be able to report their status in easily accessible biofluids, such as blood. EVs communicate among tissues using lipids, saccharides, proteins, and nucleic acid cargo that reflect the state and composition of their source cells. Currently, identifying the origin of brain-derived EVs has been challenging, as they consist of a rare population diluted in an overwhelming number of blood and peripheral tissue-derived EVs. Here, we developed a sensitive platform to select out pre-labelled brain-derived EVs in blood as a platform to study the molecular fingerprints of brain cells. This proof-of-principle study used a transducible construct tagging tetraspanin (TSN) CD63, a membrane-spanning hallmark of EVs equipped with affinity, bioluminescent, and fluorescent tags to increase detection sensitivity and robustness in capture of EVs secreted from pre-labelled cells into biofluids. Our platform enables unprecedented efficient isolation of neural EVs from the blood. These EVs derived from pre-labelled mouse brain cells or engrafted human neuronal progenitor cells (hNPCs) were submitted to multiplex analyses, including transcript and protein levels, in compliance with the multibiomolecule EV carriers. Overall, our novel strategy to track brain-derived EVs in a complex biofluid opens up new avenues to study EVs released from pre-labelled cells in near and distal compartments into the biofluid source.

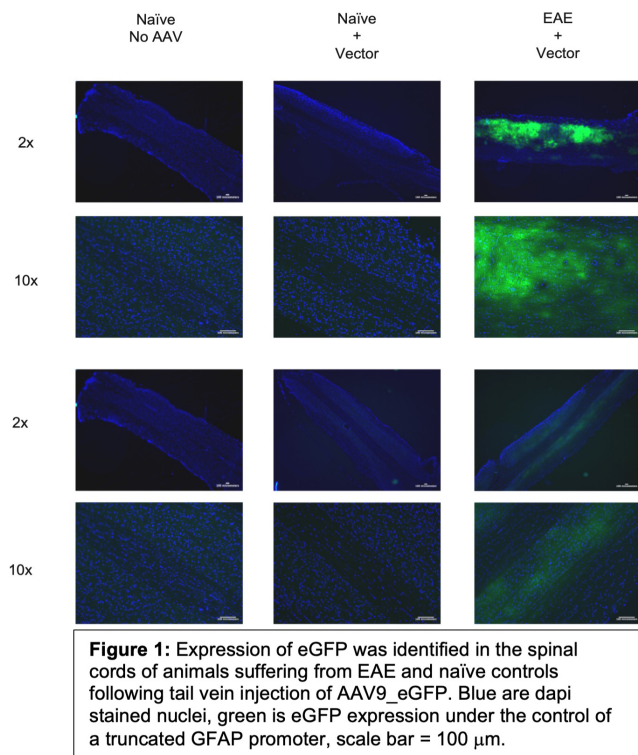
274. AAV9 Crosses the BBB Efficiently in Animals Suffering from Experimental Autoimmune Encephalomyelitis

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Demyelinating diseases are those in which the myelin sheath of neurons is damaged. Demyelination results in impaired signal conduction causing neurological problems: deficiencies in sensation, movement, or cognition depending on which nerves are affected. Multiple Sclerosis (MS) represents the most common demyelinating disease. Within MS, demyelination occurs at local foci or lesions, resulting in nerve damage due in part to chronically impaired remyelination. To date, no treatments successfully improve remyelination in patients. Promoting efficient remyelination within the CNS represents a treatment option for patients suffering from MS and other demyelinating diseases. Adeno-associated virus (AAV) represents an ideal gene therapy due to: small size, non-replicating, able to transduce dividing and non-dividing cells, non-pathogenic to humans, and capable of providing long-lasting transgene expression. In the case of treating CNS

diseases, AAV therapies have historically be hampered by inability to cross the blood-brain-barrier (BBB). In 2009, AAV9 was identified as being capable of inefficiently crossing the BBB. Subsequent new BBB crossing AAV serotypes were discovered and/or developed. AAV PHP.B showed improved efficiencies in some mice, though this serotype was identified to utilize a Ly6a mechanism and is thereby inefficient in humans. In an attempt to improve efficiencies, multiple strategies have been investigated: 1) development of new capsids, yet efficiencies remain less than optimal, 2) higher administered dose, can result in peripheral toxicity (especially in the liver), 3) different routes of administration, though these are invasive, provide localized treatment, and can result in neurotoxicity. An ideal AAV treatment for CNS diseases is one that can be given intravenously, have low toxicity, and provide efficient expression throughout the entire CNS. In the case of MS, the integrity of the BBB is compromised resulting in it being 'leaky'. Thus, we hypothesize that many of the obstacles facing AAV when treating other CNS diseases can be circumvented when treating MS and can be developed to encourage remyelination at lesion sites. In this work, initial studies were performed to determine if a 'leaky' BBB would result in increased transgene expression within the CNS. AAV9 AND AAV PHP.B, encoding eGFP under the control of a truncated GFAP promoter, was injected intravenously into animals suffering from experimental autoimmune encephalomyelitis (EAE), the animal model of MS, and controls. AAV PHP.B was selected as a secondary serotype to be tested due to Ly6a being present in the BBB of the animal model which we postulated would result in increased expression. For control animals, no appreciable expression was identified in the spinal cords of animals treated with AAV9 or AAV PHP.B (Fig. 1). In animals suffering from EAE, significant eGFP expression can be seen in animals treated with both AAV9 and AAV PHP.B (Fig. 1) as compared to controls. Interestingly, increased expression was seen in animals suffering with EAE that were treated with AAV9 as compared to EAE animals treated with PHP.B (Fig1). These studies suggest that AAV9 may represent a powerful treatment for demyelination in MS patients. Efforts are currently underway to determine the optimal dose needed for expression and to determine if liver toxicity occurs.



275. Biodistribution and Activity of a Novel AAV-hFXN Expression Vector in the Rodent CNS

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Background and objective: Friedreich's ataxia (FA) is an autosomal recessive ataxia in which a trinucleotide repeat expansion of the human FXN (hFXN) gene results in deficient expression of the frataxin protein. This frataxin deficiency results in impaired iron homeostasis and dysregulation of mitochondrial respiration and oxidative stress, which ultimately contribute to the clinical presentation of FA. FA has a heterogeneous presentation that commonly features spinocerebellar ataxia and sensory neuropathy, hypertrophic cardiomyopathy, and increased incidence of diabetes mellitus. As FA is a monogenic disease resulting from insufficient gene product, FXN gene replacement therapy is a promising therapeutic strategy for FA. We aim to utilize adeno-associated virus (AAV) as a vector for delivery of the hFXN gene to tissues critically affected in FA to restore functional levels of frataxin and prevent FA disease progression. Here we assessed the vector biodistribution and frataxin expression within CNS tissues of mice injected with a novel AAV-hFXN vector. **Methods:** We engineered a gene expression cassette containing the human FXN (hFXN) sequence under the control of a synthetic Desmin promoter and a modified hFXN

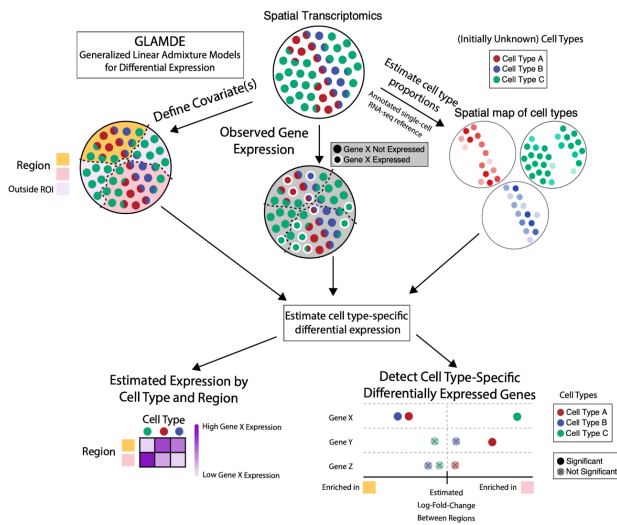
5'UTR. This construct was packaged in AAV serotype 7 for injection into the CNS of 5-week-old WT mice via a combined intraventricular and intraparenchymal route. Animals were harvested 4 weeks post-administration for biochemical and histological examination. **Results:** Combined intraventricular and intraparenchymal delivery of AAV7-hFXN resulted in broad biodistribution across multiple CNS resident cells. Importantly, this novel expression cassette achieved near-physiological frataxin expression within tissues critically affected in FA including cerebellum and dorsal root ganglia. Lastly, no gross histopathological findings have been observed following delivery of this therapeutic candidate via the studied administration routes. **Discussion and Conclusion:** Results of completed and ongoing studies strongly support further development of this promising therapeutic candidate.

276. Cell Type-Specific Differential Expression in Spatial Transcriptomics for Identification of Pathologically-Relevant Genetic Targets

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Spatial transcriptomics enables spatially resolved gene expression measurements at near single-cell resolution. The detection of genes that are differentially expressed across tissue context for cell types of interest is an essential challenge for systematic discovery of genetic targets for gene therapy. However, changes in cell type composition across space and the fact that measurement units often detect transcripts from more than one cell type introduce complex statistical challenges. Here, we introduce a statistical method, Generalized Linear Admixture Models for Differential Expression (GLAMDE), that estimates cell type-specific patterns of differential gene expression while accounting for localization of other cell types. By modeling spatial transcriptomics gene expression as an additive mixture, across cell types, of generalized log-linear functions, we provide a unified framework for defining and identifying gene expression changes for a wide-range of relevant contexts: changes due to pathology, anatomical regions, physical proximity to specific cell types, and cellular microenvironment. Furthermore, our approach enables statistical inference across multiple samples and replicates when such data is available. We demonstrate, through simulations and validation experiments on Slide-seq and MERFISH datasets, that our approach accurately identifies cell type-specific differential gene expression and provides valid uncertainty quantification. Lastly, we apply our method to characterize spatially-localized tissue changes in the context of disease. In an Alzheimer's mouse model Slide-seq dataset, we identify plaque-dependent patterns of cellular immune activity. We also find a putative interaction between tumor cells and myeloid immune cells in a Slide-seq tumor dataset. We envision that our approach can be used to identify relevant targets for gene therapy: genes in specific cell types that are spatially associated with pathology. We make our GLAMDE method publicly available as part of the spacexr R package.



277. Assessment of AAV Regulatory Cassettes with Optimal Hippocampal Neuron Expression for the Treatment of Focal Epilepsy

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Focal epilepsy is a chronic, debilitating neurologic disorder that is characterized by unpredictable seizures initiated from a specific location in the brain and affects millions of people around the world. Recurrent seizures can result in cognitive and emotional deficits, with current interventions offering limited efficacy and multiple side effects. Our approach to treat focal epilepsy is AAV-mediated delivery of a novel engineered ligand-gated ion channel (LGIC) that can subsequently respond to an exogenous ligand. Oral administration of this small molecule ligand, which is designed to only interact with our chimeric LGIC, can be finely tuned to control the aberrant activity of neurons and suppress seizures without adverse effects. To create an effective gene therapy approach for focal epilepsy, we generated a series of AAV expression cassettes that could drive optimal expression of our LGIC in target cells of the hippocampus. The first round of selection was performed *in vitro* with 23 unique expression cassettes containing various combinations of regulatory elements including enhancer, promoter, intron, 5' untranslated region, 3' untranslated region, and polyA tail. Plasmids with ITRs-flanking the regulatory cassettes driving LGIC expression were transfected in SKNAS cells, a neuroblastoma cell line, as well as HeLa cells to determine which cassettes were able to target the cells more efficiently and provide high levels of LGIC expression. Analysis by ddPCR and ELISA showed varying levels of expression, with the highest observed in cassettes containing the CMV promoter followed by those with various tissue-specific promoters. For the second round of selection, 9 expression cassettes chosen from the first round were packaged into AAV9 vectors and transduced in neonatal rat hippocampal mixed cultures. Analysis by ddPCR revealed high levels of LGIC mRNA expression from CaMKII- and Syn-driven cassettes, which were comparable to the strong, ubiquitous CAG promoter. Finally, the highest expressing cassettes from round 2 were assessed *in vivo* in male Sprague Dawley rat hippocampi by

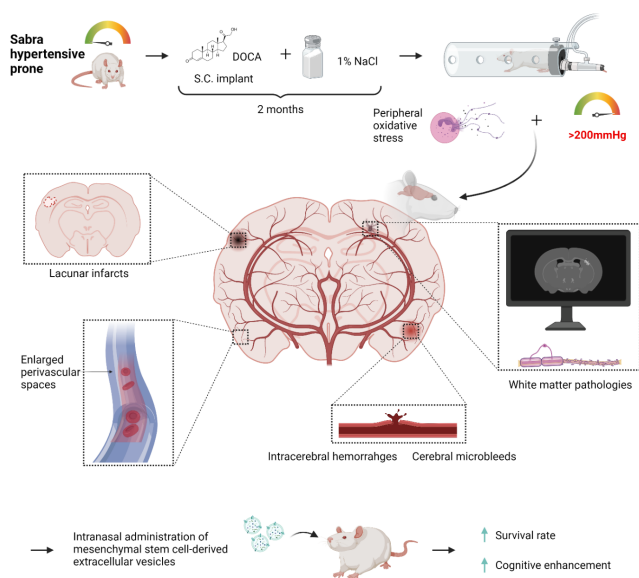
AAV9-mediated directed injection. mRNA analysis using ddPCR and immunofluorescence for cellular tropism demonstrated high levels of expression from human versions of the Syn- and CaMKII-driven cassettes. Further assessment of the two selected cassettes, one driven by the pan-neuronal hSyn promoter and the other by CaMKII, an excitatory cell-specific promoter, will be performed in an animal model of focal epilepsy to finalize the development candidate.

278. Cerebral Small Vessel Disease in Sabra Rats: A Novel Model and Proposed Therapy with Mesenchymal Stem Cell-Derived Extracellular Vesicles

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Background: Cerebral small vessel disease (CSVD) is the second most common cause of stroke and a major contributor to dementia. CSVD manifests in a variety of pathological mechanisms including cerebral microbleeds, intracerebral hemorrhages (ICH), lacunar infarcts, white matter hyperintensities (WMH) and enlarged perivascular spaces. Chronic hypertensive models have been found to reproduce most key features of the disease. Nevertheless, no animal models have been identified to reflect all different aspects of the human disease. Furthermore, no treatment is currently available for CSVD patients. Here, we describe a novel model for CSVD using salt-sensitive 'Sabra' hypertension-prone rats (SBH/y), which display chronic hypertension and enhanced peripheral oxidative stress. In addition, we suggest mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs), which are known for their therapeutic ability in various neurological disorders, as a therapy for CSVD. **Methods:** SBH/y rats were either administered deoxycorticosteroid acetate (DOCA) (referred to as SBH/y-DOCA rats) or sham operated and provided with 1% NaCl in drinking water. Rats underwent neurological assessment and behavioral testing, followed by *ex-vivo* MRI, biochemical and histological analyses. **Results:** SBH/y-DOCA rats show neurological decline and cognitive impairment, and present multiple cerebrovascular pathologies associated with CSVD such as ICH, lacunes, enlarged perivascular spaces, blood vessel stenosis, BBB permeability and inflammation. Remarkably, SBH/y-DOCA rats show severe white matter pathology as well as WMH, which are rarely reported in commonly used models. When treated with MSC-derived EVs, clinical features and cognitive deficits were ameliorated in SBH/y-DOCA rats, suggesting potential therapy for CSVD. **Conclusion:** Our model may serve as a novel platform for further understanding the mechanisms underlying CSVD and for discovery of novel therapies for this disease.



279. The Molecular and Behavioral Phenotypes Altered in CGG Knock-In Mice Edited Using CRISPR

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Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a late onset neurodegenerative disorder that is caused by a premutation allele (55-200 CGG repeats) in the 5' untranslated region of the *FMR1* gene. Mice with approximately 160 CGG repeats knocked into the endogenous *Fmr1* gene exhibit molecular phenotypes and motor and memory impairments similar to FXTAS pathology. We evaluated a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas9 based therapy for its ability to correct *FMR1* trinucleotide repeats in the KI mouse *in vivo*. Dual AAV vectors containing Cas9 and the gRNAs targeting the CGG repeat locus were injected into neonatal mouse pups at P0 to P1 bilaterally into the cerebral ventricles. Motor impairment occurs in KI mice by 28 weeks, and results in quicker falls from the apparatus than is observed in WT littermates. Mice that were treated with the CRISPR constructs performed better than untreated knock-in mice, $p = 0.0041$ and were not statistically different than their WT littermates at 28-30 weeks of age. Robust expression of SpCas9 and GFP were observed in the cortex, hippocampus, striatum, and cerebellum, of treated knock-in animals by qRT-PCR, but no significant reduction in *Fmr1* was observed. These results are consistent with only a subset of cells in these regions being transduced and edited. Long read sequencing was performed to characterize the on-target outcomes of this gene editing strategy. We identified both expected and unexpected editing events (i.e., deletion of CGG repeats and vector sequence integration, respectively), highlighting the importance of fine mapping editing outcomes of this nascent gene therapy technology. Our study is the first to demonstrate *in vivo* editing of expanded CGG repeats in *Fmr1* using CRISPR. This results in a therapeutic benefit, rescuing

motor deficits present in aged KI mice. Long read sequencing and qRT-PCR provide insights into the variation of edits being achieved and how often unexpected editing events occur at the target locus. These results are important indicators that CRISPR mediated gene-editing is worth further development for treatment of FXTAS and other Fragile X-associated disorders.

280. CRISPR-Cas9 Edited Hematopoietic Stem and Progenitor Cells for Friedreich's Ataxia

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Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disorder, caused by homozygous expansion of GAA repeats in the first intron of the frataxin gene (*FXN*). This expansion mutation reduces the expression of frataxin, a mitochondrial protein, required for respiratory complex assembly and iron homeostasis. FRDA is characterized by ataxia, areflexia, sensory loss and muscle weakness; symptoms begin between 5-15 years of age and patients are wheelchair bound by 10-15 years from onset. The predominant cause of death in FRDA patients is cardiomyopathy. Currently, there is no treatment for FRDA. We previously showed that transplantation of wild-type HSPCs could prevent the neurological, muscular and cardiac complications in the YG8R mouse model of FRDA and that tissue rescue was mediated by transfer of frataxin from HSPC-derived microglia/macrophages to diseased neurons and cardiac/muscular myocytes. With the objective of developing an autologous HSPC transplantation approach for FRDA, we have optimized *ex vivo* gene editing protocol for CD34⁺ HSPCs from FRDA patients. Because the GAA repeat mutation is in an intron and is carried by 98% of the patients with FRDA, and overexpression of frataxin is toxic, we developed a CRISPR-Cas9 strategy to excise the expansion and restore therapeutically relevant frataxin levels. Increased frataxin expression and better mitochondrial function resulted from the gene editing in patient cells including CD34⁺ HSPCs. The current study investigates the *in vivo* therapeutic potential of our CRISPR-Cas9 editing approach and its pharmacological implications in YG8s(GAA)_{>800} mice expressing human *FXN* transgene carrying greater than 800 GAA repeats. The analogous bone marrow Sca1⁺ cells isolated from YG8s(GAA)_{>800} mice were *ex vivo* gene edited with our validated gRNAs, transplanted into irradiated, diseased mice and assessed at 2- and 6-months, post-transplant. Sca1⁺ HSPCs show an average 25% hFXN gene editing efficiency, with increase in frataxin mRNA at 7 days post-editing. At 2- and 6-months post-transplant, gene editing was sustained in bone marrow cells, blood and spleen. Primary organs affected during FRDA such as heart, spinal cord and brain also show modest gene editing, confirming engraftment and migration of edited cells to the affected organs. Consistently, increased frataxin protein levels in bone marrow cells, heart and spinal cord of animals receiving edited cells compared to unedited cells was also seen. With this preliminary data, we demonstrate efficient and specific gene editing of hFXN in murine HSPCs, repopulation and proliferation of edited cells within the bone marrow niche for successful engraftment in the diseased organs. These results confirm that our CRISPR-Cas9 gene editing strategy could achieve clinically relevant frataxin levels in HSPCs for future *ex vivo* gene therapy clinical trial for FRDA.

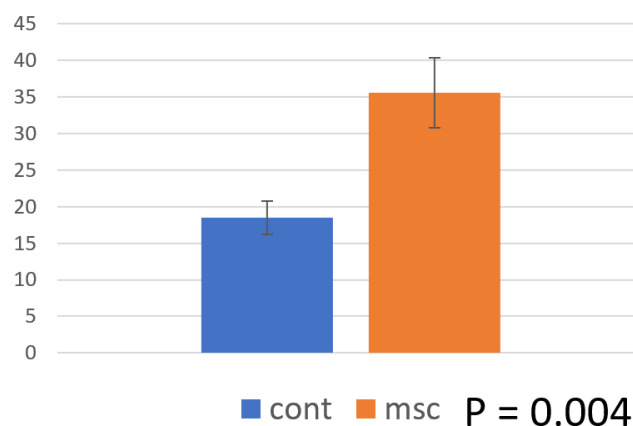
Ophthalmic and Auditory Diseases

281. AAV-Mediated Anti-VEGF Therapy Increases Corneal Allograft Survival Rate in a Model of High-Risk Corneal TransplantationWenqi Su^{1,2}, Shuo Sun¹, Xiao Ke³, Qiang Zheng³, Phillip W. L. Tai⁴, Haijiang Lin¹, Guangping Gao⁴, Bo Tian¹¹Ophthalmology, Umass Chan Medical School, Worcester, MA, ²Ophthalmology, Tianjin Medical University General Hospital, Tianjin, China, ³Chengdu Kanghong Pharmaceutical Group, Chengdu, China, ⁴Horae Gene Therapy Center, Li Weibo Institute for Rare Diseases Research, Umass Chan Medical School, Worcester, MA

Introduction: Due to the immune-privileged status of the eye, human corneal transplants (keratoplasties) generally have superior allograft survival rates compared to solid-organ transplants. Nevertheless, in “high-risk” keratoplasties, allograft rejection rates can be as high as 70% despite immunosuppressive therapy. Corneal neovascularization (CoNV) has been identified as one of the main risk factors for graft rejection. Previous studies have shown that pharmacological blockade of vascular endothelial growth factor (VEGF) or VEGF receptors (VEGFRs) can ameliorate CoNV and improve allograft survival rates in high-risk keratoplasties. However, repeated applications are required because rejection can occur even years after transplantation. In this study, we vectorize KH902 (trade name, conbercept), which is one of well-studied anti-VEGF drugs in clinical testing, and aim to determine if a single dose of AAV-mediated KH902 can increase the survival rate of corneal grafts in a rat model of high-risk keratoplasty. **Methods:** The corneal suture paradigm was used to induce CoNV and generate a rat model of high-risk keratoplasty. Sutures were placed 14-days prior to keratoplasty. At five days prior to keratoplasty, sutures were removed and either AAV-KH902 (5×10^9 genome copies) or PBS was introduced to the cornea via intrastromal injection. CoNV and graft opacity were then assessed weekly using slit-lamp microscopy and graft thickness was measured post-operatively using optical coherence tomography (OCT). Corneal graft rejection was defined as those with moderate stromal opacity where only the pupil margin is visible, and neovascularization enters into the peripheral graft. Additionally, intraocular pressure (IOP) was measured once a week as an ocular safety indicator. Potential inflammation induced by intrastromal injection of AAV-KH902 was also analyzed by quantifying cells positive for the monocyte marker, CD11b in the cornea. **Results:** We observed a significant reduction in CoNV, corneal graft opacity, and to a lesser extent, graft thickness in AAV-KH902 treated rats (N=6) compared with the control group (N=6). The survival rate of the allografts in the AAV-KH902 treated group at two months post-treatment were significantly increased by 33.3% relative to the PBS injected group. In addition, IOP remained within normal range in both groups and minimal CD11b+ cells were found in the corneas at two weeks post-intrastromal injection at this dosage of AAV vector, indicating that the treatment of vectored KH902 does not elicit IOP abnormality and inflammation. **Conclusions:** KH902 delivered by AAV vectors are safe and efficacious for the long-term inhibition of CoNV, which increases the survival rate of corneal allografts in high-risk keratoplasty.

282. Repetitive Intravitreal Administration of Human Mesenchymal Stem Cells in the Sodium Iodate Induced Retinal Degeneration Mouse ModelChang Ki Yoon¹, Kunho Bae¹, Hyunhee Seo², Hyunkyoo Lee², Young Joo Kim², Hyeong Gon Yu^{1,2}¹Ophthalmology, Seoul National University Hospital, Seoul, Korea, Republic of, ²Ophthalmology, Seoul National University College of Medicine, Seoul, Korea, Republic of

Purpose: To evaluate whether repetitive intravitreal injection of human mesenchymal stem cells (hMSC) can have protective effects on retinal degeneration without significant side effects. **Methods:** Intraperitoneal injection of sodium iodate (NaIO₃) was conducted to establish the retinal degenerative disease mouse (C57/BL6) model. Three consecutive intravitreal injection of hMSC (20,000 cells) or PBS were performed with an interval of a week. Retinal function was examined using electroretinography (ERG), and the eyes were harvested for histologic and immunohistochemical analysis. **Results:** The b-wave amplitude of ERG at one week after last injection was significantly higher in eyes with intravitreal hMSC compared to PBS injected contralateral eyes. Outer nuclear layer thickness was also significantly higher in eyes with intravitreal hMSC compared to PBS injected contralateral eyes. Any sign of inflammation was not observed in hMSC injected eye. **Conclusions:** Repetitive intravitreal injection of human hMSC can provide functional rescue of degenerating retina without side effects. These results suggest that hMSC may have therapeutic potential in retinal degeneration.

Electroretinogram b wave**283. Mutation-Independent and Allele-Specific Targeting of Autosomal Dominant Retinitis Pigmentosa Using High-Fidelity CRISPR Nucleases**Kalina Badowska¹, Laura Pezzè¹, Matteo Ciciani², Anna Cereseto², Antonio Casini¹¹Alia Therapeutics, Trento, Italy, ²Molecular Virology Lab, CIBIO, University of Trento, Trento, Italy

Retinitis pigmentosa (RP) is a wide group of genetic disorders causing the progressive degeneration of photoreceptors in the retina, leading in the vast majority of cases to irreversible blindness early in life. The molecular genetics of RP is diverse, with approximately one-third of all cases caused by autosomal dominant mutations (adRP), which are particularly amenable for treatment using CRISPR-mediated allele-specific gene KO, when toxic gain of function mutant proteins are produced. The most commonly mutated gene in adRP is RHO, which encodes the photopigment rhodopsin, however more than 200 different mutations have been identified to-date (HGMD, www.hgmd.cf.ac.uk) and targeting strategies have been reported for only a handful of these so far. We have developed an allele-specific and mutation-independent editing approach exploiting previously reported high-fidelity variants of the SpCas9 nuclease to specifically target RHO mutated alleles. Our high-fidelity variants can selectively distinguish alternative alleles of a high-frequency SNP located in the RHO gene to tag and destroy mutant rhodopsin, thereby stopping disease progression. Our strategy leverages on two basic genetic requirements, besides the knowledge of the mutation affecting RHO: i) heterozygosity for the selected SNP; ii) knowledge of the phase between the SNP's alleles and the mutation in the rhodopsin gene in order to select which of the two alleles to target. We have tested our targeting strategy in vitro using custom-built cell lines expressing two different rhodopsin mutants (P23H and P347L) demonstrating allele-specific editing and downregulation of mutant RHO only when using our high-fidelity Cas9 nucleases. In addition, we verified that no detrimental effects can be observed on the non-target wt RHO allele. Finally, we have determined the safety profile of our approach by measuring genome-wide off-targets of our best performing sgRNAs in combination with our high-fidelity nucleases by using GUIDE-seq and targeted deep-sequencing. In conclusion, we have identified and validated in vitro a safe and effective approach to specifically downregulate toxic mutant rhodopsin expression, independently of the mutation affecting the RHO gene. Further development of this strategy could significantly expand the patient population that may benefit from treatment with this single therapeutic product.

284. Lipid Nanoparticles Targeted to the Optic Nerve Lamina Region Niche as a Potential Genome-Safe Transient Epigenetic Gene Therapy Approach for Age-Related Glaucoma Mediated by Modified Polycistronic mRNA Encoding Epigenome Rejuvenating Oct4, Sox2 and Klf4 Transcription Factors

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Breakthrough retinal rejuvenation has been recently achieved by others upon AAV-mediated ectopic expression of three of the Yamanaka factors (Oct4, Sox2 and Klf4: OSK) in mouse retinal ganglion cells (RGCs). Unlike the full set of Yamanaka factors (OSKM, M: cMyc), polycistronic OSK transduction has been shown to maintain cellular identity while restoring youthful DNA methylation patterns and relevant transcriptomic landscapes, consistent with the described promotion of axon regeneration after injury and reversion of vision

loss in a mouse model of glaucoma and in aged mice. Such a stable in vivo epigenetic gene therapy achievement relies on an hit-and-run protocol in which transient expression of the long-lasting AAV OSK transgene is under the control of doxycycline, thereby prompting us to devise a genome-safe and broader strategy based on lipid nanoparticle (LNP) delivery of short-lived mRNA targeted to both RGCs and neural progenitor cells (NPCs) from the optic nerve lamina region (ONLR). The ONLR is the most anterior portion of the optic nerve, and stands as an adult NPC niche located between the eye and myelinated RGC axons. Importantly enough, the ONLR is the primary damage site in the age-related open angle glaucoma, a major eye disease, and appears to inhibit intraocular myelination and to enable postnatal optic nerve myelination of growing RGC axons, thereby suggesting that epigenome rejuvenation of the adult ONLR-NPC niche involving both NPCs and unmyelinated ONLR-RGC axons could resume an early post-natal axonal regeneration process in an optic nerve CNS background similar to the adult peripheral nervous system (PNS) process mediated by adult schwann cells. Our first approach is thus focused on intravitreal or sub-retinal inoculation of relevant lipid nanoparticles (LNP) in order to evaluate its efficiency for the clinical delivery of nucleoside-modified polycistronic OSK mRNA into both RGCs and ONLR-NPCs, and possibly into other ONLR-niche resident cell types (e.g. oligodendrocyte precursor cells). However, our current main focus is the improvement of LNP targeted specificity using selective organ targeting (SORT) and tissue/cell-specific emerging approaches, thereby providing the means to initiate pre-clinical trials on a porcine surgery model (ophthalmologist surgical training) before moving to an age-related glaucoma dog model. Our approach based on genome-safe delivery of nucleoside-modified mRNA is discussed in light of 1) the transient epigenetic/epigenomic arm of our proposed universal stem cell gene therapy platform, 2) published organ/tissue-specific delivery of mRNA/ribonucleoprotein (RNP) mediated by lipid nanoparticles, 3) induced pluripotent stem cell genesis and in vivo teratoma induction, 4) epigenetic memory and epigenetic information theory of aging and 5) potential synergistic use of senolytics and/or dietary complements (e.g. nicotinamide riboside and pyruvate).

285. Nonclinical Safety and Pharmacology of an Investigational Gene Therapy Targeting the Complement Pathway for the Treatment of Geographic Atrophy Secondary to Age-Related Macular Degeneration (GT005)

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Introduction: Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in the industrialised world, accounting for 8.7% of all cases of blindness worldwide. Complement dysregulation has emerged as a key driver of AMD pathology, with genes of the alternative pathway strongly associated with the risk of developing this disease. Here we report nonclinical safety and pharmacology data for GT005, an investigational AAV2 expressing human Complement Factor I (hCFI), a complement regulator, after subretinal delivery in non-human primates (NHP). **Methods:** GT005 was assessed in a 6-month NHP GLP toxicology study after bilateral

subretinal injection at low dose (LD) 7×10^{10} and high dose (HD) 3.5×10^{11} vg/eye (3 animals/sex/dose). Optical coherence tomography, intraocular pressure, electroretinography, vector shedding, ELISPOT, antibodies to capsid and hCFI, and hCFI levels in vitreous humor (VH) were measured throughout the study. Histopathology, vector biodistribution and hCFI expression in tissue were evaluated at termination. **Results:** There were no systemic effects of GT005 observed and no treatment-related findings outside the eye. There were histopathology findings in the injection area for both doses, with variable severity. Mononuclear cell infiltrates were found in ocular tissues, optic nerve, optic disk, VH and iris/ciliary body. Loss of RPE and photoreceptors was detected from week (wk) 13. These changes correlated with a significant anti-hCFI antibody response in serum and VH of most treated NHPs at wk 13 and 26 ($P < 0.01$). Serum levels of antibodies to AAV2 at wk 4 were weaker and remained constant at wk 13 and 26. No T cell responses to hCFI or AAV2 were found in NHPs, except for one animal in the HD group which developed a significant response against AAV2. Vector biodistribution was similar for both doses with levels highest at the dose site in ocular tissues and falling to below the limit of detection in most organs. Vector shedding was confined to tear samples up to wk 4. hCFI mRNA and protein expression was observed in the outer retina and hCFI protein was detected in VH from 2 wks up to 26 wks. Maximal levels of hCFI were found in the VH at wk 4 in the HD (1437 ± 551 ng/mL) and at wk 13 in the LD (805 ± 249 ng/mL). Reduced hCFI levels at later timepoints correlated with antibody titres to hCFI ($R = 0.83$; $P < 0.001$). hCFI mRNA expression was detected by in situ hybridization (ISH) primarily in the RPE, photoreceptor cell layer, inner nuclear layer and ganglion cells. Signal was higher in the bleb area, gradually decreasing beyond the injection area, and was absent in the far peripheral retina. CFI immunolabelling was detected in all retinal layers, from the RPE to the ganglion cells. Immunolabeling was higher in the bleb area, coinciding with high CFI mRNA expression signal, and decreased further away from the bleb area. **Conclusions:** GT005 was well tolerated with no safety findings outside the eye. GT005 led to local hCFI expression and wide-spread secretion of hCFI protein. Ocular dose-dependent inflammation-induced changes were observed, and correlated strongly with anti-hCFI antibody generation. These were deemed to be species-specific and therefore unlikely to translate to the clinic. These data supported the clinical development of GT005 for the treatment of GA secondary to AMD.

286. Development of Hydrogel Implants for the Sustained Delivery of Adeno-Associated Viruses in Ocular Gene Therapy

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Purpose: The objective of this study was to evaluate the feasibility of a hydrogel platform for the controlled delivery of AAV vector in rat eyes. **Methods:** We optimized several factors affecting the transduction efficiency and release kinetics of AAVs encapsulated in a hydrogel implant. To assess platform compatibility with AAV-GFP vectors, AAV2, AAV2.7m8, and AAV8 were exposed to hydrogel processing conditions, and infectivity with Human Embryonic Kidney (HEK293T) cells was evaluated via flow cytometry. Release kinetics were tuned by

changing hydrogel formulation parameters and assessed first with gold nanoparticles (AuNP), followed by confirmation with AAVs. Implants created with AAV2 vector with a CMV promoter-driven expression of luciferase (AAV2-CMV-Luc) in a hydrogel were injected intravitreally into both eyes of Sprague Dawley rats. These were compared to a single bolus administration of AAV2-CMV-Luc in solution at two different doses (1x and 4x). Luciferase expression was evaluated by measuring luminescence intensity using in vivo imaging system (IVIS) at various timepoints through Day 28. **Results:** Transduction efficiency of AAVs following hydrogel processing was comparable to the control vectors (Figure 1), demonstrating the compatibility of the hydrogel platform with different serotype AAVs. Release kinetics of AAV from implants could be tuned through hydrogel formulation parameters, and a formulation with limited initial release from Day 0 to 1 and complete release by Day 4 was chosen for the rat study. From the IVIS results, luminescence intensity increased over time in transduced eyes and a dose response was seen with greater luminescence in higher dose groups. Additionally, AAV2-CMV-Luc releasing hydrogel implants successfully transduced ocular tissues in vivo and demonstrated similar trends to the bolus controls. **Conclusions:** This study demonstrated a hydrogel-based sustained delivery platform with a tunable release profile was compatible with AAV vectors and capable of transducing ocular tissues in vivo. These results suggest that the use of a hydrogel platform for controlled delivery of AAVs in ocular gene therapy is feasible. **Figure 1:** Hydrogel processing effects on AAV2, AAV2.7m8, and AAV8 transduction efficiency in HEK293T cells at an MOI of $1E+5$, $1E+5$, and $1E+6$, respectively (A+B). Hydrogel formulation tunability on AuNP and AAV release kinetics (C+D).

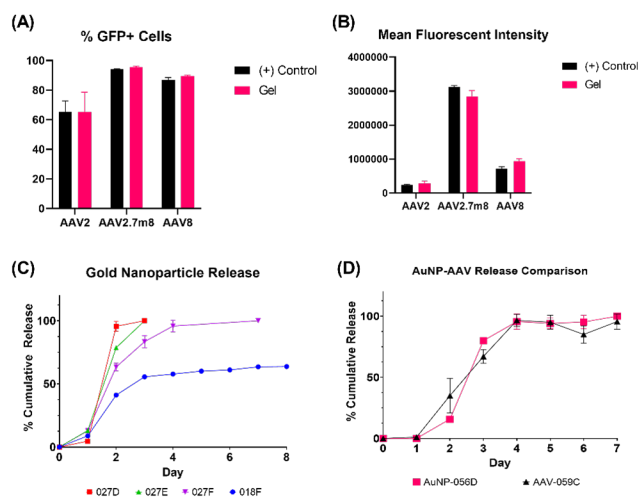
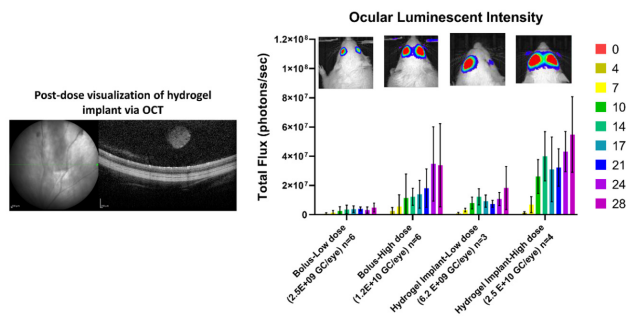


Figure 2: Luciferase expression following sustained release AAV2-CMV-Luc gene therapy from a hydrogel implant in rat eyes.



287. Role of Melanin in Phototransduction in the Eye

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Different from uveal melanocytes (i.e., iris stroma, ciliary body, choroid) that are derived from the neural crest, melanosomes in the retinal pigment epithelium (RPE) cells are derived from the neural ectoderm. The function and role of melanin in these tissues are not fully understood, though there are many reports emphasizing the protective role of melanin in different ocular diseases. It is known that melanin biosynthesis is absent in adult RPE cells and that melanin content (i.e., quantity and quality) reduces with increasing age. One of the key functions of RPE is turnover and clearance of photoreceptor outer segments (POS) which adds a strong load of free radicals to the post-mitotic RPE cells. But there is no clear evidence of how mitotically quiescent RPE cells are involved in phagocytosis and the key players behind phagocytic mechanism. We hypothesize that the presence of abundant melanin in the RPE plays a key role in phagocytosis of POS by preventing the accumulation of free radicals and in maintenance of retinal health. Our initial results show that compared with wild type (WT) mouse, the melanin concentration in the RPE is decreased in rhodopsin knockout (Rho^{-/-}) and P23H^{-/-} mice. We also observed that PKA- α and Tyrosinase are present in the retina but are absent in RPE cells in all the groups (WT, albino, Rho^{-/-}, and P23H^{-/-}). Interestingly, we found that TRP-1 is present in the RPE of Albino mice, but not in the RPE of the WT, Rho^{-/-}, and P23H^{-/-}, which suggests that it might play a compensatory role for Tyrosinase in Albino mice. We have also identified changes in the level of MerTK and Integrin, proteins that affect pigmentation and melanosome transport, in proportion with the melanin level in the RPE. Thereby, we demonstrated that melanin is one of the key factors involved in elimination of POS in the RPE cells; henceforth, they have the potential to play a therapeutic role in many retinal disorders.

288. Evaluation of rAAV Serotypes in the Murine Anterior Chamber for the Treatment of Glaucoma

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Purpose: Glaucoma affects approximately 2.9 million Americans and is characterized by retinal ganglion cell loss and subsequent optic nerve degeneration, ultimately leading to blindness. While effective medications exist for the treatment of glaucoma, they are critically undermined by patient compliance and adherence. As such, development of a singly administered, enduring treatment for glaucoma would be greatly beneficial for this patient population. Herein, we evaluate the tropism profile of nine recombinant adeno-associated viral vector (rAAV) serotypes for downstream use in the development of a glaucoma gene therapy. **Methods:** C57BL/6J mice (n=24 n=48 eyes) were bilaterally intracamerally injected with 2 μ L of either rAAV2/1, 2/2, 2/2[*MAX*], 2/2[7m8], 2/5, 2/6, 2/7, 2/8 or 2/9 vector packaging a green fluorescent protein reporter gene (GFP) driven by a ubiquitously expressing chicken beta actin promoter at concentrations greater than or equal to 2.21×10^{12} viral genomes/ml. 8-weeks post injection, animals were assessed *in vivo* using confocal scanning laser ophthalmoscopy to determine GFP expression. Images were acquired at two planes focusing on both the superficial cornea to determine expression within the three corneal layers, as well as deep anterior chamber to determine expression within the peripheral cornea and iris. After completion of *in vivo* assessments, animals were euthanized, and eyes enucleated for histological use. Half of the eyes were taken for flat mount evaluation wherein samples were stained for ZO-1 to determine endothelial cell transduction for each serotype. Contralateral eyes were processed for cryosectioning to determine tissue transduction in the anterior chamber. **Results:** cSLO imaging completed at 8 weeks post-injection revealed that rAAV2/2 showed the least amount of transduction with sparse transduction in the iris and corneal endothelium. 2/2[*MAX*] and 2/2[7m8] primarily transduced the iris particularly around the pupil. rAAV2/1, 2/5, 2/6, 2/7, 2/8, and 2/9 successfully transduced the cornea. When analyzing corneal flat mounts, we found that rAAV2/2, 2/2[*MAX*], 2/2[7m8] sparsely transduce the corneal endothelium. Serotypes 2/1, 2/7, 2/8, and 2/9 while showing some transduction in the corneal endothelium, mainly transduce the stroma. rAAV2/5 and 2/6 were the most successful in transducing the corneal endothelium with rAAV2/5 showing the strongest signal. Cryosectioning confirmed these findings. **Conclusions:** Herein we evaluate the tropism of several rAAV serotypes within the anterior chamber of C57BL/6J mice. While rAAV2/1, 2/6, 2/7, 2/8, and 2/9 show subtle transduction in the corneal endothelium, rAAV2/5 was the most successful at transducing corneal endothelial cells while minimizing transduction within iris. As the most efficient transducer of corneal endothelial cells, rAAV2/5 may prove to be an attractive candidate for the future development of gene therapies for the treatment of glaucoma.

289. Proteomic Profiling of Treatment Effects After Subretinal Gene Augmentation Therapy for *PDE6A*-Linked Retinitis Pigmentosa

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Loss-of-function mutations in the *PDE6A* gene encoding the rod photoreceptor-specific phosphodiesterase 6 (PDE6) cause autosomal recessive retinitis pigmentosa. Patients initially suffer from night blindness, followed by loss of peripheral vision and gradual visual field constrictions, eventually culminating in total blindness. Currently, no curative treatment option for RP exists. However, over the past few years great scientific effort has been made to develop a potentially curative gene augmentation approach using recombinant adeno-associated virus (rAAV) vectors. rAAV8.hPDE6A, a translatable vector that was created and optimized for specific and efficient expression of full-length human PDE6A in rod photoreceptors under the control of a human rhodopsin promoter, was previously shown to rescue rod function and photoreceptor morphology in the *PDE6A* mutant dog. In this work, we investigated treatment effects at the proteomic level in *PDE6A* mutant dogs after subretinal injection of rAAV8.hPDE6A using mass spectrometry. Proteomic profiling of untreated *PDE6A* mutant dogs revealed downregulation of proteins involved in visual perception, phototransduction, retinal cell development, and retina layer formation and upregulation of inflammatory process and autophagy proteins compared to wild-type control. Furthermore, there was also enhancement of proteins involved in processes such as axon extension and dendrite development probably indicating neuronal sprouting as a consequence of reduced retinal signal transduction. *In vivo* optical coherence tomography and electroretinography demonstrate preservation of retinal structure, restoration of rod mediated function and preservation of cone function after treatment with rAAV8.hPDE6A. This was reflected in the proteomics analysis. In particular there was normalized expression of proteins involved in visual processes such as phototransduction, visual perception and retinal development, and lower levels of proteins involved in inflammatory and neurite outgrowth processes in treated retina compared to untreated retina. In conclusion, results from proteomic analysis provide evidence for successful normalization of the pathologically changed protein expression after one-time subretinal gene therapy with rAAV8.hPDE6A.

290. Analysis of Ocular Distribution and Cell-Type Specificity for ADVM-062, Gene Therapy for Blue Cone Monochromacy

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Blue cone monochromacy (BCM) is a debilitating, rare X-linked retinal disease resulting from the congenital absence of both L- and M-opsins, that causes severely impaired color discrimination, low vision, nystagmus, and photosensitivity. Currently there is no cure for BCM, but recent progress in gene therapy in several retinal diseases makes BCM a potential indication for gene augmentation therapy. Subretinal injection of recombinant adeno-associated virus (AAV)-based gene therapy to restore opsin expression in foveal cones poses a risk to the BCM patients' fragile central retinal structure. ADVM-062 is an AAV.7m8-based vector optimized for cone-specific expression of human L-opsin via single intravitreal injection (IVT). ADVM-062 utilizes a proprietary promoter that consists of an opsin locus control region and a minimal M-opsin promoter to express human L-opsin specifically in cones and provides efficient transduction of foveal cones in primate retina when delivered IVT. The present study evaluated the intraocular biodistribution of IVT delivered ADVM-062, including the pattern of ocular expression and cone cell specificity of human L-opsin (OPN1LW) transgene using a BaseScope approach, an in-situ hybridization assay optimized to detect human L-opsin transgene mRNA and vector genomes (vgs) in the eyes of *Cynomolgus* monkeys. Due to the high similarity of nucleotide sequences of transgene and regulatory elements in non-human primates (NHPs) and humans, the assay utilized a 3'UTR- poly A signal junction, that provided a unique sequence tag for the recognition by the BaseScope assay. This approach enabled the detection of ADVM-062 expressed human OPN1LW transgene in NHPs over the nearly identical NHP OPN1LW background. Evaluation of vector genome biodistribution in ocular tissues identified vg presence in anterior and posterior tissues including retina, optic disc, ciliary body, and iris. In general, vector genome biodistribution agreed with earlier studies of AAV2.7m8 vector that utilized GFP as the reporter. In retina, vector DNA was abundant in the retinal ganglion cell layer photoreceptor layer, and to a lesser extent the inner nuclear layer in the peripheral retina. DNA removal by DNase treatment was utilized to unmask mRNA signal from the widely present vector genome signal. Following DNase treatment, OPN1LW transgene mRNA was observed exclusively within cone cells of the fovea and peripheral retina. No OPN1LW transgene was identified in other retinal cells outside of the retina. The exclusive cone specificity of ADVM-062 driven OPN1LW expression was further supported by the immunolocalization study that used AAV.7m8-MNTC-OPN1LW.myc surrogate express myc-tagged hLOpin for detection of the transgenic protein by myc immunofluorescence. The cone-restricted expression of human L-opsin driven by IVT-injected ADVM-062 supports the development of ADVM-062 as gene therapy for the treatment of BCM.

291. iPSC-Derived Retinal Pigment Epithelium Replacement Therapy for Macular Degeneration: From Bench-to-Bedside

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Induced pluripotent stem cells (iPSCs) can provide autologous and allogeneic replacement tissues, potentially all degenerative diseases. Autologous tissues have the advantage of not requiring immune-suppressive drugs that are known for their side-effects. However, feasibility of autologous iPSC-based therapies hasn't been established. Here, we developed an autologous iPSC-based therapy for age-related

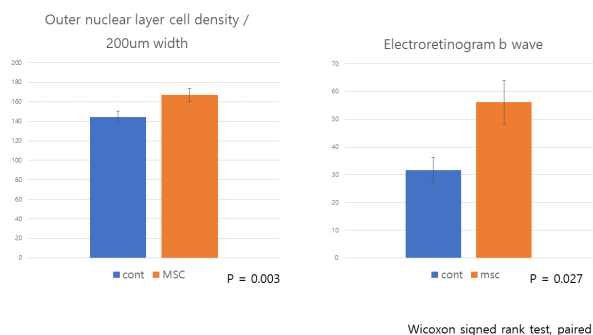
macular degeneration (AMD), a blinding eye disease that affects over 30 million people world-wide. AMD is caused by the progressive degeneration of retinal pigment epithelium (RPE), a monolayer tissue that maintains photoreceptor function and survival. Combining developmental biology with tissue engineering we developed clinical-grade iPSC-derived RPE-patch on a biodegradable scaffold. This patch performs key RPE functions like photoreceptor phagocytosis, water transport, and polarized cytokine secretion. We confirmed the safety of this patch in an immune-compromised rat model and confirmed its efficacy in a swine RPE injury model. A phase I/IIa Investigational New Drug (IND)-application for iPSC-derived ocular tissue to treat AMD was recently cleared by the FDA. This Phase I/IIa clinical trial will test safety, feasibility, and integration of an autologous iPSC-derived RPE-patch in twelve advanced AMD patients. This work is helping leverage other similar autologous cell therapies in various other degenerative diseases.

292. Intravitreal Administration of Human Mesenchymal Stem Cells in the RCS Rat Model of Inherited Retinal Degeneration

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Purpose: To evaluate whether intravitreal injection of our human mesenchymal stem cells (hMSC) can have protective effects on retinal degeneration. **Methods:** Royal College of Surgeons (RCS) 3-weeks-old rats, immunosuppressed with cyclosporine A, received intravitreal injection of hMSC (50,000 cells) or PBS. Retinal function was examined using electroretinography (ERG), and the eyes were harvested for histologic and immunohistochemical analysis. **Results:** The b-wave amplitude of ERG at 2 weeks after injection was significantly higher in eyes with intravitreal hMSC compared to PBS injected contralateral eyes. Outer nuclear layer cell density was also significantly higher in eyes with intravitreal hMSC compared to PBS injected contralateral eyes. At 4 weeks after injection, the ERG signal decreased. **Conclusions:** Intravitreal injection of human hMSC can provide functional rescue of degenerating retina, although the effects were attenuated over time in this rat model. These results suggest that hMSC may have therapeutic potential in retinal degeneration.



293. Optogenetic Gene Therapy for Treatment of Inherited Retinal Disorders in a Gene Agnostic Manner

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Introduction: Inherited Retinal Disorders (IRDs) are caused by mutation of genes leading to dysfunction and/or degeneration of the photoreceptors and retinal pigment epithelium, thereby leading to blindness. For example, vision loss in retinitis pigmentosa (RP) is due to photoreceptor degeneration whereas dysfunctional photoreceptors is the cause for vision loss in Stargardt disease. There is no available therapy for treatment of such IRDs. Recent advancements in cell and gene therapy are opening new avenue for addressing the unmet need. However, classical gene therapy requires delivery of functional copy of genes to replace the non-functional gene or correct the specific gene mutations. The process is complicated as hundreds of gene mutations are known to be associated with IRDs and thus, poses significant challenge to develop efficient therapy. Optogenetic gene therapy offers treatment in a mutation-agnostic manner by photosensitizing higher order neurons (bipolar or retinal ganglion cells) of retina. Since this approach focuses on disease phenotype versus a specific genotype deficit, it is applicable to a wide patient population with outer retinal dystrophy. Here, we report results of vision restoration in various IRD models in ambient light environment upon intravitreal delivery of AAV-carried Multi-Characteristic Opsin (MCO). **Methods:** RD10 mice mimicking slow photoreceptor degeneration, as in human disease, was used as model for RP. ABCA4^{-/-} mice were used as model for Stargardt disease, and RPE65 mutated mice were used to emulate Leber Congenital Amaurosis (LCA) conditions. AAV2 was used to deliver MCO into specific retina cells *via* intravitreal injection. The efficacy of the optogenetic MCO therapy was assessed by visually guided behavior (in a radial-arm water maze assay) and other functional assessments including Electroretinography (ERG) and Visually Evoked Potential (VEP). Further, the safety of MCO-enabled vision restoration therapy was evaluated by measurement of Intraocular Pressure (IOP), Pro/anti-inflammatory cytokines, Optical Coherence Tomography (OCT) and Immunohistochemistry. **Results:** MCO based photosensitization of cells led to fast and ambient light activation over a broad spectral range. Intravitreal delivery of AAV2-MCO in animal models of various IRDs (RP, Stargardt, and LCA) led to robust expression of targeted higher-order neurons in photoreceptor-degenerated/dysfunctional retina. Longitudinally measured visually guided behavior in radial-arm water maze at ambient light levels showed decreased latency and errors in mice treated with AAV2-MCO as compared to AAV2-controls irrespective of gene mutations in the investigated models for RP, Stargardt and LCA. Improved retinal photosensitivity after MCO-transduction was evidenced in the functional electrophysiology assessments (ERG, and VEP). In addition, AAV2-MCO treatment led to maintained retinal thickness (assessed by OCT B-Scans). **Conclusions:** By delivering MCO encoding genes, residual retinal neurons take on the photosensitizing function of the natural photoreceptors. This comprehensive study on different IRD animal models shows significant improvement in vision restoration after intravitreal AAV2-MCO

delivery. The MCO optogenetic gene therapy has potential to address the unmet need in IRDs for being gene mutation agnostic, without requiring external device for stimulation and associated phototoxicity.

295. A New Mouse Model of DFNB7/11 Recovers Broad-Spectrum Auditory Sensitivity After *Tmc1* Gene Therapy

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Transmembrane channel 1 (TMC1) forms the pore of mechanosensory transduction channels in mature cochlear hair cells in mice (Pan et al., 2013; 2018, *Neuron*) and humans. In humans, more than 65 *TMC1* mutations have been identified that contribute to recessive nonsyndromic hearing loss, DFNB7/11. Some mutations are associated with congenital deafness, which may be difficult to treat with postnatal gene therapy. Other mutations yield milder phenotypes with moderate-to-severe hearing loss that progresses during the first decade of life (Imtiaz et al., 2016, *Neurogenetics*). We generated a mouse model with a TMC1 p.N193I mutation, equivalent to the human TMC1 mutation p.N199I (c.596A>T), which causes moderate-to-severe progressive hearing loss during childhood. Unexpectedly, homozygous p.N193I mice were profoundly deaf beginning at postnatal day (P) 16, the earliest time point tested. Additionally, hair cells expressing homozygous TMC1 p.N193I on a *Tmc2*-null background exhibited lack of sensory transduction as assessed by FM1-43 uptake and electrophysiological recording from hair cells during mechanical stimulation of the hair bundle. Unlike *Tmc1*-null mice, the number of surviving hair cells in homozygous p.N193I mice at P21 was similar to that of WT mice, suggesting the possibility of a broader window for therapeutic intervention. AAV vectors that encoded human *TMC1* were injected into the inner ears of homozygous p.N193I mice at P1. Injection of *hTMC1* vectors yielded recovery of auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) to near wild-type levels. The auditory recovery was found to be durable until six months post-injection, the latest time point tested. Wild-type and mutant animals injected with AAV-*hTMC1* did not exhibit any sign of viral induced toxicity. We conclude that *Tmc1*^{N193I} mice carrying the equivalent of the human p.N199I mutation are an excellent model for studying moderate-to-severe DFNB7/11 hearing loss. Furthermore, we report unprecedented recovery of ABR and DPOAE thresholds using optimized AAV constructs encoding *hTMC1*. These results demonstrate that early and efficient gene therapy intervention can prevent hearing loss in mice, suggesting potential translation for treatment of DFNB7/11 patients.

296. Laterally Spreading AAV.SPR-hRS1 Vector for Treatment of XLRS

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Purpose: The presence of schisis cavities in the central retina has hampered safe and effective delivery of AAV-RS1 to XLRS patients. The novel AAV.SPR capsid laterally spreads well beyond the margins of the subretinal injection (SRI) bleb. This provides the ability to mediate efficient expression of RS1 in both peripheral and central retina without detaching the fovea, and in so doing avoiding schisised retina. The purpose of this study was to evaluate the performance of AAV.SPR vectored RS1 relative to benchmark AAV5. Efficacy of AAV.SPR and AAV5 vectors carrying the photoreceptor specific human rhodopsin kinase promoter (hGRK1) driving human RS1 (hRS1) were evaluated in *Rs1* knock-out (RS1KO) mice. SRI of AAV.SPR and AAV5 vectors expressing green fluorescent protein (GFP) or myc tagged human RS1 (mycRS1) were performed in cynomolgus monkey (NHP), followed by in-life and post mortem assessment of the extent of retina transduction. **Methods:** GFP, hRS1 or mycRS1 containing constructs were packaged into AAV.SPR and AAV5 by triple transfection. RS1KO mice received SRI with three different doses of vector, and retinal structure and function were assessed over 6 mos. by optical coherence tomography (OCT) and electroretinography (ERG), respectively. Cynomolgus monkeys (*M. fascicularis*) received SRI of AAV-hGRK1-mycRS1 alone or combined AAV-hGRK1-GFP + AAV-hGRK1-mycRS1 at equal concentrations. Vectors were delivered in either one or two extrafoveal blebs, and wide-field color fundus, confocal scanning laser ophthalmoscopy (cSLO) and OCT images were collected for up to 6 wks. post-injection. Immunohistochemistry (IHC) and biodistribution analysis were performed post mortem. **Results:** In treated RS1KO mice improvements in both retinal structure (resolution of schisis), and function (scotopic and photopic ERG b-waves) were observed in a dose-dependent manner. RS1 was localized to photoreceptor inner segments and to a lesser extent, bipolar cells. In NHP, GFP and RS1 (myc) were observed well beyond the bleb margins with AAV.SPR (but not AAV5) vectors. By IHC, GFP and RS1 were found in and around the majority of photoreceptors including foveal cones. Localization of vector mediated RS1 mimicked that of endogenous NHP RS1. No outward signs of inflammation or retinal damage were observed in AAV.SPR treated eyes. Finally, biodistribution of AAV.SPR compared favorably to AAV5. **Conclusion:** A novel AAV vector has been developed for the treatment of XLRS. AAV.SPR-hGRK1-hRS1 leads to therapeutic levels of RS1 in subretinally injected RS1KO mice, resulting in improvements in both retinal structure and function. In addition, AAV.SPR safely and efficiently mediated RS1 expression in the central retina of primate eyes following peripheral SRI. In comparison to previous vectors delivered intravitreally, SRI of AAV.SPR is expected to be more effective for the treatment of XLRS as it promotes highly efficient transduction of photoreceptors while avoiding surgical manipulation of schisised retina and the fovea.

297. High Throughput Screen Identifies Drugs That Rescue AMD Phenotype in iPSC Derived RPE Model of Macular Degeneration

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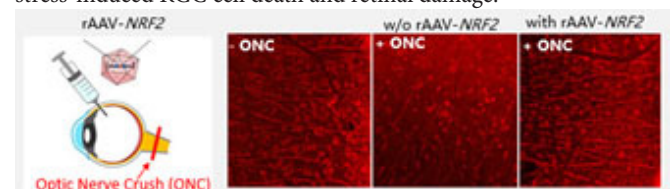
Age-related macular degeneration (AMD) is one of the leading causes of vision loss in people above 60 years. The likely cause of disease initiation arises from the dysfunctional retinal pigment epithelium (RPE), a post-mitotic layer of polarized layer of hexagonal cells that supports the maintenance of photoreceptors and choroidal capillaries. The eventual atrophy of RPE cells leads to photoreceptor cell death and capillary loss, and its dysfunction causes the sub-RPE lipoprotein-rich drusen deposits. GWAS and recent *in vitro/in vivo* studies present preliminary evidence of the role of the alternate complement pathway in AMD pathogenesis, but the exact mechanism of disease initiation and its progression is largely unknown. We developed an AMD model using iPSC-RPE to study molecular events triggered by activating alternate complement pathways through C5a and C3a during disease pathogenesis. This study aims to discover drugs that rescue alternate complement-induced AMD phenotype in iPSC-RPE, providing potential pharmaceutical intervention for the disease. **Methods:** Eleven iPSC lines from subjects of different AMD genetics were differentiated into RPE. Differentiated RPE cells were characterized for their polarity (staining with Ezrin and COLIV), junctional integrity (TER measurement) and functionality (phagocytosis). Complement Competent-Human Serum (CC-HS) was used to induce AMD like phenotype in iPSC-RPE, Complement Incompetent-Human Serum (CI-HS) served as the control. Receptor blockers Compstatin (C5aR1), PMX53 (C3aR1), and depleted sera for C5 and C3 were used to confirm the role of the complement pathway. Immunostaining was used to check APOE deposits, lipid deposits (the Nile red), epithelial phenotype (F-actin, Vimentin), and p65 localization for the NF- κ B pathway. Pathways downstream of complement were discovered using bulk RNA sequencing and corroborated by immunostaining (IF) and Western blot (WB) High throughput screen on 1280 drugs was performed to discover potential drugs that can rescue RPE cell death. **Results:** CC-HS elicited APOE and lipid deposits, eroded the epithelial phenotype, reduced TER and compromised phagocytic ability of iPSC-RPE. iPSC-RPE treated with PMX-53 and Compstatin in the presence of CC-HS or C5 and C3 depleted sera didn't show any of the AMD phenotypes. RNA sequencing revealed the activation of nf- κ b and downregulation of autophagy pathways. We confirmed these results by analyzing levels of p65, LC3, ATG5 in iPSC-RPE cells by IF, and WB. Two drugs were discovered using the HTS, L-745,870, a dopamine receptor antagonist, and aminocaproic acid (ACA), a protease inhibitor. The two drugs restored CC-HS induced reduced autophagy and suppressed NF- κ B activation in addition to reducing lipid deposits and restoring the loss of functionality in iPSC-RPE caused by CC-HS treatment. **Conclusions:** C5a and C3a induced sub RPE deposits in iPSC-RPE were rescued by two drugs discovered from the high throughput screening. The AMD model helped us in dissecting out the cell autonomous role of RPE in disease pathogenesis. It also provides a platform to study the genotype-phenotype relation in AMD.

298. Role of NRF2 as a Therapeutic Target for RGC Protection

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Introduction: Inflammation, oxidative stress and mitochondria impairment are important parameters in the pathophysiology of the major ocular diseases, such as glaucoma, ischemia optic neuropathy, optic neuritis etc. Particularly in glaucoma affecting almost 70 million people worldwide, it is characterized by the progressive death of the retinal ganglion cells (RGCs) and atrophic excavation of the optic nerves, resulting in irreversible loss of vision and ultimately blindness. Indeed, an unmet clinical need is the lack of effective neuroprotective approaches to preserve RGCs and their function. Nuclear factor erythroid 2-related factor 2 (NRF2), acting as a transcription factor and a regulator of many biological processes, plays an essential role in preventing oxidation and inflammation, and modulating both mitochondrial function and biogenesis. NRF2 has been previously demonstrated therapeutic value in preclinical models of neurodegenerative disease. Here, we investigated the potential role of NRF2 as a therapeutic target for RGC protection. **Methods:** The coding sequence of *NRF2* was codon-optimized and constructed into pAAV2 vector. The expression of NRF2 in HEK293 cells was detected by immunoblotting and the transcription of *NRF2* downstream target genes were quantified by qPCR. Mouse optic nerve crush model was used to evaluate the neuroprotective effect of rAAV2-*NRF2*-opt treatment and the expression of *NRF2* was also detected by qPCR at different AAV2 infection doses. C57BL/6 mice were administrated with rAAV2-*NRF2*-opt via intravitreal injection, and *NRF2* and its downstream target genes were detected by qPCR at 6 months post injection. Quantification of RGC numbers and H&E staining of retina tissue sections were performed to evaluate the safety of NRF2 overexpression *in vivo*. **Results:** The expression of NRF2 and the induction of its downstream target genes were detected in HEK293 cells at 72 h post AAV infection. In mouse optic nerve crush model, NRF2 expression promoted murine RGC survival in a dose-dependent manner. Long-term following up study indicated that the expression of *NRF2* and its downstream target genes were still significantly up-regulated at 6 months post infection, while the structure of the retina tissue section and RGC counts were comparable between AAV2-*NRF2*-opt treated and control mice. These findings suggest that the up-regulation of NRF2 may contribute to neuroprotection against stress-induced RGC cell death and retinal damage.



299. rAAV-Mediated *PEX1* Gene Delivery Causes Dose-Dependent Improvement in RPE Cell Function and Structure in a Mouse Model of Mild Zellweger Spectrum Disorder

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Zellweger spectrum disorders (ZSDs) are autosomal recessive disorders caused by pathogenic variants in any one of 13 *PEX* genes required for peroxisome assembly and function. More mildly affected individuals harboring the common *PEX1*-G843D missense variant consistently develop a potentially blinding retinopathy. We previously reported a proof-of-concept trial for AAV8.CMV.Hs*PEX1*.HA retinal gene augmentation therapy using our mouse model homozygous for the equivalent murine *PEX1*-G844D allele. Experimental treatment resulted in a 2-fold improvement in full-field flash electroretinogram response, near normalization of peroxisomal metabolite levels in whole retina, and a trend towards improved visual acuity. Here, we report for the first time the *in vitro* and *in vivo* effects of *PEX1* gene delivery on retinal pigment epithelium (RPE), using a re-designed clinically-translatable vector (AAV8.CBh.Hs*PEX1*). *In vitro* studies using human (immortalized) and mouse (primary and immortalized) *PEX1*-G843D RPE cell lines demonstrated dose-dependent recovery of: i) peroxisome import [visualized by immunofluorescence (IF)], ii) peroxisome enzyme receptor *PEX5* recycling [visualized by IF], iii) *PEX1* and *PEX5* protein levels [quantified by immunoblot], and iv) biochemical metabolites [ether phospholipids and C26:0 lyso-PC measured by LC-MSMS]. For *in vivo* studies, AAV8.CBh.Hs*PEX1* was administered by subretinal injection to 4 wk-old *PEX1*-G844D mice at 6 doses spanning 6.8×10^9 - 1.4×10^7 vector genomes (vg), using vehicle or AAV8.CBh.eGFP as controls. The experimental vector was well-tolerated at all doses in both homozygous *PEX1*-G844D mutants and littermate controls, with mild inflammation limited to the injection site. A dose-dependent overexpression of Hs*PEX1* protein in the RPE was quantified by immunoblot. RPE flatmounts analyzed using confocal microscopy 2 months following gene delivery showed the following dose-dependent improvements: i) preserved cell number, size, and morphology [f-actin pattern], ii) reduced inflammatory markers, and iii) reduced macrophage infiltration. Quantification of results revealed that the optimal dose of 1.2×10^9 vg expanded the preserved RPE surface area 2-3-fold compared to non-injected or vehicle-injected mutants, even at this early time point. Overall, these studies demonstrate the potential of retinal gene augmentation therapy to preserve RPE health and visual functions in people with milder forms of ZSD.

300. Evaluation of ABCA4 RNA Exon Editing and Replacement in Non-Human Primate

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Recessively inherited mutations in *ABCA4* are causal in the development of progressive forms of blindness including Stargardt disease 1 and cone-rod dystrophy 3. The 6882 bp coding sequence of *ABCA4* is too large to be delivered in its entirety by a single AAV vector. Moreover, with hundreds of disease-causing mutations found throughout the gene, a single base editing approach would not address a significant number of patients. Therefore, we set out to develop a large-scale exon editing solution by delivering a therapeutic RNA construct capable of trans-splicing into endogenous *ABCA4* pre-mRNA thereby introducing functional exons. Inducing RNA trans-splicing with a single AAV-based construct can address approximately 60% of all patient mutations. These exon editing RNA molecules were optimized using an *in vitro* synthetic RNA screening engine. Here we report the editing efficiency and tolerability of lead AAV-*ABCA4* exon editing molecules *in vivo* in African Green Monkey non-human primates (NHP) following subretinal injection. Efficient splicing between AAV-encoded therapeutic *ABCA4* exon editing constructs and the endogenous NHP *ABCA4* sequence was detected 1 month following subretinal injection. The novel exon-exon junction formed through trans-splicing is distinct in sequence from the exon-exon junction in the natively expressed NHP *ABCA4*. The levels of this new exon-exon junction were quantified by RT-qPCR. This allowed for quantitative comparison of the levels of trans-spliced RNA with native expression levels in the retina. We further utilized an N-terminal epitope tag to enable detection of the full length *ABCA4* protein product of trans-spliced RNA translation. Tagged *ABCA4* was detected in treated eyes by immunoblot, consistent with the detection of the chimeric RNA by RT-qPCR. Moreover, immunohistochemical (IHC) staining confirmed transduction of target photoreceptor cells. Importantly, all subretinal delivered AAV-mediated exon editing molecules were well-tolerated. General health assessments were normal throughout the study. There were no test article-related adverse effects and intraocular pressure remained within normal limits. To our knowledge this study is the first showing exon editing via RNA trans-splicing in NHPs. While further work is required to fully understand the long-term durability and safety of this approach, this report highlights the potential of RNA exon editing to treat *ABCA4*-related retinopathies, and other diseases for which replacement of multiple contiguous exons may provide a novel treatment strategy.

301. Chemical Reprogramming of Mouse and Human Müller Glia into Retinal Ganglion-Like Cells (ciRGCs)

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Introduction: Glaucoma is a leading cause of irreversible blindness with increasing prevalence as the population ages. Glaucoma is characterized by loss of retinal ganglion cells (RGCs), and current therapies, whether surgical, pharmacological, or neuroprotective, do not reverse the degeneration. Stem cell approaches to replace lost RGCs are a viable option. However, the use of stem cells for RGC replacement currently faces several barriers: 1) absence of a safe, non-immunogenic, and ethical stem cell source of RGCs, 2) inefficient differentiation protocols that can take more than 40 days, and 3) RGC donor integration into the degenerate host retina are major issues that need to be overcome to be considered for clinical use. A radically new approach to restoring vision for glaucoma patients that can overcome these limitations would be to reengineer a resident cell in the retina, such as Müller glia, that could serve as a reservoir for new RGCs, and avoid the need for cell transplantation. In this study, we take the first step in realizing our long-term goal of using a chemically reprogramming strategy to replace lost RGCs and restore vision. **Materials and Methods:** Primary Müller glia were isolated from mouse or human retina. Small molecules were purchased from Sigma or Cayman. For the *in vitro* studies, Müller glia were fixed after small molecule conversion with 4% PFA. Immunofluorescence staining was performed to detect RGC specific markers: Brn3a, Brn3b, ISL-1, RBPMS, and Tuj1. Total RNA was isolated and subjected for real-time PCR for detection of neuronal marker NeuN and RGC markers: Brn3a, Brn3b, ISL-1, NefH, and Nefl. **Results:** Both mouse and human Müller glia can be converted to chemically-induced RGC-like cells (ciRGCs) within 24 hours *in vitro*. Immunofluorescence staining was performed and ciRGCs expressed RGC specific markers: Brn3a, Brn3b, ISL-1, RBPMS, and Tuj1. Total RNA was isolated and subjected for real-time PCR for detection of neuronal marker NeuN and RGC markers: Brn3a, Brn3b, ISL-1, NefH, and Nefl. **Conclusion:** Our small molecule cocktail is highly efficient in converting mouse and human Müller glia to RGC-like cells *in vitro*, enabling us to generate ciRGCs in approximately one day with more than a 90% conversion efficiency. The small molecule cocktail has the advantage of bypassing the need for viral over-expression of transcription factors. Future pharmacology and retinal toxicity studies will be needed to evaluate the safety profile of the small molecule cocktail if injected in the eye for retinal regeneration.

302. Identification of GJB2's Upstream Regulatory Elements Facilitates Design of Safe, Precision AAVs and Recovery of Hearing in a GJB2-Deficient Mouse Model

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Background: Mutations in Gap junction beta protein 2 (GJB2) are the leading cause of non-syndromic, prelingual deafness (DFNB1), with an estimated US/EU prevalence of 280,000 cases. Most of these mutations are recessively inherited, loss-of-function alleles, making them attractive targets for an AAV-based gene replacement therapy. One major challenge in developing such a therapy lies in the ability to achieve the proper expression pattern in the cochlea. GJB2 is endogenously expressed in an assortment of cell types in the inner ear, and at different expression levels in each, thus requiring more sophisticated bioinformatic approaches to correctly identify a combination of genomic regulatory sequences that can replicate this complex expression pattern. Specifically, a successful DFNB1 gene therapy must achieve an optimal balance by sufficiently targeting the diverse set of GJB2-expressing cells to restore function while excluding expression from other critical sensory cells to avoid toxicity. **Methods:** Adult or neonatal mice (wildtype or GJB2 deficient mutants) received inner ear injections of AAVs driving *Gjb2* or nuclear GFP expression from a ubiquitous promoter or a proprietary regulatory element. AAVs were administered locally via the posterior semi-circular canal. Hearing function was assessed using auditory brainstem responses and distortion product otoacoustic emissions. After animal takedown, immunohistochemistry labeling was performed to assess morphology and transgene expression. **Results:** We first evaluated whether ubiquitous AAV-mediated *Gjb2* expression in the inner ear could lead to detrimental effects. Wildtype adult mice were injected with AAV CMV *Gjb2* and taken down one to two weeks later. A large fraction of the animals exhibited elevated hearing thresholds 2 weeks post-treatment. Histological analysis showed accumulation of GJB2 protein in inner hair cells after 1 week, followed by almost complete loss of these cells by 2 weeks, suggesting that ectopic *Gjb2* expression can be toxic and lead to hair cell death. In order to address this, we next sought to identify a combination of GJB2 proximal and distal regulatory regions which would allow us to more closely mirror the endogenous GJB2 expression pattern. Integrative bioinformatic analyses of bulk and single cell epigenomic datasets were used to select candidate regulatory regions, and promoter/enhancer designs were screened in neonatal cochlear explants, identifying a lead combination which successfully drove expression in GJB2-expressing cells while excluding expression from hair cells and neurons. *In vivo* experiments further confirmed the observed expression pattern in the mouse cochlea. Lastly, we evaluated whether this proprietary promoter driving *Gjb2* expression could enable hearing recovery in a GJB2 deficient mouse model. We found that delivery of our AAV gene therapy in mutant mice deficient for GJB2 led to outer hair cell preservation and improved hearing thresholds

in injected ears compared to contralateral and naïve controls. **Conclusions:** Our results underscore the importance of using a tailored GJB2 promoter to avoid toxicity 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 recovery observed in mice with our tailored vector represents significant progress towards the development of a gene therapy to restore natural hearing to DFNB1 patients, and more generally demonstrates that targeting of gene therapies to complex sets of cell types is achievable with synthetic regulatory elements.

303. Mutation-Independent RNA Editing Approach Based on RHO-Targeting Ribozyme for the Treatment of Retinitis Pigmentosa

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Retinitis Pigmentosa (RP) is the most common hereditary degenerative eye disease caused by abnormalities in the photoreceptors (rods and cones) or the retinal pigment epithelium (RPE) of the retina. Rhodopsin mutations accounts about 25~30% of autosomal dominant retinitis pigmentosa (adRP). A single Pro23His (P23H) mutation in the rhodopsin (RHO) gene is currently known to be the most common cause of adRP in USA but over 150 autosomal dominant mutations have been identified in the *RHO* gene. In this study, we have developed a specific trans-splicing ribozyme of a mutation-independent therapeutic strategy that can replace, and thus edit the endogenous RHO RNA with exogenous functional RHO RNA using AAV delivery system. Target site of rhodopsin RNA was first identified by in vitro and intracellular mapping. Sequencing analysis of trans-splicing reaction sites through mapping revealed that the most efficient target site occurred at the base (U) at the RHO RNA 5' UTR. To improve the trans-splicing specificity and efficiency of the ribozyme targeting RHO RNA, 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 cells. To verify in vivo function, AAV vector encoding the optimal RHO-targeting ribozyme was constructed and delivered by subretinal injection into the eye of human P23H/mRHO knock-in mice. The electroretinography (ERG) is a useful tool for measuring retinal responses and can be used to assess eye disease and retinal degeneration. Scotopic ERG was measured on dark-adapted mice at 4, 8, 12 wpi. The b-wave amplitude of P23H/mRHO mice decreased compared to wild-type mice. On the contrary, the b-wave amplitudes had significantly increased in eyes injected by AAV encoding the RHO-targeting ribozyme as compared to those of control injected eyes. After administration, retinas were dissected and processed for H&E staining, RT-PCR, and qPCR. Molecular and cellular analysis of retina tissue showed that P23H RNA was replaced with WT RHO with high efficacy and fidelity and ONL appeared significantly thicker in the RHO-targeting ribozyme treated eye. Of note, we observed that various mutant RHO RNAs were trans-spliced with high fidelity and restored to WT RHO RNA by the RHO-targeting ribozyme in cells. Here, we proved that the ribozyme effectively prevents the degeneration of photoreceptor cells and preserves their electrical response function in P23H/mRHO knock-in mice through specifically targeting and

trans-splicing of target RHO RNA. Taken together, these results suggest that RNA replacement based on RHO-targeting ribozyme could be potent and mutation-independent therapeutic strategy for RHO-adRP.

304. Dual Vector Mediated Gene Therapy for Restoration of STRC-Related Hearing Loss

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Stereocilin is an integral protein in outer hair cells of the inner ear and necessary to facilitate and required to convert low level sounds into a signal to the brain. Stereocilin is a 193 kDa large GPI anchored protein with several ARM-like repeats facilitating binding to extracellular glycoproteins necessary to connect the sensory organ of hair cells, the hair bundle, to the so-called tectorial membrane and provide cohesion in the bundle. In humans Stereocilin (STRC) deficiency leads to autosomal recessive deafness (DFNB16) with patients showing mild to moderate hearing loss. DB-AAV-104 aims at restoring hearing in DFNB16-patients. The affected population has been estimated to be the second most prevalent genetic auditory deficiency in the US and EU5. We generated two mouse models utilizing CRISPR/Cas9 to generate mice deficient of STRC to recapitulate the human phenotype, study disease progression and explore the feasibility of AAV gene therapy for DFNB16. The animal models show near absence in measurements targeted at outer hair cell function and highly elevated thresholds in the whole auditory pathway, mimicking the patient phenotype. At the same time sensory cells of the inner ear remain intact allowing for a long treatment window for potential gene therapies. For targeting transgenes precisely to our cell types of interest, we identified potential regulatory elements selective for outer hair cells and validated them in organ of Corti explants and in vivo in mouse studies. We later confirmed the specificity of our promoter sequences in non-human primate ears. The size of the *Strc* transgene exceeds the packaging capabilities of AAV. Therefore, we relied on dual vector technology to re-express full-lengths STRC in murine sensory cells of our deficiency model. We show hearing restoration in our animal models after delivery of dual vector AAV, and show re-expression of STRC in hair bundles of sensory cells. Our results indicate that dB-AAV-104 dual vector gene replacement therapy re-expressing full length Stereocilin can lead to meaningful auditory threshold improvements by allowing the attachment of the tectorial membrane to the outer hair cell.

305. Treatment of Ocular Neurodegenerative Diseases: An In-Depth Comparison of Anc80 and AAV9 Including Cross-Reactivity of Pre-Existing Antibodies in Human Serum

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The recently developed AAV.Anc80 vector is well known for its enhanced ability to transduce the inner ear and the retina in comparison to conventional AAV serotypes. It also can cross the blood brain barrier, making it a vector of consideration for the treatment of central nervous system disorders. While Anc80 was previously compared to AAV9 following intraventricular, intravenous, subretinal, and intra-striatal injections, limited data is available on intravitreal ocular injections and little to no information is available for these injection methods regarding transduction of the visual pathway (visual cortex (VC), superior colliculus (SC), lateral geniculate nucleus (LGN), optic tract (OPT), retina). We characterized transduction profiles of Anc80.GFP and AAV9.GFP in the visual pathway via three routes of administration. Intracerebroventricular (ICV), subretinal (SR) and intravitreal (IVT) injections were performed in C57BL/6J mice at post-natal day 1, or 2-4 months of age, respectively. Two different promoters, leading to high or moderate expression, drove GFP transgene expression. Four weeks post-injection, GFP was visualized in retinal cryosections and coronal brain sections within all regions of the visual pathway. Post-ICV administration, AAV9 transduced more neurons in the VC and LGN compared to Anc80. Irrespective of serotype, the expression patterns were similar with each promoter. In the retina, Anc80 and AAV9 transduction profiles were very similar post-ICV administration. In all vector-promoter combinations, GFP expression was primarily observed in ganglion cells with limited expression in the inner nuclear layer (INL). The high expression promoter also yielded GFP signal in photoreceptors. Post-IVT injection, although Anc80 and AAV9 transduction patterns were similar, striking differences in GFP expression levels driven by our two promoters were observed. While one led to expression in ganglion cells and Müller glia, in addition to a limited number of other INL cells and photoreceptors, the other led to restricted expression in ganglion and amacrine cells. Importantly, after AAV9 IVT injections, GFP was detected along the visual pathway in the brain, including the OPT and LGN, indicating anterograde trans-synaptic transport. Efforts to evaluate this phenomenon after IVT injection with Anc80 are ongoing. Post-SR injection, we observed higher transduction of photoreceptors with Anc80 compared to AAV9, which was consistent between promoters and aligned with previously published reports. Confirmation of transduction in other regions of the visual pathway after SR injection are ongoing with all vector-promoter combinations. Overall, Anc80 and AAV9 perform similarly in terms of overall visual pathway transduction. Because Anc80 is not a naturally occurring serotype, it is generally believed that this serotype could be used in patients that have pre-existing antibodies against other AAVs. However, the synthetic Anc80 capsid was derived by ancestral evolution, which means it might retain common epitopes the immune system may recognize. To date, Anc80 cross-reactivity has been understudied and little to no information is available from human samples. We are performing an in-depth analysis in human

serum samples positive for common AAV serotypes to evaluate cross-reactivity to the Anc80 capsid. Preliminary data indicates cross-reactivity occurs in AAV1 and AAV2 positive samples, the level and extent of which are currently under evaluation. Our data adds an important additional aspect to the characterization of Anc80, which is a highly promising candidate for the treatment of retinal, cochlear and neurological disorders.

306. Gene Therapy for Stargardt Disease Using Hybrid Dual AAV Vectors to Express ABCA4

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Stargardt Disease (STDG1), the most common autosomal recessive form of early onset macular dystrophy, is caused by mutations in the *ABCA4* gene that codes for ATP-binding cassette transporter A4, expressed in the outer segments of photoreceptor cells in the retina. Lack of *ABCA4* protein expression leads to accumulation of toxic bisretinoids, specifically N-retinylidene-N-retinylethanolamine (A2E), which further forms fluorescent lipofuscin aggregates in the retina subsequently resulting in cell atrophy and vision loss. AAV vectors have shown great potential for retinal gene therapy in the clinic; however, the large (6.8kb) size of the *ABCA4* gene is beyond the conventional packaging capacity of AAV vectors which is approximately 4.7kb. We are investigating a hybrid dual AAV strategy for *in vivo* expression of *ABCA4*. The hybrid dual AAV vectors were able to express full length *ABCA4* protein both *in vitro* in HEK293 cells and *in vivo* in photoreceptor cells when delivered by subretinal injection in both WT C57BL6 mice and in the *ABCA4* K/O mouse model. Treatment of the *ABCA4* K/O mice with the hybrid dual AAV system led to a reduced accumulation of A2E as measured in life by reduced fundus autofluorescence and by HPLC analysis of the retina at the end of the study. Subretinal injection of the hybrid dual AAV vectors in non-human primates was safe and resulted in expression of full-length *ABCA4* protein in the retina. These results show that the hybrid dual AAV vector strategy is promising and warrants further investigation as a gene therapy for Stargardt Disease.

307. Regulation of Neuroprotective Factors in Muller Glia Using Artificial Transcription Factors

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Visual impairment and blindness has a huge impact on a persons' quality of life. Irrespective of the genetic cause, photoreceptor and ganglion cell loss is the hallmark for majority of the blinding diseases in ophthalmology. The biggest challenge for any blinding disease is the availability of the treatment options. Therefore, the ongoing neuronal loss cannot be stopped and complete loss of vision is inevitable. In

order to have a treatment, novel neuroprotective approaches that will slow down or halt neuronal cell loss and at the same time maintain the function of retinal neurons are required. The main aim of this project is to develop and validate multiplex gene regulation-based therapies for neuroprotection. This unique approach will exploit gene transactivators (ATAs) that aims for overexpression of neuroprotective genes in Muller glia at different doses in retina. We also plan to test ATAs in a retinal degeneration model that has reporter expression in photoreceptors to assess the neuroprotection levels longitudinally and spatially with a minimal effort. To generate repertoire of ATAs that overexpress *Fgf2*, *Lif*, *Cntf* and other neuroprotective factors, we designed a gRNA screen that depends on expression of dCas9-VP64 in a stable Muller glia line and the corresponding vector that expresses gRNA and an eGFP reporter. This have advantages like reducing the size of the vector by several kbs and increasing the transfection efficiency of the vector that expresses gRNA. Transfected dCas9-VP64 Muller glia were FACS-sorted and overexpression levels of each gene was assessed using ddPCR method. In order to test ATAs *in vivo*, we have crossed *Rho*^{P23H/+} transgenic mouse line with rod reporter *Tg(Rho-Cre); ROSA26-LSL-tdTomato*. Crossed animals were followed up using Micron IV and the reporter expression was quantified using ImageJ at different time points. We cloned gRNAs that were designed against *Fgf2*, *Cntf* and *Lif* promoter regions. We further tested the two component system using *Fgf2* gRNAs and the Muller glia cell line that has 14 copies of dCas9-VP64. We identified several gRNAs that upregulated *Fgf2* at different doses. Similarly, we were able to find gRNAs that also overexpress *Lif* and *Cntf*. These results showed that it is feasible to control the dose of neuroprotective factors using ATAs. Moreover, early results from the rod reporter RP model showed clear reduction of tdTomato expression, which will help us to elucidate the beneficial effects of ATA-dependent neuroprotective treatment *in vivo*. We previously anticipated to have different fold levels of upregulation for different gRNAs which was important to establish dosages for neuroprotective factors. Our findings for *Fgf2* and *Cntf* were promising and suggested that the anticipated dosage adjustment is feasible. We are now cloning the corresponding *Fgf2*, *Cntf* and *Lif* gRNAs into AAV vectors and plan to test *in vivo* efficiency of ATAs in retina. Together with the *Rho*^{P23H/+}; *Tg(Rho-Cre); ROSA26-LSL-tdTomato* mouse model, we will quickly quantify fluorescence labelling of rods and thus the *in vivo* rescue levels and pattern after ATA treatments.

308. ELOVL2 Gene Therapy: Lipid Restoring Strategy to Treat Dry Age-Related Macular Degeneration

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Dry age-related macular degeneration (dry AMD) is a slowly progressing retinal disease which leads to the loss of macular 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. In the advanced stages of dry AMD, people can be deprived of 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 an elongase involved in the elongation of long-chain (C20 and C22) polyunsaturated fatty acids (LC-PUFAs). ELOVL2 converts docosapentaenoic acid (DPA)

22:5n-3 into 24:5n-3, which leads to production of docosahexaenoic acid (DHA) 22:6n-3 as well as very-long-chain PUFAs (VLC-PUFAs). Both DHA and VLC-PUFAs are critical for proper retinal function. Loss of ELOVL2 C22->C24 activity by mutation, results in diminished ERG, drusen formation and complement accumulation in young mice. Expression of ELOVL2 decreases with age due to a mechanism involving methylation of the ELOVL2 promoter. Diminishing ELOVL2 expression and the concomitant decline in DHA and VLC-PUFA, we believe is a major factor underlying pathology in AMD. To test this, we designed and constructed a series of optimized ELOVL2 expression cassettes as candidate therapeutics for dry AMD related vision loss. We began by constructing several ELOVL2 codon usage variants, from which we identified a sequence which expressed at 3X wild type levels. We then used various 5' UTR, promoter, intron, and 3'UTR combinations in conjunction with our optimized sequence and achieved a 20X expression increase over native expression. Our optimized cassette was then packaged into AAV8. One-year old C57BL/6J mice were treated with a single subretinal AAV8-optELOVL2 administration (E7 vg range) 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. Currently, we are testing second-generation constructs which are high expressing, better tolerated and may enable greater retinal protection during aging.

309. Intraocular Gene Delivery Ameliorates Retinal Structure but Not Function in a Mouse Model of Gyrate Atrophy of the Choroid and Retina

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Gyrate atrophy of the choroid and retina (GACR) is a rare autosomal recessive disease leading to degeneration of retinal pigment epithelium (RPE) and blindness in young adults. GACR is caused by mutations in the ornithine aminotransferase (*OAT*) gene, a mitochondrial enzyme expressed in the RPE and neuroretina whose deficiency leads to hyperornithinemia. A mouse deleted for *OAT* gene (*mOAT*^{-/-}) recapitulates the biochemical and retinal abnormalities observed in GACR patients and presents with hyperornithinemia, RPE degeneration, decreased outer nuclear layer (ONL) thickness and reduced retinal electrical activity. Arginine-deficient diet and vitamin B6 oral supplementation appear to slow the progression of the retinal disease in GACR patients. Gene therapy with a single intraocular administration of adeno-associated viral (AAV) vectors has the potential to restore local expression of *OAT* and its metabolic pathway in the eye and to treat the RPE disease. We developed an AAV8 vector expressing h*OAT* under the control of the ubiquitous CMV promoter. After confirming *in vitro* expression and activity of h*OAT*, the AAV8-

hOAT vector was delivered by subretinal injection in one eye of mOAT^{-/-} mice with the contralateral receiving excipient. Eyes administered with AAV8-hOAT were found to have significant thicker ONL up to 12 months of age compared to eyes administered with the excipient. The improvement in ONL thickness was observed also in distant areas from the injection site, possibly due to detoxification driven by neighboring transduced RPE cells. This is mirrored by a normalized RPE morphology across the entire retinal section. However, retinal electrical activity remained reduced similarly to retinas treated with excipient. This data suggests that hyperornithinemia, presumably unchanged by subretinal administration of AAV, impairs retinal function and that its reduction, in combination or not with local OAT delivery, is required for proper photoreceptor function.

310. Transient CRISPR-Cas Delivery for Therapeutic Gene Editing in the Retina

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Genome editing mediated by CRISPR-Cas has shown promise for the treatment of retinal dystrophies (RD). Indeed, the broad range of gene editing tools offers new therapeutic outcomes to treat all types of RD if suitable vectors are developed. Currently, adeno-associated viruses are the most widely used vector for retinal gene therapies but several limitations such as packaging capacity and long-term expression of Cas raise questions about their clinical applications. Transient delivery of gene editing tools could provide a safer alternative for delivering gene editing reagents to the retina. Indeed, successful delivery of Cas9 protein and its gRNA as ribonucleoprotein (RNP) complexes has been reported in the retinal pigment epithelium (RPE) and in the inner ear cells *in vivo* in mice. However, the feasibility of transient delivery of gene editing tools in the photoreceptors, the main target to treat early stage RD, have never been reported. **Here, we investigate the direct delivery of either Cas mRNA or Cas RNP to edit the neural retina**, composed mainly of photoreceptors in adult mice. After subretinal injection of Cas9 mRNA complexed with different lipids, no indels were detected in the neural retina and only a few indels were observed in the RPE. On the contrary, **direct delivery of Cas9 RNP generated indels in the neural retina in a dose dependent manner**. The indels reached a maximum of 0.8% in the whole neural retina compared to a maximum of 2.4% in the RPE. To improve the editing efficiency, we investigated different categories of non-viral vectors as lipids and cell penetrating peptides (CPP). These synthetic carriers were able to complex Cas RNP and increase efficacy towards RPE delivery but they did not increase indels in the neuroretina. Imaging labeled RNP complexes showed accumulation above the tightly stacked outer segments. To see if the outer segments prevented penetration of the particles into the photoreceptor layer, we assessed delivery in mice with degenerated outer segments. No improvement was noted compared to wild-type mice suggesting other barriers than the outer segment discs. Altogether our results highlight the need to develop new carrier

compounds to bring transient CRISPR-Cas delivery to the neural retina and the RPE and point to several parameters as being important in such development.

Cancer - Immunotherapy, Cancer Vaccines I

311. Development of a Minocycline Mediated Protein-Protein Displacement Platform Using an Anti-Minocycline Single Domain Antibody and a Dedicated Displaceable Peptide

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Introduction: Engineering cell therapy approaches are encompassing ever broader signalling pathways. A versatile small molecule control system is desirable to increase control and mitigate toxicity in clinical settings. We set about creating a two component small molecule control system based in disruption of protein-protein interactions. Given its wide availability, excellent bio-distribution and lack of toxicity, we developed this system around minocycline. To minimize immunogenicity, we used a single domain antibody (sdAb) as the main protein component.

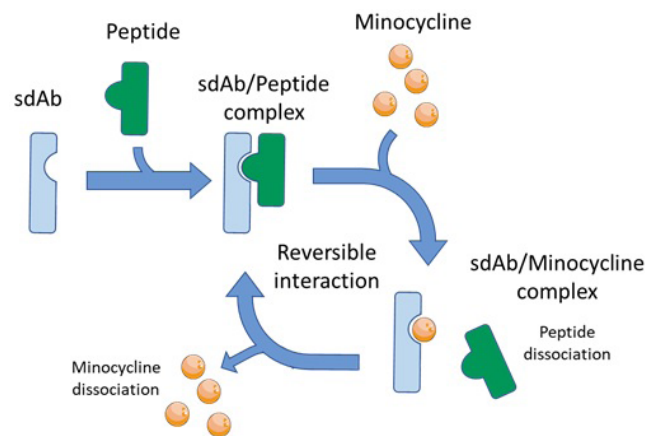


Figure 1. A minocycline binding sdAb was generated. Additionally, a peptide mimotope of minocycline was also generated. The peptide binds the sdAb but with a lower affinity than minocycline. Exposure to minocycline results in displacement of the sdAb/peptide complex. **Results:** Minocycline specific sdAb were generated by immunising an alpaca with KLH-conjugated minocycline which produced an immune response against KLH including the minocycline moiety. Phage library enrichment and selection was carried out against BSA-conjugated minocycline which yielded an estimated library size of 5×10^8 unique sequences. Using SPR analysis, the highest affinity sdAb-minocycline binding was measured at $K_D = 31 \text{ nM}$ ($k_a = 1.13 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_d = 3.6 \times 10^{-2} \text{ s}^{-1}$). A cyclic peptide which interacts with the anti-minocycline sdAb but is displaced in the presence of minocycline was isolated by

panning a cysteine-constrained heptapeptide (CX₇C) phage library against the sdAb, where bound phage was eluted using minocycline (1 μM). SPR analysis showed out of all isolated peptide sequences, ACPGWARAFC, presented the highest affinity sdAb binding at K_D=111 nM (k_a=1.55x10⁵M⁻¹s⁻¹ and k_d=1.7x10⁻²s⁻¹). Competition ELISA showed ~7-fold reduction in peptide-sdAb binding, while cell-surface and SPR binding assays showed complete displacement in the presence of minocycline (1 μM) relative to no minocycline. Peptide binding to the sdAb was also shown to be reversible post minocycline displacement. Applications of this system in adoptive T cell therapy were also explored. Firstly, we developed a minocycline mediated OFF-switch split CAR structure where transient suppression of T cell activity was achieved through displacement of the functional CAR heterodimer via minocycline administration (0.1 μM-10 μM). The effector function was tuneable in a dose-dependent and reversible manner. Moreover, cytotoxicity and cytokine release (IFNγ and IL-2) against target cells were comparable to a conventional CAR. Secondly, the KDEL amino acid retention motif was fused to the sdAb thus anchoring it to the ER/Golgi apparatus. The pro-inflammatory cytokine IL-12 was fused to ACPGWARAFC causing its cellular retention in the absence of minocycline relative to the non-KDEL control. Upon addition of minocycline (2.5 μM), the sdAb-peptide complex was displaced, enabling the dose-dependent secretion of the IL-12 (up to ~2,200 pg/mL). **Conclusions:** This work describes the development of a novel minimally immunogenic small molecule control system, controllable with the well-tolerated, and widely available antibiotic minocycline. This platform has numerous applications in controlling numerous engineered cellular therapy approach. Such control systems permit increased safety and control of engineered cell therapies.

312. Synthetic, Enzymatically Produced DNA for Use in DNA Vaccines

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The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production in gene therapy and vaccine pipelines. 4basebio has developed a proprietary, scalable synthesis process for the production of linear open DNA constructs via our Trueprime™ amplification technology. The osDNA™ 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, allowing for large scale production of linear DNA with high yield and purity in less than a week. Moreover, the DNA can be modified to modulate the immunostimulatory properties of the DNA constructs thereby improving its suitability for application in DNA vaccines. In a collaboration with Takis Biotech and Neomatrix, we have demonstrated that osDNA™ constructs can elicit a meaningful immune response and slow tumour growth in an immunocompetent mouse model. We synthesised osDNA™ constructs encoding the Neomatrix' proprietary neoantigen cancer vaccine. Adult C57Bl/6 mice were vaccinated intramuscularly at day 0 and day 14 by DNA-Electroporation (EP) using the following electrical condition: 110 V, 8 pulses, 20 msec. length, 120 msec. intervals. On day 21, mice were bled retro-orbitally to

monitor vaccine-induced immune response. PBMCs were stimulated overnight against a pool of neoantigen peptides and neoantigen-specific immune responses were measured by flow cytometry. Mice vaccinated with osDNA™ constructs showed an increase in CD8⁺IFNγ⁺, CD8⁺TNFα⁺ and CD8⁺IFNγ⁺TNFα⁺. Moreover, increases in CD4⁺IFNγ⁺, CD4⁺TNFα⁺ and CD4⁺IFNγ⁺TNFα⁺ were also observed. One month after the last immunization, mice were challenged with 3x10⁵ MC38 tumour cells. On day 9 post-tumour challenge, osDNA™ vaccinated mice showed a significant reduction in tumour growth (*p<0,05) compared to the control group. Moreover, the percentage of tumour-free mice vaccinated with osDNA™ was statistically significant compared to mice vaccinated with supercoiled pDNA at an equimolar ratio. IFN-γ ELISpot analysis at the time of sacrifice confirmed a neoantigen-specific immune response, which correlates with tumour delay. We have demonstrated that the immunostimulatory properties osDNA™ can be tailored and tuned, facilitating dose reductions compared to plasmid DNA whilst eliciting an enhanced immune response. This translates to a significant reduction in tumour growth and an increase in tumour-free mice vaccinated with osDNA™ constructs. The ability to rapidly produce synthetic osDNA™ products could greatly decrease the time required to generate personalised cancer vaccines encoding tumour specific neoantigens for patients with locally advanced or metastatic solid tumours.

313. Engineered Allogeneic CAR Natural Killer Cells Resist Tumor Microenvironment Immunosuppression by Expression of TGFBR2 Dominant Negative Receptor

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The deep and durable clinical responses seen with autologous chimeric antigen receptor (CAR) T cells in hematologic malignancies demonstrate the power and promise of adoptive immune cell therapy. However, clinical responses with CAR-T cells in solid tumors have been more limited. Furthermore, challenges with manufacturing of autologous cell therapy limit availability to patients. CAR-NK cell therapies offer new advantages, including their potential as allogeneic off-the-shelf products and their innate ability to kill tumor cells. However, clinical success in solid tumors will require overcoming the immunosuppressive tumor microenvironment (TME). TGF-β, a cytokine commonly expressed in the TME, limits the anti-tumor activities of Natural Killer cells through multiple mechanisms. Here we developed a TGFBR2 Dominant Negative Receptor (DNR) to protect the function of our engineered CAR-NK cell targeting HER2 for application in solid tumors. A TGFBR2-DNR was engineered by truncation of TGFBR2 receptor 2 to create a receptor that retains the extracellular TGFβ binding domain but interferes with endogenous receptor intracellular signaling. NK cells expressing TGFBR2-DNR resist the induction of TGF-β canonical signaling, as evidenced by a reduction of at least 75% in phosphorylation of the downstream effector SMAD2, even at supra-physiological doses of TGF-β. Whereas prolonged exposure to high levels of TGF-β reduces cytotoxicity of native NK cells, DNR expression preserves endogenous cytotoxicity.

DNR-engineered NK cells are also protected from a change in the expression of NK receptors, such as the DNAM-1-CD96 axis. Furthermore, TGFBR2-DNR acts as a trap, protecting neighboring native NK cells from TGF- β signaling. In addition to protection of NK cell cytotoxicity through innate receptors, we show that co-expression of the DNR with a HER2-CAR maintains CAR-dependent cytotoxicity in the absence or presence of TGF- β . The integration of TGF- β resistance provided by expression of the DNR with antigen-specific tumor targeting, achieved through co-expression of a CAR, provides an immediately achievable path towards novel engineered NK cell therapies with greater potential to treat solid tumors in cancer patients.

314. Tim-4-Chimeric Engulfment Receptor (CER) T Cells Elicit Phosphatidylserine-Dependent Cytotoxic and Innate-Like Function and Synergizes with Approved PARP Inhibitors in an Ovarian Cancer Model

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Background: Engineered T cells have emerged as a powerful anti-cancer therapy, mainly for the treatment of hematologic tumors. Here, we describe a solid tumor model of multi-modal CER T cell function. CER T cells recognize the stress-induced, tumor-agnostic ligand phosphatidylserine (PS) to overcome tumor-induced immune suppression by facilitating antigen capture, processing, and presentation and impart target dependent cytotoxic function. CER T cells were assessed in combination with approved small molecule inhibitors of poly (ADP-ribose) polymerase (PARP) enzymes in a PARP-sensitive ovarian cancer model. **Methods:** PS exposure on A2780 ovarian cancer cells was evaluated upon treatment with the small molecule PARP inhibitors niraparib or olaparib by annexin v staining to determine the appropriate drug dose. We then engineered novel PS targeting Tim-4 CER constructs designed to enhance cytotoxicity and target-dependent tumor cell fragment uptake. CER T cell designs were as follows: CER-1234 (Tim-4/CD28/TLR2/CD3z); CER-1236 (Tim-4/CD28/CD3z/TLR2); and CER-1250 (Tim-4 binding mutant/CD28/TLR2/CD3z). Niraparib (0.5uM) and olaparib (1.5uM) were used to expose PS on the surface of A2780 cells for CER T cell recognition and cytotoxicity, T-cell activation, and cytokine responses were evaluated. Uptake of antigen by CER-1236 T cells was modeled with human papilloma virus E7 antigen-expressing squamous carcinoma (SCC152) TMEM30A phospholipid flippase knockout cells. These cells constitutively exposed outer surface PS. E7 antigen presentation by CER-1236 T cells was assayed by surface expression of HLA-DR, a marker of T cell activation, on autologous HLA-A2-restricted E7 TCR T cells. **Results:** PARP inhibitors induced PS exposure on the outer cell surface at subtherapeutic doses in viable A2780 cells after 96 hours of treatment. Compared to untreated, 23% of A2780 cells were eliminated after addition of niraparib and 34% were eliminated after olaparib addition. CER-1234 or CER-1236 eliminated A2780 cells by 39% or 19%, respectively. Niraparib synergized with CER-1234 or CER-1236 to eliminate A2780 cells by 58% or 81% after 5 days of co-culture,

while olaparib eliminated 66% of targets with CER-1234 or 73% with CER-1236. Interferon- γ increased by 7-fold and granzyme B increased by 5-7-fold for either drug combined with CER-1234 at 96 hours compared to CER-1234 with no drug. Relative to CER-1236 T cells alone, CER-1236 T cells exposed to TMEM30A- SCC152 cancer cells increased the activation status of E7 TCR T cells 5-fold, demonstrating partial engulfment, antigen processing and presentation of HLA-A2 cognate peptide. **Conclusion:** CER T cells exhibit adaptive and innate functionality. CER-T cells have demonstrated cytolytic activity against A2780 ovarian cancer cells treated with subtherapeutic doses of PARP inhibitors and enhanced APC-like functionality demonstrated in the HPV E7 model. The combined functionality of CER T cell products provides a promising clinical application as a treatment option for patients with ovarian cancer receiving approved PARP inhibitors.

315. Abstract Withdrawn

316. Abstract Withdrawn

317. Discovery of TSC-200-A02: A Natural HPV16 E7-Specific TCR-T Cell Therapy Candidate for the Treatment of HPV-Positive Solid Tumors

Sonal Jangalwe, Daniel C. Pollacksmith, Cisem Karaca, Shubhangi Kamalia, Mollie M. Jurewicz, Kimberly M. Cirelli, Kenneth L. Jahan, Sida Liao, Alexandra L. Luther, Hannah L. Bader, Briana N. Zimmerman, Tary Traore, Maytal Bowman, Andrew S. Basinski, Anna M. Labrozzi, Amy Virbasius, Kristen Murray, Lisa Nip, Christina E. Lam, Livio Dukaj, Danielle Ramsdell, Joel W. Sher, Sadie Lee, Cagan Gurer, Qikai Xu, Yifan Wang, Antoine Boudot, Ribhu Nayyar, Gavin MacBeath

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Human papilloma virus (HPV) is an oncogenic virus responsible for over 90% of cervical and anal cancers and over 25% of head and neck cancers, which are typically incurable upon metastasis. HPV E7 oncoprotein is a compelling target for TCR-engineered T cell therapy as it is homogeneously expressed in every tumor cell, essential for tumor cell survival, and not expressed by healthy tissues. Notably, a recent clinical trial of an E7-directed TCR-T cell therapy conducted at the National Cancer Institute (NCI) showed a 50% objective response rate in heavily pre-treated patients with HPV+ cancers (1). Using TScan's proprietary *ReceptorScan* platform, we discovered 453 putative HPV16 E7₁₁₋₁₉-specific TCRs by screening 681 million naive CD8⁺ T cells from 15 unique healthy donors. We tested each TCR for expression in primary T cells and for its ability to kill T2 cells pulsed with the E7₁₁₋₁₉ peptide, using the NCI TCR as a benchmark for these studies (2). The top 3 TCRs from this screen were evaluated in depth for cytotoxicity, cytokine production, and T cell proliferation in response to a panel of HPV16+ cancer cell lines expressing varying levels of HLA-A*02:01 and E7. A lead TCR was identified that showed comparable activity relative to the NCI TCR. The lead TCR was evaluated for allo-reactivity using an array-based screen and for off-target reactivity using our

proprietary *SafetyScan* platform, which is a highly sensitive screen for off-target recognition based on supraphysiologic expression of protein fragments that span the entire human proteome. No allo-reactivity was observed to 108/110 HLAs tested, and only a few putative off-targets were identified. The TCR-T cells efficiently recognized target cells transfected with full-length E7 but showed minimal or no recognition of target cells transfected with ORFs encoding the putative off-targets, suggesting that the off-target antigens are less efficiently processed and presented on cell-surface MHC than E7₁₁₋₁₉. Furthermore, the TCR-T cells showed no reactivity to a panel of HPV-negative cancer cell lines and normal primary human cells, including cells that naturally express the putative off-targets identified in the *SafetyScan* screen. To further enhance the activity of our T cells, we designed a transposon-based vector that delivers the TCR gene, along with the genes for CD8 α / β and a dominant-negative form of TGF β R2, into both CD4⁺ and CD8⁺ T cells. We have advanced the resulting autologous TCR-T cell therapy candidate, TSC-200-A02, to IND-enabling studies. These results validate the use of *ReceptorScan*, in conjunction with *SafetyScan*, as a way to rapidly identify naturally occurring, high affinity TCRs that are suitable for clinical development. References 1) Nagarsheth NB, Norberg SM, Sinkoe AL, et al. TCR-engineered T cells targeting E7 for patients with metastatic HPV-associated epithelial cancers. *Nat Med.* 2021 Mar;27(3):419-425. 2) Jin BY, Campbell TE, Draper LM, et al. Engineered T cells targeting E7 mediate regression of human papillomavirus cancers in a murine model. *JCI Insight.* 2018;3(8):e99488

318. Development of Next-Generation, Off-the-Shelf CAR T-Cell Immunotherapies for Solid Tumors

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Despite inducing promising clinical outcomes in patients with relapsed / refractory hematologic malignancies, the adoptive transfer of engineered T cells armed with a chimeric antigen receptor (CAR) has been less effective against advanced solid tumors. Specific challenges that have emerged include lack of tumor-exclusive antigen targets, antigen heterogeneity, and functional suppression resulting from the tumor microenvironment. In addition, inherent limitations associated with patient- and donor-derived T-cell therapies limit the breadth of their therapeutic application, including the potential to reach patients earlier in care. Induced pluripotent stem cells (iPSCs) can serve as renewable starting material for the mass production of off-the-shelf, cell-based cancer immunotherapies. We have previously developed FT819, a first-of-kind off-the-shelf CAR T-cell therapy derived from a clonal master iPSC line engineered to uniformly express a novel CD19 1XX-CAR driven by the endogenous T-cell receptor (TCR) α promoter at the T-cell receptor α constant (TRAC) locus. FT819 was designed to promote antigen specificity, improved safety by knocking out the TCR to eliminate the possibility of graft versus host disease (GVHD), and enable off-shelf availability. Here, we describe a novel multiplexed-engineered, off-the-shelf CAR T-cell therapy derived from a clonal master iPSC line for the treatment of solid tumors.

The master iPSC line incorporates seven functional modalities: 1) a CAR for direct targeting of tumor antigen; 2) a cytokine receptor fusion protein for enhanced T-cell activity; 3) a high-affinity, non-cleavable CD16 (hnCD16) for enhanced antibody-dependent cellular cytotoxicity (ADCC) in combination with tumor-targeting monoclonal antibodies; 4) an engineered modality for enhanced trafficking; 5) a novel chimeric protein for enhanced functionality in response to tumor microenvironment resistance signaling; 6) CD38 deletion for enhanced metabolic fitness; and 7) TRAC-targeted TCR deletion for eliminating the risk of GVHD. Utilizing our proprietary iPSC product platform, a clonal master iPSC line was derived by CRISPR-mediated knock-in of functional modalities into the TRAC and CD38 loci. Single iPSCs were assessed and selected for specific targeted integration at the desired loci without random donor template integration; transgene copy numbers were confirmed by droplet digital PCR as well as genome stability by karyotyping and microarray analysis. We next demonstrated that T cells derived from the clonal master iPSC line exhibited improved expansion during differentiation, including without the need for cytokine support. In vitro functional studies including antigen-specific cytokine release assays, cytotoxic T lymphocytes assays, mixed lymphocyte reaction, and serial restimulation assays demonstrated enhanced efficacy. Our data validate the potential of multi-loci engineering of iPSCs to create master multiplexed-engineered iPSC lines and produce next-generation, iPSC-derived CAR-T therapies against solid tumor.

319. Sendai Virus Fusion Gene Therapy Induced Anti-Tumor Effects Mediated by RANTES

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Inactivated Sendai virus particle (or hemagglutinating virus of Japan envelope: HVJ-E) has been previously reported to possess tumor suppressive effects that activate anti-tumor immunity. Of the two glycoproteins on the viral surface, only the fusion (F) glycoprotein, and not hemagglutinin-neuraminidase (HN), when reconstituted onto liposomes showed anti-tumor activity by inducing IL-6 secretion in dendritic cells. This suggests that F protein alone can elicit anti-tumor effects. So, in this study, we examined the application of F gene therapy on the B16F10 mouse melanoma model and the potential anti-tumor effects. We delivered F gene into B16F10 tumor tissue in mice by electroporation and demonstrated that F gene therapy suppressed tumor growth, increased CD4⁺ and CD8⁺ T cell infiltration into tumors and induced tumor-specific IFN γ T cell responses. However, F plasmid-mediated anti-tumor effect was not abrogated with neutralization of IL-6R signaling. Instead, we found that F plasmid treatment resulted in significant increase in the secretion of the chemokine RANTES (regulated upon activation, normal T cell expressed and secreted) by tumor-infiltrating T cells. Neutralizing antibody against RANTES abolished the anti-tumor effect of F plasmid treatment in a dose-dependent manner. Thus, F gene therapy can elicit anti-tumor effects by upregulating the secretion of RANTES which promotes the infiltration of T cells into B16F10 tumors, and may show promise as a novel therapeutic for single or combined cancer immunotherapy.

320. Non-Viral Engineering of T Cells for Adoptive Cell Therapies Using S/MAR DNA Vectors

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T cell-based therapies with personalized or off-the-shelf T cell receptors (TCRs) are an appealing approach for the treatment of cancer. Currently, the genetic engineering of T cells is achieved through the use of viral vectors. However, their innate immunogenicity and potential for insertional mutagenesis, as well as long and expensive large-scale manufacturing protocols, remain major obstacles toward their application of TCR-transgenic T cell in cancer immunotherapy, especially in a personalized setting. We have previously described a non-viral DNA vector platform based on Scaffold/Matrix-Associated Regions (S/MAR), for the safe and efficient generation of stably engineered T cells. S/MAR DNA nanovectors (nS/MAR) are episomally maintained, and thus eliminate the risk of insertional mutagenesis, unlike platforms based on viral vectors, transposons or CRISPR systems. Furthermore nS/MAR vectors have much larger cargo capacities, allowing for the delivery of multiple or large transgenes. Finally nS/MAR vectors reduce manufacturing costs, and their stable DNA construct reduces logistical obstacles in delivery. We refined the design of nS/MAR DNA system, previously used to generate primary human Chimeric Antigen Receptor (CAR) T cells. Here, using HLA-A2⁺ human donors, we generated T cells expressing a TCR specific for the melanoma antigen MART-1, currently tested in clinical trials. We investigated the capacity of 5 strong or weak promoters driving TCR expression to modulate the functionality of TCR-transgenic T cells. We initially confirmed TCR surface expression by flow cytometry and our data demonstrates that although every promoter was able to produce detectable levels of TCR at the cell surface, the core EF-1 α promoter resulted in the highest (89%) transfection efficiency and TCR surface expression. To further evaluate the possibility of an enhanced anti-tumor function, we performed a killing assay with the MART-1/HLA-A2⁺ MeWo melanoma cell line and transgenic T cells. To date, our results demonstrate that although all promoters generated T cells competent for antigen recognition and cytotoxic activity, the core EF-1 α promoter showed the highest level of cytokine release, suggesting that it may enhance the T cell efficacy. Next, we optimized a scalable good manufacturing practice (cGMP) protocol for the rapid generation of MART-1 TCR-transgenic T cells by combining the CliniMACS[®] Prodigy and MaxCyte[®] ExPERT platforms. 4E8 T cells, isolated from peripheral blood leukapheresis product, were electroporated using the MaxCyte ExPERT GTx[®] achieving a transfection efficiency >85% with no significant loss of cell viability in a period of only 6 days. In summary, these data suggest that nS/MAR

DNA vectors are a safe technology for the development of cellular therapies with key advantages for a large-scale manufacturing of TCR-transgenic T cells, supporting their translation to the clinic.

321. Unraveling Transcriptomic Profiles of Pediatric Acute Myeloid Leukemia Cells Sensitive or Resistant to Cytotoxic Killing by Engineered TR1-like Cells

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Pediatric acute myeloid leukemia (AML) is one of the deadliest cancers among children and most patients depend on allogeneic hematopoietic stem cell transplantation (allo-HSCT) as a treatment option. Allo-HSCT can cause a life-threatening graft-vs-host disease (GvHD) while restoring the patient's immune system with stem cells from a healthy donor. Type 1 regulatory T cells (Tr1) are unique immune cells that can dampen the GvHD while killing myeloid blasts, enhancing the graft-vs-leukemia (GvL) response. However, Tr1 cells are low in frequency in peripheral blood and hard to expand *in vitro*, thus limiting their use as cell therapeutics. To overcome this, our lab generated a cell therapy product by engineering human CD4⁺ T cells to overexpress *IL10* (CD4^{IL-10}). CD4^{IL-10} functionally mimic Tr1 cells, with high suppressive capacity and cytotoxic properties against myeloid cells. We previously reported that majority of primary pediatric AML blasts are sensitive to killing by CD4^{IL-10} and some are resistant. We performed bulk RNA sequencing and selected 2 sensitive and 2 resistant blasts as well as CD4^{IL-10} cells generated from two donors to compare the transcriptional changes in both cell types before and after *in vitro* co-culture via single-cell RNA sequencing. Our analysis showed that sensitive and resistant blasts have distinct transcriptional profiles with differentially expressed markers specific to resistant blasts that could be predictive of CD4^{IL-10}-mediated killing resistance. Additionally, CD4^{IL-10} cells co-cultured with sensitive blasts upregulate different genes when compared to resistant ones. In conclusion, CD4^{IL-10} are cytotoxic cells that can kill majority of pediatric AML blasts and our data indicate a potential treatment of pediatric AML by using CD4^{IL-10} combined with allo-HSCT in the future.

322. CARs and CAR-T Engager Proteins Targeting Multiple Antigens for Acute Myeloid Leukemia and Multiple Myeloma

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Chimeric Antigen Receptor T cells targeting malignancies expressing CD19 (CAR19) have been widely successful, with products approved to treat B cell lymphomas (NHL) and acute and chronic B cell leukemia

(ALL and CLL, respectively). Similarly, CARs targeting BCMA (CAR-BCMA) have rapidly advanced for the treatment of Multiple Myeloma (MM), although relapses remain a critical issue. Of note, the CARs directed to CD19 and BCMA show prolonged persistence *in vivo*, particularly when the 4-1BB costimulatory domain is used in the CAR construct. While NHL, ALL, CLL and MM are susceptible to CAR therapeutics, Acute Myeloid Leukemia (AML) has proven to be highly resistant to CAR T treatment, with only modest efficacy seen in most trials. Antigen selection has proven challenging in AML, as some AML antigens are also expressed on normal hematopoietic stem cells. Further, AML antigen expression is very heterogeneous, with varying degrees of antigen expression on the AML blast cell population and on the leukemic stem cells (LSC) that drive relapses in these patients. The CAR T Engager (CTE) platform was developed to enable multi-antigen targeting via a single CAR domain. Here we describe two novel CTE approaches. First, we have coopted a CAR19 T cell as an ideal CAR to use for CTE-mediated therapy of AML. A CAR19 that includes both 4-1BB and CD28 costimulatory domains was created and is shown here to have excellent cytotoxic activity and capacity for restimulation. The CTE was designed to bind to CAR19 T cells via the encoded CD19 protein extracellular domain (ECD), and to bind to two AML antigens, CLEC12a and CD33. CLEC12a binding is mediated by two antibody binding domains to distinct epitopes, and CD33 binding is mediated by a high affinity antibody domain. This CTE, termed CTE-CD19.CLEC.33, binds to and kills AML cells expressing either or both antigens. The expression of CLEC12a and CD33 is sufficient to identify nearly all patient's AML, regardless of subtype. Preferential binding to dual-antigen expressing cells may bias CAR T activity to AML cells. Similarly, we created a novel CAR-BCMA, incorporating both 4-1BB and CD28 costimulatory domains. In order to target additional MM antigens, we've created several novel CTEs. CTE-BCMA.CD38 contains the BCMA ECD linked to an anti-CD38 antibody domain. A second CTE to a novel antigen will be presented, with the ultimate goal being the creation of a triple targeting CTE for MM. Finally, we can incorporate useful immunomodulatory activities into Engager proteins and will introduce one of these novel features in the context of MM targeting.

323. Ovarian Cancer Immunotherapy Through Novel Engineered Glyco Immune Checkpoint Blockade

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Ovarian cancer (OC) represents a highly lethal gynecologic malignancy. Despite the significant developments in the field of OC therapy over the last few years, recurrent OC is still associated with extremely poor prognosis. The discovery of immune checkpoints that are involved in the regulation of immune activation, and their inhibition using monoclonal antibodies (mAbs) has led to a surge in novel therapies for the treatment of solid tumors as well as hematological malignancies.

However, to date OC has benefitted little from immune checkpoint inhibitor (CPI) therapy; the overall response rate of pembrolizumab: anti-PD1 antibody and avelumab: anti-PD-L1 antibody was only 8% and 9.6%, respectively. One limitation is that most OCs often exhibit comparatively low T-cell infiltration, a characteristic which is known as being immunologically "cold" and thus poorly responsive to PD-1 blockade and T cell-based approaches. Thus, engaging the other components of the immune system may provide additional tumor control. As increasing evidence suggests that OC is receptive to natural killer (NK) cell attack, accordingly we tested antibodies developed against human Siglec 7, an inhibitory receptor present on human NK cells, as a putative NK activator. Altered surface glycosylation of tumor cells facilitates their direct binding to Siglec 7, which negatively signals to the NK cells during immune surveillance, allowing the tumor cells a free immune pass. As a countermeasure, we developed and down selected 8 human blocking mAbs against Siglec 7 based on their binding efficacy to recombinant human Siglec 7 and studied their effectiveness to overcome NK immune blockade. Three among the 8 mAbs; DB-S7-1, DB-S7-2 and DB-S7-7 showed strong binding to Siglec 7 expressed in cells, as analyzed by flow cytometry and Immunofluorescence assays. Further, we confirmed their binding to human NK cells and DB-S7-2 showed strong binding to NK cells at the concentration between 10-50 ng/ml. Further, we showed that DB-S7-1, DB-S7-2 and DB-S7-7 engaged NK cells activating them to target and killed a panel of different human ovarian tumor lines including OC BRCA mutant cells as well as cell lines resistant to chemotherapy or PARP inhibitors. For *in vivo* analysis, we developed DB-S7-1, DB-S7-2 and DB-S7-7 anti Siglec 7 antibodies as DNA launched expression cassettes (DMAbs). Sera obtained from mice inoculated with these DMAbs strongly stained human NK cells, supporting *in vivo* production of anti Siglec 7 antibodies. We when challenged NOD/SCID- gamma (NSG) mice model with OVISe tumors in the presence of human PBMCs, these mice had significantly attenuated tumor burden. The inhibition of the Siglec 7 pathway on NK cells is reminiscent of early data suggesting the importance of the PD-L1 pathway blockade for allowing T cell activation for the treatment of cancer. These studies warrant further investigation into this potentially important pathway that could complement traditional CPI through engaging NK cell pathways.

324. Cell Avidity as a New Parameter to Accurately Predict CAR T Cell Activity

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CAR-T cell therapy has seen tremendous clinical success in the treatment of haematological malignancies; however, this success has been limited to a small number of indications and only a few cancer-specific targets. One of the greatest challenges facing the field is predicting the clinical efficacy of a CAR-T product based on *in-vitro* data alone. With the increasing complexity of constructing novel methodologies to further improve the clinical outcome of immunotherapies, screening methods to quickly identify the best lead candidates is becoming even more essential. To date, it has become evident that merely measuring the affinity between a CAR and its target will not accurately predict *in vitro* and *in vivo* outcomes. On the contrary, cell avidity (or overall cellular binding strength) provides a

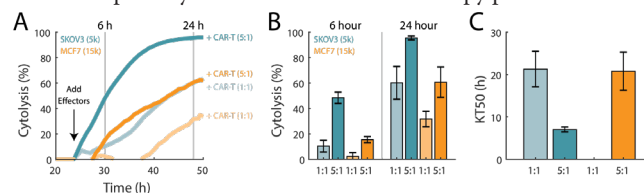
more complete and physiologically relevant picture that reflects the *bona fide* interaction between CAR T cells and tumour cells. Therefore, this interaction can better predict cellular responses *in vitro*, and drive better, more informed decisions at earlier stages for drug selection and potentially improve clinical outcomes. One of the main obstacles in the process of measuring avidity as being a critical parameter is the lack of fast, specific, and accurate tools to assess cellular avidity. We present the z-Movi[®] Cell Avidity Analyzer, our novel platform for measuring cell-cell binding strength. The z-Movi facilitates the direct analysis of CAR-T cells either against surface immobilised antigens or a monolayer of target cells. Cell-cell interactions are perturbed using resonant sound waves generated by a piezoelectric element and our proprietary cell tracking system is used to measure the required disruption force. We have used the z-Movi to measure and compare the interaction force between CAR T cells and their relevant target cells. Our analysis was compared against traditional SPR data combined with cytotoxic and cytokine release under co-culture conditions. The z-Movi measurements were shown to provide a more sensitive insight into CAR T cell activity than the competing workflow and were able to identify differences between CAR T cells more readily than the standard *in vitro* assays. We believe that avidity measurements using z-Movi have the potential of filling the knowledge gap currently present in the understanding of CAR-T and target cell interactions and complement the typical workflow with its new dimension of T cell testing thereby contributing to accelerating the development of cellular immunotherapy against cancer.

325. Comparison of Magnitude and Kinetics for HER2-Specific CAR T Cell Mediated Cytotoxicity Across Cancer Cell Lines In Vitro

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The development of immunotherapies relies on the use of *in vitro* potency assays—which are key for understanding complex interactions between immune (effector) cells and cancer (target) cells—to evaluate the function, specificity, and sensitivity of a product. A variety of *in vitro* assays are used to characterize the proliferation, cytokine release, and cell-mediated cytotoxicity of engineered immune cells, such as chimeric antigen receptor (CAR) T cells, with each representing an important aspect driving clinical efficacy of the cellular product. The desired effect of a CAR T cell is to mediate antigen-specific cytotoxicity of tumor cells, positioning immune cell-mediated cytotoxicity as the most direct method to assess potency of immunotherapy cell products. Here, we describe an *in vitro* potency assay that quantifies the antigen-specific killing kinetics of immune effector cells, and then evaluate the potency of CAR T cells against target cells with varied levels of antigen expression. First, SKOV3 and MCF7 target cells were seeded into multiwell microplates with embedded electrodes in the substrate. Electrical impedance measurements from the electrodes in each well detected the attachment and proliferation of the target cells. HER2-specific CAR T effector cells were added 24 hours after the target cells and co-cultured with the target cells for an additional 24-72 hours. Effector-only control wells confirmed that the immune effector cells did not appreciably affect the impedance measurement, ensuring that the assay readout was specific to target cell death. Cytolysis of the target cells was calculated by comparing treated wells to no treatment control

wells, and the kinetics of cell death were determined from the time required for immune effector cells to kill half of the target cells (kill time 50, KT50). Antigen-specific killing, or specific cytolysis, was evaluated by comparing SKOV3 and MCF7 cytolysis from HER2-specific CAR T cells to donor-matched mock CAR T cells and non-transduced T cells. At 24 hours post-addition of the effector cells, target cell lysis was higher for SKOV3 (95 +/- 2%) than MCF7 (61 +/- 12%) with 5:1 effector to target ratio of HER2-specific CAR T cells. The kinetic read-out from the assay indicates a KT50 of 7.1 +/- 0.6 hours for SKOV3 and 21.8 +/- 4.5 hours for MCF7 with 5:1 effector to target ratio of HER2-specific CAR T cells. Future work will further evaluate the value of specific lysis kinetics for potency assessment of immunotherapy products.



326. EG-70, A Novel Non-Viral Gene Therapy for Local Expression of Innate and Adaptive Immune Modulators for Treatment of Non-Muscle Invasive Bladder Cancer

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Background enGene is developing a non-viral gene therapy platform for repeat administration of genetic medicines to mucosal tissues, such as bladder urothelium. The first drug product candidate, EG-70, has been developed for the treatment of BCG-unresponsive non-muscle invasive bladder cancer (NMIBC). EG-70 is a nanoparticle formulation containing a non-integrative plasmid packaged in a proprietary polymer, arginine-glucose derivatized-oligomeric chitosan (DDX). The core nanoparticle is coated with an anionic methoxy-poly(ethylene glycol)-poly(L-glutamic acid) diblock copolymer. enGene has demonstrated that the reversibly PEGylated nanoparticle exhibits mucus penetration properties and improved stability in the bladder lumen for extended periods. The EG-70 plasmid DNA induces the expression of two RNA products to collectively activate the innate immune receptor, retinoic acid-inducible gene I (RIG-I), as well as a single-chain recombinant human interleukin-12 (IL-12). Direct bladder instillation of EG-70 induces a potent immune response locally in the bladder tumor microenvironment (TME), resulting in a strong anti-tumor response with no systemic toxicity. **Methods and Results** Activation of the biological pathways downstream of RIG-I agonists and IL-12 was demonstrated in cultured bladder epithelial cells. Following bladder instillation of an EG-70 mouse surrogate

(mEG-70; for production of species-relevant murine IL-12 protein), the expression of transgene products was localized to the bladder of mice and modulated in a time- and dose-dependent manner. mEG-70 resulted in transgene-specific bioactivity, including upregulation of Type I IFNs, CXCL10 and IFN γ . Two weekly instillations of mEG-70 in a murine model of orthotopic bladder cancer mediated a strong reduction in tumor burden, which lead to improved survival compared to control mice. Immune profiling of the TME demonstrated that mEG-70 treatment yielded increased NK cell activation, increased proportions of CD4⁺ and CD8⁺ T cells, and concomitantly reduced myeloid-derived suppressor cells and tumor-associated macrophages in the bladder, indicating a pro-tumor killing milieu. Antibody-mediated depletion of CD4⁺ T cells resulted in loss of anti-tumor activity whereas depletion of CD8⁺ T cells had no effect. Mice that underwent full tumor regressions were resistant to subsequent local or distal re-challenge with bladder tumor cells. Similarly, CD4⁺ T cells were critical for systemic antigen-specific response to bladder tumors while CD8⁺ T cells were dispensable. Transgene expression and bioactivity were recapitulated in cynomolgus monkeys. Results from preclinical safety studies indicated that mEG-70 and EG-70 were well-tolerated in mice and monkeys, respectively, causing only mild and reversible local findings. **Conclusion** Intravesical administration of EG-70 results in robust transgene expression in the bladder mucosa that drives profound and durable anti-tumor immunity in an orthotopic model of bladder cancer. In the preclinical setting, EG-70 was safe and well-tolerated with minimal and reversible local toxicity, which has now been recapitulated in an ongoing first-in-human Phase 1/2 clinical trial (the LEGEND study) evaluating safety and efficacy of intravesical administration of EG-70 in patients with BCG-unresponsive NMIBC. Exploratory biomarker analyses from human samples (including IL-12 transgene expression) are ongoing and may be available for presentation at the meeting.

327. Targeting SNORA38B Remodels Tumor Microenvironment and Sensitizes NSCLC to Immunotherapy

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Background Non-coding RNAs, including small nucleolar RNAs, are widely involved in cancer progression. While up to date, the underlying mechanisms and the exploration of tumor immune environment and options of convincing innovative treatment of snoRNAs in NSCLC are still warranted. **Methods** A quantitative polymerase chain reaction was performed to verify the expression of *SNORA38B* in NSCLC clinical samples and cell lines. Cytometry by the time of flight, enzyme-linked immunosorbent assay, and flow cytometry assay were used to clarify the induction effect of *SNORA38B* on the tumor immunosuppressive microenvironment. Gene set enrichment analysis, western blotting, RNA immunoprecipitation, and RNA pull-down assays were used to determine the mechanism of *SNORA38B* in the mechanistic target of rapamycin carcinogenic pathway by binding the transcription factor E2F1. The combined treatment of *SNORA38B* locked nucleic acid and immune checkpoint blockade was performed to clarify the effectiveness of targeting *SNORA38B* in NSCLC immunotherapy. **Results** *SNORA38B* was highly expressed in NSCLC tissues and cell lines, and highly expressed *SNORA38B* was associated with a worse prognosis. *SNORA38B* functioned as an oncogenic role both in vivo and in vitro

via facilitating cell proliferation, migration, invasion, and inhibiting cell apoptosis. *SNORA38B* could also recruit the CD4⁺FOXP3⁺ regulatory T cells in NSCLC immune microenvironment by triggering secretion IL-10 of tumor cells, so that reducing the infiltration of CD3⁺CD8⁺ T cells, resulting in faster tumor progression and poorer immune efficacy. We also found that *SNORA38B* highly expressed NSCLC-related genes are enriched in the mTOR pathway, followed experiments validation conformed that *SNORA38B* could activate the GRB2 associated binding protein 2/AKT/mTOR axis and its downstream effectors by directly binding to the transcription factor E2F1, which may be an essential mechanism for *SNORA38B* mediated carcinogenesis. Finally, we found that *SNORA38B* LNA was able to sensitize NSCLC to ICBs treatment. **Conclusions** In conclusion, our data demonstrated that *SNORA38B* exerts oncogenic roles on NSCLC via directly binding with E2F1, and regulating GAB2/AKT/mTOR axis. *SNORA38B* may be a critical therapeutic target for NSCLC. **Keywords** *SNORA38B*, Non-small cell lung cancer, E2F Transcription Factor 1, GRB2 Associated Binding Protein 2, Regulatory T cells, Tumorigenesis

328. Improved Antigen Uptake and Presentation of Dendritic Cells by Using Cell Penetrating D-Octaarginine-Linked PNVA-co-AA for Novel Dendritic Vaccine

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Introduction: Cancer immunotherapy using antigen-pulsed dendritic cells can induce strong cellular immune responses by priming cytotoxic T lymphocytes, however, the therapeutic efficacy could be improved. Recently, we have developed the co-polymer of N-vinylacetamide and acrylic acid (PNVA-co-AA) grafting D-octaarginine onto its backbone to improve the antigen uptake and presentation in dendritic cells by induction of macropinocytosis. In this study, we pulsed tumor cell lysates with D-octaarginine-linked PNVA-co-AA (VP-R8) into murine dendritic cell line (DC2.4) and determined the anti-tumor effect in tumor bearing mice. **Methods:** The efficiency of antigen uptake in DC2.4 was determined by fluorescein isothiocyanate (FITC)-conjugated ovalbumin with VP-R8 *in vitro*. Tumor cell lysate were obtained from murine lymphoma cell line (EL4) and pulsed into DC2.4 cells with VP-R8 for 1 hour *in vitro*. After incubation, DC2.4 cells were stained with anti-MHC class I, MHC class II, CD80 and CD86 to determine the antigen presentation and maturation. For *in vivo* study, 2x10⁵ EL4 cells were subcutaneously implanted into C57BL/6 mice (days 0). After tumor formation, DC2.4 cells pulsed with EL4 lysate and VP-R8 were subcutaneously injected into inguinal lymph node 3 times (days 7, 10, 13). To investigate the EL4 specific T cell immune responses induced by dendritic cell vaccine, spleen cells were isolated from mice and re-stimulated with mitomycin C-treated EL4 cells *in vitro*. The population of IFN- γ producing T cells were determined by intracellular cytokine staining. **Results:** The mean fluorescent intensity of FITC-ovalbumin in DC2.4

cells was significantly higher by treatment with VP-R8 than keyhole limpet hemocyanin (KLH) ($p < 0.05$), indicating that VP-R8 improve the antigen uptake in DC2.4. The expression of MHC class I, MHC class II and CD86 in DC2.4 cells were significantly increased after pulsing tumor lysate with VP-R8 compared to other treatments ($p < 0.05$). *In vivo* study, subcutaneous injection of DC2.4 pulsed with both VP-R8 and EL4 lysate ($1506.2 \pm 85.7 \text{ mm}^3$, $p < 0.05$) significantly decreased tumor growth compared to that of DC2.4 pulsed with KLH and lysate ($2159.3 \pm 154.4 \text{ mm}^3$, $p < 0.05$) and PBS control ($2967.7 \pm 175.2 \text{ mm}^3$, $p < 0.01$) 14 days after tumor injection. Intracellular cytokine staining showed that the population of CD4T cells and CD8T cells specifically producing IFN-gamma were remarkably increased by the treatment of DC2.4 pulsed with both VP-R8 and EL4 lysate compared to other treatments. **Conclusion:** We demonstrated that VP-R8 significantly improved the antigen uptake, presentation and maturation of dendritic cell line DC 2.4 *in vitro*. We also showed that dendritic cell vaccine by using DC2.4 pulsed with VP-R8 and tumor cell lysate could induce tumor-specific T cell responses and anti-tumor immunity in mice. VP-R8 is a highly biocompatible material and has minimum toxicity for mammalian cells. Our findings suggested that VP-R8 may be a promising candidate to improve the therapeutic efficacy of current dendritic cell therapy.

329. In Vitro Characterization of CD19 CAR T Cells Derived by Electroporation of mRNA

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Cancer immunotherapies reprogram the patient's immune system to mount a coordinated response against a malignant target. T cells engineered to express Chimeric Antigen Receptors (CARs) represent an effective strategy to target specific cancer cells. Electroporation of mRNA offers several advantages over viral engineering including transient expression of CAR that may limit toxicity, reduce costs associated with viral vector manufacturing and eliminate the risk of virus-associated tumorigenicity. Here, mRNA encoding an anti-CD19 CAR was electroporated into healthy, human T cells using a MaxCyte ATx[®] instrument. Flow cytometric analysis showed that T cells electroporated with mRNA encoding an anti-CD19 CAR were 86.3% viable post electroporation, and 88% of the live cells were CD3+/CAR+. These engineered cells successfully targeted and killed 100% of CD19-expressing NALM6 target cells, compared to only 20% killing with untransfected controls. In addition, CAR T cells produced proinflammatory cytokines when incubated with CD19-expressing NALM6 cells, while control T cells did not. In this study, we have used MaxCyte electroporation of mRNA to engineer CAR T cells to target CD19-expressing cancer cells. This efficient, non-viral method for generating functional CAR T cells is the foundation for future studies, including scaled-up production and the evaluation of efficacy in animal models, either alone or in combination with biologics. The ability to screen multiple candidates simultaneously would facilitate identification and selection of CARs for more rapid progression through the development pipeline supporting early drug discovery and new preclinical strategies.

330. aPDL1/41BB Switch-Receptor Enhanced CAR-T Cell Cytotoxicity and Synergize with Radiotherapy and aPD-1 Therapy in Solid Tumors

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Despite the significant efficacy in hematologic malignancies, the responses of CAR-T cells in solid tumors remain poor. The immunosuppressive tumor microenvironment in solid tumors greatly limited the infiltration, function and persistence of CAR-T cells. Here we introduced a novel aPDL1/41BB switch-receptor construct into CAR-T cells, turning the inhibitory signal of PD-L1 into activating signal. We found that in vector T cells, the cytokine release and proliferative capacity were repressed in response to PD-L1 protein, while T cells that express aPDL1/41BB switch-receptor showed further enhanced functions in response to PD-L1. *In vitro* studies, CAR-T cells with aPDL1/41BB switch-receptor exhibited better antigen-specific cytotoxicity than that of conventional CAR-T. In tumor-bearing mice, aPDL1/41BB CAR-T cells achieved better tumor regression in comparison to conventional CAR-T cells. In addition, we also explored the effect of radiotherapy as an immunologic adjuvant for CAR-T cell therapy. We found that radiotherapy promoted CAR-T cell transmigration in transwell co-culture assay and increased the expression of T-cell recruiting chemokines both *in vitro* and *in vivo*. The triple therapy with radiotherapy, aPDL1/41BB CAR-T and aPD-1 exhibited the maximized antitumor responses in tumor-bearing mice. In summary, our study demonstrated a novel aPDL1/41BB switch-receptor that augmented the function of CAR-T cells, and we also suggest that the combinatorial regime of aPDL1/41BB CAR-T cells, radiotherapy and aPD-1 could be a powerful strategy to improve the unsatisfactory clinical outcomes of CAR-T cells.

331. A Synthetic Biology Approach to Address the Immunosuppressive Tumor Microenvironment: Novel TGF-B Switch Receptors Convert Inhibitory Signals into Enhanced NK Cell Activity

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Engineered immune cell-based therapies provide an effective treatment for hematologic malignancies, such as B cell lymphomas and leukemias, and multiple myeloma. While the current generation of cell-based therapies provides highly functional tumor-targeted cytotoxic cells, they are not directly designed to address the immunosuppressive factors of solid tumor microenvironments, and clinical progress in solid tumors has been challenging. We are developing allogeneic Natural Killer (NK) cells engineered to

effectively recognize antigen-expressing tumor cells through chimeric antigen receptors (CARs) and overcome transforming growth factor-beta (TGF- β)-mediated immunosuppression through chimeric switch receptors (SRs). The TGF- β pathway is integral to the establishment, persistence, and therapeutic resistance of tumors derived from epithelial cells, such as renal cell carcinoma, ovarian cancer and other solid tumors. Tumor microenvironment-derived TGF- β also inhibits immune responses to the tumor by inducing dysfunction in NK and cytotoxic T cells as well as promoting suppressive cells such as regulatory T cells. TGF- β reduces the anti-tumor therapeutic potential of NK cells by suppressing the expression of endogenous activating receptors and interfering with NK cell growth and function. We reasoned that the ideal solution to enhancing immune cell function in TGF- β rich environments would be redesigning TGF- β signaling to confer three functions on the engineered cells: (a) protect the engineered cells from TGF- β induced dysfunction, (b) convert the TGF- β signal into an immune-stimulatory signal, and (c) locally reduce TGF- β signaling to other cells within the tumor microenvironment. To directly counteract TGF- β 's immunosuppression, we designed SRs that convert this inhibitory signal into NK cell activation signals. We replaced the endogenous Smad2/3 inhibitory signaling of TGF- β receptor 2 with ectopic signaling domains that stimulate costimulation, growth, survival, and other pathways. We devised assays to screen for TGF- β SR signaling using Jurkat reporter cell lines, then assessed the impact of TGF- β SR expression on the proliferation and survival, cytotoxic function, and cytokine production of engineered human NK cells. We discovered several TGF- β SRs that reversed TGF- β 1-mediated inhibition and promoted NK cell expansion *in vitro*. We further found that NK cells expressing these TGF- β SRs acquired enhanced effector function and cytokine secretion against solid tumor target cells when chronically stimulated with TGF- β 1. Our findings demonstrate that our TGF- β SR approach combines intrinsic relief from TGF- β immunosuppression while adding a diverse repertoire of novel beneficial signals, offering a flexible and rich design strategy for creating therapeutic NK cells capable of robust functioning within hostile tumor microenvironments.

332. CAR T-Cell Antigen-Non-Specific Killing (CTAK), CAR-T NK-Like Killing, and CD22 CAR-T Mediated Killing Are All Optimized by Bryostatin Treatment of Pre-B Acute Lymphocytic Leukemia Cell Lines

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The treatment of chronic lymphocytic leukemia (CLL), pre-B ALL cell lines, and PDX models of disease has demonstrated that bryostatin increases the surface density of CD22, and was thereby reported to increase leukemia CAR-T sensitivity. This issue is crucial, given the low levels of CD22 surface expression on leukemia targets, and the finding that reduction in CD22 surface expression can serve as an escape mechanism. We have defined a new property of CAR-T cells by which CAR-T cells, activated by the signals used to produce therapeutic cell populations, mediate non-CAR-directed cytotoxicity, termed CTAK (CAR

T-cell antigen-non-specific killing). CTAK activity may in fact account for a large part of the increase in bryostatin-mediated killing. We also found that bryostatin does not increase CD22 levels on normal B cells, and has the opposite functional effect on the Raji (Burkitt lymphoma, BL) cell line- making it resistant to CAR-T cell mediated killing. Thus, bryostatin effects are disease-type specific and cannot be assumed to universally benefit CAR-T activity, even if target cell number on the surface of the malignant cell is increased. CTAK activity is attributed in part to increased ICAM1-mediated binding, and NKG2D activity, but no single known NK ligand activity (including NKP30 and DNAM-1) predominates. The term CTAK is used to differentiate this activity from LAK cells which are generated by high levels of cytokine alone, and NK-like activity, which can be blocked using cold-target inhibition by K562 cells. Killing of BL by the NK92 cell line was not affected by bryostatin, while the killing of ALL lines by NK92 required bryostatin exposure. This indicates that bryostatin may serve as an important addition to NK-cell directed killing approaches. CD19 and CD22 were modulated in unison, being up-regulated by bryostatin and down-regulated by overnight treatment with CD22 CAR-T. Thus CAR-T cells induce modulation of target antigens in both antigen specific and general mechanisms. CD22 CAR-T mediated down-regulation of antigens was profound, yet transient, and starting expression levels, with or without bryostatin, recovered within a week. This rapid modulation may be required to see effective CAR-T cell therapy, as continual high-level antigen expression would lead to antigen-mediated exhaustion. Troglodytosis was noted, but is unlikely to be a major mechanism of antigen down-modulation and escape. Our results support previous reports of bryostatin-mediated increases in CD22 expression, and that this increase may render target cells more sensitive to CAR-T mediated cytotoxicity, even as antigen down-modulates. We also can conclude that B cell leukemias modulate B cell differentiation antigens in concert, and that epigenetic modifiers and differentiation inducing reagents like bryostatin can serve as important potentiating agents of cell-mediated cytotoxicity. However, the use of these agents may sensitize leukemia targets to alternate cell-killing mechanisms, such as CTAK, and up-regulation of target antigen numbers alone does not indicate increased sensitivity to cytotoxicity.

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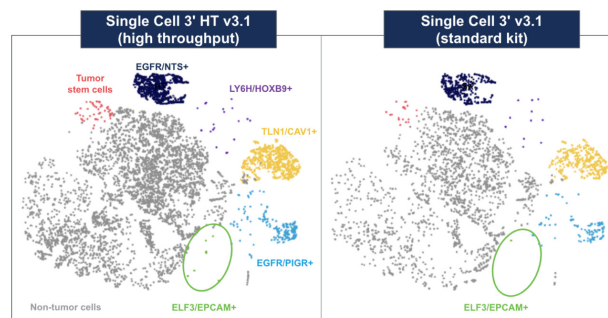
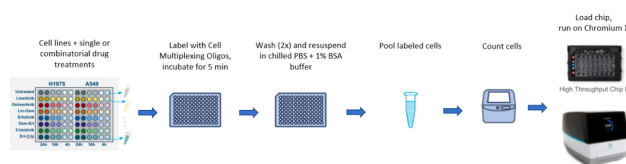
333. Maximizing Multiomic Insights with High Throughput Single Cell Screening

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Combinatorial drug treatment has been proposed as a strategy to overcome rare resistant clones, but it is challenging to find suitable combinations. Non-small cell lung cancer (NSCLC) cell lines are great models for a combinatorial drug screen as they possess multiple forms of resistant clones. CellPlex technology combined with the scale of Chromium Next GEM Single Cell 3' HT v3.1 assay is a high throughput solution on the Chromium X series instrument

that enables one-million-cell experiments across 192 independent conditions, all in one microfluidic chip (Fig. 1). An aggregation of all of the treatment and time point conditions showed that H1975 cells, bearing an EGFR mutation, are more responsive to combinatorial drug treatment than that of A549 cells, bearing a KRAS mutation. The cell cycle and DNA repair pathways were down-regulated as early as 4 h and continued until 24 h. The combinatorial treatment primarily impacts cell cycle checkpoint and DNA double-strand break repair gene networks. The impact of single treatment conditions is significantly less, suggesting that synergistic regulation of multiple pathways determines the effect on the gene network. In a separate study, we profiled single cell transcriptomes and cell surface protein markers from multiplexed primary tumor cells from seven NSCLC patients. Protein detection in conjunction with single cell transcriptome analysis enables better cell type detection and annotation. The scale of the Single Cell HT assay provides an opportunity for identifying rare tumor types (Fig. 2), which is key to understanding evolution of drug resistance and metastasis clones. This kind of study sheds light on therapeutic discovery of actionable targets. Finally, we found the common signaling pathways shared by the primary NSCLC cells and A549, indicating A549 is a great model to perform large-scale screening for the KRAS-mutated type of NSCLC. Overall, this study highlights the scalability of single cell approaches using Chromium Single Cell HT platform along with the applicability of multiomic technologies on the platform to enable high resolution study of biology. Figure 1. Experimental methods for drug screen sample preparation and Chromium Next GEM Single Cell HT Gene Expression. Two lung cancer cell lines (H1975 and A549) were each treated with four different drugs, some in combination, for a total of eight different treatments per cell line. Treated and untreated samples were collected at 4, 16, and 24 hours post-treatment, pooled and processed on Chromium X using the Single Cell 3' HT v3.1 workflow. Figure 2. Single Cell 3' HT v3.1 Gene Expression with CellPlex enables identification of rare tumor clusters. NSCLC samples from seven donors were labeled with CellPlex, pooled and processed in one channel of the microfluidic chip for Single Cell 3' HT Gene Expression assay. The ELF3+/EPCAM+ cluster in green is only present in Single Cell 3' HT v3.1 data and not identified with the Single Cell 3' v3.1 standard assay alone. ELF3+/EPCAM+ tumor cells are associated with cell growth, metastasis, and unfavorable overall survival.



334. A Unique CRISPR-Based Nuclease with a Non-NGG PAM Efficiently Targets Multiple Exclusive Genomic Sites for Immun-Oncology Based Therapy

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Chimeric antigen receptor (CAR) -T cell therapy is a recent clinically successful approach to tackling cancer, in which T cells are genetically modified to allow specific recognition and efficient killing of cancer cells via tumor associated antigens. Current CAR-based treatments require costly and time-consuming autologous cell transfer. Patients' own cells are frequently of low quality and difficult to obtain, further supporting the advantage of an allogeneic transplantation. To prevent graft versus host reactions and avoid host-mediated rejection of healthy donor-derived allogeneic cells, these cells must be adjusted by eliminating the expression of endogenous recognition components, such as T cell receptor alpha constant (TRAC or TCR) and CD3, and of HLA class I molecules such as β -2 microglobulin (B2M). In this study, we tested the feasibility of gene editing in allogeneic cells using our novel high-fidelity CRISPR-associated nuclease OMNIA4. OMNIA4 is a highly active nuclease with a unique non-NGG PAM recognition domain. The unique PAM allows gene-editing in exclusive genomic sequences that are not accessible by commonly used nucleases. Our strategy involves disrupting endogenous T cell recognition elements as well as checkpoint receptors and exhaustion genes that restrict anti-tumor T cell response. RNA guides (gRNAs) were designed for 18 such genes of interest and their editing via OMNIA4 nuclease was evaluated by NGS in HeLa cells. Ribonucleoproteins (RNPs), including OMNIA4 and gRNAs designed to target distinctive sites in either TRAC, CD3 ϵ or B2M genes, were applied to primary T cells. The resulting editing outcome was evaluated by measuring T cell receptor (TCR), CD3 ϵ or B2M surface expression by flow cytometry. In addition, GUIDE-seq analysis was performed to identify 'off-targets' edited by the nuclease. Our gRNA screening yielded at least one (and up to five) active gRNA for each gene of interest, with editing level >70%. Flow cytometric analysis showed that editing in primary T cells resulted in about 96% TCR negative cells, about 89% CD3 ϵ negative cells and about 98% B2M negative cells. An unbiased assay revealed no off-targets for neither of the guides. These findings demonstrate efficient, accurate and safe impairment of a self-presenting element and endogenous T cell recognition components. Our approach offers gene editing at unique

targets as a tool to generate universal allogeneic T cells that could be employed in the development of 'off-the-shelf' 'ready-to-use' CAR-T therapeutic agents for large-scale clinical applications.

335. Next-Generation, Inducible IL-7-Expressing, Tumor-Infiltrating Lymphocytes by Lentiviral Vector Genetic Modification for Clinical Application

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Tumor-infiltrating lymphocyte (TIL) therapy has shown some of the most favorable responses in refractory metastatic melanoma, possibly due to the immunogenicity of this cancer.^{1,2} TILs are isolated from a patient's own tumor tissues, rapidly expanded in vitro, and then adoptively transferred back into the patient. These TILs can recognize and attack cancer cells in large numbers with high specificity.³ In multiple Phase 1/2 trials of melanoma, TIL therapy has demonstrated a robust response rate of up to 50%; however, durable complete responses were only seen in about 15% of treated patients.⁴⁻¹⁰ Interleukin 7 (IL-7) is a potent stimulator of T-cell proliferation and survival. However, T-cells are incapable of producing their own IL-7 and rely on secretion from surrounding stromal cells.¹¹ We hypothesize that the introduction of an inducible IL-7 gene to TILs (next generation TIL; ADP-TILIL7) will improve their ability to engraft, proliferate, and survive, while maintaining diverse TIL specificity. This may translate into improved clinical activity and durability of response when given to patients. To investigate the ability of IL-7 to enhance T-cell functionality, we first used T-cells from healthy donor peripheral blood transduced with an affinity enhanced T-cell receptor (TCR) specific for the cancer testis antigen MAGE A4 combined with IL-7 under the control of a nuclear factor of activated T-cells (NFAT) inducible promoter as a model system (ADP-A2M4IL7). Production of inducible IL-7 by ADP-A2M4IL7 led to improved T-cell expansion and retention of functionality upon repeated stimulation in vitro, without impacting immediate T-cell activity or T-cell specificity. Next, we focused on introducing the human IL-7 gene under the control of the same NFAT inducible promoter used in the model system into patient-derived TILs to generate T-cells that secrete IL-7 upon recognition of their cognate antigen. We detail how transduction of TILs was best achieved before the rapid expansion step, and we report increased IL-7 production with the inclusion of a transduction-enhancing poloxamer. To test the functionality of the lentiviral vector, we developed an assay to determine the production of IL-7 upon non-specific TCR stimulation in vitro and found that ADP-TILIL7 reliably secreted levels of IL-7 that had been shown to increase survival in assays with ADP-A2M4IL7 cells. Finally, transduction and exposure to IL-7 did not reduce the proliferation potential or change the phenotype of the TILs, indicating that ADP-TILIL7 is as fit for purpose as conventional TILs. Based on our preclinical studies, a

single-center Phase 1 clinical trial will be initiated with ADP-TILIL7 to treat patients with metastatic melanoma. 1. Hulen TM, et al. *Immuno*. 2021;1(3):194. 2. Muul LM, et al. *J Immunol*. 1987;138(3):989. 3. June CH, et al. *Sci Transl Med*. 2015;7(280):280. 4. Rosenberg SA, et al. *Clin Cancer Res*. 2011;17(13):4550. 5. Andersen R, et al. *Ann Oncol*. 2018;29(7):1575. 6. Ellebaek E, et al. *J Transl Med*. 2012;10:169. 7. Forget M-A, et al. *Clin Cancer Res*. 2018;24:4416. 8. Dafni U, et al. *Ann Oncol*. 2019;30(12):1902. 9. Besser MJ, et al. *Clin Cancer Res*. 2013;19(17):4792. 10. Dudley ME, et al. *J Immunother*. 2002;25(3):243. 11. Mackall CL, et al. *Nat Rev Immunol*. 2011;11(5):330.

336. Process Optimisation for a 5T4-Targeted CAR-T Cell Therapy (OXB-302): Using an Iterative Small Scale Screening Approach to Identify Factors Promoting an Early Memory/Tscm Phenotype and Improved Serial Killing Capacity

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CAR-T cells have shown great promise in the treatment of lymphoid malignancies but despite high initial response rates, some patients will relapse either due to antigen loss or lack of T cell expansion and persistence. It has been demonstrated that the degree of CAR expansion correlates with clinical response and that persistence of the CAR-T cells within the patient may promote a durable response long-term. Phenotypic analysis of CAR-T cell products has suggested that an early memory or stem cell memory (Tscm) phenotype is associated with clinical responses in patients. In addition to compelling clinical data, a wealth of evidence from pre-clinical studies suggests that Tscm/early memory cells have higher self-renewal capacity while retaining the ability to differentiate into effector cells that can mediate tumour killing. We hypothesised that this property of self-renewal is likely to be key to ensuring persistence of CAR-T cells and so we aimed to enrich for cells with this phenotype in our CAR-T cell product targeting the 5T4 oncofoetal antigen (OXB-302). We used a small-scale multi-factor screening approach to identify conditions that could enhance the proportion of Tscm/early memory cells within our 5T4 CAR-T cells and enhance their serial killing capacity through multiple rounds of antigen stimulation. We adopted an iterative approach to screening multiple factors in a series of small-scale screens. We assessed the impact of different cytokines and small molecules, and other factors upon the phenotype and function of the cells using a flow cytometry panel (including CD8, CD4, CD45RA, CCR7, CD95), in vitro restimulation assays and stress tests. This analysis resulted in identification of small molecules that were potentially beneficial and highlighted the importance of a shorter process on the cellular phenotype and serial killing capacity. Surprisingly some agents that have been previously proposed to promote a favourable phenotype, did not have a positive effect in our assays.

337. AAV Mediated Expression of Mouse IL-12 Shows Potent Anti-Tumor Activity and Immune Response Alone and in Combination with Anti-PD1 in Syngeneic Murine Tumor Models

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Cytokines including Interleukin-12 (IL-12) have been recognized for their potential as potent anti-tumor agents via their activation and recruitment of multiple immune cell types and their anti-proliferative or pro-apoptotic effects. Local administration of IL-12 has been shown to activate or reactivate tumor infiltrating CD8+ T cells, improve APC and antigen presenting machinery and subsequently cause the expansion of tumor-specific CD8+ effector T cells and NK cell, which is associated with enhanced infiltration of contralateral untreated (abscopal) lesions. Despite robust preclinical activity and promising clinical data in patients with advanced cancer, development of systemic IL-12 therapies has been hampered by systemic toxicities. KT-A281 is an AAV vector with promising anti-tumor immune response that can be delivered intratumorally (IT). KT-A281 was designed to convert an injected tumor into a biofactory for continuous IL-12-driven immunostimulation of the tumor microenvironment. This allows for sufficient time to attract tumor-infiltrating lymphocytes, which provide potent local and systemic anti-tumor immune response, without systemic toxicities associated with systemic IL-12 treatment. An advantage of AAV mediated expression of IL-12 is that it eliminates the need of repeat administration as well as eliciting a mixed viral/tumor immune response seen with other approaches. KT-A281 is comprised of a nonreplicating recombinant adeno-associated virus (rAAV) with a codon-optimized expression cassette designed to drive expression of human interleukin-12 (IL-12). The *in vivo* activity of a murine version of KT-A281 (mKT-A281, AAV.mIL-12) alone and in combination with anti-PD1 was explored in three syngeneic mouse tumor models (H22, MC38 and CloudmanS91). Combination of mKT-A281 with anti-PD1 led to a marked improvement in each tumor type tested. With CloudmanS91, the combination resulted in objective responses 2 complete responses (CR) + 1 partial response in 3 of 7 mice or 43% and stable disease (SD) in the remaining 4 of 7 mice or 57%, that leads to substantial disease control rate of 100%. The combination is superior to anti-PD1 monotherapy which resulted in SD in 2 of 8 tumors, with progressive disease observed in the remaining 6 animals treated with anti-PD1 alone. The improvement in best response for the combination of mKT-A281 with anti-PD1 was reflected by a reduction in T-AUC (ns vs mKT-A281; $p < 0.05$ vs anti-PD1) and increase in MST ($p < 0.05$ vs anti-PD1). In MC38, the AAV.mIL-12 with anti-PD1 combination was superior to either mKT-A281 or anti-PD1 resulting in CR in 5/8 or 63% mice compared to only 1 CR of 8 or 13% with anti-PD1 alone. Significant improvements in AUC ($p < 0.001$ vs mKT-A281; $p < 0.05$ vs anti-PD1) and MST ($p < 0.01$ vs mKT-A281; $p < 0.05$ vs anti-PD1) were also observed. The H22 model was sensitive to anti-PD1 treatment with tumor regression observed in a majority CR (5/8) or 63% of mice and delayed tumor growth in 2/3 of the remaining animals. The mKT-A281/anti-PD1 combination was at least as effective as anti-PD1 alone, and resulted in a decrease in AUC and improvement in survival time relative to anti-PD1, but these differences did not reach statistical significance due to the heterogeneity of the model. IT delivery of mKT-A281, alone or in combination with anti-PD1, was well tolerated in each tumor

model. No significant effect on body weight or gross clinical adverse signs, outside of those commonly associated with tumor burden, were observed. These findings demonstrate that IT delivery of IL-12 via a rAAV vector is a viable therapeutic approach for cytokine-based cancer treatment. IT administration of mKT-A281 was well tolerated and resulted in durable anti-tumor immune response alone and in combination with anti-PD1 in syngeneic murine tumor models.

338. CAR T-Cell Therapy Targeting ECM Proteins for Pediatric Solid Tumors

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The outcome for pediatric sarcoma tumors remains poor and immunotherapy promises to improve outcomes since it kills tumor cells through different mechanisms. We seek to advance immunotherapy against solid tumors by using chimeric antigen receptor T cells (CAR T cells) through targeting a unique component of the microenvironment, the extracellular matrix (ECM), which contains molecules that are tumor specific and are shared among individual patients. We have developed a analytical pipeline to discover alternatively spliced exons (ASEs) and alternative expressed genes (AEGs) as targets for pediatric solid tumors and identified tumor-enriched ASEs and AEGs which could potentially serve as CAR targets. We had previously shown a promising ASE (Wagner *et al. Cancer Immunol Res. 2021*), the EDB splice variant of fibronectin (FN1), which is 100% homologous between human and mouse, can be targeted with CAR T cells in xenograft models without overt toxicity. The goal of this project was now to develop an immune competent sarcoma model, and explore if our approach could be extended to ECM proteins that are differentially expressed in solid tumors. We generated a murine EDB-CAR with a CD28z signaling domain, and demonstrated that murine EDB-CAR T cells recognize and kill a panel of EDB+ murine osteosarcoma (OS) cell lines (F420, F331, Myc29). In initial *in vivo* experiments, EDB-CAR T cells induced transient anti-tumor activity in a subcutaneous F420 model without 'on target/off cancer toxicity'. Studies are underway to evaluate EDB-CAR T cells in other immune competent OS models, and characterize tumor microenvironment (TME)/CAR T cell interactions. To extend our approach to other ECM proteins, we focused on Collagen11A1 (COL11A1), which is differentially expressed in breast cancer, pancreatic cancer, rhabdomyosarcoma (RMS), and OS. We designed a CD28.z CAR that recognizes a peptide that is specific for proCOL11A1 (COL11A1-CAR). COL11A1-CAR T cells recognized and killed human COL11A1+ OS and RMS cells *in vitro* (LM7, 143B, CRL2061, CCL136). In contrast, COL11A1-negative human fibroblasts were not killed in co-culture assays or induced cytokine production of COL11A1-CAR T cells. *In vivo*, COL11A1-CAR T cells had potent antitumor activity in a locoregional OS (LM7) model. In conclusion, our data highlights that it is feasible to target ECM proteins with CAR

T cells. Thus, ECM protein, which have been largely overlooked as CAR targets, might present a promising class of solid tumor antigen for adoptive cell therapies.

339. Durable, Multiplexed, Cell Engineering Using Zinc-Finger Guided Transcriptional Regulators Delivered via a Single Viral Particle

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The success of engineered cell therapies, such as CAR-T cells, and the continued development of genomic engineering tools points to a future of highly customized cell therapies with improved therapeutic potential and minimal adverse effects. However, most genome engineering tools, such as nucleases and base editors, are associated with shortcomings including off-target mutations, double-strand breaks, and limited editing options - only creating knockouts or a limited range of single-base changes. When nucleases are multiplexed, chromosomal translocations become a significant issue. Indeed, current efforts to develop multiplex edited CAR-T cell therapies have encountered these very issues. Furthermore, the efficiency of generating multiple independent modifications decreases significantly as the number of modifications increases. Here we describe a platform capable of efficient, durable, multiplexed, epigenetic cell engineering using zinc finger-guided transcriptional regulators delivered via a single viral particle. We have designed highly specific independent ZF-transcription factors to up-regulate or down-regulate target genes of choice. The degree of regulation is tunable, offering the possibility of partial knockdown or variable overexpression. Their compact size allows multiple ZF-transcription factors to be combined in a single viral construct to achieve highly efficient multi-gene modulation in a single transduction event and without the need for double-strand breaks. Delivery by lentivirus leverages a well-established method and does not require major change to existing manufacturing processes. As proof of concept for this novel platform, we engineer primary human T cells using multiple ZF-transcription factors encoded in a single lentivirus with and w/o a CAR, to knock down several allogeneic engineering targets or check point inhibitors. We demonstrate that ZF-transcription factors act with high efficiency and specificity on target genes of choice at both the RNA and the protein level. Multiplexed, epigenetic cell engineering using ZF-guided transcription factors offers a significantly improved approach to engineered cell therapies. It is potentially utilizable in many cell types and has the potential to be deployed in both ex vivo and in vivo applications.

340. Epitope Engineered Hematopoietic Stem and Progenitor Cells to Enable CAR-T Cell Immunotherapy for Acute Myeloid Leukemia

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Acute myeloid leukemia (AML), despite treatment advances and the use of allogeneic hematopoietic stem/progenitor cell (HSPC) transplantation (HSCT), is still associated with an unfavorable outcome for >50% of patients. While novel immunotherapies, such as CAR T cells, bispecific and toxin conjugated antibodies (Ab), demonstrated striking efficacy when targeting a dispensable lineage antigen (Ag), such as CD19 in B-ALL, the same approach cannot be exploited for AML, due to lack of a stringent leukemia-restricted Ag. The most suitable candidates are shared with healthy progenitor or mature myeloid cells, leading to immunosuppression and impairment of myeloid reconstitution. Nonetheless, anti-AML therapies targeting myeloid/HSPC antigens are currently under development, but on-target toxicities restrict their use to a limited time window, which is likely insufficient for disease eradication. To address this issue, we reasoned that precise modification of the targeted epitopes in healthy HSPC would result in loss of recognition, while allowing normal protein expression, regulation, and function. Differently from the abrogation of dispensable lineage markers (ie. CD33), epitope editing allows targeting genes essential for leukemia survival regardless of shared expression on normal HSPC, thus minimizing the risk of tumor immune escape by Ag loss or downregulation. As proof-of-concept targets, we selected the cytokine receptors *FLT3* and *IL3RA* (CD123), which are found in >85% of AML cases and whose overexpression is associated with poor prognosis. By transposon-based library screenings, we identified amino acid substitutions in the *FLT3* extracellular-domain (ECD) that preserve physiologic surface expression, ligand-binding and intracellular kinase activation but avoid detection by a therapeutic monoclonal Ab. Cells expressing this variant were resistant to CAR T killing and did not induce CAR T activation and proliferation during in vitro co-culture. Using an engineered cell line overexpressing *FLT3* from its endogenous locus, we identified a set of gRNAs that enable the introduction of our aminoacidic changes by an advanced generation adenine base editor. Similarly, we identified residues within *IL3RA* ECD that, when targeted by adenine or cytidine base editors, confer resistance to 3 different mAb clones. Electroporation of ABE8e mRNA and gRNAs into human CD34+ HSPCs achieved up to 85% and 40% editing efficiency on *FLT3* and *IL3RA* genes, respectively, either as single or multiplex targeting. After xenotransplant into NBSGW mice, edited HSPC sustained long-term multilineage hematopoiesis while maintaining editing levels comparable to input cells, thus indicating full preservation of functionality of the engineered receptor and successful editing of the most-primitive HSPC compartment. Most importantly, upon treatment with *FLT3* CAR T, we observed sparing of human HSC (CD34+38-90+), granulo-mono progenitors (GMP) and granulocytes in the bone marrow of mice engrafted with edited HSPCs compared

to *AAVS1* edited controls. Colony forming cells derived from the bone marrow of CAR T treated mice showed an increased fraction of edited cells compared to non-treated controls, thus confirming the negative selection of non-modified cells by FLT3 CAR. Transplantation of epitope engineered HSPCs endowed with selective resistance to CAR T cells or Abs is a novel approach to enable effective and safe immunotherapies for high-risk AML patients.

341. A Novel Adapter CAR™ System Based on a Human Peptide Tag Demonstrates Efficacy in Preclinical Models

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Chimeric antigen receptor (CAR) T technology has brought great advancement to the treatment of hematologic malignancies and holds great promise for the treatment of solid tumors. However, two presently unresolved drawbacks of this technology are: detrimental side effects stemming from excessive CART activation, such as cytokine release syndrome, and inability to modify the target antigen of the CAR once the drug product has been injected. We address both issues by creating a CAR (Adapter CAR) that recognizes a short human peptide epitope tag, which can be fused to a variety of biological drug products that recognize tumor antigens. This recombinant Adapter CAR system demonstrates efficacy and safety in CD20+ Non-Hodgkin's lymphoma and CD33+ acute myelogenous leukemia *in vitro* and *in vivo* models when adapters carrying the peptide tag are used. We further demonstrate that, using a tamoxifen-inducible transcriptional circuit, that adapters carrying the mutated peptide tag can be inducibly expressed by primary human T cells, and these lead to anti-tumor activity in other Adapter CAR-T cells. This work demonstrates the utility of an Adapter CAR system using short peptide tags, and the potential function of this system in producing these adapters *in situ*, ideally at the site of tumors in patients.

342. MAFF Suppresses Tumor via Regulating SLC7A11-Mediated Ferroptosis and CDKN2C-Dependent G1 Arrest in Lung Adenocarcinoma

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Introduction: In lung cancer, transcription factor MAFF (MAF basic leucine zipper transcription factor F) has rarely been explored. **Methods & Results:** Bioinformatic analyses using TCGA, GEO datasets, and 10X single-cell RNA sequencing data consistently showed that the expression of MAFF was significantly lower in tumors compared to normal tissues, and the downregulation of MAFF was significantly correlated with poor prognosis in patients with lung adenocarcinoma (LUAD). We first found that MAFF overexpression at the transcriptional level attenuated the cell proliferation *in vitro* in three LUAD cell lines, A549, PC-9, and NCI-H1975, while MAFF knockdown showed an opposite result. We next conducted an integrated analysis of the ChIP-seq data with transcriptomic data using the A549 cell line with

MAFF-knockdown and overexpression to investigate the regulatory mechanisms of MAFF. Here, SLC7A11 and CDKN2C were disclosed as two downstream effectors. Dual-luciferase reporter assays verified that MAFF could mechanically bind to the promoter regions, reducing and increasing the expression of SLC7A11 and CDKN2C, respectively. As for SLC7A11, we found that suppressing SLC7A11 by MAFF could initiate ferroptosis in LUAD cells. Moreover, we carried out a Co-IP assay followed by a mass spectrum in the A549 cell line and uncovered that MAFF could interact with UBTF, another transcription factor, to upregulate the expression CDKN2C together, inducing a G1/S phase arrest in LUAD cells. Notably, UBTF was indispensable for the regulation activity as we found that UBTF knockdown could abolish the CDKN2C-dependent cell arrest. Further analysis with Co-IP and GST pull-down assays showed that the N-terminal domain of UBTF could directly bind to MAFF. Finally, we confirmed the tumor-suppressing role of MAFF *in vivo* in LUAD patient-derived xenograft (PDX) models. On the contrary, overexpression of SLC7A11 and CDKN2C silencing could rescue the ferroptosis, cell arrest, and proliferation changes upon MAFF regulations *in vivo* and *in vitro*. **Conclusions:** Collectively, our study uncovered a previously unknown function of the transcription factor MAFF in modulating tumor progression via regulating SLC7A11-mediated ferroptosis and CDKN2C-dependent G1 arrest in LUAD.

344. Systemic Gene Therapy for Brain Malignancy Using Blood-Brain-Barrier-Penetrant AAV Vectors

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Recombinant adeno-associated viruses (rAAVs) have been increasingly explored as a vector for gene therapy in the central nervous system (CNS). Recently, we reported two new blood-brain-barrier-penetrant AAV variants, namely AAV.CPP.16 and AAV.CPP.21, by engineering AAV9 capsid (ASGCT 2021). Importantly, the superiority of AAV.CPP.16 over AAV9 translated from mice to non-human primates (macaques), showing 5-10 folds enhancement in CNS transduction with intravenous delivery. Here, as a proof-of-concept for its translational potential we report that AAV.CPP.16 could be applied in developing systemic cancer gene therapies delivering both secreted and non-secreted anti-tumor payloads for brain malignancy. In comparison with AAV9, AAV.CPP.16 expressing a secretable single-chain-variable-fragment antibody against mouse PD-L1 enhanced systemic delivery of the PD-L1 antibody to the brain in a syngeneic mouse model of glioblastoma. This viral treatment led to increased infiltration of overall T cells as well as CD8+ cytotoxic T cells in the tumor microenvironment and provided robust survival benefit with tumors eradicated in 75% of the animals. Moreover, we demonstrate that AAV.CPP.16 expressing a non-secreted payload herpes simplex virus thymidine kinase type 1 (HSV-TK1) significantly reduced glioblastoma tumor size in mice after systemic delivery and prolonged overall survival when co-administered with the prodrug ganciclovir. Future studies would examine the translational potential of combinatorial treatment of AAV.CPP.16-

HSV-TK1 and PD-L1 immunotherapy. Thus, our studies so far suggest the feasibility and prospect of developing AAV-based systemic gene therapy for combatting highly malignant brain tumors.

345. Development of Antibody and siRNA Nanocarriers to Target Wnt Signaling in Triple-Negative Breast Cancer

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Introduction Triple-negative breast cancer (TNBC) is an aggressive disease that is unsusceptible to standard therapies due to the lack of appropriate receptors. Novel targeted therapies must be designed to stop TNBC through other mechanisms. One promising approach is to inhibit Wnt signaling, which is hyperactive in TNBC cells. When extracellular Wnt ligands bind transmembrane FZD7 receptors, intracellular β -catenin molecules translocate to the nucleus to promote the transcription of various oncogenes. To suppress Wnt signaling at both receptor (FZD7) and effector (β -catenin) levels, we developed multifunctional nanoparticles (NPs) composed of gold nanoshells (NS) coated with FZD7 antibodies (anti-FZD7) and β -catenin small interfering RNAs (si β cat). Our *in vitro* and *in vivo* data show dual Wnt inhibitory NPs can greatly impair TNBC and are more effective than the mono-therapeutic NPs. **Methods** We synthesized 3 Wnt inhibitory NPs (1) Combo-NS with anti-FZD7 and si β cat, (2) FZD7-NS with anti-FZD7, and (3) β cat-NS with si β cat. NS were synthesized, coated with antibodies using heterobifunctional poly(ethylene glycol) (PEG) linkers and with siRNA duplexes using 3' -thiol sense strands, backfilled with mPEG-SH, and purified. NP hydrodynamic diameter, zeta potential, and biomolecule loading were measured using established assays. Immunohistochemistry (IHC) validated FZD7 receptor expressions in both murine 4T1 TNBC cells and orthotopic tumors. The NPs' impact on cell proliferation for 12 - 48 h was assessed *in vitro* using an EdU assay. To demonstrate proof-of-concept of the NPs' efficacy, we designed a pilot study with Balb/C mice bearing orthotopic 4T1 tumors in the 4th mammary fat pad and NPs were injected *via* tail vein weekly for 5 total times. After the 2nd NP injection, we surgically removed the tumors and monitored regrowth until a volume of 1000 mm³. Excised organs were analyzed for gold content *via* inductively plasma couple-mass spectrometry (ICP-MS) to reveal NP biodistribution. **Results** After biomolecule loading, NP mean diameter and charge increased by about 10 nm and 10 mV, respectively. Combo-NS and FZD7-NS had ~230 anti-FZD7/NS per ELISA, while Combo-NS and β cat-NS had ~2000 si β cat/NS per OliGreen assays (Fig 1A). IHC confirmed that 4T1 cells overexpress FZD7 compared to control EpH4 cells, as supported by darker pigmentation (Fig 1B), which was also seen in tumor paraffin sections. *In vitro*, FZD7-NS and Combo-NS reduced cell proliferation at 12 h post-treatment more than β cat-NS. Pilot *in vivo* studies showed that Combo-NS reduced initial tumor growth most effectively (Fig 1C) and delayed regrowth compared to the saline control. ICP-MS analysis of the primary tumors revealed that both

Combo-NS and FZD7-NS amassed intratumorally more than β cat-NS that lack antibodies (Fig 1D). **Conclusion** These data demonstrate that dual Wnt NPs are more effective than mono-therapeutic NPs in suppressing proliferation and, preliminarily, in reducing primary tumor growth. Future work will focus on elucidating gene expression mechanisms and diminishing tumor regrowth.

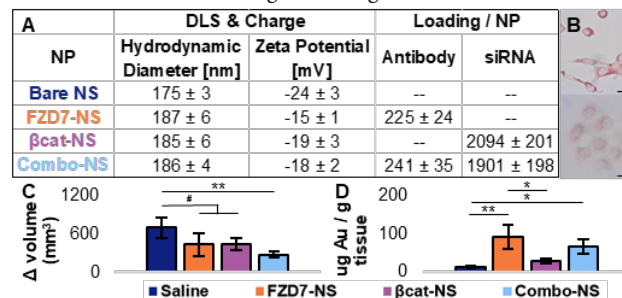


Figure 1 (A) NP Characterization. DLS/Zeta, & biomolecule loading. **(B) IHC FZD7 Expression.** Top: 4T1 cells, Bottom: EpH4 cells (scalebar = 25 μ m). **(C) Primary Tumor Growth.** Change in 1^o tumor growth from start of NP treatment to excision. **(D) Gold ICP-MS.** NP distribution in excised 1^o tumors. (# p < 0.1, * p < 0.05, ** p < 0.01).

346. Biodistribution of Human Cells Using qPCR and Engraftment Rates in the NCG Mouse Model

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The investigation of engraftment and biodistribution profiles when developing cell-based therapies is a pivotal prerequisite to allow a risk-benefit assessment during non-clinical product development. Herein, we report historical control data including biodistribution using human ALU sequences and flow cytometry in the NCG mouse model. The NCG triple immunodeficient mice on a NOD/Nju background, in which *Prkdc* and *Il2rg* genes were edited by CRISPR/Cas9, lack functional/mature T, B, and NK cells, and have reduced macrophage and dendritic cell function. These characteristics make them an ideal model for cell therapy studies. The objectives of this study were to qualify the NCG mouse model for biodistribution quantified using human ALU sequences, engraftment, toxicity and tumorigenicity assessments of cell therapies using various myeloablation regimens. CD34⁺ hHSPCs (hematopoietic stem and progenitor cells) from adults treated with mobilizing agents were used as cell product. To assess myeloablation mice were irradiated with 1.4 to 2 Gy (X-ray or Cobalt-60) or administered with Busulfex[®] via intra-peritoneal injection on Day-2 and Day -1 at 15 mg/kg for males and at 20 mg/kg for females. Following that, mice were injected 0.1-1.0 x 10⁶ cells/mouse CD34⁺ hHSPCs or PBS (control) via the tail vein and followed up to 30 weeks. Three different types of mobilized hCD34⁺ cells were injected: donors stimulated with either Mozobil[®] (MOZ), G-CSF (Neupogen[®]) or a combination of both (MOZ/G-CSF). The positive control animals received a tumor cell line (i.e. 2 x 10⁶ HL-60 cells/

mouse) after irradiation. No significant clinical signs or body weight changes were observed. In the biodistribution portion of the study, no human ALU sequences were detected in the control group animals. At the same time, human ALU sequences were detected in all tested organs of the human cell treated group animals (brain, heart, kidney, liver, lung, testes, ovaries, spleen, blood, and bone marrow). Lower levels were detected in the heart, testes, and blood with the lowest levels in the brain. Higher levels were detected in the liver, kidney, lungs, spleen, and bone marrow. Human CD45⁺ cells were observed in whole blood, spleen and bone marrow samples in groups injected with human cells, displaying a chimerism ranging between 2.2% and 91.8%. On week 18 post-injection, the percentage of human CD45⁺ cells of total lymphocytes in the peripheral blood were 48.4 ± 29 (MOZ), 56.4 ± 11.1 (MOZ/G-CSF) and 17.9 ± 12.6 (G-CSF) in comparison with 84.5 ± 9.84 when donor cells were obtained from cord blood (standard control). The expected mortalities due to tumorigenesis were observed between days 27 and 40 post-dose in a positive control group that was administrated with a cancer cell line (i.e. HL-60 cells). The biodistribution of human ALU sequences and engraftment rates, along with the absence of significant effects on the animals' body weights or survival, supports the relevance of the NCG mouse model for cell therapy efficacy and safety studies.

347. Trans-Splicing Ribozyme-Mediated RNA Reprogramming for Hepatocellular Carcinoma Gene Therapy

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Hepatocellular carcinoma (HCC) is major type of primary liver cancer, and its incidence and mortality rates are steadily increasing. However, Hepatocellular carcinoma (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. In this study, we developed group I intron based trans-splicing ribozyme that enables to sense and reprogram telomerase reverse transcriptase (TERT) RNA into therapeutic suicide gene for cancer therapy. 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. Adenovirus encoding the post-transcriptionally enhanced ribozyme improved both trans-splicing reaction and inhibition of hTERT RNA level, efficiently and selectively regressing hTERT-positive liver cancers. Adenovirus encoding the microRNA-regulated ribozyme induced selective liver cancer cytotoxicity, the efficiency of which depended on ribozyme expression level relative to the microRNA level. Systemic inoculation of adenovirus encoding the post-transcriptionally enhanced and microRNA-regulated ribozyme caused efficient anti-HCC effects at a single dose of low titers and least hepatotoxicity in intrahepatic and xenografted murine model of multifocal HCC. Highly potent anti-cancer efficacy of the ribozyme is mainly due to telomerase inhibition and suicide gene activity with induction of local/systemic immunity. In addition, combinatorial treatment of adenovirus

encoding the ribozyme with immune checkpoint inhibitor enhanced anti-tumor efficacy compared to adenovirus or immune checkpoint inhibitor monotherapy in humanized HCC mouse xenograft model. Minimal liver toxicity, tissue distribution and clearance pattern of the recombinant adenovirus were observed in normal animals administered with the virus either systemically or through the hepatic artery. Post-transcriptionally regulated RNA reprogramming strategy mediated by a cancer-specific trans-splicing ribozyme provide a clinically relevant, safe, and efficient strategy for HCC treatment.

348. CRISPR-Based Epigenome Editing Screens in Primary Human T Cells

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Genome and epigenome editing could unlock the full potential of adoptive T cell therapy (ACT) for cancer treatment through prescribed changes to DNA and underlying gene expression programs. In fact, genome editing in T cells has already entered the clinical setting with promising results in phase I clinical trials. Epigenome editing offers the unique ability to both activate and tune gene expression levels without modifying endogenous DNA sequences, but its development has lagged behind genome editing. Here, we address many of the technological challenges facing epigenome engineering in primary human T cells. First, we developed vectors to use the small Cas9 ortholog from *Staphylococcus aureus* (SaCas9) as a potent repressor for targeted gene silencing. Using our dSaCas9-based repressor, we conducted high-throughput proof-of-principle CRISPR interference (CRISPRi) screens against single gene targets (CD2 and B2M) using both flow cytometry and multi-omic single cell RNA-sequencing technologies. These screens illuminated potent sgRNAs for each gene target and important rules for optimal sgRNA design. Specifically, we identified and validated 16 sgRNAs against CD2 with a wide range of activity from 10-90% gene silencing. Moreover, sgRNAs designed against the strict PAM variant NNGRRT had substantially more activity than NNGRRV across both screens. To demonstrate phenotypic effects of gene silencing, we validated that silencing of B2M (the light invariant chain of the MHC1 complex) attenuated allogenic responses in mixed donor lymphocyte cultures. Next, we developed a novel and robust SaCas9-based activator for targeted gene activation. Prior SaCas9-based activators required either large effector domains (e.g. VPR, mini-VPR) ill-suited for lentiviral delivery to primary human T cells or smaller effector domains (VP64) with weaker activation potential. To address this problem, we hypothesized that fusing an additional copy of VP64 (only 150 bp) to the N' terminus of dSaCas9^{VP64} might enhance its activation potential without compromising lentiviral production. To test this, we generated polyclonal Jurkat lines stably expressing either dSaCas9^{VP64} or ^{VP64}dSaCas9^{VP64} and conducted parallel CRISPR activation (CRISPRa) screens tiling the promoter of the silenced IL2RA gene. Interestingly, more sgRNAs emerged as hits from the ^{VP64}dSaCas9^{VP64} screen and subsequent validation revealed that ^{VP64}dSaCas9^{VP64} markedly increased IL2RA expression (up to 10-fold) across conserved sgRNAs compared to dSaCas9^{VP64}. Moreover, we found that ^{VP64}dSaCas9^{VP64} can achieve equivalent levels of gene activation as dSpCas9-activators. Finally, we leveraged our CRISPR-based epigenetic effectors to perform CRISPRi and CRISPRa screens

in primary human CD8+ T cells to systematically screen 120 human transcription factors (TFs) and epigenetic modifying proteins for their role(s) in regulating T cell state and function. The main objective of these screens was to identify positive and negative regulators of memory T cells, which have been strongly associated with positive clinical responses to ACT. These screens revealed previously characterized TFs and epigenetic modifying proteins, as well as novel factors. We are currently characterizing these factors at the transcriptional, epigenetic, and functional level. Collectively, we have developed efficient tools for targeted gene regulation and CRISPR-based epigenetic screens in primary human T cells and applied these technologies to dissect transcriptional regulation of therapeutically relevant T cell subsets.

349. NF1 Gene Rescue via Nanoparticles Decreases Mammary Tumor Burden in NF1-Knockout Rats

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Neurofibromatosis Type I (NF1) is a multisystem genetic disorder characterized by café au lait birthmarks, Lisch nodules, skeletal abnormalities, learning difficulties, benign tumors (neurofibromas and optic pathway gliomas), as well as malignant tumors/cancers (malignant peripheral nerve sheath tumors (MPNSTs), pheochromocytomas, leukemia, breast cancer, others). Palliative treatments are limited to a combination of surgical resection, chemotherapy/radiation, and the recent approval of a MEK inhibitor (selumetinib). Pathogenic mutations in the tumor suppressor, *NF1*, lead to elevated RAS signaling and increased cell growth. Somatic cell loss of *NF1* is a contributing factor in a variety of cancers and found to be mutated in up to 33% of non-NF1 breast cancer patients. Exogenous *NF1* expression is one potential gene therapy avenue to restore NF1 and abrogate excessive cell growth. To this end, we created a constitutive knockout rat model wherein heterozygous females spontaneously develop mammary gland adenocarcinomas, driven by an underlying 14-basepair frameshift of rat *NF1* (c.3661_3674del, p.P1220fs*1223). Mammary gland tumors were treated *in vivo* with nanoparticles (NP) encapsulating the mouse *Nf1* cDNA (mNf1) and measured for tumor growth. To date, 25 tumors have been treated with either control NP (n = 9; expressing control plasmid) or mNf1-NP (n = 16) and followed for seven days. Dosing ranged from 1.5mg NP/15ug cDNA to 5mg NP/500ug cDNA and measurements were made at days 0, 4, and 7. Of the controls, eight of nine tumors increased in size by day 7 with an average increase of 417% (range = -37% to +3,297%). In contrast, by day 7, 10 of 16 mNf1-NP-treated tumors decreased in size with an average decrease of 2% (range = -98% to +199%). Together, this approach provides evidence that exogenous expression of mNf1 can slow tumor growth and decrease tumor burden, suggesting that NF1-NP may prove beneficial clinically.

350. Bioluminescent Bioassays for the Discovery and Development of Molecular and Cellular T Cell-Redirecting Cancer

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Redirected T cell therapies represent a new paradigm for cancer treatment. Two main approaches for T cell-based therapies involve molecular T cell redirection by CD3 bispecific molecules such as bispecific T cell engagers (BiTE) and cellular T cell redirection by genetic modification of T cells with chimeric antigen receptors (CAR) or transgenic T cell receptors (TCR). BiTEs redirect the cytotoxic activity of endogenous polyclonal T cells by simultaneously engaging CD3 on T cells and tumor antigens on target cells. Hitherto, BiTE potency studies have relied on primary cells, which measure target cell killing through redirected T cell cytotoxicity (RTCC) or cytokine release. However, these primary cell-based assays suffer from high donor-to-donor variability, as well as lengthy and hard to implement protocols. We have recently developed a new NanoBiT-based assay platform-based RTCC assay and cytokine immunoassays that are simple, sensitive and can quantitatively measure the potency of CD3 bispecific antibodies or BiTEs and similar biologics that induce T cell-dependent target cell killing and cytokine production. In this assay, preactivated, cytotoxic T cells (in cryopreserved, thaw-and-use format) and target cells stably expressing a HiBiT fusion protein are co-incubated with a BiTE, which results in lysis of the target cells and release of HiBiT proteins. These HiBiT proteins then bind to LgBiT in the detection reagent and form functional NanoLuc Luciferase to generate luminescence. The assay is homogenous, highly sensitive, and has a robust assay window. Furthermore, BiTE-induced cytokine production (e.g IL-2 and IFN- γ) from the activated cytotoxic T cells can be quantitatively measured in the homogenous NanoBiT immunoassays. Use of CAR-T has demonstrated promising results in treating leukemia, while the development of TCR-engineered T cells that can recognize intracellular tumor antigens, is still in early stages. To facilitate the screening and characterization of new transgenic TCRs, we used CRISPR/Cas9 to develop two TCR $\alpha\beta$ -null reporter T cell lines, which are either CD4+ or CD8+. Reintroduction of peptide-specific TCR α and β chains into TCR $\alpha\beta$ -null reporter T cell lines results in peptide-dependent TCR activation and luciferase reporter expression. The select expression of CD4 or CD8 in the TCR $\alpha\beta$ -null reporter T cell lines can enable the development of transgenic TCRs for both MHCI- and MHCII-restricted tumor antigen targets. Together, these bioluminescent bioassays represent a new set of tools for the discovery and development of T cell-based immunotherapies.

351. An Epigenetic *In Vivo* Screen Revealed That Loss of BRD7 Induces Dormant Breast Cancer Cells to Undergo Reactivation and Metastasize to the Lung

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Metastasis is a complex multi-step process and thwarting metastasis is widely regarded as the holy grail in the treatment of cancer. **Intriguingly, even though epigenetic alterations are almost a universal feature of all cancer types, relatively little is known about key epigenetic events that could potentially lead to tumor metastasis.** In order to address this gap of knowledge, we have developed and established a novel **high-throughput and flexible *in vivo* shRNA screening platform** which enables the identification of epigenetic entities that regulates the metastatic reactivation of breast cancer. Interestingly, the loss-of-function shRNA epigenetic screen revealed that BRD7 (Bromodomain Containing 7) was essential for the maintenance of the dormant state of the 4T07-TGL and D2A.1d dormancy cells *in vivo*. BRD7, a novel bromodomain-containing protein of about 75 kD, is a subunit of polybromo-associated BRG1-associated factor (PBAF)-specific Swi/Snf chromatin remodeling complexes. However, **the molecular mechanisms by which loss of BRD7 might induce dormant breast cancer cells to undergo reactivation and metastasize remains to be elucidated.** Our *in vitro* studies have indicated that CRISPR/Cas9 mediated knockout of BRD7 promotes tumor sphere formation, migration, and invasion. Interestingly, RNA-seq revealed that inactivation of BRD7 promotes the expression of genes involved in inflammation, EMT, hypoxia, metabolism and MYC function. Intriguingly, the top signatures enriched in Brd7-silenced cells were IL6-JAK-STAT3 signaling, Interferon-Alpha response, and Interferon-Gamma response, suggesting an immune mediated mechanism. Further, ATAC-seq experiments indicated that inactivation of Brd7 causes an increased accessibility of sites that were enriched for NFκB and interferon-regulated response element sites, providing further evidence that inactivation of Brd7 promotes metastatic reactivation by an immune-mediated mechanism. Additionally, to identify the effect of inactivation of BRD7 on target gene expression, we performed ChIP seq and it was revealed that active enhancers were upregulated in the BRD7 knockout cells and the GSEA analysis from the ChIP-seq confirmed the upregulation of IL-6/JAK/STAT3 signaling, and TNF-Gamma signaling pathways in the BRD7 knockout cells. Furthermore, we investigated for cytokines induced by silencing of BRD7 in breast cancer cells and our results indicated that inactivation of BRD7 induces expression of IL6, CXCL12, and CXCL10, all of which have been previously implicated in metastasis. Finally, we found that the silencing of IFNGR1, STAT3, or NFκB suppressed the capacity of BRD7-silenced 4T07 cells to metastasize to the lung, indicating that IFNγ and NFκB signaling are both required for metastatic reactivation. 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. Indeed, our potential findings could have major implications in the formulation of myriad chemo-therapeutic strategies for metastatic cancers.

352. Multi-Arming and Regulator Dial Gene Circuits to Address Key Disease Challenges in HCC

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Background: Hepatocellular carcinoma (HCC) is the 5th most common cancer worldwide and represents a major unmet medical need in clinical oncology. Successful cell therapies, including chimeric antigen receptor (CAR)-T and CAR-NK cell treatments, for liquid tumors have yet to translate into significant efficacy for solid tumors. A major obstacle for cell therapy in solid tumors is the immunosuppressive tumor microenvironment (TME), which can impair the function of therapeutic as well as endogenous immune cells. To overcome this challenge, Senti Bio is developing SENTI-301, a multi-armed CAR-NK therapy with a Regulator Dial gene circuit to strictly control the expression of the potent immune effector interleukin 12 (IL-12). Methods: SENTI-301 is an allogeneic natural killer (NK) cell engineered to express a GPC3 CAR, a calibrated release IL-15 (crIL-15), and a Regulator Dial gene circuit containing a synthetic transcription factor (TF), and a Regulator Dial TF-responsive element to control expression of crIL-12 by grazoprevir (GRZ) an FDA-approved small molecule, for precise and tunable control of dose, timing and duration of crIL-12 expression, allowing SENTI-301 to overcome the challenging TME in HCC while avoiding safety issues. The crIL-15 is tethered to the cell surface via a cleavable linker that can be cleaved by a ubiquitously expressed protease. The rate of linker cleavage can be calibrated by changing its sequence to be more or less optimal for the protease, which effectively regulates the ratio of membrane associated to secreted cytokine. Results: We designed crIL-15 to simultaneously produce membrane associated and fully secreted IL-15 to promote NK cell persistence and proliferation in an autocrine fashion, while also stimulating other immune cells in a paracrine fashion. We demonstrated that crIL-15 enhances NK cell persistence and GPC3 CAR-NK tumor killing in a serial killing assay compared to wild-type secreted IL-15. We screened 156 constructs to optimize expression of GPC3-CAR and crIL-15 (vector 1) and determine its antitumor function in a serial killing assay against different HCC cell lines. Our four lead candidates show >50% GPC3 CAR and membrane bound IL-15 expression. Furthermore, NK cells transduced with GPC3 CAR crIL-15 NK cells show higher anti-tumor function than unengineered NK cells, *in vitro* ($p < 0.0001$) and *in vivo* ($p = 0.0001$). We then screened 90 constructs to optimize the Regulator Dial gene circuit (vector 2) to control expression of the potent immune effector, IL-12. Our two lead candidates demonstrate low IL-12 basal levels in the absence of

GRZ (<100 pg/1e6 cells in 24h), while induction with 10nM of GRZ leads to an increased secretion of IL-12 by ~390 fold (30,000 pg/1e6 cells p<0.0001) in 24h. We validated the Regulator Dial gene circuit in vivo and observed ~150-fold change in IL-12 secretion (154.8 pg/mL - p= 0.02) in mice 48h post GRZ treatment (50 mg/kg). Lastly, we assessed the ability to efficiently derive SENTI-301 cells by co-transducing with vectors 1 and 2, and observe maintenance in CAR and crIL-15 expression, crIL-12 inducibility and anti-tumor functions. Conclusions: The combination of a multi-armed CAR-NK cell-based therapy with a Regulator Dial gene circuit is designed to address existing challenges of current immunotherapies for solid tumors. To our knowledge, SENTI-301 represents the first product candidate providing a potentially improved and more efficacious treatment option for HCC patients.

353. Stem-Cell Memory TCR-T Cells Targeting Hotspot EGFR, KRAS and p53 Neoantigens Generated Through Co-Expression of Membrane-Bound Interleukin-15

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Adoptive transfer of tumor-specific, self-renewing and multipotent stem-cell memory T cells (T_{SCM}) is attractive for generating long-term anti-tumor immunity and deepening clinical responses. However, to achieve necessary clinical doses for infusion, therapeutic T cells are grown *ex vivo* and become more terminally differentiated. Pro-survival homeostatic cytokines, *e.g.*, interleukin-15 (IL-15), can signal to maintain T_{SCM} pools, but systemic administration of IL-15 has been associated with intolerable toxicities and does not directly target the tumor. To address this issue, neoantigen-specific T-cell receptors (TCRs) were co-expressed with membrane-bound IL-15 (mbIL-15) to localize IL-15 to the T-cell surface in the context of transpresentation by IL-15 receptor- α to mimic physiologically augmented IL-15 activity. Neoantigen-reactive mbIL-15 TCR-T cells were generated from human peripheral blood T cells by non-viral *Sleeping Beauty* transposition of a tricistronic vector encoding mbIL-15 and TCRs specific for hotspot neoantigens in critical genes for tumor survival (EGFR, KRAS and p53) which are expressed by a variety of solid cancers and restricted by either Class I or Class II human leukocyte antigen (HLA). The mbIL-15 TCR-T cells grew comparably to conventional TCR-T cells suggesting that the larger transposon encoding both mbIL-15 and TCR was not deleterious to T cell expansion; therefore, this vector could be amenable to clinical scale manufacturing. When challenged with their cognate neoantigen, mbIL-15 TCR-T cells were highly avid and specific for target neoantigens as measured by upregulation of 4-1BB co-stimulatory receptor and secretion of IFN- γ with negligible recognition of wild type proteins. Moreover, mbIL-15 TCR-T cells specifically killed tumor targets with endogenous expression of the neoantigen and HLA. The addition of mbIL-15 to TCR-T cells changed the memory phenotype in the expanded product to contain

fewer central memory cells (CD45RA⁺CD95⁺CD62L⁺CD45RO⁺) and more effector (CD45RA⁺CD95⁺CD62L⁻CD45RO⁺) and T_{SCM} (CD45RA⁺CD95⁺CD62L⁺CD45RO⁻) populations relative to conventional TCR-T cells. Furthermore, long-term withdrawal of cytokines demonstrated significantly higher survival of mbIL-15 TCR-T cells than that of TCR-T cells lacking mbIL-15. Functional and phenotypic evaluation of persistent mbIL-15 TCR-T cells revealed that they retained their functional neoantigen specificity and potency while displaying a preponderance of T_{SCM} TCR-T cells capable of regenerating TCR-T cell effector pools. This suggested that mbIL-15 TCR-T cells could likely establish long-lived tumor-specific TCR-T cells that overcome suppression by the tumor microenvironment or other negative regulators. Thus, clinical translation of *Sleeping Beauty* transposed mbIL-15 TCR-T cells targeting neoantigens is warranted for the treatment of solid cancers.

354. Tumor-Reactive T Cell Receptors Identified by Multimodal Single-Cell Sequencing of Tumor-Infiltrating Lymphocytes

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Personalized adoptive cell therapies (ACT) utilize autologous tumor-reactive T cell receptors (TCR) to target patient specific (neo)antigens presented on human leukocyte antigen (HLA) molecules. Classically in ACT a suitable tumor (neo)antigen is first identified and used to expand and screen for antigen-specific T cells. These T cells are then sequenced to determine the sequence of the tumor binding TCR, which can then be delivered to naive autologous T cells. Current state-of-the-art protocols focus on peptide-driven T cell expansion, *i.e.* require prior knowledge of patient antigenic profile, which incurs expense in both time and cost. To circumvent these limitations, we have developed an antigen-agnostic, TCR-centric approach to identify tumor-reactive TCRs via single cell RNA and VDJ sequencing. We validated our approach by sequencing brain tumor-infiltrating lymphocytes, predicting and cloning tumor-reactive TCRs, and then confirming reactivity experimentally in co-culture assays using autologous patient-derived cell lines. Our results show that tumor-reactive TCRs indeed exhibit a unique transcriptional signature when compared to non-reactive TCRs. Surprisingly, the transcriptional signature of reactivity is similar in both CD4 and CD8 T cells. The signature was established using TILs derived from primary and secondary brain tumors - tumors growing in an immune privileged organ - and validated using datasets from various other tumor entities. Taken together, our data suggest a potential TCR-centric approach to develop a personalized antigen-agnostic ACT using tumour-reactive TCRs identified by a universal transcriptional signature.

Hematologic and Immunologic Diseases I

355. Pathophysiological Characterization of Townes Mouse Model for Sickle Cell Anemia

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Sickle cell anemia (SCA) is a debilitating inherited blood disorder. Despite current advances in treatment, SCA patients still suffer from significant morbidity and early mortality. Further understanding of the available mouse models of SCA may help investigators determine how best to evaluate promising preclinical SCA treatments. Herein we report a pathophysiological study of the Townes mouse model. We evaluated groups of 5-10 AA, AS and SS genotype Townes mice per assay. We assessed differences between younger mice (2-4 months of age) and older mice (6-8 months of age), as well as differences between sexes. SS mice displayed significant hemolytic anemia. White blood cell counts were higher in SS mice compared to AA and AS mice. In older mice, significantly higher indirect bilirubin, as well as higher lactate dehydrogenase was noted in SS mice compared to AA and AS mice. In the hypoxia *in vitro* assay, SS mouse blood displayed a high degree of sickling (85-99%) in comparison to AA and AS blood (0-10%) sickling. In the Von Frey assay, SS mice displayed a lower threshold of response than AA mice across all groups. To assess renal function urine osmolality was measured after 4 hours of water deprivation. SS mice displayed lower urine osmolality than AA mice. Younger female SS mice displayed higher urine protein/creatinine ratio than younger AA females. Echocardiography assessment on female SS mice suggested dilated left ventricles, while male SS mice displayed an increased left ventricular mass than AA males. Plethysmography under hypoxic (10% O₂) conditions revealed that SS mice displayed an obstructive lung disease phenotype and increased inflammation compared to AA mice. Histology studies performed at sacrifice (8-10 months of age) suggested vascular congestion in lungs, spleen, and kidneys, as well as increased iron deposition in kidney and liver. SS mouse spleens appeared to have a disruption in normal lymphoid architecture compared to AA genotype. Cytokine analysis using the MILLIPLEX MAP Mouse TH17 cytokine assay revealed a marked increase in IL-22, TNF-alpha, and IL-6 in SS mice. These data are important for future preclinical studies and translatability of novel interventions such as gene therapy for SCA.

356. Characterization of the Non-Cell Autonomous Effects of Oncogene Activation on Hematopoiesis as a Possible Adverse Event in HSC Gene Therapy

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Hematopoietic stem and progenitor cell (HSPC) gene therapy (GT) applications employ vectors able to insert transgenes into the cells' genome, providing therapeutic benefit upon autologous transplantation. However, the semi-random integration of genetic material into the genome of HSPCs may lead to oncogene activation and subsequently increase the risk of malignant transformation. Despite the ongoing work on developing safer vectors, as the generation of self-inactivating lentiviral vectors (SIN.LV), oncogene activation might prompt other deleterious pathways than tumor development that could negatively impact the efficacy of the therapy. We have recently reported that oncogene expression (BRAFV600E) in human HSPCs triggered oncogene-induced senescence, a program that induced the secretion of high levels of pro-inflammatory cytokines including IL-1 α , IL-1 β , and TNF α . These cells, when transplanted into immune-deficient NSG mice, provoked a profound lymphoid impairment, transferred the senescence phenotype to bystander cells, reduced the overall bone marrow (BM) cellularity, and resulted in a highly penetrant lethal BM failure. Thus, although cellular senescence is a fundamental tumor-suppressive mechanism, chronic persistence of senescent cells in tissues has a strong negative impact on hematopoiesis. Since the clearance of senescent cells greatly depends on the efficiency of the immunological system, we aimed to investigate the behavior, fate, and impact of senescent cells on hematopoiesis in the presence or absence of an active immune system. To this purpose, BM-derived mouse (m) HSPCs were transduced with SIN.LV expressing mBrafV600E, an N-truncated version of the same oncogene (mBraf trunc), both forms reportedly oncogenic, or GFP as a control. Transduced cells were transplanted into immune-deficient (NSG) and immune-competent (WT) mice and hematopoietic reconstitution of myeloid and lymphoid (B and T) cells was monitored by FACS analysis in peripheral blood (PB) over time and in BM at the time of euthanasia. NSG mice of the mBraf trunc group showed an abnormal expansion of B cells and impaired cellularity in PB and BM but did not display overt lethality. On the other hand, mice of the mBrafV600E group displayed a dose-dependent lethality characterized by lymphoid impairment and reduced cellularity in PB and BM. Compared to the humanized model, these mice presented a significantly delayed mortality induced by the treatment, and the impairment of only mBrafV600E-lymphoid cells, while bystander lymphoid cells were not impacted. Blood plasma from mice of the mBrafV600E group showed levels of secreted cytokines (IL-2, IL-12), chemokines (CCL2, CCL3, CCL4, CCL5), and growth factors (G-CSF) significantly higher than controls or mBraf trunc mice ($p < 0.05$). However, the secretory profile strongly differed from the one found in the humanized model. These data indicate that mBrafV600E expression in mHSPCs, after transplant in NSG mice, induces a less aggressive pro-inflammatory cytokine secretion profile than human cells. Characterization of the transcriptional changes occurring in oncogene-expressing (and bystander) myeloid and lymphoid cells are ongoing, as well as the study of the fate and transcriptional phenotype of oncogene-expressing cells in immunocompetent mouse models. This study could shed light on the factors that determine the resilience of senescent cells in the presence of an active immune system, suggest strategies for their elimination and ultimately improve the safety of GT.

357. Changing the Treatment Paradigm for Pyruvate Kinase Deficiency with Lentiviral Mediated Gene Therapy: Interim Results from an Ongoing Global Phase 1 Study

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Background: Pyruvate kinase deficiency (PKD) is an inherited hemolytic anemia caused by mutations in the PKLR gene resulting in impaired erythrocyte metabolism. Manifestations include anemia, splenomegaly and iron overload, which may be life-threatening. PKD represents an unmet medical need; current treatments are limited to blood transfusions, chelation therapy, and splenectomy which are associated with significant side effects. Based on compelling preclinical data, a global Phase 1 clinical trial RP-L301-0119 (NCT04105166) is underway to evaluate lentiviral mediated hematopoietic stem and progenitor cells (HSPCs)-targeted gene therapy for adult and pediatric subjects with severe PKD. **Methods:** Splenectomized patients with severe PKD (defined as severe and/or transfusion-dependent anemia) are eligible. Mobilized peripheral blood (PB) HSPCs are collected via apheresis, enriched, transduced with PGK-coRPK-WPRE lentiviral vector, and cryopreserved. Myeloablative therapeutic drug monitoring-guided busulfan is administered over 4 days and the gene therapy product is then thawed and infused. Patients are followed for safety and efficacy assessments including PB and bone marrow (BM) genetic correction, decrease in transfusion requirements, clinically significant improvement in anemia, reduction of hemolysis, and patient-reported-outcomes (PROs) for 2 years post-infusion. **Results:** As of January 2022, 2 adult patients (age 31 and 47 years at enrollment) with severe anemia have received RP-L301. Patient 1 received 3.9x10⁶ CD34+ cells/kg with mean vector copy number (VCN) of 2.73. Patient 2 received 2.4x10⁶ CD34+ cells/kg with mean VCN of 2.08. At 1-year post infusion, both patients have sustained transgene expression, normalized hemoglobin, improved hemolysis and no red blood cell transfusion requirements post-engraftment. Both patients have anecdotally reported improved quality of life which has also been demonstrated by increases in scores on both the FACT-An and SF-36 instruments, with particularly marked improvement in energy/fatigue, physical functioning, and general health components of the SF-36. **Conclusion:** Engraftment of genetically modified HSPCs (RP-L301) has resulted in normalized hemoglobin and improved hemolysis markers in adult PKD patients with a highly favorable safety profile at 12 months post-administration.

In addition to clinical benefit, improvement in PRO scores have been observed. Updated safety and efficacy data (up to 18 months post-infusion) will be available at the time of presentation.

358. Direct Cross Species Comparison of Lentiviral Vector Hepatocyte Transduction Efficiency

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Dose response studies in preclinical animal models are widely used to predict the dose range of first-in-human (FIH) studies, while results from non-human primate (NHP) studies are believed to have the most predictive value due to the high homology between NHPs and humans. However, in lentiviral vector (LV) gene therapy research, it is well known that most NHPs exhibit restrictions to LV post entry and these restrictions could significantly decrease the LV transduction efficiency. We have evaluated human-factor IX encoding LVs (LV-FIX) via systemic administration in two NHP species: *Macaca leonina* (leonina) and *Macaca fascicularis* (cyno). We observed leonina monkeys had a more than 10-fold higher FIX expression level compared to cynos, suggesting a lower degree of LV restriction in leonina than cyno monkeys. Since the degree of LV restriction in NHPs compared to human are unknown, some NHP species may not be an adequate pre-clinical model for LV FIH dose prediction. To better understand the impact of NHP LV restriction on LV transduction efficiency, LV-GFP vectors were tested in-vitro in primary human and cyno T-cells and hepatocytes. Post transduction, 100-fold and 30-fold higher percentage of GFP positive cells were detected in human T-cells and hepatocytes as compared to that of cyno cells, respectively. Vector copy number analysis in these cell types further confirmed these findings. To further assess the direct correlation of in-vivo LV transduction efficiency across species, we treated FRGN mice, a mouse model whose livers have been repopulated by transplanted murine, cyno monkey, or human hepatocytes, with LV-FIX vectors via intravenous administration. IHC analysis of liver lobe sections post dosing confirmed approximately 60-90% engraftment of donor hepatocytes in the experimental animals. Compared to the animals repopulated with murine hepatocytes, we observed higher FIX expression in mice transplanted with human, but similar FIX expression in mice transplanted with cyno hepatocytes, which correlated well with previous study results in LV-FIX treated HemB mice and cyno monkeys. In-situ hybridization RNA scope analysis also showed a 10- to 20-fold higher hepatocyte transduction in human hepatocyte repopulated mouse liver as compared to cyno hepatocyte repopulated liver, which agreed with what we have observed in previous primary hepatocyte in-vitro studies. Further studies with different human hepatocyte donors are warranted to confirm and expand these findings.

One interesting observation of the FGRN study is that LV transduction efficiency of the endogenous mouse hepatocytes was influenced by the engrafted donor hepatocytes, as we observed comparable percentage of FIX-RNA positive cells in endogenous and donor hepatocytes in all animals, although this mechanism remains to be determined. Increasing the translatability and predictive value of non-clinical studies has always been an objective in the drug development field. Our results suggest that efficacy studies of the targeted primary human cell population in-vitro and in xenograft mouse models could potentially provide meaningful insights into the design of clinical studies, rather than just relying on traditional in-vivo rodent and NHP models. While these traditional animal models can produce very useful information for FIH studies, in the case of LV research, they may underestimate the efficacy of LV gene therapy.

359. Development of a Mass Spectrometry-Based Assay for the Detection of Endogenous and Lentiviral Engineered Hemoglobin in Sick Cell Mice

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Sickle cell disease (SCD) and β -thalassemia are caused by structural defect or deficiency in the β -globin chain of adult hemoglobin (HbA). These genetic disorders are traditionally evaluated by cellulose-acetate hemoglobin (Hb) electrophoresis or high-performance liquid chromatography (HPLC). While clinically useful, these methods have limitations. We hypothesized that mass spectrometry (MS) could be used to detect hemoglobin variants in a mouse model of SCD for genotyping studies or assess therapeutic β -globin expression following lentiviral gene addition in these animals. We have engineered a lentiviral vector based on the Lenti/ β AS3-FB construct. The β -globin gene expressed from this vector contains G16D, E22A and T87Q mutations that increase affinity for α -globin and decrease its ability to bind to sickle β -globin, thereby conferring anti-sickling properties. Herein we report a novel MS-based method for the rapid, sensitive, and highly quantitative detection of endogenous and lentiviral-encoded therapeutic hemoglobins. Utilizing synthetic signature peptides for α -globin, wild type β -globin, sickle β -globin, and β -AS3 globin, we developed sample preparation methods and a MS-based assay to simultaneously detect each of these hemoglobins in cultured cells and small quantities of peripheral blood. We have successfully used these MS methods to genotype Townes AA, AS and SS mice. We have also detected lentiviral vector-encoded β -AS3-globin *ex vivo* in transduced and differentiated mouse fetal liver cells, are currently conducting a lentiviral- gene therapy study using the Townes SCD mouse model. Transduction was confirmed 6 weeks post-transplant by vector copy number assay of gDNA from peripheral blood, and our MS assay determined that 2.5% - 5% of total blood cell protein was the

therapeutic β -AS3 globin. Based on these collective results, we conclude this MS-based protocol is a viable alternative to traditional methods of newborn screening as it offers a more immediate and quantitative result. Furthermore, with several genome-editing and gene therapy approaches currently in clinical trials for severe hemoglobin disorders, this MS method will be useful for patient assessment before treatment and during follow-up.

360. Generation of RUNX1-FPD Rhesus Macaque Model for Preclinical Assessment of Hematopoietic Stem Cell Gene Therapy

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Germline loss-of-function (LOF) heterozygous mutations in the central hematopoietic transcription factor *RUNX1* gene cause the marrow failure/malignancy predisposition syndrome Familial Platelet Disorder with associated Myeloid Malignancies (FPDMM, or FPD). Patients with FPD have defective megakaryocytic development, low platelet counts, and a very high (35-50%) life-long risk of hematological malignancies, particularly MDS/AML. Murine heterozygous gene knockout models do not recapitulate the human phenotype in terms of thrombocytopenia or myeloid leukemia progression. Although gene correction of the *RUNX1* mutation in hematopoietic stem and progenitor cells (HSPCs) is being considered as a possible treatment approach, it is unknown whether mutation-corrected HSPCs will have the hoped for advantage over *RUNX1* mutant HSCs *in vivo*, likely necessary to significantly lower leukemia risk. In order to study the relative function of wildtype and *RUNX1*-mutated HSPCs *in vivo* in a model with close hematopoietic similarity to humans, we generated a rhesus macaque FPD competitive repopulation model using CRISPR/Cas9 NHEJ editing to create LOF indels in the *RUNX1* gene versus the *AAVS1* safe-harbor control locus. We transplanted mixtures of autologous HSPCs edited at the two loci (Animal 1: 75% *RUNX1*/25% *AAVS1*; Animal 2: 50% *RUNX1*/50% *AAVS1*), following conditioning with total body irradiation. Both animals engrafted tri-lineage hematopoiesis promptly following transplantation. To assess the HSPC competitiveness of *RUNX1* mutant versus *AAVS1* control and unedited WT cells we tracked *RUNX1* and *AAVS1*-mutated allele frequencies in blood cells over time via deep sequencing (Figure 1). In the infusion products (IP), allele fractions reflected the desired ratios. In both animals, *AAVS1*-edited cells dominated compared to *RUNX1*-edited cells. However, in animal 1, *RUNX1*-edited LOF cells expanded over time eventually exceeding the ratio in the IP, and in animal 2, levels of *RUNX1*-edited cells also expanded compared to *AAVS1*-edited, although at a slower rate compared to animal 1. In addition, platelet count remained below the normal range long-term in animal 1, the animal with a higher starting fraction of *RUNX1*-edited HSPCs and marked expansion of *RUNX1*-edited output over time, and below platelet counts of macaques transplanted with HSPCs edited at other loci (Figure 2). Bone marrow morphology showed some

megakaryotypic dysplasia and decreased numbers of megakaryocytes. BM-derived CD34+ HSPCs from the RUNX1-edited animals presented impaired *in vitro* megakaryocytic differentiation capacity compared to gene-edited control animals. Furthermore, polyploidization was suppressed within CD41a+ differentiated cells and multi-ploid cells were determined to have less RUNX1 editing frequency, but not AAVS1. In conclusion, we have created pre-clinical model for FPD via CRISPR/Cas editing of HSPCs in rhesus macaques. The lack of a competitive advantage for wildtype or control-locus edited HSPCs over RUNX1 heterozygous-mutated HSPCs long-term in our model suggests that gene correction approaches for FPD will be challenging, particularly to reverse the MDS/AML predisposition phenotype.

Figure 1.

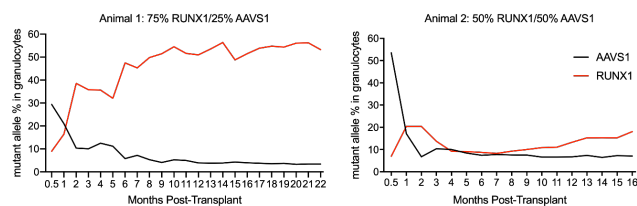
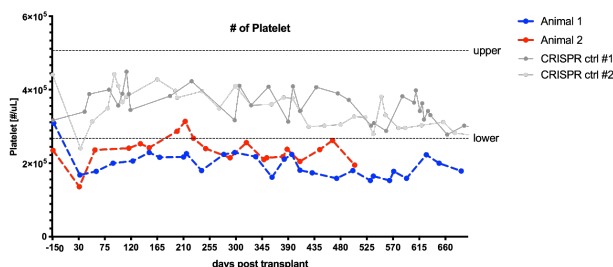


Figure 2.



361. Safety and Efficacy Evaluation for an *Ex Vivo* Selection Free CRISPR/Cas9 Based Gene Therapy for *HAX1* Associated Congenital Neutropenia

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The rare pre-leukemia bone marrow failure syndrome, severe congenital neutropenia (CN), is characterized by a severely reduced number of peripheral blood neutrophils (< 500/ μ l). Patients suffered from frequent life-threatening bacterial infections until G-CSF

was introduced as the standard of care. In approximately 10 % of the cases, neutropenia is caused by homozygous loss of function mutation in the *HAX1* gene. Patients with *HAX1* associated CN have a 20 % risk of developing MDS/AML. For CN patients with overt MDS/AML, the only therapy option is a hematopoietic stem cell transplantation with the associated risks. These factors constitute an urgent clinical need for curative gene therapy for CN patients. We have developed an approach for a corrective *ex vivo* gene therapy for the most frequent *HAX1* mutation p.W44X in primary bone marrow CD34+ hematopoietic stem and progenitor cells (HSPCs). Our approach relies on creating double-stranded breaks using the CRISPR/Cas9 system to trigger homology-directed repair (HDR). HDR outcome is controlled through a custom-developed *HAX1* DNA template, delivered to the cells through a recombinant adeno associated virus 6 (rAAV6). The delivery of CRISPR/Cas9 RNP by electroporation followed by transduction with rAAV6 was established using healthy HSPCs. The cells were transduced with the rAAV6 carrying the HDR template at an MOI of 10⁵. We observed 38,1% (\pm 1,3%) knock-in (KI) efficiency and total editing (TE) of 82,3 % (\pm 8,2%) (n=2). When the *HAX1* gene-editing process was applied to HSPCs from *HAX1*-CN patients (n = 5), we observed TE at 84,4 % (\pm 4,2 %) and a KI efficiency of 65,8 % (\pm 7,12 %). The re-expression of wild-type *HAX1* protein in edited cells was confirmed by Western Blot analysis. Restoring *HAX1* protein expression resolved the maturation arrest of granulopoiesis, as assessed by *in vitro* differentiation of gene-edited cells to neutrophils. Flow cytometric analysis showed significant (p = 0,011) increase of CD34-CD45+CD15+CD16+ neutrophils, compared to control edited cells. Morphological assessment of Wright-Giemsa stained cytopins of differentiated cells confirmed the improved neutrophilic differentiation of *HAX1* edited cells. In functional studies, we observed increased capacity to produce ROS and NETs and increased Apoptosis resistance in corrected cells compared to control edited samples. To establish a comprehensive and unbiased off-target profile for the safety evaluation of the gene editing, we performed GUIDE-Seq and CAST-Seq analyses for the utilized guide RNA in HSPCs. Observed translocations and off-target sites were infrequent, mostly intergenic, and not associated with genes known to be disease-causing. In conclusion, we describe the first-ever *ex vivo* gene therapy approach for HSPCs of *HAX1*-CN patients. The observed editing efficiencies allow for a selection-free process. Further xenotransplantation experiments and toxicological studies are needed, but our preliminary results demonstrate the feasibility of performing safe HDR-based correction of *HAX1*-mutations to cure congenital neutropenia.

362. *In Vivo* Modeling of HDR Based Gene Editing and Immune Reconstitution in CD40L-Deficient Mice

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Introduction: X-Linked Hyper IgM syndrome (XHIM) is a primary immunodeficiency caused by mutations in the *CD40L* gene. Without functional CD40L protein, there is a lack of immunoglobulin class switch

recombination and thus an inability to mount appropriate immune responses to opportunistic pathogens. Allogeneic hematopoietic stem cell transplant (HSCT) is the only curative therapy available. However, there are currently several autologous gene therapy-based strategies in development that bring the possibility of new curative therapies which avoid complications associated with allogeneic HSCT. Several groups have shown successful CD40L targeted gene replacement in XHIM patient derived CD4⁺ T lymphocytes and primary human CD34⁺ hematopoietic stem/progenitor cells (HSPCs). It has also been demonstrated that xeno-transplantation of edited human CD34⁺ HSPCs in NSG mice engraft and produce CD40L expressing CD4 T cells upon stimulation. While these are important milestones, it remains difficult to assess the immune function of edited HSPCs and downstream lineages in an *in vivo* model. Here we use the CD40L^{-/-} XHIM mouse model to evaluate restoration of CD40L expression and immune responses *in vivo* with gene modified murine T cells and HSPCs. **Methods:** Murine splenic CD4 T cells were isolated from XHIM males and edited with a sgRNA/Cas9 RNP targeting exon 1 of the murine CD40L locus in combination with an AAV to deliver a corrective CD40L cDNA donor cassette. Murine CD4s were stimulated with PMA/ionomycin and CD40L expression was assessed by flow cytometry. Murine HSPCs (Lin⁻) were isolated from the bone marrow of XHIM mice and edited using the same critical reagents. Molecular analysis for gene integration was measured by digital droplet PCR (ddPCR) in both cell types. To track engraftment of edited donor XHIM murine HSPCs in recipient XHIM mice, a CD40L^{-/-} CD45.1 mouse line was generated by crossing the commercially available CD40L^{-/-} CD45.2 mice with B6 CD45.1 mice. For murine to murine *in vivo* studies, edited adult male XHIM CD45.1 murine donor HSPCs were transplanted into irradiated adult female XHIM CD45.2 recipient mice. For immune response studies, XHIM mice transplanted with edited cells will be immunized with the T cell dependent antigen TNP-KLH. Antigen specific IgG antibody production will be measured in the serum by ELISA. **Results:** Using CRISPR/Cas9 RNP targeting exon 1 of the murine CD40L locus in combination with an AAV to deliver a corrective CD40L codon optimized cDNA donor cassette, 10.5±1.5% XHIM murine CD4 T cells carry the corrective donor as measured by ddPCR. Additionally, similar levels of CD40L expression are seen upon PMA/ionomycin stimulation by flow cytometry. In XHIM murine HSPC, we achieved 19.8±1.9% targeted gene insertion. These edited XHIM 45.1 Lin⁻ cells have been transplanted into XHIM 45.2 mice and will be assessed for engraftment and T cell production. Transplanted mice will be further studied to assess antigen specific immune responses after successful engraftment of gene modified cells. **Conclusions:** This study demonstrates the use of CRISPR/Cas9 RNP with AAV to successfully deliver a corrective CD40L cDNA cassette to restore CD40L protein expression in XHIM murine CD4 T cells and HSPCs. Transplantation of edited cells into XHIM mice represents a useful model for understanding immune reconstitution *in vivo* after gene therapy.

363. Hematopoietic Reconstitution and Lineage Commitment in HSC-GT Patients are Influenced by the Disease Background

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Lentiviral vector (LV)-based hematopoietic stem cell (HSC) gene therapy (GT) applications have shown clear efficacy and safety profiles for the treatment for a variety of genetic diseases, confirmed by high throughput clonal tracking using vector integration sites (IS) as genetic barcode distinctive of each stem cell and inherited by all its progeny. Several factors, including transplantation protocols, patients' age and the underlying genetic disease, impact on the kinetics of hematopoietic reconstitution, lineage specification, efficacy, and patients' safety. To dissect the impact of patient-specific factors and disease background on the hematopoiesis after transplantation, we studied the clonal reconstitution and multilineage potential over time in 48 patients enrolled in 3 different HSC-GT clinical trials related to distinct contexts: a neurodegenerative lysosomal storage disorder (metachromatic leukodystrophy, MLD), a primary immune deficiency (Wiskott-Aldrich syndrome, WAS), and a hemoglobinopathy (β -Thalassemia, β -Thal. Since no signs of insertional mutagenesis has been identified, we analyzed the clonal repertoire of IS to dissect the HSC activity/commitment and hematopoietic reconstitution over time. LV genomic Integration Sites (IS) were identified from the DNA of CD34⁺ cells as well as myeloid, B, T and erythroid cell lineages purified from blood and bone marrow, harvested at different time points after transplantation (longest follow up 9 years). IS analysis yielded more than 3.5 million clones and showed highly polyclonal reconstitution and multilineage marking. All patients showed a similar pattern of hematopoietic reconstitution over time, characterized by: an early phase up to 9 months, where the myeloid cells are highly polyclonal whilst T and B cells have a less complex repertoire; a second phase of 9 up to 18 months, where the polyclonality of lymphoid cells increases; and a third phase where the complexity of the lineages decreases and stabilizes. During the early phase of hematopoietic reconstitution we estimated a number of ~80,000 active HSPCs per patient that decreased to an average 11,000 after 9 months, thus highlighting the relevant role of short lived progenitors in sustaining the early phases of hematopoietic reconstitution and then exhausting after 9 months. Moreover, in all studies committed short living HSPC are significantly less represented in the pool of long lasting clones, confirming the limited life-span of committed clones. Differences across diseases were found when we analyzed the multilineage potential of individual clones and their commitment towards a specific lineage over time. In MLD patients multilineage clones reached a proportion of 66% on the total engineered clones, decreased over time to 57%, remaining stable thereafter. Myeloid-committed clones increased over time and stabilised at 40%. The decrease in multilineage clones over time was also observed in WAS and β -Thal patients. However, in WAS patients

we did not observe a concomitant increase in myeloid committed cells rather an increase of the commitment in the T-cell lineage, as expected by the selective advantage in T cells, whereas in β -Thal patients several clones showed erythroid commitment. Our data suggest that the disease condition influences the proportion and the type of lineage-committed cells over long periods of time, and that the engrafted HSPC pool dynamically respond to the disease-specific physiopathology to restore normal hematopoiesis.

364. Improving the Treatment of Hemophilia A with Directed Evolution of the Factor VIII Transgene

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Recombinant Factor VIII (FVIII) therapies were first approved by the FDA in 1992, yet the need for frequent infusions, appearance of neutralizing antibodies in many patients, and limited efficacy has led to continued research into Hemophilia A treatments, particularly gene therapy. The success of the Padua-FIX variant in Hemophilia B gene therapy raises the possibility that an improved version of the FVIII transgene might offer great promise for treating Hemophilia A patients. Specifically, a FVIII transgene with improved expression, secretion, stability, and cofactor potency as well as reduced immunogenicity could improve upon current gene therapy strategies by allowing lower doses and providing better patient outcomes. Rather than relying on the serendipitous identification of beneficial variants, we employed the CodeEvolver® directed evolution technology to engineer FVIII variants with better properties. Using CodeEvolver®, which entails high-throughput protein expression, in vitro screening with patient-derived samples, next-generation sequencing, and bioinformatics, we screened >12,000 variants of a B-domain deleted FVIII (FVIII-BDD) over eight rounds of iterative evolution. Our screens identified mutant FVIII transgenes with superior properties as compared to wild-type FVIII-BDD. Whereas wild-type FVIII-BDD loses >50% of its activity in Hemophilia A patient plasma within 48 hours, engineered FVIII-BDD variants retain >80% activity after 4 days. Furthermore, the engineered FVIII-BDD variants show >30-fold increased expression from HepG2 liver cells and >20-fold improved potency in a chromogenic FXa generation assay. During our directed evolution program, to address concerns around the risk of patients generating FVIII neutralizing antibodies, we targeted in silico-predicted major histocompatibility complex (MHC) class II epitopes, reducing the number of predicted epitopes by 30%. Our results show the promise of protein evolution when applied to transgenes and gene therapy technologies, with the goal of improving patient outcomes for patients suffering from Hemophilia A and other genetic disorders.

365. Sustained Expression of C5mAb in Presence of Murine and Human FcRn

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Adeno-associated virus (AAV) gene therapy has the potential to offer a long-term solution for diseases that rely on chronic dosing. For paroxysmal nocturnal hemoglobinuria (PNH) and other complement disorders, Homology has developed its Gene Therapy-mAb (GTx-mAb) platform, focused on a one-time dose to deliver an investigational vectorized C5 monoclonal antibody. The C5 GTx-mAb achieved dose dependent sustained expression of functional C5 mAb 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). In this study, we asked if the steady state levels achieved with our GTx-mAb platform would be influenced by the presence of human neonatal Fc Receptor (FcRn) since both NOD-SCID and humanized FRG mice express the endogenous murine FcRn. The receptor is widely expressed in humans and mice and is primarily responsible for the unusually long immunoglobulin G (IgG) half lives in circulation. FcRn protects IgGs from degradation by binding to their Fc region in the acidified endosome and recycling the IgGs back into circulation at physiological pH. While human FcRn only binds to human IgGs, murine FcRn is more promiscuous and can bind IgGs from humans and other species with high affinity. For evaluation of human/humanized mAb therapeutics, mice expressing a human FcRn transgene and lacking endogenous FcRn are considered the best translational models, as their IgG half-lives translate closely to clinical findings. We expanded our animal studies to include several strains, including immunocompromised and immunocompetent transgenic strains containing the human FcRn receptor. We found that the species-specific differences in mouse vs human FcRn did not significantly impact the overall circulating antibody levels at steady state. Circulating levels of C5 mAb in all mouse strains tested were comparable or higher than those found in NOD-SCID mice, suggesting that the continuous synthesis and secretion of antibodies by hepatocytes is the key determinant in achieving sustained antibodies levels. Furthermore, we showed that murine C5 can contribute to an *ex vivo* hemolysis assay mediated by human serum. Using a modified *ex vivo* hemolysis assay that corrects for hemolysis due to murine C5, we confirmed that *in vivo* produced C5 mAb was functional in all mouse models investigated. In conclusion, we have demonstrated that our results with the GTx-mAb platform are translatable to immunocompetent as well as mouse strains containing a human FcRn. Further, these data support additional studies to evaluate the potential of single dose C5 GTx-mAb in the development of therapeutics for PNH and other complement-related disorders.

366. A Factor VIII Variant with Elimination of the N-glycosylation Site at 2118 Reduced Anti-FVIII Immune Responses in Gene Therapy Treated Hemophilia A Mice

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Inhibitor formation is a challenging complication in hemophilia A treatment. Reduction of immune responses against FVIII will profoundly increase the effectiveness of gene therapy in hemophilia A treatment. Our previous studies have demonstrated that lower FVIII inhibitor titers were detected in hemophilia A (HemA) mice injected with FVIII plasmid containing Glutamine to Asparagine substitution in C1 domain (N2118Q) than the mice with BDD-FVIII plasmid via hydrodynamic gene delivery of FVIII plasmids. It suggested that the elimination of glycosylation in C1 domain of FVIII reduced its immunogenicity. Our aim in the study is to examine the impact of FVIII glycosylation following AAV-mediated gene therapy and further characterize the important immunogenic region in C1 domain of FVIII. HemA mice were injected with AAV-FVIII and AAV-N2118Q, followed by challenge with repeated FVIII injections. FVIII activity and inhibitor titer were examined over time. FVIII expression was initially detected in all AAV treated mice for eight weeks, however, subsequently dropped to very low or undetectable at week 12 in BDD group, whereas was only slightly decreased in N2118Q group. Following FVIII challenge, N2118Q group showed higher resistance to inhibitor formation than BDD group. To improve the therapeutic effects, codon optimized FVIII transgene was evaluated. Higher FVIII expression was detected in mice injected with codon optimized FVIII plasmid than the mice with BDD-FVIII via hydrodynamic injection. To characterize the immunogenic region around 2118 site, three sets of 15-amino-acid peptides were designed with partly overlapping and with or without mannosylation (MP1/NGP1; MP2/NGP2; MP3/NGP3). Higher proliferation rates or cytokine levels were detected in mouse cells and human PBMCs isolated from inhibitor subjects cultured with two mannosylated peptides (MP1 and MP2) but not with MP3 and all non-mannosylated peptides. Cells from control non-inhibitor subjects did not exhibit increased proliferation when stimulated with all peptides. Gene therapy for hemophilia A could be improved by reducing FVIII immunogenicity via elimination of glycosylation in C1 domain of FVIII and by increasing the FVIII expression via usage of codon optimized FVIII transgene.

367. Non-Integrating Viral Vectors for Base Editor Delivery to Hematopoietic Stem Cells In Vivo

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Background: Allogeneic hematopoietic stem cell transplant (HSCT) has proven to be the only curative therapy for genetic hematologic disorders including severe combined immunodeficiency disease (SCID), sickle cell disease (SCD), and Fanconi anemia (FA)¹. However, allogeneic HSCT requires an HLA-matched donor and carries a high risk of morbidity and mortality. More recently, *ex vivo* methods for gene correction of hematopoietic stem cells (HSCs) using CRISPR/Cas9 have been developed and shown high efficacy. These methods rely on stochastic repair of double-strand breaks (DSBs) made by the Cas9 nuclease, which can be toxic to stem cells^{2,3}. Here, we aim to deliver Base Editor (BE) to HSCs in a transient fashion to achieve comparable genetic correction without the risks associated with DSBs. To this end, we propose the use of non-integrating viruses as a delivery vector for BEs. This delivery approach of gene editing reagents takes advantage of advancements in viral vector targeting of specific cell types and efficiency of viral delivery. While many viral delivery approaches involve permanent integration of DNA cassettes, we aim to achieve transient expression of BE constructs to make permanent genetic correction of genomic DNA. Ultimately, these vectors will be used for *in vivo* editing of HSCs to eliminate the risks and costs associated with *ex vivo* manipulation.

Approach: Vectors carrying both BE and a U6 promoter driven single guide RNA (sgRNA) were generated in the following backbones: rAAV6, CD133-targeted adenovirus, and non-integrating lentivirus. A split intein approach was used to divide the ~6kb cargo between 2 separate recombinant adeno-associated virus (rAAV) constructs delivered simultaneously. Constructs were tested by plasmid transfection in immortalized cell lines before viral production. Next, viral tropism and editing efficiency will be confirmed in human mobilized peripheral blood (mPB) HSCs *in vitro*. We expect transient expression of the BE + sgRNA, as AAV and adenovirus viral DNA remains episomal, and our lentivirus harbors mutations in the integrase gene and long terminal repeats preventing integration.

Results: We have successfully developed constructs encoding BE and a targeted sgRNA for each of our viral platforms and manufactured the viruses. We expect to transduce mPB HSCs with each virus and assess editing of the beta-2-microglobulin (*B2M*) locus in the coming months. *B2M* is expressed as part of MHC class I on the cell surface, which allows its detection by flow cytometry. These proof of principle experiments demonstrating gene editing by non-integrating viral vectors can be applied to *in vivo* models of SCID, SCD, and FA as viral tropism is improved in the future.

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368. Therapeutic Base Editing of Fanconi Anemia Patient Mutations in Primary Cells for Autologous Gene Therapy

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Fanconi anemia (FA) is a rare genetic disease caused by the disruption of proteins that participate in DNA repair, leading to patient bone marrow failure and cancer predisposition. Mutations can affect any of the 22 FA DNA repair pathway genes that are essential to the repair of interstrand crosslinks (ICLs) and double-strand breaks (DSBs) through homologous recombination (HR)¹. The only proven curative therapy for FA is a hematopoietic cell transplant (HCT); however, many patients lack a matched donor, and the risks include end-organ toxicity and opportunistic infections. Targeted nucleases can induce site-specific DSBs, allowing for the correction of FA mutations through homology-directed repair (HDR). Nevertheless, the inherent deficiency in DNA repair in FA makes this approach extremely inefficient. Cutting edge ‘digital’ genome editing tools, namely the Cas9 base editors (BEs), are ideally suited for this disease, as they bypass the need for DSB induction or the requirement of an HDR DNA donor molecule. Here we report two FANCA patient mutations that were successfully edited at high rates using cytosine base editors (CBEs; C→T) and adenine base editors (ABEs; A→G). The first patient sample contained the FANCA c.3934+2T→C mutation, where a splice site was directly restored, and the second patient sample contained the most common FANCA founder mutation seen in patients with Spanish Romani ancestry (FANCA c.295 C→T)². This strategy involved changing the mutant premature STOP codon (TAG) to tryptophan (TGG), which restored the reading frame without the need to revert to the wild-type glutamine (CAG). These strategies of correction or conservative codon substitution led to phenotypic rescue, as shown by desensitization to the alkylating agent mitomycin C (MMC), a potent ICL inducer (Figure 1A, B). Further, with the latter mutation, the FANCA protein product was restored, and an intact FA pathway was shown by downstream FANCD2 mono-ubiquitination (Figure 2A, B). This strategy will enable the use of gene-corrected FA patient hematopoietic stem and progenitor cells for autologous HCT, thereby obviating the risks associated with DSBs during gene therapy and allogeneic HCT.

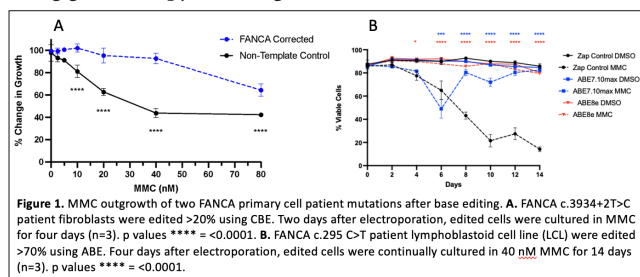


Figure 1. MMC outgrowth of two FANCA primary cell patient mutations after base editing. **A.** FANCA c.3934+2T→C patient fibroblasts were edited >20% using CBE. Two days after electroporation, edited cells were cultured in MMC for four days (n=3). p values **** = <0.0001. **B.** FANCA c.295 C→T patient lymphoblastoid cell line (LCL) were edited >70% using ABE. Four days after electroporation, edited cells were continually cultured in 40 nM MMC for 14 days (n=3). p values **** = <0.0001.

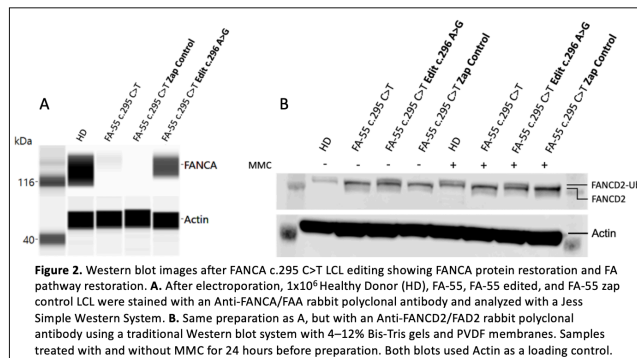


Figure 2. Western blot images after FANCA c.295 C→T LCL editing showing FANCA protein restoration and FA pathway restoration. **A.** After electroporation, 1x10⁶ Healthy Donor (HD), FA-55, FA-55 edited, and FA-55 zap control LCL were stained with an Anti-FANCA/FAA rabbit polyclonal antibody and analyzed with a Jess Simple Western System. **B.** Same preparation as A, but with an Anti-FANCD2/FAD2 rabbit polyclonal antibody using a traditional Western blot system with 4–12% Bis-Tris gels and PVDF membranes. Samples treated with and without MMC for 24 hours before preparation. Both blots used Actin as a loading control.

369. Comparison of Gene Modification Approaches for the Treatment of DOCK8 Deficiency

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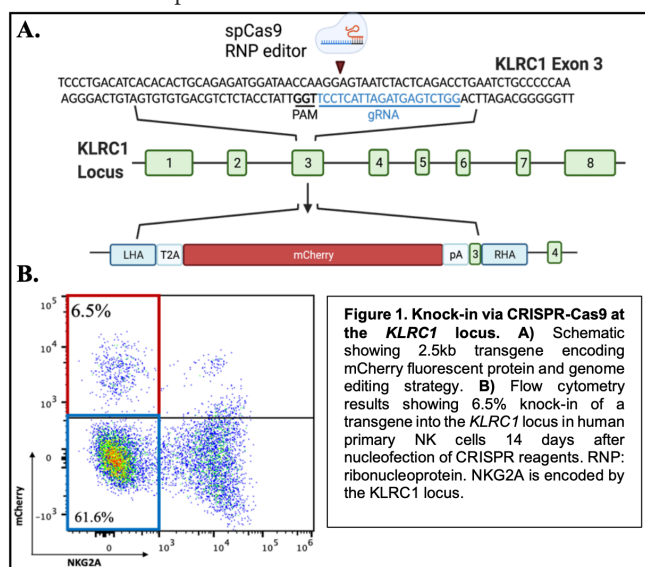
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DOCK8 deficiency is a combined immunodeficiency resulting in recurrent infections of the skin, upper and lower respiratory tract, GI tract, and a predisposition for autoimmunity and malignancy. The massive DOCK8 locus spans about 200 kb and the 48 exons in the locus total a cDNA length of 7448 bp. Located at the end of Chromosome 9, DOCK8 contains repetitious regions that can recombine to create deletions of the entire locus. Duplications of the region are also common in DOCK8 deficiency patients and the region of deletion or duplication often encompasses multiple genes. Acting downstream of T-cell receptor (TCR) stimulation, DOCK8 catalyzes the exchange of GDP for GTP in the GTPase CDC42 which then promotes actin polymerization and reorganization of the cytoskeleton. Patients lacking functional DOCK8 protein display defects in T-cell as well as B-cell proliferation and defective NK cells and granulocytes. Interestingly, allogeneic transplants have demonstrated a proliferative advantage of donor cells against those of patient origin. In this work, we designed and compared lentiviral as well as Cas9 based therapies for the treatment of DOCK8 deficiency. We created differing codon optimization schemes for our lentiviral constructs to compare their expression as well as functional activity in cell lines and primary HSPCs. In preliminary work, DOCK8 LVs transduced up to 40% of primary HPSCs, a level likely to result in clinical benefit when transplanted into patients. During this work, we optimized lentiviral production methods for viruses containing large transgenes as well as transduction protocols for primary HSPCs. In addition, a gene editing approach for the treatment of DOCK8 deficiency will also be evaluated. We identified an sgRNA targeting the 5’ region of the gene with high on-target activity in cell lines and primary HPSC with a favorable off target profile as determined by GUIDE-seq. Because the DOCK8 cDNA length exceeds the packaging capacity of AAV, we are currently testing a dual AAV strategy for targeted insertion of the full length cDNA in cells.

Cell Therapies I

370. Targeted Virus-Free Transgene Insertion into Natural Killer Cells Using CRISPR-Cas9Keerthana Shankar¹, Christian Capitini², Krishanu Saha¹¹Department of Biomedical Engineering, Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI, ²Department of Pediatrics, University of Wisconsin-Madison, Madison, WI

Natural killer (NK) cells are innate cytotoxic lymphocytes capable of killing virally infected cells and malignant tumors. Unlike T cells, NK cells are HLA-agnostic and cause little to no Graft versus Host Disease in allogeneic transfusions, making them excellent candidates for off-the-shelf cell therapeutic strategies. However, current techniques to insert the large transgenes (>1 kb; e.g., chimeric antigen receptors) into NK cells primarily employ viral vectors. Viral methods can lead to complications such as insertional mutagenesis, gene silencing, and oncogene activation. Here we have developed a fully virus-free genome editing strategy in primary human NK cells. A large transgene is encoded in a linear double stranded DNA (dsDNA) template and produced by polymerase chain reaction (PCR). The template includes homology to the intended target for insertion that is defined by a CRISPR-Cas9 ribonucleoprotein (**Figure 1A**). We show efficient (>60%) CRISPR knock-out of the inhibitory NKG2A receptor encoded by the *KLRC1* gene within primary NK cells. Optimization of electroporation timing showed that delivery of 3 μ g of donor DNA on day 6 of *ex vivo* expansion resulted in gene knock-in rates of up to 6.5% for transgenes as large as 2.5 kb (**Figure 1B**). Lastly, we report that the use of K562-mbIL15-41BBL feeder cells improves expansion of CRISPR edited cells by approximately twenty-fold. This data provides a proof-of-principle for targeted integration of a long transgene within primary NK cells via CRISPR-Cas9 genome editing. This method is likely to enable the fully virus-free manufacturing of genetically-programmed NK immunotherapies.

**371. 10-Year Stability Assessment of Cryopreserved, Engineered iPSC Banks: Genetic and Phenotypic Characterization**

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Induced pluripotent stem cells (iPSCs) represent a promising renewable source of starting material for the mass production of uniform and consistent multiplexed-engineered cellular therapies for off-the-shelf therapeutic use, including for cancer, autoimmune disease and regenerative medicine. Over the past decade, we have developed a robust proprietary iPSC platform, where cellular reprogramming, maintenance of pluripotency in the naïve state, and single-cell culture in a feeder-free environment is enabled by stage-specific, small molecule combinations to block differentiation, enhance survival, and support self-renewal of iPSCs (Valamehr et al., Stem Cell Reports 2014). We have applied our iPSC product platform to generate clonal master iPSC banks, and have used these banks for cGMP manufacture of multiplexed-engineered, off-the-shelf natural killer and T-cell product candidates now in clinical studies. Establishing a cell banking paradigm, including a bank stability program, to ensure the long-term availability and viability of clonal master iPSC banks for drug product manufacturing is critical to ensure safety, efficacy, regulatory compliance, and manufacturing demands throughout a product's life cycle. To this end, we have performed long-term stability studies of cryopreserved iPSC banks, including at 2, 5 and 10 years from formation. Our data show that different somatic cells can be efficiently reprogrammed to the pluripotent state, frozen in a controlled environment, and maintained for at least 10 years. Cryopreserved iPSC banks were thawed and tested for critical quality attributes including viability, recovery, purity, and potency. In addition, the stability of cryopreserved, multiplexed-engineered iPSC lines was examined following thaw and stress-inducing manipulations, including further rounds of genetic engineering, single-cell subcloning and expansion in feeder-free culture, and cryopreservation to establish secondary iPSC banks. The data to be presented will collectively show that cryopreserved iPSC banks maintain viability (>90%), purity (>90%), potency (tri-lineage differentiation), phenotypic and genetic stability (genome integrity and transgene expression stability) over a long period of time without significant deterioration. Therefore, clonal master iPSC banks created using our proprietary iPSC platform and maintained under our proprietary banking paradigm can serve as a renewable source of starting material over the long term for the mass production of off-the-shelf cell therapies.

372. In Vivo Generation of CAR T Cells in Presence of Human Myeloid CellsNaphang Ho¹, Shiwani Agarwal¹, Michela Milani², Alessio Cantore^{2,3}, Christian Buchholz^{1,4}, Frederic Thalheimer¹¹Paul-Ehrlich-Institut, Langen, Germany, ²IRCCS San Raffaele Scientific Institute, Milan, Italy, ³Vita-Salute San Raffaele University, Milan, Italy, ⁴Frankfurt Cancer Institute, Goethe University, Frankfurt am Main, Germany

Pre-clinical humanized mouse models are a powerful tool to evaluate efficacy and safety of novel immunotherapies. For example, CD34+ humanized NSG-SGM3 (huSGM3) mice develop more physiological levels of human myeloid cells due to transgenic expression of human SCF, GM-CSF and IL-3. Therefore, this model better reflects the human immune system than the widely used humanized NSG model (huNSG). In here, we assessed the huSGM3 model for *in vivo* generation of CD19-CAR T cells by single intravenous injection of the T cell specific lentiviral vectors (LVs) CD4-LV and CD8-LV. Overall, *in vivo* CAR T cell generation was less efficient in huSGM3 than in huNSG mice. Interestingly, the CD4-LV group showed the least robust CAR T cell development, which correlated with cytokine patterns, such as increased IL-15 and decreased GM-CSF, that are typical for monocyte and macrophage-associated activities. Further *in vitro* assays identified macrophages as potential barrier for *in vivo* gene transfer. Refining CD4-LV and CD8-LV with a less immunogenic surface by using $\beta 2M^{-1}/CD47$ overexpressing vector producer cells improved transduction efficiencies of lymphocytes cultivated in presence of macrophages. Finally, the shielded targeted LVs mediated improved *in vivo* CAR T cell generation, while maintaining T cell target specificity in huSGM3 mice. Our data emphasize the relevance of innate immune responses for *in vivo* generation of CAR T cells and we show that this model recapitulates key points of the human immune system making it ideal for testing *in vivo* gene therapy.

373. Robust T-Cell Cellular Reprogramming and Single-Cell Engineering Platform Overcomes Inconsistencies and Heterogeneity Associated with Engineering Primary T Cells

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Adoptive transfer of primary T cells expressing chimeric antigen receptors (CARs) has shown great promise in treating hematologic malignancies. However, application of CAR T-cell therapy is limited by several challenges, including cell product inconsistencies and heterogeneity resulting from engineering primary T-cell populations as part of drug product manufacture. The use of clonally-derived, master induced pluripotent stem cell (iPSC) lines is an attractive source for the renewable manufacture of precisely-engineered, homogenous CAR T-cell products that can be fully characterized, stored, and administered on-demand for broad patient access. Furthermore, generation of such master iPSC lines from donor T cells (TiPSCs) allows for the unique opportunity to use the pre-rearranged TCR locus as an ideal approach to facilitate T-cell receptor alpha constant (TRAC) locus-targeted genetic editing to enhance CAR-mediated activity. Reprogramming of donor T cells has historically been inefficient though and iPSC-derived T cells often exhibit poor functional attributes. Here we evaluate our non-integrating proprietary T-cell cellular reprogramming platform for the efficient generation of high-quality naïve TiPSCs using current good manufacturing practice (cGMP)-grade processes, procedures, and materials. Donor T cells were sourced from leukapheresis blood collections following consent and screening for infectious disease

markers. The $\alpha\beta$ T-cell population was isolated and banked in animal component-free cryopreservation medium. These parental banks were characterized for purity, safety, identity, viability, and recovery. cGMP parental cell banks from nine independent donors were assessed for reprogramming efficiency and naïve iPSC generation using our proprietary reprogramming platform (Valamehr et al., 2014). TiPSCs were efficiently generated from all nine cGMP parental cell banks regardless of donor background or attributes. The fraction of TiPSCs reached >80% by day 28 for most donors (n=7), whereas two donors exhibited lower TiPSC fraction (24 and 58%). In each case, this output of TiPSCs represented a significant increase over other donor T-cell reprogramming methods. To test the potential to precisely engineer and single-cell select TiPSCs for the generation of clonally-derived, master iPSC lines, TiPSCs from four cGMP parental cell banks were engineered by CRISPR-mediated CAR insertion into the TCR alpha constant locus. TiPSCs from all four cGMP banks showed robust knock-in efficiency (20-60%) and maintained uniform pluripotent phenotypes and genomic stability post genetic engineering. We next isolated and sorted single-cell TiPSCs into 96-well plates, and extensively characterized each clonal TiPSC population following expansion. Hundreds of TiPSC clones were successfully generated and cryopreserved for characterization and assessment. Cryopreserved TiPSC clones showed robust recovery upon thaw (viability >75%) and maintained normal karyotype (100% of clones tested). In contrast to engineering cell populations as part of each manufacturing campaign for primary CAR T-cell production, this study highlights the potential to use clonally-derived, master engineered TiPSC lines as a renewable source for the consistent and uniform manufacture of off-the-shelf CAR T cells for therapeutic applications.

374. Long-Term Stability of iPSC-Derived CD34+ Cell Banks Supports the Sustainable Manufacture of Off-the-Shelf Immunotherapies

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Human induced pluripotent stem cells (hiPSC) have the unique dual properties of unlimited self-renewal and differentiation capacity into all three somatic cell lineages. To further leverage these attributes, we have established a versatile iPSC product platform that enables multiplexed engineering of hiPSCs at the single-cell level and have developed a proprietary differentiation protocol to support definitive hematopoiesis for the derivation of CD34+ hematopoietic progenitor (iCD34) cells. We have shown that these iCD34 cells exhibit multilineage differentiation to diverse subsets of immune cells, including Natural Killer (NK) and $\alpha\beta$ T cells. The highly efficient and scalable differentiation platform is chemically-defined and cGMP-compatible, and iCD34 cells can be cryopreserved, banked and stored. Here we show the successful cryopreservation and long-term cryogenic storage (2, 4, and 6 years) of iCD34 cells, as well as the ability of these long-term stored iCD34 cells to serve as an intermediate feedstock for mass production of iPSC-derived NK and T (iNK and iT, respectively) cell therapies. A diverse set of iCD34 cells, including those engineered with a high-affinity, non-cleavable CD16 (hnCD16) and/or with an anti-CD19

chimeric antigen receptor (CAR19), were manufactured, cryopreserved, and stored in the vapor phase of liquid nitrogen at $\leq -150^{\circ}\text{C}$ for up to 6 years. To determine the impact of long-term cryopreservation on cell quality, long-term stored iCD34 lots were thawed and assessed for viability, recovery, phenotype, differentiation potential and iNK / iT cell functional potency. Post-thaw viability and enumeration was determined by trypan blue dye exclusion or acridine orange/propidium iodide staining in conjunction with Annexin V staining by flow cytometry, which was found to be similar to recently cryopreserved batches. Phenotypic flow analysis showed consistent identity and purity with pre-cryopreservation trends. To evaluate their differentiation potential, iCD34 cells were subsequently differentiated and expanded into iNK and iT cells. Flow cytometry analysis confirmed iNK and iT cell identity and purity, and differentiation yields were comparable with recently cryopreserved batches. Lastly, functionality was assessed via antibody dependent cellular cytotoxicity (ADCC) and antigen-specific cytotoxicity. In combination with monoclonal antibodies, hnCD16-iNK cells continued to show enhanced ADCC and production of the pro-inflammatory cytokines against antigen-bearing tumor cell lines. Similarly, CAR19-expressing iNK and iT cells maintained antigen-specific activity CD19+ tumor cell lines in various cytotoxicity assays. This study demonstrates that manufactured, cryopreserved, and stored iCD34 cells are stable for a minimum of 6 years in the vapor phase of liquid nitrogen and shows that cryopreserved iCD34 cells can serve as a robust starting material for mass production of iPSC-derived cell-based immunotherapies. We observed no changes in vitality, phenotype, differentiation potential or iNK and iT cell potency. Taken together, our studies show that long-term stored iCD34 cells can serve as an intermediate feedstock for rapid mass production of multiplexed engineered iNK and iT cell therapies.

375. Development of Master Multiplexed-Engineered iPSC Bank for Off-the-Shelf Cell-Based Cancer Immunotherapy with Reduced Conditioning Chemotherapy

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Induced pluripotent stem cells (iPSCs) are a unique renewable starting material for the manufacture of off-the-shelf immune cells and offer the advantages such as enhanced product uniformity, reduced cost, and on-demand availability. Current practice of both autologous and allogeneic cell-based cancer immunotherapy requires administration of systemic conditioning chemotherapy, which suppresses the patient's immune system in order to potentiate the adoptively-transferred immune cells. However, the intensity and frequency of immune suppression can also lead to increased risk of life-threatening complications, such as severe infections. New therapeutic strategies that enable potentiation of adoptively-transferred immune cells without substantially ablating the patient's immune system may significantly improve the therapeutic paradigm of cell-based cancer immunotherapy. Here, we describe a novel off-the-shelf, iPSC-derived CAR NK (CAR-iNK) cell derived from a master multiplexed-engineered iPSC line with the potential to maintain functional persistence in the background of an intact host

immune system. The multiplexed-engineered iPSC line incorporates six unique functional elements: 1) a CAR for targeting of plasma cells; 2) IL-15/IL-15 receptor fusion protein (IL-15RF) for enhanced NK cell activity; 3) a high-affinity, non-cleavable CD16 (hnCD16) for enhanced antibody-dependent cellular cytotoxicity (ADCC); 4) beta 2 microglobulin (B2M) deletion for resistance to host CD8 T cell-mediated rejection; 5) Class II transactivator (CIITA) deletion for resistance to host CD4 T cell-mediated rejection; and 6) CD38 deletion for resistance to fratricide when combined with anti-CD38 monoclonal antibody (mAb) to eliminate host allo-reactive immune cells. The multiplexed-engineered iPSC line was derived using our proprietary iPSC product platform. In the first stage, a clonal master iPSC line was established incorporating IL-15RF and hnCD16 at the CD38 locus. In the second stage, iPSCs from the clonal master iPSC line were further engineered to knock-out B2M and CIITA and knock-in the CAR. Single-cell selection was performed at each stage to assess for specific targeted integration, biallelic disruption of desired loci, and the lack of random donor template integration. Transgene copy numbers were further confirmed by droplet digital PCR. Importantly, karyotype analysis ensured genome stability of these clonal master iPSC lines, a testament to the robustness of the platform in enabling staged multiplexed engineering and single-cell selection. The master multiplexed-engineered iPSC line incorporating six functional elements was used to produce CAR-iNK cells, which were assessed for persistence and anti-tumor functionality. The successful production of these CAR-iNK cells supported the notion that the engineered modalities did not impact CAR-iNK cell differentiation and expansion (>95% CD56, >95% CAR, >95% CD16). In vitro functional studies including antigen-specific cytokine release assays, mixed lymphocyte reaction, and NK cell cytotoxicity assays in the presence of peripheral blood mononuclear cells demonstrated functional persistence from allo-reactive immune cells. In vivo studies with xenograft mouse model are ongoing. Collectively, the data provide evidence of the robust capability of our proprietary iPSC product platform to support multi-loci engineering of iPSCs at the single-cell level, the creation of master multiplexed-engineered iPSC lines, and the production of next-generation, off-the-shelf CAR-iNK cell therapies for use in minimally-conditioned patients.

376. Abstract Withdrawn

377. Evaluation of Genetic Stability of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Cell Therapy

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Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have emerged as an attractive cellular source for cardiac regeneration. It has been reported that unexpected genetic mutations can occur during in vitro subculture and differentiation process into hiPSC-CM. However, the causal relationship between genetic instability and tumorigenicity for hiPSC-CM still remains to verify before clinical

reality. Up to now, there are no specific acceptance criteria for the results of the genetic stability test on hiPSC-CMs. As a quality control and safety concern, we assessed the genetic stability of hiPSC-CM at the different stages of differentiation by using next-generation sequencing followed by potential pathogenicity analysis using multiple bioinformatics tools. In this study, we differentiated human iPSCs into myocardial cells to verify the genetic stability occurring through hiPSC-CM. The protein expression changes during differentiation into cardiomyocytes from hiPSCs were examined by Western blot and immunostaining, detecting iPSC marker (OCT4), mesoderm marker (Isl1), and cardiac-specific markers (cTnT, MHC, MLC2). To assess genetic stability during differentiation from human iPSCs to cardiomyocytes, we performed next-generation sequencing such as targeted (tumor) panel sequencing followed by Sanger sequencing at 0, 2, 4, 6 weeks after starting differentiation. We detected 29 genetic mutations including TSC1 c.3106G>T; p.(Gly1036Ter) and BCOR c.1487_1500del; p.(Ile496AsnfsTer17) that could be associated with potential tumorigenicity. From the results of Sanger sequencing, it was confirmed that the TSC1 c.3106G>T mutation occurred during the subculture of iPSCs, while the BCOR c.1487_1500del mutation occurred during the differentiation process into hiPSC-CM. We further analyzed their functional and structural pathogenicity using multiple bioinformatics tools like MutPred2, SIFT, Polyphen, PROVEAN, I-Mutant, PANTHER software. As a result of bioinformatics analysis, 27 of 29 mutations were predicted to be 'benign', but two mutations (TSC1 c.3106G>T, BCOR c.1487_1500del) were predicted to be 'pathogenic' or 'harmful'. In fact, many studies have shown the loss of the BCOR gene can cause cancer, and BCOR c.1487_1500del could be sufficiently brought about the loss of the BCOR gene, implying association with potential tumorigenicity. From these results, we demonstrated that the evaluation of genetic stability is a very important parameter on quality control and safety issues of the hiPSC-CM-based cell therapy, especially verification of unexpected genetic mutations during the differentiation process. Taken together, we suggest that a next-generation sequencing-based genetic stability test followed by pathogenicity analysis using multiple bioinformatics tools could be a useful method to predict potential tumorigenicity for the cell therapy products. These results would contribute to developing a new therapeutic source of cardiomyocyte and provide consideration points for quality control of hiPSC-CM-based cell therapy products.

378. Characteristic Analysis and Validation for Neural Stem Cells Directly Reprogrammed from Human Fibroblast

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Introduction: Neural Stem Cell (NSC) is an important source cell of neurons for the treatment of neurodegenerative disease due to its self-renewal ability and multipotent character. Direct reprogramming of human fibroblast into NSCs is a promising approach to obtain therapeutic NSCs without tumorigenic risk, bypassing pluripotent state.

Although enormous efforts have been made to enter clinical trials of cell therapies using NSCs, the evaluation methods of characterization and validation for the direct reprogrammed NSCs (iNSCs) still remain to be established. As quality control on cell therapies, one of the important issues for iNSC characterization is to obtain a consistent and reproducible quality of iNSCs by quantification of iNSC-specific marker expressions. To demonstrate characterization methods for iNSCs, we directly reprogrammed human fibroblasts into NSCs and conducted characterization analysis by using validated quantification methods for the detection of iNSC-specific marker expressions. **Methods:** To generate direct reprogrammed NSCs (iNSCs), Yamanaka factors (Oct4, Sox2, Klf4, c-Myc) were introduced into human fibroblasts using the Sendai virus vector. The genetic stability of iNSCs was verified by karyotyping and Short Tandem Repeat (STR) profiling. The expression of iNSC-specific markers and several pluripotent markers were examined by immunostaining, qPCR, and flow cytometry analysis. Validation parameters of quantitative methods such as specificity, repeatability, precision between individuals, and linearity were verified for each method according to the International Council for Harmonisation (ICH) and Korea Ministry of Food and Drug Safety (MFDS) guidelines. **Results:** We successfully generated 5 lines of iNSCs by direct reprogramming from human fibroblasts. Colonies of iNSCs showed traditional round-shaped stem cell morphology. The genetic stability was also well-maintained during the reprogramming process. We detected the expression of NSC-related markers (NCAD, NESTIN, PAX6, SOX1, SOX2) in iNSCs by immunostaining. Among NSC-related markers, we found that PAX6 and NCAD were more specifically expressed in iNSCs compared with iPSC. The pluripotent markers (NANOG and OCT4) were only detected in iPSCs, but not in iNSCs. We further quantified the expression levels of iNSC-specific markers by using qPCR and flow cytometry followed by validation according to the ICH and Korea MFDS guidelines. To demonstrate the specificity of iNSC-specific markers (PAX6 and NCAD), the expression level of each marker was assessed by spiking samples with iNSCs or iPSCs. The repeatability was verified by less than 3% RSD value from the results of three repeated experiments. There was no significant difference between the two testers ($P > 0.05$). For linearity, the positive control of each marker was analyzed by serial different concentrations, resulting in a coefficient $R^2 \geq 0.980$ of the standard curve. The limit of detection (LOD) of the pluripotent marker was 4 pg/ul and 0.1% for qPCR and flow cytometry, respectively. **Conclusion:** In this study, we demonstrated that iNSCs were successfully generated by direct reprogramming from human fibroblasts, and well characterized. From the results of method validation, we verified that all parameters of validation were met for the criteria of ICH and Korea MFDS guidelines. The characteristic analysis of iNSC-specific marker detection by qPCR and flow cytometry showed high specificity and repeatability with low LOD, suggesting accurate and sensitive methods which can be applied for the quality control of human iNSC-based cell therapy products.

379. NIH Blueprint Neurotherapeutics Network for Biologics

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Developers of biotherapeutics face numerous challenges that can be encountered while executing manufacturing runs, IND-enabling studies, IND submissions, and initiation of a clinical trial. The Blueprint Neurotherapeutics Network for Biologics (BPN-Biologics) offers researchers expert consultants and other preclinical support services, to enable late-stage therapeutics programs to progress to the clinic. This new NIH program is tailored to support a range of biotherapeutic modalities including those based on peptides, viral vectors, oligonucleotides, antibodies, cells, or other novel biotherapies. BPN-Biologics currently has two open funding opportunity announcements (FOAs): PAR-21-163 (UGH/UH3) for academic applicants, and PAR-21-133 (U44) for SBIR-eligible applicants. These FOAs are structured for investigators to acquire funding and resources for preclinical discovery and development of candidate therapeutics. Milestone-driven projects can enter at the late-discovery stage and are focused on optimizing a lead biologic. Projects then, transition to the development stage and aim to advance the through IND-enabling studies and IND submission by the end of the 5th year of funding. More advanced therapeutics programs may enter BPM-biologics directly at the development stage. Clinical trials are also supported. Successful applicants will have the opportunity to collaborate with NIH-contracted consultants, contract manufacturing organizations and contract research organizations that specialize in a number of preclinical support services, including manufacturing, pharmacokinetics, toxicology, and Phase I clinical testing. Institutions that are granted BPN-Biologics funding will be able to retain their IP rights for biotherapeutic candidates in this program. The BPN-Biologics program is supported by 11 NIH Institutes and Centers including NINDS, NIA, NIAAA, NCCIH, NIDA, NIMH, NIDCR, NICHD, NEI, NIBIB and the OBSSR. Reference: <https://neuroscienceblueprint.nih.gov/neurotherapeutics/bpn-biologics>.

380. Syngeneic Bone Marrow Transplant for the Treatment of Multiple Sulfatase Deficiency

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Multiple Sulfatase Deficiency (MSD) is an ultra-rare autosomal recessive disorder characterized by deficient enzymatic activity of all known sulfatases. MSD patients frequently carried two loss of function mutations in the *SUMF1* gene, encoding a formylglycine-generating enzyme (FGE) which activates 17 different sulfatases. MSD patients have shown common features of other lysosomal diseases (LD) like mucopolysaccharidosis and metachromatic leukodystrophy, including neurologic impairments, developmental delay, and visceromegaly. There are currently no approved therapies for MSD patients. Hematopoietic stem cell transplant (HSCT) has been applied with success in the treatment of certain lysosomal diseases. In an HSCT, donor-derived myeloid cells are a continuous source of active sulfatase enzymes that can be taken up by sulfatase-deficient host cells. Thus, HSCT could be a potential approach for the treatment of MSD. To test HSCT efficacy, we employed a new mouse model for MSD, carrying a single amino acid change (p.Ser153Pro) in the *Sumf1* gene. This amino acid substitution is equivalent to a common clinical allele (p.Ser155Pro) from MSD-patients. The B6-*Sumf1*^{S153P/S153P} mice are characterized by extremely low activity levels of Aryl-sulfatases

A, B, C, E, IDU, and SGSH, and progressive accumulation of GAG's, all hallmarks of MSD-patients. Donor bone marrow cells *Sumf1*^{+/+} were isolated from B6-*Ptprc*^{K302E} mice (CD45.1 immunoreactive) and reinfused into sublethally irradiated B6-*Sumf1*^{S153P/S153P} mice. Flow cytometric analysis at two-months post-transplant showed an average of 90% of circulating leukocytes of donor origin (*Sumf1*^{+/+}, CD45.1⁺). After 10 months post-transplant the engraftment efficiency remained stable with close to 90% of donor origin cells detected in spleen and bone marrow. Besides the elevated frequency of donor cells (*Sumf1*^{+/+}, CD45.1⁺), preliminary data on ARSA activity in brain and liver tissue did not show a significant ARSA activity increase. However, ARSA activity levels were significantly increased in the spleen of B6-*Sumf1*^{S153P/S153P} recipient mice. Since the success of an HSCT is based on the ability to achieve cross-correction, we hypothesize that the level of rescue in MSD is highly dependent on the number of donor cells resident in different tissues and their relative capacity to secrete different types of sulfatases. Additional experiments to address these questions are ongoing and will certainly contribute to understanding the mechanisms influencing the success of HSCT for MSD and other lysosomal diseases.

381. Designing for the Future of Gene and Cell Therapy Facilities

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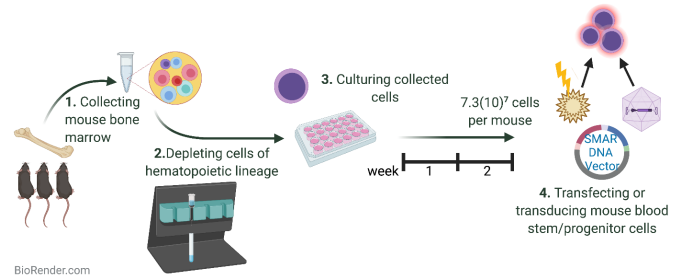
When designing and planning cell and gene therapy facilities, engaging a strategic perspective is essential to ensure these spaces will be able to accommodate future growth and development. In October 2021, various leaders in cell and gene therapy research, development, and manufacturing assembled for a virtual discussion to identify and understand shared challenges and determine what innovative solutions would look like. With representatives from nine cell and gene therapy organizations contributing to the conversation, several important discoveries emerged. First, when it comes to cell and gene therapy laboratories and manufacturing facilities, the market is advancing at such a rapid rate that the technology and production facility are often outdated before construction and validation of the space are complete. In addition, there are multiple stakeholders who have different perspectives and needs. A third reality is that startups often lack the initial capital to compete with non-profit research institutions to attract and retain key players. As a result of the open dialogue, participants agreed the need for flexibility and adaptability are priorities for creating the ideal lab environments. Flexible environments provide the greatest opportunity for innovative results and represent the future of the industry. Participants can expect to learn about market trends in GCT Facilities, where flexibility, attracting talent, with a focus on people are key. The primary target audience for this topic would be any individual that could be involved in the construction or renovation of GCT facilities.

382. A Protocol for Expanding Mouse HSPCs to High Cell Yields for Pre-Clinical Screenings of Gene Therapy Vector Constructs

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Researchers have access to human hematopoietic stem/progenitor cells (HSPCs) exclusively through a contact within the clinic or costly orders of commercial cord blood, bone marrow, or G-CSF-mobilized peripheral blood stem cells. Recently, it has been reported that Ham's F-12 medium supplemented with various reagents supports the growth of mouse HSPCs for up to 8 weeks. This has made it possible to conduct novel HSPC-based mouse experiments, which may particularly appeal to the numerous researchers lacking access to human HSPCs. This method—initiating cultures from LSK CD34⁺ CD150⁺—led to modest cell yields, despite expansion for 1 month, making it unsuitable for electroporation-based vector screenings, which requires millions of cells. We aimed to build upon this method and generate a cost-effective mouse HSPC expansion protocol for pre-clinical investigations requiring high cell yields. We developed a process in which cultures were initiated from a lineage-depleted population, and maintained an average viability of 86% with 28% LSK-like cells after an 8-day expansion. Using this method, we could produce 7.3(10)⁷ average total cells per a mouse after a 14-day expansion. Genetically modifying mouse HSPCs using non-viral DNA systems has proven to be extremely difficult, shown from the lack of publications on this specific topic. Using electroporation and our non-integrating SMAR DNA vectors—vectors that should be stably maintained—we were able to reach an average transfection efficiency of 46.4% within the LSK population along with an average viability of 9%. Transgene expression, unfortunately, only persisted for 4 days post-electroporation when using our vectors. We proposed stable maintenance of SMAR DNA vectors could be achieved by lessening the impact DNA has upon entering mouse HSPCs, which was implied by the low post-electroporation viability. LEGENDplex analysis revealed cytokine release upon DNA vectors entering mouse HSPCs. Attempting to mitigate this, we treated cells with inhibitors for DNA sensing pathways pre- and post-electroporation. While cellular INF β secretion was reduced and inversely proportional to inhibitor concentration, which may help prevent alterations to HSPC functionality, no significant improvements in viability nor transfection efficiency resulted. We have developed a protocol for generating high yields of mouse HSPCs within a 14-day period that retain a high enrichment of LSK-like cells. We have additionally made improvements in the delivery of non-viral DNA vectors into mouse HSPCs using electroporation. Work is still ongoing to optimize the stable modification of mouse HSPCs using our non-integrating SMAR DNA vectors. Nevertheless, the screening of various gene therapy vector constructs in mouse HSPCs is made possible from our findings.



383. Autologous Expanded Peripheral Blood Derived CD34⁺ Stem Cells for the Treatment of Moderate Knee Osteoarthritis

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Knee osteoarthritis (OA) affects millions of people worldwide, leading to a continuous increase in the number of total knee replacement surgeries. These surgeries improve the patient's physical mobility, but can lead to late infection, loosening of the prosthesis, and persistent pain. We are currently investigating the possibility of avoiding or delaying such surgeries in patients with moderate OA by injecting expanded autologous peripheral blood derived CD34⁺ cells (ProtheraCytes[®]) into the articular joint. *In vitro* studies demonstrated that ProtheraCytes[®] have high viability (>95%) once exposed up to 96h to synovial fluid from OA patients and after being passed through a 20G needle. In co-culture experiments with OA chondrocytes, we have also observed that ProtheraCytes[®] can modulate the expression of some chondrogenic (i.e., type II collagen and Sox9) and inflammatory/degrading (i.e., IL1 β , TNF α and MMP-13) markers at gene or protein levels. Finally, ProtheraCytes[®] survived after injection into the knee of a collagenase-induced osteoarthritis mouse model, engrafting mainly in the synovial membrane. Thanks to these promising pre-clinical data, we are now planning an exploratory clinical study to evaluate the safety and preliminary efficacy of ProtheraCytes[®] injected in 10 patients with moderate knee OA (grade II or III on the Kellgren-Lawrence). The primary objective of the study will be to assess the safety of the intraarticular injection of ProtheraCytes[®] in the symptomatic knee and secondary objectives will be to assess pain, physical exercise capacity (by self-assessed score), and prevention of cartilage degradation (by knee MRI). ProtheraCytes[®] will be manufactured using CellProthera's proprietary StemXpand system, which is an automated device for expanding and manufacturing autologous peripheral blood CD34⁺ cells. This system has been validated in our ongoing Phase 2 clinical trial in severe acute myocardial infarction (ClinicalTrials.gov Identifier: NCT02669810).

384. Agrin Overexpression Enhances Osteoblast Differentiation of Immortalized Mesenchymal Stromal Cells

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Background: Cell therapy based on the use of mesenchymal stromal cells (MSCs) is a strategy of regenerative medicine to treat large bone defects and the study of proteins from these cells that can favor bone regeneration is essential in this context. The extracellular matrix protein agrin is involved in several biological processes and it has been demonstrated that the silencing of endogenous agrin inhibits osteoblast differentiation. Thus, the aim of this study was to investigate the effect of agrin overexpression on osteoblast differentiation of immortalized mouse bone marrow-derived mesenchymal stromal cells (iMSCs).

Methods: The overexpression of agrin was done by using clustered regularly interspaced short palindromic repeats / associated nuclease Cas-9 (CRISPR-Cas9) system. Initially, iMSCs were transduced with lentiviral dCas9-VPR-Puro vector (iMSCs-VPR, control group) and then with single guide RNA (iMSCs-VPR^{agrin+}) to overexpress the gene of agrin. The agrin overexpression was confirmed by its mRNA and protein expression using real-time PCR and immunofluorescence assays. The effect of agrin overexpression on osteoblast differentiation was evaluated by the mRNA expression of its receptors and some bone markers at 5, 7 and 10 days, in situ alkaline phosphatase (ALP) activity at 7 and 10 days, and extracellular matrix mineralization at 21 days. The data were analyzed by student's T-test or analysis of variance (ANOVA), followed by the Tukey test and the level of significance was set at $p \leq 0.05$. **Results:** Agrin overexpression was validated by an increase of approximately 50% of its mRNA expression ($p=0.003$) as well as strong AGRIN labelling in iMSCs-VPR^{agrin+} compared with iMSCs-VPR ($p=0.003$). Regarding the agrin receptors, the overexpression of agrin downregulated the expression of *Dag* ($p=0.02$), without affecting *Lrp4* ($p=0.057$) and the expression of *Musk* was not detected. Moreover, it was observed that agrin overexpression increased osteoblast differentiation at both genotype and phenotype levels. At 5, 7 and 10 days, the expression of *Sp7*, *Alp*, *Bglap* and *Spp1* was upregulated in iMSCs-VPR^{agrin+} compared with iMSCs-VPR ($p=0.004$ for all mRNAs and time points). The ALP activity at 7 ($p=0.009$) and 10 ($p=0.007$) days and extracellular matrix mineralization at 21 days ($p=0.001$) were also higher in iMSCs-VPR^{agrin+} compared with iMSCs-VPR.

Table. mRNA expression of agrin and its receptors and bone markers at 5, 7 and 10 days, in situ alkaline phosphatase (ALP) activity at 7 and 10 days, and extracellular matrix mineralization at 21 days. Data are presented as mean \pm SD ($n=4$). Asterisk (*) indicate statistically significant differences ($p \leq 0.05$).

Gene (mRNA expression)	Time (Days)	iMSCs-VPR (mean \pm SD)	iMSCs-VPR ^{agrin+} (mean \pm SD)
<i>Agrin</i>	5	1.001 \pm 0.065	1.438 \pm 0.102*
<i>Dag</i>	5	1.002 \pm 0.066	0.869 \pm 0.052*
<i>Lrp4</i>	5	1.008 \pm 0.145	1.183 \pm 0.034*
<i>Runx2</i>	5	1.000 \pm 0.009	0.592 \pm 0.015*
	7	0.682 \pm 0.016	0.472 \pm 0.026*
	10	0.498 \pm 0.028	0.424 \pm 0.012*
<i>Sp7</i>	5	1.032 \pm 0.034	2.451 \pm 0.322*
	7	0.368 \pm 0.054	1.281 \pm 0.297*
	10	0.347 \pm 0.087	1.006 \pm 0.178*
<i>Alp</i>	5	1.005 \pm 0.122	1.347 \pm 0.214
	7	0.829 \pm 0.070	1.693 \pm 0.019*
	10	1.282 \pm 0.033	3.201 \pm 0.688
<i>Bglap</i>	5	1.008 \pm 0.146	14.651 \pm 2.834*
	7	1.504 \pm 0.249	17.599 \pm 1.233*
	10	9.412 \pm 0.494	21.333 \pm 0.871*
<i>Spp1</i>	5	1.028 \pm 0.299	3.259 \pm 0.178*
	7	0.932 \pm 0.147	1.855 \pm 0.470*
	10	0.630 \pm 0.132	1.216 \pm 0.067*
In situ ALP activity	Time (Days)	iMSCs-VPR (mean \pm SD)	iMSCs-VPR^{agrin+} (mean \pm SD)
Percentage of Area	7	0.916 \pm 1.009	2.816 \pm 1.210*
	10	1.377 \pm 0.427	3.374 \pm 0.574*
Extracellular Matrix Mineralization	Time (Days)	iMSCs-VPR (mean \pm SD)	iMSCs-VPR^{agrin+} (mean \pm SD)
Absorbance ($\times 10^{-2}$) 405 nm	21	2.660 \pm 0.403	3.940 \pm 0.391*

Conclusion: CRISPR/Cas9 is suitable to generate iMSCs overexpressing agrin, increasing their potential to differentiate into osteoblasts. Therefore, these genetically-edited cells could be a promising tool in cell therapy to induce bone regeneration. Sao Paulo Research Foundation (FAPESP, grant #2021/03204-2).

385. IL-7 and CCL19 Expression in Specific Peptide Enhanced Affinity Receptor T-cells Targeting MAGE-A4 Display Improved Survival and Ability to Induce Migration of Immune Cells

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Specific peptide enhanced affinity receptor (SPEAR) T-cells are engineered with human leukocyte antigen (HLA)-restricted T-cell receptors (TCRs) designed to target tumor cells with specific antigens presented on their surface. SPEAR T-cell first- and next-generation products are in early- to late-stage clinical development with responses across multiple solid tumor types,^{1,2} and further enhancements are being investigated preclinically. Interleukin 7 (IL-7) plays a role in stimulating proliferation and supporting the survival of T-cells, and

C-C motif chemokine ligand 19 (CCL19) induces the migration of immune cells.³ However, T-cells are incapable of producing IL-7 or CCL19 themselves, instead relying on other cell types to produce these elements. Here we describe a next-generation SPEAR T-cell therapy (ADP-A2M4N7X19), targeting the MAGE-A4 tumor antigen peptide presented on HLA-A*02:01, that utilizes PRIME technology to secrete IL-7 and CCL19.³ These modifications are hypothesized to enhance proliferative and survival capabilities, as well as infiltration of other immune cells into MAGE-A4-positive tumors. Together the enhancements brought by IL-7 and CCL19 may lead to the formation of tertiary lymphoid structures within the tumor. To evaluate the biological efficacy of ADP-A2M4N7X19, we determined the functional effects of IL-7 and CCL19 co-expression upon stimulation with MAGE-A4. We found that production of IL-7 by ADP-A2M4N7X19 led to an improved ability to respond to repeated MAGE-A4 stimulation and improved expansion in a MAGE-A4-dependent manner in vitro compared to T-cells expressing the TCR alone. CCL19 production by ADP-A2M4N7X19 conferred the ability to induce immune cell migration. These nonclinical data demonstrate that ADP-A2M4N7X19 has the potential for better T-cell engraftment, functionality, and immune cell infiltration into the tumor, thereby improving anti-tumor activity in the clinic. Based on these preclinical studies, a Phase 1 clinical trial will be initiated with ADP-A2M4N7X19 for the treatment of patients with advanced head and neck squamous cell carcinoma, non-small cell lung cancer, or ovarian cancer. 1. Hong DS, et al. *Ann Oncol.* 2021;32(suppl_5):S583. 2. Van Tine BA, et al. *SPEARHEAD-1: A Phase 2 trial of afamitresgene autoleucel (formerly ADP-A2M4) in patients with advanced synovial sarcoma or myxoid/round cell liposarcoma.* Paper presented at: CTOS 2021; Virtual. 3. Adachi K, et al. *Nat Biotechnol.* 2018;36:346.

386. Human Hepatocytes Expanded in a Novel Rat Bioreactor Maintain Full Functionality In Vitro and In Vivo

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Background and Aims: Hepatocyte transplantation is a potential therapy for acute and chronic liver diseases, but this approach has been limited by the availability of high-quality donor livers and well-characterized, functional hepatocytes. We previously described the development of an in vivo rat bioreactor that could be used for the large-scale expansion of functional primary human hepatocytes for clinical use. The aim of this study was to fully characterize the functionality of expanded primary human hepatocytes in vitro and in vivo and compare these to other hepatocyte sources, including iPSC-derived hepatocyte-like cells. **Methods:** Cadaveric primary human

hepatocytes were transplanted and expanded in an immune-deficient rat model of hereditary tyrosinemia type 1 (*Fah*^{-/-}, *Rag1*^{-/-}, *Il2rg*^{-/-} [FRG] rats). Purified human hepatocytes from FRG rats (HuFRG cells) were compared to cadaveric human hepatocytes and other de novo generated hepatocyte-like cells (HLCs), including iPSC-derived, in a suite of molecular in vitro analyses. In vivo transplant studies were also done to compare the engraftment, proliferation, and function of cells in preclinical models. **Results:** Purified HuFRG hepatocytes exhibited transcriptional profiles similar to freshly isolated primary human hepatocytes, including by scRNA-Seq analyses. HuFRG cells maintained their functional activities as compared to primary human hepatocytes and were significantly improved over HLCs. Functions that were indicative of mature hepatocytes included synthetic (albumin production) and metabolic functions (cytochrome P450 and ammonia detoxification), as well as the ability to attach to extracellular matrix (collagen type I). In vivo, HuFRG cells demonstrated robust engraftment and expansion in two mouse models of liver disease. In the first model, the cDNA-uPA-SCID mouse, transplanted HuFRG cells engrafted and proliferated robustly, demonstrating hepatocyte functionality for the duration of the nine week study. Next, to further demonstrate the proliferative and functional capacity of FRG rat-expanded hepatocytes, HuFRG cells and their original donor cell source were transplanted into an immune-deficient mouse model of hereditary tyrosinemia type 1 (HT1 mice). HuFRG cells proliferated at significantly increased kinetics compared to cadaveric cells, ultimately repopulating the mouse livers at enhanced levels. Importantly, HuFRG cells functioned in vivo for >4 months and normalized tyrosine and succinylacetone levels, ultimately preventing onset of liver failure in this model. Finally, to demonstrate the superiority of HuFRG cells over other de novo generated HLCs in vivo, HuFRG cells, iPSC-derived HLCs, HepaRG, and HepG2 were transplanted into HT1 mice. Only transplanted HuFRG cells engrafted, proliferated, and functioned in vivo, demonstrating the superiority of HuFRG cells over other HLCs in vivo. **Conclusion:** In summary, the in vitro and in vivo characterization of HuFRG cells described here supports the functionality of human hepatocytes expanded in FRG rats, matching or exceeding the quality of typical cadaveric donor cell lots. Therefore, we believe the development of the FRG rat bioreactor for large-scale expansion of primary human hepatocytes may provide the quantity of high-quality, and potentially superior, cells necessary for clinical testing of hepatocyte transplantation as a promising therapeutic approach for severe liver disease.

387. The Development of 'Off-the-Shelf' Manufacturing Strategies for iPSC-Based Gamma-Delta T Cells

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INTRODUCTION: $\gamma\delta$ T cells are naturally occurring immune cells that embody properties of both the innate and adaptive immune systems and represent ideal sources for developing allogeneic cell therapies for cancer, principally due to their ability to broadly recognize and kill tumor cells without initiating a graft-versus-host disease (GvHD) response. In particular, $\gamma\delta$ T cells appear to have an ability

to differentiate between healthy and tumor tissues, making them particularly attractive for adoptive cellular therapies against solid tumor cancers. Early clinical trials have revealed that adoptively transferred $\gamma\delta$ T cells are well tolerated and, so far, appear to be safe when used as immunotherapies. However, the expansion of $\gamma\delta$ T cells can be difficult. As a minor lymphocyte population, starting cell counts are lower, expansion efficiency of $\gamma\delta$ T cells varies among donors, and, in some cases *ex vivo* expansion and activation can reduce the durability of $\gamma\delta$ T cells. These limitations can be overcome using induced pluripotent stem cells (iPSCs). iPSCs possess the property of unlimited self-renewal and multi-lineage differentiation potential. Additionally, genetic modification of iPSC-derived $\gamma\delta$ T cells as well as selection and propagation of specific effector clones represent a source of near limitless and rejuvenated immune cells. We have generated iPSCs and differentiated them in a stepwise manner into hematopoietic progenitor cells (HPCs), progenitor T cells and cytotoxic T lymphocytes through a proprietary feeder-free multi-step differentiation process. **METHODS:** $\gamma\delta$ T cells obtained from healthy donors were activated and expanded from human peripheral blood mononuclear cells (PBMC) using IN8bio's protocol and reprogrammed into iPSCs using non-integrating vectors encoding Oct3/4, Sox2, Klf4, and c-Myc. Proprietary feeder-free multi-step differentiation strategies were established and applied to generate multiple effector cell populations. Cells were thereby differentiated from iPSC via HPCs to progenitor T-cells and further to $\gamma\delta$ T cell subsets. Multiplex genomic PCR assays and Sanger sequencing were performed to examine rearrangements at TCRG and TCRD gene loci. Karyotype of iPSC lines were evaluated with the G-band karyotype analysis. Flow cytometry was used to determine pluripotency markers (Tra-1-60, OCT3/4 & SSEA4), HPC markers (CD34, CD43), Progenitor T-cell markers (CD5, CD7), and $\gamma\delta$ T cell markers (CD3/ $\gamma\delta$ TCR). **RESULTS:** We were able to generate both $\gamma\delta 1$ T-iPSC and $\gamma\delta 2$ T- iPSC lines which harbor the rearrangements of the TCRG and TCRD gene regions. The $\gamma\delta 1$ T-iPSC line was identified as V $\gamma 2$ -to-J $\gamma 1/2$ and V $\delta 1$ -to-J $\delta 1$ recombination, and the $\gamma\delta 2$ T-iPSC line was identified as V $\gamma 2$ -to-J $\gamma 1/2$ and V $\delta 2$ -to-J $\delta 1$ recombination. The $\gamma\delta$ T-iPSCs lines were characterized as having normal karyotypes and highly expressing pluripotent markers (Tra-1-60, OCT3/4 & SSEA4). 98% $\gamma\delta 1$ T- iPSCs are OCT3/4 & SSEA4 double positive. 99.7% $\gamma\delta 2$ T- iPSCs are OCT3/4 & SSEA4 double positive. The early passage $\gamma\delta$ T-iPSC was expanded and went through the multi-step differentiating process. At different stages of the process, high-yield CD34+/CD43+ HPCs (72.1%, 83.1%, 83.7%), CD7+ Progenitor T-cells (81.6%, 71.2%) and CD3+/ $\gamma\delta$ TCR+ $\gamma\delta$ T cells (50.9%) were achieved. **CONCLUSIONS:** iPSC-derived $\gamma\delta$ T cells can provide an unlimited cell source to treat tumors while minimizing the risk for GvHD. We developed an "off-the-shelf" platform that enables manufacturing of different cell populations from parental iPSC lines including HPCs, progenitor T cells and $\gamma\delta$ T cells. A multi-step feeder-free differentiation process produced both V $\delta 1$ + and V $\delta 2$ + $\gamma\delta$ T cells and will pave the way toward novel genetic engineering and immunotherapies that can be applied towards various types of cancers.

Vector Product Engineering, Development or Manufacturing I

388. Forced Degradation Studies of AAVs to Generate the Full Spectrum Aggregation Toolkit

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Forced degradation studies play an essential part in all stages of development of AAV therapeutics from formulation development to the final drug product. These studies can give a probable explanation of product degradation pathways, frequently involving the formation of soluble aggregates in AAV products. Furthermore, these studies enable us to determine which analytical methods are fit for purpose and hence can be utilized as stability indicating methods. In this study, the AAV drug substance was exposed to different stress conditions: acid, base, oxidation, light, and heat. These stressed samples were analyzed with various techniques such as MFI, SEC-MALS, DLS, ddPCR, and Relative Potency. Another key aspect of the study is to subject the AAV drug substance to freeze-thaw conditions, where the samples were submitted to several cycles of freeze-thaw between -80°C and 25°C . The resulting cycles were evaluated by MFI, SEC-MALS, DLS, Titer, Potency, and visual inspection. Overall, the MFI methodology was able to detect early and subtle changes in the aggregation state induced by the stability study before a loss in titer or potency was observed. Here we have established a suitable panel to measure the full spectrum of aggregation size, with the methodology to generate and observe these species.

389. Optimisation and Scale Up of Adeno-Associated Viral Vector Production Using Transfection-Free Tet-Enabled Self-Silencing Adenovirus (TESSA) Platform

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The manufacture of AAV is regularly identified as one of the major bottlenecks to systemic gene therapy. Here we present the validation and scale up of a novel AAV manufacturing approach using an extensively modified adenoviral vector that prevents adenovirus contamination of the AAV preparation. Tet enabled self-silencing adenoviral (TESSA) vectors have the major late promoter (MLP) tightly regulated through a negative feedback loop. In the presence of inducer, the MLP is activated so both early- and late-phase adenoviral genes are expressed, and TESSA adenovirus amplification is enabled. Without the inducer, the MLP is inactive so only early-phase adenoviral genes are expressed to assist AAV production, restoring the highly efficient natural approach to AAV amplification, but eliminating generation of adenoviral particles as a by-product, as the late-phase adenoviral genes are not expressed. In order to demonstrate the ability to scale adenoviral manufacture

we optimised TESSA vector amplification in suspension cell culture. Through investigation and careful control of cell density, multiplicity of infection (MOI), process duration and scale-up conditions we achieved consistent adenoviral yields across experiments at shake flask, 1L and 10L scales, enabling transition to manufacture of TESSA vector using serum-free suspension cell culture. We then validated the ability of TESSA vectors to produce AAV. This was achieved through a production process called TESSA2.0, where two TESSA vectors are used: one encoding AAV Rep and Cap and the other encoding the AAV genome of interest. These two TESSA vectors are used to co-infect HEK293 cells for AAV production. We optimised the TESSA2.0 AAV production process for several serotypes, starting from design of experiments at 25 mL shake flask scale or on an AMBR15 platform to test a range of cell densities, MOIs and harvest time points, etc. The optimised production parameters were then tested at larger scales, which showed consistent production yield and proportion of full capsids at up to 50L scale. So far, we have produced AAV6 vectors using TESSA2.0 process with consistent upstream viral genome titres of $>5E11$ viral genome copies (GC)/mL and full capsid proportion of $>50\%$ at 25mL, 1L, 10L and 50L scales. AAV2 vectors produced using TESSA 2.0 at 25mL and 1L scales showed upstream titre of $2-3E11$ GC/ mL and $>50\%$ full capsids. The TESSA platform enables transfection-free production of AAV in serum-free suspension cell culture with high viral vector yield and quality, and improved process robustness, resulting in a novel and efficient approach to manufacturing AAV. Implementation of the TESSA 2.0 system should help to address the ongoing need to scale AAV vector production, paving a way for accelerated cell and gene therapy development, and ultimately making life-saving therapeutics more accessible for the patients in need.

390. Development of an Analytical Platform Method for Separation and Isolation of AAV Intermediate Species to Determine Their Identification and Potency

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The manufacture and purification of clinical grade recombinant adeno-associated virus (rAAV) vectors is a highly complex process. Despite having comprehensive procedural controls, product variants are often generated, such as intermediate species as defined by analytical ultracentrifugation (AUC). As the regulatory landscape for rAAV evolves, there is an increased expectation to have a more fully characterized rAAV product. One of the highly discussed and challenging aspects of AAV characterization is the potency assessment of this intermediate species. We have implemented an ultracentrifugation (UC) method on an affinity purified rAAV sample which uses a cesium chloride gradient to separate empty, intermediate, and full rAAV species on the basis of density. Further we successfully automated the collection of UC-separated rAAVs to avoid manual errors and confirmed their identity by AUC. In addition, we confirmed the presence of 2.3 kb encapsidated DNA, equivalent to the size of gene of interest in the AAV intermediates by native DNA gel electrophoresis. Finally, as a non-enzymatic potency

readout, we have demonstrated in-vitro full-length protein expression in cells transduced with intermediate species isolates by Western blot assay. Thus, we have developed a robust platform for separation and collection of rAAV intermediate species and generated data that demonstrates their therapeutic potential. The ability to precisely collect multiple rAAV particles using this platform will further enable rAAV characterization for their molecular and biophysical attributes. Future studies will involve resolution improvement between UC separated rAAV particles using purified drug substance.

391. Evaluation of PEI-Based Viral Vector Production for Research-Grade Vector Manufacturing

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INTRODUCTION With the development of novel gene therapy strategies based on viral vectors, the requirement for large-scale manufacturing of high yield vector preparations remains critical. Despite significant advancements in the clinical application, the manufacturing process still represents a critical challenge and meeting requirements for high yields remains a significant bottleneck. Viral vectors for gene delivery, such as adeno-associated viral vectors (AAVs) are frequently produced using polyethylenimine (PEI)-based transfection of HEK-293 cells. This method is commonly used in both the research setting and in Good Manufacturing Practise (GMP) for clinical application. Two novel products have recently introduced in the market, available at research and GMP grade: PEI Pro (PolyPlus) is employed in GMP manufacture with adherent cultures; and the new FectoVIR (PolyPlus) is designed for triple transfection of AAV plasmids in suspension cultures. Here we report a comparative analysis of the commonly used research-grade PEI product PEI Max (PolyScience) with the PEI Pro and FectoVIR products for research-grade AAV vector manufacturing. We assessed the properties of formed DNA:PEI transfection complexes, evaluated their efficiency in delivering plasmid DNA into HEK-293 cells and ultimately their effect on the final AAV preparation yield. **RESULTS** PEI Pro and FectoVIR required reduced PEI and DNA quantities during transfection relative to PEI Max and resulted in higher transgene expression, and increased cell viability. In addition, transfection with PEI Pro also resulted in higher final AAV yield following triple transfection on adherent HEK-293 cells. Analysis of size and distribution of the PEI:DNA complexes revealed that FectoVIR particles are small and monodisperse, while PEI Pro and PEI MAX complexes were more prone to polydispersity. **ONGOING WORK** Analysis of residual plasmid DNA (pDNA) in the AAV batches manufactured with the different PEI-based transfection reagents is currently being completed, with the aim to assess whether differences in transfection efficiency affect the packaging of extraneous pDNA and genomic DNA sequences into final the viral particles. **CONCLUSIONS** In the gene therapy manufacturing field it is critical to produce increasingly efficient transfection reagents that can reduce plasmid DNA use and contaminants, while meeting the increasing production demands, thereby reducing starting material costs and ensuring high yields. New products have been developed such as PEI Pro, a GMP-grade reagent optimised for the use on adherent cell culture. Following transient plasmid transfection and vector

production, the yield of the AAV preparation produced from adherent HEK-293 cells transfected with PEI Pro was significantly higher. For conventional transfection reagents, the incubation time is a limiting parameter as the complexes continue to form, creating larger aggregates with consequent reduction of transfection efficiency. Our results confirmed that the PolyPlus products form more stable and uniform PEI:DNA complexes over time. Overall, transfecting reagents PEI Pro and FectoVIR drive more efficient plasmid transfection and are associated to reduced cytotoxicity compared to the current standard research-grade PEI product.

392. Removal of Empty AAV Capsids to Undetectable Levels Using Orthogonal Purification Steps - Product Quality and Process Robustness Considerations

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Typical AAV Gene Therapy production systems generate cell culture harvests with a greater abundance of empty capsids (lacking DNA genomes) than full capsids (containing the desired DNA genome). Removing the empty capsids during purification is a central production challenge since empty and full capsids possess similar physio-chemical properties. Design of a robust purification process capable of completely separating empty capsids from full capsids is presented in this work. This process employs orthogonal purification steps (Ion Exchange Chromatography and Zonal Ultracentrifugation) for removing empty capsids and controlling other process and product related impurities to assure the safety of AAV products. Scalability of this process and features that allow the combination of these orthogonal steps to provide robust product quality are also presented.

393. Identification and Characterization of an ITR Deletion in a Producer Cell Line-Derived rAAV Vector Using Three Next Generation Sequencing-Based Methods

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Replication and packaging of recombinant AAV vectors (rAAV) are complex and imperfect processes whereby not only the full-length vector genome, but also truncated genomes, plasmid backbone, helper virus genome and host genomic DNA can be packaged in viral particles, posing potential oncogenicity and immunogenicity risks. AAV ITRs (Inverted Terminal Repeats), the *cis*-acting element with multiple essential viral functions, are highly unstable due to high GC content and strong secondary structures; recombination and deletion of ITRs in turn lead to reduced vector potency. Our producer cell line (PCL) platform for rAAV manufacturing allows easy scale-up and generation of high-quality vectors. As a strategy for product quality control, we

have developed next generation sequencing (NGS) methods to assess rAAV vector genome integrity, vector purity and producer cell line identity. Illumina NGS analysis on vector genomes for a candidate in our pipeline identified a 44 nucleotide (nt) deletion within the ITR of vectors derived from one of three cell lines. The deletion was visible in IGV (Integrative Genomic Viewer), but not detectable when using Bowtie 2 for alignment and FreeBayes for variant calling. The deletion eliminates the Rep binding site critical for Rep78/68 binding but showed no significant impact on potency *in vitro* due to assay variation and low deletion frequency. Multiple software have been employed to determine the deletion frequency (Array Studio, FreeBayes, VarScan, GATK and Samtools), but the results varied which prevented a firm conclusion regarding the existence of this deletion. The inconsistency is likely due to the challenges in sequencing ITRs via the Illumina platform; sequencing reads tend to be of low quality in ITRs and the deletion frequency was just above that of sequencing artifacts. The same vector was then re-analyzed using the PacBio long-read sequencing platform (AAV-GPseq) that offers enhanced polymerase processivity through the ITR region. The same 44 nt deletion was detected in the long reads, which confirmed the existence of this deletion. We hypothesize the deletion is present in a subset of integrated plasmid copies in the cell line, thus only a low percentage of vectors contains this deletion. A decrease in frequency of this deletion has been observed with passage, indicating the deletion is not stable. Targeted Locus Amplification technology (TLA) was employed to selectively enrich and sequence regions in proximity to the integration locus. Interestingly, plasmid integration was observed in chr19 at the AAVS1 site, and a complex genomic rearrangement has occurred in the region. The ITR deletion was identified as the only structural variant, suggesting the deletion could result from either plasmid fragmentation/fusion or rearrangement event during the cell line generation and integration process, which renders the deletion unstable. Taken together, the combined use of multiple NGS-based technologies confirmed the presence of a 44 nt ITR deletion in the cell line genome. Incorporation of NGS-based analyses into our quality control of viral vectors allows detection of cell line abnormalities and provides guidance on cell line selection to ensure a high degree of vector potency and safety.

394. Improvements in the 'Transgene Repression in Vector Production' (TRiP) system™ Towards Standardising Viral Vector Manufacturing and Producer Cell Line Development

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The disparity between supply and demand for clinical grade viral vector product is significant, and great effort is being made in the field to meet this need. The TRiP system™ has been developed previously (Maunder et al, Nat Comms 2017) as a vector-agnostic approach to minimise transgene effects on [1] out-put titres during Upstream phase, [2] feed quality going into Downstream concentration/purification and [3] potential immune responses to contaminating transgene protein within the final product. This translational repression system utilises

the bacterial protein 'trp RNA-binding attenuation protein' (TRAP) expressed in viral vector production cells and a 55 nucleotide TRAP binding site (tbs) inserted within untranslated regions (UTRs) upstream of the transgene initiation/'Kozak' sequence. Translation suppression is mediated by the formation of a TRAP-tbs complex blocking the scanning ribosome machinery. Repression of Cap-dependent (5'UTR) and -independent (internal ribosomal entry sites) translation initiation is possible, and consequently multiple open-reading frames can be repressed during production. The TRiP system™ therefore provides the opportunity for the development of a 'platform' process for a given viral vector/product pipeline wherein only the core vector proteins are expressed during the Upstream phase, greatly simplifying process development of vectors encoding different therapeutics. Here we report on improvements in performance of the core components of the TRiP system™ by [1] assessing preferred nucleotides within the tbs, which is a general consensus of [KAGNN]x11, [2] providing improved upstream sequences within leader/UTR sequences, and [3] optimising the position of the tbs in relation to the Kozak consensus sequence, in order to 'hide' the primary ATG codon within the TRAP-tbs complex. Combining these aspects allows 1,000 to 10,000 fold transgene repression levels in suspension (serum-free) HEK293T cells, as exemplified within rAAV vector genome constructs utilising a wide variety of constitutive promoters. In addition, we demonstrate that in the context of lentiviral vectors, aberrant splicing from the major splice donor (MSD) to strong or cryptic splice acceptors can lead to generation of transgene-encoding mRNAs that are not efficient templates for TRiP-mediated repression. Thus, in combination with a new generation of lentiviral vector genomes harbouring mutations in the MSD region, the 2nd generation TRiP technology is being utilised maximally towards the development of chimeric antigen receptor (CAR) LV producer cell lines (see poster Boura et al), which has not previously been reported in the field to date, possibly due to detrimental effects of constitutive CAR expression in HEK293(T)-based cell lines.

395. Inline Slope (abs/mm) Monitoring at 600nm of the Fermentation Process for the Optimal Cultivation of E. Coli Cells

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The increasing demand of cell and gene therapeutics (CGT) have created a need to optimize manufacturing process steps using Process Analytical Technologies (PAT) to lower costs and increase yields. Plasmid DNA (pDNA) is a precursor to the development of CGTs and is met with many challenges throughout the production process due its size, viscosity, and similarities to other impurities which can impact their yields. The generation of these plasmids in high density *E-Coli* cultures can be difficult to monitor using traditional off-line OD600 methods due to the careful sample preparation, fast growth rate, and dilutions required to be in the linear range of a standard spectrophotometer. This study proposes utilizing an inline- variable pathlength spectrophotometer in a recirculation loop during fermentation to monitor and optimize the growth of *E. Coli* cells in real-time. This provides distinct advantages vs traditional methods due to the constant modification of path lengths to create a slope-based solution that is always within the linear range of the equipment with no buffer correction required. The growth curve is tracked in

real-time using the slope (Absorbance/mm) at 600nm to measure cell density and to optimize growth performance of *E. Coli* cells. This will serve as a proof-of-concept for the optimal cultivation of *E. Coli* and production of pDNA.

396. Bioprocess Modelling of Viral Sensitizer™ Mediated Yield Enhancement on Upstream Viral Vector Production

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Viral-based cell and gene therapies comprise a key segment of the biotherapeutic landscape and are highly promising advancements for the treatment of complex and rare diseases. However, the high manufacturing costs and limited manufacturing capacity of viral vectors are insufficient to support the global demand for clinical-grade products. This can lead to delayed product development of groundbreaking treatments and, subsequently, therapies that are unaffordable and inaccessible to the general public. To mitigate these challenges, many manufacturers employ the use of process intensification strategies to increase product yield. Through bioprocess modelling, an optimized approach can be taken to thoroughly evaluate the impact of such strategies on cost of goods sold (COGS) in manufacturing processes. Virica's Viral Sensitizers (VSEs™) are upstream process additives that offer a solution to high manufacturing costs and low viral vector yields. VSEs™ are a proprietary collection of small molecules which transiently and efficiently dampen cellular antiviral defenses thereby increasing viral vector yield. VSEs™ have been shown to improve viral vector manufacturing yield in a wide variety of contexts, including both lentivirus and AAV production, where fold enhancements in yield of up to 7x were seen. Using BioSolve Process 8.3 Software (BioPharm Services Ltd.), Virica assessed the yearly upstream COGS savings associated with VSE™-mediated process yield enhancement. Analysis of the relationship between upstream yield enhancement and cost per dose has revealed that even modest 2-3 fold VSE-driven yield enhancements result in a 30-45% decrease in COGS per dose. In addition, process modifications enabled by VSE-mediated yield enhancement were explored, including the reduction in the size of bioreactor as well as the number of bioreactors required to meet a target throughput of product doses per year. Results of these models demonstrated that annual upstream COGS savings of up to 40% could be achieved. Overall, modelling results of upstream production COGS savings associated with the use of VSEs™ demonstrate the potential to produce more affordable and accessible cell and gene therapies.

397. Development of a qPCR Assay Targeting Alu Repeat Elements as a More Sensitive Method for the Detection of Residual Host Cell DNA in rAAV Drug Product

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Introduction: A variety of host cell lines are used as platforms for recombinant adeno-associated virus (rAAV) production. Within the host cells, viral genomes are replicated and packaged into the capsids of rAAV particles. During the replication process, non-viral DNA, such as the host cell's genomic DNA (gDNA), can become encapsidated. The packaging and delivery of unintentional DNA sequences to patients is a safety concern as these extraneous DNA sequences have the potential to transform transduced cells. The World Health Organization and U.S. Food and Drug Administration guidelines recommend that 10 ng/dose be the limit of residual host cell (HC) DNA in a biologic drug product. Multiple analytical methods have been used to quantify residual HC DNA, with quantitative polymerase chain reaction (qPCR) demonstrating the greatest sensitivity and specificity. Traditionally, qPCR has been performed targeting 18S ribosomal RNA (18S). The human genome contains approximately 400 copies of the gene coding for 18S. Conversely, the Alu element, is a sequence of DNA that is dispersed throughout the human genome at greater than one million copies. We hypothesized that qPCR targeting Alu would increase the sensitivity of residual HC DNA detection in rAAV. **Methods:** Purified vector underwent treatment with and without DNase to eliminate non-encapsidated DNA, then assayed by multiplex qPCR. HC DNA was measured with primer / probes specific for the Alu repeat (FAM-reporter) and 18S (Cy5-reporter). A serial reference standard (4000 pg/ μ L - 0.04 pg/ μ L) was generated from gDNA isolated from the Forge HEK293 Ignition™ cell line. DNA spike reactions and vector plus DNA spike reactions, with and without DNase treatment, were diluted 10, 40, and 160-fold. In accordance with the International Conference on Harmonisation, assay parameters set for precision, accuracy, linearity, specificity, and detection and quantitation limits were evaluated. The concentrations of hAlu and 18S in test and control samples were calculated by QuantStudio Design and Analysis software. Nanograms of Alu and 18S DNA in a therapeutic dose of 1.00E+14 vector genome copies were reported. All samples were run in triplicate, with a minimum of three independent experiments. **Results:** Singleplex and multiplex qPCR reactions demonstrated that the cycle threshold (Ct) values for Alu were 8.8 and 7.9 Cts, respectively, lower than 18S. The difference in Ct values across gDNA dilutions equates to 1000-fold more copies of the Alu amplicon than the 18S amplicon. The lower limit of quantification for Alu was 0.04 pg/ μ L and 18S was 0.4 pg/ μ L. All assays met acceptance criteria parameters: (i) linearity of DNA spike, vector, and vector plus DNA spike, at three levels, resulted in a $R^2 \geq 0.98$, (ii) accuracy, demonstrated by DNA spike recoveries, had a percent recovery of 80 - 120, (iii) precision, demonstrated by Ct values across replicates, was $\leq 5\%$, (iv) specificity, demonstrated by detection above the limit of detection (LOD) in spike samples but below LOD in NTCs. **Conclusions:** Results demonstrated that qPCR targeting Alu generated a LOD 10-fold lower than a comparable assay using 18S.

The concentrations of 18S and Alu generated using multiplex qPCR were the same as the concentrations generated using singleplex qPCR. Multiplex qPCR allowed accurate comparison of two targets from one sample. Future characterization of rAAV drug product, involving measurement of residual HC DNA utilizing the Alu element, will provide a more sensitive detection of residual DNA and a potentially safer product for patients in the field of viral vector gene therapy.

398. Strategies for Upstream Transfection Optimization in AAV5 Manufacturing Processes

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Developing safe, efficient, and cost-effective production processes for AAV is critical to ensure the continued promise of the platform as a potential therapy option for a multitude of devastating diseases. Numerous challenges plague the manufacture of AAV therapeutics, including low transient transfection yields, high empty capsid production, and subsequently poor recovery across the purification process, leading to an increased number of manufacturing campaigns and subsequently high market costs of the therapy. In order to address these challenges in upstream AAV production, Design of Experiments (DOE) was utilized with the goal of optimizing three main factors of transient transfection: plasmid ratio, DNA to transfection reagent ratios, and total DNA. Initial optimization was conducted for the plasmid ratio alone to determine that a Response Surface Model DOE was a suitable approach. Once optimization of the plasmid ratio was complete, the remaining factors were introduced into a DOE for final optimization. Using the DOE approach, an optimal plasmid ratio, DNA to transfection reagent ratio, and optimal total DNA were determined, boosting upstream viral genome yield by two-fold with minimal impact to full capsid yield. As a result of this work, it was found that the plasmid ratio plays a substantial role in viral genome yields, whereas total DNA inversely impacted viral genome and full capsids yields. Along with these observations, the optimal plasmid ratio shifted when the DNA to transfection reagent ratio was altered. Due to the complexity of individual AAV serotypes, these results are not universal, but this work exemplifies the use of DOEs within AAV-based upstream process development and its ability to improve upstream yields.

399. On-Column Stability of Recombinant Adeno-Associated Viral Vector on Affinity Chromatography

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Recombinant adeno-associated viral vectors (rAAV) are a leading delivery vehicle for gene therapy. However, rAAV vector production currently yields lower titers than desired, necessitating larger bioreactors to meet production demands. Larger bioreactors result in larger volumes of low titer clarified cell culture fluid that require longer loading times onto affinity-based capture chromatography unit operations. A good recovery in the capture chromatography operation is desired to prevent unnecessary losses while maintaining product quality. Longer loading times may raise concerns about product

stability. Although AAV instability on the column can lead to lower yields and lower product quality, it is rarely considered. In this work, we demonstrate the influence of affinity chromatography resin and operational parameters on the on-column stability of rAAV capsids and product impurities. The impact of the resin bead is decoupled from the impact of the resin ligand on rAAV stability. Furthermore, we provide mitigation strategies to promote product stability on the column. Our findings shed light on the importance of considering AAV on-column stability while designing chromatographic purification processes.

400. Clarification Studies on HEK293 Cell Lysate for AAV Production

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There is a growing interest in the development of gene therapy products that utilize recombinant adeno-associated virus (rAAV) vectors for gene delivery. The burgeoning demand for rAAV vectors has led to an increased emphasis on developing robust upstream and downstream manufacturing platforms to yield large titers of high purity vectors. Among the various methods available for the production of rAAV, transient plasmid transfection of HEK293 suspension cells is one of the most widely utilized. In the HEK293/transient transfection manufacturing process, the final production culture is usually taken through cell lysis which results in cellular debris, including cell-derived nucleic acids, proteins, and lipids. The unwanted macromolecules need to be separated from the rAAV vectors along with process-related impurities such as cell culture media components and residual components from the lysis step. A large proportion of these process contaminants can be removed through a clarification procedure. The clarification step involves the use of centrifugation and/or filtration to capture the particulate matter prior to further downstream processing. Centrifugation and bottle top filters are well suited for small-scale clarification, but depth filtration and sterile filtration are more appropriate for processing larger volumes (>5L). In this work, Forge Biologics evaluated a variety of depth filters from different manufacturers to determine their filter capacity and the recovery of rAAV across the filters. The load of the lysate in L/m² to achieve a maximal pressure target of 20 psi was determined for these different filter types. The turbidity of the material generated was measured as well as capacity studies of the resulting material through 0.2 micron PES filters. The data generated from the studies on these different depth filters will be discussed in this presentation.

401. Integral Quality Control of rAAV Batches Through a Next Generation Sequencing-Based Workflow

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With the explosive growth of cell and gene therapies over the last decade, viral vector analytical and characterization tools have rapidly

evolved. Technologies like next generation sequencing have stepped into vector analytics overcoming some limitations of classical assays while providing robust, rapid and accurate readouts. These approaches are particularly powerful to provide unbiased detection of encapsidated process- and product-related impurities in vector batches and, at the same time, even generate information of vector genome and contaminant size distribution. Here, we present the data obtained through an NGS-based workflow simultaneously evaluating the identity and integrity of DNA sequences packaged within AAV vectors. The first steps of the workflow involve DNA extraction and conversion of the rAAV genomes into double strand forms. Automated electrophoresis provides a first insight into the DNA size distribution and abundance of the native packaged DNA sequences showing a clear peak around the expected length (in our case, ~3350bp). It should be noted that sizes provided by automated electrophoresis platforms do not exactly correspond to the expected ones but there is an average deviation of $\pm 6.06\%$. Comparing this pattern to the one obtained after second-strand conversion allows for process control and confirmation of a consistent conversion of the rAAV genome to double-stranded DNA without altering the DNA fragment size distribution. The prepared dsDNA fragments are then used to generate two different sequencing libraries suitable for being sequenced on both short and long read sequencing platforms, respectively. For short read sequencing, DNA fragments are sheared and library-prepped. Analysis of the sequencing data through an in-house pipeline allows for the relative quantification of any species of interest. The majority of reads mapped to the vector genome (95.13-95.36%), followed by the plasmid backbone (2.74-2.77%) and the host cell genome (1.90-2.11%). Notably, quantifications were found to be consistent among replicates with coefficients of variation ranging from 2.57% to 3.18%. In addition, the dsDNA fragments are also used to generate an alternative library to be sequenced on the long-read sequencing platform. DNA size monitoring during library preparation confirmed that native DNA fragment sizes are preserved and this is also reflected when plotting the obtained read lengths prior to bioinformatics data mining. After data analysis, a homogenous coverage is observed throughout the whole vector sequence. Coverage drops by 50.6%, on average, within the ITRs. This may arise from both a lower performance of the sequencer to go across the ITR and also the presence of DNA fragments bearing truncated ITRs. When considering size distributions, reads aligning to the vector genome consisted of an 63.5% of full-length sequences. Notably, there was a homogenous presence of shorter fragments thus indicating the presence of eventual background noise levels. Further work is currently being performed to compare the quantification performance of the short and long read-based analysis. In summary, this workflow allows to gather information for the establishment of rAAV batch critical quality attributes related to vector genome identity and integrity, as well as quantification and size distribution of process- and product-related impurities. Additional studies are warranted in order to standardize these approaches and enable the setup of reference critical quality attribute boundaries.

402. Optimization and Characterization of the Chromatography Unit Operation for Lentivirus Production Platform

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Unprecedented growth in viral gene therapy trials warrants the development of efficient viral vector production platforms. To meet the demands of Lentiviral (LV) production for wide-scale applications, it is paramount to tackle challenges faced in the purification of high-quality LV particles. This presentation highlights process optimization aimed at improving the recovery and impurity profile of LV through the downstream chromatography unit operation. The process is based on transient transfection of HEK293 cells in suspension cell culture to produce vesicular stomatitis virus glycoprotein-pseudo typed LV with the crude harvest titer up to 5×10^8 TU/mL. Both resin and membrane-based chromatography were evaluated, in which membrane chromatography showed an order of magnitude higher dynamic binding capacity (DBC) due to larger accessible surface area, faster processing time and improved recovery in LV functional titer. The Mustang® Q membrane device was selected for further optimization. A comprehensive Design of Experiment study was conducted to evaluate the impact of feed quality, loading and elution conditions on LV recovery and impurity removal using 0.86 mL stacked disc devices. The effect of different buffer conditions on LV stability was also evaluated. The scalability of the process was demonstrated using 5 mL Mustang® Q XT5 capsule devices. Greater than 90% recovery in TU titer and 98% HCP removal were achieved with optimized operation conditions and the performance was consistent across different scales of Mustang® Q devices. This study provided insights on optimization strategy to improve capture chromatography performance for LV purification.

403. A Fast and Reproducible Method to Quantify Full and Empty Adeno-Associated Virus

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Adeno-associated viruses (AAVs) have gained significant attention in the field of gene therapy due to their capacity for highly effective delivery of therapeutic genes into human cells. Recombinant AAVs are made in mammalian cell culture which produces three types of capsids: empty, partial, and full. Empty and partial capsids are considered as impurities due to their lack of vector genome (vg) and associated therapeutic benefit. There are multiple technologies to assess AAV capsid status and impurities during AAV manufacturing, but most present challenges including low sensitivity, high cost, and laborious workflow. Therefore, we developed a faster and easier method by combining droplet digital PCR (ddPCR) and a nanoluciferase (NanoLuc)-based bioluminescent immunoassay technology to quickly determine the percentage of full AAV. With ddPCR, we were able to accurately determine the copy numbers of vector genome in samples. We then developed a NanoLuc-based AAV Immunoassay and demonstrated that it can quantify intact viral particle (vp) AAVs with high sensitivity and broad dynamic range. The robustness and reliability of both assays: ddPCR and NanoLuc immunoassay in combination,

enable the reproducible calculation of vg/vp ratio which equates to the percent of full AAV, particularly in partial purified (in-process) samples. Our combined method serves as a valuable tool to provide measurement of identity and purity in AAV viral production.

404. Process C: Next Generation Lentiviral Vector Manufacturing Process; Greater Productivity and Enhanced Purity

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The number of exciting and high-profile products based on gene and cell therapy has increased dramatically over the past few years. For over 25 years, Oxford Biomedica (OXB) has been a pioneer in the development of products and innovative technologies based on lentiviral vectors, with the company being responsible for several firsts in clinical studies based on these vectors. OXB has used this broad CMC, clinical and regulatory experience and know-how to facilitate development of a scalable, serum-free suspension manufacturing process utilising stirred tank bioreactors, both for the company's pipeline products and those of our strategic partners. With the rising demand for vector product throughout the industry as it matures, and a push to further improve product quality, even relatively recent technologies can struggle to keep pace with the expansion of therapies from ultra-rare to larger indications. This continuous trend drives the need for further innovation in vector production platforms as the success of the industry relies upon consistently generating sufficient vector quantities with the desired purity and potency. To meet the forecast on vector demand for gene and cell therapies, OXB has recently introduced a next generation lentiviral manufacturing process incorporating innovative process modifications which simultaneously enhance lentiviral process yields and improve vector quality attributes. Designated "Process C", this new process takes advantages of advances in perfusion technology to support vector production at higher cell densities than have been associated with traditional batch and fed batch production approaches for lentiviral vectors, allowing for significant process intensification without the need for increased bioreactor volume. Harvesting of lentiviral particles secreted into the bioreactor milieu via continuous perfusion also allows stabilisation of generated vector at lower temperatures, leading to improved preservation of functional lentivirus in the final product. Importantly, Process C adopts a plug and play approach facilitating incorporation of small molecule enhancers, some of which have already been identified by OXB. For example, incorporation of U1 (RNA based enhancer) during vector production using Process C led to increased cell-specific productivity. Overall, process yield improvements ranging from 2 to 10 fold have been demonstrated for a range of therapeutic lentiviral vectors with process scalability demonstrated in stirred tank bioreactors up to 200 L in GMP. In addition to the benefits of improved upstream yield, process C also incorporates an improved downstream processing approach to specifically target unwanted protein:DNA complexes implicated in vector aggregation. This combined approach has minimised process losses across sterile filtration process steps and has enabled more efficient removal of critical process impurities including plasmid and

cellular DNA. Our new manufacturing platform offers significant benefits for clinical production of lentiviral vectors and will further enable OXB to support the continuing global demand for high quality gene and cell therapy products.

405. Tetracycline Enabled Self-Silencing Adenovirus (TESSA) Enables High-Throughput Infectious Titration of rAAV

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Infectivity of rAAV vectors is a key attribute impacting the efficacy of AAV-mediated gene therapy, however, it has been challenging to determine rAAV infectious titres as titration methods generally rely on detection of a reporter marker or amplification of the rAAV genome for reliable detection within an infected cell. Use of wildtype AAVs or specific engineered cell lines (e.g. HeLa RC32) with wildtype adenovirus to supply the necessary components to replicate the rAAV genome are commonly employed for infectious titration of rAAV. However, these methods raise safety concerns associated with the use of wildtype viruses and how representative the titres are in the target cell types. To overcome these challenges, we developed a novel rAAV infectious assay based on our proprietary self-silencing adenovirus system entitled 'Tetracycline-Enabled Self-Silencing Adenovirus' (TESSA) wherein the adenovirus Major Late Promoter (MLP), primarily responsible for the expression of adenovirus structural proteins, was modified *in situ* to enable self-repression of promoter activity and truncate the adenovirus replication cycle. TESSA encoding AAV rep genes can infect a wide range of cell types as the serotype 5 adenoviruses, but only express the early-phase adenoviral helper genes and AAV Rep required for rAAV genome replication. This approach allows us to titrate various serotypes of rAAV in a wide range of target cell types, without the production of adenoviral particles, and providing a more informative and representative infectious titre. Furthermore, rAAV infectivity is frequently determined via Median Tissue Culture Infectious Dose (TCID₅₀) assay, involving the preparation of multiple replicate 96-well plates with numerous dilutions per rAAV stock. The process is laborious at scale and can be susceptible to human errors and operational variability. To streamline the process, we deployed a Hamilton liquid handling platform for automation of the workflow, further increasing efficiency, accuracy and reproducibility of the TESSA-based rAAV infectivity assay.

406. HEK293 Suspension Single Clonal Cell Line Readiness for High Productivity of Viral Vector Manufacturing

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Viral vector delivery system is the most common form of genetic material delivering method for gene therapy. Adeno-associated virus (AAV) and lentivirus have been proven to be the most popular

modality, which possess 22% (~250) and 28% (~315) cases in clinical trials, respectively. However, two major challenges of scaling up of AAV and lentiviral vector mediated gene therapy include manufacture productivity and cost. One of the solutions is to develop high producing cell line to increase the titer and yield for viral vector production. In this study, we developed a unique suspension single clonal HEK293 cell line that can produce high titer AAV and lentiviral vector for cGMP manufacturing. The development began with suspension adaptation of adherent HEK293 cells into fully chemical defined media. The cells were then subjected to recovery, stability and scalability analysis. The established polyclonal cell line showed high cell density and viability. To eliminate the potential risk of serum, one of five tested commercial serum-free media was selected as growth media. To improve the feature and develop a homogeneous suspension cell line, single cell clone was selected from polyclonal cell line. Around 3000 clones were screened based on transfection efficiency, titer, cell growth rate and low aggregation. The top two clones were banked and released for cGMP grade MCB and WCB, which were repeatedly evaluated for the best of AAV and lentiviral vector productivity. One single cell clone was established as WUXI suspension platform cell line, which demonstrated excellent recovery from thaw, robust growth, high viability, low aggregation and high AAV and Lentiviral vector production. To test the productivity of different AAV serotypes, WuXi suspension cell line was transfected with three plasmids for 12 serotypes separately. The amount of AAV production by single clonal cell line is around 2-11 folds higher than parental cell line depending on the serotypes. 9 out of 12 serotypes showed GC titer above 1.0E+11 GC/mL (AAV1, 4, 5, 6, 7, 8, 9, AAVDJ, AAVrh10), 2 serotypes showed GC titer above 6.0E+10 GC/mL (AAV2 and AAV3) and 1 serotype showed GC titer above 2.0E+10 GC/mL (AAV LK03). For lentiviral vector, WuXi suspension cell line produced significant higher infectious titer lentivirus than other producing cell lines that were evaluated. Stability study of MCB, WCB and RCB was completed up to passage 21. Cells from all three banks showed similar cell growth and viral vector productivity. To evaluate the scalability of WuXi suspension cell line, cell growth and production were tested in shaker flasks and 2L, 5L, 10L, 50L and 200L bioreactors. Key parameters were tested and optimized to achieve high titer viral vector production. WuXi suspension cell line showed robust scalability and produced high titer AAV and lentiviral vector in all scales tested. More importantly, the upstream production process including seed train, transfection and harvest has been well optimized and simplified to meet the cGMP manufacturing requirement. Shortly after the development, the cell line has been used in four client projects for AAV production, and licensed by two companies for their development studies. In summary, WuXi suspension cell line has been proven to produce high titer AAV and lentiviral vector in client and internal projects. Strikingly, it has been demonstrated that the cell line produced high titer adenovirus as well. This allows WuXi suspension cell line to be used for all three major viral vectors production for gene therapy, which will dramatically reduce the cost of labor, space, raw material, regulation and management for client projects, and accelerate drug substance and product delivery. WuXi suspension cell line provides a strong solution for large-scale cGMP manufacturing of viral vector.

407. Construction and Optimization of Novel Adeno-Associated Virus (AAV) Rep Gene Expression Control-System for Stable rAAV-Packaging Insect Cell Line

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The use of triple baculovirus expression vectors (BEV) for recombinant AAV (rAAV) production represents a well-established scalable alternative to the mammalian production system and has been approved to bring rAAV gene therapy product to market. In this platform, the double AAV Rep expression cassette is frequently adopted and has been shown to bring superior rAAV yield and quality. Here we describe the effort to adapt and optimize the control-element and molecular design of this double-cassette Rep expression protein for insect cells allowing rAAV production with single baculovirus inoculation approach. In this process, the utilization of non-canonical baculovirus expression promoters and alternative start codon is proved to be beneficial to solve the cis:trans promoter competition that has been hampering proper design adaptation into a tightly-regulated inducible expression vector. This new rationally designed double-cassette Rep also conferred significant improvements on rAAV production yield and potency compared to the single cassette one showing the possibility to generate a novel and simpler production platform to produce rAAV. Following this rational to simplify our process to a single baculovirus infection system, a single insect cell clone expressing a double-cassette Rep was isolated showing a stable expression of Rep and robust production of AAVs. To continue with these efforts a cell line development platform for isolation of the best producer is currently under development at uniQure.

408. Gene Therapy Clinical Trials, Where Do We Go? An Overview

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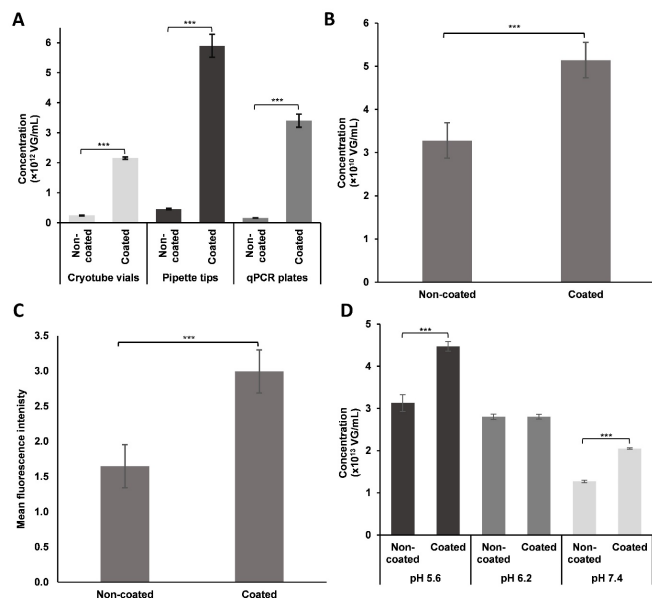
Since the introduction of gene therapy as a therapeutic modality for diseases, there are lots of up and downs. The journey of gene therapy, however, has reached to a maturity, presented as increasing number of gene therapy products that has entered the market. Looking at the currently approved and under-approval products along with numerous numbers of clinical trials in this field promises a bright future for gene therapy. The trends of changes in gene therapy strategies, vectors and targets could be sight-making for pharmaceutical companies, policy-makers and investigators. In this paper, after a brief review of gene therapy history, we reviewed the current gene therapy products along with gene therapies that could be approved in near future. Also, we studied ten-year changes in the various components of gene therapy trials strategies including used vectors, target cells, transferred genes, ex-vivo/in-vivo methods across the time and major fields that gene therapy entered. Although gene therapy first used for treating genetic disease, nowadays cancers hold the highest number of gene therapy clinical trials. Trend of changes in gene therapy strategies, especially in pioneering countries of this field, could show us the direction of future clinical products.

409. Recombinant Adeno-Associated Virus Vector Non-Specific Adsorption: A Better Way to Control

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Introduction: Non-specific adsorption of rAAV to solid surfaces, is one of the major reasons for vector loss during manufacturing, analysis, and storage. One of the most studied and used surfactants for the reduction of this issue is the FDA approved Pluronic F-68. In fact, rAAV formulations are usually diluted before being aliquoted, sampled for transfection, used in viral genome (VG) quantification analysis, or administered to patients, and this procedure results in a diluted surfactant concentration, leading to a reduction in the desired effect and increased vector adsorption. In this study, we investigated an alternative option to surfactants which are usually added to AAV formulations, aiming to provide an improved VG recovery and more accurate quantification by maintaining the adsorption prevention effect regardless of the vector formula dilution. This was achieved by application of a polyionic hydrophilic complex (PHC) polymer coating to the tools used in rAAV vector analyses [i.e., pipette tips, cryotube vials, and quantitative polymerase chain reaction (qPCR) plates]. **Methods:** Polypropylene made tools were coated with the PHC coating material. Surfactant-free and surfactant-containing rAAV vector suspension stocks including different serotypes that all express green fluorescent protein (GFP) reporter gene have been used. HeLaRC32 cells were used to assess the impact of PHC coating on the rAAV transduction efficiency. PHC-coated surfaces hydrophilicity and zeta potential were measured using contact angle measurements and a zetasizer respectively. **Results:** Recovery of the rAAV VG when using the PHC-coated tools for sample processing was 50% to 95% higher than after processing using the non-coated controls. Moreover, the tested PHC coating has remarkably decreased the negative effect of Freeze/Thaw (F/T) cycles on the recovered VG count. In addition, there was a significant difference in the rAAV adsorption rates among tested tools (Fig. A). Use of the PHC coating resulted in recovery of 157% of the nominal VG concentration of a rAAV2 reference standard stock, whereas the use of the non-coated tools resulted in recovery of almost the exact nominal VG concentration (Fig. B). Results showed that neither the serotype nor the surfactant at its usually used concentration or even at higher concentrations had an appreciable influence on the adsorption reduction ability of the PHC coating. The coating has resulted in a 1.8-fold higher transduction efficiency of the rAAV1 vector in HeLaRC32 cells (Fig. C). Assessment of the PHC coating in different pH points indicated the role of electrostatic charge repulsion with charged rAAV particles, whether positively charged at pH 5.6 or negatively charged at pH 7.4 (Fig. D). PHC-coated surfaces showed a super hydrophilic property in water besides, having an almost zero surface zeta potential. **Conclusions:** The PHC coating can substantially minimize non-specific rAAV vector adsorption to surfaces. The coating was superior to the normally used surfactant, including consistent activity and resistance of the coating to the effects of dilution. This coating is also useful in protecting against the negative impact of F/T cycles on preserved rAAV formulations.



410. Bioprocess Strategies to Mitigate the Impact of Proteases on Adeno-Associated Viral (AAV) Vector VP1 Capsid Content

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AAV vectors are currently being exploited as effective vehicles for gene therapy applications. A handful of studies have suggested that cellular and baculovirus proteases may impact the nature of insect cell-produced AAV capsids and specifically cause degradation of the vector's VP1 protein, which has been determined to be important for virus infectivity. The aim of this investigation was to detect the presence of extracellular protease activity in cell culture, and design bioprocess strategies that may inhibit protease activity, and by extension prevent the reduction of AAV VP1 levels in purified capsids. Extracellular protease activity was monitored in an insect cell-baculovirus process used to produce AAV vectors. Results showed significant enzymatic activity over the last 48 hours of cell culture, which correlated with the period of cell death and lysis. The reduction of protease activity by addition of E64, and not Pepstatin A, indicated that the majority of the enzymatic activity might be driven specifically by cysteine proteases. We attempted late cell culture process changes (i.e., temperature shifts and pH shifts) to modulate protease activity. We also tried addition of inorganic salts that may have a similar effect. During AAV production, the addition of E64 to cell culture led to an increase of normalized AAV VP1 levels by 50-70% compared to control. A similar outcome was evidenced when the cell culture was exposed to a 23°C shift. Time-course analysis of VP1 levels showed that E64 and temperature shift prevented the continuous reduction of VP1 levels, which was seen in the control condition. Moreover, the late addition of an inorganic salt at micromolar concentration had a similar effect on VP1 content. We confirmed that the salt addition also reduced extracellular protease activity within the last days of the process. Similar trends were seen during the production of a second AAV serotype. In summary, two

bioprocess strategies were identified to mitigate undesired effects of proteases released during insect cell culture, which appear to have a positive effect on the capsids of AAV vectors. More studies need to be done to understand how these potential process changes impact other process and product attributes, and how the presented results translate into product-specific *in vivo* potency levels.

411. High-Quality Purification at a Large Scale for 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 and 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. Our large-scale rAAV purification method would be applicable for considerable in vivo experimentation and clinical investigation.

412. Development of High Density & High Purity AAV Production Processes

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Despite recent advances in improving productivity and scalability of rAAV manufacturing processes, there remains significant need to improve existing AAV manufacturing platforms to achieve robust, high-yielding, scalable and cost-efficient processes. At Ultragenyx we employ a stable producer cell line process which is reproducibly scalable to 2000L and maintains AAV production at high volumetric productivity, reducing the high cost of goods typically associated with transfection-based AAV platforms. In this presentation, we demonstrate improvement on our existing platform process through process intensification to achieve a several fold increase in overall volumetric AAV yield. The intensified upstream process along with an optimized downstream process, increases AAV productivity to $\geq 3E11$ GC/mL ($\geq 3E14$ GC/L), enabling delivery of a high purity AAV product suitable for clinical use.

413. DNA-Transfection Reagent Complexes Can Undergo Size Changes During Transfection and Complex Consistency Influences the Production of Recombinant AAV

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Introduction and Objective: Transient transfection using a mixture of plasmid DNA and a cationic transfection reagent is a widely used method for the generation of recombinant Adeno associated viral particles (rAAV). The cationic transfection reagent forms complexes with the negatively charged plasmid DNA that are taken up via endocytosis by the recipient cells. These incorporated complexes escape afterwards the endosome and get transported into the nucleus where transcription takes place. The right size and the consistence of the complexes are two major prerequisites that determine the uptake and the further processing that leads to the generation of rAAVs. We investigated two different transfection reagents with respect to complex size formation in different complex formation solutions (CFS) and in growth medium and linked these data to the productivity of

rAAVs. **Methods:** Dynamic light scattering (DLS) was used for the determination of DNA-transfection reagent complex size over time in different complex formation solutions (CFS). These, in CFS pre-formed complexes were subsequently added to medium, mimicking the transfection procedure, and further analyzed for changes in size by DLS. Transient transfection of HEK293 cells with complexes generated in different CFS were performed in parallel and the production of rAAV was analyzed over time by serotype-specific Enzyme-linked-immunosorbent assay (ELISA) and digital droplet Polymerase chain reactions (ddPCR). **Results:** DLS analysis of the first transfection reagent in different CFS showed the expected size increase of DNA-transfection reagent complexes over time. Addition of these complexes to the medium led to a roughly two-fold increase of the hydrodynamic radius from ~0.5nm to ~1µm. Although two different CFS showed same complex formation in CFS and same behavior in the medium, the productivity of rAAVs doubled up for one CFS, which indicates that not the size alone but also the consistency of the complex determines the productivity. The second used transfection reagent showed same complex formation as the first in different CFS but led to no/minor changes in the hydrodynamic radius after addition to medium. Productivity with the second transfection reagent was roughly five-fold higher in comparison to transfection reagent one. **Conclusions:** Results obtained from the DLS measurement clearly indicate that complexes can increase in size when added to medium and can therefore lead to decreased uptake of the larger complexes by the cells which will subsequently reduce rAAV production. Furthermore, it was shown that complexes of the same size, but different consistency can strongly increase the rAAV yield.

414. Development of Scalable Vaccinia Virus-Based Vector Production Process Using Dissolvable Porous Microcarriers

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Vaccinia virus-based vectors have been currently investigated as oncolytic virotherapy in clinical trials. The conventional adherent culture on the cell factory system to manufacture vaccinia virus-based vectors suffers from insufficient scalability due to the intrinsic operational requirement on manpower, space, and materials. Through facilitating suspension culture of cells in an adherent manner, microcarrier cell culture enables the scalable production of vaccinia virus-based vectors, achieving large-quantity manufacturing without the need for large-capacity or heavy labor. This study herein demonstrates the development of a vaccinia virus production process using Vero cells growing on CytoNiche dissolvable porous microcarriers. The microcarriers are designed to be easily trypsinized for cell harvesting and linearly scaled-up in a large-scale bioreactor. The macropores across the microcarrier allow cell adhesion inside of particle, avoiding the effect of shear forces on cell growth. Bead-to-bead transfer and virus production were conducted in a laboratory-scale spinner and 3L bioreactor. The peak cell density was measured under 3g/L microcarrier condition, and the peak virus titer was determined by plaque assay. The results demonstrated a comparable virus titer

to adherent cell culture using cell factories with up to three-fold improvement, which provided relevant data for the next-step testing and scale-up manufacturing in large bioreactors.

415. HIP-Vax®: Solving the GMP Capacity Shortage for Viral Vector Production

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Batavia Biosciences, Leiden, Netherlands

Over the last years, there has been an ever-increasing interest in the development of Advanced Therapy Medicinal Products (ATMPs). Production processes of the viral vector component of *ex vivo* ATMPs are often either produced at small scale using labor intensive processes in-house or outsourced to a contract manufacturer. This yields challenges ranging from limited manufacturing capacity to consistency of product quality (batch-to-batch variation) due to the nature of the manufacturing process. In this presentation, we will showcase a highly intensified manufacturing platform called HIP-Vax[®] that enables the production of viral vectors with high yields on a small footprint and a low Cost-of-Goods. The process can be tailored to fit into ATMP manufacturing facilities, while only needing a small, dedicated footprint. The HIP-Vax[®] platform utilizes fixed-bed bioreactor systems and supports both adherent cell lines, as well as, suspension cell lines. This makes it an excellent platform for working with both the current transfection processes as well as it supports new stable producer cell lines. Using a standardized platform will increase the productivity of the viral vector manufacturing process and hence lead to a lower requirement of the number of batches produced to support *ex vivo* treatments, and make it less susceptible to batch-to-batch variation coming from the viral vector component. Using our HIP-Vax[®] manufacturing platform, it is possible to set-up a small-size manufacturing suite as part of a facility dedicated to the production of ATMPs. In our opinion this way of modernizing the viral vector component production helps solving the current capacity constraints in the industry.

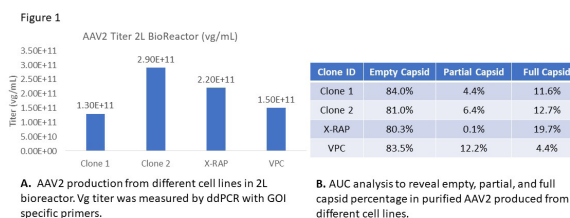
416. X-RAP Suspension Cell Line and Its Platform: Vedere's Solution for rAAV Manufacturing

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Introduction: A simplified and reliable cell line and CMC platform is needed to reduce manufacturing costs, as well as to support next generation rAAV manufacturing. As one of the most widely used rAAV production systems, the HEK293 suspension cell line-based triple transfection method supported more and more gene therapy programs. However, each of the current commercially available HEK293 suspension cell lines have different limitations: cell aggregation, high nutrient consumption, low AAV packaging efficiency, etc. Here, Vedere Bio II has successfully serum-free adapted and single-cell cloned a high-quality HEK293 suspension cell line X-RAP. Moreover, we developed a reliable suspension platform to explore the potential of this unique cell line for rAAV manufacturing. **Result and conclusion:** By using a de-novo serum-free adaptation method, Vedere

Bio II successfully generated a robust HEK293 suspension pool. Such suspension pool demonstrated better cell growth and a similar rAAV productivity when compared with commercial HEK293 suspension cell lines. However, cell aggregation persisted, which is known to impact transfection efficiency variation, large-scale bioreactor control, and cell lysate clarification. After non-serum single-cell cloning, we screened over 1100 individual clones and successfully isolated several clones without any cell aggregation. In parallel, to address our future large-scale rAAV manufacturing demands, we used a system comprised of a commercially available pHelper, wild-type AAV2 capsid, and CAG-GFP plasmids to develop a robust bench-scale production process based on Thermo's VPC cell line. Such development includes transfection reagent and plasmid ratio optimization to improve productivity, cell lysis technology improvement to meet new regulatory requirements, affinity and AEX full capsid enrichment for high-quality rAAV purification. To evaluate the potential benefit of this CMC platform, we did a side-by-side comparison in shake flasks and 2L bioreactors to evaluate the impact of isolated suspension clones against VPC on rAAV2 production. By using half of the nutrient and oxygen of the VPC's need in bioreactor, our clones demonstrated up to 2-fold higher genome titer by ddPCR. Additionally, similar step recovery in purification, up to 4.5-fold higher packaging efficiency by AUC in purified material (Figure 1 A and B). We also observed significantly higher transduction efficiency by FACS of purified rAAV produced from our HEK293 clones. Thus, we named our top suspension clone candidate as X-RAP. Subsequently, the X-RAP cell line was applied to a custom plasmid system, which showed a similar improvement in productivity and packaging efficiency. In summary, our X-RAP demonstrates an advantage in two different plasmid systems. Going forward, Vedere is focusing on further optimization of X-RAP suspension cell line and its platform, scale up to 50 L bioreactor, etc. We will offer an even better solution for rAAV manufacturing challenge in the future.



Pharmacology/Toxicology Studies or Assay Development I

417. 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 increased rapidly over the last few decades, however there are still many hurdles to overcome and further 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 manufacturing process is essential. A robust QC process expedites safe and effective commercialization of the final product. Compared to existing QC methods, next-generation sequencing (NGS) offers an effective high-throughput approach for monitoring rAAV quality, from initial construct assembly to analysis of the final encapsulated product. Both, Illumina short-read and PacBio long-read sequencing technologies offer distinct advantages including sequencing of the entire rAAV genome, with the inverted terminal repeat (ITR) regions, detection of potential mutations, truncations and contaminants. Workflows for both platforms require conversion of the single-stranded genome to double-stranded DNA prior to library preparation; however, robust protocols for this step are lacking. Additionally, an efficient bioinformatics pipeline is needed to convert the massive amounts of NGS data to interpretable results. Here, we describe our novel proprietary end-to-end workflows and present final QC results using both NGS platforms. This combined approach alleviates current constraints for high throughput rAAV sequencing and thereby enhance the overall QC process.

418. Long-Term Characterization of T Cell Product Interactions Using *In Vitro* 3D Tumor Models and the Go-Rex Platform

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T cells are complex biologics that require multiple functions to exercise killing of target cells. T cells are able to detect chemokine gradients and migrate in the direction of high chemokine concentrations to arrive at tumor sites. Additionally, once at a tumor site, T cells need to navigate and infiltrate the physical environment surrounding and within tumors. Once T cells interact with and recognize cancer cells, they exercise their key function, killing. **However, current *in vitro* assays are limited in their assessment of T cell functions: they assess each function independently and are restricted to short timeframes, thereby providing limited information about each parameter and hindering preclinical development of T cell products.** Here we highlight the development of an *in vitro* G-Rex system that can address critical biological properties of T cell products and provides a missing bridge between very short-term *in vitro* assays and long-term *in vivo* experiments. Named the Go-Rex, we found that this *in vitro* cell culture system, which has a gas permeable membrane at the base, allows for the non-disruptive, quantitative real-time assessment of the growth of genetically-engineered bioluminescent tumor models. Quantitative bioluminescence can be acquired using a common benchtop imager. We demonstrate that the growth of several different *in vitro* 3D cancer models can be optimized using the Go-Rex platform and assessed over the course of 2 weeks. Furthermore, to validate the benefit of the Go-Rex system, we tested multi-tumor associated antigen (mTAA)-specific T cell therapy that is currently being explored in the clinic. We

found that mTAA-specific T cells were able to significantly reduce the growth of a leukemic 3D tumor model in a dose-dependent manner. These results confirm the Go-Rex system can assess anti-tumor activity through its ability to coculture T cells and tumor models over several weeks. Our results demonstrate that the Go-Rex platform can serve as an important preclinical tool to bridge the gap between short *in vitro* assays and *in vivo* models. We anticipate this platform will benefit the entire field of immunotherapy as novel T cell therapeutics continue to be developed. This Go-Rex platform will advance preclinical assessment of T cell products as well as provide a novel discovery platform for new therapeutic opportunities.

419. 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 other 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 using commonly used viability 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. 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.

420. Detailed, Comprehensive and Reliable Size Distribution Analysis of Recombinant Adeno-Associated Virus Vectors

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Detailed, comprehensive and reliable characterization of virus vectors are required to ensure their efficacy and safety. We have been focusing on the development of methods for primary and higher order

structure of recombinant adeno associated virus vectors (rAAVs). The confirmation of primary structure was successfully performed by liquid chromatography mass spectrometry (LC-MS) and the ratio of VP proteins could be successfully determined by the combination approach using capillary electrophoresis SDS (CE-SDS) and LC-MS. The size distribution analysis of rAAVs can be achieved with constituent components by multiwavelength sedimentation velocity analytical ultracentrifugation (MW-SV-AUC), by which we can determine the population of free DNA, intermediate particles, aggregates as well as full and empty particles. Importantly absorption spectra for full and empty particles determined by MW-SV-AUC can be widely used for the characterizations and purity assessment of rAAV samples. References:

1. Maruno, et al., Comprehensive Size Distribution and Composition Analysis of Adeno-Associated Virus Vector by Multiwavelength Sedimentation Velocity Analytical Ultracentrifugation. *J Pharm Sci.* 110, 3375-3384 (2021).
2. Oyama et al., Characterization of Adeno-Associated Virus Capsid Proteins with Two Types of VP3-Related Components by Capillary Gel Electrophoresis and Mass Spectrometry. *Hum Gene Ther.* 32, 1403-1416 (2021).

421. Strategies for Monitoring Off-Target Integration Events Introduced by Cell and Gene Therapy Using Next Generation Sequencing Approaches

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Cell and gene therapeutic products are revolutionizing how clinicians approach previously unsolvable diseases and disorders such as cancers, blindness, metabolic disorders, and other chronic conditions. Current approaches for the delivery of gene editing products, whether intended for stable genome integration or episomal replication, have been shown to also produce unintended genomic insertions that are outside of the target region. This presents a potential risk for patient safety that must be measured and monitored. For example, inactivation of tumor suppressor genes, or the inappropriate activation of oncogenes, through insertional mutagenesis are hazardous adverse events which have been observed in previous gene therapy clinical trials. For this reason, regulatory agencies, such as FDA, have issued guidance strongly recommending annual genome-wide surveillance for off-target insertions be performed for up to 15 years post-administration depending on the delivery approach. Next-generation sequencing (NGS) is an adaptable approach which has been applied to perform deep, quantifiable characterization of single genes to complete genomes. As such, NGS approaches are well positioned to monitor and assess not only the intended insertion event, but also monitor and identify unintended insertion events that may occur post gene editing intervention. We examined several methods that fundamentally use different NGS experimental approaches to interrogate the genome to identify insertion events and assess their relative performance. NGS represents a sensitive, high-throughput and cost-effective approach for detecting insertions genome-wide immediately after and in the

years following gene or cell therapy. Here, describe several different methods to utilize NGS for this application and discuss their relative advantages and disadvantages.

422. Development of High Throughput Screening for DOE-Based Formulation Screening

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The fates of recombinant viral vectors produced for use in gene therapy are intrinsically intertwined to how they are produced, stored, and handled. Much value is derived from being able to stabilize desirable drug substance properties early in the life cycle to support development as well as evolve at a pace to sustain research and CMC activities. Unless permanently tethered to one specific delivery/modality, rapidly developing a final formulation solution is required to move at a pace that supports the production of novel vectors to be used. With the potential for creation and production of numerous capsids with highly variable properties, there is a need to evaluate, test, and incorporate DOE design and analysis in a data-directed, bottom-up platform approach to evaluate and rank various formulation components, test their efficacy, and predict (un)desirable characteristics. By developing a combination/matrix of off-the-shelf and novel screening methods fit for purpose, we successfully established a method enriching for preferred capsid attributes that was compatible with current analytical matrix testing, DOE approaches, and high throughput design.

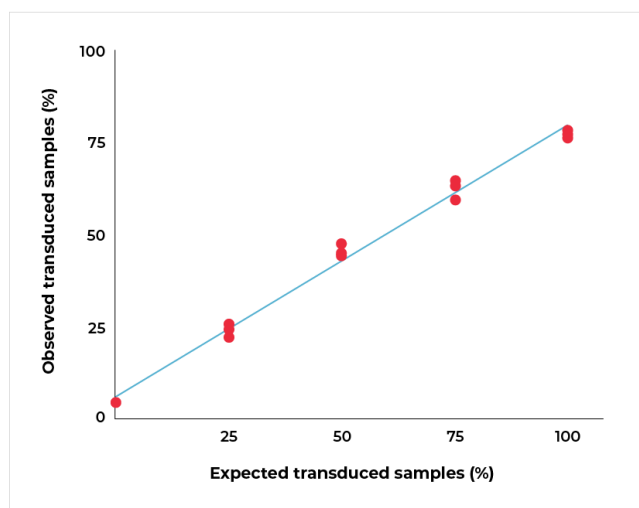
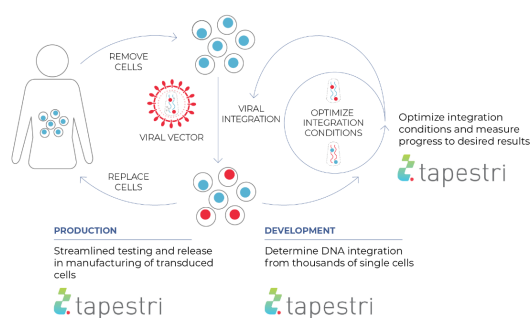
423. Precise Measurement of Transduction Efficiency at Single-Cell Resolution for Cell and Gene Therapy Development

Khushali Patel, Kathryn Thompson, Jacqueline Marin, Yilong Yang, Shu Wang, Brittany L. Enzmann, Daniel Mendoza

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Introduction: Cell and gene therapies are transformative solutions for a host of inherited and acquired diseases for which existing interventions are ineffective. Many such therapies rely on the introduction of transgenes into host cells using viral or non-viral vectors. Unlike most small molecule drugs, this approach offers long-term benefits and may even serve as a curative solution. The accurate measurement of gene transfer is critical to the development of therapeutic agents and is a key attribute for assessing their safety and efficacy. Yet, conventional methods for measuring gene transfer either report a population average (bulk) or involve time-consuming clonal outgrowth. Here, we demonstrate that single-cell DNA sequencing identifies transduced versus non-transduced cells with exceptional accuracy and precision for populations of up to 10,000 cells. **Methods:** Cells were transduced with lentivirus containing a unique genetic sequence and were then diluted with non-transduced cells to achieve roughly 0, 25, 50, 75, and 100% of positively transduced cells. Five replicates from each concentration were then quantified using Mission Bio's Tapestry Platform, an instrument that leverages droplet microfluidics to enable the targeting and sequencing of DNA in 1,000s of individual cells.

The data were analyzed using Tapestri proprietary software. **Results:** The dilution series showed excellent linearity and precision among replicates between the expected and observed transduction percentages. For the 5 replicates of non-transduced cells, the false positive rate was below 0.03%. **Conclusion:** This study showcases the ability of single-cell DNA sequencing to accurately report the percentage of positively transduced cells in a single high-throughput assay without the need for cell culture. Single-cell DNA sequencing offers exciting new capabilities for the development of *in vivo* and *ex vivo* cell and gene therapies. By precisely measuring the presence or absence of viral DNA on a per cell basis, researchers can quickly optimize gene transfer protocols during preclinical development. Additionally, single-cell analysis can be leveraged for batch release assays used in clinical trials and manufacturing.



424. A Novel VCN Assay That Detects Lentiviral Vector Integrations While Overcoming Limitations Caused by Plasmid Residuals

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Lentiviral vectors (LV) are an attractive delivery platform for the transfer and stable integration of genes to target cells, both in the

context of *ex vivo* and *in vivo* gene therapies. Analytics such as vector copy number (VCN) assays that measure the amount of integrated vector genome in target cells are important tools to assess the level of successful transductions. Furthermore, VCN and integration assays are often part of release and safety criteria as recommended by regulatory agencies. Traditionally RT-PCR amplicons are generated to the transfer gene of interest or to regions within the LV genome that are unique, such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The readouts of this method of quantification can be hindered by the amount of plasmid and nucleic acid residuals present within any viral production run, often leading to overestimations of VCN in downstream analytics even when LV have been treated with DNase enzymes such as Benzonase. To ensure accurate determination of VCN we have designed a novel assay that relies on a unique amplicon and droplet digital PCR (ddPCR) process that is specific to only reverse-transcribed self-inactivating (SIN) viral vector nucleic acids. We demonstrate that the traditional WPRE method still shows detectable VCN signal with Benzonase-treated LV even in the presence of Nevirapine, suggesting that this signal is coming from non-reverse-transcribed products such as residual plasmid nucleic acids. By contrast, this signal is completely removed in the presence of Nevirapine using the new assay design, suggesting that it detects only reverse-transcribed products. This assay is able to detect reverse-transcribed viral vector genome for both integrated and non-integrated forms of LV. Importantly, this work demonstrates the short-comings of DNase treatments and particularly the role of plasmid residuals in common timepoint readouts of transduction. Therefore, this assay allows for a more accurate determination of SIN-LV VCN. This method can, in theory, be applied to any SIN retroviral vector design, including HIV, SIV, and gammaretroviral vectors such as MLV.

425. A Quantitative Systems Pharmacology (QSP) Model for Design and Species-Translation of Bio-Distribution Studies of AAV-Based Gene Therapies

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Adeno-associated viruses (AAVs) are rapidly emerging as the leading platform for *in vivo* delivery of gene therapies for chronic and debilitating monogenetic diseases such as Hemophilia, Muscular Dystrophy, Lysosomal Storage Diseases among many others. Accurate characterization of the *in vivo* biodistribution and tissue transduction profile has become a limiting factor for the development of AAV based gene therapies since transduction can vary widely depending on AAV serotype/construct, species, target tissue, and study design. To address this gap, we have developed a first-of-a-kind Quantitative Systems Pharmacology (QSP) framework using current knowledge of the biology of AAV gene therapy pharmacology. Using analysis of compiled pre-clinical and clinical data on AAV-based gene therapy treatments, we were able to robustly characterize the serotype, construct, target tissue, and species dependencies of AAV treatment response as measured in terms of key endpoints like vector genome transduction, tissue coverage %, transcription/translation, and functional protein expression. These results guided the construction

of a minimal physiologically-based pharmacokinetic model that can explain the observed kinetics, serotype, and dose-dependence of AAV tissue biodistribution as well as transcription/translation of the target gene across multiple serotypes and species. Here we show how this QSP modeling platform can be used to design key aspects of AAV biodistribution/pharmacology studies including species, dose, and study duration even for novel AAV therapies where minimal information about the construct is available by leveraging key insights from historical data and physiological constraints. We further show how this platform can serve as an effective screening tool for capsids by providing species-specific quantitative predictions of tissue-transduction efficiency and variability that can serve as benchmarks for novel capsid performance. We anticipate that this model can serve as potential platform for projecting biodistribution/pharmacology thereby reducing the reliance on preclinical biodistribution studies and optimizing AAV dose-translation between species and clinical study designs.

426. Rapid Detection of Replication-Competent Adenovirus (RCA) in Adenoviral Vector Material

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Adenoviral vectors are a widely used delivery mechanism in gene and cell therapeutics. Testing for presence of replication-competent adenovirus (RCA) is important for ensuring patient safety and is a requirement for all biologicals manufactured using adenoviral vectors (e.g. gene and cell therapy products, adenoviral-based vaccines). The objective of RCA testing for biosafety purposes is to demonstrate the absence of RCA in manufactured material. In practice, this is translated in the requirement to demonstrate <1 RCA present in a specified quantity of production material. In the 2020 FDA guidance for gene therapy products (FDA Guidance for Industry - *Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) - January 2020*) this is determined as <1 RCA per 3×10^{10} viral particles (VP). Therefore, any assay for the detection of RCA must be able to detect a single RCA particle in a specified amount of test material. The standard cell culture based-assay using virus-induced cytopathic effect (CPE) as readout requires an extensive culture time of up to 28 days to reach this level of sensitivity. The objective was to develop a method for the detection of RCA with a shorter turnaround time compared to the extended culture-based RCA assay. As the test material consists of replication-deficient adenoviral (RDA) material it is impossible to distinguish between RDA and RCA using molecular methods alone. Therefore, a combination of a 7-day culture period on susceptible cells (A549, human epithelial lung carcinoma cells) to allow replication of RCAs present in the material, with a highly sensitive molecular endpoint was used for the rapid detection of RCA. The molecular endpoint developed targets the adenoviral E1A gene which is present in all replication-competent adenoviruses but is typically absent in replication-deficient adenoviral material. The sensitivity of the molecular endpoint was determined as ≤ 20 genome copies/reaction. Serial dilutions of Adenovirus Type 5 (Ad5) were spiked in culture medium and 5 different types of adenoviral vector preparations at various concentrations and inoculated on the A549 detector cells. After

seven days of culture cells were harvested, nucleic acids extracted, and samples analyzed by PCR. In absence of adenoviral vector material, 1 plaque forming unit (PFU) of Ad5 was consistently and reproducibly detected, demonstrating an assay sensitivity of 1 RCA. As expected, at high concentrations of adenoviral vector material, cytotoxicity and interference were observed. However, when diluted to an appropriate concentration (approximate range $5 \times 10^7 - 1.5 \times 10^8$ VP/mL), the assay was able to detect 1 RCA in all 5 vector materials after only 7 days of culture. The non-toxic/non-interfering concentration differed for each individual adenoviral vector material tested. Finally, the data demonstrated that there was little E1A background signal from residual host cell DNA from the vector-producing cells and thus unlikely to produce a false-positive result, providing assurance in the suitability of the method. For many adenoviral-based products, the RCA assay is a rate-limiting assay for lot release of manufactured batches. A rapid assay with a reduced turn-around time compared to the classical assay is likely to benefit manufacturers of adenoviral therapeutics and could help expedite patient access to therapy.

427. Primers and Probes Positions Matter: Impact on rAAV Genome Quantification and Infectivity by Multidimensional ddPCR

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Recombinant Adeno-Associated Viruses (rAAV) are the most widely used and prominent viral vectors in the gene therapy field. In the last decade, the field has rapidly grown with two products on the market and several hundred of clinical trials. Recently described difficulties have highlighted issues in the field that require to be thoroughly investigated. Critical Quality Attributes need to be evaluated and quantified with high precision, such as rAAV genome titer, genome integrity, presence of AAV aggregates and infectivity. For rAAV genome and infectious titer determination, qPCR is widely used, despite known biases, especially those arising from standard curves. Droplet digital PCR (ddPCR) uses absolute quantification, and is becoming the tool of choice for precise rAAV genome quantification. We have developed current protocols and assays by multidimensional ddPCR to bypass current qPCR limitations for rAAV genome quantification and determination of infectious titer using the available reference standard material AAVRSM8, as well as proprietary single-stranded and self-complementary rAAV1 batches. Through the partitioning in individual droplets, rAAV particles can be analysed at a single and multidimensional level. This allows for insight into the genome integrity of rAAV batches. We looked at the positions of primers and probes in the context of vector genome copy quantification and found that it had critical impact on returned absolute titers, with over 30% variation. Additionally, we were interested in the impact of accurate quantification of rAAV genomes on infectious titer determination by TCID50. We will present our data highlighting an important potential bias concerning this infectivity assay, the consideration of which leads to more reproducible and reliable infectious titer determination.

428. Development of a ddPCR Based Relative Potency Assay for APB-102

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Amotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder where some forms of the disease are due to mutations in superoxide dismutase 1 (SOD1). Many mutations in SOD1 result in protein aggregates and neuronal degeneration that leads to death of the individual most often by respiratory failure. APB-102 is an AAV based gene therapy product that expresses an artificial microRNA that selectively silences SOD1 gene expression and reduces protein expression. APB-102 is currently under clinical development. Here we describe the development of a relative potency assay used to characterize the gene therapy product. The *in vitro* potency assay measures the ability of the APB-102 drug product to infect cells, produce a microRNA directed at SOD1 to silence the expression of the SOD1 messenger RNA relative to a well characterized reference standard. The reference material is assigned a relative potency of 100%. This ddPCR based method is highly reproducible with intraday precision of 9% and interday precision of 13%. The linear range of the assay has been determined to be between 5-300% relative potency. An accelerated stability study showed that this potency assay is stability indicating and is more sensitive than a traditional TCID₅₀ infectivity assay. The assay has been revised and streamlined to simplify the method after transfer to Quality Control for use as a release assay.

429. AAV VP1, VP2 and VP3 Protein Standards - Value of Reliable Standards for AAV Vector Manufacturing

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A growing number of academic and industrial labs are using AAV vectors for the development of gene therapy products. This leads to an increase in the demand for effective and reliable analytical AAV tools for R&D and manufacturing. A crucial but often neglected factor in AAV vector manufacturing is the comprehensive monitoring of the complete manufacturing process - downstream & upstream. The establishment of a comprehensive monitoring can reduce time and money by the identification of the ideal production and purification conditions to ensure high yield and high quality of the final gene therapy product. However, the lack of reliable standards for AAV has been a major challenge for the gene therapy community. Depending on the analytical method used for AAV R&D and manufacturing, there are critical factors that need to be corrected in order to generate reliable data, e.g. differences in transfer efficiency from gel to membrane in Western Blot or labeling efficiencies with fluorescent dyes in capillary electrophoresis. For this reason, PROGEN developed suitable AAV capsid protein standards, i.e. recombinant VP1, VP2 and VP3 proteins for the determination of AAV capsid protein identity and optimization of particle yield during the initial production process. These recombinant AAV capsid proteins enable

proper comparison of VP1:VP2:VP3 ratios in an AAV preparation including the correction of artifacts with different methods. Here, we present the properties of these recombinant proteins using different techniques and demonstrate the value and importance of independently quantifiable AAV capsid protein standards for AAV vector manufacturing and quality control.

430. Development of an In-Vitro Cell-Based Assay to Measure Expression of Gene Therapy Vector for FTD

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Background and Purpose Frontotemporal dementia (FTD) is caused by a multitude of mutations, one of which is in the GRN gene, encoding progranulin, a protein important in cell growth, survival, repair, and regulation of lysosomal function. Heterozygous mutations of GRN are characterized with accumulation of TDP-43 inclusions and are linked with FTD. Studies in Grn^{-/-} murine models have shown that rAAV gene therapy vectors expressing human GRN (rAAV-hPGRN) can provide significant restoration of progranulin levels and can ultimately reduce pre-existing pathology in mice. An expression test, along with several other tests, assesses product conformance for: release testing, stability programs, and comparability studies when manufacturing changes are made. Here, we report the establishment of a sensitive and specific *in vitro* cell-based assay to measure progranulin expression from our neurologic gene therapy vector, using sandwich ELISA. **Methods** Epithelial cells were plated on to a 48-well plate and were transduced with rAAV-PGRN at multiplicities of infection (MOI) across 5 orders of magnitude. Cells were incubated for 72 ± 4 h, after which cell supernatant was collected for analysis by ELISA. Recombinant progranulin protein was used as a reference standard in the sandwich ELISA. The resulting data were analyzed using standard slope and intercept fitting. **Results and Conclusion** A sandwich ELISA was developed to measure progranulin protein in cell supernatant after infection with rAAV-PGRN. A combination of custom made polyclonal and monoclonal antibodies were used for coating and detection at concentrations of 1.0 mg/mL and 0.5 mg/mL, respectively. The level of progranulin in samples were quantified by comparison to the standard curve. During development of the assay, the following parameters were evaluated for optimization of the expression assay: selection of cell line, selection of antibodies, concentration of capture and detection antibodies, standard range, seeding density, and MOI. The assay was further qualified to assess the following parameters: precision, accuracy, LLOQ, specificity, linearity, and range. For inter-assay precision, intra-assay precision, and accuracy, high and low reference standard concentrations at 50 ng/mL and 5 ng/mL, respectively, were run in triplicates for a total of 36 times and results included % recoveries within 80-120% with %RSD < 15. Furthermore, the assay showed specificity to the protein of interest spiked in different matrices with recoveries between 80-120%. The lower limit of quantification was assessed to be 1 ng/mL for supernatant samples. The assessed parameters passed the predetermined validity criteria, indicating the assay is appropriate for use as a quantification of expression according to the ICH guidelines and will be applied to support clinical phase product release for our PGRN clinical Phase 1/2 studies.

431. Characterization of Extracellular Vesicles Associated AAV6 for Immunomodulation

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The association with extracellular vesicles (EVs) during standard production in HEK293 cell has been shown for several serotypes adeno-associated virus (AAV) vectors. These EV protected AAV vectors have several important advantages such as higher transduction efficiency and protection from pre-existing and therapy activated humoral immunity. In our preliminary studies, we particularly analyzed the ability of AAV serotype 6 to accumulate in EVs and to stimulate antigen-specific immune response. EV-AAV6 particles were measured, size distributions is ~60 nm, and the total concentration of AAV6 vesicles by model fit of measured size distribution is 2.88×10^{12} particles/mL. The EV-AAV6 size distribution measured by microfluidic resistive pulse sensing are unimodal and characteristically right-skewed to larger vesicle diameters. No additional peaks in the size distribution were observed beyond the primary peak (mode diameter ~40 nm), although standard beads 150 nm in diameter were readily detected at concentrations comparable to the EV-AAV6 concentration. Characterization of morphology by transmission electron microscopy (TEM) shows the typical 'cup-shaped' fixation artefact observed for the exosome sub-population of EVs. Initial screening for the common EV biomarkers - CD9, CD63, and CD81 tetraspanins - by single particle interferometric reflectance imaging sensing using the ExoView platform shows that EV-AAV6 vesicles are captured by each tetraspanin-specific antibody and that individual vesicles co-express comparable levels of all three tetraspanins. Western blot assays showed the presence of internalized syntenin-1 and CD9, as well as AAV capsid proteins, which also indicates that the EV-AAV6 vesicles are exosomes. At the same time, analysis of AAV titer by qPCR on the viral genome yielded 3.89×10^{10} vg/mL sufficient for further use in animal studies. We showed the EV-AAV6 expressing ovalbumin (OVA) induced significantly higher levels of specific Ova⁺ T- cell (1.43%) compared to the standard AAV-OVA (st) (0.29%) in one week after as a single intramuscular injection in C57BL6 mice. Significantly, the higher number of specific T-cell was achieved with AAV doses several logs lower than those commonly used for AAV-based immunomodulation thus demonstrating the potential of EVs as lipid-based adjuvants. Importantly, a boosting injection of EV-AAV-Ova produced greater numbers of OVA⁺ T-cells in peripheral blood compared to the standard AAV-OVA (19.5% vs 13.4% over a 6.8% baseline). The better performance of EV-AAV-OVA in a boosting injection can be explain by the protection of EV-AAV from negative inhibition by AAV specific neutralizing antibodies (NAb) formed after first injection. The ability of EVs to protect AAV was also tested in NAb assay based on transduction inhibition with mouse serum tested positive for AAV6 NAb. We showed approximately two-fold higher resistance of EV-AAV6 to NAB inhibition compared to stAAV6 since mouse serum inhibits 50% of maximum stAAV6 luciferase activity at the dilution 1:272 and EV-AAV6 at 1: 148 correspondingly. In summary, our preliminary data suggest that EV-AAV can be used as tool for immunomodulation and possible vaccine development.

432. Multiplex Digital PCR, an Analytical Method for Gene Therapy, Delivering a Broad Versatile and Meaningful Read Out

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Vector genome titer quantification using digital PCR (dPCR) is a state-of-the-art analytical method in gene therapy for determining the concentration of the product. It is common for AAV vectors to target the AAV2-ITR sequence, which represents a generic sequence applied in various AAV-based gene therapy products. Using a single target for the vector genome titer determination can provide a consistent concentration of the product, however this approach lacks important information of the DNA insert including the data of the full-length of the transgene. Multiplex digital PCR allows to quantify DNA from AAV capsids for multiple targets of the vector genome. We established a quadruplex droplet digital PCR for simultaneous quantitation of 4 targets of the transgene in one single PCR run. Our first objective was to evaluate the feasibility of the multiplex dPCR for the quantitation of vector genomes. Our next objective was to evaluate the performance of the multiplex dPCR on the expressivity for monitoring the depletion of truncated inserts during the production process on an approximate 4400 bp insert. In addition, the assessment of the presence of full-length or nearly full-length inserts in the final product was in scope. For our investigation, we designed primer-probe sets for the amplification for eight targets distributed along the DNA transgene. By using four different fluorophores for the reported signal, a variety of combinations for the multiplex dPCR arose. Equal performance of the four fluorophore dyes FAM, VIC, CY5 and CY5.5 was evaluated in singleplex and multiplex dPCR. Samples from the upstream and downstream process were analyzed to evaluate the monitoring of truncated insert depletion. As targets for the amplification, we included the sequence of the promotor, the therapeutic gene, a posttranscriptional regulatory element and an identification tag. Some elements were multiple targeted on different sequence positions. We investigated the performance of nine different process samples, including 2 samples after completed purification. For this study we analyzed one product obtained from 2 independent production runs during process development, applying equal conditions for analysis, respectively. We showed that multiplex dPCR performs in the same way as singleplex dPCR. All investigated fluorophore dyes delivered reliable results. Monitoring the different productions steps from upstream over downstream to fully purified steps, we obtained a clear picture of the purification grade of the respective sample. A change of vector genome concentration for different targets was shown to reflect the lengths of the present DNA transgene. In samples prior the depletion of empty capsids and capsids with truncated inserts we observed a higher titer of vector genomes on the 3' end of the transgene. For our final AAV product, the assay delivered information regarding full-length inserts. Data generated within our study showed that the multiplex dPCR is a suitable analytical method to further characterize the length of the DNA insert in AAV capsids. Furthermore, this method allows monitoring of successful depletion of truncated DNA strands during the production process. The method allows to assess presence of the DNA insert in its full length in the final product. We are convinced that the multiplex dPCR method is an important method to monitor the

production of gene therapy products, especially in process development but not exclusively. In addition, it delivers a good understanding on the best target for the titering assay for product release testing to deliver reliable titers for dosing. On top it might be a suitable method to further investigate the packaging model for AAV, that still leaves open questions.

Discoveries in Fundamental AAV Biology

433. Structural Characterization of Patient-Derived Anti-AAV9 Monoclonal Antibodies Generated Post-Zolgensma Treatment

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The use of vectors based on Adeno-associated virus 9 (AAV9) for gene therapy applications has grown rapidly over recent years, especially following the FDA approval of Zolgensma[®], an AAV9-based biologic for the treatment of spinal muscular atrophy. In addition, there is interest in broader AAV9 usage due to its ability to transduce cardiac and skeletal muscle, liver, pancreas, the eye and its capability to cross the blood-brain-barrier to transduce the CNS. However, the presence of pre-existing neutralizing antibodies (NAbs) against AAV9 capsids in a large percentage of the population excludes many patients from being treated. A strategy to circumvent the immune response of a patient for AAV-mediated therapeutic gene delivery is the development of modified vector capsids, able to escape antibody recognition, by either directed evolution or rational capsid design. However, in order to pursue this strategy, the antigenic regions of the capsids need to be characterized and the contact residues identified to which the NAbs bind. Previously, we and others have generated mouse monoclonal antibodies targeting the AAV capsids to simulate the immune response against the capsid and to map their major antigenic regions. However, this approach has faced criticism as mouse antibodies may not fully mimic human-derived antibodies. Here, for the first time human monoclonal antibodies are structurally characterized that were obtained from patients that received Zolgensma. Specifically, we have determined the binding sites to the AAV9 capsid by cryo-electron microscopy (cryo-EM) and three-dimensional image reconstruction of 13 antibodies obtained from two patients enrolled into a Zolgensma clinical trial. All the antibodies neutralize AAV9 transduction, and some also cross-react and neutralize a range of different AAV serotypes. Interestingly, the area located around the 2-fold symmetry axis of the AAV9 capsids appeared to be the antigenically dominant region, as the

majority of antibodies in both patients bind in this location. Structural comparisons of human and murine antibodies will be presented along with implications of the antigenic regions of the AAV9 capsid.

434. Characterization of Alternative Reading Frame Proteins Generated from AAV Cassettes

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Introduction Adeno-associated viral (AAV) vectors have tremendous potential as a delivery mechanism for gene therapy. Evidence has emerged that DNA vaccines can exhibit translation of unwanted peptides from Alternative open Reading Frames (ARF) within the cassette. Furthermore, hundreds of endogenous human genes have translated ARFs upstream of the annotated start codon, and these elements impact expression of the downstream gene. Potential for ARF translation has been previously observed in some gene therapy cassettes (Li et al, PNAS 2009). Here, we utilize ribosome profiling (Ribo-Seq) and reporter gene expression to investigate the production of ARF peptides from AAV cassettes. Methods We constructed a series of AAV cassettes that contained a CMV promoter, synthetic intron, reporter gene, and poly(A) signal sequence. The series was designed with 1 and 2 base pair deletions upstream of the reporter start codon to shift the reading frame of potential upstream ARFs. We performed Ribo-Seq on cells that were transfected with these constructs. Next generation sequencing (NGS) libraries were sequenced on an Illumina Miniseq, and ribosome protected fragment (RPF) reads were mapped to the plasmid sequence. Protein products were analyzed by western blot using an antibody against the reporter. Results We performed Ribo-Seq on cells transfected with AAV cassettes to test for the presence of upstream RPFs. We detected two RPF peaks upstream of the reporter ATG, one in the 5' exon and one in the intron of the cassette. Both peaks overlapped with the noncanonical start codons GTG and TTG, respectively. We created a set of constructs where those exonic start codons were placed to be in-frame with the reporter gene. We performed western blot analysis to directly test for the presence of a larger fusion proteins containing these upstream codons using antibodies to the reporter protein. Both alternative protein products were detected, alongside with the proper reporter protein. To further confirm that those larger proteins were due to alternative start codons, a series of plasmids with 1 and 2 base pair deletions upstream of the reporter start codon were tested in parallel. These constructs would still lead to translation of the upstream ARF, but the protein product would be out-of-frame with the reporter and undetectable on immunoblot. These deletion constructs did not lead to the same fusion protein, supporting our hypothesis. Ribo-seq also detected a peak located downstream of the proper start codon. This Ribo-Seq peak corresponded to a smaller protein on the western blot, indicating that second, intronic, start codon is also functional. Conclusion We detected the presence of functional upstream and downstream start codons in a cassette designed with commonly used promoter/intron configurations. Importantly, these start codons were not canonical ATG, but were GTG and TTG, and therefore were not predicted to support translation initiation. The fusion protein was detectable using

anti-reporter antibody when the upstream start codon was in the same frame as the reporter. These findings confirm previous observations and lend further support to the possibility that AAV transgene cassettes may express unexpected ARF products. Those non natural peptides may be immunogenic and contributing to immune responses. Since not all ARFs are predictable with current in silico approaches, Ribo-Seq analysis of clinical candidate vectors may be a valuable analytical tool.

435. Transcription, Translation, and Immunogenic Potential of P5-Associated rAAV Contaminants Post-Infection

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Adeno-associated virus (AAV) is one of the most used clinical gene therapy delivery vectors. However, it is known that AAV preparations contain nucleic acid contaminants from the production system. We examined the post-infection consequences of nucleic acid contaminants associated with the AAV2 P5 promoter used in 293-based rAAV production. By qPCR and Next-Generation-Sequencing (NGS) we observe that DNA contaminants associated with sequences upstream of the AAV P5 promoter are preferentially incorporated into rAAV particles, regardless of P5 positioning in the production plasmid. These P5-associated contaminants persist post-infection as observed by fluorescent in-situ hybridization (FISH) and NGS at 1-week and 4 months post infection. Surprisingly, we observe robust transcriptional activity corresponding to these P5-associated contaminants. To examine post-infection activity further, we replaced plasmid DNA upstream of P5 with a GFP reporter cassette and produced a ssHLPFVIII vector (AAV8^{GFP-P5}F8). FACS analysis of infected 293T cells and immunohistochemistry (IHC) of C57/BL6J mouse livers infected with AAV8^{GFP-P5}F8 show considerable detection of P5-contaminant derived protein. This protein was not detectable when the reporter cassette orientation was reversed relative to P5 (AAV8^{RevGFP-P5}F8) or with a non-reporter plasmid backbone (AAV8^{Empty-P5}F8). Infection of naive Balb/CJ mice with AAV8^{GFP-P5}F8 resulted in detection of GFP specific splenic T-cells by tetramer staining. Production of a contaminant reporter AAV8 in the absence of an ITR-flanked expression cassette (AAV8^{GFP-P5}NV) revealed the independent incorporation of these P5-associated sequences. Translation from P5-associated contaminants from AAV8^{GFP-P5}NV was again observed both in-vitro (293T cells) and in-vivo (C57/BL6J livers). Subsequently, we developed a modification to the P5 promoter (P5-HS) where a spacer sequence is inserted between the P5 TATA

and the YY1 box. This modification retains the P5 promoter dynamic of REP78 mediated autorepression and Ad5 helper gene induction. AAV produced with a P5-HS promoter retained production efficiency relative to P5 but significantly minimized the incorporation of P5-associated contaminants. NGS analysis showed AAV8 produced with P5-HS (AAV8^{P5-HS}F8) resulted in higher overall purity compared to equivalent P5 produced vectors. Side by side injection of AAV8^{P5-HS}F8 and AAV8^{Empty-P5}F8 resulted in equivalent transgene expression post-infection in-vivo for both self-complementary and single stranded AAV genome configurations. Infection of 293T cells with FVIII produced with the GFP reporter cassette upstream of P5-HS (AAV8^{GFP-P5-HS}F8) revealed significantly reduced detection of translated contaminants 72 hours post-infection. These results show P5-associated contaminants in rAAV can exhibit aberrant transcriptional and translational activity in infected cells with potential immunogenic consequences. Here we also present a system for minimizing the transfer of these sequences while retaining rAAV production efficiency. Combined, these results have implications for future clinical rAAV production design.

436. Unlocking Avian AAV Transduction in Mammalian Cells and Tissues for Immune Evasion and Redosing

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With 30-80% of adult humans producing antibodies against various AAVs, pre-existing immunity remains a significant challenge to AAV-based gene therapy. Likewise, AAV redosing is limited due to antibody cross-reactivity between AAV serotypes. Our lab previously developed a structure-guided approach to evolve novel AAV variants that can evade neutralizing antibodies. Another orthogonal approach is to utilize AAV isolates of non-primate origin, which display significant variability from capsid proteins of primate origin. As such, non-primate AAV isolates possess the potential to evade neutralizing antisera in humans. One such example is Avian AAV, with significant differences in capsid protein sequence (60% VP3 aa identity) and surface topology from primate derived AAV serotypes. However, we discovered that Avian AAV fails to transduce human cell lines as well as murine tissues in vivo. Further mechanistic investigation revealed that Avian AAV is capable of efficient mammalian cell surface binding, uptake, and nuclear localization. Moreover, capsid uncoating and second strand synthesis appear unaffected. Strikingly, Avian AAV vector genomes display impaired transcript formation in human cell lines, thereby inhibiting transgene expression. Based on these findings, we developed a rational engineering approach to unlock mammalian cell transduction by Avian AAV, yielding a novel and viable AAV variant (termed AAV.AvZ). We demonstrate that AAV.AvZ displays robust immune evasion even at high concentrations of anti-AAV8 serum, with transduction remaining virtually unchanged, while that of AAV8 was nearly fully abolished in vitro. Upon "redosing" in mice previously immunized by treating with AAV9, AAV.AvZ transduced multiple systemic tissues at levels significantly exceeding that of redosed AAV9 vectors. We also demonstrate that this approach can be broadly applied to re-engineer other AAV capsids of non-mammalian or non-primate origin, providing a roadmap to expand the portfolio of AAV candidates available for clinical use, with a particular focus on secondary dosing of human gene therapies.

437. Genome Packaging Efficiencies of Anc80 and AAV9 Vectors Using Non-Canonical Rep-ITR Combinations

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Despite recent remarkable progresses in AAV capsid engineering technologies for transduction enhancement and tissue targeting, high titer AAV vector production still remains a challenge. In this respect, in-depth understanding of the interactions between the AAV inverted terminal repeats (ITRs) and AAV replicase (Rep) proteins and their roles in AAV viral genome packaging is key to the development of novel methods to improve AAV vector yields. The standard methods for the production of AAV vectors derived from various serotypes and mutants all utilize Rep proteins and ITRs from AAV serotype 2 (i.e., Rep2 and ITR2). No study has comprehensively assessed AAV vector genome packaging efficiencies using noncanonical Rep-ITR combinations other than Rep2-ITR2. Therefore, we sought to determine whether alternative Rep-ITR combinations are capable of increasing the genome packaging efficiency and hence the vector yields. It has previously been reported that Rep3-ITR3 combination is superior to Rep2-ITR2 in AAV3 vector yields. Here we investigate various Rep-ITR combinations (Repx-ITRx, x=serotype 1-7) for genome packaging, to determine the compatibility of heterologous combinations of viral elements (i.e., the AAV2 p40 promoter, Repx and Capx) for virion formation, and report that, contrary to our expectation, none of the non-canonical combinations yields better than Rep2-ITR2. Our first approach utilized a VP3 only virion production system with the rep and cap genes provided in trans. We determined AAVxVP3 virion production levels using its own cognate, noncanonical Repx-ITRx combination (x=serotype 1,3,4,5,6 and 7) in comparison to the canonical AAVxVP3 and Rep2-ITR2 combination. The highest VP3 virion production was observed with Rep2-ITR2, rather than the cognate Repx-ITRx combination. Rep2-ITR2 could package the viral genome more efficiently than other Repx-ITRx irrespective of the type of capsid VP protein. Our second approach utilized an AAV9 and Anc80 VP1/2/3 virion production system with the rep and cap genes provided in trans. We determined AAV9 and Anc80 virion yields with different Repx-ITRx combinations (x=serotype 1, 2, 3, 4, 5, 6 and 7). All the Repx-ITRx combinations but Rep5-ITR5 and Rep6-ITR6 could support virion production with the highest yield attained by Rep2-ITR2 for both AAV9 and Anc80. Further analyses have indicated that Rep5 does not support AAV2 p40 promoter-driven VP protein expression while Rep6-ITR6 does not support viral genome packaging into the AAV9 or Anc80 capsids, resulting in the above-described observations. Our third approach involved an AAV9 VP1/2/3 virion production system with the rep and cap genes provided in cis. In this system, Repx and AAV9 capsid were expressed in cis using a pHLP-Repx-Cap9 plasmid (x=serotype 1,2, 3,4,6 and 7) while ITRx was provided in trans. Although AAV9 virion production was supported by all the Repx-ITRx combinations tested, the highest virion yield was attained again by Rep2-ITR2. Immunoblot analysis showed effective expression of the AAV9 VP proteins from all the pHLP-Repx-Cap9 plasmids, corroborating the superiority of Rep2-

ITR2 over other Repx-ITRs for genome packaging. In summary, these results demonstrate that the use of the cognate viral elements derived from the same serotype origin is not necessarily the best for high titer AAV vector production, and the Rep2-ITR2 combination likely yields the highest titer for diverse types of AAV capsids. Further studies are warranted to understand why the Rep2-ITR2 combination can package viral genomes more efficiently in a VP protein-independent manner than other Repx-ITR combinations.

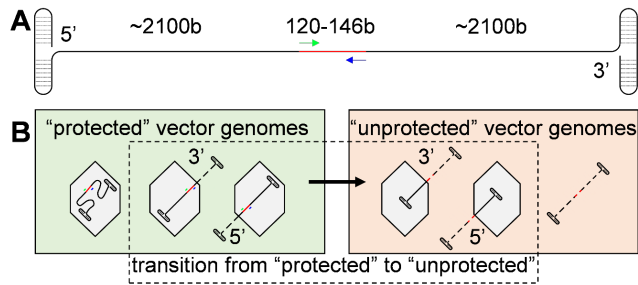
438. AAV Capsid Dynamics at the 5-Fold Pore Controls Genome Release

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The Adeno-Associated Virus (AAV) 5-fold pore has been proposed to have a critical role for genome packaging of AAV2 virions and in escape from the endo-lysosome mediated by coat viral protein 1 unique region (VP1u) externalization. Support for this hypothesis was initially derived from observations that the 5-fold capsid variant AAV2_{V221C} was defective for VP1u externalization and phospholipase A₂ (PLA₂) function when challenged with extreme heat (60 & 65 °C), along with a genome packaging and infectivity defect. Additional 5-fold capsid variants (AAV2_{V221Y}, AAV2_{L335A}, & AAV2_{L336A}) were demonstrated to not efficiently expose VP1u during endo-lysosomal trafficking *in vitro*. However, variants AAV2_{L336W} & AAV2_{L336C} have been reported to be non-infectious in VP1u externalized chimeras with PLA₂ activity, when similar VP1 deletion variants were rescued; demonstrating an uncoupling between PLA₂ activity and infectivity. Further, chemical cross-linking of the 2-fold has been shown to prevent VP1u externalization and subsequent infection, implying a critical role of this symmetry in VP1u externalization. Finally, it has been demonstrated that only genome containing virions will efficiently externalize VP1u, implying a role for the genome in the process. Despite these different observations, a causal relationship between altered VP1u dynamics, genome packaging, and infectivity in 5-fold capsid variants has not been fully established. To decouple the functional attributes of various AAV2 5-fold variants; we studied the genome maintenance of AAV2_{L336W}, AAV2_{L336C}, AAV2_{V221C}, & AAV2_{V221C&L336C} compared to AAV2. All of these variants are confirmed to be severely (≥100-fold) defective for delivery of a luciferase transgene relative to AAV2. When challenged with heat (4, 37, 45, 50, 55, 60, 65, 70, & 80 °C) model vector AAV2_{V221C} was unable to maintain genome relative to AAV2 before temperatures of expected VP1u externalization (≤60 °C), implying an enhanced conversion of full to empty virions could be the mechanism behind the previously observed VP1u heat mediated externalization defect. Upon heat challenge under divalent cation depletion there was an overall loss of ~5-10 °C of stability; with AAV2_{L336W} and AAV2_{L336C} intermediate between AAV2 and the least stable AAV2_{V221C}, while variant AAV2_{V221C&L336C} was more stable than AAV2 though less stable than in storage conditions. Under extended incubation (16hr) at 37

°C AAV2 experiences a slight (~20 %) externalization of genome only under divalent cation depletion. By contrast, 5-fold variants externalized genomes under either storage or divalent cation depletion by similar margins (~20 %) with variant AAV2_{V221C} exhibiting extreme uncoating (~50 %). In conclusion, the 5-fold pore plays a critical role in effectively maintaining packaged AAV genome configuration, and different variants can therefore display variable efficiencies of VP1u externalization, and subsequent infection.



Method Figure. A) Genome of AAV2 with the primer binding sites centrally located to generate an amplicon in red. B) Definition of “protected” vs “unprotected” genomes in relation to panel A.

439. Furin is a Host Factor Restricting Adeno-Associated Virus 4 Transduction

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Adeno-associated viruses (AAVs) are small, single-stranded DNA viruses that have emerged as a leading platform for therapeutic gene delivery. Several serotypes including AAV5, -8, and -9 are currently being investigated in clinical trials requiring systemic dosing, whereas other serotypes such as AAV4 are suboptimal due to low transduction efficiency. Understanding the mechanisms by which host factors restrict AAV transduction is critical for the continued development of improved vectors. Several AAV serotypes are known to localize to the *trans*-Golgi network and our lab has recently shown that the calcium gradient maintained by the Golgi-resident calcium pump SPCA1 is critical for AAV transduction. In the present study, we investigated whether the Golgi-localized protease furin, whose activity is dependent on calcium, plays a role in AAV transduction. CRISPR/Cas9-mediated KO of furin showed a modest effect in transduction efficiency for most AAV serotypes tested, but markedly increased transduction by AAV4. Since many viruses exploit furin for the cleavage and activation of their glycoproteins to enhance virulence and spread, we first assessed whether furin acts on the AAV4 capsid. Recombinant furin enzyme did not cleave AAV4 capsid protein VP1 *in vitro*. Next, we looked to evaluate the effect of furin KO on AAV host factors. AAV4 transduction is known to occur independently of the cognate AAVR but requires the post-entry host factor GPR108. As expected, furin KO did not affect the expression or cellular localization of AAVR, however GPR108 showed an altered intracellular localization pattern compared to normal cells. While AAV4 utilizes a currently unidentified cellular receptor, binding and transduction require O-linked α 2-3 sialic acid. Using a panel of lectins, we observed an increase in O-linked sialic

acid staining in furin KO cells. Furthermore, the increase in AAV4 transduction in furin KO cells was blocked by lectins specific for α 2-3 sialic acid. At the intracellular trafficking level, furin KO did not affect the AAV4 nucleocytoplasmic ratio; however, we observed an increase in overall AAV4 binding and cellular uptake. Based on these results, we postulate that furin regulates levels of cellular sialoglycoproteins, possibly including GPR108, thereby restricting AAV4 binding, uptake, and transduction in some tissues. In corollary, low furin expression may further explain the unique ocular and cardiopulmonary tropisms displayed by AAV4 vectors. These findings advance our understanding of the AAV transduction pathway and could inform vector design strategies to improve the clinical potential for serotypes such as AAV4.

Enhanced AAV Targeting

440. Effects of an Early Gene Therapy Targeting Oligodendrocytes in a Mouse Model of Adrenomyeloneuropathy

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Adrenomyeloneuropathy (AMN) is a late-onset axonopathy of spinal cord tracts caused by mutations of the ABCD1 gene that encodes hALDP, a peroxisomal transporter of very long chain fatty acids (VLCFA). *Abcd1*^{-/-} mice display a non-demyelinating non-inflammatory time-worsening axonal degeneration in the spinal cord that resembles AMN. Disturbed metabolic interaction between oligodendrocytes (OL) and axons is suspected to play a major role in AMN axonopathy. To develop a vector targeting OL, the human ABCD1 gene driven by a short 0.3 kb part of the human myelin-associated glycoprotein (MAG) promoter was packaged into an adeno-associated viral serotype 9 (rAAV9). Our main reason for targeting OL is their prominent role in the nurturing, myelin making and maintenance of axons, notably through the metabolic functions of their peroxisomes. *Abcd1*^{-/-} mice were given IV injection of the rAAV9-0.3 kb MAG-hABCD1 rAAV9 vector at P10 and were studied 3 weeks and 24 months later (advanced age was chosen to allow for a maximal clinical expression). Balance and motricity was studied with four different tests (ledge, hindlimb clasping, crenelated bar, rotarod). Vector treatment at P10 allowed a near normal motor performance to persist for 24 months, while age-matched untreated mice developed major defects (p 0.0005-0.003 for the 4 tests). Co-immunolabelling imaging was used to quantify cell-specific transgene expression in mouse OL and astrocytes. Almost no neurons were transduced. Transduction of OL and astrocytes by the vector was abundant in the spinal cord 3 weeks post vector: 50-54% of spinal cord white matter OL were expressing hALDP at the cervical level, decreasing to 6-7% after 24 months. In addition, 29-32% of cervical spinal cord astrocytes at 3 weeks and 16-19% at 24 months also expressed hALDP. Cerebellar OL were also abundantly transduced at 3 weeks. *Abcd1*^{-/-} mice are known to accumulate C26:0-lysoPC, a sensitive VLCFA marker of AMN, in their spinal cord and brain. C26:0-lysoPC, was lower by 41% and 50%, respectively in the spinal

cord and brain of vector-treated compared with untreated mice. The current preclinical study shows that an early gene therapy based on the rAAV9-0.3 kb MAG-hABCD1 rAAV9 vector can prevent axonopathy manifestations in the long term. Further experiments in *Abcd1*^{-/-} mice will test whether injection of the rAAV9-0.3 kb MAG-ABCD1-HA vector can halt neurological manifestations once they become significant, around 18-20 months of age. The current results provide a robust proof-of-concept not only for the gene therapy of AMN but for other CNS diseases where the targeting of OL with the rAAV9-MAG vector may be of interest.

441. Engineered AAV Capsids Exhibit Improved Transduction of the Central Nervous System After CSF Administration in Adult Cynomolgus Macaques

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Efficient gene delivery to the central nervous system (CNS) remains a major obstacle in the clinical translation of genomic medicines. Administration of AAV capsids into the cerebrospinal fluid (CSF) enables broad access to the CNS while minimizing exposure to pre-existing anti-AAV antibodies. Total vector genome doses into the CSF are also lower than those typically required for intravenous delivery in adults. Despite these advantages, the level of CNS transduction provided by natural serotypes is still insufficient for many indications. Here we applied a functional selection platform called SIFTER (Selecting In vivo for Transduction and Expression of RNA) to engineer capsids with improved neuronal transduction after CSF administration. Distinct from other transcription-dependent screens, SIFTER utilizes a divergent expression cassette and barcode look-up table to rank variant expression. The separation of capsid and barcode sequences enables implementation of unique molecular identifiers that improve signal-to-noise ratio in library selections. Moreover, universal PCR recovery primers can be used for multiplexed screening of libraries based on different parental serotypes. We conducted two rounds of neuron-specific library selection in adult cynomolgus macaques followed by evaluation of a barcoded pool with dual ranking of expression from the neuron specific human synapsin 1 (*hSyn1*) and ubiquitous human U6 snRNA (*hU6*) promoters. The highest performing capsids in these screens were then evaluated individually in adult cynomolgus macaques after intracerebroventricular administration of a neuron-specific cassette encoding a zinc finger transcription factor for targeted gene regulation. Transgene expression, vector genome biodistribution, and *in situ* hybridization analyses were conducted to benchmark capsid performance against AAV9. Engineered capsid variants mediated higher CNS transduction relative to AAV9. In particular, novel capsids STAC-102 and STAC-103 exhibited up to 100-fold enrichment in both mRNA expression and vector genome biodistribution compared to AAV9 across a majority of CNS regions including cortex, cerebellum, and spinal cord. Single cell *in situ* hybridization analysis corroborated the superior performance of these variants and underscores the

potent target engagement mediated by zinc finger transcription factor expression in transduced cells. These novel capsids have the potential to enable therapeutics for a multitude of CNS disorders.

442. A Direct Comparison of Five AAV Capsids for Intramuscular Inoculation in Non-Human Primates

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Use of adeno-associated virus (AAV) vectors in non-human primates (NHPs) is often complicated by host immune responses that can limit transgene expression. Our previous work using AAV vectors to deliver HIV broadly neutralizing antibodies to NHPs was limited by the emergence of anti-drug antibodies (ADAs). Here we compared the expression of and anti-drug antibody responses against the rhesus macaque derived, anti-SIV antibody ITS01 when delivered by five different AAV capsids. We first observed that ITS01 expressed three-fold more efficiently in mice from AAV vectors in which heavy and light-chain genes were separated by a P2A ribosomal skipping peptide, compared with those bearing F2A or T2A peptides. We then measured the preexisting neutralizing antibody responses against three traditional AAV capsids in 360 rhesus macaques and observed that 8%, 16%, and 42% were seronegative for AAV1, AAV8, and AAV9, respectively. Finally, we compared ITS01 expression in seronegative macaques intramuscularly transduced with AAV1, AAV8, or AAV9, or with the synthetic capsids AAV-NP22 or AAV-KP1. We observed at 30 weeks after inoculation that AAV9- and AAV1-delivered vectors (2.5×10^{12} vg/kg) expressed the highest concentrations of ITS01 (224 μ g/mL, n=5, and 216 μ g/mL, n=3, respectively). The remaining groups expressed an average of 35-73 μ g/mL. Notable, ADA responses against ITS01 were observed in seven of the 19 animals. Overall, our data suggest that the AAV9 capsid and the P2A peptide can improve expression of antibodies from muscle tissue in NHPs.

443. Systemic Administration of Novel Engineered AAV Capsids Facilitates Enhanced Transgene Expression in the Macaque Central Nervous System

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Recombinant adeno-associated viral vectors (rAAVs) hold great potential for therapeutic gene delivery to treat disorders of the central nervous system (CNS). Systemic administration of rAAVs is an area of particular interest, as it provides a minimally invasive means to facilitate widespread transduction across the CNS. However, CNS bioavailability and subsequent transduction by rAAVs with naturally occurring capsids are limited by inefficient delivery across the blood-brain barrier (BBB) and sequestration of viral particles in the liver. We applied a transgene mRNA expression-based directed evolution technique to perform parallel selections for novel capsids that efficiently

transduce the CNS after intravenous (IV) infusion in C57BL/6J and BALB/cJ mice and cynomolgus macaques. We utilized a self-packaging capsid variant library construct containing a random 7-mer peptide insert in the VR-VIII surface loop of the AAV9 cap gene and placed this construct under control of the neuron-specific human synapsin (hSyn) promoter to bias our selection strategy towards neurotropic variants. After two rounds of selection in mice we identified a novel variant, MDV1A, capable of superior CNS transduction in both male and female mice of both strains. MDV1A, which encodes the 7-mer sequence RSVGSVY, facilitated between a 25-fold and 160-fold increase in expression of a CMV-EGFP transgene in the brain compared to AAV9; the performance enhancement varied depending on the sex and strain. In macaques, two rounds of selection yielded a highly enriched proline-arginine loop (PAL) motif typified by the 7-mer sequence PXQGTXR. We assessed the relative performance of eight macaque-derived and four mouse-derived variants identified in this study, three mouse-derived variants described by another group, and AAV9 following IV infusion in mice and macaques. We performed within-subject comparisons by administering a pool containing equal amounts of each of the sixteen rAAVs encoding a barcoded human frataxin (hFXN) construct under control of the constitutive CBh promoter. Three of the four PAL family capsids that we evaluated enabled significantly enhanced transgene expression compared to AAV9 throughout the macaque cerebrum. These PAL capsids were additionally detargeted from the liver and dorsal root ganglia, suggesting a reduced risk of toxicity in these tissues. When assessing cross-species potential, none of the eight macaque-derived variants demonstrated increased potency over AAV9 in transducing the mouse CNS. Similarly, the seven divergent mouse-derived variants that we evaluated in this study universally failed to facilitate enhanced transduction in any region of the macaque CNS. Two of these mouse-derived variants, AAV.CAP-B10 and AAV.CAP-B22, have previously been shown to enhance transduction in the CNS of marmosets, a primate species more distantly related to humans than are macaques. Our findings therefore introduce the concerning possibility that in the context of CNS transduction, the performance of a given rAAV may vary considerably even across related primate species. These results underscore the necessity of selecting and evaluating CNS-targeted AAV capsids in an appropriate animal model with a high degree of similarity to humans. In summary, we have identified a family of variants with translational potential due to their enhanced ability to transduce the CNS of macaques while reducing virus sequestration in and transduction of the liver.

444. Astrocyte-Restricted Gene Silencing Improves the Safety of AAV9-Mediated Gene Therapy for Alexander Disease

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Alexander disease (AxD) is a rare, inherited neurodegenerative disorder affecting approximately one in one million births. Cases of AxD are most often manifested with megaloccephaly, hypomyelination, developmental delay seizures, and progressive deterioration in infancy or early childhood. Previous reports have shown that 95% of AxD are caused by dominant gain-of-function mutations in the glial fibrillary

acidic protein (*GFAP*) gene. The histopathological hallmarks of AxD are the presence of Rosenthal fibers, which are caused by GFAP protein aggregates in the cytoplasm of astrocytes in central nerve system (CNS). Currently, there is no therapy available to treat Alexander disease, because AxD is a gain-of-function disease and homozygous GFAP deficiency exhibits mild phenotypes in mice. Thus, silencing mutant GFAP could be a promising therapeutic approach. We designed an artificial mRNA (amiR) to target selected *gfap* sequences. By co-transfection of plasmids expressing amiR-*GFAP* and miRNA sensors (i.e., *GFAP* cDNAs fused with a reporter gene) in HEK293 cells, we identified multiple amiR-*GFAP* constructs that can efficiently silence the expression of *GFAP* in all three species. We then packaged self-complementary constructs of amiR-*GFAP* designs #1 and #2 expressed by the ubiquitous chicken beta actin promoter with *CMV* enhancer (*CMVen/CB*) into AAV serotype 9 (AAV9) and injected five-week-old AxD disease mice (*Gfap*^{+/*R236H*}) by intravenous administration at a dose of 2xe14 genome copies (GCs)/kg. Three weeks later, we found that both scAAV9-*CMVen/CB*-amiR-*GFAP* vectors down regulated *gfap* mRNA and protein in the brain 50% more than the control vector. In addition, histopathologic analysis of treated mouse brain showed reduction of Rosenthal fibers. To monitor long-term therapeutic efficacy and safety, we extended the treatment to three months. We found that scAAV9-*CMVen/CB*-amiR-*GFAP*#1-treated mice started dying from digestive disorders around three to four weeks post-treatment. Interestingly, *Gfap*^{+/*R236H*} mice treated with scAAV9-*CMVen/CB*-amiR-*GFAP*#2 showed improved body weights until the tenth week post-injection. During this period, the animals started losing weight, hunching, and abnormally rapid breathing. Necropsy of these animals revealed enlarged hearts, as well as reduced GFAP expression in the brain as compared to control vector-treated mice. We hypothesized that expression of amiR-*GFAP* in non-astrocyte cells might have resulted in the aforementioned side effects. To reduce off-target silencing, we replaced the *CMVen/CB* promoter with the endogenous *GFAP* promoter in the amiR-*GFAP* vector to drive astrocyte-specific expression. The second generation of amiR-*GFAP*#1 vector not only eliminated the early death, but also improved weight gains during development as early as three weeks after injection. Treatments with the 2nd gen. of amiR-*GFAP*#2 vector also improved the body weights of *Gfap*^{+/*R236H*} mice. Western blot and qRT-PCR analysis revealed that GFAP protein and mRNA levels were reduced to 30% at three weeks post-injection in the amiR-*GFAP*#2 vector treated mice. The long-term monitoring of animals that received the 2nd gen. vectors is still ongoing. Taken together, our data indicate that astrocyte-restricted gene silencing represents a promising strategy for the gene therapy of AxD and other gain-of-function diseases that affect astrocytes. (*Corresponding authors)

445. Positron Emission Tomography I-124-Labeled AAV Assessment of CSF to Blood Diffusion and Consequent Systemic Distribution of AAV Capsids Following CSF Administration of AAV Vectors

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Cerebrospinal fluid (CSF), the fluid that surrounds the brain and spinal cord, is a convenient site to administer adeno-associated virus (AAV) gene transfer vectors. While highly efficient in delivery of the AAV capsid throughout the nervous system, it is unclear how much of these vectors escape the nervous system, with consequent systemic distribution of the AAV. In contrast to the tight blood-brain barrier, the barrier between blood and CSF allows for exchange of proteins via fenestrations and pinocytosis vesicles. Consequently, there is normal blood to CSF and CSF to blood exchange of macromolecules. In this context, and with the increasing use of CSF administration of AAV vectors to treat brain and spinal cord disorders, we asked: following CSF administration of AAV vectors, is there substantial movement of AAV vectors from the CSF to blood, with consequent distribution of AAV vectors to organs other than brain and spinal cord? To answer this, we developed methods to track the AAV vector capsid biodistribution using AAV-labeled with gamma emitting Iodine-124 (I-124) isotope and positron emission tomography (PET)/computed tomography (CT) body scans to quantify the biodistribution of CSF administered AAV vectors from 1 to 120 hr post-AAV administration. To approximate the size of a small human child, we utilized African Green nonhuman primates (NHP), whose head and body length of ~45 cm allowed transverse positioning in the human Siemens PET/CT system with 4 PET rings at a spatial resolution of 5.0 x 2.0 x 2.0 mm³. PET was performed on the NHP immediately post-vector administration and on 3 successive days. We tested administration of multiple AAV vectors (AAVrh.10, AAV9, AAV-PHP.eB, AAVrh91, and AAVhu68) via CSF delivery by intracisternal (IC, to the cisterna magna) and intrathecal (IT, to L4/L5 subarachnoid space) routes. Control NHP administered NaI-124 by intracisternal delivery route exhibited rapid clearance of radioactivity to the thyroid and gut, with <0.1% remaining in the brain/CSF. Despite the PET detection of the AAVs in the CSF over several days, 64 to 88% of the I-124-labeled AAV capsids was detected systemically over the 1st 72 hr. Using the liver as a sentinel of AAV vectors in the blood, there was significant biodistribution in the liver 20.9% ± 4.0% administered dose for all vectors combined. The significant biodistribution to the liver and other systemic organs outside of the nervous system were observed for all serotypes and independent of the site of administration to the CSF (intracisternal vs intrathecal). Further evidence of the AAV vector capsids moving from the CSF to the blood was evident by induction of anti-AAV total and neutralizing antibody titers. In the NHP administered intracisternally and intrathecally, the serum total antibody titers reached 10⁵ and neutralizing titers as high as 3x10³, similar to that observed with intravenous administration. We conclude that regardless of the route

of delivery to the CSF, intracisternal or intrathecal, I-124-labeled AAV CNS administration results in significant systemic distribution of the AAV gene therapy vectors. These observations have implications for dosing and considerations of systemic adverse events that should be monitored CNS AAV gene therapy trials.

446. Identification of New AAV9 Engineered Capsids Targeting Mouse and Monkey Brains Through a Directed Evolution Approach in BALB/c Mice

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Gene therapy approaches require vectors endowed with both high transduction efficiency and specificity. Moreover, systemic delivery of the gene therapy formulation is preferable and more effective, but diffusion into the central nervous system (CNS) is limited by the blood-brain barrier (BBB) that restricts the passage of molecules. Among all the AAV natural serotype, AAV9 is known to diffuse into the CNS parenchyma after intravascular delivery. To further enhance this ability, various directed evolution approaches, have been developed. One of these approaches led to the discovery of the AAV-PHP.eB capsid with a significantly superior brain transduction in mice, which, however, is not conserved in non-human primates (NHPs). We performed an in vivo screening approach using a AAV9 display peptide library to select novel brain targeting capsid variants. The peptide library was inserted in the AAV9 capsid with an additional mutation, which is known to erase the AAV9 natural binding on galactose thus facilitating new interactions. In order to prevent LY6A binding (AAV-PHP.eB receptor), the selection was carried out using BALB/c mice, that present inactivating mutations for this locus. Upon four consecutive rounds of selection, two enriched capsid variants were identified, named AAV-Se1 and AAV-Se2. Further characterization of these variants displayed a good diffusion throughout the brain, but with prevalent endothelial cell transduction, although neurons and glia were also targeted upon tail vein injection. In order to increment neuronal cells targeting, we decided to revert the mutation, restoring galactose binding, thus incrementing neuronal transduction. The new capsids were named AAV-Se1-wt and AAV-Se2-wt, whereas their mutated counterparts were re-named AAV-Se1-mut and AAV-Se2-mut respectively. AAV-Se1-wt and AAV-Se2-wt maintained the ability to cross the BBB already displayed by the capsids bearing the mutation, but presented incremented efficiency to transduced neurons and glia. Intravenous injection of AAV-Se1-wt and AAV-Se2-wt resulted in a wider targeting of the whole brain, compared to AAV9, with AAV-Se2-wt being the best variant tested. Efficient transduction was achieved for all the area of the forebrain but also the cerebellum resulted efficiently targeted and especially Purkinje cells. To further characterize AAV-Se2-wt capsid we replicated these experiments in neonatal mice with temporal vein injection showing an increased neuronal targeting throughout the

brain. Importantly, AAV-Se2-wt showed a reduced tropism for liver compared to AAV9, and thus higher brain specificity. Ly6a mutation (BALB/c strain) did not impact the bio-distribution of Se capsids, to the exception of AAV-Se2-mut. With that in mind, we decided to proceed for analysis in marmosets, that included AAV-Se1-wt and AAV-Se2-wt in comparison to AAV9. Preliminary analysis of marmoset brains confirmed what discovered in murine models, showing diffuse brain transduction with high levels of infection in cerebellum, thalamus and striatum. Moreover, the AAV-Se2-wt variant appeared the most efficient capsid in transducing the marmoset brain. These data strongly indicate that AAV-Se1-wt and AAV-Se2-wt capsids have the ability to transduce the brain parenchyma by crossing both mouse and NHP BBB, and thus being good candidates for further exploitation in translational approaches for human CNS diseases.

Gene Editing in Cancer and Complex Diseases

447. Targeting the Hepatitis BcccDNA with a Sequence-Specific ARCUS Nuclease to Eliminate Hepatitis B Virus In Vivo

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Persistence of chronic Hepatitis B (CHB) is attributed to maintenance of the intrahepatic pool of the viral covalently closed circular DNA (cccDNA), which serves as the transcriptional template for all viral gene products required for replication. Current nucleos(t)ide therapies for CHB prevent virus production and spread but have no direct impact on cccDNA or expression of viral genes. We describe a potential curative approach using a highly specific engineered ARCUS nuclease (ARCUS-POL) targeting the Hepatitis B virus (HBV) genome. Through iterative rounds of nuclease engineering, ARCUS-POL nucleases were optimized to exhibit high levels of on-target editing with minimal off-target activity. Transient ARCUS-POL expression in HBV-infected primary human hepatocytes produced >75% reductions in both cccDNA and Hepatitis B surface antigen (HBsAg). In addition to that expressed by cccDNA, HBsAg has recently been shown to be abundantly produced from integrated viral DNA. After transient delivery of ARCUS-POL into cells containing integrated HBV DNA, >80% on-target editing was achieved with subsequent HBsAg reductions. To evaluate ARCUS-POL in vivo, novel episomal adeno-associated virus (AAV) mouse and non-human primate (NHP) models were developed containing a portion of the HBV genome serving as

a surrogate for cccDNA. Clinically relevant delivery was achieved through systemic administration of lipid nanoparticles containing ARCUS-POL mRNA. In both mouse and NHP models, a significant decrease in total AAV copy number and high on-target indel frequency was observed. In the case of the mouse model, which supports HBsAg expression, circulating surface antigen was durably reduced by 96%. Together, these data support a gene editing approach for elimination of cccDNA toward an HBV cure.

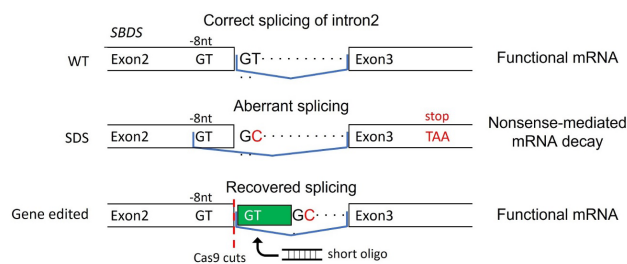
448. A Novel DNA Oligo-Based Repair Strategy for the Functional Correction of Shwachman-Diamond Syndrome

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Shwachman-Diamond syndrome (SDS) is an autosomal-recessive bone marrow failure syndrome characterized by hematopoietic dysfunction and leukemia predisposition. More than 90% of SDS patients carry the 258+2T>C splice donor site mutation in intron 2 of the Shwachman-Diamond (SBDS) gene, which leads to a frameshift and premature protein truncation. Allogeneic hematopoietic stem cell transplantation is the only curative treatment for patients with severe bone marrow failure or malignancy but is limited by matched donor availability and high risk of treatment-related morbidity and mortality. Current gene editing approaches to correct disease-causing mutations primarily rely on homology-directed repair (HDR) which is only active in the S/G2 phases. CRISPR/Cas9 based gene editing in actively dividing cells can cause chromothripsis and a 20-fold increase in chromosome structural abnormalities, with the potential of cancerous transformation. HSCs are particularly susceptible to chromothripsis due to their lifelong self-renewal and the proliferation inducing in vitro culture methods used in current protocols. Here, we established an HDR-independent short oligo-trapping strategy to reestablish correct splicing of SBDS gene. This non-homologous end joining (NHEJ) based method is active in quiescent cells, thus potentially reducing cell cycle dependent unintended edits. The benefits of editing quiescent HSCs are particularly relevant to SDS because of 1) low HSCs numbers, so preserving HSCs engraftment potential is essential, 2) increased susceptibility to accumulate chromosomal abnormalities. We co-delivered a repair short double strand DNA oligo (dsODN) with Cas9 targeting the 258+2T>C mutation site. The integration of the oligo provides a functional splicing donor sequence regardless of its orientation. To determine the best repair oligo sequence composition for efficient splicing, a partially randomized oligo was introduced into SDS patient-derived fibroblasts with homozygous 258+2T>C mutation. The selective advantage mediated by optimal splicing of SBDS led to the enrichment of oligo sequences which are similar to the human splice donor consensus sequence and different from the original splice donor. Thus, we designed 30bp oligos and achieved insertion rates of 51±0.83% in SDS fibroblasts. Using Cas9-mSA (monomeric streptavidin) tethering a 30bp biotinylated oligo, the correction rate

was further increased to $66 \pm 2.67\%$, which recovered the correct mRNA splicing and SBDS protein expression to approximately 90% of the wildtype. Experiments in healthy CD34-HSCs targeting the pseudo-gene SBDS^{P1} locus, which shares the 258+2T>C mutation, resulted in 30% dsODN insertion rate. Correction of 258+2T>C mutation in SDS fibroblasts led to a growth advantage of gene-corrected cells as the insertion rate of dsODN increased overtime. Our findings show that the most common 258+2T>C mutation in SBDS can be efficiently repaired by NHEJ-mediated insertion of dsODN. This repair mechanism is highly active in quiescent HSCs, making this approach potentially more efficient and safer than HDR-based therapies for indications where target cells are postmitotic or in a quiescent state.



449. Liver Gene-Editing Based on Nickase Cas9 for the Treatment of Primary Hyperoxaluria Type I (PH1) is More Efficient When Using an All-in-One Delivery System

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Targeted gene disruption mediated by programmable nucleases generates double-strand breaks (DSB) that are primarily repaired by error-prone non-homologous end-joining (NHEJ). NHEJ introduces random deletions or insertions (indels) that can result in frameshifts or the introduction of an early stop codon leading to the disruption of the gene expression and function. To minimize the variability of NHEJ *in vivo*, we co-injected two AAV-Cas9 nucleases carrying two different guides that target close regions in the *Hao1* gene in a mouse model of primary hyperoxaluria type I (PH1). NGS analysis showed a deletion of the sequence in between the two guide-targeted sequences, resulting in the complete elimination of the translated protein, as measured by western blot. However, this strategy might duplicate the chance of

off-target events, a rising safety concern for translation into the clinic. Thus, to generate a safer gene disruption strategy, paired gRNAs were combined with the nickase variant of Cas9. Nickase Cas9 creates nicks instead of DSB, which are sensed by high-fidelity single-strand break repair pathways. Therefore, simultaneous nicks, appropriately spaced and oriented may lead to site-specific DSB and gene knockout. Recently, we demonstrated that the simultaneous administration of two AAV-nickase Cas9, carrying the guides described above, mediated highly efficient disruption of *Hao1 in vivo*. By on-target NGS analysis, we determined that the paired nickase Cas9 system achieved similar editing efficiency of paired nuclease Cas9. Additionally, in animals treated with only one guide we observed that individual nicks were faithfully repaired, as detected by NGS, and exhibited normal *Hao1* expression, thus minimizing potential off-target events. Moreover, we proved that the minimal AAV dose for targeted gene knockout can be reduced by combining nickase Cas9 together with the two gRNAs in a single AAV vector (AAV-all-in-one). Additionally, we observed that the editing efficiency of the all-in-one system was independent of the vector dose administered. Most importantly, we established that *Hao1* disruption mediated by AAV-all-in-one, at the lowest dose tested, is therapeutically relevant in a mouse model of PH1. Finally, quantitative analysis of potential chromosomal rearrangements *in vivo* by methods such as chromosomal aberrations analysis by single targeted linker-mediated PCR sequencing (CAST-seq) would further investigate the safety and clinical feasibility of a paired nickase system for diseases that could benefit from permanent gene disruption, such as PH1.

450. Enhanced CRISPR/Cas9 Genome Editing in Heart and Skeletal Muscle with a Potent New AAV Variant

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Recombinant adeno-associated viral (AAV) vector mediated genome editing faces multiple challenges including delivery efficiency of dual vectors packaging CRISPR/Cas9 and guide RNA elements. We recently developed a newly evolved AAV variant, AAV.cc47, which displays substantially increased potency, wide spread multi-organ gene transfer, and cross-species compatibility in mice, pigs and NHPs. Here, we first observed increased gene transfer efficiency while benchmarking AAV.cc47 against AAV9 packaging Cre recombinase in the Ai9 fluorescent reporter mouse model at 3 different doses following intravenous (IV) administration. We then optimized AAV-CRISPR cassette designs for systemic genome editing at the same Ai9 locus by evaluating different promoters, gRNA vector to Cas9 vector ratios, and AAV genome composition. Increased editing efficiencies were achieved at higher gRNA to Cas9 vector ratios with greatest editing efficiencies achieved when employing a self-complementary genome to express the gRNAs. An optimized dual CRISPR vector approach comparing AAV.cc47 vs AAV9 was then evaluated in the *mdx* mouse model of Duchenne muscular dystrophy. AAV.cc47 mediated genome editing of the *Dmd* locus in *mdx* mice revealed 6-fold and 2-fold improvement in

restoration of dystrophin in cardiac and skeletal muscle, respectively, compared to AAV9. Interestingly, despite increased editing efficiencies of the *Dmd* locus with AAVcc47, vector biodistribution remains the same between the two capsids, suggesting potential improvement in transduction due to a post entry mechanism. This is corroborated by increased RNA transcript levels in AAV.cc47 treated mice. Results from ongoing NGS analysis of edited loci in tissues will be presented. We envision that the use of high potency ccAAV vectors can afford increased genome editing efficiencies at lower vector doses, thereby improving the therapeutic window when transitioning from preclinical animal models towards clinical applications.

451. A MiniCEP290 Gene Replacement Therapy to Treat CEP 290-Leber Congenital Amaurosis (LCA10)

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Purpose: Leber congenital amaurosis (LCA) is a debilitating eye disorder and is considered one of the most severe forms of retinal degeneration. Mutations in *CEP290* (LCA10) account for > 26% of all LCA cases and are the most frequent cause of LCA, which often results in severe vision loss at infancy. Adeno-associated viral (AAV) vectors are currently the most efficient vectors for gene delivery to the retina. However, the development of a gene therapy for LCA10 has been challenging because *CEP290* gene is too large to be packaged into conventional AAV vectors. Mutation specific anti-sense oligo and gene editing therapies have been previously reported. We design a mutation-independent gene therapy delivered with an AAV vector to treat LCA10. **Methods:** We designed shorter versions of *CEP290* (miniCEP290) under photoreceptor-specific promoters that are functional and can be delivered using AAV vectors. *Cep290*-mutant mice (*Cep290^{rd16}*) were injected in the subretinal space at postnatal 10 days. Photoreceptor response to light was measured by electroretinography (ERG). Retinal structure and protein trafficking were evaluated by immunofluorescence microscopy using specific marker antibodies. **Results:** We previously demonstrated that a *CEP290* minigene [*CEP290* amino acid 580-1180 domain] under the control of a ubiquitous promoter improved the function and survival of photoreceptors in neonatal *Cep290^{rd16}*. However, the effect was short-lived with degeneration ensuing after 5 weeks of age. This current study's goal was to improve the efficacy of the miniCEP290 approach and optimize protein expression in the mutant mice. We show that the expression of *CEP290*-580-1180 under the control of the rhodopsin kinase promoter improved the ERG response for both rod and cone photoreceptors by ~1.5 folds. Further, modification of the miniCEP290 gene construct with different *CEP290* domains improved the photoreceptor structural and functional rescue by ~500% in the *Cep290^{rd16}* mice. We also show that the expression of the new miniCEP290 prolonged the survival and improved the protein trafficking defects in transduced photoreceptors. **Conclusion:** Our preliminary results indicate that miniCEP290 approach with conventional AAV vectors may have the potential for mutation-independent gene therapy in LCA10 patients. These studies may also pave the way to develop new minigene therapies for retinal degenerative

diseases caused by mutations in other large genes including Stargardt disease and Usher Syndrome, which are not currently amenable to treatment using conventional AAVs.

452. A Novel Polyfunctional Editing Strategy for Adoptive T Cell Immunotherapy of Cancer

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Adoptive transfer of genetically engineered T lymphocytes has provided remarkable clinical benefits for the treatment of several types of cancer. In this context, autologous or allogenic T cells are redirected against tumor-related antigens through *de novo* expression of a transgenic TCR or a CAR. Although endowed with powerful new properties, transplanted cells often fail to exert their biological function, either because of limited tumor trafficking, premature exhaustion and/or deactivation in the tumor microenvironment. Molecular dissection of these roadblocks in pre-clinical models and clinical studies is providing a wealth of novel actionable targets that can be used to implement more effective and safer engineered T cell products. These advances, however, pose the challenge of developing innovative tools to induce orthogonal edits (i.e., knock-in/out and transcriptional modulation) at multiple genomic loci without impacting on the genomic integrity of treated cells, an aspect that may jeopardize safety of the therapeutic product. In this regard, we have developed a novel CRISPR-Cas9-based polyfunctional editor (poly-editor) to simultaneously program genetic and epigenetic edits at multiple loci without inducing reciprocal chromosomal translocations. To achieve this goal, we first focused on establishing epigenome editing procedures to efficiently silence genes in human primary T cells. In this regard, we used Engineered Transcriptional Repressors (ETRs), chimeric proteins containing a catalytically inactive Cas9 fused to one of the following epigenetic effectors: KRAB, DNMT3A and DNMT3L. Upon several rounds of platform optimization, we showed that transient delivery of ETR combinations resulted in high levels (>90%) of long-term stable (>50 days) epigenetic silencing of the *B2M* gene. Similar results were obtained by targeting two other therapeutically relevant genes. As a proof-of-concept, we also showed that epigenetic silencing of *B2M* could be reverted at will by pharmacological treatment. Then, with the aim of reducing the molecular complexity of the ETR technology, we further refined ETR architectures and developed an all-in-one fusion protein that, when transiently delivered into T cells, induced durable and near complete epigenetic silencing of *B2M* at doses lower than those used with conventional ETR combinations. Finally, by building on this improved ETR design, we constructed a poly-editor containing a catalytically active Cas9 in place of its deactivated counterpart. To achieve simultaneous orthogonal edits in T cells, we took advantage of gRNAs of different length and showed that a single administration of the poly-editor resulted in up to 40% of cells harboring both durable (>50 days) epigenetic silencing of *B2M* and targeted integration of a NY-ESO-specific TCR into the *TRAC* locus. Molecular analyses of the poly-edited cells showed lack of reciprocal chromosomal translocations between the edited loci, whereas these translocations were present in T cells simultaneously edited at the genetic level with the prototypical CRISPR-Cas9 system. We are currently expanding polyfunctional

editing to include additional therapeutically relevant targets and evaluating the functional properties of the poly-edited T cells *in vitro* and *in vivo*. Overall, therapeutic implementation of this polyfunctional editing platform may lead to safe and efficient multiplexing of T cells.

453. Generation of Efficient Lipid Nanoparticles for Liver-Directed Gene Therapy and Genome Editing

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Successful therapeutic application of CRISPR/Cas9-mediated genome editing requires a safe, organ-specific, and efficacious delivery mechanism. There is growing utilization of ionizable lipid nanoparticles (LNPs) as a non-viral strategy for the *in vivo* delivery of RNA therapeutics. However, there remains a challenge to identify LNP formulations that provide potent but selective delivery of the RNA cargo. Here we report our efforts to identify novel ionizable lipids that confer robust delivery of intravenously administered LNP-encapsulated RNA specifically to liver hepatocytes and the application of these LNP formulations for genome editing. We screened 29 LNP formulations comprising novel ionizable lipids for liver-directed delivery of firefly luciferase mRNA upon systemic administration. Five candidate formulations emerged from this screen that resulted in potent luciferase expression in the liver but not in other highly perfused organs such as spleen, heart, kidney, and lung. These formulations produced LNPs that were well tolerated in mice and physically characterized by having a diameter ranging from 95 to 160 nm and a low polydispersity index. To test the efficacy of the new LNP formulations to deliver CRISPR/Cas9 components, we produced LNPs that were co-formulated with mRNA for *S. py* Cas9 and an sgRNA targeting the mouse transthyretin (TTR) gene. We varied the ratio of mRNA to sgRNA in the formulations in an attempt to optimize the formulation conditions to achieve maximum *in vivo* gene editing. We found that a subset of ionizable lipids initially identified in the luciferase mRNA screen could produce LNPs that effectively delivered CRISPR/Cas9 components to achieve clinically relevant levels of *in vivo* genome editing accompanied by a significant reduction of serum TTR protein. The degree of serum TTR reduction and on-target DNA indel formation was dependent on both the ionizable lipid present in the formulation and the mRNA-to-sgRNA ratio. Finally, we highlight the potential of this genome editing system for gene disruption or targeted cDNA knock-in as a functional gene correction strategy.

Physical Methods and Extracellular Vesicle-Based Gene Transfer

454. DNA Sensor Palmitoylation in Mouse Skeletal Muscle Following DNA Electroporation

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High vascularization, accessibility, and the ability to synthesize and secrete therapeutic proteins make skeletal muscle a promising tissue for therapeutic gene delivery. The direct transfer of plasmid DNA (pDNA) is a straightforward but poorly efficient therapeutic gene delivery system used in skeletal muscle. By applying defined electric pulses (electroporation), the cell membrane permeability increases, facilitating the transport of pDNA into cells. However, the underlying molecular mechanisms of pDNA electrotransfer have yet to be clarified despite an increasing number of ongoing clinical trials using the technology. Several putative DNA sensors bind pDNA in the cytosol. Additionally, the cGAS-STING signaling axis, containing the synthase for the second messenger cyclic GMP-AMP (cGAS) and the cyclic GMP-AMP receptor stimulator of interferon genes (STING), may be activated, which requires palmitoylation at the Golgi for STING-dependent downstream signaling. Therefore, we aimed to investigate if putative DNA sensors are post-translationally modified by palmitoylation in mouse skeletal muscle. Plasmid DNA was injected into the caudal thigh muscles of anesthetized female C57BL/6 mice. A 2-needle electrode with a 5mm gap was placed into the muscle surrounding the injection site. Eight 20 ms pulses at a voltage to distance ratio of 100 V/cm were immediately applied using an ECM830 pulse generator. Muscle gene and protein expression were evaluated four hours after delivery by comparing a control group to groups receiving pDNA injection alone, pulse application alone, and pDNA electroporation. The production of proinflammatory molecules is implicated in the activation of DNA sensing. RNA sequencing revealed the upregulation of several proinflammatory cytokine and chemokine mRNAs. Protein levels in muscle lysates quantified by a multiplex bead array confirmed this upregulation. RNA sequencing also revealed that the mRNAs of 14 putative DNA sensors were significantly upregulated in the muscle after pDNA injection and followed by electroporation. Bioinformatical predictions revealed palmitoyl-modification sites on six DNA sensors: *Cgas* (Cyclic GMP-AMP synthase), *Ddx58* (DEXD/H-Box Helicase 58), *Dhx36* (DEAH-Box Helicase 36), *Dhx9* (DEXH-Box Helicase 9), *Ifi204* (Interferon-activable protein 204) and *Zbp1* (Z-DNA Binding Protein 1). The predictions identified high-confidence palmitoylation sites that were previously characterized by palmitoyl-proteomics studies. The predicted cysteine sites support that these sensors are palmitoyltransferase substrates. To assess the conservation of cysteine residues, we performed a Clustal Omega cross-species alignment that revealed high sequence conservation surrounding the predicted sites, which suggested the evolutionary importance of this modification. The degree of palmitoylation and the number of palmitoylation sites of the DNA sensor *Zbp1* were evaluated using the novel acyl-PEGyl exchange gel-shift assay. Two palmitoylation sites represented by a mobility shift

in the presence of PEG-5k and hydroxylamine-treated samples in the immunoblot were detected in all experimental groups. In contrast, no gel shift bands were detected in the control group. Palmitoylation has been described as necessary for DNA sensor signaling, highlighting the potential of palmitoylation sites as therapeutic targets that could differentially impact immune signaling and regulatory elements. Identifying unique post-translational modifications in the muscle will facilitate a better understanding of the underlying molecular mechanisms and the development of safety biomarkers, as well as novel strategies to improve skeletal muscle targeted gene therapy.

455. Novel Non-Thermal Plasma Based Delivery of Plasmid DNA

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In the past decade, the gene therapy field has made remarkable progress. However, the technique remains risky, including efficacy, efficiency, and toxicity. To overcome these issues, there is tremendous growth in non-viral gene delivery systems, including physical approaches such as ultrasound, hydrodynamic, and electrotransfer. A potential disadvantage of electrotransfer is the necessity of electrodes that must make contact with the target tissue to apply the necessary electric fields. It would be advantageous to utilize a similar approach that can be applied without contact. Low-temperature plasma or non-thermal atmospheric-pressure plasmas (NTAP) is one approach that can accomplish this task. NTAP can facilitate this due to the generation of reactive species, including oxygen (ROS) and nitrogen (RON). When NTAP-assisted electrotransfer is applied to the targeted site (skin) for DNA delivery, a surface modification is seen due to the generation of ions from electroporation at the tissue surface. Adjusting the amount of ion deposition on the surface of the target site has a significant advantage for gene transfer. Our group has been working on developing approaches that are particular to skin. Efficient gene transfer to the skin is convenient for various treatment options. Skin is an excellent model for gene therapy due to its simple, direct, *in vivo* method, easy accessibility, and perfect potential target. We studied the delivery of plasmid DNA with NTAP *in vitro* and then *in vivo*. First, we conducted *in vitro* studies using keratinocyte cells (HaCaT). A plasmid encoding luciferase (pLUC) was delivered using a nanosecond pulsed sliding discharge plasma device. Results from *in vitro* and then *in vivo* experiments show that plasmid DNA delivery can be accomplished using plasma. We did not see any adverse effects on cell viability, and we observed an increased expression level for the first 48 hr after treatment. From these results, it is evident that we can achieve delivery of plasmid DNA using plasma with a minimal detrimental effect on cell viability and without any adverse impact which was a positive indication for *in vivo* applications. Next, we conducted *in vivo studies* using Guinea pigs. Expression levels were observed using an IVIS whole-body imaging system. The expression levels were much higher with the NTAP device exposed sites when compared with injection alone. We are further investigating how the NTAP device can modulate the amount of ion deposition on the tissue that can control expression levels. For this experiment, we designed a novel NTAP jet. For *in vitro* studies using human keratinocyte cells (HaCaT Cells), cells were seeded

in a 12-well plate or agar/matrix gel model. The novel NTAP jet was applied at different height levels, varying duration of treatment, various voltages, and air pressure for adequate expression levels with adequate expression levels and minimum adverse effects. The approach will be further evaluated in a Guinea pig model and delivery compared to delivery with electrotransfer. The *in vitro* findings will allow us to test many parameters and reduce the number of animals. These results will lead to the development of an improved non-invasive and non-contact system for the delivery of plasmid DNA to the skin.

456. Electrotransfer Combined with Moderate Heat and Impedance Monitoring to Enhance Delivery of Agents to Multiple Tissues

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Electrically-based therapeutics have been utilized for ablative techniques (irreversible electroporation; IRE and electrochemotherapy (drug delivery); ECT) as well as for nucleic acid delivery (gene electrotransfer; GET). IRE has been approved for soft tissue ablation in the United States and ECT has been approved for use in cutaneous malignancies in Europe. With respect to GET there have been over 100 clinical trials evaluating its use for multiple indications. While these are significant achievements, the basic technology has not had any major advances in the past couple of decades. Our research group has modified the electrotransfer approach to enhance its utilization in multiple potential therapeutic applications. The new approach incorporates the application of moderate heat (43°C) to the target tissue as well the ability to monitor the tissue in a manner that enables the user to know when the therapy has been successfully administered. The addition of moderate heat has multiple advantages including: enabling a reduction in applied voltage which in turn would enable either a reduction in the applied voltage or treating a larger area with the same voltage. Heat also makes the application of the therapy more uniform across the tissue. In addition, when electric pulses are applied, the impedance of tissue will be reduced. An instrument can be used to determine when the tissue has been sufficiently permeabilized or membranes appropriately effected based on a specific reduction in the impedance. Incorporating these two physical parameters has led to enhanced delivery. Moderate localized temperature increases in skin during plasmid DNA delivery have resulted in 8-fold more expression or the magnitude of the applied pulses (voltage/field intensity) could be reduced by about 50% to achieve the same expression when compared to optimal delivery performed at ambient temperature. Similarly, adjusting pulse parameters during electrical treatment based upon real-time tissue impedance measurements has resulted in 6-15-fold increases in expression. Pulse magnitudes could also be reduced by 50% to still achieve increased expression relative to traditionally optimized conditions. One area of focus has been to demonstrate that GET was an effective tool for delivering plasmid DNA to the skin. Using this new modified GET approach we evaluated the delivery of a DNA vaccine against Hepatitis B Virus. Guinea pigs were injected intradermally with a plasmid encoding Hepatitis B viral surface antigen followed by GET with or without moderate heating. After vaccination boost, αHBSAg serum titers indicated a 20-fold increase in antibody levels up to 30 weeks post-vaccination in those animals receiving GET combined

with moderate heating compared to GET alone. This enhanced GET approach has also been evaluated for delivery to solid tumors. In a B16.F10 mouse melanoma model enhanced delivery was achieved with both small molecules as well as plasmid DNA. Delivery of plasmids encoding cytokines and/or PD1 resulted in increased regression and long-term survival of treated mice. The utilization of this enhanced approach enables a more controlled delivery and application of the pulse fields. This enables the user to compensate for differences between tissues as well as between subjects.

457. Ultrasound Mediated Gene Delivery Specifically Targets Liver Sinusoidal Endothelial Cells for Sustained FVIII Expression in Hemophilia A Mice

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Hemophilia A (HA) is a bleeding disorder in which an individual cannot produce functional clotting factor VIII (FVIII). This is a prime target for gene therapy because even minimal increases in FVIII activity can improve the quality of life. In previous experiments, we found liver sinusoidal endothelial cells (LSECs), the native production site of FVIII, could be specifically targeted with ultrasound-mediated gene delivery (UMGD) at a lower power than those used to target hepatocytes in mice. In this study, we explored energy conditions that target LSECs with UMGD to induce persistent FVIII expression in HA mice. The mouse liver was injected via the portal vein with a combination of plasmid DNA and RN18 microbubbles (MBs). Simultaneously, to enhance gene transfer via cavitation, a pulsed therapeutic US transducer was applied to the surface of the liver for one minute at 1.1 MHz frequency and 14 Hz PRF. HA mice were separated into two groups treated with different US conditions, low energy (LE; 50 W/cm², 150us PD) targeting predominantly endothelial cells or high energy (HE; 110 W/cm², 150 us PD) targeting predominantly hepatocytes. This was coupled with a new endothelial-targeting, high-expressing hFVIII plasmid, pUCOE-ICAM2-hF8/N6-X10. The APTT assay was utilized to study hFVIII activity levels in the mouse plasma and the Bethesda assay for the formation of anti-FVIII inhibitors over 86 days. Livers were harvested at various time points, sectioned at 7 μm, stained, and imaged with a Leica DM6000 fluorescent microscope. FVIII activity levels for the LE group were comparable to that of the HE group and stabilized at approximately 10% at 84 days post-treatment (Figure 1a). When assessing damage to the liver, ALT levels were transiently increased in the initial few days post-treatment and rapidly returned to the normal range in both the HE and LE groups. However, the LE mice showed a significantly smaller initial increase, indicating reduced transient liver damage compared to the HE group. Half of the HE-treated mice developed low-titer inhibitors over 84 days, while none of the LE mice had a measurable formation of inhibitors (Figure 1b). RNAscope® Multiplex Fluorescent staining showed occurrences of colocalization of hFVIII and Lyve-1 (an LSEC marker) mRNA at D7 and D120 in the LE mice. This indicates that the LE US conditions can

efficiently deliver the endothelial-specific hFVIII plasmid to the LSECs to produce persistent, therapeutic levels of FVIII gene expression with reduced transient liver damage and inhibitor formation.

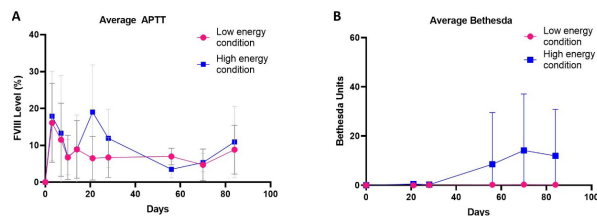


Figure 1. (A) HA mice in both the LE and HE ultrasound treatment groups had hFVIII activity levels between 5-25% with stabilization of around 10% at day 84. (B) The HE mice showed measurable amounts of anti-hFVIII inhibitors starting at day 56, while the LE had no quantifiable amount.

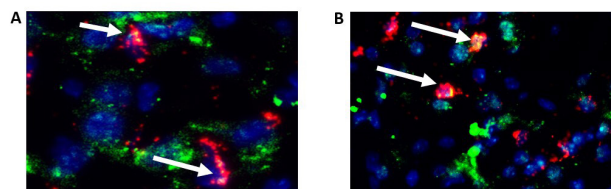


Figure 2. Livers from LE mice harvested at day 7 (A) and day 120 (B) showed expression of hFVIII mRNA shown in green, and Lyve-1 mRNA, an endothelial-specific marker, in red. Colocalization appears yellow

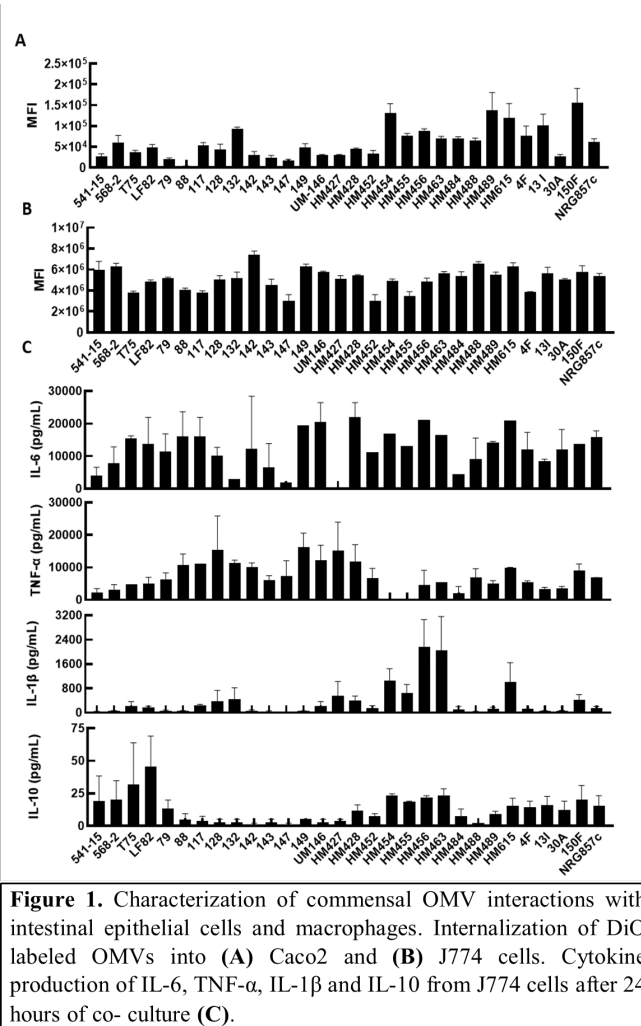
458. Assessment of Commensal E. coli Outer Membrane Vesicles for Application in a Novel Oral Delivery System

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Introduction: Gene delivery via the oral route is desirable due to the high rate of patient compliance, ease of administration and large cellular surface area present for transfection¹. To overcome challenges associated with oral gene delivery, we are developing a novel delivery system by loading outer membrane vesicles (OMVs) with plasmid DNA to create DNA-loaded OMV nanocarriers (DNA-OMV NCs). Commensal bacteria residing in the human GI tract, including *E. coli*, produce OMVs, which act similarly to exosomes by protecting and trafficking DNA, RNA, protein and other small molecule cargo². OMVs are not only a conduit of information between bacteria cells, but they can also survive gastric transit, cross the mucosal barrier within the intestine and be internalized by intestinal epithelial cells³, thus making them an ideal biomaterial for oral delivery. In preliminary work, we developed methods for loading DH5- α OMVs with plasmid DNA via electroporation. To identify new commensal OMV sources to be utilized as DNA-OMV NCs, we have screened OMVs isolated from an existing collection of 30 human gut commensal *E. coli* strains for properties such as cytokine production and internalization into an intestinal epithelial cell line (Caco2) and murine macrophage line (J774). **Methods:** All 30 *E. coli* strains were cultured as previously described⁴, and OMVs were isolated using a Vivaspin centrifugal concentrator. Total protein content of the OMVs was quantified using a BCA assay. OMVs were labeled with the lipophilic fluorescent dye DiO, co-cultured with Caco2 and J774 cells for 24 hr at 50 μg OMV/

mL and then analyzed using flow cytometry. To determine cytokine responses, OMVs were co-cultured with J774 cells for 24 hr at 50 μ g OMV/mL. Supernatants from cell cultures were analyzed for IL-1 β , IL-6 and TNF- α and IL-10 production. **Results and Discussion:** Notable differences were observed among the various OMVs in terms of their internalization rates by Caco2 and J774 cells (**Fig. 1 A and B**). Additionally, unique profiles of pro- (IL-6, TNF- α and IL-1 β) and anti-inflammatory (IL-10) cytokine production from J774 cells were observed across the 30 OMVs tested (**Fig. 1 C**). No correlations between internalization and cytokine production were found (data not shown), indicating that other factors such as surface receptors and/or cargo are potentially responsible for the differences in cytokine production observed among the strains in this screen. Lead candidate OMVs are now being used to formulate DNA-OMVs NCs and are being tested for pDNA loading efficiency, gastric protection of pDNA cargo and transfection efficiency. **Conclusions:** The data collected in this screen demonstrate that OMV properties such as internalization and cytokine production can differ between strains of bacteria. Selection of appropriate OMVs is critical for further development of our DNA-OMV NCs by improving internalization and providing the opportunity to endow our oral gene delivery system with immunomodulatory properties that act synergistically with the delivered transgene, thus allowing for customization of the DNA-OMV NC platform for a variety of oral delivery applications. **References:** 1. Farris E. *et al.*, *Curr Opin Biomed Eng* 2018 2. Kaparakis-Liaskos M. *et al.*, *Nat Rev Immunol* 2015 3. Schwechheimer C. *et al.*, *Nat Rev Micro* 2015 4. Kittana H. *et al.*, *Front Immunol* 2018



459. Extracellular Vesicle-Mediated Therapeutic Delivery of RIG-I Agonists for Immunotherapy Against Breast Cancer

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Breast cancer is traditionally regarded as a poorly immunogenic tumor type and harbors low abundance of tumor-infiltrating immune cells. Cancer immunotherapy is gaining clinical momentum but has not achieved widespread success in breast cancer. RIG-I is a cytoplasmic immunoreceptor and can be activated by RIG-I agonists to induce host immune responses. RNA molecules with 5'-triphosphate ends as RIG-I agonists have shown promise in cancer treatments. Whilst the delivery

of these molecules to tumor cell *in vivo* is efficient using hitherto polymer-based delivery system, the safety remains controversial. We have developed a novel platform for RNA therapeutic delivery using red blood cells derived extracellular vesicles (RBCEVs). RBCEVs are ideal therapeutic carriers as they are readily available, non-immunogenic and non-oncogenic. RBCEVs are easily amenable to RNA loading and surface functionalization. In this study, we delivered two RIG-I agonists, the immunomodulatory RNA (immRNA) and a bi-functional 5' triphosphorylated antisense oligonucleotide against oncogenic miR-125b (3p-125b-ASO) using RBCEVs *in vitro* and *in vivo*. We demonstrate that the delivery of immRNA and 3p-125b-ASO using RBCEVs induced RIG-I cascade activation, type I IFN production, and immunogenic cell death in breast cancer cells. Intratumoral administration of immRNA and 3p-125b-ASO in RBCEVs significantly suppressed tumor growth of mammary breast cancer, and induced immune cell infiltration and tumor cell apoptosis mediated by RIG-I activation, turning the 'cold' tumors 'hot'. Furthermore, we demonstrate that intrapulmonary delivery of immRNA-loaded RBCEVs modified with EGFR-targeting nanobodies actively enhanced the potency of immRNA, leading to suppression of tumor metastasis and elevated tumor-specific immune responses in EGFR-positive metastatic breast cancer mouse models. Thus, delivery of RIG-I agonists using RBCEVs could be a promising alternative for breast cancer immunotherapy.

460. Engineering Cells to Produce miRNA-Loaded Exosomes for Potential Biotherapeutics

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Introduction: Exosomes are extracellular vesicles that are under intense research for therapeutic applications due to their ability to deliver biomolecules to recipient cells with high efficiency and modulate many pathophysiologic processes. Recent studies suggest that many therapeutic effects of mesenchymal stem cells (MSCs), a cell type that has been investigated in >1000 clinical trials, are actually mediated by the exosomes secreted by MSCs, which make MSC exosomes attractive candidates for off-the-shelf therapies that could avoid manufacturing and safety challenges associated with whole cells. Additionally, many therapeutic effects of exosomes are associated with microRNAs (miRNAs) contained within the vesicles. This work aimed to engineer a system to actively load miRNAs of choice into exosomes using cellular machinery, to produce exosomes with enhanced therapeutic properties. The system uses expression of a minimal scaffold of the vesicular stomatitis virus glycoprotein (mVSVG) [1], a transmembrane protein that inserts into exosomes. mVSVG was fused at the C-terminus to RNA aptamer-binding domains to localize these fusions inside exosomes, and facilitate loading of transgenic precursor miRNA (premiR) containing RNA aptamers. **Methods:** The components of the transgenic miRNA loading system were expressed from plasmids (Figure 1).

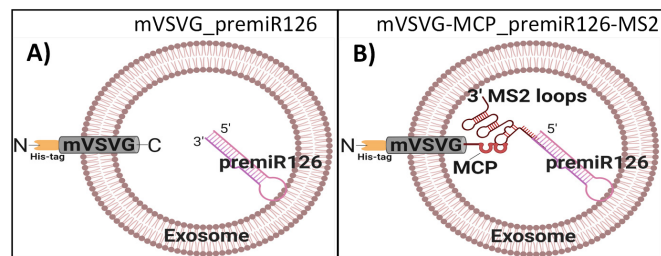


Figure 1: A) *mVSVG_premiR126* expresses mVSVG with no C-terminal fusion and premiR126 with no 3' aptamer B) *mVSVG-MCP_premiR126-MS2* expresses mVSVG with C-terminal fusion to MS2 coat protein (MCP) and premiR126 with MS2 loop aptamer. Figure created with BioRender.com HEK293T cells were transfected in 96-well plates with plasmids expressing the described system, using Turbofect reagent. After 48 hrs, exosomes were isolated from media and lysed to extract RNA. Exosomal miRNA was reverse transcribed and amplified with TaqMan miRNA cDNA Synthesis Kit prior to quantification of relative mature miR126-3p and -5p by qRT-PCR, normalized to mature miR100-5p, using TaqMan Advanced miRNA Assays. All experiments were performed in triplicate (n=3) and on duplicate days. **Results:** Transfection with *mVSVG_premiR126*, resulted in about 10-fold more mature miR126-3p and -5p in exosomes than EGFP-Luc control, indicating that increased transcription of a precursor miRNA can increase the amount of the mature miRNA in secreted HEK293T exosomes passively. However, transfection of HEK293Ts with *mVSVG-MCP_premiR126-MS2* constructs (with MS2 loop aptamers linked to either the 3' or 5' end of premiR126) resulted in about 500-fold increases in mature miR126-3p and -5p contained in exosomes, relative to passive loading with *mVSVG_premiR126*. The location of the MS2 aptamers on premiR126 (i.e. 3' or 5' linked) did not bias active loading of mature miR126-3p or -5p into exosomes. In contrast, if MS2 aptamers were linked to both the 3' and 5' ends of premiR126, little to no mature miR126-3p and -5p was loading into exosomes, relative to passive loading with *mVSVG_premiR126*. **Conclusion:** The described system is able to actively load a miRNA of interest into exosomes of transfected cells, while also allowing normal processing to yield high levels of the mature -3p and -5p miRNA species within exosomes, suggesting the system could be used to produce exosomes with enhanced therapeutic properties. Future work aims to translate this technology for engineering of exosomes produced from therapeutically relevant MSCs. **References:** [1] Meyer C, et al. *Int J Nanomedicine*. 2017.

Inborn Errors of Metabolism Gene and Cell Therapies: Proof-of-Concepts and Beyond

461. Safety and Efficacy of a Dual-Function AAV9 BCKDHA-BCKDHB Gene Replacement Vector in Murine and Bovine Models of Classic Maple Syrup Urine Disease

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Classic maple syrup urine disease (MSUD) results from biallelic mutations of *BCKDHA*, *BCKDHB*, or *DBT* that encode subunits of branched chain alpha-ketoacid dehydrogenase (BCDH), which decarboxylates ketoacid derivatives of leucine, isoleucine, and valine (BCAAs). MSUD is a volatile and dangerous inborn error of metabolism that remains a cause of death and disability worldwide. Dietary therapy is challenging to implement and punctuated by life-threatening neurological crises. Liver transplantation stabilizes BCAAs but introduces serious surgical and immunological risks. In 2018, we began collaborating to develop a dual-function precision therapy for the two common genetic forms of MSUD. Our clinical team analyzed data from 184 MSUD patients and 13,589 plasma samples to delineate salient endpoints for preclinical studies and human trials. We generated three model organisms: engineered *Bckdha*^{-/-} and *Bckdhb*^{-/-} mice and a newborn calf naturally homozygous for *BCKDHA* c.248C>T. All animals exhibit severe biochemical derangements hours after birth and die within 10 days. We designed a dual-function, AAV9 gene replacement vector co-expressing codon-optimized *BCKDHA* and *BCKDHB* cDNA. This construct restored BCDH activity to wildtype levels in *BCKDHA*^{-/-} HEK293 cells. Intravenous (IV) injection of the vector into *Bckdha*^{-/-} mice at P0 rescued lethality in a dose-dependent manner. Surviving mice grew and behaved similar to wildtype littermates, had normal or nearly normal plasma BCAAs after 16 weeks on unrestricted diet (20% protein), and tolerated a 40% dietary protein challenge for 7 days. The same AAV9 also rescued early lethality in engineered *Bckdhb*^{-/-} mice. We next tested the vector in the bovine model. As compared to humans, MSUD calves exhibit a similar neuropathological phenotype, are closely matched for size and metabolic rate, and evince the important role of muscle in human BCAA catabolism. Without treatment, they are prostrate with cerebral edema by day of life 3 and die soon after. A *BCKDHA* c.248C>T

homozygous heifer born October 2021 developed biochemical signs of classic MSUD hours after birth. Plasma leucine increased to 1314 $\mu\text{mol/L}$ (reference mean $155 \pm 18 \mu\text{mol/L}$) by 15 hours of life in association with deepening encephalopathy. Leucinosi was controlled using a combination of IV and enteral therapies until 40 hours of life, when we infused 5×10^{13} vg/kg of AAV9 bovine *BCKDHA*-*BCKDHB* intravenously without prophylactic immunosuppression. Plasma BCAAs, their molar concentration ratios, and clearance of alloisoleucine from plasma demonstrated restoration of BCDH activity *in vivo*. The calf was transitioned to normal unrestricted diet 70 days after treatment and continues to thrive 100 days post-infusion with stable BCAA concentrations at twice the upper reference limit. These data indicate the safety and efficacy of a novel AAV9 dual-function *BCKDHA*-*BCKDHB* replacement vector as one-time treatment for the most common and severe forms of MSUD, and demonstrate the power of intentional coalitions to accelerate development of genetic therapies.

462. Homology Independent Targeted Integration Leads to Highly Efficient Protein Expression and Secretion From Liver

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Liver gene therapy with adeno associated viral (AAV) vectors holds great promise to provide long term expression and secretion of therapeutic transgenes after a single administration. AAV vectors are predominantly non integrating, leading to loss of transgene expression over time due to liver proliferation or liver damage. We took advantage of AAV mediated homology independent targeted integration (HITI) by CRISPR/Cas9 to integrate transgenes of interest at the 3' end of mouse Albumin (*mAlb*) which is highly expressed in hepatocytes. Proof of concept in new born mice using the reporter gene Discosoma red (DsRed) shows higher levels of liver transgene expression with HITI than with conventional gene therapy. Exchange of DsRed with ARSB which encodes for arylsulfatase B, the lysosomal enzyme deficient in mucopolysaccharidosis VI (MPS VI), resulted in circulating supraphysiological levels of enzyme and phenotypic improvement up to 48 weeks after neonatal delivery in MPS VI mice. Overall, AAV HITI results in stable, high expression levels of a therapeutic transgene from liver with hepatocyte proliferation and should be considered for treatment of those conditions that could benefit from liver targeting and require early intervention.

463. 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 episodic hyperammonemia that can be life threatening and cause cumulative neurocognitive damage. Current medical management includes a protein-restricted diet and nitrogen scavenging agents. However, even with the best standard of care, the risk of life-threatening hyperammonemic crisis remains. DTX301, an AAV8 vector containing the OTC transgene, is being investigated for treatment of OTC deficiency.

Methods: CAPtivate (NCT02991144) is a global, open-label phase 1/2 trial evaluating DTX301 safety and efficacy in adults with late-onset OTC deficiency. The primary endpoint is incidence of adverse events (AEs). Patients received one IV infusion of DTX301: Cohort 1 received 3.4×10^{12} Genome Copies (GC)/kg; Cohort 2 received 1.0×10^{13} GC/kg; Cohort 3 received 1.7×10^{13} GC/kg, and Cohort 4 received 1.7×10^{13} GC/kg with prophylactic oral steroids. Complete responders were defined as those who discontinued all ammonia-scavenging drugs and protein-restricted diet. Responders were defined as those who had $\geq 50\%$ reduction in baseline disease management.

Results: Enrollment is complete with 11 patients dosed. No treatment-related serious AEs or dose-limiting toxicities were reported. All AEs during study were mild or moderate (grade 1 or 2), with the exception of one patient with grade 3 hyperammonemic crises, assessed as related to OTC deficiency and unrelated to DTX301, oral corticosteroids, or study procedures. Eight patients experienced treatment-related AEs. All eight experienced asymptomatic ALT increases managed with oral corticosteroids. Other treatment-related AEs reported in these patients were photophobia, headache, hypertension, and hypophosphatemia. Seven patients responded to DTX301 (4 complete responders, 3 responders). Cohort 1 had one complete responder; Cohort 2 had one complete responder and one responder; Cohort 3 had one complete responder and two responders; and Cohort 4 had one complete responder. The second patient in Cohort 4 was a responder after reducing medications and increasing dietary protein, but these changes were reversed after noncompliance with diet and medications and two viral infections. The longest-treated responder has a durable response 4 years post-treatment.

Conclusions: DTX301 has shown promising results to date. Continued

work is planned to study DTX301 as a potential new treatment for patients with OTC deficiency, and the Cohort 3 dose of 1.7×10^{13} GC/kg has been selected for study in a Phase 3 trial.

464. Genome Editing in a Canine Model for Glycogen Storage Disease Type Ia

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Glycogen storage disease type Ia (GSD Ia), the deficiency of glucose-6-phosphatase (G6Pase), is associated with life-threatening hypoglycemia as well as longer-term adverse effects including hepatocellular adenoma and carcinoma formation. Gene replacement therapy for GSD Ia has been developed, although it fails to stably reverse G6Pase deficiency. In order to more stably treat GSD Ia, genome editing with adeno-associated virus (AAV) vectors that delivers CRISPR/Cas9 and a Donor transgene encoding G6Pase were evaluated in a canine model for GSD Ia at two different ages. Adult dogs with GSD Ia were treated with genome editing at 34 months of age to demonstrate Donor transgene integration in two of 3 dogs accompanied by stable G6Pase expression and correction of hypoglycemia during fasting for over 16 months. These dogs were previously treated with gene replacement therapy during infancy to control the symptoms of GSD Ia. Gradual loss of AAV vector genomes were detected in liver as expected, although the initial gene replacement vector could be detected at 51 months of age for the adult genome editing group. Similarly, 3 newborn puppies with GSD Ia were treated with genome editing as well as gene replacement therapy to control acute symptoms, and two of 3 puppies demonstrated Donor transgene integration accompanied by stable biochemical correction for over 16 months. In the adult group of dogs, anti-Cas9 antibodies were detected prior to the administration of CRISPR/Cas9-mediated genome editing reflecting earlier exposure to *Staph aureus*. Cas9 nuclease activity was demonstrated in two of 3 adult dogs and in two of 3 puppies by Sanger sequencing, which revealed that 10-30% of alleles contained small insertions and deletions (indels) at the targeted genomic site. The detection of indels confirmed that Cas9 nuclease activity had created double-stranded breaks followed by non-homologous end joining (NHEJ). The presence of NHEJ correlated with transgene integration by homology directed repair (HDR), and the latter was confirmed by Sanger sequencing of the junction between the Donor transgene and *G6PC* gene. This study confirmed that genome editing can integrate a therapeutic transgene in the liver of a large animal model for an inherited metabolic disorder, either early or later in life, despite the presence of anti-Cas9 antibodies. Further development of genome editing is warranted to provide a more stable treatment that will more effectively prevent long-term complications of GSD Ia.

465. Liver Directed Lentiviral Gene Therapy Ameliorates the Phenotype of Progressive Familial Intrahepatic Cholestasis Type 2 in a Mouse Model

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Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is a monogenic inborn error of metabolism caused by the deficiency of the liver bile salt export pump (BSEP). BSEP is responsible for the transport of conjugated bile acids (BA) across the canalicular membrane of hepatocytes. PFIC-2 patients display cholestasis, jaundice and severe itching and usually develop liver fibrosis and end-stage liver disease before adulthood due to massive BA accumulation into the hepatic tissue. BSEP is expressed only in the liver and indeed no extrahepatic manifestation are observed in these patients. Current treatment consists of diet control, pharmacological or surgical treatments such as biliary diversions aiming at interrupting the enterohepatic circulation and decreasing BA blood concentration. However, most patients ultimately undergo liver transplantation as only curative option. Gene therapy may represent a therapeutic option for PFIC-2, holding the potential to rescue BSEP expression upon a single intravenous (i.v.) administration. In a condition such as PFIC-2, which is characterized by an early onset and a rapid disease progression, an early therapeutic intervention would be fundamental to alleviate the symptoms and limit progressive damage to the hepatocytes. Lentiviral vector (LV) mediated gene therapy would potentially allow for a stable gene transfer even in paediatric patients at the first disease stages by virtue of LV genomic integration. LV have been previously shown to allow efficient and safe liver gene transfer in small and large animal models, following i.v. administration. We assessed LV-mediated liver gene therapy in a mouse model of PFIC-2, the *Abcb11*^{-/-} mice. Here, we first characterize this PFIC-2 mouse model and show that *Abcb11*^{-/-} mice display a growth defect until weaning, a progressive impairment of liver function with accumulation of serum and liver BA and rise of circulating bilirubin, aspartate aminotransferase, alanine transferase and alkaline phosphatase (ALP) from 2 months of age. Notably, the increase in BA and bilirubin was higher in *Abcb11*^{-/-} females, thus females are more susceptible to cholestatic liver injury compared to *Abcb11*^{-/-} males. We generated and produced LV encoding a human codon optimized BSEP transgene under the control of a hepatocyte-specific cassette based on the enhanced transthyretin promoter (ET-BSEPco). We administered ET-BSEPco to juvenile *Abcb11*^{-/-} mice at a dose of 3×10^{10} transducing units (TU)/kg by i.v. injection. The treatment resulted in a significant decrease of serum BA, bilirubin and ALP biomarkers until 6 months of age, which was more pronounced in male *Abcb11*^{-/-} mice, indicating prevention of the progressive cholestatic damage. Treated animals will be monitored until 9-12 months of age for long term analyses of the PFIC-2 biomarkers, then the liver will be collected for end-point analyses. These analyses will inform about LV mediated liver transduction, histopathology, and transgene expression and potentially define therapeutic threshold for *Abcb11*^{-/-} mice. We show the first, to our knowledge, *in vivo* LV mediated gene therapy of

PFIC-2 mouse model. Our data provide evidence for therapeutic effect in this disease by LV gene therapy and encourage further development towards clinical translation.

466. Nuclease Enhancement of AAV Mediated Editing into Albumin in Neonatal Mice with Methylmalonic Acidemia (MMA)

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MMA is a rare inborn error of metabolism typically caused by a deficiency of methylmalonyl-CoA mutase (MMUT). Patients suffer from frequent episodes of metabolic instability, severe morbidity, and early mortality. Although elective liver transplantation can eliminate the devastating metabolic instability associated with this disorder, transplantation is not a viable treatment strategy for most patients. Various gene therapies have been explored in MMA mouse models as an alternative to liver transplantation with promising results. We recently reported a study of promoterless, nuclease-free editing into the 3' end of *Albumin* (*Alb*) in a transgenic murine model of MMA (MCK MMA) (PMC8252383). This approach resulted in improved clinical and biochemical phenotypes in the MCK MMA mice, but not to wildtype levels. Additionally, most parameters seen in 3' *Alb* edited MCK MMA mice did not improve until several months post-treatment, when a growth advantage caused substantial expansion of corrected hepatocytes. The use of a nuclease to increase editing at the 3' of *Alb* has yielded impressive phenotypic correction in a mouse model of Crigler-Najjar syndrome and resulted in more widespread and improved transgene expression in the mice treated with a donor and nuclease compared to donor only treated mice (PMC6693827). To investigate if 3' *Alb* editing with a nuclease could lead to an increase in the number of corrected hepatocytes and accelerate the therapeutic benefits in the MMA mice, we redesigned the previously described Alb-2A-MMUT (Alb-2MMUT) vector to incorporate longer arms of homology with a mutated PAM site, mPAM-Alb-2A-MMUT (mPAM2MMUT = donor) and designed a SaCas9 vector that also expressed a guide targeting the intronic sequence following the native stop codon of *Alb*. MCK MMA mice and controls were treated as neonates with donor (2.5e11vg/pup) or donor and SaCas9 (6e10vg/pup) vectors. The donor treated MCK MMA mice (n=3) did not have increased weight compared to untreated mice (n=9) (13.6g ± 1.0 vs. 15.0g ± 0.9; p=0.4 at 4 months). The donor plus SaCas9 treated MCK MMA mice (n=4) showed a trend of improved weight gain (17.7g ± 0.7; p=0.07 at 4 months), which was well below WT controls. In the first few months, MCK MMA mice treated with donor only had reduced plasma methylmalonic acid (pMMA) compared to untreated mice, but at 7 months, pMMA rebounded (1023µM ± 162, n=2 v. 1161µM ± 286, n=5). However, mice treated with donor and SaCas9 had improved metabolic correction, with lower pMMA (330µM ± 28, n=3, p=0.07) than the donor only treated mice. Compared to historical controls treated with the previously described Alb-2MMUT vector (n=3), mice treated with the donor plus SaCas9 vector (n=3) produced substantially more hepatic MMUT (225% ± 10 vs. 74% ± 17 of wildtype Mmut levels, p=0.001) and had more corrected hepatocytes as measured by RNA In Situ Hybridization (RNAscope) (43% ± 0.4 of hepatocytes vs. 5% ± 2, p=0.04) when studied at 8-10 months. Summary: The use of SaCas9 with the donor

vector dramatically increased the number of corrected hepatocytes compared to previous results obtained with the promoterless, 3' *Alb* targeted, integrating AAV vector (Alb-2MMUT). However, despite a greatly increased frequency of *Alb* editing the clinical and biochemical responses were not as dramatic as expected based on previous neonatal systemic AAV gene therapy studies using a more severe neonatal lethal, full knockout murine model of MMA (PMC2839224). The discrepancy between the two correction approaches suggests an intrinsic limitation of 3' *Alb* editing as a treatment for MMUT deficiency for reasons that remain unclear.

467. Updated Interim Results of Transpher A, a Multicenter, Single-Dose, Pivotal Clinical Trial of ABO-102 Gene Therapy for Sanfilippo Syndrome Type A (Mucopolysaccharidosis IIIA)

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INTRODUCTION: Sanfilippo syndrome type A, or mucopolysaccharidosis IIIA (MPS IIIA), is a fatal, neurodegenerative disorder caused by a recessive defect in the N-sulfoglucosaminase sulfohydrolase (*SGSH*) gene that leads to aberrant lysosomal storage of the glycosaminoglycan (GAG) heparan sulfate (HS). It generally manifests in early childhood with characteristic neurocognitive decline, adaptive behavioral deficits with high caregiver burden, accumulation of disease-specific biomarkers including GAGs and HS, and physiological changes including hepatomegaly and brain atrophy. MPS IIIA has no clinically approved treatments and represents a high unmet treatment need. **METHODS:** Transpher A (NCT02716246) is a pivotal clinical trial of investigational gene therapy ABO-102 (scAAV9-based vector expressing *hSGSH*) for the treatment of MPS IIIA. Patients have been enrolled across three dose cohorts: Cohort 1 (5×10^{12} vg/kg, n = 3), Cohort 2 (1×10^{13} vg/kg, n = 3), and Cohort 3 (3×10^{13} vg/kg, n = 10 patients in Cohort 3 who were treated at <2 years old or development quotient [DQ] ≥ 60). The primary endpoint includes analysis of the cognitive domain of neurocognitive development. Secondary endpoints include additional neurocognitive assessments (Mullen Scales of Early Learning, MSEL), behavioral changes (Vineland Adaptive Behavior Scale, VABS), biomarkers (GM2, GM3, HS), and magnetic resonance imaging volumetric analysis for liver and brain. Data have been collected at baseline and at months 1, 6, 12, 18, and 24 posttreatment; after month 24, patients are transferred to a long-term follow-up study for 3 years (NCT04360265). **RESULTS:** Investigational ABO-102 was well tolerated (median follow-up: Cohort 1, 65.8 months; Cohort 2, 57.4 months; Cohort 3, 28.2 months). Compared with MPS IIIA natural history data and subtherapeutic ABO-102 doses (Cohorts 1 & 2), the majority of the ten pivotal patients treated with the therapeutic dose (Cohort 3) demonstrated neurocognitive improvement, and six

tracked along normal development range (DQ ≥ 60). Preliminary VABS analyses showed overall clinical improvement in the pivotal Cohort 3 population across five behavioral domains. Cohort 3 also was associated with sustained and statistically significant reductions in disease-specific biomarkers and overall stabilization of liver and brain volumes. **CONCLUSIONS:** Investigational ABO-102 gene therapy in the youngest patients treated before advanced neurodegeneration (<2 years old or DQ ≥ 60) in Cohort 3 is associated with overall improvements in neurocognitive assessments, behavioral domains, biomarkers, and physiological volumetric changes that typically progressively decline in MPS IIIA without treatment or at subtherapeutic doses (Cohorts 1 and 2). Collectively, these data demonstrate the potential for investigational ABO-102 gene therapy to address a high unmet treatment need for patients with MPS IIIA and their caregivers.

Applications of Improved Gene Therapy Methods in Neurologic Disorders

468. Intrathalamic Delivery of AVB.PGRN Rescues Pathology in *Grn* Null Mice and Achieves Widespread Cortical Expression in a Large Animal Model without Expression in the Liver

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Background: Frontotemporal dementia (FTD) presents with changes in personality, behaviour and language and is the second most common cause of young-onset dementia. Loss of function mutations in *GRN* encoding progranulin (PGRN) causes FTD in the heterozygous state and late-onset neuronal ceroid lipofuscinosis when bi-allelic. PGRN deficiency leads to lysosomal dysfunction resulting in the accumulation of neuronal TDP-43 and exaggerated microglial reactivity that accelerate neurodegeneration. To date, effective vector delivery to the CNS for treating FTD remains a challenge. Here, we describe the intrathalamic (ITM) delivery of low dose adeno-associated virus (AAV) carrying a functional copy of the human *GRN* gene (AVB.PGRN) by exploiting the thalamocortical projection to rescue pathology in a mouse model of PGRN deficiency and demonstrate widespread cortical PGRN expression following bilateral injections in sheep. **Methods:** To minimise vector dose and to restrict PGRN expression to neurons, we engineered our AAV cassette to include (i) a codon optimised PGRN sequence, (ii) 5' and 3' flanking regions for enhanced PGRN expression, and (iii) a neuron-specific promoter. *In vitro* experimental testing of the construct was initially optimised in neuronal cultures. Efficacy studies were conducted in which AVB.PGRN was delivered via ITM injection in *grn*-deficient (*Grn*^{-/-}) mice. Biodistribution studies were carried out in sheep to evaluate ITM and intracisterna magna (ICM) delivery approaches for AVB.PGRN. **Results:** We present three key findings: (1) The AVB.PGRN vector leads to PGRN expression and secretion in rat primary neurons. (2) Bilateral ITM injection of AVB.PGRN in *Grn*^{-/-} mice suppressed neuronal lipofuscinosis in the thalamus, hippocampus and prefrontal cortex, even with the lowest

dose. (3) ITM injection of AVB.PGRN in sheep resulted in widespread cortical and basal ganglia PGRN expression without evidence of significant glial activation or neuronal loss, while ICM delivery into the cerebrospinal fluid (at 500 times the ITM dose) failed to deliver detectable PGRN expression outside of the cerebellum. CSF levels of PGRN following ITM injection in sheep mirrored the dose delivered to the cortex with PGRN levels undetectable in serum. Importantly, no significant AAV genomes were observed in the liver. **Conclusions:** Intra-thalamic delivery of AVB.PGRN rescues pathology observed in the *Grn*^{-/-} mouse model and achieves widespread PGRN expression in the cortex of a large mammal with no PGRN expression outside the CNS. Taken together, the data suggest that AVB.PGRN delivered by ITM injection constitutes a novel and promising approach to address areas of current medical need in FTD.

469. AAV-Based GDNF Expression in VTA Prevents Relapse to Alcohol-Drinking Behavior and Modifies Mesolimbic Dopamine Function in Rhesus Macaques: A Gene Therapy Approach to Treating Alcohol Use Disorder

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Background: Alcohol use disorder (AUD) exacts enormous personal, social, and economic costs globally. The few FDA-approved pharmacotherapies available for AUD are often insufficient in sustaining long-term abstinence in treatment-seeking individuals and can cause unfavorable side effects. Consequently, relapse in AUD patients is very common following treatment onset, engendering a cycle of repeated abstinence-relapse episodes. Thus, there is an urgent clinical need for a treatment that durably extends abstinence and reduces off-target effects. Aberrant function of dopaminergic reward circuits in the limbic system may be central to AUD, and lasting signal modulation in this pathway may counter the propensity for relapse to excessive alcohol use. **Methods:** To test this hypothesis, we explored the overexpression of glial cell line-derived neurotrophic factor (GDNF), a trophic agent for dopaminergic neurons, within the ventral tegmental area (VTA) as our treatment strategy. A cohort of male rhesus macaques were induced to consume alcohol and then provided unlimited access to a 4% w/v alcohol solution and water for 6 months. Animals were then forced to abstain from alcohol for 1 month, followed by surgical treatment. Using MR-guided convection-enhanced delivery, either vehicle or adeno-associated virus serotype 2 encoding human GDNF (AAV2-hGDNF) was infused bilaterally into the VTA of the macaques (n=4/group; 30 μ L/side; 1.0E+13 vector genomes). An additional 2

months of abstinence was provided to allow for surgical recovery, viral transduction, and stabilization of GDNF expression. Over a 12-month period the macaques were subjected to 6 “relapse” cycles, each comprised of 1-month of reinstated alcohol access followed by 1-month of abstinence. *Ex vivo* brain slices were used for fast scan cyclic voltammetry. **Results:** Vehicle-treated animals consumed total amounts of alcohol comparable to their pre-abstinence baseline during each relapse opportunity, while also exhibiting maximum bout sizes that escalated over successive relapse cycles, a hallmark of chronic AUD. In contrast, this pattern of escalation at relapse onset was entirely prevented in AAV2-hGDNF treated animals, and daily alcohol consumption was reduced by 80-90% versus pre-abstinence baselines. These behavioral changes correlated with neurophysiological changes within the mesolimbic pathway, whereby AAV2-driven increases in GDNF expression corrected the hypodopaminergic state observed in the mesolimbic system of vehicle-treated animals. HPLC analysis showed increased dopamine levels in VTA and increased dopamine metabolism throughout the mesolimbic system, while *ex vivo* fast scan cyclic voltammetry showed altered dopamine release dynamics in the nucleus accumbens. **Conclusion:** Our results provide evidence for therapeutic modulation of limbic reward circuits in preventing relapse of heavy alcohol consumption following attempted abstinence. These findings suggest that targeting relapse prevention with GDNF gene therapy may be a viable clinical strategy for treatment of AUD.

470. Vectorized Delivery of Tau Reduction Therapy as a Treatment Approach for Tauopathies

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Tauopathies are a group of neurodegenerative disorders characterized by the accumulation of abnormal tau protein in the brain. In humans, tau mutations can cause neurodegeneration and cognitive decline is closely correlated with tau deposition. This predicts that reduction of tau should ameliorate disease. Preclinical studies testing tau anti-sense oligos (ASOs) in the PS19 tauopathy mouse model showed a prevention of neuronal loss and a reversal of pathological tau deposition and seeding, and this treatment is being tested in clinical trials. While promising, ASOs therapies require repeated, life-long intrathecal administration. We propose an alternative 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 6 isoforms of tau found in the human or mouse brain. *In vitro* studies showed that our tau miRNAs efficiently and selectively reduced human or mouse tau expression. We then packaged the lead tau miRNA candidates into AAV9 vectors for high targeting of the brain. To assess the therapeutic benefit of our lead human-tau specific candidate (AAV9/hTau5i), we used the PS19 mouse model of tauopathy, which carries human tau with the P301S mutation. Non-transgenic (NT) and P301S transgenic littermates were intra cisterna-magna (ICM) injected with AAV9/hTau5i, an AAV9 vector carrying a control scrambled miRNA (AAV9/Scr), or vehicle at 3 months, 6 months, or 9 months of age, during early, intermediate and late stages of disease progression, respectively. Animals were assessed for body weight gain

and survival for 3 months post injection and tissues were then collected for histological and biochemical analysis. In life analysis showed no adverse effects from either AAV9/hTau5i or AAV9/Scr treatment on the survival or changes in body weight of NT and P301S mice. As part of the disease course, around 9 months of age P301S mice started losing body weight and developed progressive hind-limb paralysis that resulted in early death. We found that treatment with AAV9/hTau5i at 9 months of age significantly improved survival in P301S mice and that body weight loss was significantly attenuated in male mice. Assessments of brain tissues from mice injected at 3 months of age confirmed that ICM delivery of AAV9/hTau5i distributed vector throughout the brain and reduced human tau mRNA and protein levels. At this age (6 months), tau inclusions are minimally detectable via immunohistochemistry, so we used the cell-based tau seeding assay to measure the presence of pathological tau aggregates in brain lysate. AAV9/hTau5i treatment prevented tau aggregate formation in the mice treated at 3 months of age. Analysis of neuronal loss and pathological tau deposition and seeding are ongoing for the mice treated at 6 and 9 months of age and will be reported. In addition to our efficacy studies, we have an ongoing non-GLP safety study in WT mice, where 3-month-old mice received an ICM injection of an AAV9 vector carrying a mouse tau-specific miRNA (AAV9/mTau2i), AAV9/Scr or vehicle. As some studies have shown that the constitutive or acute knock-down of endogenous mouse tau in the brain can have adverse effects on anxiety, motor function and memory, treated animals are undergoing periodic behavioral testing while aging to one-year post-injection, at which time tissues will be collected for histopathological analysis. Interim analysis of blood chemistry, survival and body weight showed no significant differences between treatment groups, supporting that ICM delivery of miRNAs are well-tolerated in mice and that AAV9/mTau2i is not overtly toxic. Together, these data support that a one-time, vectorized delivery of a tau-specific miRNA is a potential treatment approach for human tauopathies.

471. CAP-001: Systemic AAV Gene Therapy with Next Generation Capsids for MPS II Disease

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Mucopolysaccharidosis type II (MPS II), or Hunter Syndrome, is a lysosomal storage disease caused by deficiency of iduronate-2-sulfatase (IDS) and accumulation of glycosaminoglycans (GAG) throughout the body. MPS II leads to developmental delay, neurodegeneration, and shortened lifespan, with current standard of care through enzyme replacement therapy failing to provide neurological benefits due to poor blood-brain-barrier (BBB) crossing. While naturally occurring AAV vectors exhibit poor biodistribution across the brain via traditional routes of administration, engineered AAV gene therapy delivered by a single intravenous infusion has the potential to provide long-lasting IDS expression

to treat both neurological and peripheral manifestations of MPS II. To this end, we employed a high-throughput AAV engineering platform that rapidly selects capsids with improved transduction broadly across the brain after intravenous (IV) delivery. A library of billions of capsids was generated from an AAV9 parent and injected into non-human primates (NHPs) to assess increased brain penetrance and expression through multiple rounds of screening. Through this effort, a novel engineered capsid was identified and selected for further characterization. When administered IV (2.5e13 vg/kg, n=3) in NHPs, this novel capsid packaging a human IDS transgene (CAP-001) achieved greater than 25- and 2-fold higher biodistribution than AAV9 delivered IV (2.5e13 vg/kg, n=3) or ICM (2e11 vg/g brain, n=2), respectively. CAP-001 delivered IV not only achieved better biodistribution across the brain than AAV9 through either route, but provided a significantly more uniform distribution across all brain areas with a >50-fold improvement in subcortical and deep-brain regions, an area where ICM delivery has proven to poorly distribute. In a subsequent dose-ranging study, CAP-001 showcased a dose-dependent increase in biodistribution throughout the brain with no notable safety findings. In a dose-range finding study in the MPS II rodent disease model, B6N.Cg-*Idst^{tm1Muen}/J* mice, CAP-001 increased IDS enzyme activity and reduced GAG levels throughout the body of adult mice, including throughout the brain, in a dose-dependent manner. At the higher end of the study, IDS enzyme activity was increased up to 83% of wildtype levels in the brain, while activity throughout the rest of the body was increased above wildtype levels. Taken together, the primate and murine data showcase both the efficacy and safety of the engineered CAP-001 and support the development of IV administration of CAP-001 to treat both neurological and peripheral diseases of MPS II patients.

472. Durable and Specific Rescue of UBE3A Expression in the Brain of an Angelman Syndrome Mouse Model Using an Artificial Transcription Factor

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Angelman syndrome (AS) is a rare neurogenetic disorder caused by loss of ubiquitin ligase E3A (UBE3A) gene expression in the brain. Loss of UBE3A expression leads to severe developmental delays, deficiencies in expressive communication, deficits in movement and coordination and other characteristic behaviors. The *UBE3A* gene is paternally imprinted in brain neurons, and the clinical features of AS are primarily due to the loss of maternally expressed *UBE3A* in the central nervous system. A healthy copy of paternal *UBE3A* is present in the brain, but is silenced by a long non-coding antisense transcript (*UBE3A-ATS*). Here, we demonstrate that an artificial transcription factor (ATF) can silence *Ube3a-ATS* in a mouse model of Angelman Syndrome and restore endogenous physiological expression of paternal *Ube3a*. A single injection of adeno-associated virus (AAV) into the tail vein enabled whole brain transduction and selectively re-established

UBE3A protein expression. Restoration of UBE3A expression in the brain was durable, specific, and able to rescue several motor deficits. Tolerability of a single injection of AAV-ATF therapy for Angelman Syndrome was evaluated by monitoring inflammatory response and demonstrated the use of artificial transcription factors as a promising translational approach for Angelman Syndrome.

473. Gene Therapy Mediated Cross Correction for CDKL5 Deficiency Disorder

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Cyclin-dependent kinase-like 5 (CDKL5) deficiency disorder (CDD) is a rare central nervous system (CNS) disorder affecting one in 40,000 to 60,000 births. CDKL5 deficiency causes severe neurological deficits including refractory epilepsy, hypotonia, developmental delay, intellectual disability, motor dysfunction, cortical visual impairment, and sleep abnormalities. While current therapeutic options for CDD are limited, adeno-associated virus (AAV)-based gene replacement therapy represents a promising option for restoring CDKL5 and mitigating pathology. However, a challenge for AAV gene therapy for CDD, and CNS diseases generally, is achieving therapeutic transgene expression in a sufficient number of target cells. To address this challenge, we developed a gene therapy-mediated cross correction strategy in a firmly established mouse model of CDD. This strategy used the AAV9 variant hu68 to deliver an engineered CDKL5 protein containing signal (BIP) and cell penetration (TAT) peptide sequences to enable CDKL5 to be secreted and taken up by neighboring cells, thereby increasing the number of brain cells expressing CDKL5 protein. Intracerebroventricular injection of hu68-BIP-TAT-CDKL5 into P0 or P14 male hemizygous CDKL5^{-/-} mice resulted in robust BIP-TATk28-hCDKL5 protein expression that was greater than untagged control CDKL5. Analysis of BIP-TAT-CDKL5 protein and mRNA expression showed that at 1 month post-ICV injection, 0.24 to 4.9 % of brain cells depending on the brain region and route of administration were cross corrected and positive for protein only. Robust cross correction occurred in brain regions where CDKL5 is normally expressed, such as cortex and hippocampus. We also tested if ICV hu68-BIP-TAT-CDKL5 treatment could mitigate resting state electroencephalogram (EEG) abnormalities associated with sleep and cognitive dysfunction and seizures in the mature brains of 6-7 month old seizure-prone female heterozygous CDKL5^{+/-} mice. Intracranial EEG analyses indicated that hu68-BIP-TAT-hCDKL5 significantly reduced abnormalities in delta, theta, and pro-seizure gamma frequency band power in CDKL5^{+/-} mice. These findings suggest that low levels of BIP-TAT-hCDKL5 are sufficient to mitigate established neural activity abnormalities in adult CDKL5 mutant brains. We are currently planning pharmacological and toxicological evaluation of hu68-BIP-TAT-CDKL5 in non-human primates. In summary, these data indicate that hu68-BIP-TATk28-CDKL5 cross correct cells in relevant brain regions and mitigates EEG abnormalities including the increase in gamma frequency power associated with seizure susceptibility. Importantly, these preclinical

findings suggest the remarkable possibility that the window for genetic therapeutic intervention in CDD is wider than previously recognized and may extend into adulthood.

474. Safe and Efficacious Rescue of GM3 Synthase Deficiency Mice by Spatially Regulated Gene Delivery

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GM3 synthase deficiency (GM3SD) is an infantile-onset lethal epilepsy caused by loss-of-function mutations in the ganglioside GM3 synthase gene *ST3GAL5*. The resulting enzyme deficiency leads to a complete lack of GM3 and its derivatives in all tissues of the body, particularly in the central nervous system (CNS), resulting in infantile-onset developmental stagnation, delayed brain growth, hypomyelination, intractable epileptic encephalopathy, deafness, visual impairment, irritability, and insomnia, and untimely death. GM3SD follows an autosomal recessive pattern with an estimated incidence of approximately 1 per 1,200 births in Old Order Amish communities of North America, but is found in populations worldwide. No disease-modifying treatment is available at present. Here, we hypothesize that CNS-targeted *ST3GAL5* gene replacement therapy could achieve therapeutic *ST3GAL5* expression, restore the endogenous production and trafficking of cerebral gangliosides, and thereby rescue the severe neurodevelopmental phenotypes. We first generated and validated several *ST3GAL5*-expressing constructs for their abilities to reconstitute gangliosides in induced pluripotent stem cell (iPSC)-derived cortical neurons from GM3SD patient fibroblasts. Next, we intracerebroventricularly delivered rAAV9.h*ST3GAL5* to *St3gal5*^{-/-} and *St3gal5*^{-/-}/*B4galnt1*^{-/-} mice, which recapitulated the molecular and phenotypic impairments in human GM3SD, respectively. The treatment yielded clinically relevant therapeutic benefits, including extended lifespan (median survival: untreated, 18 days; treated, 56 days), restored ganglioside production in the CNS, improved growth, and partially rescued motor function. However, when delivered systemically, the gene therapy led to fatal hepatotoxicity associated with supraphysiologic *ST3GAL5* expression in liver. We therefore designed a second-generation vector for better transcriptional and posttranscriptional regulation using a neuron-specific promoter coupled to a hepatocyte-specific detargeting microRNA. This optimized vector eliminated liver toxicity while preserving therapeutic benefits for CNS function and survival. Finally, we found that using *St3gal5*^{-/-}/*B4galnt1*^{-/-} mice might have underestimated the overall efficacy of *ST3GAL5* gene replacement alone, because AAV-mediated co-delivery of *ST3GAL5* and *B4GALNT1* led to higher proportional survival and complete normalization of behavior in these animals. Overall, we identified and overcame key hurdles in developing a GM3SD gene therapy, and demonstrated the potential of optimized rAAV.*ST3GAL5* vector as a safe and effective candidate for clinical translation. (aCo-corresponding authors)

Enhancing CAR-T Cell Efficacy

475. Leucine Zipper-Based Cytokine Receptors Augments CAR T Cell Immunotherapy for Solid Tumors

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Immunotherapy with CAR T cells presents a promising treatment option for solid tumors. However, limited CAR T cell expansion and persistence has emerged as a major roadblock. Investigators have constitutively expressed γ -chain or other cytokines in CAR T cells to overcome this roadblock, but there are concerns about systemic toxicities. To overcome this limitation, we generated chimeric cytokine receptors in which the extracellular domains of the common γ -chain and the IL-2R β chain are replaced with one (1x) or two (2x) leucine zipper domains (zipIL-2R). Structural modeling of these leucine zipper receptors (zipRs) revealed that zipIL-2R.2x forms higher order complexes through parallel and antiparallel alignments of the leucine zipper domains. Consistent with this model, zipIL-2R.2x induced significantly higher levels of pSTAT5 when expressed in primary human T cells, in comparison to zipIL-2R.1x. In addition, zipIL-2R.2x enabled T cells to survive for 21 days in the absence of exogenous cytokines without inducing autonomous cell growth. zipIL-2R.2x signaling was efficiently inhibited by ruxolitinib, highlighting the utility of a FDA-approved drug as a safety switch. Next, we genetically modified CAR T cells targeting the solid tumor antigen B7-H3 in order to test the effects of zipIL-2R.2x (zipIL-2R.CAR T cells). *In vitro*, zipIL-2R.CAR T cells retained their ability to expand in repeat stimulation assays with B7-H3+ tumor cells (A549, lung adenocarcinoma; LM7, osteosarcoma) in contrast to unmodified CAR T cells. This benefit was strictly antigen-dependent since B7-H3 KO A549 and LM7 cells did not induce CAR T cell expansion. *In vivo*, zipIL-2R.CAR T cells had enhanced antitumor activity in a systemic A549 xenograft mouse model, which resulted in a significant survival advantage of treated mice compared to unmodified CAR T cells. To demonstrate the modular nature of our zipR design, we generated additional zipRs to activate IL-7, IL-21, IL-12, and IL-18 receptor pathways. *In vitro* analysis demonstrated that these zipRs improved the effector function of CAR T cells and a detailed *in vitro* and *in vivo* comparison is in progress. In conclusion, we have generated novel leucine zipper-based cytokine receptors that improved the effector function of CAR T cells in preclinical solid tumor models, highlighting their potential to enhance current CAR T cell therapies.

476. Epigenome Editing Enables PD1 Silencing in CAR T Cells

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Introduction: The programmed cell death protein 1 (PD1) is an inhibitory receptor whose expression is associated with the exhaustion-mediated immunosuppression of Chimeric Antigen Receptor (CAR) T cells. The exhaustion state typically leads to functional unresponsiveness and proliferative impairment, thus profoundly affecting the ability of CAR T cells to battle against malignant cells. Interestingly, disrupting the interaction of PD1 with its ligand, PD1 ligand 1 (PDL1), through checkpoint inhibitors has shown to reinvigorate the immune response. As a consequence, strategies to disrupt the PD1-PDL1 interaction in CAR T cells is currently under scrutiny to enhance the immune response against tumours. **Methods:** To decrease the PD1-PDL1 interaction, we designed an approach to silence the expression of the PD1 encoding gene (*PDCD1*) in primary T cells via epigenome editing. We generated six Designer Epigenome Modifiers (DEMs) targeting the *PDCD1* promoter at different positions. Upon binding mediated by their customizable DNA binding domain, deposition of *de novo* cytosine methylation at the target site drives gene silencing. The functionality of the DEMs was initially assessed in a reporter cell line and subsequently confirmed in primary T cells using *in vitro* transcribed DEM-mRNA. We then used the *PDCD1*-specific DEMs to silence the *PDCD1* gene in two independent CAR T cell products, expressing either an anti-PSMA CAR, to target prostate cancer, or an anti-CD19 CAR, to target B cell lymphoma, respectively. The effect of epigenome editing in CAR T cell was evaluated through a set of functional assays, including cytotoxicity, cytokine release and proliferation upon antigen encounter. **Results:** Initial functional screening in a reporter cell line identified four DEMs capable of long-term silencing of the reporter gene. Delivery of the best-performing DEM (DEM#5) in primary T cells led to sustained silencing of the endogenous *PDCD1* gene with 5-fold and 3-fold reduction on the transcript or protein levels, respectively. Bisulfite sequencing of the targeted promoter confirmed that silencing was associated with increased deposition of DNA methylation. Consequently, DEM#5 was delivered to two independent CAR T cell products for the targeting of prostate cancer or B cell lymphoma models, respectively. Preliminary results from *in vitro* experiments show that in both tumour cell models the epigenetically edited CAR T cells (epiCART) maintained *PDCD1* silencing whilst key functional features, such as cytotoxicity, cytokine release profile and proliferation rate, were not impaired when compared to the unmodified CAR T cells. Additionally, CD19-targeting epiCART showed improved ability in inducing cytotoxicity of different CD19-positive cells lines, as compared to either unmodified CAR T cells or to CAR T cells in which the *PDCD1* gene has been genetically inactivated using the CRISPR-Cas9 system. **Conclusions:** Taken together, our results propose that transient expression of an epigenome editor is a novel and effective approach to modulate the PD1 inhibitory pathway in CAR T cells in a targeted manner. Initial *in vitro* characterization suggests that epiCART are functionally indistinguishable from parental

unmodified CAR T cells. Future exploitation of their efficacy *in vivo* as well as DEM-associated off-target effects will reveal the full potential of epiCAR cells as novel immunotherapeutics to overcome tumor-associated immunosuppression.

477. TNFR2 as a Target to Improve CD19-Directed CART Cell Fitness and Antitumor Activity in Large B Cell Lymphoma

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Chimeric Antigen Receptor T (CAR) cell activation and differentiation determine CAR cell fate and response to therapy. Here, we aimed to measure levels of activation-induced death receptors and ligands on CAR cells to improve fitness and clinical responses. We performed flow cytometry on *ex-vivo* stimulated, clinically annotated CAR products of patients from the ZUMA-1 clinical trial (Axi-Cel). We investigated possible correlations of surface death receptors and ligands with T cell differentiation status, post-infusion CAR T cell expansion, and response to therapy. CAR cell effector functions were measured, and apoptosis was assessed. For *in vitro* and *in vivo* functional studies, we used CART19 generated from healthy donors (HD CART19). First, we observed upregulation of death receptors and ligands in CART19 from non-responders, compared to responders after *ex-vivo* stimulation of Axi-Cel products. In an extended *in vitro* co-culture assay, where HD CART19 cells were repeatedly stimulated through the CAR, we found that tumor necrosis factor α receptor 2 (TNFR2), unlike other death receptors and ligands, was persistently elevated, suggesting a possible role for TNFR2 in long-term antigen-dependent CART19 dysfunction (Fig 1A). We further found that HD CART19 upregulate TNFR2, but not TNFR1 upon CAR stimulation (Fig 1B). While non-specific TCR activation (CD3 stimulation) of HD CART19 cells protected them from activation-induced apoptosis, antigen-specific activation through the CAR resulted in significant initiation of apoptosis within 2 hours of stimulation (Fig 1C). Having identified a possible association between TNFR2 and CART19 dysfunction, we aimed to study the impact of TNFR2 knockout on HD CART19 functions. Using CRISPR/Cas9 during CART cell manufacturing, we generated TNFR2^{ko} HD CART19 cells (50% efficiency), where TNFR2 expression in activated CART19 cells was reduced, compared to control CART19 cells (non-targeting gRNA, Fig 1D). TNFR2^{ko} CART19 cells demonstrated reduced early activation of CD25 and CD69 compared to control CART19 (Fig 1E), reduced apoptosis initiation (Fig 1F), and enhanced antigen-

specific proliferation and cytotoxicity (Fig 1G). Finally, in an *in vivo* xenograft model of CD19⁺ lymphoma, TNFR2^{ko} CART19 resulted in enhanced CART cell expansion and antitumor activity (Fig 1H). Our results indicate that TNFR2 plays a role in early activation and apoptosis initiation of CART19 following CAR stimulation with CD19⁺ target cells and present TNFR2 KO as a strategy to enhance CART19 antitumor activity.

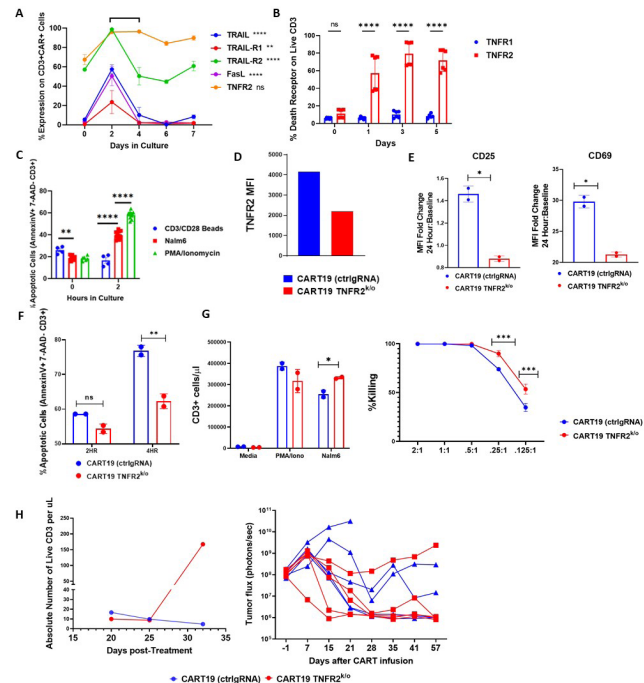


Fig 1. A) TNFR2 is persistently elevated, but not other death receptors, on CART19 cells using an extended *in vitro* culture model. CART19 cells from healthy donors were co-cultured with irradiated CD19⁺ Jeko-1 cells and repeatedly stimulated at days 0, 2, 4, and 6. Flow cytometric analysis was done in order to measure levels of death receptors during each time point (ns= not significant, ** p < 0.01, *** p < 0.0001, two-way ANOVA). **B)** TNFR2, but not TNFR1 is highly upregulated upon CART19 antigen-specific stimulation. CART19 cells were co-cultured with irradiated Naïve (CD19⁺) cells. Flow cytometric analysis was performed in order to measure TNFR1 and TNFR2 levels at days 0, 1, 3 & 5 (ns= not significant, **** p < 0.0001, two-way ANOVA). **C)** CART19 cells undergo more apoptosis upon antigen-specific stimulation in comparison to T-cell receptor (TCR) stimulation over time. CART19 cells were co-cultured with either PMA/Ionomycin, CD3/CD28 beads (TCR stimulation) or CD19⁺ cell line (naïve). Flow cytometric staining for Annexin V and T-AD is performed at 0 and 2 hours (** p < 0.01, **** p < 0.0001, two-way ANOVA). **D)** Disruption of TNFR2 on CART19 cells using CRISPR/Cas9. Representative figure showing levels of TNFR2 from CART19 (ctrlgRNA) and CART19 TNFR2^{ko} cells at day 6 of production. **E)** TNFR2^{ko} CART19 cells showed decreased levels of T cell activation markers in comparison to CART19(ctrlgRNA) cells. TNFR2^{ko} or ctrlgRNA CART19 cells were co-culture with irradiated Naïve (CD19⁺) cells. Flow cytometric staining was performed after 48hrs (right panel). *p<0.05, ** p < 0.01, two-way ANOVA. **F)** TNFR2^{ko} CART19 cells showed enhanced antigen-specific proliferation (A) and cytotoxicity (B) in comparison to CART19(ctrlgRNA) cells. CART19(ctrlgRNA) or TNFR2^{ko} CART19 cells were co-cultured with irradiated Naïve (CD19⁺) cells. PMA/Ionomycin or Media alone. Flow cytometric staining was performed in order to measure absolute number of CD3⁺ cells (left panel). CART19(ctrlgRNA) or TNFR2^{ko} CART19 cells were co-cultured with Luciferase⁺ Naïve (CD19⁺) cells and cytotoxicity was measured, via bioluminescence analysis, after 48hrs (right panel). *p<0.05, ** p < 0.01, two-way ANOVA. **H)** TNFR2^{ko} CART19 cells showed improved CART cell expansion, enhanced anti-tumor activity and proliferation *in vivo* in comparison to CART19(ctrlgRNA) cells. NSG mice were engrafted with CD19⁺ Jeko-1 and then randomized to receive either CART19(ctrlgRNA) or TNFR2^{ko} CART19 cells. Tail bleeding was done once a week in order to assess CART cell expansion *in vivo* (left panel). Bioluminescence was measured once a week in order to assess burden of disease (right panel).

478. A High Expression of IL15 Receptor Alpha (IL15Ra) in Glioblastoma Microenvironment Enables IL15-Armored CAR T Cells to Modulate Tumor Immunosuppression and Improve Survival in Syngeneic Models

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Introduction. Chimeric antigen receptor (CAR) T cells can eliminate cancer cells, with clinical success in hematologic malignancies. However, the utility of CAR-T cells in solid tumors, such as glioblastoma (GBM) (the most aggressive primary brain tumor in adults), is limited. The immunosuppressive tumor microenvironment

(TME) of GBM is a major barrier to their efficacy. Interleukin 15 (IL15) can assist CAR T cells in resisting to harsh TME of GBM, but it must be trans-presented to T cells in a complex with IL15 receptor alpha (IL15Ra). We, therefore, sought to understand which cells of the TME express the IL15Ra and may contribute to the efficacy of CAR therapy. We armored IL13Ra2.CAR T cells with IL15 and studied their impact on the TME of syngeneic models of GBM. **Methods.** RNA-seq, RT-PCR, and flow cytometry were utilized to measure the expression of IL15Ra on paired peripheral and tumor-infiltrating immune cells of GBM patients and two syngeneic murine models. Our conventional CARs targeting the tumor-associated antigen, IL13Ra2 (IL13Ra2.CAR), was modified to express either (i) soluble IL15 via T2A cleavage site (IL13Ra2.CAR.IL15sec), or (ii) IL15 fused to IL13Ra2 single-chain antibody (IL13Ra2.CAR.IL15fus). We treated GL261-IL13Ra2 or CT2A-IL13Ra2 bearing C57BL/6 mice with 1×10^6 CAR T cells via a single intratumoral injection and studied changes in gene and protein expression of TME upon treatment. We tracked animals' survival and performed histological analysis of tissues. Paired t-test, one-way ANOVA, or Log-rank test was used to determine statistical significance. **Results.** The analysis of GBM patient samples revealed a several-fold increase in IL15Ra expression in tumor-associated macrophages (TAMs), B, and dendritic cells harvested from patient tumors compared to peripheral immune cells; similar results were obtained in syngeneic models of GBM. We then investigated the gene expression profile of TME's CD45+ immune compartment *in vivo* following treatment with IL15 secreting CAR T cells. Compared to control groups, CAR-Ts administration to tumor-bearing mice resulted in a significant downregulation of genes involved in immunosuppression and monocyte migration. *In vitro*, co-cultures of CAR T cells with bone-marrow-derived myeloid-derived suppressing cells (MDSC) resulted in MDSC depletion by both IL-15-armored CARs, with the IL13Ra2.CAR.IL15fus T cells are being more efficacious. Similarly, IL13Ra2.CAR.IL15fus T cells significantly improved the survival of tumor-bearing mice in both experimental models. Furthermore, TME analysis showed that treatment with IL13Ra2.CAR.IL15fus results in higher frequencies of CD8+ T cells, NK, and B cells, but a decrease in CD11b+ cells in tumors than IL13Ra2.CAR and IL13Ra2.CAR.IL15sec T cells. **Conclusions.** Our data demonstrate that CAR T cells armored with IL15 can modulate the TME and increase the survival of mice compared to conventional CARs. Our data suggest that IL15-modified CARs act as a dual agent against glioma and TAM within the TME via IL13Ra2 and IL15Ra, respectively. Together, these results warrant further investigation of IL15-armored CAR T cells in early phase clinical trials for GBM patients.

480. Allogeneic Donor-Derived CD19-Chimeric Antigen Receptor (CAR) T Cells for Relapsed B-Cell Malignancies After Hematopoietic Stem Cell Transplantation

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Introduction: Autologous CD19 targeting CAR-T cell products are effective treatments for relapsed/refractory B-cell malignancies but have several disadvantages, including the time needed for product manufacture and the impaired function of T cells obtained from heavily treated patients. In patients who relapse after allogeneic hematopoietic stem cell transplant (allo-HSCT), donor-derived allogeneic T cells may provide a combination of improved CAR-T cell function and an additional graft-versus-leukemia/lymphoma (GvL) effect mediated by their native receptors. To discover whether these cells could indeed provide anti-leukemic benefit without inevitably precipitating severe graft-versus-host disease (GvHD) we instituted a phase 1 study in post allo-HSCT patients. **Methodology:** This is an open-label phase 1 trial (NCT02050347). Seven post allo-HSCT patients with relapsed B-cell malignancies or at high risk for relapse (5 with B-ALL and 2 with NHL) received second-generation, CD19-CD28 CAR-T cells manufactured from their allo-HSCT donors. Patients were infused with $0.1-5 \times 10^6$ T cells/kg preceded by lymphodepletion with cyclophosphamide (500 mg/m²/d) and fludarabine (30 mg/m²/d) \times 3 in the 4/7 who were not lymphopenic and had not recently received chemotherapy. The persistence of infused T cells was assessed by qPCR of peripheral blood mononuclear cells. **Results:** qPCR values peaked approximately 2 weeks post-infusion, and signal remained detectable for 3.5 to 6 years. The mean peak qPCR value was higher in patients who received lymphodepleting chemotherapy. None of the patients developed new-onset GvHD, and only two patients developed grade 1-2 cytokine release syndrome. Four patients developed grade 3 or 4 lymphopenias that were possibly or probably attributable to the infusion. No other grade 3 or 4 toxicities were attributable to the infusion. Four patients (3 B-ALL and 1 NHL) had active disease prior to infusion, 3 of whom had a complete remission (CR) after the infusion. Three patients (2 B-ALL and 1 NHL) at high risk for relapse received the infusion while in remission, with one (B-ALL) having a CR enduring for more than 7 years, but two patients experiencing a relapse less than two months after the infusion. Overall, 5 patients died of disease 3-19 months after infusion, while two remain in remission more than five years after the infusion with no chronic GvHD or other long-term adverse effects. **Conclusion:** Allogeneic donor-derived CD19 CAR T-cells in patients with heavily treated B-cell ALL or NHL patients are safe and can induce remission without risk of GvHD. However, the majority of treated patients died of disease (relapse 4/5; incomplete response 1/5), and we are now evaluating approaches to enhance both the potency and persistence of donor CAR T cells.

481. High-Affinity PD1-CD28 Chimeric Switch Receptors Enhance Costimulatory Signaling and Improve TCR and CAR T Cell Antitumor Activity

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Background: In an act of self-preservation, tumor cells express immune checkpoints such as PDL1 to dampen T cell function. It has previously been shown that a chimeric switch receptor (CSR) that combines the extracellular domain of PD1 with the transmembrane and intracellular domain of CD28 (PD1-CD28) can hijack the inhibitory PDL1-PD1 axis and turn it into a T cell costimulatory signal. Given the moderate affinity of wild-type PD1 for PD-L1 (K_D of 8.2 μ M), we sought to increase the potency of the CSR by creating a high affinity PD1-CD28 receptor (HPD1-CD28), incorporating point mutations (*Maute et al; PNAS 2015*) to increase the affinity of PD1 for PDL1 ~10,000-fold.

Results: We transduced primary human T cells with retroviral vectors encoding either PD1-CD28 or HPD1-CD28. Flow cytometry confirmed that although PD1-CD28 and HPD1-CD28 were expressed at similar levels on the T cell surface, the high affinity CSR was better able to bind recombinant PDL1 protein. When stimulated with plate-bound anti-CD3 and recombinant PDL1 protein, T cells expressing PD1-CD28 or HPD1-CD28 CSRs had higher ERK phosphorylation and IL-2 production than non-transduced T cells, indicating successful CD28 signaling. Both pERK and IL-2 were higher in T cells expressing HPD1-CD28 than PD1-CD28. To test the CSRs' ability to augment T cell therapy, we co-expressed PD1-CD28 or HPD1-CD28 with NY-ESO-1/HLA-A2-restricted TCRs or EphA2.41BBz CARs. *In vitro*, expressing PD1-CD28 or HPD1-CD28 in TCR or CAR T cells augmented cytokine production and expansion in the presence of antigen-positive tumor cells, using NY-ESO-1/HLA-A2+ A375 melanoma cells for TCR and EphA2+ A549 lung adenocarcinoma cells for CAR T cells, respectively. For both TCR and CAR T cells, these effects were more pronounced with the high-affinity CSR. Expression of PD1-CD28 or HPD1-CD28 in CAR T cells also improved their antitumor activity *in vivo* as they outperformed unmodified CAR T cells in an A549 metastatic xenograft model. Of note, the benefit of the HPD1-CD28 receptor was most pronounced after the mice were re-challenged with a second dose of tumor cells, indicating the ability of the high-affinity CSR to enhance persistence of functional T cells. *In vivo* experiments with CSR-modified NY-ESO-1 TCR T cells are in progress.

Conclusions: Our data demonstrate that PD1-CD28 CSRs provide costimulation in the presence of PDL1, and increasing its affinity improves signaling strength and functionality. Both transgenic TCR and CAR T cells expressing PDL1-triggered CSRs have enhanced effector function and antitumor activity in preclinical solid tumor models.

Gene Therapy for Immunologic Diseases

482. A Novel Engineered CRISPR-Associated Nuclease Accurately Removes ELANE Mutated Allele and Shifts HSC Differentiation Towards Neutrophils in Severe Congenital Neutropenia

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Severe congenital neutropenia (SCN) is a life-threatening disorder usually caused by dominant heterozygous mutations in *ELANE*. The resulting impaired protein, mutant neutrophil elastase, triggers apoptotic death of myeloid progenitor cells and severe reduction in circulating neutrophils. Potential genetic treatment strategies include biallelic knock-out or gene correction via homology directed repair (HDR). Such strategies, however, involve the potential loss of the essential function of the normal allele product and limited coverage of diverse monogenic mutations within the patient population, respectively. By mining bacterial metagenomic database and applying a sophisticated funnel algorithm we have identified a group of novel CRISPR-associated nucleases with diverse properties. A molecular directed evolution approach was applied on one of these newly discovered nucleases to obtain variants that could address the allele specificity requirement in targeting *ELANE*. Based on one of these optimized nuclease variants, we have developed a novel CRISPR-based mono-allelic knock-out strategy that precisely targets the heterozygous sites of single nucleotide polymorphisms (SNPs) associated with the majority of *ELANE* mutated alleles. By targeting these SNPs our strategy could potentially treat more than 75% of *ELANE*-related SCN patients. *In-vitro* studies demonstrated that patients' unedited hematopoietic CD34⁺ cells have significant abnormalities in differentiation and maturation, consistent with the hematopoietic defect in SCN patients. Selective knock-out of the mutant *ELANE* allele alleviated these cellular abnormalities and resulted in about 50-70% increase in normally functioning neutrophils (Figure 1). Genomic analysis confirmed that *ELANE* knock-out was specific to the mutant allele and involved no off-targets. An in-vivo study, conducted in an immunocompromised mouse model, depicted efficient engraftment of the edited HSCs that gave rise to multiple lineages in the mouse bone marrow and spleen. These results demonstrate the therapeutic potential of selective allele editing that may be applicable to SCN and other autosomal dominant disorders.

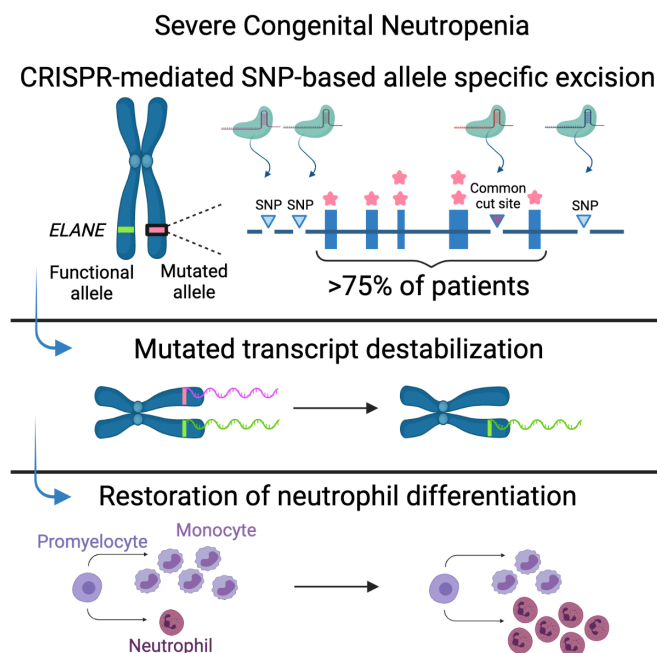


Figure 1: SCN is a neutrophil maturation disorder genetically characterized by a mono-allelic mutation in *ELANE* gene. Our strategy involves novel, highly precise CRISPR methodologies to excise mutant *ELANE* allele and destabilize its mutant transcript, while keeping the wild-type allele intact. This strategy is based on targeting heterozygous sites of SNPs, adjacent to *ELANE*'s mutations, and could potentially treat the majority of SCN patients. The resulting excision promotes neutrophil differentiation.

483. Editing T Cell Repertoire by Thymic Epithelial Cell-Directed Gene Transfer Abrogates Risk of Type 1 Diabetes Development

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Insulin is the primary auto-antigen (Ag) targeted by T cells in Type 1 Diabetes (T1D). Although biomarkers precisely identifying subjects at high risk of T1D are available, successful prophylaxis is still an unmet need. Leaky central tolerance to insulin may be partially ascribed to the instability of the MHC-InsB9-23 complex, which lowers TCR avidity, thus resulting in defective negative selection of auto-reactive clones and inadequate insulin specific Tregs induction. We developed a lentiviral vector (LV)-based strategy to engineer thymic epithelial cells (TEC) to correct diabetogenic T cell repertoire. Intra-thymic (*it*-)LV injection established persistent expression of the transgene in EpCAM+ TEC, largely beyond their turn-over time due to the transduction of TEC precursor. *It*-LV encoding for invariant chain (Ii) fused to an immunodominant portion of ovalbumin (OVA) allowed stable and

complete negative selection of OVA323-339 responsive clones in OTII chimeric mice, thus proving the efficacy of the LV platform in T cell repertoire editing. We applied this strategy to correct diabetogenic repertoire of young NOD mice, which are prone to develop T1D in few weeks, imposing the presentation by TEC of the stronger agonist InsB9-23R22E and partially depleting the existing T cell compartment. Full protection from T1D was observed in mice *it*-LV:InsB9-23R22E treated mice, while controls became symptomatic between the tenth and the twentieth week of life. We further circumscribed LV-driven presentation of InsB9-23R22E to thymic CD45- TEC by the insertion of micro-RNA142 regulatory elements, confirming the potential of TEC directed gene transfer to neutralize autoimmune prone T cell repertoire naturally selected in untreated NOD mice. The identification of InsB-specific T cell by IAg7-InsB9-23R22E tetramer revealed an expanded population of InsB-specific Tregs. TCR sequencing of T cells sorted from PLN and pancreas allowed us to identify a family of Tregs clones induced by *it*.LV:InsB9-23R22E conditioned thymic selection. Overall, these results demonstrate that our gene transfer-based prophylaxis can fine-tune central tolerance process both by negative selection and Tregs induction, thus modifying the thymic output of T cells recognizing the LV-encoded Ags and correcting an autoimmune prone T cell repertoire.

484. Therapeutic Gene Editing of T Cells Corrects CTLA4 Insufficiency

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Heterozygous mutations in *CTLA4* result in an inborn error of immunity (IEI) (also known as primary immunodeficiency) with a severe clinical phenotype. Autologous T cell gene therapy may offer a cure without the immunological complications of allogeneic stem cell transplantation. The mutational landscape and requirement for tight regulation of *CTLA4* make viral gene addition approaches unappealing. Gene editing strategies permit alteration of *CTLA4* while retaining the endogenous gene control machinery. We set out to devise a CRISPR/Cas9/AAV6 gene editing strategy to correct CTLA4 insufficiency in T cells. We designed several homology directed repair (HDR) editing strategies that would correct the genetic defect. We first assessed correction of an individual point mutation. We then evaluated several universal strategies that enable correction of most disease-causing mutations with a single edit; the first that inserts the *CTLA4* cDNA in exon 1, and a second that inserts the *CTLA4* cDNA at the 3' end of the first intron of *CTLA4*. All AAV6 HDR donor templates included a GFP reporter gene to enable easy identification of the edited cells. Superior editing efficiencies were obtained with the intronic approach compared to the other editing strategies and this strategy was then further evaluated. CTLA4 function and expression kinetics were assessed following editing using flow cytometry-based assays. Functional studies using CTLA4 transendocytosis (TE) assays, demonstrated restoration of CD80 and CD86 internalization in the edited CD4+

T cells. Following gene editing, transgene expression kinetics were comparable to healthy control CD4+ T cells. Gene editing of T cells isolated from patients with CTLA4 insufficiency restored CTLA4 expression and rescued transendocytosis of CD80 and CD86 *in vitro*. Using a similar approach, gene corrected T cells from CTLA4^{-/-} mice engrafted in immunodeficient mice at clinically relevant frequencies and tail vein bleeds were performed 1, 3 and 4 weeks post adoptive transfer. In the mice which received the GFP+ edited cells, a stable population of GFP+ cells was detectable at all timepoints demonstrating *in vivo* persistence as well as genetic stability. All mice were sacrificed 4 weeks after cell transfer. To assess lymphoproliferation, the cellularity of peripheral lymph nodes and spleen weight were analyzed. Spleen and lymph node size, lymph node cell counts, and spleen weight were all significantly lower in mice which received the edited cells (n=5) compared to non-edited controls (n=5) and there was no significant difference in these parameters between mice who had received edited CTLA4^{-/-} T cells and those who received wild type murine T cells (n=4). Analysis of lymph node and spleen cells confirmed persistence of CTLA4 expression in the edited (GFP+) cells and revealed that a higher proportion of Treg than Tconv had been successfully edited. Together these data demonstrated that CTLA4 edited T cells survived *in vivo*, expressed CTLA4 and were able to control the clinical phenotype of CTLA4 insufficiency, providing a powerful proof-of-principle of our T cell gene therapy approach. Our data provide proof-of-concept that gene editing can restore CTLA4 function in T cells demonstrating the potential of this approach to treat CTLA4 insufficiency. A similar approach could be used in other IEs that are caused by multiple heterozygous mutations.

485. A Simultaneous Knock-Out Knock-In Gene Editing Strategy in HSPCs Potently Inhibits R5- and X4-Tropic HIV Replication

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Hematopoietic stem and progenitor cell transplant of CCR5 null cells into HIV infected individuals can be a curative antiviral therapy, yet allogeneic transplant poses significant health risk and is limited by the low availability of matched CCR5 null bone marrow donors. Here, we describe a Cas9/AAV6 based gene targeting strategy for autologous HSPC transplant which allows highly efficient knock-out of CCR5 and targeted integration of a transmembrane anchored fusion inhibiting C46 peptide and/or a humanized rhesus TRIM5α containing 13 rhesus amino acids necessary for inhibition of HIV replication. We have obtained greater than 95% knock-out of CCR5 in human cord-blood derived HSPCs and primary human CD4+ T cells, with 25-35% targeted integration in HSPCs and 40-80% targeted integration in primary CD4+ T cells. Edited human peripheral blood CD34+ HSPCs were transplanted into sub-lethally irradiated NSG-SGM3 mice, and facilitated similar levels of human engraftment and multi-lineage reconstitution as unedited control cells. Human cells with targeted integration and greater than 90% CCR5 knock-out were maintained in bone marrow, spleen, and peripheral blood of all mice transplanted with edited HSPCs at the 14-week engraftment endpoint, demonstrating

long-term survival of cells with CCR5 KO and targeted integration *in vivo*. CD4+ T cells from 5 individual healthy donors were isolated, activated, and edited, then challenged with replication competent R5-tropic BaL and X4-tropic NL4-3 virus, and p24 concentration and vRNA levels were monitored in T-cell culture supernatant for 3 weeks. R5-tropic BaL challenge demonstrated potent viral replication in mock treated cells from all donors, yet p24 was undetectable throughout the course of infection from all editing conditions (both CCR5 KO alone and targeted integration). X4-tropic NL4-3 replication was similar in mock and RNP (CCR5 KO only) conditions for all donors, yet was potently inhibited in cells containing targeted integration constructs tested both individually and together. The greatest inhibition was observed in dual-targeted cells simultaneously targeted with both the C46 and TRIM constructs, for an average of 223-fold reduction in p24 concentration across all donors, and up to 733-fold reduction observed in and individual donor. Three of five T cell donors had undetectable p24 levels at the 3 week infection endpoint in at least one targeted integration condition. To our knowledge, this is the first gene editing strategy which allows inhibition of X4-tropic HIV in addition to R5-tropic HIV. Here, we have demonstrated a highly efficient gene-editing strategy for autologous HSPC transplant in HIV infected individuals which allows hematopoietic reconstitution of gene-targeted cells and potently restricts both X4- and R5- tropic HIV replication for improved transplantation safety and efficacy against non-R5 HIV strains.

486. Transcriptional Mapping of Human Hematopoietic Stem and Progenitor Cells Discriminates Chronic Granulomatous Disease Patients Able to Benefit from Gene Therapy Treatment

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X-linked chronic granulomatous disease (X-CGD) is a rare, inherited, primary immunodeficiency characterized by defective microbicidal activity in phagocytes caused by mutations in the gp91phox subunit of the NADPH-oxidase complex. This leads to increased susceptibility to recurrent, life-threatening bacterial and fungal infections as well as to inflammatory complications. A definitive cure of X-CGD patients is provided by hematopoietic stem and progenitor cell (HSPC) transplantation. Still, gene therapy (GT) constitutes a suitable alternative treatment for patients lacking an HLA-compatible donor.

A Phase I/II clinical trial based on a self-inactivating lentiviral vector, G1XCGD, driving myeloid-specific gp91phox expression has been conducted in Necker's hospital (NCT02757911) sponsored by Genethon. Among the 4 treated patients (including one treated as a compassionate-use), two showed stable engraftment of the gene-modified HSPC with a clinical benefit. In contrast, the 2 other patients showed a progressive loss of corrected cells over time. To gain insights in factors that may explain the variable levels of engraftment of gene-corrected cells between these 4 patients, we undertook a transcriptomic analysis of CGD HSPC. Single-cell RNAseq analysis allowed us to explore the most immature HSC compartment. This compartment is significantly decreased in CGD patients as well as strongly altered with increased inflammatory and interferon signatures, compared to healthy donors leading to the activation of an aberrant myeloid program in the most severe patients. This analysis revealed that a more severe inflammatory score leads to genetic and functional alterations of HSC, as demonstrated by xenotransplantation in a humanized mouse model. Altogether these results suggest that the chronic inflammatory environment in CGD induces transcriptomic alterations at the HSC level, which are reminiscent of a memory footprint comparable to what is observed in the trained immunity mechanism. These transcriptomic alterations could facilitate myeloid differentiation during a secondary challenge and drive the HSC defect by impairing the long-term engraftment ability in two out of 4 treated patients. Those identified pathways will help identify targeted treatments before GT to protect HSC functionality and allow a more efficient therapeutic approach for CGD patients.

487. Base Editing of Hematopoietic Stem Cells Rescues T-Cell Development for CD3 δ Severe Combined Immunodeficiency

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Introduction: CD3 δ severe combined immunodeficiency (SCID) is a devastating inborn error of immunity caused by a homozygous mutation in the *CD3D* gene. The predominant disease-causing substitution, *CD3D* C202T, is present in a Mennonite population, and results in the failure to produce CD3 δ protein, an essential, invariable chain of the CD3/T-cell receptor (TCR) complex. Patients living with CD3 δ SCID present with a complete absence of mature T cells and are severely susceptible to lethal infections, often leading to infant mortality. **Methods and Results:** We compared two gene editing approaches: (1) CRISPR/Cas9 homology-directed repair (HDR) with a single-stranded

oligodeoxynucleotide (ssODN) homologous donor and (2) adenine base editing (ABE)-correction, to precisely revert the *CD3D* C202T mutation and rescue CD3 expression and T-cell signaling. A CD3 Knockout (KO) Jurkat T-cell disease model, containing the C202T mutation, was developed to evaluate the two editing strategies. We rationally designed and investigated novel editing reagents, including three adenine base editor variants, to target the C202T mutation in the CD3 KO Jurkat T-cell model. Our most efficient editing reagents demonstrated up to 88%, 85%, 83%, and 55% correction of the *CD3D* C202T mutation by NRTH-ABEmax, NRTH-ABE8e, NG-ABE8e, and CRISPR/Cas9 RNP + ssODN-mediated editing, respectively. Editing efficiency correlated with functional rescue of CD3 protein complex surface expression, with results demonstrating up to 85%, 79%, 78%, and 59% of CD3 complex restoration in manipulated CD3 KO Jurkat T cells by NRTH-ABEmax, NRTH-ABE8e, NG-ABE8e, and RNP + ssODN treatment, respectively. Stimulation of edited-CD3 KO Jurkat T cells with anti-CD3 and anti-CD28 produced wildtype-level rescue of calcium flux in NRTH-ABEmax, NRTH-ABE8e, and NG-ABE8e experimental arms, while RNP + ssODN treatment restored calcium flux to 58% of wildtype levels. To validate our best-performing base editing strategy in healthy donor CD34+ hematopoietic stem and progenitor cells (HSPCs), we introduced the *CD3D* C202T disease target by lentiviral integration followed by treatment with mRNA base editor. We observed up to 75% precise and stable therapeutic base editing of the C202T mutation 15 days post-editing, suggesting similarly high levels of correction will be obtained in CD3 δ SCID patient HSPCs. We are actively assessing functional restoration of T-cell development from base-edited CD3 δ SCID patient HSPCs *in-vitro* in the artificial thymic organoid (ATO) system, which can support differentiation of CD3 δ SCID HSPCs to mature T cells, if the C202T mutation is corrected. **Conclusions:** Our studies demonstrate a novel base editing strategy for CD3 δ SCID, capable of restoring 88% of CD3 protein expression and functionally correcting CD3/TCR signaling to wildtype levels in a CD3 KO Jurkat T-cell disease model. Additionally, our approach displays high rates of precise *CD3D* C202T editing in clinically relevant CD34+ HSPCs. Altogether, these results indicate a potentially curative, novel base editing gene therapy in HSPCs for CD3 δ SCID to be translated to the clinic.

488. Chemotherapy-Free Engraftment of Gene Edited Human Hematopoietic Stem Cells Leveraged on Mobilization and mRNA-Based Engineering

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Hematopoietic stem/progenitor cell gene therapy (HSPC-GT) is proving successful to treat several human primary immunodeficiencies,

haematological and storage diseases. HSPCs are mobilized and harvested from the patient, genetically corrected *ex-vivo* by gene transfer or gene editing and infused back to the patient, after administration of partial or fully myeloablative conditioning to make space in the bone marrow for the modified cells. However, these regimens are associated with severe short- and long-term toxicity. **Here we address a major hurdle posed by the requirement for toxic conditioning and *ex-vivo* manipulation that limits safe and broad use of HSPC-GT.** We leverage on recent advances in mobilizing agents to show that they create a window of opportunity for seamless engraftment of exogenous cells as they may substantially, albeit transiently, empty bone marrow niches. We show that donor HSPCs effectively outcompete mobilized recipient HSPCs for engraftment in the depleted bone marrow niches. Indeed, recipient mobilized HSPCs express low levels of CXCR4 and other surface molecules crucial for homing/engraftment, such as ITGA4 and KIT, cleaved upon *in vivo* administration of the mobilization reagent G-CSF. However, the newly infused donor HSPCs - previously collected through mobilization but undergoing *ex vivo* culture - recover their expression and thus gained a competitive advantage. The competitive advantage endowed to donor HSPCs might be further enhanced by transient over-expression of engraftment effectors. We present proof-of-principle of the therapeutic efficacy of mobilization based - hematopoietic stem cell transplantation (M-HSCT) in a primary immunodeficiency mouse model of Hyper IgM Syndrome I (HIGM-1). We further developed our strategy using human hematochimeric mouse models of hematopoiesis, showing its applicability to human HSPCs and its versatility when coupled to genetic engineering strategies. By exploiting recently optimized RNA-based delivery, we show that *ex-vivo* manipulated HSPCs can be transiently engineered for robust but transient overexpression of key biological effectors improving their homing and engraftment features, thus providing further competitive advantage, and establishing stable long-term chimerism in hematochimeric models, following our M-HSCT strategy. Importantly, this transient enhancement of engraftment ability could also overcome detrimental impacts of *ex-vivo* gene correction strategies, such as gene editing. The M-HSCT strategy is highly versatile and can be adapted to other emerging biological reagents to achieve more complete niche depletion and exchange with exogenous cells, potentially from allogenic sources. Overall, our findings encourage the eventual disposal of conventional genotoxic conditioning in HSCT and should provide a transformative strategy paving the way to a broader and safer use HSPC-GT in a relevant number of inherited diseases.

Immune Responses to Gene Delivery and Vaccine Approaches

489. Loss of AAV-FVIII Gene Therapy in Hemophilia A Mice due to Shutdown of Hepatic Protein Production Rather Than Loss of Vector or Transduced Cells

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Adeno-associated viral (AAV) vector-mediated gene transfer to the liver has yielded promising results in ongoing clinical trials for treatment of the X-linked bleeding disorder hemophilia A. However, it is unclear why a decline of human factor VIII (hFVIII) transgene expression over time has been observed. In a murine model of hemophilia A (F8e16^{-/-} BALB/c mice), we previously observed that hepatic gene transfer with AAV8 vector expressing codon-optimized hFVIII along with administration of the mTOR inhibitor rapamycin for 8 weeks prevented antibody formation against hFVIII and viral capsid. Nevertheless, reminiscent of clinical trial results, hFVIII activity consistently declined by week 16 despite the absence of antibodies. To further understand the underlying mechanisms, we transduced additional BALB/c-HA mice with AAV8-cohFVIII vector at 2×10^{11} vg/mouse. Half of the mice received no treatment ("AAV only" group). The other half was treated with rapamycin (150 ug/mouse) twice per week for 8 weeks ("AAV+rapa" group). Blood samples were collected at 4, 8, 12 and 14 weeks after gene transfer. For both groups, 7-9 mice were euthanized at weeks 4, 10, or 14 for collection of liver tissue. Consistent with the prior experiments, mice in "AAV only" group had no detectable hFVIII activity due to inhibitory antibody formation against hFVIII. In contrast, animals in AAV+rapa group showed therapeutic levels of hFVIII activity ranging 24-225% of normal at week 4. For the AAV+rapa group, 7 of 9 animals showed sustained hFVIII activity levels ranging 20-74% of normal. The 2 of 9 animals with declining hFVIII activity levels had an initial activity of >100%. Regardless of immune suppression, vector copy numbers declined 5-fold between weeks 8 and 14, while hFVIII transcript levels only declined 2-fold. ALT levels were elevated in both groups. Immunohistochemical stains of liver sections showed widespread hFVIII protein expression by hepatocytes was well maintained throughout the experiment in AAV+rapa group (except for one animal whose initial levels of 225% had declined to 6%). In contrast, without immune modulation ("AAV only" group), hFVIII protein expression remained at similar levels at weeks 4 and 8 but was completely or nearly completely absent in the liver by week 14 despite only a modest loss in hFVIII transcript levels. In these mice, areas of residual expression showed increased CD8⁺ T cell infiltration. To investigate further, we performed single-molecule fluorescent *in situ* hybridization (smFISH) on liver sections. Interestingly, AAV genomes and hFVIII mRNA transcripts were similarly detected regardless of immune modulation at all time points in ~40-80% of hepatocytes.

Western blot analysis showed up-regulation of cellular stress markers (including GRP94, BiP, CHOP, and phospho-eIF2 α , which is known to promote translational shutdown) in livers of half of the mice in both groups by week 14. Taken together, these results indicate an immune mediated translational shutdown mechanism (connected to cellular stress in at least some animals) rather than a destructive cytotoxic response that caused the loss of hFVIII expression over time. In other experiments, the same strain of mice showed sustained supraphysiological expression of hFIX after administration of an identical dose of AAV8-hFIX; thus, the immunogenicity of hFVIII and its propensity to induce cellular stress provides a unique challenge for sustained therapy, even in the absence of antibody formation or elimination of transduced cells.

490. Novel Early Checkpoint Modifier Demonstrates Broadened and Enhanced CD8+ T Cell Responses Across Multiple Preclinical Studies

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Checkpoint inhibition by antibodies against PD-1, CTLA-4 and other immunoinhibitors has revolutionized cancer treatment. However, there are limited data on checkpoint inhibition that targets the immune cycle at earlier stages. Our research focuses on an early checkpoint that is active during T cell activation, i.e., the B and T cell lymphocyte attenuator (BTLA) on T cells that, upon binding to the herpes virus entry mediator (HVEM), expressed by antigen presenting cells (APC), dampens T cell receptor signaling. HVEM also binds with a different domain to the co-stimulator LIGHT on T cells and, upon formation of a trimolar complex between HVEM, BTLA and LIGHT, inhibition prevails. The N-terminal domain of herpes simplex virus (HSV-1) glycoprotein (gD) has high affinity to the BTLA binding site of HVEM (Figure A), thereby blocking its ligation and allowing for co-stimulation through LIGHT, when expressed on the APC surface (Figure B). Taking advantage of gD's immunomodulatory activity, we developed and tested adenoviral vectors that express antigens as fusion proteins within the C-terminal domain of gD. Using a number of different tumor-associated and viral antigens from melanoma (Figure C), SARS-CoV-2 (Figure D) or human papilloma virus (HPV)-16 (Figure E), we demonstrated that antigens, fused into gD, induce enhanced CD8+ T cell responses and broadened responses by recognizing subdominant epitopes that, in general, fail to reach the threshold for T cell activation. The broadened immune responses guard against mutational escape, which can drive tumor progression and lessens the effectiveness of traditional vaccines against viral variants. In addition, CD8+ T cells stimulated in presence of gD are less prone to become exhausted, which can limit the benefit of traditional therapeutic vaccines. The increased CD8+ T cell responses translate into improved vaccine efficacy in a mouse model of chronic hepatitis B virus (HBV) infection, based on in situ transduction of mouse livers with an AAV8 vector expressing the overlength HBV genome (Figure F). It also provides superior protection against progression of transplantable melanoma cells (Figure G) and it also induced regression of large HPV-16 oncoprotein-associated tumors in a transgenic mouse model (Figure H). In preclinical models, across different disease states, novel checkpoint modification of early T cell activation, via delivery of antigens fused

into HSV-1 gD, enhances and broadens CD8+ T cell responses to subdominant epitopes. This may be crucial for the treatment of chronic infections or progressing tumors where T cells to dominant epitopes that had been induced during the infection or cancer have likely differentiated towards terminal exhaustion.

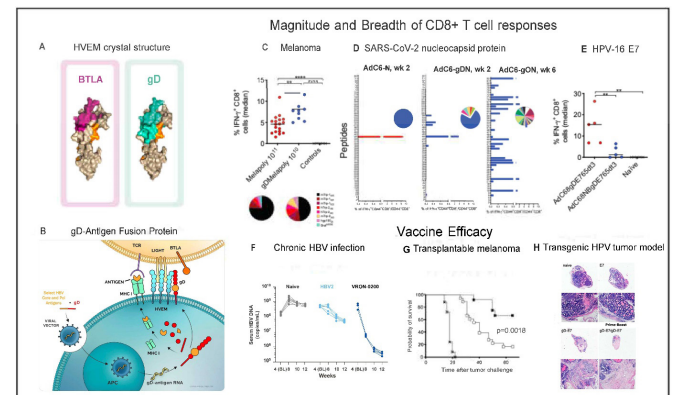


Figure Legend: [A] Crystal structure of HVEM; the BTLA and gD binding site are highlighted in purple or blue, respectively. [B] Mode of action of a viral vector vaccine that expresses a gD-antigen fusion protein. [C] CD8+ T cell responses to individual epitopes of a melanoma vaccine called Melapoly expressed by a vector with or without gD. Pie chart show proportions of responses to different epitopes. The immunodominant epitope is shown as a black slice. [D] Breadth of CD8+ T cell responses to the nucleocapsid antigen of SARS-CoV-2 expressed by a chimpanzee adenovirus vector (AdC) serotype 6 (AdC6) with or without gD and tested 2 or 6 weeks after immunization. Bar graphs show responses to individual peptides, pie chart show proportions of responses to different epitopes. [E] CD8+ T cell responses to HPV-16 E7 delivered by an AdC vector serotype 68 (AdC68) expressing gD or a non-HVEM binding version of gD (NBgD). [F] Serum HBV genome copy numbers over time in AAV8-1.3HBV injected mice vaccinated with the indicated inserts expressed by AdC6 vectors. [G] Survival rates in naive or vaccinated mice transplanted with B16.F10 melanoma cells. [H] HPV-16 E7-induced thyroid tumors in 12 months old transgenic mice that were left untreated (N), vaccinated with a vaccine expressing E7 (1X) or E7 within gD (1X or 2X).

491. Mucosal Chemokine CCL27 Adjuvant Uniquely Improves Mucosal Responses to SARS-CoV-2 synDNA Antigens Providing Heterologous Protection Against Delta Variant Challenge

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SARS-CoV-2, which causes COVID-19 has caused a global pandemic which has claimed over 5 million lives. Emerging variants of concern and waning vaccine-induced immunity continually challenge viral control. Directing vaccine-induced humoral and cell-mediated responses to mucosal surfaces could enhance vaccine efficacy and potentially reduce viral carriage in the upper airway and transmission. We immunized mice with DNA plasmids expressing a wild-type SARS-CoV-2 spike protein (pS) alone, or with the DNA-encoded mucosal chemokine adjuvant cutaneous T cell-attracting chemokine (pCTACK, CCL27). Mice co-immunized with pS and pCTACK exhibited increased spike specific IFN γ T cell responses compared to those receiving the pS alone. While both groups showed similar levels of spike receptor binding domain (RBD)-specific IgG in their serum, in the mucosa, co-immunization with pCTACK enhanced RBD-specific IgA, increased the numbers of Peyer's patches in small intestines, and cecal extracts from pCTACK co-immunized animals

neutralized pseudotyped viruses whereas extracts from pS-only animals did not. When ACE2 transgenic mice were immunized, rested for 3 months, and challenged with a heterologous SARS-CoV-2 Delta variant, co-immunization with pCTACK supported 100% survival while immunization with wild-type pS antigen alone supported 60% survival. Enhanced protection from morbidity was also observed as 100% of pCTACK co-immunized animals did not exhibit infection-induced weight loss. These studies demonstrate that mucosal chemokine adjuvants can direct vaccine-induced responses to specific immunological sites with significant impact on heterologous challenge. These data have significant implications for the design of improved vaccines targeting SARS-CoV-2.

492. An Intranasal saRNA/NLC Vaccine Induces Robust Mucosal and Systemic Immunity to SARS-CoV-2 in Mice

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Over the past year mRNA vaccines have demonstrated remarkable efficacy at preventing severe SARS-CoV-2 infection. However, global vulnerability remains high, even in highly vaccinated areas, as current vaccines only modestly reduce transmission. The inability of these vaccines to induce cross-reactive antibodies against emerging strains, waning of antibody responses after vaccination, and a lack of vaccine-induced local immunity in the respiratory tract that allows infection and transmission by vaccinated people all contribute to this sustained vulnerability. Intranasally administered (i.n.) vaccines can provide protection from respiratory pathogens by stimulating mucosal immune responses (1). Mucosal immunity, mediated by IgA antibodies and lung-resident memory T cells, effectively prevents early-stage infection and viral shedding (2, 3), minimizing chances for viral transmission. Effective i.n. influenza vaccines can also induce cross-reactive antibodies and thus provide expanded protection to multiple strains (4). I.n. administration may also improve vaccine uptake and be more feasible for pediatric vaccination. We have developed an i.n.-administered SARS-CoV-2 self-amplifying RNA vaccine that induces both potent respiratory mucosal and systemic immune responses. This vaccine, delivered by a nanostructured lipid carrier (NLC), is easily manufacturable and readily lyophilized, rendering it room temperature stable for at least six months. Preclinical studies demonstrate that, after a prime-boost dosing regimen, this i.n vaccine induces potent serum neutralizing antibody titers in C57BL/6 mice as well as robust systemic polyfunctional T cell responses, similar to responses to the intramuscular vaccine. Additionally, the intranasal vaccine induces key markers of mucosal immunity absent in the i.m. vaccine, including IgA and robust polyfunctional lung resident memory T cell (CD8+CD69+) and Tfh cell (CD8+CD154+) populations. Finally, induction of significant bone marrow resident IgG and IgA antibody secreting cell populations suggest establishment of durable immune responses. These studies indicate that i.n. administered, NLC-delivered, self-amplifying RNA vaccines have potential to induce systemic

immunity akin to i.m. RNA vaccines and to seed robust protective mucosal immune responses. The combination of this vaccine's thermostability with its potent mucosal and systemic immunogenicity may represent a key new technology for combating SARS-CoV-2 and other respiratory infections of pandemic potential.

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493. AAV-Mediated Expression of Monoclonal Antibodies Provides Protection in a Mouse Model of Marburg Virus Infection and Long-Term Expression in an Ovine Model

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Vectored monoclonal antibody (mAb) expression mediated by adeno-associated virus (AAV) gene delivery can generate protective and sustained concentrations of therapeutic mAbs in animal models for a variety of infectious diseases, including Ebola virus and HIV. Our rationally engineered AAV6 triple mutant capsid, termed AAV6.2FF, facilitates rapid and robust mAb expression following intramuscular administration. Previously, AAV6.2FF-mediated expression of murine IgG2A mAbs 2G4 and 5D2 in mice conferred 100% protection against Ebola virus challenge. We have now re-engineered our expression platform to produce human IgG1 mAbs comprised of the heavy and light chain variable domains of MR191 and MR78, two potent antibodies against the Marburg virus glycoprotein. Intramuscular injection of mice with 1×10^{11} vector genomes (vg) of AAV6.2FF-MR78 and AAV6.2FF-MR191 resulted in serum concentrations of approximately 100vg/mL and 300vg/mL of human IgG, respectively, for sustained periods of time (greater than 32 weeks). Mice receiving 1×10^{11} vg (high) and 1×10^{10} vg (low) doses of AAV6.2FF-MR191 were 100% protected against lethal Marburg virus challenge. Serum human IgG concentrations immediately before challenge ranged from 24-137vg/mL for the low dose and 383-525ug/mL for the high dose group. A dose of 1×10^{11} vg AAV-mAb yielded similar serum mAb expression levels between female and male BALB/c mice, whereas the same dose split into one injection per limb compared to a single injection in female BALB/c mice resulted in statistically significantly higher levels in the multi-injection group compared to the single injection by day 28, at 109-161vg/mL and 85-125vg/mL, respectively. A second animal study utilizing lambs was conducted to determine longevity and tolerability

of AAV6.2FF-MR191 in a large animal model. Three two-week-old male lambs were administered 5×10^{12} vg/kg of AAV6.2FF-MR191 by intramuscular injection to the rump, resulting in sustained expression for over 450 days post-administration, with peak hIgG plasma expression levels ranging between 50-120vg/mL. AAV6.2FF-mediated expression of mAbs demonstrated a lack of significant alterations in serum biochemistry or histopathological findings in this ovine model. Additionally, the three lambs revealed low levels of anti-AAV6.2FF capsid antibodies and anti-MR191 antibodies for the duration of the experiment, showcasing the potential of this platform as a one-time, prophylactic approach. AAV-mediated antibody gene transfer is a viable method for prolonging the therapeutic effect of recombinant mAbs and represents a potential alternative “vaccine” strategy for those with compromised immune systems or in possible outbreak response scenarios.

494. Adenovirus Capsid Proteins-Based Anti-Fentanyl Vaccine Attenuates Fentanyl-Induced Behaviors in Mice

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Fentanyl, a highly addictive, synthetic opioid variant of heroin that was originally developed for pain management, has become a recreational drug as well as a disguised substitute of heroin added to heroin without the user’s knowledge. With potency 80-100-fold more than morphine and a lethal dose of 2 mg, it is 15 to 20 times more dangerous than heroin and has led to >50,000 US deaths in 2020. We have developed a vaccine that targets fentanyl based on direct covalent conjugation of an analog of fentanyl to the capsid proteins of the highly immunogenic serotype 5 E1⁻E3⁻ adenovirus (dAd5). The vaccine is produced by heat and detergent disruption of the Ad5 capsid, followed by chemical crosslinking of a fentanyl analog to the capsid hexon, fiber and penton proteins. It is formulated with the Adjuplex[®] adjuvant and administered intramuscularly. The vaccine evokes antibodies that bind fentanyl, abrogating access to the fentanyl receptors in the CNS by preventing transfer through the blood brain barrier. If efficacious, the vaccinated individual would no longer evoke perceived reward by taking fentanyl and, importantly, there would be a reduced risk of overdose. The development of the anti-fentanyl vaccine requires the identification of chemical analogs of fentanyl that enable: (1) coupling to adenovirus capsid proteins; and (2) immunologically resemblance to fentanyl such that the evoked antibodies cross react in a highly specific way to fentanyl. We have evaluated carfentanil, a commercial analog, and three novel construct analogs, haptens 1, 2 and 3. Preliminary studies established anti-fentanyl potency with hapten 3 was more effective than hapten 2 and carfentanil. dAd5-Carfentanil vaccinated mice evoked anti-fentanyl antibody titers greater than 10^5 over 4 to 8 wk. The vaccinated mice had $64 \pm 30\%$ reduction of fentanyl reaching the brain ($p < 0.02$) with sequestration of the fentanyl in blood ($p < 0.001$). The dAd5-Hapten 3 conjugate vaccine, induced high anti-fentanyl antibody levels with peak 6 wk titers ranging 6.5×10^5 to 1.1×10^6 , 3- and 60-fold higher than dAd5-carfentanil and dAd5-Hapten 2, respectively. In competitive ELISA, the dAd5-Hapten

3 induced antibody had about 4-fold higher specific affinity for fentanyl compared to the vaccine based on carfentanil. Ambulatory activity in open field boxes demonstrate dAd5-hapten 3 conjugate vaccinated mice had significantly reduced fentanyl-induced hyper-locomotor activity compared to unvaccinated mice ($p < 0.02$). On a hot plate sensitivity test, fentanyl-induced antinociception was blunted (>86% reduction) in dAd5-hapten 3 vaccinated mice compared to control ($p < 0.03$). In summary, leveraging high immunogenicity of the adenovirus capsid proteins, we have developed potent anti-fentanyl vaccine that should be useful in preventing addiction reward and fentanyl overdose in addicts.

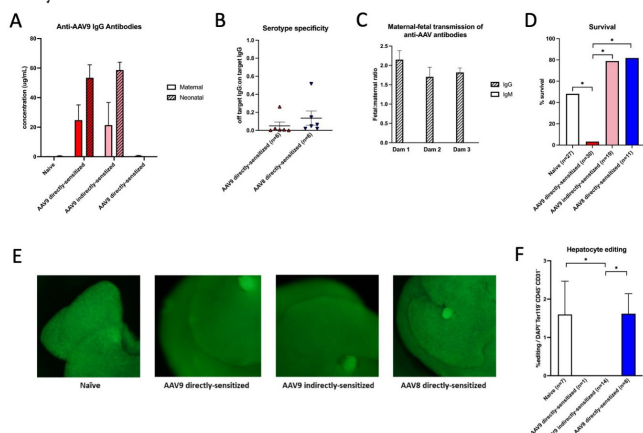
495. Pre-Existing Maternal Humoral Immunity to Adeno-Associated Virus Impairs Fetal Gene Editing in a Serotype-Specific Fashion

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PURPOSE: In utero gene editing (IUGE) is an experimental treatment for inherited metabolic liver diseases. Pre-existing antibodies to adeno-associated virus (AAV) have been shown to impair delivery of CRISPR/Cas9-mediated genome editing molecules in adults. We assessed whether pre-existing maternal antibodies to AAV are transferred to the fetus and impair gene editing in a mouse model of in utero CRISPR-NHEJ. **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). Using this model, 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 4 groups: naïve dams; dams directly-sensitized to AAV9 by intramuscular injection of AAV9.CMV.null (humoral and T cell immunity), dams indirectly-sensitized to AAV9 by adoptive transfer of serum from directly-sensitized dams (humoral immunity only); and dams directly-sensitized to AAV8. Maternal-fetal transmission of AAV-specific antibodies was assessed by enzyme-linked immunosorbent assay (ELISA) of serum collected from uninjected neonates sacrificed immediately after birth. IUGE was performed in separate litters at 16 days post-coitum via fetal intravascular injection of AAV9.SpCas9.sgloxP. Fetal survival to birth and liver editing were compared among the groups, with liver editing assessed grossly by stereomicroscopy and quantitatively by flow cytometry 14 days after injection. **RESULTS:** Intramuscular injection of AAV resulted in robust production of serotype-specific IgG antibodies (Figure 1A) with minimal cross-reactivity between AAV9 and AAV8 capsid (Figure 1B). Anti-AAV9 IgG was readily transferred to the fetus such that at birth the neonate had twice the concentration of anti-AAV9 IgG compared to its mother (Figure 1C). Neonatal anti-AAV9 antibodies were equal between neonates born to dams directly- and indirectly-sensitized to AAV9 (mean: 53.4 vs. 58.6ug/mL, $P = 0.66$). Only direct maternal sensitization to AAV9 significantly increased fetal mortality after IUGE (Figure 1D). No editing was observed by stereomicroscopy (Figure 1E) or flow cytometry (Figure 1F) among offspring born to dams either directly- or indirectly-sensitized to AAV9. By contrast, comparable editing was observed between the offspring born to naïve and AAV8 directly-sensitized dams.

CONCLUSION: Pre-existing maternal antibodies to AAV are transferred readily to the fetus and prevent gene editing in the absence of maternal T cell immunity and in an AAV serotype-specific fashion. Combined maternal humoral and T cell immunity but not isolated humoral immunity resulted in increased fetal mortality after IUGE, suggesting that maternal-fetal T cell trafficking may impact the safety of IUGE in sensitized mothers. Options to mitigate a pre-existing maternal humoral immune response include altered serotypes and non-viral delivery approaches, maternal screening for serotype-specific antibodies and cytotoxic T cells, and vector delivery earlier in gestation when maternal-fetal IgG transmission is less efficient. These require study and consideration in the context of future clinical translation.



injections of AAV vectors, and their peripheral tissues were collected 30 days post-injection, immunolabeled and analyzed by confocal microscopy. Image J and QuPath software was used to determine fluorescent intensity and to quantify mCherry expressing cell number. Vector genome copy number (VGCN) was determined using qPCR. Statistics were assessed using two-way analysis of variance with Turkey's post hoc test in GraphPad Prism 9.0 software. All animal procedures were approved by the Princeton University Institutional Animal Care and Use Committee. **Results.** Our results demonstrate that different cell types can efficiently express a transgene when transduced by AAV8-LAP2 or AAV9-LAP2 following local or systemic administration. LAP2-mediated transgene expression was compared side by side with the ubiquitous promoter EF1 α . Both AAV8-LAP2 and AAV9-LAP2 drive high level transgene expression in liver and kidney after RO injection and quadriceps, tibialis anterior, extensor digitorum longus (EDL), soleus and gastrocnemius after IM injection. Both RO and IM administration provided broad vector biodistribution and transgene expression in the lungs, but less efficiently than in other tissues analyzed. In liver and skeletal muscle over 75% of cells showed mCherry expression from AAV8-LAP2 or AAV9-LAP2, and approximately 50% of cells in kidney and lung were mCherry-positive. We next compared the vector DNA biodistribution in liver, kidney, lung, and skeletal muscle (quadriceps). AAV9-LAP2 showed a predominant tropism for liver after RO injection, with less copies found in kidney, liver, and skeletal muscle with RO and IM administration. No significant differences between the promoters were observed in terms of transgene expression or VGCN in the different tissues. **Conclusion.** The biodistribution results of this study indicate that the small LAP2 promoter can drive potent transgene expression comparable to that of the larger EF1 α promoter. After a single dose, the 404bp LAP2 promoter drives strong expression of reporter transgene with AAV8 and AAV9 in liver, kidney, lung, and different skeletal muscle. Further, these data demonstrate that the route of administration is an important variable when considering the choice of AAV serotype and promoter. These findings have important implications for the development of new therapies in diseases where a gene factor is secreted and ubiquitous expression in all cell types is required.

AAV Vectors - Virology and Vectorology II

496. The Alphaherpesvirus Latency Associated Promoter (LAP2) Drives Widespread Gene Expression in Peripheral Organs Dependent on AAV Serotype and Administration Route

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Introduction. Viral vectors have emerged as a platform for gene therapy and vaccination approaches. Adeno-associated virus (AAV) are currently used to treat a variety of genetic diseases. The efficacy of AAV as a gene delivery tool relies on regulatory elements such as promoters, critical for efficient and long-lasting transgene expression. For some therapies, such promoters should provide either cell-type-specific or ubiquitous transcription. However, the promoter of choice is limited by the size of the transgene and the limited AAV genome-packaging capacity less than 4.9 kb. To improve AAV vectors as a gene therapy tools, we validated a short, strong, constitutive promoter obtained from the genome of the herpesvirus pseudorabis virus (PRV) called alphaherpesvirus latency-associated promoter 2 (LAP2). Here we report strong transgene expression in liver, kidney, lung, and different skeletal muscles after AAV8-LAP2 and AAV9-LAP2 delivery by multiple routes of administration. **Methods.** The LAP2 and EF1 α promoter sequences were packaged into AAV8 and AAV9 vectors driving transcription of the mCherry reporter. Adult C57BL/6 mice were transduced by either retro-orbital (RO) or intramuscular (IM)

497. Epigenetic Chemical Control of AAV Transgene Expression *In Vitro*

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Adeno-Associated Virus is a widely-used gene therapy vector; there are 2 FDA approved drugs that use AAV and numerous other clinical trials that depend on AAV as the gene therapy vector. While AAV is a common gene therapy vector, very little control of the AAV-delivered transgene can be achieved after delivery. There are also scenarios in which AAV therapeutics fail during clinical trials because the desired amount of transgene expression cannot be achieved within patients. A possible solution to the lack of AAV transgene expression control once administered could be to use chemical compounds that are able to recruit endogenous epigenetic gene expression machinery to the AAV transgene promoter and increase the transgene expression in a dose dependent manner. In this work, zinc fingers that target the AAV

transgene were used in conjunction with chemical compounds that can recruit endogenous transcription activation machinery *in vitro* to achieve chemically mediated enhancement of transgene expression. These compounds are able to recruit epigenetic bromodomain protein 4 to the AAV transgene. Once there, bromodomain protein 4 facilitates increased gene expression through recruitment of other transcription activating machinery. Enhancement of AAV transgene expression with this compound was shown to be dose-dependent, thus we can regulate transgene levels with different doses of chemical epigenetic modifier activator. The effects of the compound were also reversible through wash out or chemical competition. We further demonstrated that chemically mediated transgene expression control is sustainable over time. These results could provide a method for researchers to chemically control and enhance AAV transgene expression.

498. Development of Optimized (Opt) AAVrh74 Vectors with Increased Transduction Efficiency in Primary Human Skeletal Muscle Cells *In Vitro* and in Mouse Muscles *In Vivo* Following Systemic Administration

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In a phase I/II clinical trial sponsored by Solid Biosciences using AAV9 vectors, serious adverse events such as complement activation and thrombocytopenia causing renal damage and cardiopulmonary insufficiency were reported. In a trial sponsored by Pfizer, also using AAV9 vectors, several serious adverse events such as acute kidney injury involving atypical hemolytic uremic syndrome and thrombocytopenia, and more recently, the death of a patient, were also reported. Sarepta Therapeutics reported the results of a phase I/II trial using AAVrh74 vectors with vomiting as the only adverse event, indicating that AAVrh74 vectors are safer, although a high dose of 2×10^{14} vgs/kg was used. We have previously reported that capsid-modified next generation (“NextGen”) AAVrh74 vectors (*Mol. Ther.*, 29: 159-160, 2021) and genome-modified generation X (“GenX”) AAVrh74 vectors (*Mol. Ther.*, 29: 184-185, 2021) are significantly more efficient than their wild-type (WT) counterpart. In the present studies, we combined the two modifications to generate the optimized (“Opt”) AAVrh74 vectors, and evaluated their transduction efficiency in primary human skeletal muscle cells *in vitro*, which was up to ~5-fold higher than that of the conventional AAVrh74 vectors. The efficacy of the WT and the Opt AAVrh74 vectors was also evaluated in mouse muscles *in vivo* following systemic administration. As can be seen in **Figure 1**, the transduction efficiency of the Opt AAVrh74 vectors was ~5-fold higher in gastrocnemius (GA) (A) as well as in tibialis anterior (TA) (B) muscles. Interestingly, the total genome copy numbers of either the WT or the Opt AAVrh74 vectors in GA, TA, diaphragm and heart muscles were not significantly different (C), suggesting that the observed increased transduction efficiency of the Opt AAVrh74 vectors was due to improved intracellular trafficking and nuclear transport of these vectors, which is consistent with our previously published studies with other Opt AAV serotype vectors. Taken together, these studies suggest that the use of Opt AAVrh74 vectors may lead to safe and effective gene therapy of human muscular dystrophies at reduced

doses. However, as observed in a recently published study (*Hum. Gene Ther.*, 32: 375-389, 2021), a significant fraction of the AAVrh74 vectors is sequestered in the liver, which is undesirable. Efforts are currently underway to develop liver de-targeted Opt AAVrh74 vectors for their optimal use in gene therapy of human muscular dystrophies at further reduced doses.

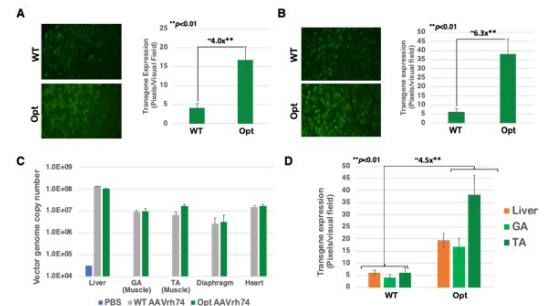


Figure 1: Transduction efficiency of WT and Opt ssAAVrh74 vectors in GA (A) and TA (B) muscles following intravenous delivery of 1×10^{12} vgs/mouse in C57BL6 mice. Various indicated tissues were harvested 8-weeks post-vector administrations, and were analyzed for vector genome copy numbers (C). Relative transgene expression in the liver and muscle was also evaluated (D). Data were quantitated using the NIH ImageJ software.

This research was supported by a sponsored research grant from Sarepta Therapeutics.

499. Development of Genome-Modified Generation Y (GenY) AAVrh74 Vectors with Improved Transgene Expression in a Mouse Skeletal Muscle Cell Line and in Primary Human Skeletal Muscle Cells

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Since the naturally occurring AAV contains a single-stranded DNA genome, and expresses the viral genes poorly, because ssDNA is transcriptionally-inactive, transgene expression levels from recombinant ssAAV vectors are also negatively impacted. We have previously reported that substitution of the D-sequence in the left inverted terminal repeat (ITR) results in generation X (“GenX”) AAV vectors, which mediate up to 8-fold improved transgene expression (*J. Virol.*, 89: 952-961, 2015). More recently, we also reported that the extent of transgene expression from GenX AAVrh74 vectors is ~5-fold higher than that from the wild-type (WT) AAVrh74 vectors (*Mol. Ther.*, 29: 184-185, 2021). We previously observed that the distal 10-nucleotides (nts) in the AAV2 D-sequence share partial homology to the the consensus half-site of the glucocorticoid receptor-binding element (GRE), and that the glucocorticoid receptor signaling pathway is activated following AAV2 infection/AAV2 vector transduction (*Mol. Ther.*, 24: S6, 2016). In the current studies, we evaluated whether substitution of the distal 10-nts in the D-sequence with the authentic GRE would lead to increased transgene expression from AAVrh74 vectors, termed generation Y (“GenY”) vectors, shown schematically in **Figure 1A**. The extent of the transgene expression from the WT and GenY AAVrh74 vectors was evaluated in a mouse skeletal muscle

cell line, C2C12. As can be seen in **Figure 1B**, the GenY AAVrh74 vectors averaged ~2-3-fold increase, compared with that from the WT AAVrh74 vectors, which was further increased by ~4-5-fold following pre-treatment with tyrphostin, a specific inhibitor of cellular epidermal growth factor receptor protein tyrosine kinase. The WT, GenX, and GenY vectors were also evaluated in primary human skeletal muscle cells, which revealed that the extent of the transgene expression from the GenX and the GenY AAVrh74 vectors averaged respectively ~4-fold and ~6-fold higher compared with that from the WT AAVrh74 vectors (**Figure 1C**). The observed increase in transgene expression was not due to increased entry of the GenX or the GenY vectors, as documented by approximately similar numbers of vector genome copy numbers quantitated by qPCR analyses of low mol. wt. DNA samples isolated from cells transduced with each of these vectors (**Figure 1D**). Studies are currently underway to evaluate the efficacy of the GenY AAVrh74 vectors in skeletal muscles following systemic administration in a murine model *in vivo*. Taken together, these studies suggest that the combined use of the capsid-modified NextGen + 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.

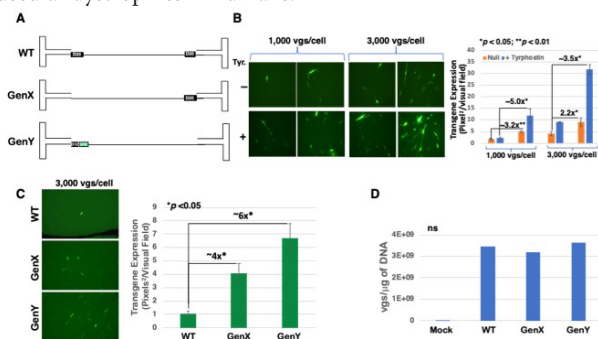


Figure 1: (A) Schematic structures of the WT, GenX, and GenY AAV2 genomes. (B) Transduction efficiency of WT and GenY AAVrh74 vectors in mouse C2C12 cells in the absence and presence of Tyr. (C) Transduction efficiency of WT, GenX, and GenY AAVrh74 vectors in primary human skeletal muscle cells. Cells were transduced with each vector at the indicated vector genome copy numbers/cell at 37°C for 2 hrs, and transgene expression was visualized under a fluorescence microscope 72 hrs post-transduction. Data were quantitated using the NIH ImageJ software. (D) Vector genome copy numbers in primary human skeletal muscle cells transduced with WT, GenX, and GenY AAVrh74 vectors.

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500. VP1 Unique and VP1/2 Shared Region Serotype Swap Hybrids Enhance Desirable AAV Properties

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AAV-based gene therapies have the potential to treat many severe diseases through a wide range of modalities, from gene addition to gene editing and modulation of gene expression. For successful delivery of the DNA payload, AAV vectors must efficiently bind to the intended target tissue, enter the cell, and traffic to the nucleus. Cell entry depends

on endocytosis followed by endosomal escape, which requires the action of the PLA2 domain located in the VP1-unique (VP1u) region of the AAV capsid, and trafficking to the nucleus, mediated by basic residues located in VP1u and the VP1/2 shared (VP1/2s) regions. With the goal of engineering capsids that maintained parental tropism with improved endosomal escape and nuclear trafficking, we designed hybrid capsids by swapping VP1u and VP1/2s from different serotypes selected based on data gathered from a previous study (Giles *et al.* ASGCT 2021). Criteria for selection included several factors such as RNA/DNA ratio and tissue-specificity of gene expression. Overall productivity of vectors was not significantly negatively impacted by the introduction of hybrid domains as measured by small scale production by triple transfection in suspension HEK293 cells. We hypothesized that VP3 is the primary driver of tropism and therefore genomic DNA biodistribution would be largely unchanged by swapping VP1u and VP1/2s regions. We anticipated that the primary differences would be seen at the RNA expression level, indicating altered endosomal escape and trafficking properties. A barcoded, AAV vector pool containing these hybrids and their parental capsids was created and administered intravenously to non-human primates. Biodistribution of vector genomes was similar to their respective parental VP3 serotypes for most of the hybrid capsids, except for those containing VP1u and VP1/2s of AAV5. The AAV5 VP1u and VP1/2s-containing hybrids had lower vector genome copy numbers in all tested tissues compared to their parental, wild type VP3 serotypes (AAV8, AAV9, and AAVrh.15); however, relative abundance of RNA transcripts from each capsid showed a liver-specific transcriptional down-regulation of AAV5-containing hybrids. For example, AAV5/AAV8 hybrid capsids have lower genome copy numbers than AAV8 in all tissues tested, but at the transcriptional level are liver de-targeted and express RNA more efficiently than wild type AAV8 in skeletal muscle, leading to an improved RNA/DNA ratio (2.5-3.5X increase over AAV8). This may indicate increased endosomal escape or nuclear trafficking abilities of AAV5 VP1u and VP1/2s-containing capsids. Furthermore, AAV5/AAV8 hybrids produce 2.5-3X more vector than their parental capsid, AAV8, in standard triple transfection of HEK293 suspension culture. Ultimately, AAV5 hybrid capsids may present a unique method of liver de-targeting at the transcriptional level and offer improved production.

501. Development of a Sensitive NanoLuc Based Bioluminescent Assay for Real Time Measurement of rAAV Transduction

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Recombinant adeno-associated viruses (rAAV) are widely used delivery vehicle for transgene expression for treatment of monogenic diseases. Key features that make rAAV a leading platform in gene therapy is tissue tropism, long-term transgene expression, and low immunogenicity. To further improve the target specificity, eliminate immunogenicity, and enhance efficacy, new engineered versions of rAAV are being designed along with optimization of transduction efficiencies in various cell types. During early development and optimization of novel rAAVs, surrogate protein expression (e.g. green fluorescent proteins (GFP) or its variants) is used to track if desired endpoint has been achieved. While widely used, the detection sensitivity of GFP is limited so its expression is only measurable 48hrs post transduction or later. In this

work, we describe an assay using NanoLuc Luciferase as a surrogate to monitor in real-time the transduction by rAAV. NanoLuc luciferase is extremely bright and therefore allows for a very sensitive detection of gene expression. In addition, use of a cell permeable NanoLuc substrate (Endurazine) enables live cell kinetic measurement of NanoLuc expression in the extended periods of several hours or days. Moreover, assays can be performed on a simple luminometer. For initial experiment, we generated AAV2 vector containing NanoLuc and transduced into HT1080 cell line (10,000 cells per well) at different measure of infection (MOI) in a 96well plate. To monitor the expression of NanoLuc, Endurazine was added to the wells and luminescence signal was measured continuously for 48hrs. A time dependent increase in luminescence signal was observed, 6hrs post transduction. Expression reached a plateau at 30 hrs and the absolute luminescence signal was dependent on MOI. Once optimized, we demonstrate the utility of the assay for two applications including: (a) rAAV transduction efficiency as a function AAV serotype and cell type and (b) screening of antibodies to inhibit rAAV transduction which we believe will be useful as detection of neutralization antibodies (nAb) against various rAAVs.

502. Separation of Full and Empty AAV Capsids by Anion Exchange

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Adeno Associated Virus (AAV) is the main vector for gene therapy and there is need for scalable, cost-efficient and robust filtration and chromatography-based purification processes. Key for a successful process are high overall yields of full capsids with empty capsid reduction and efficient impurity removal. In this study, different serotypes were produced by triple plasmid transfection of HEK 293T cells in suspension. The AAV were harvested by cell lysis and the lysate treated with DNA nuclease, clarified, concentrated and buffer exchanged before application to affinity chromatography. The affinity step does not discriminate between full and empty capsids, and a polishing step is required to remove empty capsids. Polishing to reduce empty capsid was evaluated with different ion exchange resins using the highly pure eluate from the previous affinity purification with at least 10% full capsids. Here we present how the separation can be significantly improved by using Q ligand, the presence of dextran extenders and optimized conditions. The UV260:280 ratio in the chromatograms was used to identify the full and empty capsid peaks, but the content of viral genome in the fractions were confirmed by qPCR and the total viral capsid content was assessed by ELISA. In conclusion, to maximize full and empty capsid separation, dextran extenders and optimization of the conditions such as pH, MgCl₂ and NaCl concentration is critical. Depending on serotype or AAV capsid properties, pre-screening and fine-tuning for the optimal conditions is required.

503. Strength of Subunit Association Dictates AAP Requirement for AAV Capsid Assembly

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AAV capsid assembly is mediated by viral proteins VP1, 2, 3, assembly-activating protein (AAP), and ill-defined host cellular factors. Although AAP is required for maximal capsid assembly in all AAV serotypes, AAV4, 5, 11 and rh32.33 are capable of assembling capsids even in the absence of AAP. Despite the recent remarkable progress in AAV capsid engineering technologies, the mechanisms dictating the serotype-dependent AAP requirement for capsid assembly remains elusive. Here we report that the strength of associations of the subunits of the AAV icosahedron is a key factor in determining AAP requirement. In our AAV capsid mutagenesis studies, we have identified a set of the same serotype-derived AAP-dependent and independent variants for the following serotypes, AAV4, 5, 11 and 12. We compared the predicted disorder between the variants (within each serotype) using Predictor of Natural Disordered Regions (PONDR). The disorder near the mutated region always tends to be higher in the AAP-independent variant. Since the mutated regions lie either on the 2-fold axis or 5-fold axis, we found that AAP-independent variants tend to have slightly more disorder either close to the 5-fold axis or the 2-fold axis. This could confer enhanced flexibility in these regions, promoting VP subunit associations and improving functional VP oligomerization and subsequent capsid assembly. We tested this hypothesis by determining the Association Energies (AE) and Buried Surface Areas (BSA) of these AAV variants. Using SWISS MODEL, we modelled both AAP-independent and dependent variants of each serotype against a reference template. Upon obtaining these models, we applied Viperdb's energyPlot function to determine the AE and BSA of each variant. We find that AAP-independent variants have tighter associations predominantly along the axis where the mutations reside (i.e., 2-fold for AAV4, 11, and 12; and 5-fold for AAV5), indicating greater inter-subunit associations in the AAP-independent variants. Tighter associations were indicated by relatively lower association energy (thus easier to associate) and higher buried surface areas in AAP-independent variants. In the AAV icosahedron, VP subunit association is the tightest at the 3-fold axis compared to the 2-fold and 5-fold axes. Thus, VP proteins with increased propensity to associate at the 2-fold or 5-fold axis would have a tendency to oligomerize rather than be monomers, thus protecting them from degradation. Conversely, VP proteins with relatively lower propensity to associate at the 2-fold and 5-fold axis would need non-VP factors for stabilization and therefore depend on AAP for capsid assembly. Together, our findings indicate a model where AAP aids inter-subunit associations; whereas AAP-independent AAV variants have enhanced VP subunit associations at the VP-VP interface, thereby capable of assembling capsid in the absence of AAP.

504. Strong Efficiency in Crossing Blood-Brain Barrier After Systemic Delivery of a Novel Recombinant Engineered Capsid Serotype

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Systemic intravenous delivery of adeno-associated viral vectors (AAV) has multiple advantages compared to other surgical interventions by which AAV is delivered directly into the brain parenchyma or CSF. However, the blood-brain-barrier (BBB) regulates access to the central nervous system (CNS), protecting the brain and spinal cord from non-selective exposure to circulating solutes and large molecules, such as AAV. Among all known recombinant AAV, only a few can cross the BBB and transduce brain cells. Most of these AAVs show systemic delivery efficacy in the CNS only at neonatal stages in which BBB is still immature or “leaky,” favoring the entry of both small and large drugs. To date, only AAV9 has shown convincing brain transduction in adult animals, but the mechanism by which AAV9 crosses the BBB is still not clear. In this pilot study, we evaluated the ability of a novel engineered capsid serotype of recombinant AAV, named LC.V1, to cross the BBB in adult mice. Our data shows robust and widespread cortical and subcortical distribution along the anterior-posterior brain axis including cerebellum, high levels of cellular transduction, and both neuronal and glial tropism 21 days after either tail vein or retroorbital administration at maximum dose of 8.36×10^{13} vg/mL. Transduction in spinal cord, dorsal root ganglia (DRG), and peripheral organs including liver, spleen, heart, and lungs were also investigated. Histological analysis of AAV-mediated transgene expression shows strong transduction at the DRG entry zone, as well as ventral horns and their motor neurons. Liver and heart showed positive signal while AAV-mediated transduction was not present in the other peripheral organs analyzed. Additional experiments evaluating the minimum effective dose and levels of expression at shorter survival times (1, 3, and 7 days) are currently undergoing. In summary, this novel variant has showed to be a very promising tool for investigating systemic gene delivery to target CNS diseases by crossing the BBB.

505. ADEVO: Proof-of-Concept of Adenovirus Directed EVolution by Random Peptide Display on the Fiber Knob

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Directed evolution is a method of vector development. It consists in generating a random library of vector variants and selecting the improved variants according to pre-defined selection criteria. It already led to numerous innovations in Adeno-associated virus or Lentivirus vectors, but yielded limited success in Adenovirus vector (AdV) development, 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. Here we designed novel directed evolution protocols for AdV retargeting, based on the insertion of random peptides in the fiber knob domain. In order to assess and optimize the protocols, we constructed libraries based on the commonly used adenovirus type 5 (Ad5) and tested if we could select variants able to infect A549-ΔCAR cells, that lack Ad5's primary receptor and are thus non-permissive for wild-type vectors. In protocols 1 and 2, random oligonucleotides are inserted through NEBuilder® homologous recombination inside a shuttle plasmid containing the AdV fiber gene, which is then transferred to a full AdV genome respectively by *in vitro* or *in vivo* recombination with fiber-deleted genomes. In protocol 3, the genome library is built in a single step through direct insertion of random oligonucleotides in the AdV genome. In all protocols, the initial vector library is rescued by transfection of the genome library in HEK293 cells. Variants with improved tropism for A549-ΔCAR cells are then selected by serial passage of the libraries on these cells. Libraries complexity was estimated by NGS. After co-transfections of wild-type and fiber-deficient adenovirus genomes with up to 5000 transfected genomes per cell, a dose at which high vector titers can be produced, we found that less than 3% of capsids packaged a genome different from the one that encoded them (cross-packaging). Therefore, cross-packaging should not hamper library selection. The shuttle library of ADEVO protocol 1 contained an estimated 5.4×10^6 unique clones. As a quality control, we isolated 27 of them, which all carried different insert sequences and facilitated the rescue of infectious particles when recombined into the adenovirus genome. It suggests that a large majority of inserted peptides do not hamper the functionality of the fiber protein. Protocols 1 and 3 enabled the construction of highly diverse genome and vector libraries. The vector libraries contained respectively around 1.2×10^9 and 3.8×10^8 viral particles, and an estimated 2.7×10^5 and 5.5×10^4 transducing units in A549-ΔCAR cells, showing efficient retargeting. Variants able to infect efficiently A549-ΔCAR cells were enriched during the selection phase and could be purified and characterized. On the contrary, protocol 2 proved too inefficient

and technically challenging for consideration for a wider use. ADEVO, our novel directed evolution workflow for Adenoviruses, facilitates the construction of large variant libraries and the selection of improved vectors with user-friendly protocols. We hope that it can contribute to the development of clinically relevant adenoviral vectors with more specific tropism and higher transduction ability in target cells.

506. Developing Nanopore-Based Sequencing Protocol to Evaluate AAV Directed Evolution in Human Lung Models

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Approval of multiple recombinant adeno-associated virus (rAAV)-based products validates the safety and utility of rAAV as an *in vivo* vector for clinical use. However, the specificity and efficacy of rAAV are still limiting factors hindering the wider adoption of this vector for clinical applications. Improving rAAV to target specific cells, for example, progenitor basal cells or ciliated cells in the airway epithelium would expand the utility of rAAV to treat debilitating conditions such as cystic fibrosis, COPD, ciliary dyskinesia, and asthma. Despite the limited understanding of the complex AAV capsid biology, employing AAV capsid engineering via directed evolution has led to the generation of several novel variants with improved transduction capabilities. Besides generating improved variants, monitoring AAV library selection and enrichment during directed evolution facilitates a better understanding of AAV capsid biology and informs capsid engineering in a cell-specific manner. A standard procedure to track capsid enrichment is Sanger-sequencing of single clones, which is inexpensive and simple but low in throughput. This limitation could be overcome by employing long-read sequencing like the Oxford Nanopore Technology (ONT) to capture the entirety of the enrichment process, especially for *de novo* assembly of AAV libraries that are usually randomly generated. Our aim is to develop a simple AAV library tracking protocol based on ONT, which could be employed in laboratory settings with minimal resources, to track AAV library enrichment in different *in vitro* human lung models. As proof of principle, we generated a DNA-shuffled AAV library with selected serotypes. The starting materials were PCR fragments of three AAV serotypes with different capsid gene DNA homology (phylogenetic neighbour-joining distance value), specifically AAV1 (0.0147), AAV6.2FF (0.0151), AAV8 (0.0821) as well as hBocavirus1 gene (0.451) as a control for DNA with least homology. Following DNA-shuffling, the library was cloned into an rAAV genome plasmid carrying AAV2 ITRs downstream of an AAV2 REP2 gene. Next, the rAAV library was produced in HEK29T/17 cells using a helper-free production and iodixanol centrifugation purification method. The purified rAAV was used as a PCR template to extract the DNA-shuffled capsid gene. The PCR amplicons were prepped with ONT 1D Amplicon by Ligation (SQK-LSK109) and sequenced for 8 hours using a R9.4.1 flow cell on a MinION Mk1B device. The raw MinION sequencing reads were analysed using open-source programs *Wtdbg2*, *minimap2*, *samtools* and *bedtools*. As anticipated, we observed that recombinant events in the generation of the rAAV library reflected the DNA homology between the capsid genes; thus most recombination occurs between AAV1 and AAV6.2FF DNA fragments, with a lower proportion of AAV8

fragments (Chi-square test; $p < 0.0001$). Moreover, hBocavirus1 was not detected in the shuffled library. Importantly, these findings indicate that our protocol and bioinformatics pipeline can capture the diversity of DNA-shuffled capsid genes during AAV library construction. We are currently performing directed evolution in clinically relevant *in vitro* cell culture models of both human conducting airway and human lung parenchyma, tracking the enrichment achieved using this approach. Outcomes from this study could provide insights into engineering AAV capsid for lung-based applications.

507. Multiple Generations of Capsids Engineered in NHPs Yield Improved Performance in the Central Nervous System

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The number of gene therapies in development that target indications in the CNS space has increased dramatically in recent years, driven by proof-of-concept studies established in the clinic. At the same time, the limitations of wildtype AAV serotypes to broadly and uniformly transduce cells throughout the CNS, particularly in subcortical and deep-brain structures, has been well documented (Chuapoco et al. 2022). Many indications that are amenable to genetic therapies benefit from transduction throughout the brain, which is difficult to achieve through conventional delivery methods. Engineered capsids capable of crossing the blood-brain-barrier following IV delivery and broadly and efficiently transducing cells throughout the CNS have been previously reported (Deverman BE et al. 2016, Chan KY et al. 2017), but these variants have shown poor translatability to non-human primates. As such, we sought to utilize Capsida Biotherapeutic's novel NHP-driven AAV engineering platform to develop rAAVs capable of crossing the blood-brain barrier following IV administration. Capsida's platform was employed to select AAV9-based vectors with brain enrichment and transduction of cells throughout the CNS. In both a broad and deep search of sequence space, variants spanning a range of improvements in CNS selectivity were discovered. As measured by vector genome residence, capsids were found to be greater than 25-fold improved over AAV9 across both juvenile and infant NHPs. Variants were tested additionally on iPSC-derived neurons and demonstrated improved transduction *in vitro*. These candidates are being advanced with paired cargo into preclinical disease models for clinical development.

508. Structural Elucidation and Characterization of Avian Adeno-Associated Virus Capsid

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Adeno-associated virus (AAV) vectors currently used in gene therapy trials or approved human gene therapy biologics are based on human or non-human primate AAVs. One issue undermining their broad use in the clinical setting is the high prevalence of circulating neutralizing

antibodies (NAb) in the general population targeting the virus capsids leading to vector inactivation and to a loss of treatment efficacy. To overcome this issue the capsid surface of these AAVs can be engineered to escape from Nabs. An alternative to this strategy is the utilization of AAVs that do not disseminate in the primate population and exhibit low or no reactivity with human sera and one such example is Avian AAV (AAAV). The AAAV capsid shows low sequence identities (~55-60%) to the human AAV serotypes, including the sequence and structurally diverse AAV4 and AAV5. Nonetheless, AAAV can be produced using the standard triple plasmid transfection system in HEK 293 cells. Here we present the capsid structure of AAAV determined by cryo-electron microscopy to a resolution of 2.2 Å. While the core structure of the capsid is conserved, the surface loops display unique structural features that are different to the human and primate AAV serotypes, respectively. These structural differences are located in the previously defined capsid variable regions (VR), namely VR-IV, -V, -VII, and -IX, when compared to AAV2. Many of the surface loops of the AAV serotypes have previously been identified as binding sites for NABs. Native immuno-dot-blots with human sera from 50 healthy individuals against AAAV will be presented to evaluate the capsids antigenicity relative to other AAV serotypes. This structure-function characterization will be beneficial to further expand the current repertoire of AAV vectors in human gene therapy applications.

509. Efficient Crossing of the Primate Blood-brain Barrier by Adeno-Associated Virus Serotype 4 (AAV4)

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Efficiently delivering therapeutic transgenes to widespread brain and spinal cord regions after systemic administration remains among the largest challenges in human gene therapy research. Here, we analyzed the capacity of 86 distinct barcoded capsid variants of the adeno-associated virus (AAV), subdivided into two libraries comprising 36 or 71 sequences, to cross the blood-brain barrier (BBB) in three different species: (i) in vivo in a non-human primate (NHP) - 71 sequences tested, (ii) in vitro in a model of the human BBB derived from induced pluripotent stem cells - 36 sequences tested, and (iii) in vivo in adult mice - 71 sequences tested. Strikingly, AAV4 emerged as a lead candidate in both primate models but not in mice. In the NHP, AAV4 not only showed widespread transduction in various brain regions following intravenous administration, but it was also detargeted from the liver as compared to other frequently studied AAV serotypes such as AAV9. Furthermore, AAV4 transduced the aorta and the circle of Willis in the NHP, i.e., a circulatory anastomosis at the base of the

brain, suggesting an affinity of AAV4 for endothelial cells. This study has identified AAV4 as an interesting, previously underappreciated AAV variant for transgene delivery across the primate BBB following peripheral administration, which holds potential for further capsid engineering to target and treat a variety of human brain disorders. Concurrently, our direct comparison of results obtained using primate and non-primate screening underscores the importance of assessing AAV capsid performance in species with the greatest potential for clinical translation.

510. Naturally Occurring Variations at the 501 and 706 Residues on AAVHSC16 Contribute to Reduced Liver Tropism and Slower Serum Clearance

Laura J. Smith, Lindsay Schulman, Laura Van Lieshout, Samantha Smith, Liana Behmoiras, Nancy Avila, Deiby Faulkner, Alec Tzianabos, Arnold Sengooba, Jason Lotterhand, Albert Seymour, Omar Francone

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AAVHSCs, derived from human hematopoietic stem cells, are naturally occurring adeno-associated virus (AAVs) with one to four unique variations in the capsid when compared to fellow Clade F member, AAV9. The unique variations of the panel of 15 AAVHSCs contribute to their distinct biodistributions both in mice and non-human primates (NHPs). One AAVHSC, AAVHSC16, displays lower liver tropism compared to other AAVHSCs in multiple species and does not induce any elevation of alanine transaminase (ALT) or aspartate transaminase (AST) levels at high doses, up to 1E14 vg/kg, in NHPs. AAVHSC16 has two unique amino acid variations compared to other AAVHSCs, F501I and Y706C. Both naturally occurring variations of AAVHSC16 are surface exposed on the capsid. We employed mutagenesis to determine the contribution of each naturally occurring variation to the unique biodistribution of AAVHSC16. The F501I and Y706C variation was introduced onto the AAVHSC15 capsid and biodistribution was determined through tissue specific vector genomes (vgs) for each capsid in albino C57 mice at doses of 1E13 and 1E14 vg/kg at six-weeks post-dose. Both AAVHSC15 F501I and Y706C modified capsids displayed lower liver tropism compared to wild-type AAVHSC15, but liver transduction was not as low as the parent AAVHSC16, indicating a synergistic effect of both variations on liver tropism. Mutagenesis and binding experiments indicated that the 501I variation drastically changed terminal galactose binding while the 706C variation had unaltered glycan binding. These data indicate that the lower liver tropism observed with 706C is due to an influence on non-glycan binding steps of transduction. To understand the relationship between clearance to overall biodistribution including liver tropism, we investigated the clearance from blood of AAVHSC15, AAVHSC16 and the two AAVHSC15 alternate capsids containing the 501I and 706C variations in albino C57 mice. Blood was collected at various time points over 48 hours and both whole blood and serum was assayed for vgs. AAVHSC15 displayed rapid clearance from blood while AAVHSC16 cleared at a slower rate, although both capsids reached a similar level of vgs in the blood at the latest time point, 48 hours post-dose. No differences were observed between the level of vgs between whole blood and serum, indicating that differences in

immune cell transduction in the peripheral blood was not contributing to differences in the rate of clearance between capsids. The clearance rate of the AAVHSC15 modified capsids appeared to correlate with their liver tropism, as the AAVHSC15 Y706C modified capsid had slower clearance and lower liver tropism compared to the AAVHSC15 F501I modified capsid. These data support previously published hypotheses that liver tropism and blood clearance rates are linked. The 501I naturally occurring variation appears to be contributing to the differences in liver tropism and clearance rate through changes in glycan binding due to its proximity to residues defined as key for galactose binding on AAV9. The 706C naturally occurring variation is contributing in a non-glycan binding manner. Continued structure and function studies will help to understand the relationship of the 706C residue on AAVHSC16 with the reduced liver tropism, slower clearance rate and increased safety profile in NHPs. Additionally, further characterization of the contribution of each naturally occurring variation to the tropism and transduction kinetics of each AAVHSC will allow for rational capsid selection or modulation for developing therapeutic applications.

511. The Structure of the 501 Residue on AAVHSC16 is Imperative to the Functional Binding to Cell Surface Glycans, Which is a Key Step in Successful Transduction

Laura J. Smith, Lindsay Schulman, Laura Van Lieshout, Samantha Smith, Liana Behmoiras, Albert Seymour, Omar Francone

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AAVHSCs, derived from human hematopoietic stem cells, are 15 naturally occurring adeno-associated virus (AAVs) with one to four unique variations in the capsid when compared to fellow Clade F member, AAV9. Cell surface glycans play a key role in the virus-cell interactions as glycan binding is the first step of successful transduction. We investigated how the unique capsid residues of a panel of AAVHSCs contribute to cellular glycan binding and post internalization processes. We have demonstrated that all AAVHSCs tested preferentially bind to terminal galactose with the exception of AAVHSC16. While AAVHSC16 shares the unique variation 505R with several other AAVHSCs, AAVHSC16 has two additional unique variations, F501I and Y706C. Modeling revealed that the 501 and 706 residues are surface exposed on the AAVHSC16 capsid and that the 501 residue is in close proximity to key residues that comprise the galactose binding pocket on AAV9. Phenylalanine is conserved at the 501 position among the major AAV serotypes. To determine the contribution of the two AAVHSC16 residues to altered glycan binding, we mutagenized the 501 and 706 residues on AAVHSC15. Surface exposed glycan binding and expression of the AAVHSC15 F501I and Y706C modified capsids was tested on a panel of glycan mutant CHO cell lines. Transductions were performed on ice to prevent ATP mediated internalization of bound vector genomes (vgs) and cell surface bound vgs determined after unbound vgs were removed through a series of cold PBS washes. Bound vectors were quantified by quantitative PCR post-washing and successful internalization and transgene expression assessed by flow cytometry 24 hours post-washing. Binding of AAVHSC15 F501I to terminal galactose was significantly reduced to AAVHSC16 levels while

the AAVHSC15 Y706C variation resulted in a similar glycan binding profile as wild-type AAVHSC15. While AAVHSC16 and AAVHSC15 F501I had similar levels of vgs bound to the cell surface, AAVHSC15 F501I had higher GFP expression than AAVHSC16. AAVHSC15 also has a unique T346A variation and it is possible this variation influences post internalization transgene expression. To further investigate the impact of the residue at the 501 position in the AAVHSC glycan binding, we substituted the isoleucine on AAVHSC16 with various amino acids including, leucine, threonine, valine, tryptophan and two different tyrosine codons. Each of these substitutions led to an increase in binding to terminal galactose, although full restoration only occurred with the addition of aromatic ring structured residues, suggesting aromatic ringed structures are required for strong carbohydrate recognition. The tyrosine modification at the 501 residue gave the best restoration in galactose binding most likely as it is structurally most similar to phenylalanine. Two tyrosine variants that differ at the codon level displayed differences in their binding to surface exposed glycans. Interestingly, the addition of alternative residues at the 501 position of the capsid also affected binding to non-galactose glycans including mannose and N-acetylglucosamine. While the aromatic ringed structures increased the number of vgs bound to the cell, GFP expression levels were not as high as AAVHSC15, again indicating the possible involvement of T346A on transgene expression. These data imply that the structure of the 501 residue is imperative to the functional binding to cell surface glycans. These studies also highlight the importance of the precise alterations made at key residues when investigating the structure and function relationship of the AAV capsid. Understanding of how the unique natural residues of the AAVHSCs influence their function allows for rational-based selection of capsids for developing therapeutics.

512. Structure Function Characterization of the ON and OFF State of an AAV9 Provector

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Adeno-associated virus (AAV) have been successfully utilized in numerous clinical gene therapy applications. However, broad tropism and seroprevalence present significant barriers to optimal tissue transduction, as delivery of the therapeutic transgene to off-target tissues may cause adverse effects and host immune responses may inhibit gene delivery. To address these shortcomings, AAV9 provectors with a protease activatable site were developed to target damaged tissues with elevated levels of cysteine aspartic proteases (caspases), for example, due to ischemic stroke, neurodegenerative disease, or heart failure. The AAV9 provector in the OFF state contains a peptide insertion in the capsid which prevents binding to its canonical receptor. Alternatively, when the AAV9 provector targets and interacts with the upregulated caspases, the cleavage of the inserted peptide forms the activated or ON state, which allows the vector to bind and transduce

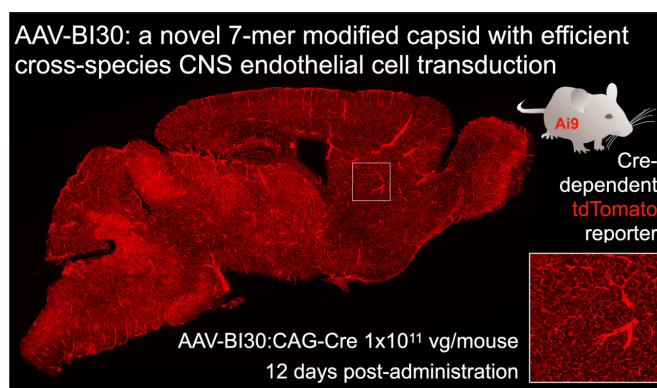
the specific target tissue. In this study we use cryo-electron microscopy to determine the high-resolution structure of two AAV9 provectors with 4 and 10 aspartic acid residues respectively, which act as a block to AAV9 receptor binding. Additionally, these provectors were also incubated with and cleaved with caspase-3 and their structures determined to $\sim 8\text{\AA}$ resolution. These low-resolution structures in comparison the pre-cleaved states have provided insights into the potential mechanism of provector activation from the OFF to ON state. Additionally, the information obtained from the structures was used to determine potential sites for AAV9 provector antibody evasion, and these sites were confirmed by immunoblots. The structure-function characterization of the AAV9 provectors in the ON and OFF state, with improved antibody evasion phenotype, will further the use of rational structural design to improve vectors used for gene therapy and reduce current therapeutic limitations.

513. A High-Efficiency AAV for Endothelial Cell Transduction Throughout the Central Nervous System

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Endothelial cells play a crucial role in neurovascular biology, and endothelial impairment is a major contributor in neurological diseases. Yet, AAV capsid engineering efforts have focused on targeting neurons or astrocytes, despite an emerging appreciation that non-neural cell types are critical for nervous system function. To address this need, we have developed and characterized AAV-BI30, a capsid which, at relatively low systemic doses (1×10^{11} vg/animal), transduced $84 \pm 4\%$ (mean \pm s.e.m.) of brain endothelial cells in adult mice, and is capable of transducing arterial, capillary, and venous endothelial cells not only in the brain, but also in the retina and spinal cord. We harnessed AAV-BI30 to achieve efficient endothelial-specific Cre recombinase activity in the brains of Cre-dependent reporter mice (**Figure 1**) and in a conditional gene knockout mouse line relevant to transcytosis at the blood brain barrier. The efficient brain endothelial cell tropism phenotype translates across mouse strains and rats in vivo as well as mouse and human brain microvascular endothelial cells in vitro. These characteristics of AAV-BI30 make it uniquely well-suited for the study of endothelial cell biology throughout the central nervous system and human cellular models, and for the development of therapies targeting endothelial dysfunction. **Figure 1. AAV-BI30 can be leveraged to achieve efficient endothelial-specific genetic manipulation throughout the central nervous system.** AAV-BI30:CAG-Cre-miR122-WPRE was intravenously administered to adult Ai9 Cre-dependent reporter mice at 1×10^{11} vg/animal and recombination was assessed via tdTomato expression after 12 days.



514. Rapid and Massively Parallel Long-Read Nanopore Sequencing of AAV Variant Libraries

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Massively parallel sequencing of complex libraries of AAV capsid gene variants is foundational to high-throughput capsid engineering. Characterizing complex variant libraries with multiple mutations spread throughout the capsid gene is essential for discovering AAV capsids with interesting properties (e.g., neutralizing antibody evasion, targeting specific tissues). However, identifying large numbers of such variants from highly diverse pools is challenging because of the limitations of current sequencing technologies. Past approaches have relied on full-capsid sequencing of each variant of interest using PacBio HiFi sequencing, an expensive and relatively low-throughput process. With this strategy a theoretical maximum of 4×10^6 variant sequences can be read using a single reagent cartridge in around 30 hours. Here we report a highly scalable method for full capsid gene sequencing of individual members of highly diverse AAV capsid libraries. This method uses rolling circle amplification to generate single-variant concatemers from individual library plasmid members or other circularized constructs, followed by Oxford Nanopore sequencing and high-confidence variant calling by alignment of the concatemers' subreads. Using a single MinION flow cell, we recovered 1×10^6 high-quality AAV variant sequences in a 48 hour experiment. Using Oxford Nanopore's PromethION instrument, these results could easily scale to provide hundreds of millions of high-quality full capsid sequences at a fraction of the cost of previous methods, thus enabling deep sequencing of complex variant libraries for capsid discovery.

515. A Collection of Enhancer-AAVs for Targeting Glial Cell Types Across Species

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Selective transgene expression is critical for studying brain cell types including their roles in brain function and disease, and their targeting for therapeutic access. However, few tools exist that can selectively target cell populations and can be applied across mammalian species. To enable specific brain cell population labeling across species, we are generating a collection of enhancer-AAV vectors that drive transgene expression in defined cell subclasses. We undertook a systematic screen to find enhancer elements capable of targeting gene expression from AAV vectors to major neocortical cell populations. In previous work, we have described enhancers for several different population of cortical neurons, and we demonstrated the maintenance of specificity in mouse and primate (Mich et al., Cell Reports 2021). Here, we show our progress generating a new collection of enhancer-AAV tools to target glial cell populations. We present a set of astrocyte- and oligodendrocyte-selective enhancer-AAV vectors, and characterize their activity across brain regions and across species. Our growing toolbox of enhancer-AAVs for selective transgene expression will be important to dissect the roles of cell populations in brain function across species.

516. Intravenous Gene Transfer Throughout the Brain of Infant Old World Primates Using AAV

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Adeno-associated viruses (AAVs) are a popular gene-delivery vector for both researchers and clinicians, as decades of research and hundreds of clinical trials indicate their potential for safe and long-term expression of genetic payloads *in vivo*. For gene-delivery to the central nervous system (CNS) however, systemic delivery of AAV

is a major pain point. The natural AAV serotypes do not traverse the restrictive blood-brain-barrier (BBB), severely limiting the transduction efficiency and coverage of AAV when using intravenous (IV) routes of administration. While the field has recently focused on developing AAV variants (e.g. AAV-PHP.B) that traverse the BBB in rodents, direct efforts in non-human primates (NHPs) are sparse. Some recently engineered capsids (e.g. AAV.CAP-B10) now enable systemic gene transfer to the brain of common marmosets (*Callithrix jacchus*), a New World primate species. But few comparable options exist for Old World primates, which are more evolutionarily related to humans compared to marmosets and are well-established animal models of human cognition, neurodevelopment, neuroanatomy, and physiology. To enable research and for greater therapeutic translatability, it is imperative that we advance AAV development for systemic gene transfer to the brains of Old World primates. Here, we identify and characterize AAV.CAP-Mac, an engineered AAV9 variant for systemic gene transfer to the infant Old World primate brain, where it transduces neurons throughout the cortex and in deep-brain regions (Figure 1). We identified CAP-Mac after 2 rounds of selection in adult marmosets followed by a final round of selection in infant rhesus macaques (*Macaca mulatta*), where CAP-Mac-delivered transgenes were enriched 10- and 6- times more than those delivered by AAV9 in viral DNA and whole RNA brain extracts, respectively. Looking across Old World primate species, CAP-Mac efficiently transduces the brains of both rhesus macaques and green monkeys (*Chlorocebus sabaeus*), achieving broader CNS distribution via IV delivery compared to intrathecal administration. We leverage this neuronal bias *in vivo* by delivering a cocktail of three fluorescent proteins to achieve Brainbow-like labeling, one of the first instances of non-transgenic, widespread genetic perturbation in Old World primates. Highlighting the opportunity for broader and more diverse study of the primate brain, we reconstruct morphology in various cell-types of disease interest, such as medium spiny neurons. Additionally, we show the potential for increased therapeutic benefit by demonstrating that CAP-Mac is over 45-fold more efficient at transducing human neurons *in vitro*. In conclusion, CAP-Mac expands the available AAV toolbox to include a vector that perturbs the Old World primate CNS, and also highlights that engineered AAVs can expand the therapeutic opportunity landscape for gene therapy to include neurological disorders, currently an unmet need.

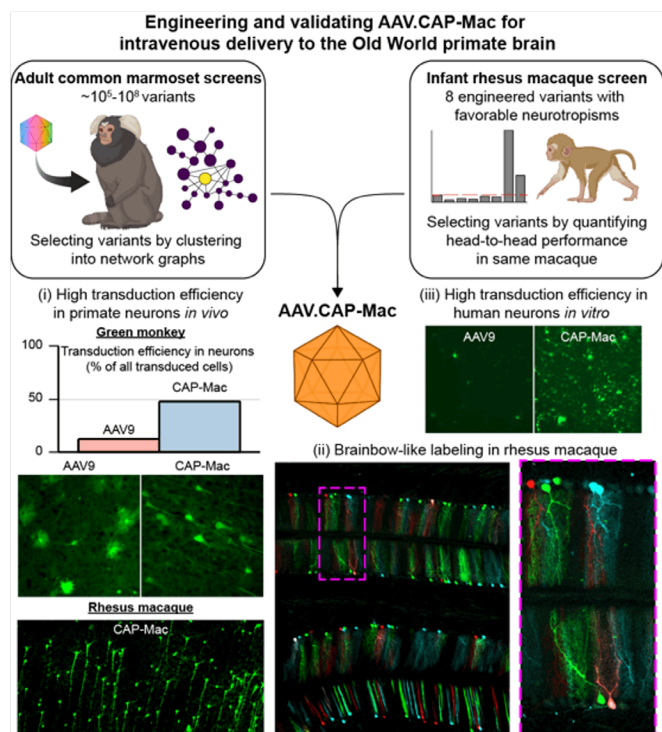


Figure 1. Engineering and validating AAV.CAP-Mac for intravenous delivery to the Old World primate brain.

517. Unbiased Screen of the Human Genome for DNA Sequences That Optimize rAAV Transcription in Liver

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Transcriptional silencing of rAAV transgenes and loss of rAAV genomes over time threaten the realization of successful long-term gene therapy. Here, we screened the human genome for cis-regulatory sequences that drive rAAV transcription in mouse liver using Self Transcribing Active Regulatory Region sequencing (STARR-seq). An AAV9 STARR-seq library with approximately 1E8 human genomic fragments was administered via mouse-tail vein injection. Poly(A+) RNA from liver tissue harvested 28 days post treatment served as the source material for first-strand cDNA production and next-generation sequencing (NGS) analysis. Sample processing and - to a lesser degree - sequencing depth determined the extent of library coverage and identification of STARR-seq candidates. Increased sample processing identified more candidates, yet using 15% of liver tissue per animal enabled the identification of the majority of robust candidates, defined as those found in all five animals from the study. Robust candidates, numbering in the thousands, resided near genes with liver function and were enriched for chromatin features associated with enhancer function such as DNase I hypersensitivity (DHS) and histone 3 lysine 27 acetylation (H3K27ac). A follow-up study examining 2000 robust STARR-seq candidates confirmed enhancer activity for most in liver at day 28-post-vector administration. Current studies aim to identify sequences that confer durable transcription to therapeutic transgenes in NHP liver and potentially other tissues.

AAV Vectors - Preclinical and Proof-of-concept Studies II

518. Human Liver Tissue Equivalents (hLTEs): A Novel Platform for Studying the Impact of AAV Gene Therapy on the Liver

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Clinical trials for hemophilia A employing AAV vectors have been hindered by unanticipated immunological/inflammatory responses and lower levels of transgene expression. The poor correlation of transduction efficiency observed *in vitro* vs. that *in vivo* and the species-species differences in AAV-vector tropism raise the question of the accuracy with which animal models will likely predict tropism/vector transduction efficiency and eventual treatment success in humans. Human liver tissue equivalents (hLTEs) are comprised of major cell types in the liver in physiologically relevant frequencies and possess the ability to recapitulate the biology and function of native human liver. The objective of this study was to utilize hLTEs to define the safety and efficacy of human liver-directed AAV gene therapy. hLTEs were fabricated by self-aggregation using 75% hepatocytes, 10% stellate cells, 10% Kupffer cells, and 5% liver sinusoid-derived endothelial cells in ultra-low attachment Elplasia[®] plates (96 x 79(micro) wells). On the day of fabrication (day 1), hLTEs were transduced (MOI: 10⁵ vg/cell) with the clinically relevant serotypes, AAV5 or AAV3b, both encoding a GFP reporter. Transmission electron microscopy of hLTEs showed that all groups exhibited microvilli and bile canaliculus-like structures, demonstrating the formation of a rudimentary biliary system and, more importantly, providing evidence that hLTEs resemble native liver. Incucyte[®] Live-Cell Imaging System was used to track the aggregation and GFP expression which showed impaired aggregation in AAV5 and AAV3b groups (57.57 ± 2.42 and 24.57 ± 4.01 spheroids/well, respectively) compared to control hLTE(-) (74.86 ± 3.8 spheroids/well) and most efficient transduction in AAV5 group compared to AAV3b (fold change in GFP expression compared to control: 2.73 ± 0.09 and 1.19 ± 0.03 for AAV5 and AAV3b, respectively). Liver function biomarkers measured from day 6-10 was normalized to urea concentration. Both transduced groups showed decreased albumin and alpha-1-antitrypsin at the earliest time points when compared to hLTE(-). Albumin levels recovered by day 10 but alpha-1-antitrypsin levels remained significantly lower in both transduced groups. ALT was significantly elevated in AAV5 group on day 6, and AST was elevated on day 6 in both AAV3b and AAV5 groups. However, both ALT and AST fell to match control levels by day 7 and 9, respectively. RT-qPCR analysis of genes involved in inflammation and hepatotoxicity revealed significant dysregulation of hepatotoxicity-related genes in both AAV5 and AAV3b groups, while only AAV3b group exhibited significant dysregulation of genes involved in inflammation. Histological staining and RT-qPCR were used to investigate potential AAV-induced fibrosis. ImageJ[®] analysis of Masson Trichrome and Sirius

Red staining showed no significant increase in fibrotic area compared to control hLTEs, and no significant dysregulation of genes that were pro-fibrotic was observed with RT-qPCR. In conclusion, data show successful recapitulation of native liver biology and demonstrate that AAV5 transduces hLTEs more efficiently than AAV3b. No compelling indication of AAV-induced fibrosis was observed. However, impaired self-aggregation and significant dysregulation of genes involved in hepatotoxicity and inflammation was observed which warrants further investigation. Data collected thus far show the potential for hLTE to provide critical knowledge regarding the efficacy and safety of AAV gene therapy in human liver.

519. Inhaled Administration of SP-101 Followed by Doxorubicin Leads to Durable hCFTRΔR Transgene Expression in the Airways of Wild-Type and CF Ferrets

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Individuals with cystic fibrosis (CF), an autosomal recessive genetic disease, may benefit from gene therapy targeting loss of function mutations in the cystic fibrosis conductance regulator (CFTR) gene. CFTR gene replacement therapy is expected to work in all people with CF, independent of the type of mutations. SP-101 (AAV2.5T-SP183-hCFTRΔR) is a novel recombinant adeno-associated virus (AAV) gene therapy vector intended for inhalation in people with CF who do not benefit from the treatment with small-molecule modulators (10-20%). To evaluate the potential efficacy of SP-101, the vector was evaluated *in vitro* in human CF airway epithelia (HAE) and *in vivo* in wild-type and CF ferrets. When delivered to the apical surface of CF HAE cultures of various Class I or II CF genotypes, SP-101 restored forskolin-induced CFTR-mediated chloride conductance to levels similar to, or even greater than, non-CF epithelia or CF epithelia treated with modulators. Chloride conductance increased with increasing multiplicity of infection (MOI) and by co-administration of a small molecule, doxorubicin, that has been shown to enhance the translocation of AAV genomes into the cell nucleus, resulting in enhanced transgene expression. Ferrets were chosen for *in vivo* studies since SP-101 capsid tropism for ferret airway has been previously demonstrated. Furthermore, CF ferrets develop spontaneous lung disease resembling many features of CF in humans. SP-101 was delivered to wild-type and CF ferrets via inhalation using a nebulizer attached to a plenum exposure system, followed by nebulized doxorubicin or saline. RNA was isolated from different regions of the airways and hCFTRΔR mRNA expression was determined by RT-qPCR, optimized for removal of viral genome DNA, and normalized to total RNA. Robust hCFTRΔR mRNA expression was observed in the respiratory tract of wild-type and CF ferrets. hCFTRΔR mRNA expression increased with increasing doses of SP-101/doxorubicin and was highest in lung tissues. Expression was evident as early as 48 hours after exposure and lasted for at least 12 weeks, the latest timepoint investigated. Similar hCFTRΔR mRNA expression was evident in the respiratory tract of both wild-type and CF ferrets, indicating successful airway transduction regardless of

preexisting mucus accumulation in the respiratory system of CF ferrets. In summary, these data strongly support continued development of SP-101 for the treatment of CF.

520. AAV Mediated Liver Directed Gene Therapy for Gyrate Atrophy of the Choroid and Retina

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Gyrate atrophy of the choroid and retina (GACR) is a progressive blinding chorioretinal degeneration involving the retinal pigment epithelium (RPE) and caused by pathogenic variants in the gene encoding the ornithine aminotransferase (OAT). Affected patients present with night blindness and progressive constriction of vision fields leading to blindness. Patients have increased ornithine concentrations in blood and other body fluids. OAT is expressed in the liver and murine studies have shown that elevated blood ornithine is a necessary factor to induce retinal degeneration. Current therapies are unsatisfactory and better treatments are highly needed. In a mouse model of OAT deficiency (*Oat^{rhg}*) that recapitulates the biochemical and retinal changes detected in GACR patients, we investigated the efficacy of an intravenously injected AAV8 vector expressing *OAT* under the control of a liver-specific promoter (AAV-OAT) at the dose of 1×10^{13} genome copies/kg. Following injections with AAV-OAT, *Oat^{rhg}* mice showed sustained reductions of blood ornithine concentrations compared to control mice injected with a vector expressing the green fluorescent protein (AAV-GFP). The reduction in blood ornithine concentrations was associated with improved electroretinogram (ERG) response, suggesting preservation of retinal function. In contrast to control mice injected with AAV-GFP, *Oat^{rhg}* mice injected with the AAV-OAT vector showed partial restoration of the retinal structure on pathology with improvements of RPE structure at 1-year post-injection. In summary, hepatic OAT expression by AAV8 was effective at reducing blood ornithine concentrations and improving both the function and the structure of the retina. In conclusion, this study provides a proof-of-concept of the efficacy of liver-directed AAV-mediated gene therapy for GACR.

521. A Novel Peptide Insertion into VR-IV or VR-VIII of AAV9 Improves Transduction Strength and Penetration Depth Upon Intravitreal Injection

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AAV-based gene therapy is ideal for treating many severe ocular diseases with a single dose, one-time treatment. Intravitreal (IVT) injection of AAV vectors is a simple and minimally invasive procedure that is being assessed in clinical trials but requires high doses of vectors that may cause toxicity. With the goal of improving the ability of AAV vectors administered intravitreally to reach and transduce the posterior segment of the eye, we developed AAV9-derived capsids containing a peptide insertion in VP3 variable regions IV (VR-IV) or VIII. We have characterized two lead candidates, AAV9.SAY01 and AAV9.SAY02 that contain a 10-mer peptide insertion after Q588 or S454 of VP1, respectively. The candidates were compared to the parental AAV9 and an AAV2-variant capsid. A CAG-eGFP expression cassette was packaged, and a total of 2×10^9 and 1×10^{10} vector genomes were administered intravitreally in the eyes of adult male *C57BL6/J* mice. Time course of eGFP transgene expression in the retina were evaluated weekly by fundus autofluorescence imaging using a Phoenix MICRON IV retinal imaging microscope. Eyeballs (left eyes), retina and RPE samples (right eyes) were collected at three weeks post-injection (3 WPI). Transgene expression was detected and characterized by immunohistochemistry, and eGFP levels were measured by ELISA. At 2×10^9 vg/eye, AAV9.SAY01 started to have stronger eGFP transgene expression at 1 WPI by fundus autofluorescence than AAV2-variant capsid. At 1×10^{10} vg/eye, both AAV9.SAY01 and AAV9.SAY02 vectors mediated widespread and high-level retinal (retinal ganglion cell, neuron in inner nuclear layer, photoreceptors, muller cell/astrocytes) and RPE transduction following intravitreal administration compared to wild type AAV9 (limited expression in photoreceptors and RPE) at 3 WPI. Ongoing studies in mice and nonhuman primates (NHP) will provide more information about these novel vectors pertaining to cell-specific tropism and maintenance of ocular structural integrity.

522. AAV.U7.ex44 Mediates Efficient Exon Skipping, Protein Restoration & Phenotype Rescue -Pre-Clinical Intramuscular & Dose Escalation Study for a Mutational Hotspot of the Duchenne Muscular Dystrophy (DMD)

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Exon skipping, a promising approach to treat Duchenne muscular dystrophy (DMD) is based on restoring the reading frame of the *DMD* pre-mRNA and to produce truncated but functional dystrophin. Conventional exon skipping using antisense oligonucleotides has some limitations - a need of repeated injections due to less stability and limited tissue tropism in major affected organs (e.g., heart). To overcome these, we and others used AAV.U7 antisense delivery. U7 small nuclear RNA carrying antisense sequence improves the stability and adeno-associated virus (AAV) increases tropism. This approach is currently being tested in a clinical trial for *DMD* exon 2 duplication.

In this study, we applied this vectorized exon skipping strategy (VES) approach to a mutational hot spot in the *DMD* gene: the exon 44. We evaluated our lead candidate using intramuscular (IM) and systemic (IV) pre-clinical dose escalation study. Humanized *DMD* mice with exon 45 deletion were used. We evaluated exon skipping by RT-PCR, proteins restoration using immunostaining, muscle function using force transducer, muscle co-ordination and strength using rotarod&hang wire, behavior test with open field apparatus. 3-months post-IM, around 85% of exon skipping was achieved, resulting in around 90% of truncated dystrophin expression and more than 50% improvement in eccentric contraction. Inflammation (macrophage mediated) was significantly reduced in the treated muscle. 3-months post-IV dose escalation, around 80% of exon skipping was observed in heart and 30-60% in diaphragm, TA and gastrocnemius muscles. Dystrophin expression and improvement in force generation in dose-dependent manner was noted. Macrophage infiltration, muscle fibrosis, rotarod, hang wire and open field test revealed improvement after treatment with the highest IV dose. To conclude, our lead candidate induces efficient *DMD* exon 44 skipping, resulting in dystrophin production and muscle strength improvement in major muscle groups affected in *DMD*. This AAV.U7-exon 44 skipping vector represents a promising candidate for ~6-12% of *DMD* patients.

523. Development of BBP-818 (AAV9-CB6-Galt) for the Treatment of Classic Galactosemia

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Classic Galactosemia is an autosomal recessive disorder caused by the deficiency of galactose-1 phosphate uridylyltransferase (GALT). Absence of GALT activity results in the accumulation of toxic metabolites that, if left untreated, can be lethal. Current treatment strategies are centered on dietary modification to reduce lactose/galactose content, but endogenous galactose production still leads to accumulation of disease-progressing metabolites. Even with early intervention long-term complications such as speech delay (56% of patients), intellectual deficits (45%), motor functions deficits (tremors & cerebellar ataxia) (18%), and primary ovarian insufficiency (POI) (91% of females) exist and highlight the urgent need for a therapy. We have designed an adeno associated virus (AAV) based gene therapy approach that could potentially treat the disease by restoring GALT activity throughout the body. This potential therapy, AAV9-CB6-Galt (aka BBP-818) is being developed for the treatment of Classic Galactosemia. The *Galt* gene trapped mouse model is an excellent model for preclinical studies in Classic Galactosemia. The model is sensitive to galactose challenge at birth and develops a subfertility phenotype reminiscent of POI and shows signs of motor function defects allowing for multiple disease-relevant phenotypic readouts. Additionally, the model shows consistent elevation in metabolic markers of the disease, such as galactose 1-phosphate (gal-1P). Initial studies in mice given a single intravenous dose of BBP-818 ranging from 5×10^{12} - 5×10^{13} vg/kg showed biodistribution profiles similar to other AAV9-based vectors with excellent expression in the liver and penetration into the brain. Protein analysis via western blot

demonstrated that GALT protein expression was restored to ~20-70% of wild type levels across the doses range at eight weeks post dosing. Expression of GALT was associated with normalization of gal-1P levels in liver and brain tissue at all doses tested demonstrating functional restoration of the enzyme to these tissues. Studies in wild-type mice at BBP-818 doses of up to 1×10^{14} vg/kg have been well tolerated, and expression of transgene is persistent for up to six months in target tissues including the brain, liver, and ovary. These studies support the ongoing development of BBP-818 to further characterize its impact on phenotypes in the *GalT* gene trapped mouse and safety in IND-enabling toxicology studies.

524. Comparison of Adeno-Associated Viral Capsids for Gene Transfer into Non-Human Primate Liver

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Recombinant adeno-associated virus (rAAV) based liver-targeted gene transfer is a strategy to correct many adult liver-based human genetic diseases. However, preclinical studies evaluating rAAV vectors have shown that the vector uptake and transduction varies in different test species and hence the use of appropriate species is critical for translatability to humans. In this study, we compared AAV3B and AAV5 capsids (both already used in clinic) to Sanofi capsid, AAV.SNY001 for gene transfer into NHP liver. The latter capsid is a next generation capsid derived from a shuffled capsid library. All rAAV vectors contained an expression cassette with a liver-based promoter encoding eGFP and all were generated, purified and titered in a comparable manner and contained >80% full particles. The vectors were administered by IV route using slow infusion into male cynomolgus monkeys (prescreened for pre-existing NABs to each capsid). Two weeks later, sera and selected organs were collected. Of the three capsids, AAV5 generated the highest neutralizing antibody titer two weeks post administration while AAV3B had the highest pre-existing human IVIG titer. At two weeks post-delivery, the ranking in liver by vector DNA was AAV.SNY001 > AAV3B > AAV5. For mRNA and eGFP protein (ELISA, IHC) the ranking was AAV.SNY001 ≥ AAV3B > AAV5. Recombinant AAV3B and rAAV5 based vectors showed high level of vector DNA in spleen that was comparable to levels detected in the liver. All vectors were detected at relatively high levels in adrenal glands while rAAV5 vector DNA was present at higher levels in dorsal root ganglion cells and brain tissues compared to the other two capsid vectors. No expression was observed in muscle or heart with rAAV3B- and rAAV.SNY001-vector treated animals as expected for the liver-based promoter. However, mRNA and eGFP protein was observed in muscle and heart of rAAV5 vector treated animals. In summary, we evaluated three AAV capsids for NHP liver transduction as a model for gene transfer to human liver and demonstrated robust and consistent transduction with rAAV.SNY001 vector with little transduction in other tissues. Additionally, we have made an interesting observation on the impact of capsid on tissue specificity our liver-based promoter.

525. Dissecting Messenger RNA Expression and Cellular Localization of a AAV-Derived Reporter Gene in Mouse Eyes by RNAscope™ In Situ Hybridization

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Viral vector distribution, tissue tropism, and transgene expression are among the key pre-clinical considerations when designing and selecting vectors for gene therapies. Standard bioanalytical molecular techniques such as qPCR/RT-qPCR can provide an overall dissemination profile of viral genomes and mRNA levels for a particular tissue sample, whereas the use of DNA/RNA *in situ* hybridization (ISH) analysis technologies can deliver precise vector localization and transgene expression across multiple layers of a complex organ down to single-cell resolution. RNAscope™ as a specific ISH technology, utilizes multiple target specific probes to produce signals and prevent unintended cross-hybridization. It permits the unique opportunity to monitor cellular localization to determine which cell layers are more permissive to the virus transduction and the vector promoter activity. To aid in identifying tissue distribution and transgene expression of a novel viral vector intended for use in AAV ophthalmic therapies, we employed the RNAscope™ techniques to visualize and semi-quantitate the ocular cell layers that are transduced in a sodium iodate induced mouse model of retinal degeneration. Mice were intravitreally injected with an AAV2 vector encoding GFP or GFP fused to the HIV Tat protein, and eye globes were collected 28- and 42-days post sodium iodate dosing. The HIV Tat protein has the capability of secreting into the extracellular space from expressing cells and penetrating into neighboring cells, therefore, allowing for a broader functional effect throughout the expressing tissue. To enhance the signal to noise ratio, up to 20 ISH probe pairs per target were designed against the GFP and Tat-GFP transgene mRNA, and the antisense region of the vector promoter. Tandem binding of each probe in the probe pair must occur to generate a binding site for the pre-amplifier and subsequent chromogenic enzyme, thereby increasing the specificity of the assay. Fast red dye was selected for the chromogenic staining as it offers high contrast and is more readily identifiable on highly pigmented retina sections. Formalin fixed, paraffin embedded (FFPE) mouse whole globes for all treated mice were sectioned, quality checked for RNA integrity, and assay controlled using the murine PPIB housekeeping and *dapB* bacterial genes. Tissue from all vehicle-treated study control mice exhibited no staining or background signal when subjected to each of the three target probe pair sets. Analysis of FFPE 28-day post sodium iodate mouse whole globes from both GFP treatment groups demonstrated that vector distribution was confined to the intraretinal space with vector uptake primarily residing in the ganglionic layer. Importantly, GFP mRNA was readily detected and identified predominantly in ganglion cells with some expression observed in deeper layers suggesting effective absorption and expression of the AAV2 viral vector to some extent beyond the ganglia. The level of vector expression remained consistent upon examination of the 42-day post sodium iodate dosed animals from the two study arms, indicating persistent vector activity. Assessment of immunofluorescent images (GFP funduscopy) from the same animal cohorts during the in-life portion of the study confirmed results that GFP expression followed the same expression pattern as revealed by the RNAscope™ results and remained throughout the course

of the study. Interestingly, animal tissues that demonstrated a lower percentage of RNAscope™ positive cells also exhibited a reduction of GFP protein, further providing support for the use of RNAscope™ as an informative and reliable tool for the examination of viral vector therapies in pre-clinical studies.

526. AAV-Mediated Phenotypic Correction of Very Long-Chain Acyl-CoA Dehydrogenase (VLCAD) Deficiency in Mice

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Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is a rare, autosomal-recessive disorder of fatty acid oxidation with an incidence rate of ~ 1:40,000. 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 associated disease is clinically heterogeneous. However, 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. Prior gene therapy studies in VLCAD-deficient (VLCAD^{-/-}) mice have shown promising biochemical and phenotypic correction suggesting the potential for a disease-specific therapy for patients with VLCAD deficiency. In preparation for the clinic, the authors modified prior vector constructs to increase transgene expression and stability, as well as reduce the potential for immunogenicity. VLCAD-deficient mice were treated systemically with various doses of adeno-associated virus (AAV)-ACADVL (gene encoding human VLCAD protein) vector constructs. Biochemical changes in blood glucose and long-chain fatty acyl were observed as early as 2 weeks post dosing. Observed changes were maintained through 8 weeks when animals were subjected to a cold-fast challenge. This disease specific phenotype was assessed by monitoring the animal's ability to thermoregulate and maintenance of euglycemia after a fasting cold challenge. Internal body temperatures of untreated VLCAD^{-/-} mice dropped below 20°C and animals were hypoglycemic, resulting in mice becoming lethargic and euthanized moribund. In contrast, the treated VLCAD^{-/-} mice and WT mice were able to maintain body temperatures and treated VLCAD^{-/-} mice maintained euglycemia. VLCAD protein expression in target tissues (liver, cardiac muscle, and skeletal muscle) was observed via Western Blot analysis with mRNA expression also being quantified. Overall, the results from this study further demonstrate proof-of-concept for an AAV-mediated gene therapy to correct key biochemical and phenotypic

aspects of VLCAD deficiency. Additional IND-enabling studies will be performed to evaluate safety and identify the potential therapeutic dose-range for first-in-human clinical trials.

527. Development of Novel Epigenetics-Guided Promoters to Target Central Nervous System and Peripheral Tissue and Cell Types for Canavan Disease Gene Therapy

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It is common practice to design recombinant adeno-associated virus (rAAV)-based gene therapy constructs with ubiquitously active promoters, as seen in Zolgensma, a treatment for spinal muscular atrophy. This vector platform, like many others, utilizes a *CMV-C β A* hybrid promoter. While efficacious, these expression cassettes are active in all cell and tissue types that are transduced by the vector, which may lead to supra-physiological levels of the gene product. Alternatively, tissue-specific regulatory sequences can direct the expression of therapeutic transgenes to mimic endogenous transcript levels and locations, potentially eliminating side effects attributed to ubiquitous overexpression. We previously developed an rAAV-based therapy for Canavan disease (CD), an inherited leukodystrophy caused by the loss of aspartoacylase (ASPA) function, particularly in oligodendrocytes. The treatment delivers a *CMV-C β A* promoter-driven human *ASPA* transgene and mitigates disease phenotype in CD mice. However, treated animals also exhibited off-target effects. These results led us to re-evaluate the benefit of a ubiquitously active promoter for CD treatment. We analyzed the mouse *Aspa* (*mAspa*) promoter using publicly available DNase hypersensitivity and histone modification datasets, and identified sequences that appear to regulate *mAspa* in a tissue-specific manner. Based on these findings, we developed novel promoters to drive native-*Aspa*-like expression. Expression plasmids containing these epigenetics-guided promoter designs were first tested in cultured HEK293s and human glial hybrid cells (MO3.13), which have characteristics of oligodendrocytes. We identified constructs that produced high levels of expression, similar to those achieved by the *U1a* ubiquitous promoter. Importantly, the promoters demonstrated preferential activities in MO3.13 cells. Neonatal mice treated with rAAV9 vectors containing *mAspa* promoter-derived regulatory sequences demonstrated patterns of tissue restricted expression that mimicked native *Aspa* expression, as assessed by digital droplet PCR and immunofluorescence microscopy. Specifically, reporter transgene levels in tibialis anterior and heart muscle were negligible, while expression in the brain and liver were similar to those observed in mice treated with a vector harboring the *CMV-C β A* promoter. In the brain, *mAspa* promoter-derived regulatory sequences drove distinct regional patterns of expression compared to the *CMV-C β A* promoter as determined by immunofluorescence. Our ongoing experiments seek to define the mechanisms underpinning the tissue-specific activities achieved by *mAspa* promoter elements. This work will help to further refine novel regulatory cassette designs for physiologically regulated CD gene therapy vectors. *Co-corresponding authors

528. Development of an Optimized AAV Gene Therapy for Autosomal Dominant Optic Atrophy

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Introduction: Autosomal Dominant Optic Atrophy (ADOA) is an inherited blinding disease characterized by progressive bilateral visual loss, degeneration of retinal ganglion cells (RGCs) and the atrophy of optic nerve. ADOA is associated predominantly with mutations in the *OPA1* gene and has variable onset and severity. The *OPA1* gene is a mitochondrially targeted GTPase that plays a pivotal role in mitochondrial health by regulating the mitochondrial membrane dynamics and cell survival. The GTPase and the coiled-coil domains of OPA1 are hotspots of ADOA mutations, leading to severe mitochondrial dysfunction and disease progression. Here, we investigated rAAV-mediated gene therapy to compensate OPA1 protein as a therapeutic strategy for the treatment of ADOA. **Methods:** The coding sequence of *OPA1* isoform 1 was codon-optimized and constructed into pAAV2 vector. The expression of exogenous OPA1 in HEK293 cells was detected by western blot with an OPA1 antibody. *In vitro* efficacy study of rAAV2-*OPA1*-opt treatment was evaluated in *Opa1*-null mouse embryonic fibroblasts (MEFs). Mitochondrial morphologies were assessed by immunofluorescence to examine if OPA1 reconstitution can improve the mitochondrial network defect. Cell proliferation and cellular ATP level in galactose medium were measured to examine whether OPA1 re-expression can rescue the impaired mitochondrial respiration and cell survival. *In vivo* expression and safety studies were performed by Western blot and immunohistochemistry in mouse eyes after intravitreal injection of rAAV2-*OPA1*-opt. **Results:** We found codon optimization enhanced the expression level of OPA1 by around two folds in HEK293 cells. Mitochondrial morphology defects of *Opt1*-null MEFs were rescued by the transduction with rAAV2-*OPA1*-opt but not AAV2-GFP control. Cell proliferation and cellular ATP level of *Opa1*-null MEFs in galactose medium were much lower than those of wide-type MEFs due to impaired mitochondrial function, whereas AAV2-mediated OPA1 expression can rescue these phenotypes. Exogenous OPA1 expression was investigated in mouse retina at 4 weeks post intravitreal injection, and the structure of the retina tissue sections and RGC numbers were comparable between rAAV2-*OPA1*-opt treated and control mice. **Conclusion:** Our results suggest that rAAV2 mediated *OPA1* gene therapy is a potential safe and effective treatment for *OPA1* mutation associated ADOA.

529. An AAV9 Encoding Human ABCD1 (SBT101) Shows Functional Improvement Following Spinal Cord Delivery in a Rodent Model of Adrenomyeloneuropathy

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OBJECTIVE: The objective of the current study was to evaluate the dose dependent effect of intrathecally administered AAV9-h*ABCD1* (SBT101) on the behavior and function of *Abcd1/Abcd2* Double Knock-Out mice (*Abcd1*^{-/-}/*Abcd2*^{-/-}; DKO) in comparison to AAV9-Null vector-injected DKO and wild-type (Wt) control mice. **BACKGROUND:** X-linked adrenoleukodystrophy (ALD) is an inherited neurodegenerative disease caused by mutations in the gene encoding the ATP-Binding Cassette sub-family D member 1 (*ABCD1*) gene. The *ABCD1* gene encodes a peroxisomal membrane protein that transports very long-chain fatty acids (VLCFA) into the peroxisome. In the absence of functional ABCD1, affected cells accumulate increased VLCFA levels which can result in oxidative stress at the cellular level and inflammatory demyelination in the spinal cord. Adrenomyeloneuropathy (AMN) is the most frequent clinical manifestation of ALD affecting virtually all males and >80% of females. AMN is characterized by a slowly progressive spastic paraparesis in adults leading to loss of mobility, incontinence, and debilitating pain. So far there is no therapy available for AMN, leaving the patients with progressive neurodegeneration and lifelong disability. We are developing SBT101, an adeno-associated virus serotype 9 (AAV9)-based gene therapy vector capable of delivering a functional copy of the human *ABCD1* (h*ABCD1*) gene, for use as a treatment for AMN. Utilizing the DKO mouse model of AMN which exhibits key behavioral features of the human disease and increased VLCFA levels, we evaluated the effect of SBT101 following IT delivery of the h*ABCD1* gene in DKO mice as compared to AAV9-Null vector-injected DKO and Wt control mice. **DESIGN/METHODS:** DKO male mice aged 7 - 8 months received a single lumbar intrathecal bolus administration of SBT101 (3.3E10 vector genomes/animal (vg/an) or 3.3E11 vg/an), and control Wt and DKO male mice received AAV9-Null (1.5E11 vg/an). Animal grip strength was evaluated at 15 and 18 months of age, followed by post-mortem analysis of VLCFAs in lumbar spinal cord. **RESULTS:** Intrathecal administration of SBT101 at doses up to 3.3E11 vg/an, and AAV9-Null at 1.5E11 vg/an were well tolerated by both DKO and Wt mice, respectively. Four-paw grip strength at 15 months of age was significantly increased as compared to AAV9-Null treated DKO mice and was maintained at 18 months of age in DKO mice administered SBT101 at 3.3E10 vg/an and 3.3E11 vg/an. Evaluation of VLCFA in the lumbar spinal cord of DKO mice administered SBT101 showed an apparent dose dependent decrease of VLCFA compared to DKO animals administered AAV9-Null vector of 5% (3.3E10 vg/an)

and 16% (3.3E11 vg/an), respectively, at 10-11 months post dosing. **CONCLUSIONS:** Intrathecal delivery of SBT101 to DKO mice demonstrated a dose dependent improvement of both 4-paw grip strength and VLCFA. These data support further preclinical development of AAV9-hABCD1 as a potential treatment of AMN.

530. The Effect of Recombinant Adeno-Associated Viral Vector Stability on Anion Exchange Chromatography Operations

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Recombinant adeno-associated viral vector (rAAV) production and purification processes can generate a heterogenous array of product-associated impurities such as rAAV capsids with no transgene or with the fragmented or truncated transgene. Furthermore, capsid charge variants and aggregates that lack infectivity may pose undesired immune responses. These undesirable product-associated impurities need to be reduced along with the DNA impurities that are originating from both the product and the host cells. Ion exchange chromatography (IEX) is a reliable method not only for the separation of proteins based on their charge but also for the separation of aggregates. Furthermore, DNA impurities may also be separated with IEX due to their negative charge. IEX has also been successfully employed for the purification of AAV vectors which are protein capsids surrounding a single-stranded DNA. In the present work, we demonstrate the impact of resin architecture/chemistry on impurity separation and product stability. In particular, we evaluated the on-column stability of rAAV capsid variants on ion exchange resin. The influence of buffer specifications on product stability was also evaluated. Although the chromatographic performance of rAAV on ion exchange resins is capsid-dependent, our approaches can be leveraged in assessing the selectivity and stability of a wide variety of capsid serotypes.

531. PR001 Gene Therapy Increased GCase Activity and Improved Gaucher Disease Type 1 Phenotypes in Animal Models

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Gaucher disease (GD) is caused by biallelic mutations in the glucocerebrosidase (*GBA1*) gene, with severe mutations leading to neurological manifestations (neuronopathic Gaucher disease; nGD) and more moderate mutations leading to a primarily peripheral disease (Gaucher disease type 1; GD1). The *GBA1* encoded enzyme glucocerebrosidase (GCase) is a key lysosomal enzyme required for the normal metabolism of glycolipids. GCase deficiency causes the accumulation of glycolipid substrates, resulting in lysosomal dysfunction that leads to inflammation and other pathological changes. With our investigational gene therapy, PR001, we aim to increase GCase activity in GD1 patients in order to ameliorate lysosomal

dysfunction and treat the disease manifestations with a one-time treatment. Using mouse models of GD, we evaluated the therapeutic effects of PR001 administered via intravenous (IV) injection. Post-treatment, animals were assessed for GD-like phenotypes in the periphery, including decreased GCase activity, increased glycolipid substrate levels, and inflammation. PR001 delivery via IV injection resulted in broad biodistribution and dose-dependent increases in GCase activity, reductions in glycolipid substrate accumulation, and reductions in inflammation-associated endpoints. Long-term studies evaluating the persistence of PR001, and its therapeutic effects revealed durability out to at least one-year post-administration. Overall, PR001 demonstrated long-lasting efficacy in two independent mouse models of GD supporting further development of PR001 for patients with GD1.

532. Evaluating miR-Target Sites as a Strategy to Allow AAV Vector-Based De-Targeting of Gene Expression in the Inner Ear

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MicroRNAs are short, single-stranded, non-coding RNA molecules with a key role in negative regulation of gene expression. Each mature microRNA binds to its specific target site to mediate translational repression or mRNA degradation. The target-site sequence is specific to a microRNA, but any gene can include the coding information for multiple target sites to be regulated by numerous microRNAs. In the development of AAV gene therapy vectors, a goal is to generate safe and efficacious product candidates that confer transgene expression where it is needed to have the desired effect, while limiting transgene expression where it may not be well tolerated. In recognition of this transgene-dependent need, the potential use of microRNA target site (miR-TS) incorporation in AAVAnc80 vectors was explored for de-targeting transgene expression in different cell types of the cochlea. Using previously published datasets from neonate mouse cochleae, multiple microRNAs with differential expression patterns in cochlear cells were identified. Their respective target sites were then incorporated in an AAVAnc80-delivered transgene, together with a ubiquitous promoter, and the transgene expression in vitro and ex vivo was evaluated in search of distinct expression profiles. Using an in vitro model, expression of transgene mRNA and protein in the absence of the target sites was demonstrated. Decreased transgene levels were observed once a target site was included. Using a cochlear explant model, miR-TS that abolished expression in both hair cells and supporting cells were identified. Sequences that promoted hair cell de-targeting while preserving supporting cell expression were also identified. Transgene de-targeting may be beneficial for AAV gene therapy vectors when expression may not be well tolerated in a subset of cells. The work described here identified several miR-TS that enable differential expression in cochlear cells. Future in vivo evaluation of various miR-TS combinations will guide the selection of regulatory sequences for selective, physiologically relevant transgene expression.

533. Non-Clinical Data Support Efficacy and Tolerability of a Human Equivalent Dose of 6E10 vg/eye of ADVM-022 for the Treatment of Neovascular Age-Related Macular Degeneration

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Intravitreal (IVT) administration of anti-vascular endothelial growth factor (VEGF) (such as aflibercept) is the standard of care for neovascular age-related macular degeneration. A shortcoming of these therapies is the need for frequent injections associated with fluctuation in central subretinal fluid ultimately impacting best corrected visual acuity. Therefore, there is an urgent need to develop new VEGF long-acting therapeutic modalities. To date, a single IVT injection of ADVM-022 has resulted in robust levels of aflibercept at therapeutic concentrations out as long as 104 weeks, as demonstrated in an ongoing, long-term clinical study, OPTIC. Both the aforementioned study and the current, non-clinical study described here utilize ADVM-022, an adeno-associated virus (AAV) gene therapy vector encoding aflibercept packaged in the chimeric AAV.7m8 capsid. ADVM-022 has been shown to reduce annual injection frequency by >80%, and in most patients eliminate the need for any additional injections of anti-VEGF. Previous non-clinical studies demonstrated a nearly flat dose response suggesting a lower dose could also yield efficacious aflibercept levels. To assess whether lower doses of ADVM-022 provide relevant ocular aflibercept levels we performed a good laboratory practice (GLP) toxicology study using non-human primates (NHP). In this study, NHP subjects were administered bilateral IVT ADVM-022 at either 3E10 vg/eye or 1E11 vg/eye (n=4/group), or vehicle (n=2/group). Vitreous (VH) and aqueous humors (AH) were collected for aflibercept quantification on days 30, 60 and 98 post dose. In addition, ophthalmic examinations and tonometry were performed once pretreatment, and throughout the study. Electroretinography (ERG) and optical coherence tomography (OCT) were performed once pretreatment, and at weeks 4 and 12. Administration of ADVM-022 at both doses resulted in meaningful aflibercept levels with peak aflibercept levels at approximately 4-weeks post dose. Mean peak aflibercept levels at the 3E10 vg/eye dose were 942 ng/mL for VH and 535 ng/mL for AH. At the 1E11 vg/eye dose, mean peak aflibercept levels were 1339 ng/mL for VH and 834 ng/mL for AH. These peak levels were comparable with aflibercept levels observed in previous studies utilizing higher doses of ADVM-022. Overall IVT administration of ADVM-022 in NHP subjects was well tolerated. No adverse systemic clinical signs were observed during the 3-month study. In life observations were dose-dependent and limited to non-adverse slight to mild dose-dependent ocular inflammation characterized by pigment and cells in the VH. No abnormalities of the anterior segment or lens were found, and no iris changes were observed by transillumination. Microscopic findings were limited to minimal mononuclear cell infiltrations, considered non-adverse based on minor severity, in agreement with absence of abnormal findings in ocular physiology and function in animals. In summary, the findings from

the non-clinical NHP study suggest administration of ADVM-022 at doses as low as 3E10 vg/eye (human equivalent dose of 6E10 vg/eye) may provide therapeutic levels of aflibercept in the clinic.

534. Ex Vivo and In Vivo Suppression of SARS-CoV-2 with Combinatorial AAV/RNAi Expression Vectors

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The SARS-CoV-2 pandemic has sparked an unprecedented race to develop and deploy novel generations of antiviral vaccines, yet it has concurrently also illustrated the urgent need to devise clinically relevant modalities to combat viruses on a genetic level. This is best exemplified by the observed rapid evolution of SARS-CoV-2 variants such as Omicron (B.1.1.529) that are increasingly resistant to prophylactic intervention, prompting the need for original strategies that not only suppress the virus but also counteract its mutational escape. As an RNA virus that also transcribes numerous mRNAs, SARS-CoV-2 is highly susceptible to inhibition and degradation through RNA interference (RNAi). Here, we have thus investigated Adeno-associated virus (AAV) vector-based expression of short hairpin (sh)RNAs as an antiviral strategy for prevention or treatment of SARS-CoV-2 infections. To this end, we initially screened shRNAs targeting the viral RdRp and N genes for their ability to knock down SARS-CoV-2 in Vero E6 cells. Strikingly, when tested individually, none of the three shRNAs that most robustly reduced viral infection and replication could prevent the rapid escape of SARS-CoV-2 by acquisition of single point mutations in the respective shRNA binding sites. Importantly, though, we were able to fully prevent this mutational escape by multiplexing the best shRNAs in a single AAV vector called SAVIOR (**SARS-CoV-2 viral repressor**). Next to Vero E6 cells, SAVIOR also markedly inhibited SARS-CoV-2 replication in human gut organoids as well as in chronically infected Caco-2 cells. Finally, intranasal application of AAV9-SAVIOR to wild-type mice prior to infection with a mouse-adapted SARS-CoV-2 strain (MA10) resulted in a reduction in viral load in the lungs and milder disease symptoms as compared to a control vector. The combinatorial and versatile AAV/RNAi vector platform reported here enabled us to demonstrate the high *ex vivo* and *in vivo* susceptibility of SARS-CoV-2 to RNAi-based intervention as a direct antiviral strategy. Concomitantly, our studies revealed the great propensity of SARS-CoV-2 to escape from mono-shRNA suppression by mutational escape and showcase the great potential of AAV-SAVIOR to suppress this adverse effect. Combined with the encouraging clinical safety profile of AAV vectors and our repertoire of advanced technologies for

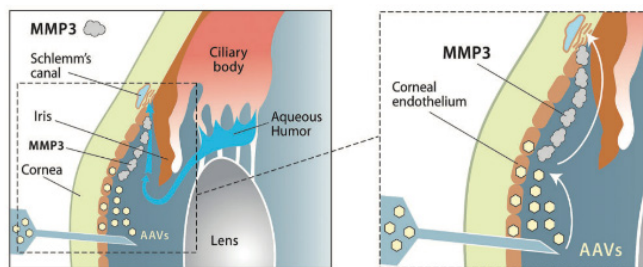
AAV engineering and manufacturing, this raises hopes that our new stratagem can be translated into humans and contribute to ongoing global efforts to control the COVID-19 pandemic, including future SARS-CoV-2 variants.

535. A Gene Therapy Approach Utilising a Matrix Metalloproteinase Secreted from the Corneal Endothelium to Treat Glaucoma

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The primary risk factor for glaucoma is ocular hypertension, which is caused by increased resistance to the outflow of aqueous humour (AH) from the eye. Outflow resistance derives from extracellular matrix (ECM) deposition in the trabecular meshwork. As such, gene therapies targeting trabecular meshwork ECM hold promise for decreasing intraocular pressure; these therapies offer the additional advantage of built-in compliance, which is a problem with eyedrops. Here we demonstrate that intracameral delivery of AAV9 expressing matrix metalloproteinase 3 (MMP3) - a therapeutic modulator of ECM - is safe and efficacious in mouse models, and well tolerated in the non-human primate. Normotensive mice injected intracamerally with AAV9 encoding murine MMP3 demonstrated elevated levels of both MMP-3 protein and MMP-3 activity in the aqueous humour. Additional efficacy studies were performed using AAV9-MMP3 in two murine models of glaucoma: both studies resulted in decreased intraocular pressure (IOP) by approximately 2 mmHg, with a concomitant 50% increase in outflow facility. Further to this, ultrastructural images of the outflow tissue revealed optically empty spaces that indicate reduced ECM at key sites. Based on the encouraging data obtained in the murine models, a preliminary safety study was performed in non-human primates (NHP). AAV9 encoding hMMP3 (1.2×10^{12} vg in 50 μ L) was administered intracamerally in one eye, while the contralateral eye was injected with vehicle (n=7 animals). At 2-week intervals over 6 months, ophthalmic examinations were performed using specular microscopy, pachymetry, tonometry, slit lamp biomicroscopy, anterior segment imaging and anterior segment OCT, alongside routine general wellbeing assessment and body weight measurements. Corneal thickness measurements were similar for AAV9 and vehicle injected eyes at all time points. Corneal endothelial cell area and cell density remained unchanged. Immunohistological imaging showed exclusive and efficient transduction of the corneal endothelium in AAV9-treated eyes. In addition, we observed an open iridocorneal angle and no evidence of inflammation or cellular damage. This pre-clinical data demonstrates the potential of genetic intervention in the anterior chamber to generate a "protein factory" for the treatment of anterior segment conditions such as glaucoma.



AAV9 mediated MMP-3 expression in the corneal endothelium. AAV9 transduces the corneal endothelium upon intracameral inoculation (left). MMP-3 molecules are secreted into the AH from this location and are transported toward the outflow tissue by the natural flow of the aqueous (right).

536. Tropism of AAV-rh32.33 for Skin Endothelial Cells and Fibroblasts

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There are approximately 500 known skin diseases with 80% of genetic skin disorders being caused by a single gene defect. Recent advances in designing gene delivery tools, manufacturing, and administration have facilitated gene therapy for monogenetic disorders. Adeno-associated virus (AAV) is a well characterized and thriving gene delivery modality with two FDA and one EMA approved products. Recent studies have shown successful cutaneous transduction using AAV in both ex vivo and in vivo. However, further examination is needed to study the tropism of multiple AAV capsid variants and characterization of the target cells after in vivo administration. Herein, we injected C57BL/6J mice with several AAV capsid variants, AAV1, AAV2, AAV5 and AAV-rh32.33 expressing GFP via intramuscular and intravenous routes. Cells were isolated from dermis and epidermis of ear and tail and were stained for multiple surface markers. Characterization by flow cytometry revealed GFP expression only in the dermis of AAV-rh32.33 and AAV1 injected animals with higher expression in i.v. injections. GFP expressing cells in AAV-rh32.33 injected animals expressed CD49f and CD31, or CD90 which characterize endothelial and fibroblast cells. Moreover, a small population of GFP⁺ cells expressed MHCII and CD11b, suggesting the transduction of antigen presenting cells. Further analysis ruled out transduction of keratinocytes and melanocytes. GFP-expressing cells in AAV1-injected animals expressed CD90 and CD8, indicating the transduction of CD8⁺ T cells. This study indicates the potential of AAV-rh32.33 for gene transfer to endothelial and fibroblast cells. Further investigations are ongoing to study the functionality and feasibility of AAV-rh32.33 as well as other AAV capsid variants in the treatment of genetic skin diseases.

537. An Endonuclease-Free Genome Editing Technology Provided Long-Term Efficacy and Benefits in a Mouse Model of Tyrosinemia Type 1

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Tyrosinemia Type 1 (HT1) is a neonatal-onset metabolic disease caused by loss-of-function mutations in fumarylacetoacetate hydrolase (FAH). Standard of care (SoC) for HT1 patients consists of daily nitisinone (NTBC) intake and life-long strict dietary management. While effective, the SoC also imposes a significant burden to the patients and their families. GeneRide™ is a novel AAV-based, nuclease-free, genome editing technology that leverages the natural process of homologous recombination to site-specifically insert a therapeutic transgene into the genome. Compared to conventional promoter-driven AAV gene therapy, GeneRide™ features durable transgene expression regardless of tissue growth or tissue regeneration caused by injury. In addition, for certain genetic diseases (e.g., Methylmalonic acidemia, Wilson Disease, HT1) where the underlying mutations cause liver damage, we have observed in preclinical animal models that the GeneRide-corrected hepatocytes were healthier than mutant cells and thus the edited hepatocytes naturally expanded (have a selective advantage) leading to therapeutic levels of transgene expression. Using a mouse model of HT1 (*FAH^{-/-}*), we have demonstrated that GeneRide-edited hepatocytes rapidly repopulated the entire liver within four weeks post dose and corrected disease phenotypes. All GeneRide-treated HT1 mice in that study reached normal body weight and achieved NTBC-independence even on a normal diet (ESGCT 2021). In the current study, we have further demonstrated that the speed of selective advantage and the level of GeneRide-edited hepatocyte repopulation were independent of initial vector dosage. GeneRide treated animals showed undetectable succinyl acetone (SUAC), a toxic metabolite, in plasma and normalization of tyrosine levels despite a normal diet. HT1 patients also suffer from a life-long high risk of hepatocellular carcinoma (HCC) believed to be due to accumulation of mutagenic toxic metabolites. To assess the durability of GeneRide treatment as well as mitigation of HCC risk, we treated pediatric *FAH^{-/-}* mice with either GeneRide or NTBC and monitored the mice up to one year of age. We believe the data from this study supported the potential durability of GeneRide genome editing for HT1. In addition, GeneRide-treated animals showed a rapid decrease of alfa-fetoprotein (AFP), a clinically validated biomarker for HCC, to levels comparable to healthy heterozygous littermates. Compared to the current standard of care, treatment with GeneRide vectors in HT1 mice resulted in superior succinyl acetone reduction, better tyrosine management and a lower alfa-fetoprotein level. Taken together, these encouraging preclinical data illustrate the potential of the GeneRide approach in a mouse model of HT1. The preclinical data package support further development of GeneRide therapy for treating HT1 patients.

538. Gene Replacement of Mutant Presenilin1 Normalizes Gamma-Secretase Function in Models of Autosomal Dominant Alzheimer's Disease

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We are developing an AAV gene therapy for the treatment of Autosomal Dominant Alzheimer's Disease (ADAD), an early onset form of AD (onset at <65 years of age). ADAD is mainly caused by mutations in the presenilin 1 (PSEN1) gene, which encodes the catalytic subunit of γ -secretase complex and is responsible for the cleavage of amyloid precursor protein (APP). This cleavage produces A β of varying lengths, with longer peptides like A β 42 being more prone to aggregation than shorter peptides such as A β 40. Of the >150 distinct, 100% penetrant PSEN1 mutations associated with ADAD, most result in a significant loss of γ -secretase function, resulting in decreased production of A β 40 and an increase in the ratio of A β 42:A β 40. Aggregation of A β peptides into amyloid plaques in the brain is a hallmark of ADAD. We are employing a gene replacement approach to normalize γ -secretase function. Using an AAV vector, we have successfully expressed a functional copy of *PSEN1* in mouse and patient-derived cellular models of presenilin dysfunction. We have demonstrated that treatment with AAV-PS1 in mouse models and patient-derived cell lines effectively normalizes γ -secretase function. Our AAV vector can be broadly distributed in brain tissue and provides attractive expression of PSEN1. We have developed a vector that enables broad expression of WT PSEN1 and is capable of normalizing γ -secretase function in *in vitro* and *in vivo* models of ADAD.

539. Extracellular Vesicle Associated Adeno Associated Virus for Efficient Inhaled Gene Transfer to the Lung

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We have previously reported that adeno-associated virus serotype 6 (AAV6), unlike other serotypes, possesses a unique ability to penetrate the sticky mucus layer covering the lung airways to provide widespread and efficient pulmonary transgene expression following intratracheal administration. On the other hand, extracellular vesicle (EV) has been recently shown for its ability to penetrate mucus, albeit in intestinal mucus, while being extensively demonstrated as a delivery vehicle to enhance therapeutic delivery to various tissues and cells. Here, we thus tested our hypothesis that EV-associated AAV6 (EVAAV6) would further enhance the localized delivery and transduction efficacy of AAV6 in the lung. AAV6 and EVAAV6 were produced by transfecting HEK 293T cells with AAV-producing plasmids. The cell culture media and lysate were used for EV and AAV6 preparation, respectively. Morphological analysis with Transmission Electron Microscopy revealed that EVAAV6 exhibited both internal and

external associations of AAV6 with EV, whereas EV+AAV6 showed no association between AAV6 and EV (Fig. 1A). Hydrodynamic diameters of AAV6, EV, and EVAAV6 were measured by Dynamic Light Scattering to be 22 ± 2 , 115 ± 11 , and 136 ± 12 nm, respectively, on average (Fig. 1B). The motion of EV and EVAAV6 in sputum from CF patients were tracked by fluorescent microscopy and quantified by mean square displacement (MSD) values which represent diffusion rates. EV efficiently penetrated human airway mucus with diffusion rates only ~15-fold lower than their theoretical rates in PBS, and the rates were greater on average compared to those with AAV6 (Fig. 1C). We also found that diffusion rates of EV and EVAAV6 were virtually identical. For in vitro test, human bronchial epithelial cells were treated with AAV6, EVAAV6, or EV+AAV6 at varying multiplicity of infection (MOI). After 72 h, luciferase activity of EVAAV6 group was significantly greater than both AAV6 and EV+AAV6 at three incrementing MOI (Fig. 1D). For in vivo test, saline, AAV6, EVAAV6, or EV+AAV6 were intratracheally administrated to C57BL/6 mice. After 2 weeks post-treatment, EVAAV6 exhibited significantly greater luciferase activity in mouse lungs compared to both AAV6 and EV+AAV6 (Fig. 1E). Also, representative confocal images revealed that while all treatments provided widespread YFP transgene expression in the whole lung lobe, the fluorescence intensity appeared to be markedly greater for EVAAV6 compared to AAV6 and EV+AAV6 (Fig. 1F). We discovered that EVAAV6 provides enhanced mucus penetration and cellular transduction compared to AAV6. The association of EV and AAV6 at the production stage appeared to play a critical role in improving the delivery and transduction, since all performances of EV+AAV6, devoid of direct association of EV and AAV6, were similar to those of AAV6. The mucus penetrating ability of EVAAV6 was preserved with the externally associated AAV6, which could perturb beneficial surface properties of EVs. To this end, we speculate that inherent ability of AAV serotypes to resist adhesive interactions with mucus, as the case of AAV6, may be important for developing efficient EVAAV-based delivery platforms for inhaled or other mucosal applications.

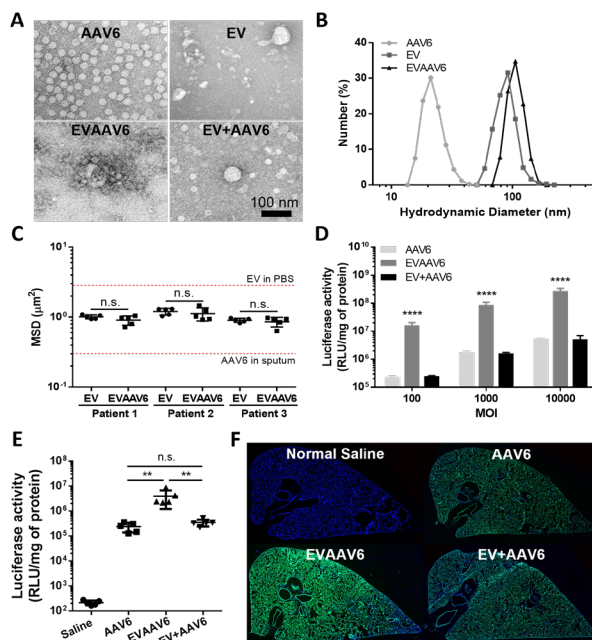


Figure 1. EVAAV6 provide enhanced in vitro and in vivo delivery and transduction efficacy compared to AAV6. (A) Representative TEM images for AAV6, EV, EVAAV6 and EV+AAV6. (B) Hydrodynamic diameters of AAV6, EV and EVAAV6. (C) Median MSD values of EV and EVAAV6 in CF sputum. Transduction efficacy of EV, EVAAV6, and EV+AAV6 in (D) HBE cells and (E) mouse lungs determined by luciferase assay. ** $p < 0.01$, **** $p < 0.0001$. (F) Representative confocal images of whole left lung lobes of mice intratracheally treated with normal saline, AAV6, EVAAV6 or EV+AAV6.

540. Dual AAV Vector Restores Dysferlin in Dysferlin-Deficient Murine Model

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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 the present work, we present evidence that overlapping AAV vectors are capable to deliver dysferlin gene cDNA to restore dysferlin protein expression. **Methods.** The experiment was carried out on a sample of fifteen 4-6-month-old Bla/J mice and involved an intravenous or intramuscular dual AAV system delivery of a codon-optimized dysferlin cDNA, respectively. Muscle samples were obtained 4 weeks post-delivery. The transgene delivery was confirmed using PCR, RNA detection by RT-PCR, and protein detection by ELISA and immunohistochemistry (ICC). Histopathologic 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. **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. In addition to that, muscle tissue structure showed no obvious toxicity. In further studies, we will use larger animal groups and perform functional tests along with additional toxicity assays.

541. A Novel AAV-Based Gene Therapy for Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder characterized by progressive muscular weakness and hypotonia as a consequence of the loss of lower motor neurons. Based on the age of onset and the severity of the neuromuscular symptoms, SMA can be generally classified into four subtypes SMA I-IV. The most severe form, type I SMA, is a devastating childhood condition also known as Werdnig-Hoffmann disease. The gene responsible for most cases of SMA, *survival motor neuron (SMN)*, was identified at the chromosomal locus 5q13. The human gene is duplicated with telomeric and centromeric copies, *SMN1* and *SMN2*, respectively. SMA is caused by mutations or deletion of the *SMN1* gene, leading to depletion of SMN protein, because *SMN2* fails to generate sufficient amounts of full-length protein. Gene therapy has been successfully developed for SMA. Onasemnogene abeparvovec (Zolgensma), which was approved by FDA as a one-time intravenous administration to SMA patients below the age of 2 years, is an adeno-associated virus (AAV) vector-based gene therapy that delivers a fully functional copy of human *SMN* gene into the target cells. Zolgensma has been used to treat >1,000 patients and demonstrated significant clinical benefits including prolonged event-free survival and motor milestone achievement. However, current gene therapy faces various challenges including, for example, insufficient expression level of the *SMN* gene in the target organs/tissues and off-target toxicity. Thus, there is a need for improved gene therapies for SMA with tissue specific *SMN* expression and reduced off-target toxicity. Here we report that EXG001-307, a novel AAV-based gene therapy product that is designed to express *SMN* gene specifically in neurons, has demonstrated a better extended survival, greater motor improvement and significantly reduced toxicity in a mouse model of SMA.

542. AAV1-Mediated Airway Epithelial Progenitor Cell Transduction in Ferret Lung

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The domestic ferret has been a useful model to study chronic pulmonary diseases including cystic fibrosis (CF) and alpha-1 antitrypsin deficiency (AATD). Both CF and AATD ferrets acquire lung disease phenotypes very similar to patients with these disorders and thus may be useful for testing therapeutics, including recombinant adeno-associated virus (rAAV)-mediated gene therapy. Gene editing of airway stem cells is a promising gene therapy strategy to provide durable CFTR expression. We have previously demonstrated that rAAV serotype 1 (rAAV1) was capable of apically transducing polarized ferret and human airway epithelia *in vitro*, and the conducting airways and lung parenchyma of neonatal ferrets *in vivo*. rAAV1-mediated transduction of ferret airway epithelial progenitors has not been previously evaluated. In this study rAAV1Cre delivery was tested in transgenic ferrets harboring a CRE recombinase-responsive reporter (LoxP-tdTomato-stop-LoxP)-EGFP cassette at the ROSA26 locus (ROSA-TG ferrets). In this model, the fluorescence conversion of Tomato to EGFP expression marks the rAAV1Cre-transduced cells to enable fate mapping of long-lived progenitor cells. To this end, we intratracheally dosed both neonatal and adult ferrets with 10E13 DRP/kg of rAAV1Cre virus in the presence of 200 mM of the proteasome inhibitor doxorubicin (Dox). The 1-week-old neonatal ferrets were evaluated 6 weeks post-dosing, while the adults were evaluated 2 weeks post-dosing. Results demonstrated that the rAAV1 vector could efficiently transduce airway epithelial cells in both neonatal and adult ferrets. Long-term persistence of EGFP-positive epithelial cells appeared to expand in a clonal-like fashion and were observed in the trachea, main bronchus, intralobal bronchi, bronchioles, and alveoli of the lungs of juvenile ferrets transduced as neonates. Abundant rAAV1Cre-mediated EGFP positive cells were also observed in adult ferret airway epithelial cells after transduction. Notably, morphometric quantitative analysis demonstrated a proximal-distal gradient of AAV1-mediated transduction with more abundant EGFP-positive epithelial cells in the tracheal relative to the intrapulmonary airways in adult ferret, consistent with our previous finding that bronchioalveolar lavage fluid from adult ferrets contains an inhibitory factor that blocks rAAV1 transduction of airway epithelial cells *in vitro*. Cellular phenotyping of EGFP positive epithelial cells in juvenile ferrets infected as neonates demonstrated the existence of marked ciliated cells (acetylated alpha-tubulin), secretory cells (MUC5AC), club cells (SCGBA1), alveolar type 2 cells (SPC), and basal cells (KRT5). In the adult ferrets the pattern of cellular transduction in the trachea was similar with the exception that marked progenitor basal cells (KRT5) was rare. These results demonstrate that rAAV1 transduces ferret airway epithelial cells in both neonatal and adult ferret airways, with age-dependent regional difference in tropism. The apparent targeting of multipotent long-lived epithelial progenitors in 1-week-old neonatal ferrets suggests that the ROSA-TG ferret model may be useful for evaluating gene editing strategies targeting airway progenitors in models of chronic lung disease.

543. Plakophilin-2 Gene Therapy Prevents and Rescues Arrhythmogenic Right Ventricular Cardiomyopathy in a Novel Mouse Model Harboring Patient Genetics

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a fatal cardiac disease that causes sudden death in young adults and athletes. ARVC patients are classically characterized by early electrical defects (arrhythmias) that progress to a structural phase as ARVD/C hearts are characterized by fibro-fatty replacement of the myocardium, which leads to ventricular dysfunction and failure. ARVC is termed a genetic cardiac “disease of the desmosome” as 40-50% of mutations in ARVC patients are found in desmosomal genes, with plakophilin-2 (PKP2) being the most frequently mutated desmosomal gene. Studies of PKP2 ARVC populations suggest that a majority of mutations impact PKP2 protein levels via diverse mechanisms. No effective treatments or cures for ARVC exist, thus, strategies targeted at elevating PKP2 protein levels represents a clinically relevant avenue to treat a large portion of ARVC populations. We hypothesize that PKP2 protein dose is a critical driver of ARVC, and that PKP2 gene therapy via adeno-associated virus (AAV) strategies can prevent and rescue ARVC development. Through CRISPR-Cas9 we generated a novel knock-in mouse model harboring a PKP2 mutation that impacts PKP2 RNA splicing, PKP2 protein levels, and is sufficient to selectively impact the heart and recapitulate all classic ARVC disease features. PKP2 homozygous mutant (PKP2 Hom) mice are viable at birth yet display classic adult hallmarks of ARVC leading to sudden death starting at 4 weeks of age. PKP2 Hom mice display reduced PKP2 protein levels, which resulted in early and selective disruption of cardiac desmosomal and gap junction proteins that was instrumental to the onset of disease features. We show that a one-time early stage (neonatal mice at day 2) administration of AAV-PKP2 was sufficient to restore PKP2 protein to wild type levels and completely prevent (i) cardiac desmosomal and gap junction dissolution (ii) cardiac right and left mechanical dysfunction, (iii) cardiac electrical dysfunction and (iv) pathological cardiac tissue remodeling (fibrosis) in adult PKP2 Hom mice at 4 weeks of age. Survival analyses further highlighted 100% survival of adult PKP2 Hom mice up to 6 months of age with early stage AAV-PKP2 administration. In addition, we show that a one-time late stage (4 week old mice) administration of AAV-PKP2 had immediate benefit as it was sufficient to rescue cardiac desmosomal (including PKP2) protein dissolution as well as cardiac right and left mechanical dysfunction in adult PKP2 Hom mice at 6 weeks of age (2 weeks post-AAV injection). Kaplan-Meier survival curves highlighted long term benefits of this strategy as 100% of adult PKP2 Hom mice survived up to 5 months of age (4 months post AAV injection), with late stage AAV-PKP2 administration. We provide a novel mouse model that harbors PKP2 patient genetics that serves as an ideal platform to evaluate therapies for ARVC. Early and late administration of PKP2 via AAV represents an effective and clinically relevant approach to prevent and rescue ARVC disease development.

544. A New Gene Therapy Platform for Hereditary Blood Coagulation Factor VII (FVII) Deficiency

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INTRODUCTION: FVII deficiency is a rare autosomal recessive bleeding disorder resulting from variants in the gene encoding FVII (F7) with variable clinical symptoms ranging from severe to mild. The prevalence is approximately 1:300,000-500,000 individuals in the general population of western countries. Currently, no optimal treatment is available for patients suffering from this disorder. The only treatment option is a recurrent and lifelong factor concentrate infusion to prevent bleeding, which is both a burden for the patients and a high cost for the healthcare system. Aim of the project is to develop a liver-directed gene therapy treatment for FVII deficiency, which has the potential to be a one-time, durable and more efficient cure than current treatment options. **METHODS:** Our AAV-based gene therapy platform is designed to deliver recombinant FVII cDNA into hepatocytes. The recombinant cDNA will then be transcribed, translated and released into the circulation as functional therapeutic protein. We are currently developing this platform by: 1) adapting liver specificity, as a result of a rationally design capsid engineering approach; 2) improving efficiency and specificity of the therapeutic expression cassette by developing a rationally designed new liver-specific promoter; and 3) adapting the therapeutic efficacy of the recombinant FVII by evaluating multiple codon optimization strategies. Combining these three adaptations should result in an efficient AAV platform for liver-mediated gene therapy. **RESULTS:** Analysis of a first group of rationally designed liver-specific capsids showed that two out of five different candidates have high in vivo hepatic localization. Furthermore, our in vitro selection of novel liver-specific promoters also showed multiple candidates with high liver-specificity as resulted by transient transfection in HepG2 cells as opposed to HEK293 cells. Finally, we have also compared three to four different versions of codon optimization for FVII cDNA which were evaluated by transient in vitro protein production with the Expi293 expression system. Preliminary ELISA analysis revealed that one of the version is more efficiently expressed when compared to wild type FVII. **CONCLUSIONS:** Our results showed that we have established a novel liver-specific gene therapy platform that can successfully be used to express FVII in vivo. We are currently further optimizing the platform and extending the technology to other liver-specific disorders.

545. Evaluating Pharmacology and Efficacy of Delandistrogene Moxeparvec in Young and Aged DMD^{MDX} Rats

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Gene replacement therapy is a promising treatment for patients with Duchenne muscular dystrophy (DMD). Delandistrogene moxeparvec (SRP-9001) is an investigational gene transfer therapy developed for targeted skeletal and cardiac muscle expression of microdystrophin, a shortened, functional dystrophin protein. The purpose of this preclinical study is to evaluate the myocardial efficacy and safety of commercially representative delandistrogene moxeparvec material in DMD^{MDX} rats. DMD^{MDX} rats are a valuable alternative animal model of DMD, as they demonstrate cardiac dysfunction that recapitulates DMD patient cardiac dysfunction. We performed systemic, intravenous delivery of commercially representative delandistrogene moxeparvec 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 1.33×10^{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, commercially representative delandistrogene moxeparvec material increased ambulation and vertical activity in young DMD^{MDX} rats and demonstrated cardiac function improvements. 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 safety and efficacy of delandistrogene moxeparvec. Further evaluation of cardiac disease phenotypes at 12 and 24 weeks post-systemic delivery utilizing several indicators of cardiac function will also be presented.

546. CNS Expression of ABCD1 Corrects Basal Forebrain Neurons and Hind Limb Claspings in a Mouse Model of X-Linked Adrenoleukodystrophy

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X-linked Adrenoleukodystrophy (X-ALD) is caused by mutations in peroxisomal half-transporter *ABCD1* and represents the most common metabolic cause of hereditary spastic paraparesis. Here, analysis of the endogenous *ABCD1* expression pattern in normal mice reveals high expression in periaqueductal gray matter, basal forebrain and hypothalamus. In X-ALD mice (*Abcd1*^{-/-}) these structures reveal alpha synuclein and other signs of neurodegeneration. Similarly, human brain shows high expression of *ABCD1* in deep gray nuclei and in autopsy

specimen of human X-ALD patients these structures show high levels of phosphorylated tau, gliosis and complement activation. This suggests that basal ganglia pathology results from a loss of peroxisomal transport activity and could potentially contribute to motor impairment in X-ALD. As cholinergic neurons in basal forebrain express high levels of *ABCD1*, we investigated *ABCD1* silencing in SHSY5Y neurons and found impaired functional proteins as well as a decrease in acetylcholine levels similar to that seen in plasma of the X-ALD mouse model. We also provide evidence that hind limb claspings exhibited in the X-ALD mouse model can be corrected by intraventricular adeno-associated virus (AAV)-vector-mediated delivery of *ABCD1*. A comparison to lumbar intrathecal delivery of vector identifies basal forebrain neurons as critical to behavioral rescue. The data support the contention that rescuing peroxisomal transport activity in basal forebrain neurons might represent a therapeutic strategy for X-ALD.

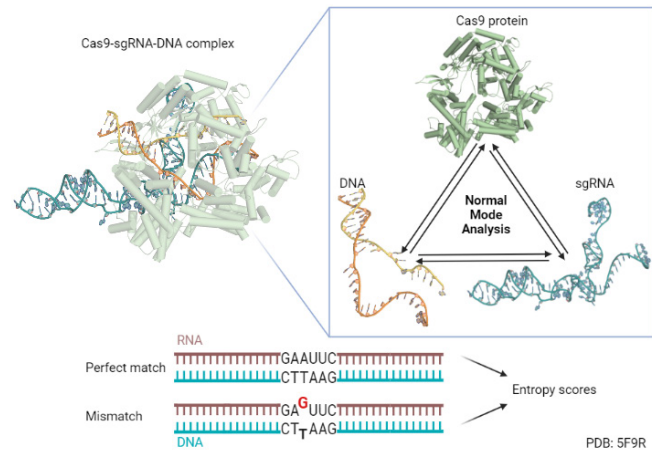
547. Ocular Pharmacokinetics of Recombinant and AAV Mediated Expression of Adalimumab After Intravitreal Injection in Mice

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Introduction Similar to the brain, the eye is known to be isolated from systemic circulation by the blood-aqueous barrier and the blood-retina barrier. The two barriers together tightly restrict the movement of therapeutic proteins and other molecules between the systemic and ocular compartments making it challenging to treat many ocular diseases with systemic therapies. Published literature indicates that only ~1% of the total dose of a therapeutic protein (e.g., ranibizumab) reaches systemic circulation after intravitreal injection in humans and higher species (e.g., non-human primates); however, ocular concentrations are not reported for mice (Gaudreault 2007 Retina). Therefore, a representative therapeutic protein adalimumab (Humira[®]) was chosen to establish ocular pharmacokinetic profiles after intravitreal (IVT) injection in severe combined immunodeficiency (SCID) mice. The pharmacokinetic profile of recombinant adalimumab was then utilized as a basis to characterize the PK of an IVT administered AAV2 vector encoding for adalimumab. **Methods** For studies with adalimumab, mice received a 3.2 and 1.6 ug dose on days 1 and 7 respectively as a 0.5 µL bilateral IVT injection. Serum samples and ocular tissues were collected at 15 mins and on days 1, 3, 7 (prior to 2nd dose), 8, 10, 14 and 21 post injection. For studies using AAV vector, mice received a single 1E9 or 1E10 vg bilateral IVT injection (0.5 µL) of an AAV2 vector encoding for adalimumab. Serum samples and ocular tissues were collected on weeks 2, 4, 8, and 12. Additional serum samples were collected on weeks 1, 6, and 10. Adalimumab concentrations were quantified using a commercially available antibody ELISA kit (cat# ab237641). Animals were observed twice per day in accordance with Institutional Animal Care and Use Committee and facility standard operating procedures and body weights were measured daily. **Results** In mice that received adalimumab recombinant protein, the clearance of adalimumab from the ocular compartment was rapid with an estimated half-life of ~4 hours after IVT injection. Although a small concentration remained detectable throughout the duration of the study this was likely due to redistribution of adalimumab from the central compartment. Contrary to what is found in humans after IVT injection of an antibody, mice had

substantially higher serum concentrations within 24 hours although the systemic clearance remained similar to what was reported for adalimumab in mice, indicating an interspecies difference in the distribution of the antibody between the compartments. In mice that received AAV2-adalimumab, ocular concentrations elevated over 8 weeks and plateaued by 12 weeks. Similar to what was observed after injection of adalimumab, adalimumab concentrations were highest in the serum as compared to what was observed in the eye. **Conclusion** While the movement of large therapeutic proteins is known to be generally restricted between central and peripheral compartments, our data suggest that in mice, adalimumab moves rapidly from the eye and into systemic circulation with most (> 80%) of the antibody found in serum 24 hours after injection. For reference, other large therapeutic proteins in humans are reported to have an ocular half-life of 6 to 9 days (Mandal 2018 Adv. Drug Deliv. Rev.). Although mice are a common species for preclinical evaluation of drug response, the rapid clearance from the eye and systemic accumulation may confound interpretation of safety and efficacy of AAV gene therapies expressing a secreted protein.



Gene Targeting and Gene Correction II

548. Computational Simulation of CRISPR-Cas9 Activity and Specificity Using Normal Mode Analysis

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The CRISPR-Cas system is a microbial adaptive immune system repurposed as a powerful genome-editing tool. Since being discovered, the novel genome-engineering technique transformed the field of gene editing, creating opportunities from repairing disease-causing mutations to crops engineering. Existing computational tools aim to predict the on-target efficacy and potential genome-wide off-targets by scoring a particular gRNA according to an array of gRNA design principles or machine learning algorithms based on empirical results of large numbers of gRNAs. However, such tools are unable to predict the editing outcome by variant Cas enzymes and can only assess potential off-targets related to reference genomes. Moreover, assessing the activity and specificity of variant enzymes is time and labor intensive. Here, we demonstrate the feasibility of normal mode analysis (NMA) to depict the structure-function association of SpyCas9, complexed with sgRNA and its target DNA. Our results demonstrate the validity of NMA to predict the activity and specificity of SpyCas9 in the presence of mismatches by comparison to empirical data. Furthermore, despite the absence of their exact structures, this method accurately predicts the enzymatic activity of known high-fidelity engineered Cas9 variants. The ability to computationally simulate the enzymatic function may lay the groundwork for generating future gene-editing tools and technologies such as off-targets assessment tools and engineering of novel Cas variants.

549. Development of a Prime Editing Strategy to Treat Mutations in the *Crumbs Homologue 1 (CRB1)* Gene

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Purpose Mutations in *Crumbs homologue 1 (CRB1)* gene cause chronic and disabling autosomal recessive retinal dystrophies, including Leber congenital amaurosis 8 (LCA8) and retinitis pigmentosa 12 (RP12). Currently, there are approximately 80,000 *CRB1* patients worldwide and there is no available treatment to date. The mouse and human retina contain three major *CRB1* isoforms, *CRB1-A*, *CRB1-B* and *CRB1-C*. *CRB1-A* and *CRB1-B* have predominately cell-type specific expression making the choice of gene augmentation strategy currently unclear. Gene editing with prime editing (PE), that is capable of installing all types of edits, including transitions, transversions, small deletions and insertions, may be a viable alternative for the amelioration of *CRB1* associated retinal degenerations. In this study, we tested the efficacy of PE for the correction of *CRB1* mutations in patient induced pluripotent stem cell (iPSC) lines. **Approach** To begin optimizing PE for correction of *CRB1* mutations, 30 combinations of pegRNA and nicking sgRNA design were tested per mutation on the corresponding *CRB1* patient iPSC lines using nucleofection or Stem Cell lipofectamine. Combinations showing efficient editing were taken forward for further optimization. Editing efficiency of different PE designs was evaluated by NGS or Sanger sequencing followed by ICE analysis. Currently, we are adapting the PE to a virus system, in which we have optimized promoters to highly express PE machinery in postmitotic retinal cells, using human iPSC-derived retinal organoids as a screening tool. Human retinal organoids were differentiated using a slightly modified version of the Cowan *et al.* 2020 differentiation protocol. **Results** Based on our analysis of the *CRB1* LOVD database and our cohort of *CRB1* patient iPSC lines we chose to initially develop prime editing for p.(Cys948Tyr) (Most prevalent *CRB1* mutation) and p.(Gly1103Arg) (8th most prevalent

CRB1 mutation) mutations. We found that *CRB1* patient iPSC lines were amenable to prime editing. With editing efficiencies as high as 72% dependent on the combination of pegRNA (primer binding site (PBS) and reverse transcription template (RTT) length) and nicking sgRNA for a particular mutation. This system is now being modified to a viral system, which can successfully transduce retinal organoids and express GFP in photoreceptor cells and/or Müller glial cells. **Conclusions** Prime editing is amenable for the correction of *CRB1* patient mutations and achieves high editing efficiencies dependent on optimization of the pegRNA and the nicking site used. Further, we have developed a viral system, which we are adapting for our PE strategy to treat our patient mutations. Together this work provides a platform for the testing of *CRB1* therapeutic editing in *CRB1* patient iPSC-derived retinal organoid disease models.

550. Highly Efficient Somatic Gene Editing in Murine Lung

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We have established methods for effective AAV-based CRISPR/Cas9 delivery and somatic gene editing in murine lung epithelial cells, including alveolar type II (AECII) cells and distal airway epithelial cells. A first approach utilizing separate delivery of Cas9 and gene specific guides, or through a split-intein Cas9 format enabled efficient *in situ* somatic editing in a reporter mouse model through a dual-vector and repeat dosing strategy. Repeat dosing was necessary for maximized editing in distal airway epithelial cells but not AECII cells. We then demonstrated highly efficient editing through AAV-mediated sgRNA expression in a Cas9-transgenic background, leading to a significant down-regulation of a protein of interest in AECII cells in one study and a prominent transdifferentiation phenotype in distal airway epithelial cells in the other study. During the course of these studies, we observed negligible inflammatory responses. We also found that CRISPR editing occurred in concert with AAV vector integration at sites targeted for double-stranded DNA breaks, highlighting a potential safety risk and complicating factor for clinical applications that rely on AAV-mediated delivery of DNA nucleases. In sum, the distinct potency of these methods will enable rapid drug target validation and generation of mouse models for lung disease.

551. High-Throughput RNA Sequencing Directly from Cell Lysates Enables Reproducible Phenotypic Profiling for CRISPR Treatment Phenotyping and Cell Response Screening Applications

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Gene editing has the potential to revolutionize genetic approaches to personalized medicine and enable a new era of gene-based therapeutic treatment options for heretofore untreatable diseases. Recent advancements in targeted gene editing and genome editing techniques allow for highly-specific, targeted DNA modifications to genes of interest. This includes the CRISPR/Cas9 system, which can be used for gene knockout, gene mutagenesis and gain-of-function gene knock-in approaches to engineer cell lines and potential therapeutics. Downstream discovery of CRISPR editing effects relies heavily on high-throughput screening assays for measurement of phenotypic responses which can range from very targeted assays to wide-ranging unbiased approaches. Standard whole transcriptome RNA-seq is a preferred method for unbiased measurement of phenotypic responses but is often overlooked due to limited scalability and high sample screening costs. To address these challenges, we have developed a high-throughput gene expression (HT-GEx) assay that combines a variety of strategies to enable a low-cost and high-throughput alternative to standard RNA-seq. First, a simplified workflow removes upstream RNA isolation steps and tags transcripts directly from cell lysate. This is achieved by incorporating both a sample barcode and a unique molecular index (UMI) during the reverse transcription reaction. Next, we leverage a 3' end counting approach to enable a reduction in sequencing depth coverage (i.e. sample cost) without compromising gene detection sensitivity. With these key advances, a high-plex and high-throughput screening assay is achievable at a reduced cost by removing the need to purify RNA, tagging transcripts early in the workflow to allow pooling of samples and reducing sequencing depth. We have combined these high-throughput and cost-saving strategies and present results which confirm HT-GEx offers results on par with standard RNA-seq. Gene detection sensitivity based on number of genes detected per millions of reads is highly similar, with gene detection sensitivity saturated at approximately 2 million reads. Additionally, the number of genes detected between replicates for RNA samples and lysate samples demonstrate strong linear correlation, implying highly reproducible results. Thus, high-throughput gene expression is an ideal method for phenotypic screening and has applications in CRISPR edited cell lines following stand-alone or combined compound treatments.

552. Selective Silencing of Mutant C9orf72 Transcripts

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Amyotrophic lateral sclerosis (ALS) is a motor neuron degenerative disorder that affects the upper and lower motor neurons. The loss of motor neurons in the brain and corticospinal tract leads to muscle atrophy and paralysis. The most common genetic cause of familial ALS is an expanded hexanucleotide repeat in the first intron of chromosome 9 open reading frame 72 (*C9orf72*) gene. The transcribed expanded repeat containing *C9orf72* mRNA causes cellular toxicity due to RNA foci and dipeptide repeat proteins production, both underlying the pathogenesis of the disease. A next generation of therapeutic

microRNAs was developed based on our miQURE® platform to selectively silence mutant C9orf72 transcripts. The candidate silencing efficacy was tested on luciferase reporters harboring C9orf72 mRNA target sequences. Additionally, the potency of the therapeutic microRNAs was tested in the presence of the repeat expansion by using an expression vector encoding C9orf72 mRNA with 90 hexanucleotide repeats. A strong knockdown (>80%) was observed in microRNA-treated cells. The efficacy of the next generation microRNAs in the presence of the repeat expansion was also confirmed *in vitro* with up to 70% C9orf72 mRNA lowering. In conclusion, we show strong mutant-specific knockdown of C9orf72 transcripts *in vitro* by expressing our next-generation microRNAs. Ongoing pre-clinical proof-of-concept studies in ALS rodent models will provide data on the feasibility of using AAV encoding our next generation microRNAs selectively targeting the mutant C9orf72 as an attractive approach to target ALS.

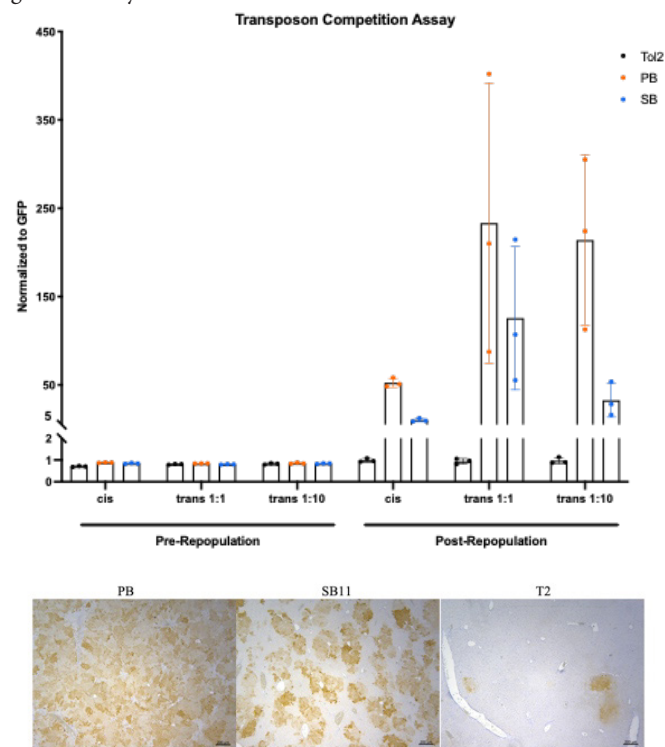
553. Comparative Assay of Barcoded Transposon Vector Systems *In Vivo* Reveals Advantages of PiggyBac

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Mobile DNA elements known as transposons enable transposition of DNA cargo by a “cut and paste” mechanism. Transposon systems have translational applications to disrupt, modify, or rescue gene functions via insertion into the genome. Consequently, transposons are being used for *in vivo* and cell culture-based genetic screens and for gene therapy. We and others have performed gene therapies in mice using hydrodynamic tail vein injection of *Sleeping Beauty* (SB11) transposons to restore FAH expression in a fraction of the hepatocytes of *Fah*^{-/-} mice, which is followed by liver repopulation by the corrected cells. We have used this gene therapy paradigm to conduct genetic screens *in vivo* within the liver. The capacity to perform screens is proportional to the number of clonal events. We hypothesized that a more efficient transposon system would lead to more clonal events in the liver. The *PiggyBac* (PB) and *Tol2* (T2) are alternative transposon systems that have never been directly compared to the SB11 in an *in vivo* mouse system. To directly compare these transposon systems, we generated four DNA plasmid vectors: SB11, PB, T2, and a no-transposase GFP control. Each contained the transposon ends (or none in the control), the FAH gene as the cargo, and a unique nucleic acid barcode to enable identification of each vector by sequencing. The four vector types were then pooled together in three separate conditions: the transposon and transposase on the same plasmid (*cis*), the transposon and the transposase of different plasmids (*trans*), and tenfold the transposon plasmid for each transposase plasmid (*trans 10:1*). The three separate pools of vectors were then screened in *Fah*^{-/-} mice using hydrodynamic tail vein injection in a parallel gene transfer assay. One month after injection, the liver tissues were harvested, bulk DNA was extracted, and the region of the barcode was amplified to generate sequencing libraries. Using high throughput sequencing, we were able to track the efficiency of gene delivery for each vector system condition. Our results indicate that the PB transposons were greatly enriched in the

repopulated mice. They were detected 200-fold more often than the no-transposase condition, and up to 10-fold more than SB11, across all conditions (figure 1). The PB and SB11 plasmids delivered in *trans* had superior enrichment over the *cis* condition. Tol2 had similar efficiency to the results to the basal no-transposase condition. In *Fah*^{-/-} mice repopulated separately with each transposon system, staining for FAH indicated more extensive liver repopulation with the PB condition (figure 2). We verified the insertion of the transposons into the mouse genome using high throughput sequencing method. Overall, we conclude that *PiggyBac* is the most efficient transposon system for gene delivery to the mouse liver.



554. Multiplex Cell Engineering of Six Target Genes in Primary T Cells Using a Single Gene Silencing Construct

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Gene-modified cell therapies offer promising solutions to treat and potentially cure a variety of conditions, including cancers, infectious and auto-immune diseases, as well as certain inherited disorders. However, numerous challenges remain with respect to achieving optimal treatment safety and efficacy. Additionally, these treatments are still cost-prohibitive for most, and thus the need for strategies to facilitate broad access are a key focus. Some of these aspects can be addressed through improved efficiencies in gene-modification, as well as the introduction of additional gain and loss-of-function characteristics. However, this adds complexity to gene and cell engineering practices, which thus relies heavily on the use of a safe and efficient molecular strategy. We have developed a substantially

optimized microRNA (miRNA) gene construct to achieve highly efficient, simultaneous gene silencing of multiple targets. Our aim was to demonstrate the use of this single construct to silence expression of up to six target genes relevant to immunotherapeutic applications, and to measure the consistency of gene silencing over long-term *in vitro* assays. Using in-house developed software, we first prioritized a range of target sequences against each gene of interest, cloned these into our miRNA backbone as single hairpin configurations, and screened for efficiency of target gene silencing. All constructs were delivered to primary T-cells via lentiviral vector transduction. Best performing miRNAs, with respect to target gene silencing, were then cloned into multi-hairpin constructs to assess the ability to simultaneously silence multiple target genes. Our data demonstrates successful and high efficiency gene silencing of relevant cell surface receptors. We also show efficient gene modification of primary T-cells and multiplex silencing of up to six target genes from a single construct and transduction step. Notably, we observed comparable gene silencing efficiencies relative to control constructs carrying single miRNA hairpins against corresponding target genes. Finally, we also show durable gene silencing of all target genes over long-term *in vitro* assays. We have developed a novel gene construct able to silence the expression of multiple target genes in a single gene modification step. The technology is widely applicable, and can be applied in both gene and gene-modified cell therapy products. It can be delivered using viral vectors, homology-directed repair (gene editing) or transposons; and used to augment the therapeutic capabilities of immune effector cells, including CAR and TCR-engineered T-cells, tumor infiltrating lymphocytes, and viral specific T-cells.

555. CRISPR-Associated Gene-Editing Systems Discovered in Metagenomic Samples Enable Efficient Genome Engineering in Multiple Primary Immune Cell Types

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Gene-editing technology has revolutionized molecular therapeutics, enabling DNA-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 novel type II and type V gene-editing systems from metagenomic data, the characterization of these systems *in vitro*, and a demonstration of their activity in primary T cells. Here, we substantially advance this work, applying our gene-editing technology to NK cells, B cells, HSCs, and iPSCs. Gene-editing efficiency (indel formation) in all such primary cell types was typically greater than 95%. Similarly, we demonstrate efficient transgene integration and expression in these cell types. Further, we edit primary cells with one additional novel nuclease and one PAM-engineered nuclease so as to expand our genomic targeting density. Extending our T cell work, we achieve greater than 90% efficient simultaneous double knock-out of the alpha and beta chains of the T cell receptor as well as triplex editing in combination with knock-out

of beta-2 microglobulin. To add additional beneficial phenotypes to T cell therapy products we performed guide screens for TIGIT, FAS, and PDCD1 (PD-1) finding highly efficient guides for each gene. Finally, as our gene-editing systems are sourced from microbes found in environmental samples and from the non-pathogenic human microbiome, we expected 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 to either of our lead nucleases in a panel of serum samples from fifty randomly selected donors. In all, we show that these new gene-editing systems have the activity, specificity, and translatability necessary for broad use in cell therapy development.

556. Challenges and Inconsistencies in Type II CRISPR-Associated Nuclease Subtype Classification

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CRISPR-associated nucleases were first found and classified as a component of the bacterial immune defense system, designed to combat foreign DNA. The discovery of SpCas9, and its repurpose as a genome editing tool led the path to the discovery of additional distinct nucleases with diverse properties that are employed in a variety of applications. Initial categorization of CRISPR-related nucleases was based on a narrow pool of nucleases from limited origins and could not anticipate the heterogeneity of nucleases that is known today. The current subtype classification is based on several methodologies; however, no differential weight was assigned to each classification method, and in several cases, the discrepancy between the methods resulted in subjective and/or arbitrary classifications. In the current study, we employed three different commonly used classification methods of type II nucleases, namely: Loci architecture, Cas9 phylogeny and Cas1 phylogeny. For each classification method, the distribution of nucleases by subtype was studied and the agreement between the methods was measured. Out of the nucleases analyzed, about 30% were inconsistently classified. A similarity matrix revealed a diverse correlation between the methods used. In some cases, the nucleases did not fit with any of the established subtype classifications. Overall, these findings demonstrate the challenges in CRISPR-associated nuclease classification. They also question the present paradigm of affiliating nucleases into distinct, allegedly homogenous groups with shared properties and functions. Accordingly, we propose that newly discovered and/or engineered nucleases should be regarded as distinct elements and carefully characterized prior to being confined with existing classifications.

557. shRNA-Mediated Gene Therapy for the Treatment of Angelman Syndrome

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Angelman Syndrome (AS) is a severe neurodevelopmental disorder caused by the deficiency of functional Ubiquitin Protein Ligase E3A (UBE3A) resulting in intellectual deficits, ataxic gait, lack of speech and frequent laughter. *UBE3A* expression is regulated through genetic imprinting whereby the maternal allele is expressed and the paternal allele is silenced in a neuron-specific manner. Paternal silencing is induced with a portion of a large, non-coding antisense transcript specific to *UBE3A* (*UBE3A-ATS*). Previously it has been demonstrated that anti-sense oligonucleotides and CRISPR/Cas9 targeting *UBE3A-ATS* elevated paternal expression of *UBE3A* and improved behavior deficits in an AS mouse model. In this study, we tested the hypothesis that shRNA-induced inhibition of *UBE3A-ATS* could induce paternal *UBE3A* expression. First, we screened siRNA candidates targeting *UBE3A-ATS* in a human neuroblastoma cell line. We discovered several candidates which reduced *UBE3A-ATS* while concomitantly increasing *UBE3A* transcript levels. We also differentiated induced pluripotent stem cells (iPSC) from AS patients into neurons and found that *UBE3A-ATS* expression increased at each stage during the development into neurons. Top siRNA candidates were converted into shRNA oligonucleotides and cloned into a self-complementary construct plasmid. We transfected lead candidate shRNA plasmids into iPSC-derived neuronal progenitor lines. Preliminary data based on immunolabeling of *UBE3A* suggests that shRNA plasmid candidates reactivated paternal *UBE3A* expression. This study demonstrates initial proof-of-concept to use an AAV vector to treat AS via shRNA-mediated knock-down of *UBE3A-ATS*. Conceptually, using an AAV-based approach may provide a one-time dosing regimen to confer permanent paternal *UBE3A* expression. This work was funded by Taysha Gene Therapies, Inc.

558. SAFE-T: A Novel T Cell Platform to Define Biological Effects of Genome Editing

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Although methods for defining CRISPR-Cas genome editors unintended genome-wide off-target activity have become increasingly advanced and sensitive, interpreting biological consequences of this activity and predicting the long-term impact on safety remains challenging. Thus, we sought to develop SAFE-T (Serial analysis of functional edits in T cells), a platform to define functional consequences of genome editing in therapeutically relevant human

T cells. We hypothesized that genome-edited cells with functional off-target mutations may gain a proliferative advantage, increasing the chance they could accumulate additional transforming mutations. To rapidly assess changes in on- and off-target activity over time, we optimized a streamlined and scalable version of GUIDE-seq (GUIDE-seq-2), which simplifies library preparation with a Tn5 tagmentation-based protocol. We directly compared GUIDE-seq and GUIDE-seq-2 at 10 Cas9 sgRNA targets across 6 therapeutically relevant *loci* in human primary T cells (*CCR5*, *TRAC*, *PDCD1*, *CTLA4*, *AAVS1* and *CXCR4*). We observed strong correlations between the GUIDE-seq and GUIDE-seq-2 read counts at 9 out of 10 targets ($R^2 > 0.9$). To detect off-target mutations that affect cell proliferation over time, using GUIDE-seq-2 we measured the genome-wide off-target activity of Cas9 targeted to 20 therapeutically-relevant targets across 12 *loci* in human primary T cells (*AAVS1*, *B2M*, *CBLB*, *CTLA4*, *CCR5*, *CXCR4*, *DNMT3A*, *FAS*, *LAG3*, *PDCD1*, *PTPN2* and *PTPN6*) at 5 timepoints (from day 7 to day 30), with 6 replicates per timepoint. Across 2,648 unique off-targets, we identified evidence of both positive and negative selection of off-target mutations, suggesting a subset have likely functional impacts on cell growth. We are currently performing follow-up multiplex targeted sequencing (rhAmpSeq) to quantify the off-target editing activity over time at sites identified by GUIDE-seq-2. Preliminary results for 260 off-target sites associated with *AAVS1* site 11, a relatively non-specific target, revealed that 4.6% of off-targets significantly increased over time and 76% significantly decreased over time (FDR < 0.05). Taken together, our data demonstrates a novel framework to rapidly define functional off-targets by quantifying genome-wide activity over time and may provide more general insights into predicting the safety of genome editors for therapeutic applications.

559. The Mito-DdCBE System as a Mitochondrial Gene Therapy Approach

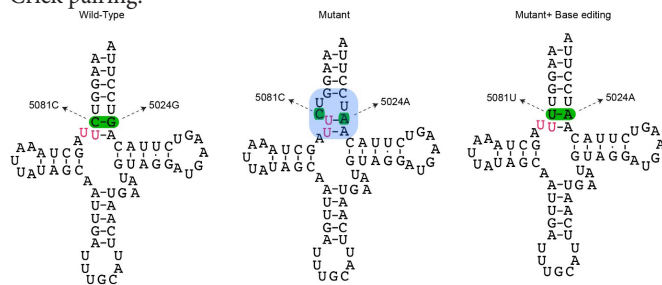
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Introduction and Methods The mitoDdCBE system was recently developed to edit mitochondrial DNA. It is based on a deaminase from *Burkholderia cenocepacia* which is capable of deaminating cytosine residues in double-stranded DNA. Because mitoDdCBE only edits cytosines "C" preceded by a thymine "T", the number of pathogenic mutations that it can target at the moment is limited. To test whether this approach can be used for gene therapy *in vivo*, we took advantage of one of the few existing animal models of heteroplasmic mtDNA mutations, the m.5024T mutant mouse, which develops a mild cardiomyopathy with aging. The m.5024T mtDNA mutation affects the stability of the mouse mitochondrial tRNA alanine, likely by disrupting a Watson & Crick (WC) base pair in the tRNA cloverleaf secondary structure. Although the m.5024T cannot be edited, we found that the position opposite in the tRNA structure (m.5081C) can be edited as it is preceded by a thymine. In the context of the m.5024A tRNA mutation, an m.5081C>U edited base would restore WC base pairing and likely improve tRNA^{Ala} stability and function (Fig.1). **Results** To test this hypothesis, we attempted to isolate a cell line homoplasmic for the m.5024T mutation. To do so, a heteroplasmic MEF line was treated with 125ng/ml Ethidium Bromide for 50 days to

block mtDNA replication and reduce the mtDNA levels. Isolated clones were characterized for the levels of the mtDNA mutation. Through this manipulation, we obtained a line with ~90% mutant mtDNA. We then designed *in silico* a pair of mitoDdCBEs to target the m.5081C, electroporated the plasmids carrying the two monomers into the 90% m.5024T line and determined their expression and editing efficiency. Immunocytochemistry performed 2 days after electroporation showed that more than 90% of the cells expressed the mitoDdCBE monomers tags and they were localized to mitochondria. Western blots confirmed the expression and expected molecular sizes. Initial editing at position m.5081C was approximately 20%. Therefore, we decided to both perform sequential rounds of mitoDdCBE electroporation followed by treating 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 screened by a RFLP-based approach. Twenty-two clones homoplasmic for the m.5024T have been isolated from these experiments. They harbored different levels of heteroplasmy for the edited m.5081C, ranging from 14% to 90% with a mean of 52%. We are currently using these clones to assess whether the tRNA^{Ala} stability is restored as a consequence of the m.5081C edit. **Conclusions** We have successfully modified a cytosine in the mouse mtDNA that may act as a second site suppressor. We will characterize the biochemical phenotype of edited cells in culture and if the results are positive, treat mouse tissues *in vivo* with AAV vectors. A positive result would demonstrate the viability of this system as a gene therapy approach in patients with mtDNA diseases.

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 propose to edit the m.5081C to a U, thereby restoring the original Watson and Crick pairing.



560. A Compact Zinc Finger Architecture for High-Efficiency Base Editing in Human Cells

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Introduction: Nucleobase editors are an emerging technology that enables precise single-base changes to the genomes within human

cells¹. Delivering the required reagents to the proper tissues for therapeutic interventions can be challenging for conventional CRISPR base editors since existing high-efficiency base editors tend to be too large to package in a single AAV construct for therapeutic delivery. Here we demonstrate that zinc fingers (ZFs) fused to split cytidine deaminase domains derived from bacterial toxins along with a ZF-targeted DNA nickase are capable of high-efficiency base editing in human cells. In addition, all components of our ZF base editing system are compatible with packaging in a single AAV construct. **Results and Conclusions:** Recently a new class of cytidine deaminases that do not require CRISPR/Cas9-mediated DNA unwinding to achieve reasonable levels of activity have been reported². In addition to mitochondrial DNA editing, the authors were able to demonstrate up to ~27% base editing in the nucleus of human cells by fusing split halves of the DddA deaminase to engineered TALE domains. Here we build upon the work of Mok *et al.* by replacing the TALE DNA-binding domain with arrays of engineered ZF. Each 34-residue TALE repeat recognizes a single basepair of target DNA, while each 28-residue ZF recognizes three basepairs. Replacing the TALE domains with engineered ZFs thus yields constructs that are substantially smaller and less repetitive. In order to increase the editing efficiency, we also included a ZF nickase^{3,4} which is a dimeric ZF-nuclease where one of the two FokI domains is catalytically inactive so only one strand of DNA is cut. In an effort to keep the overall construct small enough for AAV packaging, we fused one deaminase construct and one copy of the FokI DNA cleavage domain to opposite ends of one of our three ZF arrays. In our initial characterizations, we confirmed that constructs containing the DddA deaminase domain employed by Mok *et al.* had target sequence limitations (5'-T required adjacent to target C). Furthermore, a characterization of an initial ZFP-DddA fusion yielded multiple off-targeting events with base editing frequencies above 10% in a nuclear editing context. To improve the performance of our ZF base editors, we explored deaminase domains derived from other bacterial toxins. One such deaminase domain enabled up to ~60% base editing in human T-cells with minimal indels, an improved specificity profile, and good cell viability. The edited cells demonstrated the expected gene knockout phenotype and the entire ZF base editing system is compatible with packaging in a single AAV vector, thus showing promise for potential therapeutic applications.

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561. ARCUS Gene Editing to Eliminate MELAS-Associated m.3243A>G Mutant Mitochondrial DNA

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Mitochondria, the ATP-producing organelles present in nearly all eukaryotic cells, contain their own multi-copy genome that is susceptible to mutation. Pathogenic mitochondrial DNA (mtDNA) mutations are commonly heteroplasmic, with both wild-type and mutant genomes co-existing in the same cell. It is not until the level of mutant mtDNA exceeds a particular disease threshold that clinical symptoms manifest. Diseases resulting from these mutations are multi-system, complex disorders with no curative treatment options. In recent years, gene editing technologies have been developed to take advantage of the heteroplasmic nature of these mutations and the lack of efficient double-strand break repair within mitochondria. This therapeutic approach aims to selectively cleave and eliminate mutant mtDNA while leaving wild-type mtDNA to repopulate the cell, resulting in a shift in heteroplasmy. Here we report on the use of a mitochondrial-trafficked engineered ARCUS nuclease (mitoARCUS) to target one of the most common pathogenic mtDNA mutations, m.3243A>G, which is responsible for >80% of cases of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). This point mutation resides in the mitochondrial tRNA^{Leu(UUR)} gene and impacts tRNA modification and aminoacylation, which consequentially impairs the cell's ability to produce mitochondrial proteins, use oxygen, and generate sufficient ATP. It was observed that fusion of a mitochondrial-targeting sequence to the N-terminus of mitoARCUS efficiently trafficked the nuclease to the mitochondria, resulting in undetectable levels of nuclear off-target editing. As the m.3243 mutation only differs from the wild-type sequence by one base pair, the ability of the nuclease to discriminate against the wild-type mtDNA sequence is paramount. Nuclease optimization enhanced specificity of the enzyme to prevent cutting of the wild-type mtDNA sequence while maintaining activity against the mutant mtDNA sequence, allowing for robust shifts in heteroplasmy in a variety of heteroplasmic cell lines. Using these different cell lines, the wild-type mtDNA threshold necessary for proper respiratory function was established. Compared to untreated heteroplasmic cells, it was observed that mitoARCUS-induced shifts in heteroplasmy above the determined wild-type mtDNA threshold produced improvements in cellular oxygen consumption rates and mitochondrial ATP production. Together, these data support the development of mitoARCUS as an *in vivo* gene editing therapeutic for the treatment of disease-causing heteroplasmic mtDNA mutations.

562. Understanding the Educational Needs of United States Physicians Related to Gene Therapy

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Introduction: Gene therapy, including both gene addition and gene editing approaches, is rapidly evolving and being investigated for a range of diseases. The goal of gene therapy is to alter the course of the target disease. To understand current awareness, knowledge, and attitudes related to gene therapy, a survey was conducted among primary care physicians (PCPs), internal medicine subspecialists (including hepatologists, gastroenterologists, and endocrinologists), and medical geneticists.

Methods: The survey was developed and distributed via email to United States physicians in January 2021. To be eligible for the survey, physicians were required to see at least one patient per week and to have treated at least one patient with a rare genetic disease at some point in their clinical practice. Responses were collected and analyzed from a total of 101 PCPs, 175 subspecialists, and 81 medical geneticists.

Results: Respondents were asked about their previous education on gene therapy; 21% of PCPs and 17% of subspecialists reported that they never had education on gene therapy. The majority of respondents sought education on their own after formal training. All respondents reported low familiarity with currently approved gene therapies and approximately half of PCPs and subspecialists were unaware that there was more than one gene therapy approved for use in the United States. Further, nearly half of PCPs and subspecialists reported they were not aware of any disease for which gene therapies were currently being investigated. Most medical geneticists were aware that some gene therapy vectors are derived from viruses because they are recognized by receptors on the cell membrane, but only about half of PCPs and subspecialists were familiar with these aspects of gene therapy vectors. Only 27% of PCPs, 34% of subspecialists, and 65% of medical geneticists were aware of differences between integrating and non-integrating viral vectors for gene therapy. Over half of medical geneticists and subspecialists and 27% of PCPs manage some or all aspects of metabolic disorders in patients with inborn errors of metabolism. Physicians believe gene therapy may be effective in treating inherited monogenic diseases and perceive it will be less effective in multifactorial disorders. As previously noted, overall awareness of emerging therapies was low. **Conclusion:** This study highlights gaps in awareness and knowledge regarding gene therapy, particularly among PCPs and non-geneticist subspecialists. Future continuing medical educational initiatives should incorporate information on the current state of gene therapy, including gene therapy mechanisms and processes, as well as efficacy and safety data for currently approved and emerging gene therapies.

563. Active CRISPR-Associated Transposases from Natural Environments

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Mobile genetic elements are widely used in genetics, biotechnology, and therapeutics to introduce large fragments of DNA into genomes for exogenous gene expression and therapeutic gene delivery. DNA transposons are either indiscriminate in their integration (e.g. Tc1/

mariner) or able to integrate in only a single, well-conserved site (Tn7). With either of these integration preferences, the ability to deliver DNA payloads in a programmable manner is not possible. However, CRISPR-associated Transposase (CAST) systems combine the transposition capability of Tn7-like proteins and the RNA-guided targeting of Cas proteins for efficient programmable and site-specific transgene integration. The simplest CASTs are those of the Cas12k-associated systems that, in addition to the effector, contain three protein components, TnsB, TnsC and TniQ, as well as a guide RNA and a cargo sequence flanked by terminal inverted repeats (TIR). While there are only a handful of well-characterized Cas12k CAST systems from reference genomes, we hypothesized that novel active Cas12k and associated transposase genes could be identified from metagenomic sequences of environmental samples. To characterize these CAST systems functionally, we developed an *in vitro* screening method for rapid identification and engineering of active systems. We show that Cas12k CASTs make up a phylogenetically diverse transposase family. Here we describe one active CAST family for its PAM preferences, the preferences of its integration spacing, and preferred orientation. *In vitro* screening enabled the rapid minimization of single-guide RNAs and testing of TIR size to ultimately improve therapeutic delivery. Finally, by introducing these systems into *E. coli*, we achieve high rates of on-target integration across multiple endogenous and engineered loci. Translation of these CAST systems to mammalian cells will further expand the already valuable CRISPR gene-editing toolbox.

564. Rapid Genome Editing by Cas9-POLD3 Fusion

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Precision CRISPR gene editing relies on the cellular homology-directed DNA repair (HDR) to introduce custom DNA sequences to target sites. The HDR editing efficiency varies between cell types and genomic sites, and the sources of this variation are incompletely understood. Here, we have studied the effect of 450 DNA repair protein-Cas9 fusions on CRISPR genome editing outcomes. We find the majority of fusions to improve precision genome editing only modestly in a locus- and cell-type specific manner. We identify Cas9-POLD3 fusion that enhances editing by speeding up the initiation of DNA repair. We conclude that while DNA repair protein fusions to Cas9 can improve HDR CRISPR

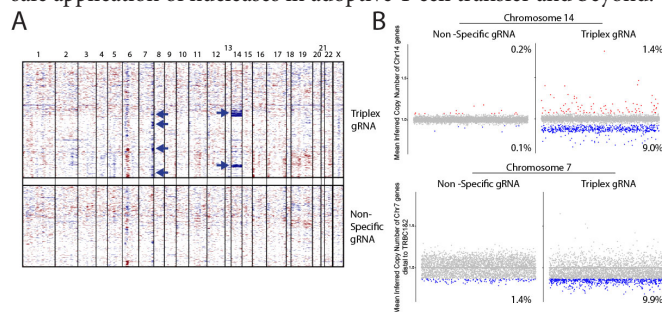
editing, most need to be optimized to the cell type and genomic site, highlighting the diversity of factors contributing to locus-specific genome editing outcomes.

565. Frequent Aneuploidy in Primary Human T Cells Following CRISPR-Cas9 Cleavage

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Multiple clinical trials use site-specific nucleases to disrupt T cell receptor (TCR) genes for allogeneic T cell therapy. In particular, the first U.S. clinical trial using CRISPR-Cas9 entailed the targeted disruption of the TCR chains and programmed cell death protein 1 (PDCD1) in T cells of refractory cancer patients. Here, we applied single-cell RNA sequencing to human T cells, transfected with CRISPR-Cas9, using the same guide RNA sequences. 4 days post-transfection, we found a loss of chromosome 14, harboring the TCR α 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 TCR β 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 post-transfection, 0.93% of T cells still had a chromosome 14 loss. Therefore, aneuploidy and chromosomal truncations are frequent outcomes in human T cells targeted using CRISPR-Cas9 and clinical gRNA sequences. This highlights potential oncogenic risks and underscores the need for mitigation strategies in order to allow the safe application of nucleases in adoptive T cell transfer and beyond.



A. A heat map depicting gene copy numbers inferred from scRNA-seq analysis following treatment with either the non-specific or the combination of TCR α , TRBCs and PDCD1-targeting gRNAs (Triplex). The chromosomes are ordered in columns and the color coding indicates an increase (red) or decrease (blue) in copy number. Blue arrows indicate loss of chromosome 14 or chromosome 7 segments. B. Each dot represents the mean inferred copy number of genes coded on the relevant chromosome in each cell treated with a non-specific gRNA (left) or the combination of TCR α , TRBCs and PDCD1-targeting gRNAs (Triplex, right). For chromosome 7, the mean inferred copy number of genes coded distal to the TRBC1&2 is analyzed. Cells are marked with red and blue dots, corresponding to a gain or a loss, respectively, if their mean inferred gene copy number is >2 standard deviations away from the population's mean. $n=8619$ and 6326 for cells treated with the non-specific gRNA (left) or the combination of TCR α , TRBCs and PDCD1-targeting gRNAs (right), respectively.

566. Development of AAV-GBA1 Gene Replacement Therapy for IV Delivery via Blood Brain Barrier Penetrant AAV Capsid

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The GBA1 gene encodes the lysosomal enzyme β -glucocerebrosidase (GCCase). Homozygous GBA1 loss of function (LOF) results in a deficit of GCCase activity, cellular build-up of glycosphingolipid-substrate, and manifests as Gaucher disease. While enzyme replacement therapy can have significant clinical impact in the periphery, such therapy fails to adequately cross the blood-brain barrier (BBB). Interestingly, LOF mutations in GBA are also one of the greatest genetic risk factors for Parkinson disease and Lewy Body dementia. Adeno-associated viral (AAV)-based gene replacement therapies using a BBB-crossing capsid can be used to achieve sustained correction of lysosomal storage disorders affecting the central nervous system. Here, we describe the design and characterization of multiple transgenes for optimal GBA1 transgene expression in both in vitro and in vivo model. We designed and tested several transgenes for the expression of GCCase. Following expression validation in vitro, we packaged GBA1 transgene into AAV for testing in patient-derived cells and demonstrated target engagement. We observed dose-dependent increases in GCCase activity, and normalization of GBA protein levels and substrate compared to healthy control cells. Using single IV-dosing of our AAV-GBA1 vectors in both wildtype and GBA1 LOF mouse models, we observed widespread CNS biodistribution and increased GCCase activity. In summary, we have designed and characterized novel GBA1 expression, and demonstrated target engagement and delivery therapeutically relevant delivery of GCCase into the CNS in animal models using IV delivery of a BBB-penetrant vector.

567. CRISPR/Cas9-Mediated Base Editing Efficiently Repairs Limb Girdle Muscular Dystrophy Type 2A Causing Mutations

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Limb girdle muscular dystrophies (LGMD) are progressive and rare genetic disorders of which LGMD2A/R1 is the most frequent. LGMD2A is caused by mutations in CAPN3, the gene encoding the cysteine caspase 3 and characterized by progressive muscle weakness and atrophy leading to loss of ambulation. More than 600 mutations in CAPN3 are described with varying prevalence. There is no treatment. CRISPR Cas9-based adenine base editing (ABE) enables the precise targeted conversion of adenine into guanine and is considered a relatively safe tool. We aimed to establish *ex vivo* base editing in G:CtoA:T mutations in CAPN3. We selected three CAPN3 mutations:

CAPN3 c.245 CtoT, CAPN3 c.2243 GtoA, CAPN3 c.1469GtoA based on their relative frequency among LGMD2A patients and availability of primary muscle stem cells (MuSC) or induced pluripotent stem cells (iPSCs) from these patients. Using ABEmax and an NRCH-PAM on the antisense strand, we were able to repair 20% of the CAPN3 c.245CtoT in MuSCs. CAPN3 c.2243GtoA is targeted with a vector encoding ABEmax together with a proper sgRNA after screening with ABEmax, and yielded the same editing efficiency. CAPN3 c.1469GtoA was targeted in iPSCs using the appropriate sgRNA selected after a screen together with ABE7.10 using the NGG PAM. Here, repair efficiency was CAPN3 c.1469GtoA 73%. Of note, the targeting sequence carries an additional adenine at protospacer position 8 within the ABE activity window. In iPSCs, the second adenine was not edited. In conclusion, base editing in CAPN3 mutations is feasible but requires optimization before clinical application becomes reality.

568. Engineering of CRISPR Cas12i to Enable Therapeutic Genome Editing

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CRISPR-Cas type V-I systems (Cas12i) feature diverse small effectors (1033aa -1093aa) capable of processing pre-crRNAs and cleaving target dsDNA without tracrRNA. We apply an unbiased mutational scanning approach to engineer Cas12i2 which shows low activity in mammalian cells and identify single substitutions that significantly improve indel activity. The engineered combination variant, ABR-001, exhibits broad genome editing capability in human cell lines, primary T cells, and hematopoietic stem cells, with both robust efficiency and high specificity. In addition, ABR-001 achieves a high level of genome editing when delivered via AAV vector to HEK293T cells. Finally, we demonstrate that ABR-001 also functions as an efficient chassis for precision editing applications. This work establishes ABR-001 as a versatile, specific and high-performance genetic medicine platform for *ex vivo* and *in vivo* gene therapy.

569. Targeted Epigenetic Editing for X-linked Intellectual Disabilities

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Introduction: Intellectual disability (ID) disorders are a group of neurodevelopmental disorders defined by limitations in adaptive behavior and cognitive abilities. One to three percent of the global population are estimated to have some degree of ID. The X chromosome accounts for about 15 percent of currently known ID-associated genes. X-linked intellectual disabilities (XLID) are caused by mutations in genes on the X chromosome. In female somatic cells, one X chromosome becomes randomly transcriptionally inactive in each cell, ensuring an X-linked gene dosage compensation between females and males, known as X chromosome inactivation (XCI). This causes a mosaic expression of neurons, where half of the cells express the healthy copy of the gene and the other half express the mutant copy. Interestingly, an estimated 15-30% of X-linked genes escape XCI. These

escape genes are associated with specific epigenetic signatures linked to active chromatin. Due to random XCI in females, the presence of the healthy allele in cells presents an opportunity to reactivate the healthy allele from the inactive X chromosome with targeted epigenetic editing.

CRISPRa Library Construction: In this project, we aimed to understand how genes associated with XLID could be regulated. A CRISPR library for nine XLID genes, containing ten sgRNAs per gene, was constructed by cloning individual Weismann CRISPRa sgRNAs, with a single Golden Gate cloning reaction, into U6 expression plasmids and sequence validated. The XLID CRISPRa library was then packaged in lentiviral particles and transduced in HEK293T cells. Target genes were analyzed for gene regulation using RT-qPCR. Following the library screen, target genes and single guide RNAs (sgRNAs) could be selected for reactivation studies.

CASK Gene Studies: As a proof-of-concept for a gene in the XLID CRISPRa library, CASK was selected for further analysis. De novo loss of function mutations in the X-linked gene CASK disrupt brain development and cause severe symptoms such as severe learning impairments, abnormal repetitive behaviors, and recurrent seizures. The initial sgRNA screen was followed by the identification of sgRNA combinations to increase efficacy of gene regulation. Split dCas9 plasmids on the AAV2 backbone expressing C terminal (C-) MPH and N-terminal (N-) VP64-p65-Rta (VPR) tripartite activator construct (MPH/miniVPR) were co-transfected in HEK293T cells with individual CASK sgRNAs. qRT-PCR was used to determine CASK mRNA upregulation. sgRNA 1 significantly upregulated CASK expression when coupled with MPH/miniVPR. To assess if combinations of sgRNA would lead to synergistic effects for gene regulation, combinations of up to three sgRNAs were transfected with MPH/miniVPR in HEK293T cells. sgRNA combinations were semi-randomly selected equally tiled across the promoter. A combination consisting of three sgRNAs resulted in a significant upregulation in CASK expression. All experiments were done in biological triplicates and repeated three independent times using dCas9 no effector that has been co-transfected with the same sgRNA as a negative control.

Conclusion: In summary, using the XLID CRISPRa library, we identified a gene that can be regulated with CRISPR/dCas9. sgRNA for CASK will be further tested for epigenetic editing using a C-TET1CD and N-VPR split dCas9 constructs for targeted demethylation. Future studies will use CASK variant containing iPSC-derived neuronal stem cells and patient-derived cells to assess reactivation and functional recovery.

570. Combining Rational Engineering with High Throughput Screening of Suppressor tRNAs to Enable Translational Readthrough of Premature Termination Codons

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Nonsense mutations causing premature termination codons (PTCs) are responsible for more than 2400 human genetic disorders and account for 20% of disease-causing single-base substitutions in gene coding

regions. Therapeutic strategies that promote read-through of PTCs during translation to produce a full-length protein are a promising approach to treat these genetic diseases. However, current approaches, such as aminoglycoside small molecules, have yielded poor outcomes in clinical trials, possibly due to inefficient read-through or non-specific insertion of any amino acid at the PTC site. To address these limitations, we developed RNAskip™ - a tRNA technology platform that combines rational engineering with high throughput screening of suppressor tRNAs to identify molecules that recognize in-frame PTCs and insert the cognate amino acid, thereby enabling translation of the wild-type, functional protein. RNAskip™ suppressor tRNAs offer several therapeutic advantages. They are specific for each class of nonsense mutation (e.g. Arg>X), versatile in their ability to treat a given class of nonsense mutation across any disease target, and amenable for consistent and controlled cellular expression via AAV delivery. Here, we present the development of a platform for engineering suppressor tRNAs and its specific application towards Arg>X nonsense mutations in Rett syndrome, a rare X-linked neurodevelopmental disorder caused by mutations in methyl-CpG-binding protein 2 (MeCP2). We first developed novel fluorescence and luminescence-based reporter assays that enable several thousand candidate suppressor tRNA molecules to be efficiently screened and rank-ordered for potency. Top candidates were then packaged into viral delivery vectors and tested in mouse neurons containing MeCP2*R168X and *R255X mutations, as well as human iPSC-derived neurons from Rett syndrome patients. Flow cytometry-based screening demonstrated that the engineered tRNAs were highly effective at suppressing disease-causing MeCP2 nonsense mutations, achieving >50% read-through in these disease-relevant pharmacology models. Our results highlight the potential of RNAskip™ as a powerful technology that can be combined with advances in targeted AAV delivery to develop next-generation gene therapies that are broadly applicable for nonsense suppression of genetic diseases.

571. Enhanced Dystrophin Gene Editing and Cas9 Inactivation via AAV Re-Administration in a Mouse Model of Duchenne Muscular Dystrophy

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In vivo gene editing is showing great promise for correcting dystrophin expression and muscle pathophysiology in Duchenne muscular dystrophy (DMD). While robust dystrophin-correction and pathophysiological improvements have been well-documented following local treatment of affected muscles using multiple editing approaches, whole-body treatment via systemic delivery of editing components has proven much less efficient. Some potentially limiting variables include suboptimal availability of editing components within myofibers due to limitations in effective deliverable vector dose, and host immune responses raised against AAV capsids and expressed editing components; both of which may cause loss of edited muscle tissue over time and significantly hamper long-term therapeutic benefits. Strategies aiming to excise mutation containing exons using multiple sgRNAs appear more affected than single sgRNA strategies that target exon splice sites and can likely be partly attributed to asynchronous DNA cleavage between the intended cut sites. As both

sites need to be cleaved simultaneously to remove the intervening mutation-containing genomic segment and correct the dystrophin open reading frame, indel formation following asynchronous cleavage and repair ensures that these sites cannot be cut again and renders the affected genomes uncorrectable using the same target sites. Here we build on our previously described muscle-specific dual-AAV gene editing system to correct dystrophin expression in DMD mice by re-administering a third AAV vector to re-target previously uncorrected genomes and boost overall dystrophin-editing efficiency without affecting already corrected genomes. Concomitantly, the initial AAV vectors were modified to contain the genomic sequences recognized by the re-administered AAV target vector at strategic locations to enable their inactivation and thus limiting prolonged expression of Cas9 in transduced muscles. While some alternative strategies to enable temporal control over delivered editing components trade one foreign, and potentially immunogenic, gene product (e.g., Cas9) for another (e.g., rtTA for Tet-on/off), or sequester Cas9 protein to the cytoplasm (e.g., Cas9-ER[T]); this approach makes maximum use of administered components and aims to leave as small of a footprint as possible via vector self-inactivation. Following administration into immunodeficient Rag1^{-/-};mdx^{4cv} double-mutant mice, this approach simultaneously resulted in significantly enhanced dystrophin-correction levels and robust inactivation of the initial nuclease- and target vectors. Our results suggest that this approach can successfully be employed to improve treatment efficiency while restricting prolonged expression of editing components.

572. Characterization of Type V-K CRISPR-Associated Transposases

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CRISPR-associated transposases (CASTs) are naturally occurring RNA-guided systems capable of creating programmable insertions of large payloads in bacteria. CASTs show promise as a therapeutic solution for providing large insertions into mammalian loci. Three distinct classes of CAST systems have been shown to be functional to date, in which catalytically inactive Type I-B, I-F, or V-K CRISPR nucleases were exapted by distinct families of Tn7-like transposase proteins. The most minimal of these systems, Type V-K, utilizes a sgRNA bound to a Class 2 CRISPR nuclease (Cas12k) to direct three Tn7-like transposase proteins (TnsB, TnsC, and TniQ) to incorporate a DNA cargo sequence downstream of a programmable target site. Through extensive bioinformatic searches of bacterial and metagenomic sequences, we have identified a diverse set of putative Type V-K CAT systems. We characterized these putative systems using a bacterial screen coupled with next-generation sequencing and discovered several which display high integration rates and on-target specificity in bacteria. Focused efforts with these top systems revealed that these systems display a level of modularity and can retain significant activity when individual components are swapped between homologous systems. Additionally, we have identified protein fusions that retain significant activity levels

for a subset of these systems. These results inform our efforts to utilize CAST systems to make programmable DNA insertions in mammalian contexts.

573. Improved Methods and Optimized Design for CRISPR Cas9 and Cas12a Homology-Directed Repair

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CRISPR-Cas proteins - specifically *S.p.* Cas9 and *A.s.* Cas12a - can be used to introduce double-stranded breaks (DSBs) at targeted genomic loci in mammalian cells. Generating edits that mediate specific changes at targeted loci requires homology-directed repair (HDR). To this end a DNA template during repair allows for precise introduction of a desired mutation via the HDR pathway. Here, we describe comprehensive design considerations and optimized methods for highly efficient HDR using single-stranded oligodeoxynucleotide (ssODN) donor templates for several CRISPR-Cas systems including *S.p.* Cas9, *S.p.* Cas9 D10A nickase, and *A.s.* Cas12a delivered as ribonucleoprotein complexes with synthetic guide RNAs. Features relating to guide RNA selection, donor strand preference, and incorporation of blocking mutations in the donor template to prevent re-cleavage were investigated and were implemented in a novel online tool for HDR donor template design. Additionally, we employ chemically modified HDR donor templates in combination with a small molecule to boost HDR efficiency up to 10-fold. These findings allow for highly efficient, precise repair utilizing HDR in multiple mammalian cell lines. Finally, we feature these design rules in a case study to generate multiplex SNP changes via HDR.

574. Abstract Withdrawn

575. Engineering an Improved Protein-Based HDR Enhancer

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One of the most common methods for achieving precise genome editing is by creating a double-strand break using a targeted nuclease and providing a single or double-stranded DNA sequence containing a desired modified sequence as a template for the endogenous cellular double-strand break repair machinery. The cell has two primary methods of repair of double-strand breaks, non-homologous end joining (NHEJ) in which broken DNA ends are directly joined together and homology directed repair (HDR), which encompasses a number of different pathways that use a DNA template to facilitate repair. The efficiency of HDR varies by cell type and target site within the genome. One of the methods for improving rates of HDR is by biasing the cell towards using HDR rather than NHEJ for double-strand break repair. A common way to bias repair toward HDR is by using small molecule inhibitors of a key protein complex in NHEJ called the DNA-dependent protein kinase (DNA-PK), however these inhibitors often have known

off targets including phosphatidylinositol 3-kinase and can cause growth inhibition or toxicity in primary cells. Here we describe the engineering of an improved version of a short peptide-based HDR enhancer targeting the human p53-binding protein 1 (53BP1). 53BP1 is recruited to sites of double-strand breaks and inhibits end resection, a key step in HDR. A small peptide was recently described that blocks 53BP1 recruitment to broken DNA ends and has been shown to enhance HDR with no observable toxicity. The affinity of the original peptide for 53BP1 is weak, and while the peptide has been shown to improve HDR rates when delivered as mRNA, it does not function well as an HDR enhancer when co-delivered as protein alongside Cas9 ribonucleoprotein complexes. We performed a two-hybrid screen of the peptide with a fragment of 53BP1 to examine the effect of all possible single amino acid changes across the peptide sequence on the interaction with 53BP1. We identified a combination of mutations that dramatically improves the affinity of the peptide for 53BP1. Improved affinity peptide variants demonstrated an improved ability to enhance HDR when delivered as protein. This improved peptide targeting 53BP1 is a valuable new tool to facilitate genome editing.

576. A Systemic Optimization of Non-Viral Engineering of Human B Cells

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B cells are able to differentiate into long-lived plasma cells, producing large quantities of antibody for many years and even decades. This makes B cells attractive candidates for novel engineered cellular therapies. Recombinant adeno-associated viral vectors (rAAV) have been extensively used for delivering a homology directed repair (HDR) DNA template for CRISPR/Cas9 mediated transgene insertion in immune cells, including B cells¹⁻³. A major drawback of rAAV is the small cargo capacity, which hinders large transgene DNA template delivery (Figure 1). In addition, the high cost and long turnaround time of viral vector production highlights an urgent need for non-viral approaches to B cell engineering. Plasmid vectors accommodate larger cargo, cost less than virus, and can be rapidly manufactured, making them an attractive platform for DNA donor template delivery. Here, we demonstrate a systematic optimization of a plasmid-based approach for B cell engineering. First, we tested whether B cells can tolerate a GFP-expressing plasmid transfection. We observed up to 80% GFP-positive B cells with 45% viability, suggesting B cells can tolerate plasmid transfection, albeit with reduced viability. NanoplasmidTM (NP) is a compact circular plasmid with a minimized, 500bp backbone lacking traditional bacterial and antibiotic-resistant genes, reducing cellular toxicity and inflammation of the plasmid-transfected cells. We constructed optimized NP vectors carrying a promoterless GFP reporter to compare HDR, hybrid HDR_homology mediated end joining (HDR_HMEJ), or HMEJ integration mechanisms. The HDR and the hybrid mechanisms utilized 1000-bp homology arms (HAs), while the HMEJ mechanism utilized 48-bp HAs⁴. Previously described⁴, synthetic gRNA target sequence was incorporated on either side of the homology arms of the HMEJ constructs to liberate a linear integration template, which is required for the HMEJ mechanism. We observed similar integration efficiency across repair mechanisms

(25-30% GFP+). As seen previously, B-cell health was affected by the plasmid approach (45-50% viability). We hypothesized that the presence of hypomethylated CpG oligonucleotide used for B cell stimulation may sensitize B-cells to foreign nucleic acid via TLR9 ligation, and that the use of a non-TLR9 activation would improve B-cell health during non-viral engineering. To test this hypothesis, we acquired a proprietary B-cell expansion media (Immunocult) that does not activate TLR9 pathway for B-cell culture. Moreover, since HDR and HMEJ are highly active during the late G1_S_G2 phase^{5,6}, we performed a cell cycle analysis to determine optimal engineering timing post activation. We found that B cells cultured in Immunocult for 48-, 60- and 72-hour showed the highest prevalence of G1_S_G2 phases, in which HDR and HMEJ are highly active. Next, we tested engineering B cells at these times and observed efficient engineering frequencies (15-20%). Interestingly, unlike CpG containing media, B-cell health in the Immunocult media was not drastically affected by plasmid-engineering. This indicates that the non-TLR9 activation media ameliorated low B-cell health post non-viral engineering. This study demonstrates that plasmid can be used as a non-viral approach for B cell engineering, and a non-TLR9 activation circumvents the issue of plasmid inducing low B-cell health.

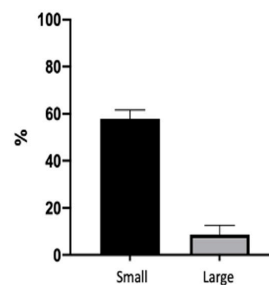


Figure 1. A large construct with reduced homology arms resulted in drastically decreased efficiency of a viral-based engineering approach. The homology arms (HAs) are reduced in expense of a larger transgene construct in order to package into a small rAAV vector. The small transgene insertion showed significantly higher engineering frequency (58%) than the larger construct (5%).

577. A Novel Cas9 System with Robust Genome and Epigenome Editing in Human Cells

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The discovery of CRISPR-Cas9 systems has revolutionized biotechnology and has considerable potential for therapeutic genome and epigenome editing. However, there are a limited number of Cas9 effectors that function robustly in human cells. The relatively limited number of systems available has in turn limited the options for avoiding pre-existing immunity, targeting any sites in the genome, enabling diverse delivery vehicles, allowing concurrent multiplexing of genome editing applications and modalities, and overcoming other hurdles in the translational pathway. To increase the number of available systems,

we have predicted a set of 47 Cas9 proteins and cognate sgRNA sequences originating in organisms that have not been identified as pathogenic or commensal species in humans and tested their activity in human cells. To first characterize the DNA-targeting capabilities of these systems, we generated constructs to test epigenetic repression by mutating the nuclease active sites and fusing a KRAB domain to the C-terminus. We then designed pools of sgRNAs targeting the HBE promoter, and tested activity by transducing K562 cells containing an mCherry reporter for HBE expression. This revealed a previously uncharacterized Cas9 with potent repressive activity against HBE when measured either by flow cytometry or RT-qPCR. Further, re-instating the nuclease active sites and targeting the TRAC gene in HEK293T cells showed editing rates of 40%. Taken together, this suggests that we have characterized a new Cas9 that can robustly function in human cells. Ongoing studies include testing this novel system in primary cells with alternative delivery vehicles relevant to gene and cell therapy. With a coding sequence of 3.3 kb and an origin in an organism not known to infect humans, this new Cas9 is a useful addition to the panel of Cas9 effectors for genome and epigenome editing.

578. Reversible Epigenome Editing of *PCSK9* as a Therapeutic Strategy

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Among the best-established causal risk factors for cardiovascular disease is the blood concentration of low-density lipoprotein cholesterol (LDL-C). Proprotein convertase subtilisin/kexin type 9 (*PCSK9*), an antagonist to the LDL receptor, has emerged as a promising therapeutic target for the prevention of coronary heart disease. Current *PCSK9* inhibitors are administered via subcutaneous injection and their effects are short-lived. An alternative “one-and-done” strategy using genome editing to disrupt *PCSK9* at the DNA level has been demonstrated in preclinical animal models, including non-human primates. However, concerns about the permanence and irreversibility of the genomic changes have been raised and might limit the acceptance of the therapies. Recently, a set of CRISPR-based epigenome editing tools, CRISPRoff and CRISPRon, were reported to regulate gene expression via site-directed methylation and demethylation of gene promoters, respectively. We hypothesized that these epigenome editing tools could durably and reversibly induce methylation changes in the *PCSK9* promoter and thereby modulate its expression. We first screened CRISPRoff guide RNAs (gRNAs) targeting the *PCSK9* promoter, individually and in dual combinations, for their ability to reduce *PCSK9* expression in the human HuH-7 hepatoma cell line. We then performed long-term experiments with the lead candidate gRNAs. We found that these CRISPRoff gRNAs induced profound increases in methylation at CpG dinucleotides in the *PCSK9* promoter, with up to 80% decreases in *PCSK9* expression. These methylation increases and gene expression decreases have endured through >56 cell divisions so far, with only mild attenuation over time. Using the same gRNAs with CRISPRon in cells previously treated with CRISPRoff,

we observed moderate decreases in methylation and increases in *PCSK9* expression. Having established these effects *in vitro*, we are similarly assessing the durability and reversibility of epigenome editing using the lead gRNAs in a *PCSK9*-humanized mouse model. Overall, this work provides a proof of concept of precise gene regulation via methylation and demethylation and suggests a potential new therapeutic approach for protection against cardiovascular disease.

579. Modeling and Correction of *RAG2* Severe Combined Immunodeficiency by CRISPR-Cas9 and rAAV6 in Human CD34+ HSPCs

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Severe combined immunodeficiencies (SCIDs) are a set of life threatening genetic diseases in which patients are born with mutations in one of more than twenty known genes and are unable to develop functional immune systems. While allogeneic bone marrow transplantation can be curative for these diseases, there remain significant limitations to this approach. In contrast to using viral vectors to deliver transgenes in an uncontrolled fashion, we are working towards developing CRISPR genome editing to correct the *RAG2* disease-causing mutations by precisely modifying the genome. Here we first developed a *RAG2*-SCID disease model using CRISPR-Cas9 and rAAV6 gene-editing through biallelic knock-out in healthy donor (HD)-derived CD34+ HSPCs. Additionally, we established a knock-in/knock-out (KI-KO) strategy to develop a proof-of-concept gene correction in HD CD34+ HSPCs. Lastly, we show successful gene correction of *RAG2*-SCID patient-derived CD34+ HSPCs which developed into CD3+ T cells with diverse TCR repertoires. Our system outlines an approach for the study of human lymphopoiesis and will allow researchers to determine the optimal configuration for CRISPR-Cas9 gene correction for SCIDs and additional recessive primary immunodeficiency and blood disorders.

580. Screening Cas13d Guides in Angelman Syndrome

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CRISPR/Cas13 can target RNA directly which makes an attractive therapy tool for a variety of diseases, including genetic disorders affecting the brain. One of them is Angelman Syndrome (AS). It arises from the genetic loss of the maternal *UBE3A* gene in brain neurons, causing severe communication and motor deficits, seizures, cognitive and physical impairments, and disrupted sleep. Due to a brain-specific long non-coding RNA transcript that is called the *UBE3A* antisense (*UBE3A-ATS*), the paternal *UBE3A* remains silenced. Inactivation of the *UBE3A-ATS* RNA by RfxCas13d could therefore restore *UBE3A* expression in the brain. Building on our previous work with CRISPR/Cas13 in AS mice, we are screening for guides in the rat model of Angelman Syndrome and in humans. Preliminary data using HEK293T cells that

express the target regions along with GFP will be presented. The next step will be transduction experiments with the best guides in AS rat primary neurons and in Angelman patient-derived iPSCs.

581. Correction of DMD Proximal Hotspot Mutations Using Homology-Independent Targeted Integration

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Duchennemuscular dystrophy (DMD) is fatal muscle disease that occurs in ~1:5000 newborns males. DMD manifests early in childhood as difficulty walking, rapidly progresses to loss of ambulation in adolescence, and leads to death from cardiac or respiratory failure early in adulthood. DMD is caused by absence of the muscle-specific structural protein dystrophin resulting in poor muscle cell integrity. Individuals with DMD experience a decline in muscle mass, strength, cardiac performance, and ventilation over time due to chronic cycles of muscle degeneration. Adeno-associated virus (AAV) vectorized gene editing with CRISPR-Cas9 has recently emerged and shown great potential for restoring partially functional mutant dystrophin isoforms in animal models of DMD. However, these mutant isoforms have unknown clinical benefits. Moreover, most DMD patients carry single or multiple exon deletions and thus knock-in of the missing exon to restore full-length dystrophin expression is highly desirable. However, knock-in gene editing for DMD is limited by the low efficiency of homology-mediated DNA repair within non-dividing cells like muscle. A recently described CRISPR-Cas9-based technology called homology-independent targeted integration (HITI) drives knock-in through the non-homologous end joining DNA repair pathway, which is highly active in muscle. Here, we designed a HITI gene editing approach to replace exons 1 - 19 with a single "mega-exon" of ~2.5 kb comprised of the coding sequence of exons 1 - 19 without the intervening introns and driven by an MHCK7 promoter. Using a CRISPR guide RNA that targets a conserved region of intron 19, we tested HITI replacement of exons 1 - 19 in a mouse model of DMD carrying an exon 2 duplication. We found that we could induce knock-in of the ~2.5 kb mega-exon into intron 19 with the highest efficiency of up to ~0.8% in the heart. Despite this modest gene editing efficiency, up to 12% of heart dystrophin transcripts were derived from the knock-in and up to 9% normal levels of dystrophin protein were restored in the hearts compared to healthy mice. This work provides proof of concept data in support of a HITI gene editing approach to replace exons 1 - 19 that could benefit ~25% of DMD patients with mutations that lie upstream of intron 19. Future work will focus on improving efficiency and assessing safety of HITI gene editing to restore full-length dystrophin.

582. LgRNA as Next Generation Guide RNAs for Precise Gene Editing

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Among various subtypes of CRISPR-CAS systems, type II has attracted prominent interest in their potential therapeutic applications, particularly Cas9. Natural CRISPR-Cas9 is a dual guide system comprising a crRNA and a tracrRNA. Gene editing efficiencies of

dual gRNAs are typically lower in eukaryotic cells than an artificial sgRNA, and potential future drug products of dual gRNAs are complicated in their formulations. sgRNA is a single molecule guide RNA comprising a truncated crRNA and tracrRNA joined together by a tetraloop (GAAA) [1]. Development of the dual CRISPR-CAS9/sgRNA system has revolutionized many aspects of biological research with unlimited therapeutic potential. It has long been recognized that chemically modified guide RNAs exceed unmodified sgRNAs in its many properties such as increased cellular stability and eliminated immunogenicity. A primary challenge towards manufacturing chemically modified sgRNAs for SpCas9 gene editing therapies is due to their long length, which is typically 100 nt, and is even longer for effectors such as prime editors, and many others. Standard nucleic acid synthesis on solid supports suffers from poor crude yields (typically 10%-25% depending on the sequence and scale) and low overall product purity (60%-80% by HPLC) [2], while liquid phase synthesis still has a long way to go even for much smaller RNAs such as siRNAs. The content of full-length products of synthetic sgRNAs are constantly around 40% as shown by HPLC in combination with MS analysis [3]. Synthetic errors at the critical spacer segment cause extra guide-dependent off-target cleavages, which unfortunately increase as the synthesis progresses towards the 5'-end of the long strand. Guide RNAs direct CRISPR CAS proteins to their action sites in targeted genes and are critical in determining both efficacy of on-target gene editing and the extent of off-target effects, and thus precise gene editing. Guide RNAs of better quality is needed even for better understanding the existing data and also the development of RNP-based clinical products. We and others reported a chemically ligated guide RNA (LgRNA), comprising chemically modified RNA segments and non-nucleotide linkers [4-6]. LgRNAs are guide RNAs of next generation, which are not only cost-effective to manufacture, but also give access to high quality validated full-length products with much fewer synthetic errors at the critical spacer segment than sgRNA and enable cost-effective various chemical modifications for better efficacy and selectivity, stability, targeted delivery by molecular tagging, and so forth. References: 1. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012 Aug 17;337(6096):816-21. 2. Rees HA, Minella AC, Burnett CA, Komor AC, Gaudelli NM. CRISPR-derived genome editing therapies: Progress from bench to bedside. *Mol Ther*. 2021 Nov 3;29(11):3125-3139. 3. Superior Quality and Editing Performance of Synthetic sgRNAs. *Synthego Application Note*. 4. Zhong MH. Chemically ligated RNAs for CRISPR/Cas9-LgRNA complexes as antiviral therapeutic agent. US Patent 10059940B2. 5. He K, Chou ET, Begay S, Anderson EM, van Brabant Smith A. Conjugation and Evaluation of Triazole-Linked Single Guide RNA for CRISPR-Cas9 Gene Editing. *Chembiochem*. 2016 Oct 4;17(19):1809-1812. 6. Taemaitree L, Shivalingam A, El-Sagheer AH, Brown T. An artificial triazole backbone linkage provides a split-and-click strategy to bioactive chemically modified CRISPR sgRNA. *Nat Commun*. 2019 Apr 8;10(1):1610.

583. A Consensus Model of Homology-Directed Repair Initiated by CRISPR/Cas Activity

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The mechanism of action of ssODN-directed gene editing has been a topic of discussion within the field of CRISPR gene editing since its inception. Multiple comparable, but distinct, pathways have been discovered for DNA repair both with and without a repair template oligonucleotide. We have previously described the ExACT pathway for oligo-driven DNA repair, which consisted of a two-step DNA synthesis-driven repair catalyzed by the simultaneous binding of the repair oligonucleotide (ssODN) upstream and downstream of the double-strand break. In order to better elucidate the mechanism of ExACT-based repair, we have challenged the assumptions of the pathway with those outlines in other similar non-ssODN-based DNA repair mechanisms. This more comprehensive iteration of the ExACT pathway better described the many different ways where DNA repair can occur in the presence of a repair oligonucleotide after CRISPR cleavage, as well as how these previously distinct pathways can overlap and lead to even more unique repair outcomes.

584. Massively Parallel gRNA Screening and Machine Learning to Enable Efficient and Selective RNA Editing with Endogenous ADAR

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We present data to support the use of high-throughput screening and machine learning to advance ADAR mediated RNA editing as a therapeutic modality. Our approach utilizes the natural enzyme ADAR to correct G->A mutations, control splicing, or modulate protein expression and function. Delivery of a guide RNA (gRNA) with full or partial complementarity to a target RNA can redirect ADAR's deaminase activity, converting the target adenosine to inosine, which is read by cellular machinery as guanosine. One major challenge is that ADAR does not naturally act on all RNA sequences with equal efficiency and is prone to promiscuously engage and edit multiple adenosines within dsRNA structures. A small fraction of natural ADAR substrates are edited with high selectivity and efficiency to recode at the amino acid level or alter RNA splicing. These substrates lack perfect complementarity within the dsRNA duplex and contain multiple secondary structures that promote a high degree of specificity. Akin to natural substrates, we hypothesized that the use of optimal secondary structures within the gRNA/target RNA complex will be necessary to achieve the high level of efficient and selective editing needed for a therapeutic intervention. To test this hypothesis, we built a massively parallel screening platform that assesses up to 500,000 structurally unique gRNAs against any clinically relevant target sequence. Data generated from our high-throughput screening platform for seven clinically relevant targets highlights the impact that secondary structure can have on the efficiency and selectivity of gRNAs. Furthermore, a panel of gRNA designs that enable potent and highly specific editing for each target, including those with a 5' guanosine neighbor, which ADAR strongly disfavors, were identified (Figure

1). Yet 500,000 gRNAs represents only a tiny fraction of all possible gRNAs. To explore the secondary landscape even further, the massive data sets were used for machine learning and a generative model was built to identify gRNAs predicted to be even more potent and selective. Empirical testing confirmed that the machine learning generated gRNA libraries significantly increased on-target editing and decreased off-target editing compared with the original libraries. In summary, the data highlights the use of massively parallel high throughput gRNA screening combined with deep learning as a powerful approach to iterative gRNA discovery and optimization supporting the clinical application of ADAR mediated RNA editing.

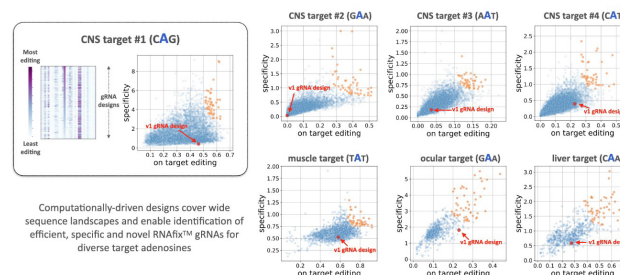


Figure 1 RNAfix™ HTS explores a diverse secondary structure landscape, enabling discovery of gRNAs for optimal RNA editing efficiency and specificity of disease-relevant targets with diverse sequence contexts. Highlighted are the editing efficiency and selectivity of RNAfix™ HTS libraries targeting seven disease-relevant targets. For each target, a panel of lead gRNA designs (orange) were identified that are superior to the canonical A-C mismatch design (red).

Oligonucleotide Therapeutics II

585. Pharmacokinetics, Biodistribution, and CNS Penetration of a PATROL™- Enabled Investigational Genetic Therapy for Myotonic Dystrophy-Type 1 Following Systemic Administration in Mice

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Initial studies using a PATROL™ platform-enabled peptide nucleic acid (PNA) pharmacophore combined with a novel delivery technology in transgenic animal models demonstrated pharmacologic activity in both brain and muscle following systemic administration. Patients with DM1 suffer from cognitive deficits and muscle pathology caused by a trinucleotide expansion in the *DMPK* gene. An exploratory radiolabeled biodistribution study of the delivery module administered intravenously in nonhuman primates showed distribution to brain, muscle, and heart, the major organs affected in DM1. A single dose of our PATROL™ DM1 selected candidate was administered subcutaneously (10 or 30 mg/kg) or intravenously (30 mg/kg) in BALB/c mice to evaluate the pharmacokinetics and biodistribution and examine central nervous

system (CNS) penetration. Blood and tissues were collected over a time course ranging from 0.5 hours to 28 days. Serum and tissue compound concentrations were quantified by liquid chromatography-tandem mass spectrometry. Pharmacokinetic parameters were estimated using Phoenix WinNonLin. Following subcutaneous administration, compound maximal plasma concentration (C_{max}) and area under the plasma concentration-time curve (AUC_{0-t}) were approximately dose-proportional; bioavailability was ~46%. Plasma C_{max} and AUC_{0-t} after intravenous dosing were 2490 ng/mL and 8655 hours*ng/mL, respectively. Compound plasma total body clearance and volume of distribution following subcutaneous or intravenous administration were ≥ 4 -fold greater than mouse glomerular filtration rate (GFR) and ~110-fold greater than mouse blood volume, respectively, suggesting primarily GFR clearance and wide tissue distribution. Data will be presented supporting distribution of our investigational PNA therapy targeting DMPK pre-mRNA conjugated to a novel delivery technology to the brain and throughout the body following systemic administration in BALB/c mice, consistent with previously observed CNS pharmacologic activity.

586. Splice-Switching Antisense Oligonucleotides for the Treatment of Cystic Fibrosis Caused by Class I CFTR Mutations

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Cystic Fibrosis (CF) is an autosomal recessive disease affecting more than 70,000 people world-wide. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *CFTR* encodes an anion channel that, when mutated, results in the buildup of mucus in tissues such as the lung and pancreas, disrupting proper organ function. Class I *CFTR* mutations introduce premature termination codons (PTCs) that result in nonsense-mediated decay of the mRNA and the production of a truncated protein from any translated mRNA. These mutations account for ~11% of CF patients and are associated with severe forms of the disease as they usually result in low *CFTR* expression. Because of the low *CFTR* expression, current therapeutics for CF, which work to increase protein function, are not effective in patients with these types of mutations. One approach that holds promise as a therapeutic strategy involves antisense technology. Here, we tested splice-switching antisense oligonucleotides (ASOs) that modify gene expression by directly modulating pre-mRNA splicing. We examined the effects of ASOs designed to induce skipping of exons that contain PTCs introduced by nonsense mutations, thereby stabilizing *CFTR* mRNA and correcting the open reading frame to allow for translation to the natural C-terminus. We show that skipping of exon 23 produces a *CFTR* protein with retained function when co-treated with FDA-approved *CFTR* modulators. Further, ASO treatment in primary CF-patient derived bronchial epithelial cells successfully induces exon 23 skipping and recovers *CFTR* function disrupted by the common *CFTR-W1282X* mutation that resides in exon 23. These results provide substantial evidence for the use of ASOs in treating CF patients with *CFTR* class I mutations that result in truncations of the *CFTR* protein, who currently do not have available treatment options.

On a broader scale, our results further validate ASO-mediated exon skipping strategies for open reading frame correction disrupted by mutations in other genetic diseases.

587. Production of *In Vitro* Transcribed mRNA Using Synthetic, Enzymatically Produced Linear DNA

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The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA and viral vectors for use in gene therapy and vaccines. 4basebio has developed a proprietary, scalable synthesis process for the production of linear closed DNA constructs via our Trueprime™ amplification technology. The osDNA™ 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, allowing for large scale production of linear DNA with high yield and purity in less than a week. Here, we compared the synthesis of *in vitro* transcribed (IVT) mRNA using osDNA™ versus linearised plasmid DNA, and achieved equivalent mRNA yields and capping efficiency with a range of caps. Proinflammatory cytokine/chemokine levels *in vitro* were comparable to mRNA produced from linearised plasmid, as was 3' heterogeneity of the transcripts. Luciferase expression across a range of cell lines (HEK293, C2C12, primary human chondrocytes and primary human hepatocytes) was comparable to Luciferase mRNA produced from linearised plasmid. Moreover, we were able to generate linear DNA sequences with polyA tails ranging from 30-120 bp in length. We have demonstrated that osDNA™ templates can be used for the production of IVT mRNA, which could greatly accelerate gene therapy therapeutic development. 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.

588. Oligonucleotide-Based Telomere Disruptor for Prostate Cancer Immunotherapy

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Telomerase (TERT) is an enzyme expressed in ~90% of human cancers including prostate cancers, which is critical for tumorigenesis and tumor survival. Recent studies demonstrated that nucleoside analogs such as 6-thio-2'-deoxyguanosine (6tdG) can induce telomere uncapping and induce accelerated cancer cell senescence compared to earlier telomerase inhibitors. The telomere-associated DNA released from dying cancer cells was also shown to be immunogenic. Unfortunately, 6tdG can also interfere with telomerase-dependent T cell proliferation and thus limit the generation of antitumor immune responses and long-term immunity. To improve the safety and efficacy of telomerase inhibition, we developed a novel strategy for targeted delivery of 6tdG into cancer cells and myeloid immune cells, but not T cells, using a synthetic oligodeoxynucleotides incorporating 6tdG molecules (6tdGOs). The 6tdGOs showed comparable or better *in*

in vitro cytotoxicity than 6tdG alone against a NCI-66 panel of human and mouse cancer cells. Prostate cancer cells showed high sensitivity to killing by 6tdGOs at low nanomolar IC_{50} values: 29 and 83 nM for human PC3 and DU145 cells, and 21, 63, 61 nM for mouse RM9, Tramp-C1 and Tramp-C2 cancer cells, respectively. In contrast to 6tdG alone, 6tdGOs have not affected proliferation of activated T cells. The cytotoxic effects of 6tdGOs correlated with the levels of telomerase activity in prostate cancer cells or in T lymphocytes. As verified using human, primary and TERT-transformed epithelial cells, 6tdGOs showed selective cytotoxicity only against TERT-positive cells and not against untransformed cells. To assess whether 6tdGOs induce immunogenic cancer cell death, we incubated mouse dendritic cells (DCs) with 6tdGO-pretreated or untreated RM9 cells. The 6tdGO-treated RM9 cells were significantly more effective in stimulating DCs to produce type I IFNs than 6tdG alone or untreated cancer cells. Such immunogenic effects likely rely on STING signaling in target immune cells. The activation of Interferon Regulatory Factor (IRF) promoter fragment in reporter immune cells cocultured with dying 6tdGO-pretreated cancer cells required was abrogated in STINGKO reporter cells. Finally, 6tdGOs demonstrated potent antitumor immune responses against syngeneic tumor models in mice. Repeated systemic administration of 6tdGO arrested growth of aggressive, TERT^{hi} *Ras/Myc*-driven (RM9) prostate tumors significantly more effectively than 6tdG alone or in combination with CpG oligonucleotide. The antitumor effects of 6tdGO correlated with DC activation and the increased CD8 to regulatory T cell ratio in tumors. The immune-mediated effects of 6tdGOs were further verified using dual prostate tumor models in mice. Local, intratumoral injection resulted in T cell infiltration and inhibited growth of non-injected, contralateral RM9 tumors. Similar abscopal effect were observed in mice bearing the Tramp-C2 tumors. Our results suggest that 6tdGO strategy generates a double whammy effect resulting from the direct cytotoxicity to telomerase-positive cancer cells and the induction of potent cGAS/STING-driven antitumor immune responses.

589. Efficient In Vivo GalNAc Conjugated siRNA-Mediated Knockdown of Human Hepatocyte Complement C5 in Liver-Humanized FRG Mice

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Introduction: Preclinical models to test the efficacy and toxicity of non-viral liver-targeted therapeutics are currently lacking. We therefore evaluated the utility of FRG[®] liver-humanized mice for GalNAc-siRNA-mediated gene knockdown. For these studies, complement C5 was targeted as it is highly expressed in hepatocytes and activated by all complement pathways. Inappropriate regulation of complement contributes to the pathophysiology of numerous human diseases and has an increasingly recognized role in liver disease, including non-alcoholic steatohepatitis and hepatocellular carcinoma. **Methods/Results:** Mouse and human plasma C5 protein levels were measured in our non-humanized and liver-humanized mouse models, respectively, using species-specific ELISA. Both the FRG and FRGN strains express functional mouse C5 whereas our recently developed congenic

FRGN strain that is homozygous for the NOD Hc0 mutation does not (hereafter referred to as FRGN C5KO). Accordingly, plasma from FRGN C5KO mice lacks classical complement activity in a sensitized sheep red blood cell hemolytic assay. In liver-humanized FRG, FRGN and FRGN C5KO strains, human plasma C5 protein is present at levels ~10-fold above that of mouse plasma C5 protein in non-humanized mouse complement-sufficient strains. Consistent with this large difference in C5 protein levels, hemolytic activity in the plasma from liver-humanized mice, including liver-humanized FRGN C5KO mice, is ~28 fold higher than in plasma from non-humanized, complement-sufficient mice. These findings suggest that even in complement-sufficient mouse hosts, the overwhelming majority of complement activity in liver-humanized mice is of human origin. To begin to assess whether efficient *in vivo* delivery of GalNAc-conjugated molecules to human hepatocytes in xenografted mice is possible, the expression of the asialoglycoprotein receptor (ASGPR1) in primary human donor cells prior to transplant and following stable liver engraftment in FRG strains was evaluated. Flow cytometry revealed that cell surface ASGPR1 expression is present in ~80% of the primary cryopreserved hepatocytes from each of our donors tested. Importantly, ≥95% of engrafted donor human hepatocytes express high levels of cell surface ASGPR1 for at least 150 days after transplant. To knockdown human C5 expression, liver-humanized mice were subcutaneously dosed with 5-10mg/kg of a C5-targeted siRNA containing a triantennary GalNAc conjugate and enhanced stabilization chemistry. A single dose of 5mg/kg or 10mg/kg of C5 siRNA was sufficient to knockdown human plasma C5 protein levels ~70% by day 10 post-injection. In 5 of 7 of singly dosed animals, knockdown of human C5 protein in plasma was maintained for at least 25 days. We are currently evaluating whether multiple doses of the siRNA in liver-humanized mice is toxic to human hepatocytes by measuring human-specific ALT1 expression using a custom ELISA that we developed. **Conclusion:** The high levels of expression of ASGPR1 in human hepatocytes in chimeric livers combined with the efficient and durable knockdown of human C5 achieved with a single dose of galNAc siRNA demonstrate that FRG[®] liver-humanized mice are an ideal preclinical model for evaluating the targeting efficiency and toxicity of GalNAc-conjugated therapeutics.

590. Functional Transfer of Engineered MicroRNAs Through Extracellular Vesicles: An In Vitro Proof-of-Mechanism Study in Huntington's Disease Models

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Huntington's disease (HD) is a devastating neurodegenerative disorder triggered by a single mutation in the Huntingtin (HTT) gene. The mutant huntingtin protein misfolds and aggregates, causing cellular toxicity which culminates, over the years, in permanent neuronal loss. As therapeutic strategies for disease modification in HD, several HTT targeting microRNA (miRNA)-based therapies are currently in development, including AAV5-miHTT. Considering the extent

of neuropathology in HD, widespread biodistribution of engineered miRNAs is a key aspect to increase efficacy. Extracellular vesicles (EVs) carry a wide variety of molecules, including proteins and endogenous miRNAs. We have previously shown that miHTT is present in EVs, but the extent to which engineered miRNAs are loaded into EVs and their contribution as therapeutic spreading agents to modulate gene expression in far distant cells, is not well understood. To investigate the loading, secretion, uptake and therapeutic properties of our engineered miRNA miHTT through EVs, we developed an *in vitro* non-contacting co-culture transwell system. To exclude possible AAV-based vector genome cross-contamination amongst donor and recipient cells, we generated a stable cell line overexpressing miHTT (HEK293-miHTT). HEK293-miHTT cells were used as donor cells in a series of experiments in which different human cell lines, not expressing miHTT, were used as recipient cells. A time-response cell-to-cell transwell culture was first performed using HEK-293T cells as recipient cells, showing an increase in miHTT levels in receiver cells after 4, 6 and 9 days of co-culture, suggesting that miHTT can be secreted and taken up by recipient cells. To evaluate the efficacy of the uptaken miHTT, we measured target engagement (HTT mRNA levels). As expected, donor cells showed endogenous HTT mRNA lowering at all timepoints investigated, while in recipient cells, HTT lowering was observed after 6 and 9 days of co-culture. In the other cell models investigated, we also observed an increase of therapeutic miHTT molecules in the recipient cells in comparison to controls, which were sufficient to induce a robust reduction in the endogenous HTT mRNA levels, suggesting that there is a mechanism of transfer that may involve the use of EVs. To confirm this hypothesis, we analyzed the presence of miHTT in EV-enriched fractions isolated from the cell culture media, confirming the presence of miHTT in EVs. Altogether, our findings indicate that our engineered miRNA, miHTT, can be loaded, secreted and transported, presumably by EVs, uptaken by recipient cells, and able to maintain its therapeutic properties - a relevant mechanism for intercellular transfer of miRNAs, and supporting the sustained therapeutic spread of AAV5-miHTT upon one-time administration.

591. Alternative Elution Buffers for Recovery of mRNA from the POROS OligodT(25) Affinity Resin

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POROSTM Oligo dT(25) resin was developed to address the selectivity and capacity requirements for the large-scale manufacturing of mRNA used in vaccine and gene therapy applications. The resin consists of a 50-micron cross-linked poly(styrene-divinylbenzene) POROS bead that is functionalized with a poly(dT)25mer with a proprietary linker. The Oligo dT(25) resin enables the purification of mRNA from a crude transcription mixture in a single capture step. This simplified workflow maximizes the removal of plasmid DNA and other IVT components without the need for subsequent polish steps. mRNA is typically bound to the Oligo dT resin with high salt and neutral pH buffer and eluted from the column using water. Although water worked well for most mRNA constructs, high recoveries are not always achieved and there is a need to identify alternative elution buffers for the POROS Oligo dT(25) resin. In this study, we identified alternative elution buffers by execution of a Design of Experiments (DoE) by evaluation of a series of

elution buffers using a high throughput screening method. The elution buffers that were tested included Citrate, Tris, and EDTA at various pH conditions and concentrations. The buffers that showed similar or improved recoveries to water were verified using a 1 mL Oligo dT(25) column. The results from this study will enable a broader understanding of what elution buffers can be used for the optimal recovery of mRNA from the POROS Oligo dT resin.

592. Antisense Oligonucleotide-Mediated Exon 27 Skipping of Dysferlin for the Treatment of Dysferlinopathy

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Introduction: Dysferlinopathies are a family of disabling muscular dystrophies caused by mutations in the *DYSF* gene, which encodes dysferlin protein. Dysferlin is a key player in sarcolemmal homeostasis and plasma-membrane resealing. It is a modular protein with multiple calcium-dependent C2 lipid binding (C2) domains. Based on reports of patients with very mild and late-onset phenotypes, dysferlin lacking one or more of the repetitive C2 domains retain (at least partial) functionality. This provides the rationale for the development of exon skipping therapies for dysferlinopathies. Exon skipping therapy uses DNA-like synthetic molecules called antisense oligonucleotides (AONs) to modulate splicing, allowing exons harboring or near genetic mutations to be removed and the open reading frame corrected. Previous investigations have suggested that skipping single or multiple exons could be a promising therapeutic approach for dysferlinopathies. In this study, we are developing exon 27 skipping therapy for patients with dysferlinopathies. **Methods:** The patient-derived cells' mutation status was assessed using Sanger sequencing, reverse-transcriptase PCR (RT-PCR), and western blotting. We then utilized a computational tool to design three AONs targeting exon 27 of *DYSF* to be tested in a patient muscle cell line with a splice site mutation that leads to exon 26 skipping. We then evaluated the efficiency of the AONs in immortalized myoblasts and myotubes. Exon skipping efficiency of the AONs and restoration of in-frame gene transcripts with a deletion of exons 26-27 were evaluated by RT-PCR. We analyzed the rescue of dysferlin protein using western blotting. Finally, we assessed the membrane resealing ability of the AON-treated patient cells using a laser-wounding assay. **Results:** Analysis of the mutation status of the patient-derived cells suggested that exon skipping of exon 27 would restore the reading frame. As measured by RT-PCR, all three AONs efficiently skipped *DYSF* exon 27 at 10 μ M dose, with a skipping efficiency of up to 90% in both myoblasts and myotubes. Western blotting analysis of the AON-treated myoblasts and myotubes revealed that the treatment with AONs rescued over 40% of normal dysferlin expression levels in these cells. In addition, membrane wounding assay using a two-photon laser showed functional recovery of membrane wounding in the myotubes with exon 27 skipping. **Conclusions:** This study showed that skipping of *DYSF* exon 27 and restoration of in-frame transcripts with exons 26-27 deletion rescues expression of dysferlin and membrane resealing ability in patient-derived cells. This study paves the way for

future *in vivo* work that would help establish a foundation for the future clinical implementation of antisense-mediated exon skipping for dysferlinopathy.

593. Targeted Exon Skipping of NF1 Exon 52 as a Mutation-Specific Therapeutic for Neurofibromatosis Type I

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Modification of pre-mRNA splicing using specific antisense oligonucleotides (ASOs) can be exploited to skip one or more exons carrying pathogenic DNA sequence variants. We investigated the feasibility of utilizing an exon skipping approach as a genotype-dependent therapeutic for neurofibromatosis type 1 (NF1) by determining which *NF1* exons might be skipped while maintaining neurofibromin function. Human neurofibromin is well-known as a GTPase activating protein (GAP), but outside of its GAP-related domain (GRD), it is unclear how critical other regions are for function. Subsequent *in silico* analysis predicted exons that can be skipped with minimal loss of neurofibromin function. Utilizing a novel *Nf1* cDNA system, we performed a functional screen to determine the effects of deletion of predicted exons on *in vitro* neurofibromin expression and GRD function. Deletion of exons 17 or 52 results in both the highest neurofibromin levels and the greatest suppression of Ras activity. We designed antisense phosphorodiamidate morpholino oligomers (PMOs) to skip exon 52 and evaluated them in human cell lines harboring exon 52 NF1 mutations introduced via CRISPR/Cas9. These exon 52 mutant cells do not express NF1 and have elevated GTP-Ras and pERK/ERK ratios. The designed PMOs efficiently induced skipping of exon 52 with a low IC₅₀ of 45 nM and restored NF1 expression and Ras signaling function. Further, homozygous deletion of exon 52 in a novel mouse model appears compatible with viability and a grossly healthy and fertile animal, providing proof of concept that exon 52 may not be essential for murine neurofibromin function. Hence, exon skipping holds therapeutic potential for intragenic NF1 exon 52 mutations that affect a significant portion of individuals.

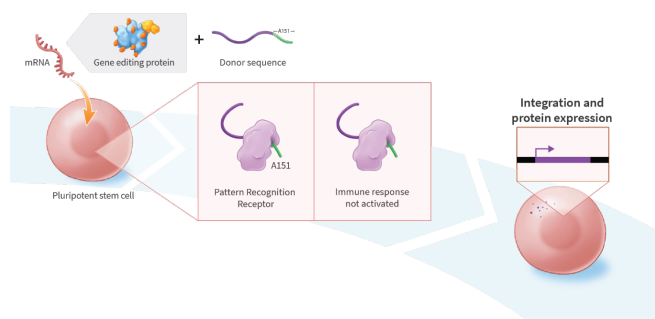
594. The Immunosuppressive TTAGGG Motif Improves Homology-Directed Insertion of DNA Sequences in Human Primary and Induced Pluripotent Stem (iPS) Cells

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Synthetic oligodeoxynucleotides (ODNs) have been used as repair templates in gene-editing applications to insert transgenic sequences into defined genomic loci, albeit with low efficiency. Cells engineered in this way are of interest for many therapeutic applications, including allogeneic NK and T cells engineered to express stealth proteins, cytokines, and chimeric antigen receptors (CARs) for the treatment of a variety of cancers. To increase the efficiency of integration, gene-editing proteins can be co-expressed to create a double-strand break at the target locus. However, recognition of dsODNs by pattern recognition

receptors activates signaling cascades resulting in the production of cytokines, including type I interferons such as IFIT1-3 and IFN- β . This immune response can lead to cell cycle arrest, differentiation, and apoptosis and may contribute to low insertion efficiency observed in primary and iPS cells. It has been shown in human immune cells that co-delivery of a short ODN comprising the immunosuppressive motif, TTAGGG, which is found in mammalian telomeric DNA, inhibits the activation of the damage-associate molecular pattern (DAMP) pathway in response to cytosolic DNA. This ODN competitively binds to inflammasomes, and reduces the secretion of proinflammatory cytokines. We hypothesized that the presence of the TTAGGG motif would decrease dsODN-related activation of a pro-inflammatory response in human cells, leading to higher transgene insertion efficiency. We incorporated the TTAGGG motif either at the 5' end of dsODNs, or delivered it separately on a short single-stranded ODN (A151). Human primary fibroblasts, iMSCs and iPSCs were electroporated with a dsODN encoding a GFP reporter and containing an SfoI restriction site. Upregulation of pro-inflammatory markers including IFIT1-3, was measured by RT-PCR. We observed 29-fold higher expression of IFIT1 and IFIT3 in cells electroporated with dsODNs than in untreated controls. Interestingly, including TTAGGG motifs at the 5'-ends of the dsODNs limited the upregulation of IFIT1 and IFIT3 to 10- and 15-fold, respectively, while co-delivery of the TTAGGG motif prevented their upregulation altogether. We then used a gene-editing endonuclease targeting the AAVS1 safe-harbor locus on chromosome 19 to investigate the impact of the TTAGGG motif on the insertion of transgenes at this site. The TTAGGG motif (whether incorporated in the dsODN or co-transfected in the form of the A151 ODN) resulted in approximately 50% higher viability and approximately 50% more GFP-positive cells than when the motif was not present. We show that immunosuppressive sequences can increase ODN insertion efficiency and improve cell viability, and may therefore be a powerful tool for therapeutic knock-in applications, including the generation of knock-in iPS cell lines.



595. Systemic Delivery of RNA Encoding Partial Dystrophins and Expression in Skeletal Muscle

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Duchenne muscular dystrophy (DMD) is a devastating, lethal muscle disease caused by mutations in the *DMD* gene that result in the absence of dystrophin protein expression triggering rapid, severe muscle wasting. The large size (11kDa) of the dystrophin coding region has made systemic full-length protein (427kDa) replacement unachievable to date. Becker muscular dystrophy is a milder dystrophy where patients express truncated dystrophin protein and exhibit increased lifespans suggesting expression of shortened versions of dystrophin may induce partial function and slow disease progression. Current therapies are targeting the re-expression of truncated versions of dystrophin (micro-dystrophin) via gene replacement or exon skipping strategies using adeno-associated virus (AAV) delivery systems however, these methods are hampered by reduced cloning capacity and (viral) immunogenicity that limits redosing capabilities. At Orna Therapeutics, we are developing a protein-coding circular RNA technology (oRNA™) that, coupled with our LNP delivery system, exhibits improved durability, protein expression, non-liver tissue distribution, and multi-dosing capabilities. In addition, our platform offers unprecedented payload capacity that can accommodate significantly large constructs, opening new opportunities in therapeutic areas such as muscle disease. We are developing a delivery solution that can deliver RNA payloads to a broad set of muscles including skeletal, heart, and diaphragm. In combination with our high capacity oRNA™ technology we have successfully delivered and expressed oRNA™ encoding both a micro (167kDa) and a mini (228kDa) version of dystrophin in primary human skeletal muscle myotubes. Additionally, micro dystrophin-encoding oRNA™ formulated into LNPs and delivered systemically via intravenous injection to the *mdx* mouse model of DMD (dystrophin null) demonstrated protein expression with correct localization to the muscle sarcolemma of several different muscles. Our oRNA™ technology represents a scalable, re-dosable, durable protein replacement gene therapy with a cargo capacity that has the potential to one day express full-length dystrophin and provide a currently unmet therapy for Duchenne Muscular Dystrophy.

596. Engineering More Efficient Therapeutic miRNAs for FSHD Gene Therapy

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Background: Facioscapulohumeral muscular dystrophy (FSHD) is among the most common muscular dystrophies. The disorder leads to weakness in skeletal muscles, most commonly of the face, shoulder girdle, and upper arms but effects can be widespread. There is no effect on lifespan, but the disease can be debilitating. No treatments exist and therapy development remains a critical unmet need. FSHD is a dominant disorder caused by de-repression of the myotoxic gene *DUX4*. Previously, our lab demonstrated proof-of-principle for an RNA interference-based gene therapy. We engineered 36 microRNAs (*miDUX4*) capable of targeting and inhibiting *DUX4* to varying degrees. When packaged into AAV and delivered to *DUX4*-expressing mice, these miRNAs protected the muscles from *DUX4*-induced damage. However, some miRNAs also led to muscle toxicity potentially due to improper processing. Our goal here was to better understand artificial miRNA processing to ultimately improve design of therapeutic inhibitory RNAs to treat various dominant genetic diseases. **Objectives:** The original *miDUX4* constructs have flanking regions 40-50 nucleotides long at both the 5' and 3' ends, resulting from the polylinker sequence at which the *miDUX4* constructs were cloned. Secondary structure predictions showed base pairing between the flanking sequences (FS). We hypothesized that optimizing the sequences immediately flanking the mature miRNA, which are required for binding and properly positioning the key RNase III enzyme Drosha and Dicer, could improve predictability and efficiency of processing to a mature miRNA form, and ultimately improve potency and safety of our miRNA systems by helping support the use of lower miRNA doses. To test this, we created new versions of multiple *miDUX4* miRNAs including our lead therapeutic sequence (*mi405*). **Methods:** We tested the inhibition efficiency of the new *miDUX4* constructs using a Dual-Luciferase Reporter assay and western blot. We tested our best candidates with redesigned flanking regions *in vivo* in a dose de-escalation study using AAV vectors. AAV.*miDUX4* constructs were co-injected for 8 weeks at 1.00E+08, 5.00E+08, 5.00E+09 DNase Resistant Particles (DRP) with 5.00E+09 DRP of AAV.*DUX4* in the tibialis anterior (TA) muscle of C57BL/6 mice. **Results:** First, we engineered seven variations of *mi405* by designing seven different combinations of 5' and 3' FS and tested their ability to reduce *DUX4* expression. Three top constructs called *mi405F*, *mi405G* and *mi405H* with specific short FS at the 5' and/or the 3' end, had the best silencing efficiency, with 40-fold greater potency than the original *mi405*. The incorporation of the same pro-silencing FS into other *miDUX4* sequences did not enhance their silencing efficiency of *DUX4*. Our *in vivo* dose de-escalation experiments showed that *mi405G* and *mi405H* were the most efficient in protecting muscle from *DUX4*-induced toxicity even at 1.5 logs lower dose than the original *mi405*. Surprisingly, *mi405F* was less efficient. **Conclusions:** By testing multiple FS on all of our *DUX4*-targeted miRNAs, we were able to enhance the inhibition efficiency of only one miRNA (*mi405*). Our results show that enhancing the inhibition efficiency of a miRNA does not only depend on the 5' and 3' FS but is also specific to the miRNA sequence. Thus, although FS play an important role in miRNA processing and efficiency, in our work there was no universal approach that could be translated to all sequences. *In vitro* and *in vivo* empirical testing remains important. Finally, our work shows the need to pay special attention to the miRNA FS when designing miRNA-based gene therapies.

597. Novel RNA Therapeutic Strategy to Optimize RNA Targeting in Brain Cancer

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Individuals diagnosed with glioblastoma multiforme (GBM) have a short life expectancy of 12-15 months. Current strategies are often limited by the blood-brain barrier. This project is to develop therapies to bypass challenges to effective and continuous drug delivery to the brain, targeting cancer-driving genes. Tumor blood vessel formation depends on vascular endothelial growth factor receptor 2 (VEGFR2), while tumor cell proliferation is stimulated by epidermal growth factor receptor (EGFR). Both are important for tumor cell survival. In our lab, we are developing an innovative therapy that can bypass the blood brain barrier by developing RNA therapies to alter the splicing mechanism of the VEGFR2 and EGFR genes to reduce or block their activation, thus stop tumor cell angiogenesis and growth. Our approach uses an adeno-associated virus gene transfer vector encoding RNA therapeutics targeting critical elements of the EGFR and VEGFR2 pre-mRNA transcript. The 'pre-mRNA structure' can be used to uncover and determine the accessibility of targetable regions. Our approach has the potential to deliver one single dose of gene therapy directly to the GBM tumor environment and block the production of EGFR and VEGFR2 and activate the expression of a stable therapeutic isoform of the two genes. To advance our therapeutic strategy, we have analyzed the secondary structure of the two genes using selective 2' hydroxyl acylation and primer extension followed by mutational profiling (SHAPE-MaP). SHAPE-MaP reactivity profiles were generated revealing the structure of splicing and cryptic polyadenylation signal (PAS) elements within the targeted region. We identified enhancer binding motifs surrounding the 5' splice site and hidden elements of the cryptic PAS. Based on these structural profiles, we generated RNA therapies to unravel the hidden PAS to activate expression of the short therapeutic isoform. This research has the potential to impact future strategies in RNA targeted therapeutics.

598. Development of Dicer Substrate RNA Nanostructures for Enhanced *In Vivo* Gene Silencing

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Three-arm junction RNA nanostructures (Y-RNA) have enhanced *in vitro* and *in vivo* RNA interference (RNAi) potency compared to canonical siRNA. Y-RNA was structurally optimized to be Dicer substrate and load into RNA-induced silencing complex (RISC) in the proper orientation. In addition, fluorescence polarization (FP) assay was used to determine binding affinity with RISC loading complex (RLC) components such as TRBP, Dicer, and Ago2. TRBP is an integral cofactor of Dicer, which distinguishes Dicer substrate RNA for effective loading and transfer to Ago2. We found that Dicer substrate RNA binds to TRBP with a 35-fold higher affinity than siRNA. This allows that Dicer substrate RNA to form active RISCs at much lower concentrations. Further, we have developed a universal chemical modification for Y-RNA to be applicable clinically. The position-specific

chemical modification of Y-RNA did not interfere with binding to the RLC components. In addition, it offered the enhancement of serum stability and *in vivo* RNAi potency. When the chemically modified Y-RNA (Y-mod) was delivered using lipid nano particles (LNP), the EC50 was reduced up to 8 times compared to canonical siRNA.

599. WNT Modulating Gene Silencers as Novel Gene Therapy for Osteoporosis and Critical Sized Bone Defect

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The WNT signaling pathway is a pivotal regulator of bone formation that mediates the augmentation of bone quantity and the maintenance of bone remodeling. Bone remodeling is a continuous bone replacement regulated by serial action between bone-forming osteoblast and bone-resorbing osteoclast, and crucial for utmost bone quality and proper fracture healing. Since antiresorptive drugs have shown impaired bone replacement after long-term treatment of osteoporosis and delay bone remodeling in treatment for fracture healing, anabolic drugs have become attractive pharmaceutical treatment in osteoporosis and fracture healing. Accordingly, several molecules that modulate WNT signaling pathway have been proposed as potential targets for new anabolic drugs. Sclerostin (SOST), an antagonist of the WNT signaling competing with WNT ligands, is the most investigated WNT modulator, and anti-sclerostin antibody is available in clinical practice. Romosozumab, a humanized monoclonal antibody, significantly increased bone mineral density with increase in levels of bone-formation markers over the first 6 to 9 months of treatment in postmenopausal women. Despite the evident effectiveness of this drug, the treatment more than 1 year is not recommended because of the decreased level of bone-formation markers that is lower than initial after 9 months of treatment, and there also has been a concern about potential cardiovascular event. Schnurri-3 (SHN3), endogenous suppressor of WNT signaling pathway by inhibiting ERK MAPK activity, is a critical regulator of osteoblast-mediated bone formation. Although limited to animal experiments, conditional deletion of SHN3 in osteoblast-lineage cells prevents osteoporosis by promoting bone formation. However, therapeutic interventions are needed to provide optimal WNT activation specific to the skeleton. Here, we identify bone-specific recombinant adeno-associated virus (rAAV)-mediated silencing of WNT antagonists as a fine-modulator of WNT signaling, effective for bone formation during bone remodeling and fracture healing. As expression of two WNT antagonists, SHN3 and SOST, is connected via a negative feedback mechanism, dual silencing of both factors further enhanced WNT signaling and bone accrual in healthy and osteoporotic mice. Remarkably, the dual silencing of SHN3 and SOST in osteoblasts inhibited osteoclastogenesis via upregulation of a key osteoclast suppressor osteoprotegerin (OPG) while *in vivo* osteoblast activity also wanes quickly. As bone regeneration and remodeling are both required for fracture repair, dual silencer-treated mice showed a significant delay of fracture healing. By contrast, a single silencer targeting SHN3 or SOST sustained an increase of osteoblast

activity along with little to mild decrease of osteoclastogenesis, promoting fracture healing process. Finally, transplantation of a synthetic bone or decellularized isograft expressing a single silencer targeting SHN3 or SOST, by directly attaching bone-specific rAAV to hydroxyapatite, was effective to promote healing of critical-sized bone defect. Collectively, our proof-of-concept studies demonstrate the potential of bone-specific WNT-modulating gene silencers to treat skeletal disorders associated with low bone mass and impaired fracture repair.

600. Identification of Potent Small Interfering RNAs Targeting the SARS-CoV-2 Genome

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Background: SARS-CoV-2 (CoV2) is responsible for over 5.5 million deaths as of February 2022, and it continues to circulate globally despite large scale vaccination. There are currently few effective treatments for CoV2 and as the virus continues to change, new treatments will be needed to combat emerging variants. Small interfering RNAs (siRNAs) are a class of drugs with great therapeutic potential, especially against respiratory viral infections such as CoV2 and they could be used intranasally at the beginning of the infection. Like other coronaviruses, CoV2 has a positive stranded RNA genome composing one large open reading frame (ORF1ab) followed by several subgenomic mRNAs (sgRNAs) with a common 3' end. While targeting the 3' end of the viral genome would cover the full-length genomic RNA and all sgRNAs, the abundance of sgRNAs could also counteract siRNA activity. To identify optimal target sites and potential siRNA therapeutics we have screened several siRNAs for activity against CoV2. **Methods:** We identified 96 siRNAs targeting different regions of the CoV2 genome using the NCBI reference sequence Wuhan-Hu-1 (NC_045512) and different criteria including high sequence conservation in circulating strains, limited sequence homology with the human transcriptome, and sequence features known to enhance siRNA activity or stability. To evaluate the activity of these siRNAs we have cloned all sub-genomic genes into enhanced green fluorescent protein (EGFP) expression constructs and have divided the long ORF1ab and the S gene into smaller fragments fused to EGFP. Toxicity of the siRNAs was evaluated using a WST-1 metabolism assay and efficacy against CoV2 viral replication was determined by measuring the ability of the siRNAs to inhibit cell death caused by CoV2 isolate RIM-1 (GenBank accession number: MW599736), also measured using a WST-1 metabolism assay. **Results:** At a screening concentration of 20 nM, several siRNA candidates showed activity against their targets conjugated to EGFP and against CoV2 replication with negligible toxicity. There was a very poor correlation between efficacy results obtained using the EGFP constructs and the live virus, possibly due to major differences in target site accessibility resulting from different RNA secondary and tertiary structures. Only four out of 40 siRNAs targeting sgRNAs inhibited viral replication by more than 50% (10% success rate), with

all four targeting the N gene. In contrast, 16 out of 56 siRNAs targeting ORF1ab inhibited viral replication by more than 50% (29% success rate) and the six most effective siRNAs all targeted ORF1ab, suggesting that ORF1ab may be a more effective siRNA target compared to the sgRNAs. The top two siRNAs inhibited CoV2 replication by more than 90%. **Conclusions and future directions:** The very poor correlation between siRNA efficacy using EGFP reporter constructs and the live virus suggest that reporter constructs containing parts of the CoV2 genome are not useful for identifying effective anti-CoV2 siRNAs. Our results also suggest that ORF1ab is a better target compared to the sgRNAs. From our screen we identified several highly accessible siRNA target sites in the CoV2 genome. We will use the target sites we identified to design more potent siRNAs with advanced chemistries that can be developed for use in the clinic.

601. A Phase I/II Study of NS-089/NCNP-02, Exon 44 Skipping Drug, in Patients with Duchenne Muscular Dystrophy

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The rapid pace of precision medicine development is illustrated by the progress of new therapies for the incurable condition Duchenne muscular dystrophy (DMD), which has wide-ranging implications for genetic neuromuscular diseases. The current state-of-the-art is an antisense oligonucleotide-based gene therapy designed to skip specific exons to restore the expression of shorter but functional dystrophin. NS-089/NCNP-02 is a novel phosphorodiamidate morpholino oligomer that consists of a novel sequence design involving connected antisense oligonucleotides targeting different parts of exon 44 discovered by NCNP and Nippon Shinyaku Co., Ltd. NS-089/NCNP-02 targets exon 44, to serve as an effective treatment for DMD patients amenable to exon 44 skipping. The trial was designed in two parts. First-in-human part 1 (phase 1) is a stepwise dose-finding stage (4 weeks). Part 2 (phase 2a) is a 24-week evaluation period based on the dosages determined during Part 1. In Part 1 of the study, Cohort 1 (three patients) will initially receive NS-089/NCNP-02 at dosing Level 1 (1.62 mg/kg QW) for two weeks and at dosing Level 3 thereafter (40 mg/kg QW) for two weeks. Cohort 2 (three patients) will initially receive NS-089/NCNP-02 at dosing Level 2 (10 mg/kg QW) for two weeks and at dosing Level 4 thereafter (80 mg/kg QW) for two weeks. In Part 2 of the study, two different doses of NS-089/NCNP-02 (determined from the results of Part 1) will be intravenously administered QW for 24 weeks. Patients who have completed Part 1 are allowed to participate in Part 2, and total 6 subjects were assigned to this study. The primary

endpoints are safety, and the secondary endpoints include expression of dystrophin protein, motor function assessments, exon 44 skipping efficiency, plasma and urinary NS-089/NCNP-02 concentrations, and changes in blood creatine kinase levels. In parallel, we collected urine-derived cells (UDCs) from all the subjects enrolled for the trial and obtained MYOD1-UDCs. We then compared the efficiency of exon 44 skipping induced by NS-089/NCNP-02 in differentiated MYOD1-UDCs (UDC-myotubes) and muscle biopsy samples from all the subjects. Our first-in-human studies shall provide critical data of safety, efficacy and pharmacokinetics of NS-089/NCNP-02 for subsequent clinical development of NS-089/NCNP-02, with the ultimate aim of expanding treatment capacity for DMD and other neuromuscular conditions.

Synthetic/Molecular Conjugates and Physical Methods for Delivery I

602. Efficient LNP Delivery of mRNA In Vivo and In Vitro to T and NK Cells

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Chimeric antigen receptor (CAR) T cell therapy has demonstrated tremendous therapeutic potential in the treatment of some malignancies. However, autologous CAR T cell therapy requires the manufacture of bespoke products on an individual patient basis, and current-generation allogeneic CAR T cells require stringent patient lymphodepletion to achieve clinical responses comparable to autologous products. *In vivo* delivery of mRNA encoding therapeutically relevant transgenes such as CARs has great potential for transiently reprogramming patient immune cells in an autologous manner without the need to manufacture the cells *ex vivo*, and without the undesirable effects of stringent lymphodepletion or the added risk of allogeneic product rejection. Lipid nanoparticles (LNPs) are an efficient platform for *in vivo* delivery of RNA via intramuscular injection or through intravenous (IV) administration to the liver. Unlike viral vectors, LNP delivered mRNA could be re-dosed, and the shorter duration of protein expression allows for repeat dosing with various antigen targeting CARs in the case of antigen negative relapse or heterogeneous tumors. However, significant optimization of LNP identity and formulation is required for efficient *in vivo* delivery of RNA to immune cells using IV administration. Here we describe *in vivo* and *ex vivo* LNP-mediated delivery of mRNA encoding reporter proteins to T and NK cells. Over 1,000 chemically distinct LNPs were screened *in vivo*, resulting in the identification of three lead LNPs that target T and NK cells efficiently. The biodistribution and pharmacokinetics of LNP1, LNP2, and LNP3 were assessed by delivery of cre recombinase mRNA in a cre-reporter mouse model (Ai14), which expresses the fluorescent protein tdTomato under a constitutive CAG promoter following cre-mediated gene editing. We confirmed dose-dependent tdTomato expression in T and NK cells isolated from murine blood, bone marrow,

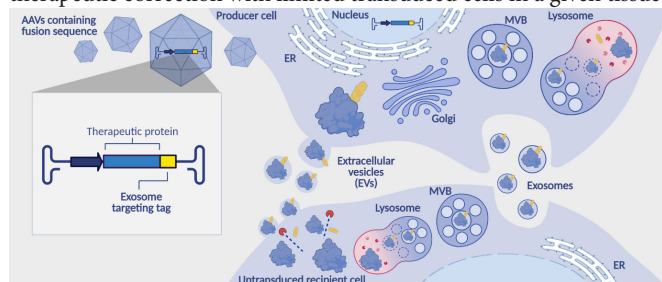
and spleen at levels between 10-40% following a single LNP dose. We next confirmed the LNP transfection of human cells *In vitro* and in hu-NSG mice and cynomolgus macaques. *In vitro* transfection of human cells at non-toxic LNP doses yielded reporter protein expression in over 90% of human T cells, and over 70% of NK cells. In a humanized NSG mouse model, we observed reporter expression following a single LNP dose in human T and NK cells at approximately 10% and 20% in bone marrow, respectively; and up to 50% in the spleen. Due to low levels of NK cells in hu-NSG mice, subsequent studies are planned to evaluate delivery to hu-NSG or humanized BLT mice with engrafted NK cells. In NHPs, we observed reporter mRNA transfection following LNP delivery to nearly 7% of bone marrow T cells. Taken together, these data demonstrate the feasibility of LNP-mediated delivery of mRNA to T and NK cells enabling transient therapeutic transgene expression, providing a promising delivery platform for an *in vivo* approach to CAR-T and -NK cell production.

603. Engineering Exosome-Mediated Cross-Correction for Next-Generation Gene Therapies

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Adeno-associated virus (AAV) gene therapy is emerging as a revolutionary modality for the amelioration of genetic disease. Currently, AAVs can only target a small fraction of target tissue cells. Without transgene protein product secretion and uptake into untransduced cells, most cells will remain untreated. Engineering cross-corrective mechanisms directly into the transgene is a method for overcoming this limitation. This can be accomplished by engineering secretion signals, receptor-mediated uptake tags, and mutations that improve stability in circulation. Here, we present a new modality for cross-correction by directing the therapeutic protein to extracellular vesicles (EVs). EVs, including exosomes, are natural protein cargo vessels that are secreted by virtually all cell types. Naturally produced exosomes have advantages in biodistribution, cargo protection, and immune evasion and are known to cross the blood-brain barrier - a significant challenge for neurological indications. We engineered various peptide tags that mimic natural mechanisms for exosome targeting of viral and cellular proteins. These short exosome-targeting tags are introduced as N-terminal or C-terminal fusions to the protein-of-interest. For screening targeting tags, we generated fusions with mCherry and transfected these constructs into various cell types. We demonstrate evidence of secretion and uptake in tissue culture via flow cytometry and confocal microscopy. When these plasmids were electroporated into mouse embryonic brains, they exhibited evidence of cross-correction both within the target organ (brain) and distal somatic organs (liver), suggesting systemic biodistribution. By iteratively engineering, we have created a library of motifs for exosomal targeting. A successful subset of targeting tags was then applied to multiple therapeutic proteins (lysosomal storage disorder targets, as well as cytoplasmic and nuclear targets). Our proprietary engineered

tags can be incorporated into a transgene sequence and enhance cross-correction of a therapeutic protein without the need for *in vitro* production/purification of exosomes. This may enable more complete therapeutic correction with limited transduced cells in a given tissue.



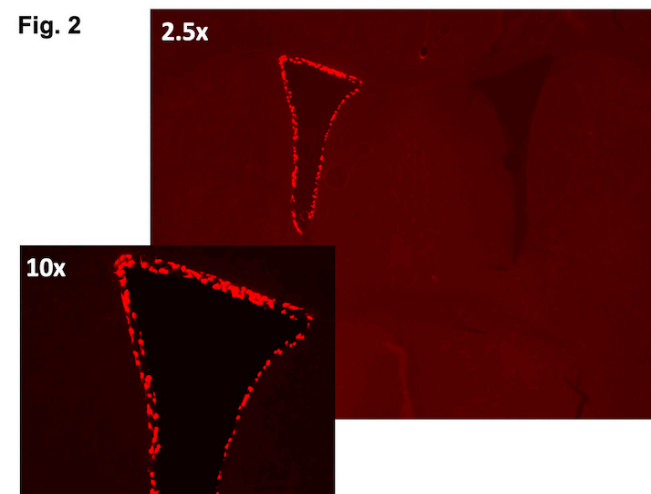
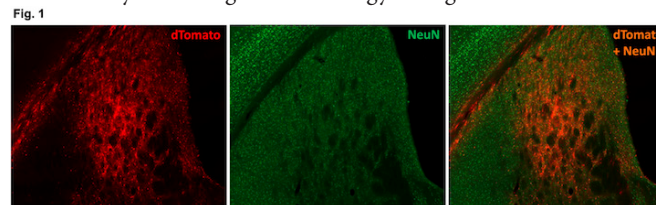
604. Development of a Monoparticle-Based Gene Editing Technology for Neurological Applications

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Introduction. CRISPR-based gene editing of the brain has the potential to revolutionize the treatment of neurological diseases. A large number of incurable brain diseases, such as Huntington's, Alzheimer's, and Parkinson's disease, are caused by the over-expression of pathogenic proteins and could be treated with CRISPR-based therapeutics. Despite its potential, developing CRISPR-based therapeutics for the brain has been challenging because of delivery problems. In particular, two key challenges need to be solved before gene editing in the brains of large animals and in humans is possible. First, strategies for efficiently and safely delivering Cas9 and gRNA into neurons, after an intracranial injection, need to be developed. Second, strategies that can enable a large volume of brain tissue (> 1 cm) to be transfected after intracranial injection of CRISPR reagents must also be developed. The overall goal of this project is to develop formulations of Cas9 ribonucleoprotein (RNP) enzymes that can edit large volumes of brain tissue after intracranial injections. **Material and Methods.** Our team generated a series of different formulations of Cas9-RNPs. A nuclear localization signal (NLS)-rich Cas9 nuclease protein was combined with sgRNAs targeting the Ai9 cassette repressing TdTomato reporter expression. We screened dozens of RNP-based particles featuring diverse compositions: different type and density of NLS, reversible surface PEGylation, and/or inclusion of delivery peptides. Our *in vivo* testing involved injecting lead Cas9-RNPs formulations into the striatum of Ai9 mice (7 μ L per striatum) via convection-enhanced delivery (CED). Three weeks later animals were euthanized, and their brains were processed for immunohistological staining (double immuno-fluorescence for TdTomato and NeuN markers) and image analysis (percentage of edited/dTomato-positive neurons). The distribution of the injected Cas9-RNPs within the target structure, striatum, was calculated by the Cavalieri method. **Results and Conclusion.** We have tested dozens of various Cas9-RNP formulations and evaluated their efficiency of neuronal editing in the Ai9 mouse brain. Our formulations contained

small particle sizes that corresponded to the RNP diameter (≤ 20 nm), which may have contributed to improved tissue distribution. Our best performing formulation consisted of NLS-rich Cas9-RNP enzymes and amphiphilic delivery peptides and was able to edit nearly 50% neurons (Fig. 1) within the site of injection. In addition, when this formulation was administered into the CSF (lateral ventricles), complete editing of ependymal cells within the choroid plexus was observed (Fig. 2). We are currently advancing this technology to large animals.



605. Novel Lipid Nanoparticle Delivery Reagent and Rapid Manufacturing Workflow to Accelerate Preclinical Development of RNA Vaccines

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Introduction: The recent SARS-CoV-2 pandemic has seen an unprecedented drive to develop vaccines with RNA vaccines at the forefront of this effort. Due to the rapid degradation of RNA and low cellular uptake, an essential element of RNA vaccines are lipid nanoparticle (LNP) delivery systems. However, limited access to ionizable lipids and LNP compositions, and the difficulty in scale-up production of RNA-LNPs remain challenges in the field. Therefore, we have developed a novel LNP reagent for vaccine applications, and established a rapid workflow for RNA-LNP production using a scalable microfluidic platform.

Methods: PolyA, messenger RNA (mRNA) and self-amplifying mRNA (saRNA) encoding for EGFP, luciferase (FLuc) and SARS-CoV-2 spike protein were used. RNA-LNPs were produced on the NanoAssemblr® Ignite™ microfluidic system using GenVoy-ILM™ and MC3 benchmark lipid mix. Size and polydispersity (PDI) were measured by DLS, and encapsulation efficiency (EE) by Ribogreen™. *In vitro* activity was assessed in HEK293 cells. Biodistribution and expression were performed by IM injection of BALB/c mice and IVIS® imaging. Mice were immunized by IM injection, with a booster on day 28 and culling on day 42. IgG titers were determined by ELISA. **Results:** RNA-LNPs produced with different mRNA and saRNA were of high quality with particle sizes of 60 - 85 nm, PDI <0.1 and EE >85%. RNA-LNP uptake and protein expression was observed *in vitro* for EGFP mRNA and saRNA. *In vivo* biodistribution and expression was investigated after IM injection. The RNA-LNPs were localized to the injection site, with fluorescent DiD signal observed up to 14 days. Expression of FLuc mRNA and saRNA was high, peaking within 1 day and 6 days, respectively. Immunization of mice with SARS-CoV2 saRNA LNPs resulted in an IgG specific response of almost 10⁵ ng/ml. Analysis of the IgG2a/IgG1 ratio showed an IgG2a-skew which is indicative of Th1-biased immune response. **Conclusions:** We have developed a novel LNP reagent for vaccine applications and established a rapid workflow for RNA-LNP production using a scalable microfluidic platform. These technologies should have utility for a wide-range of preclinical drug development activities, including antigen screening, and *in vivo* proof-of-concept studies.

606. Miniature Devices for Controlled Airway Surface Perturbation in Rats: Which Device Produces the Best Lentiviral Vector Gene Transfer?

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Introduction: Natural airway defences impede the efficacy of viral vector-mediated airway gene therapy. Airway surface preparation methods performed prior to vector delivery can disrupt some of these barriers, improving viral vector access to target receptors and airway stem cells. In this study we assessed and quantified the histological and gene transfer effects of physical perturbation devices in rat airways to identify the most effective approach. **Methods:** We evaluated the histological effects (n=3 rats/group) of a Brush, flexible wire baskets with varying configurations (Cook Medical: NCircle®, NCompass®, NGage® and Storz: 11582M Stone Extractor), biopsy forceps, and a balloon catheter. Rats were anaesthetised, intubated, and controlled physical perturbation performed to the trachea via the endotracheal tube. Ten minutes later, tracheas were excised and processed to produce hematoxylin and eosin stained sections. Regions where epithelial cells were flattened to one cell layer thick, or completely devoid of cells on the basement membrane were measured. Devices that produced a statistically significant increase in

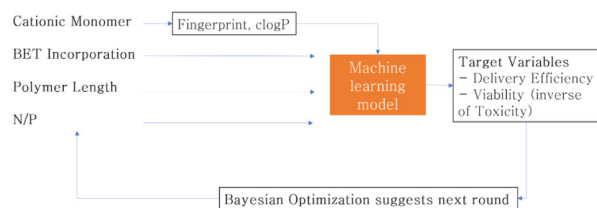
epithelial cell removal compared to the unperturbed control group were subsequently tested to assess whether they could enhance gene transfer (n=3-6 rats/group). Ten minutes after perturbation, 50 µL of lentiviral vector carrying the *LacZ* reporter gene (5 x 10⁹ TU/mL) was delivered. After 7 days, excised tracheas were X-gal processed to quantify the area of *en face* *LacZ* staining. Data was analysed using one-way ANOVA (Dunnnett's post-hoc) or Kruskal-Wallis test (Dunn's post-hoc) with comparisons to the unperturbed control group. **Results:** Significant tracheal epithelial removal was produced by the Brush, and the NCircle®, NCompass® and NGage® wire baskets (p<0.05). These four devices were selected to assess their ability to enhance LV-*LacZ* gene transfer. Quantification of *LacZ* staining found that only the NGage® wire basket significantly increased gene expression (p=0.0003), showing an 8-fold increase in the proportion of *LacZ*-stained area when compared to rats that did not receive perturbation. **Conclusion:** We found that the NGage® wire basket was the only device to significantly increase *LacZ* staining, even though other devices also produced significant epithelial cell removal. These findings suggest that factors other than cell removal contribute to perturbation-enhanced gene transfer, such as the location and pattern of disturbance, or type and viability of cells remaining following perturbation. The potential for improved gene delivery shown here supports further development of physical perturbation approaches.

607. Polymer-Mediated Gene Delivery Development Driven by Machine Learning

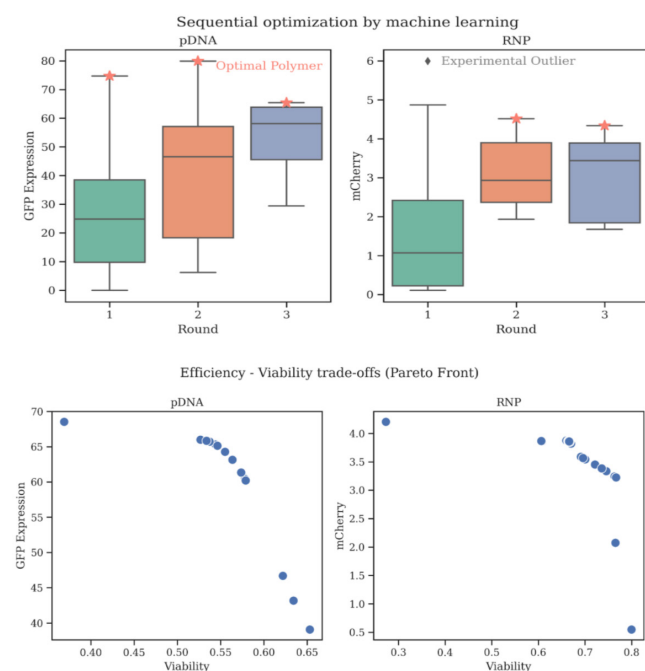
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Functional delivery of biological payloads, such as plasmids (pDNA) or ribonuclear proteins (RNP), requires precise design and optimization of polymer vehicles. Choosing an optimal polymer vehicle requires exploring a large combinatorial space. This large parameter space may be prohibitively expensive to navigate with heuristic or design of experiments approaches. In this work, we developed a machine learning (ML) model to optimize a tunable polymer platform for effective gene-delivery. For two cargos, we demonstrate various experimental rounds of *in vitro* sequential optimization, along with the *in vivo* delivery feasibility of our vehicles. **Methods:** Bayesian optimization is an ML algorithm to optimize black-box functions efficiently. In the batch setting, the BO algorithm models the relation between the design variables and the target variable using a probabilistic model; during each batch, data is collected from experiments, the model is updated, and then used to suggest next promising samples. In this work, we adapt the BO algorithm to polymer optimization, in particular we are interested in selecting optimal cationic monomers, polymer length, molar incorporation of the BET monomer, and N/P ratio. Figure 1 illustrates our model, which encodes the cationic monomers based on chemical fingerprints and hydrophobicity to fit the categorical-continuous design space correctly.



We use this model to suggest optimal combinations of polymer variables in sequential experimental rounds, with the aim of optimizing payload delivery efficiency. These suggestions are experimentally synthesized through stepwise post-polymerization modification of a designed polymer scaffold. Each polymer is chemically characterized through ¹H NMR and SEC-MALS, physically characterized, and biologically characterized to understand expression when delivering the biological therapeutics of pDNA and RNP. We demonstrate that our approach leads to optimized polymers. Subsequently, we use the probabilistic model to estimate the best trade-offs for delivery efficiency and cellular viability (toxicity) in each payload, the so-called Pareto fronts. Finally, we demonstrate the feasibility of our best performing polymers by in vivo pDNA luminescence measurements in mice via hydrodynamic injections. **Results & Conclusions:** For pDNA, we find an optimal set of polymer design variables after 3 in vitro experimental rounds and a total of 93 measurements. This constitutes only 2.5% of the possible combinatorial space, confirming the experimental efficiency of our approach. In the RNP case, we are able to optimize a subset of polymers, however low signal to noise ratio limits our performance compared to pDNA. Figure 2 (top) summarizes these trends. Subsequently, we use closed-loop multi-objective optimization to find the optimal trade-off between delivery efficiency and viability. This trade-off is summarized in the Pareto-front plots of Figure 2 (bottom). After in vivo pilot measurements, we demonstrate 1 order of magnitude superior delivery performance compared to commercial polymer baselines (Jet-PEI).



608. Bioengineering Multifunctional Extracellular Vesicles for Targeted Delivery of Biologics to T Cells

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Engineering T cells is an area of active investigation for the development of therapeutics to treat cancer, autoimmunity, and infectious disease, such as HIV. Recent advances in HIV treatments have greatly increased the quality of life for infected individuals, but there remains an unmet need for a cure for this lifelong condition, a major barrier to which is the existence of the latent viral reservoir. Gene editing technologies, such as CRISPR/Cas9, hold great promise for being able to remove or damage HIV proviruses such that they can no longer replicate, implementing a functional cure for these infections. However, there exists a key unmet need for clinically relevant delivery vehicles that enable targeted, intracellular delivery of therapeutic biomolecules. A promising potential solution is the use of extracellular vesicles (EVs) to package and deliver Cas9 to latently infected cells. EVs are nanoscale lipid particles secreted by all cells that naturally encapsulate and transfer proteins and nucleic acids between cells. The ability to engineer these vesicles to load therapeutic cargo makes them an attractive platform for creating targeted, fusogenic drug delivery vehicles. Toward this end, we built a high-affinity, scFv-based targeting construct and achieved specific binding of EVs to CD2 on T cells with efficiencies exceeding a 100-fold increase over a non-targeted control. We developed a method for actively loading protein cargo into EVs via protein tagging to direct trafficking into vesicles during EV biogenesis. By displaying viral glycoproteins derived from the measles virus or vesicular stomatitis virus on the EV surface, we demonstrated increased uptake and fusion with recipient T cells. In combination, these targeting, loading, and fusion techniques were used to functionally deliver Cas9 to primary human CD4⁺ T cells to edit the CXCR4 locus as validated by NGS. In addition, we used these techniques to demonstrate that, for certain vesicle populations, receptor engagement influences EV trafficking in a way that increases functional delivery. We anticipate these engineering approaches will be broadly applicable for targeting vesicles to a range of cells to deliver biomolecular cargo in a variety of systems.

609. Peptide-Mediated Delivery of Gene Editing Modalities

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The field of gene editing can currently offer tools for permanent cures for a plethora of inherited genetic diseases. Recent developments have made safe gene editing possible, for example, by the use of base-editing Cas9 fusion proteins. However, the methods for their delivery have not yet reached the efficiency and safety needed for widespread clinical adoption. The delivery is further complicated by

the large size of Cas9 and its fusion proteins. Gene-editing agents are most commonly delivered via viral vectors, which have developed considerably since their first use in humans over 30 years ago. These vectors still suffer from safety concerns and expensive production. Viruses also generally only transfer nucleic acid-based moieties and are limited in their cargo capacity. Being able to deliver gene-editing moieties in a protein format, such as Cas9 ribonucleoprotein (RNP), has many advantages, such as reduced off-target effect and no risk of genome integration. Non-viral vectors enable the delivery of more varied cargo, both in terms of size and type. The most clinically advanced non-viral delivery method is lipid nanoparticles (LNPs). LNPs are approved by the FDA for siRNA delivery and are currently being clinically evaluated for Cas9 mRNA delivery. However, dosing of LNP at the levels likely necessary for therapeutic gene editing is not without issues; both cortisone and antihistamines are needed to treat side effects following IV injection due to side effects to the LNP. Here we hypothesized that a family of nucleic acid delivering cell-penetrating peptides could be repurposed for the delivery of Cas9 RNP. This should be possible due to electrostatic interactions between the cationic CPP and anionic guide RNA of the RNP. A fluorescent stop-light reporter cell line was used for the optimization of RNP-CPP delivery and was evaluated using flow cytometry. Early results showed that the RNP-CPP complexes enabled productive RNP delivery. Further optimization of molar ratio, buffer, and additives resulted in a roughly tenfold increase in transfection ability, reaching gene-editing values over 50% at below 0.06 nM Cas9. The editing was further corroborated by genomic analysis and editing of the endogenous gene HPRT, which also showed similar levels. To test our original hypothesis of an electrostatic CPP-cargo interaction, we attempted to deliver Cre recombinase to a traffic-light reporter cell line to investigate the possibility of delivering proteins without a large anionic element. To our surprise, Cre was successfully delivered into several cell types, including hard-to-transfect cell lines such as B16F10 mouse melanoma cancer, T47D human breast cancer, and Raw264.7 mouse macrophages. The delivery of Cre was generally less efficient than that of Cas9 when normalized to the concentration of gene editing agents in the wells. The successful delivery of Cas9 and Cre, two very different proteins, points towards the fact that these CPPs can deliver a varied protein cargo, independently of size and charge, which can lead to new applications. We are in the process of evaluating the peptides for *in vivo* delivery.

610. Tumor-Targeted Delivery of IL-15 Derived Transgenes by Transmorphic Phage AAV Particles

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Immunotherapy has established a new era in cancer treatment and among its various modalities is the use of cytokines as therapeutic genes. The interleukin IL-15 cytokine has recently gained attention in several preclinical and clinical trials. Similar to the clinically approved IL-2, IL-15 can stimulate natural killers and cytotoxic T lymphocytes, lacking, importantly, the capability to activate the immunosuppressive regulatory T cells, which makes IL-15 a more attractive therapeutic candidate. Yet, such a promising cytokine requires to be matched with

delivery methods that allow accurate on-target therapeutic action. Our group has developed M13 derived phagemid based vectors, named transmorphic phage AAV particles (TPA), displaying the RGD4C peptide in their pIII coat protein, making them highly specific towards tumor cells and associated tumor vasculature after *in vivo* systemic administration. In the current study, we have used this platform to deliver different versions of the mouse IL-15 cytokine: native IL-15, ^{IgK}IL-15, fused to the IgK signal peptide (sp), and ^{IgK}IL-15Ra/IL-15 as a hybrid between IL-15 ligand, IL-15 receptor and the IgK sp. The transgenes were cloned into the TPA vector between the AAV2 ITR sequences. The TPA^{IgK}IL-15 transgene was cloned by inserting the mouse IgK sequence upstream of the mouse IL-15 gene. For the vector encoding the fusion cytokine, TPA^{IgK}IL-15Ra/IL-15, the mouse IL-15Ra sushi domain was cloned downstream of the IgK peptide followed by a linker peptide sequence and the IL-15 gene. For each type of transgene, both tumor cell targeting (RGD4C) and untargeted vectors, without RGD4C, were produced. Murine B16F1, B16F10 melanoma and CT26CL25 colon carcinoma cells were either transfected with the corresponding plasmids or transduced with TPA particles at 5x10⁵ or 1x10⁶ TU/cell. The conditioned medium was analyzed by ELISA to evaluate cytokine secretion and bioactivity using a lymphocyte proliferation assay on CTLL2 cells. *In vivo* efficacy of the TPA^{IgK}IL-15 vectors was then evaluated on a CT26CL25 colon cancer model in BALB/c mice by assessing their biodistribution through IL-15 mRNA quantification and their therapeutic efficiency on tumor size reduction, tumor viability and animal survival. Analysis of the B16F1, B16F10 and CT26CL25 transfected cell conditioned medium shows that the newly designed mouse ^{IgK}IL-15 transgene improves secretion over the native version of the cytokine while maintaining its biological activity as proven by its ability to stimulate CTLL2 cell proliferation. Transduction of the same cells by TPA^{IgK}IL-15 particles demonstrated a clear tumor-specificity by the targeted particles, which induced cytokine secretion in a vector concentration-dependent manner. The ability of these vectors to systemically deliver the therapeutic cytokines was then studied *in vivo* in a CT26CL25 model, which further confirmed the specificity of RGD4C displaying vectors to target tumors while sparing other healthy tissues as observed from the IL-15 mRNA quantification data. Moreover, treatment with the targeted vectors resulted in tumor regression and eradication in 50% of the treated mice. In sight of these results, an additional IL-15 variant was designed by incorporating the IL-15Ra sushi domain. This fusion is hypothesized to increase its bioactivity by bypassing the need to complex with free IL-15Ra prior receptor binding. *In vitro* analysis of these TPA^{IgK}IL-15Ra/IL-15 particles have shown successful expression of a bioactive cytokine. We have used our novel transmorphic phage AAV (TPA) particles to deliver a highly secreted ^{IgK}IL-15 cytokine in a safe and targeted fashion after systemic administration achieving clear outcomes in preclinical tumor models. Future studies are being planned to test efficacy of the fusion ^{IgK}IL-15Ra/IL-15 cytokine.

611. Extracellular Vesicles from Induced Nephron Progenitor Cells Reduce Urinary NGAL in Mice with Ischemia/Reperfusion Kidney Injury

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Acute kidney injury (AKI) is associated with morbidity and mortality, necessitating the development of new therapies. The nephron progenitor cells of the embryonic kidney have renal identity, making them an ideal target cell type for development of therapies to regenerate the kidney. While stem/progenitor cell treatments have promise, extracellular vesicles (EVs) or other biological products derived from progenitor cells may exert similar effects. EVs or exosomes have practical advantages over live cell products for treatment of time-sensitive conditions such as AKI because of the potential to add therapeutic RNA or protein payloads to the particles. Human nephron progenitor cells cannot be directly isolated from adult kidneys because nephrogenesis ceases shortly before birth, exhausting this progenitor population. In previous work, we found that tubule cells (HK-2) could be reprogrammed into induced nephron progenitor cells via expression of the transcription factors *SNAI2*, *EYA1*, and *SLX1*. We harvested EVs from the conditioned media derived from a population of clonal HK-2-derived induced nephron progenitor cells. Polyethylene glycol-based enrichment was used for the isolation of EVs at a final concentration of 8%. The isolated EVs had an average size of 85.4 ± 5.1 nm and concentrations of $2.49 \times 10^8 \pm 3.47 \times 10^7$ particles/ml by nanoparticle tracking analysis. Either EVs or saline were administered to FVB/N mice via the tail vein 24 h following ischemia/reperfusion clamping injury of one kidney and unilateral nephrectomy surgery of the other kidney. Blood urea nitrogen and serum creatinine were increased, confirming AKI. Urinary NGAL, a marker of distal tubular injury, was decreased in mice treated with EVs (~ 40 ug/ml) as compared to saline (~ 100 ug/ml) by one week following surgery ($p < 0.05$). The survival rate of mice was improved by EVs (67%) compared saline (50%). Morphologic assessment of kidney injury two weeks after surgery suggested the EVs had beneficial effects on tubular dilation, cast formation, and fibrosis, but these trends did not reach significance. In conclusion, administration of human induced nephron progenitor cell-derived EVs may decrease severity of AKI in mice.

612. A Novel Scalable Electroporation Platform for the Manufacturing of Gene Modified Hematopoietic Stem and Progenitor Cell Therapies

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Electroporation is an established gene delivery method that enables the therapeutic modification of many cutting-edge cell and gene therapies. While the platform is well grounded, significant time and energy must still be dedicated to fine tune parameters as more novel cell types and indications arise. Thus, the optimization of electroporation conditions, such as the pulse profile and buffer, is critical to achieve the best drug product possible. However, scalability is a challenge when electroporation needs to be translated from an R&D to development and cGMP manufacturing environment. Recently, CTS Xenon Electroporation System has been launched; the design of this device is based off the R&D Neon Transfection System, but suited for the needs of a clinical scale, regulated environment. The Xenon/Neon systems are open programmable electroporation platforms where parameters such as voltage (V), pulse width (ms), number of pulses, and pulse interval (last one being specific to Xenon, to help reduce processing time for larger volumes) can be explicitly controlled by the end user. Several publications have demonstrated the efficiency of the Neon transfection system in blood and immune derived cells (CD34⁺ and T cells), providing promise in the suitability of the Xenon to be used for scale-up. Based on vendor-provided optimization parameters, 13 programs were tested for gene modification of CD34⁺ cells, using the Neon 100 uL tip format. Cell viability was determined by AO and DAPI staining, while gene editing efficiency was assessed by the presence of indels (insertion or deletions) and analyzed with our internal bioinformatics tool. The program selected to evaluate the scalability from Neon 100 uL tip, showed average cell viability and gene editing higher or equal to 80%, comparable to 1 mL single-shot cartridge on Xenon (Figure 1A). We then evaluated the scalability from 100 uL tip on Neon transfection system to 1 mL single-shot and 5-25 mL multi-shot cartridges in a beta version of Xenon electroporation system, prior to its launch. Cell viability during cell culture post-electroporation was comparable; at the same time, gene editing stayed high with an average of $75\% \pm 5$ for 1 mL single-shot and average of $70\% \pm 6.5$ for 5 mL multi-shot, while using the prototype consumables. Nonetheless, it was lower than 100 uL tip which average was $87\% \pm 1$ (Figure 1B). In light of this positive data, Xenon electroporation system appears to be a promising tool for the clinical scale manufacturing of gene modified HSCs. In addition, the inclusion of the Xenon in Thermo Fisher's Cell Therapy System (CTS) portfolio of products, provides great confidence in meeting quality and regulatory environments for manufacturing and the potential for workflow integration of additional CTS portfolio items.

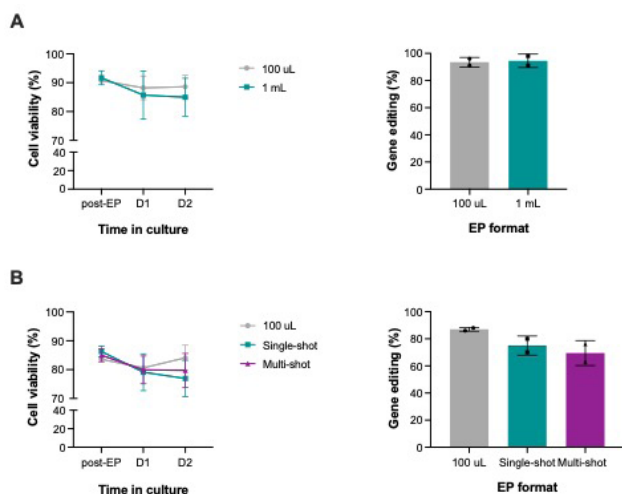


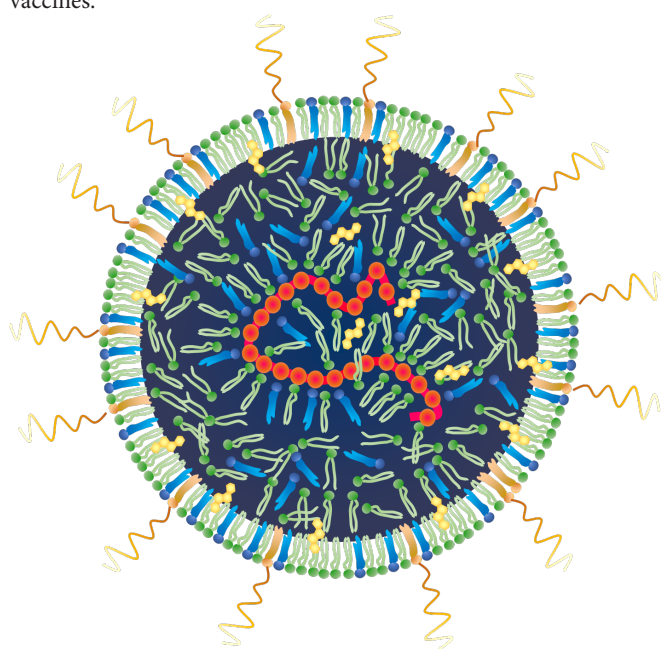
Figure 1. CTS Xenon electroporation system generates biological material with high cell viability and gene editing. **A**, comparability in cell viability and gene editing was observed from 100 uL tip on Neon transfection system to 1 mL single-shot cartridge on Xenon electroporation system, when payload was electroporated using program 12 and electroporation buffer R. **B**, single and multi-shot performance on Xenon electroporation system beta-device is comparable for cell viability and gene editing.

613. Ionizable Lipid Nanoparticle Platform for mRNA Therapeutics with Room Temperature Stability

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The recent FDA approval of COVID-19 vaccines composed of mRNA loaded ionizable lipid nanoparticles (mRNA-LNP) has increased interest in novel application of this modality including cancer immunotherapy. However, mRNA applications have been limited by immunogenicity, poor drug product stability and low in vivo expression. Here we report on our efforts to develop COATSOME® SS-Lipid: an mRNA-LNP composed of biodegradable ionizable lipids with improved safety profile, increased gene expression and room temperature stable lyophilized drug product. Previously we demonstrated that SS-lipids can be engineered to degrade in response to specific intracellular signals yet provide extended stability in circulation. Biodegradable lipids also demonstrate excellent safety profile in rodents with limited immunogenicity at total lipid doses as high as 160 mg/kg. For the vaccine application, we identified two new SS-Lipids “SS-EC” and “SS-E” with high macrophage activation in an in vitro interferon- β production assay. We next assessed antibody response in mice immunized with OVA mRNA-LNP vs conventional immunization with OVA protein and Poly I:C adjuvant. Although IgG induction required multiple doses and sufficient titer was seen only after 20 days, anti-OVA IgG titers were approximately 2X higher in the LNP group vs the OVA protein immunized group. These results suggest that for mRNA LNP T-Cell immunity emerges first and B-cell immunity is delayed for several weeks, possible due to inefficient antigen presentation. Examination

of draining lymph nodes showed SC injected LNP appeared within 24 hours, and flow cytometry analysis of the cells that had taken up the LNPs showed that the SS-E LNPs were largely taken up by macrophages and a small number of dendritic cells. We found that the transient deletion of CD169+ macrophages, unexpectedly enhanced the activity of the vaccine. Finally, we evaluated the interim stability of a lyophilized drug product of the SS-Lipid hEPO mRNA LNP stored under different temperatures at the 12 week timepoint. The mRNA expression activity in vivo of the lyophilized samples was comparable to that of the fresh LNP across all storage conditions. Conclusion: We showed evidence of robust T-cell responses with a single shot mRNA-LNP vaccine, consistent with presentation of exogenous mRNA antigens in the MHC-I complex. Our mRNA-LNP was able to induce antibody responses exceeding that of a conventional OVA subunit vaccine. Furthermore, we have developed a lyophilized drug product with room temperature stability that overcomes many of the cold-chain handling limitations of currently approved mRNA vaccines.



614. Generation of Potent and High-Yield Exosome-Associated AAV (ExoAAV) Using Engineered Exosomes

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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 all patients following an initial dose (preventing re-administration). Encapsulation 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 loading AAV into exosomes using camelid nanobodies. Engineered exoAAV yields are multi-log higher than endogenously loaded exoAAV while still retaining potency and resistance to nAb characteristic of exoAAV. **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 12 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 five days before exoAAV 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 and HEK293 cells to assess transgene expression. **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. **Conclusions:** Engineering exosomes to carry an AAV-binding ligand can dramatically increase the amount of loaded AAV. We observe that many of the VHH sequences tested here were able to successfully increase AAV loading into exosomes but significantly inhibited the ability of AAV to transduce recipient cells. Out of our initial panel of 12 VHH proteins, we identified 3 distinct 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 through the use of optimized engineered exosomes.

615. Tumor-Targeted Multifunctional Exosomes as Drug Carrier for Lung Cancer Therapy

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Chemotherapy continues to be the frontline treatment for lung cancer patients. However, treatment-related toxicity and off-target effects limit the use of chemotherapy. Therefore, improvements in delivering chemotherapeutics with reduced toxicity to normal tissues is in need.

In the present study, we combined nanotechnology with exosome technology to produce tumor-targeted multifunctional exosomes (tt-Mfn-Exo) as a drug carrier for cancer therapy. The tt-Mfn-Exo was formulated by exogenously loading exosomes with gold nanoparticle (GNP) conjugated to cisplatin (CDDP) *via* pH-sensitive coordination ester linkage. Attached to the outer surface of drug-loaded exosomes is the transferrin ligand for targeting transferrin receptor (TfR) overexpressing lung cancer cells. The tt-Mfn-Exo were 138.2 nm in size, and exhibited greater drug release kinetics at pH 5.5 compared to pH 7.2. Tt-Mfn-Exo significantly reduced cell viability of A549 (TfR high) lung cancer cells compared to HCC827 (TfR low) cells and non-targeted Exo. Tt-Mfn-Exo also induced higher level of apoptosis and DNA damage in A549 and HCC827 cells compared to control groups. Finally, tt-Mfn-Exo-mediated cytotoxicity was minimal in normal MRC-9 lung fibroblast and HEK293 kidney cells compared to free CDDP. Our study showed tt-Mfn-Exo exerted selective and enhanced tumor-targeted cell killing *in vitro* and provides an opportunity for developing exosomes-based drug carriers for cancer therapy. However, testing of tt-Mfn-Exo in *in vivo* lung tumor models is warranted prior to advancing to clinical translation.

616. Cell Membrane Coating Improves In Vivo Systemic Retention and Infectivity of Oncolytic Viruses

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Oncolytic viruses have, in past studies, undergone notable failures in their late stage clinical trials, demonstrating that while they are safe, there is still a gap in the desired therapeutic efficacy. However, the solution lies beyond simply increasing the dosage or the regimens as many of the most efficacious viruses remain staunchly immunogenic and will be rapidly cleared upon administration. Initial doses will lead to the development of humoral immunity, which can dampen the response of subsequent doses. To overcome this, we have developed a cell-membrane based material, which when applied, encapsulates the oncolytic viruses within it. This generates a virus bearing nanoparticle that is indistinguishable from the cell whereby the membrane was obtained. Our preliminary studies show that the combination of material and method is virus agnostic, and works for many of the oncolytic viruses. Once coated, the virus exhibits (1) **improved immune evasion capabilities in vivo in an immune-competent mouse**, and (2) **improved infectivity against cancer cells in tumor-bearing mice**. Together, this leads to a many-fold improvement in the tumor suppression capabilities of the original virus, and provides a means to improve upon the therapeutic efficacy of the viruses without the need to make modifications to it.

617. A Novel Chimeric Nanosystem for Piggyback Delivery of mRNA Vectors

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Vaccines based upon mRNA have emerged as a transformative technology to address emerging pandemics. In this regard, the rapid development, and deployment, of mRNA vaccines for SARS-CoV-2 proved pivotal in limiting the most direct consequences of COVID-19. The most salient advantage of the mRNA vector technology is the rapidity by which a specific vaccine can be derived for a new pandemic agent. Based on this distinguishing characteristic, considerable efforts are being currently directed towards advancing the pharmacologic aspects of mRNA to improve its utility, especially for the context of emerging pandemic threats. To this point, the application of mRNA vaccines has been based upon direct delivery of the naked nucleic acid vector or a complexing of the mRNA with lipid nanoparticles (LNP). These vector platforms, however, limit the delivery of mRNA vaccines to a very restricted repertoire of routes. In this regard, key advantages of mucosal immunization have become apparent, especially for respiratory pathogens. Critically, available vector technologies for mRNA vaccine implementation are of limited utility for mucosal immunization. Thus, despite the many key advantages of mRNA vaccines for emerging infectious agents, current vector vehicles for mRNA limit the possibility of achieving the full vaccine gains that would accrue delivery via the mucosal route. To address this limit, we have progressed the technical development of a novel nanostructure in which mRNA is complexed to a vector substrate optimized for effective gene delivery for the achievement of mucosal immunization. Utilizing bioengineering principles for design and synthesis, we have enabled “piggyback” gene delivery via mRNA complexed to the capsid of adenoviral vectors (Ad) exploiting an approach based upon SpyTag/SpyCatcher “molecular glue”. In this design, the Ad component subserves two key functions relevant to the achievement of effective mucosal immunization: (1) flexible tropism determinants of the Ad component of our novel nanostructure allows enhanced gene delivery to key immune effector cells; (2) transgene expression deriving from the Ad component primes the mucosal microenvironment to facilitate effective immunization via the delivered mRNA. Here we thus advance this concept via a novel design that exploits bioengineering principles to enhance mRNA delivery while simultaneously modulating the mucosal microenvironment to induce effective immunization. The realization of our novel nanostructure will expand the repertoire of available defenses for emerging pathogens by facilitating effective mRNA vaccination via the mucosal route, which may promote new strategies for controlling respiratory and possibly other pathogens. The advent of SpyTag/SpyCatcher has proven a powerful tool for the rational advancement of our nanosystem design. Most importantly, the compelling mandates of emerging pandemics has provided a new driving impetus to advance technology that can fully capture the vaccine promise of mRNA technology.

Cardiovascular and Pulmonary Diseases

618. Derepression of the IL-10 Gene via Genome Editing Across Species Towards Clinical Translation in Lung Transplantation

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Introduction: Optimizing donor organs by genome editing is a promising approach to expand the donor pool and improve outcomes in lung transplantation. In particular, persistent immunomodulation in donor lung leveraging genome editing holds promise in ultimately obviating the need for post-transplant systemic immunosuppression after lung transplantation. Towards realizing this therapeutic vision, we explored an efficient and clinically applicable genome editing strategy to upregulate IL-10, an immunomodulatory cytokine. Given that IL-10 expression is physiologically suppressed at a steady state, we hypothesized that genetic disruption of the regulatory region of IL-10 could induce persistent upregulation. To prove this concept, we started by evaluating the efficacy of this genome editing approach *in vitro* using cell lines which are originated from animal species used in the translational path, and humans. **Methods:** We designed a series of gRNAs to bind to a locus between 1-500 bp upstream of the IL-10 gene in the genome of each species, and screened for their activities of IL-10 upregulation. *Staphylococcus aureus* Cas9 nuclease (SaCas9) and a gRNA were delivered to the human kidney cell line (HEK293), porcine kidney cell line (PK15), and rat lung epithelial cell line (L2) by plasmid transfection. The expression of the endogenous IL-10 gene after 48 hours of transfection was measured by qPCR. **Results:** Screening of five gRNAs designed for each species identified one specific gRNA that effectively upregulates the IL-10 gene. Mutating the *cis*-regulatory region of the IL-10 gene using SaCas9 nuclease and the selected gRNA significantly enhanced the expression of endogenous IL-10 gene in cell lines derived from different species; genome editing increased an 11.8±0.73-fold increase (p=0.0013) in HEK293 cells, a 134.3±49.7-fold increase (p=0.038) in PK15 cells, and a 2329±194.8-fold increase (p=0.0005) in L2 cells, compared to the negative control (Figure). **Conclusion:** We have achieved upregulation of IL-10 gene through simple mutagenesis at the regulatory region. The potential mechanism underlying this finding is derepression of the native IL-10 gene that could be conserved among species tested. Our findings could make donor organ immunoregulatory optimization possible, with clinical practicality, thus bring this innovation to the bedside in organ transplantation.

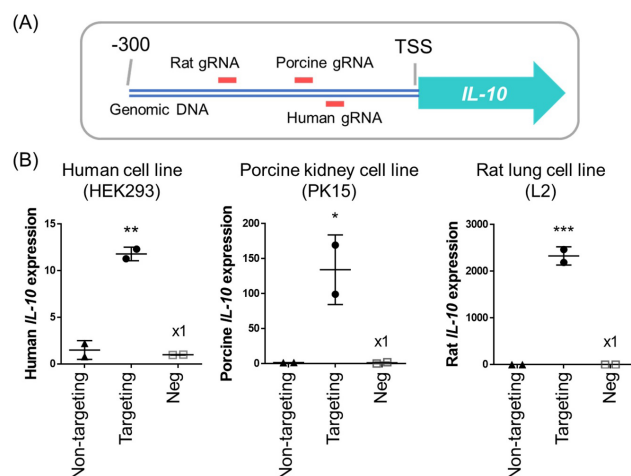


Figure. Derepression genome editing in cell lines from different species. (A) Genomic loci targeted by each gRNA. (B) SaCas9 and were delivered to human, porcine, and rat cell lines by plasmid transfection. Endogenous *IL-10* gene expression at 48 hours were measured by qPCR. One-way ANOVA followed by Bonferroni correction is used in comparison to non-targeting group. N=2, biological replicates. Symbols indicate *: $P \leq 0.05$, **: $P \leq 0.01$, and ***: $P \leq 0.001$.

619. Utilizing CRISPR-GNDM® Mediated Gene Activation of the Extra-Large Gene Titin for the Treatment of Dilated Cardiomyopathy and Other Titinopathies

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For diseases suspected to be related to haploinsufficiency mediated by a single mutant allele coding for a protein with impaired or a loss of function, enhancing the expression of the healthy allele can be a viable approach to correct the disease phenotype. Unfortunately, conventional gene therapy utilizing viral delivery of the gene product has several limitations with regards to large genes and their resulting expression constructs. Here we evaluated the ability of CRISPR-GNDM® mediated gene activation technology to target extra-large proteins which would otherwise be impossible to express by conventional gene therapy modalities. Titin is the largest human protein (> 4000kDa) and is encoded by a 363 exon gene (*TTN*) with a coding region spanning > 100,000bp. Titin's primary function is within muscle tissue where it is localized to the sarcomere and acts as a spring to the contractile unit. The size of titin renders conventional expression and delivery techniques ineffective or impossible. Titin has been implicated in a variety of diseases and phenotypes, otherwise called titinopathies, largely related to impaired titin expression or function in muscle. Mostly notably, are dilated cardiomyopathy and congenital muscular dystrophy associated with titin mutations resulting in proteins with impaired function or truncations suspected to lead to haploinsufficiency. With regards to dilated cardiomyopathy, no curative therapy exists beyond a heart transplant. Titin is therefore an ideal example of an extra-large gene which cannot be delivered to cells and overexpressed with conventional gene therapy approaches but by which CRISPR-GNDM® can potentially activate expression. We performed an epigenetic analysis of the human and murine titin regulatory regions as well as an RNA-seq analysis using

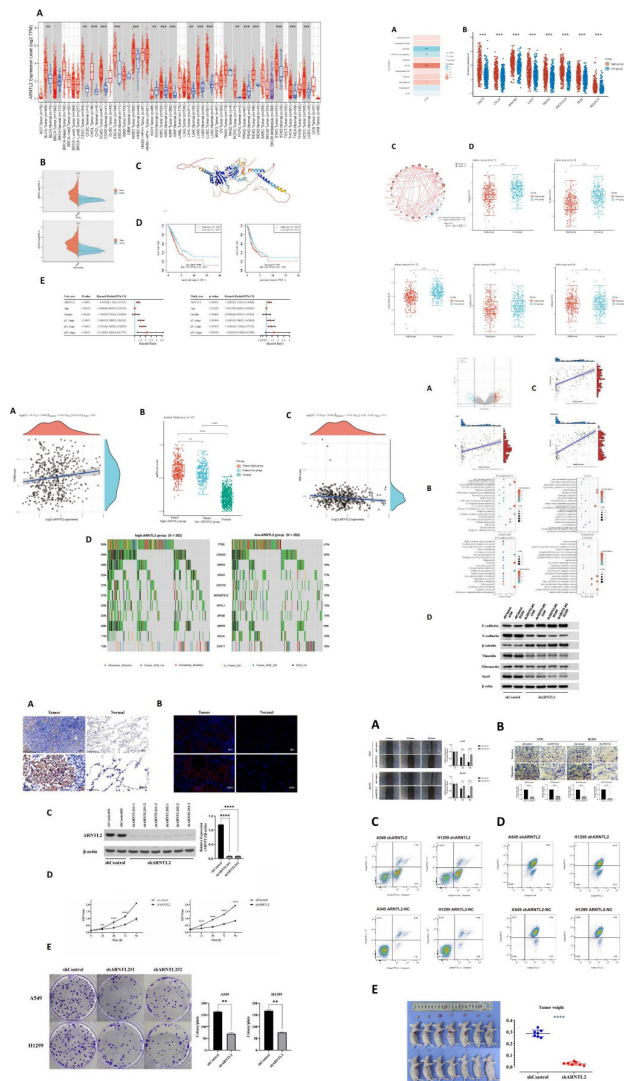
publicly available data from human heart tissue and relevant cell lines. We identified cell models with similar titin expression and isoform distribution to that of human heart tissue from healthy and dilated cardiomyopathy donors. Leveraging these data, we designed sgRNAs and conducted proof of concept CRISPR-GNDM® guide screens in HEK293, C2C12, human myotubule, and primary skeletal muscle cells to identify sgRNAs which could enhance the expression of *TTN*. In the *TTN* null expression cell line HEK293 we identified sgRNAs capable of inducing *TTN* expression > 300-fold relative to untreated baseline. Several of these sgRNA effects could then be recapitulated in *TTN* expressing cell models such as myotubule cells and primary skeletal muscle cells. These data demonstrate the ability to activate the expression of *TTN* in human cells utilizing CRISPR-GNDM®. This modality is highly advantageous as it also allows for the packaging of tissue specific promoters, CRISPR-GNDM® and candidate guides into adeno-associated virus for systemic delivery. Utilizing this technology extra-large genes like titin can potentially be activated in human cells.

620. Targeting the ARNTL2 Gene as a Potential Strategy for Lung Adenocarcinoma

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Objectives: By investigating the effects of ARNTL2 on the development, clinical features, molecular characteristics, and immune microenvironment of lung adenocarcinoma and carrying out related experiments to validate, we seek to provide a theoretical foundation for future mechanistic studies and possible personalized treatment. **Methods:** The Cancer Genome Atlas (TCGA) database's multi-omics data were downloaded using the Xena browser. Based on the expression levels of ARNTL2, patients with lung adenocarcinoma from TCGA were divided into two groups: those with high ARNTL2 expression and those with low ARNTL2 expression. AlphaFold2 predicted the structure of ARNTL2 protein. ARNTL2 was studied for its effects on lung adenocarcinoma's clinicopathological, genomic, and immunological characteristics. Furthermore, in vivo and in vitro assays were used to confirm the impact of ARNTL2 knockdown on lung adenocarcinoma cells. **Results:** ARNTL2 is highly expressed in lung adenocarcinoma and was found to be an independent predictor of a poor prognosis in patients with lung adenocarcinoma. Notably, ARNTL2 expression in lung adenocarcinoma is linked to tumor immunity, ferroptosis, MSI, and drug sensitivity. ARNTL2 could also play a role in tumor oncogenesis, metastasis, EMT, and angiogenesis. **Conclusions:** This study is the first to conduct a comprehensive multi-omics analysis of the effect of ARNTL2 on LUAD, revealing that ARNTL2 may be a potential biomarker for lung adenocarcinoma, providing a theoretical foundation for future mechanistic studies and possibly personalized treatment.



621. A High-Throughput Cell Culture Model of Idiopathic Pulmonary Fibrosis (IPF) for Gene Therapy Applications

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Idiopathic Pulmonary Fibrosis (IPF) is a chronic, progressive, and lethal disease of the lower respiratory tract. Emerging evidence suggests that repeated injury to the alveolar region could result in irreversible Epithelial to Mesenchymal Transition (EMT), activation of pulmonary fibroblasts and pathological deposition of extracellular matrix (ECM). This accumulation of ECM and fibronectin leads to stiffness in the lungs making it increasingly difficult to facilitate gas exchange, leading to respiratory failure. We would like to develop a gene therapy for IPF but the lack of suitable models to recapitulate this complex disease is limiting and the underlying cellular/molecular mechanisms remain

elusive. Use of the bleomycin-induced mouse model has identified some potential therapeutic compounds but few have shown beneficial effects in clinical trials. The standard TGF β 1-induced cell model makes only a limited contribution to the fibroblast activation signature and fails to upregulate some hallmarks of the disease including Actin Alpha 2 (*ACTA2*), relevant Mucin genes such as Mucin1 and Mucin16 (*MUC1*, *MUC16*) and Matrix Metalloproteinase 7 (*MMP7*), thought to be key effectors of collagen deposition and biomarkers of disease progression. Our goal is to generate an *in vitro* model that can better represent key features of IPF and can be used for investigating new therapeutic modalities. We previously developed an air-liquid interface (ALI) cell culture model based on human lung adenocarcinoma cell line (H441) that recapitulates human lung parenchyma markers (Munis et al 2021 PMC7782204). Here, we adapt this model to study pathophysiological mechanisms of IPF. Human H441 cells grown in transwells with basal culture media lacking dexamethasone were air-lifted to differentiate at the ALI to allow the cells to acquire a phenotype similar to alveolar Type II (ATII) cells in the lung parenchyma. An IPF phenotype was induced via exposure to cytokines relevant to tissue repair mechanisms (IL-25, IL-33, TSLP) as well as TGF β 1. RNASeq studies were performed (1E+05 cells per group, n=6 replicates) and libraries prepared and sequenced (NextSeq, Oxford Genomics Centre) using paired-end 150 bp reads. Differential expression analysis was then performed to compare the induced samples (IL-25, IL-33, TSLP \pm TGF β 1) against the naïve control. As expected, RNAseq data revealed that addition of TGF β 1 can upregulate some key pathways involved in the progression of IPF such as EMT, Wnt and Notch. Importantly, the addition of type 2 cytokines (IL-25, IL-33, TSLP) upregulated many hallmarks of the condition including: *ACTA2*, *MUC1*, *MUC16*, *PCOLCE* and *FOXA1* genes (Log2 fold change 0.42, 0.72, 1.56, 0.46, and 0.29, with adjusted p-values of 4.50E-02, 6.12E-15, 5.91E-17, 2.67E-04, and 1.05E-04 respectively) compared with the naïve control, which TGF β 1 alone failed to upregulate. Interestingly, with addition of type 2 cytokines there was also a trend for upregulation of MMP7 (log2 fold change 0.35), which is a profibrotic mediator and important predictive biomarker for IPF disease progression. Key changes in RNA levels are currently being validated by qPCR assay through the $2^{-\Delta\Delta CT}$ method and protein levels such as fibronectin will be assessed by western blot. In summary, a well characterised and patho-physiologically relevant model is needed for understanding IPF. Our human ALI culture model of lung parenchyma provides a new platform for modelling aspects of the formation and progression of IPF at the molecular (RNA and protein) level. It may also provide an opportunity to develop opportunities for screening of novel antifibrotic agents and validating gene therapy interventions.

622. Developing a DWORF Micropeptide Gene Therapy for Heart Failure

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Heart failure is a prevalent and chronic disease in which the heart cannot pump enough blood to meet the body's needs. Defective intracellular calcium homeostasis with reduced SERCA (sarco/endoplasmic reticulum calcium-ATPase) activity plays a crucial role in contractile

dysfunction in the failing myocardium. Dwarf open reading frame (DWORF) is a muscle-specific micropeptide that enhances SERCA activity by displacing SERCA inhibitors such as phospholamban (PLN). Indeed, overexpression of DWORF in transgenic models as well as with delivery using adeno-associated virus (AAV) has been demonstrated to correct calcium cycling abnormalities, cardiac contractility defects, and reverse adverse ventricular remodeling in multiple mouse models heart failure, highlighting the potential of DWORF as a therapeutic for heart failure. Tenaya Therapeutics has developed a series of AAV cassettes designed to express varying amounts of DWORF at a given viral dose and demonstrated the tolerable threshold of DWORF expression in healthy mice. We have tested our gene therapy candidates in a well-characterized dilated cardiomyopathy (DCM) mouse model due to genetic deletion of the muscle-specific LIM domain protein (MLP). AAV-delivered DWORF mitigated the contractile dysfunction and attenuated pathological remodeling in this DCM models. AAV:DWORF cassettes expressing higher, but well-tolerated, levels of DWORF supporting the greatest degree of efficacy that was durable out to 24 weeks. Our preclinical results suggest DWORF gene therapy may represent a novel means of normalizing calcium homeostasis and limiting progression.

623. Strategies to Increase Endothelial Cell-Specific Expression of an APOAI Transgene for Gene Therapy of Atherosclerosis

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Background: Gene therapy for atherosclerosis requires high-level transgene expression to increase therapeutic efficacy and to allow use of lower vector doses (minimizing vector-related toxicity). We previously showed that transduction of endothelial cells (EC) with a helper-dependent adenoviral (HDAd) vector that expresses apolipoprotein A-I (APOAI; a protein that initiates cholesterol removal from blood vessels) from the CMV promoter can prevent and reverse atherosclerosis in carotid arteries of fat-fed rabbits. However, the effects on atherosclerosis were partial and need to be increased before clinical application. Here we tested several strategies to increase APOAI expression from HDAd: use of EC-specific expression cassettes based on the *EDN1* gene (these cassettes provide EC-specific expression); addition of enhancer sequences reported to be active in EC; addition of computationally identified EC-specific cis-regulatory modules (CRMs) to the *EDN1*-based cassettes; and insertion of the rabbit APOAI gene at the transcription start site (TSS) of genomic sequences associated with genes that are highly expressed in EC. Methods: We searched the literature to identify sequences that increase transcription in EC and to identify genes that are highly expressed in EC. We then used a bioinformatics-aided search of the human genome to identify cis-acting sequences that enhance transcription of the highly expressed EC genes. We focused on transcriptionally active sequences within 100 kb of the TSS of four genes that are highly and preferentially expressed in cultured EC (*CDH5*, *EFEMP1*, *THBS1*, *VWF*). These sequences were cloned from human DNA and inserted upstream of our *EDN1*-based expression cassettes that express rabbit APOAI. We also constructed

expression cassettes in which the rabbit APOAI gene was inserted into the TSS of the 4 highly expressed EC genes. The new expression cassettes were screened *in vitro* by plasmid transfection into bovine aortic EC. Some were screened by plasmid transfection into human aortic EC and human umbilical vein EC. Some of the cassettes were also incorporated into HDAd vectors and tested *in vivo* in rabbit carotid arteries. Results: Addition of a known shear-stress-response element did not increase APOAI expression either *in vitro* or *in vivo*. Addition of 1-5 copies of a *Mef2c* enhancer sequence increased APOAI expression from a cassette containing a minimal *EDN1* promoter/enhancer sequence; however, adding even one copy of the *Mef2c* enhancer to an expression cassette that contained a larger and more-highly expressing *EDN1* promoter/enhancer sequence decreased APOAI expression. The bioinformatics-based strategy identified 11 potential CRMs (55-352 bp). Three of the CRMs increased APOAI expression from the minimal *EDN1* promoter/enhancer in at least 1 of 3 types of EC (2-7-fold; $P < 0.05$); none of the CRMs increased expression from the larger *EDN1* promoter/enhancer. Insertion of the rabbit APOAI gene into the TSS of genomic sequences of genes highly expressed in EC did not increase expression above levels obtained with the minimal *EDN1* promoter/enhancer. Conclusions: Addition of known transcriptionally active sequences did not reliably increase APOAI expression. An unbiased bioinformatics-based approach identified CRMs that increased expression from a minimal *EDN1* promoter/enhancer. Our current expression cassette containing the *EDN1* promoter and multiple *EDN1* enhancers outperforms all of the new experimental cassettes, affirming its value. Future strategies that modify our bioinformatics-based approach based on new *in vivo* EC-specific expression data from scRNA-seq studies may have promise for obtaining higher levels of HDAd-mediated APOAI transgene expression *in vivo* in EC.

624. Allele-Specific Silencing as a Potential Therapeutic Strategy for Treating Restrictive Cardiomyopathy with Non-Truncating FLNC Variant

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Restrictive cardiomyopathy (RCM) is among the rarest forms of cardiomyopathy with a poor clinical prognosis. Genetically determined RCM is associated with mutations in sarcomeric and cytoskeletal muscle proteins. Among the recently identified genetic causes is actin-binding protein filamin-C (gene *FLNC*) which is crucial for sarcomere integrity and Z-line organization. *FLNC*-associated RCM is characterized by a high risk of sudden children's death

due to heart failure. A unique *de novo* heterozygous *FLNC* variant c.7416_7418del was recently identified in a Russian child with RCM. The mutation leads to the substitution of two amino acids for one (p.Glu2472_Asn2473delinsAsp) in the ROD2 domain of the protein. Pathomechanism of these non-truncating mutations in the ROD2 domain is presumably mediated through protein misfolding and aggregate formation. In this study, we aim to develop a personalized gene therapy strategy for the RCM patient with *FLNC* c.7416_7418del. Considering the likely dominant-negative effect of the mutation, we propose allele-specific silencing of a mutant *FLNC* allele through AAV-mediated RNA interference as a potential therapeutic approach. We designed seven shRNA constructs targeting mutant *FLNC* and screened them in a dual-luciferase reporter assay. Efficacy and allele-specificity of shRNA sequences were verified in the hemizygous test system with the full-length *FLNC* protein expression. Based on the performance in both assays, two shRNA lead candidates were selected, further optimized, and moved forward for validation in patient-specific iPSC-derived cardiomyocytes. To ensure efficient delivery, shRNA vectors were packaged in engineered serotype AAV-DJ, which outperformed natural serotypes 1, 2, 6, and 9 in the transduction of cardiomyocytes *in vitro*. Following treatment with control *FLNC*-directed AAV-shRNA, the endogenous *FLNC* was downregulated by 90% at the mRNA and protein levels. In turn, allele-selective AAV-shRNAs reduced the abundance of mutant *FLNC* transcript in cardiomyocytes by more than 50% and only moderately affected wild-type *FLNC* (up to 25%). To sum up our pilot study, AAV-RNAi is a feasible strategy for the suppression of *FLNC* c.7416_7418del in the patient's cardiomyocytes, though further optimization of the constructs is required. Testing our approach in the animal models is critical for obtaining evidence that reduction of the mutant *FLNC* is sufficient to slow down the cardiomyopathy progression.

625. Development of Novel Vectors Based on Human Adenovirus Serotype 4 as an Alternative to Adenovirus Serotype 5

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Adenoviral vectors are among the most frequently used vectors for gene therapy and cancer treatment. Most adenoviral vectors were derived from human Adenovirus (AdV) serotype 5. But its applicability is limited due to frequent preexisting immunity within the human population and unfavorable liver tropism upon systemic delivery. Apart from few exceptions, the other 103 known human serotypes remained largely unused. To study potential use of other AdV serotypes we established and screened a library of reporter gene expressing AdVs representative of the natural human AdV diversity in order to identify novel vector candidates with favorable features for gene- and or tumor therapy based on these naturally occurring serotypes. The infectivity and transgene expression of different AdV serotypes was assessed by transducing various Human Papillomavirus (HPV) induced cancer cell lines, as well as established cardiac cell lines, iPSCs derived cardiac cells

and primary neonatal cardiac cells. 24-48 hours post transduction we quantified AdV mediated GFP fluorescence or luciferase activity within the cells. To further test oncolytic activity, we measured cell viability in cancer cell lines. Chosen candidate or oncolytic vectors were tested on HPV induced cancer cells derived mini-organoids, which better mimics the three-dimensional tumor environment than monolayer cultures. We found that AdV4 transduced and expressed its reporter genes more efficiently in cardiac cells than AdV5. Moreover, despite low transduction efficiency, AdV4 showed some oncolytic activity, efficient gene expression, genome replication and progeny virus production in tumor organoids derived from HPV related cancer cell lines. Based on the previous findings we constructed a first generation AdV4 vector platform by serial deletion of the E1 and E3 genes using recombeneering technology. The E1-E3-deleted AdV4 vector is now able to package transgenes of up to 6.5kb. Subsequently we were able to rescue the E1 and E1-E3-deleted AdV4 vectors by serial passaging in HEK293 cells, whereas other cancer cell lines including Hela and Siha were not permissive. Therefore, we assume that AdV5 E1, that is expressed by HEK293 cells can complement for AdV4-E1, enabling the rescue of E1-deleted AdV4 vectors. The E1 and E1-E3-deleted vectors transduced murine cardiac cells as well as the parental vector. In order to increase AdV4 uptake into Papillomavirus induced cancer cells but retaining its proliferative ability, we replaced the AdV4 fiber knob with the AdV5 fiber knob. However, pseudotyping did not alter vector infectivity nor replication. E1-E3-deleted AdV4 vectors could be useful tools for improved gene delivery to the heart. Moreover, due to its ability to replicate in HPV induced cervical cancer tumor spheroids we suspect them to be useful as tumor gene therapy/oncolytic vectors for these tumors. Future work will focus on testing different applications for cardiac gene therapy such as biological pacing as well as oncolytic/tumor gene therapeutic applications for these new AdV4 vectors. We hope that our work will lead the way for the development of further improved vectors based on a broad range of different AdV serotypes for various applications.

626. 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 of novel AAV capsids with improved properties. We have established an in-house AAV capsid engineering platform and successfully screened over 30 diverse, proprietary AAV libraries (rational design, peptide insertion, variable region, chimeric, scanning, etc.) representing more than one billion of unique capsids in multiple *in vitro*, *in vivo*, and *in silico* models to discover novel AAV capsids that can target the different

types of cells in the heart through different routes of administration. Targeting cardiomyocytes following systemic delivery is critical to enable gene therapy treatments for many cardiac conditions and AAV9 has become the workhorse capsid for this type of gene transfer. By combining directed evolution and candidate validation in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), mice, and non-human primates (NHPs), our initial efforts identified a proprietary capsid, TNC-CM3, that has three-fold better cardiomyocyte transduction measured by pooled RNA-based assay in NHPs and eight-fold better heart transduction measured by individual protein-based assay in mice, as well as better heart-to-liver transduction ratio in both NHPs and mice, compared to AAV9. Moreover, TNC-CM3 boosts efficacy of MYBPC3 gene therapy in a *Mybpc3*^{-/-} model of disease at the clinically relevant dose of 3E13 vg/kg compared to AAV9. Our more recent efforts include directed evolution in NHPs with AAV libraries built on multiple parental capsids and have identified next-generation capsids with further improved properties including two-fold better cardiomyocyte transduction on top of TNC-CM3 and greater than ten-fold heart-to-liver transduction ratio improvement compared to AAV9. Together, these superior novel AAV capsids will enable more efficacious and safer gene therapies for cardiac disorders.

627. Development of 3D In Vitro ACE2-Overexpressing Models for SARS-CoV-2 Kinetics Study

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In spite of the vast widening of scientific knowledge regarding COVID-19 since the beginning of the pandemic, adequate therapeutic approaches to the disease are still lacking. The influence of the ACE2 receptor, which composes viral entrance into human cells, for example, has not been completely understood yet, which hinders the establishment of solid therapeutic solutions. Our work aims to bridge this gap by proposing an innovative model of study of SARS-CoV-2 infection in pulmonary bronchial epithelial (BEAS-2B) cells. 3D cell cultures were selected as our main study method, as these models, in contrast with 2D, precisely represent the in vivo environment, providing more data accuracy in terms of cell-to-cell interactions, response to stimuli, and drug metabolism. Moreover, it represents a study strategy that bypasses the usage of the laborious, complicated and scarce animal models. We introduce the development of spheroids, the most promising scaffold-free model. To investigate SARS-CoV-2 infection in pulmonary cells' spheroids, we created BEAS-2B overexpressing ACE2 (BEAS-ACE2) through nucleofection with pCEP4-myc-ACE2 (Addgene #141185). Spheroids were developed using micromolded nonadhesive agarose hydrogel and were characterized over time. Live and dead assay with calcein and 7AAD was performed to quantify the viability of the spheroids. After characterization, the spheroids were infected with SARS-CoV-2 (0.2 MOI) isolated from a Brazilian patient (EPI_ISL_413016). Viral load was quantified by qPCR. Spheroids were monitored for 14 days. BEAS-ACE2 spheroids exhibited increased radius (1.4 fold, $p \leq 0.01$) and higher volume at day 1 (2.3 fold,

$p \leq 0.0001$), 7 (1.9 fold, $p \leq 0.05$) and 14 (2.5 fold, $p \leq 0.001$). Sphericity was very similar between the groups over time (Figure 1). Interestingly, the area of BEAS-2B(2D) was 1.25 fold ($p \leq 0.05$) higher than BEAS-ACE2. Both groups presented similar viability one day after cultivation (Figure 2). After SARS-CoV-2 infection, BEAS-ACE2 spheroids presented a 640 fold higher intracellular viral load ($p \leq 0.01$) than control (Figure 2), which, therefore, demonstrates that the ACE2 receptor positively influences SARS-CoV-2 interaction with pulmonary cells. Altogether, our results show that spheroids are a suitable model for SARS-CoV-2 kinetics study. Our data have the potential to enhance the yet limited comprehension of COVID-19 harm on the organism, paving the way towards the development of drugs that restrict disease progression.

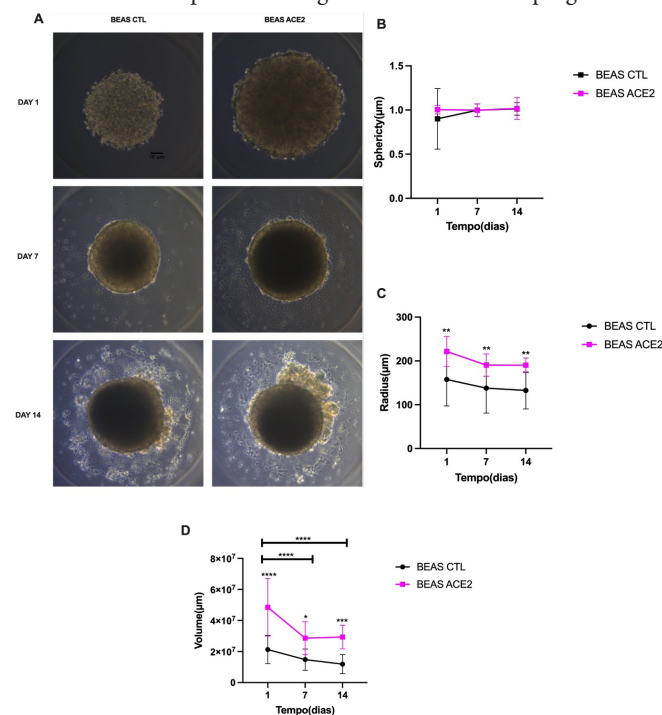


Figure 1 | Characterization of pulmonary BEAS-CTL and BEAS-ACE2 spheroids. Morphology data showing bright field images of the spheroids on days 1, 7 and 14. (A) Comparison of spheroids' sphericity (B) radius (C) and volume (D). Measures were determined using ImageJ. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale bar: 50µm

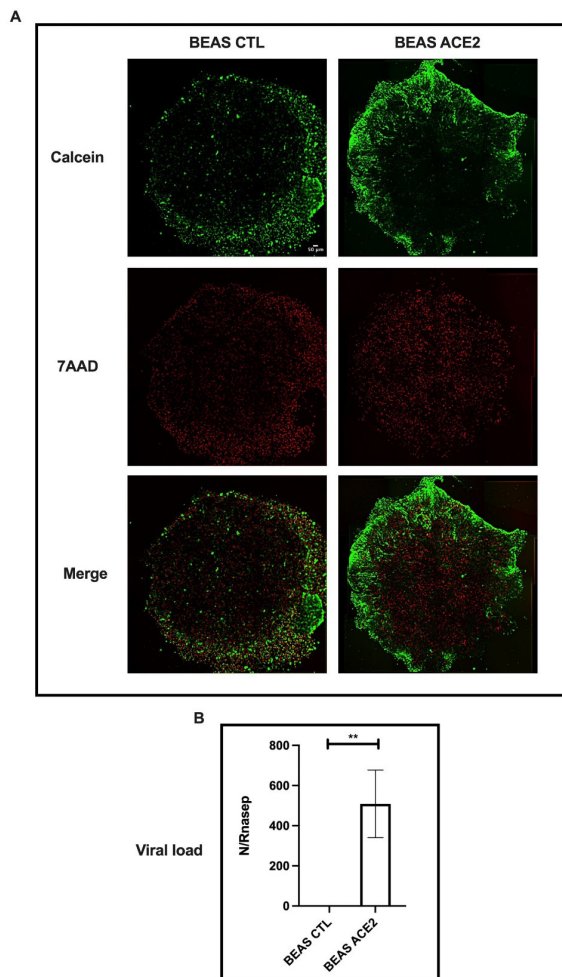


Figure 2 | Cell viability assay and intracellular viral load of BEAS-CTL and BEAS-ACE2 spheroids (A) Live and dead assay of the spheroids (B) Viral load of the spheroids. Scale bar: 50 μ m. ** $p \leq 0.01$

628. Optimizing Cardiac Gene Therapy for CPVT

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BACKGROUND: Catecholaminergic polymorphic ventricular tachycardia (CPVT), is an inherited cardiac arrhythmia characterized by adrenergically triggered ventricular tachycardias, often leading to sudden death. Current medical care is inadequate in treating all CPVT effectively and preventing recurrent life-threatening arrhythmias. CPVT usually arises from gain-of-function mutations in the Ryanodine receptor 2 (RyR2), which governs the release of calcium from the endoplasmic reticulum, essential for excitation-contraction coupling in the heart. RyR2 mutations are unmasked in the presence of catecholamines released during stress, thus activating downstream kinases that enhance cardiomyocyte Ca^{2+} handling by phosphorylating

numerous targets. Among these kinases is Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). Here, we optimize CaMKII inhibitor gene therapy to suppress arrhythmia in CPVT mouse models to further advance gene therapy for CPVT from proof-of-concept towards clinical translation. **METHODS/RESULTS:** Building on a previous proof-of-concept vector, we optimized each component for improved expression, enhanced CaMKII inhibition and reduced off-target effects. We optimized promoter elements to restrict expression to the heart using a competitive expression paradigm. The combination of a CASQ2 enhancer with the full cTnT promoter demonstrated superior expression in the heart with little extra-cardiac expression. We then assessed the *in-vivo* efficacy of six inhibitors based on two parental inhibitory peptides, AIP and the high potency peptide CN19o. The inhibitors were expressed in cardiomyocytes using AAV9, a potent cardiac-tropic virus. Arrhythmia burden was evaluated in mice with a known CPVT mutation (RyR2R4650I/+) by non-invasive electrophysiology testing. We identified an optimized AIP derivative with superior arrhythmia suppression without significant proarrhythmic effect, suggesting that peptide optimization is critical for improved efficacy. The effects of our peptides on CaMKII inhibition were tested in *in-vitro* kinase assays with purified single and optimized inhibitors. Finally, we selected the optimized AAV capsid by barcoded transgene expression in transduced animals. **CONCLUSIONS:** Previous work showed that AAV-mediated delivery of a CaMKII peptide inhibitor to the heart was effective in suppressing arrhythmias in a murine model of CPVT. In this study, we further optimize CaMKII inhibitor gene therapy for CPVT by optimizing the promoter, inhibitor, and capsid.

629. Nanoparticles for Correction of a Rare Cystic Fibrosis Variant

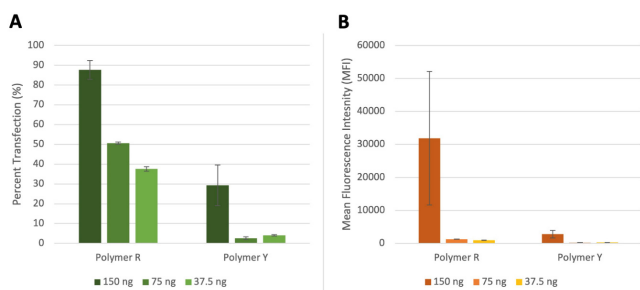
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Cystic fibrosis (CF) is a life-shortening genetic disorder characterized by defective function of the cystic fibrosis transmembrane receptor (CFTR) anion channel, leading to altered mucous rheology. Current treatments for CF primarily focus on modulator drug therapies designed to correct malfunctioning CFTR protein. However, these modulators are ineffective for the 10% of CF individuals with variants that do not allow protein production. Among these is the splice variant 3120+1 G to A, the second most common CF-causing mutation in African Africans. Correction of this variant by genome editing technology would allow production of CFTR protein and enhancement of function using available CFTR modulators. In CRISPR-based approaches, a double strand break can lead to indel formation and off target genome instability. We propose an alternative method using modified CRISPR-Cas9 base editors which initiate repair by a single-strand nick, allowing for lower off target effects and more efficient editing. Nanoparticle-mediated delivery of mRNA has been shown to be highly successful, with safety and efficacy for systemic delivery as demonstrated by the next-generation SARS CoV-2 vaccines. The newly developed polymer R enables high transfection efficacy in a

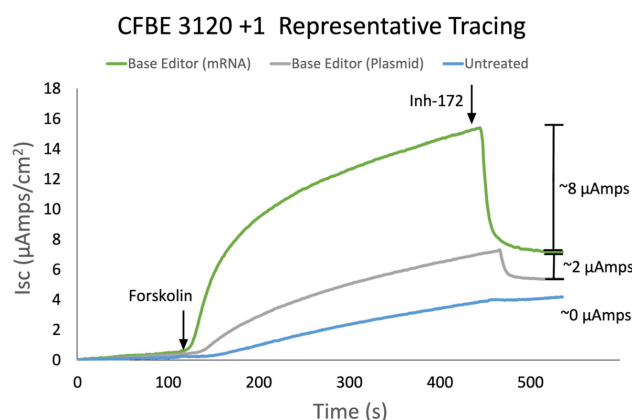
CF bronchial epithelial (CFBE) cell line bearing an expression minigene with the 3120+1 variant. Transfection efficiency, cell viability and mean fluorescence intensity (MFI) were tested at three dosages (150, 75, 37.5 ng mRNA) and four w/w ratios (60, 40, 30, 20) across four different formulations. An optimal and nonoptimal subset of these are shown in **Graphic 1**. Our current formulations achieve greater than 85% transfection (**Graphic 1a**) with 80% viability. MFI was used to quantify expression, showing Polymer R achieves expression nearly 100X higher than other formulations (**Graphic 1b**).

Graphic 1



Reconstitution of CFTR function can be measured by short circuit current. After stabilization of transepithelial current, forskolin is added to stimulate activation of CFTR, followed by administration of CFTR inhibitor-172 to block CFTR-mediated currents. The corresponding drop in current is a CFTR-specific measurement of function. **Graphic 2** demonstrates significant gain of function in CFBE cells transfected with Polymer R nanoparticles, base editor, and guide RNA when compared to untreated cells.

Graphic 2



We have been able to demonstrate an optimized nanoparticle formulation containing base editor and guide RNA can correct splicing of isogenic cells bearing the 3120+1 variant, resulting in recovery of CFTR function. Next steps will optimize delivery and editing in primary human nasal & bronchial epithelial cells bearing the 3120+1 variant. If successful, our work could inform development of revolutionary therapies for minority CF patient populations who currently have no treatment options.

630. Cardiac AAV:PKP2 Gene Therapy Reduces Ventricular Arrhythmias, Reverses Adverse Right Ventricular Remodeling, Improves Heart Function, and Extends Survival in a *Pkp2*-Deficient Mouse Model of Arrhythmogenic Right Ventricular Cardiomyopathy

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a familial cardiac disease associated with ventricular arrhythmias and an increased risk of sudden cardiac death. ARVC has an estimated prevalence in the general population of 1:2000 with the mean age of presentation before the age of 40 years old. ARVC is an important cause of sudden cardiac death in young people. Currently there are no approved treatments that address the underlying cause of this disease. Desmosome gene *Plakophilin-2* (*PKP2*) is the most frequently mutated gene responsible for 45% of ARVC cases. Our goal is to examine whether adeno-associated virus (AAV)-mediated delivery of the wild-type (WT) transgene prevents ARVC development in a cardiac specific knock-out mouse model of *Pkp2*. Both *PKP2*-deficient human iPSC-derived cardiomyocytes and a *Pkp2-cKO* mouse model were developed to identify the molecular, structural, and functional signatures that recapitulate human disease phenotypes. A single dose of cardiac AAV:PKP2 gene delivery significantly improves life span of *Pkp2-cKO* ARVC mice by 1) restoring expression of desmosome components; 2) preventing and reversing adverse right ventricular remodeling; 3) maintaining and improving ventricular functions; 4) preventing cardiac fibrosis and 5) reducing ventricular arrhythmia event frequency and severity. Safety evaluation of AAV:PKP2 in WT mice shows no adverse effects on cardiac functions and no changes in tissues examined. Our preclinical results suggest that cardiac AAV:PKP2 gene therapy may be a promising therapeutic approach to treat ARVC patients with *PKP2* mutations, which Tenaya is currently advancing into IND-enabling studies.

Neurologic Diseases II

631. Development of Platforms to Target HTT In Vivo Using Base Editing Technologies

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Huntington's disease (HD) is an autosomal dominant disorder characterized by a progressive decline in cognitive and motor function that affects ~1 in 10,000 individuals and is caused by the expansion of a polyglutamine-encoding CAG repeat within exon 1 of the huntingtin (HTT) gene. The expansion of the CAG repeats leads to the formation of mutant protein aggregates in the brain, which are toxic for medium spiny neurons in the striatum and cortical neurons. Deletion of the mutant HTT (mHTT) gene in rodent models of the disease can reverse HD-associated abnormalities and halt disease progression. This can be accomplished with CRISPR-Cas nucleases, a gene editing approach that can effectively introduce targeted modifications in genomic DNA of living cells. However, their reliance on creating double stranded breaks (DSBs) in DNA to achieve editing limits their therapeutic potential due to safety concerns associated with DSBs. Alternatively, base editors (BEs) consisting of a deaminase domain fused to a Cas9 nickase, circumvent this problem by their ability to introduce C>T or A>G nucleotide conversions at targeted sites without creating DSBs. While BE tools show great promise for therapeutic applications, their large size poses a challenge for in vivo delivery by adeno-associated virus (AAV), a non-integrating viral vector system commonly used in gene therapy capable of infecting a wide range of cell types but with limited packaging capacity. We have overcome this problem by utilizing a split-BE architecture that is compatible with AAV technology and capable of in vivo base editing. In this work, we demonstrate the application of split-BEs for editing the human HTT gene in a mouse model of Huntington's disease. It has been postulated that cleavage of the mHTT protein at the predicted caspase-6 cleavage site ("EIVLDG" motif) spanning the exon 12-13 junction generates highly toxic mHTT derivatives that are responsible for the development of symptoms characteristic of HD. Our hypothesis is that skipping exon 12 or 13 of the HTT gene by base editing will decrease mHTT toxicity. We tested this hypothesis by first creating base editing systems tiling the splice acceptors of HTT exons 12 and 13, analysing the editing rates in genomic DNA and then characterizing the HTT splicing patterns resulting from modifications introduced with the most efficient base editing systems. Further, we delivered the corresponding split-BE by AAV to the striatum of a mouse model of HD via stereotactic injection and quantified the molecular and cellular changes resulting from base editing. Our results demonstrate that BEs can efficiently edit the splice acceptor

of HTT exon 13 in cultured cells, leading to HTT exon 13 skipping, which effectively removed the caspase 6 cleaving site from HTT. In vivo, while AAV9 efficiently transduced striatal neurons at the site of injection, the overall transduction of the striatum was limited. Nonetheless, our data shows that this approach facilitated survival of edited neurons, which was supported by increase of editing rates at the target site in striatal neurons at 10 months post-injection. Collectively, these results illustrate the potential of our AAV encoded split-BE technology to permanently reduce mHTT protein in vivo and promote the survival of striatal neurons.

632. Identification of Arfaptin-2 as a Potential Therapeutic Target for Amyotrophic Lateral Sclerosis (ALS) Using iPSC-Derived Motor Neurons and Zebrafish as Models of ALS

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Rationale and Hypothesis: The pathogenesis of ALS is multifactorial; however, a key pathological feature is the presence of neuronal protein aggregates. Studies in Huntington's disease (HD) show that Arfaptin-2 (ARFIP2) localises in aggregates and regulates protein aggregation. Furthermore, its C-terminal (HC-ARFIP2), or the phosphorylation of Ser²⁶⁰ on the C-terminal, exhibits neuroprotective effects. Preliminary data in our lab showed that in ALS, ARFIP2 colocalized with SOD1 in aggregates whereas expression of its C-terminal showed a diffuse distribution throughout the cytoplasm. Furthermore, it was shown that HC-ARFIP2 significantly enhanced motor neuron survival. Moreover, the pathway by which HC-ARFIP2 may exert neuroprotective effects showed the involvement of the Akt pathway and autophagy. This indicates that HC-ARFIP2 may rescue proteasome activity, at least partially through autophagy. Consequently, we hypothesize that ARFIP2 may be a potential therapeutic target for ALS and expressing HC-ARFIP2 may ameliorate the disease phenotype. **Methodology and Results:** To assess the expression and distribution of endogenous ARFIP2 in control and ALS biosamples, we quantified its expression in C9ORF72-iPSC-derived motor neurons (MNs) and human brain tissue sections. Analysis of data obtained using iPSC-derived MNs showed that the levels of endogenous ARFIP2 are elevated in ALS (n=3, 3 technical repeats). Human cerebellum sections and neocortex sections from the frontal lobe stained for the expression and distribution of ARFIP2 showed that the ALS sections were stained slightly darker, especially in the Purkinje cells. Analysis of the sections showed that the intensity of the staining for endogenous ARFIP2 was significantly higher in the neocortex of sporadic ALS patients and those with the C9ORF72 mutation and in the cerebellum for those with C9ORF27 mutation, compared to healthy controls. With the HC-ARFIP2 showing neuroprotective properties, we sought to assess whether HC-ARFIP2 affects disease development *in vitro* and *in vivo* in models of ALS. For *in-vitro* analysis, a lentivirus expressing HC-ARFIP2 (LV-HC-ARFIP2) was made and optimised for efficient delivery to C9ORF72-iPSC-derived MNs. iPSC-derived MNs transduced with the LV-HC-ARFIP2 showed a dose-dependent expression of the C-terminal of ARFIP2 in all cell lines while the cells still looked healthy at 4 days post-transduction.

Assessment of the effect on toxic dipeptide repeat proteins (DPRs) in C9ORF72-iPSC-derived MNs showed that when treated with LV-HC-ARFIP2, the amount of DPRs in ALS decreased by 70%, to levels observed in control healthy patient lines (n=3, 3 technical repeats). For *in-vivo* analysis using zebrafish, transgenic lines expressing the shorter variant of the protein have been successfully established. These transgenic lines will be crossed to C9ORF72 zebrafish models to determine the effect of HC-ARFIP2 on the pathogenicity and motor performance in zebrafish. **Conclusion:** Results obtained indicate an elevated level of endogenous ARFIP2 in ALS and support our hypothesis for targeting this protein in ALS. Furthermore, the C-terminal of ARFIP2 is believed to exert neuroprotective effects and the data on DPRs in ALS suggests that HC-ARFIP2 may ameliorate this phenotype in our *in vitro* model.

633. Patient-Derived iPSC-Neurons and Cerebral Organoid for Modeling Friedreich's Ataxia Disease

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Friedreich ataxia (FA) is an autosomal recessive neurodegenerative disease. Most patients carry homozygous GAA expansions in the first intron of the frataxin gene causing reduction in frataxin (*FXN*) expression. *FXN* is a mitochondrial protein, and its deficiency leads to mitochondrial iron overload, defective energy supply and generation of reactive oxygen species. In this study, our aim is to establish a new human model system to advance the understanding of FA pathogenesis. We differentiated multiple FA patient-derived induced pluripotent stem cell (iPSC) lines to neurons, and cortical organoids as well as control lines. All iPSC lines have been characterized using well-established immunostaining assays and were found to be karyotypically normal. Interestingly, when we differentiated iPSCs into neurons, we observed a striking phenotype in *FXN*-deficient neurons. We identified no homogenous microtubule staining along the neurites or in neuronal cell bodies of FA neurons, and instead noted periodic breaks of neuronal immunostaining called blebbing while only few blebbing could be observed in the control cell lines. Similar results were obtained in the FA cortical organoids as compared to the control organoids. Dendritic blebbing is a feature of apoptotic neurons. In addition, Cleaved-caspase-3, a marker for apoptosis, was increased in both FA neurons and organoids compared to controls. Mitochondrial function was also found abnormal in these two FA models. Overall, this study provides a platform to advance the understanding of FA pathogenesis.

634. Ultra-Sensitive MR Tracking of AAV Gene Therapy with Enzymatically Activated MR Probes

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AAV gene therapy has shown transformative outcomes in treating lysosomal storage diseases (LSDs). For most LSDs, restoration of 10%-20% of normal enzyme level is sufficient to correct most aspects of the disease in affected organs. Considering that, enzyme levels in blood do not reflect levels of central nervous system (CNS) or even peripheral tissues, the field lacks a non-invasive way to assess magnitude and duration of gene expression in target organs. We previously reported measurement of β -galactosidase (β -gal) enzyme activity after AAV gene therapy encoding lysosomal β -gal using an MR-based β -gal responsive probe in CNS and periphery of GM1 (β -gal^{-/-}) mouse. This gadolinium-based probe has an exposed arm that is the enzymatic target of the therapeutic, which, upon cleavage, results in MR signal enhancement. Strong correlations were shown after AAV treatment between enzyme activity and MR enhancement in brain (AAV:1E10 vg, thalamus; Contrast: 1.5 μ mol, intrathecal) and liver (AAV:3E11 vg, intravenous (IV); Contrast: 5 μ mol, intraperitoneal (IP)) of GM1 mice. β -gal levels up to 100X of wild type (WT) were detected. Here we report application of a new agent for detection of β -glucuronidase (β -gluc) and a more sensitive version of the β -gal probe, enabling detection of enzyme levels well below normal. For β -gal, dose-response studies are underway to determine the optimal dose of contrast and time required for optimal enhancement after cerebrospinal fluid (CSF) administration (0.6 μ mol-0.15 μ mol, intracerebroventricular (ICV)). Parenchymal penetration of contrast agent increases in uniformity ~120 min after injection (Figure: A-G). The optimal dose of contrast agent for CNS detection is being tested in heterozygote β -gal^{+/-} (50% WT activity) and in AAV treated β -gal^{-/-} mice (6E10 vg ICV or 3E11 vg IV). Preliminary data indicates an optimal contrast agent dose between 0.6 μ mol and 0.3 μ mol. Similarly, the enhancement properties of the contrast agent in peripheral organs, will be determined in β -gal^{+/-} and AAV-IV treated β -gal^{-/-} mice (1E12; 5E11; 1E11 vg IV). Administration of IV-contrast is a clinically relevant method, and we expect to need lower doses of contrast as compared to IP-contrast (5 μ mol) used in previous study. For the β -gluc probe, enhancement of peripheral organs in WT mouse showed a similar enhancement profile as the β -gal probe after IP injection in preliminary experiments (Figure: H-K). Experiments in AAV treated β -gluc^{-/-} mice are planned and contrast will be administered IV for β -gluc probe. These data show that this class of contrast agents can be modified to interact with active sites of other enzymes independent of treatment approach. Considering the number of gene therapies in phase I/II clinical trials, development of non-invasive methods to assess efficacy of gene therapy is crucial to the future of the field.

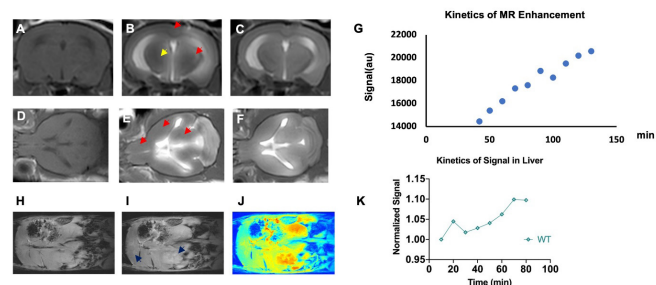


Figure. Activation of contrast in WT mouse. **A-G) Brain, β -gal probe** (0.3 μ mol). **A&D)** pre-contrast, **B&E)** 40 min, **C&F)** 120 min post ICV-contrast injection. Arrows point to regions showing uniform

enhancement in C&F at ~ 120 min. G) Kinetics of MR enhancement, 40-120 min post contrast, in region shown by yellow arrow in B. **H-K) Liver, β -gluc probe.** H) pre, I) post IP-contrast ~ 90 min. Arrows point to enhanced regions in abdomen. J) heat map of WT abdomen ~ 90 min post contrast. K) Kinetics of MR enhancement, liver 10-90 min post contrast.

635. Developing Allele-Specific Gene Editing Technologies for Silencing or Precise Correction of GFAP Mutations Causing Alexander's Disease

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Alexander disease (AxD) is an autosomal dominant neurodegenerative disorder caused by missense mutations in the glial fibrillary acidic protein (GFAP) gene. Mutations are mainly clustered in two hotspots affecting arginines at positions 79 and 239 of the structural helical coiled-coil rod domains, which play a crucial role in filament assembly. Accumulation of GFAP aggregates in Rosenthal fibers leads to impairment of proteasomal activity and hyperactivation of the stress response, thus compromising astrocyte functions and altering the homeostasis of the central nervous system (CNS) in AxD patients and murine models. Currently, this orphan disease lacks a cure. Our long-term goal is to develop novel, single-dose therapeutic treatments exploiting allele-specific gene editing strategies to target GFAP mutation hotspots and recover pathological phenotypes. Here, we report the optimization of *in vitro* models and reagents that are key for the project. We engineered cell lines to express mutated and wild-type (WT) murine *Gfap* coding sequences fused to reporter genes for a fast screening of single guide RNAs recognized by different Cas9 nucleases. We identified CRISPR/Cas9 systems efficiently inducing the allele-specific knock-out of *Gfap* sequences harboring the R76H and R236H mutations, homologues of the human mutation hotspots. Also, we identified adenine base editors to correct R-to-H hotspot R76H mutation avoiding the potential activation of DSB-induced DNA damage response. These editing platforms are currently tested in *in vitro* disease models. Results of these *in vitro* screening will pave the way to translational studies aimed at delivering safely and effectively the selected editing systems in the CNS of AxD murine models by means of AAV vectors or nanoparticles. Novel editing platforms for *in vivo* targeting of CNS astrocytes could benefit AxD and other diseases characterized by primary astrocyte degeneration or dysfunctional/maladaptive astroglia.

636. Targeted Gene Knockdown in the Brain and Spinal Cord by CRISPR-Cas13

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Cas13 nucleases are a class of programmable RNA-targeting CRISPR effector proteins that are capable of silencing target gene expression in mammalian cells. Among the several different Cas13 subtypes that have been discovered is the Cas13d nuclease from *Ruminococcus flavefaciens* XPD3002, a Cas13 ortholog with favorable characteristics to other family members and is small enough to fit within a single adeno-associated virus (AAV) vector with a crRNA expression cassette for *in vivo* gene transfer. Given these features, we sought to determine whether RfxCas13d could be delivered to the central nervous system (CNS) to silence the expression of genes whose mutations are associated with debilitating gain-of-function phenotypes. To this end, we have demonstrated that RfxCas13d can be deployed to the mouse spinal cord and brain to knock down the genes causative for two progressive neurodegenerative disorders: amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). More specifically, intrathecally delivering an AAV vector encoding RfxCas13d with a crRNA programmed to target superoxide dismutase 1 (SOD1), a protein whose mutation can cause ALS, could reduce SOD1 mRNA and protein in the spinal cord by >50%, an outcome that we found results in decreased muscle atrophy, improved neuromuscular function, and a slowing in the overall progression of the disease in a mouse model of the disorder. Furthermore, we have demonstrated that RfxCas13d can be programmed to target huntingtin (HTT), a protein that, when mutated to carry an abnormal expansion of a polyglutamine (polyQ)-encoding tract, causes HD. Intrastratially delivering RfxCas13d to a mouse model of the disease led to a potent reduction in HTT protein and its toxic aggregates. Our results thus establish RfxCas13d as a versatile platform for knocking down gene expression in the CNS.

637. Hematopoietic Stem Cell Gene Therapy For Mucopolysaccharidosis Type IIIC

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Mucopolysaccharidosis type IIIC (MPSIIIC) is a genetically inherited severe lysosomal storage disease (LSD) characterised by the accumulation the glycosaminoglycan (GAG) heparan sulphate. MPSIIIC is a progressive childhood neurodegenerative disease caused by loss of function of transmembrane lysosomal protein Heparan- α -glucosaminase N-acetyltransferase (HGSNAT). This disease presents early in life with consequences like missing developmental milestones, hyperactivity, autistic features, neuronal cell loss and loss of motor function, culminating with early death normally from overwhelming dementia and neurodegeneration in adolescence. There is no available treatment for this disease. Our group has previously shown that transplantation of hematopoietic stem and progenitor cell (HSPC) could rescue cystinosis, another LSD due to a transmembrane lysosomal protein. The main mechanism of rescue involved lysosomal cross-correction from HSPC-derived macrophages to the disease cells via long tubular protrusions called tunnelling nanotubes (TNTs). We believe that the same principles could be used to treat MPSIIIC. We generated a new MPSIIIC mouse model by knocking-out exon 2

which introduces multiple early stop codons shortly after this exon in the *HGSNAT* gene. The MPSIIIC mice have displayed disease phenotypes such as accumulation of GAGs in the spleen and kidney, enlarged liver, distended bladder, increase in urine volume in the bladder at sacrifice, and the presence of disease specific non-reducing end carbohydrates biomarkers of MPSIIIC. The mice also present with kidney defects such as glomerular hyaline bodies, glomerular sclerosis, glomerular fibrosis, and dilated tubules. As the first proof of concept, we have transplanted MPSIIIC mice with HSPC from wild-type GFP mice. Our preliminary results show engraftment of HSPC-derived cells in tissues and a trend decrease in liver and bladder size as well as a trend decrease in bladder urine content at sacrifice. These initial results are based on low number of mice analysed so far but more groups will be analysed in the very near future. In parallel, we have generated self-inactivated (SIN)-lentivirus vector containing human *HGSNAT* cDNA which will be used for autologous gene corrected HPSC transplants. Previous studies of MPSIIIA, a similar LSD, have reported limited improvement in the MPSIIIA mice following allogeneic wild-type HSPC transplant, while strong therapeutic benefits were obtained with the gene therapy approach. Overall, our results show that we have successfully generated an MPSIIIC mouse model with characteristic MPSIIIC phenotypes that can be used to test the efficacy of HPSC gene therapy. We have also found preliminary results that suggest that HSPC transplant may ameliorate some of the MPSIIIC mouse disease phenotypes.

638. Improving Neuronal Gene Transfer to the Brain After CSF Administration of AAV9 in Juvenile Non-Human Primates

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One of the more significant barriers for effective gene therapy treatments for neurodevelopmental and neurodegenerative disorders is the need for broad neuronal transduction across the brain. While adeno-associated virus serotype 9 (AAV9) and some AAV9 variants have been shown to cross the blood-brain barrier after systemic administration, this approach yields gene transfer in only a very small percentage of neurons. Conversely, when AAV vectors are injected directly into the brain parenchyma, a high percentage of neurons and other cell types at the injection site are transduced, but gene transfer is far lower beyond the injection site. AAV vector administration via the cerebrospinal fluid (CSF) more broadly targets the brain than parenchymal injections and yields significantly more transduced neurons than systemic administration. In an effort to devise the most effective AAV9 CSF-dosing strategy, we performed three progressive studies in cynomolgus macaques, testing various factors to potentially improve neuronal gene delivery throughout the brain. In these studies, a single-stranded AAV9 vector carrying an enhanced green fluorescent protein (eGFP) reporter gene was administered to juvenile non-human primates via the CSF. Variables included different promoters, routes of CSF administration, Trendelenburg positioning, supplemental co-

administered drugs, and high-volume flush. Two weeks after dosing, tissues from subjects were collected and analyzed for vector genome presence and eGFP protein expression, with a focus on understanding how efficient neuronal transduction was within different regions of the brain. Half of the brain was flash frozen to analyze vector genome by qPCR and eGFP protein by ELISA, while the other half was fixed and processed for immunostaining. Using these methods, we found that neuronal expression was highest when a synapsin promoter was used instead of a constitutively active promoter, that injection into the cisterna magna improved transduction over lumbar intrathecal, and that a high-volume flush of artificial CSF after vector dosing dramatically decreased eGFP expression. Together, these studies have informed our strategy for optimal delivery of AAV9 therapeutic candidates to neurons in the brain.

639. Transduction Efficiency of Adeno-Associated Virus 2, 5, and rh10 to Neural Stem Cells in Gerbil Dentate Gyrus

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[Introduction] Adeno-associated virus (AAV) vectors are promising tools for gene transduction. The difference of amino acid sequences between serotypes determines the difference of tropism. Because the tropism to the neural stem cells is not well-known, we compared the transduction efficiency of three different AAVs, such as AAV2, 5, and rh10, into the neural stem cells of gerbil hippocampi. [Methods] Four-week-old male gerbils were injected with 1.5×10^{10} viral genomes of AAV, such as AAV2, 5, or rh10, carrying green fluorescent protein (GFP) into the right hippocampus ($n = 3$, each). The control animals were administered with the same amount of phosphate-buffered saline (2 μ l) into the right hippocampus. One week later, the animals were decapitated for the immunohistochemical analysis. [Results] AAV5 showed the largest number of double positive cells for GFP and Sox2, a marker for the neural stem cells, compared to the AAV 2 and rh10 (AAV2: 0.0 ± 0.0 , AAV5: 24.4 ± 1.6 , AAVrh10: 15.4 ± 3.5 , $p < 0.001$ and $p < 0.05$ for AAV5 compared to the control and AAVrh10, each). On the contrary, AAVrh10 showed the largest number of double positive cells for GFP and NeuN, a marker for mature neurons, compared to AAV2 and 5 groups (AAV2: 6.4 ± 3.3 , AAV5: 13.6 ± 5.9 , AAVrh10: 143.4 ± 20.0 , $p < 0.001$ for AAVrh10 compared to the control). [Conclusions] Our data demonstrated that AAV5 showed the highest transduction efficiency to the neural stem cells. Overall, we should choose an appropriate serotype of AAV, which depends on the purpose of the project.

640. Gene Therapy Treatment in Young SLC13A5 Deficient Mice

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SLC13A5 Deficiency is a rare, severe pediatric epileptic encephalopathy originating from mutations in the SLC13A5 gene, which encodes a sodium dependent citrate transporter. These mutations cause a partial or complete lack of citrate transport through the plasma membrane that results in elevated citrate levels in blood, urine, and CSF. This disorder is characterized by severe seizures that begin in patients a few days post parturition. In addition to seizures, patients also experience hypotonia, ataxia, and hypodontia in conjunction with expressive language, motor, and cognitive developmental delays. The effects of these seizures are devastating, with some patients succumbing to the disease. To-date, no therapies exist that target the underlying cause of disease and anti-epileptic drugs have variable success in controlling seizures. Gene replacement therapy is a potential treatment approach for SLC13A5 Deficiency. We developed a self-complementary adeno-associated virus (AAV) vector carrying a codon-optimized human SLC13A5 coding sequence whose expression is driven by a ubiquitous promoter. To assess vector efficacy, we used *Slc13a5* knockout (KO) mice. KO mice do not exhibit an overt phenotype; however, animals do have elevated citrate levels, abnormal EEG activity, including infrequent tonic-clonic seizures, and greater susceptibility to chemically induced seizures. Previously we showed that young adult KO mice treated with 8.00×10^{11} vg AAV9/hSLC13A5 at ~3 months of age via intra-cisterna magna (ICM) or intrathecal lumbar puncture (IT) delivery routes showed treatment benefits in disease-relevant indicators as compared to vehicle treated KO mice. The current study expanded on those findings and tested the safety and benefit of vector treatment when given at a younger age. To assess safety of early vector delivery, wildtype (WT) animals were injected on postnatal day 10 (P10) with 8.00×10^{11} vg (high dose) or 2.00×10^{11} vg (low dose) AAV9/hSLC13A5 or vehicle as part of a non-GLP toxicology study. This study showed that both doses were well-tolerated as assessed by clinical blood chemistry, weight gain, survival and histopathology. To test the efficacy of early treatment, KO and WT littermates received an IT injection of high or low dose vector or vehicle at P10. Like treatment in adult animals, treatment in younger KO mice demonstrated no adverse effects on weight or survival at either dose tested. At ~3-4 months of age EEG activity was recorded, and mice were then subjected to a pentylenetetrazol (PTZ) seizure induction paradigm to test seizure susceptibility. Unexpectedly, we found that younger WT and KO mice had more variable and less severe EEG activity than older WT and KO mice and that the younger KO mice were also less susceptible to seizure induction. Despite these caveats, we found that unlike vehicle treatment, vector treatment at both doses protected KO mice against worsening seizure severity with repeated PTZ dosing. Additionally, animals treated with the low dose showed reduced seizure susceptibility and latency to seizure over the course of PTZ injections. Tissues were then collected for biochemical and histological assessments. Vector biodistribution showed that IT P10 delivery achieved widespread, dose-dependent levels of transduction in the CNS and periphery. In the adult treated group, ICM delivery resulted in greater and more widespread vector expression in the brain

as compared to IT injected animals; however, the level and spread of SLC13A5 expression in the brain was lower in the adult ICM treated animals as compared to younger animals that received the IT low dose. Overall, these results support the potential safety and benefit of treating SLC13A5 Deficiency at a younger age and with a lower vector dose.

641. Dopaminergic Abnormalities Within the Substantia Nigra Identified in Murine Models of Jordan's Syndrome

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Dopaminergic Abnormalities within the Substantia Nigra Identified in Murine Models of Jordan Syndrome. Intellectual disability (ID) affects nearly 1% - 3% of the population worldwide and can range from mild to profound in severity. ID and its severity are defined using three primary criteria which include (i) deficits in intellectual functioning (ii) deficits in adaptive functioning (iii) deficit onset during early childhood development. In severe cases of ID, causality is commonly linked with an underlying genetic defect. PPP2R5D-related ID disorder, also known as Jordan's syndrome (JS), is a rare monogenic form of ID that is characterized by moderate to severe ID and neurodevelopmental delay along with other comorbidities including hypotonia, macrocephaly, seizures, and autism spectrum disorder. JS is caused by de novo missense mutations that occur within *PPP2R5D*. *PPP2R5D* encodes an isoform of the regulatory B subunit, B56 δ , for the protein phosphatase 2A (PP2A) holoenzyme. PP2A is a major serine/threonine phosphatase that regulates dephosphorylation within many cells. PPP2R5D is enriched within the CNS and plays a vital role in negatively regulating cell growth and proliferation. Mutations within *PPP2R5D* have been shown to affect substrate specificity, subcellular localization, and the catalytic activity of PP2A- B56 δ . Additionally, previous research has shown that PPP2R5D mutations affect the AKT-mTOR signaling pathway resulting in increased cell growth and proliferation. Moreover, recent clinical case reports have identified five patients with JS concurrently diagnosed with early-onset parkinsonism. In these reports, patients were described as levodopa-responsive suggesting dopamine dysfunction in association with *PPP2R5D* mutations. Here, we utilize novel murine models of JS containing the equivalent human pathogenic mutations in *Ppp2r5d* to examine the pathophysiological effects of *Ppp2r5d* mutations in brain tissue. In our study we examine the immunoreactivity of tyrosine hydroxylase (TH), a conical marker associated with Parkinson's, within the substantia nigra. Furthermore, through immunohistochemistry and immunoblotting, we also examine select substrates of PP2A-B56 δ within the substantia nigra and striatum, and whole-brain respectively, in JS mouse brain tissue. In summary, our group is the first to report dopaminergic abnormalities within the substantia nigra of JS mice. These data support previous clinical case reports of dopamine dysregulation in association with *PPP2R5D* mutations and warrant further investigation.

642. Safety and Biodistribution Assessment in Non-Human Primates (NHPs) of a miniMECP2 AAV9 Vector for Gene-Replacement Therapy of Rett Syndrome

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Background: A miniMECP2 gene packaged in a self-complementary AAV9 vector (TSHA-102) and utilizing the miRARE platform, designed to regulate MeCP2, is in development as an investigational gene-replacement therapy for Rett syndrome. **Methods:** 24 Female NHPs aged approximately 2 years were assigned to receive either a single intrathecal administration of vehicle or a total dose of TSHA-102 (2.88x10¹³, 5.77x10¹³, or 2.31x10¹⁴ vg/animal, corresponding to 2.5x10¹⁴, 5x10¹⁴, 2x10¹⁵ vg human equivalent dose [HED], respectively). Assessments were performed at day 90 and 180 post TSHA-102 administration for each group. Clinical pathology, anatomic pathology, behavior, clinical signs, body weight, and food consumption were assessed throughout. **Results:** At the 90-day timepoint there were no TSHA-102-related abnormal clinical observations, or changes in clinical pathology, body weight or food consumption, or neurobehavioral signs noted throughout. Animals receiving TSHA-102 demonstrated minimal to moderate hepatic infiltration of lymphocytes and macrophages without functional deficiency. Dose-related minimal to moderate nerve degeneration in dorsal tracts of the cervical, thoracic, and lumbar spinal cord was observed. Minimal nerve fiber degeneration was present in the sciatic, tibial, and radial nerves in mid- and high-dose groups, with minimal mononuclear cell infiltrate in the cervical and lumbar dorsal root ganglion. These findings were considered non-adverse. While all vector-treated animals developed anti-AAV9 antibodies, only one demonstrated an AAV9 capsid antigen-specific T cell response. Two animals developed anti-miniMeCP2 antibodies by day 90, but none showed a corresponding T cell response. **Conclusion:** Single IT administration of TSHA-102 doses of up to 2.31 x 10¹⁴ vg/animal (HED 2x10¹⁵ vg) were well tolerated in NHPs, with no adverse treatment-related findings.

643. A Study of Intracisternal Administration of LYS-GM101 in Children with Infantile GM1 Gangliosidosis: Preliminary Results of the Safety Cohort

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GM1 gangliosidosis is a fatal autosomal recessive disease caused by mutations in the GLB1 gene encoding the lysosomal acid beta-galactosidase (beta-gal) enzyme. The resulting enzymatic deficiency leads to accumulation of GM1 ganglioside in neurons and progressive neurodegeneration. P1-GM-101 (NCT04273269) is an adaptive clinical

trial assessing safety and efficacy of intracisternal administration of LYS-GM101, an AAVrh10 vector carrying the GLB1 cDNA, in children with early onset (type 1) and late onset (type 2a) infantile GM1 gangliosidosis. In the first stage of this trial (safety cohort), 2 patients with early and 2 patients with late infantile GM1 gangliosidosis will be enrolled. The gene therapy vector LYS-GM101 is administered at a dose of 2.0E+12 vg/mL of CSF by injection into the cisterna magna. The safety cohort data will be reviewed by an independent Data Safety Monitoring Board before initiating the confirmatory phase (Stage 2) with the objective to enroll at least 12 additional patients with early or late infantile GM1 gangliosidosis. Preliminary safety and biomarker data from the first enrolled subjects will be presented during this conference.

644. A Human-Ready Regulated AAV9/miniMECP2-miRARE Gene Therapy (TSHA-102) Improves Survival and Respiratory Health After Dosing Translationally Relevant Treatment Ages in Mice Modeling Rett Syndrome (RTT)

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Background: MeCP2 is an X-linked transcription regulator whose loss-of-function or duplication respectively mediates two equally severe neurodevelopmental disorders: Rett syndrome (RTT) and MECP2 duplication syndrome. For nearly a decade, the dose sensitivity of MeCP2 presented a challenge for gene therapists seeking to create a safe treatment for mosaic heterozygous RTT patients. This challenge was met by miR-Responsive Autoregulatory Element (miRARE), a miRNA target panel that was designed to regulate MECP2 transgene expression. Insertion of miRARE into the 3'UTR of a miniMECP2-myc viral genome was previously shown to improve the safety of miniMECP2-myc gene therapy in wild-type (WT) mice without compromising efficacy in *Mecp2*^{-/-} knock-out (KO) mice. **Methods:** TSHA-102, a clinical-candidate version of the AAV9/miniMECP2-miRARE vector (lacking the myc epitope tag) was assessed in a multifactorial dose-ranging pharmacology study in KO mice. This study included intrathecal (IT) administration at translationally relevant ages (P7, P14, or P28). The IT study evaluated doses in the presence and absence of an immunosuppressant. Assessments reported here include body weight (BW), measurements of respiration, and survival. **Results:** IT administration of TSHA-102 significantly improved survival, BW, and respiratory health across treatment ages. After P28 administration, the maximum dose (8.8x10¹¹ vg/mouse; HED 2.86x10¹⁵ vg) permitted a >50% survival extension. Earlier administration (P7 and P14) of TSHA-102 permitted survival extensions with a 10-fold lower dose. In general, the best improvements in KO BW were observed when TSHA-102 was administered at an early age or in conjunction with an immunosuppressant. Finally, unrestrained whole-body plethysmography showed that TSHA-102 improved respiratory patterns across multiple IT doses and treatment ages. After P28 administration, TSHA-102 decreased apnea frequency and apnea

duration across a four-fold dose range. The highest dose decreased apnea frequency in KO mice by >50%. Earlier administration (P7 and P14) of TSHA-102 decreased apnea frequency with a 10-fold lower dose. This improved respiratory health is important from a translational perspective as the respiratory dysfunction of Rett syndrome in humans causes significant burden to patients and families. Conclusions: TSHA-102 offers a path to clinical testing by meeting the following list of criteria: (1) The pre-clinical gene therapy uses a capsid that has been in extensive clinical use; (2) The gene therapy achieves behavioral efficacy at translationally relevant treatment ages; (3) The gene therapy is well-tolerated in KO as well as WT mice as miRARE allows for regulation of gene expression; (4) Key behavioral efficacy conclusions relied on unbiased, objective measures; and (5) The viral genome faithfully packages within the size constraints of self-complementary AAV.

645. Intravenous Gene Therapy Using AAVPHP.eB for Metachromatic Leukodystrophy

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Metachromatic leukodystrophy (MLD) is a rare, autosomal recessive disease caused by deficient activity of the lysosomal enzyme arylsulfatase A (ARSA), resulting in sulfatide accumulation and subsequent demyelination and neuronal loss within the central peripheral nervous systems (CNS, PNS). Three clinical forms of MLD have been described, based on the age of symptom onset. Among them, early-onset forms comprise a continuum between late-infantile MLD (LI-MLD, most frequent accounting for 50-60% of MLD patients) and early juvenile MLD (EJ-MLD). In these early-onset forms, first symptoms typically develop between 1 and 4 years of age and progress rapidly towards severe motor and cognitive regression and premature death. There are currently no approved therapies for early onset MLD once patients are symptomatic. Hematopoietic stem cell transplantation (HSCT) gene therapy with lentiviral vector aiming at overexpressing the ARSA enzyme in bone marrow-derived microglia was shown to be safe and most treated patients exhibit preservation of motor and cognitive functions, associated with protection from CNS demyelination and PNS involvement. Unfortunately, no significant effect was observed once symptoms are already present. Here we proposed a gene therapy approach based on intravenous delivery of an AAVPHP.eB encoding the human ARSA gene with an HA tag. We demonstrated a broad transduction of brain and spinal cord leading to a complete correction of sulfatide storage both in brain and spinal cord in symptomatic animals (ARSA KO animals) as well as a significant improvement of neuroinflammation in mouse model of the disease. A dose response study has been done in ARSA KO mouse model. More importantly in term of translational study to establish a phase I/II clinical trial, we will present the impressive data of expression in the Non human primate that we recently obtained.

646. Characterization and Restoration of Sodium Channel Stop Codon-Opathies

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Introduction. The *SCN2A* gene encodes the alpha subunit of the neuronal voltage gated sodium channel NaV1.2, which is necessary for action potential generation and propagation. Allelic variants of *SCN2A* are associated with *SCN2A* Syndrome, which features autism spectrum disorder (ASD), intellectual disability, and seizure disorders. Mutations that result in loss of function are closely linked with ASD and developmental delay. Nonsense mutations, like Cys959x, create a premature termination codon (PTC) in the reading frame of the sodium channel and result in loss of function. Therapeutic repair of these nonsense mutations could restore NaV1.2 sodium channel expression. Previously, treatment of PTCs was limited to using small molecules with to promote ribosomal readthrough of the nonsense codon in a nonspecific fashion. These therapies would likely offer minimal clinical improvement since the NaV1.2 channel is relatively intolerant of mutations. Defective channel activity could cause serious neurological side-effects. Here we take a different approach. Rather than reading through the PTC, we used anticodon engineered transfer RNAs (tRNA) to correct PTCs. With this approach, the final NaV1.2 channel contains the correct amino acid. Edited tRNAs also have limited interaction with the native translation termination codons. Here we show that this edited tRNA system rescues PTCs in a neuronal context. **Methods.** HEK293T cells are used for tRNA dosing experiments. The plasmids in this study contained mOrange2 engineered to contain a PTC and an anticodon engineered tRNA to correct the mOrange2 mutation. Plasmids were transfected using PolyJet and cells were analyzed for fluorescence using a plate reader 48 hours after transfection. Induced pluripotent stem cells (iPSCs) were generated from a patient with a Cys959x mutation in *SCN2A* and a healthy control. We used dual-SMAD inhibition to differentiate iPSCs into neural progenitors, and then differentiated progenitors into neurons in BrainPhys medium using the manufacturer's protocol. Patient-derived neurons reach electrophysiological maturity and have functional ion channels six weeks after neuronal induction. Neurons in this study are six weeks old at minimum. Plasmids containing mutated mOrange2 and the corresponding edited tRNA were transfected into the patient neurons using Lipofectamine STEM transfection reagent. **Results.** Our HEK293T experiments suggest that increasing the copy number of edited tRNA does not correlate with increased plasmid rescue. We also observed rescue of an exogenous plasmid-encoded PTC in human iPSC-derived neurons in both the control and Cys959x lines. We observed mOrange2 fluorescence in live-cell microscopy of neuronal cell bodies and neurites without the use of immunofluorescence, suggesting robust PTC rescue. This study is the first of its kind to show that edited tRNA can be expressed in the context of human neurons. These results broaden the potential therapeutic applications of edited tRNAs to treat loss of function mutations associated with genetic disorders by targeting other PTCs with anticodon engineered tRNAs. Future directions include transitioning from plasmids to viral vectors such as AAV, which have greater translational potential, and attempting to correct endogenous PTCs in both human and mouse models.

647. Treatment with ROS Detoxifying Gold Quantum Clusters Alleviates the Functional Decline in a Mouse Model of Friedreich Ataxia

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Friedreich ataxia (FRDA) is caused by the reduced expression of the mitochondrial protein frataxin (FXN) due to an intronic GAA trinucleotide repeat expansion in the *FXN* gene. Although FRDA has no cure and few treatment options, there is research dedicated to finding an agent that can curb disease progression and address symptoms as neurobehavioral deficits, muscle endurance and heart contractile dysfunctions. Since oxidative stress and mitochondrial dysfunctions are implicated in FRDA, we demonstrated the systemic delivery of catalysts activity of gold cluster superstructures (Au₈-pXs) to improve cell response to mitochondrial reactive oxygen species (ROS) and thereby alleviate FRDA-related pathology in mesenchymal stem cells from patients with FRDA. We also found that systemic injection of Au₈-pXs ameliorated motor function and cardiac contractility of YG8sR mouse model that recapitulates the FRDA phenotype. These effects were associated to long-term improvement of mitochondrial functions and antioxidant cell responses. We related these events to an increased expression of frataxin, which was sustained by reduced autophagy. Overall, these results encourage further optimization of Au₈-pXs in experimental clinical strategies for the treatment of FRDA.

648. Sialidosis: From Gene Editing to Gene Therapy

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Sialidosis is a rare lysosomal storage disease caused by mutations in the *NEU1* gene that encodes the lysosomal sialidase neuraminidase 1 (NEU1). Deficiency in NEU1 function causes accumulation of sialylated glycopeptides and oligosaccharides in tissues and body fluids, resulting in cell and organ dysfunction. Sialidosis is categorized by the presentation and severity of symptoms as either Type I or Type II (most severe). Early onset Type II patients present

with severe psychomotor and developmental delay, coarse faces, bone deformities, hepatosplenomegaly, and bilateral macular cherry red spots. Normosomatic Type I forms have a milder phenotype, characterized by abnormal gait, progressive visual loss, myoclonus, and ataxia, but no neurological involvement. To date, there is no effective treatment for sialidosis. Our team is developing an adeno-associated viral (AAV) gene therapy strategy to treat sialidosis through testing different vector designs encoding *NEU1* in small and large animal models. Preliminary results of an intravenous injection of an AAV9 vector encoding *NEU1* in wild type mice showed vector expression in various tissues and 100-87% increased activity of NEU1 compared to PBS controls. Next, we will further refine our selection to the most efficacious vector design, route, and dose in a large and small animal model of sialidosis. However, no large animal model of sialidosis exists. Therefore, we aim to generate a sheep model using CRISPR-Cas9. We achieved ~80% gene editing through electroporation of early-stage sheep embryos with SpyCas9 protein and guide RNAs targeting exon 5 and 6 of *NEU1*. Edited embryos were implanted in ewes and lambs were born with NEU1 activity ranging from 0-56% of normal and phenotypes including, hepatosplenomegaly, severe spine curvature, bone deformities and blindness. One lamb, S4, was determined to have a germline 3 base pair deletion that overlaps with a nonsynonymous mutation detected in human patients (A319V). S4's 3 base pair deletion was detected in 30% of embryos that were sired from S4, making him the best candidate for generating a sialidosis flock. Further characterization of these animals as well as those that will be born in late spring 2022 are ongoing. Together, these studies in parallel with natural history studies in sialidosis patients will inform future clinical trials for this devastating disease.

Sheep #	Sex	Embryo #	Embryo Stage at Electroporation	Guide	DOB	Genotype	% Date	Phenotype
S1	Male	241	3 one-cell, 2 two-cell	Exon 5	2/21/21	Affected Mouse: 50% in frame 5bp deletion 50% in frame 5bp deletion	2/23/21	Very sick, blind, wide stance, enlarged kidneys, severe spine curvature, generalized fluid, died of sepsis
S2	Male	229	3 one-cell, 2 two-cell	Exon 5	2/25/21	Normal sequence		Alive, normal behavior
S3	Male	229	3 one-cell, 2 two-cell	Exon 5	2/25/21	Affected Mouse: Variety of mutations: No partial sequences Missed/offset 80% normal sequence 20% in frame 5bp deletion	2/25/21	Died in womb (anhydriotic) - 2 days prior to birth
S4	Male	226	3 one-cell, 2 two-cell	Exon 5	2/25/21	Affected Mouse: 50% frameshift 11bp deletion 50% frameshift 8bp insertion	2/25/21	Alive, normal behavior, germline mutation of 5bp deletion
S5	Male	226	3 one-cell, 2 two-cell	Exon 5	2/25/21	Affected Mouse: 50% frameshift 11bp deletion 50% frameshift 8bp insertion	2/25/21	Died shortly after birth, enlarged kidneys and liver, pleural effusion, contracted tendons, severe spine curvature and contractures
S6	Male	254	3 one-cell	Exon 5	4/26/21	Normal Mouse: Missed several codons of mutation. Further analysis pending.		Alive, normal behavior
S7	Female	254	3 one-cell	Exon 5	4/26/21	Affected Mouse: possible Large deletions, further maternal analysis pending.	4/26/21	gastrocnemius, enlarged liver and kidney, severe spine curvature, died shortly after birth
S8	Male	232	3 one-cell	Exon 5	4/27/21	Affected: possible Large deletions, further maternal analysis pending.	4/28/21	Anhydriotic - died in womb
S9	Male	232	3 one-cell	Exon 5	4/27/21	Affected Mouse: Large deletions in some lanes further maternal analysis pending.		Alive, been very small, blind, bilateral contractures, spinae, contract bones
S10	Male	232	3 one-cell	Exon 5	4/27/21	Affected Mouse: possible Large deletions, further maternal analysis pending.		Alive, been very small, possibly blind, bilateral contractures, spinae, contract bones
S11	Male	76	2 one-cell, 1 three-cell & 1 four-cell	Exon 6 gfp	1/12/22	Affected: deletion - further analysis pending		Alive, been very small, restricted feedings, high sodium, enlarged kidneys, hydrocephalus, poor motor activity, contractures (PDA)

649. VecTrons: Targeting Misfolded Proteins in Neurodegenerative Diseases

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The appearance of insoluble protein aggregates is considered to cause many neurodegenerative diseases. Pathology associated with aggregation of TDP-43 is observed in 97% of patients suffering from sporadic Amyotrophic Lateral Sclerosis (ALS) and antibody-based therapeutic interventions targeting TDP-43 aggregation have been shown to ameliorate ALS related symptoms in mice. Although expression of intracellular binders such as antibody

fragments may remove misfolded or aggregated proteins, technologies to more effectively target intracellular antibody/target protein complexes for proteolytic aggregate degradation are needed. We have designed a small library of protein-based motifs and domains that can be fused to antibody (fragments) and have named these VecTrons. VecTrons consist of one part antibody, specifically targeting the disease-causing misfolded protein, and one part degrader or degron, which are in frame expressed via an adeno-associated virus (AAV). VecTrons have been generated to target three specific cellular degradation machinery pathways: (1) the Ubiquitin Proteasome System (UPS) pathway, (2) the Chaperone Mediated Autophagy (CMA) pathway, and (3) the Macroautophagy pathway. In vitro testing has shown that the VecTron technology can increase the ability of antibodies to remove TDP-43 aggregates. It was also shown that VecTrons targeting all three proteolytic pathways can alter TDP-43 aggregate turnover. Importantly, VecTrons have been designed to specifically target misfolded or aggregated TDP-43 species without interfering with the normal function of properly folded TDP-43 which is essential for neuronal health. In summary, the modular VecTron technology enables the therapeutic targeting of any intracellular protein form and, in combination with an AAV, in any diseased cell type.

650. A Novel Rhesus Macaque Model of Huntington's Disease Recapitulates Key Neuropathological Changes Along with Progressive Cognitive and Motor Decline

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To create a nonhuman primate model of the neurodegenerative brain disorder, Huntington's disease (HD), we injected adult rhesus macaques with a 1:1 mixture of AAV2 and AAV2.retro expressing a fragment of mHTT bearing 85 CAG repeats into the caudate and putamen. In previous work we demonstrated that this injection strategy leads to the expression of mutant HTT protein, and the formation of hallmark HTT+ inclusions, throughout the striatum as well as dozens of cortical and subcortical brain structures due to the strong retrograde capability of AAV2.retro (Weiss et al., 2020). Here, we queried the disruption of cortico-basal ganglia circuitry for 20-months post-delivery of this mHTT construct (HTT85Q, n=6), a control HTT construct bearing 10 CAG repeats (HTT10Q, n=6), or PBS buffer (n=5). We characterized the emergence of motor and cognitive phenotypes to link behavioral changes with disruptions in cortico-basal ganglia circuitry using multi-modal neuroimaging techniques (structural MRI; diffusion tensor imaging, DTI; resting state functional connectivity, rsfMRI; PET imaging of D2/D3 receptors and glucose metabolism; [18F] Fallypride and [18F]FDG). To achieve this, we evaluated animals using complex behavioral tasks to assess fine motor coordination (Lifesaver Retrieval Task), gross motor function (NHP-specific neurological rating scale), working memory (Spatial Delayed Response) and object recognition (Delayed Non-Match to Sample) to complement our neuroimaging battery. Compared to controls, animals treated with AAV2:2retro-HTT85Q showed a progressive development of mild orofacial dyskinesia, aberrant forelimb posture, forelimb

chorea, incoordination, hindlimb slowness (bradykinesia) and/or tremor, which were exacerbated by the dopamine receptor agonist apomorphine. Moreover, HTT85Q animals also exhibited impaired fine motor coordination and spatial working memory, but preserved object recognition, in relation to controls and to pre-surgical baseline measures. Voxel-based brain image analysis revealed many white and gray matter regions with alterations in fractional anisotropy (FA), suggesting that mHTT expression resulted in microstructural changes throughout the cortico-basal ganglia circuit. Similar approaches applied to the volumetric (T1w/T2w) data using Tensor Based Morphometry revealed areas of the striatum with significant atrophy. Data from our rsfMRI studies revealed reductions in functional brain connectivity among cortico-striatal networks; and using Positron Emission Tomography (PET) we identified significant perturbations in the dopamine system (F18-Fallypride) as well as regional changes in glucose metabolism (F18-FDG) in many of the same brain regions. These data demonstrate the feasibility of generating AAV-based models of HD in nonhuman primates that exhibit the hallmark motor and cognitive behavioral phenotypes, as well as neuropathological manifestations of HD. Using a combination of AAV2 and AAV2.retro allowed for the expression of mHTT throughout the cortico-basal ganglia circuit, versus just the caudate and putamen, leading to the creation a NHP model of this disease that more closely depicts the neuropathology observed in human HD patients. This work sets the stage for developing novel biomarkers, as well as testing promising new mHTT lowering therapeutics. This approach will be critical to advance HD treatment options for this devastating disorder.

651. Autophagy and Neurodegeneration- A Neuron Specific Model of TBCK Encephalopathy

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TBCK syndrome is an autosomal recessive disease caused due to biallelic loss-of- *TBCK* gene. Children affected by this syndrome develop severe neurodevelopmental disorder causing profound developmental delay, brain atrophy without microcephaly, abnormal white-matter signal, seizures, diminished respiratory function, and distinctive faces. The TBCK protein comprises three functional domains: an N-terminal Serine/Threonine kinase domain, a central TBC domain, and a C-terminal rhodanese homology domain (RHOD), and the exact function of the TBCK protein is not known. Preliminary studies predict that the TBC domain functions as a GTPase -activating protein for small Rab GTPases, but the role of the other two domains is not known. Studies from patient-derived fibroblasts show that the loss of *TBCK* modulates the mechanistic Target of Rapamycin (mTOR) pathway and, fibroblast showed an increased accumulation of autophagosome, providing evidence that autophagic-lysosomal dysfunction may play an essential role in the disease's progression. In the present study, we used a neural progenitor cell line (ReNcells), which can differentiate into neurons and astrocytes as a model to understand TBCK patient brain. We have used lentivirus shRNA transductions followed by a single-cell clone selection method to generate stable TBCK knockdown ReNcells. Primary analysis on two different clones indicates that these clones have a similar phenotype at the mTOR signaling and autophagy. We observed TBCK knockdown in ReNcells affected their differentiation

towards both neurons and astrocytes. TBCK KD neurons have small axons, show a higher percentage of broken axons, and also found abnormal-shaped neurons. Further, we observed a higher number of LC3 positive vesicles accumulated along the axons. These results from knockdown cells predict neuronal and glial cell loss in TBCK patients.

652. Gene Therapy Candidate Restores Dopamine Levels in Lesch-Nyhan Syndrome

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Introduction: Lesch-Nyhan syndrome (LNS) is a rare X-linked recessive neurological condition. Patients with LNS present with hyperuricemia as well as an unusual neurological syndrome of generalized hypotonia with superimposed dystonias and compulsive, self-injurious behavior. The neurological manifestations of LNS may be related to defective projections of dopaminergic neurons of the substantia nigra (SN), which is apparent on positron emission tomography studies of LNS patients. LNS is caused by mutation or deletion of the Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene resulting in an enzyme deficiency. HPRT is an enzyme that functions to recycle nucleotides; however, it is not known how the disruption of this pathway leads to dysfunction of dopaminergic neurons and LNS. Currently, there is no approved treatment for LNS. We have utilized Adeno-associated virus (AAV) gene replacement of HPRT as a strategy for enzyme replacement and restoration of dopamine in a mouse model of LNS. **Results:** We have tested an AAV vector expressing an HPRT transgene in HPRT knockout (HPRT KO) mice. HPRT KO mice do not have any detectable HPRT activity in the brain and have ~50% reduction of normal dopamine levels. These mice have no overt phenotype. Intravenous administration of AAV.PHP.eB. HPRT to 3-week-old and 3-month-old HPRT KO mice resulted in robust expression of HPRT and restoration of dopamine levels in the striatum to wildtype levels. HPRT enzyme activity in the brain of injected 3-week-old mice was approximately 4-fold the level of activity in a WT mouse. Having confirmed that introduction of HPRT to the brain using an AAV with broad transduction can rescue dopaminergic cell function, we sought to investigate how more direct targeting of HPRT expression to the substantia nigra would affect dopamine levels. Ongoing studies in the HPRT KO mice will evaluate how different direct delivery approaches impact substantia nigra transduction and restoration of dopaminergic neuron function. **Conclusion:** We have shown that injection of an AAV HPRT transgene in adult HPRT KO mice restores enzyme function and dopamine levels in the brain. We are now optimizing delivery approaches to achieve similar rescue of dopaminergic neurons with localized AAV delivery. Further work will include studies to assess vector toxicity, and expression following intraparenchymal delivery to the brain in nonhuman primates. Overall, this gene replacement strategy shows that postnatal correction of the dopaminergic defect is possible and could potentially result in alleviation of symptoms in LNS patients.

653. Ultrasound and Cationic Microbubble Assisted Gene Delivery in the Brain for Fragile X Syndrome Therapy

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Fragile X syndrome (FXS) is the most common cause of hereditary intellectual disability (ID) and is caused by a mutation in the *FMR1* gene. At present, there is no treatment for this disease, only symptomatic treatments to reduce the effects of the disease are proposed. Gene therapy is a promising therapeutic strategy; however, an efficient, noninvasive and safe gene transfer method must be put in place. The use of pulsed ultrasound (1 MHz, 109 or 145 kPa, 5% duty cycle, 1 sec period) combined with cationic microbubbles transiently permeabilized the blood-brain barrier (BBB) allowing targeted delivery of plasmid DNA bound at the surface of microbubbles. We have developed a homemade gene transfer platform in mouse brain by focused ultrasound using cationic microbubbles. We used cationic microbubbles in interaction with 30 µg of luciferase or FMR1-eGFP encoding plasmid DNA. The mix was injected in the tail vein followed by targeted ultrasound stimulation in the left hippocampus area. Gadolinium uptake was observed up to 2 hours after sonoporation in T1 MRI showing that we were able to open the BBB transiently. No gadolinium uptake was observed 24 h after treatment. The T2* MRI images did not show any bleeding or damage in the brain after treatment. Histology analysis of the brain 24h after treatment confirms the BBB opening area at the pointed location. Luciferase activity was specifically measured in the targeted area compared to control area 24 hours after sonoporation. The use of FMR1-eGFP encoding plasmid on FMR1KO mouse model showed FMRP expression in neurons and astrocytes by immunohistochemistry. These results show the potential of this technique for brain gene therapy for intellectual disability disorders.

Cancer - Immunotherapy, Cancer Vaccines II

654. Humanized Bi-Specific Antibody Shows a Potent Activity in Primary and Therapy-Resistant Glioblastoma in Experimental Models of Disease

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Background. Glioblastoma (GBM) is the most aggressive brain tumor in adults. GBM invariably recurs following standard of care treatments,

and recurrent GBM respond poorly to additional treatment. Therefore, novel therapeutic approaches for treating primary and recurrent GBM are needed. We have developed a bi-specific antibody that bridges T cells with glioma cells through IL13R α 2, a tumor-associated antigen, and have recently demonstrated its potential for treating GBM patients in association with results from experiments involving preclinical mouse models of GBM. In order to advance this treatment approach for testing in GBM patients, we humanized a single-chain antibody (scFv) to IL13R α 2 and fused it with fully human scFv to T cell CD3 ϵ . In this study, therapeutic properties of the humanized bi-specific T cell engager, designated as hBiTE^{IL13R α 2ON}, have been investigated in the context of primary and recurrent human GBM. **Methods.** A recurrent tumor derivative of a patient-derived xenograft, referred to as GBM6r, was generated by treating mice with treatment-naïve GBM6 intracranial tumor using 2Gy/day irradiation and concurrent 10 mg/kg/day TMZ for 5 consecutive days, with two additional 5-day cycles of TMZ upon indication of tumor recurrence as indicated by bioluminescence imaging. The treated recurrent intracranial tumor was excised and established as a subcutaneous xenograft that is sustained by serial passaging. We investigated the ability of hBiTE^{IL13R α 2ON} to engage and activate T cells in co-cultures with cells from GBM6 and GBM6r through use of Cr51 release assay. Flow cytometry was used to assess the tumor cell expression of IL13R α 2 and T cell expression of activation markers. For in vivo study of hBiTE^{IL13R α 2ON} anti-tumor activity, intracranial PDX were established in NSG MHCII KO mice (Jackson Labs) reconstituted with 10⁷/mouse donor PBMCs, and mice were subsequently treated i.p. with 2.5mg/kg x 4 days of therapeutic BiTE for three weeks. Animals were followed for survival, and the brains of long-term surviving mice were analyzed for the presence of residual disease and T cell infiltration. **Results.** GBM6 and GBM6r TMZ response were analyzed in vitro by MTT assay and in vivo through treatment of mice bearing intracranial tumors. The expression of therapeutic target protein IL13R α 2 on GBM6r cells was similar to that determined for treatment-naïve GBM6 from which it was derived. Both GBM6 and GBM6r cells showed hBiTE^{IL13R α 2ON} dose-dependent T cell-mediated cell cytotoxic response. No cell killing was observed when treating co-cultures with hBiTE^{IL13R α 2OFF}, which lacks the binding for IL13R α 2. T cell activation by hBiTE^{IL13R α 2ON} was confirmed by analysis of granzyme B, IFN γ expression and by increases in the proportion of CD25+CD69+ CD8 T cells in cultures treated with hBiTE^{IL13R α 2ON}. Therapeutic BiTE treatment showed in vivo anti-tumor activity against intracranial GBM6r tumor and against treatment-naïve GBM6, with most mice surviving long term irrespective of whether engrafted with resistant or naïve tumor cells. Histologic analysis of brain tissues confirmed the response of intracranial tumors to hBiTE^{IL13R α 2ON} treatment. **Conclusion.** Our data demonstrate the efficacy of humanized BiTE^{IL13R α 2ON} protein in both treatment naïve and TMZ-resistant patient-derived GBM, and our results support the investigation of this novel therapeutic in a clinical trial setting. Funding: R01 NS122395-01 and R01 NS095642

655. OXB-302: 5T4-Targeting CAR-T Cells Are Cytotoxic Against Low-Expressing Cells and Successfully Eliminate Leukaemic Stem Cells *In Vitro*

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Trophoblast glycoprotein (5T4) is a transmembrane oncofoetal antigen that has been shown to be expressed on a wide range of solid tumours but has limited expression on normal tissue. In addition, 5T4 has also been shown to be expressed on cancer stem cells (CD34+CD38-), allowing for the targeting of both cancer stem cells and the tumour making it an excellent target for cancer immunotherapy. Despite data showing expression of 5T4 on most solid tumours, very little is known about the expression of 5T4 on haematological malignancies. To investigate this, we screened samples collected from patients with Acute Myeloid Leukaemia (AML) for cell-surface expression of 5T4 by flow cytometry. Our data demonstrate that AML patients have 5T4 expressing leukaemic cells. We have shown that 5T4 is expressed on bulk AML blasts and the more primitive leukaemic stem cell population (CD34+CD38-). Using our proprietary Lentivector[®] technology we have developed OXB-302 to generate CAR-T cells specifically targeted towards cells expressing 5T4. In preparation for embarking on non-clinical and clinical development, we utilised 5T4-expressing AML cell lines to demonstrate the functional activity of 5T4-targeting CAR-T cells *in vitro*. We determined that the sensitivity of detection and cytotoxic killing of 5T4 expressing cells by OXB-302 cells exceeds detection by flow cytometry methods. In addition, we demonstrated the elimination of CD34+CD38- leukaemic stem cells during co-culture of CAR-T cells with Kasumi-1 AML cell line. Furthermore, we showed that pre-incubation of CAR-T cells with Kasumi-1 cells inhibited growth of leukaemic stem cells in a colony-formation assay. Together, these data evidence the fact that 5T4-targeted CAR-T cells show specificity and efficient of cell killing. This suggests that OXB-302 could successfully kill 5T4-expressing leukaemic blasts from AML patients and further eliminate 5T4-expressing leukaemic stems cells, which would help to prevent relapse.

656. Development of an *In Vitro* Assay for Assessment of IL-2 Independent CAR T Proliferation

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The rapid expansion of CAR (chimeric antigen receptor) therapies has revolutionized cancer immunotherapy. While only a few T cell-based CAR (CAR T) therapies are currently approved for B cell lymphomas, new technologies and platforms provide promise for treating a multitude of potential malignancies. During the generation of many CAR T cell therapies, expression of the CAR construct is induced by

viral transduction methods. The use of specific viral lineages during this transduction process have raised concerns regarding persistence of the CAR T cells beyond the therapeutic window. The presence of these transduced, actively proliferating CAR T cells, may lead to side effects and pathological expansion within the patient. Evaluating the potential for a transformation event that may contribute to negative outcomes such as unregulated persistence and expansion can help support the safety assessment of these products during regulatory filings. To test for cytokine-independent proliferation, we have developed an *in vitro* kinetic assay to track CAR T cell viability and proliferation. We cultured commercially available, lentiviral-transduced CAR T cells in the presence or absence of IL-2 for a period of 30 days, enumerating cells and tracking viability every 3-4 days. Additionally, we co-cultured CAR T cells with a titration of GFP-expressing (GFP+) Jurkat cells, an IL-2-independent T cell line, in the absence of IL-2 as a positive control. Using the GFP marker, spiked Jurkat cell outgrowth was tracked by flow cytometry. In the presence of IL-2, CAR T cells proliferated for a period of 2 - 3 weeks followed by a slow loss in viability and total cell number. In the absence of IL-2, CAR T cells rapidly decreased in viability and total cell number. Jurkat cells spiked at low total cell numbers into IL-2 negative wells containing CAR T cells resulted in the characteristic decrease in CAR T cell number followed by subsequent expansion of a GFP+ cell population, indicating that identification of low numbers of initial proliferative cells, such as potentially transformed CAR T cells, can be captured using this assay. When developing a cell-based therapeutic, it is essential to identify potential pathological complications during early phase studies before initiating expensive and time-consuming pre-clinical and clinical studies. In order to meet client needs and regulatory interest in this space, we provide an *in vitro* assay of CAR T cell proliferation to assess development of IL-2 independent growth.

657. Tumor Adjacent IL-2 Cytokine Factories for Eradication of Various Solid Tumor in Mice Through Cytotoxic T-cell Activation with Safe and Predictable Dosing in Non-Human Primates

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Recombinant IL-2 has been approved by the FDA for the treatment of metastatic melanoma and renal carcinoma. However, effective cytokine therapy is limited by its short half-life in circulation and the severe adverse effects associated with high systemic exposure. In an attempt to overcome the toxicities with systemic administration, intraperitoneal

(IP) administration of rIL-2 has been studied in multiple cancer types. Edwards et al. studied IP administration of rIL-2 in platinum-resistant and platinum-refractory ovarian cancer with an ORR of 25%, however, the use of in-dwelling peritoneal catheter led to a significant number of patients having catheter obstruction or infusion pain. To overcome these limitations, we developed a clinically translatable localized cytokine delivery LOCOcyte™ platform composed of polymer encapsulated human epithelial cells that produce potent immune effector molecules for local delivery with temporal regulation. AVB-001 is engineered to produce native hIL-2, for the treatment of peritoneal cancer. Administration of AVB-001 in mice showed that IL2 local concentration (IP space) was 100x higher than the systemic concentration demonstrating the ability of the LOCOcyte™ platform to deliver native cytokines *in vivo* and create a high local concentration of cytokines with limited peripheral exposure. Additional studies in mice demonstrated a dose-dependent levels of IL-2 in the IP cavity in mice. Treatment of peritoneal solid tumors using a single administration of IL-2 producing cytokine factories demonstrated complete responses as monotherapy and provided sustained eradication of tumors in ID8 ovarian cancer and MC38 colon cancer mouse model. Our data in mice confirmed that AVB-001 leads to a local increase in activation (CD25+CD8+) (4.5 fold) and proliferation (Ki67+CD8+)(10 fold) of cytotoxic T cells within the IP space in comparison to sham mice. To determine whether administration of AVB-001 elicits local and systemic antitumor immune response capable of targeting distal tumors we assessed the establishment of tumor-specific memory responses. We designed a tumor rechallenge experiment where mice with IP tumors that were treated with AVB-001 were challenged with a second MC38 tumor injection at a distant location 35 days after the first MC38 tumor injection. None of the AVB-001 treated mice developed a subcutaneous tumor in contrast to the control naïve animals. Finally, we studied the pharmacokinetics, pharmacodynamics, and safety of AVB-001 in non-human primate (NHP). Administration of AVB-001 led to therapeutic levels of IL-2 in the IP space in NHP and produced local and systemic T cell biomarker profiles that predict efficacy. In addition, administration of AVB-001 cytokine factories at various doses in NHP was well tolerated with no abnormal clinical observations by all animals. Our findings demonstrate that the LOCOcyte™ platform is dose adjustable, safe, and efficacious in preclinical animal models. Avenge Bio aims to pursue a Phase 1 study of AVB-001 in ovarian cancer patients. The LOCOcyte™ platform enables itself to deliver a diverse set of cytokines alone or in combination which is presently explored.

658. Assessment of Rituximab as a Pretreatment Strategy for Augmentation of CAR/CXCR5 T Cell Therapy in SIV-Infected Rhesus Macaques

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During chronic HIV or SIV infection, the majority of viral replication occurs within B cell follicles in secondary lymphoid tissues. We have demonstrated that infusion of CAR T cells which express the follicular homing receptor, CXCR5, leads to localization of the cells within follicles where they have the potential to track down and kill the virally infected cells. A problem with this potential cure strategy is the lack of persistence of the CAR T cells in the treated animals. We hypothesized

that temporary disruption of follicles with Rituximab, prior to CAR T cell infusion, would create space for the CAR T cells to engraft and lead to increased persistence. SIV-infected, ART-suppressed rhesus macaques were treated with 7 mg/kg Rituximab. Seven days later, CAR/CXCR5 T cells were infused and the animals were released from ART. Paired animals, serving as controls, were pre-treated with Rituximab but were not infused with CAR/CXCR5 T cells at the time of ART release. The first animal was treated with a dose of 2×10^8 CAR T cells per kg. Two days after the infusion, the animal experienced apparent cytokine release syndrome (CRS) and was sacrificed. Subsequent animals were pre-treated with Siltuximab in order to prevent CRS and were used in a CAR T cell dose escalation study in order to assess safety of lower doses. The animals were infused with CAR/CXCR5 T cell dosages of 1 to 8.3×10^7 cells per kg at the time of ART release. The infused cells expressed high levels of CAR and CXCR5 (88-93% co-expression), were primarily central memory (77-85%), and showed specific migration to CXCL13 (50-66%) in vitro. Preliminary IHC analysis of lymph nodes biopsied at 9 and 13 days post-Rituximab treatment (days 2 and 6 post-infusion) showed few small follicular-like structures in lymph nodes. Preliminary RNAScope analysis of CAR/CXCR5 T cells and vRNA in lymph nodes, show increasing quantities of CAR/CXCR5 T cells with dose, expansion of CAR/CXCR5 T cells from day 2 to 6, and a sharp decline of cells at day 14, all occurring prior to the detection of recrudescing vRNA. No impact on viral loads was detected early after infusion in the treated animals. Thus, preliminary data from our ongoing study suggests that Rituximab pretreatment does not lead to increased persistence of CAR/CXCR5 T cells. Nonetheless, this study is yielding important information for the cellular immunotherapy field.

659. PD1 Blockade of Tumor-Primed CD4 T Cells Initiates Protective Antitumor Immunity

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Blockade of immune checkpoint PD1 in cancer patients leads to remarkable therapeutic benefits. Uncovering mechanisms behind PD1 blockade-mediated antitumor immunity is important to identifying biomarkers for predicting responses to this therapy and developing innovative PD1 blockade combined therapeutic strategies for broader clinical efficacy. While rejuvenation of preexisting intratumoral 'exhausted' T cells and recruitment of peripheral antitumor T cells into tumors have been proposed to explain how PD1 blockade works, mechanisms of action of PD1 blockade remain to be fully revealed. In a clinically-relevant mouse model of spontaneous metastatic breast cancer 4T1.2-Neu, our studies suggest that effective and durable anti-PD1 therapy depends on systemic immunity, including type 1 conventional dendritic cells (cDC1: CD8 α ⁺/CD103⁺DC), B cells, and CD4/CD8 T cells. Also, elevated systemic and/or intratumoral IL12⁺CD103⁺DC and IL2⁺CD4/CD8 T cells are associated with the response to PD1 blockade. Further, anti-PD1 treatment results in tumor-primed CD44⁺IL2⁺CD4 T cells in the periphery regardless of

cDC1. In the breast cancer model with adoptive cell transfer, PD1 blockade-driven CD44⁺CD4 T cells infiltrate into tumors and ignite protective antitumor immunity in an IL2-dependent and tumor-specific manner. Together, these data suggest the prominent effect of PD1 blockade on tumor-primed CD4 T cells in instigating tumor-specific antitumor immunity.

660. Assessing Heterogeneity of CD8⁺ CAR-T Cell Subpopulations Using Single-Cell RNA Sequencing

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Many cancer therapies rely on immunotherapeutic approaches. In particular, chimeric antigen receptor T cell (CAR-T) immunotherapies have transformed treatment options for many cancer patients. The cellular composition of the CAR-T cells, including the proportions CD8⁺ T cell subpopulations, is a critical success factor for efficient CAR-T immunotherapy. CD8⁺ T cells play a crucial role in eliminating tumor cells, with various subpopulations playing different roles in tumor response. Single-cell RNA-seq profiling can serve as an analytical tool to capture cell-to-cell variability in CAR-T cells and identify the right cell types to ensure cell expansion, persistence, and anti-tumor activity. Here, utilizing the Genedata Selector[®] software, we analyzed a public single-cell RNA-seq dataset that was generated from the CAR-T infusion products and blood of patients with durable persistence to immunotherapy. We aimed to identify and characterize three different CD8⁺ T cell subpopulations using previously reported marker genes: naïve/memory, cytotoxic, and exhausted CD8⁺ T cells. We hypothesized that these different CD8⁺ T cell subpopulations would contribute to a differential therapeutic response and altered biological processes. Subpopulations of naïve/memory and cytotoxic CD8⁺ T cells were identified in both the CAR-T infusion products and blood of patients with favorable response to the immunotherapy. Genes expressed in these subpopulations were found to be involved in the tumor cell response and regulation of T cell-mediated cytotoxicity. Interestingly, there was no detectable expression of exhausted CD8⁺ T cell subpopulations as indicated by the diminished levels of immunosuppressive checkpoint genes such as *CTLA-4*, *LAG3*, *HAVCR2*, and *TIGIT*, all of which can dampen anti-tumor activities of T cells and lead to T cell exhaustion. Moreover, we identified three genes, *ENO1*, *LADH*, and *GZMB* to be highly expressed in the cytotoxic CD8⁺ T cells relative to the naïve/memory T cells, which could serve as potential markers for anti-tumor activities. In this study, the persistence of the naïve/memory and cytotoxic CD8⁺ T cells, in addition to the lack of exhausted CD8⁺ T cells, were indicative of positive therapeutic response in these patients. Overall, utilizing the automated single-cell RNA-seq workflow, as implemented in the Genedata Selector[®] software for this study, enabled the rapid testing of our hypothesis by profiling CD8⁺ T cell population before and after infusion and identifying transcriptional profiles associated with positive therapeutic outcome to CAR-T immunotherapy.

661. Preclinical Development of LYL797, a ROR1-Targeted CAR T-Cell Therapy Enhanced with Genetic and Epigenetic Reprogramming for Solid Tumors

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Chimeric antigen receptor (CAR) T-cell therapy has produced profound results in certain hematologic malignancies, however treatment of solid tumors has not been as successful. Studies have suggested that T-cell exhaustion plays a role in limiting the ability of CAR T cells to eradicate solid tumors. Additionally, stem-like qualities of T cells have been associated with improved outcomes in patients treated with cellular therapies, including CAR T cells. Therefore, maintaining stem-like qualities and overcoming T-cell exhaustion may be key to improving clinical efficacy of CAR T cells in solid tumors. LYL797 is a novel, ROR1-targeted CAR T-cell product that incorporates genetic and epigenetic reprogramming technologies, Gen-R and Epi-R, designed to overcome barriers to CAR T-cell therapy in solid tumors. Gen-R is an ex vivo genetic reprogramming technology in which CAR T cells are engineered to overexpress the activator protein 1 (AP-1) transcription factor c-Jun. Dysregulation of the AP-1 family of transcription factors has been implicated in CAR T-cell exhaustion, and previous studies have demonstrated that overexpression of c-Jun can reduce functional exhaustion, enhancing both anti-tumor efficacy and persistence in preclinical hematologic and solid tumor models. Epi-R, a proprietary epigenetic reprogramming protocol, optimizes the T-cell manufacturing process resulting in a T-cell product that maintains stem-like phenotype. Preclinical studies of LYL797 in ROR1+ tumors have demonstrated the favorable impact of the combination of Gen-R and Epi-R on CAR T-cell characteristics and functionality. First, addition of Epi-R resulted in higher proportions of stem-like T-cell populations and demonstrated improved functional activity in the presence of ROR1+ tumor cells compared to ROR1 CAR T cells without Epi-R. Further modification of CAR T cells using Gen-R maintained high potency in clearance of ROR1+ tumor cells and improved ROR1-specific cytokine production in vitro. An in vitro chronic antigen stimulation model was developed to characterize the effects of Gen-R on CAR T-cell exhaustion. In this model, the ROR1 CAR T cells without Gen-R displayed characteristics of exhaustion such as expedited loss of cytokine secretion and increases in multiple exhaustion-related cell subsets and gene signatures; LYL797, however, demonstrated prolonged persistence and cytokine production. The LYL797 findings also revealed a reduction in exhaustion-related cell subsets and gene signatures as well as an enrichment in c-Jun binding motif, indicating persistent c-Jun transcription factor binding activity following chronic antigen stimulation. Taken together, these data support the hypothesis that the c-Jun overexpression in LYL797 effectively reduces T-cell exhaustion and loss of function in the presence of repeated antigen

stimulation. Additionally, LYL797 demonstrated overall improved survival and expansion in the peripheral blood of ROR1+ tumor-bearing animals, which correlated with improved anti-tumor activity in an established human ROR1+ NSCLC mouse xenograft model. These studies collectively demonstrate that Gen-R and Epi-R technologies can give rise to enhanced and prolonged anti-tumor functional capacity of CAR T-cell therapy in solid tumors. Based on these promising preclinical data, LYL797 is being studied in a phase I clinical trial to assess the safety, pharmacokinetics, immunogenicity, efficacy, and duration of effect for patients with ROR1+ TNBC and NSCLC.

662. Engineering Plasma Cells to Secrete Anti-Cancer Biologics in Humanized Mice

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Advances in genome-engineering have enabled the generation of plasma cells (PCs) that secrete large quantities of therapeutic proteins. However, *in vivo* modeling of unmanipulated and engineered human PCs has largely been limited to short-term studies following adoptive transfer of PCs into immunodeficient mouse models including NSG mice. Human PCs are challenging to study in immunodeficient mouse models, in part, due to deficiencies (missing or lack of cross-species reactivity) in key factors provided by human bone marrow stromal and myeloid cells. In this study, we tested the hypothesis that immunodeficient mice humanized by engraftment of human CD34+ hematopoietic peripheral stem cells (NSG-huCD34) would increase the engraftment potential of genome-engineered human PCs. Further, we predicted that this approach might help to elucidate critical interactions between human PCs and human host BM-resident cells (myeloid cells and lymphocytes). Consistent with this concept, autologous genome-engineered plasma cells engrafted more efficiently in NSG-huCD34 mice than in NSG control mice. Further, genome-engineered PCs in NSG-huCD34 mice secreted substantially higher levels of antibodies (>100 ug/mL for over 80 days) and engrafted in additional lymphoid compartments. Genome-engineered PCs migrated to and localized in the bone marrow and spleen within 2 days of transfer and were retained in these locations based upon luciferase-based *in vivo* imaging, ex vivo flow analysis and histopathology. Taken together, these data demonstrate that the presence of autologous human hematopoietic cells in NSG-huCD34 mice permits establishment of a robust model for studying the *in vivo* biology of genome engineered, long-lived human PCs. This system is likely to permit assessment of functional activity of drug products produced by edited PCs as well as drug mediated manipulation of immune cell interactions in malignancy models. In support of this postulation, we will present proof-of-concept data showing the potential of PCs engineered to secrete anti-cancer therapeutics.

663. Establishing a Cell Library Screening Strategy for Identifying Novel NanoCARs

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Chimeric antigen receptor (CAR)-T cell therapy targeting CD19 through genetically modifying T cells have enhanced the treatment of B cell malignancies such as acute lymphoblastic leukaemia (B-ALL). While complete responses are observed for CD19-CAR-T therapy, long-term efficacy remains poor among most CAR-T clinical trials for haematological and solid tumours. Replacing the conventional single-chain variable fragments (scFv) with camelid-derived single-domain antibodies (also known as VHH or Nanobodies) for CAR-T targeting elements has shown good long-term efficacy in clinical trials for BCMA-targeting CAR-T and are now being investigated for numerous tumour targets. Nanobodies offer several advantages over the scFv-based binding element, including smaller size, access to different epitopes than scFvs, and low immunogenicity, due to high sequence similarity with human VH domains. However, like scFv-CARs, current discovery strategies for identifying functional nanobody-based CAR molecules (nanoCARs) are time-consuming, laborious, and expensive. We report the development of a polyclonal nanoCAR cell library screening strategy, wherein nanobody sequences are polyclonally transferred into a modular CAR backbone, followed by lentiviral production and generation of a polyclonal CAR-Jurkat library. Downstream, clonal nanoCARs are isolated through stimulation with target-expressing cells and several rounds of cell sorting. We are now testing this screening strategy with a multi-antigen adeno-based immunization strategy to quickly identify multiple novel CAR binding elements targeting CD19, CD20, and MUC1. Immunised llama serum showed cell binding to target-specific cells compared to pre-immune llama serum. After the generation of the polyclonal multi-antigen targeting CAR plasmid library, stable cell lines were made using lentiviral transduction or an alternate CRISPR-mediated insertion strategy to create a CAR-Jurkat cell library. Cell sorting of the polyclonal nanoCAR Jurkat cell library in the absence of target cells demonstrated that it was possible to group cells according to different activation profiles. Sanger sequencing again confirmed polyclonality of the initial plasmid library and after the polyclonal nanoCAR Jurkat cell library generation. The next step will involve sorting against target-specific and target-knockout cell lines to isolated binders for specific target antigens. Additionally, next-generation sequencing will be performed to gain a deeper understanding of how different sequences could predict nanoCAR functionality as a single-antigen molecule and as part of a multi-antigen CAR. Ultimately, through this cell-based screening approach, we should be able to isolate lead candidates for therapeutic development and binders demonstrating varying activations profiles for the development of multi-antigen nanoCARs.

664. CD4⁺ T Cells Engrafted with MHC Class II-Restricted T Cell Receptors Provide Help for T Cell Therapy of Hepatitis B Virus Infection and Hepatocellular Carcinoma

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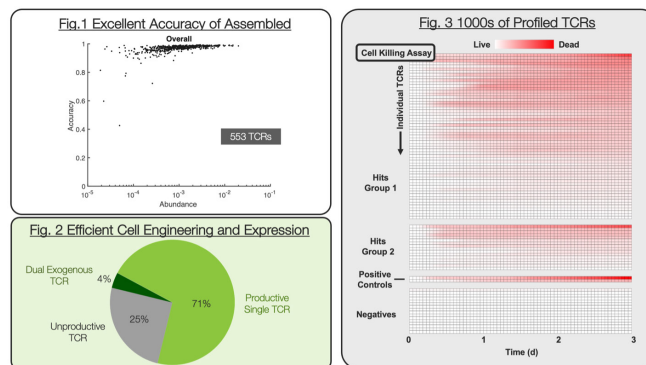
Hepatitis B virus (HBV) infection remains a major global health problem with 296 million chronically infected individuals and an estimated 820000 deaths in 2019, mostly related to hepatitis B-induced liver cirrhosis or hepatocellular carcinoma. Current therapeutic options require lifelong medication and rarely achieve viral clearance. In the natural context of resolved infection, virus eradication is associated with a strong T cell response, whereas chronically ill patients typically display scarce and dysfunctional T cells. Therefore adoptive T cell transfer has emerged as a promising alternative treatment approach. We recently demonstrated that CD8⁺ T cells grafted with T cell receptors (TCR) restricted by MHC class I have the potential to cure HBV infection *in vitro* and *in vivo* when combined with HBV entry inhibitor Myrcludex B. Nevertheless, CD4⁺ T cells are also known to play an important role in the natural course of resolved HBV infection, since they were shown to be indispensable for the induction of a functional virus-specific CD8⁺ T cell response in HBV-infected chimpanzees. Mechanistic details of their helper function, however, or their benefit for adoptive T cell therapy remain poorly understood. We identified, cloned and characterized a library of 20 MHC class II-restricted TCRs from HBV-specific CD4⁺ T cells, restricted by nine different MHC II molecules and specific for eight different epitopes from the HBV envelope, core or polymerase protein. The HBV core-specific TCR 1C11 and envelope-specific TCR 1E1 were chosen for further functional studies, based on their broad therapeutic applicability, strong functional avidity and their HLA-DR1 restriction, which allows for *in vivo* analysis in an HLA-A2/DR1-transgenic mouse model. In a triple co-culture system, where HBV peptide- or protein-loaded monocyte-derived dendritic cells served as antigen-presenting cells, HBV-specific TCR-transduced CD4⁺ T cells exerted a specific helper effect towards CD8⁺ T cells, measured through CD8⁺ T cell proliferation and IFN- γ secretion. This effect was observed in particular at lower target concentrations. Upon co-culture with HBV-expressing hepatoma cells, HBV-specific MHC class II-restricted CD4⁺ T cells were able to enhance CD8⁺-mediated killing, especially when CD8⁺ T cells were transduced with a low-affinity MHC class I-restricted TCR. These data suggest that HBV-specific MHC II-restricted CD4⁺ T cells can exert help towards CD8⁺ T cells with a potential benefit for T cell therapy of viral infection or HBV-induced hepatocellular carcinoma. In addition, they could be used as an experimental tool for elucidating the mechanistic details of CD4⁺ T cell help in HBV infection.

665. Massively Parallel TCR Gene Synthesis and Unbiased Functional Screening: From Decoding Immune Interactome to Fully Personalized TCR-T Therapy

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Identifying and understanding the tumor reactive T cell population is fundamental for advancing cancer immunotherapy. However, discovering these tumor reactive T cells and their corresponding T cell receptors (TCRs) has been limited by current technology, namely due to their low and variable frequencies in the tumor and periphery. Rapid and systematic identification of these tumor reactive TCRs will enhance our understanding of tumor immunity, lead to fully personalized TCR-T cell therapies, and potentially new “off-the-shelf” TCR-T cell therapies. With the advent of scRNA-Seq, >1,000 α - β paired TCR sequences from a tumor sample can be routinely obtained. Unfortunately, one cannot easily discover the rare tumor reactive TCRs from this collection yet. To do so, one would ideally synthesize and test all TCRs sequenced. Using existing technology this is cost prohibitive, exceeding \$100,000 in synthesis cost alone (~\$100 per TCR). To address this challenge, we have developed a massively parallel TCR gene synthesis technology, named PathFinder™ that enables orthogonal synthesis of 1,000s of TCRs at a time for a cost of **under \$1 per TCR**. Our DNA assembly technology takes advantage of inexpensive, custom-made oligonucleotide pools, but makes a critical innovation that allows for many kilobase-long fragments to be assembled in a single compartment. Using our proprietary, biophysics-driven approach we can generate junction sequences in coding regions that are sufficiently orthogonal and allow for the assembly of highly related sequences such as TCRs. NGS analysis showed extremely high fidelity of our assembly as well as high overall accuracy and uniformity (Fig. 1), thus demonstrating our capabilities in this extremely challenging context of TCR assembly. To further enable our personalized and off-the-shelf TCR workflows, we developed a high-throughput screening platform. The pooled TCR sequences from our assembly are transferred to and functionally expressed in T cells while ensuring each T cell only expresses a single TCR (Fig. 2). Pooled TCR T cells can then be screened for tumor reactivity by co-culturing with tumor or other antigen-expressing cells, selected based on an activation marker, and sequenced to identify the tumor reactive TCRs. This is our core discovery engine, and to date we have synthesized more than 20,000 TCRs from various tumor samples and have identified dozens of tumor-reactive TCRs (see Fig. 3 for examples). With our core discovery engine, we have built the foundation for our first personalized cell therapy program, called DisTIL. This therapy starts from primary tumor resections and results in a truly personalized, polyclonal and highly potent TCR-T therapy. From the patient TIL, thousands of TCRs are synthesized and screened for tumor reactivity and only those reactive TCRs are infused back to the patient. With this growing catalogue, we’re building a database of TCR reactivity, how that relates to the original T cell phenotype, and discovering their target antigens to decode the immunome at unprecedented levels. This uniquely enables highly potent personalized TCR-T therapies, novel off-the-shelf TCR-T products against the most relevant targets, and greater immunological insights.



666. Investigating the Role of T-Cell Immunoglobulin- and Mucin Domain-Containing (TIM)-3 on Natural Killer Cell Metabolism and Cytotoxicity Against Glioblastoma Multiforme

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Glioblastoma (GBM) is the most common and fatal type of primary brain cancer, yet there is no curative regimen. While immunotherapy has made substantial progress in the treatment of many cancers, advancement in GBM therapy has been lackluster due to the multifaceted challenges GBM poses. Among immunotherapeutic approaches, adoptive cell therapy using Natural Killer (NK) cells has emerged as promising due to NK cells’ inherent safety, ability to target tumors without prior antigen sensitization, and potential for allogeneic use. Though few in numbers, NK cells can infiltrate brain tumors and exert powerful effector functions. However, the Warburg effects due to metabolism of cancer cells in the tumor microenvironment can rapidly exhaust NK cells and drastically impair their cytotoxicity. One way to remedy NK dysfunction is through *ex vivo* persistent metabolic reprogramming, by enabling the cells to not only effectively compete for nutrients, but also maintain their cytotoxic functions. As NK cells’ activities are regulated by the balance of their surface receptors, a receptor that uniquely lies at the intersection between immunometabolism and functions is TIM-3. Previously, we have reported that lower TIM-3 expression is correlated to lower IFN- γ production against GBM, and others have shown that downregulation of this receptor is also related to decreased phosphorylation of mTOR, among others. As cytokine production reflects immunometabolic states, we hypothesize that expression of TIM-3 drives activation in NK cells to promote increased target lysis and IFN- γ secretion by engaging NK cells in a metabolically competitive state against GBM targets. NK cells were isolated from healthy adult donor peripheral blood and expanded *ex vivo* through a K562-based feeder system. TIM-3 *knock-out* was generated using electroporation of CRISPR/Cas9 ribonucleoprotein complexes, and *knock-out* cells were challenged against patient-derived glioblastoma targets for evaluation of cytotoxicity. Changes in phosphorylation of key proteins such as mTOR, Akt, and rS6 were measured by phospho-flow cytometry. We found that TIM-3 *knock-out* NK cells have lower cytotoxicity that those of the *wild-type*, and that this behavior is consistent with those of GBM patients, where the tumor infiltrating NK cells have lower TIM-

3 expression than trafficking peripheral blood NK cells, that later of which are expected to have an activated metabolism. Our data suggests that TIM-3+ NK cells are metabolically resilient, and the presence of this receptor can be beneficial in assisting NK cells to overcome the acidic and fatty GBM tumor microenvironment.

667. A Vaccine Targeting The Recurrent Driver Mutation H3K27M Induces A Mutation Specific T- and B- Cell Response in Patients with Diffuse Midline Gliomas

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A recurrent point mutation at position 27 in the histone-3 gene (H3K27M) defines a distinct subtype of highly aggressive diffuse midline gliomas (DMG) characterized by high mortality and morbidity rates. Despite the high clinical need and several clinical trials focusing on novel treatments, therapeutic interventions remain limited. Immunotherapeutic approaches are increasingly investigated in patients with malignant gliomas. A vaccine targeting the neoepitope H3K27M has been shown to induce a mutation-specific CD4⁺ T-cell response and confer tumor control in an MHC humanized mouse model. We have developed a protocol to expand neoantigen-reactive T cells from vaccinated patients, and recover T-cell receptors (TCR) using single cell RNA sequencing. We established a medium-throughput pipeline to clone TCRs from expanded populations of T cells and test their neoepitope specificity. Applying a novel DNA assembly process, we could significantly decrease costs, allowing us to test large numbers of TCRs for neoepitope specific reactivity using a co-culture assay with immortalized patient-derived cell lines. In addition to confirming the presence of mutation-specific CD4⁺ TCR upon vaccination of DMG patients with a long H3K27M peptide we have identified activated B cells in a cerebrospinal fluid sample from a vaccinated patient. We validated these B cells to encode H3K27M antibodies by cloning and testing BCR recovered from single B cells. Taken together, our data shows that a neoepitope vaccine targeting H3K27M is not only able to mount a CD4⁺ specific T-cell response but further induces a mutation-specific B-cell response with development of H3K27M-targeting antibodies.

668. iPSC-Derived CD38-Null NK Cells in Combination with CD38-Targeted Antibody Represent a Novel Therapeutic Strategy to Avoid Host Immune Cell Rejection for Off-the-Shelf Cell-Based Cancer Immunotherapy

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Conditioning chemotherapies that temporarily suppress a patient's immune system are commonly used in both autologous and allogeneic cell-based cancer immunotherapy. However, the intensity and frequency of lympho-conditioning can impact immune reconstitution and increase a patient's susceptibility to adverse outcomes, such as severe infections. Genetic deletion of cell-surface human leukocyte antigen (HLA) molecule expression has long been known to abrogate T-cell alloreactivity. Loss of class I HLA elicits NK cell-mediated recognition and attack though, and therefore additional immunomodulating strategies must be applied. To this end, mouse models using immune cells expressing certain inhibitory molecules, such as HLA-E and CD47, have been shown to abrogate NK cell alloreactivity. However, in the human system, HLA-E is the canonical activator of NKG2C, a dominant activating receptor found on human NK cells. Likewise, the expression of signal regulatory protein alpha (SIRP α), the major interactor for CD47, is mostly restricted to human macrophages and dendritic cells and not human NK cells. In this study, we provide details of a novel therapeutic strategy that combines engineered iPSC-derived NK (iNK) cells that uniformly lack CD38 surface expression with anti-CD38 antibodies to avoid host immune cell alloreactivity for off-the-shelf cell therapy. When testing iNK cells engineered with knockout of beta-2-microglobulin (B2M KO) to ablate HLA class I expression, B2M KO iNK cells were depleted over time in mixed lymphocyte reaction (MLR) assays containing peripheral blood mononuclear cells (PBMCs), suggesting activation of a "missing self" response by PBMC-containing NK cells. To overcome this mechanism of NK cell alloreactivity, we also genetically knocked-out CD38 (CD38 KO) to derive CD38 KO / B2M KO iNK cells having the potential to avoid anti-CD38 antibody-mediated fratricide. When combined with CD38-targeted antibody, the depletion of B2M KO CD38 KO iNK cells was abrogated as expected, and B2M KO CD38 KO iNK cell numbers were increased by 3.5-fold, comparable to the iNK cell numbers cultured without PBMCs. In contrast to these observations seen with B2M KO CD38 KO iNK cells, the combination of anti-CD38 antibody with B2M KO iNK cells resulted in fratricide and reduction of iNK cell counts. B2M KO iNK cells impaired expansion of PBMC-containing T cells over the duration of co-culture, resulting in 50% lower fold T-cell expansion at the peak of the control response, while B2M WT iNK cells stimulated T-cell activation and depleted iNK cells over time. However, parallel co-cultures maintained in the presence of anti-CD38 antibodies

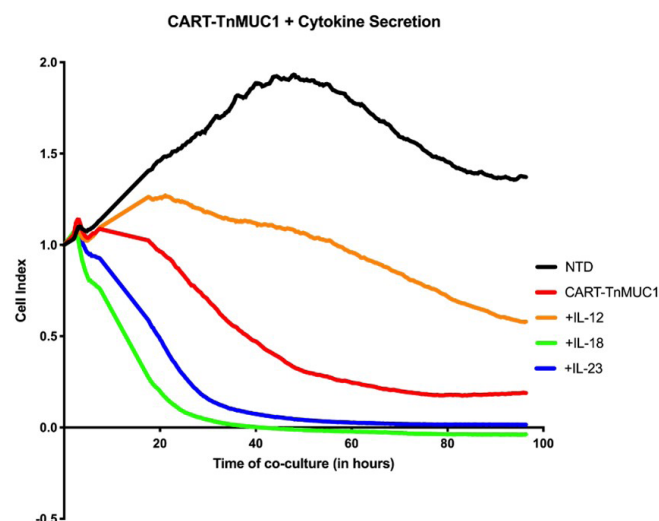
showed complete iNK cell resistance (both B2M KO and B2M WT iNK cells) to allogeneic PBMC attack, suggesting that anti-CD38 antibodies affords some control of allogeneic T-cell responses as well. Ongoing *in vivo* studies suggest that co-administration of anti-CD38 antibodies can significantly enhance the persistence of iNK cells in the presence of allogeneic PBMCs as seen in the blood, spleen and bone marrow. These data demonstrate the potential advantages of combining iPSC-derived CD38-null NK cells with anti-CD38 antibodies as a novel therapeutic strategy for reducing conditioning chemotherapy, depleting alloreactive lymphocytes, and promoting off-the-shelf cell therapy.

669. Effects of Constitutive Secretion of Immunostimulatory Cytokines on CAR-T Cells

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The durable responses of chimeric antigen receptor (CAR) T cells in hematological tumors have inspired development of CAR T cell therapies for the treatment of solid tumors. Fourth-generation CAR T cells that target tumor-associated antigens and secrete soluble factors, such as bi-specific T cell engagers or cytokines, can eradicate solid tumors when compared to CAR T cells alone through multiple mechanisms, including enhancements to T cell differentiation, proliferation, survival, effector functions, and engagement of endogenous immunity to overcome tumor heterogeneity. To improve the efficacy of anti-TnMUC1 CAR T cells, we generated fourth-generation CAR T cells that utilize the variable domains of the anti-TnMUC1 antibody 5E5, are co-stimulated by CD28, and constitutively secrete the following immunostimulatory cytokines: a single chain IL-12, IL-18, and a single chain IL-23. IL-12 and IL-18 secretion have been shown to increase the proliferation, survival, and IFN γ secretion of CAR T cells. In our study, we confirmed the secretion of IL-12p40 (the subunit of IL-12 not shared with IL-23), IL-18, and IL23, respectively, and observed enhanced secretion when T cells were stimulated by PMA and ionomycin or the triple-negative breast cancer cell line MCF7. Impedance-based cytotoxicity assays demonstrated enhanced killing of multiple tumor cell lines by CAR T cells secreting IL-18 and IL-23, while cells secreting IL-12 show decreased killing as compared to CAR T cells alone (Figure 1). CAR T cells constitutively secreting IL-12 exhibited a significant decrease in the number of central memory T cells (CCR7+, CD45RO+), with a shift toward more effector memory-like cells (CCR7-, CD45RO-), and enhanced activation of STAT4, while CAR T cells secreting IL-23 showed robust activation of STAT3. Future directions include RNA sequencing to determine the differential gene expression induced by constitutive cytokine secretion as well as evaluation of cytokine-secreting CAR T cells in a xenograft model of triple-negative breast cancer.



670. Efficient Non-Viral Engineering and Large-Scale Expansion of Polyclonal $\gamma\delta$ T Cells

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Introduction: $\gamma\delta$ T cells are a subset of lymphocytes that recognize non-peptide antigens via canonical $\gamma\delta$ T cell receptors (TCRs). Because of their diverse functionality and MHC-independent cytolytic activity, $\gamma\delta$ T cells have garnered increasing interest as a potential allogeneic immunotherapy. However, their relative infrequency within peripheral blood, combined with inefficient genetic engineering and expansion of populations bearing polyclonal $\gamma\delta$ TCR repertoires, has limited their clinical application. Here we describe a novel procedure for the large-scale production of non-virally engineered $\gamma\delta$ T cells possessing potent anti-cancer activity. **Methods:** To select the optimal antibody for $\gamma\delta$ T cell expansion and TCR heterogeneity, peripheral blood $\gamma\delta$ T cells were isolated and stimulated with a variety of plate-bound antibodies, along with soluble CD28 as a cofactor. Cells were then subjected to a series of culture and electroporation conditions, yielding an optimized two-stage protocol for $\gamma\delta$ T cell engineering (Fig. 1A). Cas9 base editing was used to inactivate genes responsible for immune cell inhibition, including *CISH* and *PD1*. Cytolytic activity was further enhanced via transposon-based integration of a CD19 chimeric antigen receptor (CAR) construct. Cytotoxicity against tumor lines was evaluated using *in vitro* co-culture assays. **Results:** $\gamma\delta$ T cell populations stimulated with plate-bound pan- $\gamma\delta$ TCR and soluble CD28 yielded >250-fold expansion by day 11, and continued to expand >10,000-fold following a second 11-day cycle (Fig. 1B). These $\gamma\delta$ T cells exhibited significantly greater TCR heterogeneity than populations produced using zoledronate-based expansion methods (Fig. 1C), and retained both cytolytic activity and effector functions even after repeated stimulation. When electroporated 48 hours after initial stimulation, $\gamma\delta$ T cells experienced highly efficient gene knockout with Cas9 base editor (>90%, Fig. 1D) and transposon-mediated CAR integration (>45%, Fig. 1E). $\gamma\delta$ T cell populations engineered to express

a CD19 CAR possessed potent cytolytic activity *in vitro* (Fig. 1F), yielding >97% clearance at a 1:1 effector:target ratio after 48 hours. **Conclusions:** The high cytolytic activity and potent effector functions of $\gamma\delta$ T cells make them a prime candidate for use in cancer immunotherapies. We developed a scalable, clinically-relevant method of expanding non-virally engineered human $\gamma\delta$ T cells with enhanced cytolytic activity. $\gamma\delta$ T cell populations generated using this process achieved >10,000-fold expansion across a 22 day time course, and, when engineered with a CD19 CAR, displayed highly-efficient killing against target cell lines. Our approach substantially improves the feasibility of developing allogeneic CAR T cell banks capable of supporting large numbers of patients, with broad implications for the treatment of human cancers. Ongoing experiments are being conducted to determine *in vivo* anti-tumor activity, and to further increase functional activity via multiplex base editor engineering.

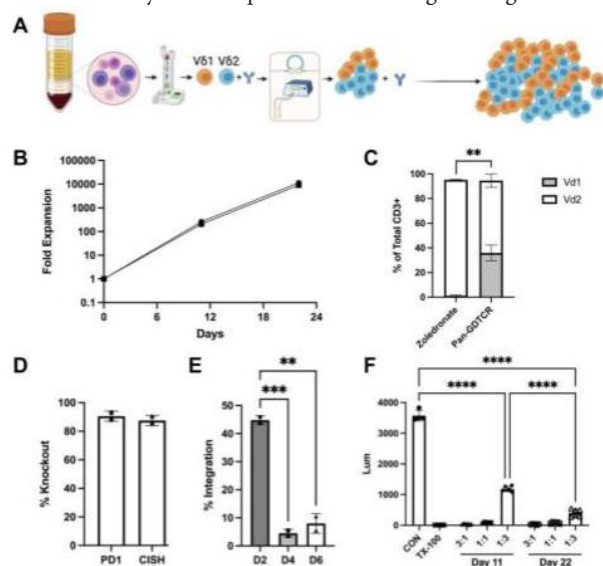


Figure 1. Non-viral engineering and large-scale expansion of $\gamma\delta$ T cells. (A) Procedure for large-scale expansion of engineered $\gamma\delta$ T cells. Purified $\gamma\delta$ T cells were stimulated with pan- $\gamma\delta$ TCR antibody and soluble CD28, engineered after 48 hours, re-stimulated on day 11, and harvested at day 22. (B) Outgrowth of purified $\gamma\delta$ T cells following pan- $\gamma\delta$ TCR stimulation. (C) $\gamma\delta$ T cells stimulated with pan- $\gamma\delta$ TCR possess higher TCR diversity than those produced from zoledronate. (D) Base editor knockout of *CISH* and *PD1* in $\gamma\delta$ T cells transfected 48 hours after initial stimulation. (E) CAR integration as a product of electroporation day. (F) $\gamma\delta$ T cells engineered with a CD19 CAR display highly efficient killing of tumor lines. Luminescence of Raji-Luc target cells was measured after 48 hrs. $P^{***} > 0.01\%$, $^{****} > 0.001$, $^{*****} > 0.0001$.

671. In Vitro Modeling of CAR T Cell Induced Cytokine Release Syndrome Validates Two Genetic Targets to Mitigate the Condition

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Chimeric antigen receptor (CAR) T cells are genetically engineered to recognize and eradicate cancer cells. CAR T cell therapy shows promising results in treating patients with hematological malignancies but it can also lead to undesirable side effects, such as Cytokine Release

Syndrome (CRS). CRS is triggered by CAR T cell-based activation of monocytes, which leads to massive release of cytokines, especially IL-1b and IL-6. The monocytes are activated by direct interaction with the CAR T cells via the CD40 ligand (CD40L) - CD40 receptor axis or via uptake of GM-CSF released from the activated CAR T cells. Current mouse xenotransplantation models are able to predict the efficacy of CAR T cells but not necessarily CRS. To overcome this limitation, we established a cell-based *in vitro* assay that involves the co-culturing of leukemic B cells with primary CD19-targeting CAR T cells and monocytes from the same donor to monitor both CAR T cell activity and cytokine release. We show that upon activation, the CD19-targeting CAR T cells upregulated CD40L and GM-CSF expression, which in turn activated the isogenic monocytes to secrete IL-1b and IL-6. CRISPR-Cas9 based disruption of the CD40L and/or GM-CSF encoding loci in CAR T cells did not affect the antitumor activity of the engineered lymphocytes but significantly reduced IL-1b and IL-6 secretion from the monocytes. We conclude that our cell-based assay is able to model CRS *in vitro*, allowing us to evaluate gene-edited CAR T cells pertaining their antitumor activity as well as their propensity to trigger CRS.

672. Intranasal Administration of Multi-Epitope Long Peptide with D-Octaarginine-Linked PNVA-co-AA as a Novel Drug Delivery System for Cancer Vaccine

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Introduction: Recent advances of cancer immunotherapy greatly contribute the clinical success, however, the drug delivery system such as administration route and antigens can be improved and simplified. Mucosal immunization such as oral, nasal or sublingual vaccination could induce systemic cellular immune responses effectively by stimulating mucosa-associated immune system. In this study, we investigated the therapeutic efficacy of intranasal administration of cancer vaccine with multi-epitope long peptide and cell penetrating D-octaarginine-linked co-polymer of N-vinylacetamide and acrylic acid (PNVA-co-AA) as novel cancer vaccine. **Methods:** Four CD8 epitopes from human papillomavirus (HPV) type 16 E6E7 proteins were linked by arginine linkers to synthesize HPV E6E7 long peptide. For *in vivo* study, 10 micrograms of HPV E6E7 long peptide was mixed with D-octaarginine-linked PNVA-co-AA (VP-R8) and intranasally administrated into C57BL/6 mice once a week for 3 weeks (n=5). After vaccination, spleen cells were isolated and T cell immune responses were determined by using intracellular cytokine staining, MHC class I tetramer assay and LDH-release cytotoxicity assay. To investigate anti-tumor immune responses, one hundred thousand TC-1 tumor cells, which were stably expressing HPV E6E7 protein, were subcutaneously injected into mice. Intranasal administrations were conducted as mentioned above once a week for 4 weeks. To expand the feasibility for the nasal vaccination with VP-R8, 4 kind of

T cell epitopes from Wilms' tumor 1 (WT1) protein were intranasally administered into mice and the anti-tumor immunity was determined. **Results:** The intranasal vaccinations of HPV E6E7 long peptide with VP-R8 significantly increased the number of HPV E6E7-epitope specific CD8T cells and IFN-gamma secretion compared to long peptide alone ($p < 0.05$, respectively). The *in vitro* cytotoxicity against TC-1 cells of spleen cells was significantly increased by the intranasal vaccination compared to long peptide alone at effector:target ratio 40:1 ($p < 0.05$). The intranasal vaccinations of HPV E6E7 long peptide with VP-R8 significantly decreased the tumor growth of TC-1 after 4 times of vaccination compared to long peptide alone ($p < 0.05$). The intranasal administration significantly prolonged the survival compared to intraperitoneal administration of long peptide with incomplete Freund's adjuvant (IFA) ($p < 0.05$). For WT1 study, intranasal administration of WT1 long peptide remarkably decreased tumor growth and prolonged survival against C1498-mWT1 tumor cells, which were stably expressing mouse WT1 protein, compared to current WT1 peptide vaccine using mono-epitope with IFA in mice. **Conclusion:** We demonstrated that intranasal vaccinations of multi-epitope-linked long peptide with VP-R8 could induce tumor-specific anti-tumor immune responses in mice. Intranasal administration with cell penetrating polymer could be less invasive and more efficient route than current cancer vaccines to deliver cancer antigen. Our findings have significant implication for nasal cancer vaccines that deliver antigen to nasal-associated lymphoid tissues and elicit strong anti-tumor T cell immunity.

673. Targeting Macrophages with CAR-T Cells Delays Solid Tumor Progression and Enhances Anti-Tumor Immunity

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Tumor-associated macrophages (TAMs) are one of the most abundant cell types in many solid tumors and typically exert protumor effects. This has led to an interest in macrophage-depleting agents for cancer therapy, but approaches developed to date have had limited success in clinical trials. Here, we report the development of a strategy for TAM depletion in mouse solid tumor models using chimeric antigen receptor (CAR) T cells targeting the macrophage marker F4/80 (F4.CAR-T). F4.CAR-T cells effectively killed macrophages *in vitro* and *in vivo* without toxicity. When injected into mice bearing orthotopic lung tumors, F4.CAR-T cells infiltrated tumor lesions and delayed tumor growth comparably to PD1 blockade, and significantly extended mouse survival. Anti-tumor effects were mediated by F4.CAR-T-produced IFN- γ , which promoted upregulation of MHC molecules on cancer cells and tumor-infiltrating myeloid cells. Notably, F4.CAR-T promoted expansion of endogenous CD8 T cells specific for tumor-associated antigens and led to immune editing of highly antigenic tumor cell clones. Antitumor impact was also observed in mouse models of

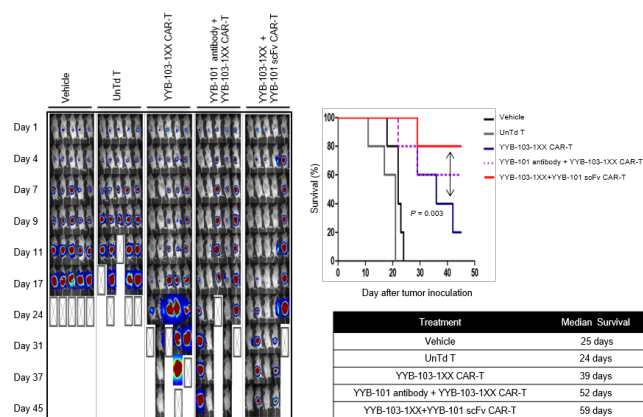
ovarian and pancreatic cancer. These studies provide proof-of-principle evidence to support CAR-T targeting of TAMs as a means to enhance antitumor immunity.

674. A Novel CAR-T Cell Therapy Strategy to Inhibit Hepatocyte Growth Factor in Solid Tumor

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Background: Chimeric antigen receptor (CAR) T cell therapy has recently attracted attention as one of the most promising advances in cancer immunotherapy, particularly targeting hematological malignancies. However, CAR-T cell therapy for solid tumors has not been nearly as successful and remains very challenging. One of the reasons limiting the efficacy of CAR-T in solid tumors is that various factors in the tumor microenvironment inhibit the activity of immune cells including CAR-T cells. Hepatocyte growth factor (HGF) is a multifunctional cytokine that promotes the proliferation, invasion, metastasis and angiogenesis by its autocrine and paracrine signaling in diverse solid tumors including breast, lung, glioblastoma and ovarian tumor. In addition to these cancer-promoting properties of HGF, recent studies reported that HGF interacts with CD8 cytotoxic T cells expressing its receptor c-MET, resulting in reduced cytotoxicity and decreased infiltration to tumor region. Here, we investigated whether anticancer efficacy of CAR-T is improved in combination with anti-HGF monoclonal antibody, YYB-101. We also developed a YYB-101 single chain variable fragments (scFv)-secreting CAR-T and investigated whether it could modulate tumor microenvironment in solid tumor. **Results:** To examine efficacy of YYB-103 CAR-T and/or anti-HGF monoclonal antibody, *in vivo* experiment was performed in orthotopic xenograft mouse model using HGF-overexpressing ovarian cancer cell line A2780/IL13Ra2. The efficacy of IL13Ra2-directed CAR-T (YYB-103 CAR-T) cells was improved in combination with YYB-101 compared to YYB-103 CAR-T alone. Next, we generated YYB-103 CAR-T cells secreting YYB-101 scFv (YYB-103-1XX+YYB-101 scFv CAR-T) and confirmed whether the anticancer activity was increased. We found that YYB-103-1XX+YYB-101 scFv CAR-T cells showed increased cytotoxicity and IFN-gamma release compared to wild-type YYB-103 CAR-T. Interestingly, *in vivo* efficacy of YYB-101 scFv-secreting YYB-103 CAR-T was better than combination of YYB-103 CAR-T cells with YYB-101 antibody in orthotopic xenograft model. This improved efficacy of YYB-101 scFv-secreting CAR-T may probably due to overcoming the immunosuppressive environment caused by HGF in the tumor microenvironment by blocking HGF released from the target cells. **Conclusion:** Our experimental results showed that inhibition of HGF could be one strategy to improve the efficacy of CAR-T in solid tumors. Further study is ongoing to confirm experimental evidences that our new type of CAR-T, YYB-101 scFv-releasing CAR-T, actually acts on the immunosuppressive tumor microenvironment of solid tumors.



675. Gene Therapy with IL-2 and IL-12 in Murine CT26 Colon Carcinoma

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Gene therapy is becoming an important approach for cancer treatment. An example is the introduction of genes encoding immunostimulatory cytokines such as interleukin 2 (IL-2) and interleukin 12 (IL-12) into the tumour cells. In this way the tumour microenvironment could be modified by recruitment and activation of tumour-specific immune cells, thus leading to tumour eradication. Electroporation represents a safe and effective delivery method for the introduction of genetic material (gene electrotransfer) inside the cell. The aim of our study was to determine the effects of gene electrotransfer of plasmids encoding IL-2 (pIL-2) and IL-12 (pIL-12) *in vitro* and *in vivo*. First, the cytotoxic effect of gene electrotransfer of both plasmids individually and combined was determined *in vitro* in CT26 murine colon carcinoma cell line. Two electroporation protocols (EP) were tested: EP1 (600 V/cm, 5 ms, 1 Hz, 8 pulses) and EP2 (1300 V/cm, 100 μ s, 1 Hz, 8 pulses). Cell viability was afterward measured using PrestoBlue assay and the expression profile of IL-2 and IL-12 in cells was determined by qRT-PCR and ELISA. Moreover, we also performed *in vivo* gene electrotransfer of pIL-2 and pIL-12 in CT26 murine colon carcinoma model (permission #U34401-35/2020/8). Tumor growth delay and the concentrations of different cytokines in tumor samples using Magpix assay were determined. In the *in vitro* experiment, the percentage of viable cells after gene electrotransfer of plasmids with EP2 pulse protocol was significantly higher compared to EP1 pulse protocol. In contrast, the mRNA and protein expression of IL-2 and IL-12 in cells after gene electrotransfer was significantly higher using EP1. Based on the *in vitro* results, the EP1 was selected for the *in vivo* experiment. Tumor growth was delayed (up to 11 days) in all groups of mice treated with gene electrotransfer of the individual or combined pIL-2 and pIL-12 plasmids, as well as control plasmid. In comparison to the control group, increased concentrations of several analytes such as CCL5, MIG,

MCP-1, M-CSF, IP-10, GM-CSF and G-CSF were observed only in tumors treated with gene electrotransfer of pIL-12. Interestingly, IL-2 and IL-12 were not detected in any of the treatment groups. Thus, our results suggest that gene electrotransfer of plasmids encoding IL-2 and IL-12 in murine CT26 colon carcinoma had a minor effect on the tumor growth, therefore further studies on optimization of the treatment protocol are needed to obtain a better antitumor response.

Cancer - Oncolytic Viruses

676. Retargeting of Adenoviral Particles by Position-Specific Attachment of Small Epidermal Growth Factor Receptor (EGFR)-Targeting Affilin Molecules to the Vector Capsid

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Introduction: Treatment of head and neck squamous cell carcinomas (HNSCC) by oncolytic adenoviral vectors hold promise as an efficient anti-cancer therapy. Here, the epidermal growth factor receptor (EGFR) represents an attractive target receptor since it has been shown to be frequently overexpressed in many types of HNSCC. **Methods:** To achieve EGFR-specific targeting by human adenovirus type 5 (HAdV5) based vectors, a position-specific and covalent attachment of the EGFR affinity ligand Affilin to either the fiber or hexon protein of the vector capsid was performed. *In vitro* and *in vivo* studies investigated EGFR-specific cancer cell transduction, susceptibility to natural sequestration mechanisms, pharmacokinetics and biodistribution profiles of Affilin-decorated vectors. **Results:** Affilin-decorated vectors showed remarkably enhanced and EGFR-specific cancer cell transduction *in vitro* and less susceptibility to known sequestration mechanisms of HAdV-5 particles. However, *in vivo*, neither systemic nor intratumoral vector administration resulted in an improved transduction of EGFR-positive tumors. Comprehensive analysis indicated hampered EGFR-targeting by Affilin-decorated vectors caused by rapid vector particle consumption due to binding to murine EGFR, insufficient tumor vascularization and poor target accessibility for Affilin in the solid tumor caused by a pronounced tumor stroma. **Conclusion:** *In vitro* studies yielded proof-of-concept results, demonstrating that covalent attachment of a receptor-specific Affilin to the adenoviral capsid provides an effective and versatile novel tool to address cancer-specific target receptors by adenoviral vectors. Regarding EGFR, off-target transduction and hindered receptor accessibility within the tumor tissue prevented efficient tumor transduction by Affilin-decorated vectors, rendering EGFR a difficult-to-address receptor for adenoviral vectors.

677. GoraVir, a New Gorilla-Derived Oncolytic Adenovirus Vector: A Promising Candidate for the Treatment of Pancreatic Ductal Adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy in which the majority of patients presents with unresectable, advanced stage or metastatic disease at the time of diagnoses. In addition, PDAC shows woeful therapeutic resistance due to its considerable genetic and cellular heterogeneity, dense extracellular matrix, and an immune-suppressive tumor microenvironment. Human adenoviruses (Ads) have demonstrated considerable potential as oncolytic agents. However, their efficacy in clinical studies is generally moderate and presents with considerable patient-to-patient variation. This may in part be attributable to variable preexisting neutralizing immunity in patients which can impact the anti-tumor efficacy and lead to response heterogeneity. Non-human primate Ads of the Human Adenovirus B (HAdV-B) species may serve as an alternative for the development of oncolytic Ad vectors by virtue of their limited preexisting neutralizing immunity in humans. We have generated a new oncolytic derivative of a gorilla-derived HAdV-B Ad harboring a deletion in one of the Rb-binding domains of *E1A*. This new vector, named GoraVir, has demonstrated increased oncolytic potential compared to human adenovirus type 5 (HAdV-C5) in various tumor types *in vitro*. Here, we show that GoraVir is lytic in both PDAC cancer cells as well as cancer-associated fibroblasts (CAFs). Similar to several human Ads of the HAdV-B species, GoraVir was shown to make use of complement receptor CD46 as its main entry receptor, which is highly expressed in many tumors. Interestingly, GoraVir maintained its oncolytic potential in BxPC-3 tumor spheroids in contrast to HAdV-C5. Based on histology analyses this superiority could be attributed to an increased lytic potential rather than viral spread. Finally, single-dose intratumoral administration of 1×10^8 plaque-forming units of GoraVir in a BxPC-3 xenograft model resulted in a significant reduction in tumor growth compared to PBS control mice as well as mice treated with the oncolytic human Ad- $\Delta 24$. Collectively, these data demonstrate that the new gorilla-derived oncolytic Ad is a potent oncolytic vector candidate which seems promising regarding future clinical development.

678. Oncolytic Adenovirus-Mediated Expression of an Anti-PD-L1 scFv Induces Recruitment and Immune Evasion Inhibition

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Oncolytic virotherapy is an emerging therapeutic approach that could offer an attractive combination of tumor-specific cell lysis together with immune stimulation, therefore acting as potential *in situ* cancer vaccines. Furthermore, arming an oncolytic virus with immunomodulatory transgenes such as immune checkpoint inhibitors (ICIs) or other molecules can increase the virus ability to target the tumor microenvironment (TME) and, therefore, enhance immune-virotherapy. Adenoviruses (Ads), especially, oncolytic adenoviruses (Onc.Ads), can kill cancer cells in different ways by inducing immunogenic cell death. To increase their anticancer activity, we armed the previously published Ad5D24 to add the ability to block tumor immune evasion. The main pathway involved in immune evasion implicates the interaction between Programmed death ligand 1 (PD-L1) and its receptor PD-1. The PD-1/PD-L1 interaction inhibits CD8+ T cell proliferation turning off the antitumoral T cell-mediated response. We previously isolated a single-chain variable fragment antibody (scFv) that binds to both human and murine PD-L1 with high affinity and developed an Onc.Ad $\Delta 24$ (Onc.Ad $\Delta 24$ -scFv-anti-PD-L1) that expresses the scFv joining the blockage of PD-1/PD-L1 interaction with the antitumoral activity of Onc.Ads. Firstly, we evaluated Onc.Ad $\Delta 24$ -scFv-anti-PD-L1 on B16.OVA melanoma cells, demonstrating the reduction of cell survival *in vitro* and slow down tumor growth *in vivo* in a melanoma mouse model. In addition, we observed an increase in the tumor-infiltrating lymphocytes (CD8+) in tumors from treated mice, indicating a favorable immune profile. Subsequently, we evaluated the effect of Onc.Ad $\Delta 24$ -scFv-anti-PD-L1 on TOM-1-GFP-Luc, a murine lung cancer cell line. At this aim, we developed an *in vitro* tumor model generating 3D spheroid to better mimic tumor structure; preliminary data reveal a significant reduction of spheroids diameter after Onc.Ad $\Delta 24$ -scFv-anti-PD-L1 infection. Altogether, these data suggest that this innovative approach could be effective in the treatment of different tumors supporting and potentiating the available current therapy and, in addition, the combination of immune cells and 3D spheroids can get us closer to obtaining an *in vitro* model that mimics the complexity of an *in vivo* model.

679. New Strategy of Oncolytic Herpesvirus for Retargeting Cancers: Double Targeting oHSV-1 Exhibits Antitumor Activity Against EpCAM-Expressing Cancers

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Background: Oncolytic virus (OV)-based cancer therapy is one of the emerging tools to destroy the cancer cells without damaging normal cells. The various oncolytic herpes simplex virus (oHSVs) harboring tumor-killing efficacy have been studied in many different tumor models with *in vitro* and *in vivo*. It has been a difficult task to completely retarget HSV to cancer-specific antigens in the plasma membrane of selected tumors while maintaining the fully lytic potential of HSV. The present retargeting strategies of oHSV have been employed with the incorporation of single-chain antibodies (scFv) against specific receptors of cancer cells, into the viral glycoprotein D, gB, or gH. We developed a new double retargeting strategy carrying both glycoprotein H modified with scFv that attach to the specific receptor of cancer cells and bi-specific soluble adapter which consists of HveA⁸², a part of gD receptor, and scFv against the specific receptor on the cancer cells.

Results: In the present study, we designed a self-retargeting system of bi-specific adapters that are expressed within infected tumor cells in which EpCAM adapter gene (scFvEpCAM-HVEM) is incorporated into the oHSV genome. In addition, scFvEpCAM was inserted into gH to complete the double retargeting vector with adapter molecules. Consequently, we constructed an EpCAM-double targeting oHSV to maximize retargeting modality without deletion/attenuation. In our results, we first showed that the initial infection and subsequent spreading of EpCAM-double targeting oHSV depend on the cellular EpCAM expression *in vitro*. A preliminary systemic toxicity assessment was conducted following intravenous injection of double targeting virus at a maximum dose (2×10^8 PFU). Neither illness nor significant body weight loss was observed in the EpCAM-double targeting virus-treated group. A single intratumoral dose of EpCAM-double targeting oHSV was administered in MDA-MB-453 breast cancer xenografts, that rendered mice tumor-free. Moreover, intravenous injection of the virus significantly reduced tumor growth according to dose-dependent manner. These data indicate that EpCAM-double targeting oHSV could be more effective than a single agent for both local and systemic treatments of cancer.

Conclusions: Our research indicates that double targeting platforms by self-retargeting could be a potent treatment of tumor associated antigens-overexpressing cancers.

Keywords: Oncolytic virus, EpCAM, self-retargeting, adapter, double targeting, bi-specific

680. Biosynthesized Cobalt Ferrite Nanoparticles Combined with Oncolytic Newcastle Disease Virus Enhances Cancer Virotherapy Against Breast Cancer Cells

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Oncolytic virotherapy is one of the promising cancer therapies approaches. It shows augmented killing effect through combination therapy by attacking cancer cells across multiple mechanisms of action to overcome cancer cells resistance. In previous studies by our group, we noticed a synergistic anti-tumor effect when combining cobalt ferrite (CoFe₂O₄) nanoparticles and Newcastle Disease Virus (NDV) AMHA1

strain against breast cancer cells. Another study by our team found that rhizomes extract of *Rheum ribes* enhances cancer virotherapy using AMHA1 NDV against murine mammary adenocarcinoma *in vivo*. The current study was designed to biosynthesize cobalt ferrite (CoFe₂O₄) nanoparticles (NPs) using methanol and aqueous extract of *R. ribes*. We found that some of the phytoconstituents of the *R. ribes*, especially Rutin, succeeded in biosynthesizing CoFe₂O₄ NPs when used alone and linked with NPs by bonds. These results were approved by FTIR analysis. Rutin showed to help in biosynthesizing CoFe₂O₄ NPs with 68.83 nm in size. The newly biosynthesized CoFe₂O₄ NPs characterized by atomic force microscopy (AFM), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transform Infrared (FTIR), X-Ray Diffraction (X-RD), and UV-Vis spectroscopy. The biosynthesized CoFe₂O₄ NPs (BioNPs) were studied *in vitro* for their cytotoxicity properties against cancer and normal cells as a single drug or with Newcastle Disease Virus (NDV) as combination therapies on different hormone-dependent (MCF-7) and hormone-independent (AMJ13) breast cancer cells and measuring safety in normal cells. The results found that each BioNPs alone and Newcastle disease virus (NDV) alone had a clear anticancer activity against (AMJ-13 and MCF-7) breast cancer cell lines at 72hr exposure time. The higher killing rate was 78% at 0.5 mg/ml concentrations for Bio-NPs alone, while NDV alone induced a 79.9% killing rate at one MOI. We found that normal human fibroblast cells were not affected by BioNPs or NDV alone. The combination therapy of BioNPs with NDV showed higher anticancer activity against AMJ13 and MCF-7 breast cancer cell lines. The highest inhibitory rate was 86 % at a concentration of NDV at 0.05 MOI combined with BioNPs at 0.0625 mg/ml. BioNPs-NDV combination therapy had no considerable killing effect against normal cells. The highest killing rate was 10.7 % at a concentration (NDV: 0.05 MOI with BioNPs: 0.0156 mg/ml). Chua-Talalay combination analysis showed synergistic interaction between the two agents against breast cancer cells. In contrast, all combination points showed antagonism in normal cells. In conclusion, the present study introduced unique and novel combination therapy of Biosynthesized CoFe₂O₄ NPs and NDV AMHA1 strain as a promising breast cancer treatment.

681. Targeting of Oncolytic Adenoviruses to Cancer Cells Using a DARPIn Lipocalin-2 Fusion Protein

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Oncolytic viruses are a promising technology to attack cancer cells and more importantly to recruit immune cells to the tumor site. Since the Lipocalin-2 receptor (LCN2R) is expressed on epithelial cancer cells, we took advantage of the interaction of this receptor with its ligand Lipocalin-2 (LCN2) to target oncolytic adenoviruses (Ads) to cancer cells. Therefore, we produced a designed ankyrin repeat

protein (DARPin) adapter that was fused to LCN2 and could bind the knob of Ad type 5 (knob5) in order to retarget the virus towards the LCN2R. We generated an *in vitro* model based on Chinese Hamster Ovary (CHO) cells stably expressing the LCN2R. Adapter tests were performed on this model and on 20 cancer cell lines (CCLs) using an Ad5 vector encoding luciferase and green fluorescent protein (GFP) as reporter genes. Luciferase assays conducted in CHO cells showed a ten-fold increased viral uptake for virus incubated with the LCN2 adapter (LA) in cells expressing LCN2R and in cells not expressing the LCN2R. In contrast, we observed decreased uptake with a blocking adapter (BA) as control that consists of a knob5-binding DARPin without LCN2 fused to it. In most CCLs an increased viral uptake of LA-bound virus compared to BA-bound virus controls was observed, while comparison to unbound virus led to varying results. To confirm luciferase assay observations, fluorescent assisted cell sorting (FACS) analysis was performed to detect GFP expressing cells indicating viral infection. Most tested CCLs showed increased values after LA-bound virus infection compared to BA-bound virus infection, while unbound virus infected cells showed similar percentages. Because both assays were conducted 24 h post-infection, additional hexon stainings are being done, to analyze the adapter effect on early viral uptake 3 h after infection. Furthermore, virus spread was studied in 3D cell culture models that better represent the tumor environment than monolayer culture. Nine CCLs showed increased and earlier GFP fluorescence for LA-bound virus compared to BA-bound virus and a similar fluorescence signal compared to unbound virus. We conclude that the novel DARPin adapter LA-increases viral uptake in most cell lines compared to the BA and might even increase viral uptake compared to unbound virus. Note, that in the CHO model experiments, we found that the adapter effect seems to be independent of LCN2R expression, suggesting that LA-bound vectors use a different uptake mechanism.

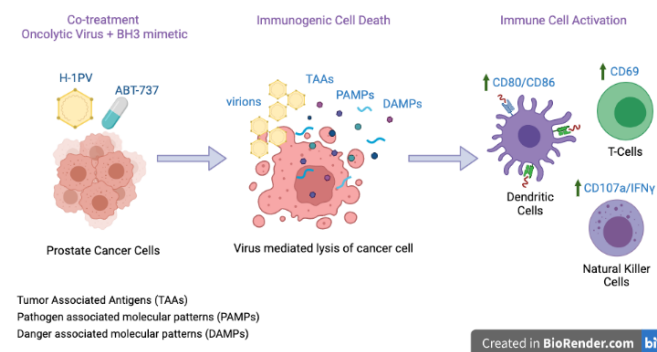
682. Oncolytic H-1 Parvovirus in Combination with Pro-Apoptotic Drug ABT-737, Shows Improved Cytotoxicity and Immune Activation in Prostate Cancer Cells

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In the past decade, novel methodologies have been developed facilitating the use of immune activation against cancer. This has greatly improved the overall survival and prognosis for various malignancies. However, not all cancer patients benefit from these treatments, so there is an unmet need to find agents that could potentiate the efficacy of immunotherapy. One such field of research is oncolytic virus mediated immunotherapy. More than 40 oncolytic viruses (OVs) are presently undergoing clinical testing alone or, increasingly, in combination with other immunotherapeutic agents. OVs specifically target cancer cells,

induce tumor cell lysis, tumor associated antigen release and adjuvant immune response through immunogenic cell death and cytokine induction. H-1 Parvovirus (H-1PV) is one such “clinically relevant” OV, which has been extensively studied in various cancer models and evaluated in phase I/II clinical trials for the treatment of patients with glioblastoma multiforme and pancreatic ductal adenocarcinoma. It has been shown to be safe, non-toxic, and capable of inducing favorable immune modulation of the tumor microenvironment. However, H-1PV, as a monotherapy, is still not sufficient to completely eradicate the tumors. The development of combination strategies based on H-1PV, and other anticancer agents thus seems to be a rational approach to improve efficacy. Previous data from our laboratory has shown great promise in using the combination of H-1PV with pro-apoptotic BCL-2 inhibitors in different cancer models. In this study, we evaluated whether it is possible to enhance the oncolytic activity of H-1PV by using the pro-apoptotic BH3 mimetic ABT-737, in prostate cancer cells. Our data show that the combination shows improved killing of prostate cancer cells in a synergistic manner. We also provide first evidence that this cell death is immunogenic, by showing a strong induction of cell surface calreticulin, a classical marker of immunogenic cell death (ICD). To investigate whether the induction of an ICD event also brings about the activation of the dendritic cell (DC)/T-cell axis we have established multiple co-culture systems using the prostate cancer cell line PC3 and primary immune cells. Firstly, we see the maturation and activation of DCs through the upregulation of CD80 and CD86 expression. Furthermore, we see peripheral activation of T-cells through the upregulation of early activation marker CD69. The combination also shows an improved activation of natural killer cells through an increase in degranulation marker CD107a and intracellular IFN γ . Our study thus shows that a combination treatment of H-1PV and ABT-737 shows promise in enhancing direct tumor cell killing as well as anti-cancer immunity in the context of prostate cancer.



683. Oncolytic Measles Virus Encoding a Bispecific Killer Engager Activates NK Cells and Promotes Anti-Tumor Cytotoxicity

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Oncolytic virotherapy with measles vaccines (MV) leads to increased intratumoral infiltration of immune cells, including natural killer (NK) cells. However, NK cells may preferentially act against virus-infected cells and not bystander cells. We hypothesized that directing NK cells towards tumor cells via bispecific killer engagers (BiKEs) can enhance bystander killing and anti-tumor efficacy. Therefore, we generated MV encoding a BiKE that binds CD16A on NK cells and carcinoembryonic antigen (CEA) as a tumor surface antigen. Replication and cytotoxicity of MV-BiKE are comparable to parental MV. MV-encoded BiKEs are functional, as shown by binding and tumor cell killing assays. We tested MV-BiKE activity in cocultures of colorectal or pancreatic cancer cells with primary human NK cells. The results show that MV-BiKE treatment of tumor cells leads to secretion of effector cytokines, degranulation and specific NK cell anti-tumor cytotoxicity against bystander cells. Cocultures with primary patient-derived pancreatic cancer cultures indicate that MV-BiKE efficacy may correlate with viral permissiveness of individual tumors. This study provides proof-of-concept for MV-BiKE as a novel oncolytic therapy harnessing virus-activated NK cells as anti-tumor effectors.

684. Exploring Novel Viro-Immunotherapies Using “Armed” Oncolytic Adenoviruses and Adoptive T-Cell Therapy to Target Pancreatic Ductal Adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related mortalities and is projected to become second by 2030. Although major advancements in cancer treatments have improved outcomes for many cancers, the survival rate for PDAC has not improved in nearly four decades despite tremendous effort. As a result, there is an urgent need for the development of more efficient therapeutics targeting PDAC. Two primary features make PDAC refractory. 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. Transforming growth factor beta (TGF- β) plays a significant role by activating immunosuppressive immune cells and inactivating anti-tumor immune cells. Second, desmoplasia causes formation of fibrotic tissue within and around tumor tissue, leading to compression of blood vessels. This creates a physical barrier for infiltration of anti-tumor immune cells and therapeutics. In addition to playing a role

in the highly immunosuppressive TME, TGF- β also contributes to desmoplasia by activating cancer-associated fibroblasts. The goal of this project is to develop viro-immunotherapies against PDAC that target the immunosuppressive TME and desmoplasia by utilizing “armed” oncolytic adenoviruses (OAd) and adoptive T cell therapy (ATCT). I have selected three transgenes to “arm” my OAd constructs: i.) TGF- β blocker, ii.) interleukin-7 (IL-7), and iii.) interferon gamma (IFN- γ). Preliminary has shown that localized expression of either TGF β blocker or IL-7 using adenovirus vectors significantly decreases PDAC tumor growth in syngeneic mouse models. I hypothesize that my “armed” OAd and ATCT will favorably alter the immunosuppressive TME and breakdown physical barriers from desmoplasia to elicit potent anti-tumor responses against PDAC. The findings of this proposal 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, which is desperately needed for this deadly disease.

685. Engineered Vaccinia Virus for Lung Cancer

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Oncolytic virus, especially vaccinia virus (VV) is a promising cancer treatment option for effective cancer immunotherapy. Non-small cell lung cancer (NSCLC) is likely to respond to immunotherapy. Recently, we developed pan-cancer specific novel oncolytic vaccinia virus (NOV) expressing TRAIL and Ang1. Here, we applied the viruses to CT26 mouse colon cancer and LLC1 mouse lung cancer syngeneic model and observed the virus treatment showed attenuated tumor growth, which was in accordance with the results of percent survival measurement, CD8 expression analysis, and TUNEL staining with advanced genetic engineering. NOV induces cancer tissue apoptosis and anti-tumor immunity and may constitute a highly advantageous therapeutic agent especially for next-generation colon and lung cancer virotherapy and high biosafety.

686. Pharmacokinetics and Biodistribution of VSV-GP Using Methods to Decouple Input Drug Disposition and Replication Dynamics in Mice

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Introduction: The ability of oncolytic viruses (OVs) to replicate affords their desirable therapeutic properties yet poses challenges for interpreting their pharmacokinetics (PK) and biodistribution (BD). Replication impacts both concentration-time (PK/BD) and concentration-effect (PK-PD) profiles by increasing apparent concentrations, often in a pharmacodynamic (PD) dependent

manner. This results in a bidirectional PK-PD relationship which obscures modeling and dose prediction approaches common to other drug modalities. Here, we decouple the PK profile of VSV-GP, a model oncolytic vesiculovirus, to independently study its primary input disposition (PK¹) and secondary replication (PK²). **Methods:** To study PK¹, we generated a replication-incompetent tool virus using UV irradiation (VSV-GP^{UV}). Analytical characterization was performed to examine UV impacts on virus structure, function, and innate immunogenicity. Next, we dosed BALB/c mice with VSV-GP and VSV-GP^{UV} at equivalent genomic doses via bolus i.v. injection. Blood and tissues were collected 1min through 168h and subjected to RT-qPCR and TCID₅₀ analysis to measure total and infectious genomes. The resulting concentration-time profiles were analyzed using noncompartmental analysis to derive PK¹ and PK² parameters. Replication in blood and tissues was then quantified by comparing exposure ratios of VSV-GP and VSV-GP^{UV} (AUC_{Live/UV}). To further examine replication dynamics in tissues, we developed strand-specific *in situ* hybridization (ISH) to facilitate independent tracking of antisense genomes/genomic replication (input genomes only in the case of VSV-GP^{UV}) from sense transcripts at a cellular resolution. **Results:** VSV-GP^{UV} retained all critical qualities of VSV-GP that we suspected might impact PK (e.g., target binding, structural integrity, and innate immunogenicity), except for its ability to replicate. Both VSV-GP and VSV-GP^{UV} rapidly cleared the blood compartment. Thereafter, we observed a secondary peak in blood concentration for VSV-GP, whereas VSV-GP^{UV} cleared with a biphasic elimination profile. VSV-GP replication impacted all PK parameters in the late phase (3h-168h). The predominant distribution sites were liver and spleen, which sequestered similar total genomes per tissue mass; however, the spleen was more permissive to replication. Transient replication was detected in all tissues to varying degrees, as measured by AUC_{Live/UV}. Despite impacting all PK and BD parameters, the replication in healthy tissues did not appear to be productive, as infectious virus cleared with no secondary peak. Using ISH, we tracked the source of splenic replication to the marginal zone macrophages where we observed amplification of both antisense and sense RNAs. By contrast, in liver macrophages there was only a brief burst of sense RNA transcripts with no increase in antisense genomic RNA. **Conclusions:** Our study demonstrates unique tools for interpreting OV PK/BD to help address the unique PK considerations of a replicating virus. The utility of decoupling OV PK into primary (PK¹) and secondary (PK²) profiles is demonstrated, which enabled quantification of viral replication and its impact on PK. Additionally, examination of sub-tissue-level replication with ISH enabled tracking viral and cellular responses. We are currently applying these approaches to characterize PK and BD in tumor-bearing and healthy mice to help inform OV pharmacology and safety assessments.

687. Identification of an Antiviral Drug as a Novel Potentiator of H-1PV-Mediated Oncolysis

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The rat protoparvovirus H-1 (H-1PV) is a small non-enveloped virus with a natural oncolytic capacity. Replication of the virus in human cells is strictly dependent on a transformed phenotype in the host cell, including a dependence on cellular S-phase factors. This has made H-1PV an attractive treatment option for human cancers. The oncolytic activity of H-1PV has been extensively tested *in vitro* against a wide range of cancer cell lines, including brain cancer, lymphoma and leukemia, pancreatic ductal adenocarcinoma (PDAC), and breast cancer. In the clinics, H-1PV as a monotherapy was shown to be safe and well-tolerated for the treatment of glioblastoma and PDAC, but was unable to eradicate tumours under the regimes used. Thus, there is a clear need for improvement of oncolytic H-1PV therapy. Here, we identify a novel combination of H-1PV with an antiviral drug that potentiates the oncolytic capacity of the virus *in vitro*. We carried out a high-throughput screening of 1443 FDA-approved drugs in combination with H-1PV in glioma cells to identify drug candidates able to potentiate the oncolytic abilities of H-1PV. From this screening, we identified the Hepatitis C Virus inhibitor ledipasvir as a potentiator of H-1PV-mediated oncolysis, and confirmed this *in vitro* in a variety of cellular backgrounds, including glioblastoma, PDAC, lung cancer, and ovarian carcinoma (Figure 1). We further show that ledipasvir can potentiate the cytotoxic effects of the H-1PV non-structural protein NS1 in a virus-free system. However, a cellular thermal shift assay showed that it is unlikely that ledipasvir directly interacts with NS1. Functional kinome profiling (PamGene) in glioma cells co-treated with ledipasvir and H-1PV revealed a number of kinase pathways that are differentially activated in the presence of ledipasvir and H-1PV co-treatment. We are currently validating these pathways by independent methods, as well as investigating the effect of ledipasvir on H-1PV replication. Our data demonstrates for the first time that ledipasvir is able to potentiate the oncototoxicity of H-1PV and is a promising candidate for combination therapy in the clinics.

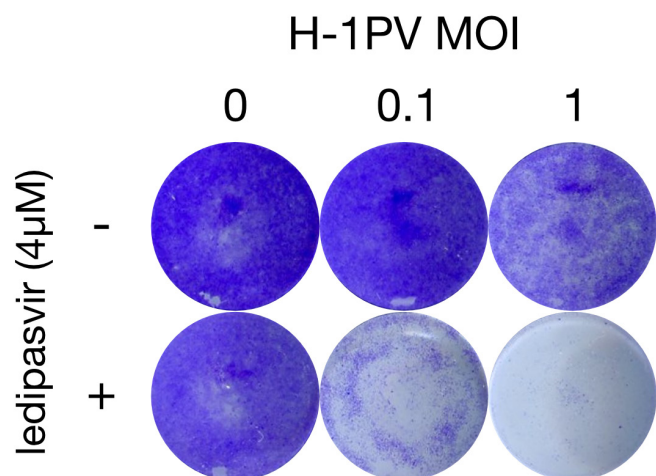


Figure 1. Crystal violet staining of U373-MG glioblastoma cells after infection with various multiplicities of infection (MOI) of H-1PV for 7 days in the presence or absence of ledipasvir (4 μ M).

688. UPLC Method for Oncolytic Coxsackievirus Viral Protein Separation and Empty to Full Capsid Quantification

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Oncolytic virus immunotherapy is emerging as a novel therapeutic approach for cancer treatment. Immunotherapy clinical drug candidate V937 is currently in phase I/II clinical trials and consists of a proprietary formulation of Coxsackievirus A21 (CVA21), which specifically infects and lyses cells with over-expressed ICAM-1 receptors in a range of tumors. Mature Coxsackievirus virions, consisting of four structural virion proteins VP1, VP2, VP3 and VP4, and the RNA genome, are the only viral particles capable of being infectious. In addition to mature virions, empty procapsids with virion proteins VP0, VP1 and VP3 and other virus particles are produced in V937 production cell culture. Viral protein VP0 is cleaved into VP2 and VP4 after RNA genome encapsidation to form mature virions. Clearance of viral particles containing VP0, and quantification of viral protein distribution are important in V937 downstream processing. Existing analytical methods for the characterization of viral proteins and particles may lack sensitivity or are low-throughput. We developed a sensitive and robust reverse-phase ultra-performance chromatography (RP-UPLC) method to separate, identify and quantify all five CVA21 virion proteins. Quantification of virus capsid concentration and empty/full capsid ratio was achieved with good linearity, accuracy, and precision.

689. Combination Therapy of GM101 and Histone Deacetylase Inhibitors Elicits Potent Antitumor Efficacy

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Oncolytic adenoviruses (oAds) are promising cancer therapeutics due to their many advantages such as excellent cancer specificity, exponential amplification of therapeutic transgene, and minimal insertional mutagenesis rates. Despite the first oncolytic Ad (oAds) being commercialized in China as early as 2005, no Ad-based therapeutic has been approved for clinical use in Europe or the USA to date, due to inadequate therapeutic benefits. Subsequent investigations have revealed that insufficient tumoral infection of oAd is one of the limitations contributing to suboptimal therapeutic efficacy in clinics. In detail, variable expression of the coxsackie-adenovirus receptor (CAR) in heterogenic tumor populations and ablation of CAR expression with disease progression in a subset of tumor cases can severely hamper CAR-mediated infection and the spreading of the adenovirus (Ad) across the tumor tissues. Further, aberrant expression of extracellular matrix (ECM) can be a physical barrier of Ad to penetrate within the tumor. To this end, we investigated whether combination therapy of GM101, an ECM degrading oAd, and histone deacetylase inhibitors (HDACis) elicits potent antitumor efficacy. In our present study, HDACi was shown to increase CAR expression levels of cancer cells and increase the internalization of Ad into cancer cells via upregulating key factors in CAR-dependent or dynamin-dependent clathrin endocytic pathways. Moreover, the combination therapy of GM101 and HDACi elicited ECM destruction and induction of apoptosis, ultimately leading to the augmentation of the cytolytic and antitumor effects of oAd. Therefore, the combination therapy of Ad and HDACi is an effective strategy to maximize the potent antitumor efficacy of each monotherapy.

690. Decorin Attenuates Intratumoral Hypoxia by Suppressing HIF and Extracellular Matrix Components

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ECM in tumor microenvironment possesses a complex network consisting of cancer cells, inflammatory cells, and neo-vessels with enriched angiogenic factors. Further, as excessive ECM contributes to hypoxia and acidosis in tumor tissues, leading to increased risk of metastasis. In present study, we demonstrated that the expression of DCN, an ECM degrading molecule can efficiently attenuate hypoxic burden in the tumor milieu, and thus effectively leads to reduction in metastatic properties of cancer. Although there are several reports demonstrating ECM degrading property of DCN in tumor

microenvironments this is the first study to demonstrate the molecular link between DCN-mediated ECM degradation and attenuation of hypoxia within the tumor microenvironment.

Hematologic and Immunologic Diseases II

691. Myeloid Conditioning with CAR-T Cells to Enable Donor Stem Cell Engraftment without Chemotherapy or Irradiation

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Hematopoietic stem cell (HSC) transplantation is a beneficial therapeutic option for malignant and monogenic diseases. The recipients of allogeneic hematopoietic stem cell transplants typically require myeloablative conditioning prior to HSC transplantation to allow the donor stem cells to engraft and proliferate in the patient's bone marrow. The current clinical approach to conditioning involves high-dose chemotherapy and/or whole-body irradiation. This is highly myeloablative, yet it also carries a significant risk of morbidity and mortality, which makes it a suboptimal and often unusable strategy for many patients such as older patients, patients with confounding medical conditions, and those with non-malignant diseases. Therefore, new targeted approaches to bone marrow conditioning are required to improve the safety and efficacy of HSC transplantation. In this project we aim to determine whether our unique Adapter CARTM T (AdCAR-T) cell approach is capable of effective myeloablation *in vitro*. To this end, we initially optimized the AdCAR T cell system towards the targeting of HSCs. *In vitro* cytotoxicity measured by flow cytometric analysis, colony-forming assays, and MACSplex cytokine assays were used to determine the optimal effector to target ratio, adapter concentration, incubation time and media type for efficient killing of HSCs. Our results indicated that TexMACS MediumTM was more suitable to maintain the viability and functionality of AdCAR-T cells *in vitro* without compromising HSC stemness. Furthermore, to reach an efficient level of target cell lysis, an effector to target cell ratio (E:T) of 10:1 should be utilized in the presence of 10 µg/ml of adapter for at least 24h. With the aforementioned conditions, we were able to show that the AdCAR-T cells are functional by secretion of specific cytokines such as GM-CSF, TNFα, and IFNγ using MACSplex cytokine assays. In a second line of experiments, we used adapters directed against a broad range of HSC surface antigens such as CD34, CD33, CD117 and CD135 to determine their utility as future targets. Preliminary results of the cytotoxicity assays indicated that efficient lysis of HSCs ranging from 50%-90% could be achieved after 24h or 48h, which was also

dependent on the HSC donor. We consistently observed more efficient killing of HSCs in the presence of the CD34 adapter, ranging from 40%-90% whereas cytotoxicity assays with CD33, CD117 and CD135 adapters were less efficient compared to the CD34 adapter. Data derived from CFU assays mostly corroborated results from the flow cytometric analyses, with a decrease of colony count that often exceeded the level of HSC lysis observed by flow cytometry. *In vitro* kinetic analysis of the aforementioned adapters for 24h, 48h and 72h indicated that at least 10% of HSCs survive even after 72h of incubation in the presence of the adapter, suggesting that a certain percentage of HSCs is resistant to lysis and that the system reaches a plateau phase during which no further killing of HSCs is observed. We are currently investigating the reasons for the HSC resistance using the MACSima Imaging Platform and NGS-based transcriptome analysis. Future experiments in mice will indicate if the HSC lysis observed *in vitro* can be successfully translated into complete myeloablation *in vivo*.

692. A Lentiviral Vector Expressing Human IL7alpha Receptor Rescues IL7alphaR Deficiency

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Deficiency of the α-chain of the Interleukin 7 (IL7) receptor (IL7Rα) causes 10% of severe combined immunodeficiency (SCID) cases, and is a T^B⁺NK⁺ SCID. Mice lacking IL7Rα, *Il7ra*^{-/-}, lack both T and B cells (Peschon, JJ, *et al. J Exp Med.* 1994). IL7 signaling leads to STAT5 phosphorylation and proliferation of developing T and B cells. In mice and humans, IL7 signaling is required for T cell receptor β gene rearrangement and T cell maintenance. In *il7r*^{-/-} mice, B cell development is halted at the pre-pro-B cell stage. A prior attempt to rescue murine IL7Rα deficiency utilized a retroviral vector and promoter with the murine *Il7ra* gene (Jiang, Q, *et al. Gene Therapy.* 2005). This strategy did restore T cells, had variable restoration of B cells, but led to a myeloproliferative condition. We evaluated a novel gene therapy for IL7Rα SCID using human *IL7Rα*. To prevent lineage skewing, we sought to limit ectopic expression of *IL7Rα* utilizing putative enhancers and promoters of *IL7Rα* with high sequence conservation and DNA hypersensitivity (DHS). We utilized the proximal DHS alone or with the constitutive phosphoglycerate kinase promoter (PGK) to generate lentiviral vectors (LV), vDHS-hIL7Rα and vPGK-DHS-hIL7Rα vectors, respectively. Here we present human *IL7Rα* gene addition can rescue murine *Il7ra*^{-/-} immunodeficiency. Transduction of *Il7ra*^{-/-} bone marrow cells with *IL7Rα* encoding LV rescued the formation of lymphocyte precursors from murine bone marrow cells in pre-B colony forming unit (CFU) assays, with the most robust response seen with vPGK-DHS-hIL7Rα. Mouse bone marrow from *Il7ra*^{-/-} animals transduced *ex vivo* engrafted in lethally irradiated (8 Gy) *Il7ra*^{-/-} opposite gender recipients and there were no significant aberrations in neutrophil or platelet count, hemoglobin, or organomegaly. Preliminary studies in a couple of long-term animals (6 months post-bone marrow transplant) indicated that vector copy number (VCN) in bone marrow from the vPGK-DHS-hIL7Rα treatment was 7.6 compared to 0.3 in the vDHS-hIL7Rα treatment, despite starting at similar VCN at 1 month in blood (10.9 vs 10.6).

Transduced mice had a 127-fold increase in thymocytes compared to untransduced mice and were similar to wild type (3.04×10^7 vs 2.89×10^7 , respectively). Spleen morphology was rescued in vPGK-DHS-hIL7Ra, with restoration of white pulp, follicle architecture and T-cell zones. In contrast, vDHS-hIL7Ra treatment had little effect on white pulp, no T-cells were observed in the spleen and B-cells were much reduced compared to the PGK containing vector and wild type (Figure). Additional mice are being evaluated to characterize different subsets of lymphocytes, T and B cell function in response to immunization, VCN and expression in different hematopoietic lineages, and correlation of these parameters to the vector utilized and phenotypic correction. Further evaluation in *IL7Ra* deficient human cells is warranted. This novel approach to *IL7Ra* gene replacement has the potential to be a therapeutic and expedient option for those without a matched donor given the risks of GVHD with mismatched donors and the risk of infection and death without HSCT. Additionally, this would be an ideal disorder for conditioning with less toxic, HSC-targeted strategies given gene-corrected lymphocytes and progenitors will expand post-transplant.

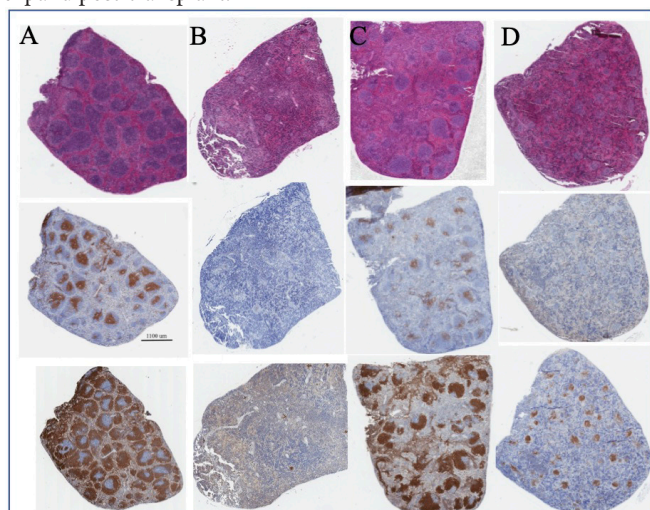


Figure: From top to bottom: H&E stain, CD3 and B220 immunohistochemistry of spleen from *il7ra*^{-/-} recipients 6 months after hematopoietic stem cell transplant with bone marrow cells from C57BL/6 (A), *il7ra*^{-/-} (B), *il7ra*^{-/-} treated with vPGK-DHS-hIL7R LV (C), or *il7ra*^{-/-} treated with vDHS-hIL7Ra LV (D).

693. Cellular Analysis After Systemic Delivery of AAV8-GFP Under Control of Liver-Specific Promoter Elements Shows Transgene Expression in Hepatocytes and Liver Sinusoidal Endothelial Cells (LSECs)

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Liver-targeted adeno-associated virus (AAV)-mediated gene therapy is actively in clinical development for hemophilia A (HA), an X-linked bleeding disorder caused by a deficiency in factor VIII (FVIII). Current transgene constructs for AAV delivery of FVIII utilize minimized promoter elements derived from hepatocyte specific promoters that direct expression to hepatocytes, the native site of synthesis for most coagulation factors. The human alpha-one antitrypsin/ApoE promoter-enhancer (hAAT/ApoE) (Pasi 2020) and the transthyretin (TTR) promoter (George 2021) are used in HA clinical studies to achieve liver-specific FVIII expression. Within the past decade, several studies demonstrated that the major endogenous site of FVIII synthesis is liver sinusoidal endothelial cells (LSECs). The cellular expression pattern in LSECs and hepatocytes after AAV-mediated gene therapy using these promoter elements is unknown. We aimed to determine the pattern of transgene expression in hepatocytes and liver sinusoidal endothelial cells (LSECs) after systemic delivery of AAV8-hAAT/ApoE-GFP and AAV8-TTR-GFP. The hAAT/ApoE (Nathwani 2006) and modified transthyretin (TTR) (Elkouby 2021) promoter element were incorporated into AAV8 constructs expressing green-fluorescent protein (GFP). AAV8-TTR-GFP (n = 4) and AAV8-hAAT/ApoE-GFP (n = 3) were administered intravenously to HA-CD4KO (1×10^{11} vg/mouse). To compare GFP biodistribution in a different viral vector system, the TTR promoter was inserted in lentiviral vectors (LV-TTR-GFP) and injected in C57BL/6 mice (5×10^8 TU/mouse). Single cell suspensions of LSECs and hepatocytes were analyzed for GFP expression by fluorescent activated cell sorting (FACS). AAV8-TTRm-GFP expressed the transgene in $83.3\% \pm 12.8\%$ of hepatocytes (albumin⁺) as well as $71.8\% \pm 15.9\%$ of LSECs (CD146⁺/CD31⁺). Similarly, AAV8-hAAT/ApoE-GFP drove transgene expression to both hepatocytes ($76.2\% \pm 15.4\%$) and LSECs ($87.9\% \pm 6.4\%$). LV-TTR-GFP delivery showed GFP expression in 13% of hepatocytes and 45.7% of LSECs (CD146⁺/CD309⁺). Livers of AAV-treated and LV-treated mice were also evaluated by immunofluorescence (IF). IF confirmed GFP expression after AAV8-TTRm-GFP, AAV8-hAAT/ApoE-GFP, and LV-TTR-GFP administration in both hepatocytes and LSECs (LYVE1⁺). In conclusion, we demonstrate that expressing a transgene under the control of hAAT/ApoE or TTR promoter results in expression in LSECs as well as hepatocytes. Further studies into the significance of transgene expression in LSECs and hepatocytes after AAV-mediated gene therapy targeting the liver are necessary not only to

understand if LSEC-derived FVIII alters efficacy and immunogenicity, but additionally, how promoter design affects specificity of transgene expression in cells.

694. Correction of Congenital Dyserythropoietic Anemia Type II Using Lentiviral Gene Therapy

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Congenital dyserythropoietic anemia type II (CDAIL) is a rare inherited disorder that affects red blood cell development. CDAIL patients suffer from anemia of variable degrees often accompanied by jaundice and splenomegaly. Bone marrow of CDAIL patients is hypercellular, with erythroid hyperplasia and presence of binucleated erythroid cells. CDAIL is caused by mutations in the *SEC23B* gene. *SEC23B* is part of coat protein complex II (COPII), which is involved in protein processing and Golgi-reticulum trafficking. Management of CDAIL is generally limited to blood transfusion, iron chelation and splenectomy. Allogeneic hematopoietic stem cell transplant (HSCT) represents the only curative option for this disease. Autologous HSCT of genetically corrected cells can offer a definitive treatment for CDAIL, overcoming the limitations of allogeneic HSCT. To develop a gene therapy for CDAIL, we have developed two different lentiviral vectors carrying either wild type or codon optimized versions of *SEC23B* cDNA (*wtSEC23B* LV and *coSEC23B* LV, respectively) under the control of the human phosphoglycerate kinase promoter (PGK). *SEC23B* knock-out human hematopoietic progenitors (*SEC23B^{KO}* CD34⁺ cells), generated by CRISPR/Cas9 gene editing, were transduced with both lentiviral vectors at different MOIs. We observed a clear correlation between MOI and vector copy number (VCN) in individually picked colony forming units (CFUs) derived from transduced *SEC23B^{KO}* CD34⁺ cells, without affecting clonogenic capacity. Furthermore, *SEC23B^{KO}* CD34⁺ cells were subjected to *in vitro* erythroid differentiation. Transduction with any of *SEC23B* LVs was able to increase *SEC23B* protein expression, improved terminal erythroid differentiation to values equal to wild type cells and slightly reduced the number of binucleated cells. Similarly, the *in vivo* erythroid differentiation of these transduced *SEC23B^{KO}* progenitors, when were transplanted into immunodeficient NBSGW mice, showed a selection of the transduced cells throughout erythroid differentiation process, accompanied by a reduction in the number of binucleated cells.

In parallel, peripheral blood-derived hematopoietic progenitors (PB-CD34⁺ cells) from CDAIL patients were transduced with *wtSEC23B* LV at MOI 25 and *in vitro* differentiated to erythroid cells. Improvement in the expression of *SEC23B* protein was observed in the erythroid cells. More importantly, percentage of binucleated erythroid cells generated *in vitro* after *wtSEC23B* LV transduction diminished to wild type levels. In summary, *SEC23B* LVs compensate the *SEC23B* deficiency in *SEC23B^{KO}* and in CDAIL hematopoietic progenitor cells, paving the way for gene therapy of autologous hematopoietic stem and progenitor cell as an alternative and feasible treatment for CDAIL.

695. Parvoviral Inverted Terminal Repeat (ITR) Sequences for Non-Viral Gene Therapy

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Recombinant adeno-associated viral (rAAV) vectors are the predominant gene therapy modality currently being used in clinical trials. AAV vectors are integrase-free protein capsids with tailorable tropism and efficient nuclear-delivery of their single-stranded DNA (ssDNA) cargo. The inverted terminal repeat (ITR) sequences within the delivered ssDNA enables the long-term persistence of transgene expression as episomes. AAV vectors have demonstrated clinical efficacy and favorable safety profiles for a handful of disease targets, however, several limitations warrant the development of alternative modalities. First, an integrase-free strategy is associated with the viral vector dilution in regenerating tissues. Second, a humoral immune response induced by viral capsid prevents re-administration and disqualifies the pediatric patients. Third, pre-existing neutralizing antibody further reduces eligible adult patient population and more importantly, fourth, AAV capsid has a limited packaging capacity of ~5 kb transgene size, which limits its use to genetic diseases with a smaller transgene. Taken together, AAV-specific limitations are associated with the viral capsid and unlike ITRs, capsid is not required for long-term persistent transgene expression. However, limited information is available on parvoviral ITRs other than AAV in driving long-term persistent transgene expression. In this study, we explored the efficacy of ITRs from different parvoviruses including, AAV2, human erythrovirus B19, and Goose Parvovirus (GPV) in driving long-term persistent expression of coagulation factor VIII (FVIII) in hemophilia A (HemA) mice following systemic administration of ssDNA. Expression constructs comprising of a human BDD-FVIII cDNA under the control of liver-specific promoter and flanking parvoviral ITRs were used to generate ssDNA. Following ITR-ITR restriction digestion, the digested double-stranded DNA products were heat denatured at 95°C followed by exposure to 4°C to allow the palindromic ITR sequences to form hairpins. The resulting ssDNAs were then systemically administered *via* hydrodynamic injections in human FVIII tolerized FVIII^{R593C}+/+/HemA mice. More than 6 months of follow-up showed persistent expression of therapeutic levels of FVIII at 0.8 - 1.2 mg/kg of ssDNA containing different parvoviral ITRs. We also demonstrated the significance of ITR's pre-formed hairpin structure on long-term persistent FVIII expression compared to the linear double-stranded DNA (dsDNA) and minicircle DNA. To further optimize the ITRs, different variants were generated considering the thermostability and specific elements required for long-term persistence of parvoviral

genome in their respective hosts. Each engineered ITR was then tested with BDD-FVIII cDNA *in vivo* by systemic administration of ssDNA. More than 6 months of follow-up showed persistent FVIII expression in all the modified parvoviral ITRs treatment groups albeit with varying levels. Amongst different variants of GPV and B19 ITRs tested, B19 minimal ITRs demonstrated the long-term persistent FVIII expression at 0.8 mg/kg of ssDNA indicating an ITR-dependent episomal stability and persistence of FVIII expression cassette *in vivo*. Thus, our *in vivo* efficacy studies validate the functionality of different parvoviral ITRs and support the use of modified ITRs to drive long-term stability as well as persistence of transgene expression for non-viral gene therapy.

696. A Universal Correction Strategy for α -Thalassemia Using CRISPR/AAV-Mediated Genome Editing

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Alpha-thalassemia is one of the most common monogenic diseases in the world, and the global health burden is increasing due to population growth, human migration, and advances in treating milder forms of the disease. While multiple gene therapy/genome editing trials have demonstrated clinical efficacy for treatment of β -thalassemia, no such therapies exist for α -thalassemia, indicating a major unmet medical need. Due to the similarity to β -thalassemia—lack of functional hemoglobin tetramers and formation of toxic globin aggregates in absence of the corresponding binding partner—we believe CRISPR/AAV-mediated genome editing may be used to create a universal correction strategy for α -thalassemia in patient-derived hematopoietic stem cells (HSCs). To do so, we will knock in a full α -globin (*HBA1*) transgene into the start codon of the β -globin (*HBB*) locus to restore α -globin production and normalize the globin chain imbalance in patient-derived red blood cells (RBCs). First, we screened an array of Cas9 guide RNAs (gRNAs) in non-coding regions of *HBB* in the HUDEP-2 cell line and identified effective gRNAs in intron 1 and intron 2 (insertion/deletion frequency over 76%). We then developed AAV6 DNA repair donors to mediate insertion of constitutive GFP cassettes with homology arms facilitating integration from the start codon of *HBB* start codon to either intron 1 or intron 2. Following editing in primary WT human HSCs by electroporation of Cas9 RNP and subsequent AAV6 transduction, we found that homology-directed repair was most efficient using the intron 1 gRNA and homology arms (16.8% vs. 12.9% GFP⁺ cells; $P < 0.01$). We then developed promoterless cassettes to deliver an *HBA1* transgene followed by a 2A-YFP reporter to the start codon of *HBB*. To determine UTR regions that yield the highest frequencies of editing and transgene expression, we compared *HBA1*-2A-YFP vectors flanked by *HBB*, *HBA1*, or *HBA2* UTRs. Following editing in WT HSCs and subsequent *in vitro* RBC differentiation, we found that all three cassettes yielded high frequencies of erythroid-specific expression, ranging from 44.2–63.0% YFP⁺ RBCs. Because *HBA2* UTRs yielded the lowest levels of expression among YFP⁺ cells (1.9×10^4 vs. 3.3×10^4), we focused on *HBB* and

HBA1 UTR cassettes for clinical development by removing the 2A-YFP reporter. We found that WT HSCs edited with both vectors efficiently differentiated into RBCs *in vitro* and yielded high frequencies of editing (36.6% and 42.3% targeted alleles for *HBB* and *HBA1* UTR vectors, respectively). Next, we sought to test these vectors in patient-derived HSCs. To do so, we developed a novel workflow to isolate CD34⁺ HSCs from fetal liver tissue from an α -thalassemia patient. We then edited these cells with *HBB* and *HBA1* UTR cassettes and performed *in vitro* RBC differentiation. We found that both edited conditions efficiently differentiated into RBCs and yielded high frequencies of editing (24.1% and 18.5% targeted alleles with *HBB* and *HBA1* UTR vectors, respectively). We are currently performing follow-up experiments to determine the ability of these vectors to restore α -globin production and hemoglobin tetramers to patient-derived RBCs. We also are planning mouse experiments to ensure edited HSCs are capable of long-term engraftment and hematopoietic lineage reconstitution *in vivo*. Overall, we believe this work represents a significant advancement toward a universal *ex vivo*/autologous transplantation strategy for correction of this disease.

697. An Enhanced Hemostatic Factor VIII Variant for Hemophilia A Gene Therapy

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Introduction: Though AAV-mediated factor (F) VIII gene transfer for hemophilia A (HA) has demonstrated phenotypic amelioration in clinical trials, sustained and stable FVIII expression in all participants at levels sufficient to eliminate bleeding remains an unrealized goal of HA gene therapy. In contrast, stable, near-curative expression levels and successful management of dose-dependent capsid immune responses have largely been achieved in hemophilia B gene therapy by use of a gain of function FIX variant, FIX-Padua. Correspondingly, we hypothesized an enhanced hemostatic FVIII variant-transgene for HA gene transfer may achieve hemostatic efficacy at lower AAV vector doses thereby overcoming vector dose-dependent safety and efficacy limitations and reducing vector manufacturing demands while eliminating bleeding. We previously characterized a FVIII variant (FVIII-R336Q/R562Q or FVIII-QQ) that is resistant to inactivation by activated protein C (APC) with a normal specific activity. In recombinant protein experiments, FVIII-QQ demonstrated ~5-fold enhanced hemostatic function *in vivo* relative to wild-type FVIII (FVIII-WT) (Wilhelm et al. Blood 2021). We sought to investigate the hemostatic efficacy of FVIII-QQ for HA gene transfer in murine studies. **Methods:** Two different codon-optimized cassettes were used to manufacture AAV8-FVIII-WT or AAV8-FVIII-QQ vectors for a total of 4 different vectors. 8–12 week old male HA/CD4KO mice received vector doses of 1×10^{10} – 1×10^{12} vg/mouse to express FVIII-WT or FVIII-QQ. 8 weeks post-vector, FVIII activity was measured by Chromogenix COAMATIC FVIII kit and FVIII:Ag was determined by ELISA. 9–10 weeks post-vector, the hemostatic effect of FVIII-WT or FVIII-QQ transgene expression was determined by tail clip assay, which measures blood loss following 3mm diameter tail transection. **Results:** Consistent with or better than prior recombinant protein studies, on a vector dose basis, FVIII-QQ demonstrated 5–10-fold enhanced hemostatic activity relative to FVIII-WT (Figure 1, Table 1). As expected, FVIII-QQ and FVIII-WT activity

and antigen were approximately 1:1 demonstrating that transgene-derived FVIII-QQ similarly does not have enhanced specific activity in a plasma based functional assay. Interestingly, relative steady-state FVIII activity and antigen values of FVIII-QQ were 3-5 fold higher than FVIII-WT using the same vector and dose, suggesting either enhanced expression or a modified steady-state half-life of FVIII-QQ relative to FVIII-WT. **Conclusion:** These data demonstrate FVIII-QQ expression imparts a 5-10 fold enhanced therapeutic benefit over FVIII-WT for HA gene therapy. Work is ongoing to understand the mechanism of higher relative steady-state FVIII-QQ expression. These preliminary data support further investigation of FVIII-QQ as a possible FVIII transgene for HA gene therapy clinical translation. **Figure 1:** Transgene-derived FVIII-QQ hemostatic function is significantly better than FVIII-WT. Data are mean \pm SEM (n = 6-12 mice/dose cohort)

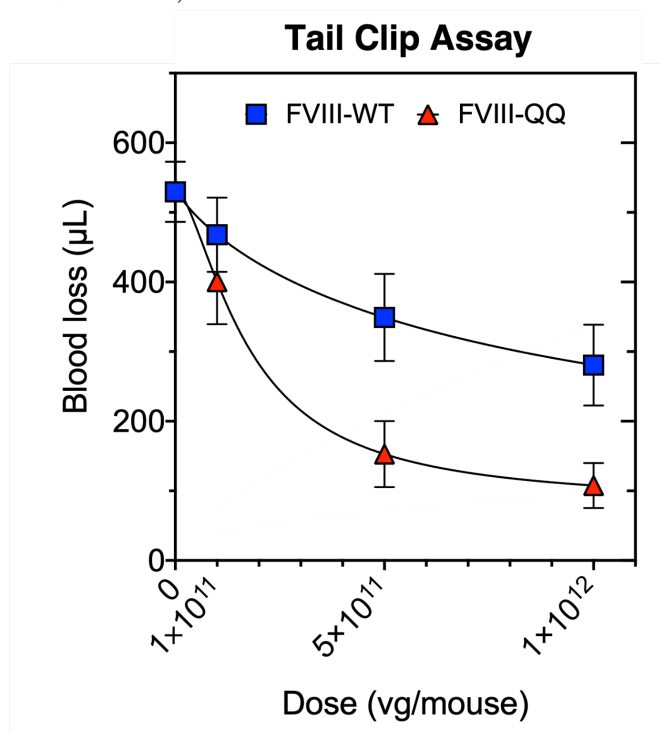


Table 1: AAV expression of FVIII-QQ has 5-10-fold enhanced hemostatic effect

	EC ₅₀ (vg/mouse)	EC ₈₀ (vg/mouse)
FVIII-WT	8.18 × 10¹¹	4.17 × 10¹²
FVIII-QQ	1.73 × 10¹¹	4.19 × 10¹¹
FVIII-QQ fold improvement	5X	10X

698. Characterization of DSG3-CAART Cells Prior to & Following Adoptive Transfer in Mucosal Pemphigus Vulgaris

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Background: Mucosal-dominant pemphigus vulgaris (mPV) is a painful blistering mucosal disease mediated by anti-desmoglein 3 autoantibodies (anti-DSG3 Ab). The current standard of care for mPV includes broadly immunosuppressive therapies (corticosteroids, MME, & rituximab) that are not curative, require chronic administration & have risks of serious or life-threatening infection. Ideally, therapy would selectively eliminate pathogenic memory B cells that are DSG3 specific while sparing non-autoreactive immune cells. As chimeric antigen receptor engineered T cells (CAR-T) have demonstrated long lasting remission of B cell-mediated cancers, we developed engineered chimeric autoantibody receptor T cells (CAART) to assess if remission of B cell mediated autoimmune disease is possible. Currently, gene-modified autologous DSG3 specific CAART cells (DSG3-CAART) are being evaluated in patients with mPV in an open-label dose escalation Phase I study (NCT 04422912). Here, we report on the phenotypic & functional characteristics of the DSG3-CAART cell infusion product along with phenotypic studies of T cells & sera from mPV patients treated with DSG3-CAART cells. **Methods:** Flow cytometric analyses were performed on the infusion product & on post-infusion PBMC samples to assess transduction efficiency & memory phenotype. DSG3-CAART cell cytotoxicity assays were performed *in vitro* using the IncuCyte® platform. Engineered T-cell persistence was assessed by qPCR for the vector in post-infusion PBMC samples. Serum cytokines were measured via a multiplexed MSD immunoassay. Finally, anti-DSG3 Ab levels were evaluated on pre- and post- infusion serum samples via ELISA (MBL International). **Results:** Manufacturing success rate was 100%. The mean transduction efficiency across all products was 50.7% (range: 40.3%-61.2%). 80% of products exhibited a CD4 dominance with a mean CD4:CD8 ratio of 2.6 (range: 0.5-6.2). Immunophenotyping of the products showed a heterogeneous mixture of effector (CD45RA⁺CCR7⁻), central memory (CD45RA⁺CCR7⁺), & stem cell memory (CD45RA⁺CCR7⁺) phenotypes in all products. DSG3-CAART products from all subjects exhibited antigen-specific lysis of anti-DSG3 surface immunoglobulin-expressing NALM6 cells *in vitro*. Gene-modified T cells were detected by qPCR in the blood of all patients within the first 29 days post-infusion with a dose dependent increase in peak vector concentration & AUC within the first 29 days following infusion. Flow cytometric analyses revealed that persisting post-infusion DSG3-CAART cells were either of the central memory or stem cell memory phenotype. For the vast majority of patients, no significant changes were observed in serum cytokines following adoptive cell transfer. There was no clear pattern in anti-DSG3 Ab levels from pre-infusion to post-infusion. **Conclusions:** DSG3-CAART cells are a novel investigational precision cellular therapy being evaluated

in a first in human study in autoimmune disease. We have shown that functional DSG3-CAART cells can be manufactured successfully from mPV patient apheresis material. In the absence of lymphodepletion, engrafted DSG3-CAART cells exhibited a stem cell or central memory phenotype with a strong positive correlation between the dose of gene modified T cells and post-infusion persistence to day 29. These data suggest that DSG3-CAART cells are not being eliminated by the pre-existing anti-DSG3 immunity present in mPV, and support the exploration of higher DSG3-CAART doses.

699. Using Recombinant Adeno-Associated Viral Vectors for Long-Term Expression of a Hyperactive Human Factor IX Mutant in Hemophilic Mice and Comparison of AAV-LK03 and AAV-KP1 in Nonhuman Primates

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We used CB 2679d-GT - a single-stranded recombinant adeno-associated virus (rAAV) vector with a strong liver-specific promoter to express a next generation human coagulation Factor IX (huFIX) variant, in a hemophilic mouse model as well as in rhesus macaques. CB 2679d-GT carries the same huFIX sequence as the subcutaneously delivered dalcinonacog alfa, a high potency FIX variant developed using rational design with three amino acid substitutions. Daily subcutaneous delivery raised FIX levels in severe hemophilia B patients into the mild hemophilia range in a phase 2b clinical study (Mahlangu et al., *Haemophilia*, 2021). A novel chimeric capsid, KP1, was used to package the CB 2679d-GT rAAV construct. KP1 was shown to transduce human and mouse hepatocytes with high efficiency in a chimeric humanized xenograft mouse model (Pekrun et al., *JCI Insight*, 2019). We injected groups of 3 or 5 hemophilic mice with 1×10^{11} or 1×10^{12} vector genomes (vg)/kg respectively of an rAAV vector expressing the mouse codon optimized CB 2679d-GT sequence under control of the ApoE-HCR-hAAT promoter. Over 3 months, antigen and activity levels for this construct were considerably higher than in a previous study employing the DJ-8 capsid and a self-complementary expression construct (Nair et al., *Blood* 2021). Specific huFIX activity in plasma from mice injected with CB 2679d-GT was approximately 10- or 2-fold higher than mice injected with wild-type or Padua huFIX rAAV, respectively. We subsequently infused capsid seronegative juvenile rhesus macaques with 3.5×10^{12} vg/kg of a human codon-optimized and CpG depleted version of CB 2679d-GT packaged with either KP1 or LK03 capsid (N=3 per group). LK03 had previously been shown to selectively transduce human hepatocytes when tested in a xenograft mouse model (Lisowski et al., *Nature*, 2014) and successfully delivered a huFVIII expression vector in a clinical study (George et al., *NEJM*, 2021). Animals injected with KP1-CB 2679d-GT had up to 10-fold lower peak expression levels of the variant huFIX than those injected with LK03-CB 2679d-GT. Two animals from each group showed sustained huFIX expression at 3 months post-administration with antigen levels ranging from 2% to 4% of the normative human range

and activity levels between 50% and 80% of the normative human range, consistent with the enhanced potency of this huFIX variant. One animal from each group showed declining circulating huFIX antigen levels beginning after day 28, coinciding with the emergence of high anti-huFIX antibody levels. Transduction as measured by 3-month liver DNA and mRNA levels was approximately 10-fold lower in the KP1 group compared to the LK03 group. However, the data obtained from this rhesus study does not rule out that human liver may be transduced more efficiently using the KP1 capsid, as both capsids provided similar levels of human hepatocyte transduction in the xenograft mouse model. Our study demonstrates that combining a next generation AAV vector with the potency enhanced FIX variant CB 2679d-GT has the potential to improve transgene expression and effectively lower the viral dose needed to achieve therapeutically relevant FIX activity levels when administered systemically. This would substantially reduce the cost of rAAV based FIX gene therapy, the potential for liver toxicity and the risk of adverse immune responses with high doses.

700. EBT-101 Achieves Robust Intended Editing of HIV without Detectable Off-Target Editing

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Safe and effective viral excision requires pairs of CRISPR-Cas guide RNAs (gRNAs) that specifically cleave HIV, but have minimal similarity to sites in the human genome. Bioinformatics facilitate selection of HIV-specific gRNAs with fewer nominated (potential) sites output in the human genome than when targeting human sequences. Here, we describe this bioinformatics, as well as our results from multiplex amplicon sequencing that identified high levels of viral excision without detecting unintended indels or recombinations. This work extends our previous specificity studies of EBT-101, a CRISPR-based therapeutic candidate entering clinical trials as a potential one-time cure for HIV, and extends our validation of HIV excision in humanized mice and NHPs studies that demonstrate biodistribution, safety and SIV excision in relevant tissues. According to recent estimates, ~1 million people in the US and 36.9 million people worldwide live with HIV/AIDS (D'Souza, 2019). The current standard of care is a combination of antiretroviral therapies, which may control the disease but are not curative as they are unable to target integrated HIV-1 genomes (Hu, 2014). Gene editing to remove proviral DNA can provide the opportunity to eradicate the disease, advancing beyond the current standard of care. **EBT-101** is an adeno-associated virus (AAV) vector expressing the SaCas9 nuclease and 2 gRNAs targeting 3 sites in HIV: two well conserved regions in the 5' and 3' long terminal repeats (LTRs) and one in the Gag gene. Excising these large sections of the integrated HIV-1 genomes disrupts the viral life cycle and is intended to reduce latent HIV-1 patient reservoirs. The efficacy of EBT-101 has been demonstrated in humanized mouse models (Dash, 2019).

To assay for potential off-target Cas9 activity, the human reference genome was scanned for the 21 bp EBT-101 LTR-1 and GagD guide sequences with NNGRRT, the SaCas9 protospacer adjacent motif (PAM), using software adapted from Cas-OFFinder. There were no identical matches, as there are no target sites in human cells without HIV. There were also no candidate off-target sites nominated with one or two differences (mismatches and/or bulges).

Table. Only one nominated human chromosomal site in the bioinformatics output with up to three differences from either target site.

Differences	Search settings		Nominated Genomic Sites	
	Mismatches	Bulges	GagD	LTR-1
Zero	0	0	-	-
One	1	0	-	-
"	0	1	-	-
Two	2	0	-	-
"	1	1	-	-
Three	3	0	-	1
"	2	1	-	-

The human reference genome was searched using bioinformatics software to nominate locations similar to EBT-101 guide RNA sequences and the SaCas9 PAM (NNGRRT). Sites were then scored and assigned by the number of nucleotide mismatches and bulges. Only one DNA sequence was output with three differences from the LTR-1 target site. No sites were identified with up to three differences to the GagD target site.

Cas, CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeats; LTR, long terminal repeat; PAM, protospacer adjacent motif; SaCas9, *Staphylococcus aureus* Cas9.

Only one site was nominated with three or fewer differences (including 2 mismatches in the seed region) to the LTR-1 gRNA. To provide an exhaustive list of nominated sites for use in high-throughput sequencing studies, bioinformatic searches were expanded to allow sequences with more differences and those predicted to be poorly tolerated. We demonstrate that the lower number of nominated sites contrasts markedly to the numerous highly homologous sequences nominated when targeting therapeutic sites in human genes (Chaudhari. 2020). To demonstrate HIV excision and confirm the predicted lack of off-target edits, U1 cells, a pro-monocytic, human myeloid leukemia cell line containing 2 integrated copies of the HIV genome, were treated with LTR-1 and GagD gRNAs coupled with SaCas9. High-throughput multiplex amplicon sequencing detected high levels of HIV editing and excision, but not unintended editing or recombination. In the specific case of EBT-101, these results highlight the feasibility of this novel approach for a curative HIV therapy.

701. Design of Novel Non-Viral Vectors for the Ex Vivo Gene Therapy for β -Hemoglobinopathies

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β -Thalassemia and Sickle Cell Disease are the most common monogenic diseases worldwide, with >317,000 newborn/year, caused by defects of β -globin expression or structure. They lead to transfusion-dependent anemia, multi-organ damage and early death.

Ex vivo gene therapy (GT) is an extremely promising approach for β -hemoglobinopathies. GT for β -thalassemia provided a remarkable clinical efficacy and benefit for β -thalassemic patients with non- β^0/β^0 genotype, but failed to control the disease in complete absence of endogenous β -globin, due to an insufficient expression of therapeutic β -globin driven by the LentiGlobin vector. Based on these results, ZYNTEGLO™ by Bluebird Bio, i.e. autologous CD34+ cells transduced with the LentiGlobin, is the first GT medicinal product approved in Europe for transfusion-dependent β -thalassemic non- β^0/β^0 patients. This partial clinical efficacy is mainly due to a suboptimal β -globin production, from a limited number of erythrocytes, and to inner LV restraints. In addition, the inability to reach a consensus around a fair pricing of the therapy with European authorities highlighted the need for more cost-effective therapeutic strategies for β^0 -thalassemia and SCD, which still remain a crucial unmet clinical need. To overcome these limitations, we designed a GT strategy for β -globin defects based on a gene-cassette optimization for enhanced production of a potent anti-sickling (AS) β -globin, embedded in a hyper-functional transposon-vector (TV) to be delivered as minicircle (MC) DNA, for a cost-effective, scalable and standardized vector-production. A first TV expressing the AS β -globin under the control of a large regulatory region of ~6kb, including the HS1-2-3-4 elements of the β -globin Locus Control Region (LCR), was produced as MC DNA (HBBAS3-VP1), together with an identical TV containing the GLOBE cassette (G-AS3) as a reference. We set the conditions for an efficient and non-toxic nucleofection of these large MCs into human CD34+ cells derived from G-CSF-mobilized peripheral blood of healthy donors, achieving the integration of multiple vector copies without impairment of cell clonogenic potential in vitro. Nucleofected cells were in vitro differentiated in mature erythrocytes before evaluating the AS β -globin mRNA expression by real-time PCR. Preliminary data showed a comparable AS β -globin mRNA expression from the two TVs in bulk populations of CD34-derived erythrocytes, overall significantly lower than the LV GLOBE performance at comparable vector copy numbers. We are currently developing a second generation of β -globin expression cassettes containing different combination of small, epigenetically-identified core enhancer elements derived from the 30-kb long β -globin LCR, optimized in size and sequence to be delivered as TVs. Once identified the optimal vector for a superior production of therapeutic AS β -globin in donor-derived HSPCs, it will be further validated in vitro in patient-derived HSCs and in vivo in humanized immunodeficient mice in an efficacy/safety preclinical study.

702. ZF-Nuclease AAV6-Mediated Factor IX (FIX) Insertion into B Cells Results in Sustained Therapeutic FIX-Levels *In Vivo* After Using a GMP-Like Differentiation Culture System

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The development of precise gene editing techniques opens a therapeutic opportunity with genetically modified B cells. In such an approach, the differentiation of B cells into long-living plasma cells (PCs), natural factories producing high amounts of proteins, can be leveraged. B cells could be genetically engineered so that resulting PCs produce a predefined therapeutic protein in high quantities. Moreover, thanks to the engraftment of the engineered PCs into a bone marrow niche, the production of therapeutic protein could last for decades. This innovative autologous approach could deliver sustained and high doses of therapeutic proteins and not be limited by the downsides of AAV-mediated *in vivo* gene therapy, such as pre-existing immunity to the AAV capsid, unfavourably impacting the product efficacy, challenges for the re-dosing and unfavourable liver conditions such as in hepatitis or pediatric patients, limiting the patient population that could benefit from such therapy. Furthermore, engineered B cell therapy could be applied for various indications such as protein deficiency diseases, prophylactic treatment in infectious diseases or immuno-oncology. In this study, we show that highly specific editing of B cells is feasible using Zinc Finger Nuclease (ZF-nuclease) technology and human Factor IX Padua variant (FIX-R338L). First, B cell engineering was performed by combining electroporation of ZF-nuclease mRNA with AAV6 donor template delivery, to achieve site-specific insertion of a GFP cassette at multiple *loci*. Furthermore, we established an advanced *in vitro* culture system using a serum free GMP-compatible medium, optimizing cell viability, expansion rate and promote efficient differentiation of B cells into plasmablast/PCs. Using this optimized *in vitro* culture protocol, FIX-engineered B cells (FIX-B cells) consisting of a mix of memory B cells and over 50% of plasmablast/PCs were obtained by targeting the silent and safe TRAC locus with a specific TRAC ZF-nuclease and an AAV6-FIX-Padua vector. *In vitro* analysis of FIX-B cells revealed high levels of secreted human immunoglobulins (huIg) and up to 30ng/ml of FIX-Padua suggesting fully functional engineered B cells producing significant amount of FIX. To further assess the therapeutic potential of FIX-B cells *in vivo*, FIX-B cells were injected into the NOD SCID gamma (NSG) mice model pre-engrafted with human memory T cells (Tm) (Ishikawa Y. *et al.* (2014)). Then, engraftment efficiency of FIX-B cells, huIg and FIX secretion were monitored for four weeks. Human B cells were detected in the mouse bone marrow indicating successful and potential long-term engraftment. Engrafted PCs were CD38^{hi}CD138⁺ expressing highly CD27, BLIMP-1, XBP1 and low level of CD20 as expected. Additionally, FIX-B cells were detected in the spleen suggesting short-term living PCs. In the blood, FIX-B cells were not detectable at any time. Moreover, a stable and functional engraftment of FIX-B cells is suggested by the secretion of high levels of huIg (up to 2500µg/mL IgM) and therapeutically relevant FIX-Padua levels in mouse blood (up to 90ng/mL). The FIX-level would correlate with about 15% of FIX activity. In summary, we demonstrated that ZF-nuclease mRNA combined with rAAV6 donor DNA allowed efficient site-specific insertion of human FIX transgene into the B cell TRAC locus, resulting in therapeutic levels of FIX-Padua expression *in vivo*. This study highlights the therapeutic potential of engineering B cells as a new technological platform to treat a variety of protein deficiencies, blood disorders, infectious diseases, or cancers.

703. Development of a Synthetic AAV Vector for Gene Therapy of Hemophilia in Children

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In all previous or currently ongoing clinical trials for gene therapy of hemophilia, children have not been enrolled since traditional liver-directed AAV gene therapy is unlikely to work for the following reasons: (i) Hepatocytes in the growing liver are undergoing rapid cell divisions, and with every cell division, the AAV vector genomes would be expected to be diluted out due to the episomal nature of the AAV genome; and (ii) Repeat vector dosing is not an option since following the first administration of the vectors, neutralizing antibodies will be generated, making subsequent vector delivery difficult. We have previously reported the development of a synthetic AAV vector, termed No-End (NE) AAV DNA, which is devoid of AAV capsid proteins, which upon transfection into human hepatic cells, leads to sustained expression of a reporter EGFP gene for up to 35 days (*Mol Ther*, 29: 178, 2021). In the present studies, an optimized NE-DNA containing the human Factor IX (hF.IX) gene expression cassette under the control of a human liver-specific transthyretin (TTR) promoter (optNE-TR75-TTR-hF.IX), was generated. This NE-DNA was transfected into a human hepatocellular carcinoma cell line, HepG2. The same expression cassette as a linear DNA was used as an appropriate control. Following transfections, cells were passaged for up to 28 days. At day 7, day 14, and day 28, cell extracts were prepared from replicate cultures and equivalent amounts of total cellular proteins were analyzed on Western blots using a hF.IX-specific monoclonal antibody. These results, shown in **Figure 1**, indicate that ~6-fold higher hF.IX levels were expressed from the optNE-TR75-TTR-hF.IX cassette compared with that from its linear counterpart for up to 4-weeks post-transfection. These data are consistent with our previous studies with NE-DNA containing a reporter gene, suggesting that the observed increased hF.IX gene expression is due to prolonged stability of the NE-DNA. Studies are currently underway to generate optNE-TTR- DNA containing the human clotting factor VIII (hF.VIII). In future studies, encapsidation of these optNE-DNAs in liver-targeted synthetic liposomes may provide a viable approach for repeated delivery, and thus, the potential gene therapy of hemophilia B as well as of hemophilia A in children.

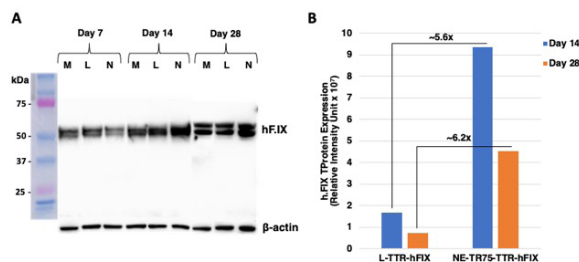


Figure 1: Linear- and NE-TR75-TTR-hF.IX DNA mediated expression of hF.IX protein in human hepatic cells. Human HepG2 cells were either mock-transfected (M) or transfected with linear-TTR-hF.IX (L) or NE-TR75-TTR-hF.IX (N) DNAs. Equivalent amounts of total protein samples prepared at the indicated time-points were analyzed on Western blots (A) using a monoclonal anti-hF.IX antibody. (B) Quantitation of the mock-adjusted data from (A).

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704. A Novel Strategy for Platelet-Specific Gene Therapy for the Treatment of Hemophilia A via Intranasal Delivery Lentiviral Vectors Containing Factor VIII

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Hemophilia A is a disease caused by absent or deficient FVIII protein. Gene therapy targeting hematopoietic stem cells is an attractive approach to treating this disease. Emerging evidence points to the lung being a reservoir for hematopoietic progenitors/stem cells (HSCs), in addition to bone marrow. Our previous data demonstrated that specific promoter GP1ba (G) can drive external factor VIII (FVIII) gene and achieve long-term expression of FVIII protein in megakaryocytes by transducing HSCs in mice. FVIII protein stored in platelets can be released to facilitate clotting upon platelet activation. Thus, intranasal (IN) delivery of lentiviral vectors (LVs) targeting FVIII gene expression in megakaryocytes in the lung has the potential to correct the HemA mice phenotype. HemA mice were conditioned with N-acetylcysteine and dexamethasone to reduce lung mucus and the immune response, respectively. They were then administered a LV with a titer of 1.1×10^9 ifu/mL intranasally. The LV contained a megakaryocyte-specific Gp1ba-promoter driven FVIII gene to target expression to platelets. Using flow cytometry, we found that IN delivery of $24 \mu\text{L}$ G-GFP-LVs (2.5×10^9 ifu/mL) produced up to 0.075% GFP expressing-platelets after 4 days. When a vector containing the FVIII gene (G-F8-LV) was delivered intranasally (2.5×10^9 ifu/mL), FVIII expression in platelets was detectable by ELISA at a level of $0.4 \text{ mU}/10^8$ platelets. Additionally, rotational thromboelastometry (ROTEM) assays using whole blood from IN gene therapy group showed that the mice had functional improvements. The clotting time was significantly shortened from HemA control mice at day 7 and 30, and the maximum clot firmness and alpha angle value were significantly different 7 days after treatment. Additionally, FVIII colocalized in CD41+ cells in the lung using immunohistochemistry, indicating FVIII is being expressed in cells of the megakaryocyte lineage. These data demonstrate that we have succeeded in producing FVIII expressing platelets and partially corrected HemA phenotype in mice via IN delivery of G-FVIII-LVs in the lung. The benefit of FVIII expression and storage in platelet α -granules is to protect FVIII from naturalizing antibodies and to decrease the possibility of inducing inhibitory antibodies. The IN delivery of LVs encoding FVIII gene targeting lung would be a promising option for therapeutic treatment of hemophilia patients.

705. Therapeutic Platelet-Targeted FVIII Engraftment Was Altered in a FVIII-primed System Preconditioned with Busulfan Alone Through Cytotoxic CD8 T Cells

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Background: The development of anti-FVIII inhibitory antibodies (inhibitors) is a problem in FVIII protein replacement therapy in hemophilia A. We have developed a platelet-specific FVIII (2bF8) gene therapy protocol that can restore hemostasis and induce immune tolerance in FVIII^{-/-} mice even with inhibitors. Our previous studies have demonstrated that busulfan preconditioning alone can result in sustained therapeutic levels of platelet-FVIII expression in FVIII^{-/-} mice that received 2bF8-transduced hematopoietic stem cells (HSCs) in the non-inhibitor model but not in the inhibitor model. **Objective:** Here we explored the mechanism of platelet-FVIII loss upon busulfan conditioning in the inhibitor model. **Methods and results:** FVIII^{-/-} mice were immunized with FVIII protein to establish the inhibitor model. Animals received busulfan preconditioning followed by transplantation of either whole bone marrow (BMT) or Sca-1⁺ cells (HSCT) from 2bF8 transgenic (2bF8^{Tg}) mice. After BM reconstitution, platelet-FVIII expression level in the 2bF8^{Tg}-BMT group was $7.19 \pm 8.59 \text{ mU}/10^8$ platelets, which were significantly higher than in the 2bF8^{Tg}-HSCT group ($0.55 \pm 1.02 \text{ mU}/10^8$ platelets). When CD8 T cells were depleted in addition to busulfan preconditioning, platelet-FVIII expression was significantly enhanced in FVIII-primed mice that received 2bF8^{Tg} Sca-1 cells ($2.14 \pm 2.25 \text{ mU}/10^8$ platelets) and sustained during the study period. We then explored which subset of cells from 2bF8^{Tg} mice could activate FVIII-primed CD8 T cells using the mouse IFN γ ELISpot assay. FVIII-primed CD8 T cells were stimulated with platelets, Sca-1⁺ cells, or megakaryocytes (Megs) sorted from either 2bF8^{Tg} or FVIII^{-/-} mice. We found that FVIII-primed CD8 T cells were efficiently activated by 2bF8^{Tg}-Sca-1⁺ cells and secreted IFN γ but not by platelets or Megs, suggesting that 2bF8^{Tg}-Sca-1⁺ cells could be a potential target for FVIII-primed CD8 T cells. To explore whether the loss of platelet-FVIII expression in the inhibitor model relies on antibody-dependent cellular cytotoxicity (ADCC), we transplanted 2bF8^{Tg}-Sca-1⁺ cells into FVIII-primed B-cell deficient μMT mice preconditioned with busulfan. We found that no platelet-FVIII was detected in μMT recipients, suggesting that the loss of platelet-FVIII expression in the inhibitor model is not mediated by the ADCC pathway. In **conclusion**, pre-existing anti-FVIII immunity can alter the engraftment of 2bF8-genetically-manipulated Sca-1⁺ cells via the cytotoxic CD8 T-cell killing pathway. Sufficient eradication of FVIII-primed CD8 T cells is critical for the success of platelet gene therapy in hemophilia A with pre-existing immunity.

706. Optimization of In Vivo HSC Transduction in Rhesus Macaques Using a Desmoglein 2-Targeting HDAd5/F3+ Vector and Transient Over-Expression of cxcr4

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We developed a new *in vivo* HSC gene therapy approach that involves only intravenous injections and 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 (HDAd) 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. So far, we have used CD46-targeting HDAd5/35++ vectors for *in vivo* HSC transduction in rhesus macaques (PMID: 35036470). In these studies, we identified two limiting factors: *i*) the sequestration of injected HDAd5/35++ vectors by NHP erythrocytes (which, in contrast to humans, are CD46-positive) and *ii*) the preferential return/survival of mobilized transduced HSCs to/in the spleen, a secondary hematopoietic organ. To address the first problem, we searched for alternative HSC-targeting receptors. Desmoglein 2 (DSG2) is known to be an integral component of desmosomal epithelial junctions. DSG2 is also the primary attachment receptor for some species B human adenovirus (Ad) serotypes including Ad3, 7, 11, 14, and 55. More recently, DSG2 is gaining recognition for its ability to exist outside of desmosomes and to regulate additional biological processes. We report here that DSG2 is highly expressed on primitive HSCs in humans and rhesus macaques. A helper-dependent, chimeric adenovirus vector carrying Ad3 fibers with enhanced affinity to DSG2 (HDAd5/3+) efficiently transduced HSCs *in vitro*. Notably, DSG2 is not expressed on NHP erythrocytes and not accessible in epithelial tissues which gives a rationale for *in vivo* studies with HDAd5/3+ vectors. For a 7-day study, two rhesus macaques were mobilized with G-CSF/Plerixafor and intravenously injected with 0.4×10^{12} vp/kg HDAd5/35++ vector and 0.4×10^{12} vp/kg HDAd5/3+ vector containing the same $\text{mgmt}^{\text{P140K}}$ /GFP expression cassette. Animals were pretreated with the IL1R and IL6-blockers Anakinra and Tocilizumab to prevent innate toxicity associated with high-dose intravenous HDAd injection. For the HDAd5/3+ vector, 3.57% and 2.10% GFP-positive HSCs (CD34⁺/CD45RA⁻/CD90⁺ cells) were found at day 7 in the bone marrow and spleen, respectively. For the HDAd5/35++ vector, 0.35% and 1.05% GFP-positive HSCs were measured in the bone marrow and spleen, respectively. This indicates that the HDAd5/3+ vector more efficiently transduced HSCs that returned to the bone marrow. To further increase the return of transduced, mobilized HSC to the bone marrow, we transiently expressed the CXC motif chemokine receptor 4 (cxcr4) from our vector under a CMV promoter that would be active early in mobilized HSCs and then silenced. In the context of an HDAd5/35++ vector, transient cxcr4 expression mediated efficient return of (total) mobilized HSCs to the bone marrow, thereby doubling the percentage of GFP-positive bone marrow HSCs. In an ongoing study, we combined the HDAd5/3+ capsid platform with transient cxcr4 expression and followed GFP-positive HSCs and PBMCs long-term after *in vivo* HSC transduction in a rhesus macaque with and integrating HDAd5/3+-GFP-cxcr4 vector and *in vivo* selection. Results will be reported.

Immunological Aspects of Gene Therapy and Vaccines I

707. 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 human tissues. However, the immune response against this vector 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 readministration. 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. We also investigated complement activation by AAV capsids by measuring increases in C3a and C5a in whole blood. In order to investigate the role of pre-existing anti-AAV antibodies on cytokine release and complement activation, these experiments were conducted in both seropositive and seronegative donors. The results showed that AAV vectors could trigger low but detectable levels of cytokines and chemokines (MCP-1, MIP-1a, MIP-1b, IL-8, IP-10, IL-1b, TNF-a, IFN-g, IL-6, IL-2 and IL-10) in whole blood from a proportion of the AAV-seropositive donors tested, but not in all of them. Among the panel analyzed, IP-10 (CXCL-10) was the only chemokine detected in blood from seronegative donors following incubation with AAVs. Complement activation occurred in an AAV dose-dependent manner and only in 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 immunomonitoring 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 pro-inflammatory and safer AAV vectors.

708. ImmTOR Tolerogenic Nanoparticles Blunt AAVrh32.33 Capsid-Specific Antibody and CD8 T Cell Immune Responses in C57BL/6 Albino Mice

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Background: Adeno-associated virus (AAV) is a promising gene delivery vector, however capsid specific immune responses may limit therapeutic efficacy and prevent redosing. Animal models have been used to characterize the adaptive response to AAV and test immune modulation strategies. One capsid variant AAVrh32.33, elicits a strong capsid specific antibody response and CD8+ T-cell response. When delivered intramuscularly (IM), it can be used as a valuable model to evaluate both arms of the adaptive immune response. ImmTOR (tolerogenic nanoparticles encapsulating rapamycin) and methotrexate (MTX) are immune modulators being evaluated in AAV gene therapy studies. ImmTOR promotes the generation of tolerogenic dendritic cells, the induction of antigen-specific Tregs and inhibits the formation of AAV specific antibodies in preclinical models. MTX has been reported to induce immune tolerance by promoting the generation of IL10- and TGF β -secreting B cells, as well as blocking a variety of other innate and adaptive cell signaling pathways. This study evaluated the efficacy of MTX and ImmTOR to mitigate the AAVrh32.33-specific B- and T-cell responses in a pre-clinical gene therapy setting. **Methods:** C57BL/6 albino mice (n=12) received an IM injection of 5X10¹⁰ vector genome copies of AAVrh32.33/Luc in the anterior tibialis muscle; meanwhile 4 animals served as controls and were given buffer only. Mice were either co-dosed with AAV+ImmTOR or AAV+MTX and compared to AAV+vehicle controls. ImmTOR was administered the same day as the vector with 200 μ g administered via intravenous injection, as well as 100 μ g admixed with vector and injected IM. MTX (5 mg/kg) was delivered by intraperitoneal injection as two cycles (2 doses per week for 4 weeks). *In vivo* imaging to detect luciferase transgene expression was performed on days 7 and 21 after vector injection. Serum was collected pre-dose, day 14, and day 28, and the levels of anti-capsid IgG measured by ELISA. Splenocytes were evaluated for interferon- γ (IFN- γ) secretion by ELISpot, and AAVrh32.33-specific CD8 T-cell responses were assessed using flow cytometric readouts of Db dextramers loaded with SSYELPYVM peptide on day 28. **Results:** No significant differences in transduction levels were observed *in vivo* when AAVrh32.33 vector was administered alone or in combination with either ImmTOR or MTX. ImmTOR effectively inhibited the formation of AAVrh32.33-specific IgG antibodies on day 28 in all animals, while MTX treatment did not significantly decrease antibody levels compared to AAVrh32.33 controls. Moreover, IFN- γ secretion by splenocytes was markedly reduced in ImmTOR recipients equivalent to buffer only controls, while MTX recipients showed varied IFN- γ response. Flow cytometric analyses of Dextramer+CD8+ T cells revealed that not only was the

frequency of AAVrh32.33 capsid-specific CD8 T cells reduced in ImmTOR recipients but the proliferative (%Ki-67+Dextramer+CD8+ T cells) and cytotoxic (%Dextramer+Perforin+CD8+) potential was also significantly reduced compared to AAVrh32.33 controls. **Conclusions:** Our results suggest ImmTOR blunts both AAVrh32.33 capsid-specific IgG secretion and CD8+ T-cell splenocyte responses. We believe multi-functional mechanisms of ImmTOR action make it a superior candidate compared to MTX in the efforts to modulate AAV specific adaptive immune responses.

709. Attenuation of AAV Humoral Immune Responses with Glucocorticoids and Rapamycin in Non-Human Primates

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Viral vectors based on adeno-associated viruses (AAV's) have been utilized to deliver therapeutic genes to treat several monogenic disorders. Pre-existing immunity directed to the viral capsid antigens poses a major challenge for AAV directed gene therapy. AAV neutralizing antibodies (NAbs) have been demonstrated to block vector transduction in both animal models and in clinical trials. Often patients with preexisting immunity are excluded from clinical trials limiting patient inclusion. However, even dosing naïve subjects carries the risk of complement activation via the induction of de novo capsid-specific antibodies and the formation of immune complexes that target the vector in circulation. Strategies that reduce the burden of de novo capsid antibodies may improve the safety of AAV vectors. In the present study, we evaluated an immunosuppression strategy to reduce the burden of de novo antibodies following vector administration. Cynomolgus macaques (n=3/group) were systemically administered with an AAV vector in the presence of immunosuppressants (rapamycin and methylprednisolone). An additional group received either methylprednisolone alone or no treatment and served as controls. Serum samples were collected at different time points post vector administration and evaluated for the presence of anti-AAV NAbs, as well as total IgG and IgM capsid binding antibodies (BAbs). A humoral immune response to AAV capsid protein was evident by increased NAb and BAb titers in all vector treated animals. However, we observed a significant decrease in NAb antibodies in animals that received the highest dose of rapamycin along with methylprednisolone when compared to the control groups. The decrease in NAbs was associated with a decrease in levels of binding IgG antibodies. A significant decline in the levels of anti-capsid IgM antibodies was not observed. These findings are consistent with the known mode of action of rapamycin. In conclusion, our study demonstrates that the combination of rapamycin and methylprednisolone can be used to dampen the humoral immune response following AAV vector administration.

710. Absence of Dorsal-Root Ganglia Pathology in Non-Human Primates Following MR-Guided Brainstem Administration of AAV2-hAADC

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Adeno-associated virus (AAV) based vectors are widely used for introduction of transgenes into nervous tissue in preclinical and clinical gene therapy. AAV administration has utilized systemic (IV), cerebral spinal fluid (CSF), and parenchymal infusions to target neurons. Recent published findings suggest that IV and CSF administration of AAV vectors hosting either a self-recognized or foreign transgene are linked to aberrant pathological findings within the dorsal root ganglia (DRG) in non-human primates (NHP). The DRG houses the cell bodies of the peripheral sensory neurons and is thus a key component in sensory transmission, conveying peripheral sensory information to the spinal cord. While the observed DRG pathologies appear clinically silent in NHPs, their presence has raised concern for IV and CSF delivery of gene therapy. However, parenchymal delivery of AAV vector had not previously been evaluated for effects on the DRG. Gene therapy utilizing convection enhanced delivery (CED), allows for the delivery of viral vector into parenchymal tissue with broad coverage of target structures. Gadolinium contrast agent co-infused with the vector allows real time feedback via magnetic resonance (MRI), assisting with targeting accuracy and delivery monitoring. Compared to systemic or CSF delivery, parenchymal MR-guided CED yields a more contained delivery within the brain, while also minimizing leakage of vector into the CSF or bloodstream. Thus, parenchymal delivery should avoid the DRG toxicity associated with CNS-targeted AAV delivery. To evaluate this hypothesis, we performed histological and histochemical analyses of the DRG from NHPs that received MR-guided parenchymal CED infusions of transgene-carrying AAV or sterile PBS/saline to the brainstem. We also assessed the DRG from NHPs that received AAV vectors either via IV or CSF delivery. H&E staining, unbiased software-based cell counting, and immunohistochemical analyses were utilized to evaluate the DRGs. We confirmed the presence of DRG pathology in NHP following CSF delivery of AAV, as previously reported. However, we found no significant pathology in the DRG following parenchymal delivery of the tested vector infusate as compared to controls. Further work is needed examining infusion of other serotypes, transgenes, and CNS structures to reach a general conclusion. Nevertheless, our results suggest that AAV2-hAADC parenchymal delivery into the brainstem avoids the DRG toxicity associated with IV and CSF delivery, demonstrating safety for this therapeutic intervention.

711. Effect of Tolerogenic ImmTOR Nanoparticles on the Formation of Anti-AAV8 Antibodies in Mice, Nonhuman primates, and Healthy Human Volunteers

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We previously described the ability of tolerogenic ImmTOR nanoparticles, encapsulating the small molecule immunomodulator rapamycin, to mitigate the formation of anti-AAV antibodies and enable vector re-dosing in mice and nonhuman primates. Here we describe the results of a randomized, placebo-controlled, double-blind experimental medicine trial in 23 healthy volunteers who were administered a single dose of 2E12 viral particles (vp)/kg of an empty AAV8 capsid formulation (EMC-101), either alone (n=8) or in combination with an immediate single-dose pre-treatment of 0.15 or 0.30 mg/kg ImmTOR nanoparticles (n=15). Subjects showed baseline anti-AAV8 neutralizing antibody (NAb) titers of <1:5 at screening and were followed for 90 days after dosing. The combination treatment was safe and tolerated in healthy volunteers with no serious adverse events. AAV8 empty capsids elicited a strong immune response with peak median anti-AAV8 NAb titers of 1:6875. ImmTOR showed dose-dependent inhibition of NAb with median Day 30 titers of 1:25 and 1:5 in the 0.15 mg/kg and 0.3 mg/kg ImmTOR cohorts, respectively. All 6 subjects who received 0.3 mg/kg of ImmTOR exhibited an anti-AAV8 neutralizing antibody titer of 1:25 or less, and 4 of 6 had a titer of 1:5 or less at 30 days. At Day 90, 2 of 6 subjects in the 0.3 mg/kg cohort showed sustained control of neutralizing antibodies with titers of 1:5 and 1:25. However, the other four subjects showed delayed development of NAb, with titers reaching levels similar to those of control subjects receiving AAV8 capsid alone by Day 90. Nonclinical studies of C57BL/6 mice dosed with a single dose of ImmTOR combined with a comparable dose of 2e12 vp/kg of the same empty capsid preparation showed breakthrough anti-AAV8 antibodies in only 1 of 6 mice through Day 84; however, mice dosed with a 10-fold higher dose of empty capsid (2E13 vp/kg) showed delayed induction of high titer anti-AAV8 antibodies in 3 of 6 mice. At both empty capsid doses, the administration of two additional monthly doses of ImmTOR, on Days 28 and 56, resulted in sustained reduction of anti-AAV8 antibodies in all mice. Similar results were observed in nonhuman primates (NHP), where 2 of 5 animals treated with a single dose of ImmTOR with 2E12 vp/kg AAV8-SEAP showed sustained reduction of anti-AAV8 antibodies through 3 months, while the other three animals showed delayed development of antibodies. Interestingly, the distribution of NAb titers at Day 84 in NHP receiving a single dose of ImmTOR was similar to that observed in the 0.3 mg/kg ImmTOR cohort of the human volunteer study at Day 90. However, the administration of two additional doses of ImmTOR, on Days 28 and 56, resulted in sustained reduction of NAb titers <1:5 through Day 84 in monkeys. These results show for the first time that empty AAV8 capsids can elicit a robust NAb response in humans and that co-administration of a single dose of ImmTOR can substantially

inhibit NAb formation through Day 30. The delayed formation of NABs observed in most subjects suggest that additional monthly doses of ImmTOR may be required to durably inhibit NAb antibody formation.

712. Efficacy of Immunomodulatory Regimens to Prevent Anti-AAV Humoral Response and to Enable AAV Re-administration in Mouse Gene Transfer Model

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Introduction Adeno-associated virus (AAV) continues to be a vector of choice for therapeutic gene delivery due to its satisfactory safety and efficacy profile that was established in various gene therapy clinical trials. Nevertheless, adverse reactions potentially related to AAV immunogenicity have been reported at times that may limit durable benefits of AAV-based gene therapy in patients. Once AAV-transduced cells are rejected by the host immune system, redosing of the therapy is not possible due to the development of broadly cross-reactive AAV neutralizing antibodies in recipients of the treatment. It is therefore of interest to investigate immunomodulatory (IM) strategies by which cellular and humoral immune response against AAV vectors could be prevented. Such approach, if successful, should facilitate sustained transgene expression following systemic AAV-based gene delivery and/or facilitate repeated AAV dosing if needed. Here, we tested effects of anti-IL-6R mAb, anti-IL1 β mAb, Etanercept, Anakinra and Rapamycin, alone or in combination, on the anti-AAV humoral response prevention and efficacy of AAV re-administration.

Experimental Design Starting from day -2 through day 28 mice were treated with 9 different IM regimen combinations of 5 listed drugs or no IM (control). Mice received intravenous injection of AAV-Spark100-hFIX on Day 0 and AAV-Spark100-Luc at week 10. At week 14 all animals were sacrificed. Both transgenes, human Factor IX and secretable luciferase were monitored in the plasma over time. Transduction efficacy and transgenes expression were measured in the terminal livers. To determine immune responses related to AAV injections in different treatment groups, development of anti-AAV IgMs and IgGs and early post AAV-infusion cytokine levels in the circulation were assessed. **Results and Conclusion** Among tested IM regimens, anti-IL-6R mAb exhibited the best efficacy to lower the adaptive immune response against AAV-Spark100 capsid, at both IgG and IgM levels, and enabled modest luciferase expression after vector re-dosing. Combination of anti-IL6R MAb with other IM drugs, including Rapamycin, did not provide additional advantage. Interestingly, although Rapamycin alone decreased formation of anti-AAV-Spark100 IgG antibodies following the first vector infusion, it caused prolonged neutralizing anti-AAV-Spark100 IgM formation, presumably due to prevention of immunoglobulin class switch in B cells. As a result, Rapamycin has not enabled transduction with the second AAV injection. Anakinra (IL1-R blockade), similarly to anti-IL-1 β mAb lowered to some extent anti-capsid antibody formation, although the effect was not consistent across all animals in the group and only few mice had modest luciferase expression after vector re-dosing. Overall, our results demonstrate the critical role of IL-6

signaling pathway in mounting immune response to AAV vector and warrant further investigation of such immunomodulatory strategy in the context of gene transfer.

713. Deep Immune Profiling of Candidate Endogenous Anellovirus Gene Therapy Vectors

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Despite their immense therapeutic potential, the commensal human virome remains largely understudied. Anelloviruses, which establish ubiquitous and persistent infections, represent the major constituent of the human virome and yet little is known about their immunobiology¹. In this study, we employed the massively multiplexed phage immunoprecipitation sequencing (PhIP-Seq) assay² to characterize the immunogenicity of anelloviruses and the antigenic profile of anellovirus proteins. We designed and constructed a novel, large and diverse “AnelloScan” library comprising more than 32,000 peptides representing ORF1, ORF2, ORF3 and TAIP protein sequences of more than 800 anelloviruses from all three human genera. We screened serum samples from 156 subjects to examine the anellovirus peptides recognized by antibodies in these samples. Antibody reactivities in the AnelloScan library (median number of reactivities per subject: 124) were lower compared to the total reactivities observed in a separate, independent screen of the 156 subjects against the VirScan library³ (median number of reactivities per subjects: 1741.5), which contains peptides from more than 200 species and 1000 strains of human viruses. We observed that the vast majority of anellovirus peptides were not antigenic and did not demonstrate an antibody reactivity in any of the subjects tested (n \sim 28,000; \sim 85% of the library). The peptides that did demonstrate antibody reactivity were predominantly derived from the C-terminal region of the putative capsid protein, ORF1, an observation that is consistent with previous studies⁴. To understand how the immune system responds to a newly acquired anellovirus infection, we obtained longitudinal AnelloScan data from a blood-transfusion donor(s)-recipient cohort. We observed that most anelloviruses that were transmitted from blood donors did not elicit an antibody response in the recipient (29 out of a total of 40 transmitted anelloviruses) and that those that did, elicited a delayed response (\sim 100 days after transfusion). The commensalism and immune evasion displayed by anelloviruses support their utility for medical applications, including delivery of therapeutic payloads. To date, human immune responses to recombinant adeno-associated virus (rAAV) vectors have been a major obstacle. Our data indicate that anellovirus-based gene therapy vectors may have the potential to be administered to an expanded pool of patients in multiple doses. **References:**1. Kaczorowska, J. & van der Hoek, L. Human anelloviruses: diverse, omnipresent and commensal members of the virome. *FEMS Microbiol. Rev.* **44**, 305-313 (2020).2. Larman, H. B. *et al.* Autoantigen discovery with a synthetic human peptidome. *Nat. Biotechnol.* **29**, 535-541 (2011).3. Xu, G. J. *et al.* Comprehensive serological profiling of human populations using a

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714. Anti-AAV9 Immunity Results in an Unconventional T Cell Response in Rats and Healthy Human Donors

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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. Varying degrees of immunotoxicity have been reported when gene transfer protocols were translated from animal models to patients. Two levels of immunogenicity must be taken into consideration: the unspecific innate immune response related to the recognition of the vectors as “modified viruses” and the adaptive immune response specific to the viral capsid and/or the transgene product. Whereas pre-existing humoral response to AAV capsid is well documented, the anti-capsid specific T cell responses still need to be studied and characterized. In the present study, we focused on the anti-capsid cellular immune response on healthy human donors to two serotypes currently used in clinical trials: AAV8 and AAV9. A prevalence study performed on these donors suggest a higher prevalence for AAV9 than AAV8 (n=83). Moreover, using multiparametric assays not limited to the secretion of IFN γ after *in vitro* PBMC restimulation on a small cohort (n=6-8), we evidenced a heterogeneity of the cellular immune response to AAV8 with IFN γ secretion associated to IL2 and/or TNF α , whereas in all donors tested for AAV9, no IL2 secretion was detected suggesting an unconventional T cell immunity for this particular serotype. The singularity of the anti-AAV9 immune response was also highlighted in a rat animal model. Indeed, animal models are usually not predictive of an anti-AAV cellular immune response. In our model, we injected intravenously (IV) rats with an ssAAV9-CAG-GFP vector (1 \times 14vg/kg). We analyzed the IFN γ -secreting cells by ELISpot and surprisingly, 6 out of 8 animals developed a cellular immune response to AAV9 in opposition to rats injected IV with an AAV8. A more extensive characterization of these cells in healthy donors and our rat model is currently ongoing. Moreover, the rat model will provide a relevant animal model to develop immunomodulatory strategies to prevent the anti-AAV immune response. Altogether, these results suggest that immunity to AAV may vary depending on the serotype and support the necessity of using multiparametric monitoring methods to better characterize anti-capsid cellular immunity and predict its impact in rAAV-mediated clinical trials.

715. Investigating the Role of Innate Immune Signaling in AAV-Mediated Gene Transfer in hiPSC-Derived CNS Models

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Gene therapy with adeno-associated viral (AAV) vectors is gaining ground as a promising treatment option for genetic neurodegenerative diseases for which there is currently no cure. Nonetheless, bypassing the blood-brain barrier, achieving broad central nervous system (CNS) transduction and rescuing all the affected areas remains a challenge that requires the administration of high AAV vector doses for clinical efficacy. Importantly, the administration of high vector doses has recently been associated to lethal adverse events and to neurotoxicity in clinical trials and non-human primate studies, raising great concern regarding high-dose AAV toxicity. Links between innate immunity, cell toxicity and transduction efficacy are emerging, but the mechanisms of innate immune sensing and potential antiviral restriction of AAV remain largely unknown. We have exploited here human iPSC-derived models to assess AAV tropism and innate sensing in cells of the human CNS. AAV2 and AAV6 serotypes showed superior transduction efficacy compared to AAV1, AAV9 and AAV5 in human iPSC-derived neurons and astrocytes. Interestingly, although all serotypes carried the same expression cassette, we observed that the expression of AAV5 was particularly affected in both neurons and astrocytes, as increasing vector genome copies did not correlate with transgene expression levels, differently from other serotypes. This data suggests the presence of serotype-dependent interactions between the AAV capsid and host factors that have an impact on transgene expression. Overall, neurons and microglia were less permissive to AAV transduction compared to astrocytes, and more susceptible to vector-induced DNA damage. On the other hand, both astrocytes and microglia showed signs of inflammatory activation in terms of pro-inflammatory gene upregulation upon exposure to AAV. Genome-wide transcriptomic analysis of AAV-transduced neurons and astrocytes revealed the induction of several pathways including DNA damage response, cytokine-cytokine receptor interaction and apoptosis that potentially contribute to the observed toxicity. Studies are currently ongoing to assess the impact of AAV-mediated glia activation on neurotoxicity in 2D cultures and cerebral organoids. Together, our studies will shed light on AAV sensing and innate immune restriction in the CNS and provide critical insight for the development of more stealth gene therapy and gene correction strategies.

716. Stabilin-2 Promoter Modulates the Immune Response to FVIII After Lentiviral Vector Delivery in Hemophilic Mice

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Background: Liver sinusoidal endothelial cells (LSECs) are highly specialized endothelial cells with unique morphology and functions. The high clathrin-mediated endocytic capacity, let them stand out as one of the most effective scavengers of blood waste macromolecules in the body. However, their function in immunity is only partially explored. The scavenger function widely belongs to the ability of stabilin-1 and stabilin-2 (STAB2) in mediating the binding of several ligands, including coagulation products; indeed it mediates VWF-dependent FVIII endocytosis. LSECs, as main source of FVIII, are an optimal cell target for gene therapy of hemophilia A, while STAB2 promoter can be used for directing specific expression of FVIII in LSECs upon lentiviral (LV) delivery. **Aims:** To investigate *in vivo* the role of LSECs in the modulation of the immune response against FVIII transgene. **Methods:** First, we studied the activity of the STAB2 promoter in driving transgene expression by injecting LV carrying the GFP reporter gene into C57BL/6 or Balb/c mice and by assessing its expression in organs by immunofluorescence (IF) at several timepoints. To evaluate the modulation of the immune response to the transgene regulated by the STAB2 promoter, Jedi GFP-specific splenic CD8 T cells were adoptively transferred in mice previously injected with LV. Expression of GFP was then verified 4 days after CD8 T cells injection by IF, while CD8 T cells recruitment and activation were assessed by flow cytometry. STAB2 promoter activity has been further *in vivo* evaluated by injecting LV carrying FVIII transgene in several strains of hemophilic mice followed by assessment of FVIII activity and neutralizing antibodies formations. **Results:** After LV.STAB2-GFP injection in C57BL/6 and BALB/c mice, GFP expression was mainly restricted to LSECs and stably maintained for up to three months. Then, when we performed the adoptive transfer with Jedi CD8 T cells, we observed GFP⁺ cells in the livers of all mice injected with LV.STAB2-GFP while no GFP⁺ cells were detected in mice receiving LV.PGK-GFP. Lentiviral delivery of FVIII transgene driven by the STAB2 promoter led to the transgene expression mainly in LSECs as well as high and long-term FVIII activity (up to 25 %) without inhibitor formation up to one year. **Conclusions:** Expression of GFP under the control of STAB2 promoter resulted in a strong expression into LSEC. When Jedi GFP-specific CD8 T cells were employed, LSEC were able to prevent immune reactions against the foreign protein. Moreover, FVIII transgene expression driven by the STAB2 promoter supported a durable partial correction of the bleeding phenotype in a mouse model of hemophilia A (HA). Overall, these data support the hypothesis that LSEC could play a main role in promoting immune tolerance and indicate that the STAB2 promoter represents a good candidate for a potential application in HA gene therapy.

717. STRV5, a Liver Detargeted AAV Capsid Demonstrates Improved Safety Profile After Intravenous Administration of High Doses in Non-Human Primates

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STRV5 is a novel AAV capsid being developed as a vector for gene therapies in CNS diseases. Toxicity concerns for AAV-based gene therapies administered intravenously at high doses ($\geq 1 \times 10^{14}$ vg/kg) have resulted in clinical holds for several gene therapy trials. Furthermore, elevated liver enzymes, thrombocytopenia, and/or complement activation have been reported in several animal models and some animals required early euthanasia. The mechanism underlying these acute toxicities remains unclear, but an immune-mediated toxicity is one potential hypothesis. Here, the safety of STRV5 was evaluated in NHPs and compared to AAV9 after intravenous administration of 1×10^{14} vg/kg with both capsids packaging GFP reporter cassettes. All animals enrolled to the study were seronegative based on anti-capsid IgG ELISA and transduction inhibition/neutralizing antibody assay prescreening results and were treated with an immunosuppressive regimen including methylprednisolone, cyclosporin A, and MMF to dampen anti-capsid (or anti-transgene) immune responses post-treatment. All animals dosed with STRV5 and AAV9 were clinically normal for the duration of the study. However, two of three AAV9-treated animals had increased serum ALT levels (3.6-12.1x) and lower platelet counts (0.50.6x) on Day 8 compared to pre-treatment levels. Evaluation of anti-capsid IgG and IgM responses post-treatment showed similar kinetics across both capsids suggesting anti-capsid antibodies did not mediate liver enzyme increases or platelet decreases. NHPs dosed with STRV5 had approximately 2 log lower vector genome copies, GFP mRNA copies, and GFP protein levels in the liver compared to AAV9. Overall, STRV5 has an improved safety profile relative to AAV9 after systemic administration highlighting the importance of liver detargeted capsids for high dose AAV-based gene therapies.

718. Enzymatic Strategies for Eliminating Neutralizing Antibodies Prior to Administration of AAV Based Gene Therapies

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Neutralizing antibodies against viral capsids are formed during exposure to common viral pathogens and are abundant in the population. Adeno associated viral vectors (AAVs) are commonly used as delivery vehicles for gene therapies and the transduction of new genetic material may be hindered by the presence of pre-existing antibodies. Pre-existing neutralizing antibodies are used as an exclusion criterion for gene therapies, causing large groups of patients to be excluded from potential life changing gene therapy treatments. Recently, enzymatic strategies to overcome the issue by treating a patient with IgG specific proteases prior to administration have shown promising results. However, enzyme derived from human pathogens are immunogenic and presence of antibodies against the

enzyme itself may impact the digestion efficiency in the patient. In this work, we describe a novel IgG specific protease derived from a streptococcal strain that does not infect humans. We characterize the efficiency of the enzyme to digest human and other species of IgG and demonstrate low cross-reactivity with natural occurring antibodies in human sera. In combination with the new IgG protease, we have developed methods for quantification of enzyme levels in complex samples using enzyme specific antibodies. Taken together, we present a new IgG specific protease with a differentiated profile and methods for the quantification of commonly used IgG proteases in complex samples. Our results address the problem of pre-existing neutralizing antibodies both against the viral capsid and against IgG proteases and supports further development of enzymatic strategies to enable gene therapies to broader patient populations.

719. Pre-Existing Humoral Immunity to AAV Capsids: Species and Age-Related Differences in Anti-Capsid ELISA and Neutralizing Antibody Assays

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Pre-existing immunity to AAV is a major challenge to gene therapy. Depending on the serotype and age of patients, up to 70% can be seropositive which may affect the success of the therapy, demanding the need for accurate assays to assess the presence of anti-capsid antibodies prior to therapy. Whether one prescreening assay, an anti-capsid IgG ELISA or a cellular transduction inhibition assay (commonly referred to as a neutralizing antibody, or NAb assay), provides more immunologically relevant information is often debated. In actuality, the two assays provide distinct but complementary information that together build a more complete understanding of pre-existing immunity, and both assays are essential to evaluate during initial development. Further, potential cross-species differences with these assays and associated parameters have not been explored to date. Here, naïve serum samples were collected from NHPs and pigs and screened by anti-capsid IgG ELISA and NAb assay against AAV9 and our newly evolved capsid variant, STRV5. To compare the assays, we utilized the same firefly luciferase reporter packaged virus for both methods. Our results show a clear correlation ($R^2 = 0.839$) between the level of anti-capsid IgG antibodies in the ELISA (measured as higher relative luminescent unit signal) and the percent transduction inhibition in the NAb assay with NHP sera. Setting an appropriate MOI is critical for determining neutralizing potential. We noted that for many sera samples, transduction inhibition observed at a lower MOI was decreased at higher MOIs, suggesting greater sensitivity is obtained at lower MOIs. However, it is unclear how *in vitro* MOI parameters translate to *in vivo* conditions and warrants further exploration. For the ELISA, a range of signal significantly above an established negative control was detected in NHP sera dilutions at which NAb assay inhibition was not observed indicating the ELISA was more sensitive for detecting pre-existing immunity. In Yorkshire/Landrace hybrid pigs such a trend was not observed for AAV9 or STRV5 ($R^2 = 0.325$ and 0.510 , respectively); instead, we observed a correlation with the age of the animals. Older pigs had higher ELISA baseline signals and their sera

was more likely to be neutralizing. For AAV9, the average ELISA signal increased 6-fold from newborn (2-weeks) to juveniles (2-months), and then increased an additional 2-fold from juveniles to sexually mature pigs (>5 months). For STRV5, the average ELISA signal for juveniles increased 2.4-fold over newborns and sexually mature pigs had 4.7-fold greater signals than juveniles. Interestingly, the higher baseline ELISA signal and NAb assay neutralization in older pigs may be associated with increases in total serum immunoglobulin levels as pigs age. The implications of AAV neutralizing activity from increased baseline immunoglobulin levels in pig sera are unknown but could suggest higher vector doses will be necessary to achieve optimal transgene expression in older pigs compared to newborn piglets. A number of sera samples showed low ELISA signal and high neutralization, which was not observed in NHP sera, suggesting that there may be other factors affecting neutralization in pig sera. Additionally, differences between AAV9 and STRV5 responses were observed. Overall, we observed that the signal strength of the anti-capsid IgG ELISA correlates with NAb assay inhibition in NHPs for both AAV9 and STRV5, but less so for Yorkshire/Landrace hybrid pigs.

720. Validation of the Novel Serotype-Universal AAV Neutralizing Antibodies Assay in Human Samples

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The pre-existing humoral immunity against AAV vectors due to natural infection in humans remains one of the major challenges, resulting in the exclusion of a significant cohort of patients from possible clinical benefits. Thus, the lack of an individualized approach to match AAV serotype with personal needs leads to poor access to advanced treatment such as AAV-based gene therapy for large populations of patients. Therefore, accurate assessment of serotype-specific anti-AAV neutralizing antibodies (NAB) is necessary for further AAV-based drug development and their clinical advancement. Previously developed assays for evaluating AAV seroprevalence are lacking standardization across multiple AAV serotypes currently used in clinical trials. To address the need for universal neutralizing antibodies assay (NAA), we recently developed a method to standardize infection of HEK293 cell with ten commonly used AAV serotypes by pre-treating cells with a selective inhibitor of AMP-activated protein kinase (AMPK) Dorsomorphin, also known as Compound C (CC). Utilization of CC in our assay enhances the infection of HEK293 cell by all AAV serotypes, including hard-to-transduce AAV serotype 8, which otherwise hardly infectious *in vitro* with even a very high MOI. Consequently, our findings resulted in the development of a universal protocol for the analysis of AAV-specific NAB for commonly used serotypes based on inhibition of AAV-mediated luciferase expression by serial dilutions of the serum samples. In the current study, we further validate the assay by assessing NAB titer in random human donor samples. To perform analysis fast and with maximum efficiency, the following strategy was applied. In the initial screening, the samples were tested at three dilutions 1:1, 1:8 and 1:32 in their ability to inhibit reporter luciferase encoded by ten commonly used AAV serotypes. The usage of three dilutions allows performing the screening in one 96-well plate. Importantly, our method applies equivalent infection

conditions of 10 μ M CC and MOI 2000 vg/cell to each AAV serotype used in the assay and allows to analyze AAV NAB titer within 24 hrs after infection, although extension to 48 hrs is beneficial for overall level of luciferase activity. For these samples with NAB higher than 1:32, the assays were repeated with range of dilutions from 1:2 to 1:4096 to build saturation curve. AAV NAB titer was determined as the dilution of the tested serum sample at which 50% of the chemiluminescence signal was inhibited in comparison with the maximum at plateau. Our results suggest the opportunity to rapidly evaluate immunoprevalence and NAB titer across different AAV serotypes in identical experimental conditions. Thus, our assay is not only useful for the accurate assessment of AAV NAB for clinical enrolment but also for the selection of the appropriate AAV serotype for a specific patient. Acknowledgements: This project is supported by NIH R01HL131586 FDA R01FD007483 funding. Dr. Mark Brantly, University of Florida and Alpha One Foundation, provided human samples.

721. Universal AAV Amplification and DNA Sequencing of Plasma Derived and Recombinant Factor VIII and Factor IX Concentrates

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Background and Objectives: Neutralizing antibodies (NABs) to adeno-associated virus (AAV) may exclude individuals from AAV-based gene therapy. Increased AAV seroprevalence has been observed in some patients exposed to plasma products. The aim of this work was to analyze a range of commercially available plasma and recombinant Factor VIII (FVIII) and Factor IX (FIX) products to determine if traces of AAV sequences could be detected by developing an assay to enable highly sensitive detection of AAV gene sequences. **Methods:** Plasma-derived FVIII and FIX concentrate lots (n=11) and recombinant FVIII and FIX concentrate lots (n=4) were sourced according to commercial availability. A nested and tailed PCR primer approach was utilized for amplification ahead of short-read DNA sequencing. Oligonucleotides were designed in locations conserved across multiple AAV serotype reference sequences. The best performing assay was selected through in-silico analysis and functional performance evaluations. Spike-in controls based on an AAV4 reference sequence and synthetic sequence tags were developed for quality control and detection of cross-contamination. A spike-in control was utilized during the first experiment which produced positive plasma AAV detection results. **Results:** One plasma-derived concentrate lot produced 5 positive results from 7 reactions to amplify AAV. DNA sequencing reads were high-quality and consistent across all positive replicates. While the AAV sequence was distinct from our control material, the amplified region was too short to unambiguously match to a single serotype. A best match was found by Basic Local Assignment Search Tool (NCBI BLAST™) search of the viral subset that had 94% homology to an AAV2 sequence reported in a French AAV2 natural history study. The concentration was estimated to be 2,000 to 7,000 genomic copies

per mL based on the ratio of number of mapped reads from the test sample to the spike-in control. Other samples did not produce positive results within the sensitivity and reproducibility limits of the method utilized. **Discussion:** This exploratory work was conducted to determine whether there could be a mechanistic basis to a previously reported clinical observation. This observation reported increased seroprevalence of NABs to AAV in some patients who had received plasma-derived blood products. A highly sensitive assay was developed and our preliminary results indicate a potential for the presence of AAV and other viral serotypes in some of the plasma-derived blood products. Further analysis to determine the consequences of these findings is encouraged and a more extensive study that includes a wider range of concentrates would be required to confirm the hypothesis that this exploratory study has generated.

722. Immunosuppression Improves AAV2.5T Transduction of Ferret Lungs Following Repeat Dosing

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We have previously shown that repeat administration of adeno-associated virus (AAV) vector 2.5T (rAAV2.5T) to ferret lungs leads to reduced transgene expression and higher levels of neutralizing antibodies (NABs) compared to a single-dose rAAV2.5T administration. To improve the efficiency of repeat dosing, we tested two immunosuppressive (IS) strategies. Regimen-1 involved daily administration of three immunosuppressants (tri-IS), cyclosporine, methylprednisolone (MP), and azathioprine. Regimen-2 consisted of a daily administration of MP. We found that both strategies effectively improved transgene expression following a second intratracheal administration of rAAV2.5T to ferret lungs. Four-week-old ferrets were initially dosed with AAV2.5T-fCFTRdR (ferret CFTRdeltaR) via an atomizer. Four weeks later (day 28), the ferrets were administered AAV2.5T-gLuc (secreted gaussian luciferase). The immunosuppressed ferret groups received tri-IS or MP daily starting at 2 days prior to the AAV2.5T-fCFTRdR administration and ending at the day administration of the AAV2.5T-gLuc. Transgene expression (gLuc) was monitored in the blood over the next 14-days (observation period) and in the bronchiolar lavage fluid (BALF) at necropsy, 14 days post-dosing of the AAV2.5T-gLuc vector. The tri-IS and MP regimens led to gLuc levels in the plasma that were 79.6-fold (tri-IS) and 28.3-fold (MP) higher than repeat-dose animals with no IS. Similarly, gLuc levels in the BALF were 85.8-fold (tri-IS) and 19.1-fold (MP) higher than the control animals that were repeat dosed with AAV2.5T but had no IS. Our IS strategies did not prevent the generation of capsid-specific humoral immunity elicited by the 1st dose of the AAV2.5T vector or activation of that response, as demonstrated by the increase of NABs in the plasma after the 2nd dose of AAV2.5T vector. However, the levels of the NABs in tri-IS or MP repeat-dose groups were lower in the plasma (2.6- and 2.3-fold, respectively) and in the BALF (4.2- and 4.4-fold, respectively)

than animals without IS at 14-days following AAV2.5T redosing. We found that plasma IgG levels were significantly inhibited by tri-IS or MP, compared to redosing without IS. This suggests that understanding, and suppressing, IgG responses may be important to avoid lower levels of transduction upon repeat administration of AAV2.5T. Furthermore, we assessed the AAV2.5T-specific cellular immune responses by ELISpot. We found no significant differences in T-cell responses between animals of the IS or repeat-dosing control groups. We also quantified the splenocyte derived cytokines, IL2, IL4, IL6, IFN γ , IFN α , IFN β and TNF α by RT-qPCR. The mRNA levels for IL4 and IFN α from the tri-IS group, not the MP group, were significantly inhibited compared to the non-IS AAV2.5T redosing control group, suggesting that the Th2 and pro-inflammatory immune response may have been affected by tri-IS. Collectively, our data suggest that tri-IS or MP treatment may improve transgene expression following AAV2.5T redosing. The administration of IS to facilitate a sustained therapeutic benefit following AAV2.5T vector redosing in airway is currently being investigated.

723. Process-Related Impurities in the ChAdOx1 nCov-19 Vaccine

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Introduction To fight the SARS-CoV2 pandemic, two adenoviral vector-based vaccines encoding for the SARS-CoV2 spike protein have been approved: ChAdOx1 nCov-19 marketed by AstraZeneca is based on chimpanzee adenovirus Y25 and Ad26.COVS2.S marketed by Johnson&Johnson is based on human adenovirus type 26. The latter was approved by the FDA and the EMA, while ChAdOx1 nCov-19 was approved only by the EMA. Following vaccination with the vaccines, rare cases of vaccine-induced immune thrombotic thrombocytopenia (VITT) have been reported, occurring more often with ChAdOx1 nCov-19. The initial trigger for these serious adverse events has not been determined. **Methods** Initially intended to exclude any product-related issues, we analyzed the ChAdOx1 nCov-19 and the Ad26.COVS2.S vaccines by biochemical and proteomic methods. **Results** Unexpectedly, we found that ChAdOx1 nCov-19, in addition to the adenovirus vector, contained both non-structural viral proteins and substantial amounts of human host cell proteins (HCPs). Protein amounts demonstrably exceeded the specification limit per vaccine dose set by the EMA. Among the human proteins, heat-shock proteins and cytoskeletal proteins were particularly abundant. It is possible that the often-observed strong clinical reaction one or two days after vaccination might be associated with the detected protein contaminants. A linkage to later immune related adverse events is also conceivable. In contrast, in lots of Ad26.COVS2.S only negligible amounts of HCPs were detected, confirming that excellent purification of clinical grade adenovirus vaccines is possible at an industrial scale. **Conclusion** The here reported identification of specific classes of protein impurities in the ChAdOx1 nCov-19 vaccine should guide and accelerate efforts to improve the purity of adenoviral vector-based vaccines and quality assessment methods to increase safety and efficacy.

Cell Therapies II

724. Towards Improved Human Mesenchymal Stromal Cell-Based Therapies: Enhancing Cell Transduction with HAdV-5 Vectors

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Human multipotent mesenchymal stromal cells (hMSCs) are of significant therapeutic interest for different clinical applications due to their inherent immunomodulatory properties and their ability to migrate to inflammatory sites and tumor tissue. To further improve their clinical potential, hMSCs can be genetically engineered to express, for example, therapeutic transgenes or deliver oncolytic viruses to target sites. Viral vectors based on human adenovirus type 5 (HAdV-5) are promising candidates for such approaches. However, this endeavor is hampered by the inefficient transduction of hMSCs with unmodified HAdV-5 vectors. We identified several transduction enhancers that, using a very simple protocol, impressively increased HAdV-5-mediated gene transfer into bone marrow- and adipose tissue-derived hMSCs. Since most of the identified transduction enhancing agents share a positive charge, we, in a next step, introduced positively charged amino acids into the viral capsid by genetic engineering. While most modifications of adenoviral capsids focus on the protruding Fiber, we altered distinct surface-exposed amino acids in Hexon, the most abundant protein in the adenoviral capsid. Strikingly, one of the generated vectors (HAdV-5-HexPos3) transduced hMSCs more efficiently than any other adenovirus-based strategy published so far. Here we show that transduction enhancers and HAdV-5-HexPos3 are valuable tools for engineering hMSCs to enable a plethora of applications. We demonstrate that (i) high therapeutic transgene expression is feasible (here tumor necrosis factor-inducible gene 6 protein (TSG-6) as an example was used), (ii) efficient virus replication of wild-type or conditionally replicating HAdV-5 vectors in hMSCs can be achieved, and (iii) migration of efficiently transduced hMSCs can be accurately tracked upon intravenous administration in mice.

725. Long-Term Stability Assessment of iPSC-Derived T and NK Cells Support the Feasibility of Off-the-Shelf Therapeutic Applications

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Cell-based immunotherapies have shown remarkable promise in the fight against various cancers. Induced pluripotent stem cell (iPSC)-derived natural killer (NK) and T (iNK and iT, respectively) cells can be mass produced and administered off-the-shelf to patients, and several iPSC-derived cell-based cancer immunotherapies are now undergoing human clinical testing. Our iPSC product platform leverages the use of clonal master iPSC lines that serve as the starting

material for the manufacture of multiplexed-engineered, cell-based cancer immunotherapies that can be fully characterized, stored, and administered on-demand to patients. Here we demonstrate long-term stability (1, 3 and 6 years) of our engineered iNK and iT cell drug product candidates following long-term cryogenic storage. Multiple iNK and iT cell product candidates, including those engineered with a high-affinity, non-cleavable CD16 (hnCD16) and/or with an anti-CD19 chimeric antigen receptor (CAR19), were manufactured, cryopreserved and stored in the vapor phase of liquid nitrogen for up to 6 years. To assess the impact of cryogenic storage on these product candidates, cryopreserved lots were thawed and assessed for cell health, recovery, identity and functionality. Post-thaw recovery and viability was evaluated using acridine orange/propidium iodide staining and Annexin V staining by flow cytometry, and we found cell health was consistent with newly frozen cells suggesting that stability was maintained over years of cryogenic storage. Phenotypic analysis via flow cytometry was used to monitor product identity and purity and shown to be similar to pre-cryopreservation cells. Further, functionality was evaluated via pro-inflammatory cytokine production, antigen-specific cytotoxicity, and antibody-dependent cellular cytotoxicity (ADCC). Tumor necrosis factor-alpha and interferon-gamma production and secretion were examined in response to PMA/ionomycin or tumor cell lines via intracellular flow cytometry and electrochemiluminescence immunoassays and demonstrated consistent pro-inflammatory cytokine production. Notably, cryopreserved CAR-expressing iNK and iT cells maintained antigen-specific cytotoxicity against leukemia and lymphoma tumor cell lines in cytotoxicity assays, and hnCD16-expressing cells continued to show enhanced ADCC toward tumor cell lines when combined with tumor-targeting monoclonal antibodies. As there is no accelerated method established to model the shelf-life of cryopreserved cell therapies, periodic examination of stored drug product against release criteria provides the most thorough and accurate assessment of the feasibility of long-term storage. This study demonstrated that iNK and iT cells, including those that are genetically engineered, have a minimum shelf-life of 6 years when stored in the vapor phase of liquid nitrogen. No significant changes in viability, cell recovery, phenotype and tumor-killing potency were observed. Collectively, the data illustrate that engineered iNK and iT cell products can be mass produced, cryopreserved, and long-term stored for use as off-the-shelf cell-based cancer immunotherapy.

726. The Safety of Mesenchymal Stem Cells Engineered with Herpes Simplex Virus Thymidine Kinase

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Human mesenchymal stem cells (MSCs) are multipotent stem cells that have been intensively studied as therapeutic tools for a variety of disorders. To enhance the efficacy of MSCs, therapeutic genes are introduced using retroviral and lentiviral vectors. However, serious

adverse events (SAEs) such as tumorigenesis can be induced by insertional mutagenesis. We generated lentiviral vectors encoding the herpes simplex virus thymidine kinase (HSV-TK) gene and transduced MSCs to obtain MSC-TK. Transduction of lentiviral vectors encoding the HSV-TK did not alter the proliferation capacity, mesodermal differentiation potential, or surface antigenicity of MSCs. The MSC-TK cells were genetically stable, as shown by karyotyping and responded to ganciclovir. MSC-TK cells were not tumorigenic in immune compromised mice. The results indicate that MSC-TK cells appear to be pre-clinically safe for therapeutic use. We propose that genetic modification with HSV-TK makes allogeneic MSC-based ex vivo therapy safer by eliminating transplanted cells during SAEs such as uncontrolled cell proliferation.

727. Resolving the Heterogeneity and Dynamic Changes of Hematopoietic Stem-Progenitor Cells in Gene Therapy Drug Products

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Human CD34+ hematopoietic stem/progenitor cells (HSPC) are key components of *ex vivo* lentiviral gene therapy (GT) drug products (DP) for the treatment of systemic monogenic disorders. Despite its longstanding clinical application, little is known about the impact of *ex vivo* manipulation and expansion on the functionally heterogeneous pool of CD34+ HSPC. The surface markers commonly used for assessing CD34+ cells composition do not reflect accurately the dynamic changes occurring upon therapeutic modification; consequently, a full understanding of the composition of the DP prior to infusion into patients has remained virtually inaccessible until now. To overcome these issues, we combined multiparametric flow cytometry and next generation single-cell (sc) technologies for a systematic characterization of the main sources of human HSPC and their fates upon genetic engineering. We first investigated the transcriptional landscapes of CD34+ cells obtained from healthy donor derived bone marrow (BM), umbilical cord blood (CB), single- or double-mobilized peripheral blood (G-CSF and G-CSF+Plerixafor MPB, respectively), unveiling at high resolution (60,900 sc) previously unappreciated cell source-specific differential gene expression patterns and cell cycle states. We focused on G-CSF+Plerixafor MPB as the most clinically relevant source, and FACS-sorted and individually cultured 7 classes of HSPC subsets, capturing for the first time at the single cell level (100,578 sc) the transcriptional response to cytokine cocktail stimulation of individual progenitors. Using a combination of whole transcriptome sequencing (RNA-seq), droplet digital (dd-)PCR and flow cytometry RNA detection (PRIMEFLOW) we are now validating at the bulk CD34+ and sorted HSPC level, novel candidate molecular markers which would allow detecting and reliably quantifying the content of long-term hematopoietic stem cells that are retained in the DP at the end of the genetic engineering process. In addition, we sought to characterize and cross-compare the dynamics of CD34+ cells that were genetically modified and expanded *in vitro* in different culture conditions. By the mapping of single cell states of bulk HSPC and of FACS-sorted hematopoietic stem cells (HSC) (total of 191,980 sc) over 7 days of *in vitro* expansion, we unveiled potential early effects of the HSC-agonist UM171 on CD34+ cells priming and differentiation. Notably, we are now combining these single cell data with the molecular

profiling of viral integration sites to assess how the culture conditions might affect chromatin accessibility to viral insertion sites selection. We are also currently expanding the application of this profiling to the analysis of CD34+ single cell states from bone marrow aspirates of gene therapy patients before and after treatment. This approach provides a view of the dynamics of HSPC composition in humans upon autologous transplantation. To our knowledge, this analytical platform represents the most comprehensive transcriptional characterization of CD34+ cells upon genetic engineering with potentially broad application spanning manufacturing, pre-clinical and clinical development.

728. Induced Nephron Progenitor Cells for Cell Therapy of Ischemia/Reperfusion Kidney Injury

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Few therapies are currently available for acute kidney injury, and its association with morbidity and mortality urgently requires the development of regenerative treatments. In the mammalian embryonic kidney, nephron progenitor cells give rise to the nephrons, the basic functional unit of the kidney. Methods for the generation of nephron progenitor cells are crucial to the success of kidney regenerative strategies. As previously described, HK2 cells were reprogrammed to a nephron progenitor-like state using an integrated *piggyBac* transposon to express three key reprogramming transcription factors (*SNAI2*, *EYA1*, and *SIX1*). The doxycycline-inducible reprogramming cassette includes mCherry to monitor reprogramming effectiveness. Reprogramming of the HK2 cells was induced with doxycycline and valproic acid. After two days, the valproic acid was withdrawn. On day five, the cells were transitioned to a nephron progenitor cell-specific media, NPSR. Reprogramming was monitored via mCherry expression. On day eight, cells were lifted with accutase, washed, and resuspended in sterile buffer for injection. Either 1x10⁶ induced nephron progenitors or saline buffer was administered via intraperitoneal injection to six-week-old immunocompromised NSG mice 24 h following unilateral nephrectomy and ischemia/reperfusion clamping injury. Blood and urine were sampled at 24 h, 72 h, and two weeks post-surgery, prior to sacrifice at two weeks. The kidney markers blood urea nitrogen and serum creatinine were used to confirm kidney injury. Animals treated with induced nephron progenitors showed a reduction in blood urea nitrogen and serum creatinine levels between the 24 h and 72 h time points that did not reach statistical significance. Histologic and additional kidney marker studies are ongoing. In conclusion, induced

nephron progenitors injected following the induction of kidney injury may show promise as a method for improving biomarkers associated with AKI. However, further study is needed.

729. Systematic Conversion of Natural Receptors into Modular Synthetic Biosensors for Programming Cell Therapies

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Cell-based therapies represent an exciting frontier in design-driven medicine, leveraging the natural capabilities of cells to sense, process information, and produce and secrete therapeutic molecules in situ. Synthetic receptor systems enable engineered mammalian cell-based therapies to sense physiological cues and produce therapeutic responses. There now exist many examples of synthetic receptor systems that can sense surface-bound and soluble extracellular targets and signal through either natural signaling pathways or synthetic gene circuits. Synthetic sensors have the advantage of minimally disturbing or being regulated by native cellular processes, yet it remains laborious to generate new synthetic receptors for soluble ligands of interest. Although natural receptors exist for many soluble ligands, no systematic strategy has been developed to convert natural receptors into synthetic receptors that signal orthogonally from native pathways. Towards addressing this goal, we investigated how natural receptor domains and their corresponding biophysical mechanisms can be leveraged and incorporated into a synthetic receptor architecture. We employed the Modular Extracellular Sensor Architecture (MESA), a synthetic receptor system that signals via proteolytic release of a transcription factor upon receptor dimerization. This signaling mechanism enables customized transcriptional output upon detection of the target ligand. We systematically characterized surface expression and signaling performance for MESA receptors derived from three different types of human cytokine receptors. This process generated multiple novel, high performing synthetic cytokine receptors. We also identify mechanisms that render this conversion challenging or infeasible. We propose design rules for extending this engineering approach to other natural receptor systems. Finally, we validate these novel receptors by building therapeutic programs for sensing and responding to soluble cues in the tumor microenvironment. Ultimately, these reagents and insights will benefit the field of engineered cell-based therapies.

730. Ex-Vivo Tunability of the Ratio of Helper (CD4+) T-Cells to Cytotoxic (CD8+) T-Cells Expanded by Dissolvable Hydrogel Microspheres Conjugated with Specific Costimulatory Antibody Clones

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Introduction: Adoptive Cell Therapies (ACT) are becoming a vital therapeutic tool for cancer treatment. With five CAR T-cell therapies already on the market, and several more in the pipeline, robust *ex-vivo* T-cell expansions are a crucial developmental steppingstone towards

successful commercialization of these immunotherapies. Although there are several products that have demonstrated effective T-cell activation and expansion, a consensus has yet to be reached on an ideal phenotype/ratio of helper (CD4⁺) T-cells: cytotoxic (CD8⁺) T-cells necessary for basic research and successful applications. Herein, we demonstrate the tunability of the CD4⁺:CD8⁺ ratio via the use of a dissolvable alginate particle (Cloudz™) capable of bi-valent binding to the T-cell receptor and the crucial costimulatory receptor, CD28. While keeping the anti-Hu CD3 antibody constant, different Cloudz prototypes were functionalized with various clones of the anti-Hu CD28 antibody and evaluated for their ability to expand CD3⁺ cells in G-Rex and T-flask cell culture protocols. At the end of the culture period, specific prototypes led to a CD4⁺ (60%) rich phenotype while other prototypes led to a CD8⁺ rich phenotype (94%), exhibiting the tunability of this ratio via the Cloudz platform. **Methods:** Cloudz prototypes 1, 2, and 3 were developed via simultaneous conjugation of anti-Hu CD3 and three different clones of anti-Hu CD28 respectively. The prototypes were cultured with CD3⁺ cells in an expansion protocol combining 24 well plates and T-25 flasks (protocol 1) and alternatively in a low-touch G-Rex6M protocol (protocol 2). In addition, conditions were compared to two commercially available kits, a competitor's kit and a Cloudz T-Cell expansion kit. Experiments were conducted in serum-free, xeno-free Xcellerate medium with IL-7 and IL-15 and allowed to run for 9 days. Flow cytometry analysis was used to assess T-cell activation, expansion, and CD4⁺:CD8⁺ ratio. **Results:** In protocol 1, when looking at the Day 2 T-cell activation levels (CD25⁺CD69⁺ co-expression) of the different Cloudz, prototypes 1 and 2 resulted in similar CD25⁺CD69⁺ co-expression, while prototype 3 demonstrated a significantly lower co-expression level. Additionally, when assessing the CD4⁺:CD8⁺ ratio after 9 days of culture (Fig. 1A), prototype 1 demonstrated a significantly higher CD4⁺ T-cell % (~60%), while prototype 3 favored CD8⁺ T-cells (~94%). When prototype 1 was selected for further testing and dosed at varying amounts with CD3⁺ cells, a clear positive correlation between the Cloudz dose and the CD4⁺:CD8⁺ ratio was observed. The highest dose (1X) resulted in the highest CD4⁺:CD8⁺ ratio (~60%), while the lowest dose (1/4X) resulted in the lowest CD4⁺:CD8⁺ ratio (~45%). Furthermore, prototype 1 displayed its capacity for large scale culture when cultured in protocol 2. After 9 days with the 1X dose, prototype 1 resulted in 50-60X expansion of CD3⁺ cells (Fig. 1B), while maintaining its higher CD4⁺:CD8⁺ ratio. **Conclusions:** The antibody clone used to activate the CD28 receptor on CD3⁺ cells can have a significant impact on the ratio of expanded CD4⁺ and CD8⁺ T-cells. By conjugating multiple antibody clones to the dissolvable hydrogel microspheres (Cloudz) a useful reagent is prepared for research and clinical applications where control over the CD4⁺:CD8⁺ ratio of dosed cells is important.

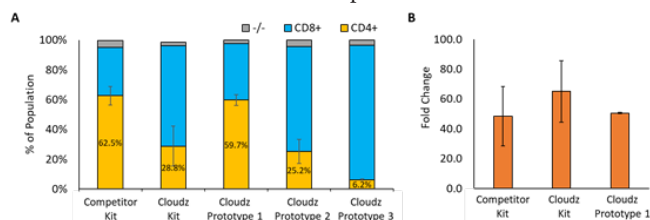


Figure 1: Post-Cell Culture Parameters Assessed (A) Comparison of the CD4⁺/CD8⁺ Ratio Elicited by Different T-Cell Activators Tested in Protocol 1. (B) Comparison of the CD3⁺ Fold Expansion of Selected T-Cell Activators Cultured in Protocol 2.

731. Developing an iPSC-Based Therapeutic Approach to Reverse MPS IIIA Neurodegeneration

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Mucopolysaccharidoses (MPS) are a rare family of lysosomal storage disorders, which have a frequency of 4.5 in 100,000 live births. They are caused by genetic mutations which lead to the progressing accumulation of long-chain sugars, ultimately determining a multi-organ dysfunction and both somatic and brain degeneration. MPS type IIIA, one of the most common types of MPSs, does not present major somatic symptoms, yet extended neurological impairment and behavioural abnormalities, which only manifest during the first years of life. As a consequence, diagnostic delay is quite frequent, with some cases of MPS III misdiagnosis as attention deficit/hyperactivity disorder. The combination of the latter together with a progressive cognitive decline makes MPS IIIA particularly challenging, to the point that no treatment is currently available. For this reason, we hypothesise that a brain-targeting therapy aiming at regenerating the CNS could be the only option to rescue such advanced neuronal damage. In this respect, we generated induced pluripotent stem cells from MPS IIIA compound heterozygous (R245H/c1284del11) patients dermal fibroblasts and a control cell line by a single transfection with oriP/EBNA-based episomal vectors. Following complete characterisation, we differentiated these into neural progenitor cells, in which the parental mutation was genetically corrected using lentiviral vectors (LVs) overexpressing the missing enzyme (SGSH) and luciferase. Interestingly, the untreated MPS IIIA NPCs seem to undergo loss of PAX6 when kept in culture, as opposed to our healthy NPCs; this is not only followed by acquisition of abnormal shape, but also by hampered neuronal formation and development. In addition, gene correction proved not to be effective in reversing this phenomenon. Finally, we have also performed a double intracranial injection of our genetically corrected NPCs into the striatum of our highly immunodeficient mouse model of the disease (MPS IIIA/NSG), and we are currently analysing both neuropathological and behavioural outcomes.

732. Functional Assessment of 3D Bio-Printed Primary Human and Stem Cell-Derived Hepatocytes

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Introduction: Liver disease resulting in liver failure is a global health concern that primarily affects the main parenchymal liver cells, the hepatocytes. The major challenges of developing a cell therapy for the treatment of liver failure are the insufficient availability of high-quality hepatocytes and the lack of a robust technology to deliver them to patients. Although primary human hepatocytes can be isolated from livers that do not qualify for transplantation, their donor specificity,

variable quality, and finite supply prevent the rapid development of large-scale allogeneic cell transplantation methodologies. Recent development in stem cell differentiation and generation of hepatocyte-like cells have shown promise for production of more metabolically functional cells that could be on par with primary human hepatocytes. In addition, 3D bioprinting shows exciting potential to provide a scaleable and consistent process for generating implantable grafts containing hepatocytes encapsulated in unique biomaterials for therapeutic applications. In this study, we tested the hypothesis that utilization of a novel microfluidic 3D bioprinting technology promotes maturation of human embryonic stem cell (hESC)-derived hepatocytes and benchmarked them against similarly printed primary human hepatocytes and a hepatic cell line. **Methods:** Using an established, patent-pending protocol, hESCs were differentiated in 2D towards hepatocyte-like cells (immature hepatoblasts, and mature zone I and zone III hepatocytes), and primary human hepatocytes and HepG2 cells were obtained from commercial vendors. All cell types were aggregated for 2-3 days in 3D, combined with alginate-based biomaterial and printed in core-shell structures using the Aspect RX1 bioprinter. Printed aggregates were compared *in vitro* and assayed for viability and protein secretion (e.g. albumin (ALB) and alpha-fetoprotein (AFP)). Hepatic gene expression and functional metabolism in culture will be tested in the future, in addition to *in vivo* implantation in immunodeficient animals to assess graft viability, function and durability. **Results:** All evaluated cell types displayed high levels of ALB secretion and viability following 3D printing in alginate-based biomaterial. As expected, primary human hepatocytes were fully mature and functional and displayed decreased AFP secretion. In contrast, HepG2 cells maintained high levels of fetal AFP secretion. Interestingly, 3D printed hESC-derived hepatocytes displayed increased levels of maturation relative to unprinted aggregates and HepG2 cells. This was evident by a reduction in AFP secretion and an increase in ALB secretion. Additional experiments are currently underway to determine *in vitro* and *in vivo* function of the 3D printed hESC-derived hepatocytes as compared to primary hepatocytes and HepG2 cells. **Summary:** Although hESC-derived hepatocytes do not completely replicate the high-level functionality of primary human hepatocytes, our data shows that by aggregation and printing in 3D, additional maturation cues are provided that increases their function and maturity. Further, high level viability and ease of printing makes hESC-derived hepatocytes highly desirable for use in solid organ cell therapy. Together, these qualities demonstrate that combining unique bioprinting approaches with hESC-derived hepatocytes has the potential to enable the next generation of liver cell-containing therapeutic implants.

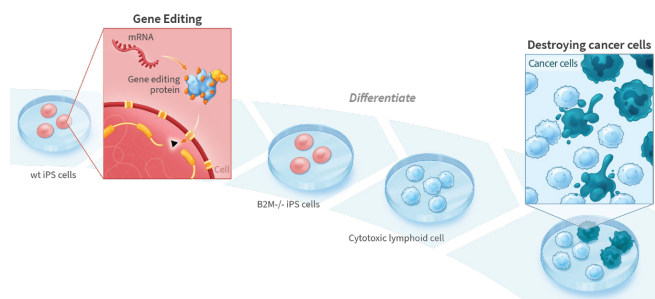
733. Cytotoxic Lymphocytes Derived from B2M-Knockout iPSCs Show Enhanced Expansion and Cytokine-Controlled Cytotoxicity In Vitro

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Cytotoxic lymphocytes, including T cells and NK cells, are being developed as allogeneic, “off-the-shelf”, cell therapies for the treatment of hematological and solid tumors. Allogenic lymphocyte therapies

face challenges, however, including limited expansion potential and limited *in vivo* persistence due to host immune rejection. To address these challenges, we developed an mRNA-reprogrammed iPSC line with a biallelic knockout of the beta-2 microglobulin (B2M) gene, a key component of MHC class I molecules, using an mRNA-encoded chromatin context-sensitive gene-editing endonuclease. We differentiated these B2M-knockout iPSCs using a novel, fully suspension process that replaces specialized micropatterned culture vessels with a spheroid culture step. The resulting lymphocytes were characterized for surface markers via flow cytometry and incubated with cancer cells to assess tumor cell engagement and cytotoxicity. Notably, we observed consistently higher yields of lymphocytes from the B2M-knockout iPSC line than from the parental wild-type iPSC line. Both wild-type and B2M-knockout lymphocytes cells killed 75-90% of K562 cells after 24 hours (effector to target (E:T) ratio of 5:1). Interestingly, cytotoxic lymphocytes derived from B2M-knockout iPSCs exhibited greater K562 cell killing with the addition of IL15 and IL2, while killing by wild-type cells was not controlled by these activating cytokines. Cancer cell killing activity was maintained through cryopreservation, albeit at a reduced level (15-40% reduction in activity). These results suggest that B2M-knockout iPSCs may serve as an ideal source of cytotoxic lymphocytes for the development of “off-the-shelf” allogeneic cell therapies for the treatment of cancer.



734. Anti-c-kit CAR-T Cells Enable HSC Engraftment in a Humanized Model of Stem Cell Transplant Conditioning

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Each year in the U.S. over 8,000 patients are treated with myeloablative conditioning therapy prior to hematopoietic stem cell (HSC) transplant. Conditioning regimens typically consist of high doses of genotoxic radiation or busulfan that can lead to life-threatening post-transplant complications. This has prompted the investigation of more targeted approaches to specifically deplete HSCs in the bone marrow (BM). To address this need, we have used the piggyBac® Gene Delivery System to develop CAR-T cells targeting human c-kit, which is highly expressed on HSCs, as well as myeloid malignancies such as acute myeloid leukemia. In addition to the CAR gene, the transposon encodes a selection gene for generation of an entirely pure CAR+ cell product, and an inducible, fast-acting safety switch gene to enable rapid clearance of the reactive CAR-T cell product prior to donor HSC transplant. Here

we demonstrate, for the first time in a humanized mouse model, the use of CAR-T cells as a preconditioning agent to enable HSC transplant. To first establish the ability of anti-c-kit CAR-T cells to target and kill human cells in the bone marrow, we administered the CAR-T cells to humanized mice and measured the number of human c-kit⁺ or CD34⁺CD90⁺ (HSPC) cells four days after treatment. Compared to mice receiving no CAR-T cells, mice conditioned with anti-c-kit CAR-T cells showed an 88% reduction in c-kit⁺ cells and a 97% reduction in HSPCs in the bone marrow. To determine if this level of conditioning was sufficient to enable engraftment of new human stem cells, we generated a humanized mouse model of autologous transplantation. To avoid complications related to HLA mismatch, we isolated T cells and CD34⁺ cells from the same donor. First, NSG mice were humanized with donor CD34⁺ cells, attaining an average peripheral blood human chimerism of 43% by 8 weeks post-transplant. Next, we conditioned the humanized mice for a second transplant using either anti-c-kit CAR-T cells or 40mg/kg busulfan as a control. Following safety-switch mediated clearance of the CAR-T cells, the second transplant was performed using lentiviral-transduced CD34⁺ cells expressing a high level of GFP to distinguish the newly transplanted cells from those unmarked human cells previously engrafted. We measured modified and unmodified human chimerism in the blood and BM at various times after the second transplant. As validation of our model, mice receiving busulfan were effectively repopulated by cells from the second transplant—more than 90% of the human graft in the bone marrow twelve weeks post-transplant was comprised of GFP⁺ cells, establishing the high level of gene marking and engraftment capacity of the modified CD34⁺ cells used for the second transplant. In mice conditioned with CAR-T cells we observed a robust population of GFP⁺ cells arising from the second transplant, making up approximately 40% (range 25%-70%) of the total human graft in the BM and demonstrating the ability of our CAR-T cells to clear space in the human stem cell niche. Importantly, two weeks after conditioning, we observed significant differences in blood cell lineage subsets between groups. There was a marked reduction in CD33⁺ myeloid cells in the blood of mice conditioned with busulfan, compared to CAR-T cells alone, suggesting the latter may more specifically target BM resident stem cells, while sparing progenitors, resulting in less toxic, safer levels of conditioning. Our data indicate that anti-c-kit CAR-T cells can be used as an alternative and less toxic conditioning regimen to facilitate engraftment of autologous HSCs. Future studies aim to further develop our humanized transplant model to assess their use in an allogeneic HSC transplant setting.

735. Evaluation of CAR/CXCR5-T Cell Immunotherapy in Humanized HIV-Infected DRAGA Mice

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It is estimated that 37.7 million people are living with HIV worldwide. To date, no effective treatment eradicates HIV infections. Although antiretroviral therapy has been used to treat HIV infections, it is not sufficient to eradicate the HIV reservoirs. Previous studies have reported that B-cell follicles in the secondary lymphoid tissues are the preferences of HIV replications. Chimeric antigen receptor T (CAR T) cells that target HIV-producing cells are being developed as a potential means to achieve durable remission of HIV infections. We have developed HIV-specific CAR T cells that target follicular reservoirs of HIV via the expression of the follicular homing receptor (CXCR5). Our previous work revealed that the infusion of CAR/CXCR5-T cells led to CAR/CXCR5 T cell accumulation in lymphoid follicles and was associated with the reduction of SIV viral loads in the SIV-infected rhesus macaque model of HIV. In this study, we evaluated the use of humanized HIV-infected DRAGA mice to evaluate the efficacy of CAR/CXCR5 T cell immunotherapy. We hypothesized that CAR/CXCR5-T cell immunotherapy in humanized HIV-infected DRAGA mice would lead to reductions in HIV viral loads and preserve human CD4 T cells. To address this, fourteen female DRAGA mice were irradiated and infused with the same donor of human hematopoietic stem cells (HSCs). The fourteen mice were divided into three groups: CAR/CXCR5-T cell production group (no HIV infection; n=2), HIV-infected group (controls; n=6), and HIV-CAR/CXCR5 group (treated cases; n=6). Prior to HIV-1 infection, all mice were assessed for the level of human T cell reconstitution. We found that DRAGA mice were successfully reconstituted with human hematopoietic cells, identified by human CD45⁺ staining. These included human CD45⁺CD3⁺ cells (29.9±8.51% of the lymphocytes), human CD45⁺CD3⁺CD4⁺ cells (24.9±6.7% of the lymphocytes), and human CD45⁺CD3⁺CD8⁺ cells (2.69±1.33% of the lymphocytes). Twelve mice (controls and treated) were subcutaneously injected with Depot Medroxyprogesterone Acetate (DMPA) 7 days before being exposed to 10,000 TCID₅₀ of HIV-1 BaL through intravaginal inoculation. HIV-1 viral loads and CD4 T cells levels were monitored biweekly. Meanwhile, the spleens of the two mice not infected with HIV were used to produce CAR/CXCR5-T cells. Co-expression of the CAR and CXCR5 was 71.1 % on the transduced cells. Six mice were intravenously infused with 2.0 x 10⁵ cells/gram of the transduced cell product through the tail vein. The peak time of plasma HIV-1 viral load varied among the mice (ranging from 2 to 6 weeks post-infection), and the average concentration at the peak was 1.43 x 10⁷ copies/ml. Preliminary findings early after infusion show no difference in HIV viral loads and CD4⁺ T cell counts

between the treated cases and controls. Thus, this exploratory study is yielding important insights into CAR/CXCR5 cell immunotherapy in humanized HIV-infected DRAGA mice.

736. A Phenotype Lock to Enhance the Safety and Efficacy of Treg Cell Therapies

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Regulatory T cells (Tregs) are potent and broad spectrum modulators of the immune response. Thus, Treg cell therapy has potential to induce immune tolerance in an array of inflammatory disorders from transplantation to autoimmune disease. The transcription factor, FOXP3, is considered the master regulator of Tregs; directly promoting expression of Treg functional markers such as CD25 and CTLA-4 and repressing expression of pro-inflammatory cytokines such as IL-2. A significant safety concern in Treg cell therapies is the stability of FOXP3 expression; loss of FOXP3 from Treg results in cells acquiring a pathogenic phenotype that could exacerbate inflammatory conditions. This is an even greater concern in antigen-specific Treg cell therapies. Here we show that transduction of bona fide Tregs with the FOXP3 gene drives high expression of FOXP3 but does not impact cell activation potential, proliferation or survival. Importantly, we show that under conditions that can drive loss of FOXP3 *in vitro* and *in vivo*, constitutive expression of FOXP3 maintains Treg phenotype and function. Moreover, we demonstrate that exogenous FOXP3 expression in CD3+CD25- T cells, one of the common contaminants of Treg drug products, reduces inflammatory pathogenicity of these cells. In addition to safety features offered by exogenous FOXP3 technology, antigen-specific suppressive function of engineered CAR-Tregs can be improved by the enhanced expression of FOXP3. Overall, we show that engineering bona fide Tregs to constitutively express FOXP3 offers a safety mechanism to “lock in” a Treg phenotype, providing a significant benefit for clinical application of antigen-specific Treg products.

737. A One-Time Treatment to Continuously and Permanently Deliver Lysosomal Enzymes to the CNS

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Human neural stem cells (hNSCs) from fetal cortex are highly migratory, especially on myelinated fibers. As they migrate, they differentiate into neurons and glia and seamlessly and stably integrate into the CNS tissue. The hNSCs will be engineered to overexpress and secrete a specific lysosomal enzyme by a lentiviral vector *ex vivo*. The resulting cells will be deposited bilaterally in the major white matter tracts across corona radiata, internal capsule, corpus callosum, and cerebellar tracts

of the brain to achieve maximum cell spreading with a minimal set of cannula penetrations. The cells will migrate to all corners of the CNS where they will provide not only their innate neuroregenerative stimuli but also pump out the missing enzyme for the life of the patient. With temporary immunosuppression, each engineered hNSC will be an allogeneic cell line capable of treating hundreds of thousands of patients. As a proof-of-principle demonstration, a pilot stable hNSC line from a MPS IIIA iPSC has been engineered to overexpress and secrete SGSH, the enzyme missing in MPS IIIA, by *ex vivo* gene therapy with a replication-defective lentivirus. The genetically engineered hNSCs were then administered directly into normal healthy adult dog brain parenchyma by intracerebral infusion of the cell suspension, using clinically intended stereotaxic method. Here we describe the surgical procedure and the resulting distribution of cells in the brain and SGSH levels in the CSF after 2-month survival under tacrolimus immunosuppression.

738. Novel Notch-Like Receptors with Increased Potency and Fidelity for Synthetic Gene Circuits in Primary Human T Cells

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Introduction: Notch-like receptors that bind to specific extracellular antigens and induce the expression of a second receptor allow for AND-gated cellular therapies. The first receptor or “prime receptor” binds to antigen A, causing the release of a transcription factor that induces the expression of a CAR specific to antigen B. In this way, the engineered cell only kills tumor cells in the presence of antigens A AND B. Previous iterations of this type of synthetic gene circuit relied on a transmembrane domain (TMD) and juxtamembrane domain (JMD) from the Notch family of human proteins. The TMD and JMD are connected in series to the transcription factor DNA-binding domain (DBD) and play an important role in the induction capacity as well as specificity of the gene circuit. In this project, we performed an agnostic search of ~20,000 TMD/JMD pairs to find novel prime receptor architectures that exceed the potency and fidelity of previous Notch-like circuits. **Methods:** TMD and JMD receptor domains comprising 20,000 unique pairs were sourced from the Uniprot protein database, codon optimized for mammalian expression, and ordered as an oligo library. The library was barcoded via PCR with degenerate codon primers and amplicon sequencing was performed to link unique prime receptors with specific barcodes. Primary human T cells were edited with this plasmid library using electroporation-based site specific gene insertion. Edited T cells were co-cultured with target tumor cell lines expressing antigen A, B, or neither and amplicon sequencing with barcode counting was performed to measure induction of CAR for each prime receptor. Top prime receptors were then assessed head to head for levels of CAR induction via flow cytometry and on-target/off-target killing with plate based cytotoxicity assays. **Results:** Pooled screening yielded >20 TMD/JMD pairs that outperformed the fidelity of the benchmark Notch-like prime receptor. These new prime receptor pairs induced CAR at varying levels, forming a toolbox for future genetic circuit tuning. Arrayed

screening revealed multiple new TMD/JMD pairs with higher fidelity and comparable killing to the benchmark receptor. A novel TMD/JMD pair was found with exceptional specificity and slightly decreased induction capacity. The addition of a downstream, constitutively expressed protein can boost T cell survival, but often decreases the expression of other proteins in the circuit. Multiple novel JMDs from the screen increased CAR induction and cytotoxicity capacity over benchmark levels in circuits with this additional constitutive protein.

Conclusions: These novel prime receptor TMD/JMD pairs allow for genetic circuit tuning in future cell therapy products. The high fidelity prime receptors will increase the safety of these products by limiting off target killing and could open the door to inducing payloads that may be toxic if delivered systemically but safe and potent if delivered locally. The high inducing prime receptors will allow for larger gene circuits with additional protein components, such as constitutive receptors that promote T cell survival and proliferation.

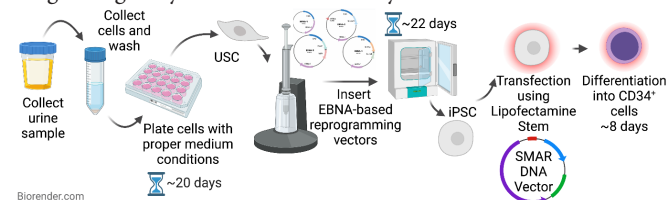
739. Utilizing Urinary-Derived iPSCs to Generate SMAR DNA Vector Modified CD34⁺ Cells

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Introduction Human induced pluripotent stem cells (iPSCs) have a promising outlook for the treatment of various diseases. They can repair functionally defective tissues and can, theoretically, differentiate into any human cell type. iPSCs are generated by reprogramming a differentiated cell, collected from a patient or healthy donor, using specific transcription factors. Non-invasive methods for the collection of these cells of origin, such as through urine samples, are very attractive as they are generally less stressful for donors, quicker, and more cost-effective compared to invasive methods, which could lead to more diverse collections of HLA types within iPSC banks. Genome integrating technologies are frequently used for stem cell gene therapies as they provide stable transgene expression in mitotically active and differentiating cells. One adverse consequence of using randomly integrating systems is their risk of genotoxicity. We intended to avoid this risk while maintaining the same efficacy as these systems by developing a non-integrating stably maintained DNA vector system. In this work, we aimed to develop a process for generating CD34⁺ cells from urinary-derived human iPSCs stably modified with our non-integrating SMAR DNA vectors, which could essentially be used to treat monogenic blood diseases. **Results** Urinary stem cells were isolated from healthy donor urine samples and subsequently reprogrammed into iPSCs using EBNA-based reprogramming vectors. These iPSCs could differentiate into cells from the three germ layers, and express alkaline phosphatase as well as the pluripotent stem cell markers Lin28, Tra160, Oct4, and Nanog. We stably modified these urinary-derived iPSCs using our non-integrating SMAR DNA vectors, and transgene expression persisted after trilineage differentiation. We next targeted iPSC differentiation towards hematopoietic stem/progenitor cells (HSPCs), which is a useful cell population for treating various blood diseases. FACS analysis confirmed we could generate HSPC-like cells (CD45⁺ CD43⁺ CD34⁺ CD38⁻ CD90⁺ cells) from these urinary-derived iPSCs; further characterization is currently ongoing. **Conclusion** With this work, we have taken steps towards a potential

off-the-shelf iPSC-derived therapy for monogenic blood diseases, as well as an intermediate step in the development of T-cell or NK-cell immunotherapies. By using our non-integrating DNA vector system, this therapy may possess safety benefits over cell therapies using genome integrating technologies. We have shown that urinary-derived cells are a good cell source for reprogramming, and we have added to the growing body of research on urinary-derived iPSCs.



740. Superior Expansion and Cytotoxicity of Human Primary NK and CAR-NK Cells Using Feeder Cell or Non-Feeder Cell Expansion Systems

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Clinical success of chimeric antigen receptor (CAR) T cell immunotherapy requires the engineering of autologous T cells, which limits the broader implementation of CAR cell therapy. The development of allogeneic and universal cell products will significantly broaden their application and reduce costs. Allogeneic natural killer (NK) cells can be used for universal CAR immunotherapy. Here, we develop an alternative approach for the rapid expansion of primary NK and CAR-NK cells with superior expansion capability and *in vivo* cytotoxicity from various sources (including peripheral blood, cord blood, and tumor tissue). We apply a human B-lymphoblastoid cell-line 721.221 (hereinafter, 221)-based artificial feeder cell system with membrane-bound interleukin 21 (mIL-21) to propagate NK and CAR-NK cells. The expansion capability, purity, and cytotoxicity of NK cells expanded with 221-mIL-21 feeder cells are superior to that of conventional K562-mIL-21 feeder cells. RNA sequencing (RNA-seq) data show that 221-mIL-21 feeder cell-expanded NK cells display a less differentiated, non-exhausted, limited fratricidal, memory-like phenotype correlated with enriched metabolic pathways, which explains underlying mechanisms. Thus, “off-the-shelf” NK and CAR-NK cells with superior functionalities and expansion using a genetically modified 221-mIL-21 feeder cell expansion system will greatly support clinical use of NK immunotherapy.

741. Generation of Human Myeloid Derived Suppressor Cells from Induced Pluripotent Stem Cells (iPSC) for Graft versus Host Disease Therapy

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Allogeneic hematopoietic stem cells transplantation (aHSCT) is a widely used treatment for hematological disorders. Graft versus host

disease (GVHD), a donor anti-host cell tissue destructive response, is a life-threatening aHSCCT complication. Front line pharmaceutical treatment is not uniformly effective and has toxic side-effects. Myeloid derived suppressor cells (MDSC) are a heterogenous population of immature myeloid cells which are immunosuppressive. Their potential in GVHD therapy has been proven in different rodent models. However, a high MDSC to T cell ratio and multiple doses of MDSC are needed for GVHD therapy. But in vitro generation of MDSC limits its clinical usage by low yield and the need for personalized patient products. To remove the barriers that limit its application, we developed a method to generate MDSC in vitro from human iPSC derived CD34+ cells. We achieved a large-scale of cell expansion using OP9-DLL4 mouse stromal cells, cytokines to support CD34 differentiation followed those for myeloid lineage. In 19 days, 205-1085 iMDSC were derived from one CD34 of which 98% of iPSC derived MDSC (iMDSC) were CD45+CD33+, typical for a myeloid lineage phenotype. As with peripheral blood (PB) MDSC human iMDSC generated in our culture system are composed of two major populations: monocytic MDSC (51% CD45+CD33+ CD14+) and granulocytic MDSC (11% CD45+CD33+CD66b+). In contrast, PB MDSC expansion was < 5-fold. MDSC possess immunosuppressive function, key for a successful GVHD therapy. To investigate this property of iMDSC, we applied our iMDSC in a T cell proliferation assay and compared the potency of the immune suppressive effect to PB MDSC. iMDSC inhibited anti-CD3/28 bead driven proliferation of CD4 and CD8 T cells by 56% and 57%, respectively, at 1:2 (iMDSC:PBMC) while a correspondently lower % suppression was seen for CD4 (39%) and CD8 (30%) cells. Suppression was contact-dependent and associated with increased inhibitory receptor, resulting in decreased T effector cell proinflammatory cytokine expression. We next investigated the suppressive capability of monocytic CD14+ vs granulocytic CD14- iMDSC subsets. We found that the %proliferation of CD4 (57%) and CD8 (55%) T cells suppressed by CD14+ iMDSC compared favorably to CD14- iMDSC for CD4 (19%) and CD8 (9%) at the same ratio. Previous studies have shown that LPS and ATP which are released due to tissue injury in the GVHD environment will induce inflammasome activation that we reported subverted in vivo suppression of murine GVHD lethality by bone marrow derived MDSC. To our surprise, iMDSC still retained 95% of their suppressive function after the LPS and ATP induced inflammasome activation while PB MDSC lost 77% of their suppressive function. Since MDSC have been reported to be sensitive to freeze-thaw that will be important for off-the-shelf clinical application, we investigated the function of iMDSC from frozen stock and found that iMDSC remained 86% of their function after the freeze thaw cycle. In summary, we developed a method for generating MDSC from human iPSC on a large scale. These iMDSC had potent immunosuppressive function and were resistant to inflammasome and freeze-thaw induced function loss facilitating the use of iMDSC as a potential alternative to or adjunct for GVHD therapy.

Vector Product Engineering, Development or Manufacturing II

742. Structural Comparison of AAV9 Derivatives Produced on Two Platforms and Characterization of Their Empty Capsids

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The structural complexity of adeno associated virus-based therapeutics is influenced not only by the cell lines used to express the final product but also the purification schemes employed, with the potential to lead to significant batch to batch variability. Differences in both the macro and microscale structure may impact critical product attributes such as stability, safety, and efficacy. As a significant product related impurity, the levels of empty capsids will also need to be monitored and controlled. The structure of an rAAV9 produced using both insect and mammalian cell lines was examined in detail to determine if any differences influence the above-mentioned critical attributes. Additionally, a series of rAAV9 variants were analyzed and compared with their empty equivalents, to distinguish structural features that can be exploited to quantify and remove the empty species. A full analytical toolkit was employed to examine attributes including titer, transgene size, occupancy, VP ratio, surface charge, heterogeneity, aggregation state and activity. The capsid macroscopic structure remained similar between the two platforms, including the empty preparations. Subtle differences were detected between both sets of material using certain analytical approaches, where the sf9/BV material gave higher titers and % full values. Interestingly the empty capsids displayed a higher propensity to form sub- μm aggregates, but the cause of this is yet to be established. Based on the similarity of the empty and full variants other approaches outside charge differentiation should be exploited to enhance their separation.

743. The ELEVECTA® HEK293 Platform - An Innovative, Fully Stable and Helper Virus-Free AAV Production System

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Background and novelty: To address the growing demand for industrial scale AAV vector production, we have recently launched an innovative stable, helper-virus free AAV production platform called ELEVECTA. The ELEVECTA stable producer cell lines are the core of our newly developed platform and harbor all relevant components for AAV production stably integrated into the cellular genome. Addition of an inducing agent enables high-titer AAV production with a simplicity comparable to current monoclonal antibody production processes. Initially, our platform was based on immortalized human amniocytes (CAP cells). Here, we describe the successful transfer of the ELEVECTA system to the HEK293 cell substrate, a well-established cell line for the industrial scale production

of AAV-based gene therapy products by transient transfection. **Experimental approach:** Adherent HEK293 cells were adapted to suspension growth mode under serum-free and chemically-defined culture conditions. A clonal cell line was isolated from the resulting HEK293 suspension cells and served as base cell substrate for ELEVECTA cell line development. Stable integration of Rep as well as adenoviral helper genes under control of a Tet-inducible promoter system resulted in polyclonal ELEVECTA HEK293 alpha cells. Subsequently, these cells were used for the generation of a clonal alpha cell line. In a conclusive step, capsid and transgene elements were genetically integrated into the alpha cell genome to yield a proof-of-concept HEK293 producer cell line. **Results and discussion:** Adaptation of adherent HEK293 cells to serum-free suspension growth resulted in the generation of a clonal high-performance cell line showing growth to high cell densities and advantageous production characteristics in comparison to various competitor HEK293 cell lines in a fully transient setting. The first phase of ELEVECTA cell line development resulted in the generation of monoclonal HEK293 alpha cells. These cells produce AAV to high titers after transient transfection of packaging as well as transgene elements with excellent stability over 100 cell doublings. Finally, we were able to successfully generate highly productive monoclonal HEK293 producer cells, proving the feasibility of our ELEVECTA HEK293-based production system.

744. Large-Scale Production, Purification, and Concentration of Therapeutic Lentiviral Vectors Using Single-Use Supplies

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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 $2-6 \times 10^7$ TU/mL for a clinically relevant vector. 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.

745. Adaptation of Multi-Plasmid Transfection-Based AAV Production to High Density Cell Respirator Bioreactors Yields Functional Vector

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The multiplasmid transfection process for AAV production is a mainstay for gene therapy, providing a helper virus-free solution and easy mixing-and-matching of genes-of-interest and serotypes. When carried out in a stirred tank bioreactor (STR), oxygenation constrains cell density (mid- 10^6 cells/mL) and consequently limits volumetric productivity ($\sim 10^{13-14}$ vg/L). For high systemic doses of AAV (10^{16} vg), a massive 1000 L bioreactor run can be needed to generate the vector. While workable for very small trials, there simply is not enough STR bioreactor volume globally to meet even a fraction of the projected gene therapy demand. Intensification of the AAV production process is therefore a necessary step to support the continued growth of the gene therapy field. The high density cell respirator (HDCR) is a new generation of intensified bioreactors that supports mammalian cell expansion up to 10^8 cells/mL through a gas-media decoupled architecture. We sought to adapt the conventional multi-plasmid transfection process to the HDCR and validate the functionality of vector produced as a step towards intensified AAV production. HEK293 cells were seeded into an HDCR bioreactor at mid- 10^6 cells/mL and expanded over 4 days to $\sim 10^7$ cells/mL under perfusion with DMEM+10% FBS+1% PS. Prior to transfection, the bioreactor was purged with DMEM-only media. A PEI-based transfection cocktail was prepared in DMEM (3ug PEI:1ug DNA; 3ug DNA/ 10^6 cells; 1.3 pHelper+pAAV2RepCap: 1 pAAV-CBA-Luciferase), injected into the mixing loop, and circulated for 30 min to mix with cells before resumption of regular media perfusion. Cells were harvested from the HDCR bioreactor 72 hours post-transfection (4E13 vg/L in crude by qPCR) and AAV was purified by CsCl gradient. The functionality of purified AAV2-CBA-Luciferase was first validated *in vitro* by transduction of HeLa cells followed by luciferase imaging (+72 hours). Vector (10^{11} vg/mouse) or PBS was subsequently tail vein injected into mice (C57BL/6J (B6(Cg)-Tyr^{-2/J}); n=4 each). Luciferase activity was assessed at Day 21, 30, and 37 and was robust and uniform across vector-injected mice and background level in PBS-injected mice. This confirms that HDCR-produced AAV vector is functional *in vivo*. This proof-of-concept study establishes that multi-plasmid transfection-based AAV production can be adapted to high density cell culture to generate functional vector. With a volumetric productivity of mid- 10^{13} vg/L, the process is comparable to traditional processes and puts down a marker for subsequent improvements through optimization. While cell viability is well-supported in the HDCR, the uniformity

of the transfection needs to be improved to ensure all cells become productive AAV factories. Various strategies are being investigated to take full advantage of the higher cell density supported by the intensified bioreactor.

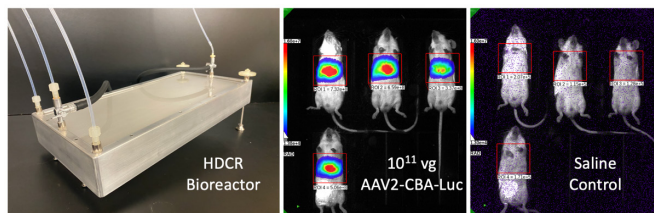


Figure 1: (left) High density cell respirator bioreactor. (middle) Luciferase imaging, 1 month post-injection with HDCR-produced vector and (right) PBS control.

746. AEX Enrichment of AAV5 Full Capsids with Dual Salt Elution Step, Linear, and Hybrid Elution Gradients

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The increasing use of AAV-based therapies for the treatment of rare and devastating genetic diseases demonstrates their potential to significantly benefit the lives of patients and their families. The AAV production process yields both full and empty capsids, with the latter representing a product-related impurity with no clinically demonstrated therapeutic benefit. Therefore, one of the primary downstream objectives of AAV manufacturing is the removal of these empty capsids to provide patients with as much potentially therapeutically active product as possible. Various strategies have been employed to address full capsid enrichment, from density gradient ultracentrifugation to chromatography. In this work, a commonly used beaded strong anion exchange (AEX) chromatography media was used to achieve empty and full capsid resolution for the AAV5 serotype. A salt screen of multiple mono- and divalent salts in gradient mode was performed to determine optimal peak resolution, full capsid isolation, aggregate reduction, and step yield. The evaluated salts were narrowed, and dual salt effects were evaluated on the same product and process attributes. A dual salt approach was selected for further gradient development in linear, step, and hybrid elution modes. Multiple elution strategies were evaluated to select the optimal method which provided robustness against potential charge heterogeneity from the AAV production process and variability in buffer preparation. Further baseline peak resolution was achieved by assessing gradient hold setpoints prior to product elution. With this work, it was determined that a dual salt gradient using a beaded strong AEX resin-based chromatography media provided superior yield, process robustness, and product quality over previously implemented monolith chromatography at manufacturing scale. The AAV5 strong AEX polishing step provides 3X enhancement of full capsids with near baseline separation of empty capsids, reduced HMW species formation due to the combination of salts selected, and an overall vector genome step yield of greater than 70%.

747. Upstream Manufacturing Process Development of Recombinant AAV Vectors for Ocular Applications

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The AAV serotypes AAV2, AAV5, and AAV8 are central in the field of ocular gene therapy. Luxturna is a gene therapy based on recombinant AAV2 (rAAV2) which was FDA-approved in 2017 for treatment of Leber congenital amaurosis type 2 (LCA2) retinal disease. Further, vectors based on rAAV5 are currently tested in clinical trials to treat LCA and rAAV8 and rAAV5 are investigated for treatment of retinitis pigmentosa. Here, we identified critical steps in the upstream manufacturing process development of rAAV vectors for ocular applications. AAV serotypes 2 and 8 were produced via dual or triple transfection method in the adherent cell line HEK293TN (System Biosciences). For viral vector harvest, cells were detached using trypsin (0.05 %), pelleted and lysed using 0.1 % T-X-100 final concentration. After downstream purification using affinity chromatography followed by IDX-centrifugation, we tested vector purity via silver stain of 1E10 vector genomes (VG) separated via SDS-PAGE. For both rAAV2 and rAAV8, bands of AAV capsid proteins VP1, VP2, and VP3 were observed in the expected size and ratio. However, an additional band between the VP2 and VP3 corresponding bands at ca. 64 kDa was observed for both rAAV2 and rAAV8. To test if this additional protein is of VP origin or a process related impurity, we performed western blot analysis using a VP1-3 detecting antibody (Progen). Primary antibody specific bands corresponding to VP1, VP2, and VP3 were observed, alongside the additional band at ca. 64 kDa. We concluded that the additional band is of VP origin and likely cleaved VP protein. We observed the cleaved VP protein in independent rAAV2 (n=5) and rAAV8 (n=7) productions using various transgene expression cassettes, therefore, we hypothesized that residual trypsin in the upstream process could cleave AAV VP proteins during harvest. This hypothesis was supported by comparative analysis of the amino acid sequence of VP1 from AAV2, AAV5 and AAV8, showing that there is a trypsin cleavage site (lysine containing motif at position 164 in VP1) in AAV2 and AAV8. However, this motif is absent in AAV5, and congruently, the additional AAV VP band was never observed in rAAV5 productions (n=6). Further, we incubated 2E12 VG of purified rAAV8 preparations (n=3) with 0.1 µg trypsin at 37 °C for 1 hour. Compared with untreated rAAV8 samples, the additional AAV VP band which was visualised by silver stain, intensified after trypsin treatment. To test our hypothesis, we produced rAAV2 and rAAV8 in the suspension cell line HEK293F (Thermo Fisher Scientific) and performed harvest and downstream purification as in adherent cells, but without the now redundant trypsin. After SDS-PAGE and silver stain, only bands corresponding to VP1, VP2 and VP3 were observed and no additional bands (n=7). These findings led us to move to an alternate process using suspension cells to avoid the use of trypsin during manufacturing of rAAV vectors for

our ocular platform. We concluded that the use of trypsin during the upstream process can influence AAV VP integrity and speculate VP1 cleavage could lead to decreased vector functionality due to loss of the VP1 nuclear localisation sequence. Ultimately, our results caution against the use of trypsin in rAAV vector manufacturing and emphasise the importance of quality control.

748. Lysis and Clarification Strategies for AAV Suspension Processes

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Our lead AAV gene therapy candidate, FLT180a, is planned to be manufactured for commercial use on an adherent large-scale manufacturing platform (the iCELLis® 500 system), which was chosen for optimal potency and quality of vectors. However, vectors to support Phase 3 and commercial production for subsequent programs, which will likely require higher vector doses, will be manufactured on a new suspension manufacturing platform; this suspension manufacturing platform will support scaling up of our manufacturing capacities for AAV gene therapy, to meet anticipated patient demand. The suspension platform was developed and optimized for quality comparable to, and yields improved over, that of the iCELLis® platform. Furthermore, the suspension manufacturing platform was developed with the advantage of being free of animal-derived components. The choice of cell lysis method is important to achieve high yields and minimize any negative impact on critical quality attributes of recombinant AAV vectors. We have explored freeze-thaw, mechanical, as well as chemical lysis methods with various surfactants and salts. The criteria used for selecting a successful lysis method were lysis time, scalability, and yield recovery. The advantages and disadvantages of each lysis method, as well as supportive recovery data for each method tested, will be presented. In addition, a one-stage clarification step following lysis was developed and optimized. This clarification step utilizes depth filtration and demonstrates high filter loading capacity and high AAV recovery of 70-90%. In summary, mechanical lysis combined with a one-stage clarification step achieved the best recovery yields compared with other lysis methods tested. This combination showed linear scalability from 2L to 50L of input harvest material, and potentially larger harvest volumes can be processed by linear scale-up of the filters. The optimal lysis and clarification strategy identified in this study can simplify further downstream processing using the suspension cell platform, while enabling increased vector yields and reduced cost per dose during manufacturing compared to other approaches. Furthermore, our study shows that the optimal lysis and clarification strategy can contribute to the ability of our animal derived component-free suspension manufacturing platform to achieve potency and quality attributes comparable to those achieved by our adherent iCellis® platform.

749. Investigating the Oxygen Control Before and After Fixed Bed in the iCELLis® Nano Bioreactor to Create a More Robust Scale-Down Model for the iCELLis® 500 Bioprocess

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A commercial-scale platform for manufacturing adeno-associated virus (AAV) gene therapies has been developed using the iCELLis® 500 system in conjunction with a proprietary plasmid system for our lead clinical program (FLT180a), which provides very high cell-specific productivity, vector potency, and reduced levels of replication-competent AAV. In the manufacturing process, it is important to carefully control oxygen supply to the cells to ensure optimal cell growth, maintenance, as well as product formation. In an iCELLis® 500 bioreactor, two probes are present to measure the dissolved oxygen percentage (DO%) before and after the fixed bed for providing information on how much oxygen is consumed by the cells within the fixed bed. The dissolved oxygen level is regulated according to the measurement after the fixed bed to guarantee that a sufficient oxygen level for the cells is reached inside the bed. For the iCELLis® nano scale-down model, a probe port for measuring the DO% after the fixed bed does not exist as a biomass probe is occupying the corresponding site. Oxygen supply is therefore regulated before the fixed bed. To mimic the large-scale process (iCELLis® 500) more closely in the iCELLis® nano, by regulating the oxygen supply after the fixed bed in the iCELLis® nano bioreactor, we developed a customized adapter for the biomass sensor port to apply a second DO% probe after the fixed bed. A primary comparison of the oxygen concentration before and after the fixed bed showed a large oxygen gradient within the iCELLis® nano fixed bed. This led to a substantial DO% reduction after the fixed bed when oxygen was regulated using the measurement before the bed. However, when DO% was controlled based on the oxygen measurement after the fixed bed, oxygen concentration was kept at a high level within the whole fixed bed, revealing an oxygen supply pattern comparable to the large scale iCELLis® 500. When comparing an iCELLis® nano unit controlled before fixed bed with an iCELLis® nano controlled after the fixed bed, no differences in cell growth were observed. Analysis of the respective rAAV material showed a slightly elevated vector genome (vg) and capsid (cap) titer for the unit controlled after the fixed bed. In addition, no impact on the impurity profile (plasmid-derived as well as host-cell derived impurities) was observed and no differences in potency were found between both tested sensor arrangements. In conclusion, a two-probe DO% measurement in the iCELLis® nano was established to regulate the oxygen supply after the fixed bed to further optimize the iCELLis® nano model toward a more accurate representation of the large-scale iCELLis® 500 process. This work further supports process robustness and validation on the small scale iCELLis® nano.

750. Characterization of Lentiviral Vector Quality Attributes Using Capillary Electrophoresis (CE) Platform Technology

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Cell and gene therapy products have the potential to offer curative approaches for many disease indications, and they are an area of intense focus for the biotechnology and biopharma industry. Over 89% of cell and gene therapies in development use viral vectors for gene delivery.⁽¹⁾ The lentivirus vector (LVV) is now one of the most commonly used viral vector platforms in pre-clinical development. It offers many advantages over other gene delivery technologies, including (but not limited to) stable integration of genetic material into the host genome and the ability to deliver larger genetic sequences, up to 9 kb.⁽¹⁾ While these benefits make LVV an attractive technology for cell and gene therapy applications, the inherent complexity of the lentiviral particle along with the scarcity of analytical methods to support engineering, manufacturing and quality assessment of cell therapy products create challenges for the clinical development of LVV-based therapeutics. Here, we address a platform-based approach that uses capillary electrophoresis (CE) technology to mitigate the technical difficulties of characterizing LVVs for the assessment of multiple quality attributes. Two critical attributes for LVV that need to be monitored are the p24-based physical titer and the genome integrity assessment. Sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) coupled with high-sensitivity laser-induced fluorescence (LIF) detection is commonly used as an advanced alternative to SDS-PAGE for protein characterization. Additionally, gel-based CGE technology with LIF detection can be used for size-based separation of nucleic acids. Here, we show how a single CE-based platform can be used for 1) the separation of viral proteins for protein profiling and simultaneous p24 quantification (physical titer) determination with demonstrated comparability to ELISA using SDS-CGE, and 2) genome integrity analysis by separation of the intact transgene of interest from impurities or truncated gene forms using gel-based CGE.

751. Optimization of HEK293 Suspension Platform for Improved rAAV Titers

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Recent clinical and non-clinical success with recombinant adeno-associated virus (rAAV) as an *in vivo* gene delivery vehicle, is leading to an increase in demand for high purity and efficacious viral vectors of various natural and engineered serotypes. The current rAAV production in human embryonic kidney (HEK) 293 cells uses helper plasmids instead of a helper virus and is referred as a helper virus free system. The triple plasmid transfection HEK293 platform is routinely used for rAAV production and utilizes AAV cis plasmid encoding the gene of interest flanked by the AAV2 inverted terminal repeats

(ITRs), AAV trans plasmid comprising capsid and replicase genes, and helper plasmids. However, commonly used HEK293 manufacturing platforms yield low titers and are not scalable. There is a need for next-generation HEK293 production platforms to increase rAAV titers and scalability while also reducing cost. In this study, we evaluated effect of various transfection and cell lysis conditions on engineered rAAV2 titers using two different suspension cell lines that have been clonally derived from a parental HEK293 cells to optimize rAAV production. We used different transgenes, serotypes and cell lysis methods to evaluate the effect of each variable during rAAV production. Our results demonstrated a significant increase in titer, up to 10-fold, based on choice of the cell line, transfection reagent and plasmid molar ratio used for rAAV production. Our results also revealed transgene size and gene product directly impacted rAAV titers. Overall, these results are applicable to serotypes that necessitate a HEK293 production system for improved potency and larger scale in support of human trials.

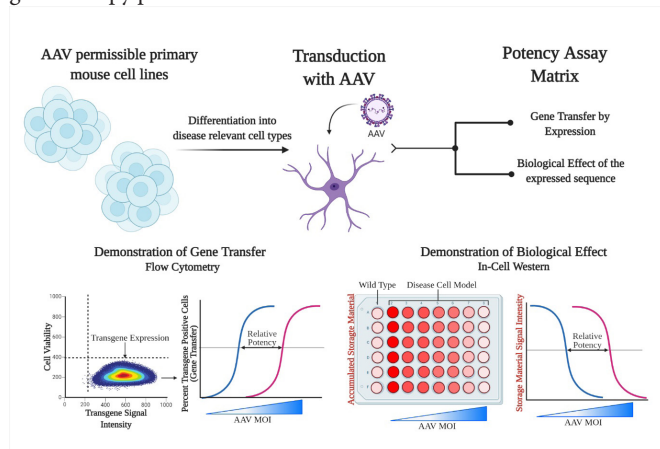
752. An In Vitro Potency Platform for AAV Gene Therapies

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For the clinical manufacture of gene therapies, regulatory agencies require a demonstration of drug substance potency. The assessment of potency must represent the product's mechanism of action which may be dependent upon multiple biological activities. Regulatory guidance suggests that gene therapy potency assays should include assessment of gene transfer, expression of the therapeutic target, and biological effect of the expressed transgene. Here, we describe a novel two-part potency assay matrix that assesses gene transfer by expression of the therapeutic transgene and its biological effect. This strategy required identifying a suitable cell line that was highly amenable to AAV transduction, recapitulated disease relevant phenotypes, and is expandable into GMP cell banks. To this end, we have developed a method for deriving primary cells from mouse models that meet all of these requirements. To demonstrate AAV vector gene transfer, we transduced cells with an AAV, identified the transgene expressing cells by immunocytochemistry, and quantified expressing cells by flow cytometry. Immunolabeling of the human transgene with a human specific antibody demonstrates transfer and expression of the transgene and increasing percentages of transgene expressing cells in a dose dependent manner. This strategy provides a common platform by which any number of gene therapies can be assessed, so long as there is an antibody available to detect the transgene and the promoter is compatible with the primary cell line. Demonstration of biological effect of the expressed sequence is dependent upon both the specific transgene and the cellular disease phenotype the expressed sequence is intended to correct. In the case of lysosomal diseases, a common phenotype is the accumulation of storage material in the lysosomes. We have derived cell lines from Cln3-, Cln6-, and Fabry mouse models that have well characterized histopathological storage material phenotypes that are recapitulated in our cell lines. Using in-cell westerns that target components of this storage material, we demonstrate the biological effect of our expressed transgene by the reduction or prevention of storage material accumulation in a dose dependent manner. We have successfully used these platforms to demonstrate potency of our AAV

gene therapy vectors for three separate lysosomal storage diseases, CLN3 Batten, CLN6 Batten, and Fabry disease, and these assays are currently being qualified for use as release assays for our manufactured gene therapy products.



753. Overcoming Barriers in Viral Vector Manufacturing: Small Molecule Targeting of Antiviral Defences

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Viral-based cell and gene therapies are scientifically promising for rare and complex diseases. However, these therapies' cell-culture based manufacturing is complex, 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. The cellular innate antiviral immune pathways that remain partially, if not fully, intact in producer cells are often overlooked and underappreciated. These create a bottleneck in cell culture-based manufacturing of viral vectors. These well-conserved pathways are triggered via the detection of foreign nucleic acid and/or molecular bi-products of viral replication/assembly. These result in a blunted viral vector yield in both infection and transfection-based manufacturing strategies. Due to the breadth of cellular innate antiviral defenses, even manufacturing cell lines genetically designed to attenuate the cellular antiviral defenses cannot overcome all major antiviral signaling hubs. Virica's Viral Sensitizers (VSEsTM) 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, VSEsTM can be combined and formulated for specific uses across various viral and cellular platforms. Using Virica's unique high-throughput methodology, a custom VSETM formulation was developed to produce 3rd generation Lentiviruses in adherent HEK293T cells. Treatment of HEK293T cells with an optimized VSETM

formulation resulted in a 5-7-fold enhancement compared to untreated cells. VSETM enhanced viral vector production was also demonstrated using serum-free suspension HEK293 cells to produce AAV-2 based vectors. Six of eight individual VSEsTM tested resulted in at least 2-fold improvement in AAV-2 functional titre. Ultimately, Virica's VSEsTM address the often-neglected cellular antiviral defenses to uniquely enhance viral vector manufacturing yields.

754. Evaluating Mass Photometry as a Platform Analytic Tool for Determining Relative Amount of Empty and Full Capsids in Inprocess and Purified AAV Samples

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Recombinant adeno-associated virus (rAAV) manufacturing processes produce heterogenous population of AAV capsids ranging from full capsids (containing approx. 4.5kb of DNA flanked by ITR) to empty capsids (without any DNA payload). The amount of empty capsids in the final formulated dose in gene therapy trials may negatively impact the efficacy and tolerability of the drug product. Advances in process analytics can support the identification and quantification of impurities and help in maintaining consistent quality of the manufactured product. Efficient purification process can be developed using data from rapid determination of the relative amount of empty and full capsids present in AAV sample. Optimized purification improves the product quality and safety profile. In this study, feasibility of mass photometry technology as a platform process analytic tool was evaluated for determining the relative abundance of empty and full capsids in purified AAV samples. Recombinant AAV produced from both Sf9 insect cells and HEK293 mammalian cells were included as part of the evaluation, and the assay robustness was evaluated by testing samples from different matrices. The mass photometry data was compared to analytical ultracentrifugation (AUC) as an orthogonal method and found to be in agreement within 1-8% of full capsid data from AUC. Overall, mass photometry provides valuable information and supports the process analytics with faster turnaround time, higher sensitivity and reduced material requirement compared to traditional methods such as AUC.

755. Development of a Scalable Platform Process for Manufacturing High Quality AAV9 Vector Using a Baculovirus Expression System in Insect Cells

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Neurogene was founded to bring life-changing genetic medicines to patients and families affected by rare, devastating neurological disorders by using Adeno-associated Virus (AAV) gene therapy vectors. While AAV based gene therapy is a very promising treatment modality, high quality, scalable, and economically viable AAV-based manufacturing is an important area of development. To address this challenge, Neurogene has developed a scalable AAV production platform using

suspension-based insect cells (rhabdovirus free, Sf-RVN) and a baculovirus expression vector system (BEVS). The process entails using two different recombinant baculovirus constructs, one containing *Rep* and *Cap9* genes and the second containing the transgene of medical importance. The process was first optimized for obtaining stable, high yielding, and infectious recombinant baculovirus (rBV) particles for both constructs. For AAV production, upstream process development efforts were aimed to increase volumetric productivity by modifying factors including the production medium, feed medium, infection viable cell density (ICD), time of infection/harvest, and multiplicity of infection (MOI). Once the process was optimized for AAV production at small scale, development was focused on ensuring a scalable process with expected yields in a single-use Stirred Tank Bioreactor (STR). The downstream process optimization efforts aimed for high recovery, while enriching the full/empty ratios and reducing any impurities during the affinity and AEX chromatography steps. To demonstrate that this process could serve as a platform process for multiple transgenes, three different transgenes were tested and scaled up to a 50L scale. These studies resulted in reproducible and scalable process yielding titers from 0.6- 1e12 vg/mL at harvest, with final process recovery of 25-35% across multiple products. Moreover, critical quality attributes, for the final bulk drug substance and drug product including strength, purity, and safety demonstrate that this platform process generates product with a high full/empty ratio, high infectivity, and low residual impurity levels. In conclusion, our baculovirus based AAV production process is a robust and scalable process with high yield and high product quality.

756. Quantification of Lentiviral Vectors Using Quantitative Real-Time PCR vs Digital Droplet PCR

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We are developing viral vectors for the treatment of a range of acquired and inherited respiratory disorders. We have previously described a minimal, self-inactivating, replication-defective recombinant simian immunodeficiency virus (rSIV) vector, pseudotyped with the F and HN glycoproteins from the murine form of human parainfluenza virus (known as Sendai virus). The rSIV.F/HN vector platform (Alton *et al*, 2017; PMID: 27852956) has high tropism for multiple cell types found within both the conducting airway epithelium and the gas-exchanging alveolar regions of the mouse and human lung. To facilitate clinical development, we routinely produce a large number (5E9 TU Lot per week) of highly purified (anion-exchange/TFF), high-concentration (target >1e9 TU/mL) research-grade preparations of rSIV.F/HN vectors. We typically use two assays to assess vector yields: (i) We use a viral particle (VP) assay (reported as VP/mL) derived from the concentration of viral RNA found within vector Lots which is evaluated using RT-qPCR. (ii) We use a Transducing Unit (TU) assay (reported as TU/mL) determined from the concentration of vector-derived genomic DNA within cells that have been transduced with dilutions of viral Lots which is evaluated using real-time qPCR. These assays are used to normalise vector dose between *in vivo* (non-clinical) studies. We have recently developed Droplet Digital (dd) PCR methods to assess both VP and TU values. The ddPCR methods have the distinct advantage of needing no ongoing standard curve validation. The ddPCR approaches

are highly specific, and have the ability to detect as little as 31 copies of the target DNA sequence in the assay matrix background. It is anticipated that employing ddPCR should improve both inter-assay and intra-assay variation, as well as detection range. To compare the methods, several different purified lentiviral vector Lots were sampled and analysed. A standardized dilution series of vector was used to transduce suspension-adapted Gibco™ Viral Production Cells. At 72 hours post-transduction, cellular DNA was extracted using a standard column-based approach (Qiagen 96 well DNeasy kit) and quantified for levels of both WPRE (vector target) and human CFTR (as a cellular reference gene) using both qPCR and ddPCR. In parallel, RNA was directly extracted from vector samples (using Qiagen RNeasy mini kit) and quantified for levels of WPRE (vector) using both RT-qPCR and RT-ddPCR. A paired, parametric t-Test of TU/mL values obtained by qPCR and ddPCR methods for the same samples showed no significant difference between titres ($P=0.007$). In contrast, The VP/mL values obtained from RT-qPCR analysis were consistently ~5-fold higher than reported by RT-ddPCR. We continue to investigate the reaction chemistry to further understand these observed differences. Historically our qPCR-based TU/mL method has had difficulty providing TU values of 1E6TU/mL or less, as many of the individual qPCR replicates had experimental values below the lower-limit of quantification. Our ddPCR-based TU/mL assay offers an extended assay range which could provide a solution for this issue. In summary, while we continue to investigate differences observed in our VP/mL assay by the qPCR and ddPCR approaches, under our assay conditions, both qPCR and ddPCR approaches generate equivalent TU/mL values and therefore both approaches are viable options for quantifying the most widely used dose-defining lentiviral vector QC parameter.

757. Modeling System Contributions from Chromatographic Workstations on Ion-Exchange Chromatography

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Chromatographic workstations can have a significant impact on column chromatography peak shape and retention time. Variations in system contributions can lead to misinterpretation during column validation, process transfer, and process scale-up, in which a different system flow path or a different chromatographic workstation altogether may be used. In this work, we developed two mathematical models that combine system contributions from commercial purification units (AKTA Avant 25 and AKTA Pilot 600R) with mechanistic ion-exchange column models. System contributions were added in the form of plug flow and continuous stir tank reactors. We provide system models for two differently scaled chromatographic workstations spanning a total system hold-up volume in the order of ~ 1mL to ~100mL. The applicability of these system models was demonstrated across columns of 2.5 mL to 127 mL column in both binding and nonbinding mode. The combined model can be used to simulate the impact of foreseeable system variations, saving resources.

758. Transient Transfection and Scale-Up of AAV Production from 1L to 500L in Bioreactors

Steven Wesel, Danielle Sexton, Mason Bonitz, Pooja Madan, Ashley Craddick, Adam Davis, Frank Agbogbo, David Dismuke

Process Development, Forge Biologics, Grove City, OH

In order to satisfy the promise of gene therapies that utilize recombinant adeno-associated virus (rAAV) vectors, robust commercial-ready processes are needed for rAAV manufacturing. Like most biological products, the production processes consist of an upstream process, a downstream process, and final formulation/fill. The major approaches used in the upstream process for the production rAAV include transient plasmid transfection of HEK293 cells, infection of HEK293 (or BHK21) cells with recombinant herpes simplex virus (rHSV), infection of *Spodoptera frugiperda* (Sf9) with baculovirus expression vectors, and use of stable producer cell lines. Transient plasmid transfection can be performed with adherent HEK293 cells in a variety of platforms, including using roller bottles, HyperStacks, or fixed-bed bioreactors. The production vessels for suspension cultures of HEK293 cells are typically either stainless steel or single-use bioreactors (SUBs). Transient transfection of HEK293 in suspension culture is a standard approach used at Forge Biologics to produce rAAV vectors. Forge Biologics has developed a robust upstream HEK293/transient transfection process for high productivity and product quality of rAAV vectors to overcome industry-wide challenge of scalable rAAV manufacturing. After optimizing the process at 1L and 5L flask level, the process was optimized in SUBs at the 1L and 5L level. The results from these experiments were used to scale the production to 40L, 50L and 500L SUBs. The representative harvest material was taken through standard clarification, including depth filtration, affinity chromatography, full particle enrichment (anion exchange or CsCl ultracentrifugation), and final formulation. The rAAV vectors produced from shake flasks and bioreactors at the different scales were compared for GOI titer by ddPCR, residual plasmid by ddPCR, residual host cell protein, purity by SDS-PAGE, genome integrity by alkaline gel electrophoresis, empty:full particle ratio, and potency. The results demonstrate the utility and scalability of the transient transfection platform at Forge to produce high quality rAAV vectors for a variety of serotypes and types of transgenes. An overview of Forge's transient transfection upstream processes and lessons learned from scaling from 1L to 500L will be discussed.

759. Downstream Process Development for AAV Manufacturing from 1L to 500L Batch Sizes

Corben Davis, Blake Gursky, Elijah Bodey, Bryant Yung, Michael Swenor, Ashley Craddick, Adam Davis, Frank Agbogbo, David Dismuke

Process Development, Forge Biologics, Grove City, OH

Recombinant adeno-associated virus (rAAV) vectors are one of the most promising technologies to deliver therapeutic transgenes for gene therapy. Nanoparticles of rAAV are composed of viral capsids but are devoid of viral genes; these vectors can traverse the cell membrane and deliver the recombinant DNA transgenes to the nucleus of the cell.

For optimal potency and safety, rAAV vectors must be highly purified from cellular and process contaminants during the downstream manufacturing steps. The crude bulk harvest material obtained following the upstream production process contains the rAAV vector in a milieu of cellular debris, including host proteins, host cell nucleic acids, membrane lipids, as well as empty rAAV capsids. In addition, any transfection reagents, endonucleases, detergents, and other process additives are also typically unwanted in the final product. These process contaminants are removed during downstream purification unit operations to yield a highly pure concentration of rAAV vector (the active ingredient) in a defined final formulation buffer. The purification process typically begins with the separation of cellular debris by centrifugation and/or filtration to clarify the rAAV vector going on to the chromatographic step. Chromatography begins with a capture step (typically using an affinity resin) followed by a polishing process to separate full capsids from empty capsids. The process parameters for the 1L harvest were determined and then scaled increasingly in larger batch sizes. Forge Biologics has developed a suite of downstream processes that can efficiently purify 1L to 500L of harvest material from clarification through to final formulated material. The rAAV purified from 1L to 500L batch sizes were compared for GOI titer by ddPCR, residual plasmid DNA by ddPCR, residual host cell DNA, residual host cell protein, purity by SDS-PAGE, genome integrity, empty:full particle ratio, potency, and endotoxin. The data from the development of platform downstream processes will be presented.

760. Platform Process Development for AAV Production

Frank Agbogbo, Steven Wesel, Corben Davis, Danielle Sexton, Mason Bonitz, Noah Weyrick, Blake Gursky, Elijah Bodey, Bryant Yung, Pooja Madan, Adam Davis, David Dismuke

Process Development, Forge Biologics, Grove City, OH

Recombinant adeno-associated viral (rAAV) vectors are one of the powerful tools in gene delivery for the treatment of human diseases. Several platforms are commonly used to produce rAAV, such as transient plasmid transfection of HEK293 cells, infection of HEK293 (or BHK-21) cells with recombinant herpes simplex virus (rHSV), infection of *Spodoptera frugiperda* (Sf9) with baculovirus expression vectors, and stable producer cell lines with induction of gene expression with doxycycline addition or Adenovirus infection. While each of these platforms can be used for rAAV production, optimization is needed for increased vector yield and high product quality. Forge Biologics has developed a transient transfection platform using the Ignition™ HEK293 suspension cells in single-use bioreactors (SUBs) for the manufacturing of rAAV vectors for research, clinical, and commercial use. The advantages of developing a platform process of this nature helps in decreasing time and effort required in process development and allows projects to move into cGMP production more rapidly. Data will be presented on work that has been executed at small-scale (1L), mid-scale (50L), and large-scale (500L) for a variety of rAAV serotypes. An overview of a process steps (seed train, cell growth and transfection in the bioreactor, lysis, clarification, chromatography, and final formulation will be shared. In addition, analytical data for rAAV vectors produced in our production platform will be presented. The

vector characterization includes a number of assays, including GOI titer by ddPCR, residual plasmid DNA by ddPCR, residual host cell DNA, residual host cell protein, purity by SDS-PAGE, genome integrity by alkaline gel electrophoresis, aggregation by DLS, empty:full particle ratio, potency, and endotoxin.

761. AAV Manufacturing Platforms Enabled by Predictive Modeling

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Adeno-associated virus (AAV) is a well known viral vector in clinical in commercial use. While AAV has received a significant share of attention in the gene therapy space, the process technologies to express this viral vector are still developing and maturing. Due to the complexity of AAV as a product, understanding and predicting the multivariate relationship of process parameters to yield and product quality is challenging. Currently, transient triple transfection is a common method for producing AAV. In this process, the transfection step is sensitive to multiple variables including the mass of plasmid DNA, the mass ratio of transfection reagent to the DNA, and the molar ratios of the plasmids. Design of experiments (DoE) can be used to understand and predict how these factors affect the productivity of a viral vector production process. This understanding and predictive power allows the application of this process as a manufacturing platform across multiple capsid serotypes, such as AAV2 and AAV8. In this study we conducted a multi-factor DoE focused on the transfection process design space, comparing how the transfection process parameters affect the process yield. Critically, this impact is understood not just an “one-factor-at-a-time” (OFAT) basis, but is understood throughout a representative region of the design space. The multifactorial approach, when applied to multiple unit operations in the transient triple transfection production process, drives understanding and predictive models for AAV manufacturing platforms.

762. A Novel Dual Plasmid Platform Provides a Scalable Transfection Process Yielding Improved Productivity and Packaging Compared to Triple and pDG Dual Plasmids

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Transient transfection of mammalian cells using plasmid DNA is a standard method to produce adeno-associated virus (AAV) vectors allowing for flexible and scalable manufacture. Typically, three plasmids are used to encode the necessary components to facilitate vector production, often referred to as triple transfection. One plasmid contains the inverted terminal repeats (ITRs) flanking an expression cassette with a gene of interest (GOI), another plasmid contains the

RepCap sequences *in trans* and a third plasmid contains the adenovirus helper genes required to facilitate replication. Over two decades ago, a dual plasmid system termed pDG was introduced, which added the RepCap and helper genes onto a single plasmid, demonstrating sequences could be combined resulting in comparable productivity to triple transfection with the obvious benefits of using two plasmids rather than three. We have developed novel dual plasmid designs, and found that one of the resulting dual plasmid transfection systems provides substantial increases in AAV vector genome (VG) productivity and calculated full capsids in crude lysate compared to triple transfection. These findings are highly reproducible across multiple distinct ITR constructs produced in 2L bioreactors, demonstrating up to 222% increase in VG productivity and up to 214% increase in calculated percent full vectors. The platform exhibits improved productivity across a variety of common serotypes. Furthermore, the dual plasmid platform is scalable from shake flasks to 2L, 50L and up to 2,000L bioreactors. Purified drug substance showed a consistent product quality profile, across a wide range of analytical methods and in line with triple transfected vectors, except for a substantial improvement in intact genomes packaged using the dual transfection system. The new dual plasmid design represents innovation in AAV manufacturing resulting in significant process gains while maintaining the flexibility of a transient transfection platform.

763. Fully-Integrated Digital PCR System for Robust and Consistent Viral Titer Quantification

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Adeno-associated virus (AAV) is a popular vector for use in gene and cell therapy research due to its high transduction efficiency, lack of pathogenicity and demonstrated efficacy and safety profile in several clinical trials and preclinical research. Recombinant AAV are produced in bulk quantities, and accurate quantification of the viral titer is essential in biopharma research for both production and tracking across the manufacturing process. Digital PCR (dPCR) has emerged as a leading technology for accurate and precise absolute quantification of nucleic acid targets. Researchers are using dPCR for robust viral titer quantification since it does not require a standard curve. Unlike other dPCR methods that rely on stochastic processes to generate micro-reactions by droplet formation, microfluidic array plate (MAP) technology facilitates automated sample distribution consistently into over 20,000 microchambers and utilizes >99% of the bulk reaction to minimize sample loss. Here we demonstrate how the increased robustness and consistency possible with MAP technology improves AAV viral titer quantification by performing comparison experiments using the Applied Biosystems™ QuantStudio Absolute QTM Digital PCR system, as well as a comparison performed using a droplet digital PCR instrument. We found MAP-based digital PCR performed highly efficient digitization of the sample into 99% ($\pm 0.03\%$ STDEV) of available microchambers compared to generation of 90% ($\pm 14.01\%$ STDEV) of anticipated droplets on a droplet-based dPCR system. Paired with MAP technology, the Applied Biosystems™ QuantStudio Absolute QTM Digital PCR system is a fully integrated,

single instrument digital PCR system which has a simple qPCR-like workflow and can generate data in under 90 minutes. Reduced hands-on-requirements, consistent reagent digitization and fast time to answer serve the need for rapid, highly accurate and reproducible viral titer quantification required in the recombinant AAV production workflow.

764. Changes to Culture pH and Dissolved Oxygen Can Enhance CAR T-Cell Generation and Differentiation

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Chimeric antigen receptor (CAR) T-cell therapy is growing clinically and commercially as a powerful new approach to treat cancer. Understanding how key culture conditions such as pH and dissolved oxygen (DO) affect CAR T-cell generation and function is important in developing better CAR-T manufacturing processes and CAR T-cell therapies for patients. We used the automated mini-bioreactor (AMBR) 15 platform to assess how differences in pH and DO affect CAR T-cell transduction, proliferation, and differentiation. We found that higher pH can significantly improve CAR T-cell transduction and proliferation, and also biases CAR T-cells away from an effector memory and towards a more central memory phenotype. Both high and low DO negatively affect CAR T-cell generation, with both hypoxic and hyperoxic conditions reducing T-cell transduction into CAR T-cells. Collectively, this data underscores how pH and DO can significantly affect CAR T-cell expansion and differentiation, and provides insight into the optimal culture conditions to enhance CAR T-cell yield and phenotype in clinical and commercial processes.

765. Adeno Associated Virus Adsorption on Different Surfaces Relevant to Production of Pre Clinical and Clinical Material

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Throughout the 40-year history of the gene therapy field, recombinant Adeno-Associated Virus (AAV) vector has emerged as a leading drug platform for delivering treatment to patients. During the AAV production process, capsids get exposed to and interact with various surface types including bioreactors, resins, tubing, and storage containers. These interactions potentially impact vector concentration due to surface adsorption. When evaluating drug product in both *in vitro* and *in vivo* studies, the concentration of the purified AAV vectors (typically 1E12-1E14 vg/mL) should be consistent. Maintaining vector concentration accuracy is critical for successful evaluation of pre-clinical and clinical-stage studies, particularly for lower doses where small changes in concentration have a larger impact on the performance of the drug. Therefore, it is of interest to investigate the effect of contact layer adsorption on AAV concentration over time. To understand the effects of material surface on concentrations for two widely used serotypes, AAV8 and AAV9, this study evaluated the extent of product loss on 10 commonly used contact materials: polypropylene, polystyrene, flint glass, borosilicate glass, crystal zenith, high-density polyethylene, polyethylene terephthalate glycol, polypropylene

copolymer, polycarbonate, and silicon. In addition, the study examined the effect of a non-ionic detergent, commonly used in process or in storage formulations, on adsorption. Samples and buffer controls for the two serotypes were stored in different materials in the presence and absence of the detergent. Sample optical density (OD) measurements were used to calculate the changes in total and in the percentage of empty capsids over time. Noticeable differences in adsorption were observed for different serotypes and different contact layers over time when normalized to the contact area. Additionally, serotype and contact surface interactions had an impact on the percentage of empty capsids in the solution, and polypropylene showed the largest influence. Inclusion of detergent had a variable effect depending on the surface and serotype. The information gained from this experiment will help determine materials and agents used throughout the AAV manufacturing process, in order to preserve accurate and consistent AAV concentration during pre-clinical and clinical studies.

766. Enhancement of rAAV Productivity Utilizing HDAC Inhibitors on Helper-Free HEK293 Suspension Cell Culture Process

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Adeno-associated virus (AAV) is a non-enveloped, non-pathogenic virus and is replication incompetent without helper virus. With its unique characteristics, recombinant AAV (rAAV) is now one of the most widely used *in-vivo* gene delivery vectors for clinical treatment, however, due to poor vector yield there is a growing concern with its increasing demand. In the helper-virus free rAAV production process, upstream process steps incorporate costly materials such as transfection reagents and plasmid DNAs. The purpose of this study was to look at variables in the manufacturing process that would improve productivity and reduce the cost of goods. In this study, a series of histone deacetylase (HDAC) inhibitors were evaluated for its impact on rAAV productivity, capsid protein titer and cellular cycle of host HEK293 cells in search of successful production protocols applicable to different serotypes (AAV2, AAV8 and AAV9), different HEK293 cell lines, and different transfection reagents (Figure 1 and Figure 2). Here we demonstrate the versatile application of a series of HDAC inhibitors (Valproic acid, sodium butyrate suberoyl bis-hydroxamic acid (SBHA), suberoylanilide hydroxamic acid (SAHA), FK228, and Trichostatin A (TSA) which offers a relatively simple and powerful method to significantly improve not only rAAV vg titers but also capsid titers up to log-scale and alternates cellular cycle to more preferable phase for rAAV production.

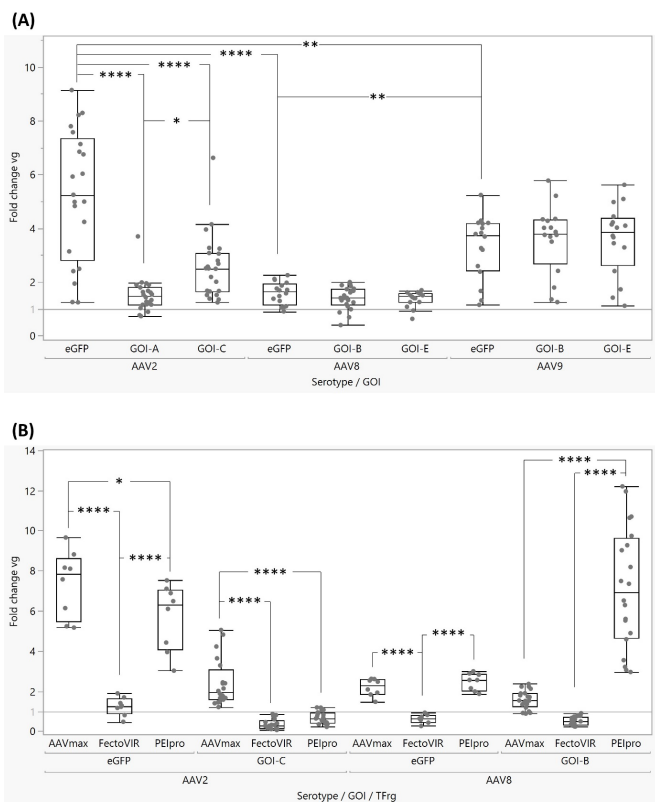


Figure 1

Figure 1. Addition of HDAC inhibitors significantly increased rAAV vg titers with serotype, GOI and transfection reagent specific manner. Plot (A) shows data on Serotype and Gene of Interest (GOI) specific upregulation of rAAV vg titers. Fold change vg titers from all the tested HDAC inhibitors and Enhancer using AAVmax transfection reagent was plotted over serotypes and GOIs. Box plot was generated from two to four different effective concentrations of each reagents. Plot (B) shows data for Transfection reagent specific upregulation of rAAV vg titers. Fold change vg titers from all the tested HDAC inhibitors and Enhancer was plotted over serotypes, GOIs and transfection reagents (TFrg).

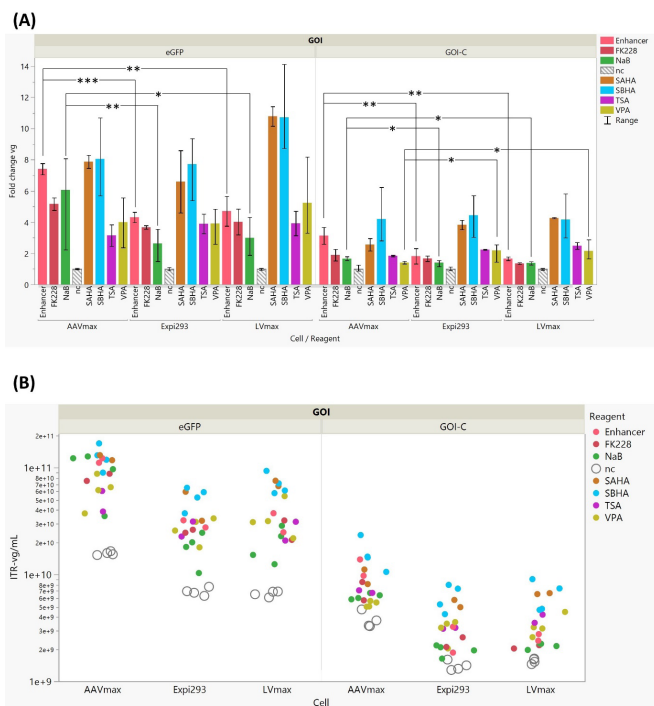


Figure 2

Figure 2. Addition of HDAC inhibitors significantly increased rAAV vg titers with cell line specific manner. Plot (A) shows data on Cell line specific enhancement of rAAV titers by HDAC inhibitor and Enhancer addition. Fold change vg titers was plotted over three HEK293 cell lines, reagents and two GOIs using AAVmax transfection reagent. Error bars are shown as mean \pm ranges obtained from two to four different effective concentrations of each reagent. Statistical analysis was performed for reagents with more than three data sets. Plot (B) shows data on rAAV vg titers compared between different HEK293 cell lines by HDAC inhibitor and Enhancer addition. Log scale rAAV vg titers were plotted over three cell lines, two GOIs and reagents (defined color in legend) using AAVmax transfection reagent. nc, negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (This work was supported by funding from Astellas Pharma, Inc.)

767. Introducing the Aura+ for Gene and Cell Therapy Aggregation, Particle Analysis, and ID

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Aura+ is the latest instrument designed specifically to detect, count, and characterize subvisible aggregates and extrinsic materials for product quality measurements in both gene and cell therapy applications. Aura+ outputs images, count, size, particle identification and morphological information with 100% sampling efficiency requiring as little as five microliters of sample volume through Backgrounded Membrane Imaging and Fluorescence Membrane Microscopy (FMM). FMM identifies aggregates as cellular, protein, or extrinsic so you can quickly know what is in your sample. Specifically, Aura+ has the capability to detect SYBR® Gold stain, which is used to detect the presence of DNA

in AAV aggregation to understand the role of leaky capsids in subvisible particle formation often leading to reduced AAV transduction efficiency and adverse patient responses.

768. High-Titer Herpes Simplex Virus Type 1-Based Vector Manufacturing Using Univercells Technologies' Production System

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Recombinant herpes simplex virus type-1 (HSV-1) has been widely investigated as oncolytic virotherapy for treating cancer. Conventional HSV-1 production could suffer from limited scale-up capability due to cell culture using cell factories or roller bottles. This study herein is based on the HSV-1 production with adherent Vero cells culturing on fixed bed bioreactors for clients to meet NMPA's and FDA's standards. OBiO has developed an upstream process with high-titer, high-quality manufacturability and flexible scalability utilizing Univercells Technologies' scale-X™ hydro system. In this study, the results for 2.4 sqm fixed bed bioreactor are shown, in which the peak cell density and peak virus titer were measured. Critical quality attributes including residual host cell DNA and host cell protein were determined. The results indicated that the virus yield from a 2.4 sqm fixed bed culture is comparable to traditional roller bottle production with operational simplification on manpower, material, and space requirements, providing potential scalability for large scale manufacturing of HSV-1. These results are very relevant for the coming scale-up manufacture in 30 sqm scale-X™ carbo system and 600 sqm scale-X™ nitro in the NevoLine™ Upstream platform.

769. A Novel Platformable Approach for the Reproducible and Scalable Enrichment of Full AAV Capsids

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Adeno-Associated Virus (AAV) is the leading vector for gene therapy applications. The use of AAV involves meeting stringent standards for impurity thresholds in AAV sample formulations. Clearance of "empty" AAV capsids which lack the gene of interest reliably and at preparative purification scale presents an ever growing challenge for process scientists. Our lab has recently developed a novel method of purifying full capsids using Mustang Q anion exchange membrane chromatography. High capacity, easy validation, single use nature, fast flow rate and remarkable ease of use are some of the advantages of membrane chromatography as compared to other modalities. Hence, we believe that our novel method which features 1 mS/cm steps to selectively elute empty and full AAV capsids presents a breakthrough in purification of full AAV capsids and has a great potential in providing a highly competitive solution. Here we showcase the progress in understanding platformability, reproducibility and scalability of the conductivity step elution method. We developed this technique using AAV5, AAV8, and AAV9 with Mustang Q 0.86 and 5 mL devices. Absorbance measurements (260 nm vs. 280 nm) are used to determine

the elution conditions for obtaining optimal amounts of full capsids from an affinity purified pool. The majority presence of full capsids in these elution fractions was confirmed with ddPCR and intact capsid ELISA. Using the stacked 0.86 and 5 mL devices. We show that using this technique we can achieve a greater than 6-fold enrichment of full AAV 5, 8 and 9 capsids whilst still maintaining a greater than 60% yield of full capsids. The elution method can be used as a rapid process development tool to screen for conditions to improve separation as well as identify conditions to elute empty and full capsids with just two conductivity steps. We will show reproducibility data, to show the purification is robust across varying feedstreams. Additionally we will show a scaled up process to demonstrate the feasibility of including this approach in a complete AAV manufacturing process. Here we present a novel solution to AAV empty and full capsid separation. This is a current big painpoint to process scientist and engineers in the field. Here we show new data on different serotypes as well as new developed technique from rapid process development, to scale up.

Pharmacology/Toxicology Studies or Assay Development

770. Ghost Cytometry as a Novel Approach for Label-Free Evaluation and Sorting in the Manufacturing of CAR-T Cells and Other Cell Therapy Products

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Cost reduction and standardization of processes in manufacturing of cell therapy products such as CAR-T cells, stem cells, and tissue engineering products are important for sustainable adoption of such therapies. There still remain unmet needs for non-destructive testing for quality controls (QC) and bioprocess automation. Ghost cytometry (GC) is a novel AI-based flow cytometry approach that combines unique optical designs with machine learning methods to rapidly analyze morphological features of cells in the form of temporal waveform signals, bypassing image reconstruction. Recently, an advancement in GC to include phase image information has been developed enabling label-free evaluation and sorting of cells. Here we show that label-free GC has potential applications in the manufacturing process of cell therapy products. First, we evaluated the performance of GC in predicting QC metrics for cell therapy products. We were able to achieve high accuracy in cell counting and viability of CAR-T cells (i.e., discerning live cells from dead and apoptotic cells) without any molecular labels. Furthermore, we evaluated the performance of GC in predicting cell viability during the manufacturing process of CAR-T cells. We collected data using GC by sampling a small amount of cells at each step of the CAR-T cell manufacturing process, from upstream to downstream (1. Thawed PBMCs (Day0); 2. Purified T cells (Day0); 3. Activated T cells (Day2); 4. Transduced T cells (Day3); 5. Expanding CAR-T cells (Day6); 6. Expanded CAR-T cells (Day7); 7.

Thawed CAR-T cells after cryopreservation (Day7)). We obtained a prediction error of less than approximately 5% for samples collected from each step after the purification of pan-T cells from PBMCs, by training data from multiple lots across the entire production process. These results indicate that GC technology will be useful for monitoring cell viability without the need for manual labelling manipulation during the manufacturing process after the T cell purification step.

771. Host Cell DNA Impurity Sizing in rAAV by an 18S rRNA Gene-Based ddPCR Approach

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Introduction: Adeno-associated virus (AAV)-encapsidated host cell-derived DNA (HCD) impurities are an undesirable byproduct of vector manufacturing consisting of heterogeneous fragments of DNA packaged from the producer cell line. The potential expression of oncogenes, immunogenic polypeptides, or other potentially harmful DNA sequences could be a risk to patients who receive recombinant AAV (rAAV) gene therapy. Thus, regulatory authorities require characterization of the quantity and size of HCD impurities. Since the heterogeneity of HCD makes testing of all potential nucleic acid sequences a challenge, high copy number genes or repeat elements are commonly used as surrogates for analysis. The 18S rRNA gene located in the 45S open reading frame is arranged in tandem repeats on multiple chromosomes and is therefore a suitable target for HCD quantification. Due to its size of 1871 bp, it is also a preferable target for HCD sizing approaches as compared to other commonly used HCD targets such as Alu elements, which contain sequence repeats smaller than 300 bp. The research described here was conducted to identify a method meeting regulatory requirements for routinely assessing HCD in rAAV products. **Method:** An 18S rRNA gene-based HCD sizing assay that assesses five gene fragments from 69 bp up to 1514 bp was developed. Smaller fragments of up to approximately 500 bp were measured by singleplex ddPCR using two primers and one probe. Larger fragments were analyzed by duplex ddPCR. In this approach, two primer-probe sets labelled with different fluorophores were positioned at distances from one another of 1040 bp and 1514 bp, respectively. Droplets that are positive for both fluorophores are indicative of 18S rRNA gene sequence fragments of the respective size. **Results:** Comparable results for the singleplex method and the duplex method were observed by measuring a 572 bp fragment with both methods. Furthermore, it was demonstrated that packaging frequency decreases as the size of the fragment increases, which is in agreement with long-read next-generation sequencing (NGS) data that have been generated for one rAAV batch. Three large-scale batches of one rAAV product manufactured using our iCELLis® production platform were analyzed by this sizing assay and the respective HCD sizing profiles were compared. Results showed that the size distribution profile observed was consistent across all three batches tested. **Conclusions:** The method described here meets regulatory requirements for analysis, in a controlled QC environment, of the sizing of HCD impurities which do become packaged. Furthermore, the assay enables the assessment of the effects of different process parameters on the HCD impurity

sizing profile. The data generated from this method can form part of a comprehensive assessment of the potential risk to patients from packaged HCD.

772. Development of a High-Throughput, Miniaturized, Semi-Automated Rapid Transduction Inhibition Assay (TIA) for the Characterization of Anti-AAV Antibodies in Gene Therapy

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Adeno-associated virus (AAV) vectors have become the vector of choice for gene therapy in large part due to their non-pathogenic nature. However, most people will be exposed to at least one strain of AAV during their lives and anti-AAV antibodies that develop during this exposure can reduce or eliminate the efficacy of AAV-based gene therapy. The activity of anti-AAV antibodies is reliably assessed by a transduction inhibition assay (TIA), which is a functional cell-based assay that estimates the extent of AAV neutralization in patient plasma, regardless of its anti-AAV antibody quantity. TIA, therefore, remains the gold standard in determining patient eligibility for AAV gene therapy. We previously described the development of 6 and 24-hour 'rapid' TIA methods, which have subsequently been validated. While this assay is suitable for determining titers of neutralizing antibodies in prospective patients, throughput limitations hinder use for population-based seroprevalence studies for clinically relevant and novel capsids. A high-throughput version of the rapid TIA was developed through miniaturization and automation. First, an additive protocol was developed that leverages contact-free dispensing of all assay components including plasma sample, AAV vector, cells, and luciferase reagent into 384-well plates. This method builds a 2-fold, 7-step sample titration series that enables testing of 8 samples per plate while maintaining all assay conditions of the validated method (3 samples per plate) starting with as little as 100 nL. The optimal number of cells per well was tested and it was found that the same single-digit multiplicity of infection (MOI) as established in the validated method resulted in robust assay signal ($Z' > 0.5$) and quality (coefficients of variation typically $< 25\%$) for AAVS3, our novel AAV gene therapy capsid. To enable comparison among different AAV serotypes and cell-line combinations without changing TIA assay parameters, the assay signal was further increased by using an MOI of 50 (10-fold higher than manual method). The use of a bright luciferase reporter enabled similar assay performance regardless of capsid serotype differences in transduction efficiency. Testing of 8 plasma samples with a broad range of TIA titers for AAVS3 and intravenous immunoglobulin (IVIG) showed overall no significant changes in IC_{50} at the two MOIs. Finally, freezing of plates pre-dispensed with plasma samples did not change the measured IC_{50} s vs "uninterrupted" assays, and enabled the "printing" of an entire library of 96 healthy donor plasma samples into 384-well plates before completing the TIA assay. This protocol enabled a ~6-fold improvement in throughput compared with the manual method. The automated 384-well plate rapid TIA protocol consistently replicates all steps of the validated rapid TIA assay. A modification of the MOI increases the versatility of this protocol by enabling testing of

different serotype and cell-line combinations in support of detailed seroprevalence studies. This work shows that once cumbersome functional cell-based assays such as TIA can be upgraded to expand their role in both pre-clinical and clinical studies of gene therapies facilitating their development and represents a first step toward their standardization.

773. Dose-exposure Relationships of AAV9 in Nonhuman Primate CNS and Liver with Different Routes of Administration: Leveraging Published Datasets to Gain Insights

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Introduction: Adeno-associated virus (AAV) has proven to be a useful tool for delivering payloads aimed at treating genetic disorders. AAV serotype 9 (AAV9) has become a workhorse capsid, and is involved in many clinical trials and several marketed gene therapy products targeting the central nervous system (CNS). Despite recent breakthroughs, a significant challenge remains of quantitative dose exploration in larger species, such as nonhuman primates (NHPs). Due to resource demands and ethical concerns, individual NHP studies typically utilize a small number of animals and limited doses and timepoints, making it difficult to ascertain dose-exposure relationships, especially between different routes of administration (ROAs). Comparison of on-target CNS exposure and off-target liver exposure following intra cerebral spinal fluid (CSF) dosing and intravenous (IV) dosing is of particular interest. To leverage existing NHP data sets, digitized biodistribution data from multiple published NHP studies utilizing AAV9 dosed intrathecally (IT), intra cisterna magna (ICM) or IV was used to create dose-exposure plots. Metrics from the included studies were standardized to allow for data integration and analysis.

Results: *CSF administration:* IT and ICMIT administration of AAV9 based gene therapies in NHPs shows a linear dose exposure relationship in both brain and liver. ICM dosing resulted in higher brain exposures than IT dosing, and the available datasets suggest a fairly steep dose exposure relationship for doses up to 1E13 vg/kg. The dataset includes one study that used a higher ICM dose (2E13 vg/kg) yet resulted in a lower brain exposure, comparable with studies using 10x lower doses. More data is needed to investigate if this result is an outlier or if ICM dosing indeed results in a bell shaped dose response curve. For the liver, a similar dose exposure relationship for ICM dosing was observed as for IT. Overall, the data available suggest a lower brain to liver exposure ratio for increasing ICM doses, however more data is needed to strengthen this relationship. Both CSF ROAs resulted in similar exposure in all segments of the spinal cord and DRG. Both CSF ROAs resulted in high liver exposure, which has been previously reported. *IV administration:* The characterization of the dose exposure relationship of AAV9 after IV administration in comparison to the CSF ROAs is limited by the lack of reported studies for doses below 1E13 vg/kg. For IV dosing, the dose exposure relationship is nevertheless shifted relative to CSF dosing, resulting in much lower brain exposure, as expected. What is interesting however, is that liver exposure appears to also be less than the CSF ROAs at doses lower than 2E13 vg/kg. This finding has not been previously reported to our knowledge, and suggests that our understanding of dose-exposure relationships of AAV are incomplete and may be highly dose-dependent.

Conclusions: Our analysis found very different dose-exposure relationships for the three investigated ROAs. Interestingly the data suggests that the majority of CSF administered vector escapes to peripheral organs including the liver, resulting in higher liver exposures after CSF administration dosing than after IV administration for doses lower than 2E13 vg/kg. This dataset highlights the potential for revealing key trends in AAV NHP dose-exposure with sufficient animal numbers. This analysis shows that consistent dose-exposure data can be derived when integrating different studies despite the interindividual variability observed in single studies. Therefore increased data transparency and standardization in data reporting for these types of studies could advance the field of AAV gene therapy by facilitating comparative cross-study data analytical approaches.

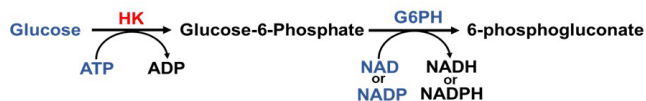
774. Measurement of AAV1-Mediated Glucokinase Function in Mouse Skeletal Muscle

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Type 1 diabetes (T1D) is a chronic autoimmune disorder resulting in β -cell failure, insulin deficiency and severe hyperglycemia. Despite decades of research, no cure for T1D exists. The only available therapy for T1D is exogenous insulin, administered to patients through injections or ports. Alternative approaches are being explored, including gene therapy to replace exogenous insulin with expressed insulins and other factors to enhance glucose disposal. In healthy humans, skeletal muscle is responsible for the disposal of ~70-80% of circulating post-prandial plasma glucose. In T1D skeletal muscle glucose uptake and metabolism is disrupted due to diminished circulating insulin and downregulation of transporters and metabolic enzymes. We are investigating an experimental therapy to enhance glucose uptake and metabolism in T1D patients utilizing AAV1-mediated expression of human insulin (AAV1-hIns) and glucokinase (AAV1-hGck) in skeletal muscle. To evaluate the strategy, precise assessment of functional glucokinase activity in skeletal muscle *in vivo* is critical. A standard assay for detection of glucose metabolism by hexokinases depends on the conversion of glucose to Glucose-6-Phosphate (G6P), coupled to conversion of G6P to 6-phosphoconate (6PG) through an enzymatic reaction producing NADH or NADPH, detectable at 340 nM. Mammalian skeletal muscle contains multiple isoforms of hexokinase (HK1 and HK2), with reported Km values for glucose in the 30-50 μ M (HK1) and 100-400 μ M (HK2) range. The catalytic activity of both isoforms reaches Vmax in the presence of >5 mM glucose. Glucokinase (HK 4 or GcK), as a liver-predominant isoform, has a Km of 8-10 mM with a catalytic Vmax >50 mM glucose. Measurement of glucose phosphorylation at the Vmax of both HKs and GcK is necessary to accurately dissect the activity of the individual enzymes. In the presence of 100 mM glucose, the substrate concentration ensures achievement of the Vmax of catalytic activity of both HKs and GcK. On the other hand, we found that low (<1 mM) glucose concentrations do not achieve the Vmax of HKs;

there is no detectable GcK activity. We optimized homogenization conditions to secure the catalytic activity of both HKs and GcK, and measured glucose phosphorylation activity in the presence of 1 mM or 100 mM glucose in muscle homogenates. A conversion factor of 1.36 was calculated to estimate the activity of HKs in the presence of 100 mM glucose (HKs-V_{max}) using the activity of HKs in the presence of 1 mM glucose and assessed GcK activity in the presence of 100 mM glucose (GcK-V_{max}) by subtracting HKs-V_{max} from total glucose phosphorylation activity. This assay system allows us to quantify the relative contribution of endogenous HKs and transduced GcK to the glucose phosphorylation rate from gastrocnemius and quadriceps muscle homogenates of AAV1-hGcK injected mice. We were able to unambiguously confirm functional expression of the therapeutic GcK transgene as >2-fold over background in flash-frozen muscle samples from C57BL/6 mice 6 weeks post vector administration. This work provides an opportunity to assess the contribution of AAV1-mediated GcK activity to glucose metabolism in treated mice, and to quantify the amount of enzyme activity required for efficacy at therapeutically relevant doses of AAV1-GcK.



775. Comparison of PCR- and Non PCR-based Vector Integration Site Analysis Approaches for Vector Safety Evaluation

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Current regulatory guidances require the preclinical and clinical assessment of integration profiles for *in vivo* or *ex vivo* therapies involving integrating vectors. Despite this not being required for non-integrating vectors, adenoassociated viral vectors have lately been in the focus as exhibitors of potential genotoxicity and, therefore, drug developers are increasingly including these analyses in their safety studies. This scenario has evidenced the need for a deeper understanding and standardization of integration site analysis methods performance. Here we investigate a PCR- and non-PCR based approach for lentiviral integration site retrieval and quantification using a reference material. To this purpose, we used the reference sample developed by the WHO for lentiviral vector integration site analysis, where 10 pre-defined integration sites (IS) are to be detected. This reference sample was analyzed using our previously validated 'Shearing Extension Primer Tag Selection Linear-Mediated PCR' (S-EPTS/LM-PCR) and a customized Target Enrichment Sequencing (TES) protocol. In addition, in a deeper method characterization, this reference material was used to generate samples of known clonalities by spiking it into a polyclonal background in order to evaluate performance in terms of IS quantification. Both S-EPTS/LM-PCR (PCR-based) and TES (non PCR-based) successfully detected the 10 expected IS in all replicates of the reference material. Those 10 IS accounted, on average, for 89.91% of all IS

sequencing reads for S-EPTS/LM-PCR and 87.75% of all IS sequencing reads for TES. Two additional IS were detected by both approaches in all replicates thus indicating that they could be present in the sample, in line with WHO's sample description, rather than being representative of background noise levels. A 0.03% and 1.45% of the sequencing reads for S-EPTS/LM-PCR and TES, respectively, were not homogeneously detected among replicates or approaches being likely indicative of false positive events and thus showing the low background noise levels of both techniques. Interestingly, all 10 IS were retrieved at different relative frequencies ranging from 0.01% to 14.56% for S-EPTS/LM-PCR and from 2.60% to 13.18% for TES. These divergent individual relative frequencies might most likely reflect the different performance in IS retrieval and identification. On average, TES yielded relative IS frequencies only 0.34% lower when compared to the PCR-based method with deviations increasing at lower relative contributions. Next, we generated samples of known clonalities by spiking the reference material into a LV transduced polyclonal background at 70%, 30% and 5% ratios in order to mimic nominal individual IS relative frequencies of 5.83%, 2.50% and 0.42%. For S-EPTS/LM-PCR, CV values ranged from 3.70% to 30.80% for the highest and lowest spike-in, respectively, thus showing low replicate variability. Average accuracy values were found to be 5.50% for the 70% spike-in, 24.31% for the 30% spike-in and 42.57% for the 5% spike-in. The sample set has also been analyzed by TES and data analysis is currently ongoing and will be provided to enable a side-by-side comparison between both integration site analysis approaches. In summary, the data shows that both PCR-based and non PCR-based approaches exhibited similar IS retrieval performance from a reference material and it also highlights the need for the development of reference materials also allowing for the quantitative evaluation of the different method's performance.

776. Novel Method for Measuring Infectious Titer of Adeno-Associated Virus Serotype 9 Using ddPCR

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With the increasing number of clinical trials testing *in vivo* gene therapy with adeno-associated virus (AAV), the development of suitable quality control methods is critically needed for accurate vector characterization. Among the quality criteria, infectious titration of AAV drugs is needed to determine vector efficacy and hence requested by multiple regulatory agencies. One major method to measure AAV infectivity *in vitro* is the median tissue culture infective dose (TCID₅₀), which is currently qPCR-based, requires a standard curve and has poor precision. ddPCR has emerged as a powerful alternative to qPCR that offers better precision, accuracy, and sensitivity than qPCR. Additionally, ddPCR can directly quantify viral DNA copies without the need of a standard curve. In the present study, we have developed a novel ddPCR-based TCID₅₀ method to accurately measure the infectious titer of AAV. This method has shown robust infectious titers that are comparable to qPCR-based TCID₅₀ infectious titers with improved sensitivity at higher dilutions leading to more accurate titer results. In conclusion, ddPCR-based TCID₅₀ methods can be a suitable alternative of qPCR-based methods, providing an improvement in sensitivity and accuracy without the need of a standard curve.

777. Development of a Conducive and Hands-Off Automated Solution for Viable Therapeutic TILs Results in Deep Immune Cell Profiling and an Increase in Quality and Complexity

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¹Curiox, Woburn, MA, ²Charles River Laboratories, Worcester, MA

Many gene-and-cell therapy based cancer treatments and tumor infiltrating therapies work by inducing apoptosis through an extrinsic pathway. To use TILs as a therapy, the focus is to overcome the hurdles in the tumor environment, which includes expanding the TILs and engineering them with certain attributes to focus on the viability and metabolic functionality. However, many current assay formats reveal a limited snapshot of viability in functionality and efficacy studies. This may not be adequate for monitoring the overall quality of cells intended for clinical use, where injection of nonviable cells could lead to safety issues. Systematic studies have been conducted over cancer cells and infiltrating lymphocytes alike to identify targetable metabolic vulnerabilities, despite heterogeneity in metabolic profiles of cancer cell lines. Laminar Wash™ technology emphasizes a gentle, reduced hands-on upstream of preparing your sample, allowing the enhanced identification of the target cell population, but also removal of dead cells, debris and doublets while allowing the cells to remain healthy. This creates a conducive and front-end automated solution that results in Deep Immune Cell Profiling to increase in quality and complexity.

778. New Insights into the Toxicity of AAV-Mediated Overexpression of Frataxin

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Friedreich's ataxia is a rare genetic disorder resulting from deficiency in frataxin, a mitochondrial protein implicated in the synthesis of essential iron-sulfur cluster cofactors. Preclinical studies in mice have shown that gene therapy is a promising therapeutic approach for Friedreich ataxia. However, a recent report provided evidence that AAVrh10-mediated overexpression of frataxin could lead to cardiotoxicity associated with mitochondrial dysfunction. While evaluating an AAV9-based frataxin gene therapy approach using a chicken b-actin promoter, we showed that toxic overexpression of frataxin in mice was achieved with dose levels ranging from 10¹³ to 10¹⁴ vg/kg. In a mouse model of cardiac disease, these dose levels corrected the cardiac dysfunction partially and transiently, and led to adverse findings in the liver. Further studies in wild-type mice showed that toxicity mediated by frataxin overexpression was associated with the primary function of frataxin in iron-sulfur cluster biogenesis. Together, our data provide

key understandings of the intrinsic toxicity of frataxin overexpression *in vivo* that should be considered in the development of an effective and safe gene therapy for Friedreich's ataxia.

779. From Cell Culture to Sterility, Applications for Rapid Microbiological Methods Across the Entire CGT Production Process

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Reducing the lead time for sterility testing is a clear bottleneck for the release of cell and gene therapies to patients. Rapid microbiological methods have been implemented in the past to help reduce the detection time of potential contaminations with varying levels of success. But, expanding their use beyond sterility testing has been a challenge for many quality control groups, commonly validated with limited scope, focusing on specific sample types and individual applications and often conforming to limitations of the technology being utilized. This has led to many quality control laboratories failing to apply a single rapid method across multiple in-process points along the process beyond just final product release sterility testing, despite the clear advantages they provide. When producing cell therapies, there is an increased need for rapid microbiological methods to detect potential contaminations across the manufacturing process as quickly and confidently as possible to protect batch yields and patient safety. In this presentation, critical in-process control points and how an ATP bioluminescence rapid detection method can be applied to them will be covered. Studies and data will be presented to demonstrate specificity and signal detection in a wide variety of samples and quality control testing points, including rapid testing cell containing samples commonly used in cell therapy production, Incoming QC testing of growth media and water, testing of column chromatography resins, product release in as little as 3 days using a risk-based approach. The rapid microbiological detection technology, compatible with multiple sample types, can be applied along upstream and downstream processing steps to help accelerate the development, production, and release of safer cellular therapies to patients.

780. Safety of Cell Therapy Products: In Vitro Methods to Assess the Tumorigenicity of Human Cell-Based Therapeutic Products

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Human pluripotent stem cells (hPSC) have the potential to revolutionise regenerative medicine. However, there are concerns associated with hES/iPSC-derived products, in particular, the possibility of residual undifferentiated PSCs persisting in the final product, which could lead

to tumorigenicity. Currently, there is no globally accepted consensus on the evaluation of methods for tumorigenicity *in-vivo* or *in-vitro*. This results in a high variability of data presented in regulatory submissions and difficulty in interpretations. To address this challenge, the Health and Environmental Sciences Institute (HESI) Cell Therapy - TRacking, Circulation, & Safety (CT-TRACS) committee, convened international experts in the field, from multiple sectors and geographic areas, to critically review currently available *in-vivo* and *in-vitro* testing methods for tumorigenicity evaluation against expectations in international regulatory guidelines. The outcome of this effort was recently published in a position paper (Sato *et al.* 2019), which highlighted that the establishment of robust methods, internationally harmonised for tumorigenicity assessment of cell therapy products, is critically important, not only for product developers, but also for regulatory authorities and patients. Here, we will present the follow-up activity of the committee: the launch of an international collaborative project to evaluate *in-vitro* testing methods focusing on the detection of residual undifferentiated PSCs using ddPCR. The two objectives, among many, for the multi-site study are to develop better, standardised *in-vitro* models for predicting tumorigenicity and to aid researchers, developers and regulators to assess the safety of products with more confidence and contribute to faster/earlier decision-making.

781. Real Time MRI Guided Convection Enhanced Delivery for Parenchymal and Ventricular Brain Administration in NHPs

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Recent advances in convection-enhanced delivery (CED) have allowed for unprecedented delivery accuracy to targeted brain regions using cell and gene therapies. These methods are now routinely used in non-clinical models to assess safety and efficacy of cell and gene therapies in development and help to ensure the highest translational value. Considering this, we have developed real-time intraoperative magnetic resonance imaging (MRI) with a stereotactic navigation system to deliver novel neurological therapeutics in a non-human primate (NHP) model. Real-time MRI scanning confirmation of dose site accuracy and dose diffusion monitoring was made by visualization of a gadolinium-containing solution used as a contrast agent. Infusions into the targeted brain structure was planned by neuroscientists and surgeons and achieved a dosing accuracy of <0.5mm radial error from the planned target. Real-time MRI scanning was used to assess distribution relative to the targeted brain structures. Further post-hoc analysis using custom bilateral masks outlining target subcortical neurological structures was used to calculate the volumetric coverage of the structures. Sequential dosing paradigms were used in the preclinical model to cover structures bilaterally given anatomical constraints. During recovery from surgery, animals typically presented mild and expected procedure-related clinical signs (e.g. mild tremors) which generally resolved within four days. Histopathological assessments also identified the expected neuropathological changes indicative that surgical procedures were well tolerated. Since the use of NHP models is particularly important for

testing the delivery of cell and gene therapies, this approach provides a reproducible preclinical methodology for targeted brain CED with high structural accuracy but also remarkable translational value.

782. Development of a New Transduction Assay to Evaluate *In-Vitro* Relative Infectivity of ASC618, a Second Generation AAV8-Based Bioengineered Factor VIII Gene Therapy

Chengtao Zhang, Avital Gilam, Yin Yang, Tanvee Sawant, Eliza Dewangan, Rong Chen, Ruhong Jiang, H. Steve Zhang, Zoya Gluzman-Poltorak

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Since rAAV viral vectors are the leading platform for gene delivery for the treatment of a variety of human diseases, the development of suitable and accurate assays for qualifying the quality attributes of manufactured AAV vectors is in urgent need. For the titration of infectious units of an rAAV stock, one of the most widely used methods is TCID₅₀, which needs a special packaging cell line (expressing AAV *rep* and *cap* genes) and a helper virus to fulfill the rAAV replication. TCID₅₀ method was originally developed with serotype AAV2 but requires further development to improve accuracy for other serotypes, like AAV8, with much lower infectivity in the packaging cell line. Here, we developed a new analytical assay for the titration of infectious units in rAAV8 stock through the detection of intracellular vector genomes in a permissive cell, independent of *rep* and *cap* gene expression and a helper virus. ASC618, an advanced AAV8-based gene therapy vector carrying an engineered novel human Factor VIII transgene targeting liver tissue for the treatment of Hemophilia A, was evaluated for the development and pre-qualification of the new assay and its transduction in the host HepG2 cells (a human liver cancer cell line). The new assay, called Transduction Assay, has three major steps: 1) Development of the viral infection protocol; 2) Development of tests assessing the intracellular viral genome copies; and 3) Statistical analysis. During the viral infection, special treatments of both viral vectors and the host cells were performed to enhance the transduction efficiency of vector genome in HepG2 cells. ASC618 vector infected HepG2 cells at about 7 MOI levels in the range of 7E+2 - 8E+3 vg/cell at Day 0. On Day 6, the supernatant culture media was collected for FVIII ELISA assay and the cell cultures were treated with Trypsin-EDTA to dissociate cells and remove the surface-binding rAAV particles. After adequate washing of the collected cells with DPBS, DNA was extracted from the cell pellets and the intracellular ASC618 genome DNA copies were quantitated by a ddPCR-based method. The measured value of intracellular ASC618 DNA copies in HepG2 exhibited a strong correlation to the ELISA-measured FVIII protein concentration from the same cell culture. The relationship between ASC618 MOI levels and the measured transduction values (intracellular ASC618 DNA copies/cell) showed linear correlation coefficient $R^2 > 0.985$ in Log(x)-Log(y) scale. In pre-qualification experiments, ASC618 Engineering Lot was applied as Reference Standard and used to prepare QC samples with target ratio of infectivity (TRI) of 0.5 (50% QC), 1 (100% QC), 1.5 (150% QC) and 2 (200% QC) respectively. Both Reference Standard and the prepared QC samples were applied to infect HepG2 cells at 7 MOIs. The transduction data were collected and the relative transduction of each QC sample to the Reference Standard was analyzed using parallel

line assay in the log-log scale. The evaluated relative transduction is 0.595 for TRI=0.5 sample, 1.090 for TRI=1 sample, 1.84 for TRI=1.5 sample, and 2.43 for TRI=2 sample. Since relative errors in these measurements are within 30% for all tested QC samples, we suggest that this new Transduction Assay has an acceptable accuracy to evaluate the relative infectivity of an ASC618 test lot as a reference standard. This approach was presented in the ASC618 IND submission and the program received IND clearance from the U.S. FDA and a phase 1/2 clinical trial to evaluate the safety, tolerability, and preliminary efficacy of ASC618 (NCT04676048) will be conducted.

783. Pharmacology, Toxicology and Safety Studies of ASC618, a Second Generation Factor VIII Gene Therapy for Hemophilia A

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ASC Therapeutics, Inc has developed a second generation of AAV-based Gene Therapy for Hemophilia A, called ASC618. A series of developmental in silico, in vitro and in vivo studies were conducted to design an efficient HCB-ET3-LCO (ASC618) construct. As a result of these studies, the ASC618 construct was developed, which to our knowledge represents the shortest vector genome in development for Hemophilia A. It encodes a liver specific codon optimized (LCO) bioengineered BDD hFVIII (ET3) under a synthetic Hepatic Combinatorial Bundle (HCB) promoter (HCB-ET3-LCO). This transgene sequence demonstrated 10-fold or greater increased secretion efficiency compared to a standard BDD hFVIII transgene with a 14 amino acid linker (HSQ). ASC618 pharmacology studies at dose levels from 5E10 to 5E12 vg/kg in a hemophilia A mouse model, humanized liver mouse model and wild type mice and monkeys were well tolerated and showed therapeutic levels of $\geq 20\%$ normal hFVIII (0.2 IU/ml) at all tested doses in all animal models. ASC618 liver infectivity, liver mRNA expression and ET3 hFVIII plasma levels expressed in the humanized liver mouse model, wild type mice and monkeys were compared to support toxicology studies design as well as subsequent dose translation and First in Human initial dose selection. ASC618 liver infectivity demonstrated similar dose dependence in human, mouse and monkey liver cells, while ET3 mRNA expression was detected at significantly lower levels in both humanized liver model and NHP compared to C57BL/6J mice. The difference in mRNA expression levels between human and NHP liver cells and mouse liver cells was approximately 10-fold. A GLP toxicology study was conducted to evaluate the safety of ASC618 following a single IV administration in healthy male C57BL/6J mice. ASC618 was well tolerated at the doses of 2E11, 2E12 and 2E13 vg/kg. There were no statistically significant differences in mean body weights, food consumption, clinical pathology, including liver enzymes, macroscopic or microscopic histopathology in ASC618 treated dose groups throughout the study when compared to the vehicle control group. The no-observed-adverse-effect level (NOAEL) of ASC618 (under conditions of this study) has not been established and the NOAEL of ASC618 is expected to be

higher than 2E13 vg/kg. The no-observed-effect level (NOEL) has also not been established since the lowest tested dose 2E11 was found to be efficacious. Based on these results and dose translation approach the program received IND clearance from the U.S. FDA and a phase 1/2 clinical trial to evaluate the safety, tolerability, and preliminary efficacy of ASC618 (NCT04676048) will be conducted.

784. Digital Trace: Universal Molecular Tool Box for Monitoring Allogenic Stem Cells Using Digital PCR

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We have commercialized the Digital TRACE analysis system as an alternative technological approach to genomic mixture analysis by short tandem repeats (STR). Digital TRACE utilizes digital PCR, targeting 80 bi-allelic insertion/deletion polymorphisms in the human genome, and has been developed to discriminate HLA-matched sibling donors and recipients involved in hematopoietic stem cell transplants (HSCT). This system has a greater than 99% chance in identifying 2 informative loci between HLA-matched sibling donor : recipient pairs involved in HSCT. Digital PCR also enables facile monitoring of multiple allogeneic donors in a recipient, whereas STR is extremely challenged with more than 1 donor. With an increasing number of candidate allogeneic pluripotent stem cell therapies being developed, there is a need for universal, standardized, sensitive methods for monitoring the engraftment, proliferation and persistence/half-life of these cells for research, development, and manufacturing purposes. Digital TRACE affords an out-of-the-box molecular analysis toolbox which can be utilized universally for allogeneic stem cell monitoring.

785. Novel Cytometry Based Characterization of Lysosomal Disease Affected and Gene Corrected Patient's Cells

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Wolman disease (WD) is a severe lysosomal storage disorder (LSD) caused by deficiency of the lipid metabolic enzyme, Lysosomal Acid Lipase (LAL), involved in the lysosomal hydrolysis of cholesterols and triglycerides. Due to the imbalance of lipids homeostasis, WD patients suffer from hepatosplenomegaly, hepatic failure or adrenal calcification resulting in a premature death within the first year of age. Here, we explored the combination of different cytometry modalities to characterize the phenotype of WD patients' fibroblasts and its correction via gene therapy (GT). In particular, we analysed fibroblasts stained with Nile Red (NR), marker of neutral lipid, and Lysogreen (LG), marker of lysosomes, on wide field fluorescence microscope, confocal microscope, conventional and image flow cytometer. Wide field fluorescence microscopy analysis showed higher staining intensity in impaired cells for both LG and NR. By conventional flow cytometry, we confirmed the LAL-D phenotype and observed that WD cells had a higher granularity than healthy cells. To understand

if the higher staining intensity is due to an increase in size or in number of lysosomes, we analysed cells using 3D confocal microscopy. Interestingly, we noticed that both fluorescence intensity and the number lysosomes were augmented in WD fibroblasts. To increase the throughput and the speed of the analysis, we explored image flow cytometry, which allows localization and labelling intensity quantification within the cell. Analysing hundreds of cells, we confirmed that WD fibroblasts had a higher fluorescence intensity and granularity as well as an increased number of NR and LG spots. For the first time, we demonstrated that this spot counting could differentiate WD and healthy fibroblasts better than currently used imaging technique. Moreover, lentiviral vector mediated integration of a functional LIPA gene fully correct the lipids accumulation in WD fibroblasts making this pipeline interesting to assess GT efficacy. However, fibroblasts are not easily accessible cells for clinical evaluation and assessing GT efficiency compared to peripheral blood mononuclear cells (PBMC) that are already used for clinical assessment of LAL-D. In absence of patient blood cells due to rarity of WD, we generated 2 LAL KO cell lines from T lymphocytes (Jurkat) and monocytes (U-937) by deleting the exon 4 of the LIPA gene using the CRISPR-Cas9 system. We first confirmed an almost complete LAL KO by DNA sequencing and enzymatic activity assay and, then, we analysed KO cells by flow cytometry and image flow cytometry upon NR and LG staining. Similarly, to our previous observation using WD fibroblasts, KO cells showed a significant increase of lipid and lysosome vesicles. These generalize our observation to multiple different cells and illustrate that PBMCs can be used for cytometry evaluation of WD. Overall, these analyses can be applied to diagnose the disease and to monitor overtime the phenotypic progression and correction upon treatment. We expect that this sensitive analysis pipeline could be used to evaluate the success of gene correction strategies and implemented to study and diagnose other lysosomal storage disorder (LSD).

Gene and Cell Therapy Trials in Progress

786. Effects of BCL11A shmiR-Induced Post-Transcriptional Silencing on Distributions of Fetal Hemoglobin and Hemoglobin Polymer Inhibition in Single Red Blood Cells in Sickle Cell Disease Gene Therapy

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Fetal hemoglobin (HbF) is a critical modulator of sickle cell disease severity due to its capacity to inhibit sickle hemoglobin (HbS) polymerization. We previously reported a successful gene therapy (GT) trial (DOI:10.1056/NEJMoa2029392, NCT 03282656) which used a lentivirus shmiR technology allowing the post-transcriptional silencing of BCL11A, a master regulator of γ -globin synthesis, increasing HbF while simultaneously reducing HbS by reversing the fetal to adult hemoglobin (Hb) switch. Here we report, using single-cell quantification of HbF per red blood cell (RBC) and Hb polymer inhibition, a massive and broadly distributed HbF synthesis in BCL-targeted subjects (BCL^{sub}). At a variety of oxygen tensions (O₂) mimicking physiologic tissue levels, the RBCs of BCL^{sub} had HbS polymer levels lower than for hydroxyurea (HU)-treated patients with HbF>6%. Single-RBC Hb polymer and single-RBC HbF level measurements were combined to show that, polymer formation was also inhibited to a greater degree than for RBCs from HU-treated patients. At venous oxygen tension of 4.7% the HbF mass required to prevent HbS polymer *in vitro* was 5.8 ± 1.9 pg/cell for BCL^{sub} compared to 9.5 pg for HU, levels reached in 65% and 37% of RBCs, respectively. At 3.7% O₂ found in the microcirculation, 52% of RBCs from BCL^{sub} demonstrated detectable polymer formation vs. 66% for HU (median) (p = 0.02) (Fig 1A). At 1.7% O₂ found in renal medulla and bone marrow, these proportions were 68% of RBCs from BCL^{sub} and 79% for HU (p<0.001) (Fig 1B). RBCs containing >6 pg HbF/cell also showed a clear selective advantage for survival (Fig 2). In summary, BCL11A

down-regulation was associated with high and broadly-distributed HbF and favorable distributions of single-RBC Hb polymer at multiple physiologic oxygen tensions compared to HU. These data confirm the clinical benefits of this GT strategy at the cellular level and describe a target HbF protective threshold.

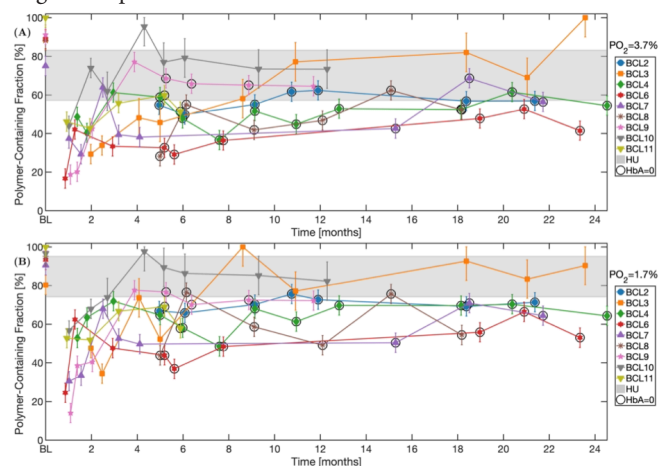


Figure 1: Panels (A) and (B) show the fraction of RBCs with detectable levels of Hb polymer at oxygen tension of 3.7% and 1.7%, respectively, that were measured in blood samples from BCL^{mb} (n=9) for up to 23 months following treatment. Black circles identify data points with 0% HbA (untransfused). Other data points represent fractions scaled by (100-HbA%). The gray region shows the interquartile range of fractions measured for HU-treated patients (n=14).

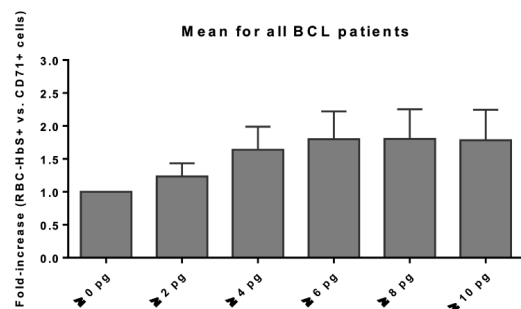


Figure 2: Mean fold increase between %HbS-containing RBC and %CD71⁺ cells in BCL^{mb} according to their minimal HbF content per cell.

787. Long-Term Safety of Intraparenchymal Administration of AAV2-GDNF and Gadolinium in Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disease characterized in part by progressive loss of dopaminergic (DA) neurons of the substantia nigra pars compacta resulting in denervation of caudate nucleus and the putamen. Neurotrophic factor research has provided potential neuroprotective remedies directed toward delaying or preventing neuronal loss and to slowing or arresting the course of disease. We have shown that delivery of AAV2-GDNF vector, using an MR-guided convection-enhanced delivery (CED) method into the putamen of stable parkinsonian MPTP monkeys, was safe and efficacious for at least 2y. Such results led to our launch of a Phase I clinical trial at NINDS to

investigate the safety, tolerability, and clinical effects of AAV2GDNF in advanced PD participants. Thirteen advanced idiopathic PD participants received CED (450 μ L/hemisphere) co-infusions of AAV2-GDNF and gadoteridol (an MRI tracer) into the bilateral putamen (52 total infusion trajectories). Three escalating vector genome (vg) doses were evaluated: 9×10^{10} vg (n=6); 3×10^{11} vg (n=6); and 9×10^{11} vg (n=1). Intraoperative MRI allowed real-time visualization of the CED. Pre-operatively, and at 6-12-month post-operatively, the Unified Parkinson's Disease Rating Scale (UPDRS), levodopa equivalent daily dose (LEDD), and [¹⁸F]FDOPA positron emission tomography (PET) scanning evaluated clinical motor and striatal DA functions, respectively. Regardless of the vector dose received, UPDRS scores and LEDD remained relatively stable throughout the 5-year study, and increased [¹⁸F]FDOPA uptake in the CED regions was noted bilaterally in 10/13 participants at 6 months and 12/13 participants at 18 months post-treatment. Pre-, intra- and post-operative (up to 5y) DICOM MR-scans (\pm contrast) were evaluated from all 13 participants enrolled in the Phase I AAV2-GDNF trial, so as to determine the long-term safety and tolerability our MR-based CED co-infusion methods in advanced PD participants. Pre-operative MR-imaging defined the putaminal targeting trajectories via bifrontal approaches. Intra-operative MR-images re-confirmed the brain anatomy, target registration and proposed CED cannula trajectories. Serial post-operative MR-images allowed visualization of the perfused putaminal volumes and surrounding brain parenchyma for signs of toxicity. Each participant's MR DICOM datasets were qualitatively reviewed by two independent neuroradiologists for signs of parenchymal changes proximal to the CED. Quantitative volumetric assessment of the putaminal volumes and of delivery cannula tracks (when visible) were documented on serial postoperative imaging. No post-operative parenchymal toxicity was evident in the regions of CED infusion on MRIs over the 5-year study period. MR-imaging did not detect signs of a localized inflammatory response to the therapeutic co-infusions, up to 5 years following CED (Figure 1). Cannula track volumes were consistently present but significantly reduced by 36-, 48- and 60-month MR assessments. Co-infusion of gadolinium with AAV2-GDNF was safe and well-tolerated in 13 advanced PD participants, up to 5 years post-treatment, without evidence of parenchymal toxicity.

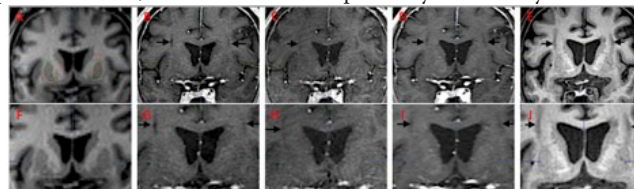


Figure 1: Example of coronal set of serial scans (Left to right: Day 0, 6m, 18m, 36m, 60m). Black arrows indicate cannula tracks

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788. Long-Term Phenotypic Correction of Fanconi Anemia-A Patients Treated by Gene Therapy in Early Stages of the Bone Marrow Failure

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In this study we report the results obtained 2-6 years after lentiviral-mediated gene therapy of patients with Fanconi anemia, subtype A (FA-A). Data correspond to the FANCOLEN-I and the companion long-term follow-up clinical trials onto which patients were enrolled after completing or discontinuing the parent study. Patients were infused with mobilized CD34⁺-enriched cells after transduction with the PGK-FANCA.Wpre* lentiviral vector without any conditioning regimen. The number of CD34⁺-enriched cells infused in the patients ranged from 7.3x10⁴ to 1.9x10⁶ CD34⁺ cells/kg. Vector copy numbers (VCN) in colonies derived from the manufacturing products ranged from 0.2 to 0.9 VCN/cell. Progressive engraftment of gene-corrected cells was observed in six out of the eight evaluable patients. In five patients the proportion of corrected cells reached values ranging from 10% to 70% in either BM or PB at 2-6 years post-gene therapy, and none of these engrafted patients showed signs of HSC exhaustion during long-term follow-up. In all instances progressive increases in the proportion of corrected cells were associated with increases in MMC-resistance in BM progenitors, and also with a reduction in the chromosomal instability in PB T cells exposed to diepoxybutane. Four of the eight evaluable patients who were treated at advanced stages of BMF, and/or infused with low numbers of corrected CD34⁺ cells, showed progressive evolution of BMF, requiring alternative treatments, including transfusions and/or allogeneic transplantation. Nonetheless, two of the patients among those receiving higher doses of corrected CD34⁺ cells had faster and higher engraftment levels, and showed stabilized and subsequently improved PB cell counts. The results obtained in these phase I/II and long-term follow-up clinical trials demonstrate the importance of performing FA gene therapy in early stages of the disease, and reveal the efficacy of this therapeutic approach

when significant numbers of corrected CD34⁺ cells are infused prior to BMF progression. Based on these results, global phase II trials are currently ongoing under the sponsorship of Rocket Pharmaceuticals Inc., with the goal of treating FA patients in early stages of disease to avert BMF.

789. Utility of a Semi-Mechanistic Model in Ex Vivo Gene Therapy for Sickle Cell Disease to Predict Clinical Results: Preliminary Results from the PRECIZN-1 Study

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Background: Fetal hemoglobin (HbF) is the major hemoglobin present during gestation and declines progressively after birth, where it is replaced by HbS in patients with sickle cell disease (SCD) and is associated with symptom onset by 6-12 months of age¹. Patients with SCD and genetic variants resulting in the persistence of high HbF levels (20-30%) due to hereditary persistence of fetal hemoglobin (HPFH) are asymptomatic throughout life². Disruption of the BCL11A gene in an animal model is shown to prevent switching to HbS production, maintaining high levels of HbF and preventing the pathologic characteristics of SCD³. BIVV003 is a novel, non-viral gene therapy that uses a pair of zinc finger nucleases to disrupt BCL11A expression and increase HbF production to mimic the natural condition of HPFH. **Objectives:** PRECIZN-1 is an ongoing Phase 1/2, first-in-human, open label, single arm, multi-site study currently actively enrolling approximately 8 adults with severe SCD, 18-40 years of age, for infusion with BIVV003/SAR445136 (NCT03653247). The objective is to build and validate a semi-mechanistic model to predict HbF response after BIVV003/ SAR445136 infusion in SCD patients. **Methods:** A semi-mechanistic model was developed to describe the erythropoietic system. A base model was built using data from healthy individuals and extrapolated to SCD patients. The model describes the kinetics of hematopoietic stem cells and erythroid progenitors, and markers of stem cell recovery (platelets). The erythropoietic system comprised a feedback loop driven by erythropoietin modulation at various Hb levels. Virtual SCD patients were created by modifying red blood cell (RBC) life span and then calibrating model parameters. The model was validated using existing literature (SCD transplantation⁴, CTX001 SCD^{5, 6}) and ongoing BIVV003 Phase 1/2 data (includes first 4 subjects treated with BIVV003). Simulations (grid of 100 patients) were performed with a range of 2-6x10⁸ CD34⁺ cell dose and 10-40% editing efficiency to identify the most sensitive parameters for achieving higher HbF levels at 1 year. **Results and Conclusions:** Stem cell treatment in SCD patients is only now emerging, thus available literature and in-house clinical data to support model validation is limited. Due to the small sample size and short follow up in the PRECIZN-1 study, the model may require further evaluation. Platelet levels indicated the degree of myeloablative conditioning (suppression of stem and progenitor cells pretreatment) and recovery rate of stem and progenitor cells. Model simulations indicate that conditioning affects HbF levels while RBC transfusion

(post treatment) does not. Higher conditioning leads to increased HbF levels. Predicted HbF levels were similar with or without RBC transfusion. Stem and progenitor cell levels and their kinetics were validated using SCD transplantation data⁴. CTX001 data^{5,6} suggested that early levels of engraftment (% edited peripheral cells at Week 13) can be predictive of HbF production over time. The number of edited peripheral cells at Week 13 strongly correlates with HbF level as observed in our preliminary clinical results. **References:**¹ Dong M, McGann PT. *Clin Pharmacol Ther.* 2021 Jan;109(1):73-81. ² Weatherall D, Clegg J. *Historical perspectives: The Thalassemia Syndromes* 4th Edition. Blackwell Scientific, Oxford UK, 2001. ³ Xu J, et al. *Science.* 2011 Nov 18;334(6058):993-6. ⁴ Hsieh M M, et al. *N Engl J Med.* 2009 Dec 10;361(24):2309-17. ⁵ Frangoul H, et al. *N Engl J Med.* 2021 Jan 21;384(3):252-260. ⁶ Frangoul H, et al. *ASH,* December 6, 2020, virtual.

790. Interleukin-15 Co-Expression Induces Robust *in vivo* Expansion and Antitumor Effect of Glypican-3-Specific CAR T Cells in a Patient with Liver Cancer

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Background: Glypican 3 (GPC3) is an attractive immunotherapeutic target because it is overexpressed in several solid cancers but not in non-malignant tissues. We have previously treated 12 patients with T cells expressing a GPC3-CAR at two dose levels (DL1: 1×10^7 ; DL2: $3 \times 10^7/m^2$). Although no dose limiting toxicities occurred, there was minimal CAR T cell expansion and antitumor responses were limited to stable disease by RECIST criteria. Preclinical studies have demonstrated that GPC3-CAR T cells co-expressing IL15 have superior *in vivo* expansion and antitumor activity in hepatocellular carcinoma (HCC) xenograft models compared with GPC3-CAR T cells. Thus, we hypothesized that IL15 co-expression in GPC3-CAR (15.GPC3-CAR) T cells would induce higher CAR T cell expansion and boost antitumor activity in patients. **Methods:** The AGAR (NCT04377932) study is a phase 1 clinical trial to define the safety, expansion, persistence, and antitumor activity of 15.GPC3-CAR T cells in patients with relapsed/refractory solid tumors. Autologous T cells are generated using two vectors that encode i) the GPC3-CAR and ii) the inducible caspase 9 (iC9) safety switch with IL15 (iC9.IL15). Patients receive lymphodepletion on Days -4, -3 and -2 with cyclophosphamide and fludarabine followed by T cell infusion on four dose levels (DL1: 3×10^7 , 1×10^8 , 3×10^8 , $1 \times 10^9/m^2$ CAR+ cells). Toxicity is monitored using the Common Terminology Criteria of Adverse Events v5. *In vivo* persistence is quantified using quantitative PCR and flow cytometry. Antitumor activity of CAR T cells is defined at week 4-6 post-infusion by standard 3D imaging and quantification of changes in serum alpha-fetoprotein (AFP) levels. **Results:** We infused $3 \times 10^7/m^2$ 15.GPC3-CAR T cells into a 20-year-old male with relapsed, therapy resistant, metastatic HCC. We detected robust expansion of lymphocytes that peaked at week 2 as measured via Quantitative PCR (9.7×10^9 CAR transgene copies/ml) and flow cytometry (2935.6 CAR+ T cells/ μ L; Fig 1). Expression of the CAR alone or together with iC9.IL15, but not iC9.IL15 alone,

resulted in increase of transduced T cells in blood. This rapid CAR T cell expansion was associated with grade 4 cytokine release syndrome which was not controlled by IL6, IL1 or Tumor necrosis factor-alpha inhibition, but was rapidly relieved by a reduced dose (0.04 mg/kg) of rimiducid, activator of iC9. At week 4 post-infusion, the primary liver mass and multifocal lung metastases showed marked decrease in size corresponding with a 94% decrease in serum AFP (Fig 2). **Conclusions:** GPC3-CAR T cells are safe and well tolerated. Results from patient 1 on the AGAR study suggest that co-expression of IL15 with GPC3-CAR in T cells can induce robust expansion and antitumor activity of transduced effectors and that associated toxicities can be mitigated with partial iC9 activation selectively removing the IL15 co-expressing T cell subset.

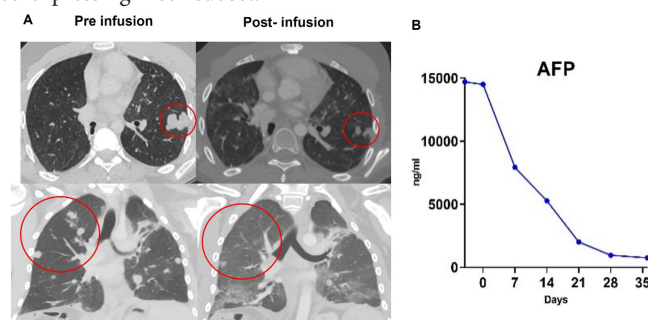


Figure 2: Treatment efficacy of 15.GPC3-CAR T cells in AGAR Patient 1. A. Chest CT pre- and four weeks post-infusion show responses of lung metastases. B. Serum AFP measured at indicated time points post-infusion, day 0 = CAR T cell infusion.

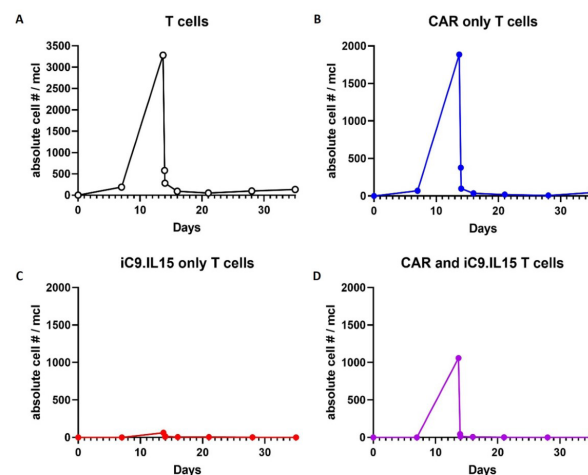


Figure 1: Expansion of 15.GPC3-CAR T cells *in vivo*: Frequency of CAR T cells was quantified by flow cytometry at indicated timepoints and absolute values were calculated from absolute lymphocyte count measured as part of complete blood count. A. CD3+ T cells; B. GPC3-CAR T cells; C. iC9.IL15 T cells (measured by the expression of truncated neural growth factor receptor including the transgene and D. GPC3-CAR and iC9.IL15 dual transduced T cells.

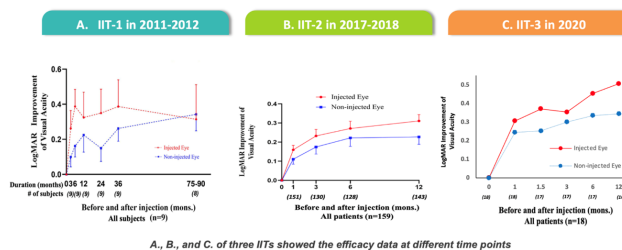
791. Safety and Efficacy of First China-Manufactured Gene Replacement for Leber's Hereditary Optic Neuropathy

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Purpose: There is no effective treatment for Leber's hereditary optic neuropathy (LHON), a mitochondrially inherited disease characterized by acute or subacute painless central vision loss in young adults. We investigated the safety and efficacy of intravitreal (IVT) administration of a recombinant adeno-associated viral vector serotype 2 expressing the human *ND4* codon-optimized gene (rAAV2-*ND4*) in a total of three investigator-initiated trials (IITs). Here, we report these IITs supporting a seamless Phase 1/2/3 registrational trial of rAAV2-*ND4* (NR082) with manufacturing improvement. **Methods:** From 2011 to 2020, 186 subjects aged between 6 and 60 years old with LHON carrying the *ND4*-G11778A mutation (*ND4*-LHON) were enrolled in three prospective, open-label gene therapy trials (NCT01267422, NCT03153293, and CTR2000038570) evaluating the safety, tolerability, and efficacy of a single 1.5×10^9 vector genome (vg) to 1.0×10^{10} vg/eye unilateral IVT injection of rAAV2-*ND4*. The primary endpoints were the incidence of adverse events (AEs) or serious adverse events (SAEs) with some patients being followed up to 90 months post-treatment. The main secondary endpoint was change from baseline in best-corrected visual acuity (BCVA) quantified by using the LogMAR chart test when compared to various timepoints. **Results:** Of the 168 evaluable subjects who have been followed up between 12 months and up to 90 months in these three IITs, no AEs such as uveitis, vitreous inflammation, keratitis, crystal damage and immune response were observed. There were no drug-related SAEs reported. 99 out of 168 evaluable subjects (59%) maintained clinically significant (defined as ≥ 0.3 LogMAR, three lines for a total of 15 letters) BCVA improvement of the injected eyes after 12 months of treatment. The third IIT using improved manufacturing triple-transfection process demonstrated 12 out of 16 evaluable subjects (75%) had ≥ 0.3 LogMAR BCVA improvement at the 12-month follow-up visit. **Conclusions:** A total of 186 *ND4*-LHON subjects (including 10 subjects from Argentina) achieved consistent safety and efficacy across ethnicities after receiving the IVT injection of rAAV2-*ND4*, and no drug-related SAEs were observed among 3 IITs conducted in over 10 years. With a follow-up period of up to 90 months, no late toxic effect from the therapy were reported. rAAV2-*ND4* gene therapy in *ND4*-LHON subjects resulted in sustained, clinically significant BCVA improvement. This China-manufactured rAAV2-*ND4* gene therapy product was granted orphan drug designation for the treatment of LHON by US FDA and EMA. A seamless Phase 1/2/3 clinical trial IND of rAAV2-*ND4* (NR082) has been cleared by the China National Medical Products Administration. Recently, NR082 IND was also cleared by the FDA.

Figure 1: BCVA Improvement After rAAV2-*ND4* IVT Injection in Three IITs



792. First Gene Transfer Clinical Study in Leber's Hereditary Optic Neuropathy Associated with Mitochondrial *ND1* G3460A Mutation

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Purpose: Leber's hereditary optic neuropathy (LHON) is a common inherited disease caused by mutation in the mitochondrial DNA (mtDNA) that manifests with progressive degeneration of the optic nerves, leading to central vision loss. The G3460A mutation in *ND1* gene accounts for about 13% of all LHON cases. Previously, we showed that NFS-02 (a recombinant adeno-associated viral vector serotype 2 expressing human mitochondrial *ND1* codon-optimized gene; rAAV2-*ND1*) with higher translation efficiency in *in-vitro* model. Dose-dependent *ND1* expression in the retinal ganglion cells (RGCs) of the rabbit retinas were observed after 6 months of the NFS-02 intravitreal (IVT) injection at 5.0×10^9 vg/eye with no adverse effect. NFS-02 is based on allotopic expression of the codon-optimized *ND1* gene in the nucleus of the RGC, which leads to the production of the *ND1* proteins that redirected into the patient's RGC mitochondria. Here, we report the first AAV-*ND1* clinical study evaluating the safety, tolerability, and efficacy of NFS-02 for the treatment of LHON associated with *ND1* mutation. **Methods:** Using the same approach of the *ND4* gene replacement therapy for *ND1*, 12 subjects aged between 5 and 65 years old with LHON carrying the *ND1*-G3460A mutation (*ND1*-LHON) were enrolled in this prospective, open-label gene therapy trial (CTR2000041574) of a single 1.5×10^8 vector genome (vg) and 1.5×10^9 vg in 0.05 mL/eye unilateral IVT injection of rAAV2-*ND1* (NFS-02). The incidence of adverse events or serious adverse events, including liver and kidney function in plasma, best-corrected visual acuity (BCVA) quantified by using the LogMAR chart test, optical coherence tomography (OCT), computerized visual field, and retinal nerve fiber layer (RNFL) were compared before and after treatment at 1-, 3-, 6-, and 12-month intervals. **Results:** Six *ND1*-LHON subjects were enrolled at each dose cohort for a total of 12 subjects. The mean age of these subjects was 24.17 ± 5.61 years [Min: 13; Max: 33]. None of the subjects in this study reported drug-related serious adverse events. While the study is ongoing and has not reached 12-month post-treatment timepoint, the preliminary data based on 6-month follow-up visit suggested that 2 out of 6 subjects (33.3%) at 1.5×10^8 vg/0.05 mL/eye and 4 out of 6 subjects (66.7%) at 1.5×10^9 vg/0.05 mL/

eye reached clinically significant BCVA improvement (defined as ≥ 0.3 LogMAR, three lines for a total of 15 letters). Bilateral improvement of BCVA was also observed after the unilateral IVT injection of NFS-02; a similar contralateral effect was also reported in three investigator-initiated trials of ND4-LHON subjects in China by our team and four other comparable AAV-ND4 clinical trials developed for ND4-LHON subjects in the United States and Europe. **Conclusions:** Similar to the previous reporting of three China rAAV2-ND4 gene therapy clinical trials in 186 subjects, we found that a single IVT injection of NFS-02 (rAAV2-ND1 gene replacement therapy) in ND1-LHON subjects also demonstrated consistent BCVA improvement without acute toxicity. This first AAV-ND1 clinical study validates the potential of gene replacement therapy as a promising treatment for LHON. Recently, NFS-02 has been granted orphan drug designation for the treatment of LHON associated with ND1 mutation by the US FDA.

793. A Phase 1 Trial of Targeted DSG3-CAART Cell Therapy in Mucosal-Dominant Pemphigus Vulgaris (mPV) Patients: Early Cohort Data

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Background: mPV is a painful blistering mucosal disease mediated by anti-desmoglein 3 (DSG3) autoantibodies (Abs). Standard of care including rituximab, steroids and other immunosuppressive agents is not curative, requires chronic administration and is associated with serious infections due to persistent immunosuppression. The ideal therapy would selectively eliminate pathogenic anti-DSG3-expressing B cells while sparing healthy B cells. Based on the long-lasting remission of B cell cancers with chimeric antigen receptor T (CART) cells, we developed chimeric autoantibody receptor T (CAART) cells to target B cell-mediated autoimmune diseases, using the same construct but switching the targeting domain to the autoantigen. For mPV patients, autologous T cells have been genetically modified to express the DSG3 autoantigen (DSG3-CAART) and target only the anti-DSG3 B cells. **Methods:** The objective of this ongoing Phase 1 open-label trial (NCT04422912) is to determine the maximum tolerated dose of DSG3-CAART in adult subjects with active, anti-DSG3 Ab positive, biopsy confirmed mPV inadequately managed by ≥ 1 standard therapy. The primary endpoint is incidence of adverse events (AEs), including dose-limiting toxicities (DLTs), such as cytokine release syndrome (CRS) and neurotoxicity, related to DSG3-CAART within 3 months of infusion. Secondary endpoints include CAART persistence (qPCR), anti-DSG3 Ab levels (ELISA) and disease activity (PDAI). After discontinuing or tapering immunosuppressives, subjects in cohorts 1-4 received 2×10^7 , 1×10^8 , 5×10^8 and 2.5×10^9 DSG3-CAAR-transduced cells as fractionated infusions and without lymphodepletion. **Results:** 12 subjects, 3 per cohort, have been treated; 9 have completed 3 months follow up after DSG3-CAART infusion. Characteristics of the 9 subjects at screening (median (range)): 67% female; age 53y (32-70); disease duration 4.3y

(0.4-15.4); PDAI 12 (2-20); anti-DSG3 Ab 104 U/mL (51-169). Prior medications included prednisone (9), rituximab (6) and mycophenolate (6). No DLTs, serious AEs or clinically relevant AEs within 3 months of DSG3-CAART infusion were observed. With cohort 3, there was a dose dependent increase in DSG3-CAART persistence through day 29. Anti-DSG3 Ab levels through month 3 were increased, stable (+/-20%) or decreased in 3, 5 and 1 subjects, respectively. 4/9 subjects stopped or tapered immunosuppressives prior to DSG3-CAART infusion; increased Ab levels and disease activity occurred in subjects who stopped/tapered therapies or received lower doses. Disease activity was clear or almost clear (PDAI 0-1) in 0/9 subjects at screening, 1/9 pre-infusion, 2/9 at month 1, 5/9 at month 2 and 3/9 at month 3 after treatment. **Conclusions:** Early cohort data from the first-in-human trial of DSG3-CAART, a novel investigational precision cell therapy for the autoimmune disease mPV, demonstrate that DSG3-CAART was well-tolerated with no CRS/neurotoxicity or clinically relevant AEs. Dose-dependent increase in persistence indicates that DSG3-CAART cells were not eliminated by soluble anti-DSG3 Ab; peak persistence in cohort 3 was 10-1000x lower than observed with CART therapy in B cell cancers, likely due to differences in target frequency and lymphodepletion. The favorable DSG3-CAART safety profile and absence of DLTs allow the study to evaluate higher doses and a manufacturing enhancement, intended to increase in vivo presence of DSG3-CAART, to reach deep and durable remission for mPV patients without generalized immunosuppression.

794. IND-Enabling Studies Towards an Open Phase I/IIa Trial to Evaluate the Safety and Efficacy of Anti-HIV DuoCAR-T Cell Therapy

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Anti-HIV chimeric antigen receptor (CAR) T cell therapies are candidates to functionally cure HIV infection in people with HIV (PWH). Our previous preclinical studies demonstrated the potent therapeutic efficacy of anti-HIV duoCAR-T cell therapy against broad strains of HIV and its ability to eliminate HIV-infected cells and protect against CD4⁺ T cell loss in humanized mice with HIV. To this end, IND-enabling studies have led to initiation of a phase I/IIa clinical trial to evaluate the safety and efficacy of anti-HIV duoCAR-T cell therapy in PWH (NCT04648046). To participate in the study, ART-suppressed individuals with HIV must be between the ages of 18 and 65 and have a CD4⁺ T cell count of greater than or equal to 500 cells/mm³ at the time of screening to satisfy eligibility requirements. The primary objectives of the trial are to assess safety, tolerability, and efficacy. The secondary objectives are to evaluate persistence and biodistribution of anti-HIV duoCAR-T cells as well as their impact on the HIV reservoir. In preparation for the clinical trial, we developed and validated a GMP-complaint, 8-day CAR-T cell manufacturing process. Here,

we demonstrate our ability to successfully manufacture high-quality anti-HIV duoCAR-T cells at clinical scale from the T cells of PWH. CyTOF analyses of pre-infusion duoCAR-T cell products revealed a predominant CCR7⁺ stem cell-like/central memory (T_{SCM}⁺/T_{CM}⁺) phenotype with some effector-like characteristics consistent with the product's in vivo anti-HIV effector function. In conclusion, this work demonstrates our clinical readiness and further supports translation of anti-HIV duoCAR-T cell therapy to PWH in our presently open clinical trial.

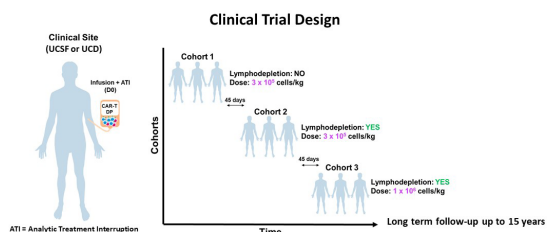


Figure 1. Clinical trial design for the anti-HIV duoCAR-T cell therapy study

795. High-Resolution Cellular and Molecular Follow-Up of Lysosomal Disease Patients Treated with Autologous Hematopoietic Stem Cell Lentiviral Gene Therapy

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AVROBIO is developing investigational therapies to treat Lysosomal disease (LD) patients with *ex vivo* lentiviral (LV) gene therapy (GT) using Plerixafor and G-CSF mobilized peripheral blood (PB) CD34⁺ cells combined with precision busulfan conditioning. Monitoring the dynamics and stability of the engineered cells after transplant is crucial for assessing safety and efficacy of LV GT. Standard cellular and molecular follow up in safety studies are mostly based on the analysis of bulk populations providing valuable but often insufficient information on the dynamics of LV GT in patients. To overcome these limitations, we have developed a high-throughput platform based on the combination of high-resolution cellular and molecular assays that allow tracking of the clonal stability of engineered cells, with unique granularity, across multiple stages of hematopoietic differentiation. We designed and conducted exploratory studies on bone marrow (BM) and PB samples from LD patients before and after LV GT, and analyzed the composition of the whole white blood cells in both BM and PB at baseline and at timepoints after GT, by multicolor flow cytometry. We also developed a high-resolution sorting by FACS to isolate with high purity (average purity 95%) multiple cell subsets from both BM and PB. After a pre-selection by ficoll-gradient and microbeads, BM CD34⁺ cells went through a three-step FACS-sorting for the isolation of 9 subpopulations. BM CD34⁻ cells were also sorted into 6 different lineages for a total of 15 BM subsets isolated from a single BM sample at multiple differentiation stages. A two-step FACS-sorting

was then used for the isolation of 7 PB mature cell types. We ran cell subsets composed as low as a few hundred cells through a custom pipeline for ddPCR for vector copy number evaluation and integration site (IS) analysis, which allowed us to monitor the stability of the engrafted drug product in its main niche, as well as along different lineages of the hematopoietic reconstitution. Notably, because we were able to isolate baso/eosinophils from BM and PB, we have a surrogate marker for measuring the previously inaccessible clonal output of genetically engineered megakaryoid/erythroid precursors. In the BM, we analyzed, for the first time, the cellular composition of LD patients, and included the sialomucin CD164 (from Pellin, Loperfido et al. Nat Commun 2019) as a novel complementary marker for defining the content of primitive vs. committed progenitors within the CD34⁺ cell population before and after LV GT. Using insertional clonal tracking data, we could combine 3 separate methods for abundance estimation and non-linear regression models, to explore the dynamics of each blood cell population after GT. We then designed network analyses to estimate progenitor cell clonal output by means of IS sharing based on the most recent concepts on hematopoietic hierarchies. Lastly, we implemented high-throughput scRNA-Seq analyses of BM CD34⁺ cells isolated before and after LV GT (to date 132,513 single-cell transcriptomes), achieving a unique granular view of hematopoietic progenitor states before and after transplantation as well as providing the first look into the composition of hematopoietic progenitors in different LD backgrounds. In conclusion, we developed a novel analytical platform for investigating, at high-resolution, the fate and activity of genetically engineered CD34⁺ cells and their progeny after LV GT, and in the process, generated new information on the composition of the hematopoietic system in LD patients.

796. Safety and Preliminary Clinical Findings of Neurosurgical AAV2-GDNF Delivery for Parkinson's Disease

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Introduction: Glial cell line-derived neurotrophic factor (GDNF) has been pursued as a disease altering treatment for Parkinson's disease (PD) because of its established role in the development and maintenance of dopaminergic neurons. Optimization of delivery and ability to maintain therapeutic GDNF levels in targeted brain regions have been challenges in earlier studies. Improvements in gene therapy infusion techniques, such as increased infusion volumes and intraoperative MRI-monitored convection-enhanced delivery (CED), may address these limitations. **Methods:** This ongoing open-label Phase 1b study examines the safety of neurosurgical AAV2-GDNF gene delivery and preliminary clinical impact on PD symptomatology, assessed by the MDS Unified Parkinson's Disease Rating Scale (MDS-

UPDRS) Part III motor examination and levodopa equivalent dose (LED). Six participants diagnosed with early-stage (<5 years; MDS-UPDRS III OFF <32) and 4 participants with moderate-stage (>4 years; MDS-UPDRS III 33-60) PD received up to 1.8mL of AAV2-GDNF (3.0E+12 vg/mL)/gadoteridol (2mM ProHance) co-infusions per hemisphere utilizing iMRI-monitored CED to target >50% of each putamen. Brain MRI scheduled 6 months post-treatment were qualitatively reviewed for treatment emergent changes. MDS-UPDRS III motor scores are reported as mean absolute change from baseline.

Results: All 10 participants tolerated the neurosurgical procedure well, were discharged home one day post-operatively and have completed >9 months of clinical follow up. Putaminal coverage was 62.5% ± 2.4% and comparable in both cohorts. No AAV2-GDNF associated SAEs have been reported. One asymptomatic procedure-related SAE was identified on a scheduled 6-month MRI scan as a small, well-circumscribed area of T1 hypointensity adjacent to a single infusion site and remains without clinical sequelae. All other scheduled 6-month MRIs demonstrated anticipated residual cannula tracks and expected putaminal T2 hyperintensities. Most AEs were transient, occurred peri-operatively, or were associated with underlying PD. No significant changes in LED requirement at 6 months reported for either cohort. **Early Cohort:** PD duration from diagnosis of 2.1 ± 0.6 years. Baseline UPDRS III OFF score 19.5 ± 3.3 points (pts), UPDRS III ON score 7.7 ± 0.99 pts, with an LED of 541mg/day. Six-month post-treatment assessments showed relative stability with UPDRS III change of -2.7 ± 2.3 pts in the OFF state, and -1.6 ± 1.2 pts in the ON state. **Moderate Cohort:** PD duration from diagnosis of 8.3 ± 0.7 years. Baseline UPDRS III OFF score 41.5 ± 3.5 pts, and UPDRS III ON score 24.0 ± 3.4 pts, with an LED of 840mg/day. Six-month UPDRS III showed improvement of -13.0 ± 3.7 pts in the OFF state and -6.8 ± 5.4 pts in the ON state. Twelve-month data from both cohorts is pending and will be presented. **Conclusions:** Intrapataminal, iMRI-guided CED infusions of AAV2-GDNF were well-tolerated by participants with both early- and moderate-stage PD. Asymptomatic, procedure-related radiographic findings were identified at 6 months without clinical sequelae. Although interpretation of clinical outcomes in small open-label studies is limited by the placebo effect, we believe these results showing stability of UPDRS III OFF/ON scores in the Early Cohort, and improvements in the Moderate Cohort are encouraging. Ongoing clinical follow-up and planned controlled studies are needed to confirm these early clinical observations.

797. Abstract Withdrawn

798. STEADFAST: A First-in-Human Study Assessing HLA-A02-Chimeric Antigen Receptor Regulatory T Cells in Renal Transplantation

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Renal transplantation is a lifesaving therapy for patients with end-stage renal disease (ESRD) but requires lifelong immunosuppressive treatment to prevent rejection of the graft. However, immunosuppressive

therapy is frequently associated with adverse effects such as a high risk of infections, cardiovascular disease, malignancy and nephrotoxicity. Therefore, the creation of a tolerogenic environment, enabling reduction of immunosuppressive therapy whilst maintaining allograft acceptance, is an important goal in transplantation medicine. TX200-TR101 is a genetically modified cell therapy product consisting of *ex vivo* expanded autologous naïve regulatory T cells genetically modified to express a chimeric antigen receptor (CAR) specific to the mismatched donor HLA-A02 antigen on their surface cell membrane of the graft. The CAR is composed of a single-chain variable fragment (ScFv) from a humanized antibody that recognises the HLA-A02 antigen, coupled to a transmembrane domain and the activation domain of human CD28 and human CD3 zeta. The lentiviral vector used for the transduction of the Tregs is based on a third-generation, self-inactivating (SIN) lentiviral vector. In preclinical testing, it was demonstrated that HLA-A2 CAR-Tregs (TX200-TR101) can be specifically activated through their HLA-A2 CAR, exert antigen-specific immunological suppression, proliferate *in vitro*, and maintain a stable (functional) phenotype. *In vivo* mouse model data further provided evidence that TX200-TR101 was able to efficiently inhibit human peripheral blood mononuclear cell (PBMC) engraftment, as well as onset of Graft-versus-Host Disease (GvHD) in NSG mice. TX200-TR101 was also able to release immune-modulatory cytokines, which shows its potential to lead to a tolerogenic environment. In this First-in-Human (FIH) phase I/II study, HLA-A02 negative transplant recipients awaiting a kidney graft from an HLA-A02 positive, living donor will undergo leukapheresis to collect autologous Tregs for the manufacture of TX200-TR101. Several weeks after transplantation TX200-TR101 will be administered via a single intravenous infusion. It is planned to test 3 weight-adjusted doses of TX200-TR101 in single ascending dose-cohorts. A small control cohort of HLA-mismatched transplant recipients with similar immunological risk will also be recruited. All transplant recipients will be followed up for 84 weeks post-transplant. In addition, transplant recipients will have the opportunity to enter a separate study afterwards in order to account for a total of 15 years follow-up post TX200-TR101 administration, according to regulatory guidelines for lentiviral gene-modified cell therapy products. The primary endpoint of the study is the incidence and severity of treatment-emergent adverse events within 28 days post-TX200-TR101 infusion. Other endpoints include additional safety parameters, as well as clinical and renal outcome parameters, with endpoints regarding graft dysfunction or rejection based on established diagnostic methods including biopsies at pre-specified timepoints. The evaluation of a range of biomarkers will determine the localisation of TX200-TR101 in the graft and the effects on graft and peripheral immune responses. The primary goal of this FIH trial is to establish a safe and tolerable dose of TX200-TR101 for subsequent clinical testing. TX200-TR101 holds great promise to prevent immune-mediated graft rejection and induce immunological tolerance following HLA-A02-mismatched renal transplantation. This FIH study is currently enrolling patients at several centres in Europe, and is expected to be the setting for the first ever dosing of a human with a CAR-Treg investigational gene-modified cell therapy product.

799. A Phase I Clinical Trial of Osteoarthritis Gene Therapy (NCT02790723)

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INTRODUCTION Local gene delivery to joints is a promising approach to treating arthritis and other arthropathies. Not only does this strategy overcome the pharmacokinetic barriers to intra-articular therapy but, by targeting individual joints, it also lowers the amount vector administered thereby increasing safety and reducing manufacturing cost and complexity. Osteoarthritis (OA) of the knee has few effective therapies, is associated with high morbidity and, as the leading cause of disability, represents a major burden on public health. Interleukin 1 (IL-1) has been implicated by a number of biochemical, genetic, and animal model studies as a major contributor to OA. Here we report data from a Phase I clinical trial in mild-to-moderate OA of the knee using an intra-articular AAV vector encoding the human interleukin-1 receptor antagonist (IL-1Ra) (NCT02790723). **STUDY DESIGN** Following screening and appropriate informed consent, subjects with mid-stage OA of the knee (Kellgren-Lawrence score 2 or 3) were treated with a single dose of 10^{11} vg, 10^{12} vg, or 10^{13} vg AAV.IL-1Ra (n=9 total subjects; 3 per cohort). Individuals in each cohort received an ultrasound-guided, intra-articular injection of the vector and were monitored for 1 year. The primary outcome measure is safety, defined as an absence of drug-related serious adverse events. Additional measures include levels of circulating viral genomes, immune response to the vector, blood and urine analysis and physical examination. Where possible, synovial fluid was withdrawn from knee joints under ultrasound and IL-1Ra concentrations measured by validated ELISA. Although this study was not powered for efficacy and had no control group, patients reported pain via VAS (0-10) and pain and function via WOMAC. Knee joints were imaged by X-ray and MRI upon study entry and after 1 year. **RESULTS** Intra-articular injection of AAV.IL-1Ra produced no severe adverse events; blood chemistries and hematology remained normal during the 12-month follow-up period, with no evidence of neutropenia. There were no vector-related adverse events in 8 of 9 subjects, whereas one subject experienced a mild/moderate knee effusion following injection which resolved with ice and rest. Subjects developed various degrees of anti-AAV neutralizing antibodies after injection of vector, as seen in pre-clinical studies. Small amounts of viral genomes were found in peripheral blood, beginning one day after injection and clearing within 4 weeks. Injection of vector was associated with increased concentrations of IL-1Ra in synovial fluid. Pain and function scores improved following injection of the vector. **CONCLUSION** The promising results from this phase 1 trial justify further clinical trials of AAV.IL-1Ra for the local gene therapy of OA.

800. The Treatment of Wounds Associated with Recessive Dystrophic Epidermolysis Bullosa with Local Injections of Gene-Corrected, Collagen VII-Expressing Autologous Human Dermal Fibroblasts

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Recessive dystrophic epidermolysis bullosa (RDEB) is a genetic disorder caused by mutations in the COL7A1 gene encoding type VII collagen (COL7). This study examined the feasibility of correcting these mutations by providing a wild type COL7A1 gene. Local injection of COL7-expressing autologous human dermal fibroblasts (D-Fi, also known as FCX-007) led to wound healing in patients with RDEB. Six subjects (9-38 years of age) with various COL7A1 mutations resulting in undetectable COL7 expression and a lack of intact anchoring fibrils enrolled in this Phase 1/2 clinical study. Subject fibroblasts, isolated from their respective skin biopsies, were transduced with a third-generation self-inactivating lentiviral vector encoding the wild type COL7A1 gene, and expanded. Eligible treatment wounds were chronically present prior to treatment and excluded those that had evidence of infection, were previously treated or located on mucous membranes, face, hands and feet. Intradermal injections with gene-corrected fibroblasts were completed on Day 1 and comparator wounds were not treated. Four of the six subjects received a second treatment 4 to 52 weeks after the initial injection; all were to be followed through 52 weeks. At 12 weeks, 80% (8/10) of treated wounds (4.3 cm² to 34.1 cm²) demonstrated complete wound healing, compared to 0% of the untreated comparator wounds. Linear COL7 expression at the dermal-epidermal junction and restoration of AF was confirmed in treated sites. No treatment emergent serious adverse reactions were reported. No replication competent virus nor significant antibody responses to COL7 were detected through 52 weeks. The local injection of gene-corrected autologous fibroblasts to the wounds of RDEB appears to be generally safe, and complete wound healing is typically exhibited. The durability of wound healing needs to be evaluated over a longer period.

801. AAV Intravitreal Optogenetic Therapy for Vision Restoration in Patients with Severe Retinal Dystrophy

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Introduction: Optogenetics therapy offers the potential for vision restoration in advanced retinal dystrophy patients by photosensitizing higher order neurons. Since this approach focuses on disease phenotype versus a specific genotype deficit, it is applicable to a wide diseased

population. Existing tools utilize opsins that are sensitive to narrow band of light and do not generate adequate current in ambient light requiring an external device for stimulation. The objective of this study is to evaluate safety and efficacy of intravitreal AAV Optogenetic therapy targeting ON-bipolar cells. **Methods:** Multi-Characteristic Opsin (MCO) is an engineered opsin that has fast ON and OFF response as well broadband activation spectrum allowing stimulation at different light wavelengths. Further, cells sensitized with MCO are activated at ambient light levels, thereby avoiding the need for an external amplifying device and associated phototoxicity. Red fluorescence reporter (mCherry) was used downstream of MCO to allow visualization of gene transduction. Through the delivery of opsin encoding genes, residual retinal neurons take on the photosensitizing function of the photoreceptors. Targeting bipolar cells with MCO by use of cell-specific promoter-enhancer allows potential for greater spatial resolution. Modified RepCap Plasmid was used to efficiently produce AAV2 and to transduce bipolar cells with intravitreal injection. Advanced retinitis pigmentosa subjects received prophylactic oral steroids prior to a single intravitreal injection of AAV2-MCO (vMCO). Safety and exploratory efficacy of intravitreal vMCO dose escalation were evaluated to identify a safe dose. **Results:** Utilization of mCherry in the active drug product led to enhanced photocurrent as well as assessment of gene transduction. Intravitreal vMCO delivery did not lead to detectable increase in neutralizing antibody level or lymphocyte levels in the serum. vMCO was well tolerated with no reported serious adverse events. Ocular adverse events were limited to inflammation and intraocular pressure rise, which was controlled with topical medications. No patient required topical therapy at the end of the study at 52 weeks. Furthermore, exploratory efficacy endpoints demonstrated improvement in visual function, measured by Freiburg visual acuity, ambulatory mobility tests, and shape discrimination, following a single vMCO dose. The improvement in measured vision correlated well with patient reported outcome (assessed by Visual Function Questionnaire). **Conclusions:** vMCO is well tolerated with no serious adverse events. Higher dose vMCO appears to improve visual acuity and visual function compared to baseline measurements and demonstrates tremendous promise in restoring vision for patients with retinal degeneration or dysfunction of outer retinal cells.

802. 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 ≥ 4 months of age receive one image-guided RGX-111 injection to the CNS with 104 week follow-up for safety, tolerability, and efficacy. Assessments include cerebrospinal fluid, plasma, and urine biomarkers; cognitive, language, and motor neurodevelopmental scales; and imaging. Two subjects have been dosed at Dose 1 and enrollment continues at Dose 2 (1.0×10^{10} and 5.0×10^{10} genome copies/gram brain mass, respectively). Interim results of this trial will be presented. Additionally, two-year 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.

803. Gene Therapy and Newborn Screening: A Resource to Prioritize Candidate Conditions

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For over sixty years, newborn screening (NBS) has enabled the early diagnosis and treatment of genetic disease on a population basis. In the United States, NBS programs list screening panels totaling 80 conditions, 61 of these on a recommended uniform screening panel (RUSP) either as a core or secondary condition. The pipeline of conditions that are candidates for NBS based on novel therapies and new screening methods, including genomics, is growing rapidly. As the technology and availability of gene and cell therapies advance, NBS offers a mechanism to expand screening to potentially thousands of candidate conditions. Recent discussion of NBS expansion has proposed that there are currently hundreds of genetic disorders that could potentially benefit from NBS followed by gene and cell therapy. To help the NBS community prioritize research efforts, we led an effort to identify which conditions are candidates for expansion, and we present a data tool to prioritize the almost 7,000 conditions that are candidates for NBS followed by gene and cell therapy. The Newborn Screening Translational Research Network (NBSTRN) is a key component of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Hunter Kelly Newborn Screening Research Program. The American College of Medical Genetics and Genomics (ACMG) operates the NBSTRN through a contract from NICHD. Our mission is to facilitate newborn screening-related research to discover novel technologies, treatments, and interventions through the development of resources, tools, and expertise. In addition to the 80 conditions screened in the US, there are thousands of rare disorders that may be candidates for NBS. The Newborn Screening Conditions Resource (NBS-CR) provides centralized resource of

facts and statistics on both screened and candidate conditions. The NBS-CR is designed to be an interactive resource for researchers, clinicians, parents, and families to learn more about these disorders, and links to National Library of Medicine (NLM) resources including the National Center for Biotechnology Information (NCBI). NCBI provides access to biomedical and genomic information and maintains MedGen, NCBI's portal to information about human disorders and phenotypes having a genetic component. The tool enables a user to sort conditions by nomination status and RUSP category. Several conditions are considered candidates for RUSP nomination, and the NBS-CR currently includes 37 of these. NBSTRN updated the approach to include eleven additional sources to identify potential candidate conditions and expanded the annotations to a total of twenty-six data points. As gene and cell therapies advance, the NBS-CR will become a useful resource for the research community to prioritize and disseminate efforts.

804. A Randomized Trial to Determine the Efficacy and Safety of FCX-007 for the Treatment of Recessive Dystrophic Epidermolysis Bullosa -DEFI-RDEB

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Recessive dystrophic epidermolysis bullosa (RDEB) is a debilitating genetic disorder caused by mutations of the type VII collagen gene, COL7A1. Mutations of this gene result in a reduction or absence of a functional collagen VII (COL7) protein, the primary component of anchoring fibrils (AFs) in the basement membrane zone between the dermis and epidermis. Absent or reduced AFs results in separation of the epidermis from the dermis in response to minor skin trauma resulting in mechanical fragility of the skin and recurrent blister formation. DEFI-RDEB is a multi-center, randomized, controlled open label Phase 3 study designed to evaluate the safety and efficacy of FCX-007 (also known as D-Fi) for the treatment of RDEB wounds. The overall study design and selected endpoints were chosen after considering results from an earlier Phase 1/2 study demonstrating complete wound healing in 80% of the evaluated subjects. While no serious adverse reactions were reported in that study, the current study is of longer duration which will allow a more complete analysis of long-term benefits and risks. After screening and verification of genetic mutation of COL7A1, fibroblasts are isolated from the subjects' skin biopsies, transduced with a third-generation self-inactivating lentiviral vector encoding the wildtype COL7A1 gene and expanded for local injection. Following a 12-week observation period to characterize the wounds, confirming their persistence and duration, investigators identify up to 3 pairs of wounds ranging from 10-50 cm². One wound in each pair will serve as a target wound for treatment, while the other serves as an untreated control. Autologous gene-corrected, collagen VII-expressing fibroblasts (FCX-007) are injected intradermally in the randomly assigned target wounds on Day 1 and Week 12. Injections are repeated at Weeks 24 and 36 at the investigator's discretion. Efficacy assessments will include evaluation of complete wound closure by a blinded assessor (primary endpoint is serial assessments of first wound pair at Weeks 22 and 24), durability of response, change in wound surface area and pain level (via Wong-Baker FACES[®] Pain Rating Scale). COL7 expression in selected treated wounds will be assessed by

immunofluorescence (IF) and immunoelectron microscopy (IEM) in a subset of subjects. Safety assessments include adverse events (including the presence of neoplasms), replication-competent lentivirus (RCL), COL7 antibody and clinical laboratory testing. Subjects who have received at least one treatment injection will continue in the long-term safety follow-up period through 15 years.

805. A Phase 1, First in Human (FIH) Study of Adenovirally Transduced Autologous Macrophages Engineered to Contain an Anti-HER2 Chimeric Antigen Receptor (CAR) in Subjects with HER2 Overexpressing Solid Tumors

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Background: Adoptive T cell therapies have led to remarkable advances among patients with hematologic malignancies, but with less success in those with solid tumors. Macrophages are actively recruited into, and are abundantly present in the solid tumor microenvironment (sTME). Tumor associated macrophages typically display immunosuppressive behavior. However, macrophages may be an ideal vector to administer adoptive cellular therapy in solid tumors, when engineered to be proinflammatory. Furthermore, insertion of a CAR on the macrophages allow them to selectively recognize and phagocytose antigen overexpressing cancer cells. CAR macrophages reprogram the sTME and present neoantigens to T cells, leading to epitope spreading and immune memory. Human Epidermal Growth Factor Receptor 2 (HER2) overexpression promotes tumorigenesis in many solid tumors (Table 1). CT-0508 is a cell product comprised of autologous monocyte-derived proinflammatory macrophages expressing an anti-HER2 CAR. Pre-clinical studies have shown that CT-0508 induced targeted cancer cell phagocytosis while sparing normal cells, decreased tumor burden, prolonged survival, and were safe and effective in a semi-immunocompetent mouse model of human HER2 overexpressing ovarian cancer. **Methods:** This FIH Phase 1 study is evaluating safety, tolerability, cell manufacturing feasibility, trafficking and preliminary evidence of efficacy of investigational product CT-0508 in 18 subjects with locally advanced (unresectable) or metastatic solid tumors overexpressing HER2, with progression on available therapies, including anti-HER2 therapies when indicated. Filgrastim is being used to mobilize autologous hematopoietic progenitor cells for monocyte collection by apheresis. The CT-0508 CAR macrophage product is manufactured, prepared and cryopreserved from mobilized peripheral blood monocytes. Group 1 subjects receive CT-0508 infusion split over D1, 3 & 5. A Safety Review Committee will review dose limiting toxicities. Group 2 subjects will receive the full CT-0508 infusion on D1. Pre and post treatment biopsies and blood samples will be collected to investigate correlates of safety (immunogenicity), trafficking (PCR,

RNA scope), CT-0508 persistence in blood and in the tumor, target antigen engagement, TME modulation (single cell RNA sequencing), immune response (TCR sequencing) and others.

Tumor	HER2 Overexpression (%)
Bladder	8 - 70
Salivary duct / mucoepidermoid	30 - 40 / 17.6
Gastric	7 - 34
Ovarian	26
Breast	11 - 25
Esophageal	12 - 14
Gallbladder / Cholangiocarcinoma	6.3 - 9
Colorectal	1.6 - 5
Cervical	2.8 - 3.9
Uterine	3
Testicular	2.4

806. CEDAR Trial in Progress: A First in Human, Phase 1/2 Study of the Correction of a Single Nucleotide Mutation in Autologous HSCs (GPH101) to Convert HbS to HbA for Treating Severe Sickle Cell Disease

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Background: SCD is a recessive monogenic disease caused by a single point mutation in the human beta-globin gene (HBB) leading to the production of abnormal globin chains (HbS) that polymerize and cause erythrocytes to sickle. This results in hemolytic anemia, vaso-occlusion and organ damage, which leads to lifelong complications and early mortality. GPH101 is an investigational, autologous, hematopoietic stem cell (HSC) drug product (DP) designed to correct the SCD mutation in the HBB gene *ex vivo* using a high efficiency homology directed gene editing platform combining high fidelity Cas9, AAV6 donor DNA template delivery precomplexed with chemically modified guide RNAs, and stem cell culture optimization. This approach has the potential to restore normal adult hemoglobin (HbA) production while simultaneously reducing HbS levels. **Study Design and Methods:** CEDAR (NCT04819841) is a first-in-human, open-label, single-dose, multi-site Phase 1/2 clinical trial in participants with severe SCD designed to evaluate safety, efficacy and pharmacodynamics (PD) of GPH101. Approximately 15 adult (18-40 years) and adolescent (12-17 years) participants will be enrolled

across 5 sites, with adolescent enrollment proceeding after a favorable assessment of adult safety data by a Safety Monitoring Committee. Participants must have a diagnosis of severe SCD ($\beta\text{S}/\beta\text{S}$), defined as ≥ 4 severe vaso-occlusive crises (VOCs) in the 2 years prior and/or ≥ 2 episodes of acute chest syndrome (ACS), in 2 years prior with at least 1 episode in the past year. Participants on chronic transfusion therapy may be eligible if required VOC and ACS criteria are met in the 2 years prior to the initiation of transfusions. Key exclusion criteria include availability of a 10/10 HLA-matched sibling donor, or prior receipt of HSCT or gene therapy. After eligibility confirmation including screening for pre-treatment cytogenetic abnormalities, participants will undergo plerixafor mobilization and apheresis, followed by CD34+ cell enrichment and cryopreservation, undertaken locally at each trial site before shipment to a centralized manufacturer for GPH101 production. After GPH101 release, participants will undergo eligibility reconfirmation prior to busulfan conditioning and DP infusion. The primary endpoint for this study is safety, measured by the kinetics of HSC engraftment, transplant related mortality, overall survival and frequency and severity of adverse events. Secondary endpoints will explore efficacy and pharmacodynamics, including levels of globin expression as compared to baseline, gene correction rates, clinical manifestations of SCD (including VOC and ACS), and changes in packed red blood cell transfusion needs. Key exploratory endpoints include evaluation of patient-reported outcomes, erythrocyte function, characterization of gene correction rates, and change from baseline in select SCD characteristics and organ function.

807. An Update on LYS-SAF302 Gene Therapy Study (AAVance) in Children with Mucopolysaccharidosis Type IIIA

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Mucopolysaccharidosis type IIIA (MPS IIIA) or Sanfilippo syndrome type A (OMIM 252900) leads to early-onset neurodegeneration and premature death. AAVance is a Phase 2-3, single-arm, international study of AAVrh.10 carrying the human SGSH cDNA (LYS-SAF302, olesanasufligene relduparvovec), delivered directly to the brain via intraparenchymal infusion, for the treatment of MPS IIIA (NCT03612869). Eight clinical sites in the US and Europe are participating in this trial. The trial is fully enrolled, with 19 subjects dosed from February 2019 to March 2020. The primary objective of AAVance is to assess the drug efficacy in improving or stabilizing the neurodevelopmental status of patients compared to the expected

evolution based on natural history data. Safety, tolerability, biomarkers, effect on behavior, sleep and quality of life are secondary endpoints. An update on the status of the study will be presented.

Presidential Symposium and Presentation of Top Abstracts

808. Short- and Long-Term Hematopoietic Reconstitution After Transplantation is Stem Cell-Driven and Stochastic: Implications for Gene Therapy

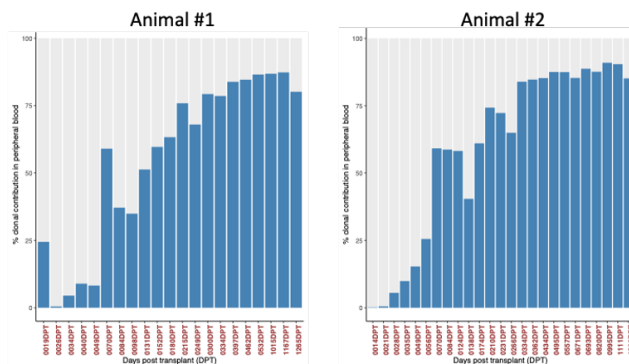
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Recovery after hematopoietic stem cell (HSC) transplantation is thought to be bi-phasic, with short-term progenitors driving recovery within the first 6-9 months and multipotent HSCs providing long-term repopulation. This model has been presumed for decades, with recent data from human gene therapy trials using retroviral integration site analysis (ISA) to track gene-marked cells seeming to confirm this concept (Biasco et al. 2016, Cell Stem Cell; Six et al. 2020, Blood). While ISA is reliably detecting highly abundant clones, low sensitivity and high error rates require significant data exclusion and sophisticated statistical tests to ensure data reliability (Adair et al. 2020, Molecular Therapy MCD). Lack of sensitivity and loss of low abundance clones can be overcome by increasing the frequency of sampling (high density). However, limited material available from patients does not allow such in depth analysis. Here, we performed high-density sampling for ISA in nonhuman primates (NHPs) where we can perform the necessary in-depth analysis of samples and thus overcome the limitations of ISA in patients. We followed the animals for 4.5 years to collect HSC clone signatures and identify HSC contribution in very early samples to determine the onset of HSC contribution and investigate the kinetics of hematopoietic recovery. Finally, observed changes in clonal diversity *in vivo* were used to inform a simulation of hematopoietic reconstitution to determine the temporal involvement of HSCs. In contrast to the current biphasic model, contribution of multipotent HSCs clones was detected during neutrophil recovery. Most surprisingly, multipotent HSCs became the dominant source for mature blood cells in the peripheral blood as early as 50 days post-transplant. Simulations predicted that hematopoietic recovery is primarily HSC driven and HSC contribution follows a stochastic pattern. Finally, to confirm that HSCs proliferation and differentiation is a stochastic process, *in vitro* experiments in colony-forming cell (CFC) assays were carried out. As predicted, the decision of individual HSCs to either grow into a larger pool or differentiate and get lost followed the same kinetics as observed *in vivo*. Here, we show evidence that hematopoietic reconstitution after myeloablative conditioning and transplantation is entirely driven by HSCs. Most importantly, observed changes in the clonal diversity during early recovery suggest a stochastic HSC engraftment and differentiation pattern, rather than a bi-phasic reconstitution initially

driven by short-term progenitors. Early blood production is driven by HSC clones that are not predetermined but randomly contribute to a limited number of lineages before they disappear - a population of cells previously incorrectly identified as lineage-restricted short-term progenitors. These findings have major implications for HSC transplantation and particularly for HSC gene therapy. The ability to directly modify a highly enriched CD34+CD90+ cell population to accomplish both short and long-term engraftment would substantially reduce the typically large and expensive amount of vectors or editing agents needed for the genetic engineering of HSCs.

Contribution of multipotent 5 lineage clones (blue) to the peripheral blood



809. Correction of the Sickle Cell Mutation by *In Vivo* HSC Prime Editing in a Mouse Model

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We developed a new *in vivo* hematopoietic stem cell (HSC) gene therapy approach that does not require myeloablation/conditioning and HSC transplantation. In this approach, HSCs are mobilized from the bone marrow (BM) into the peripheral bloodstream 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 BM and spleen and persist there long-term. At this stage, expansion of gene-modified HSPCs and high-level marking in blood cells are achieved by *in vivo* selection using an *mgmt*^{P140K} gene that confers resistance to low dose- methylating drugs such as O⁶BG+BCNU. Here, we used this approach for *in vivo* HSC gene therapy of sickle cell disease (SCD). SCD is a monogenic disease caused by a single nucleotide mutation in the beta-globin gene. Current gene therapy studies are mainly focused on lentivirus vector-mediated gene addition or CRISPR/Cas9-mediated fetal globin reactivation, leaving the root cause unfixed. We developed a prime editing system that is delivered *in vivo* to HSCs by helper-dependent adenovirus (HDAd5/35++) vectors and that can directly repair the A>T sickle mutation. We designed and produced a panel of HDAd vectors expressing various versions of prime editors (PE3, PE3max, PE5max). Among these vectors, HDAd-PE5max with engineered guide RNA sequences showed the highest activity in cell lines and lineage-minus (Lin⁻) cells from a murine model of SCD (Townes/CD46). *Ex vivo* transduction of Lin⁻ cells from Townes/CD46 mice followed by

transplantation into irradiated recipient mice led to over 90% bi-allelic correction of the sickle cell mutation (GTG>GAA; V6>E) in peripheral blood cells. In an *in vivo* HSC transduction setting, involving HSC mobilization by G-CSF/AMD3100, intravenous injection with HDAd-PE5max, and short-term selection with low doses of O⁶BG/BCNU, an average of 31% (range 17-46%) correction of the sickle mutation was achieved. Deep sequencing of week 16 BM samples revealed that most of the edited alleles contained the expected GTG>GAA conversion. Less than 2% byproducts (mostly short deletions) around the guide RNA-targeting region were detected, consistent with the property of prime editors to function independently of double-stranded DNA breaks. Correction of phenotypic SCD hallmarks were observed in treated Townes/CD46 mice, including reticulocyte counts, spleen sizes, hematological and histological parameters (iron deposition and extramedullary hematopoiesis). No obvious adverse effects related to *in vivo* transduction/selection were observed. 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 28.1% and 12.5% of colony-forming stem cell progenitors had monoallelic and biallelic correction, respectively. The editing levels remained stable at week 8 after transplantation (ongoing). We are currently analyzing genome-wide off-target effects by CIRCLE-Seq and investigating the activity of our vectors in patient CD34⁺ cells. For the first time, we report here vectorized prime editors for *in vivo* HSC genome editing. Our 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.

810. Generation of Islet-Specific Engineered Regulatory T Cells (EngTregs) for Immune Tolerance Induction in Type 1 Diabetes Using a Novel Dual-Editing Strategy

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Adoptive regulatory T-Cell (T_{reg}) therapies represent a potentially transformative cell-based therapy to promote immune tolerance following stem cell or solid organ transplantation, and in autoimmune diseases including type 1 diabetes (T1D). Key technical hurdles, however, may limit broad clinical application including the rarity of natural T_{reg}, the requirement for antigen-specificity to modulate tissue-specific disorders, and requirement to compete *in vivo* for engraftment and survival. To address all three challenges, we have developed a dual-HDR-based gene editing approach to simultaneously induce constitutive expression of the T_{reg} master transcriptional regulator, FOXP3, and replace the endogenous T-cell receptor (TCR) with a tissue-specific TCR. As previously described, this approach introduces a ubiquitous promoter (MND) into the FOXP3 locus immediately downstream of the T_{reg}-specific demethylated region (TSDR), resulting in high level FOXP3 expression leading to a stable T_{reg} phenotype and function. Concurrently, editing within the first coding exon of the TRAC locus deletes the endogenous TCR and replaces it with a defined TCR. Finally, to simultaneously select for

cells with successful integration of both HDR templates (in vitro and in vivo), we designed donor cassettes also containing a heterodimeric, chemically-induced signaling complex (CISC) that mimics IL-2 signaling in response to an exogenous dimerizer. In this study, we focused on generation of engineered T_{reg} (EngTregs) products for prevention and/or treatment of T1D. We utilized a TCR specific for the islet antigen, IGRP, previously identified from clonally expanded CD4⁺ Teff cells in a T1D patient. Using CRISPR-based tools, we observed initial FOXP3/TRAC dual-editing rates ranging from 2% to 15% in healthy donor derived CD4⁺ T cells. Following expansion in dimerizer (rapamycin), dual-edited cells were enriched to >85% purity based on expression of both FOXP3 and the islet TCR. Droplet digital PCR analysis before and after rapamycin enrichment confirmed positive selection for each on-target editing event, and negative selection for low-frequency chromosomal translocations (resulting from double-strand breaks at both chromosomes X and 14). Enriched cells were cryopreserved, and subsequent analysis demonstrated a robust T_{reg} immunophenotype and secretome switch from pro-inflammatory to immunosuppressive cytokines. Functionally, dual-edited Ag-specific EngTregs strongly suppressed the activation and proliferation of T_{eff} cells expressing a matched islet TCR demonstrating potent direct suppression. Importantly, Ag-specific EngTregs also suppressed T_{eff} cells expressing a different islet TCR in a mixed activation co-culture, demonstrating significant bystander suppression. Thus, dual-edited, Ag-specific, CISC expressing EngTregs represent a promising cell-based therapy for T1D simultaneously predicted to mediate tissue-specific bystander suppression and CISC-mediated enhanced *in vivo* survival and engraftment. Finally, this dual-editing platform is readily adaptable for use with alternative TCR (or CAR) cassettes designed to target a range of tissue-specific autoimmune or inflammatory diseases.

811. AAV-Meganuclease-Mediated Gene Targeting Achieves Efficient and Sustained Transduction in Newborn and Infant Macaque Liver

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We describe a genome-editing approach for treating ornithine transcarbamylase deficiency (OTCD), which can cause lethal episodes of hyperammonemia in infancy. The goal of genome editing is for the therapeutic effect to be durable and achievable in all OTCD patients, independent of their mutation. We propose to accomplish this goal by treating surviving newborns with two AAV vectors; one to deliver a nuclease to create a double-stranded break in a safe harbor site and a second to deliver an OTC minigene for knock-in at this site. Our assumption is that dividing hepatocytes of the newborn liver will be conducive to efficient knock-in of the OTC gene. Furthermore, this process will eliminate the non-integrated input vector genomes during liver proliferation. We used the PCSK9 gene as a safe harbor site and a

PCSK9-specific ARCUS meganuclease to target the site. This strategy was informed by our previous work in adult macaques, which showed safe, efficient, and stable reductions of PCSK9 following AAV delivery of ARCUS. We performed the initial studies of genome editing for OTCD in an OTC-deficient mouse that was rendered susceptible to PCSK9 ARCUS through germ-line modification of exon 7 of the endogenous *Pcsk9* gene. Injecting the two vectors into newborn mice efficiently knocked-in the human *OTC* minigene and protected against lethal hyperammonemia when challenged with a high-protein diet. In preparation for clinical studies, we evaluated key safety and efficacy parameters in newborn and infant macaques. A total of 24 animals were treated with AAV vectors. Analyses included examination of liver biopsies at 3 and 12 months. In these studies, we evaluated the impact of the following parameters on editing efficiency and toxicity: transgene (human factor IX and human *OTC*), promoters driving ARCUS nuclease expression, Clade E capsids, length of donor flanking the transgene, and age of the macaque at time of dosing. Here, we report preliminary data of 16/24 animals that includes, at a minimum, 3-month biopsy results. We found that injecting AAV vectors was well tolerated with no evidence of transaminase elevations or liver histopathology in any ARCUS-treated animals. The key measure of efficacy in the primate model is transduction efficiency measured by *in situ* hybridization and immunostaining to detect cells expressing the human *FIX* or *OTC* mRNA and protein, respectively. We obtained the highest and most consistent results with vectors using a novel clade E capsid driving ARCUS with a TBG promoter in the first vector and 500 bp flanking homology arms on the donor vector. With this combination, we achieved $11.3 \pm 6.6\%$ (N=6) transduction efficiency in hepatocytes, which is higher than the ~5% OTC-expressing cell threshold for substantially benefitting patients. Preliminary data suggests that the level of editing is stable over one year. Moreover, we can achieve efficient targeted insertion by injecting macaques up to 3 months of age. In summary, the substantial unmet need of the neonatal onset form of OTCD warrants consideration of experimental therapies such as the ARCUS-based genome editing approach as described in this report.

RNA Virus Vectors

812. Development of a Lentiviral Vector Mediated B Cell Gene Therapy Platform for the Delivery of the Anti-HIV eCD4-Ig Immunoadhesin

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Key barriers to effective gene therapy for many diseases include inadequate gene modified target cells producing sufficient therapeutic

protein for the desired effect. B cells are an attractive target for foreign protein expression as they differentiate to long-lived plasma cells specialized for protein secretion. B cells can travel to and reside in lymphoid, bone marrow or vascular compartments, and secrete large quantities of antibodies, ideal for gene therapy-mediated approaches for genetic and infectious diseases. However, despite the potential of B cells for gene therapy applications, their use has been limited by technical challenges including inefficient gene delivery, stable and lineage specific transgene expression, and limited expansion and differentiation in culture. Lentiviral vectors (LVs) appear to offer a safe integration profile combined with stable transgene expression. However, VSV-G pseudotyped LVs are inefficient at transducing primary human B cells. Alternative LV pseudotypes can achieve higher B cell transduction rates, but may suffer from low viral titers, which may limit inclusion of larger transgenes and challenge large-scale production. Here, we report the development and evaluation of a human B cell gene delivery platform using the potent, HIV-1 entry inhibitor immunoadhesin, eCD4-Ig, which binds and neutralizes HIV-1 with nanomolar potency. To overcome the low transduction efficiency associated with VSV-G LV bicistronic eGFP-eCD4Ig primary B cell delivery, a measles-pseudotyped LV (MV-LV) packaging methodology was optimized achieving B cell transduction efficiency of up to 75%. Sustained and lineage restricted eCD4Ig expression was achieved using the B cell specific E μ B29 enhancer/promoter. We improved the eCD4-Ig protein for B cell expression by mutating the CH3 Fc IgG domain of eCD4-Ig (knob-in-hole, KiH) which reduced eCD4-Ig/B cell IgG heterodimerization and resulted in increased eCD4-Ig-KiH HIV-1 neutralizing potency of 1.5-4-fold depending on the HIV-1 isolate. E μ B29 regulated eCD4-Ig-KiH expression was stable and found to increase with B cell differentiation to plasma cells, eventually outperforming the strong constitutive viral MND promoter in plasma cells. E μ B29 regulated eCD4-Ig-KiH production in B cells reached the lower level of IgG expression; an encouraging observation given that much of the protein-synthesizing capacity of plasma cells are committed to intrinsic immunoglobulin production. Finally, unlike previously reported AAV eCD4-Ig delivery to non-human myocytes, HIV-1 eCD4-Ig produced in B cells did not require co-gene delivery of TPST2, a tyrosine sulfation enzyme required for eCD4-Ig function. These findings imply that TPST2 was present (confirmed by RNAseq) and sufficient in B cells for optimal eCD4-Ig HIV-1 neutralizing function, allowing for a simpler and safer vector design. Our LV strategy and improvements resulted in the development of a robust B cell gene delivery platform that can be applied to other therapeutic needs. In summary, our findings provide support for further evaluation of LV E μ B29-eGFP-eCD4-Ig engineered B cells in appropriate animal models for the amounts and longevity of eCD4-Ig in sera and an anti-HIV-1 protective effect. Additionally, our findings support further testing of an LV-based B cell regulated gene therapy platform for therapeutic protein production for against human diseases.

813. Taking a Good Look Under the Hood of Engineered Lentiviruses: Nanoview's Novel LentiView Technology Allows the Quantitative Profiling of Pseudotyped Lentiviral Particles

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Gene-therapy relies on the ability to modify the genome of a target cell, by inserting new genes and/or replacing and repairing disease-causing genes. One of the most heavily used vehicles for gene and also protein (e.g. CRISPR/Cas) delivery, are lentiviruses. While lentiviruses exert high transduction efficiencies into dividing and non-dividing cells, they are also able to deliver large transgene payloads and through custom-pseudotyping highly specific targeting of specific cell types can be achieved. One of the challenges that the Gene- and Cell-therapy community is still facing when dealing with the generation of virus preparations like lenti- or other commonly used viruses is, that there is really no good technology available that can (1) link physical and functional titer, (2) determine the degree of contaminating non-functional particles that may pose a severe safety concern while potentially compromising drug efficacy and target binding, and (3) carry out quantitative pseudotyping to determine the level of target-specific viral particles. To close this gap and provide the community with an easy, low-sample volume and fast to-use assay, here we present Nanoview's newest assay - the LentiView. To this end Nanoview Biosciences' ExoView platform, which uses a combination of interferometric and 4-color fluorescence imaging, has enabled researchers to characterize and size small lipid nanoparticles like EV, in their native state from culture as well as complex biofluids and without the need of further purification or enrichment. The LentiView allows the quantitative characterization of specific lentiviral surface (e.g. VSV-G) as well as luminal (e.g. p24) markers down to the single-epitope level, while providing the ability to link proteomic information to functional virus titer. Further, unlike other commonly used analytical technologies such as ELISA, this assay can be used as a tool to distinguish empty from full viral particles, virus fragments or aggregates from functional and correctly pseudotyped virus. Here, we present our most recent data on the characterization and pseudotyping-profiling of engineered lentiviruses containing custom-designed surface receptors. We demonstrate our ability to identify discrete non-viral particles (e.g. exosomes and other vesicle-like particles), as well as particles that express the viral envelope marker of choice (e.g. VSV-G) but lack p24 capsid. In the same sample we can detect a second discrete population of p24 capsid- and surface marker-positive viral particles sizing around 85 nm of the correct pseudotype. Along with functional titer data, we could demonstrate that this new assay is highly predictive of both vector performance and the ratio of functional and non-functional viral particles generated. In sum, the LentiView assay enables researchers to dissect the overall composition of a virus sample into its sub-populations, and hence represents a fast and accurate tool to assess the quality, purity and functionality of any given lentivirus sample.

814. Investigating Liver Tissue Dynamics to Improve *In Vivo* Gene Therapy with Lentiviral Vectors

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Liver-directed *in vivo* gene therapy has shown promising results in the context of metabolic diseases and coagulation disorders in adult patients. Adeno-associated viral (AAV) vectors have been extensively exploited for this purpose, however their non-integrating genome is diluted in proliferating cells and therefore post-natal liver growth represents a challenge for application of AAV gene therapy to pediatric patients. We have previously developed lentiviral vectors (LV), which integrate into cell genome and target transgene expression into hepatocytes, and achieved stable coagulation factor IX (FIX) and factor VIII expression in adult mice, dogs, and non-human primates, following intravenous (i.v.) administration. Here, we performed an in-depth analysis of maintenance of LV-transduced hepatocytes following post-natal liver growth and homeostasis and of the impact of age at treatment on liver-directed LV gene therapy in mice. We first investigated clonal proliferation of hepatocytes in Alb-CreERT/Rosa26-Confetti mice up to 1 year of age and observed high proliferation rate of ~25% of hepatocytes during the first week after birth, which generated more than 90% of the adult liver. Hepatocytes' proliferation rate appeared to be reduced already from the 2nd week of age and was minor in adult mice. Transduced hepatocytes showed a similar behavior compared to non-transduced ones, both in terms of percentage of proliferating cells and proliferation rate at different ages, indicating that transduction does not alter proliferation of hepatocytes. Given the metabolic and structural differences existing in the liver at different ages, we investigated whether LV transduction efficiency was affected by the age at treatment. We observed that i.v. administration of LV in mice before the 3rd week of age (around weaning) leads to a reduced uptake of vector particles by non-parenchymal cells that allows higher transduction of hepatocytes. Moreover, we observed that LV distribution in the liver lobule is affected by the age at treatment, with a switch from peri-central to peri-portal transduction bias observed during growth, which is then maintained during the entire life-span of the mouse. By exploiting FIX as a secreted reporter, we observed that the lower transduction of hepatocytes in adult-treated mice resulted also in a lower transgene output compared to young-treated mice. Despite we did not observe differences in terms of transduction between newborn (2-day-old)- and juvenile (2-week-old)-treated mice, the latter showed a 2-3 fold higher transgene output compared to the former. Interestingly, in mice receiving a second dose of LV expressing mCherry when 2-week-old, after being treated as newborns with a first dose of LV expressing GFP we observed a lower percentage of co-transduced hepatocytes than in mice simultaneously administered with the 2 LV. These data suggest that different hepatocyte subsets are transduced at different age of treatment. Moreover, we observed that, by changing promoter, we achieved higher transgene output in newborn-treated mice compared to juvenile-treated mice, suggesting differences in promoter activity in these hepatocytes' subsets. Overall,

our results show that dynamic changes during the growth of the liver impact LV transduction efficiency, spatial distribution of transduced hepatocytes and transgene output. These studies will inform further development of liver-directed LV gene therapy towards application to pediatric patients and may shed light on mechanisms of post-natal liver growth that are relevant also for genome editing strategies.

815. CD90-Targeted Viral Vectors of Hematopoietic Stem Cell Gene Therapy

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Hematopoietic Stem Cell (HSC) gene therapy is a promising route to curing patients with a variety of hematologic diseases and disorders. HSC gene therapy is currently performed *ex vivo* and requires rare cleanroom infrastructure, which is a significant obstacle to patients in resource poor areas. Application of gene therapy agents directly in the patient (*in vivo*) would overcome this bottleneck. However, specifically the *in vivo* use of integrating viral vectors to deliver therapeutic transgenes can lead to unwanted off target effects in non-target tissues. Attempts to target viruses to CD34, a common HSC marker, failed due to the lack of viral fusion. The Kiem lab has previously shown that CD90+ HSCs are crucial for long term engraftment in the nonhuman primate and mouse xenograft model. Here we designed CD90-targeted measles-pseudotyped lentiviral vectors (MV) for the delivery of transgenes into HSCs. We initially developed and attached a CD90 scFv to the hemagglutinin of MVs (CD90-MV). To increase target specificity, CD90-MVs were further modified to knock out the native targeting of CD46 and CD150 (CD90-MV-KO). Functionality of the CD90 scFv on MVs was determined with an in-house developed read-out testing the binding of a custom-designed fusion protein consisting of the extracellular domain of CD90 and mCherry (CD90ex-mCh). Interaction of MVs with CD90ex-mCh was confirmed on a Zetaviewer capable to measure the size and fluorescence of particles. Successful interaction of CD90ex-mCh was observed with CD90-MVs as indicated by the size increase of particles and labeling with mCherry. Next, we evaluated the uptake of MVs on CD90-expressing Jurkat cells. CD90-MV as well as CD90-MV-KO both successfully transduced Jurkat cells confirming that the CD90-scFv does not impact the functionality and uptake of MVs. Of note, CD90-MV-KO showed specific transduction of CD90-high expressing Jurkat cells, whereas CD90-MV uptake was diffuse across all cells. Finally, we tested the MVs on human CD34+ cells *ex vivo* to determine the target specificity. Similar to experiments with Jurkat cells, diffuse GFP expression across all progenitor subsets was seen with CD90-MVs, whereas CD90-MV-KO did lead to a higher GFP expression in phenotypically more primitive cells. GFP-expressing cells from both conditions were further introduced into colony forming cell (CFC) assays to confirm their multilineage differentiation potential. As expected, cells transduced with CD90-MV-KO demonstrated increased CFC potential in primary assays, and most importantly, were capable to generate colonies in secondary CFCs, a feature limited to HSC. Here we show that CD90-targeted measles-pseudotyped lentiviral vectors are functional and taken up by the target cells. Targeted MVs showed greater specificity for CD90 high-expressing Jurkat cells as well as CD90+ HSCs *ex vivo*. Experiments in humanized mice are ongoing to test the targeting efficiency of vectors *in vivo*. In contrast to other

approaches, this new viral vector will be capable of improving *ex vivo* therapies and overcoming the hurdles *in vivo* therapies still have. The development of targeted viral vectors has the potential to increase the on-target efficiency and safety of *in vivo* HSC gene transfer and will be a crucial step in democratizing access to gene therapies.

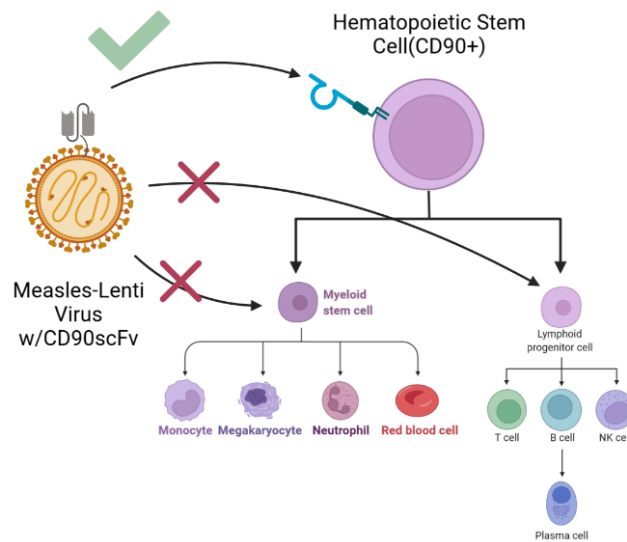


Figure 1: Schematic Showing CD90 virus targeting.

816. SARS-CoV-2 Spike Protein-Pseudotyped Lentiviral Vectors (S-LV) for In Vitro and In Vivo Modelling of Emergent SARS-CoV-2 Variants

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) remains a public health concern with the COVID-19 pandemic driven by the continued global emergence of variants of concern (VOC). The public health response is complicated by the ability of VOC to evade immunity derived from vaccines based on the original Wuhan Hu-1 isolate of the SARS-CoV-2 Spike (S) glycoprotein (Garcia-Beltran, *Cell* 2021; 184(9): 2523). Native SARS-CoV-2 must be handled in high bio-containment facilities which severely limits routine access for research. Therefore, we developed a range of pseudovirions (PSVs) where the S protein from multiple clinically relevant SARS-CoV-2 variants was used to pseudotype an HIV-1-based lentiviral vector (termed S-LV). The variants, including Wuhan Hu-1, G614, BetaCoV/Australia/VIC01/2020, B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta), and B.1.1.529 (Omicron), were combined with or without mutations that confer mouse (m)ACE2 co-permissiveness (Q498Y, P499T ± N501Y). The S-LV variants were manufactured under standard laboratory containment conditions using multiple-plasmid transfection of serum-free, suspension-adapted, HEK293T/17 cells, followed by anion-exchange purification and tangential flow-filtration concentration. To facilitate titration of our S-LV vectors we created ACE2+ & TMPRSS2+ HEK293T/17 cells, to show that truncating the

C-terminus of the S protein ($\Delta 19aa$) improved PSV manufacturing yields up to ~ 10 -fold ($P = 0.0252$). For S-LV variants expressing EGFP, we observed $\sim 5E6$ infectious units (IU)/mL at harvest and $\sim 7E9$ IU/mL after ~ 1000 -fold volumetric concentration. Alternatively, S-LVs expressing luciferase resulted in $\sim 2E12$ relative light units (RLU)/mL after ~ 1000 -fold volumetric concentration. S-LV titres were further improved 3-4-fold when SARS-CoV-2 Nucleoprotein was co-expressed ($P = 0.0004$). Importantly, we demonstrated that S-LVs created from VOC/emerging strains retained important pathological/biological properties. For example, the B.1.351 ($+\Delta 19aa$) S-LV escaped neutralization by the commercial antibody R001, whilst the B.1.1.7 ($+\Delta 19aa$) S-LV escaped antibody MM43 neutralization. Additionally, we observed potent neutralization of the B.1.1.7 ($+\Delta 19aa$) S-LV by plasma from COVID-19 convalescent patients (dilution range 1:10-1:6250) compared with the G614 ($+\Delta 19aa$) S-LV. When normalizing cellular infections by HIV-1 p24 level, as a surrogate of viral particles, S-LV incorporating B.1.1.7 $+\Delta 19aa$, B.1.351 $+\Delta 19aa$, and mACE2 co-permissiveness via Y498, T499, Y501, G614 $+\Delta 19aa$ had 2.5-7-fold higher infectivity than S-LV incorporating G614 $+\Delta 19aa$ or Y498, T499, G614 $+\Delta 19aa$ ($P < 0.0001$ - 0.0162), thought to be driven predominantly by the N501Y mutation. We also used S-LV to evaluate new therapeutic interventions *in vivo*; and showed that expression of the anti-SARS-CoV-2 antibody NC0321, delivered by recombinant LV or Adeno-associated viral vectors (AAV), could substantially reduce infection of the G614 ($+\Delta 19aa$) variant S-LV ($P = 0.004$ - 0.0124) (Du, *Thorax* 2022; manuscript accepted). In summary, our library of S-LV variants is a useful tool to support ongoing SARS-CoV-2 research. By modelling of single-cycle infection kinetics, we can distinguish whether or not an emergent VOC or variant of interest has increased potential to infect via its strain-defining S glycoprotein. This S-LV resource can be used to test new therapeutic antibodies, to evaluate response to therapeutic interventions in plasma samples from recovered patients, and to aid surveillance efforts to help curb the COVID-19 pandemic.

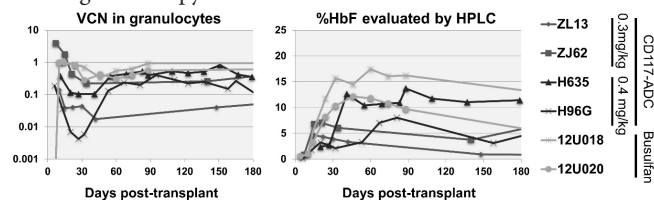
817. A Single Injection of CD117 Antibody-Drug Conjugate Allows for Efficient Engraftment of Gene-Modified CD34+ Cells in a Rhesus Gene Therapy Model

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Hematopoietic stem cell (HSC) gene therapy is curative for multiple genetic diseases; however, it is limited by morbidity and mortality from chemotherapy-based conditioning. Therefore, we developed a pre-clinical antibody-drug conjugate (ADC) targeting CD117 (c-KIT) using a DNA-binding payload to specifically deplete both HSCs and progenitor cells. In our preliminary rhesus study, 0.2 mg/kg ADC

conditioning resulted in $>99\%$ bone marrow depletion, detectable engraftment of gene-modified cells (vector copy number per cell (VCN) ~ 0.01), and minimal toxicities (ASH 2019). In this study, we investigated escalating doses of ADC to determine the optimal dose to enable engraftment of gene-modified CD34+ HSCs in rhesus macaques. We administered a single injection of CD117-ADC at 0.3 mg/kg (ZL13 and ZJ62) and 0.4 mg/kg (H635 and H96G), compared with busulfan (Bu 5.5 mg/kg x 4 days) (12U018 and 12U020). Mobilized rhesus CD34+ cells (ADC $3.8 \pm 1.9e7$ vs. Bu $2.9 \pm 0.2e7$, n.s.) were transduced with a lentiviral vector encoding *BCL11A*-targeting shmiRNA and a truncated human erythropoietin receptor for stable fetal hemoglobin (HbF) induction (Sci Transl Med. 2021). These cells (*in vitro* VCN 10.1 ± 3.8 vs. 10.2 ± 7.3 , n.s.) were transplanted into autologous animals 6 or 10 days after ADC conditioning (0.3 or 0.4 mg/kg, respectively) or 1 day after Bu conditioning. Granulocyte (Gr $>500/\mu\text{l}$, day 6-9 vs. day 8-9), reticulocyte ($>50,000/\mu\text{l}$, day 10-14 vs. day 11), and platelet ($>30,000/\mu\text{l}$, day 2-8 vs. no reduction) recoveries were similar between ADC and Bu conditionings, respectively. Only ADC conditioning resulted in a reduction of platelet counts as well as a transient rebound in all major lineages. Two months post-transplant, efficient gene marking (VCN in Gr 0.32 ± 0.14 vs. 0.47 ± 0.11 , n.s.) was observed in 3 of 4 animals in ADC conditioning (ZJ62, H635, and H96G) (Left panel in Figure). Robust and durable HbF induction was detected by both HbF+ percentages (F-cell $7.4 \pm 1.1\%$ vs. $11.6 \pm 4.1\%$, n.s.) and HPLC-quantitated HbF amounts ($6.2 \pm 2.8\%$ vs. $9.3 \pm 4.0\%$, n.s.) in these 3 animals (Right panel in Figure). In ZL13 (1 of 2 animals in 0.3 mg/kg ADC), lower gene marking (VCN in Gr 0.03) was obtained, along with low HbF induction (F-cell 2.5% and HbF amounts 1.7%), suggesting that 0.3 mg/kg ADC is marginal, and 0.4 mg/kg ADC is sufficient for robust engraftment of gene-modified cells. Importantly, CD117-ADC conditioning resulted in minimal toxicities, unlike Bu conditioning. In summary, we demonstrated that a single dose of CD117-ADC allows for efficient engraftment of gene-modified CD34+ HSCs and robust HbF induction in a rhesus gene therapy model, achieving a similar level as myeloablative Bu conditioning. This targeted approach for safer conditioning could improve the risk-benefit profile in HSC gene therapy.



818. Bioinformatic-Guided Design of a Lentiviral Vector for X-Linked Chronic Granulomatous Diseases Recapitulates Endogenous *CYBB* Gene Regulation and Expression

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Introduction: X-Linked Chronic Granulomatous Disease (X-CGD) is a primary immune deficiency caused by mutations in the *CYBB* gene resulting in the inability of phagocytic cells to produce anti-microbial oxidase, leading patients to suffer from recurrent life-threatening infections. **Methods:** We employed a bioinformatic-guided approach to elucidate the endogenous genomic elements that regulate the *CYBB* locus to design a physiologically regulated lentiviral vector (LV). Our analysis revealed 15 putative elements contained within a 600kb topologically associated domain (TAD) regulating the *CYBB* gene. A LV library was constructed to functionally validate each putative enhancer element and genomic databases were deployed to elucidate the minimal functional boundaries of each key enhancer. When designing the new core elements, many variables were considered including DNaseI-accessibility, transcription factor binding, epigenetic histone modifications, and sequence conservation. This led us to generate a 5.9kb LV, MyeloVec, with optimized hematopoietic stem and progenitor cell (HSPC) gene transfer, titer and expression. **Results:** Using MyeloVec to express a mCitrine reporter, we demonstrated strict *in-vivo* lineage specific expression by xenografting MyeloVec transduced healthy donor (HD) HSPCs into NSG mice. At 18-weeks post-transplant, we observed high-level expression in mature neutrophils, moderate expression in B-cells, and negligible expression in both T-cells and HSPCs. To demonstrate the disease-modifying activity of MyeloVec, murine X-CGD HSPCs were transduced with either MyeloVec or a X-CGD LV currently in clinical trials (pCCLchim) (PMID: 20978475) at an equal vector dose of 1×10^7 TU/mL and were transplanted into X-CGD mice. MyeloVec treated mice restored 76% DHR+ neutrophils with physiological levels of anti-microbial oxidase activity, while pCCLchim treated mice had 63% DHR+ neutrophils with sub-physiological levels of oxidase ($p < 0.01$). At 11-weeks post-transplant, mice were experimentally infected with *B. cepacia*. All MyeloVec treated mice survived the infectious challenge and regained bodyweight while 80% of the pCCLchim treated mice developed a fatal infection ($p < 0.05$). To assess MyeloVec's ability to correct X-CGD patient cells, X-CGD patient CD34+ HPSCs were transduced with MyeloVec or pCCLchim and differentiated *in-vitro* to produce mature neutrophils. MyeloVec-transduced cells restored gp91^{phox} to HD levels, while the pCCLchim-transduced cells restored gp91^{phox} to 40% of HD levels at equivalent VCNs of 1.63 and 1.64, respectively ($p < 0.01$). Furthermore, oxidase-positive neutrophils had HD levels of oxidase activity when transduced with MyeloVec while pCCLchim-transduced

cells showed 33% of HD levels ($p < 0.05$). **Conclusion:** We utilized a bioinformatic-based approach to design MyeloVec, a physiologically regulated LV driven by endogenous regulatory elements of *CYBB* locus. When compared to a LV currently in clinical trials for X-CGD, MyeloVec showed improved expression, superior gene transfer to HSPCs, corrected an X-CGD mouse model leading to complete protection against *B. cepacia* infection, and restored HD levels of oxidase activity in neutrophils derived from X-CGD patient HSPCs. Our findings validate the bioinformatics-based design approach and have yielded a novel lentiviral vector with clinical promise for the treatment of X-CGD.

AAV Developments in Liver, T-cells, and Toxicity

819. Alteration of ITR Sequences for Attenuating the AAV Toxicity in Human Embryonic Stem Cells

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The inverted terminal repeats (ITRs) are the only genetic elements of adeno-associated virus (AAV) present in recombinant AAV (rAAV). Previous reports have demonstrated that a p53-dependent DNA damage response (DDR) induced by the ITR of serotype 2 (ITR2) occurs following rAAV transduction in human embryonic stem cells (hESCs); however, the mechanism of vector cytotoxicity is not well understood. Moreover, no reports exist regarding ITR engineering to attenuate the observed ITR2-induced toxicity. In the present study, transgenic genomes flanked by ITR2 or a rationally designed synthetic ITR (synITR) were evaluated for rAAV transduction efficiency and the induced DDR in differentiated cell lines and in hESCs. Current data demonstrates no significant differences in transduction efficiency or obvious cytotoxicity in differentiated human cell lines following treatment with ITR2 or synITR-based vectors. However, hESC cell toxicity was observed in an ITR sequence-dependent manner. Notably, a synITR, in which selected guanine-cytosine (G-C) rich regions were substituted with adenine-thymine (A-T) nucleotides, showed substantially less cell toxicity compared to ITR2 in hESC WiCell H9 cells upon transduction. In addition, colony expansion was significantly inhibited in the ITR2 rAAV infection, while effects on colony growth inhibition were less evident using a synITR vector. Furthermore, synITR rAAV transduction demonstrated minimal influence in the WiCell H9 potential to form embryoid bodies following transduction, while an ITR2 based vector exhibited a strong negative effect. To characterize the ITR-dependent AAV toxicity in hESCs, induction of DDR pathways, innate immune responses, and cell stemness markers following rAAV infection were examined at the cDNA and/or protein level. The data revealed a high basal level of expression of DDR pathway genes and proteins in H9s, such as tumor protein 53 (p53) isoforms, and it is noteworthy that activated and total full-length p53 protein levels were downregulated following synITR rAAV transduction when compared to ITR2 vectors. Activation of other DDR proteins including

H2AX, ATR, ATM, CHK1 and CHK2 were also evaluated following rAAV transduction with ITR2 or synITR sequences. The results demonstrated a rAAV vector-specific DDR induction following ITR2-based ssAAV or scAAV vectors, as well as a unique response attributed to the synITR. The collective data demonstrate that modulation of ITR sequences influences rAAV cell toxicity, and activation of the host cell DDR, thus representing a crucial optimization target for further elucidating AAV and rAAV virology, with the potential for developing enhanced and safer AAV-based gene therapy drugs.

820. Preclinical Evaluation of AAV Vectors in an *Ex Vivo* Human Whole Liver Explant Confirms the Potential of Bioengineered AAVs as Clinically Relevant Hepatotropic Vectors

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Bioengineering the adeno-associated virus (AAV) capsid facilitates the generation of novel variants with desirable properties, such as specific tropism and the ability to evade recognition by pre-existing neutralizing antibodies (NAbs). Recently, we have reported the identification of a set of novel AAV variants (AAV-SYDs) enabling high transduction of primary human hepatocytes in a xenograft mouse model. However, the ability of the xenografted mouse models to predict the performance of AAV vectors in clinical settings remains a topic of ongoing discussion. Here, we report the development and validation of a whole human liver explant model using *ex-vivo* normothermic machine perfusion as a preclinical model for the evaluation of gene therapy vectors. Two livers were perfused using a red cell-based perfusate under normothermic conditions (36°C) for six and nine days, respectively. Human plasma samples used for the formulation of the perfusate were analyzed for the presence of anti-AAV NAbs. Stable liver function was demonstrated by lactate clearance, bile production, and synthesis of coagulation factors. Graft integrity was confirmed with immunofluorescent analyses of liver zonation markers. We used an expression cassette containing unique 44-mer barcodes to enable side-by-side comparison of multiple AAV variants. The barcoded cassettes were packaged into fourteen natural and bioengineered AAVs, including clinically tested AAV5, AAV8, AAV9, and AAV-LK03, as well as bioengineered and natural variants such as AAV-SYD12 and AAV-hu.Lvr06. The equimolar mix of the vectors was injected directly in the portal vein of the liver explant and tissue samples were harvested

at various time points for vector copy and functional analysis. Under non-neutralizing conditions, AAV-SYD12 and AAV-LK03 were the most functional variants at the level of cellular uptake and transgene expression. When working with human plasma containing anti-AAV NAbs, vectors of human origin (AAV2/AAV3b derived) were widely neutralized, whereas an AAV8-based bioengineered variant containing variable regions VI-VIII from AAV7 was the best performer. Finally, to be able to relate these results to the murine preclinical models, we evaluated the performance of the same barcoded AAV mix in naïve and xenografted mice engrafted with human and non-human primate hepatocytes. Our data suggest that results obtained from animals presenting high levels of repopulation with human hepatocytes are the most predictive of data obtained from human whole liver explants. Importantly, in addition to enabling functional studies of gene therapy vectors, the explant human liver model can prove highly useful when studying critical translational parameters such as therapeutic vector doses and vector-induced cellular toxicity.

821. Optimized Novel AAV Capsids Selected for Improved Homology-Dependent Repair in Human T-Cells

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T-cells are promising targets for the development of gene therapies for immune-deficiencies and cellular immune-therapies for malignant tumors. The latter has been successfully applied in the context of chimeric antigen receptor (CAR) T-cell therapies. Conventionally, lentiviral vectors have been used to semi-randomly insert the CAR coding sequence into the T-cell genome. To improve safety and minimize exhaustion of CAR T-cells, novel approaches utilizing targeted gene editing with the help of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 were developed. This approach, pioneered by Eyquem *et al.*, 2017, uses CRISPR/Cas9-driven homology-dependent repair (HDR)-mediated targeted insertion of a CAR cassette delivered by a recombinant adeno-associated viral (rAAV) vector. However, we hypothesized that because AAV6, the natural serotype frequently used to target human primary T-cells, has not evolved to specifically drive such biomedical applications, it may not be the optimal vector to mediate targeted gene editing in those cells. We have developed a novel directed evolution AAV bioengineering platform that allows for selection of AAV variants based on their ability to support on-target CRISPR/Cas9-mediated HDR into the *TRAC* locus in human T-cells. We used this new platform to perform a screen of diverse AAV capsid libraries on primary human T cells and other *ex vivo* models of the immune system. Applying a custom-designed Illumina-based high-throughput screen we were able to identify and validate several novel candidates

that show significantly improved levels of on-target HDR, when compared to AAV6, with low donor to donor variability (**Figure 1**). As anticipated, high HDR of a given candidate correlated well with improved nuclear entry of the AAV, but interestingly did not strictly correlate with higher mRNA expression from a conventional promoter-driven reporter. Thus, our data indicate that high transgene expression and successful HDR may be driven by different mechanisms and/or interactions between the capsid and the cellular machineries. We will present the selection platform, functional data on the new identified variants as well as results of currently ongoing additional validation studies that aim to further characterize the variants and to gain an insight into the mechanisms involved in AAV-driven HDR.

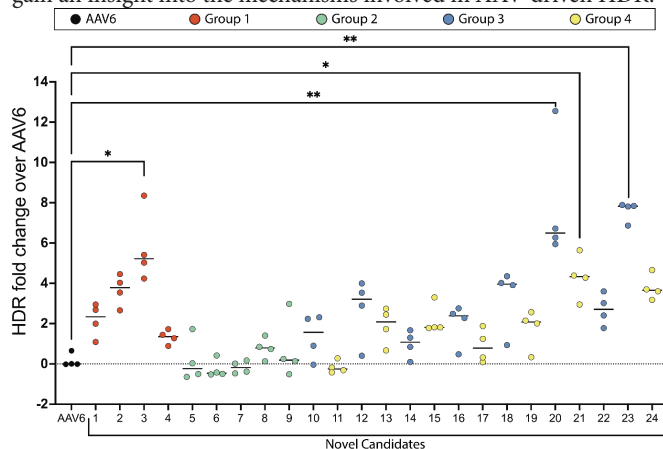


Figure 1. Next-generation sequencing (NGS)-based on-target homology directed repair (HDR) comparison of novel AAV capsids. Twenty-four novel AAV capsids were compared to AAV6 in their ability to drive nuclease-mediated HDR in primary human T-cells of four different donors. The cells were transduced with an equimolar mix of the 25 AAVs and the performance was measured by amplification of a unique barcode inserted into the *TRAC* locus.

822. Selection of Engineered AAV Capsids with Enhanced Incorporation into Extracellular Vesicles and Stable Liver Transduction In Vivo

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While adeno-associated virus (AAV) is defined as a non-enveloped virus, several groups have observed that a population of AAV vectors in producer cell-conditioned media can be found encapsulated in extracellular vesicles (EVs). These enveloped AAV (called exo-AAV) vectors have distinct properties from conventional non-enveloped AAV vectors, including resistance to antibody-mediated neutralization. To advance the system towards clinical development, improvements in yield of exo-AAV is desired. Here, we hypothesize that capsid/cellular protein interactions will allow AAV packaging into EVs. We produced an AAV9-based 7-mer peptide display library in 293T cells and collected EV-rich fractions using column chromatography. Collected library fractions were serially passaged on 293 cells to rescue capsids that were enriched in EV fractions. After 3 rounds

of selection, we chose two peptides, exo-Cap^a and exo-Cap^m which were increased in frequency by 72 and 272-fold, respectively, over the unselected library. Using size exclusion chromatography, the two capsid variants were increased in the EV fractions by 2 to 3-fold compared to parental AAV9, suggesting that there was enhanced EV-associated of the engineered capsids. In cultured cells we compared transduction of enveloped and standard versions of each capsid. This revealed that transduction enhancement over standard AAV9 (up to 40-fold) was dependent on both the capsid variant as well as the EV component. We then intravenously injected BALB/c mice with exoAAV9, exoCap^a, and exoCap^m vectors encoding firefly luciferase and performed bioluminescence imaging over a 12-week interval. We found that exoCap^a mediated a remarkably stable liver transduction compared to the other two vectors. For example, while exoAAV9 displayed an average 53-fold decline in whole-body bioluminescence from weeks 1 to 12, exo-Cap^a-mediated bioluminescence increased by 2-fold over the same time period. Currently, we are comparing non-enveloped (standard AAV) and enveloped exo9 and exo-Cap^a *in vivo* to better understand the mechanism for stable liver transduction, which includes identification of transduced cells in the liver by each vector as well as vector genome biodistribution. In all, our approach shows promise for improving the yield and transduction properties of eAAV vectors.

823. Receptor and Antibody Interactions of AAV by cryo-EM and Tomography

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Most AAV serotypes are dependent on membrane protein AAVR for endosomal cell entry. Cryo-Electron Microscopy (cryo-EM) at up to 2.5 Å resolution has revealed interactions of several serotypes with single PKD domains of AAVR. AAV5-like viruses bind PKD1 at a site that is distinct from the PKD2 site at which most other AAVs interact. Epitopes on several serotypes, recognized by a dozen antibodies have previously been mapped by cryo-EM. It is now surprising to see high spatial correlation between neutralizing epitopes and the respective AAVR binding sites that would be expected if neutralization was by interference with receptor-mediated cell entry. This was not anticipated, because neutralization mechanisms were thought predominantly to involve glycan attachment or a post-entry step. Only single domains of AAVR are bound to AAV with sufficient homogeneity to be revealed by high resolution cryo-EM. Cryo-Electron Tomography (ET) has been used to visualize complexes of AAV2 and AAV5 with two-domain fragments of AAVR. The second domain, PKD1 for AAV2 and PKD2 for AAV5, can be seen (at ~3 nm resolution), each in several different configurations extending away without significant interactions at the AAV surface. Low-pass filtering of cryo-EM maps of AAV5-PKD12 complexes to reveal less-ordered structure show strands of polypeptide adjacent to PKD1 near the AAV5 surface. Side chains are too disordered for identification, but the possibilities are either another AAVR subunit or part of the hitherto unseen N-terminal region of the AAV capsid protein. The cryo-ET of the AAV2-PKD12 complex shows partially ordered

features on the inside surface near the 2-fold axis. Similar features have previously been interpreted as the N-terminal region of capsid proteins. Its presence is correlated with reduction in the occupancy of β A in high resolution cryo-EM of some (but not all) preparations of AAV2 complexed with AAVR, but not others, and not AAV5. Thus, this appears to be a finely balanced conformational equilibrium, explaining why it has been difficult to rationalize the presence or not of these features on the inside surface in past structural studies.

824. Gene Expression from AAV Vectors in the Liver: A Comparative Study Across Species, Promoters and AAV Serotypes

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One of the earliest clinical uses of adeno-associated viral (AAV) vector was for Hemophilia B targeting the liver using AAV2 vector with a hepatocyte-specific expression cassette (*NCT00076557*). Over the years, there have been several clinical trials using similar strategies, but different AAV serotypes, in an attempt to enhance the expression and these had varying outcomes. The results obtained in many *in vitro* systems as well as rodent models do not translate well into larger animal models like non-human primates (NHP) and ultimately humans. Here, we describe our efforts to understand and document the differences in gene expression in the liver between mouse, rat, FRG mice (mice whose livers are reconstituted with human hepatocytes) and non-human primates. AAV8 vector expressing an anti-plasma kallikrein (anti-pkal) antibody from a liver specific promoter (human alpha anti-trypsin promoter with ApoE enhancer) was delivered intravenously into C57BL/6J mice, Wistar rats, Cynomolgus monkeys and FRG mice at a uniform dose of 1×10^{13} vg/kg body weight. The study durations varied for different species. The mouse, rat and FRG mouse studies lasted for one month and NHP studies ran for 3 months post vector administration. Serum anti-pkal antibody levels were monitored bi-weekly for all studies. At study termination, the anti-pkal antibody levels in the serum of NHPs were around 3000-fold lower compared to C57BL/6J mice. The vector copy numbers in liver for both mice and NHP were similar, but transgene transcript levels were significantly lower in NHP and mirrored the reduction seen in protein levels. Experiments in Wistar rats showed lower vector copies when compared to mice and NHP and lower transgene transcripts resulting in lower protein concentration relative to mice. RNAscope analyses of the livers from mouse and NHP supported these findings showing abundant vector DNA with little or no RNA in NHP hepatocytes. FRG mouse livers showed robust RNA expression in mouse hepatocytes with no or limited expression in human hepatocytes in the presence of AAV vector genome. We evaluated different promoters and AAV serotypes in NHP with similar results in the liver as with AAV8, but the RNA levels in other tissues, like skeletal muscle, had expected vector copy number- transcript correlation. This indicated that the transcription repression is largely restricted to liver

in NHP. RNAseq analysis of liver from mouse and NHP studies and *in vitro* experiments are ongoing to decipher the pathways involved. To investigate further, we conducted studies in FRG mice using a different transgene (eGFP) under a universal promoter (CAG), packaged as a single stranded or self-complementary AAV genome into different AAV serotypes (AAV5, AAV6, AAV8, AAV9, AAVrh73). The liver sections were evaluated by RNAscope and immunofluorescence analyses. In this study, we could recapitulate what we observed with AAV8-anti-pkal antibody vector in this animal model. The key finding from these studies is that, although the extent of repression varied between transgenes and promoters, this phenomenon is mostly transgene and promoter independent, and occurs with AAV serotypes commonly used for liver targeting.

825. Evolution of a New AAV Variant with Murine T Lymphocyte Tropism Using the MHC-Ib Molecule H2-Q7 as a Receptor

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Murine models remain an important staple for developing gene, cell-based and immuno-therapies. To better manipulate murine T-cells either by transient gene delivery or site-specific knockins, we evolved capsid libraries based on AAV serotype 6 as a template on C57/Bl6 T-cells *ex vivo*. One dominant clone, Ark313, was enriched over 200,000-fold from the parental library. Sequence analysis of top sequenced and enriched reads reveals a consensus 4-amino acid motif comprised of hydrophobic residues in multiple lead capsids. Ark313 displays increased transduction in C57/Bl6 T-cells *ex vivo*, outcompeting other natural serotypes and displays both mouse strain and species-specific tropism. Further, we demonstrate the utility of Ark313 to generate site-specific knockins by both GFP reporter and a 1928z-CAR into the TRAC locus. Using the Cas9 mouse and a single Ark313 vector packaging both guide and homology repair template, we attain transfection free knockin of 1928z-CAR into the TRAC locus resulting in log increase in viable T-cell yield and up to a 45% knockin rate. To elucidate the mechanism underlying enhanced transduction displayed by Ark 313 in murine T cells, we performed a genome wide CRISPR screen on primary murine T-cells, which revealed the receptor for Ark313 as H2-Q7, the murine HLA-G ortholog. Knockout of either H2-Q7 or the MHC-I associated beta 2 microglobulin (b2m) ablates transduction of Ark313 on C56/Bl6 T-cells. Cleavage of GPI anchored H2-Q7 from T-cells results in lower binding and uptake of Ark313. Together this data presents a new tool for use in genetic manipulation of murine T-cells and the first AAV based CRISPR screen performed in primary T cells. Additionally, use of MHC-Ib as a receptor for AAV transduction represents a novel infectious pathway that can potentially inform the rational design of AAV capsids with selective tropism.

New Gene Editing Technologies and Applications

826. MitoTALENs as Genetic Tools to Reduce Mutant mtDNA Levels in the CNS of a Mouse Model Carrying a Heteroplasmic mtDNA Mutation

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Diseases caused by mutations in the mitochondrial DNA can manifest as debilitating neuromuscular disorders, but there is currently no effective treatment. In recent years, mitochondrial DNA editing enzymes have been evaluated to shift mtDNA heteroplasmy by reducing the levels of mutant mtDNA. We and others have successfully used mitochondria-targeted systems: restriction endonucleases (mitoREs), zinc-fingers nucleases (mitoZFNs), transcription activator-like effector nucleases (mitoTALENs) and more recently meganucleases (mitoARCUS™) to shift mtDNA heteroplasmy in cell culture and in muscle of a mouse model carrying an heteroplasmic m.5024C>T mutation in the mt-tRNA^{Ala} gene. Mice with relatively high levels of mutant mtDNA have a mild cardiomyopathy only detectable at old age but show decreased mt-tRNA^{Ala} levels at earlier ages. We now show that mitochondria-targeted mitoTALENs expressed from AAV-PHP.eb (to enhance CNS transduction) with a neuron-specific synapsin (syn) promoter was able to specifically target mtDNA in neurons, mainly in cortex, cerebellum and to a lesser extent in hippocampus and spinal cord. Neither expression nor change in heteroplasmy were observed in skeletal muscle, heart, kidney or liver of the systemically (i.v.) injected 18 days-old heteroplasmic mice. Tissues with high expression of the AAV-PHP.eb-syn-mitoTALENs showed a significant shift in heteroplasmy levels decreasing the m.5024C>T mutation load when compared to the levels of heteroplasmy in kidney or tibialis anterior (TA) where no mitoTALEN expression was observed. This shift was observed at 6, 12 and 24 weeks post-systemic injection with no significant reduction in the mitochondria copy number. We can conclude that mitoTALENs can efficiently target neurons without causing mtDNA depletion. These studies open a door for future treatment of patients with heteroplasmic mtDNA mutations in the CNS.

827. Lowering Huntingtin Gene Expression by Targeted Base Editing of Cis-Regulatory Elements in its Promoter Sequence

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Huntington's disease (HD) is a progressive neurodegenerative disorder resulting from an expansion of a CAG trinucleotide repeat within exon 1 of the huntingtin (HTT) gene. This expanded CAG repeat

leads to the production of mutant huntingtin (mHTT) protein, which forms toxic aggregates and ultimately triggers neuronal dysfunction. Given the causal link between the mHTT protein and HD, strategies capable of lowering its expression hold the potential to slow the clinical progression of the disease. While technologies such as CRISPR interference (CRISPRi) could in theory be used to interfere with the expression of the mHTT gene, they must continuously engage with a target sequence to sustain an effect, which could pose challenges for translation. In the present study, we demonstrate that CRISPR base editors, a technology capable of precisely introducing targeted single-base substitutions in DNA but in the absence of a DSB, can be used to lower the expression of the HTT gene by permanently disrupting cis-regulatory elements (CREs) in its promoter sequence. To identify actionable CREs for base editing, we conducted a CRISPRi screen using a deactivated Cas9 (dCas9) variant with 30 sgRNAs designed to tile the human HTT promoter. This screen revealed that a 100-bp region upstream of the HTT transcriptional start site contains multiple regulatory sequences, which we then targeted for mutagenesis with cytosine base editors (CBEs). Our results revealed that the most effective of these CBEs lowered HTT expression by up to 55% compared to control cells. Additionally, we determined the ability of the top performing CBE to reduce HTT expression *in vivo* by delivering dual adeno-associated virus (AAV) vector particles encoding a split-intein CBE to the R6/2 mouse model of HD, a commonly utilized model of HD. When compared to control animals injected with a non-targeted CBE, we found that treated mice had a ~10% increase in survival and a ~20% decrease in mHTT aggregates in the brain. Collectively, our results demonstrate that disruption of CREs by targeted base editing can effectively and permanently lower gene expression.

828. Long-Read Nanopore Sequencing Reveals Outcomes of AAV-CRISPR Editing in the Brain of Transgenic Mouse Models

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We are developing AAV-CRISPR strategies to treat spinocerebellar ataxia type 2 (SCA2), a neurodegenerative disease caused by a CAG trinucleotide repeat expansion in *ATXN2*, as well as broadly applicable methods to reliably assess editing after *in vivo* delivery. Standard methods analyze editing with PCR amplification, a strategy that introduces biases. Furthermore, PCR of expanded repeat sequences is unreliable for assessing editing near repeats. We therefore developed a targeted CRISPR enrichment strategy followed by long-read Oxford Nanopore Technology sequencing of native genomic DNA from transgenic SCA2 mice previously co-injected with AAV-SpCas9 and AAV-gRNA in the brain. Nanopore MinION sequencing runs resulted in reads with a mean length of ~10 kb and 2500-5500x target coverage (~1% aligned reads) after alignment to the mouse genome and transgenic allele. In two transgenic SCA2 mouse models treated with AAV-CRISPR expressing dual-gRNAs targeting sequences flanking the CAG repeat, CRISPR-enriched Nanopore sequencing detected intended deletions of the expected size range at frequencies of 2-4%. AAV vector integrations were found at on-target gRNA sites at frequencies of 10-20% and ranged from fragments to full-length genomes (2.3-4.8 kb). CRISPR-enriched

sequencing revealed AAV integrations containing inverted terminal repeat (ITR) sequences that were corroborated by digital droplet PCR. While there was no evidence of CRISPR-mediated induction of longer repeat expansion, there were large deletions and rearrangements of CAG repeat sequences between tandem transgene copies—an unintended consequence of editing in a transgenic model. For comparison we performed PCR-based Nanopore sequencing, and detected deletions and only short AAV sequence integrations, primarily ITR sequences similar to previously reported studies, and no full-length genomes. In summary, CRISPR-enriched Nanopore sequencing allowed unbiased assessment of native and edited genomic sequences. Target sequencing coverage, and on-target deletion and AAV integration frequencies correlate with transgene copy number, an important variable for interpreting sequencing and editing in transgenic mice. By combining long-read sequencing with polymerase-free enrichment, we captured large deletions between transgenes and full-length AAV genome integrations—outcomes missed by standard methods. To our knowledge this is the first study detecting full-length AAV genome integrations of clinically relevant sized vectors in the brains of animals after the delivery of editing machinery. Our results are important for development and safety considerations of CRISPR therapies for SCA2 and other neurodegenerative disorders.

829. Optimization of Pre-mRNA Exon Editing for Efficient Rescue of Protein Expression

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Exon editing is a novel therapeutic strategy for correcting disease-causing mutations by inducing *trans*-splicing between a synthetic RNA molecule and an endogenous pre-mRNA target, forming repaired or wild-type mRNA and protein. Exon editing via *trans*-splicing allows for the replacement of large stretches of exons, and thus provides a potential solution to the well-documented AAV capacity limit for genes with coding sequences (CDS) greater than 5 kb. In addition, RNA exon editing can address multiple disease-causing mutations with a single therapeutic construct, as well as maintain precise gene expression by interacting specifically with pre-mRNA, without changes to DNA. While exon editing by *trans*-splicing has been previously demonstrated in principle for disease genes, these attempts were hampered by low levels of efficiency or lack of translation in relevant preclinical models. *ABCA4* is an ideal target for exon editing via *trans*-splicing as hundreds of recessively inherited mutations found throughout the entire 6.8 kb CDS have been reported to cause retinal degeneration and progressive

vision loss. Here we report the discovery of exon-editing molecules that can successfully replace a significant portion (>20 exons) of the *ABCA4* CDS *in vitro*. With the goal of identifying improvements in exon-editing design in a non-biased and high-throughput manner, we developed a screening platform with the capacity to test thousands of molecules in human cell culture and pre-clinical animal models. First, we describe a human cell line that drives expression of mutant *ABCA4* to enable screening and optimization of exon-editing molecules. Next, utilizing synthetic biology, next-generation sequencing, and bioinformatics we screened over 2,000 molecules *in vitro* to identify a lead series of *ABCA4* exon-editing molecules. Following several rounds of iteration, we validated individual molecules and demonstrated up to 38% successful exon editing of endogenous *ABCA4* mRNA, which resulted in an almost complete rescue in *ABCA4* protein expression *in vitro*. Results from the library screens and individual construct validation data are enabling sequence- and structure-based *in silico* prediction models and RNA design tools. Finally, we demonstrate successful proof-of-concept *in vivo* screening of exon-editing libraries in non-human primate retinas. While additional work is required to unlock the full therapeutic potential of exon editing, this report - the first rescue of *ABCA4* using pre-mRNA *trans*-splicing - demonstrates the viability of exon editing as a therapeutic strategy, and the RNA screening platform described here will enable the discovery of additional novel exon-editing molecules for other genetic targets unable to be sufficiently addressed by conventional gene therapy or editing strategies.

830. Promoterless AAV Vectors with Homology Arms Can Integrate and Express from Transcriptionally Active Sites in Non-Targeted Loci

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The classical gene therapy approach using recombinant (r)AAV to supply a functional gene is suitable for treating older children and adults with inherited disease. However, treatment of pediatric patients is challenging due to the dilution of rAAV episomes during organ growth and development, which causes a loss of therapeutic gene expression. To overcome this obstacle, gene editing and genomic integration approaches have the potential to achieve lifelong expression after single vector administration. Our lab has developed a non-disruptive, nuclease-free promoterless rAAV-based homologous recombination approach (AAV-HR) using *Albumin* locus homology arms surrounding a P2A self-cleaving peptide and transgene protein coding sequence (Barzel et al., Nature 2015). After integration of the GeneRide vector a chimeric mRNA is made that is translated into albumin as well as the transgene protein product after ribosomal skipping. Here we describe studies targeting rAAV to alternative loci in the mouse liver for the purpose of comparing targeting efficiencies of various genomic loci. To do this, we generated AAV-HR vectors targeting the murine *ApoC1*, *ApoE*, and *Gapdh* loci. Surprisingly, we were unable to detect any fusion chimeric mRNAs derived from on-target integration by qPCR, despite ELISA and IHC confirmation of transgene protein

expression. Subsequent 5'RACE and Sanger sequencing analysis of transgene-specific mRNA revealed these targeting vectors produced mRNA fused to Alb mRNA, at various exons, as well as fusions to other non-targeted loci. We next injected mice with an *ApoC1*-targeting vector expressing TdTomatoRed and isolated TdTomato+ hepatocytes using FACS. We then examined the global chimeric mRNA transcriptome using 5'RACE with a primer specific for TdTomato coupled with Nanopore high-throughput sequencing. We found 1,148 independent gene transcripts fused to tdTomato mRNA. Fusions to mAlb were the most abundantly sequenced transcript and no on-target fusions to ApoC1 mRNA were detected. To validate our findings, we performed limited Sanger sequencing of the 5'RACE products after topocloning and were able to confirm five of the most abundant independent fusion transcripts, all of which were in-frame with the endogenous mRNA's ORFs. Our next study examined this phenomenon in the Hepa1-6 cell line using the *mAlbumin*-targeting vector, AAV-mAlb-GFP. Following serial enrichment of GFP+ cells, we again found hundreds of independent gene transcripts fused to the GFP reporter mRNA. We have also conducted integration mapping in this cell culture model and in mice using non-restrictive LAM-PCR, which has uncovered hundreds of off-target integration sites. These datasets and the sequences of the fusion mRNAs are being studied to establish the mechanism of off-target integration. To estimate on-target rates in the serially enriched GFP+ Hepa1-6 cells, we used qPCR to determine ~5.5% of all *Albumin* mRNA was comprised of Alb:GFP fusion mRNA. This surrogate targeting rate was supported by direct Nanopore sequencing of the Albumin target site using genomic DNA from GFP+ Hepa1-6 cells, which determined ~6% of all Albumin alleles contained GFP due to on-target homologous recombination. We are currently performing ribosomal pull-down experiments to establish which of the fusion mRNAs are being translated into functional GFP protein. These experiments will help establish which type of integration events result in protein production and define the origin of the majority of protein producing transcripts. Taken together, this data supports the notion that AAV-HR vectors integrate into non-targeted regions of the genome and produce functional chimeric mRNAs, resulting in transgene expression.

831. SLEEK: A Method for Highly Efficient Knock-In and Expression of Transgene Cargos for Next-Generation Cell-Based Medicines

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Editas Medicine, Cambridge, MA

Inefficient knock-in of transgene cargos limits the potential for cell therapy treatments. Insertion of clinically relevant payloads has been dominated by lentivirus and transposon technologies, which insert

randomly, resulting in heterogenous cell populations and with few methods to enrich for expressing cells. As a potential solution to this challenge, we hypothesized that a target site within an exon of an essential gene could drive efficient knock-in through a selection mechanism. A homology directed repair (HDR) donor template could be designed such that the correct in-frame integration via HDR would retain essential gene function and any other desired cargo would simultaneously be integrated. Cells with incorrectly edited alleles, such as disruptive indels or with incorrect integration events, would undergo negative selection. As *GAPDH* is both a highly and constitutively expressed essential gene, no exogenous promoter was needed, and we were able to drive expression of multicistronic transgenes using a P2A cleavable peptide. We termed this method SLEEK (SeLection by Essential-gene Exon Knock-in) and achieved HDR efficiencies >90% in clinically relevant cell types without impacting long-term viability or expansion. We show this with several clinically important cargos including CARs and Allo shields. We further show that SLEEK can be adapted to other essential genes allowing for tunability of cargo expression based on different promoter strengths in specific cell types. Notably, SLEEK is more efficient than *TRAC* knock-in with AAV6 in T cells. SLEEK improves current autologous and allogeneic T cell and NK cell transgenic processes. SLEEK also enables constitutive and robust expression in iPSC-derived cell types where transgene promoter silencing is a major challenge during the differentiation process. As a clinical application, SLEEK was used to insert CD16 and mbIL-15 cargos into iPSCs which generated NK cells with significantly improved tumor killing and persistence in animals. These properties have led us to use SLEEK in EDIT-202, our first iPSC-derived NK (iNK) cell oncology program. Taken together, SLEEK has the potential to be the strategy of choice for achieving robust multi-transgene knock-in for the next generation of cell-based medicines.

832. Programmable Deletion, Replacement, Integration and Inversion of Large DNA Sequences with Twin Prime Editing and Site-Specific Recombinases

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The targeted deletion, replacement, integration, or inversion of DNA sequences at specified locations in the genome could be used to study or treat many human genetic diseases. Here, we describe twin prime editing (twinPE), a method for the programmable replacement or excision of DNA sequence at endogenous human genomic sites without requiring double-strand DNA breaks. TwinPE uses a prime editor (PE) protein and two prime editing guide RNAs (pegRNAs) that template the synthesis of complementary DNA flaps on opposing strands of genomic DNA, resulting in the replacement of endogenous DNA sequence between the PE-induced nick sites with pegRNA-encoded sequences. We show that twinPE in human cells can perform precise

deletions of at least 780 bp and precise replacements of genomic DNA sequence with new sequences of at least 108 bp. By combining single or multiplexed twinPE with site-specific serine recombinases either in separate steps or in a single step, we demonstrate targeted integration of gene-sized DNA plasmids (>5,000 bp) into safe-harbor loci including *AAVS1*, *CCR5*, and *ALB* in human cells. To our knowledge, these results represent the first RNA-programmable insertion of gene-sized DNA sequences into targeted genomic sites of unmodified human cells without requiring double-strand breaks or homology-directed repair. Twin PE combined with recombinases also mediated a 40 kb inversion at *IDS* that corrects a common Hunter syndrome allele. TwinPE expands the capabilities of precision gene editing without requiring double-strand DNA breaks and synergizes with other tools to enable the correction or complementation of large or complex pathogenic alleles in human cells.

Novel Therapeutic Targets to treat CNS Disorders

833. Hematopoietic Stem and Progenitor Cell Gene Therapy Uniquely Benefits Multiple Sclerosis in the Animal Model

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Multiple sclerosis (MS) is a debilitating neurodegenerative disease characterized by an abnormal immune response mediated by auto-reactive activated T cells inducing chronic inflammatory demyelination in the central nervous system (CNS). Current strategies are mainly aimed at controlling symptoms and do not provide a stable remission of the disease. Transplantation of autologous hematopoietic stem and progenitor cells (HSPCs) along with the administration of an immune and myeloablative regime has been explored to eliminate self-reacting clones and induce tolerance upon renewal of the immune system. Despite some benefit observed in selected patients, its use still is controversial. Here, we designed and tested an innovative lentiviral-based HSPC gene therapy approach for MS aimed at enhancing HSPC immune-regulatory properties by their genetic engineering for human factor programmed death-ligand 1 (hPD-L1). Several evidence suggest that the PD-1/PD-L1 axis plays a key role in the restraint of self-reactive T cells in target organs, inhibiting T cell activation and favoring their exhaustion. We hypothesized that overexpression of PD-L1 in HSPCs could favor reprogramming the auto-reacting immune system towards a *de novo* self-tolerant immune repertoire, restoring a physiological immune system function. Thus, we investigated in the

experimental autoimmune encephalitis (EAE) MS animal model, the ability of PD-L1 overexpressing HSPCs to mitigate the clinical course upon transplantation. To properly design such an approach for clinical translation, we coupled to the transplant protocol innovative intra-CNS routes of cell administration, including intrathecal lumbar injection (ITL), adopted here for the first time for HSPC delivery in a MS animal model. Yet, we explored the usefulness of administering a low dose pre-transplant conditioning regimen intended at extending the persistence of donor-derived cells, while limiting CNS toxicity. Interestingly, transplantation of hPD-L1 over-expressing HSPCs resulted in a significant benefit in EAE mice, consisting in mitigation of the severity of the disease over its course and shorter duration of the disease acute phase/faster recovery of treated mice as compared to controls. Consistently, PD-L1 HSPC transplanted mice showed a reduction of inflammatory cell infiltration in the CNS, reflecting an overall lower CNS inflammation. Interestingly, ITL cell transplantation resulted more effective in limiting EAE disease than the standard intravenous cell delivery. Benefit associated to PD-L1 HSPC transplantation exceeds the benefit of mock/untransduced HSPCs. Therapeutic benefit was profound in the absence of a pre-transplant conditioning regime and was confirmed also following a shorth, sub-myeloablative busulfan course aimed at preventing disease recurrence and relapse. The actual value of the latter is being assessed in models of relapsing remitting MS. Overall, these data provide the first evidence that PD-L1 HSPC gene therapy may represent a valuable therapeutic option for MS, possibly enhancing the immune-modulatory role exerted by autologous HSPCs, thus paving the way for the development of the approach towards clinical testing.

834. A Self-Regulating Gene Therapy for Rett Syndrome

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Introduction: Rett syndrome (RTT) is a neurological disorder caused by mutations in the X-linked *MECP2* gene. *Mecp2*-null mice recapitulate the cardinal features of the disorder and gene reactivation studies using conditional alleles lead to robust phenotypic correction. Whilst this makes RTT an attractive gene therapy target, *MECP2* is a dosage sensitive gene, with both animal studies and the human duplication disorder suggesting that MeCP2 levels need to be kept within a narrow range to achieve efficacy while avoiding overexpression related toxicity. These challenges are magnified by the variable biodistribution pattern of commonly used AAV vectors, which lead to hotspots of expression as well as excessive transgene expression in sensitive cell types. **Approach:** To overcome these challenges, we have developed a single gene circuit in which transgene expression is regulated by a miRNA-based feedforward loop (termed EXACT). This circuit provides a cell autonomous mechanism to prevent overexpression in strongly transduced cells whilst still allowing expression of therapeutic protein levels in more modestly transduced targets. Importantly, the miRNA sequence is not based on any existing mammalian miRNA, thus preventing interference with endogenous miRNA-mRNA gene regulation in transduced cells. **In-vitro testing:** In order to test the

ability of the EXACT circuit to regulate protein levels and tune the setpoint of expression, we designed a cell-based fluorescent screening assay in which candidate *MECP2* transgenes were fused to a reporter, whilst a second reporter in a separate transcriptional unit acted as a surrogate for gene dose. For unregulated constructs, MeCP2 levels increased proportionally with plasmid dose, whilst for regulated constructs, protein levels displayed relative dosage insensitivity and were maintained within a much narrower range or setpoint. **Efficacy and Safety:** To demonstrate efficacy *in vivo*, we delivered either regulated or unregulated *MECP2* constructs to male *Mecp2* knockout mice using AAV9. This severe mouse model displays reduced lifespan (median survival = 11.7 weeks) and prominent respiratory and motor impairments. Mice dosed by neonatal intracerebroventricular (ICV) injection with 1×10^{11} vg/mouse of the lead regulated *MECP2* construct showed significant improvement in survival (median survival = 22.9 weeks) and a concomitant improvement in RTT-like clinical score. In contrast, mice dosed with the unregulated constructs did not show any improvement in survival, possibly due to overexpression toxicity. At a higher dose of 3×10^{11} vg/mouse, mice treated with the regulated lead construct showed a profound improvement in lifespan (median survival > 35 weeks) and significant amelioration of RTT-like phenotypes. At this higher dose, mice treated with the unregulated construct showed severe signs of MeCP2 overexpression and most were euthanised at ~3 weeks. These data demonstrate the ability of the EXACT circuit to enable strong efficacy and significantly improve the safety profile of an *MECP2* gene therapy vector. In separate toxicity studies, early in-life safety was demonstrated in non-human primates treated with the lead construct. **Conclusion:** Overall, these data support the use of the EXACT technology in the further development of an effective gene therapy for Rett syndrome that avoids the safety concerns with conventional unregulated gene replacement.

835. A New Gene Therapy Approach to Treat Niemann-Pick Type C2 Disease

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Niemann-Pick type C2 (NPC2) disease is a rare autosomal recessive lysosomal storage disorder caused by deficiency of *NPC2*. This protein is involved in the egress of unesterified cholesterol from the endo-lysosomal compartment. NPC2 deficiency leads to pathological accumulation of cholesterol in lysosomes, which results in cellular damage and eventually cell death. Patients develop a severe, progressive neurological disorder -including cerebellar ataxia, dysarthria,

dysphagia, and progressive dementia- together with relatively mild peripheral pathology. In the end, affected patients usually die during the first or second decade of life. Nowadays, an efficient therapy for NPC2 disease represents a highly unmet medical need. An *in vivo* gene therapy based on a single intra-cerebrospinal fluid (intra-CSF) administration of adeno-associated vectors (AAV) may offer the possibility of lifetime treatment. Here, we first developed a new mouse model of NPC2 disease, generated by targeted disruption of *Npc2* gene, that recapitulated the main hallmarks of the disease. Next, we assessed the therapeutic efficacy of intra-CSF delivery of AAV-*Npc2* vectors in *Npc2*^{-/-} mice. AAV-mediated gene transfer resulted in significant decrease of unesterified cholesterol storage and correction of lysosomal pathology in the CNS, leading to increased myelination and a reduction in neurodegeneration (Purkinje cells) and neuroinflammation. After AAV-*Npc2* delivery into the CSF, liver was efficiently transduced, providing a long-lasting source of therapeutic protein that allowed to correct the peripheral cholesterol storage and lysosomal pathology of NPC2 disease. Finally, AAV-*Npc2* treatment also resulted in normalization of locomotor deficits, improved body weight and considerably prolonged survival. Altogether, our results demonstrated that intra-CSF administration of AAV-*Npc2* vectors led to widespread correction of both CNS and peripheral pathology in a NPC2 mouse model. Thus, this study lay the foundation for the clinical translation of AAV-*Npc2*-based gene therapy to treat human patients.

836. Comparison of Therapeutic Efficacy and Durability of Gene Therapy for Tuberous Sclerosis Type 2 with Standard of Care Everolimus

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by a hereditary loss of function mutation in one of two tumor suppressor genes, *TSC1* and *TSC2*, encoding for hamartin or tuberin respectively. These proteins form a complex that constitutively inhibits the mammalian target of rapamycin (mTOR) signaling pathway. In TSC-related lesions, the loss of either proteins due to a somatic mutation in the normal allele in susceptible tissues causes over activation of mTOR signaling, subsequently leading to cellular proliferation and overgrowth in many vital organs, most commonly affecting the brain, kidneys, skin, heart and lung. Neurological features of the disease include seizures, cognitive impairment and autism. We have recently demonstrated in a mouse model, that gene therapy using an adeno-associated virus (AAV) vector carrying a “condensed” form of human tuberin (cTuberin) is a promising therapeutic strategy for TSC2. Here, we compare and contrast our gene therapy strategy to the current standard of care for TSC patients, the mTOR inhibitor everolimus. A mouse model of TSC2 generated by AAV1-Cre recombinase disruption of homozygous *Tsc2*-

floxed alleles at birth (P0) via intracerebroventricular injections has a shortened lifespan (mean 50 days) and brain pathology consistent with TSC, including overgrowth of ependymal/subependymal tissue and enlarged ventricles. When these mice were then single injected intravenously at post natal day 21 (P21) with an AAV9 vector encoding cTuberin, most survived for more than 120 days (ongoing). Further immunostaining assays in this model showed that AAV9 vector transduced cells throughout the brain, including subependymal cells, astrocytes and neurons. Post treatment neuropathologic assessment resulted in near normal brain with reduction in ventricular volume and abnormal overgrowths. Interestingly, continuous treatment with everolimus, used in TSC patients, extended survival for up to 75 days but failed to maintain life after discontinuation. These studies demonstrate the potential of treating life-threatening TSC2 lesions with a single intravenous injection of AAV9-cTuberin as compared to the alternative drug treatment currently in use clinically.

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837. A Novel Gene Therapy for Rett Syndrome through Reactivation of the Silent X Chromosome

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Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder caused by loss of function of the transcription factor Methyl-CpG-binding protein 2 (MeCP2). RTT patients are mainly female and experience a loss of previously achieved developmental milestones and a progressive loss of function. By adulthood most patients are wheelchair bound, requiring 24/7 care, and typically die in middle age. X chromosome inactivation (XCI) plays an important role in determining the phenotype of RTT patients. XCI is a developmentally regulated process in females, wherein one X chromosome per cell is inactivated through accumulation of XIST RNA. This process leads to a mosaic of cells with an active X chromosome expressing either the mutant MeCP2 gene or the healthy copy of MeCP2. Importantly, all cells that express the mutant non-functional copy of MeCP2 contain a healthy copy of the gene on the silenced chromosome. Thus, therapies that reactivate the X chromosome containing the healthy copy of MeCP2 could modify the progression of RTT and improve its clinical manifestations. Dr. Bhatnagar and her lab discovered that the microRNA miR106a is an important regulator of X chromosome inactivation, and that knockdown of this miRNA leads to re-activation of the silent X chromosome. To achieve continuous miR106a loss-of-function in vitro and in vivo, the Bhatnagar lab engineered a miR106a-specific sponge RNA harboring multiple tandem repeats of miR106a binding sites. We have developed an adeno associated virus serotype 9 (AAV9) carrying a miR106a sponge (miR106asp) construct as a treatment for X-linked dominant disorders diseases. Treatment with

AAV9.miRNA106asp significantly ameliorated RTT-like behavioral and motor phenotypes in a RTT female mouse model and significantly improved survival, further underlining the therapeutic potential of this approach. Additionally, treatment with this vector was shown to be safe in wild type mice and female non-human primates. Importantly, given its mechanism of action, AAV9.miRNA106asp could potentially be applicable to other X-linked dominant disorders affecting females thus providing an efficient therapeutic platform.

838. Second Generation AAV-Mediated Gene Therapy to Mitigate Risk for Alzheimer's Disease in APOE4/4Homozygotes

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Alzheimer's disease (AD), a progressive degenerative neurological disorder affecting over 35 million people worldwide, is associated with a strong genetic risk in polymorphisms of the apolipoprotein E (*APOE*) gene. There are 3 common *APOE* alleles, E2, E3 (the most common) and E4. There is extensive data demonstrating toxic effects of the *APOE4* allele, and epidemiological studies demonstrate that *APOE4/4* homozygotes have a 14.5-fold increased risk of developing AD, while *APOE2/2* homozygotes are protected. The knowledge that the presence of *APOE2* in *APOE2/4* heterozygotes markedly reduces the *APOE4* risk led to the ongoing clinical trial of a 1st generation preventative AD gene therapy using adeno-associated virus (AAV) to deliver *APOE2* to the brain of *APOE4/4* homozygotes. We hypothesized that a 2nd generation gene therapy expressing *APOE2* but also including artificial microRNAs (miRNA) targeting endogenous *APOE4* may further mitigate the risk and limit AD development of *APOE4/4* homozygotes. As a first step to test this hypothesis, repetitive copies of an artificial miRNA targeting *APOE* were incorporated into the promoter or in the 3' untranslated region of the expression cassette. To determine the efficiency of silencing endogenous *APOE*, 293T and U87-MG cells were transfected with pAAV expression plasmids containing *APOE*-targeting miRNAs. While all arrangements of *APOE*-targeting miRNAs significantly silenced endogenous *APOE*, the pAAV expression plasmid with multiple 5' copies of *APOE*-targeting miRNA showed the most significant silencing of *APOE* in 293T and U87-MG cells (both $p < 10^{-4}$). To test the ability of the *APOE*-targeting miRNAs *in vivo*, AAV9-mCherry-mir*APOE*, an AAV9 vector expressing the *APOE*-targeting miRNAs, was administered to the right hippocampus of TRE4 mice, a mouse model with murine *Apoe* gene deleted and replaced with human *APOE4*. When compared with AAV9-mCherry, the *APOE*-targeting miRNA downregulated hippocampal h*APOE4* mRNA levels by 2.8-fold suggesting that an AAV-mediated gene therapy incorporating *APOE*-targeting miRNA into the expression cassette can silence human h*APOE4* in the CNS. Silencing human *APOE4* could serve as part of a therapeutic approach in a 2nd generation gene therapy for the treatment of *APOE4/4* homozygous individuals at high risk for the development of Alzheimer's disease.

839. Modulation of miR-181 Influences Dopaminergic Neuronal Degeneration in a Mouse Model of Parkinson's Disease

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Parkinson's disease (PD) is caused by loss of dopaminergic (DA) neurons in the substantia nigra (SN). Although PD pathogenesis is not fully understood, studies implicate perturbations in gene regulation, mitochondrial function, and neuronal activity. MicroRNAs (miRs) are small gene regulatory RNAs that inhibit diverse subsets of target mRNAs, and several studies have noted miR expression alterations in PD brains. For example, miR-181a is abundant in brain and is increased in PD patient brain samples; however, the disease relevance of this remains unclear. It is also noteworthy that several pesticides previously linked to risk of PD manifestation have been shown to elicit robust increases (~3-4-fold) in miR-181 levels in various experimental settings. Herein, we show that miR-181 target mRNAs are broadly down-regulated in aging and PD brains. To address if the miR-181 family plays a role in PD pathogenesis, we generated adeno-associated viruses (AAV) to overexpress (pri-miR) and inhibit [tough decoy (TuD) / sponge] miR-181 isoforms. After co-injection with AAV overexpressing alpha-synuclein (aSyn) into mouse SN (PD model), we found that moderate miR-181a/b overexpression strongly exacerbated aSyn-induced DA neuronal loss, as determined by staining for the cell-specific marker tyrosine hydroxylase, TH (Figure panel A, aSyn Lo + miR-181 Lo, $p < 0.001$). Also, prolonged miR-181 overexpression in SN alone elicited measurable neurotoxicity, hallmarked by ~50% DA neuronal cell loss ($p < 0.001$, data not shown), which coincided with an increased immune response. By contrast, miR-181 inhibition was profoundly neuroprotective (Figure panel B, aSyn + TuD-181, $p < 0.01$), relative to a scrambled stem-loop RNA control (U6-Ctrl). Insight into likely complex mechanisms of action was gained by RNA-seq analyses, which revealed that miR-181a/b inhibits a broad set of genes involved in synaptic transmission, neurite outgrowth, and mitochondrial respiration, along with several genes having known protective roles and genetic links in PD. Overall, this work provides the first proof-of-concept demonstration that AAV-mediated miR-181 inhibition is neuroprotective in a mouse model of neurodegenerative disease. The results support the need for additional pre-clinical studies in other animal models of PD, and perhaps other neurodegenerative diseases, and also to explore testing of synthetic anti-miR oligonucleotides in parallel.

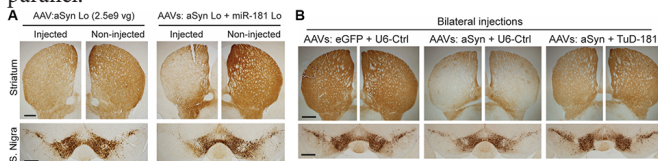


Figure. Histological analyses done on mouse brains harvested 16 weeks after (A) unilateral co-injection with either AAV:aSyn plus or minus AAV:miR-181 (each at a low of 2.5e9 vg) or (B) bilateral injection with either AAV:GFP or AAV:aSyn (1.3e10vg) in combination with AAV:U6-Ctrl (scrambled control) or AAV:TuD-181 (each at 1e10 vg). Representative photomicrographs of immunohistochemical staining for TH+ DA neuronal cells in substantia nigra and their projections in striatum are shown.

Harnessing Innate Immunity for Cancer Immunotherapy

840. Multiplex Base Editing of NK Cell to Enhance Cancer Immunotherapy

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Natural killer (NK) cells are uniquely capable of killing transformed cells lacking major histocompatibility complex (MHC), and have garnered extensive attention in immunotherapy. However, their intransigence to genetic modification poses a major obstacle for high-efficiency engineering. Conversely, base editing (BE) technology has achieved successful multiplex base substitution in many types of immune cells without the drawbacks of multiple nuclease based editing. Previously, we reported high-efficiency multiplex engineering in T cells using BE, and applying a similar concept in NK cells may offer a chance to overcome the editing obstacles. We selected a panel of genes that play a critical role in NK cell function, including AHR, CISH, KLRG1, TIGIT, KLRC1, PDCD1, and CD16A. CD16A encodes an Fc receptor responsible for antibody-dependent cellular cytotoxicity (ADCC) and is cleaved upon NK cell activation. Non-cleavable CD16A improves ADCC and can be achieved through single-base substitution with BE. Using ABE8e, we report editing efficiencies, both in single and multiplex applications, approaching 100% and 98%, respectively, both at genomic and protein levels (Figure 1). Functional analysis also demonstrates improved NK cell cytotoxicity and functionality. Next, we designed a pair of juxtaposed nickase gRNAs to introduce a double-stranded break (DSB) at AAVS1 locus for integration of a CD133 chimeric antigen receptor (CAR), and achieved approximately 50% integration (Figure 2). Finally, we combined multiplex base editing with CAR integration and further improved NK cell functionality. Our goal is to develop a high-efficiency multiplex editing platform in CAR-NK cells, to improve CAR-NK cell activity and toxicity in future applications of immunotherapy.

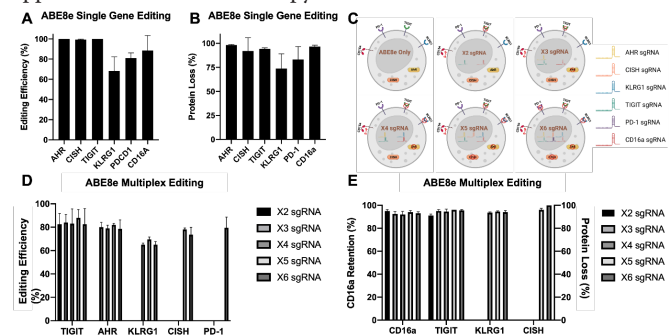


Figure 1. A) Single gene editing efficiencies of each target genes at genomic level. B) Single gene editing efficiencies of each target genes at protein level. C) Diagram showing the design of multiplex editing. D) Multiplex editing efficiencies of each target genes at genomic level. E) Multiplex editing efficiencies of each target genes at protein level. *All data points have at least three independent healthy donor replicates.

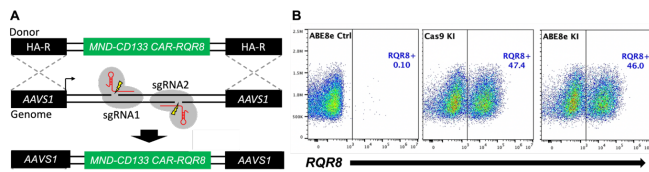


Figure 2. A) Diagram design of ABE8e CD133 CAR knock-in at AAVS1 locus. B) A comparison of CD133 CAR knock-in efficiencies at AAVS1 locus in between traditional spCas9 and ABE8e. *With at least three 3 independent healthy donor replicates.

841. Engineered Induced Pluripotent Stem Cell-Derived Natural Killer Cells Reactively Co-Target TIGIT and CD73 in the Glioblastoma Tumor Microenvironment

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Solid tumors such as glioblastoma multiforme (GBM) are particularly difficult to treat, being largely resistant to traditional treatments. Though natural killer (NK) cells have emerged as promising effectors to target GBM and other solid tumors, immunosuppressive conditions within the tumor microenvironment (TME) and unfavorable interactions between tumors and NK cell activating and inhibitory receptors complicate NK cell-based treatments. Among these receptors, the receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT) is expressed on NK cells and interacts with CD155 to induce immunosuppression of NK cell cytolytic functions. We have found that in GBM, TIGIT is an NK cell co-receptor that is responsive when targeted in combination with a secondary inhibitory receptor. Further, within the TME tumor cells release high levels of ATP extracellularly. While intracellular ATP is necessary for cell metabolism, extracellular ATP is converted into adenosine (ADO) by ectonucleotidases CD39 and CD73, both overexpressed on GBM. We have found that extracellular ADO not only has a negative prognostic value in GBM, but it induces immunometabolic suppression of NK cells through binding with A2A adenosine receptors (A2ARs) on NK cells, suppressing cytokine secretion, proliferation, and other functional activities. In order to effectively target immunometabolic reprogramming induced by CD73-produced adenosine and the immunosuppressive TIGIT-CD155 axis, we have engineered NK cells to concomitantly target CD155 and CD73-induced immunosuppression on GBM using a novel tumor-responsive genetic construct based on the synNotch signaling system. The construct is capable of blocking the immunosuppressive CD155/TIGIT interaction, and, upon binding, release a CD73-blocking scFv to inhibit the accumulation of extracellular ADO and mitigate immunosuppression of NK cells. Such localized response enhances specificity and reduces off-target effects of NK-based targeting. To advance the allogeneic use of engineered NK cells, we engineered induced pluripotent stem cells (iPSCs) to express the synthetic TIGIT-synNotch construct and differentiated them into functionally and phenotypically mature NK cells using our fully chemically defined differentiation process. iPSC-NK cells can functionally express the construct upon maturation and expansion and can mediate powerful anti-tumor functions against patient-derived GBM models in vitro and in vivo. Such locally-activated iPSC-NK cells are able to specifically target the local GBM TME while sparing healthy tissues, promote restored NK cell metabolism and drive TIGIT signaling away from immunosuppression. Overall, co-targeting CD155 and CD73 in a

localized, responsive manner can dampen immunosuppression and significantly enhance the killing potential of engineered iPSC-NK cells against aggressive patient-derived GBM tumors as a programmable, off-the-shelf immunotherapy.

842. Consistent Expansion and Activation of Autologous Non-Genetically Modified Natural Killer Cells with Enhanced Cytotoxicity (SNK01) from Heavily Pre-Treated Patients with Advanced Solid Tumors

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Background: Natural killer (NK) cells play a key role as the main effector cells toward cancer in innate immunity. Thus, a leading approach is to boost NK-cell mediated anti-tumor activity using adoptive transfer of ex vivo activated NK cells. NK cells have always been challenging to grow ex vivo especially when derived from heavily pretreated donors, thus most have focused on universal allogeneic donor derived products. SNK01 is a first-in-kind, autologous non-genetically modified NK cell product with significant anti-tumor cytotoxicity and over 90% activating receptor expression that can be consistently produced even from heavily pre-treated cancer patients (pts). SNK01 has been found to have strong pre-clinical activity against both liquid and solid tumors. We hypothesized that SNK01 could be consistently produced from any heavily pre-treated pt, would be safe without need for lymphodepletion, and might have potential activity against solid tumors. **Methods:** In this Phase 1 dose escalation study (NCT03941262), SNK01 was administered intravenously (IV) weekly for 5 consecutive weeks using a 3+3 design in pts with advanced solid tumors. The starting dose was 1×10^9 SNK01 cells and the highest dose was 4×10^9 SNK01 cells. Primary endpoint was safety and secondary endpoints included tumor response per RECIST v1.1. Individual NK cell expansion was characterized for increases in cytotoxicity and changes in activating receptor expression. **Results:** As of Feb 1, 2022, 10 pts with advanced refractory solid tumors have been enrolled. Median age is 50 (range 32 - 75) and 6 were male. Pts had a median 5.5 lines of prior therapy (range 2-10). The subtypes were 4 leiomyosarcoma, 1 chondrosarcoma, 1 NSCLC, 1 small round cell tumor, 1 colorectal, 1 synovial cell sarcoma, 1 angiosarcoma. NK cells were successfully activated and expanded from all heavily pre-treated pts. Average cytotoxicity was increased over 450% and average activating receptor expression was greater than 90%. There were only two Grade 1 adverse events reported in the 50 total doses given. Best objective response of SD was demonstrated in 7 pts. Overall median PFS and OS have not yet been reached. **Conclusion:** SNK01 with high cytotoxicity and activating receptor expression can be consistently produced from heavily pretreated patients. SNK01 was very safe and appears to have some clinical activity against heavily pretreated solid tumors. SNK01 will be studied further as adjuvant monotherapy and in various combination regimens.

843. Multifunctional Natural Killer Cell Engager Releasing CXCL10 Augments Natural Killer Cell Recruitment and Anti-Tumor Efficacy Against Glioblastoma

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Background: Natural killer (NK) cell-based cell therapy has emerged as a powerful weapon in the treatment of multiple malignancies. However, the inadequate infiltration of the therapeutic NK cells into solid tumors remains a challenge to their clinical utility. As a synthetic strategy to drive recruitment and enhance NK cell activity in tumors, natural killer cell engagers, have been developed to specifically engage functions that can enhance the efficacy of NK cell-based immunotherapy. However, many solid tumors are characterized by heterogeneous immunosuppressive mechanisms, requiring a multi-specificity of targeting mechanisms to achieve durable responses. Here, we present our work on developing a novel class of NK cell engagers that multi-specifically addresses recruitment of NK cells via the CXCR3-CXCL10 axis, cytokine-mediated activation of NK cells, and tumor-specific target recognition. **Methods:** We isolated tumor-infiltrating NK cells from GBM patient tumor samples and compared the CXCR3 expression on those cells to NK cells from peripheral blood of the same patient or other healthy donors. The level of CXCR3 was also measured on ex vivo expanded NK cells in response to GBM cell lines in vitro. The effect of exogenous CXCL10, one of the ligands of CXCR3, on NK cell viability, migration, and cytotoxicity was also established. We further investigated the regulatory mechanisms of the CXCR3-CXCL10 axis on NK cells and tumor cells by establishing CXCR3-knockdown primary NK cells and CXCL10-overexpressing GBM cells using lentiviral transduction. The role of CXCL10 overexpression on tumor growth and NK cell anti-tumor activity was also measured in vivo using a xenograft patient-derived GBM model. Driven by these findings, we designed, synthesized, and characterized a novel natural killer cell engager which not only targets Nkp46 on NK cells and IL13Ra2 on tumor cells, but also specifically releases CXCL10 at the tumor sites while sustaining NK cell activation via production of IL-15. NK cells recruitment and anti-tumor efficacy of the engager with the CXCL10-releasing moiety were evaluated in vitro and in vivo. **Results:** CXCR3 was highly expressed on NK cells in peripheral blood from either GBM patients or healthy donors. Although the number of tumor-infiltrating NK cells in GBM patients was low, the CXCR3 expression on these NK cells was up-regulated. After co-culture with GBM cells in vitro, NK cells downregulated the expression of CXCR3. CXCL10 induced NK cells migration via CXCR3 but did not affect the viability and cytotoxicity of NK cells. On the other hand, CXCL10 overexpression on GBM cells showed no effect on tumor cell growth both in vitro and in vivo but resulted in enhanced NK cell migration and infiltration into tumor sites and, in turn, enhanced the anti-tumor activity of adoptively transferred NK cells in vivo. Furthermore, our novel natural killer cell engager activated NK cells by binding Nkp46 and recognizing GBM tumor cells by binding IL13Ra2, promoting contact between NK cells and tumor cells. Additionally, incorporation of the locally CXCL10-releasing domain, which is activated in the local tumor microenvironment (TME), into our engager favors the specific increase of the local CXCL10 at tumor sites, which leads to improved NK cell trafficking and homing in the TME. **Conclusions:**

Our results demonstrated that the CXCR3-CXCL10 axis contributes to the recruitment of NK cells to tumor sites without effect on the anti-tumor capacity of NK cells. Our novel natural killer cell engager within a specific locally-cleavable CXCL10 domain not only induced enhanced NK cell migration and infiltration into tumor sites but also boosted NK cell anti-tumor activity, representing a promising strategy to facilitate the recruitment of NK cells and therapeutic efficacy against solid tumors.

844. Logic Gated FLT3 or CD33 Not EMCN CAR-NK Cell Therapy (SENTI-202) for Precise Targeting of AML

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Background: More effective therapies against acute myeloid leukemia (AML) are needed, which has a 29.5% 5-year survival rate and is curable only by hematopoietic cell transplantation (HCT). Using a logic gated chimeric antigen receptor (CAR) approach with Natural Killer (NK) cells, we designed an allogeneic CAR-NK cell therapy (SENTI-202) to overcome current AML CAR-mediated treatment challenges, including: (1) lack of tumor-associated target antigens expressed across all AML subpopulations, including leukemic stem cells (LSCs), and (2) lack of AML-exclusive antigens to prevent off-tumor toxicity. **Methods:** For precise targeting of AML, we incorporated two types of logic gated CARs into our gene circuit:

(1) a bivalent FLT3 OR CD33 activating CAR (aCAR) to maximize clearance of both LSCs (via FLT3) and bulk AML tumor (via CD33), and

(2) a NOT EMCN inhibitory CAR (iCAR; NOT GATE) to protect FLT3+ healthy hematopoietic stem cells (HSCs) from off-tumor toxicity.

Results: First, to show that the FLT3 OR CD33 aCAR portion of our gene circuit has the potential for improved AML clearance, we demonstrated that FLT3 OR CD33 CAR-NK cells possess: (a.) significantly greater in vitro cytotoxicity activity against leukemia cells than traditional single FLT3 ($p < 0.05$) or CD33 ($p < 0.01$) CAR approaches, (b.) significant in vitro cytotoxicity activity against multiple leukemia cell lines, (c.) up to ~80% cytotoxicity activity at E:T ratio of 1:1 against primary AML cells, and (d.) potent in vivo killing potential that reduced tumor burden and improved mouse survival in MOLM13 ($p < 0.05$) and MV4-11 ($p < 0.01$) xenograft AML models. Second, we developed a NOT EMCN iCAR gene circuit component with the aim of protecting HSCs from off-tumor toxicity. Initial in vivo proof-of-concept experiments using HER2 as a model iCAR target antigen demonstrated significant ($p < 0.01$) NOT GATE-mediated protection of HER2+ target cells from CAR-NK cell toxicity. For the SENTI-202-specific iCAR, we combined an scFv that recognizes EMCN (an HSC-enriched surface protein not found on AML) with intracellular domains containing immunoreceptor tyrosine-based inhibitory motifs and identified 9 different NOT GATE architectures that significantly ($p < 0.05$ - 0.001) protect EMCN+ target cells from CAR-mediated cytotoxicity, with validation studies showing up to 67% protection of FLT3+ EMCN+ (HSC-

like) cells from FLT3 aCAR-mediated cytotoxicity ($p < 0.01-0.001$). To replicate a clinical setting more closely, we demonstrated the complete gene circuit engineered FLT3 OR CD33 NOT EMCN CAR-NK cells: (1) possess the same ($>80\%$) in vitro cytotoxicity activity against AML cells as bivalent aCAR only CAR-NK cells (suggesting the iCAR does not exhibit basal inhibition of tumor killing), and (2) provide up to 47% protection to primary HSC-enriched cells from aCAR-mediated off-tumor toxicity. **Conclusion:** SENTI-202 is a CAR-NK cell product candidate engineered with OR logic gating with the aim to increase AML LSC/blast tumor clearance, prevent single antigen tumor escape, and provide a deeper disease remission. In addition, the NOT logic gate intends to protect healthy HSCs from off-tumor toxicity, thus potentially improving post-treatment regeneration of a healthy hematopoietic system and mitigating the need for HCT. Combining logic gating technologies has the potential to not only create more efficacious and safer cell therapy products, but also to enable targeting of tumor-associated antigens that were previously avoided due to concerns about antigen escape or off-tumor toxicity, thereby potentially expanding the therapeutic application of these cell therapies to previously unaddressable patient populations.

845. FT536: A First-of-Kind, Off-the-Shelf CAR-iNK Cell Product Candidate for Solid Tumors Designed to Specifically Target MICA/B Stress Proteins and Overcome Mechanisms of Tumor Evasion

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The advent of chimeric antigen receptor (CAR)-T cell therapies has revolutionized the treatment of hematological malignancies; however, broader therapeutic success has been challenged by observed toxicities, including on-target, off-tumor engagement of non-cancerous cells, limited tumor antigen expression and availability, and the ineffectiveness of single-antigen targeted CAR T cells to eradicate heterogeneous tumors. In addition, the inherent variability that arises from the use of patient- and donor-sourced T cells and the engineering of these T-cell populations as part of each manufacturing campaign results in significant drug product inconsistencies, which can impact safety, efficacy, and therapeutic reach. We have developed FT536, a first-of-kind, induced pluripotent stem cell (iPSC)-derived NK (iNK) cell product candidate that expresses a novel CAR and ubiquitously targets cancer cells through canonical stress ligand recognition (Figure 1A). FT536 recognizes the conserved $\alpha 3$ domain of the pan-tumor associated MICA and MICB (MICA/B) stress proteins, a novel targeting strategy that mitigates a key tumor immune evasion mechanism. In addition

to the CAR, FT536 is derived from a clonal master iPSC line that incorporates multiple genetic edits to enhance NK cell effector function, persistence, and multi-antigen targeting. Starting with a clonal master iPSC line, the cGMP manufacturing process of FT536 is analogous to pharmaceutical drug product development, and consistently and uniformly delivers greater than 4×10^7 cellular fold expansion per manufacturing campaign. FT536 drug product can be cryopreserved, stored, and delivered to clinical sites for thaw-and-infusion to patients in the out-patient setting. Preclinical assessment of the product candidate's unique CAR modality demonstrated potent antigen-specific cytolytic activity against an array of solid and hematological tumor cell lines. FT536 is also armed with a high-affinity, non-cleavable CD16 (hnCD16) Fc receptor, which provides the potential to target additional tumor antigens in combination with tumor-targeting antibodies. In addition, FT536 demonstrated significant tumor growth inhibition in multiple solid and liquid *in vivo* xenograft models (Figure 1B). An Investigational New Drug (IND) application for FT536 was allowed by the U.S. Food and Drug Administration (FDA) in December 2021, and a first-in-human clinical study of FT536 as monotherapy and in combination with tumor-targeting monoclonal antibody therapy for the treatment of multiple solid tumor indications is expected to commence in mid-2022.

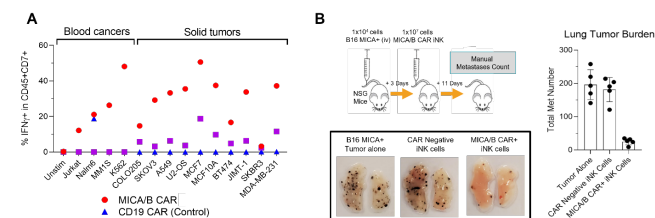


Figure 1: FT536 contains a novel pan-tumor targeting CAR and displays significant in vivo efficacy

(A) MICA/B CAR containing primary T cells demonstrated pan-tumor reactivity and superior IFN γ cytokine responses compared to control and NKG2D CAR containing primary T cells. (B) FT536 significantly reduced the number of lung and liver (not shown) metastases compared to CAR negative iNK control cells in a murine metastatic melanoma model using B16/F10 cells engineered to overexpress human MICA.

846. IFNalpha by In Vivo-Engineered Macrophages Abates Liver Metastases and Triggers Counter Regulatory Responses Limiting Efficacy

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The liver hosts an immune suppressive environment favouring metastatic seeding and proliferation of cancer cells. Pharmacological

treatments, including immunotherapies, fail in the presence of liver metastases (LMS). Therefore, identifying new interventional tools and key targetable players involved in the immunosuppressive environment is of pivotal importance. Vesicular stomatitis virus G protein-pseudotyped lentiviral vectors (LVs) delivered systemically to mice and non-human primates efficiently transduce liver cells, including resident macrophages termed Kupffer cells (KCs). Building on these findings, we developed a novel LV-based platform, termed KC-LV, to selectively engineer KCs in vivo with the goal of delivering bio-therapeutics specifically to LMS. To this aim, the KC-LV design exploits a reconstituted mannose receptor c type 1 (MRC1) promoter, active in macrophages, including KCs. To further fine-tune KC specificity, the KC-LV also includes microRNA target (miRT) sequences that prohibit off-target transgene expression in liver sinusoidal endothelial cells and hepatocytes. Upon systemic delivery of the KC-LV, we observed selective transgene expression in KCs which was enhanced in macrophages located in areas surrounding LMS. We then equipped the KC-LV with an IFN α -coding sequence, a cytokine with pleiotropic immune effects. Long term analysis in mice showed LV dose-dependent, sustained and well-tolerated IFN α expression. To investigate the therapeutic efficacy, we employed a colorectal cancer (CRC) organoid-based syngeneic mouse model of LMS containing molecular and histopathological hallmarks of the human disease. IFN α LV treatment significantly delayed LMS growth reaching a complete response in up to 50%. Single cell omics of LMS from IFN α LV-treated mice showed upregulation of IFN α -responsive genes, macrophage skewing to an antigen presenting (M1-like) polarization state, and expansion as well as reduced exhaustion of LMS-associated antigen specific CD8 T cells. Employing spatial transcriptomics, we found that the interface between LMS and liver parenchymal tissue was the major site of IFN α action, which was associated with enhanced immune activation and antigen presentation. When comparing LMS of treatment responsive to resistant mice, we found accumulation of activated CD8 T-cells in responsive lesions and instead a high number of immunosuppressive T regulatory type 1 (TR1)-like cells in resistant mice. Molecular analyses suggest that TR1-like cell infiltration was associated with increased IL10 signaling in resistant LMS. Furthermore, we found a positive correlation between IFN α and TR1-like cell signatures in human LMS and primary CRC thus supporting the link between IFN α activation and expansion of TR1-like cells in cancer. In summary, we developed an innovative gene-based platform that upon a single well-tolerated intravenous LV infusion rapidly promotes a protective therapeutic response against LMS through enabling immune activation. However, we also found that TR1-like cells might promote tumor immune evasion in presence of IFN α signaling in this setting, suggesting targeting of TR1-like cells when facing resistance to cancer immunotherapies that trigger IFN α signaling.

Cell-Based Cancer Immunotherapies II

847. Characterization of the Transcriptomic and T-Cell Receptor (TCR) Clonal Heterogeneity of Tumor-Infiltrating Lymphocyte (TIL) Therapy Infusion Products by Single-Cell Sequencing and Correlative Analyses With Clinical Efficacy in Patients with Advanced Cutaneous Melanoma

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Introduction: Autologous TIL products made from tumor digests produced high clinical response rates (67% overall response rate; 19% complete responses) and a safety profile consistent with lymphodepletion and high-dose interleukin-2 in a retrospective analysis of a single-center compassionate use clinical series of 21 patients with advanced cutaneous melanoma (Hawkins RE, et al. *Cancer Res.* 2021;81[13 suppl]:LB150). Using single-cell and bulk sequencing techniques, this translational subanalysis characterizes TIL therapy infusion product composition, mediators of cell-cell interactions, TCR repertoire, and correlates these findings with clinical efficacy. **Methods:** Suitable patients underwent resection of ≥ 1 cm³ of tumor tissue for TIL production. Quantitative disease assessments were conducted and analyzed retrospectively per Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 when feasible. TIL products were characterized using RNA-based bulk TCR sequencing and paired single-cell RNA and TCR sequencing techniques. TCR repertoire clonality was assessed using multiple metrics, including Gini coefficient. Descriptive statistical testing was performed using Wilcoxon test; *P* values were not adjusted for multiplicity. **Results:** As of December 31, 2019, 21 patients underwent treatment; data are reported for patients with assessable products (n=20 for RNA-based bulk TCR sequencing, and n=18 for paired single-cell RNA and TCR sequencing). Analysis of the single-cell RNA sequencing data identified several cell subpopulations with distinct transcriptional signatures previously undescribed in TIL products, including MX1+OAS1+ T cells, 2 subsets of mixed gamma/delta CD8+ T cells, and CD8+ regulatory T cells. The combined frequency of MX1+OAS1+ T cells and apoptotic CD8+ T cells was significantly lower among responders versus nonresponders (*P*=.0012). Transcriptional state of the MX1+OAS1+ TIL subpopulation may be maintained by the IRF7 transcription factor, as suggested by gene regulatory network analysis. Among all patients, analyses of molecules involved in cell-cell interaction identified potentially detrimental signaling interactions, including the TGF β , galectin, Fas ligand, and MIF pathways, among different TIL product subpopulations. Analysis of the bulk TCR sequencing data suggested that higher clonality of TIL product TCR repertoire (α , β , γ , δ) was associated with response (all *P*≤.02). TIL products made from different patients shared little similarity in TCR repertoire, and overall, the majority of TCRs were previously undescribed. Joint analysis of the single-cell RNA and TCR sequencing data suggested that higher

clonality was observed among EOMES+ TIL product subpopulations and that higher clonality among CD62L+ TIL product subpopulations was associated with response ($P=.0012$). **Conclusions:** This subanalysis characterized TIL therapy infusion products using single-cell and bulk sequencing techniques and may identify actionable TIL therapy improvements, which may improve outcomes for patients with advanced cutaneous melanoma. Collectively, these findings suggest that a significant fraction of the clonotypes contained in TIL infusion products are unique to each patient, highlighting a potential benefit of TIL therapy versus more targeted T-cell therapies. These hypothesis-generating data may identify TIL therapy improvement opportunities and potential response biomarkers that warrant further study.

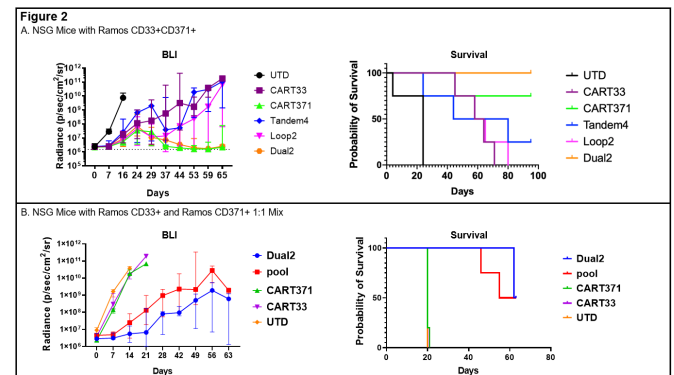
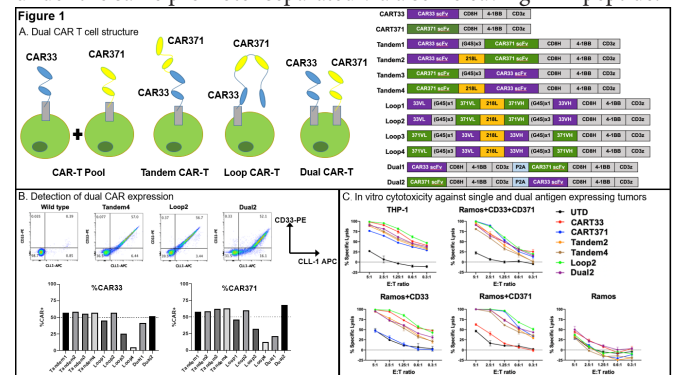
848. Dual CD33/CLL-1 Targeted CAR T Cells for Treatment of Acute Myeloid Leukemia

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Introduction: Chimeric antigen receptor (CAR) T cells are effective against hematological malignancies, but relapse is common due to antigen escape. Dual CD33/CLL-1 targeting CAR T cells can mitigate this problem by targeting tumors with mixed antigen expression. CD33 and CLL-1 (CD371) were selected as targets as these are expressed on the majority of acute myeloid leukemia (AML), and CD33-targeting CAR T cells (CART33) and CLL-1-targeting CAR T cells (CART371) are under evaluation in human clinical trials.

Methods/Results: Human CAR T cells were generated from healthy donor T cells by anti-CD3/28 bead stimulation and lentiviral gene transfer. We designed ten different CAR constructs targeting both CD33 and CD371 in several configurations to determine the most effective structure (Fig 1A). Binding of each CAR to fluorescently labeled CD33 and CD371 proteins was measured by flow cytometry (Fig 1B). In vitro cytotoxicity was measured by incubating CAR T cells with THP-1, a myeloid cell line that expresses both CD33 and CD371, at a range of effector to target (E:T) ratios. Similar assays were performed using Ramos, a lymphoid cell line engineered to express either CD33, CD371 or both antigens (Fig 1C). From these tests we determined Tandem4, Loop2, and Dual2 to be the most effective and were selected for further evaluation. We injected 5×10^5 Ramos+CD33+CD371 cells IV into NSG mice, followed by treatment with 5×10^6 CART33, CART371, Tandem4, Loop2, and Dual2 CAR T or untransduced T cells (UTD) ($n=5$ /group). Ramos cells expressed click beetle red/green fluorescence protein (CBR/GFP) to enable tracking of tumor cells by longitudinal bioluminescent imaging (BLI) and flow cytometry. While all CAR T treated mice had delay of tumor progression, only Dual2 treated mice had complete tumor clearance and long-term tumor-free survival (Fig 2A). Therefore, Dual2 was deemed the most effective dual-antigen targeting construct. To ensure that the Dual2 CAR T cells were able to control tumor cells expressing only CD33 or CD371, we injected a 1:1 mix of Ramos+CD33 and Ramos+CD371 single antigen expressing cells (5×10^5 cells total) into NSG mice, followed by treatment with 3×10^6 CAR T cells: UTD, CART33, CART371, CART33+CART371 pool (mixed at 1:1 ratio), and Dual2 ($n=5$ /group). As expected, we found that CART33 or CART371 were ineffective at controlling a mixed population of tumor cells, and tumor progression was similar to UTD-treated

mice. While pooled CART33+CART371 had efficacy against mixed tumor, we observed the best anti-tumor effect with Dual2 (Fig 2B). **Conclusion:** Dual CD33/CLL-1 targeting CAR T cells were effective at controlling tumors expressing both targets on the same cell in addition to mixed tumors cells each expressing single targets in vivo. Optimal targeting was achieved by expressing two full-length CAR constructs under the same promoter separated via a self-cleaving P2A peptide.



849. Off-the-Shelf Natural Killer Cells Derived from HIPSC via Genetic Modifications

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Human induced pluripotent stem cells (hiPSC)-derived allogeneic Natural Killer (NK) cells are emerging as a promising off-the-shelf cell therapy for cancer. However, host-versus-graft rejection limits the allogeneic NK cell durability in patients, especially when the drug is applied in a redosing regimen. Numerous efforts have been applied to engineer “hypoimmunogenic” immune cells, with limited clinical success. Therefore, a novel genetic combination remains to be developed and validated in a relevant preclinical model to warrant clinical success. In this study, we first established a Non-Human-Primate system for allogeneic NK transfusion and rejection. We then engineered a small library of monkey iPSCs, each one carrying 3-21 genetic modifications. Subsequently, we differentiated engineered monkey iPSCs into NK cells and conducted *in vivo* screening. We identified that QH-X001 had significantly longer persistency in the allogeneic monkey recipient, compared with 3-edit NK cells (MHC1 KO, MHC2 KO, CD47 KI) as the control. We then applied the edits on

GMP-grade human iPSCs and demonstrated that QH-X001 does not impact NK differentiation or function, and has robust resistance towards human T cells, NK cells, and complement challenges *in vitro*. Taken together, we identified that the novel genetic combinations of QH-X001 could render iPSCs-derived NK cells hypoinmunogenic *in vivo*. We anticipate that this could be a useful asset to greatly enhance cell therapy efficacy.

850. Targeting the C Domain of Tenascin C with CAR T Cells for the Immunotherapy of Pediatric Brain and Solid Tumors

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Cellular immunotherapy with T cells expressing chimeric antigen receptors (CARs) has been successful for hematological malignancies but has limited activity against pediatric brain and solid tumors due to multiple factors. One significant roadblock is the limited array of targetable antigens. We have developed a pipeline that identifies cancer-specific exons upregulated in pediatric solid and brain tumors for CAR targeting to overcome this barrier. From this pipeline, we identified the C domain of Tenascin C (C.TNC), an extracellular matrix (ECM) protein that contributes to the malignant phenotype of cancer, as a potential target for pediatric high-grade glioma and osteosarcoma (OS). We focused here on OS, the most common pediatric bone tumor whose prognosis remains poor in the recurrent and metastatic setting. We first demonstrated that C.TNC is expressed in OS cell lines and pediatric patient-derived xenografts. We then generated a prototype C.TNC-CAR utilizing an scFv specific for C.TNC and a CD28 ζ signaling domain. We generated C.TNC-CAR T cells by retroviral transduction from CD3/CD28-activated peripheral blood T cells, and the mean transduction efficiency was 66%. In coculture assays, C.TNC-CAR T cells recognized C.TNC-positive pediatric OS cell lines (LM7, HOS, U2OS, 143B) as determined by cytokine production and had cytolytic activity against these targets in luciferase-based cytotoxicity assays. In contrast, T cells expressing a nonfunctional C.TNC-CAR had no cytolytic activity, confirming antigen-specificity. *In vivo*, C.TNC-CAR T cells had transient antitumor activity in a locoregional model of OS (LM7 i.p.). Based on these encouraging results, we have now developed a panel of C.TNC-CAR constructs with different hinge, transmembrane, and signaling domains to optimize our CAR. Initial *in vitro* cytotoxicity studies have identified C.TNC-CARs with improved activity, and T cells that express these CARs are currently being evaluated in our *in vivo* models. In conclusion, we have demonstrated that a secreted ECM protein that binds to the tumor cell surface like C.TNC can be targeted with CAR T cells. Our encouraging results warrant further active exploration for the immunotherapy of other pediatric tumors that express C.TNC.

851. A Phase 1 Dose Escalation Study of GCC19CART a Novel CoupledCAR® Therapy for Subjects with Metastatic Colorectal Cancer

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Background: Chimeric antigen receptor (CAR) T-cell therapy has shown remarkable clinical efficacy in hematologic malignancies but limited success in solid tumors. GCC19CART, the first clinical candidate from the CoupledCAR® solid tumor platform, is designed to overcome the limitations of conventional CAR T-cells in solid tumor malignancies by pairing solid tumor CAR T-cells with CD19 targeting CAR T-cells to amplify proliferation and activation of the solid tumor CAR T component. GCC19CART targets guanylate cyclase-C (GCC) which is expressed in the metastatic lesions of 70%-80% of subjects with colorectal cancers. A Phase 1 investigator-initiated clinical trial is underway in China for patients with relapsed or refractory metastatic colorectal cancer who have received at least 2 prior lines of therapy. Based on a data cutoff on December 13, 2021, 21 subjects have been enrolled in 2 dose escalation groups at 5 hospitals in China. Methods: Subjects are screened for GCC expression by immunohistochemistry. Eligible subjects undergo leukapheresis, a single dose of lymphodepleting chemotherapy (fludarabine 30mg/m² and cyclophosphamide 300mg/m²) 3 days prior to infusion, and then administration of a single infusion of GCC19CART at one of two preassigned doses: 1x10⁶ or 2x10⁶ CAR T-cells/kg. Endpoints are safety and preliminary evidence of efficacy as determined by CT or PET/CT per RECIST 1.1 or PERCIST 1.0. All responses were confirmed by an independent third-party imaging contract research organization (CRO). Results: 13 subjects have been enrolled to dose level 1 (1x10⁶ cells/kg) and 8 subjects have been enrolled to dose level 2 (2x10⁶ cells/kg). The most common adverse events were cytokine release syndrome (CRS) in 21/21 subjects (Grade 1 19/21 (90.48%) or Grade 2 2/21 (9.52%)) and diarrhea in 21/21 subjects (Grade 1 6/21 (28.57%) Grade 2 5/21 (23.81%) Grade 3 9/21 (42.86%) or Grade 4 1/21 (4.76%)). Neurotoxicity was observed in 2/21 (9.52%) subjects at Grade 3 or 4 and resolved with corticosteroids. The combined overall response rate (ORR) for both dose levels was 28.6% (6/21). For dose level 1, the overall response rate (ORR) per RECIST 1.1 was 15.4% (2/13). Two subjects demonstrated a partial response (PR) while 3 additional subjects had partial metabolic response (PMR) on PET/CT with stable disease (SD) or progressive disease (PD) per RECIST 1.1. For dose level 2, The ORR per RECIST 1.1 was 50% (4/8). 4 subjects demonstrated a PR (3 at month 1, 1 at month 3 after being SD at month 1) and 2 additional subjects had PMR on PET/CT with SD per RECIST 1.1. Conclusions: GCC19CART demonstrated meaningful

dose dependent clinical activity and an acceptable safety profile in relapsed or refractory metastatic colorectal cancer. This trial is ongoing and updated data will be presented. A United States based Phase 1 trial of GCC19CART is anticipated for mid-2022.

852. Discovery of a Novel C07:02-Restricted Epitope on MAGE-A1 and Pre-Clinical Development of an Enhanced TCR-T Cell Therapy Candidate for the Treatment of Solid Tumors

Nancy Nabils, Jenny Tadros, Teagan Parsons, Melissa Carr-Reynolds, Kenneth Jahan, Sida Liao, Dalena Nguyen, Andrew Ferretti, Hannah L. Bader, Briana Zimmerman, Tary Traore, Maytal Bowman, Antoine Boudot, Qikai Xu, Cagan Gurer, Yifan Wang, Gavin MacBeath

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Immune checkpoint therapy enhances the anti-tumor activity of T cells and has led to significant and durable responses in a subset of cancer patients. Identification of the T-cell receptors and cancer antigens that drive these positive anti-tumor responses are of great interest as it allows them to be developed into TCR-T cell therapy candidates, thus extending these anti-tumor benefits to patients that share the same tumor antigens but lack the appropriate T cells. Using our proprietary *TargetScan* genome-wide screen, we interrogated patient-derived TCRs that underwent clonal expansion in response to immune checkpoint therapy. Among other shared antigens, the cancer testis antigen MAGEA1 was identified in a *TargetScan* screen which evaluated multiple expanded TCRs from a Head & Neck cancer patient who exhibited substantial tumor reduction after only 8 weeks of therapy. Notably, two distinct TCR clonotypes recognized a novel C*07:02-restricted epitope, suggesting that a polyclonal response to a single MAGEA1 epitope may have contributed to the tumor reduction of this patient. Engineered T-cells expressing each TCR exhibited significant activation, cytokine production and cytotoxicity against a panel of HLA-C*07:02-positive cancer cell lines expressing varying levels of MAGEA1. To ensure that these TCRs do not exhibit problematic off-target recognition, each candidate TCR was individually screened against a genome-wide library comprising sequences and prevalent SNPs from every protein in the entire human proteome. This identified TCR-41 as suitable for clinical development. Additional safety assessments revealed no allo-reactivity relative to a panel of 110 HLA-I molecules, and no discernable recognition of a diverse collection of C*07:02-positive primary normal cells. To further enhance the activity of our T cells, we designed a transposon-based vector that delivers the TCR gene, along with the genes for CD8 α/β and a dominant-negative form of TGF β R2, into both CD4⁺ and CD8⁺ T cells. We have advanced the resulting autologous TCR-T cell therapy candidate, TSC-204-C07, to IND-enabling studies. These results validate the use of *TargetScan* to identify relevant tumor antigens and TCRs from the expanded T cell repertoires of patients exhibiting strong and durable responses to immune checkpoint blockade.

853. Asymmetric Cell Division for Fate Induction of Chimeric Antigen Receptor (CAR) T Cells

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Early expansion and long-term persistence predict efficacy of genetically-engineered T cells. While this is thought to reflect successful induction of effector and memory T cell populations to provide both short-term clearance and long-lasting remission, the cellular mechanisms of fate induction after T cell activation through synthetic receptors are unknown. A better understanding of such processes could improve therapeutic outcome. Here we show that human T cells engineered to express chimeric antigen receptors (CARs) undergo asymmetric cell division (ACD) with distinct proximal and distal daughter cells that adopt effector and memory phenotypes, respectively. Using molecular proximity labeling to distinguish first division proximal and distal daughter cells, we demonstrate that target-engaged CAR molecules remain on the proximal first division daughter cell and establish cellular asymmetry between daughter cells in size, proliferative pace, transcriptional program and metabolism. The single cell transcriptional program of proximal first division daughter cells is driven by c-myc, mTORC1, and JAK-STAT3 activation (each with adjusted $p < 0.001$) resulting in primarily glycolytic energy production, features consistent with effector T cell differentiation. Conversely, distal daughter cells utilize BACH-2, ETS-2, and KLF2 (each with adjusted $p < 0.001$) to shape their transcriptome, indicating a memory precursor phenotype that was accompanied by an overall muted metabolic activity and predominance of oxidative phosphorylation. Surprisingly, despite their memory precursor phenotype, first division distal daughter cells maintain similar cytolytic activity as proximal daughter cells for up to 48 hours after cytogenesis, uncovering a transient state of increased target sensitivity. This period of 'target readiness' is followed by a substantial decrease in cytotoxicity in distal, but not proximal daughter cells, highlighting functional plasticity as a hallmark of early memory CAR T cell differentiation. In vivo characterization of first division daughter cells in 2 separate xenograft leukemia mouse models confirms superior leukemia elimination ($n=14$, $p=0.0004$) and long-term persistence ($n=12$, $p=0.0051$) by distal daughter cells, functionally establishing these cells as memory precursors responsible for long-term efficacy of human CAR T cells. Collectively, these studies establish ACD as a novel framework for understanding mechanisms of CAR T cell differentiation and influencing therapeutic outcomes.

Engineered Cell Therapies

854. Primary Human Hepatocytes, Genetically Engineered Ex Vivo to be Hypoimmunogenic, Can Rescue a Model of Metabolic Liver Disease

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Background: Orthotopic liver transplantation is the only established treatment for patients with severe liver disease; however, its application is limited due to a shortage of donor organs and need for life-long immunosuppression. Alternative therapies have been pursued to address the huge unmet medical needs of liver diseases, with primary human hepatocytes (PHH) transplantation demonstrating clinical efficacy in multiple liver indications. However, two major factors have limited the widespread use of hepatocyte transplantation: a shortage of high-quality hepatocytes and longitudinal loss of transplanted cells due to immune recognition and clearance of allogeneic cells. In this study, we hypothesized that PHH could be engineered ex vivo to be hypoimmunogenic and that these engineered hepatocytes could be expanded in an in vivo bioreactor to generate high quality “universal” hepatocytes suitable for therapeutic applications in many liver disease indications. **Methods and Results:** Utilizing clinically validated genetic delivery modalities, a platform was optimized to efficiently engineer PHH ex vivo. >80% double-editing efficiency was achieved for both knock-out and knock-in engineering events consistently in PHH ex vivo. To induce hypoimmunogenicity in PHH, HLA class I expression was blocked by CRISPR/Cas9 nuclease-mediated B2M gene knockout to prevent CTL recognition (PHH do not express HLA class II molecules). In addition, several decoy genes, including HLA-E and CD47, were overexpressed with lentiviral vectors (LV) to inhibit NK cell clearance. The engineered PHH demonstrated protection against CTL and NK cells from mismatched donors in killing assays in vitro, while maintaining normal human hepatocyte function. Finally, to demonstrate the in vivo functionality and expandability of these engineered hepatocytes, the hypoimmunogenic cells were transplanted into an immune-deficient mouse model of hereditary tyrosinemia type 1 (FRG mouse). The hypoimmunogenic engineered human hepatocytes proliferated and readily repopulated the FRG mouse, demonstrating the ability to expand the quantity of engineered cells in vivo >100-fold. Importantly, engineered hepatocytes expanded in vivo at comparable kinetics to non-edited cells, and thus rescued the HT1 mice at levels comparable to normal functioning, non-edited hepatocytes. **Conclusion:** The data generated clearly demonstrate efficient (>80%) genetic engineering of primary human hepatocytes to render them hypoimmunogenic, and their subsequent expansion. This is the first report of fully functional universal human hepatocytes. We expect that combining this engineering approach with our ongoing

development of the FRG rat bioreactor for large-scale expansion of primary human hepatocytes will provide large quantities of high-quality hypoimmunogenic hepatocytes. This would allow the treatment of a much wider spectrum of liver disease patients using allogeneic hepatocytes without immune suppression.

855. Kinase p38 Regulates Macrophage Adaptation in the Lung Upon Pulmonary Macrophage Transfer

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In the last decade, the interest in macrophages (MΦ), as an attractive cell type for cell-based therapies, has greatly increased. The discovery, that most tissue-resident MΦ are (I) of embryonic origin, (II) have self-renewal capacity, and are (III) long-lived, render them a promising candidate for novel regenerative therapies. Several studies highlighted the therapeutic effects of pulmonary MΦ transfer (PMT) in the context of different lung diseases, including pulmonary infections. However, a deeper understanding of the driving factors, which promote MΦ adaption post PMT is still missing. Using single-cell analysis of MΦ post-PMT, we demonstrated a stepwise adaptation process of bone marrow (BM)-derived MΦ towards alveolar MΦ (AM). In a time-dependent process, transferred BM-MΦ adapt an AM-like surface marker profile and AM-associated transcriptome, which was underlined by a population of MΦ expressing Ki67, indicating local proliferation and adaptation of transferred cells. To gain a deeper understanding of this adaption process, we performed CRISPR/Cas9 mediated knockout (KO) of various candidate genes, including the kinase p38, known to regulate self-renewal in hematopoietic stem cells (HSC) but also stress response, differentiation and MΦ function. *In vitro* differentiation studies showed no impact of p38 KO on MΦ differentiation, proliferative potential, morphology and surface marker expression of BM-MΦ. In a competitive BM transplantation, we observed superior engraftment of (GFP⁺dTom⁺) p38 KO HSC compared to (GFP⁺eBFP⁺) wildtype (WT) HSC in the BM. However, AM in the lung were mainly GFP⁺eBFP⁺ WT-derived, suggesting a role of p38 in AM biology. Since p38 KO did not affect myelopoiesis in the BM, we adoptively transferred a 1:1 mixture of WT and p38 KO BM-MΦ directly into the lungs of a *Csf2rb* deficient mouse model, which lacks endogenous AM. Engraftment and adaptation of both donor cell populations was observed, illustrated by an AM-like surface marker profile (CD45⁺CD11c⁺SiglecF^{mid}CD11b⁻). In addition, an improved disease phenotype such as improved turbidity and lower protein and cholesterol levels in the broncho-alveolar lavage fluid of transplanted *Csf2rb* deficient mice indicated proper AM function. However, in contrast to the competitive BM transplantation experiments, the ratio of WT:p38 KO cells changed to the advantage of p38 KO cells,

which comprised 65-75% of the engrafted cells. These results suggest a beneficial effect of p38 KO on the overall fitness of AM under stress conditions, which may be abolished in steady state.

856. *Ex Vivo* Transduced Macrophages Engraft in the Lung Following Transplantation and Produce Therapeutic Levels of Secreted Proteins

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We are assessing the feasibility of cell therapy-based treatments for respiratory diseases and have optimised transduction of murine bone marrow-derived macrophages (BMDM) with our proprietary F and HN pseudotyped lentivirus (rSIV.F/HN). We have shown similar transduction efficiencies compared to VSV-G pseudotyped SIV lentivirus (F/HN: $10.7 \pm 1.9\%$, VSV-G: $12.9 \pm 5.6\%$, MOI ~50). We next assessed whether gene modified BMDM can function as factories for production of secreted proteins following transplantation to murine lungs. Successful macrophage therapies have thus far relied on the depletion or absence of endogenous alveolar macrophage compartments to facilitate transplanted cell engraftment. Here, BMDM of ly5.1/*Ptprca* donor mice were transduced with rSIV.F/HN carrying Gaussia luciferase (Gluc) cDNA (MOI 20) and transplanted into lungs of wildtype mice through oropharyngeal delivery ($3e6$ cells/mouse, $n=6$ /group). Gluc expression in the lungs was stable for up to 16 weeks (control: 21.6 ± 8.2 RLU/ μ l, week 2: 12027 ± 2251 RLU/ μ l, week 16: 21983 ± 3763 RLU/ μ l, in broncho-alveolar lavage fluid [BALF]) indicating transplanted cells can be retained in the lungs even in the presence of an established alveolar macrophage compartment. We next performed proof-of-concept studies in a mouse model of auto-immune pulmonary alveolar proteinosis (aPAP). aPAP is caused by the development of anti-GM-CSF antibodies, which leads to defective surfactant clearance and deposition in alveoli, resulting in breathing difficulties. BMDMs from donor mice were transduced with rSIV.F/HN carrying GM-CSF or Gluc cDNA (MOI 20) and transplanted into the lungs of PAP mice ($6.3e6$ cells/mouse, $n=6$ /group). Four weeks post transplantation, the turbidity of BALF was significantly ($p<0.05$) reduced (optical density: GM-CSF: 2.2 ± 0.7 , Gluc: 4.1 ± 0.9) and we also detected a trend in reduction of surfactant protein-D in BALF (GM-CSF: 2504 ± 3084 ng/ml, Gluc: 4987 ± 1852 ng/ml) indicating a clearance of the surfactants. In summary, we have demonstrated that macrophage engraftment in mouse lungs is feasible following pulmonary transplantation in the presence of endogenous alveolar macrophages. Transplantation of gene-modified BMDM then led to correction of biomarkers in a mouse model of PAP establishing their potential as vehicles for secreted protein expression in the lungs.

857. Scalable Generation and Tailored Design of Human iPSC-Macrophages for Novel Immunotherapies Targeting Bacterial Infections

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Macrophages can be found in various tissues and play an important role in organ function by sensing and eradicating pathogens, regulating immune responses and contributing to tissue homeostasis and repair. Nowadays, increasing numbers of macrophage-based cell therapies are entering (pre-) clinical studies, e.g. for the treatment of lung or liver cirrhosis, chronic inflammation or cancer. We have also previously introduced a novel immunotherapy concept utilizing the adoptive transfer of macrophages to target pulmonary infections caused by different Gram positive- and -negative pathogens. However, the diverse functions, activation stages and overall plasticity of macrophages, highlight the necessity to establish the robust production and tailored design of specific macrophage subsets for individualized macrophage-based therapies. We here demonstrate the efficient and continuous production of induced pluripotent stem cells (iPSC)-derived macrophages (iMac) in stirred-tank bioreactors, which represent a highly pure CD45⁺CD11b⁺CD14⁺CD163⁺ population, exhibit classical functionality and show a transcriptional profile similar to blood-derived macrophages though with a more primitive fingerprint. After contact with Gram-negative *Pseudomonas aeruginosa* or Gram-positive *Staphylococcus aureus*, iMacs remodeled their transcriptome towards the signature of activated macrophages, secreted important pro-inflammatory cytokines and efficiently phagocytosed and killed these bacteria. Interestingly, iMac demonstrated a stronger pro-inflammatory response to the bacterial stimulus when compared to blood-derived macrophages. We next evaluated different strategies in order to further tailor the functionality of iMac and to increase their antimicrobial activity. First, we utilized polarization with IFN γ to imprint a proinflammatory M1 phenotype in the iMac. M1(IFN γ)iMac showed an upregulation of CD86, CD64 and HLA, and gene sets e.g. associated with inflammatory response or chemokines and secreted elevated levels of CXCL10 and IL6. Moreover, they displayed an enhanced reaction to a LPS used as a secondary inflammatory stimulus. In addition, we performed a preloading of iMac with different antibiotics to enhance the killing capacity. We show that iMac efficiently take up Gentamicin and Levofloxacin and demonstrate an improved killing of viable *P.*

aeruginosa or *S. aureus* bacteria *in vitro*. Interestingly, Gentamycin remained intracellular in the iMac for > 24 hours, hinting to a direct role in the enhanced intracellular killing. In contrast, Levofloxacin was quickly released from iMac into the medium, suggesting a possibility to utilize preloaded iMac as a carrier to deliver drugs to the site of infection - in addition to the intrinsic antimicrobial potential of the cell. In summary, we demonstrate the scalable generation and tailored design of human iPSC-macrophages as a new immunotherapy targeting bacterial infections.

858. Bioprinting of Organotypic Aggregates of Hepatocytes and Mesenchymal Cells as a Platform for Liver Cell Therapy

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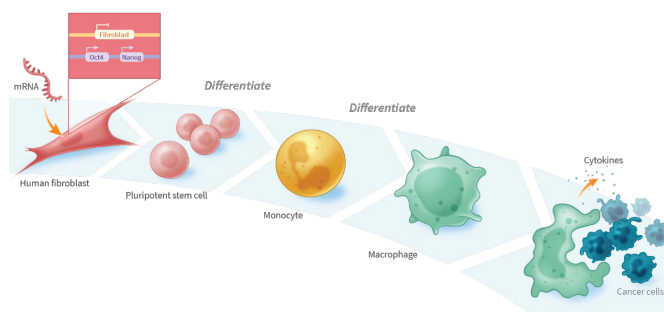
Background: Severe liver failure is currently treated with liver transplantation. Organ transplantation is made difficult due to the limited supply of organs; and the complexity, and invasiveness of the surgery. Liver transplant recipients must receive lifelong immunosuppression therapy which increases susceptibility to infections and some forms of cancer. Transplantation of isolated hepatocytes has recently been demonstrated to treat pediatric acute liver failure. Long term viability and function of grafted hepatocytes remains a challenge. Herein we present data that our unique bioprinting technology is capable of generating hepatocyte implants that maintain high viability and function both *in vitro* and *in vivo* after implantation within the intraperitoneal (IP) space. **Methods:** Hepatocytes and CD166 positive Mesenchymal Stem Cells (MSCs) were aggregated into spheroids and then printed in an alginate-based fibre using the Aspect RX1 bioprinter. Using this approach, cells are placed within a cell friendly core material surrounded by an immune isolating shell. After printing, viability, synthetic, and metabolic function was tested *in vitro* using a Calcein AM stain, ELISAs (Albumin, A1AT) and colorimetric assays (ammonia). Hepatocyte implants containing 1 million cells were also inserted into the IP space of NSG mice. Plasma was collected over 28 days and analyzed for the presence of human proteins including A1AT and Albumin. Fibers were retrieved at the end of the study to analyze for viability and function. **Results:** Following aggregation, hepatocyte and MSC implants cultured *in vitro* were able to synthesize Albumin at ≥ 20 ug / million cells / day. Cells containing implants with 1 million hepatocytes implanted into NSG mice showed human plasma albumin at ≥ 2000 ng/mL with levels remaining steady for 28 days. Following retrieval, implants maintained >75% viability and retained the ability to detoxify ammonia and produce albumin *in vitro*. **Conclusion:** Our data indicates that bioprinted implants containing hepatocytes and MSC are a promising option for future use in treatment of liver diseases. In future studies, we aim to demonstrate the efficacy of these implants for extending survival of small and large animal models of acute liver failure.

859. iPSC-Derived Monocytes Generate Functional M1 and M2 Macrophages with Enhanced Cytokine Secretion and Tumor Cell-Killing Activity

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Cancer immunotherapy has advanced rapidly over the past two decades, with several autologous chimeric antigen receptor (CAR)-T cell therapies approved for the treatment of hematologic cancers. However, CAR-T cells have shown limited activity against solid tumors, in part due to the immunosuppressive nature of the tumor microenvironment preventing CAR-T cell infiltration. This has led to investigation of other immune cells as alternatives to T-cell-based therapies, including monocytes and monocyte-derived macrophages, which exhibit innate tumor-infiltration properties. We developed a process for differentiating pluripotent stem cells along a myeloid lineage, and generated populations of cells with characteristics of monocytes and M1 and M2 macrophages, including cytokine secretion and tumor cell-killing activity. mRNA-reprogrammed human induced pluripotent stem cells (iPSCs) were differentiated into monocytes using a 28-day monolayer protocol. Beginning on day 14, cells were harvested every 3-4 days. CD14+ isolation yielded >95% CD14+ cells with an average yield of 4.1×10^4 cells per cm^2 per harvest. iPSC-derived monocytes were compared to peripheral blood mononuclear cell (PBMC)-derived monocytes for expression of key hematopoietic and myeloid-lineage markers CD11 β , CD14, CD33, CD45, CD80, CD163, CD206, and SIRP α . iPSC-derived monocytes showed similar expression of CD11 β , CD14, CD33, CD45, and CD163 compared to PBMC-derived monocytes, and increased expression of markers indicative of an activated state: CD80 and CD206. Compared to PBMC-derived monocytes, iPSC-derived monocytes showed both higher viability in culture and superior recovery from cryopreservation. iPSC-derived monocytes were further differentiated into macrophages by exposure to MCSF for 3-4 days, and were assessed for their ability to polarize, secrete pro- and anti-inflammatory cytokines, and for cytotoxic activity when co-cultured with cancer cells. M1 macrophages were polarized with interferon gamma (IFN- γ , 50 ng/mL) and lipopolysaccharide (LPS, 10 ng/mL) for 48 hours, while M2 macrophages were treated with IL-4 (10 ng/mL) for 48 hours. iPSC-derived monocytes differentiated into macrophages with >90% efficacy, as assessed by cell adherence, morphology, and surface marker expression (CD14, CD45, CD163). M1 and M2 polarized iPSC-derived macrophages secreted similar levels of TNF α , IL-12p70, and IL-10 compared to PBMC-derived macrophages. iPSC-derived macrophages killed 45% of U2OS cancer cells *in vitro* after 24 hours at an E:T ratio of 5:1. We demonstrate a process for differentiating mRNA-reprogrammed iPSCs into cytotoxic macrophages. The mRNA reprogramming and differentiation processes are virus-free and DNA-free, avoiding any potential risk of vector integration. These results suggest that mRNA-reprogrammed iPSCs may represent a viable source of macrophages for the development of therapies to treat various indications, including solid tumors.



860. Transplantation of Gene Edited Upper Airway Basal Stem Cells in Immunocompromised Mice Using Fibrinogen Based Scaffolds

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Cystic fibrosis (CF) is a fatal genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride channel. CF results in chronic lung infections that cause lung failure. Although CFTR modulators have benefited many patients significantly, some patients are affected by mutations that are not responsive to modulators. Gene therapy approaches that restore CFTR function have the potential to treat all CF patients. However, delivery of the CFTR gene *in vivo* using viral and non-viral strategies have been unsuccessful. Recognizing these challenges, we are working to develop an *ex-vivo* gene corrected autologous airway stem cell therapy for CF. We have previously reported the use of CRISPR/Cas9 and adeno-associated viruses (AAV) to correct the F508del mutation and to insert the full-length CFTR cDNA in the endogenous CFTR locus. Airway basal stem cells corrected using these approaches restore CFTR function to ~40-70% of the CFTR function seen in non-CF controls. The transplantation of the corrected airway stem cells is the next technical hurdle that needs to be resolved for the clinical translation of this approach. The transplantation of cells into the lower airways poses many safety concerns. Recognizing this, we identified the upper airways (nose and sinus) as a readily accessible source of upper airway basal stem cells (UABCs) which can be used to treat CF sinusitis. This approach enables us to treat CF sinusitis while minimizing safety concerns to patients and will inform future efforts to transplant airway stem cells into the lower airways. In preliminary *in vivo* experiments, we discovered that the delivery of cells in saline into the nasal cavity of mice resulted in their expulsion. Therefore, we attempted to identify biomaterial scaffolds that will adhere to the tissue and facilitate the engraftment of the gene corrected airway stem cells. We evaluated the ability of several materials including type I collagen, laminin foam-gel, fibrinogen, alginate, hyaluronan and dextran to support the survival and proliferation of UABCs. We used basement membrane extract from mouse tumors (MatrigelTM) as controls. Among the materials tested, UABCs seeded in laminin and fibrinogen showed 5±1 and 9±6 fold expansion respectively compared to 4±4 fold expansion in Matrigel. In addition, over 90% of the UABCs cultured in laminin and fibrinogen gels maintained the expression of

cytokeratin 5 after 4 days in culture. We then evaluated the ability of these materials to facilitate the transplantation of UABCs from mice that endogenously express firefly luciferase into immunocompromised NOD scid gamma (NSGTM) mice. In preliminary studies, the upper airways of NSG mice were injured using 2% povidone iodine and transplanted with UABCs using human fibrinogen and recombinant laminin. These mice exhibited stable bioluminescence for over 60 days. We then attempted to transplant human UABCs into NSG mice using fibrinogen gels. Human UABCs were genome edited using CRISPR/Cas9 and AAV to express NanolucTM and GFP. UABCs expressing GFP were enriched using flow cytometry to over 80% purity and transplanted into mice. Preliminary experiments show that the transplanted human UABCs produce a stable bioluminescent signal for ~120 days. Studies to evaluate the differentiation of the transplanted human UABCs *in vivo* are ongoing.

Vector Manufacturing and Engineering 3: Improving Vector Design and System Performance

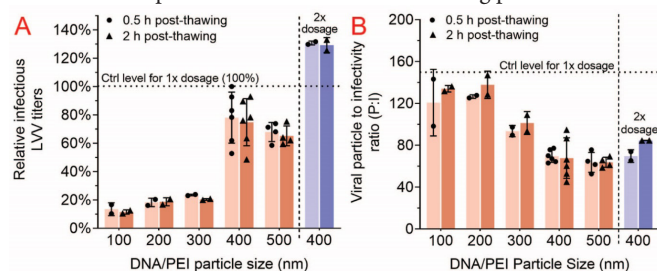
861. Size-Optimized and Shelf-Stable Plasmid DNA Particles for Production of Viral Vectors

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The quality of the plasmid DNA (pDNA) particles packaged with a pDNA cocktail and poly(ethyleneimine) (PEI) is critical to their ability to transfect HEK293 cells for production of lentiviral vectors (LVVs). With an increasing need to produce viral vectors with higher yield, lower cost, and better quality control, process development for pDNA/PEI particle assembly will offer tangible benefits to increasing transfection efficiency and LVV yield and improving the reproducibility and shelf-stability of pDNA/PEI particles. Here we identified the size of pDNA/PEI particles as a key determinant for a high transfection efficiency with an optimal size of about 400 nm, due to a cellular uptake-limited mechanism. We developed a kinetics-based approach to assemble stable 400-nm particles using preassembled sub-100-nm nanoparticles as building blocks in a bottom-up manner. This approach is based on charge screening and ionization on particle surfaces that introduce either controllable particle size growth or stabilization, respectively. Because it depends solely on modulation and reversal of solvent compositions, it was implemented into a compartmentalized, stepwise mixing procedure using confined impinging jet (CIJ) mixers with capabilities of continuous and scalable manufacturing. The procedure was validated to produce particles at a pDNA concentration of 50 µg/mL, synthesized at a flow rate of 100 mL/min with a single device. Critical quality attributes (CQA) were established based on particle size, particle polydispersity, pDNA concentration in suspension and stability upon freeze-thaw cycle. The synthesized particles were cryopreserved at -80 °C and could stay stable for at least 4 months. They could also stay stable at room temperature upon thawing for up to 48 h without losing transfection potency, which is a preferred property to streamline the LVV production process.

We used a four-plasmid lentiviral vector (LVV) system for validation. The particles were synthesized in a university research lab and shipped frozen to a corporate partner located in another city to be tested in in-house developed stirred tank (STR) bioreactors. The LVV yield from stable 400-nm particles matched the highest yield from industrial standard (Fig. 1A). The high stability at ambient temperature for 2 h after thawing and nearly 70% increase in LVV titer at a 2-fold DNA dose were features previously unattainable with particles prepared using the standard method. Owing to co-encapsulation of all plasmid species at desired ratios in *pDNA*/PEI particle assembly, transfection by these stable particles resulted in better co-expression of all viral components, reflected as more efficient LVV assembly thus a lower viral particle-to-infectivity ratio (*i.e.*, less empty LVVs and higher purity, Fig. 1B). These results showcased that the assembly method to generate size-optimized, shelf-stable *pDNA*/PEI particles can be developed into a platform technique that would potentially standardize and improve the transfection step in the viral vector manufacturing processes.



862. The Primate Selective Transduction of rAAV-LK03 Vectors is Related to Variation in Histone and Histone Post-Translational Modifications on the Viral Genome in the Host Nucleus

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There is little concordance comparing transduction between species when using recombinant AAV vectors. This makes it hard to select the optimal vectors for human clinical trials. Recombinant AAV-LK03 was shown to robustly transduce human but not mouse hepatocytes in a humanized mouse model¹. Furthermore, this capsid is currently being evaluated in several clinical trials². We aim to better understand the observed discordance between rAAV transduction amongst different species, not only to gain a better understanding of basic AAV virology but allow better means to predict more robust rAAV vectors for human gene therapy. We compared AAV-LK03 transduction in multiple mouse and human cell lines. Transduction defined by transgene expression was 100x higher in the human vs mouse lines. We next compared transduction steps such as 1) internalization to the cell, 2) nucleus, 3) stable double strand proviral DNA genomes. To our surprise, we found only small differences (less than 2-fold) between species, which could not explain the large difference in transduction based on transgene expression. Using Cut&Tag methods, we explored the enrichment of histones and histone modifications associated with the viral genome. We tested three core histones, H3, H2A and H4 and four post-translational

modifications (PTMs) of H3. H3K4me2 and H3K27ac are generally associated with actively transcribed chromatin; H3K9me3 and H3K27me3 are repressive histone marks. We found that histone H3 and its active PTMs, H3K7me3 and H3K27ac were depleted on the viral genome transduced by LK03 inside mouse cells. We have also engineered a point mutant in the LK03 capsid that increases the efficiency of transduction in mouse cell lines and found H3 and its active PTMs enriched on the viral vector. These results make us hypothesize that the capsid proteins that uncoat the viral genome in the nucleus interact with host proteins to help set up the epigenetic state of the viral episome and that these interactions differ between species. We are currently investigating host proteins associated with capsid or viral vector by Mass Spectrometry, to better understand the mechanism of transduction by LK03 in both species. Unraveling these mechanisms will allow for better predictions of how to select optimal vectors in human clinical trials. (1) Lisowski L., et al., Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature* 2014; volume 506, pages382-386. (2) George L.A., et al., Multiyear Factor VIII Expression after AAV Gene Transfer for Hemophilia A. *N Engl J Med* 2021; 385:1961-1973.

863. Development and Characterization of Highly Optimized Monoclonal Producer Cell Lines (PCLs) for the Treatment of CDKL5 Deficiency Disorder (CDD)

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CDKL5 deficiency disorder (CDD) is a rare, X-linked dominant, neurodevelopmental, early infantile epileptic encephalopathy (EIEE) caused by monoallelic pathogenic mutations in the *CDKL5* gene. CDD is characterized by infantile onset of seizures and significant developmental and neurological delays. Ultragenyx has developed a producer cell line (PCL) manufacturing platform for production of recombinant AAV (rAAV) gene therapy products, which has demonstrated scalable 2000L production in an industrial setting to support ongoing early Phase I/II and planned Phase III clinical trials. Utilizing our PCL manufacturing platform, we developed a novel rAAV vector for the treatment of CDD, followed by the generation and characterization of highly optimized monoclonal PCLs to facilitate large scale production. PCL generation was initiated via transfection of a single plasmid containing the AAV rep-cap genes, a rAAV CDKL5 transgene cassette flanked by AAV ITRs and an antibiotic resistance gene, followed by application of selective pressure to isolate stably integrated cells. Three parental pools of stable integrants were isolated, single-cell seeded via Verified In-Situ Plate Seeding (VIPS™) and monitored for monoclonality with cell imaging analysis during outgrowth. High-throughput screening of monoclonal stable integrants for rAAV productivity identified 6 top producers generated from 3 parental cell lines, with final productivities >1.5E11 GC/mL, which were confirmed in an automated bioreactor system. Following isolation and banking, top monoclonal PCLs were subjected to a comprehensive 16-week stability study designed to mimic the growth requirements

of a successful 2000L production run. Phenotypic analyses of the CDKL5-rAAV producing PCLs demonstrated stable cellular growth, rAAV productivity and banking recovery during the stability study, and genotypic analyses demonstrated stable integrated genome copies, integration sites and genomic fidelity. Finally, quality assessments of rAAV purified from the monoclonal PCLs showed >70% full particles with low intermediates and undetectable subgenomic fragments via alkaline DNA gel electrophoresis. Taken together, these data suggest we have developed a robust monoclonal PCL for efficient, large-scale manufacture of rAAV for the treatment of CDD.

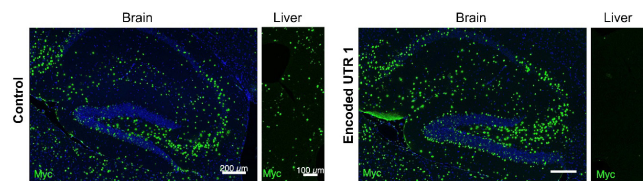
864. Optimized Human Regulatory Sequences Achieve Targeted Expression in CNS and Decreased Liver Expression in Mice

Mitchell Lopez, Serena X. Liu, Steven A. Tan, Tulasi I. Solanki, Ben Zhao, Pujya Dhanota, Andrew Kim, Simon Levine, James S. Griffin, John McLaughlin, Gregory Lucey, Kenneth McCullough, Saki Shimizu, Chi Zhang, Maria Candidia Vila, Kartik Ramamoorthi, Stephanie Tagliatela, Raghavendra Hosur, Annie Tanenhaus, Martin Moorhead

Encoded Therapeutics - South San Francisco, CA, South San Francisco, CA

Adeno-associated virus (AAV)-mediated gene therapy (GT) has demonstrated transformative potential in treating genetic disorders, however targeted transgene expression is essential to its safety and efficacy. Achieving appropriate cell-specific expression in target tissues while minimizing off-target transgene expression in the liver is key due to high hepatocyte tropism of clinically validated AAV serotypes. Human genomic regulatory elements (REs) can produce exquisite control of gene expression, but efficient identification of sequence elements that confer cell-selective expression remains a challenge. We have developed a RE discovery platform that leverages expression-based functional screening and computational modeling to rapidly and iteratively discover optimized sequence elements to regulate transgene expression that can be applied to various cell and tissue types. Here, we have applied our high-throughput next generation sequencing (NGS)-based screening method to simultaneously test thousands of genomic elements in vivo to uncover 3'UTR sequence elements that selectively decrease liver expression while maintaining central nervous system (CNS) expression. In a first-generation (Gen1) screen, over 10,000 3'UTR variants comprised of human-derived sequence elements were tested in a multiplexed AAV library concomitantly delivered systemically and via intraparenchymal injection to the CNS in mice. The top 10 3'UTR sequence candidates maintained robust expression in target brain tissue and showed an up to 100-fold reduction in transgene expression in the liver compared with control 3'UTR element vectors (Figure 1). We then generated a follow-up AAV library using multiple combinations as well as sequence variants of these top 3'UTR sequences, with lead variants showing dramatically improved liver-selective de-targeting activity. In addition, using the functional data from the Gen1 screen, we built predictive sequence-based models, which were used to inform sequence selection for a second-generation (Gen2) screen. Compared with the Gen1 screen, the distribution of activity profiles of the 10,000 REs measured in the Gen2 screen showed significant further reduction in liver expression

in mice, with unchanged expression in the brain. The specificity of expression achieved demonstrates that the RE prediction model of our discovery platform is able to discover genomic sequences that modulate gene expression selectively in targeted cell types. We have applied this approach to the discovery of various sequence-encoded genetic elements - enhancers, promoters and UTR elements - to achieve desired transgene expression profiles to improve the safety and efficacy of GT for a broad range of monogenic and non-monogenic diseases.



865. Development of Next Generation Vaccine Platform with Self-Amplifying mRNAs

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Introduction: The COVID-19 pandemic remains challenging to control due to the 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 mRNA-based vaccines for human use have shown progress in producing protective immunogenicity against the variants of SARS-CoV-2. Self-amplifying mRNA (saRNA) has emerged as a newly designed mRNA platform that can replicate itself, allowing a substantially lower dose for effective immune response. Herein, we demonstrated the preclinical success of our saRNA-based vaccines encased by lipid nanoparticles (LNPs) that were scaled-up by microfluidic technology. **Methods:** Novel ionizable lipids were selected from a library after assessing the physicochemical profiles and biological read outs for its use in LNP. saRNAs were synthesized from in vitro transcription. Full-length SARS-CoV-2 spike protein-encoded saRNA encapsulated LNPs were manufactured using NxGen microfluidic technology platform and characterized for hydrodynamic size, polydispersity, loading, and encapsulation efficiency. The lead saRNA-based vaccine candidates were screened and assessed for protective immunogenic responses including humoral and cell-mediated immunity, following a prime-boost model 4 weeks apart in Balb/c mice. Viral neutralization titres were further analyzed. Finally, protective immunogenic responses of our saRNA-based vaccine candidates were evaluated in a hamster virus challenge model against SARS-CoV-2 virus. **Results:** saRNAs-encapsulated LNPs produced were in the desired range of 60-80 nm with narrow PDI and with high encapsulation efficiency. Our

repeated preclinical investigations exhibited the generation of robust humoral and cell-mediated immune response in both mouse models. In both animal models, no clinical symptoms and adverse effects were observed. Vaccine candidates protected 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. **Conclusion:** Overall data provide a clear insight that our NxGen microfluidic technology platform is fully capable of scaling-up large volumes of LNP-based saRNAs vaccines by retaining the Critical Quality Attributes (CQA) of the drug product. In addition, the obtained protective immunity in both animal models from our preclinical studies strongly suggests pre-clinical evidence for exploring the development of saRNA LNP based vaccine platform along with mRNA LNPs.

866. Long Term Stability Profiles of AAV Vectors at Ambient Temperature Within a Film Matrix

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Significant progress has been made in the development of gene therapy vectors with products receiving approval by the EMA and U.S. FDA. Despite this, like COVID vaccines, they are stored and shipped at ultralow temperatures. This poses significant logistic and economic barriers with respect to global access to these therapeutics. To address this important issue, we developed a novel method for stabilizing live viruses in a peelable film matrix that can be stored at ambient temperature. Stability of AAV9 CBA-Luc was evaluated in high (F1S) and low (F2S) viscosity films. Virus embedded in each formulation and stored at 4°C for 5 months retained 100% of its initial titer. In addition, high- and low- viscosity formulations maintained 90% and 85% of the initial infectious titer after storage at 25°C for 6 months respectively. Infectious virus was not detected after 4 months in the currently marketed formulation stored at 25°C. *In vivo* studies in mice demonstrated that biodistribution and transgene expression profiles of vector dried within the film matrix, stored for 150 days at 4°C, shipped from Texas to North Carolina without dry ice/temperature regulation were equivalent to those of vector stored frozen in the commercial formulation when given via tail vein at a dose of 1×10^{11} vg/mouse. To further evaluate the robustness of this approach, transgene expression in tissues obtained from animals given virus prepared in films stored at 25°C for 100 days and shipped from Texas to North Carolina in the same manner remained unchanged or were slightly reduced for the majority of tissues with respect to those obtained from mice given, fresh frozen virus at doses of 1×10^{10} and 1×10^{11} vg/mouse. This suggests that storage of AAV in a film matrix facilitates easy transport of vector to remote sites without compromising *in vivo* performance. Companion

studies using AAV vectors of different serotypes/capsid compositions will also be discussed as well as perspective of utility of this stable formulation modality for other gene therapy delivery systems.

867. Combination of Advanced Plasmid Design, Transfection Reagent and Design of Experiment (DOE) Achieves High-Yield, High-Quality and Potent AAV Vectors in Scalable Suspension HEK293 Cells

Jing Liao, Hans Reuter, Carmen Wu, June Deng, Yinxing Chen, Noel Walsh, William Lee, Shiliang Hu, Nannan Jia, Priyanka Vaishampayan, Ruohong Zhou, Amy Bastille, John Xiong, Nicholas DiGioia, Jeremy Kerrick, Lauren Drouin, Matthias Hebben

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Manufacturing cost of goods is one of the major challenges in the gene therapy field. Here, we manufacture our AAV products using the transient transfection of suspension HEK293 cells, which is known to offer scalability and the potential for flexibility of quickly shifting to other therapeutic genes. Our development is focused on increasing vector yields in the upstream process in order to reduce the per patient cost of manufacturing. To address these goals, we have developed an optimized process combining novel plasmid design, a new transfection reagent and improved transfection conditions determined by design of experiment (DOE). Compared to the triple transfection process which uses standard plasmids and polyethylenimine (PEI), we have seen our process improved crude harvest titers by ~15 to ~30-fold for various capsids. Moreover, we have observed the full capsid percentage at harvest to be slightly higher with our improved process. Then, we focused on a synthetic capsid to further evaluate the scalability using our optimized process. In the crude harvests, the titer reaches up to 7×10^{11} vg/mL in shake flask, bench scale 250 mL bioreactors, 2 L bioreactors and 50 L single use reactors. In addition to the yield improvement, we performed extensive analysis on the quality of the purified vectors. The results of these analysis showed low host cell genomic DNA, significantly reduced residual plasmid DNA and undetectable replication competent AAV (rcAAV). The potency of the vectors was assessed both *in vitro* and *in vivo* and compared to vectors manufactured using standard plasmids with PEI. In summary, by improving key raw materials and process parameters of the AAV manufacturing process, we observed that the combination of these improved conditions from plasmid design, transfection reagent and DoE resulted in high-yield, high-quality and potent AAV vectors. The more than 15-fold increase of vector yield at harvest has the potential to reduce dramatically the per patient cost of goods.

Pharmacology/Toxicology Studies or Assay Development I

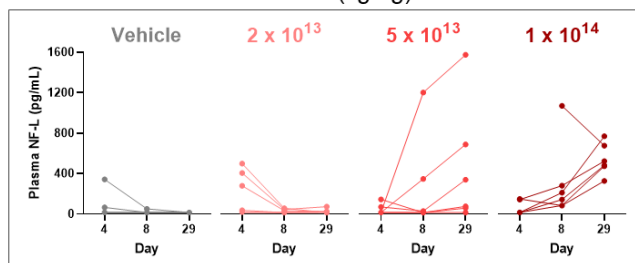
868. Circulating Neurofilament Light Chain as a Promising Biomarker of AAV-Induced Dorsal Root Ganglia Toxicity in Nonclinical Toxicology Species

Kelly A. Fader¹, Ingrid D. Pardo², Ramesh C. Kovi³, Christopher J. Somps¹, Helen H. Wang³, Vishal S. Vaidya⁴, Shashi K. Ramaiah³, Madhu P. Sirivelu³

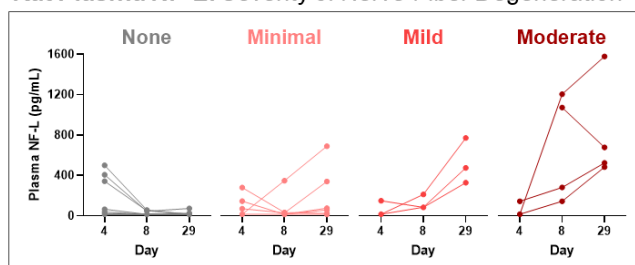
¹Worldwide Research, Development and Medical; Drug Safety Research and Development, Pfizer Inc, Groton, CT, ²Biogen, Cambridge, MA, ³Worldwide Research, Development and Medical; Drug Safety Research and Development, Pfizer Inc, Cambridge, MA, ⁴Worldwide Research, Development and Medical; Early Clinical Development, Pfizer Inc, Cambridge, MA

Adeno-associated virus (AAV)-induced dorsal root ganglia (DRG) toxicity has been observed in several nonclinical species, where lesions are characterized by neuronal degeneration/necrosis, nerve fiber degeneration, and mononuclear cell infiltration. During the recent meeting held by the U.S. Food and Drug Administration (FDA)'s Cellular, Tissue, and Gene Therapies Advisory Committee, a clear consensus emerged among panelists regarding a paucity in our current toolkit for evaluating DRG toxicity and the critical need for novel non-invasive safety biomarkers. Based on biological relevance, reagent availability, antibody cross-reactivity, and DRG protein expression, neurofilament light chain (NF-L) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) emerged as promising biomarker candidates. Following 'fit-for-purpose' validation, temporal biomarker profiles were evaluated in two nonclinical studies using an in-house duplex immunoassay (Meso Scale Discovery) and then compared with microscopic DRG lesions on Day 29 post-dose. In the rat study, an AAV9 neurotropic variant expressing the survival motor neuron-1 (*SMN-1*) transgene (AAV-PHP.B-CBh-SMN1) was administered to male Wistar Han rats by intravenous bolus injection at doses of 0, 2×10^{13} , 5×10^{13} , or 1×10^{14} vg/kg. In a separate study, male cynomolgus monkeys were administered a proprietary AAV9 vector via intrathecal lumbar injection according to the following dosing regimens: i) three injections of 3.3×10^{13} vg/dose (total = 1.0×10^{14} vg/animal), ii) single injection of 8.7×10^{13} vg, or iii) single injection of 3.0×10^{13} vg. In animals exhibiting DRG toxicity, plasma/serum NF-L was strongly associated with the severity of neuronal degeneration/necrosis and nerve fiber degeneration, with elevations beginning as early as Day 8 in rats ($\geq 5 \times 10^{13}$ vg/kg; see figures below) and Day 14 in monkeys ($\geq 3.3 \times 10^{13}$ vg/dose). Consistent with the unique positioning of DRGs outside the blood-brain barrier, NF-L in cerebrospinal fluid was only weakly associated with DRG findings. The utility of UCH-L1 as a DRG biomarker is currently unclear as levels were below the detection limit in most samples assessed. In summary, circulating NF-L is a promising biomarker of AAV-induced DRG toxicity in nonclinical species, while translation to humans remains to be determined.

Rat Plasma NF-L: AAV Dose (vg/kg)



Rat Plasma NF-L: Severity of Nerve Fiber Degeneration



869. Development of Probe-Based qPCR Assays for the Detection of Replication-Competent Lentiviral Particles

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Safety monitoring of lentiviral cell and gene therapy products comprises testing for the presence of replication-competent lentivirus (RCL) during production and patient follow-up, including materials such as master and working cell banks, vector supernatants, end of production cells or patient samples. As literature points out for the infused cell product, for example, no evidence of RCL presence has been reported and this highlights the importance of having assays available exhibiting extremely low false positive rates. In this line, we developed and qualified three highly sensitive and accurate quantitative (q)PCR assays for RCL detection. Two assays consist of singleplex hydrolysis probe-based qPCRs amplifying sequences within the vesicular stomatitis virus glycoprotein (VSV-G) and the group-specific antigen (gag) regions. Our third assay is a duplex qPCR approach combining both targets in a single test. Assays were established and characterized using a synthetic control DNA bearing RCL target sequences. After establishment and optimization, assay performance was characterized on calibration standards spanning from $1 \text{E}+7$ to 10 copies. All samples were generated by spiking the synthetic control DNA into a blank matrix of untransduced human genomic DNA. Considering these calibration standards, both singleplex assays exhibited a dynamic range from $1 \text{E}+7$ to 10 copies and PCR efficiencies were 97.8% and 95.1% for the VSV-G and the gag assay, respectively. All calibration standards fell within a $\leq 5\%$ precision and $\pm 15\%$ accuracy thresholds. Similarly, a set of 3 quality control (QC) and a lower limit

of quantification (LLOQ) samples were analyzed confirming the assays' performance throughout the dynamic range and setting the LLOQ values at 50 copies for the VSV-G assay and 30 copies for the gag assay. Additional experiments were performed in order to define the limit of detection (LOD) and this was established at 3 VSV-G and 5 gag copies. In order to minimize the time and resources required, a duplex qPCR assay was developed combining both singleplex assays. The conditions of the duplex assay were optimized in order to achieve individual PCR efficiencies of 94.8% and 94.6% for the VSV-G and gag amplifications. In this case the dynamic range spanned from $1E+7$ to 75 copies, with an LLOQ at 75 copies and an LOD at 10 copies. The duplex assay performance was still within the acceptable precision and accuracy thresholds thus providing a robust quantification. Therefore, despite a slightly reduced dynamic range, this duplex assay provides a sensitive RCL detection. In summary, here we present a set of qPCR assays suitable for the accurate and highly sensitive detection of RCL particles within human samples. In particular, the duplex qPCR exhibits a suitable performance while simultaneously detecting two different RCL targets.

870. Sonication Linker Mediated-PCR (SLiM-PCR), an Efficient Method for Quantitative Retrieval of Vector Integration Sites

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¹SR-TIGET, Milan, Italy; ²GlaxoSmithKline, Stevenage, United Kingdom; ³Tettamanti Research Center, Monza, Italy; ⁴Dr. von Hauner Children's Hospital, LMU, Munich, Germany; ⁵Institut de Genetique Moleculaire de Montpellier, CNRS, Montpellier, France

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. To validate our approach, we set up an experimental framework in which the genomic DNA from a monoclonal diploid cell lines harboring one lentiviral vector (LV) IS in a known genomic position (CEM1) was mixed at different ratios (70-0%) with that of a bulk-transduced cell line with LV IS randomly distributed in the genome. By applying SLiM-PCR and other PCR techniques on the different DNA mixes, we calculated the relative abundance of the CEM1 IS genomes versus the expected value. SLiM-PCR resulted ~10-fold more efficient in IS retrieval than the other techniques tested and showed a high correlation between the number of retrieved and expected IS ($R2 \approx 0.9$). Indeed, the observed values of CEM1 IS matched the expected (observed/expected = 0.96 ± 0.1) for all the dilutions up to the detection limit of 0.16% of CEM1 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.

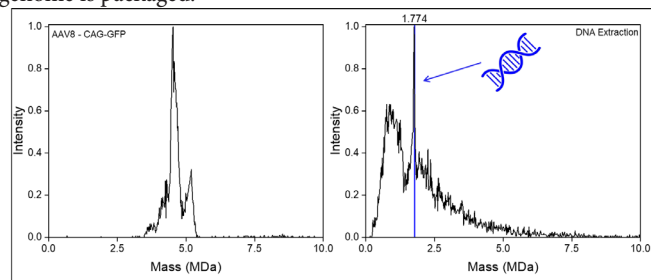
871. CDMS Analysis of DNA Released from rAAV Gene Therapy Vectors

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Introduction: Recombinant adeno-associated virus (rAAV) is a commonly used gene therapy delivery platform. AAV consists of a capsid (a protein shell) that surrounds a single-stranded DNA genome of up to around 4.7 kb. During production, empty capsids and capsids with a partial genome are formed in addition to capsids that have packaged a full genome of interest (GOI). Heterogeneous DNA can also be packaged or co-packaged with the GOI. Characterizing the DNA content of AAV viral vectors, beyond simple empty-full quantitation, is an analytical challenge. In this work, charge detection mass spectrometry (CDMS) is used to measure the mass distributions of DNA released from rAAV viral vectors. **Methods:** A variety of protocols were explored to optimize release and purification of the DNA from rAAV vectors. The extracted DNA samples were buffer exchanged into ammonium acetate solution with micro-biospin P6 columns and

then electrosprayed. Except for short polynucleotides, the analysis of DNA and RNA by conventional electrospray mass spectrometry is hindered by their high charge and heterogeneity (due to counter ions). In this work, mass distributions were recorded using charge detection mass spectrometry (CDMS) where masses are determined for individual ions from simultaneous measurement of their charge and m/z . The prototype CDMS instrument used in this work employs an electrostatic linear ion trap (ELIT) consisting of two end caps with a detection cylinder located between them. **Preliminary Data:** Initially we performed a series of measurements for rAAV vectors incubated at temperatures up to 80°C. The MW of empty AAV is around 3.7 MDa and full AAV, with a genome close to the packaging capacity, has a MW around 5.2 MDa. For unincubated samples, the charge of empty and full capsids and particles with a partial genome are all around 150-165 e (elementary charges). The charge provides insight into the physical size of the ion; extended structures are more highly charged than compact near spherical ones. That all the particles have similar charges (in spite of their different masses) indicates that the genome is inside the capsids. As the temperature is raised, the charge on some of the particles with full and partial genomes increases substantially attributed to extrusion of the genome. Furthermore, there is a corresponding increase in abundances of ions with masses <3 MDa. The low charged species are likely capsid fragments. However, many highly charged ions form narrow peaks at the mass expected for the genome indicating that some of the genomes are completely released. In addition to peaks at the mass expected for the ssDNA genome, there are peaks at twice the genome mass. AAV packages a single-strand DNA genome, however, both positive and negative sense DNA strands are packaged in different AAV particles. When released, the positive and negative strands can base pair in solution to form double stranded DNA which has a mass of twice the average mass of the ssDNA. The ssDNA/dsDNA ratio is influenced by solution conditions. In most cases the mass of the dsDNA agrees with the expected mass. However, in at least one case the mass is significantly less than the expected mass indicating that a truncated genome is packaged.



872. The Safety and Biodistribution Profiles of Systemically Delivered Oncolytic Adenovirus in Pigs

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Oncolytic adenoviruses (OAd) are powerful tools in cancer treatment as they can directly lyse tumor cells as well as exert multiple antitumor functions including the induction of innate and adaptive

immune responses against tumor cells. The most studied and used OAd backbone, adenovirus type 5 (OAd5), targets to coxsackie and adenovirus receptor (CAR), which expression is profoundly low in many cancer types. One of the approaches to overcome this limitation is development of the serotype chimeric OAd5/3 viruses with surface receptor-binding protein (fiber) or its distal part (knob) from adenovirus type 3, which use human desmoglein-2 (DSM2) and CD46 as cell receptors. Systemic delivery of both types OAd, which is necessary for the treatment of metastatic disease, remains a major challenge in the field, owing to the lack of animal models that 1) possess the appropriate receptors for viral entry; 2) support OAd replication. The most broadly used murine models do not meet these requirements as OAd do not replicate in mice. Moreover, none of the rodent models (including semi-permissive Syrian hamsters) are suitable for evaluation of OAd5/3 and other group B targeted vectors since rodent cells lack CD46 and DSG2 receptors. We have recently demonstrated that pigs fully support the replication of both OAd5 and OAd5/3 vectors and provided the *in vivo* evidence that pigs can be used as a model for preclinical study of OAd. Here we present the first full evaluation of biodistribution and toxicology profiles of OAd5 and OAd5/3 in pig model upon systemic delivery. Eighteen Yorkshire pigs were systemically injected with 5×10^{11} vp of the virus (OAd5 or OAd5/3) or PBS (n=6) and sacrificed on day 1 and 7 post infection. Liver, lung, spleen, pancreas, mesenteric lymphatic nodes, kidney, and heart from all pigs were collected for biodistribution analyses and histology evaluation. Hematology parameters, blood chemistry, and coagulation factors were determined at 0, 1, 6 hours and on day 1, 2, 3, 4, 7 p.i. The detailed clinical pathological and histopathological analyses did not reveal any signs of adenovirus-induced toxicity. No negative effects of adenovirus treatment on hematology or biochemistry were reported. As the biggest concern of OAd in rodent models is liver tropism, we assessed the possible liver toxicity by analyzing 9 different liver zones from each pig. OAd administration did not alter the level of liver specific enzymes and damage markers (ALT, AST, LDH, ALP, GGT, and bilirubin) compared to control pigs. To analyze OAd biodistribution, viral DNA copy numbers were measured by qPCR utilizing Ad E4 primers. The levels of both OAd5 and OAd5/3 in liver were negligible ($\sim 10^2$ and 10 copy numbers / μg of total DNA on days 1 and 7, respectively). The highest viral copy numbers were recovered from the spleens of both OAd5 and OAd5/3-treated pigs ($\sim 10^4$ and 10^3 copy numbers / μg of DNA on days 1 and 7) and lungs ($\sim 10^3$ and 10^2 copy numbers / μg of DNA). Interestingly, OAd5/3 showed significantly lower virus copy numbers in all organs compared to OAd5 on day 1, which can suggest its higher overall clearance relative to OAd5. These data conclude that unlike in rodent models, the main organs of OAd trapping in pigs are spleen and lungs but not liver. The role of particular macrophage subsets, which are responsible for the OAd uptake in pigs, and their correspondence to those in humans, need future investigation.

873. Electrophysiology and Soluble Biomarker as Translational Tools to Monitor Adeno-Associated-Virus Related Ganglionopathy

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University of Pennsylvania, Philadelphia, PA

Delivering AAV vectors into the central nervous systems (CNS) of nonhuman primates (NHP) via the blood or cerebrospinal fluid can lead to dorsal root ganglia (DRG) toxicity, which is characterized by neuronal degeneration, mononuclear cell infiltration, and secondary axonal degeneration of central and peripheral axons of the dorsal spinal cord and peripheral nerves, respectively. We previously reported this dose-dependent toxicity with a variety of capsids and transgenes, with vectors purified by gradient or column chromatography, and in animals receiving different immunosuppressive regimens. The toxicity is triggered by high rates of transduction in a subset of DRG sensory neurons, which leads to stress from overproduction of the transgene product. Our efforts to study AAV-mediated ganglionopathy have been hindered by the lack of available biomarker or monitoring during the in-life phase, and the difficulty to perform comprehensive neurological evaluation in nonhuman primates. We hypothesized that sensory nerve amplitude (SNAP) recordings and levels of Neurofilament Light chain (NfL), would be modified upon the loss of sensory neurons and degeneration of corresponding myelinated fibers. We incorporated sensory nerve conduction velocity and action potential testing of the median nerve in all our recent NHP studies, and we submitted frozen samples of cerebrospinal fluid (CSF) and serum from past studies for NfL levels quantification using a sensitive digital immunoassay (Quanterix Simoa NF-Light™). We observed that the median nerve SNAP amplitude decreases 1 month post-AAV dosing correlated with the severity of axonopathy and perineurial fibrosis of the nerve. Grade 3 severity and above (corresponding to approximately 50% of axonal loss) led to a sharp drop or complete loss of the wave, which then remained abnormal for all subsequent recordings; the maximum follow up was 6 months post-AAV dosing. In animals that had histopathological findings of DRG pathology 2-to-3-months post-AAV administration, we observed that NfL levels were elevated in both CSF and serum three to four weeks post dosing and returned to low baseline values subsequently. The extent of the NfL spike observed at 1 month correlated remarkably well with the severity of spinal cord dorsal axonopathy seen at necropsy several weeks later, thereby offering a unique opportunity to monitor the extent of DRG pathology during the in-life phase. Interestingly, the observation of a NfL spike 3-to-4-weeks post dosing at peak transgene expression, followed by subsequent resolution confirms our previous observations that DRG pathology is non progressive after the initial neuronal degeneration event.

874. Application of Single-Cell Transcriptomics to Assess rAAV-Delivered Transgene Expression

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With the advancement of gene therapies has come the need to better understand the biology guiding rAAV-delivered transgene expression and, ultimately, therapeutic effectiveness. Most of the commonly used techniques to assess transgene expression lack the resolution to adequately address questions surrounding the biological context of rAAV-delivered transgene expression, as they often utilize “bulk” expression level values generated from tissue-derived total RNA samples. Other techniques with higher resolution, such as RNA-ISH, often supply only qualitative data and lack the throughput to provide a full representation of the target tissue. Because of the need to better understand the fate of the expression patterns of rAAV-delivered transgenes, we set out to employ a higher-throughput and higher-resolution technique to measure transgene. Recent advances in single-cell technologies have made it possible to investigate transcriptional trends on a cell-by-cell basis from tens of thousands of cells at once, allowing previously unseen levels of detail across different tissues and cell types. With this in mind, we utilized single-cell transcriptomics to assess rAAV-delivered transgene expression in individual cells from the liver of both a mouse and non-human primate model. Using this approach, we were able to demonstrate a dose-dependent increase in transgene expression across increasing vector doses in mice at day 14, with 60% of cells expressing transgene at the highest dose and average transgene expression within the top 1.5% of all expressed genes in the liver. In the NHP samples included in this analysis, though levels of transgene were lower than observed in mouse, we still detected a range of expression levels, with 16% of cells positive for transgene at the highest dose 21 days post-injection. Because our approach yielded transcriptome-wide data for each cell, we also investigated the biological context of transgene expression. To accomplish this, we first performed a robust characterization of the cell types of the liver. Overlaying transgene expression on top of these cell type classifications allowed us to identify specific clusters of cells with significantly higher transgene expression. Interestingly, while the highest levels of transgene expression in mice clearly colocalized with markers of perivenous hepatocytes, NHP samples showed increased transgene expression in periportal hepatocytes, supporting previously reported observations of inverse transgene expression patterns in these two model systems. Taken together, our data shows that the use of single-cell transcriptomics is a powerful tool in the assessment of AAV gene therapy. Moving our current methods of detecting and assessing transgene expression towards the utilization of more robust and high-throughput techniques, such as single-cell transcriptomics, will allow us to have a better understanding of the impact of gene therapies at a molecular level, and hopefully guide the design of more targeted and more effective treatments in the future.

RNA Virus Vectors

875. Fusosome-Targeted Gene Transfer to Human Hepatocytes

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Targeted gene delivery has the potential to significantly improve the safety and accessibility of *in vivo* gene therapy for patients. We have developed a novel gene therapy platform using retargetable viral fusogen proteins - the "fusogen platform" - that can deliver transgenes (or other payloads) directly to specific cell types through systemic administration of a fusosome. A fusosome is an integrating viral vector pseudotyped with a paramyxovirus fusogen that has been blinded to its native ligand and engineered for targeted binding to a receptor that is specifically enriched on the surface of a given cell type. Such fusosomes enable specific delivery of the encapsulated payload to the cytoplasm of the target cells. Using this approach, we have been able to successfully target diverse cell types such as different T cell sub populations and hepatocytes. Here, we present early proof of principle data showing efficient delivery of a reporter transgene to human hepatocytes *in vivo* using a humanized liver mouse model. Several fusogens engineered to target receptors enriched on hepatocytes were screened for efficiency and specificity of delivery *in vitro* on cell lines and primary cells. Fusogen candidates with the highest efficiency and specificity were further optimized to improve the efficiency of targeting to hepatocytes while maintaining specificity. The fusosomes, harboring optimized fusogens, were tested *in vivo* in the FRG (*Fah^{-/-} Rag2^{-/-} Il2rg^{-/-}*) humanized liver mouse model. Fusogen performance on primary human hepatocytes (PHHs) *in vitro* was well correlated with human hepatocyte transduction *in vivo*. We observed nearly 50% transduction of human hepatocytes *in vivo* using fusosomes engineered with our top fusogen candidate in a dose-dependent manner. Furthermore, we could achieve a nearly 50% human hepatocyte transduction in the FRG model via repeat dosing a lower dose. Three successive fusosome doses given 24h apart were well-tolerated and showed no overt histopathological changes compared to saline treated controls. Repeat dosing can provide alternative strategies to achieve high *in vivo* transduction. We have demonstrated high efficiency hepatocyte targeting with the Sana fusogen platform, enabling the potential to specifically deliver a number of payloads, including integrating gene therapies and other genome modification reagents. These approaches may lead to treatments for disease states such as inborn errors of metabolism amongst others.

876. Improved Gene Therapy for Metachromatic Leukodystrophy

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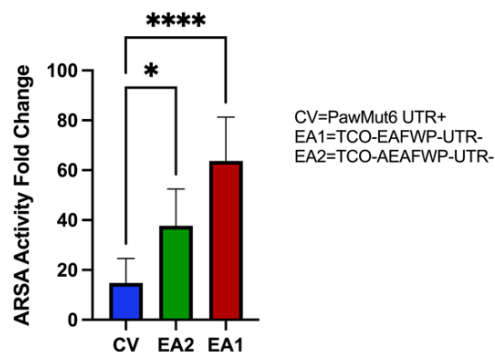
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Metachromatic Leukodystrophy (MLD) is an autosomal recessive lysosomal storage disease characterized by a decreased Arylsulfatase A (ARSA) enzymatic activity. The most common form, late infantile MLD, universally results in rapid loss of neurologic function in early childhood. Ex-vivo hematopoietic stem cell (HSC) gene therapy using a lentiviral vector (LV) can improve clinical outcomes by supplying a functional copy of the ARSA cDNA (Aiuti A, et al, Lancet 2022). This approach has a limited therapeutic window in pre- and minimally symptomatic individuals. We hypothesize vectors resulting in higher ARSA activity would broaden the therapeutic window allowing the treatment of early symptomatic children. The only clinical vector (CV) approved to treat MLD patients, PawMut6, includes the human ARSA cDNA gene under the control of the human Phosphoglycerate Kinase promoter (Biffi A, et al, Science 2013). To increase expression of ARSA cDNA at a single integration level, we generated 6 novel LVs that include a codon modified ARSA gene, additional regulatory elements, the human Elongation Factor 1 α promoter and insulators to optimize ARSA expression and enhance safety. Our top performing vectors showed 2.5X and 4X more ARSA activity compared to PawMut6 (**Fig. 1A**). We also explored the addition of a key coactivator of ARSA, Formylglycine-generating enzyme (FGEs) role in enhancing enzymatically active levels of ARSA. We demonstrated transduction of FGE in cell lines already transduced with ARSA produced more ARSA enzyme activity than ARSA transduced alone (**Fig. 1B**). This has further led to the creation of a dual vector including FGE to further maximize ARSA enzymatic activity. We also detected a superior ability of our vectors to secrete functional ARSA enzyme into the culture media of transduced primary MLD patient fibroblast cells, which is a critical modality for transfer of functional ARSA from microglia to oligodendrocytes. Extracellular vesicle isolation, purification, and immunoblot analysis has demonstrated small vesicle secretion is the primary modality by which ARSA is secreted, having significant implication for how we approach treatment of MLD. To exclude potential toxicity, we performed bone marrow transplants on WT animals with HSCs transduced with vectors expressing the highest levels of ARSA, with up to 13 integrations per genome. Mice transplanted with high VCN transduced bone marrow did not show bone marrow failure, abnormal hematopoiesis or signs of distress. Preliminary experiments using *Arsa*-KO MLD mice (generated using CRISPR-Cas9) demonstrate reversal of the MLD disease phenotype. Additional ongoing analysis includes pathological sections of the CNS, brain lysate collection and sulfatase activity assays. Our studies are currently focused on completing in-vivo validation and toxicity

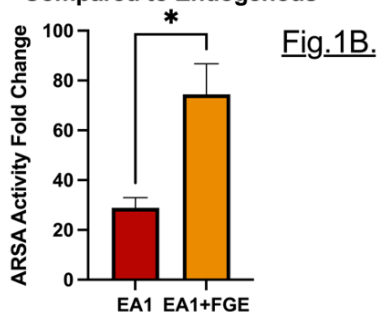
assays to move our best vector to the pre-clinical and IND application. The accumulated data on our novel vectors imply new mechanistic considerations for treatment of MLD and demonstrate utility as a strong approach for treating early symptomatic patients.

Fold Change in ARSA Activity Compared to Endogenous

Fig.1A.



Fold Change In ARSA Activity +/-FGE Compared to Endogenous



877. Development of an *Ex Vivo* Hematopoietic Stem Cell Lentiviral Gene Therapy for Multiple Sulfatase Deficiency

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Multiple Sulfatase Deficiency (MSD) is a rare, inherited lysosomal storage disorder (LSD) caused by mutations in the gene *SUMF1*, which encodes formylglycine-generating enzyme (FGE). FGE activates all 17 sulfatases in the cell and the additive effects of each inactive sulfatase lead to the clinical features of MSD. Patients present with symptoms of neurological regression and psychomotor retardation followed by loss of motor skills, speech, hearing, and vision. Currently, there are no approved disease-modifying therapies for MSD. However, *ex vivo* lentiviral gene therapy with hematopoietic stem cell transplant is approved by the EMA for metachromatic leukodystrophy (MLD) and is being developed for other related single-sulfatase LSDs (i.e. MPSIIIA and MPSII). Therefore, we aim to develop an *ex vivo* hematopoietic stem cell lentiviral gene therapy approach for MSD. We first optimized *SUMF1* lentiviral vector design to maximize sulfatase activation *in vitro*. We generated lentiviral vectors encoding human *SUMF1* under the control of the human elongation factor 1 alpha (EF1-alpha) promoter. In addition, these vectors include a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) after

the 3'-self inactivating long terminal repeat, which has been proven to enhance the performance of viral vectors. Using these vectors, we transduced two MSD patient fibroblast cell lines and found 1) increased FGE protein expression and 2) increased arylsulfatase A (ARSA) and arylsulfatase B (ARSB) activities. These findings indicate rescue of FGE function, as ARSA and ARSB are activated by FGE. While replacing *SUMF1* alone increases sulfatase activity, co-expressing *SUMF1* and key target sulfatases may further enhance activity. To examine this, we co-transduced MSD fibroblasts with two lentiviral vectors - one expressing *SUMF1* and one expressing ARSA, which encodes the ARSA protein. This co-transduction led to greater ARSA activity in MSD cells compared to transduction with *SUMF1* alone. Furthermore, the increased ARSA activity corresponded to a dose-dependent increase in ARSA protein expression in the cells. This suggests that co-expression of both *SUMF1* and key target sulfatases may augment therapeutic outcomes in MSD. Our findings support the generation of a dual-transgene lentiviral vector expressing both *SUMF1* and key target sulfatases to optimize sulfatase activity in MSD cells. The larger packaging allowance of lentiviral vectors, as opposed to AAV vectors, enables us to encode both transgenes in one vector. We will examine the efficacy of *SUMF1*-only and dual-transgene vectors in a MSD mouse model with bone marrow transplant of corrected syngeneic hematopoietic stem cells. We hypothesize that *ex vivo* gene therapy with hematopoietic stem cell transplant in MSD mice will increase sulfatase activities, reduce lysosomal storage material, and correct disease phenotypes. If we observe a therapeutic benefit in a MSD mouse model, this work could justify future translational studies in MSD patients.

878. Auto-Replicative Vectors Expressing Galectin-3 Inhibitors New Tools to Fight Pediatric Osteosarcoma

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Osteosarcoma is an aggressive bone tumor, primarily arising in pediatric age. Despite years of intensive research, the outcome for metastatic and non-responder patients is very poor and has not improved in the last 30 years. These tumors harbor a highly immunosuppressive environment, making the existing immunotherapies ineffective. Inhibition of galectin-3 (Gal3), a protein involved in immunosuppression, adhesion of tumor cells and metastases, has demonstrated to reduce tumor progression in different tumor models, including osteosarcoma. On the other hand, virotherapy based on recombinant Semliki Forest Virus (SFV), a self-replicating RNA virus, has shown therapeutic effect in orthotopic osteosarcoma mouse models. With this premise, we constructed different recombinant SFV vectors expressing Gal3 inhibitors based on truncated forms of this protein. These constructs included the Gal3 carboxy-terminal domain (SFV-Gal3-C) and

its amino-terminal domain by itself (SFV-Gal3-N) or fused to the Gal3 inhibitor peptide C12 (SFV-Gal3-N-C12) and the C12 peptide (SFV-C12). We observed *in vitro* that Gal3-N and Gal3-N-C12 were able to block binding of Gal3 to the surface of IL-10-activated CD8 and CD4 T cells. We selected the murine osteosarcoma cell lines K7M2 and MOS-J for *in vivo* studies because of their robust expression of Gal3. Orthotopic osteosarcoma tumors, induced in mice by intratibial injection of K7M2 or MOS-J cells, were treated with SFV vectors expressing Gal3 inhibitors or SFV expressing luciferase (SFV-Luc) and PBS as controls. SFV-Gal3-N-C12 vector showed the highest antitumoral activity, and significantly reduced tumor growth in comparison with control mice that received PBS. In fact, this vector was able to prolong animal survival, leading to 47% and 30% of complete regressions in K7M2 and MOS-J osteosarcoma mouse model, respectively. Further studies in the K7M2 osteosarcoma model also showed a reduction in the number of spontaneous lung metastasis in mice treated with SFV-Gal3-N-C12. Interestingly, transcriptional analysis showed a significant down-regulation of osteosarcoma-related pro-metastatic genes after treatment with SFV-Gal3-N-C12 compared with SFV-Luc. Mechanistic assays in K7M2-bearing mice revealed an increase of CD8 and CD4 Foxp3⁺ cells infiltration in both primary tumors and lung metastasis of mice treated with SFV-Gal3-N-C12. Moreover, these tumor-infiltrating cells presented a less exhausted phenotype after treatment with SFV-Gal3-N-C12 compared to SFV-Luc. Nevertheless, treatment with SFV-Gal3-N-C12 showed a modest protection against tumor rechallenge in cured mice. The absence of a strong immune memory response suggest that this therapy might benefit from combinatorial immunostimulatory approaches. In summary, we believe that the SFV-Gal3-N-C12 vector could constitute a promising therapeutic strategy for patients affected by osteosarcoma expressing high levels of Gal3.

879. VivoVec: A Novel Lentiviral-Based *In Vivo* CAR T Cell Generation Platform with Viral Particle Surface Engineering Incorporating T Cell Activating and Co-Stimulatory Ligands

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Adoptive cell therapies featuring *ex vivo* expanded autologous T cells engineered to express tumor-targeting chimeric antigen receptors (CAR Ts) have revolutionized the treatment of B cell malignancies, leading to long-term remission in 30-40% of certain patient populations. Despite the promising clinical efficacy of CAR T cells in hematologic malignancies, major limitations hinder their widespread application, including challenges to patient access, complex manufacturing, and high cost. To overcome these challenges, we have developed VivoVec, an engineered lentiviral particle-based platform harboring a CAR transgene that is being developed for off-the-shelf use for the generation of CAR T cells *in vivo*. To achieve specific and efficient *in vivo* transduction, VivoVec particles are pseudotyped

with the Cocal fusion glycoprotein and engineered to express T cell binding, activating, and costimulatory ligands. We evaluated multiple novel surface engineering approaches, including incorporation of an anti-CD3 single chain variable fragment combined with a panel of T cell costimulatory ligands, such as CD80, into the particles' surfaces to initiate T cell activation in conjunction with co-stimulation and particle binding to facilitate efficient transduction. CAR T cells generated with VivoVec particles exhibited a less-differentiated, central memory-like phenotype and CAR-antigen specific polyfunctionality, including proliferation and tumor cell killing *in vitro*. Finally, we observed that VivoVec particles generated CAR T-cells *in vivo* with potent antitumor activity in a humanized NSG mouse model of B cell malignancies. Overall, our results suggest that the collective mechanism of action of VivoVec particles to initiate *in vivo* CAR T cell generation and subsequent anti-tumor immune responses is enabled by particle surface-displayed ligands that promote T cell binding, activation, and costimulation, rendering T-cells competent for transduction while optimizing their immunophenotype and function.

880. Heterologous Signal Peptides Enhance Protein Secretion for Respiratory Gene Therapy

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Introduction: Pseudotyping recombinant SIV lentiviral vectors with sendai virus F and HN proteins (rSIV.F/HN) allows for robust and sustained transduction of murine lungs. However, transduction of the human lung will require large amounts of virus. Technologies that improve protein secretion could decrease the necessary dose and even modest increases (2x) could both reduce manufacturing costs by half and reduce the potential for toxicity. Signal peptides are heterogenous sequences at the N-terminus of secreted proteins that can be modified to improve protein secretion. Here we explore if synthetic and endogenous signal peptides are a viable strategy for boosting the secretion of therapeutically relevant proteins such as alpha-1 antitrypsin (AAT) and granulocyte-macrophage colony-stimulating factor (GM-CSF) into the lungs. **Methods:** To select candidate signal peptides, bioinformatic analysis was performed to find proteins expressed in the lungs and secreted in high concentrations into the blood stream or epithelial lining fluid of the lungs. HEK293T cells were transfected (n = 8-16 wells per signal peptide) with plasmids encoding fusions of these signal peptides and either AAT or GM-CSF. Secreted protein concentrations were measured in the resulting cell culture media by ELISA. Human air-liquid interface cultures (n=12 cultures per signal peptide) were transduced with VSV-G pseudotyped lentiviruses encoding signal peptide fusions and protein concentrations were measured by washing the apical surface and performing ELISA on the resulting wash fluid. Bioinformatic analysis of proteins secreted from the lungs produced five candidate signal peptides from: tetranectin (CLEC3B), cartilage acidic protein 1 (CRTAC1), alpha-2-macroglobulin (A2M), uteroglobin (SCGB1A1) and pulmonary surfactant associated protein A2 (SFTPA2).

Additionally, a strong synthetic signal peptide, secrecon, was selected from the literature. In HEK293T cells, secrecon reduced secretion of AAT from 41.1 (IQR 38.6 - 44.1) to 16.8 (IQR 13.0 - 17.9) ng/mL (0.4x) and GM-CSF from 213.7 (IQR 196.4 - 247.9) to 33.7 (IQR 31.4 - 47.5) pg/mL (0.2x) compared to the endogenous signal peptides. However, CRTAC1 significantly ($p < 0.05$) increased GM-CSF secretion from 213.7 (IQR 196.4 - 247.9) to 383.7 (IQR 339.0 - 431.8) pg/mL (1.8x). In human air-liquid interface cultures CRTAC1 and SCGB1A1 significantly ($p < 0.05$) increased GM-CSF secretion 4.2x (IQR 2.0 - 7.4) and 5.3x (IQR 3.8 - 8.5), respectively. However, secrecon reduced GM-CSF secretion to 0.3x (IQR 0.2 - 0.4) of that secreted with the endogenous signal peptide. **Conclusions:** Signal peptides are a viable strategy for increasing protein secretion following gene transfer to the lungs. Although the synthetic signal peptide secrecon reduced protein secretion in all models tested, CRTAC1, A2M, and SCGB1A1 increased protein secretion. Further investigation of these signal peptides *in vivo* could identify an optimal signal peptide for respiratory gene therapy.

881. Gene Editing of Hematopoietic Stem Cells Restores the Cytotoxic T Cell Response in a Murine Model of Familial Hemophagocytic Lymphohistiocytosis Type 3

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Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome characterized by excessive activation of T cells and macrophages, and a life-threatening cytokine storm. In 1/3 of cases, a rare inborn error of immunity predisposes to this syndrome, with familial HLH type 3 (FHL3) accounting for 30% of all inborn HLH cases worldwide. The underlying mutations in the *UNC13D* gene result in functionally impaired or absent Munc13-4 protein, with the consequence that cytotoxic vesicles in lymphocytes are not processed properly, compromising T and NK cell mediated killing of antigen-presenting cells. Current treatment protocols, including allogeneic hematopoietic stem cell (HSC) transplantation, still show 30-40% mortality, warranting the exploration of genome editing strategies to treat this disorder. The *Jinx* mouse model reflects the human FHL3 phenotype well. *Jinx* mice harbor a cryptic splice site in intron 26 of *Unc13d*, leading to premature termination of Munc13-4 translation. Here, we employed CRISPR-Cas technology to excise the cryptic splice site in intron 26. To validate this approach, *Jinx* mice derived HSCs were edited with CRISPR-Cas9 ribonucleoprotein complexes (RNPs), and then transplanted into conditioned *Jinx* mice to assess engraftment, differentiation and functional restoration. Flow cytometry confirmed >95% engraftment and successful establishment of a donor-derived immune system. Genotyping based on NGS, CAST-Seq and digital droplet PCR performed on genomic DNA of cells isolated from transplanted mice confirmed efficient gene editing (>95%) and absence of off-target effects. *Unc13d* transcription levels of edited and

non-edited cells were comparable. Challenge of the transplanted mice with lymphocytic choriomeningitis virus (LCMV), which triggers HLH in *Jinx*, resulted in rapid virus clearance and protection from HLH in *Jinx* mice transplanted with edited HSCs that was comparable to wildtype mice. In sum, we established a highly efficient CRISPR-Cas RNP-based editing protocol for murine HSCs, which allowed us to fully restore the functionality to cytotoxic T cells in a preclinical disease model of familial HLH.

882. Scaling from Transient to Stable Lentiviral Manufacturing Approaches Using Consistent Packaging Vector Sets

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Lentiviral vectors (LVV) present significant potential for cell and gene therapy, however, challenges remain with process robustness and scalability. Whilst most manufacturing systems currently use transient transfection for lentiviral manufacture, we aimed to develop a platform that would allow transition from transfection-based manufacturing to a fully stable producer cell line system using the same vector set. This would, in theory, reduce potential product comparability issues at later stages of clinical development. Accordingly, regions of DNA homology, that might represent sites of inter-chromosomal recombination in stable cell lines, were eliminated where possible in a transient lentiviral production vector set, termed here XLenti. We then conducted high-throughput screening to identify HEK293 single cell clones that would support high lentiviral productivity. In combination, the optimised plasmid system and improved clonal cell line achieved transient production of lentiviral vectors up to 2E8 TU/mL (eGFP as GOI), and the production process showed good scalability up to 200L scale in a stirred tank, suspension, serum free disposable bioreactor. We then transitioned the transient plasmid system into a stable cell line context, regulating the VSV-G and GagPol components to be doxycycline inducible and, also, incorporating Rev in a second cell line engineering step. For lentiviral production, the XLenti packaging platform only requires transfection of the LV genome and the supplementation of the inducing agent to activate VSV-G and GagPol expression. This approach yielded lentiviral titres up to 6E7 TU/mL (eGFP as GOI). To further streamline the process and remove the dependency on transfection entirely, we stably integrated the LV Genome cassette into the packaging cell line to develop XLenti producers cell lines. This enables transfection-free lentiviral production, increasing process development flexibility and improving manufacturing robustness. Ongoing work aims to increase the yields of these stable systems to allow scalable and cost-effective lentiviral vector manufacture for both cell and gene therapies.

883. Regulated Expression of Lentiviral Vectors Following Administration of an Inducing Molecule

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The UK Respiratory Gene Therapy Consortium, has developed a lentiviral vector pseudotyped with Sendai virus envelope proteins F and HN (rSIV.F/HN) to achieve efficient pulmonary gene transfer. However, not all target diseases require constitutive expression of the therapeutic gene and diseases such as pulmonary alveolar proteinosis (PAP) may only require short intermittent bursts of granulocyte macrophage colony stimulating factor (GM-CSF) to ameliorate disease phenotypes. Here, we aimed to generate a lentiviral vector carrying a regulatable expression cassette, which will initiate gene expression following administration of an inducing molecule. To establish proof-of-concept that lentivirus-mediated expression can be regulated, a commercial mifepristone-inducible system (GeneSwitch, Invitrogen) was cloned into a lentiviral backbone and VSV-G vectors generated. The commercial expression cassette was kept in the original configuration, encoded by two vectors (2V), and additionally cloned into one vector (1V). First HEK293T cells were transduced with 1V or 2V, carrying the *GM-CSF* cDNA and expression induced by administration of the drug mifepristone 24 hours post-transduction (10^{-8} M, MOI 10, n=6 wells/condition). Mifepristone administration led to a significant ~500- and ~1200-fold increase in GM-CSF expression with the 1V and 2V systems, respectively (1V p=0.004 and 2V p<0.0001, Kruskal-Wallis, Dunn's multiple comparison test). Similarly, human air-liquid interface cultures (ALIs) were transduced with VSV-G GM-CSF vectors and expression induced with mifepristone (10^{-8} M, MOI 100, n=3 ALIs/condition). Mifepristone administration to 1V transduced ALIs generated a median peak in GM-CSF expression of 27.8 pg/ml (range 19.7-170.0 pg/ml) with undetectable baseline expression. Conversely, 2V transduced ALIs showed a median peak in GM-CSF expression of 575.9 pg/ml (range 265.0-739.3 pg/ml) from median baseline expression of 25.1 pg/ml (range 9.5-37.9 pg/ml). To investigate regulation of gene expression in primary cells, murine bone marrow-derived macrophages (BMDMs) were transduced ex vivo with VSV-G GM-CSF. Expression was induced with mifepristone 48 hours post-transduction (10^{-8} M, MOI 10, n=6 wells/condition) and GM-CSF expression measured after 24 hours of stimulation. Macrophages transduced with 1V demonstrated a median peak in GM-CSF expression of 31.9 pg/ml (range 0.2-60.9 pg/ml) with undetectable baseline expression, while 2V generated a median peak in GM-CSF expression of 660.0 pg/ml (range 367.7-764.5 pg/ml) with undetectable baseline expression. In summary, we have demonstrated that lentivirus-mediated gene expression can be regulated in a human airway model and BMDMs, with higher induction of transgene expression achieved with the 2V system.

884. Development of Molecular Assays to Determine Lentiviral Vector-Mediated Transduction Efficiency in Airway Epithelial Cells

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Introduction: Quantification of transduction efficiency is an important reference point in lung gene therapy studies. Here, we refined droplet-digital (dd)PCR-based methods to allow quantification of lentiviral vector transduced cells in large cell pools ($\sim 10^5$ cells, bulk), as well as in small cell pools (50 cells) and single airway epithelial cells, with the aim to develop methods that allow more accurate estimation of the number of transduced airway epithelial cells. **Methods:** Bronchial epithelial cells were transduced with our proprietary lentiviral vector (rSIV.F/HN) carrying either EGFP or codon-optimised Cystic Fibrosis Transmembrane Conductance Regulator (coCFTR) cDNA at an MOI of 10-30 and differentiated into air-liquid interface cultures (ALIs). 21 days after transduction ALIs were dissociated and analysed as bulk sample ($\sim 2.5 \times 10^5$ cells) or sorted into single cells and small cell pools. ddPCR and RT-ddPCR were performed with primer/probes specific to a sequence in the vector (WPRE), the vector-derived CFTR (coCFTR) or the endogenous CFTR (hCFTR). **Results:** DNA analysis of bulk samples showed no difference in integrated vector copy number between vectors [EGFP: 0.61 ± 0.25 , coCFTR: 0.56 ± 0.32 copies/cell, n=7/group] confirming that both vectors led to similar transduction efficiency. RNA analysis of bulk samples showed that EGFP mRNA levels were significantly higher than coCFTR mRNA levels [26,850 \pm 13,528 and 7117 \pm 4459 copies mRNA/ng RNA, respectively]. Despite reduced levels of CFTR compared to EGFP mRNA, expression of coCFTR was $\sim 10x$ greater than endogenous hCFTR levels in the bulk sample. Quantification by FACS and single cell RT-ddPCR in rSIV.F/HN-EGFP transduced ALIs detected a similar number of transduced cells [$15.8 \pm 7.7\%$ and $13.2 \pm 6.3\%$ cells positive, respectively, n=5/group], validating the single cell assay. EGFP mRNA expression levels varied 10-fold between individual cells [12-150 copies/cell, 53-63 single cells analysed/sample]. Single cell analysis of rSIV.F/HN-CFTR transduced ALIs showed that mRNA levels per cell also varied [12-75 copies/cell, 59-65 single cells analysed/sample] and that the percentage of mRNA positive cells was lower than after transduction with SIV.F/HN-EGFP [$6.5 \pm 3\%$ cells positive, n=5]. Given that VCN analysis of bulk DNA samples indicated that both vectors led to similar transduction efficiency, the lower number of cells expressing mRNA may be due to cells falling below the detection limit of the assay. **Conclusions:** Quantification of transduction efficiency based on quantification of vector-derived mRNA in single airway epithelial cells is feasible. This assay will provide a useful tool for analysis of future clinical trial samples.

885. Lentiviral Vector Mediated *In Vivo* Gene Transfer into Liver Organoid Forming Cells in Mice and Non-Human Primates

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Liver gene therapy with adeno-associated viral (AAV) vectors delivering clotting factor transgenes into hepatocytes has shown multi-year therapeutic benefit in adults with hemophilia. However, the mostly episomal nature of AAV challenges their application to paediatric patients. Moreover, their ability to maintain efficacy in the context of damage-driven proliferation remains to be addressed. We have developed a gene therapy strategy based on integration-proficient lentiviral vectors (LV) that specifically express their payload in hepatocytes upon i.v. administration, and showed safety and efficacy in mice, dogs and non-human primates (NHP). Stable expression of the transgene product was observed throughout long-term follow-up also upon neonatal administration, confirming the advantage of vector integration into the cell chromatin. Here we set out to investigate whether LV gene transfer also occur to liver cell types that have been shown to regenerate the liver following damage. Among them, cholangiocytes have been described to generate self-renewing liver organoids *in vitro* and *trans*-differentiate into hepatocytes *in vivo*. We systemically administered LV ubiquitously expressing GFP to newborn mice and showed efficient transduction of intrahepatic cholangiocytes that remained stable for at least 3 months after LV administration. Transduced cholangiocytes retained their ability to form liver organoids *in vitro*, although at a lower efficiency compared to the untransduced counterparts. In contrast, *ex vivo* transduced cholangiocytes did not show reduced organoid formation efficiency, excluding LV transduction as a cause for the lower outgrowth and rather suggesting preferential *in vivo* transduction of a subset of ductal cells with an intrinsically lower organoid formation potential. Indeed, gene expression analysis on organoids derived from *in vivo* transduced or untransduced cholangiocytes showed increased expression of hepatocyte-specific genes in the former, supporting this hypothesis. We then performed transcriptomic analysis on GFP-positive or GFP-negative cholangiocytes and found around 1000 differentially expressed genes. Genes upregulated in the former highlighted biological processes typical of hepatocytes, such as cholesterol metabolism, coagulation cascade, bile secretion and amino acid metabolism, indicating an intrinsic priming of these ductal cells toward hepatocyte lineage. Further investigations are underway to better characterize the *in vivo* *trans*-differentiation potential of these cell populations. We also derived organoids from the liver of LV-treated NHP and showed LV marking in around 2% of the obtained organoids. These organoids can be cultured on different substrates to skew differentiation towards hepatocytes or

mature cholangiocytes and produce the transgene encoded by the LV that was systemically administered to the NHP. Finally, we showed similar engraftment of organoids derived from the liver of either treated or untreated NHP in immunodeficient mice. Our work suggests that *in vivo* LV transduction of the liver may allow persistent gene transfer even in the context of disease or tissue damage that trigger extensive hepatocyte turnover and may also shed light on the role of different cell populations in liver regeneration.

886. Efficient and Safe Delivery of Multiple mRNA Using Non-Integrative Bacteriophage-Chimeric Retrovirus-Like Particles for *In Vivo* Applications

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Gene therapy approaches show that there is no universal delivery tool for all therapeutic strategies. Compared to DNA delivered-therapies mediated by integrative lentiviral vectors and AAV, RNA therapies are expected to be more versatile, cover a broad range of applications with minimal regulatory concerns and thus address a large variety of diseases. The technology targets applications in which a transient expression is expected. Once engineered, patient's cells become the effective medicine. As a game-changing RNA carrier, LentiFlash[®], a non-integrative bacteriophage-lentivirus chimera, can efficiently and safely deliver multiple RNA species that are transiently expressed into the cell cytoplasm directly available to be translated into protein. RNA delivery mediated by a lentiviral particle is an attractive approach as it combines most of the inherited properties of lentiviral vectors (cell entry and tropism) without the potential adverse effects from long-lasting expression or genomic integration. From a therapeutic perspective, a great advantage of such system is its ability to carry different RNA species. RNA-mediated expression level and duration must be managed according to applications. *In vivo* injection may also impact delivery requirements. Here, we show that LentiFlash[®] delivers different RNAs after multiple routes of administration for different *in vivo* animal models. These properties, and the ability to produce LentiFlash[®] using our own lentiviral production platform already compliant with the cGMPs, offer additional safety considerations making it certainly the most versatile, flexible, and safe mean for human therapy today.

887. Integration Site Analysis Using a Biomek 4000 Automated Workstation

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Lentiviral gene therapy offers the permanent correction of cells for many disease entities. Due to the risk of insertional mutagenesis, the clonal complexity of the gene-modified cell population has to be monitored. Vector-genome junctions can be analyzed by integration site analysis. In 2001, Manfred Schmidt described a form of ligation-

mediated PCR to detect and sequence the vector insertions, which became a standard in the field of integration site analysis. Over time, this method was developed further, and today many different techniques to amplify and directly sequence integration sites are available. In 2017, the Bushman lab published the integration site pipeline for paired-end reads (INSPIRED). The wetlab and NGS-based bioinformatic solutions provide a highly reproducible and quantitative measurement of the clonal repertoire of vector integrations in preclinical and clinical samples. Here, we describe the integration site analysis using a Biomek 4000 automated workstation. Except for the ultrasound fragmentation, we implemented robotic protocols for all other steps, including bead purification, end-repair-, ligation-, and on-deck PCR reactions. We present results for the world health organization (WHO) lentiviral integration site standard 18/144 from the national institute for biological standards and controls (NIBSC). With little adaptation, the Biomek scripts could be transferred to other laboratories setting up a Biomek 4000 workstation with a similar configuration. This could further increase the quality, speed, and throughput of integration site analysis.

888. CD4-Targeted CRISPR/Cas9 RNP Delivery for Anti-HIV Gene Editing

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Increased capabilities for *in vivo* gene editing would represent significant advances for many therapeutic applications, but lack of cell-type specific delivery and reduced ability to modulate cell status *in vivo* limits possibilities. For example, although VSV-G pseudotyped lentiviral (LV) vectors are highly effective when used *ex vivo* with stimulated cell cultures, they cannot efficiently transduce resting lymphocytes *in vivo* due to lack of receptor expression, low endocytosis rate, and the presence of dominant antiviral restriction factors. Moreover, using LV vectors to express nucleases such as CRISPR/Cas9 from integrated lentiviral genomes also raises concerns that long-term expression could lead to unwanted immune responses or increased off-target editing. As an alternative, we are developing lentivirus-based vector-like particles (LVPs) that can both target specific cell types and package CRISPR/Cas9 ribonucleoproteins (RNPs) with high efficiency. Our goal is to allow delivery to resting lymphocytes without long-term expression of the Cas9 protein. Cell targeting was achieved by replacing VSV-G with engineered CD4-targeted paramyxovirus glycoproteins. Incorporation of CRISPR/Cas9 was compared using (1) passive incorporation of RNPs expressed in producer cells, (2) chemical-induced incorporation using the FRB-FKBP12 interaction to package Cas9, and (3) by exploiting an aptamer/ABP interaction to package RNPs via the guide RNA component. At the same time, additional expression of guide RNA was evaluated from the LV genome, using an optimized scaffold sequence. Gene editing can be directed against several cellular and viral targets for anti-HIV applications. As proof of principle, we targeted CCR5, an essential co-receptor for viral entry. We observed that the aptamer/ABP approach resulted in up to 6-fold higher RNP incorporation into LVPs than the passive packaging strategy, allowing near 100% CCR5 disruption in the CD4+ reporter cell line, TZMbl, compared

to less than 10% gene disruption with other packaging strategies. When transducing unstimulated PBMC in culture, to mimic *in vivo* conditions, we found that using CD4-targeted paramyxovirus glycoproteins provided significantly higher rates of delivery to resting CD4+ T cells than VSV-G. Interestingly, switching from VSV-G to CD4-targeted glycoproteins resulted in a 2-fold reduction in Cas9 incorporation, which could be restored by co-expressing a truncated, non-functional, VSV-G stem protein. The LVPs also retain the possibility of exploiting the vector genome to incorporate donor sequences for homology-directed repair. Taken together, these findings provide a guideline for selecting the best delivery strategy for gene editing in resting CD4+ T cells.

889. In Vivo Lentiviral Gene Transfer to Airway Surfaces for Cystic Fibrosis is Improved by Magnetic Guidance of Particles

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Introduction: Gene vectors designed to treat cystic fibrosis lung disease should be targeted to the conducting airways for the greatest therapeutic benefit. Transduction efficiency is directly related to the vector residence time, but gene vectors naturally rapidly spread to the alveoli during inspiration. Extending residence time within the conducting airways is important, but hard to achieve. We proposed that gene vector conjugated magnetic particles (MP) that can be guided to the conducting airway surfaces could improve targeting. However, the behaviour and effects of small MP on the airway surface in the presence of an applied magnetic field are poorly understood, as is the efficacy of MP-guided vector delivery on conducting airway gene transfer. **Methods:** Synchrotron phase contrast X-ray imaging (PCXI) was performed at the BL20XU beamline at the SPring-8 Synchrotron in Japan. The motion of a range of MP was visualised *in vitro* and in live rat trachea, to examine the dynamics and patterns of individual and bulk MP behaviour. In subsequent rat studies informed by the results from the PCXI visualisation experiments we then delivered a lentiviral (LV) LacZ reporter gene vector conjugated to MP both with and without an external magnetic field, to assess whether this approach increased transduction efficiency in the rat trachea. **Results:** PCXI revealed the behaviour of magnetic particles in stationary and moving magnetic fields, both *in vitro* and *in vivo*. During delivery, MP deposition was focused within the field of view where the magnetic field was the strongest. MP could be concentrated and aligned by magnetic field manipulation, but MP could not be dragged along the live airway surface. Transduction efficiency in rat trachea was improved six-fold when the LV-MP were delivered in the presence of a magnetic field. **Conclusions:** These results show that conjugating gene vectors to MP may be a valuable approach for improving gene vector targeting to the conducting airways *in vivo*.

890. Intranasal Administration of Multifunctional Polymer-Conjugated Lentivirus - Efficient Gene Replacement Therapy for Epilepsy

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Epilepsy, a neurological disorder frequently characterized by abnormal circuit activity in the brain resulting in seizures, is often resistant to medication and other therapies that helps in decreasing the frequency of seizures. Therapeutic approaches to restore gene function, using anti-sense oligonucleotides or regulatory elements to increase the expression of normal gene copy, are underway. However, efficient delivery of transgene-vehicles to brain remains a major limit to gene replacement. To overcome these obstacles, we constructed a novel chemically modified lentivirus-expressing wild type deoxyhypusine synthase (DHPS^{WT}) to compensate for the mutated DHPS forms found in neurodevelopmental disorders with epilepsy. Additionally, DHPS^{WT} expressing lentivirus was chemically conjugated with pH- and reduction-responsive copolymer [poly(ethylene glycol)-poly(bioreducible β -aminoester)-arginine-grafted polyethyleneimine (PPA)] to generate lentivirus-PPA complex enabling efficient nose-to-brain delivery. Each chemical crosslinking steps for the generation of lentivirus-PPA complex were optimized through several analytical assays. Optimized lentivirus-PPA complex was effectively delivered to the brain tissues via intranasal administration without significant immunogenicity. Our novel intranasal delivery of a lentivirus-PPA complex may offer a promising gene therapy tool to treat epilepsy and other neurological disorders.

891. Improvement in Baseline Lentivirus Production Using Design of Experiment (DOE) Tool and Scale-Up in Bench-Top Bioreactors

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Lentiviral vectors (LV) have gained popularity as modified gene therapy and gene delivery vehicles for treating a variety of acquired and inherited diseases. There is a growing demand for highly purified and concentrated viral vector products. To meet the market demands as research studies progress into pre-clinical and clinical phases, scalable manufacturing poses a big challenge. One of the areas that can help alleviate this challenge is optimization of transfection conditions to achieve higher viral titers. During lentivirus production processes using suspension-adapted HEK293T cells, 4 plasmids are complexed with a PEI-based cationic polymer and added to the cells at a specific cell density. Key factors for optimal transfection include transfection cell density, DNA to cationic polymer ratio, plasmid DNA molar ratio, transfection complex volume and transfection complex formation

time. The work in this poster reflects the successful exploration of these factors using statistical software - JMP[®] and DOE approach to achieve higher lentivirus harvest titer. After obtaining optimal level for each factor, findings were confirmed in shaker flasks and bench-scale Mobius[®] 3 L Single-Use Bioreactors.

AAV Vectors - Virology and Vectorology III

892. Intraparenchymal Administration to the Striatum of a Barcoded AAV Library for the Characterization of Capsid Tropisms in Rodents and Non-Human Primates

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Adeno-associated viral (AAV) vectors are increasingly used for gene therapy in the central nervous system (CNS) and there is a need for identifying optimal capsids for specific targets. In this study, we evaluated the tropism of a library of 118 AAV capsids following intraparenchymal delivery. This library consisted of approximately equal concentrations of 56 NAV[®] Platform vectors, 49 engineered AAV variants, and 13 commonly used AAVs. Each AAV vector was produced individually using a unique cis plasmid expressing GFP under the control of the universal CAG promoter and a unique barcode sequence allowing for measurement of the relative abundance of each capsid's genomes and transcripts in different tissues using next-generation sequencing (NGS). The library was administered intraparenchymally to the striatum of non-human primates (*Cynomolgus* macaques, MRI-guided injection with convection enhanced delivery) and mice (C57BL/6, stereotaxic injection). These studies allowed for the direct comparison of each capsid in the pool and translation of findings between species. Biodistribution analysis revealed vector genomes and transcripts were most prominently found at the injection site and brain regions anatomically connected to striatum (e.g. cortex, globus pallidus, amygdala, thalamus, substantia nigra) but were almost undetectable in brain regions that have no connections with the striatum (e.g. hippocampus, cerebellum, spinal cord). Within these connected brain regions, NGS revealed that some capsids were not present at all whereas other capsids were preferentially abundant. Specifically, AAV5 was found to have larger spread at the injection site and better retrograde axonal transport than any other vector. However, AAV5 was found to yield poor transcription, despite its high vector DNA. In contrast, one of our newly engineered capsids was found to yield higher RNA expression than other capsids in most brain regions, ~4-fold better than AAV9, which was observed in both mice and primates. This new capsid is a promising candidate for improving transgene expression in gene therapies using intraparenchymal injections. Taken together, these

findings highlight the importance of direct, simultaneous comparison of the performance of AAV vectors and reveal promising variants for specific therapeutic targets within the CNS.

893. A Novel Engineered Capsid Serotype of Recombinant Adeno-Associated Viral Vector with Enhanced Transduction Efficiency and Widespread Distribution in the Brain

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Introduction. Adeno-associated viral (AAV) vectors are often used in gene therapy for neurological disorders because of their safety profile and promising results in clinical trials. One challenge to AAV-based gene therapy is effective transduction of large numbers of the appropriate cell type. The brain has been a particularly challenging site for effective transduction. What are needed are new vectors that can efficiently transduce neural tissue and the brain. Our team has engineered a new recombinant AAV capsid based on previously described Rec2 that showed high transduction efficiency in adipose tissue and liver. By additional modification of the Rec2 capsid, we generated a novel AAV serotype, LC.V1, with a highly neuronal tropism as well as transduction and distribution efficiency. **Material and Methods.** To evaluate the performance (distribution, transduction efficiency, and trans-neuronal transport) of LC.V1-GFP in the brain, we injected the vector into the thalamus and ventral tegmental area (VTA) + substantia nigra of 3 nonhuman primates (NHP) via MRI-guided Convection-enhanced Delivery (CED). Animals were euthanized after 3 weeks and the brains were processed for immunohistochemical staining to assess the efficiency of distribution and transduction. Double fluorescence staining against the transgene, GFP, and neuronal marker, NeuN, was used to determine the percentage of transduced neurons. **Results.** 1) Intracerebral delivery by CED of LC.V1 into the thalamus of a NHP demonstrated near-complete coverage with 67% of neuronal transduction efficiency based on transgene expression (GFP) at the site of injection of only 124 μ l of the LC.V1 vector (Fig. 1A). 2) LC.V1 was transported retrogradely from the site of injection (thalamus) to cortical regions where it transduced pyramidal neurons of prefrontal and frontal cortex (Fig. 2) layer V with 50% efficiency. 3) LC.V1 was transported retrogradely from the site of injection (thalamus) to the hippocampus and transduced neurons of the subiculum (53% efficiency). 4) LC.V1 was transported anterogradely from the site of injection (midbrain: VTA and substantia nigra) to the striatum (both caudate nucleus and putamen) where numerous GFP positive fibers were observed. 5) Distribution of LC.V1 within the brain parenchyma delivered via CED was monitored by real-time MRI. By including the MRI contrast agent, ProHance, one can track the convective movement of LC.V1 viral particles by continuous monitoring of distribution of ProHance (Fig. 1B/1C). **Conclusion.** LC.V1 vector is extremely efficient in the distribution and efficiency of transducing large areas of the brain. This new AAV capsid has the

potential to substantially improve gene therapy for neurodegenerative diseases affecting large areas of the brain (Alzheimer's, Parkinson's, lysosomal storage disorders).

Fig. 1

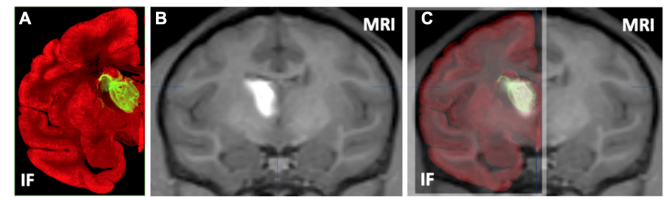
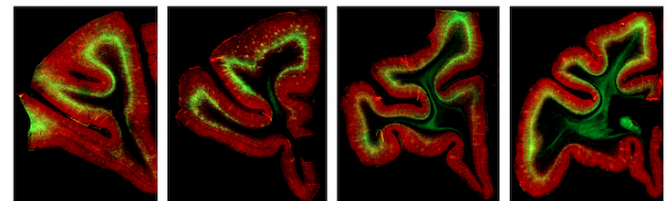


Fig. 2



Green fluorescence – reporter gene, GFP
Red fluorescence – neuronal marker, NeuN

894. A Longitudinal, Comparative Analysis of Transgene Expression Durability via Different Promoters in the Striatum of Mice Delivered by Intraparenchymal Injection of rAAV9

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Adeno-associated viral (AAV) vectors are being used therapeutically to deliver transgenes to the brain. Neuron-specific promoters allow targeted expression of transgenes, but the durability and kinetics of these promoters' activity has not been extensively characterized. Previously, transgene expression driven by the human synapsin (hSyn) promoter has been suggested to be repressed over longer time periods¹. Here, we directly characterize the temporal profile of transgene expression after bilateral stereotaxic injections of rAAV9 encoding GFP into the striatum of mice via the neuronal-specific promoters hSyn and CamKII, in comparison to the ubiquitous pan-cellular promoter CAG. Brains were harvested three weeks, three months, and six months after rAAV9 administration. One hemisphere was processed for histological analyses, whereas the other hemisphere was used to extract DNA and RNA from the striatum for ddPCR analyses. Luminance analysis of native GFP fluorescence demonstrated that GFP expression driven by the CAG promoter was highest at all three timepoints, CamKII was the lowest, and hSyn was in the middle. The RNA-to-DNA ratios from ddPCR analyses showed similar trends to the luminance quantification for all three promoters in terms of relative strength of expression. Interestingly, all three promoters showed increasing levels of transgene expression through six months post-injection. These data demonstrate that transgene expression driven by the hSyn promoter is not repressed over the course of six months; in fact, this expression increases over this time in the CNS of mice. Therefore, the hSyn promoter is capable of providing strong, durable neuron-specific transgene expression, indicating suitability as a promoter in therapeutic applications.

¹Jackson, KL, Dayton, RD, Deverman, BE, and Klein, RL (2016) Better Targeting, Better Efficiency for Wide-Scale Neuronal Transduction with the Synapsin Promoter and AAV-PHP.B. *Front Mol Neurosci* 9: 116.

895. Post-Translational Modifications of Adeno-Associated Virus Assembly Activating Protein (AAP)

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Although adeno-associated virus (AAV)-based vectors have emerged as successful gene delivery tools, they remain prohibitively expensive. It is imperative that we discover new methods of vector production to mitigate costs. AAV capsids are made of the *cap* gene products VP1,2,3, assembly activating protein (AAP) and numerous host cellular factors. Since AAP is necessary for maximal vector production across all AAV serotypes, our lab has been studying AAP in detail. In this study, we report our initial explorations into the post-translational modifications (PTMs) of AAP. Although PTMs of AAV VP proteins have been studied to understand AAV tissue transduction and host immune response, there has been no study examining the PTMs of AAP. AAP is a multi-domain protein, and some domains have particular functions attributed to them, for instance the basic region (BR) of AAP is important for appropriate nucleolar localization of AAP. Understanding PTMs of AAP could reveal how each domain of AAP carries out its function. The observed molecular mass of AAP is higher than its predicted molecular mass, which might be due to PTMs, further deepening our interest in identifying the PTMs of AAP. Using biochemical approaches of histidine-ubiquitin pulldowns, we have previously reported that AAP is poly-ubiquitinated and can be regulated by the cellular de-ubiquitinase USP36. To identify additional PTMs of AAP, we purified FLAG-AAP2 and 9 from HEK293 cells transiently transfected with plasmids coding for CMV-driven FLAG-AAP2 and 9. Purified protein samples were reduced, alkylated, trypsin digested and cleaned up prior to liquid chromatography-mass spectrometry (LCMS) analysis employing peptide fragmentation to identify novel and serotypespecific PTMs. The data were searched for small PTMs (oxidation, methylation, phosphorylation, and acetylation), glycans, and larger proteins (ubiquitin, SUMO, and NEDD8). This bottomup proteomics approach has so far yielded poly-ubiquitination (congruous with our previous biochemical approach where we identified poly-ubiquitination of AAP) and various sites of potential phosphorylation and O-glycosylation. We plan to validate these further by a top-down proteomics approach and assess the presence of combinatorial PTMs on various AAP proteoforms. In summary, we present the first study to identify unknown PTMs of AAP. We hope to glean information on how these PTMs change in the absence or presence of VP proteins, and gain insights into the functional roles of these PTMs.

897. Identification of Plasmid Backbone-Derived Antisense RNAs in AAV Transduced Animals

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Introduction. Adeno-associated viral (AAV) vectors have tremendous potential as a delivery mechanism for gene therapy. While a generally safe therapeutic strategy, some patients have adverse responses to AAV treatment, including inflammatory and immunological responses and hepatotoxicity. In an effort to make gene therapy vectors as safe as possible, it is critically important to understand the full spectrum of protein and RNA products that are generated from the AAV drug product. Here we describe an unbiased next-generation-sequencing (NGS) based approach to characterize all RNA transcripts generated from AAV vectors. The method does not rely on the presence of 5' cap or 3' poly(A) tails on the RNA targets, allowing the identification of all RNAs independent of RNA polymerase or post-transcriptional processing events. **Methods.** Mice were dosed IV with 3e11 vg/animal of different recombinant AAV vectors. Four weeks after dosing, total RNA was isolated from mouse livers. RNAs were decapped and converted to 5' monophosphate. RNA fragments were converted to NGS libraries using the Illumina TruSeq small RNA protocol. Libraries were paired-end sequenced on the Illumina platform, and reads were mapped to the plasmid sequence that the AAV vector was derived from. **Results.** We generated unbiased NGS libraries from AAV transduced mouse liver RNA samples. 30 - 50 million sequence reads were obtained per library; of these, 5000 - 10000 reads mapped to the plasmid sequence used in AAV production. Of sense orientation reads (relative to the transgene), >90% mapped to the transgene transcriptional unit, while < 5% mapped to the plasmid backbone outside of the ITRs. In contrast, for reads that mapped antisense to the transgene direction, >40% mapped to the plasmid backbone. Backbone reads for both orientations were concentrated in the ColE1 origin of replication and the Kanamycin resistance gene. Antisense reads that mapped within the transgene cassette were concentrated in the promoter region, though small islands of reads were present throughout the transgene coding region. **Conclusion.** We detected ectopic transcripts in both orientations in AAV transduced mouse liver. Transcripts that originated from the plasmid backbone were presumably derived from co-packaged plasmid fragments. It should be noted that the number of backbone reads were disproportionately high compared to the level of bacterial DNA contamination in AAV vector preparations. The source of transcriptional activity is also unclear as this backbone does not contain known mammalian promoter elements. The presence of sense and antisense RNA could lead to dsRNA and concomitant activation of innate sensing pathways. Furthermore, strategies to eliminate the plasmid backbone from AAV manufacturing processes, such as the use of synthetically generated close ended linear duplex DNA, may prevent much of the dsRNA product and reduce innate activation. We are focused on contributing close ended linear duplex DNA source material in clinical AAV manufacturing which should mitigate these potential events.

898. Abstract Withdrawn**899. Evaluation of a Human Neurovascular Model to Complement a Parallel Non-Human Primate Selection for Blood Brain Barrier Penetrant AAV Capsids**

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Delivery of genomic medicine to the central nervous system (CNS) is a major hurdle for clinical applications of gene therapy. The blood brain barrier (BBB) limits the brain distribution of virtually all intravenously administered macromolecules. Several AAV serotypes, most notably AAV9, distribute to the brain after intravenous administration but require high doses to achieve limited expression. AAV capsid engineering has produced novel variants that are superior to their parental serotypes and have progressed into the clinic for several indications. However, translation of clinical programs from pre-clinical models to humans remains a challenge for the entire gene therapy field including capsid engineering efforts. Two factors for a stringent selection campaign have emerged: library designs that incorporate functional cellular transduction pressure, and selection of appropriate *in vitro* and/or *in vivo* models. We employed SIFTER (Selecting In vivo for Transduction and Expression of RNA) to engineer capsids with improved CNS transduction following intravenous administration in *Cynomolgus* macaque using several parental capsids and four library designs. This platform is distinguished from other selection systems by using a barcode look-up table that tracks capsid evolution through expression of an RNA barcode from an engineered therapeutically relevant vector. The results from a round one naïve library screen identified several capsid variant families across the library designs reinforcing that transcription-dependent library designs are critical to AAV capsid engineering. Although non-human primates mostly exemplify the genetics and physiology of the human CNS, there are still differences that can imperil translation in humans. Many *in vitro* models of the blood-brain barrier have shortcomings including the use of immortalized or non-human cells, poor tight junction integrity, and lack of polarization. These limitations are likely major contributors to discordant capsid performance observed *in vitro* vs *in vivo*, and between species. To address these challenges, we implemented an all-human cell model of the BBB that recapitulates many of these key BBB properties. This human *in vitro* BBB model has the potential to be used as an orthogonal selection model and/or validation platform for variants from our round one naïve library screen *in vivo*. We have optimized this system to assess distribution of AAV vectors and found that the levels of transcytosis across the endothelial monolayer are consistent with macromolecule distribution in non-human primates (0.1 - 0.2% of injected dose). Capsids arising from both *in vitro* and *in vivo* selection campaigns have the potential to provide intravenously administered CNS delivery solutions for a broad range of therapeutic indications.

900. Next-Generation Automated AAV Engineering Platform for Rapid Identification of Efficient and Specific Capsids in Non Human Primates

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The rapid development of gene therapies in recent years has highlighted the critical need for more efficient and specific delivery vectors for targets throughout the human body. Previous engineering platforms which focused on high-throughput screening and directed evolution in lower-order animal models led to dramatically improved capsids for a variety of targets, including organ-level and cell-type-level specificity (Deverman BE et al. 2016, Kumar SR et al. 2020). However, the translatability of these engineered capsids has proven challenging (Hordeaux et al. 2018, Liguore WA et al. 2019). To bypass the challenges of translating capsid engineered *in vitro* or in lower-order species, while still leveraging the success of directed evolution in a high-throughput and high-capacity manner, Capsida Biotherapeutics has developed an automated AAV engineering platform capable of interrogating targets through the body, directly in non-human primates and human cell lines at a rapid pace. This platform has generated several iterations of improved capsids for targets in the CNS and is now being leveraged for targets throughout the body. In addition to increased capacity and throughput, the automated engineering platform has simultaneously improved data quality by increasing the volume of primate tissues interrogated, enabling greater predictive analysis in the bioinformatics platform and more rapid identification of improved capsids.

901. Targeted In Vivo Manipulation of Murine T-Cells Using a Newly Evolved AAV Capsid Mutant

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Manipulation of T-cells in mouse models *in vivo* has allowed for interrogation of immune mechanisms and pathways. However, generation of conditional T lymphocyte specific genetic changes in mouse models can be labor intensive, requiring embryonic manipulation and challenging to control temporally. To better study and manipulate T-cell biology in mouse models for gene therapy and immunotherapy applications, we have generated a novel AAV capsid mutant Ark313, capable of efficient and targeted gene transfer to murine T-cells *in vivo*. Delivery of transiently expressed genes is feasible as demonstrated by a self-complementary GFP cassette delivered using Ark313. We observed ~10% of CD3+, but no significant transduction of CD3- splenocytes at a systemic vector dose of 5E12 vg/kg. A slight bias for CD8+ over CD4+ T-cell resident splenocytes was also noted. At a single IV dose of 5E13 vg/kg in the Ai9 fluorescent reporter mouse model, we show Ark313 expressing Cre recombinase can achieve permanent genetic changes in ~25% of murine T-cells

in vivo. Additionally, Ark313 appears to display a liver detargeted phenotype relative to parental AAV6. Analysis of T-cell subtypes shows Ark313 significantly transduces naïve, effector, and memory with slight preference for effector and memory T-cells. Results from ongoing studies in CRISPR based mouse model systems will be presented to demonstrate the broad spectrum of applications made possible by this novel AAV variant in manipulating mouse T-cell biology *in vivo*.

902. Identification and Validation of Target Receptors for AAV9

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Adeno associated virus (AAV) vectors are the major delivery vector utilised in gene therapy. Unfortunately, treatment of neurological disorders with the use of AAV gene therapy is hampered by their exclusion from the central nervous system (CNS) by the blood brain barrier (BBB) as well as the limited neurotropism of many serotypes. Of the numerous AAV capsid serotypes identified, AAV serotype 9 (AAV9) shows strong neurotropism as well as the ability to cross the BBB, and is therefore the leading serotype for CNS associated gene therapy. However, the exact mechanism by which AAV9 interacts with and transduces neuronal cells is not well understood. In an attempt to identify protein targets necessary for AAV9 transduction we performed microarray analysis of porcine endothelial brain cells (PEBCs) infected with AAV9. Dysregulated targets were further validated by qPCR. Our preliminary data revealed AAV9 infection resulted in the upregulation of Syndecan-4 (SDC4) and Solute Carrier Family 7 Member 2 (SLC7A2). We therefore hypothesise that these targets may facilitate AAV9's wide neurotropism. To investigate whether SDC4 and SLC7A2 are involved in AAV9 transduction we established a transduction efficiency assay in AAV9 permissive cells using scAAV9-EGFP. Expression of SDC4 and SLC7A2 was then modulated by targeted siRNA. Using AAV receptor (AAVR) knockdown as a positive control for reduced transduction efficiency, we found that targeted siRNA knockdown of SDC4 significantly reduced AAV9 transduction efficiency in permissive cells, while targeted knockdown of SLC7A2 did not. These data identify SDC4 as a potential protein target required for AAV9 transduction and help to further our understanding of AAV9's neurotropism which could assist future improvements to the capsid and the development of enhanced variants.

903. A Novel Self-Complementary AAV Vector Enhances Transgene Expression

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Recombinant adeno-associated virus (AAV) has become a promising tool for DNA delivery. Long-term, stable therapeutic gene expression is required for gene therapy. AAV inverted terminal repeats (ITRs) form the hairpin conformations, by which interacting with the MRN complex (Mre11, Rad50, and Nbs1), blocking access to transcription factors to adjacent, severely limiting the gene expression and replication of AAV and rAAV vectors. Here we present a new self-complementary

AAV (scAAV) vector. It contains a normal ITR and a covalently-closed linear end. To form this covalently-closed end, the mutation ITR in conventional scAAV vector was replaced with a TelN protelomerase 56 bp palindromic recognition sequence. Recognition of this sequence by TelN enzyme results in covalently-closed ends formed at the site of cleavage. To prevent MRN-mediated inhibition of transgene expression, the promoter is adjacent to the covalently-closed end and not the ITR. The new scAAV vector yield is comparable to that of conventional scAAV vector. The yield of the novel vectors was not significantly different from the conventional vectors. Furthermore, the transgene expression of this new scAAV vector was higher 1.5~3.5 folds than that of this conventional scAAV vector. For new scAAV-EGFP-TelN and scAAV-Gluc-TelN, the transgene expression was not obviously affected by MRN inhibitor KU55933 or Mirin. The number of EGFP positive cells or Gluc activity has no significant change at 0-20 μ M of KU55933 or 0-40 μ M of Mirin. However, EGFP expression or Gluc activity from the two conventional vectors increased with increasing dose of KU55933 or Mirin. *In vivo* assay showed that hFIX expression was significantly different between scAAV-hFIX and scAAV-hFIX-TelN, the later showed a higher hFIX expression level in the long term. In conclusion, this new scAAV vector shows more efficient transduction efficacy *in vitro* and *in vivo*, without sacrificing productivity.

904. Identification of an N-Terminal Degron and its Leucine Residue in the AAV AAP Protein Critical for its Promoted Degradation

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Recombinant adeno-associated (AAV) vectors are successful FDA-approved gene delivery tools that have broad-spectrum applications. However, AAV vector production costs still remain a bottleneck for its widespread use. AAV vector viral capsid assembly requires VP capsid proteins, assembly-activating protein (AAP) and a multitude of unknown host cellular factors. Although AAP binds VP proteins and promotes capsid assembly, AAP has not been found as a structural protein of the capsid. This has led us to a hypothesis that AAP has a degron motif and undergoes rapid degradation during the capsid assembly process. To lend support to this hypothesis, we have previously discovered that the hydrophobic region (HR) potentially contains a degron. Here we propose an N-terminal degron model in which a leucine residue in the HR at the N terminal end of AAP plays a critical role in promoting AAP degradation in the process of capsid assembly. We have previously shown that, among AAP serotypes 1 to 12 (i.e., AAP1 to AAP12), the HRs derived from all AAP serotypes but those from AAP4 and AAP11 drive effective degradation of a reporter protein. By taking advantage of this serotype-dependent proneness to AAP degradation, we sought to identify an amino acid residue(s) within the AAP HRs that is shared in each of the two groups, the degradation-prone AAPs (AAP1, 2, 3, 5, 6, 7, 8, 9, 10, and 12) and the degradation-resistant AAPs (AAP4 and 11), and investigate its functional significance. An amino acid sequence alignment study of AAPs revealed position 21 to have P in AAP4 and 11, while the other serotypes have either L, K or Q (note: AAP positions are those

corresponding to those of AAP2). At position 25, AAP4 and 11 have T, while other serotypes primarily have L. We then created mutations in the HR region of AAP9 and assessed the degron function of each mutant. To this end, we constructed AAP9Q21P-HR-FLAG-GFP and AAP9L25T-HR-FLAG-GFP and compared their GFP expression against AAP9-HR-FLAG-GFP. Whereas AAP9-HR-FLAG-GFP and AAP9Q21P-HR-FLAG-GFP showed a substantial decrease in GFP expression, AAP9L25T-HR-FLAG-GFP retained GFP expression albeit at a level slightly lower than that of our FLAG-GFP control. This indicated that the leucine (L) residue at position 25 in AAP9's HR plays a critical role in the degron activity. To examine if positioning the HR region at the N-terminal end is critical as has been demonstrated in N-terminal degrons, we made a GFP construct with a FLAG tag fused on the N terminus of AAP9HR (FLAG-AAP9HR-GFP). Unlike AAP9-HR-FLAG-GFP, FLAG-AAP9HR-GFP restored GFP expression partially, indicating that a FLAG-tag on the N-terminus of the HR can partially abrogate its degron function. We are currently investigating if a FLAG-AAP9L25T-HR-FLAG-GFP construct could synergistically rescue the GFP expression to the control GFP levels. In summary, our observations strongly support a model that the AAP HR is an N-terminal degron whose positioning and its leucine residue at position 25 drive its degron function. Further studies are warranted to understand the functional significance of the AAP's degron activity in AAV capsid assembly and what types of post-translational modifications and pathways mediate AAP degradation.

905. Combinatorial Engineering Across Multiple Surface Exposed Loops of AAV2 and AAV9 Yields Capsids with High Degrees of Enrichment and Specificity for Target Tissues

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Concerns for patient safety are at the forefront of the gene therapy field due to current dosing regimens which include IV administration of large boluses of vector genomes and highly invasive delivery modalities to target organs (Maurya et al. 2022). The continued development of gene therapies with applicability beyond ultra-rare indications is gated by the ability to deliver genetic therapies more efficiently and specifically to targets of interest throughout the human body. To that end, Capsida Biotherapeutics has continued to innovate AAV capsid diversification strategies, and has developed a high-throughput method for simultaneous enrichment of target tissues and de-enrichment of non-target tissues, thereby achieving organ-level and cell-type specificity. Many CNS indications require broad delivery of genetic therapies throughout the brain and/or spinal cord. IV delivery of engineered capsids capable of crossing the blood-brain-barrier is well-suited to achieve broad and uniform transduction of the CNS. However, there are several ongoing clinical trials focused on CNS or muscle therapeutic areas that have observed severe adverse events related to liver toxicity due to the high viral burden of wildtype AAV serotypes on the liver. As such, we employed our combinatorial engineering strategy to evolve novel AAV capsids that are simultaneously enriched in the CNS and de-enriched in the liver following IV administration

in NHPs, thereby significantly improving the therapeutic window. Following multiple rounds of screening, we identified lead candidates with >100-fold enrichment in the CNS and >10-fold de-enrichment in the liver relative to the parent capsid AAV9, and are advancing these lead candidates into IND-enabling studies.

906. Conserved Receptors for Enhanced Blood-Brain Barrier Crossing by Engineered Adeno-Associated Viruses

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Efficient transcytosis of the blood brain barrier (BBB) is a key bottleneck in the development of molecules to study and treat the brain, particularly biologics requiring active transport. Our work on adeno-associated virus (AAV) engineering has yielded diverse capsid variants with enhanced central nervous system (CNS) infectivity upon systemic delivery in mice¹, marmosets², and macaques³. However, the directed evolution methods by which we identified these capsids do not provide much insight into the mechanisms by which enhanced tropisms are conferred, complicating the application of engineered AAV both across model organisms and in potential human gene therapies. This dilemma is perhaps best illustrated by AAV-PHP.eB, whose enhanced CNS tropism was found to be restricted to mouse strains expressing membrane-localized Ly6a^{4,5} after unsuccessful application in non-human primates and select mouse strains. Here, we used surface plasmon resonance to assemble a panel of previously identified engineered AAV with enhanced CNS tropism that do not interact with Ly6a. Administration of these capsids to the genetically diverse 129S1/SvImJ, CBA/J, DBA/2J, and NOD/ShiLtJ mouse strains showed enhanced CNS infectivity patterns consistent with non-Ly6a mechanisms for BBB transcytosis. We developed a cell culture screen of candidate membrane proteins selected based on their single-cell RNA sequencing profiles in C57BL/6J mouse cortex and tested a diverse panel of engineered AAVs. Using this screen, we identified potential novel roles for multiple proteins as molecular receptors in the BBB that enable enhanced CNS infectivity by engineered AAVs. These include receptors that complement previously identified Ly6a-interacting AAVs as research tools in diverse mouse strains and receptors that are broadly conserved across species, including humans. The latter present a promising new target for rational engineering of AAV for species-specific enhanced CNS infectivity. References: 1. Ravindra Kumar, S. et al. Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types. *Nat. Methods* 17, 541-550 (2020). 2. Goertsen, D. et al. AAV capsid variants with brain-wide transgene expression and decreased liver targeting after intravenous delivery in mouse and marmoset. *Nat. Neurosci.* 25, 106-115 (2022). 3. Chuapoco, M. R. et al. Intravenous gene transfer throughout the brain of infant Old World primates using AAV. <http://biorxiv.org/lookup/doi/10.1101/2022.01.08.475342> (2022) doi:10.1101/2022.01.08.475342. 4. Huang, Q. et al. Delivering genes across the blood-brain barrier: LY6A, a novel cellular receptor for AAV-PHP.eB

capsids. PLOS ONE 14, e0225206 (2019).5. Hordeaux, J. et al. The GPI-Linked Protein LY6A Drives AAV-PHP.B Transport across the Blood-Brain Barrier. Mol. Ther. 27, 912-921 (2019).

907. Enhanced Transduction Efficiency of AAV Vectors Mediated by Polyvinyl Alcohol and Human Serum Albumin is Serotype- and Cell-Type Specific

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Human serum albumin (HSA) has been reported to increase the transduction efficiency of several AAV serotype vectors in human hepatocellular carcinoma cell line, Huh7 (*Gene Ther*, 24: 49-59, 2017). We have reported that polyvinyl alcohol (PVA) increases the transduction efficiency of AAV6 vectors in both human erythroleukemia cell line, K562, and in primary human hematopoietic stem cells, and that the observed increase is due to direct interaction between PVA and AAV6 capsid (*Mol Ther Nucl Acids*, 20: 451-458, 2020). In the current study, the following two questions were addressed: (i) Does PVA also increase the transduction efficiency of AAV3 serotype vectors in view of their remarkable tropism for human liver (*Hum Gene Ther*, 31: 1114-1123, 2020); and (ii) Do PVA and HSA have an additive effect on the transduction efficiencies of AAV3 as well as AAV6 vectors given their high-efficiency transduction of primary human hematopoietic stem cells (*Sci Rep*, 6: 35495, 2016). Transduction efficiencies of AAV2, AAV3, and AAV6 vectors, with and without preincubation with PVA, HSA, or PVA+HSA, were evaluated in HeLa, Huh7, and K562 cells, respectively. PVA failed to increase the transduction efficiency of AAV2 vectors, and no increase with HSA alone, or PVA+HSA together was observed in HeLa cells. The transduction efficiency of AAV3 vectors was significantly increased by both PVA and HSA, but no additive effect was observed in Huh7 cells. Similarly, no additive effect was seen in the transduction efficiency of AAV6 vectors in K562 cells. We also evaluated whether PVA could increase the transduction efficiency of AAV3 vectors expressing a therapeutic gene. Human HepG2 cells, known to express and secrete human clotting factor IX (hF.IX) were either mock-transduced or transduced with scAAV3-hF.IX, with and without preincubation with HSA or PVA. As shown in **Figure 1**, HSA had no effect, but preincubation with PVA led to ~5-fold increase in mock-adjusted hF.IX protein expression without PVA in HepG2 cells. Taken together, these data suggest that PVA/HSA-AAV interactions occur by different mechanisms, and that enhanced transduction efficiency of AAV vectors mediated by PVA and HSA is serotype- and cell type-specific. The use of PVA is an attractive strategy to further improve the efficacy of clinically relevant AAV serotype vectors, which has important implications in the optimal use of these vectors in the potential gene therapy in humans.

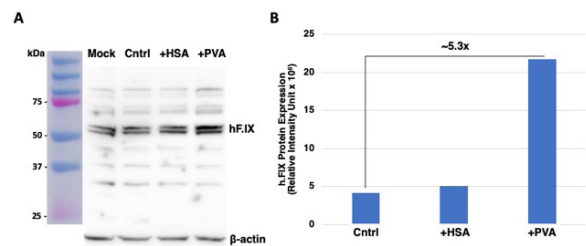


Figure 1: Effect of HSA and PVA on hF.IX protein expression in scAAV3-hF.IX vector-transduced human hepatic cells. HepG2 cells were either mock-transduced or transfected with scAAV3-hF.IX vectors with or without preincubation with HSA or PVA. Equivalent amounts of total protein samples prepared 72 hrs post-transduction were analyzed on Western blots (A) using a polyclonal anti-hF.IX antibody. (B) Quantitation of the mock-adjusted data from (A).

908. Widespread Brain Distribution of a Novel Recombinant Engineered Capsid Serotype After CSF Delivery

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Widespread AAV-based distribution of therapeutic genes at non-toxic and effective doses throughout the central nervous system (CNS) is still challenging. Local CNS delivery using MR-guidance or other systems can achieve certain level of distal distribution depending on the capsid serotype and the anatomical structure targeted, but in the cases where global transduction is required parenchymal delivery can present limitations. In contrast, systemic delivery is an alternative by which the vector is injected into the blood stream. However, crossing of the blood-brain-barrier, which prevents the majority of the available AAV serotypes to enter the CNS, still needs to be overcome. As an alternative, CSF delivery has been shown to achieve wide CNS distribution for a few AAV serotypes. Intrathecal, cerebellomedullary or intraventricular delivery of AAV9 or AAV7, among others, is extensively used in pre-clinical and clinical development specially in lysosomal storage disorders. Engineering of capsids with enhanced properties is currently under development. In this study, we analyze the brain distribution, level of expression and tropism after CSF delivery of a novel engineered capsid serotype of recombinant AAV, named LC.V1. Large (25 μ L) and small (5 μ L) volumes were infused into mouse lateral ventricle in two different cohorts at 1 μ L/minute. Histological assessment using DAB immunohistochemistry evinced extensive brain distribution along the anterior-posterior axis in all the tested groups. GFP-positive cells were found in the cortex (layers II to VI), as well as different anatomical subcortical structures including the basal ganglia, hippocampus, and cerebellum at an 8.36E+13 vg/mL dose. NeuN immunostaining confirmed the neuronal tropism, while GFAP staining showed very low astrocytic transduction 21 days after delivery. Both infused volumes yielded similar brain distribution with differences in levels of transduction. In contrast, histological analyses of peripheral organs, spinal cord and DRG revealed volume-based vector biodistribution

differences. In summary, this novel variant shows very promising results. Ongoing experiments will evaluate brain distribution and levels of transduction in NHP.

909. A Biophysical Study of AAV Genome Ejection

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Adeno-associated Virus (AAV) are non-enveloped, single-stranded DNA viruses. The AAV are non-pathogenic, can be produced and purified in therapeutic quantities, transduce many cell types, and have shown long term expression of packaged transgenes. These characteristics have contributed to the establishment of AAV as the most widely studied viral vector for clinical gene therapy applications. Consequently, a significant amount of research has focused on understanding all aspects of the virus life cycle. In brief, an AAV infection is initiated by receptor/co-receptor binding, clathrin coated-mediated endocytosis, endo-lysosomal (pH 7.4 - 4.0) transport and escape to the cytoplasm, nuclear localization, and entry into the nucleus via the nuclear pore, where genome release/ejection occurs in the nucleus at pH 7.4. Genome release/ejection is therefore the final critical step for replicating and expressing the therapeutic gene, yet there is limited data available on the environmental conditions that affects this process. In this study we present the development of a thermal assay to probe this process using two structurally diverse AAV serotype, namely AAV2 and 5. Previously, differential scanning fluorometry studies determine the thermal melt temperatures (T_m) of AAV2 and 5 in PBS as 68 and 89 °C, respectively. We have shown, using these thermal assay that both AAV2 and 5 loss of ~50% of their packaged genome at ~55 °C, in PBS. This data confirmed by cryo-EM micrographs of the virus at these temperatures. Examination of negative stain EMs reveal conformational changes in the AAV capsid at temperatures > 55 °C, ~5 °C, before the capsid T_m , when all the capsids are stain penetrated (empties) with 'strings' of exposed DNA exiting/ejecting the capsid. This data would imply that the trigger for genome ejection is independent of the AAV serotype, but the rate required for all genomes to be released is dependent on the thermal stability of the capsid. These thermal assays can therefore be used to screen other environmental conditions experienced by the capsids and their effect on genome ejection, and ultimately to see how the rate of genome ejection affects the rate of expression of the therapeutic gene.

910. Discovery of Highly Epistatic AAV Capsid Variants Through Machine-Guided Navigation of the Fitness Landscape

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Understanding a protein's fitness landscape is a challenging but highly promising approach towards efficiently and effectively optimizing its function. Natural Adeno-associated Virus (AAV) capsids were not evolved for therapeutic use, and therefore have great potential to benefit from engineering to optimize them for in vivo gene delivery. To systematically engineer AAV capsids, a principled approach is to first construct a model of its landscape topography. Thanks to new technologies this is now possible through high-throughput sampling and computational modeling. We quantitatively sample 520k sequences and assess their AAV2 packaging efficiency. This is the largest landscape of its kind, and includes modifications to the primary sequence of up to 29 mutations, including insertions, within a single 28-aa segment. Our sequence designs focus on 15 distinct regions of the sequence space around identified pseudo-peaks, variants that were measured to be high-efficiency packagers. This allows a detailed characterization of the landscape around each peak, and further enables computational models to interpolate between these regions in the sequence space. Our analyses show that the landscape has significant regularities, including an unexpectedly high degree of local additivity (i.e. independence between effects) among mutations. We also observe ubiquitous sequence-level epistasis, where mutations have idiosyncratic effects in distinct backgrounds. Despite this, we find that the global shape of the landscape is similar across different regions. We observe the existence of a "neutral-network" of viable paths between the pseudo-peaks, and further explore the space with machine learning models, discovering viable sequences in epistatic regions of the space which independent-sites and rational-design methods do not explore. With this detailed and comprehensive map of the AAV capsid fitness landscape, we analyze the implications for machine guided and rational design. More importantly, we use our diverse dataset to elucidate the current strengths and weaknesses of various machine learning models for the design of AAVs. These results show how machine learning models can accurately extrapolate and design sequences with many more mutations compared to those collected in the training data. We show that robust models can be built using different types of training data, for example sampled by evolutionary methods or random-mutagenesis. Designing sequences with superior properties compared to the samples in the training data is the key challenge which protein engineers aim to solve. We assess this challenge quantitatively and propose machine-guided approaches to bridge current gaps. This work expands the state of the art in applying machine-learning methods to optimizing AAV capsids for more effective therapeutic gene delivery.

911. Host Transcription Factors and Co-Opted Signaling Pathways Orchestrate Epigenetic Modulation of the AAV Vector Genome

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Gene therapies based on recombinant AAV vectors often exhibit low initial transgene expression and waning expression over time, despite the persistence of viral genomes within the target tissue. Following cellular transduction, the AAV capsid is trafficked to the nucleus, where the vector genome is released and persists as an episome. However, multiple studies in preclinical models and in the clinic show diminishing transgene expression in the liver due to multiple overlapping mechanisms, despite the persistence of vector genomes in the tissue. Particularly striking is the observation that less than 10% of episome-containing cells express transcripts derived from the vector genome. While episomal dilution (from hepatocyte division) and the role of innate/adaptive immune responses continue to be explored, the mechanistic basis for this silencing has remained elusive. Overcoming this problem requires a better understanding of the host mechanisms that can influence viral genome persistence and transcriptional activation following transduction. We previously applied a high-throughput pooled CRISPR activation (CRISPRa) screen targeting ~1,500 putative transcription factors (TFs) to identify subsets of TFs that influence transgene expression, with further analysis of the mechanisms by which this transcriptional modulation occurs. We provide an in-depth analysis of two candidate TFs - ZNF431 and ZNF225, which exhibited a common effect of transgene activation or suppression, respectively, across all promoters tested. For both of these TFs, we show that the transduction phenotypes are due to transcriptional modulation and are independent of viral capsid. Furthermore, we have identified epigenetic regulation of chromatinized viral genomes as a likely mechanism, and we demonstrate that signal transduction pathways associated with these TFs may be co-opted to exert positive effects on viral genome transcription. We postulate that insights obtained from these studies can potentially be utilized to de-repress AAV vector genomes and achieve enhanced therapeutic transgene expression.

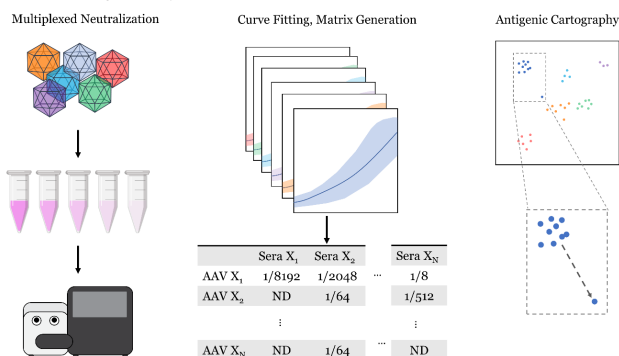
912. Constructing an Antigenic Cartography of AAV Capsids by Massively Multiplexed Neutralization Assays

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Pre-existing immunity against the adeno-associated virus (AAV) capsid remains a barrier towards the development and translation of AAV-based therapies. Neutralizing antibodies (NAbs) can reduce the transduction efficiency of candidate gene therapies and even modest titers of NAbs can be an exclusion criterion in clinical trials.

Consequently, researchers are continually motivated to understand the potential of human polyclonal antisera to neutralize clinically relevant AAV capsids, as well as to discover antigenically distinct AAV capsids. In this work we present a barcode-based multiplexed neutralization assay to profile human polyclonal antisera for their potential to neutralize over 32,000 vectors in parallel. First, we validate this massively multiplexed assay by demonstrating that it recapitulates findings from clonal, *in vitro* neutralization assays. We then demonstrate how this approach can be used to construct an antigenic cartography to quantify and visualize the antigenic relationships between families of AAV capsids. Antigenic cartographies have been constructed in other viruses (such as influenza and sarbecoviruses) but have not previously been applied to the study of AAV capsids due to the limited throughput of existing neutralization assays. By this approach, we observe that libraries of ancestrally reconstructed AAVs (AncAAVs) are generally distributed within the antigenic space defined by naturally occurring AAV capsids. Moreover, we identify preliminary subpopulations within our AncAAV libraries which appear distant from the centroid of the rest of their library members. Although these mutants are still within the antigenic landscape defined by naturally occurring capsids, these subpopulations as well as the broader approach may be useful towards informing future efforts to identify or engineer capsids with distinct antigenicity.



Graphical Abstract depicting the approach. All representations are strictly for illustrative purposes only and do not convey any actual data.

913. Engineered AAVs for Non-Invasive Functional Transgene Expression in Central and Peripheral Nervous Systems Across Rodent and NHPS

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Gene therapy offers great promise in addressing neuropathologies associated with the central and peripheral nervous systems (CNS and PNS). However, genetic access remains difficult, reflecting the critical need for the development of effective and non-invasive gene delivery vectors across species. To that end, we evolved adeno-associated viral (AAV) capsid, serotype-9, in mouse models and used M-CREATE to identify a family of novel AAV capsids that were enriched towards PNS areas, and detailed the properties of two such selected AAVs, AAV-MaCPNS1 and AAV-MaCPNS2, in rodents and NHPs. We provide *in vivo* validation of their tropism in mice and demonstrate their potential applications with studies for functional readout and manipulation of the sensory ganglion which has been challenging to achieve with systemic AAV. We used AAV-MaCPNS1 in mice to systemically deliver: (1) the neuronal sensor GCaMP8s to record calcium signal dynamics in nodose ganglia and evaluate vagal function in a gastric assay, and (2) the neuronal actuator DREADD to dorsal root ganglia and mediate pain. In addition to finding improved PNS-targeting capsids, we sought to address a fundamental question of the translatability of capsids selected in mouse models. To this end, we examined the translatability of these capsids across four species commonly used in basic through pre-clinical applications: mice, rats, marmosets, and rhesus macaques. The variants discussed in this study show improved efficiency and specificity towards the PNS, and translate their potent behavior across mammalian species, transducing the SNS and ENS efficiently in both marmosets and macaques. Interestingly and potentially due to the heterogeneity of the BBB, these variants also show the efficient crossing of the BBB in NHPs. In both marmoset and rhesus macaque, the variants transduce neurons and glia cells with higher efficiency compared to AAV9 following intravenous delivery. With potent PNS tropism across mammals, and improved CNS transduction in NHPs, these new tools, AAV-MaCPNS1 and AAV-MaCPNS2, will open doors for gene delivery in applications ranging from basic research to clinical gene therapies. The new vectors' potential for functional readout and manipulation of the PNS through non-invasive systemic administration will substantially ease experimental execution and expand the reach of gene delivery to surgically hard-to-access areas.

AAV Vectors - Preclinical and Proof-of-concept Studies III

914. A Highly Promising Adeno-Associated Viral (AAV) Gene Replacement Therapy for the Treatment of Neurofibromatosis Type 2 Effectively Delivers High Protein Expression in the CNS

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Neurofibromatosis type 2 (NF2) is a rare autosomal dominant disorder affecting 1 in 60,000 people worldwide that originates from aberrant inactivation of the *NF2* tumor suppressor gene. NF2 is a severe debilitating condition characterized by bilateral vestibular schwannomas and other nervous system tumors such as meningiomas or ependymomas. Although these tumors are usually benign and slow-growing, tumor burden in the brain and spine ultimately leads to increased morbidity and reduced life expectancy. Most NF2 patients carry a loss-of-function mutation in one *NF2* allele throughout the entire body. However, tumor formation occurs only upon biallelic *NF2* inactivation, often through loss of heterozygosity (LOH) in the second healthy allele. Current "gold standard" treatment options for NF2 include surgery and radiotherapy, but these invasive procedures often cause further nerve damage and malignant transformations in additional tissue types. Moreover, successful treatments are short-lived as more tumors may still develop when previously unaffected cells incur a second hit to inactivate *NF2*. The high emotional and financial cost of repeated tumor treatments highlights the urgent need of this patient population for therapies that could effectively halt disease progression. In recent years, gene therapy has emerged as a particularly promising treatment option for rare monogenic disorders and tumor entities for which only procedures with very limited curative potential had previously been available. AAV vectors have been demonstrated to effectively target neurons and other cell types of the nervous system and have demonstrated a good safety profile due to their non-pathogenic, low immunogenic, and non-integrating properties. As such, the development of gene therapy approaches may provide substantial advancement to treatment of NF2. Here we describe a gene replacement strategy for NF2 loss-of-function pathology wherein a healthy *NF2* gene copy is delivered to cell types that are highly susceptible to *NF2* downregulation. We have generated several AAV vectors with truncated *NF2* promoters to identify the optimal sequence that will allow efficient viral packaging and transgene expression in relevant cell types. We used *NF2* promoter-green fluorescent protein cDNA reporter (AAV.NF2.GFP) and *NF2* promoter-*NF2* cDNA (AAV.NF2.NF2 cDNA) expression cassettes which were further cloned in self-complementary and single stranded AAV plasmids. *In vivo* testing of the AAV.NF2.GFP constructs in wild-type mice followed by confocal Z-stack imaging identified two highly promising promoter segments among our candidates. These showed high co-localization of the GFP reporter transgene and relevant markers in multiple brain regions which are

implicated in NF2 pathology. The top two AAV.NF2.GFP candidates also showed efficient targeting of Schwann cells in peripheral nerves of injected mice. Further, injection of the corresponding AAV.NF2.NF2 cDNA constructs in wild-type mice at maximal dose confirmed upregulation of NF2 expression in whole brain lysates. In addition, we have also generated phosphorylation-resistant AAV.NF2.NF2 cDNA constructs for increased stability of the expressed protein. Preliminary analysis in wild type mice did not reveal any safety concerns. Further efficacy and safety testing is currently ongoing for our two lead candidate vectors using *in vitro* and *in vivo* systems.

915. Impact of Genome Accessibility and Long-Term Expression of Adeno-Associated Virus 5 Produced in Mammalian (HEK293) and Insect (Sf) Cell Lines

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Understanding the mechanistic factors affecting long-term transgene expression is vital for recombinant adeno-associated virus (rAAV) gene therapy. In particular, determining the optimal cell line production system for rAAVs, which achieves durable expression for commercialization would advance gene therapy applications. The two leading approaches are transiently transfected human HEK293 cells and baculovirus infection of *Spodoptera frugiperda* (Sf9) insect cells. Human HEK293 cells are predominantly used for rAAV vector production and may package larger genomes better while an Sf-based system offers a more scalable alternative to producing standard-sized (<4700 bp) rAAVs, though data regarding their capacity to efficiently produce larger rAAVs is limited. Here, we compared the long-term durability of expression in mice treated with “oversized” (4970 bp) and “standard-sized” (4600 bp) rAAV5 human alpha-1 antitrypsin (rAAV5-hA1AT) vectors produced in HEK293 or Sf cells. C57BL6 wildtype mice were treated with 6×10^{13} vg/kg of either vector at 8 weeks of age. Blood was collected serially through week 57 for the assessment of circulating hA1AT. In addition, livers were collected from separate take down cohorts at weeks 1, 3, 12, 24, and 57 for vector genome analysis by ddPCR and ATAC-Seq. Mice treated with standard-sized vectors had higher serum hA1AT protein levels compared to the oversized vectors produced in both HEK293 and Sf platforms at week 57, respectively ($P = 0.0015$, and $P = 0.0232$). For standard-sized vectors, there was no significant difference in hA1AT expression between HEK293 and Sf-produced vectors from weeks 12-57. In contrast, mice treated with Sf-produced oversized vector had slightly higher circulating hA1AT level from weeks 12-57, at 77% higher level ($P < 0.001$) by week 57 when compared to mice treated with HEK293-produced oversized vector. In all groups, serum hA1AT protein levels peaked by weeks 12-24 and declined 47%-63% by week 57. Analysis of liver transgene RNA showed similar expression pattern as circulating hA1AT (Fig.1). For the HEK293-vector treated mice, liver transgene DNA declined from peak (week 12/24) to final time point (week 57) and while liver transgene DNA levels remained similar from peak to final time point in

the Sf cohort (Fig.2a). Furthermore, ATAC-Seq analysis demonstrated decrease in genome accessibility from peak to final time point in Sf-vector treated mice and not in HEK293-vector treated mice (Fig.2b). Overall, rAAV5 vectors produced in HEK293 or Sf cells showed similar long-term durability of expression in mice, and Sf cells produced oversized vectors may represent a viable alternative to HEK293 cells for production of larger rAAV constructs. In Sf cells, determinants, and dynamics of genome accessibility, including transcription factors binding and epigenetic modifications may distinctly impact durable transgene expression for rAAV vectors.

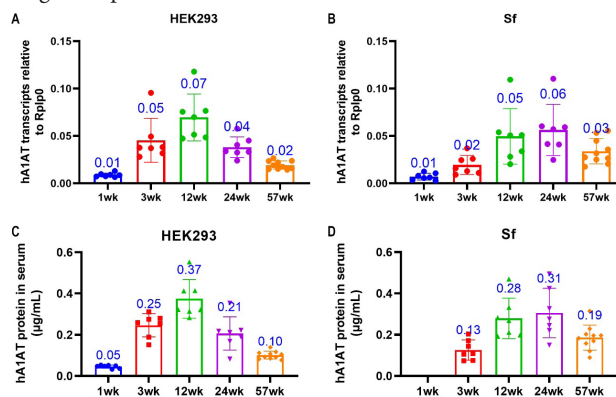


Figure 1. Liver hA1AT transcripts and circulating hA1AT protein levels in AAV5-hA1AT transduced mice. A) Liver hA1AT transcripts normalized to the housekeeping control Rplp0 in mice treated with oversized vector produced in HEK293 cells, and B) Sf cells. C) Serum hA1AT protein levels in mice treated with oversized vector produced in HEK293 cells, and D) Sf cells. Wk=week, hA1AT, human alpha-1 antitrypsin; HEK, human embryonic kidney; Sf, *Spodoptera frugiperda*.

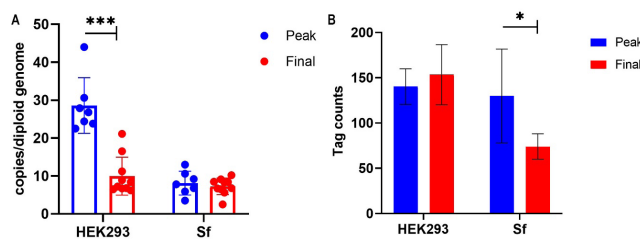


Figure 2. Liver transgene levels and genome accessibility in HEK293 and Sf-vector treated mice. A) Vector genome levels in mice treated with HEK293- and Sf-produced vector from peak (week 12/24) to final time point (week 57). B) ATAC-Seq analysis of peak (week 12/24) to final time-point (week 57) of mice treated with HEK293- vs. Sf-produced oversized vectors. Tag counts are peak regions (MACS 2.1.0) in the AAV5 promoter spanning 1-660 bp and corrected for sequencing depth and normalized to vector genome copies.

916. An AAV Vector Targeting Oligodendrocytes in Macaca Fascicularis CNS

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A large number of gene therapy proofs-of-concept in mouse models fail to be transferred to humans, due to the barrier species. Since transduction of OL in the spinal cord white matter of non-human primates does not occur when a CAG, CBA or MBP promoter is used, we developed a vector targeting OL. To do so, the human ABCD1 (hABCD1) gene driven by a short 0.3 kb part of the human myelin-associated glycoprotein (MAG) promoter was packaged into an adeno-associated viral serotype 9 (rAAV9). Our main reason for targeting OL is their prominent role in the nurturing, myelin making and maintenance

of axons, notably through the metabolic functions of their peroxisomes. One male *Macaca fascicularis*, 3.2 kg, negative for anti-AAV2/9 antibody received intrathecal injection of 2E13 vg/kg of ss rAAV9-MAG-hABCD1-HA vector. Injection was performed by lumbar puncture into the subarachnoid space of the lumbar thecal sac. The subject was placed in the lateral decubitus position and the posterior midline injection site at ~L2/3 level. A 21G spinal needle with a 25G catheter bound to a Sprötte canula was introduced and gently pulled up to the highest possible cervical level and 1ml of vector solution was immediately injected (0.5ml/min) with a Hamilton syringe. The NHP was kept in Trendelenburg position for 15 minutes. Three weeks after the intrathecal (IT) administration of the vector, abundant hALDP expression was observed in numerous OL of the white matter at the cervical, thoracic and lumbar levels of the spinal cord and in cerebellum. In the cervical spinal cord, 43% of total cells were transduced, and 58% of OL expressed hALDP. The proportion of OL expressing ALDP was 40% in thoracic and 30% in lumbar spinal cord. In the cerebellum, 56% of OL expressed ALDP. Less than 2% of corpus callosum OL expressed hALDP, and no OL in the cerebral cortex. The proportion of GFAP(+) astrocytes of the spinal cord expressing hALDP was 33% at cervical level, 41% and 40% at the thoracic and lumbar levels, 27% in cerebellum and <2% in corpus callosum. hALDP expression was not seen in microglia over all studied regions of the primate CNS. Very few neurons, including Purkinje cells, were found to express hALDP. The abundant transduction of OL and astrocytes in the cerebellum of the studied primate is encouraging for the gene therapy of AMN patients displaying cerebellar disease. Liver, heart, or adrenal glands did not show any hALDP expression. Based on the current observation, we postulate that the rAAV9-0.3kbMAG promoter-hABCD1 vector may find applications not only in oligodendroglipathies, a group of rare diseases for which there is currently no specific gene therapy, but in several CNS disorders where dysfunction of OL is postulated.

917. Correction of a Knock-In Mouse Model of Acrodysostosis with Gene Therapy Using a rAAV9-CAG-Human PRKAR1A Vector

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Heterozygous mutations in the type 1 regulatory subunit of cAMP-dependent protein kinase alpha (*PRKAR1A*) gene cause acrodysostosis, a rare autosomal dominant skeletal dysplasia. The first mutation (p.R368X) identified was a nonsense mutation in the last exon of *PRKAR1A* which later proved to be recurrent. In a knock-in mouse model (*PRKAR1A*^{wt/mut}) expressing one copy of the recurrent R368X mutation, we tested the effects of a rAAV9-CAG-human *PRKAR1A* (h*PRKAR1A*) vector intravenously administered at 4 weeks of age. The specific objective of the current proof-of-concept study was to use a gene therapy approach to activate the cAMP/PKA pathway in bones and kidney, and to study whether our vector could correct postnatal growth and renal cAMP production. Caudal vertebrae and tibial growth plates contained 0.52±0.7 and 0.13±0.3 vector genome per cell (VGC), respectively, at 10 weeks of age and 0.22±0.04 and 0.020±0.04 at 16 weeks while renal cortex contained 0.57±0.14 and

0.26±0.05 VGC. Six weeks after vector injection to *PRKAR1A*^{wt/mut} mice, vector-mediated h*PRKAR1A* expression was found in growth plate chondrocytes, osteoclasts, and osteoblasts. 30% of tubular cells in the renal cortex also showed h*PRKAR1A* expression, with a more widespread expression in the inner layer of the cortex, reaching up to 50-70% of tubular cells. Expression was not detected in glomeruli, nor in renal medulla. Tibial growth plates were markedly modified in *PRKAR1A*^{wt/mut} mice as previously observed in newborn mice, with lower density of chondrocytes, increased acellular spaces, decreased hypertrophic zone. Vector treatment normalized all aspects of growth plate architecture. The ratio of hypertrophic/proliferative zones, which was 0.49±0.03 in untreated *PRKAR1A*^{wt/mut} mice was 0.65±0.04 in vector treated *PRKAR1A*^{wt/mut} mice ($p<0.05$), compared with 0.62±0.02 in WT mice ($p<0.05$ vs untreated). Body length, tail length, and body weight were improved in vector treated mice. The decrease in urinary cAMP was very significant in vector treated mice at 8 weeks ($P<0.0001$) and 16 weeks of age ($p<0.0002$). At 8 and 16 weeks of age, mean plasma PTH had increased only by 2.1 and 2.6 fold, respectively in vector treated mice, compared with 3.9 ±0.5 ($P<0.005$) and 6.2±1.4 fold in untreated *PRKAR1A*^{wt/mut} mice. PTH increase was comparable in treated *PRKAR1A*^{wt/mut} and WT mice (1.4 and 2 fold at 8 and 16 weeks, respectively). In conclusion, gene therapy with h*PRKAR1A* improved skeletal growth and kidney dysfunction, the hallmarks of acrodysostosis in R368X mutated mice and humans.

918. AAV-Mediated APOE Gene Silencing for Alzheimer's Disease

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Alzheimer's Disease (AD) is a multifactorial neurodegenerative disorder and the most common form of dementia in people over 65 years (~50 million), manifesting as progressive loss of memory and cognitive function. This is due to the extracellular accumulation of amyloid- β ($A\beta$) plaques and intracellular neurofibrillary tangles in the brain leading to neurodegeneration. Most cases of AD occur sporadically in people over 65 years old. The strongest genetic risk factor for late onset AD (LOAD) is a variant of the gene encoding Apolipoprotein E (APOE). This is a multifunctional lipoprotein expressed mainly in the liver and the brain and is involved in facilitating lipid transport and homeostasis. Three common variants of the APOE protein (E2, E3 and E4) result from different haplotypes at the *APOE* locus; $\epsilon 2$ (7.3% of the population), $\epsilon 3$ (79%), and $\epsilon 4$ (13.3%). The genotype-related changes of the structure of APOE variant proteins influence lipid and receptor binding abilities and are also associated with modulation of $A\beta$ aggregation and clearance and tau-mediated neuroinflammation. For example, the E4 variant is present in 45-60% of all AD cases and is associated with increased risk and decreased age of onset of AD, whereas E2 is associated with reduced risk and increased age of onset. The potential of protective variants was shown in preclinical studies in humanized (h) APOE-AD animal models in which a reduction of hAPOE4, as well as expression of hAPOE2,

could prevent neurodegeneration at various stages of AD pathology. uniQure is developing an AAV gene therapy product to treat patients with LOAD. Our approach is to silence toxic APOE variants and to overexpress a protective APOE variant. Therefore, we first have tested a series of miRNAs against APOE based on our miQURE™ platform. The miRNAs were screened *in vitro* for their ability to knockdown luciferase reporter constructs and endogenous APOE mRNA and protein in human-derived cells. The best candidates were selected for AAV production and the potency of APOE silencing was tested in a transgenic mouse model carrying hAPOE. In addition, we tested various protective APOE overexpression variants for transgene expression and secretability in human-derived cells. In conclusion, we report effective silencing of APOE by expressing miQUREs. These potent miQUREs could be implemented in a gene therapy approach for Alzheimer's disease knocking down toxic APOE and overexpressing an APOE protective variant individually or in combination.

919. Young Mice Administered Adult Doses of AAV5-hFVIII-SQ Achieve Therapeutic Factor VIII Expression into Adulthood

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Hemophilia A (HA) is an X-linked genetic bleeding disorder caused by a deficiency in the activity of coagulation factor VIII (FVIII). Individuals with severe deficiencies (i.e. <1 IU/dL) experience spontaneous bleeding internally into joints or muscles. Hemarthrosis typically develops before 2 years of age in children with severe HA and, if untreated, leads to recurrent bleeds with musculoskeletal deformity and loss of mobility. Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) gene transfer provided reduced bleeding for adult clinical trial participants with severe hemophilia A. However, pediatric clinical feasibility and outcomes are unknown. Previous preclinical research in neonatal mice and non-human primates with AAV vectors indicates robust liver cell proliferation early in life can prevent stable transgene expression. Using a mouse model of hemophilia A, we investigated the effect of vector dose on transgene production and persistence in neonatal vs adult mice. We dosed AAV5-hFVIII-SQ to neonatal and adult mice based on body weight or at a fixed dose and assessed human factor VIII SQ-variant (hFVIII-SQ) expression through 16 weeks. AAV5-hFVIII-SQ dosed per body weight in neonatal mice did not result in meaningful plasma hFVIII-SQ protein in adulthood. When treated with the same total vector genomes per mouse as adult mice, neonates maintained hFVIII-SQ expression into adulthood, though plasma levels were 3- to 4-fold lower vs mice dosed as adults (Figure 1). Treated neonatal mice initially exhibited high hFVIII-SQ plasma levels and maintained therapeutically meaningful levels into adulthood, despite a partial decline potentially due to age-related body mass and blood volume increases. Quantitative polymerase chain reaction (qPCR) showed neonatal mouse livers were capable of taking up the same amount of vector genomes as adults when

given the same absolute dose. The majority of total vector genomes in the liver disappeared over the first few weeks following infusion, as expected given typical AAV-vector processing kinetics; however, the slope of decline was similar between young and adult animals, despite liver growth in the neonatal cohort. In addition, the total number of hepatocytes that stained positive for hFVIII-SQ DNA remained stable in neonatal mice through adulthood, suggesting that hFVIII-SQ vector genomes are not lost due to hepatocyte mitosis in neonates. Rather, the in-situ hybridization staining intensity per cell decreased over time, suggesting that the degradation of vector genomes in each transduced hepatocyte contributed to the overall loss of vector genomes. Further, the kinetics of vector genome trafficking differed between neonatal and adult mice. hFVIII-SQ vector genome can be detected in hepatocyte nuclei of neonatal mice by 24 hours post AAV5-hFVIII-SQ treatment compared with a delay of 3 to 8 weeks for adult mice, which may explain the more rapid expression of hFVIII-SQ protein observed in the younger mice. No features of hepatotoxicity or endoplasmic reticulum stress were observed (Figure 2). These data suggest that young mice require the same total vector genomes as adult mice to sustain hFVIII-SQ plasma levels.

Figure 1: hFVIII-SQ plasma protein concentration in mice treated with AAV5-hFVIII-SQ as neonates vs adults

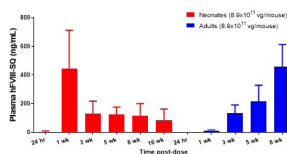
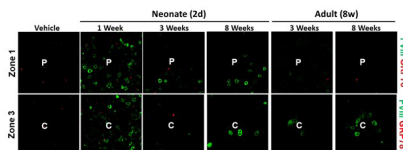


Figure 2: AAV5-hFVIII SQ does not induce ER stress in Neonates



920. Prevention of Cardiac Disease Features Observed in a Conditional Knockout Mouse Model of Friedreich's Ataxia Treated with a Novel AAV FXN Gene Therapy (AVB-202)

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Friedreich's ataxia (FA) is the most common form of hereditary ataxia. It is caused by autosomal recessive inheritance of GAA repeat expansions in the first intron of the frataxin (FXN) gene resulting in reduced levels of this mitochondrial protein. FA is a systemic disorder with hallmark features including cardiomyopathy (primary cause of mortality), gait ataxia, and sensory loss among other central nervous system manifestations. Gene replacement is a promising therapeutic modality for replacing FXN across disease-relevant tissues, thus preventing progression or reversing the course of disease. We performed a preclinical study to evaluate efficacy of AVB-202, a novel AAV9 gene therapy product expressing a codon optimized FXN

gene with endogenous 3' UTR, using a conditional knock out mouse model of FA that develops a cardiac phenotype and early mortality. Untreated animals exhibit abnormalities on echocardiogram as early as 6 weeks of age and mortality by 9-11 weeks of age. Neonatal animals were given a single intravenous injection of AVB-202 at varying doses. Regular physical exams and echocardiograms were performed to evaluate survival and cardiac function in animals up to 30 weeks of age. Treatment at various doses resulted in delayed weight loss, extended lifespan, and delayed onset of cardiac abnormalities. At 21 weeks of age, animals from four of five doses demonstrated echocardiogram results similar to healthy controls. Additional molecular analysis is ongoing to characterize recovery of frataxin protein levels and of mitochondrial function in heart tissue. Our findings demonstrate strong positive effects on survival and cardiac function in this FA model and support continued development of AVB-202.

921. Development of an *In Vitro* Potency Assay for Galactose-1-Phosphate Uridyltransferase (GALT) Enzymatic Activity in AAV-Transduced Cells

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Classic galactosemia is a rare, autosomal recessive genetic disorder caused by mutations in the galactose-1-phosphate uridylyltransferase (GALT)-encoding gene, with an incidence between 1/40,000 to 1/60,000 births. A GALT enzyme deficiency inhibits the metabolism of galactose and is associated with multiple clinical manifestations that present in childhood, especially in infants who often rely on milk (a precursor of glucose and galactose) as a sole source of nutrients. Despite early detection by newborn screening and current standard of care, a galactose-restricted diet, most patients grow to experience a broad constellation of long-term complications. A gene therapy vector carrying a functional *GALT* gene has the potential to restore GALT activity and help alleviate some of the most serious complications of the disease. As adeno-associated virus (AAV)-based gene therapy products progress from preclinical development to GMP manufacturing and clinical trials, a series of potency assays are required for drug lot release testing. Phase-appropriate validated potency assays are required from early to late phase clinical trials. As a candidate treatment for galactosemia, BBP-818 uses AAV serotype 9 as a vector for delivering a functional *GALT* gene to galactosemia patients to restore GALT activity. Here, we describe proof-of-concept development of an *in vitro* enzymatic activity assay for evaluation of BBP-818 potency via quantification of GALT activity in transduced HeLaRC32 cell lysates. The GALT enzyme catalyzes the reversible reaction between co-substrates galactose-1-phosphate (gal-1P) and glucose-uridine-diphosphate (glu-UDP), wherein the uridine-monophosphate group is transferred from glu-UDP to gal-1P to form co-products galactose-uridine-diphosphate (gal-UDP) and glucose-1-phosphate (glu-1P). An LC-MS/MS method was developed for the quantification of all substrates and products: gal-1P, glu-UDP, gal-UDP, and glu-1P. To measure GALT activity, modified substrates were utilized to overcome the issue of the endogenous GALT reaction resulting in substrates and products having the same molecular weight. Substrate concentrations

were optimized to saturate enzymatic velocity and several cell lines were screened for the appropriate background to conduct the assay. The accumulation rate of gal-UDP, normalized by total protein concentration, was used as the basis of calculating GALT activity rates in units of nmole-mg⁻¹·min⁻¹. Dose-dependent activity was measured in BBP-818-transduced cell lysates after 48hr of transduction, where a 2-fold increase of MOI correlated to an approximate 2-fold increase in GALT activity whereas no product formation was detected in non-transduced control cells. These early findings of dose-dependent GALT activity in transduced cells underlie the foundation of a potentially robust and qualifiable assay that can be used to ensure the potency and stability of manufactured lots of this promising gene therapy program.

922. Optimization of Intrathecal Delivery of an Infused AAV9 Vector for Delivery of a Gene Therapy Candidate for Adrenomyeloneuropathy (AMN) in Non-Human Primates (NHP)

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OBJECTIVE: Evaluation of intrathecal administration (IT) parameters of an adeno-associated virus serotype 9 (AAV9) to produce widespread gene transfer/biodistribution within the spinal cord (SC) and dorsal root ganglia (DRG), for gene therapies targeting the spinal cord. **BACKGROUND:** Adrenomyeloneuropathy is a form of X-linked Adrenoleukodystrophy caused by mutations in the gene encoding the ATP-Binding Cassette sub-family D member 1 (*hABCD1*), characterized by a dying-back axonopathy affecting SC tracts and ultimately leading to loss of mobility. We are developing SBT101, an AAV9-based gene therapy encoding a functional *hABCD1*, for use as a treatment for AMN. Prior research in mice indicates that 24-hour IT-Lumbar administration delivered the transgene to the entire SC while minimizing biodistribution to the periphery as compared with other routes of administration. We have therefore assessed multiple infusion parameters in NHPs to enhance widespread SC/DRG distribution following intrathecal delivery of a vector. **DESIGN/METHODS:** Preclinical studies were carried out to assess delivery of an AAV9 vector containing the deoxyribonucleic acid (DNA) sequence for a green fluorescent protein (GFP) reporter gene (AAV9-GFP) to further understand intrathecal delivery infusion parameters and the effect on biodistribution throughout the SC/DRG in NHPs. Animals received either a bolus (20 min) or extended (6 or 24-hour) infusion of AAV9-GFP at one of two sites (cervical or lumbar) utilizing doses of 1.1E13 vector genomes/animal (vg/an) to 3.4E13 vg/an as measured by droplet digital polymerase chain reaction (dd-PCR) (previously reported as 3.25E13 and 9.75E13 vg/an as measured by qPCR), with a total delivered volume between 2.5-10 mL followed by a 14-day in-life period. The biodistribution of the vector in tissues was analyzed by two independent approaches: immunohistochemistry (IHC) and quantitation of vector genome distribution by dd-PCR. **RESULTS:** The administration of AAV9-GFP via the IT route over 20 minutes through 24 hours resulted in a distribution of AAV9 vector genomes that was highest in liver, followed by cervical, lumbar, and thoracic SC+DRG at 2 weeks post-infusion. Distribution of AAV9-GFP in heart was minimal.

Intrathecal lumbar infusion over 24 hours delivered widespread biodistribution to the entire SC and DRG compared to either cervical or bolus delivery. Further it was found that a 6-hour lumbar infusion was equivalent to the 24-hour infusion for biodistribution to the SC+DRG, while total volume delivered did not significantly impact biodistribution. **CONCLUSIONS:** These results provide evidence that a 6-hour intrathecal lumbar infusion of an AAV9 vector can produce widespread biodistribution to the SC+DRG comparable to a 24-hour and potentially more extensive than bolus infusion, at doses predicted to be potentially clinically relevant in patients.

923. Comprehensive Particle Characterization of Adeno-Associated Virus Vector by Analytical Ultracentrifugation

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Adeno-associated virus (AAV) vectors are one of the most promising platforms for in vivo gene therapy. In the manufacturing of AAV vectors, the impurities such as empty particles (EPs) without DNA and intermediate particles (IPs) with fragmented DNA are often generated in addition to the full particles (FPs) containing the therapeutic gene. These impurities can compete with the FPs by interacting with receptors on the surface of the target cells, which not only reduces the therapeutic efficacy of the FPs, but can also cause immunogenicity. The aggregates of AAV vectors may also be one of the factors that reduce the therapeutic efficacy. Therefore, it is important to understand the particle characteristics of AAV vectors accurately during and after the manufacturing process, however the comprehensive particle characterization of the components of AAV vectors is not fully established. In this study, we developed the strategy for the characterization of the EPs, FPs, IPs and aggregates in AAV vector samples using sedimentation velocity analytical ultracentrifugation with multiwavelength detection method. The ultraviolet (UV) spectra of capsid protein and encapsulated DNA was obtained from the size distribution analyses using the multiwavelength detection data. The spectral deconvolution analysis using the UV spectrum data of capsid protein and encapsulated DNA enabled the determination of the nucleic acid-protein ratio of all components detected in SV-AUC. A comprehensive particle characterization method we have established here is applicable not only for AAV but also for other viral vectors.

924. Characterization of a Novel AAV Gene Replacement Therapy, VY-NPC101, In Vitro and In Vivo for the Treatment of Niemann-Pick Disease Type C1

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Niemann-Pick Disease Type C1 (NPC1) is a rare, autosomal recessive, neuro-visceral disease caused by a loss of function (LOF) mutation in the NPC1 gene. These mutations result in a non-functional NPC1 protein, which causes abnormal cholesterol and sphingolipid metabolism in the periphery and CNS, and eventual disease. Gene replacement therapy of a functional NPC1 protein has the potential of being the first treatment to change the disease progression for patients battling this terminal disease. However, as the functional NPC1 gene is 3.8Kb, the AAV maximum packaging capacity of approximately 4.8 Kb leaves little room for genetic control elements. Therefore, we designed a potent NPC1 transgene following a comprehensive evaluation of multiple promoters and transgene enhancement features that could be vectorized and delivered via AAV. When testing within multiple disease relevant human cell lines, we uncovered multiple enhancements that resulted in robust transgene expression while remaining within the maximum packaging capacity. We also confirmed that levels of exogenous NPC1 correlated with improved cholesterol metabolism in patient derived cell lines. Evaluation of several of these optimized transgenes in wildtype mice using AAV9 transduction showed robust and persistent NPC1 expression within the CNS and the periphery at 4-, 8-, 12- and 26-weeks following dosing. We then tested our lead constructs in a rodent efficacy model that carried a LOF mutation in NPC1 and measured key disease-associated phenotypes, including peak body weight and survival. Robust improvements in these efficacy readouts were observed and a minimal efficacious dose was determined. These results led to the identification of VY-NPC101. In summary, the rational design of VY-NPC101, and validation in a NPC1 disease-relevant cells lines and an NPC1 mouse model support its continued development and translation into the clinic.

925. Para-Retinal Administration of AAV204, a Novel AAV Capsid for the Treatment of Inherited Retinal Dystrophies, in Non-Human Primates

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The use of viral vectors has revolutionized the treatment of previously intractable inherited diseases. Inherited retinal dystrophies represent a unique opportunity for therapeutic development due to the eye's relatively small size and immune privileged status, and the availability of non-invasive imaging methods. There are currently, more than 20 active clinical trials using adeno-associated viruses to deliver a therapeutic transgene for treating ocular diseases. Most active INDs use natural serotypes to package their transgenes, with AAV2, 5 and 8 being the most common. Novel capsids developed through either rational design or directed evolution make up a small percentage of currently in-clinic drugs. The development of novel AAV capsids has expanded over the past decade due to several considerations, including improved cell or tissue targeting/detargeting, ability to evade immunity to naturally occurring serotypes, and improved manufacturing and isolation. Historically, AAV administration to the eye has been performed by subretinal injection between the neural retina and underlying retinal pigmented epithelium. While this method provides the benefit of positioning the vector directly next to its cellular target, it requires a retinal detachment, an operating room, and a trained retinal surgeon. A therapy that is administered directly into the vitreous of the eye could

provide a safer and more feasible approach. Here, we characterize a novel AAV capsid that facilitates robust transduction and resulting gene expression in both the inner and outer retina after intravitreal administration in mice and non-human primates (NHPs). Recently, clinical trials using intravitreally administered AAV drugs have resulted in unacceptable levels of inflammation, sometimes resulting in vision loss. A novel method for ocular delivery, para-retinal administration, is performed by layering the virus on top of the retina between the vitreous and the inner limiting membrane, thus not creating a subretinal detachment. The manuscript describing this method used AAV8 and showed expression in the macula but did not show scanning laser ophthalmoscopy (SLO) images due to the use of a non-reporter transgene construct. We administered AAV204.CBh.GFP or AAV8.CBh.GFP to four NHP eyes per vector by para-retinal injection at a total dose of 1×10^{11} vg/eye. SLO imaging of AAV204.CBh.GFP-dosed eyes 28 days post-injection showed intense GFP expression in the macula, the papillomacular bundle, and retinal nerve fibers. Immunohistochemistry analysis confirmed SLO images with high levels of GFP expression in the macula and foveal pit, as well as retinal ganglion cells and the associated retinal nerve fibers extending to the optic nerve. Alternatively, AAV8.CBh.GFP-injected animals showed very little to no GFP expression in the macula or optic nerve. The dose used in this experiment is at least one log lower than those currently being used with other intravitreally injected AAVs. Despite being slightly more invasive than a standard intravitreal injection, with the appropriate capsid para-retinal injection could prove to be an efficient route of administration for drugs that target either the macular or optic nerve/retinal ganglion cell layer.

926. Abstract Withdrawn

927. Correction of the I22I Inversion Mutation of the Factor 8 Gene in Hemophilia A

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Hemophilia A is an X-linked bleeding disorder caused by a lack of functional clotting Factor VIII (FVIII). It is treated by regular infusions of recombinant FVIII. Approximately half of all hemophilia A patients have a large inversion at intron 22 that results in the relocation of exons 23 to 26 to upstream of the FVIII promoter. This inversion is commonly referred to as I22I and results in the production of a truncated FVIII protein that is deficient for clot formation. Current AAV-based gene therapies for hemophilia A deliver episomal copies of a modified FVIII gene to mature, differentiated liver cells. However, given cell turnover over the lifetime of the patient coupled with the episomal nature of therapeutic AAV genomes, the long-term durability of this approach is unknown. Gene editing offers the potential to permanently correct genetic mutations at the genome level such that all progeny cells would inherit the correction. Here we developed a gene editing strategy to precisely insert exons 23 to 26 of the FVIII gene downstream of exon 22 to restore expression of the full length FVIII protein using AAVHSC editing vectors. To facilitate evaluation of correction, the editing vectors additionally inserted a FLAG tag at the C-terminus of FVIII and a promoterless mCherry expression cassette. Flow cytometric analyses showed an editing efficiency of approximately 15-25% based

upon mCherry expression. Targeted integration analysis followed by Sanger sequencing confirmed precise and seamless insertion of exons 23-26 at the end of exon 22 in both primary human liver sinusoidal endothelial cells (LSEC) and hemophilia A patient-derived I22I patient B-lymphoblastoid cells (B-LCL). No AAV inverted terminal repeats or insertion or deletion mutations were ever observed. Western blot evaluation revealed that AAVHSC edited wild type LSECs showed overexpression of the full length FVIII protein as well as the FVIII light chain. Restoration of expression of full-length FLAG-tagged FVIII protein and the FVIII light chain was observed in AAVHSC-edited but not unedited hemophilia A patient-derived I22I B-LCLs. Our results demonstrate that targeted insertion of exons 23 to 26 at the end of exon 22 corrects the inversion mutation responsible for approximately half of all hemophilia A cases. Thus, our genome editing strategy may form the basis for a permanent curative therapy for I22I hemophilia A.

928. Scalable Production of Recombinant Adeno-Associated Virus in Different Single-Use Bioreactor Platforms

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Background and novelty Viral vectors are increasingly used for their applications in gene therapies, with recombinant adeno-associated virus (rAAV) as one of the most used vectors. One significant challenge in the industry is to establish large-scale production processes, and a trend is to move away from adherent cell culture and towards suspension cell culture approach. Here, we describe a scalable, robust process for production of rAAV using suspension cells in single-use bioreactors. **Experimental approach** We have utilized a design of experiments (DoE) methodology to optimize rAAV production in HEK293T suspension cells. To verify the performance of our process, batches of rAAV5 using Xcellerex™ XDR-10 and WAVE™ 25 bioreactors were run in the 10 to 20 L scale. We describe the production process — from cell inoculation and expansion in single-use bioreactors — to transfection and harvest. Analysis of harvest material included qPCR for viral genomes (VG)/L and ELISA for viral particles (VP)/L. The percentage of full capsids was calculated as a ratio between VG/L by VP/L. **Results and discussion** Data from the rAAV5 production batches shows that we reached our criteria for harvest material, with viral particles of 10^{14} VP/L, viral genomes of 10^{13} VG/L, and a percentage of full capsids of more than 10%. The consistency between batches indicates that we have generated a robust, reproducible production process that can be utilized with rocking-motion bioreactor systems as well as stirred-tank bioreactors. We also present data for production of rAAV serotypes 2, 8, and 9. The same method was applied using both shake flasks, as well as the XDR-10 and WAVE™ 25 bioreactors, where we achieved similar titers as for rAAV5. Our results show a scalable, robust, and reproducible rAAV5 production process in the single-use XDR-10 and WAVE™ 25 bioreactors, with enhanced viral titer productivity and percentage of full viral capsids. The process we describe is capable of use with natural or engineered serotypes with minor modifications.

929. Development and Scale-Up of a Transient AAV Production Process Using a Suspension-Adapted HEK293 Clone

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Adeno-associated virus (AAV) has become an increasingly popular viral vector for gene therapy. Currently, there are two approved AAV-based gene therapies and the number of clinical trials is steadily increasing. As with most viral vector therapies, their production is labor intensive and expensive due to the use of adherent cell culture production processes. For process intensification, the industry has begun to utilize bioreactors. Here, we highlight our development of a suspension based AAV upstream production process to provide a more efficient and cost-effective bioprocessing solution for large scale production.

930. Development of an *In Vitro* Model for the Evaluation of Adeno-Associated Virus Delivered Microdystrophin Transgenes

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Duchenne Muscular Dystrophy (Duchenne) gene therapy using recombinant Adeno-Associated Virus (rAAV) to deliver a truncated Dystrophin protein (μ Dys) has shown promise in recent clinical trials. However, developing a functional potency release assay for Duchenne has proven difficult and led to delays in proceeding directly from Phase I/II to III. Ideally, for Phase III and commercialization, the potency release assay is *in vitro*, quantitative, and shows that dystrophin has proper function. An important aspect to the function of dystrophin is that it forms a complex with various binding partners and is localized to the sarcolemma, which provides integrity to the muscle fibers and protects against contraction-induced damage. One of those binding partners is beta-dystroglycan (β -DG), an integral membrane protein. We sought to develop an *in vitro* assay that quantifies binding partner interactions as a method to assess dystrophin function. Additionally, assays that evaluate muscle health provide supporting evidence that restoration of the binding partners and the dystrophin-associated complex has a biologically relevant functional effect. To address this question, we first developed a Duchenne Muscular Dystrophy knock out (KO) muscle cell line (DMD^{KO}), which lacks dystrophin and exhibits several key phenotypes of dystrophic disease, including: 1) disassembly of the dystrophin-associated complex, 2) impaired myotube fusion and 3) aberrant release of cytosolic components such as Creatine Kinase (CK) under electrical stimulation. We next utilized three *in vitro* methods to assess the activity of our rAAV- μ Dys vectors: 1) an electro-chemiluminescent ELISA and 2) an immunohistochemical (In-Cell Western) assay, which both provide quantitative readouts of protein expression, such as μ Dys and β -DG; and 3) an *in vitro* model of muscle contraction and induced cellular stress utilizing electrical pulse stimulation (EPS).

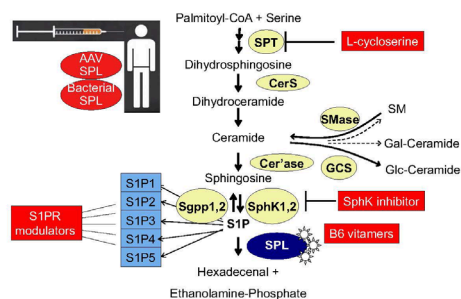
However, a current challenge to robust measures of μ Dys/ β -DG in these assays has been low transduction efficiency of myotubes by rAAV *in vitro*. To address this challenge, we screened several pharmacological agents to identify compounds that could enhance rAAV vector transduction and expression. These pharmacological agents were added to the differentiated DMD^{KO} cell culture prior to transduction, either individually or in combination, and μ Dys expression was measured by an *in vitro* MSD ELISA assay and an automated Western Blot system for high throughput screening. Cell toxicity was monitored visually using the Incucyte live cell imaging system. Daunorubicin or etoposide treatment increased μ Dys expression the most, up to 26-fold, while the other agents had only modest effects. These two compounds enhanced the delivery of rAAV- μ Dys to the DMD^{KO} cells, resulting in restored expression and re-assembly of dystrophin-binding partner β -DG at the cell membrane. This approach also provided protection against stimulation induced CK release, indicating improved membrane integrity and reduced susceptibility to induced cellular stress. In summary, we established *in vitro* methods to evaluate μ Dys activity, and identified potent enhancers for increasing *in vitro* transgene expression, enabling the future development of an *in vitro* functional potency release assay.

931. Adeno-Associated Virus Mediated SGPL1 Gene Therapy as a Treatment for Sphingosine Phosphate Lyase Insufficiency Syndrome (SPLIS)

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Sphingosine-1-phosphate lyase insufficiency syndrome (SPLIS) is a rare metabolic disorder caused by inactivating mutations in SGPL1, which is required for the final step of sphingolipid metabolism. SPLIS features include steroid-resistant nephrotic syndrome (SRNS) and impairment of neurological, endocrine, and hematopoietic systems. Many affected individuals die within the first two years. No targeted therapy for SPLIS is available. We hypothesized that SGPL1 gene replacement would address the root cause of SPLIS, thereby serving as a universal treatment for the condition. As proof of concept, we evaluated the efficacy of adeno-associated virus 9-mediated transfer of human SGPL1 (AAV-SPL) given to newborn Sgpl1 KO mice that model SPLIS and die in the first weeks of life. Treatment dramatically prolonged survival and prevented nephrosis, neurodevelopmental delay, anemia, and hypercholesterolemia. STAT3 pathway activation and elevated pro-inflammatory and fibrogenic cytokines observed in KO kidneys were attenuated by treatment. Plasma and tissue sphingolipids were reduced in treated compared to untreated KO pups. SGPL1 expression and activity were measurable for at least 40 weeks. In summary, early AAV-SPL treatment prevents nephrosis, lipidosis and neurological impairment in a mouse model of SPLIS. Our results suggest that SGPL1 gene replacement holds promise as a durable and universal targeted treatment for SPLIS.



932. Transcriptomic Disparities Between Male and Female Non-Human Primates Related to AAV Transduction Efficiency

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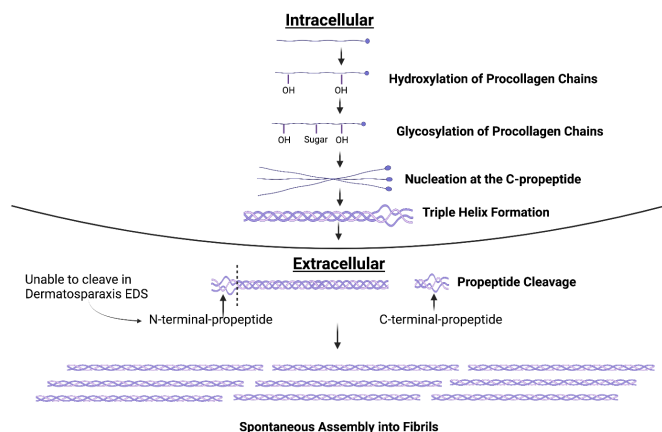
Adeno-associated virus (AAV) vector transduction efficiency and tissue tropism have been reported to vary in some animal models between males and females, yet relatively little has been published investigating the cause of these disparities. The clinical relevance of these observations has yet to be established; human disparities in AAV transduction dynamics would have significant implications for therapy designs and clinical trials. We have investigated AAV biodistribution and transgene expression levels in the central nervous system (CNS) of macaques (*Macaca fascicularis*) using 20 AAV capsid serotypes. Using barcode labeling and high-throughput sequencing technology, each vector was distinguished, and the data was analyzed for CNS AAV tropism using two different injection techniques. In this study, we performed a male to female comparison of transcriptomic data obtained from the temporal lobe, midbrain and cerebellar cDNA of cynomolgus macaques. We then compared this data to the AAV serotype expression data for these same brain regions comparing by gender. AAV biodistribution and gene expression for each individual serotype was cross analyzed to establish capsid-by-capsid differences. This non-human primate analysis provides insight for future studies investigating the clinical relevance of sexual dimorphism on AAV tropism and trans-gene expression in the setting of CNS delivery.

933. Development of Gene Therapy for Dermatosparaxis Ehlers-Danlos Syndrome

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Dermatosparaxis type Ehlers-Danlos syndrome (dEDS) is an autosomal recessive connective tissue disorder found in humans, cattle, sheep, dogs, cats, rabbits, and mink. The disorder is caused by a mutation in ADAMTS2 which encodes for procollagen I N-proteinase, the enzyme responsible for the extracellular cleavage of the N-terminal-propeptide of type I, II, III, and V procollagen. In dEDS, the N-propeptide is not cleaved from procollagen chains, resulting in collagen fibrils with aberrant morphology and ultimately, lower tensile strength (Figure 1). Humans with dEDS present with premature placental rupture of the membranes, severe skin fragility and redundant skin, hernias, craniofacial deformities, short stature, easy bleeding and bruising, and visceral fragility. To date, there is no definitive treatment for dEDS or any other type of EDS. This form of EDS is amendable to AAV 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) since ADAMTS2 is expressed ubiquitously throughout the body and the procollagen targeted by ADAMTS2 does not have any known function, we hypothesize that global secretion of ADAMTS2 will be efficacious in treating this form of EDS. A knockout ADAMTS2 mouse model will be used for therapeutic development. Following screening of AAV capsids (AAV9, AAVrh74, AAV8 and a proprietary capsid) for best stromal cell tropism, intravenous AAV-mediated gene therapy will be employed. We will test 3 AAV vectors encoding promoters of various strength. Three groups of *Adamts2*^{KO} (n=8) and control (n=8) mice will be used. Group 1 will be treated with AAV at birth, group 2 will be treated post-symptomatically at 2 months of age, and group 3 will not be treated. Two endpoints, 2 months and 1 year, will be employed for each group. Clinical signs, necropsy findings, transmission electron microscopy, biochemical electrophoretic analysis of procollagens, qPCR analysis of ADAMTS2 expression and vector genomes will be used to select the best vector. Following selection of this vector we will test the gene therapy strategy in the naturally occurring dog model to further inform on efficacy. This study represents the first gene therapy program for the spectrum of EDS disorders.



934. Determining Cell-Type Specific Distribution of AAV Variants in NHP Retina Using Single-Nuclei RNA Sequencing

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Machine-learning designed recombinant AAV (rAAV) variants are rapidly being developed to meet the growing demands for effective and targeted rAAV based gene delivery systems. Coupled with high throughput sequencing efforts, these endeavors are generating variants with greatly improved transduction efficiencies and tissue tropism. However, the ability to assess cell-type specific tropism of these variants has remained limited and often confined to extremely low throughput approaches. Single-cell RNA sequencing has been previously demonstrated to allow characterization of cell-type specific tropism of barcoded rAAVs (Brown et al., *Front. Immunol.*, 2021). However, obtaining single cell suspension from certain tissue types and/or flash frozen samples from externally-sourced non human primate (NHP) studies can be extremely challenging. We developed an approach that combines single-nuclei RNA sequencing (snRNA-Seq) with targeted amplicon sequencing to reliably detect cell-type specific transduction from up to 50-100 barcoded rAAVs with minimal sequencing depth, with an initial focus on tissues of the eye. To implement this approach we have: 1) developed optimized protocols for isolation of high quality single nuclei suspensions from flash frozen NHP retina, 2) used the 10x Genomics Chromium platform to encapsulate these nuclei and generate gene expression libraries for reliable identification of cell types, and 3) leveraged the 10x feature barcode kit to selectively amplify (for sequencing) barcoded viral transcripts that were captured using the 10x CS1 feature designed into the viral genomes. Using this approach we investigated cell-type specific tropism of multiple rAAVs in the NHP (*cynomolgus macaque*) retina. Our snRNA-seq gene expression analysis identified all the major retina cell types including therapeutically relevant cells such as Rods, Cones and Retinal ganglion cells. Viral transduction events, as assessed from our targeted library sequencing, were detected in almost all clusters and we could successfully quantify differences in transduction rates between rAAVs and benchmarks. Overall, we demonstrate that snRNA

sequencing can be used to both effectively determine cell-type specific tropism of barcoded rAAVs, and quantitate relative transduction between multiple rAAVs in a single experiment. These developments open up opportunities for further designing and validating rAAVs capable of cell-type specific targeting for gene therapy. Additionally, this approach enables a medium throughput identification of rAAVs with desired properties for further study.

935. Adeno-Associated Vector Discovery Platform for Inner Ear Disorders

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The World Health Organization estimates around 466 million people worldwide have hearing loss, and over 700 million people - or 1/10 people - will have disabling hearing loss by 2050. More than half of the congenital non-syndromic deafness cases have a genetic cause, and 80% are inherited in an autosomal recessive fashion. There are to date no approved curative therapies for genetic hearing loss, cochlear implantation remaining the only option proposed to patients. Even though this solution improves the quality of life and language acquisition, hearing recovery is limited and thus more targeted treatments are overall unmet medical needs. Adeno-associated virus (AAV) is a vector of choice for *in vivo* gene therapy. Many natural or synthetic serotypes are emerging, increasing the number of possible indications. The inner ear is a closed bony system that contains a small and non-expandable volume. It is a diverse mosaic of highly specialized cells including sensory hair cells (about 3,500 inner hair cells and 12,000 outer hair cells) but also supporting cells, and spiral ganglion neurons, all of which play an important role in the process of hearing. Each subpopulation of inner ear cells is associated with one or more genetic disorders and could therefore be selectively targeted by gene therapy. Then, the production of an AAV-based gene therapy product, especially for the inner ear, requires the development of novel tissue-targeted capsids and cell-specific expression cassettes aiming to reach a therapeutic effect using a minimal dose. Here we describe Sensorion AAV platform, allowing fast screening of therapeutic candidates for inner ear indications. First, we established an *in vitro* high throughput selection process allowing us to sort in a few weeks cell-specific capsids and regulatory elements among tens of candidates, developed from targeted *in silico* studies or existing repositioned libraries. Using an *ex vivo* approach, the platform uses cultures of rodent cochlear and vestibular explants, either infecting them with AAVs or electroporating them with plasmids of interest. The imaging acquisition has been automated for efficient data analysis and quantification, (3D and live imaging). Selected candidates are further validated by *in vivo* injection, either in rodents and non-human primates, into the inner ear using canalostomy, round window injection or specific inner ear gene delivery approaches. Afterward, inner ear functions are assessed via Sensorion audiology platform which includes auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) recordings.

Models for testing include genetic models, noise-induced hearing loss, synaptopathy, and vestibular disorders are supported by morphological and immunohistochemical examination. The gene therapy platform allows Sensorion to identify and validate novel AAV variants of suitable tropism for inner ear disorder therapies, as well as cell-type-specific expression cassettes of interest for the development of new therapeutic products to treat inner ear disorders.

936. Medium-Throughput Characterization of Engineered AAV Transduction Properties in NHP Retina

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Datasets of property measurements from highly diverse capsid libraries are a cornerstone of capsid engineering workflows. However, High Throughput (HT) libraries require pooled synthesis and production, introducing the potential for artifacts due to mutation and decoupling of genotype and phenotype. In addition, certain capsid properties cannot be measured in highly complex libraries, for example when downstream assays are unable to scale to the point of measuring any variant more than once. In capsid engineering workflows, Medium Throughput (MT) pooling and characterization approaches are a valuable compromise between the efficiency of HT libraries, and gold-standard, but resource-intensive, single capsid studies. We designed a MT characterization experiment to validate and expand upon HT bulk transduction property measurements through the use of orthogonal manufacturing methods and snRNA-seq. We focused our study on variants identified in HT datasets with improved transduction of the retina and trabecular meshwork. First, we selected variants from an ocular NHP HT dataset based on an algorithm that optimizes for capsid performance while balancing diversity and measurement uncertainty. Next, we synthesized 86 variants and benchmarks, and produced these separately with co-purification, balancing representation of individual variants to be within a defined range of abundance in the final vector preparations. Variants were paired with genomes bearing identifying barcode sets as well as diverse random sequence IDs for quantification of transduction events in bulk data. Pools were introduced by intravitreal and intracameral injection into *Cynomolgus* macaques. 4 weeks following injection, transduction was measured by bulk barcode-seq as well as snRNA-seq. The data enabled us to observe correlations between HT and MT property measurements, to compare transduction efficiency between bulk and single-cell measurements, and to determine transduction rates for high-performing variants across major cell types within the eye. In addition to identifying highly promising capsids for improved ocular gene therapy delivery, this high-resolution MT dataset provides validation of HT measurement quality and valuable input data for future machine-guided design directed at improving cell transduction, specificity and tissue distribution.

937. ICV Delivery of AAV9-NGLY1 Gene Replacement Therapy Shows Dose Dependent Improvement of Phenotypic and Biomarker Endpoints in *Ngly1* Deficient Rats

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GS-100 is an AAV9 gene replacement therapy that delivers a functional copy of the full-length human NGLY1 (N-glycanase) gene coding region to treat NGLY1 Deficiency. NGLY1 Deficiency is a devastating, ultra-rare, autosomal recessive disease caused by loss of function mutations in NGLY1. Patients suffer from global developmental delay, intellectual disability, a hyperkinetic movement disorder, elevated liver transaminases, (hypo)alacrimal, and peripheral neuropathy. No approved therapy for NGLY1 Deficiency currently exists. Previous studies of GS-100 in *Ngly1* deficient rats demonstrated GS-100 is efficacious when delivered using the intracerebroventricular (ICV) route of administration. To investigate GS-100 dose-dependent toxicity and treatment effect following ICV administration, a 13-week dose range finding study was performed in *Ngly1* deficient rats. We observed widespread biodistribution of human NGLY1 DNA that correlated with expression of NGLY1 mRNA and NGLY1 protein throughout the CNS. Treatment with GS-100 significantly reduced levels of the NGLY1 biomarker of disease, GNA (GlcNAc-Asn; aspartylglucosamine) in cerebrospinal fluid (CSF) and brain tissue in a dose-dependent manner within four weeks after dosing. The highest dose tested reduced GNA levels 38% in brain tissue compared to vehicle treated animals. GNA levels in the CSF significantly correlated with GNA levels in brain tissue, demonstrating that CSF GNA levels can serve as a surrogate pharmacodynamic marker of GS-100 activity in brain parenchyma in a clinical setting. GS-100 treated *Ngly1* deficient rats displayed a sustained dose-dependent trend in behavioral and locomotor improvements as early as 5 weeks, during a period where untreated rats continued to decline. Improvements in treated rats included increases in latency to fall in rotarod assessments and significant improvement in the rearing number during open field locomotor activity testing. A dose-dependent, inverse correlation was observed between behavioral/locomotor improvements and the GNA biomarker in CSF or CNS tissue. These correlations provide evidence for the use of CSF GNA as a predictive surrogate marker of clinical endpoint improvement in the clinical setting. GS-100 was well tolerated at all dose levels in the *Ngly1* deficient rats and no significant increases in liver transaminases were observed at any time point during the 13-week study. There were no macroscopic pathology findings attributable to GS-100. Histological changes in the brain were noted in close proximity to the needle track, but did not worsen over time and were not dose-dependent. GS-100 treatment did not have a negative impact on dorsal root ganglion (DRG) neuron viability. The biodistribution, expression, and GNA biomarker data suggest that delivery of GS-100 by ICV administration leads to expression of functional NGLY1 that can reverse molecular and behavioral NGLY1 Deficiency disease phenotypes. These data provide evidence that ICV delivery of GS-100 as a therapy for NGLY1 Deficiency is well tolerated

and highly effective in the rat model of the disease. These data will help determine the dose of GS-100 that may be safe and efficacious for human clinical trials.

938. Improving Efficiency of an AAV-CRISPR Platform for Lung Gene Editing

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Pediatric pulmonary disorders that are genetic in etiology are often detected early in childhood. Severe subsets of these monogenic lung diseases (MLDs) can lead to respiratory distress and death in the afflicted patient. Surfactant protein disorders are a group of MLDs that affect surfactant production in the distal lung, which is necessary to maintain lung surface tension and proper function. Surfactant protein B deficiency is an autosomal recessive disease that is often fatal within the first year of life. Current treatment relies on exogenous surfactant delivery until a lung transplant is performed, but this is often not successful, therefore novel therapeutic strategies are needed. Genetic intervention through gene delivery can treat the root cause of this disease and viral vector-based gene therapy is one method that can be applied to achieve targeted gene expression. Harnessing the innate infectivity of viruses for gene therapy requires safe, tissue tropic viral vectors with an absence of pathogenicity. A viable candidate that fulfills these requirements is adeno-associated virus (AAV). AAV-vectors can mediate long term transgene expression in post mitotic cells; however as cells turnover, transgene expression eventually wanes. This is further implicated in the developing environment of the pediatric lung, and therefore advances to the duration of gene therapy treatments must be considered. Permanent genome alterations through gene editing techniques, such as CRISPR-Cas9, can introduce genetic edits that are lineage lasting and when applied in combination with AAV cell targeted effects can be achieved. Nevertheless, the benefits possible from these gene edits are limited by the low efficacy, as seen in previous attempts with CRISPR-Cas9, to reach these permanent corrections in vivo. In this study we investigate the impact of age and dose of AAV on the integration efficiency of our AAV-CRISPR-Cas9 platform designed to edit cells in the murine lung. Here, repair and nuclease templates were packaged separately into the lung tropic AAV6.2FF capsid and administered intranasally to the lungs of C57BL/6 mice. GFP reporter gene expression, which is only detectable upon targeted gene integration, was quantified 3 weeks post vector administration. C57BL/6 mice were administered the dual vector platform at either 4, 10 or 20 weeks of age with a moderate (3e11 vg) or high dose (1e12 vg) of the repair template and a fixed dose of the Cas9-gRNA vector (1e11 vg). Control mice received repair template only or were left untreated. Gene insertion, measured as GFP expression, was quantified by flow cytometry after lung dissociation. The number of GFP positive lung epithelial cells ranged from 4-8% in the moderate dose group and approximately 12-20% in the high dose group suggesting

that increasing the amount of repair template increases integration efficiency. There was a trend toward reduced integration efficiency in older mice, with significantly less gene edited GFP positive lung epithelial cells in the 20-week-old mice compared to the 4-week-old mice, suggesting that gene editing efficiency is age dependent. These data provide important insights into the therapeutic window for this technology and repair template dosing requirements.

939. Effect of Age on Intrathecal AAV9 Transduction in Mice

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Adeno-Associated Viral (AAV) vector-mediated delivery of gene therapies has emerged as a promising way to treat neurological diseases. AAV9 is the most commonly used capsid to target the central nervous system due to its ability to cross the blood brain barrier and its high transduction efficiency of both neurons and glia. For high targeting of the brain, intrathecal (IT) delivery into the subarachnoid space is frequently used, with the least invasive route occurring via classic lumbar puncture between L4 and L5 of the spine. Currently, multiple clinical trials are approved using IT delivery of AAV9 for a range of inherited disorders (NCT02362438, NCT02725580, NCT04798235, NCT03381729, and NCT05152823) and many more treatments are in the preclinical phase of development. To better understand the distribution and transduction efficiency of IT lumbar puncture-delivered AAV9 at the preclinical stage, wild-type C57BL/6 mice were injected during development and as adults. We used a self-complementary AAV9 vector packaging a GFP reporter protein whose expression was driven by the ubiquitous CBh promoter. Mice were injected with a single bolus injection of AAV9/CBh-GFP at post-natal day 10 (P10), P14, P21, P42 or P84-88. The same volume and dose were delivered at each age point. All mice tolerated the injection, and no signs of overt toxicity were observed. Three weeks post injection, mice were euthanized, and brains were collected for analysis. GFP staining showed robust and widespread transduction throughout the brains of mice injected at P10. Notably, the number of GFP positive cells began decreasing when delivered even 4 days later at P14 and was substantially reduced in adult mice when delivered at P84-88. This indicates that in mice there is a rapid reduction in IT-delivered AAV9 brain transduction with development and aging. The loss of transduction efficiency may result from increased body weight and size during this development period but could also reflect changes in cellular factors, such as receptor expression. Interestingly, there was a markedly greater number of glial cells transduced at P10 as compared to P21. Elucidating the exact mechanisms involved in this age dependent phenomenon could open further supplemental targets to improve the delivery of AAV for neurological diseases and disorders. Immunohistochemical analyses are ongoing to fully characterize and quantify AAV9 brain transduction in the neonate to adult injected mice. This analysis includes cell type and regional differences. Whole body biodistribution is also ongoing to determine how AAV9 CSF escape and peripheral transduction changes with the age of delivery. Together, results from these studies support that IT AAV delivery is more efficient in younger mice as compared to older animals, with a marked decrease in transduction in adult mice. Furthermore, these results highlight the

careful consideration that should be taken by researchers for the age of delivery in preclinical AAV gene therapy studies in mice. A lack of consideration for the age of delivery could overcompensate or under appreciate a proposed AAV treatment. It remains to be determined if the age of larger animals significantly impact IT-injected AAV transduction efficiency as this may change how GLP-toxicology studies are designed for pediatric versus adult disorders.

940. Evaluation of Novel AAV Gene Replacement Therapy in Mouse Model of Friedreich's Ataxia

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Friedreich's Ataxia (FRDA) is an autosomal recessive disorder caused by a GAA trinucleotide repeat expansion which reduces expression of the mitochondrial protein frataxin (FXN). FRDA is characterized by cardiomyopathy, progressive neurodegeneration, glucose intolerance, and skeletal deformities. Currently, the only treatments available focus on symptom management. By utilizing a novel gene replacement approach with the ubiquitous and constitutive phosphoglycerate kinase (PGK) promoter, we successfully expressed the FXN transgene *in vitro* and *in vivo*. The mouse model, FA MCK, harbors a conditional FXN allele, a null FXN allele, and a transgene expressing cre-recombinase driven by a muscle creatine kinase (MCK)-specific promoter, resulting in the ablation of FXN expression in cardiac and skeletal muscle. FA MCK mice have progressive weight loss and functional cardiac deficits leading to morbidity by 10-12 weeks of age (WOA). In this study, FA MCK mice were given a single dose of an AAV serotype 8 (AAV8) vector containing the coding sequence of human (hFXN) or mouse frataxin (mFXN), administered at 1E14VG/kg or 3E13VG/kg respectively, via intravenous injection in the tail vein at 6 WOA. In addition to measuring FXN expression in the heart, other endpoints such as survival, body weight (BW) and echocardiograms were evaluated to assess the functional rescue of the FA MCK mouse phenotype. A significant difference in disease progression between control and treatment groups was observed, most notably a dramatic increase in BW and median survival (Figure 1) of FA MCK mice that received mFXN or hFXN vectors. Significant rescue was observed in the heart weight normalized by BW ($P < 0.0001$), and the ejection fraction ($P < 0.0003$) between FA MCK mice that received vehicle and mice that received mFXN or hFXN mice at the doses tested. Heart transgene FXN expression ranged from 3.7 to 23-fold greater in treated mice compared to WT mice, as detected by a quantitative ELISA method. Immunohistochemistry with HALO analysis confirmed FXN protein expression in the heart. The percentage of cells exhibiting FXN expression was similar in lower dose mice (58.64%) compared to WT (54.43%), and increased overall at the higher dose level, 1E14VG/kg (78.79%). Western blot analysis revealed expression of the mature form of FXN in cultured mouse cells and in tissues harvested from AAV-treated FA MCK mice. A myocardial damage marker, myosin light chain, was reduced in the serum ($P < 0.01$) of mice that received

AAV vector compared to vehicle-treated FA MCK mice. No toxicity or fibrosis in the hearts of FA MCK mice were observed 4 weeks after receiving the AAV FXN vector. Progression of the cardiac phenotype that FA MCK mice normally develop was prevented by AAV vector administration as evidenced by an increase in median survival and improved cardiac function. These results demonstrate the potential of AAV8 gene replacement therapy as a novel therapeutic for the treatment of FRDA.

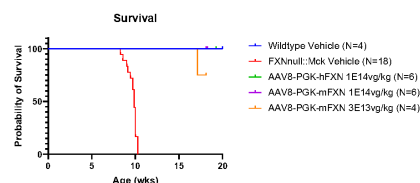


Figure 1: Probability of survival

Animals were dosed at 6 weeks of age. Survival data are represented as the age mice were euthanized if the mice displayed $>20\%$ BW decrease, signs of respiratory distress, unresponsiveness to meaningful stimuli, and/or overall poor body condition prior to scheduled necropsy. The probability of survival was analyzed in all treatment groups up to 20 weeks of age, with the probability of survival significantly improved ($P < 0.0001$) in all treatment groups.

941. Intrathecal Delivery of AAV9 Vectors Favors Peripheral Organs and Spinal Cord Over Brain Biodistribution Following Single Dose Administration in Young Cynomolgus Macaques

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AAV-based gene therapy holds tremendous potential to treat congenital or acquired disorders, especially the ones affecting the central nervous system (CNS). Intra-CSF AAV administration has the purported advantage of allowing direct access to the CNS by minimizing the off-target transduction of peripheral tissues that may result from systemic intravascular delivery route. Several groups have in the past reported that AAV9 delivery into the CSF leads to transduction of both neurons and glia cells throughout the CNS in non-human primates studies, thus touting this approach for moving forward into clinic gene therapies for myriad of complex brain diseases. In the context of advancing the clinical development of several of our gene therapy programs for brain diseases, we carried out a nonclinical study aimed at evaluating the biodistribution of self-complementary adeno-associated virus-9 (scAAV9)-chicken β -actin promoter-green fluorescent protein (GFP) in young cynomolgus macaques infused via lumbar puncture or intracisterna magna routes (1.0×10^{13} or 3.0×10^{13} vg/animal). Our study comparatively assessed CNS and peripheral organs biodistribution of vector genome, mRNA and GFP protein using various analytical tools such as ddPCR, RNAscope *in situ* hybridization and MSD ELISA as well as immunohistochemistry. There were no overt differences in vector genome biodistribution and GFP expression between lumbar

puncture or intracisterna magna administration locations. Overall, the vector genome biodistribution was widespread but variable and GFP expression greatest in the spinal cord, dorsal root ganglia (DRG), and select systemic tissues. By comparison, lesser concentrations were detected in brain regions. Transduction and expression in the brain tissue were observed primarily in perivascular astrocytes, with a paucity of transduction or expression in the neurons. Despite direct cerebrospinal fluid (CSF) administration, greater transduction and expression were detected in hepatocytes, striated myocytes (skeletal muscle), and cardiomyocytes. This data indicate that intrathecal delivery into the CSF favors vector transduction in the spinal cord, lower motor neurons, DRG neurons, and selected systemic tissues, over key brain structures, potentially targetable for neurologic diseases.

942. Identification of the Therapeutically Beneficial Intravenous Dose of AAVrh.10hFXN to Treat the Cardiac Manifestations of Friederich's Ataxia

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For *in vivo* adeno-associated virus (AAV) gene therapy to be successful, it is critical to identify a minimal effective clinical dose, setting the therapeutic goal for a human clinical trial. Friedreich's ataxia (FA), a life-threatening disorder for which there is no disease-modifying therapy, is characterized by neurologic and cardiac dysfunction. Most FA patients are homozygous for a GAA trinucleotide expansion within the first intron of the frataxin (FXN) gene, which causes transcriptional silencing, leading to a reduction in the levels of FXN mRNA and protein. While progressive neurologic disease limits mobility, cardiomyopathy is the cause of death in nearly two-thirds of individuals. From prior studies in experimental animals, it is known that an adeno-associated virus serotype rh.10 expressing human FXN (AAVrh.10hFXN) effectively treats the cardiac manifestations of the disease in murine models of FA, but there is a therapeutic window, with high doses of the hFXN transgene associated with toxicity. This leads to the question: what minimal dose will effectively treat the cardiac manifestations of FA? To answer this question, based on the knowledge that FA heterozygotes with one normal FXN allele have no clinical manifestations of FA, we first estimated the level of FXN necessary to convert the heart of an FA homozygote to that of a heterozygote. Analysis of the average FXN levels in autopsy samples of the hearts of 5 individuals demonstrated normal levels of FXN in the human heart are 59 ± 5 ng/mg protein. Based on the knowledge that FA heterozygotes have 30-80 % of normal levels (19-50 ng/mg), we estimated that, to be effective, an AAV vector would need to provide >9 ng/mg for effective therapy (heterozygote 19-50 ng/mg - FA endogenous 10 ng/mg = 9-40 ng/mg). To determine a dose of AAVrh.10hFXN necessary to produce >9 ng/mg in the heart, we administered intravenously 1.8×10^{11} , 5.7×10^{11} or 1.8×10^{12} gc/kg (qPCR determined) of AAVrh.10hFXN to 6-7 wk old MCK (creatinine

kinase *-/- fxn* deficient) mouse, a model of severe cardiac disease that completely lacks murine FXN protein in cardiac and skeletal muscle and shows progressive disease deterioration with death in untreated animals by week 11. Assessment by echocardiography demonstrated that a dose of 1.8×10^{12} gc/kg led to a beneficial outcome with significant improvement in ejection fraction and fractional shortening ($p < 0.05$, both comparisons) compared to untreated MCK mice. This dose mediated a 21.5 % improvement in mortality ($p < 0.001$ compared to untreated controls). A mild 4% improvement in mortality was seen with 5.7×10^{11} gc/kg dose, the minimal effective dose. To determine if the significantly effective dose of 1.8×10^{12} gc/kg could achieve heterozygote levels in a large animal model, this dose was administered intravenously to African Green nonhuman primates. After 12 weeks, the levels in the heart were 21.9 ± 4.9 ng/kg, comparable to levels in the range estimated necessary to convert the FA homozygote to an FA heterozygote. Together these data identify a therapeutically effective dose that has a potential to be clinically relevant for the treatment of the cardiac manifestations of Friedreich's ataxia.

943. The Effect of Prophylactic Corticosteroid Treatment on Adeno-Associated Virus-Mediated Gene Therapy and Potential Mechanisms of Action

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Adeno-associated virus (AAV)-based gene therapy can initiate host-immune responses to the AAV vector components or the transgenic protein product which could reduce AAV-mediated expression. Data from preclinical studies suggest that corticosteroid treatment may limit these immune responses and improve gene therapy outcomes. In an earlier study, prednisolone treatment initiated one week after valoctocogene roxaparvovec (AAV5-hFVIII-SQ) administration had no impact on FVIII expression in mice. In a subsequent study we investigated if corticosteroid treatment initiated before AAV administration could improve transgene expression in mice. Mice were dosed daily for 4 weeks with prednisolone (2 mg/kg) or water starting 1 day or 2 hours before administration of 6×10^{13} vg/kg of an AAV5-HLP-hA1AT vector expressing human $\alpha 1$ -antitrypsin (hA1AT) as a reporter. Prednisolone-treated mice had significantly higher serum hA1AT protein levels (1.5- to 2.2-fold) starting 6 weeks after AAV dosing and through study end at 12 weeks. Furthermore, mice treated with prednisolone had a higher percentage of hepatocytes stained positive for vector DNA as well as higher liver full-length vector genome DNA and transgene RNA compared to the non-prednisolone-treated groups. This demonstrates that corticosteroid treatment before AAV administration can improve liver-directed AAV5 expression in mice. To better understand potential mechanisms involved in this beneficial effect of prophylactic steroid treatment on AAV transduction we further investigated early events after AAV5 dosing in mice with or without prophylactic corticosteroid treatment. RNAseq analyses of liver samples were performed to evaluate differential expression 2 hours and 24 hours

following AAV5-HLP-hA1AT administration in mice treated with and without prophylactic prednisolone. These analyses showed that prophylactic steroid treatment reduces acute innate immune responses to AAV. For example, serum IL-1b protein levels were increased 2 hours and 24 hours after AAV administration which were suppressed with prednisolone treatment. Moreover, RNAseq showed an upregulation of the AAV5 cell surface co-receptor, PDGFRa, and downregulation of its competitive ligand PDGFa 2 hours after AAV administration in prednisolone treated mice compared to mice not treated with prednisolone. This suggests that prophylactic corticosteroid treatment may increase AAV5 capsid uptake by upregulation of the receptor and downregulation of the ligand. In summary, prophylactic corticosteroid treatment before AAV5 administration improved transgene expression potentially through multiple mechanisms, including suppression of the innate immune response and increased AAV uptake by cells.

AAV gene therapy products. However, the results of this study show that the immunosuppressants used in AAV gene therapy trials are commonly studied in ways that do not align with their existing FDA-approved USPIs/labels. Therefore, there are a number of important regulatory implications to be considered for implementing the use of immunosuppressants in combination with AAV gene therapy products. Most notably, if clinical evidence indicates that these immunosuppressants are required to be co-administered with the AAV gene therapy products, one major regulatory implication for pursuing marketing authorization for the gene therapy could be the potential for meeting the regulatory definition of a combination product, which may require consideration of additional factors to support a successful marketing application. Falling within this definition, however, may be dependent on the interpretation of the manner in which the immunosuppressant drugs are being utilized.

AAV Vectors - Clinical Studies

944. A Systematic Analysis and Interpretation of Regulatory Implications of Immunosuppressant Use in Combination with AAV Gene Therapy Products

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Introduction This work is intended to assess the increasing usage of immunosuppressant drugs in combination with AAV gene therapy products. The study offers particular consideration of the potential application and interpretation of established US regulations and regulatory guidance. Specifically, this study analyzes the usage of immunosuppressant drugs in AAV gene therapy development and outlines potential regulatory implications that sponsors may need to consider for utilizing these products in combination during clinical trials as well as in support of marketing applications. **Methods** A systematic review of public information was conducted to quantitate and classify immunosuppressant use in US AAV gene therapy clinical trials over a 10-year period. The review analyzes the use of various immunosuppressant drugs and subclassifies with respect to: a) combined use with various AAV capsids, b) utilization over time, and c) usage in various stages of clinical development. A sub-analysis of the Phase 3/pivotal studies incorporating one or more immunosuppressant drug was conducted to determine the prescribed duration of immunosuppressant therapy, dose(s), as well as the defined populations of patients and systematically compares these points against the most recent FDA-approved label(s), i.e., USPI(s), for the respective immunosuppressant(s). Diversions from the FDA's prescribing information were evaluated according to relevant FDA guidance documents, regulations, and recent public statements in order to characterize the regulatory implications for immunosuppressant use in combination with AAV gene therapy products. **Results and Conclusions** With increasing evidence that immunomodulation may improve the safety profile and/or durability of response for AAV gene therapy products, there has been an increasing trend for sponsors to incorporate prophylactic or therapeutic immunosuppression into various stages of clinical development for

Gene Targeting and Gene Correction III

945. Abstract Withdrawn

946. Genome Orthogonal Zinc Finger Proteins for the Development of Genomic Medicines

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Zinc finger proteins (ZFPs) are the most abundant DNA-binding proteins found in the human genome and can be engineered to precisely target desired genomic sequences. Fusing a ZFP to an effector domain of a transcription factor (ZF-Transcription Factors, ZF-TFs) enables up- or down-regulation of a target gene. ZF-TFs hold great promise as genomic medicines due to their potency, tunability and compact size which allows for packaging into AAV for delivery. A key requirement for effective genomic medicines is specificity - the ability to regulate a target gene without perturbing other genes. Transcriptional profiling methods such as microarray platforms and RNAseq are powerful techniques used to measure transcriptome wide ZF-TF specificity. Specificity is measured by comparing ZF-TF treated samples to a control ZF-TF which binds at a defined alternative genomic locus. A designed genome orthogonal ZF-TF is a preferable control for specificity analysis. To identify ZFPs that do not bind to pre-clinically or clinically relevant genomes, we designed a panel of engineered ZFPs to specifically target DNA sequences several mismatched bases away from any sequence found in the mouse, two non-human primate species or human reference genomes. The engineered ZFPs were screened for DNA binding by ELISA with potent binding to their intended target sites observed for 60% of the designed ZFPs. Using the Affymetrix microarray platform, we measured the transcriptome-wide specificity of a panel of ZFPs fused to the KRAB repressor domain from the human transcription factor ZNF10 in human fibroblasts, and found a subset of ZF-TFs with minimal to no off-target transcriptional activity. Their specificity was further evaluated in mouse primary neurons and human iPSC-derived neurons transduced with AAVs encoding the

engineered ZF-TFs. Minimal to no dysregulation of any genes in the AAV-transduced neurons was observed by microarray and RNAseq, further confirming the specificity of these genome orthogonal ZF-TFs. These proteins will be valuable controls for measuring ZF-TF specificity and may also find use in other genome engineering applications.

947. A New Destabilizing Domain for Gene Therapy Enables Tighter Control of Protein Abundance

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Current gene therapy methods primarily rely on the delivery of a constitutively expressed transgene to a tissue of interest. While this approach may be appropriate for certain loss-of-function diseases, the potential for cellular negative feedback loops and transgene phenotoxicity limit the utility of this strategy. As a result, the need for conditionally regulated and reversible transgene expression may be a key factor for revolutionizing current gene therapy methods. Destabilizing domains (DDs) are inherently unstable, small molecule-regulatable protein domains that are a promising chemical biology tool for controlling protein abundance and potentially in gene therapy applications. DDs can be fused to a therapeutic protein of interest, thereby allowing the ability to turn its stability 'on/off' with the addition or removal of a small molecule ligand. A commonly used DD is derived from *E. coli* dihydrofolate reductase (ecDHFR), which binds to the pharmacological ligand and commonly used antibiotic, trimethoprim (TMP) for stabilization. While promising, the efficiency with which the non-stabilized conventional ecDHFR DD is degraded by the proteasome remains a significant roadblock that must be addressed before utilization in gene therapy. In this study, we addressed this issue by developing a new ecDHFR DD with enhanced basal degradation capabilities and more widespread applicability. Beginning with the wild-type ecDHFR DNA template, we generated a random library of ~1200 new mutants fused to YFP with a mutagenesis rate of ~8-10 mutations/kb. After introducing this library into CHO cells, we used flow cytometry to select for clones that demonstrated no YFP fluorescence under basal conditions, but strong fluorescence after TMP addition. In the process, we identified 6 new and unique variants that are degraded significantly more efficiently than the original ecDHFR DD. Moreover, these new variants were stabilized by TMP to similar degrees as the conventional ecDHFR DD. Within this new group, a particular ecDHFR variant designated as 'C12' exhibited the largest increase in basal protein turnover, piquing our interest as the most promising new ecDHFR DD for downstream applications. We have observed that the C12 ecDHFR variant is applicable as both an N or C terminal DD, and undergoes higher basal turnover at lower culturing temperatures (30°C) than other conventionally used ecDHFR DDs. We have also demonstrated its usability *in vivo*, in which mouse eyes injected with C12.ecDHFR.YFP AAV showed strong fluorescence after mice were fed TMP water. These improved features of the C12 ecDHFR variant highlight its potential for increasing the application of destabilizing domains, not only as a chemical biology tool but for gene therapy avenues as well. Currently, we are working on investigating biochemical features of the C12 ecDHFR DD that allows for its enhanced degradation in comparison to the conventional ecDHFR DD,

as well as using the C12 ecDHFR DD to control transcription factors whose careful regulation, rather than constitutive overexpression, is optimal for homeostasis.

948. Split dCas9 AAV for Knockdown of HTT

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Huntington's disease (HD) is a rare, autosomal dominant neurodegenerative disorder caused by a trinucleotide expansion in exon 1 of the Huntingtin gene (HTT), which leads to neuronal dysfunction and cell death. Healthy HTT is implicated in axonal trafficking and trophic factor regulation, making targeted allele-specific reduction important. This study aimed to target single nucleotide polymorphisms (SNPs) found in HD patient-derived cells and transgenic animal models to selectively reduce expression of mutant HTT using CRISPR epigenome editing. Heterozygous SNPs near regulatory regions of the HTT promoter were confirmed in HD patient cells, allowing for the design of allele-specific gRNAs. gRNA screen was initially conducted in patient-derived fibroblasts, followed by assessment in iPSC-derived neural stem cells. Knockdown was induced through targeting multiple loci in the HTT gene and significant downregulation was achieved using several of our gRNAs that were designed to be allele-specific. We also assessed HTT knockdown using different effector domains, including those that induce histone methylation as well as DNA methylation. We saw significant downregulation of HTT in both patient-derived fibroblasts as well as iPSC-derived neural stem cells. The delivery of large proteins into the CNS continues to be a barrier to success when using these gene-editing tools. By using a split dCas9 AAV system, large proteins such as dCas9 can be split into two domains fused to inteins that are able to recombine and create a full-length Cas9 in target cells. Previously established gRNAs targeting HTT were cloned into the split dCas9 AAV vector system containing dxCas9 fused to KRAB. These AAV were injected into the striatum of YAC128 mice that harbor the full-length human HTT locus. Knockdown of HTT was assessed via qPCR, western blot, and immunohistochemistry (IHC). We also conducted behavioral assays which included rotor rod and open field to assess functional recovery of the mouse models after treatment. These studies support the potential of targeted allele-specific strategies paired with split dCas9 AAV delivery for CNS disorders.

949. Achieving CRISPR/dCas13b Site-Directed RNA Editing of Pathogenic Adenosine Nucleotides in iPSC-Derived Neuronal Cells

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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 achieves site-directed RNA editing when fused to the ADAR2 deaminase domain (ADAR2DD). A guide RNA (gRNA) sequence forms a dsRNA substrate for dCas13b-ADAR2DD binding at the complementary target while enabling RNA editing at a single base. However, the CRISPR Cas13 system has yet to be employed for RNA editing of G-to-A mutations in neuronal cells or multiple pathogenic adenosine variants within the same transcript. Our lab created induced pluripotent stem cell (iPSC) from fibroblasts of individuals with pathogenic G-to-A mutations in exon 5 (E198K) of protein phosphatase 2, regulatory subunit B δ (*PPP2R5D*) which is 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-moderate off-target editing in the *PPP2R5D* transcript. We demonstrate gRNA length as an important factor in on-target editing efficiency. We have developed neuronal models to understand the consequence of G-to-A mutations on *PPP2R5D* targets at the phosphoproteomic and transcriptomic levels by differentiating isogenic and E198K variant NSCs into midbrain and forebrain neurons. 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 while providing an alternative editing technology for neurodevelopmental disorders.

950. Find and Cut-and-Transfer (FiCAT) Mammalian Genome Engineering

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The gene editing toolbox has expanded over the last few years and it opened an exciting novel therapeutic landscape. Gene editing is based on engineered endonucleases that induce a double-strand break (DSB) into the genome sequence of interest. Cells repair the DSB through one of two major pathways: Non-Homologous End-Joining (NHEJ) or Homology Directed Repair (HDR). Recently editing independent on DSB has been developed, taking advantage of the site specific binding machinery Cas9 in fusion with a protein, such as deaminases in base editors or reverse transcriptase in prime editors, responsible for different DNA modifications. While multiple technologies for small allele genome editing exist, robust technologies for targeted integration of large DNA fragments in mammalian genomes are still missing. Here we develop a gene writing tool (FiCAT) combining the precision of a CRISPR-Cas9 (find module), and the payload transfer efficiency of an engineered piggyBac transposase (cut-and-transfer module). FiCAT combines the functionality of Cas9 DNA scanning,

targeting and cleaving DNA, with piggyBac donor DNA processing and transfer capacity. PiggyBac functional domains are engineered providing increased on-target integration while reducing off-target events. We demonstrate efficient delivery and programmable insertion of small and large payloads in cellulo (human (Hek293T, K-562) and mouse (C2C12)) reaching up to 22% of programmable DNA insertion. We compare FiCAT with other means of doing programmable integration of full CDS encoding DNA by either HDR or Homology-Independent- Targeted-Insertion (HITI), and we showed significant increase in efficiency of FiCAT especially for large payloads. We also demonstrate FiCAT performance with a variety of Cas proteins from different Classes. Finally, we evolve more efficient versions of FiCAT by generating a targeted diversity of 394,000 variants and undergoing 4 rounds of evolution. In this work, we develop a precise and efficient targeted insertion of multi kilobase DNA fragments in mammalian genomes.

951. Characterization and Deployment In Vivo of FiCAT Genome Writer

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Find Cut-and-Transfer (FiCAT) is a robust gene writing platform, combining the precision functionality of Cas9 DNA scanning, targeting and cleaving 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 mouse models liver using different transduction reagents via systemic intravenous injection. JetPEI transduction reagent and hydrodynamic injection allow us to efficiently deliver FiCAT to mice liver targeting Rosa26 genomic safe harbor, together with a reporter transposon in DNA form. Stable transduction efficiency was confirmed by qPCR analysis of the inserted transgene and at 4-5 weeks after injection in order to remove the episomal signal. However, these reagents compromise the safety and enhance the immune response in mice pointing out the need for the emergence of an alternative way of delivery. Lipid nanoparticles (LNPs) have been extensively validated both pre-clinically and clinically for the delivery of different nucleic acid cargos. We first validate the on target cutting capacity of our technology in traffic light reporter (TLR) cell lines. Once optimized FiCAT mRNA-based LNP formulation, specific cut capacity in mice liver was analyzed by targeted NGS. In this work, we were able to successfully deliver mRNA cargos via LNPs in vivo, and optimize DNA cargos delivery for codelivery with FiCAT machinery; for enabling gene writing in vivo. Our work validate the feasibility of doing viral free stable gene transfer in vivo; which is one of the challenges in our desire of deliver safely and efficiently therapeutic gene into the target cells and achieve precise and efficient in vivo targeted insertion of DNA fragments in mammalian genomes.

952. Enabling the Engineering of iPSC Based Cell Therapies Using MAD7, a Novel CRISPR Nuclease

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CRISPR nucleases have dramatically improved the process of precise genome engineering of mammalian cells, allowing for targeted gene deletions and insertions at high efficiencies. Although these advancements initially impacted the basic research community, they are now rapidly being applied to therapeutics, especially in engineered cell therapy programs utilizing primary cells and induced pluripotent stem cells (iPSCs). MAD7 is a class 2, type V CRISPR nuclease that utilizes a short gRNA without the requirement of a tracrRNA and utilizes a PAM site that is thymidine rich. Although MAD7 and Cpf1 share similar activity and structure, they only possess around 30% sequence identity. We have developed a production process and biophysical analysis assays to produce and characterize recombinant MAD7 for use in ribonucleoproteins (RNPs) in our iPSC gene editing platform. Our process yields a protein that is homogenous and monomeric in solution after formulation. In addition, protein stability is maintained at -80°C for 6 months as measured by biophysical and functional characterization. The activity of recombinantly produced MAD7 is equivalent to Cpf1 with regard to knock-outs (KO) and homology directed repair (HDR) efficiencies at multiple loci in iPSCs. We have generated and tested multiple gRNAs targeting different sites in the genome and demonstrated that MAD7 does not induce any structural anomalies as determined by orthogonal genetic characterization assays. The data indicate that MAD7 CRISPR nuclease can be efficiently expressed in a recombinant expression system, purified and formulated to enable robust and precise engineering of mammalian cells as a ribonucleoprotein (RNP). We are currently using our MAD7 optimized process to generate MAD7 RNPs to enable the genetic engineering of iPSC-derived NK and T cell product candidates with multiple gene edits.

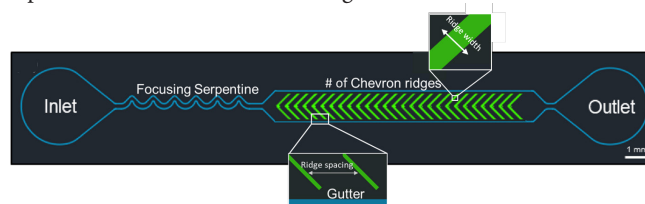
953. Novel Microfluidic Delivery Device for CRISPR Cas9 Editing of T Cells for Adoptive Cellular Therapy

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CRISPR/Cas9 genome editing has revolutionized the field of genetic engineering. However, the current delivery approach of editing molecules via electroporation can negatively affect the viability of the target cells. This can be challenging in the CRISPR/Cas9 editing of primary T cells used in adoptive cellular therapies, especially when multiple genome edits are required. Here, we describe a novel microfluidic platform, called volume exchange for cell transfection (VECT) mechanoporation, which is a non-viral, biomechanical approach that enables efficient delivery of genome editing products

into the cell interior. It has the potential to permit multiple CRISPR edits with high transfection efficiency and viability, while being gentle enough to avoid detrimental damage to therapeutic cells. VECT mechanoporation has shown low damage to the nucleus of T cells and therefore, offers a route to produce more proliferative therapeutic T cells. As proof of concept, we utilized this technology to develop chimeric antigen receptor (CAR) T-cells against T-cell malignancies. CAR T-cell manufacturing has been difficult in T-cell disease due to the lack of a cancer specific T-cell antigen, resulting in fratricide among T-cell antigen directed CAR T-cells. Editing of the target antigen enables the development of fratricide-resistant CAR T cells; furthermore, disruption of the T-cell receptor alpha chain (*TRAC*) locus allows for creating an 'off-the-shelf' allogeneic CAR T-cell product. In this report, we applied a VECT mechanoporation device to deliver cas9/gRNA complexes targeting CD5, a pan T-cell antigen, and the *TRAC* locus into Jurkat T cells and primary T cells. Based on the mechanics of the cell volume exchange phenomenon, we designed and tested VECT devices with different ridge widths, number of ridges, and ridge spacings (Fig. 1) to optimize its performance for gene editing in Jurkat T and 24-hr stimulated primary T cells. We achieved high delivery efficiency comparable to standard electroporation (58% vs 57% delivered population) and reached over 50% CD5 knockout in Jurkat T cell line. In a proof-of-concept experiment in primary human T cells, we achieved >30% CD5 and TCR knockout in primary T cells. Most importantly, cells processed by microfluidic devices maintained high viability (90%) and unaltered proliferation rate (30-fold growth over 5 days) in Jurkat and primary T cells, while cells processed by electroporation showed reduced viability (50-60%) even after 5 days and impaired proliferation (5-fold growth). The impact of the two methods on primary T cell memory was assessed using expression of CCR7 and CD45RA, and result indicated similar population composition of stem memory T cells compared to the negative control, suggesting that mechanoporation had minimal negative impact on memory. To summarize, we showed an alternative delivery method for safe and robust multiplexed genetic engineering of primary T cells. This method preserves T cell viability and proliferative ability compared to another clinically-relevant Cas9 delivery methods. Our result is directly translatable to adoptive cellular therapy development and will result in improved CAR T cell manufacturing for T-cell disease.



954. CRISPR-Editing of Macrophages for Muscle Regeneration in Volumetric Muscle Loss Injuries

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Massive muscle loss places a huge burden on the health care system as well as the individual who must re-learn to walk with partial to

majority muscle loss. Volumetric muscle loss (VML) is loss of at least 20% of a muscle's mass and can occur from high impact car accidents or battle-injuries in military personnel. This injury often leads to fibrotic scarring and impaired muscle function. Recently, the role of immune cells in muscle healing has been a focus of regenerative muscle research, finding that macrophages are necessary for muscle regeneration. Macrophages provide two roles in muscle healing, first as a pro-inflammatory state and second as a pro-regenerative state. When skeletal muscle maintains a prolonged pro-inflammatory states, the regenerative process fails, causing fibrotic scarring and loss of function. Macrophages are the main modulator in muscle regeneration and have a broad range of applications in regenerative medicine. The ability to manipulate macrophages for specific applications will prove invaluable in the regenerative space. We propose that editing macrophages towards a pro-regenerative state post-injury will improve muscle regeneration and improve major muscle loss injury outcomes. One issue with attempting to edit macrophages is their response to foreign objects. We will determine the appropriate delivery vehicle for macrophages that reduces inflammatory response and optimizes transfection efficiency. To determine the most efficient delivery vector, we will use a TNF- α ELISA assay to detect macrophage inflammation and flow cytometry to determine transfection efficiency in RAW264.7s. We will test both viral and non-viral delivery methods. For viral delivery, we will test AAV, adenovirus, and lentivirus and for non-viral methods, we will use lipid-nanoparticles. Using CRISPR activation and CRISPR inhibition, we will modulate gene expression in macrophages to promote muscle regeneration. In stimulated macrophages, we will use dCas9-KRAB to down-regulate pro-inflammatory cytokines, namely IL-10 and NF κ B. In the future, we plan to determine an effective gene target for a pro-regenerative state through a CRISPRa genome-wide library screen in the RAW264.7 cell line using the epigenetic modifier VPR. We also plan to move toward editing human iPSC-derived macrophages. We believe that immunomodulatory therapy using gene-edited macrophages will improve muscle regeneration in major injuries.

955. Sickle-HUDEP2 BFP Reporter Cell Model to Monitor HBG Regulation for Treating β -Hemoglobinopathies

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Introduction: β -hemoglobinopathies, including sickle cell disease (SCD) and β -thalassemia, are inherited blood disorders caused by mutations in the β -globin gene (*HBB*), which result in abnormal adult hemoglobin (HbA). Fetal hemoglobin (HbF) is the major hemoglobin in the fetus, which is suppressed after birth. Currently, there are clinical and pre-clinical studies on CRISPR/Cas9 based gene-editing approaches to induce HbF for therapeutic purposes as elevated HbF was shown to reduce morbidity and mortality among patients with β -hemoglobinopathies. However, studies on the relationship between genetic changes and HbF induction have been limited since conventional flow cytometry method for analyzing intracellular HbF levels requires fixation of live cells and laborious sample preparation, and significant cell loss may occur during washing steps. Here, we aim to establish a fluorescent reporter cell model that enables monitoring HbF expression in a living cell to investigate mechanisms of HbF induction by various CRISPR/Cas9-based gene editing strategies.

Materials and Methods: Sickle-Human Umbilical cord Derived Erythroid Progenitor 2 (S-HUDEP2) is an immortalized CD34⁺ hematopoietic stem and progenitor cell line with a sickle mutation in the *HBB* gene. We knocked in the mTagBFP2 gene at the C-terminus of *HBG1/2* by delivering HiFi *SpyCas9/gRNA* targeting the *HBG* stop codon and a double-stranded DNA donor P2A-mTagBFP2-polyA linked by P2A flanked by homology arms. Bulk-edited cells were sorted based on the BFP protein expression to establish a mixed population of S-HUDEP2 expressing BFP and *HBG* under the *HBG* promoter. We confirmed targeted integration of mTagBFP2 by in and out PCR followed by Sanger sequencing. Bulk sorted BFP⁺ cells were erythroid differentiated and analyzed with flow cytometry to demonstrate the correlation of increased BFP expression and HbF production levels with the expected increase in HBG expression throughout erythroid differentiation. We quantified the changes in BFP and HBG expression due to gene editing by a gRNA targeting the *HBG* promoter that has proven to induce robust HbF production in HUDEP-2. We confirmed the increase in HbF expression by staining HbF with FITC-labeled antibody. All experiments were conducted with biological replicates. We confirmed precise in-frame targeted integration of mTagBFP2 in sorted BFP⁺ cells by in and out PCR across the expected knock-in junction followed by Sanger sequencing. **Result and discussion:** We found that unedited S-HUDEP2 had 10.08% HbF(FITC)⁺ cells, however in sorted BFP⁺ S-HUDEP2s the HbF⁺ cells increased to 61.40 \pm 1.33%. After expansion, we confirmed that 56.13 \pm 0.11% of BFP⁺ in sorted BFP⁺ S-HUDEP2s. 97.04 \pm 0.76% of BFP⁺ cells had co-expression of HbF and BFP. We found that 9.54 \pm 1.05% of sorted BFP⁺ S-HUDEP2s were BFP-FITC⁺ which requires further investigation. After gene disruption of the *HBG* promoter, the percentage of HbF(FITC)⁺ cells increased to 88.53 \pm 0.86%. Interestingly, the percentage of BFP⁺ cells decreased to 24.25 \pm 0.77%. We hypothesis that this decrease is due to an intergenic 5kb large deletion between *HBG1* and *HBG2* induced by gRNA targeting the promoter of both *HBG1* and *HBG2* simultaneously due to the high sequence homology between *HBG1* and *HBG2*. We will validate this hypothesis by sorting BFP⁺ cells and quantifying the rate of intergenic deletion. We will also establish single-cell clones with confirmed genotype that shows a distinct increase in mean fluorescence intensity (MFI) along with HbF induction. **Conclusion:** We demonstrated that the engineered S-HUDEP2 cell mode with BFP at C-terminus of *HBG* can be used to monitor *HBG* promoter activity. Further development of this cell model will provide a useful tool for quantifying HbF induction in living cells by flow cytometry, thus aiding the mechanistic studies of *HBG* regulation, and genetic screening to discover new *HBG* regulators.

956. Development of a Long-Read Sequencing Workflow to Characterize the Precision and Efficacy of Gene Editing in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is an X-linked recessive monogenic disorder, which makes it an excellent candidate for gene editing applications. It affects 1 in 5000 males and the life expectancy is 26 years of age. The principal symptom associated with DMD is severe muscle weakness, which ultimately leads to cardiomyopathy

and compromised respiratory function. CRISPR-mediated genome editing is sustained for over one year in mouse models of DMD and partial restoration of muscle function has been observed. There are important safety considerations that remain unanswered and present as barriers to clinical translation, including the elicited immune response and CRISPR-induced genetic variants. The aim of this work was to develop a workflow to assess genome editing outcomes, using long-read sequencing, at the genomic DNA and transcript levels. A reliable method is needed to assess the unintended on-target and off-target modifications that have been observed. These aberrant events include multi-exon skipping and viral fusions that have been observed by targeted RNA sequencing. Diverse RNA isoforms are difficult to detect with short-read sequencing, and thus cDNA analysis using a reliable long-read sequencing workflow will also give insight into unexpected editing outcomes. In this study, NIH3T3 and C2C12 cell lines were transfected with dual-guide RNAs for targeted deletion of exon 23. A protocol was developed to analyze on-target and off-target editing efficiencies using long-read sequencing (Oxford Nanopore). A 10 kilobase gDNA amplicon and 3 kilobase cDNA amplicon was sequenced and exon 23 deletion was detected as the most prevalent editing event. However, several kilobase deletions were also detected as well as well as cryptic splicing events, pseudoexons, alternate splice donors, and a duplication. Mouse samples, treated with the dual-guide system are also being sequenced in order to further optimize variant characterization using long-read sequencing. We anticipate being able to detect vector integration events in addition to many of the variants detected *in vitro*. The next step of this work will include exploring amplification-free enrichment methods that allows for sequencing of larger amplicons and free of intrinsic biases associated with PCR. Future steps will also include increasing the number of samples that are sequenced to verify the repeatable nature of the unintended editing events that were observed.

957. Efficient Immune Cell Editing with Engineered CRISPR-Cas12i for *Ex Vivo* Cell Therapy

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CRISPR RNA-guided nucleases have gained considerable interest for their role in revolutionizing existing *ex vivo* approaches to engineered cell therapies, but the toolbox of gene editing systems for the generation of such therapies remains relatively limited. Here we present a novel engineered Type V-I CRISPR system (Cas12i), ABR-001, as an alternative to the widely used Cas9 and Cpf1 CRISPR nuclease systems for gene edited cell medicines. ABR-001 is the result of a high-throughput evaluation of engineered variants of Cas12i and yields dramatically increased (~40-fold) activity over the wildtype Cas12i system. Rigorous characterization of ABR-001 revealed therapeutically meaningful attributes including compact size (<1100 amino acid protein, tracr-less guide RNA), high fidelity, and distinct editing outcomes. To evaluate the potential of ABR-001 for *ex vivo* cell therapies, we delivered RNPs targeting therapeutically relevant genes,

such as *B2M* and *TRAC*, to primary human T cells and CD34+ HSPCs, and observed robust editing activity (>80% indels) across multiple targets and donors. The large deletion pattern of ABR-001 suggests that ABR-001 may be uniquely positioned to disrupt cis-regulatory elements. To this end, we used ABR-001 to disrupt a region of the intronic *BCL11A* enhancer in CD34+ HSPCs and observed induction of fetal hemoglobin *in vitro* and in *in vivo* mouse engraftment studies. Taken together, these data suggest that ABR-001 produces robust and distinct editing outcomes, which uniquely differentiates its capabilities to generate complex engineered cell therapies.

958. OMG Assembly: A New Technique Enabling High-Throughput Manufacture of Complex, Repetitive or AT-Rich DNA Sequences for Gene Therapy Applications

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DNA synthesis - a core part of synthetic biology - has revolutionized the speed and reach of not only basic molecular biology research, but also gene therapy and even personalised DNA therapeutics. However, current gene synthesis techniques cannot manufacture highly repetitive, AT-rich or otherwise complex DNA sequence - despite these being a common feature in the many disease associated genes and enhancer regions that are of particular interest for gene therapy applications. Furthermore, gene therapy vectors themselves contain complex motifs complicating their manufacture, including long terminal repeats (LTRs) in viral vectors and AT rich S/MAR sequences within episomally maintained DNA vectors. OMG Assembly is a new method developed for the rapid, efficient synthesis of such complex, repetitive Or AT-rich DNA Sequences. As a proof of concept we manufactured the ApoB S/MAR sequence that has recently been shown to enhance the episomal maintenance of therapeutic DNA vectors (Bozza et al 2021). The 916bp ApoB S/MAR sequence is highly repetitive, containing a central domain of 34 repeats of a 10bp motif separated by alternating linkers, and has an average AT richness exceeding 80%. As a result of this complexity the ApoB S/MAR is not available from commercial suppliers, and is unstable when amplified from the Human genome by PCR. Using an early beta version of OMG Assembly we were able to generate the complex ApoB S/MAR sequence correctly in 93% of tested clones. We have gone on to improve the OMG Assembly process, and have manufactured thousands of DNA sequences of varying complexity, including high-throughput production of TCR, BCR and CAR sequences relevant for personalised medicine. In conclusion we have demonstrated that OMG Assembly enables the easy manufacture of complex repetitive DNA sequences for gene therapy applications, whether present within gene delivery vectors (viral LTRs, AT rich S/MARs), disease genes containing repeat domains (such as polyglutamate repeat disorders), gene editing vectors (tandem sgRNA arrays), or - in a basic research setting - gene expression reporter constructs containing repeated transcription factor binding sites.

959. Gene Editing on Primary and Stem Cells Using Lentiflash RNA Delivery Approach/ Key Safety Considerations for Gene Therapy Clinical Applications

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Genome editing tools (GETs) hold great potential for the care of many diseases including cancer, auto-immune disorders, or genetic diseases. Somatic therapy (*in vivo* GETs delivery) and cell therapy (*ex vivo* GETs modified cells) are really promising. Nevertheless, their safety and efficacy are still being explored by many researchers and in clinical trials. Various risks must be minimized for successful clinical application. Especially, the extensive use of GETs in research has revealed much unpredictability, both off and on target, in the outcome of their application. Absence of deleterious genomic alterations must thus be evaluated. Others risks like the impact of the transduction method, vector toxicity, immunogenicity or long-term or high expression of the GET are also to be reduced. The mode of delivery is one of the main factors that can be tackled to reduce these risks but also provide efficient cells targeting. For example, with adenovirus or AAV viruses, insertion of viral sequences may occur in time. Critically, these delivery systems may lead to long-term expression of the GET and therefore increase risk of genomic alterations. Compared to DNA delivered-therapies, RNA therapies are expected to be more versatile, cover a broad range of applications with minimal regulatory concerns and thus address a large variety of diseases. RNA technologies enable to reduce the risk of genomic alterations because it is devoid of any recombination events in the host genome. As a game-changing RNA carrier, LentiFlash[®], a non-integrative bacteriophage-lentivirus chimera with the same cell specificity as a classical lentivirus, can efficiently and safely deliver multiple RNA species that are transiently expressed into the cell cytoplasm. Here, we show the potential of LentiFlash[®] particles for gene therapy clinical applications. We delivered CRISPR-Cas9 components with this method in Human primary and stem cells. Our results showed that LentiFlash[®] particles provided efficient gene editing without observing toxicity, without affecting cell phenotype nor stem cell differentiation capacity, and with a very low impact on genotoxicity. These properties, and the ability to easily produce LentiFlash[®] particles using existing lentiviral production platforms, that are already validated for clinical trials (GMP grade), provide a very promising method for safe and efficient therapy in Humans. It offers additional safety considerations compared to other therapeutic approaches.

960. An RNA Switch-Regulated Single AAV-CRISPR/Cas System for In Vivo Inducible Gene Editing Therapy

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Inducible CRISPR/Cas systems for temporally controlled genome engineering have a wide range of scientific and medical applications. In a recent study, we developed a highly efficient self-cleaving ribozyme-based RNA switch for *in vivo* regulation of therapeutic transgene expression (Zhong G, et al. *Nature Biotechnology*. 2020). The exceptional regulatory range and protein-independent nature of this switch system makes feasible precise *in vivo* control of an AAV-delivered transgene. Here, by leveraging the ability of Cpf1/Cas12a to process its own CRISPR RNA from Pol-II transcripts (Zhong G, et al. *Nature Chemical Biology*. 2017) and built on the basis of our ribozyme-based RNA switch, we've engineered a multilayer-regulated inducible CRISPR/Cas system that can be delivered by a single AAV vector (manuscript in preparation). We demonstrated in cell culture that the system had minimal editing activity before induction and can be efficiently induced by a steric-blocking antisense oligonucleotide inducer. Using a single AAV vector to deliver the entire system that encodes a Cas protein, its CRISPR RNAs, as well as the switch elements, we achieved efficient and temporally controlled gene editing activity *in vivo*. The system we developed here has the potential to improve the safety, as well as efficacy, of AAV-delivered gene-editing therapies. We are currently testing in mouse models for the application of this highly compact inducible CRISPR/Cas system to treating a fatal genetic disease, Duchenne Muscular Dystrophy.

961. Engineering B Cells to Express Antibodies with Modified Fc Functions

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Recent advances in genome editing at the immunoglobulin (Ig) locus are allowing B cells to be re-programmed to express custom antibodies which retain essential features of natural antibody production. These include the regulated expression of both an antigen-responsive cell surface B cell receptor (BCR), as well as the secreted antibody isoform. Given the complex heavy (H) plus light (L) chain design of antibodies, most Ig editing approaches rely on the site-specific insertion of a cassette comprising both a complete L chain and the variable (VH) region of the H chain. Insertion of this cassette upstream of the remaining constant regions of the H chain results in expression of both antibodies and BCRs with the programmed specificity. However, the constant (Fc) regions of such engineered antibodies retain the sequence of the endogenous locus. We have previously reported an alternate strategy to engineering the Ig locus, that mimics the design of the H chain only antibodies from Camelids. By targeting CRISPR/Cas9 editing to introns in the constant

region of IgG1, we are able to insert new antigen-specific domains that become spliced to the remaining Fc exons of the locus and thereby result in a custom H chain only antibody. Importantly, insertion of the cassette downstream of the CH1 exon excludes the domain that pairs with an L chain partner, preventing the formation of heterologous H plus L pairings and allowing H chain only antibodies to be secreted. In a further innovation, we have now identified editing strategies that also allow us to incorporate changes to the Fc domain of the antibody, opening up the possibility of enhancing antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC). These novel Fc editing strategies required considerable optimization, including identification of appropriate target sites, manipulation of the homology donors to appropriately direct site-specific insertions, and other customizations to ensure the desired splicing events. Our editing protocols took advantage of conditions we had previously optimized for B cells using CRISPR/Cas9 RNPs and AAV6 homology donors. Studies are currently underway to evaluate the impact of including such ADCC-enhancing mutations in H chain only antibodies.

962. *getools* Enables Ultra-Fast and Accurate Quantification of Gene-Editing Outcomes with Both Bulk and Single-Cell Data from Genome Editing Experiments

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The gene-editing systems as represented by CRISPR/Cas and their derivatives enable genome-wide targeted editing in humans and a wide variety of other organisms by either DNA or RNA level modulation. The expanding usage of gene editing in gene and cell therapy brings hopes and choices for patients suffering from serious diseases like genetic disorders and tumors while quantifying the on- and off-target editing outcomes and profiling the mixed complex mutational spectrum *in vitro* and *in vivo*, in bulk or even at the single-cell level, at high accuracy and sensitivity becomes an urgent need for both industry and clinical studies. However, the vast diverse mutations with variable frequency plus multiple-target gene-editing makes systematical enumerating the editome and cataloging rare but relevant co-occurring events and monitoring the single-cell level clonal dynamics very challenging in the real world, especially for the interpretation of the noisy amplicon deep sequencing data. Despite of many efforts, the existing pipelines and analytic tools are either defective in processing high depth multiplexed amplicon data or do not support single-cell data analysis. To address these challenging issues and attempt to standardize the gene-editing data analysis, we have developed *getools*, a native integrated gene-editing data analysis software toolkit for determining editing efficiency and cataloging outcomes at 'per-read' level from next-generation sequencing data. *getools* recognizes and splits typically pooled libraries and single-cell libraries by both primer match and sequence alignment based on a novel reading/processing framework, and enables fast and error-tolerant in-place processing of pooled data from thousands of samples each with hundreds of targets. *getools* improves in pair-end data merge, using kmers as well as overlap strategy with a precomputed optimal minimal overlap region length for each amplicon, to prohibit false-positive insertion brought by greedy sliding in repeat regions as adopted by FLASH. Both merged read pairs and unmerged reads are firstly deduplicated respectively to minimize the memory

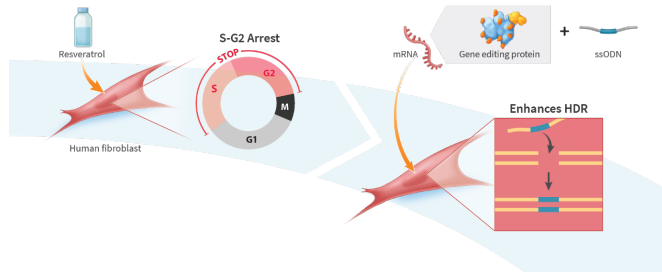
consumption and downstream computation, and then aligned to the known reference with optimized Smith-Waterman local alignment algorithm with a customized scoring strategy to make cleavage site aware. The alignments are further filtered to remove amplification and sequencing artifacts with unsupervised clustering strategy using the alignment score and variant count, while hard filters could also be applied according to the sequence count and allele frequency threshold. *getools* calculates mutation frequency and outcomes for all types of edit events, including snv, insertion, deletion, deletion-insertions (delins), separately or by any combination, in single sample mode or paired with a supplied control sample. *getools* is implemented as a high-performance C/C++ standalone software for localized deployment without any data security concerns, and provides industry-level bam, bcf, json, tsv output plus vivid JavaScript-based interactive HTML report with processing details and summary metrics for whole cycle data tracing. As benchmarked with several public datasets of Cas9 and Cas12a editing, *getools* outperforms other known pipelines in accuracy and speed, with generally 6 to 30X faster than CRISPResso2, and 10 to 70X faster than ampliCan, demonstrating the power of *getools* as a robust and efficient tool in analyzing gene editing data.

963. Resveratrol Treatment Increases Homology-Directed Repair in Primary Human Cells

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Gene editing technology, which enables the precision modification of DNA in living cells, is being developed for the treatment of various diseases, including genetic diseases and cancer. Gene editing commonly employs sequence-specific endonucleases to create double strand breaks in genomic DNA, and relies on the cell's DNA repair mechanisms to apply the desired changes. Precise sequence modifications, such as single-base changes, rely on the homology directed repair (HDR) mechanism. Despite its essential role in gene repair, HDR occurs at a very low frequency in many cells compared to other repair mechanisms. Here, we evaluate the impact of resveratrol, a small molecule extracted from grape skin that has recently been shown to promote the expression of key HDR factors and induce cell cycle arrest at S phase in porcine fetal fibroblasts, on single-base editing efficiency in primary human fibroblasts. Following treatment with resveratrol, fibroblasts were co-transfected with mRNA encoding a chromatin context-sensitive gene-editing protein targeting the AAVS1 safe-harbor locus and a single-stranded DNA repair template designed to introduce a SfoI restriction-enzyme site through a G-to-C mutation. Single-base-editing efficiency was determined by restriction fragment length polymorphism (RFLP) analysis. Resveratrol treatment prior to transfection increased the S and G2-phase population 2.3-fold and increased HDR efficiency 2-fold compared to untreated cells. Application of resveratrol after transfection (i.e. no cell cycle synchronization) yielded further improvement in single-base editing efficiency (> 2-fold), suggesting that the effects of resveratrol on HDR are not confined to cell-cycle control. Resveratrol treatment provides a straightforward method for improving HDR efficiency in primary human fibroblasts, and may serve as a useful tool in the development of HDR-based gene-editing therapies.



964. PACE Modifications Enhance the Stability and Editing Yields of Guide RNAs for Cas9 Editors

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CRISPR gene editing and control systems continue to emerge and inspire novel clinical applications. Advances in CRISPR performance such as optimizing the duration of activity in cells, tissues, and organisms, as well as limiting off-target activities while maximizing on-target activities have been extremely important for expanding the utility of CRISPR-based systems. Initial studies demonstrated that chemical modifications in guide RNAs such as 2'-O-methyl (M), 2'-O-methyl-3'-phosphorothioate (MS), or 2'-O-methyl-3'-thiophosphonoacetate (2'-O-methyl-3'-thioPACE, or MSP) placed at positions determined to be compatible with guide RNA activity significantly enhance CRISPR activity in cells.¹ Indeed, since this demonstration, gRNAs with multiple MS modifications at the 5' and 3' ends have become commonly used in the field. By further investigating the effects of various chemical modifications in guide RNAs at defined positions and combinations we demonstrate that 2'-O-methyl-3'-PACE (MP) modifications can be substantially more effective than 2'-O-methyl-3'-phosphorothioate (MS) modifications at the 3' ends of sgRNAs to promote high editing yields, in some instances showing an order of magnitude higher editing yield in human cells. MP-modified 3' ends are especially effective at promoting the activity of guide RNAs co-transfected with mRNA encoding the Cas enzyme, as the gRNA must persist in cells until the Cas protein is expressed. Our results demonstrate performance advantages for using MP compared to MS to modify the 3' ends of sgRNAs for creating indels using Cas9 and for editing targeted nucleotides using a BE4 cytidine base editor. We also demonstrate that MP at the 3' ends of prime editing guide RNAs (pegRNAs) co-transfected with PE2 mRNA can promote maximal prime editing yields. To simulate harsher cellular environments that CRISPR-Cas components may encounter when delivered *in vivo* (as by nanocarriers or other cell-penetrating formulations), we co-transfected Cas9 mRNA with sgRNA into cells that were isolated from culture media but not rinsed with PBS to remove residual serum. In the presence of serum,

sgRNAs with MP-modified 3' ends show marked improvements in editing efficiency over sgRNAs with MS-modified 3' ends delivered with Cas9 mRNA and are also effective at enhancing the activity of transfected ribonucleoprotein (RNP) complexes. Given the importance of high editing yields in many therapeutic settings, whether cells are edited *ex vivo* or *in vivo*, our results suggest that MP should be considered as a performance enhancing modification for the 3' ends of synthetic gRNAs, especially in situations where the guide RNAs may be susceptible to exonuclease-mediated degradation.

¹ Hendel, A., Bak, R. O., Clark, J. T., Kennedy, A. B., Ryan, D. E., Roy, S., Steinfeld, I., Lunstad, B. D., Kaiser, R. J., Wilkens, A. B., Bacchetta, R., Tsalenko, A., Dellinger, D., Bruhn, L., and Porteus, M. H. (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.* 33, 985-989.

965. Enhanced Cas9 Activity with a Human Cell-Based Directed Evolution Platform

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Engineered variants such as Cas9-NG (Nishimasu et al. 2018) and SpRY (Walton et al. 2020) have greatly expanded the targeting range of Cas9 by recognition of alternate protospacer adjacent motifs (PAMs). However, their genome editing activity in mammalian cells remains lower than wild-type Cas9 at mutually accessible targets, suggesting an opportunity for general improvement. Here we describe a human cell-based directed evolution platform to discover Cas9 variants with expanded targeting ranges and improved activities. We transduced human U2OS cells with a library of Cas9 variants and targeted *HPRT1* to confer resistance to purine analogue 6-thioguanine (6-TG). We found that targeting an *HPRT1* site with a canonical *NGG* PAM enriched for active Cas9 variants more than 100-fold when the input of active Cas9 is 1%. With variant libraries of Cas9 targeting *HPRT1* sites at non-canonical PAMs such as *NTT* and *NGA*, we observed activity 25-fold higher in cells surviving 6-TG selection. After sequencing Cas9 variants in surviving human cells, we cloned several promising candidates to test for PAM specificity in a high-throughput PAM specificity assay. One variant we identified significantly improved editing of targets with alternate *NAN*, *NCN*, *NGN*, and *NTN* PAMs compared to SpRY by 34%, 23%, 25%, and 26% respectively. We envision that iterative directed evolution in a human-cell based context may enable further discovery of variants with substantially improved cellular activities.

966. Genome Editing in Human Haematopoietic Stem Cells for the Treatment of X-Linked Agammaglobulinemia

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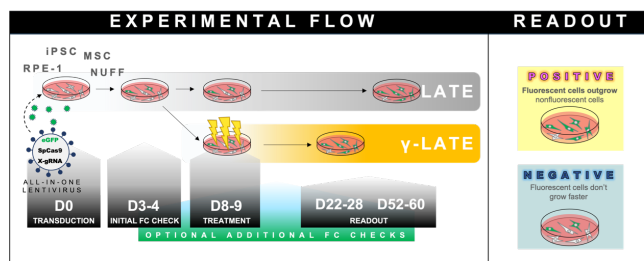
In recent years, CRISPR gene editing techniques have improved, and human trials of this technology have been reported in cell-based cancer therapies and haemoglobinopathies. These techniques also have the potential to rescue genetic defects in monogenic immunodeficiencies through correction and transplantation of autologous haematopoietic stem cells. Here we trial a gene-editing technique on stem cells from patients with X-Linked Agammaglobulinemia (XLA). This disease is caused by mutations in Bruton's Tyrosine Kinase (BTK) which causes a cessation of B-Cell development and an absolute antibody deficiency. XLA patients are less able to fight pathogens and therefore they acquire frequent and severe infections. Conventional treatment with costly Immunoglobulin Replacement Therapy (IRT) involves regular intravenous infusions and is not available in some developing countries. Despite IRT, severe and chronic breakthrough infections result. Although conventional gene therapy has been shown to restore B cell development in a mouse model, the risk of insertional mutagenesis and dysregulated expression of the transgene remain. Our gene editing strategy mitigates these risks by inserting a codon optimised version of the BTK minigene in the endogenous gene location. This universal strategy would correct almost all XLA causing gene mutations with the transgene remaining under the control of local regulatory factors. We first employed nucleofection to introduce the cas9 ribonucleoprotein targeting exon 2 of the BTK gene into cells. An Adeno Associated Virus was then used to deliver the BTK cDNA flanked by 300bp of the BTK last intron into the target genome. Initially we optimised our gene editing technique in a BTK knockout version of a B cell line (DG75). We showed successful integration and restored BTK expression using In-Out PCR and western blot analysis, respectively. The expression of BTK followed a physiological pattern as no BTK was detected in edited clones of the Jurkat T cell line. We next recruited 3 XLA patients to undergo peripheral blood stem cell donation via G-CSF mobilisation and apheresis. We attempted our gene editing protocol on stem cells from these patients and showed that the editing procedure can restore the ability of these cells to differentiate into immature CD19+IgM+ B cells in vitro by using a specialised feeder free culture protocol employing IL-6, IL-7 and ICAM1. Cutting efficiencies of 70% and editing efficiencies of up to 60% were achieved (as assessed by droplet digital PCR). The results show that our editing method can restore a physiological pattern of BTK expression in cell lines and rescue B cell development in XLA stem cells. Further work in an NSG mouse model will be performed to provide in vivo data of our method's effectiveness.

967. LATE 2.0 - Improved Cellular Assay to Assess Potential Genotoxicity of CRISPR/Cas9 System

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CRISPR/Cas9 discovery and subsequent research have resulted in the extremely fast and broad application of genome-editing techniques in various fields of biotechnology. That includes medicine, with a persistently increasing number of clinical trials involving CRISPR/Cas9 use. Notwithstanding its enormous potential, the process of translation of genome editing toward clinical application is hampered by several factors, including the high likelihood of immune responses towards bacterial proteins as well as possible "off-target" activity that might impact growth-control genes. "Classical" CRISPR/Cas-based genome editing involves the generation of double-stranded DNA breaks, potentially toxic lesions that trigger DNA damage response (DDR). Multiple cuts might result in faulty repair, i.e., induction of potential chromosomal aberrations, or exhaustion of repair capabilities resulting in cell cycle arrest, programmed cell death (PCD), or, in the worst case, malignant transformation of affected cells. Therefore, suitable assays are required that allow thorough testing of novel genome-editing techniques regarding potential adverse effects. Recently, we have developed a long-term adverse treatment effect (LATE) in vitro assay that addresses the potential toxicity of designer nucleases by assessing cell transformation events. We already provided proof of concept demonstrating that the LATE assay facilitates detection of unwanted growth promotion due to Cas9 off-target cutting in TP53 as well as other tumor suppressor genes based on the following concept: (1) cell transduction with all-in-one lentiviral vectors, (2) checking baseline transduction rate and follow-up for up to 8 weeks by flow cytometry, and (3) positive readout, with increasing numbers of transduced cells as an indicator of an acquired growth advantage in the process of genome editing (confirmed by digital PCR) [Glow et al. 2021]. To further improve the LATE assay and broaden its applicability, we have now introduced several modifications. For that, we have shown that the LATE assay enables the detection of transformation events in iPSCs - a highly relevant cell type for cell and gene therapies. Moreover, we significantly shortened the readout of the LATE assay (from 8 to 4 weeks) by subjecting target cells with other DDR-causing treatments such as gamma-irradiation to inhibit the growth of non-transformed cells. We show here that the positive γ -LATE readout remains fully dependent on the acquisition of genomic changes by the genome-editing step before the treatment. Additional de novo mutations acquired due to gamma-irradiation treatment could readily be detected by NGS. The workflow of LATE and γ -LATE is schematically presented in Fig. 1. In conclusion, we suppose that the improved LATE-assay might become very useful for preclinical toxicity assessment in the expanding field of clinical genome editing.



968. CRISPR-Cas9 T Cell Editing for Finnish Founder Diseases

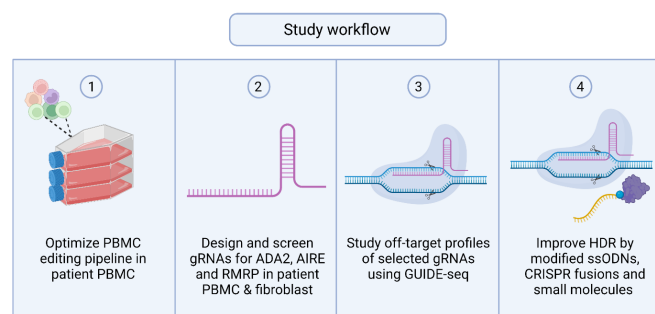
Katariina Mami¹, Zhuokun Li¹, Ganna Reint¹, Nail Fatkhutdinov¹, Frida Haugen¹, Thea Gjerdingen¹, Monika Szymanska¹, Kornel Labun², Johanna Olweus¹, Emma Haapaniemi¹

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Introduction: In this study, we present optimized CRISPR-Cas9 gene editing reagents for correcting three Finnish founder mutations that cause the following syndromic primary immunodeficiencies: ADA2 deficiency, Cartilage-Hair Hypoplasia (CHH), and Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy (APECED). In addition, we present an optimized pipeline for editing and expanding patient T cells from small blood sample volumes. The developed methods are useful for *in vitro* studies of primary immunodeficiency diseases and lay a foundation for future gene correction therapies for these conditions. **Methods:** To obtain optimal editing efficiency, patient peripheral blood mononuclear cells (PBMC) were stimulated for 3 days and expanded for additional 4 days after electroporation. 7-18 gRNAs for each locus were designed and screened *ex vivo* in patient PBMCs and fibroblasts. On-target editing by homology-directed repair (HDR) and non-homologous end-joining (NHEJ) was assessed by Droplet Digital PCR (ddPCR) and Next-generation amplicon sequencing containing Unique Molecular Identifiers (UMIs). *In vitro* gRNA performance data were compared to available online gRNA prediction tools. Finally, we optimized GUIDE-seq method for quantifying off-target editing in patient PBMCs. To improve HDR editing outcomes in primary cells, we screened published chemicals, Cas9 fusion proteins and repair template modifications for HDR improvement. **Results:** During the 8-day stimulation and expansion period, CD4+ and CD8+ T cells are enriched in stimulated PBMC, direct use in experiments without the need to extract T cells from PBMC. Our data demonstrates that it is essential to validate gRNAs *in vitro* for optimal precision gene editing outcomes, and that *in silico* gRNA prediction tools are limited in prediction accuracy. For early tool optimization patient PBMC and fibroblasts can be used interchangeably as gRNA performance correlates between the cell types. We show that selected chemical modifications to ssODN repair DNA template can improve HDR in primary PBMCs and fibroblasts. **Conclusion:** In this study, we present a robust pipeline for patient PBMC expansion and editing for selecting optimal gene editing tools for mutation correction. Although established for ADA2, AIRE and RMRP loci, this pipeline can be used for *in vitro* studies for virtually any other monogenic mutation. The

established methods optimized directly in patient cells allow rapid tool optimization for a wide range of diseases for gene therapy development aimed at clinical translation.

Selected immunodeficiencies for Finnish founder mutations in ADA2, AIRE and RMRP loci				
Locus	Mutation	dbSNP ID	Immune symptoms	Other symptoms
ADA2	C->T (R169Q)	rs77563738	Aplastic anemia, large granular lymphocyte leukemia and vasculitis	-
AIRE	C->T (R257X)	rs121434254	Autoimmune polyendocrinopathy	-
RMRP	T->C (A70G)	rs199476103	Viral infections and immunodeficiency-associated cancers	Short stature, gut malformations



969. National Institute of Neurological Disorders and Stroke's Ultra-Rare Gene-Based Therapy (URGenT) Network

Chris Boshoff, Mario Skiadopoulos, Tjerignimin Adissa Silue, Amir Tamiz

NIH - National Institute of Neurological Disorders and Stroke, Rockville, MD

The National Institute of Neurological Disorders and Stroke (NINDS) Ultra-Rare Gene-based Therapy (URGenT) network supports the development of gene-based and transcript directed treatments for ultra-rare neurological diseases to improve patient outcomes since the current standard-of-care for many of them remain at the symptomatic level, with consequent life-long disabilities and early mortality. The network is a late-stage preclinical therapy development program that aims to fund projects that will develop and deliver gene-based therapies (e.g. oligonucleotides and viral-based gene therapies) for ultra-rare neurological or neuromuscular disorders to individuals while standardizing and harmonizing best practices, encouraging innovation in clinical trials. The URGenT network is not only central to NINDS' plans to have a critical role in providing the funding, resources, and structure necessary to link academic and nonprofit communities to the point-of-industry adoption but to also support translational research that spans the gap between basic research findings and clinically impactful therapies. The URGenT Network's process incorporates the planning stage, during which access to specialized consultants is available as needed; the therapeutic development stage, during which appropriate contract partners are provided to aid with different stages (including manufacturing, IND-enabling PK/toxicology studies, and IND submission), and the clinical stage,

during which the transition to clinical trials is facilitated. All aspects will be overseen by an external consulting board while a centralized data management ensures facilitative sharing of data, resources, and practices with other projects in the network. The URGenT Network aims to provide a robust and readily accessible infrastructure with a network comprised of experts to provide design, conduct, and data analysis built around submitted potential therapeutic agents through the PAR-22-020 and PAR-22-028 funding announcements.

References: <https://www.ninds.nih.gov/Current-Research/Research-Funded-NINDS/Translational-Research/urgent-network>

970. A High-Throughput Screen for CRISPR-Cas9-Mediated Exon Deletion

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Exon skipping or deletion has proven to be a powerful strategy for the correction of genetic diseases, where removal of an exon can correct reading frames distorted by aberrant splicing or other mutations. Antisense-oligo therapies that force exon-skipping are now approved drugs, yet their effects are transient. Gene editing stands to revolutionize exon-skipping strategies given that a single dose could yield a sustained benefit for patients. To implement this, CRISPR-Cas9 can be directed to cleave the intronic regions on either side of an out-of-frame exon, enabling the NHEJ repair process to permanently remove the exon. gRNA choice is critical given the lower efficiency of this repair process. However, gRNA choice is complicated by the large size and complexity of intronic regions. Moreover, the most efficient individual gRNAs do not always form the most efficient deletion pair. Here we report a high-throughput screen to discover gRNA pairs that mediate high efficiency exon deletion. The approach hinges on a novel strategy for detecting exon deletion: as each gRNA pair generates a different deletion junction, junction sequences can serve as a readout of deletion efficiency for all pairs simultaneously. In this approach, cells expressing SaCas9 are transduced with lentiviral libraries of gRNA pairs. After editing, genomic DNA is harvested from the cell pool and novel junctions are enriched with biotinylated probes and detected with Illumina-based sequencing. For our initial experiments we designed a library of 2,080 gRNA pairs for deletion of DMD exon 51 in HEK293T cells. We ranked the gRNA pairs based on the frequency with which their corresponding junctions were identified, normalizing for initial gRNA abundance and bias introduced by probe hybridization. All 92 gRNAs present in the screen were identified as participating in at least one deletion, while 563 of the 2,080 pairs had no deletions detected. There was no indication that the smaller deletions are more efficient in this experimental design. The screen identified novel high-efficiency gRNA pairs, and in subsequent validations we observed an increase in deletion efficiency for gRNA pairs with higher normalized counts in the screen. Further validation of the HEK293T screen in both DMD patient myoblasts and hDMDΔ52/*mdx* mice is ongoing. Since this screening method relies only on genomic DNA as an output, it does not require a reporter and can be applied on any locus, in any cell type. Thus, it can be easily adapted to optimize deletion efficiency for any genetic disease where targeted deletion is a viable therapeutic strategy.

971. Utilizing Selex to Identify Novel Functional gRNAs to Probe gRNA Structure-Function Relationships and Expand the Universe of DNA Sequences That Can be Efficiently Targeted for Editing

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The efficient and specific genome editing generated by CRISPR systems has rapidly accelerated the technology to the forefront of genome engineering. Unfortunately, due to the inherent ability of gRNAs to form multiple folded structures, low editing efficiency can result. In particular misfolding of the gRNA can result from intramolecular interactions formed between the guide RNAs two functional domains: its Cas9 binding domain sequence (an RNA aptamer that binds the protein) and its DNA targeting domain sequence (an antisense RNA that binds to DNA). We have utilized SELEX (Systematic Evolution of Ligands by Exponential Enrichment) to identify a multitude of variant/alternate guide RNA aptamer domains that can bind to Cas9. Many of these RNA aptamer-domains selected for binding do not appear to support DNA cleavage. Thus we modified our high through-put selection strategy to identify gRNA aptamer variants that support Cas9-mediated DNA cleavage. This functional gRNA aptamer selection yielded numerous gRNAs with altered Cas9 binding domain sequences that still support efficient DNA cleavage activity in vitro. Moreover, these novel guide scaffolds are capable of forming cleavage-competent complexes in cell culture, and can engender high editing efficiencies at sites in a target gene where the standard wild type guide RNA aptamer scaffold is not efficient. Thus, this set of novel functional Cas9 binding RNA aptamer domains can be paired with different DNA targeting antisense domains to generate gRNA variants with domain combinations that can efficiently cleave many more DNA sites than when using the wild type aptamer domain. These results highlight the importance of guide RNA folding for effective targeting of DNA sequences and indicates that a more detailed understanding of the structure-function relationship between the gRNAs Cas binding aptamer domain and the DNA targeting antisense domain allows the repertoire of DNA sequences that can be efficiently targeted and edited to be greatly expanded.

972. ncRNA-a3 Mediated Regulation of Erythroid Specific *TAL1* Gene Expression

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Recent advances in genomic research have identified long non-coding RNAs (lncRNAs) that regulate protein coding gene expression. lncRNAs interface with target regions of the genome and form ribonucleoprotein complexes with chromatin modifiers or transcriptional machinery to regulate gene expression. A subset of lncRNAs, termed activating non-coding RNAs (ncRNA-a), promote the transcription of neighboring genes. In breast cancer cells, activating lncRNA, ncRNA-a3, transcribed from an enhancer region of the *TAL1* gene, activates *TAL1* transcription. The *TAL1* transcription factor is a critical regulator of hematopoietic development. *TAL1*

target genes direct erythroid and megakaryocyte development and differentiation. Precise control of *TAL1* levels is essential to prevent malignant hematopoiesis, as ectopic *TAL1* expression is associated with T-cell leukemogenesis. Thus, it is imperative to understand the molecular mechanisms modulating *TAL1* levels during hematopoiesis and erythroid differentiation. In this study, we examined the regulation of *TAL1* by ncRNA-a3 during normal erythropoiesis. We used RNA interference to deplete ncRNA-a3. Knock down of ncRNA-a3 led to a substantial decrease in *TAL1* protein and transcript levels. Transcriptome changes suggest that ncRNA-a3 works as a *cis* regulatory element. Further, chromatin immunoprecipitation (ChIP) revealed reduced histone H3 lysine 27 acetylation (H3K27ac) along the *TAL1* locus marking transcriptional inactivation. Consistently, cells showed aberrant changes in chromatin accessibility genome wide. The cells also lost promoter-enhancer interactions at the gene locus. To determine the mechanism by which ncRNA-a3 regulates the transcription, we checked its association with *TAL1* DNA and transcription machinery proteins. ncRNA-a3 binds to the gene locus and interacts with co-activators CBP/P300 to regulate gene expression. To access the functional role of ncRNA-a3, we induced erythroid differentiation in ncRNA3 depleted CD34+ hematopoietic stem cells. By day 14, we observed a significant reduction of fully differentiated erythroid cells. Our results elucidate the regulatory mechanism of ncRNA-a3 in *TAL1* expression. Co-activator binding shows a new mode of action and suggests that ncRNA-a3 functions as an enhancer RNA. We also demonstrated the functional relevance of ncRNA-a3. Interestingly, since non-coding RNAs have served as biomarkers for prognosis, understanding the functional and mechanistic roles of ncRNAs is of therapeutic importance.

973. TALE Writer: Design and Assembly of Custom TALE-Based Technologies

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Transcription activator-like effector-based technologies (TALE-based technologies) have been at the forefront of the mitochondrial genome editing revolution. These technologies lack the requirement for guide RNA molecules, in contrast to the CRISPR systems widely utilized for nuclear genome editing. At present, no system for the efficient import of nucleic acids into mitochondria has been identified. In contrast, protein import into mitochondria is well-characterized. Thus, the proteinaceous nature of TALE-based technologies enables their utilization as mitochondrial genome editors. Mitochondrially targeted TALEs (mitoTALEs) can be fused to effector molecules, such as nucleases or deaminases, in order to induce a desired change in the mitochondrial genome. MitoTALE pairs bind specific sequences in mitochondrial DNA (mtDNA), bringing an effector molecule in close proximity to its target locus. This modular and programmable model has facilitated different modalities of mitochondrial genome editing. Presently, utilizing TALE-based technologies, our group has achieved mtDNA editing via targeted deletions, and base editing. Targeted

deletions are possible through a combination of mitoTALEs and mitoTALE-nickases. Base editing is done via mitoTALE base editors, which mediate C-to-T edits through base deamination followed by mtDNA replication. Additionally, other groups have reported a targeted degradation approach in which mtDNA molecules are degraded after double-strand break induction via mitoTALE nucleases (mitoTALENs). This toolbox of TALE-based technologies can be deployed in a multitude of studies, such as the development of gene therapies, the generation of disease models, and genetic screenings. However, TALE assembly is a nontrivial problem. Different groups have developed different assembly libraries, including our own: FusX, a rapid one-step TALE assembly system. We have enhanced this protocol using TALE Writer, a script for the efficient design of TALE-based technologies, coupled with an automated liquid-handling laboratory automation tool. This enhancement allows manufacturing TALE-based technologies at a larger scale than manually and minimizing human-derived technical errors. Recently, we utilized TALE Writer to design mitoTALE base editors for the generation of premature termination codons in protein-coding genes in the human and zebrafish mitochondrial genomes. Additionally, we are currently leveraging TALE Writer for the design and assembly of mitoTALEs, mitoTALE-nickases, mitoTALENs, and next-generation mitoTALE base editors. In conclusion, TALE Writer facilitates the efficient design and automated assembly of custom TALE-based technologies, allowing us to write new tales in the revolutionizing chapter of mitochondrial genome editing.

974. Improved Fluorescent CRISPR Fusion Proteins Allow for Enrichment of Edited Cells by Fluorescence Activated Cell Sorting

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It is often desirable to visualize CRISPR enzymes in live cells as a means to verify efficient delivery of CRISPR reagents. The most common method to accomplish this goal with protein cargo is to make an in-frame fusion to fluorescent proteins like GFP or RFP. Here we present an improved set of CRISPR reagents that have been carefully fine-tuned to allow visualization in live cells while still retain near-wildtype levels of on-target activity. We demonstrate that fluorescent CRISPR enzyme fusion proteins and fluorescently labeled tracrRNAs can be used in combination with fluorescence-activated cell sorting to achieve levels of editing greater than with unlabeled CRISPR enzymes. We expect that these reagents will be a valuable resource in research.

975. Novel Gene Supplementation, Genome Editing and Cellular Therapeutic Approaches to Treat Glutaric Aciduria Type I

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Glutaric Aciduria type I (GA-1) is a rare metabolic disorder caused by mutations in the gene encoding Glutaryl-CoA dehydrogenase (*GCDH*), the last enzyme of the lysine catabolism pathway. Its deficiency causes an increased level of toxic metabolites leading to striatal neurodegeneration and devastating neurological problems in patients with GA-1. Similar to many other inborn errors of metabolism, disease management by dietary restriction and carnitine supplementation is the only current option available for patients with GA-1. As dietary lysine is mainly catabolized by hepatocytes, we propose therapeutic approaches in the liver, a more accessible organ to gene therapy than the brain. Aminoadipate-Semialdehyde Synthase (AASS) is the first enzyme in the lysine catabolic pathway. In this study, we exposed both *Gcdh*^{-/-} single knockout, the animal model of GA-1, and *Gcdh*^{-/-} *Aass*^{-/-} double knockout mouse strains to casein diet and observed that all mice from the *Gcdh*^{-/-} group deceased within five days of high protein diet exposure while all *Gcdh*^{-/-} *Aass*^{-/-} mice survived. To generate mechanistic insight, we transplanted *Gcdh*^{-/-} hepatocytes into the livers of *Gcdh*^{-/-} *Aass*^{-/-} double knockout mice, and exposed again to a high protein diet. All mice succumbed within a few days after high protein exposure, indicating that a liver directed therapy can potentially rescue GA-1 mice. In our first approach to treat the livers of *Gcdh*^{-/-} mice, we introduced *mGcdh* cDNA expressed under a liver specific promoter by using an Adeno-Associated Viral (AAV) vector. With this treatment, we could rescue adult but not neonatal mice, possibly due to hepatocyte proliferation and loss of episomes. As GA-1 is a disease that develops in early childhood, our second therapeutic approach based on metabolic reprogramming aimed to delete the hepatic *Aass* gene using CRISPR/Cas9 technology delivered by AAV. This approach successfully rescued GA-1 mice upon high protein exposure. Finally, we were also able to rescue GA-1 mice by transplantation of normal hepatocytes, which also provided further insight into the amount of corrected hepatocytes needed to rescue GA-1 mice. Mice from all treatment groups had reduced levels of toxic metabolites in the serum, and most importantly, showed no neurological abnormalities in their motor behavior in comparison to wild type mice. This work demonstrates that modulation of the lysine catabolism in the liver, rescues lethality without emergence of a neurological phenotype, and thereby opens new therapeutic vistas for GA-1.

977. Novel Engineered MMLV Variants with Enhanced Activity as Primer Editor

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Prime editor (PE) is a fusion of SpCas9 nickase and MMLV reverse transcriptase that enables precise genome editing with minimized double-strand DNA breaks. The targeting space of PE is versatile with the potential to correct the vast majority of disease-associated mutations. An engineered MMLV variant with robust activity is required for efficient editing by PE. However, the specific MMLV variant utilized by the current PE construct contains patented mutations from multiple sources, which may hinder the application of PE due to patent complexity. To address this issue, we isolated a set of novel point mutations of MMLV with enhanced activity. These mutations improved the performance of MMLV in the context of both one-step RT-qPCR for COVID detection and prime editing. The optimized MMLV variant with multiple stacking mutations enabled robust prime editing at comparable efficiency to the published PE constructs. We anticipate our proprietary MMLV variant will simplify the patent landscape and thereby enabling broader application of prime editing in human therapeutics.

978. Spacer-Nick: Precise Gene Correction in Human Primary Cells with Minimal Off-Target and Unintended On-Target Mutations

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The CRISPR/Cas9 system has been applied for developing gene therapy for monogenic hematopoietic disorders. However, off-target and on-target mutations are the main concerns for clinical application. On-target DSBs can potentially cause translocations, large deletion, and DNA rearrangement. Here, we establish a 'spacer-nick' gene correction approach that combines the Cas9D10A nickase with a pair of PAM-out sgRNAs at a distance of 200-350 bp. Combined with adeno-associated virus (AAV) serotype 6 template delivery, our approach led to efficient HDR in human hematopoietic stem and progenitor cells (HSPCs) and T cells, with minimal NHEJ-mediated on-target mutations. Using spacer-nick, we developed an approach to repair disease-causing mutations in the *HBB*, *ELANE*, *IL7R*, and *PRF1* genes. We achieved gene correction efficiencies of 20-50% at the targeted loci, with minimal NHEJ-mediated on-target mutations. Based on the in-depth off-target assessment, we found that frequent classical CRISPR/Cas9-induced unintended genetic alterations were significantly reduced or absent in the HSPCs treated with spacer-nick. Thus, the spacer-nick provides improved safety and suitability for gene therapy.

979. Targeting DNA Polymerase I to DNA Double-Strand Breaks Refines CRISPR/Cas9-Induced Insertions and Deletions

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CRISPR/Cas9 endonucleases induce various insertions and deletions (INDELs) in human cells. Although templated 1-bp insertion (4th nucleotide upstream of the PAM) and <25-bp small deletions are most common, other insertions and unpredictable on-target long DNA deletions (>500 bp) can be observed. The possibility of generating long on-target DNA deletions poses safety risks to somatic genome editing, and makes the outcomes of genome editing less predictable. Using high fidelity Cas9 versions does not resolve this issue and methods refining CRISPR/Cas9-induced INDELs are currently unavailable. We hypothesize that targeting *E. coli* DNA Polymerase I to DNA double-strand breaks can refine CRISPR/Cas9-induced INDELs via increasing templated insertion and decreasing large deletions induced by extensive DNA resection. Here we targeted *E. coli* DNA polymerase to DNA double-strand breaks by fusing *E. coli* DNA polymerase I to Cas9 and examined its effects on CRISPR/Cas9-induced INDELs. We observed a significant increase of 1-bp deletions and a significant decrease of insertions and large deletions. Importantly, long deletions (500 bp~2 kb) are also greatly suppressed. In addition, templated 1-bp insertions relative to other insertions were increased, and this effect depended on targeting the polymerase activity to the double stranded breaks. Further, total INDEL rates were significantly increased in human primary cells. Finally, targeting DNA polymerase to double-strand breaks did not increase off-target rates or base substitution rates around the cleavage sites. The suppression of large deletions was the results of counteracting DNA resection, a process that MRE11 and CtIP are involved. These effects were observed in human primary cells targeting multiple target sequences. Our data show that targeting DNA polymerase I to DNA double-strand breaks is able to refine CRISPR/Cas9-induced INDELs and increase gene disruption efficiency. Importantly, doing so improves safety by reducing the risks of generating unpredictable large on-target DNA deletions.

980. Synergistic Engineering of CRISPR-Cas Nucleases Enables Robust Mammalian Genome Editing

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The naturally occurring CRISPR-Cas systems in prokaryotes provide abundant valuable resources for the development of new genome editing tools. However, most of the prokaryotic Cas nucleases exhibit poor editing efficiency in mammalian cells which significantly limit their utility. Here, we developed a method, termed Improving Editing Activity by Synergistic Engineering (MIDAS), that can exert a synergistic effect on improving the mammalian genome editing efficiency of a wide range of CRISPR-Cas systems by enhancing the interactions of Cas nuclease with the protospacer adjacent motif

(PAM) and the ssDNA substrate in the catalytic pocket simultaneously. MIDAS-engineered variants of Cas12i, Cas12b and CasX showed significantly increased editing efficiencies in human cells, up to 137-, 11-, and 465-fold, respectively. Notably, a Cas12i variant, Cas12i-Max, exhibited robust activity with a very broad PAM range (NTNN, NNTN, NAAN and NCAN) and higher efficiency than the currently widely used Cas nucleases (AsCas12a, BhCas12b v4, SpCas9, SaCas9 and SaCas9-KKH). We further engineered Cas12i-Max-HF variants, enabling minimal off-target risks while maintaining similar on-target editing efficiency of Cas12i-Max. Collectively, our work provides an effective and expandable method to identify Cas nuclease variants for robust mammalian genome editing.

981. APOE-Targeted Epigenome Therapy for Alzheimer's Disease

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Background: There is an urgent need to refocus Alzheimer's disease (AD) drug discovery on new targets and shifting the paradigm of AD drug development towards precision medicine. Apolipoprotein E gene (*APOE*) is the strongest and most reproducible genetic risk factor for late-onset Alzheimer's disease (LOAD), and thus holds promise as a potential therapeutics target for LOAD. In this study we developed an epigenome therapy platform to reduce *APOE* expression generally and *APOE*e4 allele specifically by targeted modification of the epigenome landscape within *APOE* locus. **Methods:** Our gene therapy strategy is based on CRISPR/deactivated (d)Cas9 editing technology fused with an effector molecule and delivered by viral-based vehicles. Our gRNAs were designed to target regulatory elements within the *APOE* promoter/intron 1 region and in exon 4 sequence overlapping the SNP that defines the *APOE*e4 allele. We evaluated our epigenome therapy platform *in vitro* using human hiPSC-derived neurons and *in vivo* by stereotactic injection of reporter gene into the hippocampus of mice. **Results:** The viral dCas9-repressor vector showed decreased *APOE*-mRNA and protein overall levels in hiPSC-derived neuronal model. To specifically target the *APOE*e4 allele we utilized the VRER-dCas9 protein. Evaluation of the system specificity showed a reduction in *APOE*-mRNA levels in the hiPSC-derived neurons with the e4 allele while there was no effect in the *isogenic* hiPSC-derived neurons homozygous for the e3 allele. Moving onto *in vivo* studies in mice, administration of the viral dCas9-repressor vector and the GFP reporter gene into the hippocampus showed a significant decrease in GFP expression with strong repression effect, demonstrating promising preliminary data. Collectively, our results provided *in vitro* and *in vivo proof-of-concept* for the utility and efficacy of the *APOE*-targeted epigenome therapy. **Conclusions:** Our epigenome therapy strategy for fine-tuning of *APOE* 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.

Synthetic/Molecular Conjugates and Physical Methods for Delivery II

982. Ultrasound-Responsive Nanobubbles Loading Nucleic Acids for Cancer Therapy

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Introduction: Ultrasound exposure is known to increase cell membrane permeability and facilitate the delivery of drugs or genes into cells. A combination of ultrasound exposure and contrast agents, microbubbles, further increases cell membrane permeability even when using weak intensity ultrasound, leading to enhanced drug and gene uptake. This system is less invasive and has attracted attention as a site-specific delivery by ultrasound exposure. We have developed nano-sized bubbles (NBs), which were polyethylene glycol-modified liposomes that contain echo-contrast gas. NBs are able to function as gene and nucleic acids delivery tools in response to ultrasound exposure *in vitro* and *in vivo*. However, when applied to systemic delivery, nucleic acids injected intravascularly are rapidly degraded, removed from circulation, and might not be co-localized with bubbles, functioning as the driving force of transfection in blood vessels. Based on these reasons, there are concerns that the systemic administration of the mixed solution with free nucleic acids and NBs reduces transfection efficiency. Then, we previously developed nucleic acid-loaded NBs using cationic lipids. NBs containing cationic lipids were easy to load pDNA, siRNA, and miRNA with electrostatic interaction and release by ultrasound exposure. Nucleic acid-loaded NBs could improve the effectiveness of systemic delivery. However, cationic lipid caused instability of gas retention in NBs. Therefore, we focused on anionic NBs, which were shown to be stable in the retention of echogenicity, to improve the efficiencies of nucleic acids delivery and ultrasound imaging. In this study, we developed novel simple method to efficiently load nucleic acids onto anionic NBs. Furthermore, we investigated its function as a nucleic acid delivery tool and a US contrast agent *in vitro* and *in vivo*. **Methods:** To form anionic NBs, liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), and N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE-PEG) were used and entrapped echo-contrast gas by the sonication. We added methyl glycol chitosan (MGC), which is a kind of cationic polysaccharide, to anionic NBs and assessed the coating of NBs by a change in the zeta potential. To examine the nucleic acid-loading ability, the interaction of MGC-coated NBs (MGC-NBs) with fluorescent-labeled miRNA were analyzed by flow cytometry. The effects of MGC-NBs on ultrasound imaging and intracellular delivery were also evaluated. To investigate the therapeutic effects of miRNA-coated MGC-NBs, we evaluated the tumor growth of the tumor-bearing model after the transfection of miR-145 known to be a tumor suppressor. **Results and Discussion:** The zeta potentials of the anionic NBs changed from negative to positive charge with increasing the amount of MGC, indicating that surface coating with MGC is possible. In addition, MGC-NBs possessed the ability to load miRNAs and showed the effect of intracellular delivery

by the combination with the ultrasound. Furthermore, the transfection of miR-145 showed a cell-killing effect *in vitro* and a tumor growth inhibitory effect *in vivo*. These results indicate that miRNA-coated MGC-NBs may be a useful carrier for nucleic acid systemic delivery.

983. Therapeutic In Vivo Base Editing with Minimal Off-Target Activity via RNP Delivery

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Methods to deliver gene editing agents *in vivo* as ribonucleoproteins could offer safety advantages over nucleic acid delivery approaches. We report the engineering and application of eVLPs, engineered DNA-free virus-like particles that efficiently package and deliver base editor or Cas9 ribonucleoproteins. By engineering VLPs to overcome cargo packaging, release, and localization bottlenecks, we developed fourth-generation eVLPs that mediate efficient base editing in several primary mouse and human cell types. Using different glycoproteins in eVLPs alters their cellular tropism. Single injections of eVLPs into mice support therapeutic levels of base editing, reducing serum Pcsk9 levels 78% following 63% liver editing, and partially restoring visual function in a mouse model of genetic blindness. *In vitro* and *in vivo* off-target editing from eVLPs was virtually undetected, an improvement over AAV or plasmid delivery. These results establish eVLPs as promising vehicles for therapeutic macromolecule delivery that combines advantages of both viral and non-viral delivery.

984. Abstract Withdrawn

985. Tri-GalNAc Conjugation to AAV Capsid Enhances Transduction of Human Hepatocytes *In Vitro*

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Although recombinant adeno-associated virus (rAAV) is arguably the most successful *in vivo* gene delivery platform, AAV capsid discovery for higher tropism remains a fruitful research direction for translational gene therapy. Chemical modification emerges as a versatile capsid engineering approach, powered by the advances in the field of chemical and synthetic biology. In this study, we aim to leverage the well-established tri-GalNAc-mediated hepatocyte targeting mechanism to develop potent rAAV for liver gene delivery. We first incorporated a click chemistry handle to individual AAV5 and AAV8 capsid residues in the variable loop regions by genetic code expansion. A subset of AAV mutant vectors were successfully packaged and capable of being conjugated via click chemistry, as demonstrated by successful coupling with a reporter dye (IRD800). Next, we conjugated a subset of AAV mutants with tri-GalNAc, a ligand binding to the asialoglycoprotein receptor (ASGPR) specifically expressed in hepatocytes. Successful tri-GalNAc conjugation was determined by molecular weight shifting of capsid proteins on an SDS-PAGE gel. Encouragingly, AAV5-GalNAc vectors showed 3- to 10-fold higher transduction than AAV5 vector in human primary hepatocytes with or without adenovirus coinfection. Milder enhancement was observed in two human hepatocyte cell lines (Huh7 and HepG2) and HEK293 cells transiently expressing ASGPR, but not in control HEK293 cells, supporting a GalNAc-dependent mechanism. However, liver transduction of AAV5-GalNAc vectors was inferior to AAV5 vector when tested in mice in pilot experiments. Possible reasons underlying the discrepancy are being studied and will be discussed.

986. Multi-Step Engineering of Gene-Edited CAR T Cells Using a Novel RNA Lipid Nanoparticle Platform

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Introduction: Traditional autologous chimeric antigen receptor (CAR) T therapies utilize patient cells and can be limited by cell quality, and the high manufacturing burden of viral vectors. As such, there is a need for allogeneic, “off-the-shelf” CAR T cells to make these transformative treatments available to a wider population. However, allogeneic therapies require multiple genetic engineering steps to express CAR and to eliminate proteins responsible for graft-versus-host disease. Messenger RNA (mRNA) is a promising new approach for expression of therapeutic proteins and gene editing nucleases. While mRNA is often delivered to cells using electroporation, lipid nanoparticles (LNPs) represent an attractive alternative. In this

work, we demonstrate the utility of a novel lipid nanoparticle (LNP) reagent to enable multi-step engineering of gene-edited CAR T cells. **Methods:** LNPs encapsulating Spy-Cas9 mRNA, TCR and CD52 guide RNA (sgRNA), and CD19 CAR mRNA were produced using a scalable microfluidic production platform. We optimized the LNP protocol, including the packaging, cargo ratios, and sgRNA combinations. Purified human primary T cells were cultured, activated, and expanded in serum-free media. Single or double TCR and CD52 knockout cells were generated by addition of the corresponding LNP to activated cells. Gene-edited CAR T cells were achieved by purification of residual TCR+ cells and addition of the CAR LNPs. The TCR-/CD52-/CAR+ T cells were co-cultured with leukemia cells to determine cytotoxic killing efficiency. Gene knockout, CAR expression, viability and cell killing were assessed using flow-cytometry. **Results and Conclusion:** TCR and CD52 were selected as clinically relevant gene knockout targets. Following TCR and CD52 LNP addition to T cells, the onset of gene editing was within 48 hours, reaching single target knockout efficiencies of $75.0 \pm 5.8\%$ (TCR) and $69.6 \pm 9.1\%$ (CD52), and double knockouts of $61.1 \pm 10.8\%$. As proof-of-concept for multi-step engineering, TCR-/CD52- T cells were further modified with CAR LNPs, achieving >70% of cells expressing CD19 CAR. Cell viabilities above 90% were maintained at all steps. These “off-the-shelf” gene-edited CAR T cells were functionally equivalent to non-edited cells in a cell killing assay, efficiently clearing over 80% of leukemia target cells at a 1:1 ratio. Overall, these results showcase the potential of this novel lipid nanoparticle reagent to enable the development of next generation cell therapies.

987. Abstract Withdrawn

988. A Simple Kinetic Model to Rapidly Assess the Therapeutic Potential of Nucleic Acid Formulations

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A major bottleneck in the clinical translation of nucleic acid drugs is the inefficiency in testing formulations for therapeutic potential. The inability to test these drugs in an expedient manner stems from the lack of predictive capacity when transitioning from *in vitro* studies to more complex *in vivo* models and ultimately clinical applications. To this end, we have developed a mathematical framework that can reliably predict siRNA-mediated gene silencing with as few as one experimental data point as an input. The model consists of only essential rate-limiting steps and parameters with easily characterizable values of the RNAi process, enabling facile identification of which parameters play dominant roles in determining the potency of siRNA. Predictions from our kinetic model are in close agreement with both *in vitro* and *in vivo* experimental results across a retrospective analysis of multiple peer-reviewed data sets. Most importantly, critical information needed to design efficient dosing regimens (*i.e.*, maximum gene silencing efficiency, the time point at which this maximum gene silencing occurs, and the duration of silencing) are accurately captured by our framework. Our findings suggest that siRNA dilution is the primary determinant of gene-silencing kinetics, and that this dilution

rate is governed by different parameters *in vitro* (cell dilution) and *in vivo* (clearance from target tissue), highlighting a key reason why *in vitro* experiments do not always predict *in vivo* outcomes. Although this effort focuses on siRNA, we anticipate that the framework can be modified and applied to other nucleic acids therapeutics, such as mRNA-based interventions, that rely on similar biological pathways.

989. Making Bionanoparticles at the 10kL Scale: The Evolution of a Perfusion Cell Culture to Increase Supply of Exosomes

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The promise of exosomes as a new drug modality is to deliver the right therapy to the right cells. In just 6 years, Codiak has progressed from bench scale to cGMP manufacturing, and has successfully completed GMP campaigns to supply exosomes to three phase 1/2 clinical studies. This presentation highlights the development of a continuous upstream process for exosome production and details some of the challenges and lessons learned from operation at large-scale GMP manufacturing. To improve productivity, Codiak shifted its manufacturing technology from 2000L fed-batch suspension cell culture to 500L perfusion. Perfusion cell culture provides increased yield and a reduction in protein impurities as compared to fed-batch. Transitioning from fed-batch to perfusion however, created new challenges, with scale-dependent phenomena observed at large scale. To improve manufacturing-scale operations, a scale-down model was established to study key parameters affecting cell culture health. Hollow fiber recirculation rate in the cell retention device was identified as an important parameter that affects cell culture health and product yield. Optimization of the parameters led to improved cell culture performance in a subsequent manufacturing run but limited the maximum cell density. Upon investigation, the pressure drop from the longer, hollow fiber filters required at scale was identified as the root cause. To further improve the perfusion process, a re-design of the filter assembly was performed and resulted in a 50% decrease in pressure drop relative to the previous run, leading to higher cell viability, and reduced permeate turbidity. When combined with additional observations and process insights, the process changes increased robustness, enabling a longer process duration and increased exosome productivity by 90%. Manufacturing ample vector has been a persistent challenge for viral vector-based gene therapy. Exosomes share many structural attributes with viral vectors such as AAV and lentivirus. Therefore, the lessons learned in establishing this large-scale manufacturing platform may apply not just to exosomes but can support capacity unlocks for the broader gene therapy field.

990. Lipid Nanoparticle Formulation Enhances Gene Silencing, Immunostimulatory Properties and In Vivo Efficacy of CpG-STAT3siRNA Against Human B Cell Lymphoma

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Recent advances in cancer immunotherapy highlighted the need to target both positive and negative regulatory pathways governing antitumor immune responses. Our earlier studies focused on eliminating tolerogenic effect of STAT3 signaling in the tumor microenvironment while triggering innate and adaptive immune responses through Toll-like Receptor-9 (TLR9) stimulation. To achieve such dual function, we have utilized oligonucleotide design combining STAT3 siRNA with TLR9 agonist (CpG oligonucleotide). CpG-STAT3siRNA proved effective against multiple tumor models in mice and resulted in the recently opened clinical trial in patients with relapsed/refractory non-Hodgkin lymphoma using local, intranodal treatments. To further optimize this strategy by enhancing molecule stability and potency in terms of gene silencing and immunostimulation, we used lipid nanoparticles (LNP) since such delivery systems emerged in the recent decade as an attractive tool for gene therapy. Here in this study, we demonstrated the feasibility of using ionizable lipid, MC3-based formulation for the delivery of oligonucleotides with dual gene silencing and immunostimulatory functions. The MC3-based LNPs are commonly used for gene therapy rather than in immunotherapeutic applications. To enable delivery of immunostimulatory CpG-STAT3siRNA to target immune cells, we generated and tested LNPs with various N/P ratios and PEG content to retain optimal gene silencing without losing immunostimulatory properties of the conjugate. We found that CpG-STAT3siRNA encapsulated in slightly negatively charged LNP-02 (~100nm in diameter) were selectively taken up by human and mouse monocytes, macrophages and dendritic cells. As expected, the LNP-02 formulation prolonged stability of CpG-STAT3siRNA in human serum (4hr vs. >24hr), which permits systemic administration and less frequent dosing. In addition, the selected LNP/CpG-STAT3siRNA formulation strongly enhanced potency of STAT3 silencing in target cells such as human OCI-Ly3 B cell lymphoma cells as well as in U251 glioma cells. Importantly, the sequestration of CpG-STAT3siRNA in the LNP-02 has not abolished immunostimulatory activity of this oligonucleotide. We observed up to three-fold increased activation of NF- κ B transcriptional activity using reported gene assays in mouse macrophages. Healthy donor PBMCs showed a robust type I IFN production (5-10 ng/ml of IFN α) compared to the naked oligonucleotide (<100pg/ml) as measured by ELISA. Finally, in our initial *in vivo* study, we have assessed the efficacy of LNP-02 encapsulated CpG-STAT3siRNA vs. equivalent molar amount of the naked oligonucleotide (at suboptimal 0.42 nmole dosing) against xenotransplanted B cell lymphoma using local, intratumoral injections. These studies indicated ~10-fold increase in the potency of our oligonucleotide in local administration resulting in lymphoma growth arrest. While further studies in syngeneic lymphoma models are ongoing, our data suggest that the new LNP-02 formulation of CpG-STAT3siRNA can provide an attractive new therapeutic option for the immunotherapy of B cell lymphoma and potentially certain other malignancies.

991. High Efficiency of Histidylated-Based Lipid Formulations for mRNA Transfection of Human Schwann Cells

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Schwann cells are the principal glial cell type of the peripheral nerve myelin sheath. They play critical roles in the development, maintenance, function, and regeneration of peripheral nerves. In fact, Schwann cells are involved in various neurological diseases, many of which do not have effective treatments including Charcot-Marie-Tooth disease, multiple sclerosis, several malignant nerve sheath tumors, and neurofibromatosis. The great potential of messenger RNAs (mRNAs) has been revealed by the recent approval of the two very efficient COVID-19 mRNA vaccines. In addition to the use of mRNA as a vaccine platform, they have been widely explored in several therapeutic approaches such as protein replacement therapy. As an example, neurofibromatosis (NF) is an inherited disorder due to several types of mutations within the ~8,0 kb coding gene sequence of the NF1 tumor suppressor located in Schwann cells. Neurofibromatosis treatment is limited to surgical resections, chemical drugs that are invasive and poorly efficient. In this context, it is interesting to develop a new mRNA-based approach to produce Nf1 protein and restore all of its biological functions. This study aims to determine and optimize the delivery and expression of mRNA in human Schwann cells (HSCs), using a panel of polymeric and lipidic vectors including our histidylated original lipids. In both wild-type and mutated HSCs, our original lipid-based formulations made with both modified and non-modified mRNA achieved a high percentage of transfected cells (~90%) without inducing any cytotoxicity. By contrast, polymeric vectors as PEI and mRNA-fect were not efficient at all. More interestingly, our histidylated lipid-based formulations allowed a better transfection efficiency compared to the gold standard vector Lipofectamine Messenger Max[®]. Ongoing experiments concern the evaluation of this mRNA delivery-based approach using intradermal injections in fresh human skin explants, a highly relevant organotypic model. Interestingly, we observed an efficient luciferase mRNA expression in skin explants for up to 5 days using our lipid-based formulation. To the best of our knowledge, this study shows for the first time the feasibility of Schwann cells transfection with mRNA. As mentioned above, it is of interest to explore further as they are implicated in several neuro-disorders. Thereby, this work will pave the way to a therapeutic paradigm shift based on protein replacement by mRNA delivery in HSC.

992. Investigation of Human Bone Marrow Mesenchymal Stem Cell-Derived Extracellular Vesicles as Therapeutic Agents for Facioscapulohumeral Muscular Dystrophy (FSHD)

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common muscular dystrophies worldwide estimated to affect around 870,000 individuals. Both genetic and epigenetic factors can lead to FSHD, but the underlying mechanism for all types is caused by aberrant expression of the myotoxic transcription factor DUX4. Therefore, DUX4 inhibition is considered the most direct route to FSHD therapy. However, clinically, this protein has proven to be extremely problematic to detect with high variability and overall low abundance in skeletal muscles at any given time. These factors make development of targeted therapies such as Antisense Oligonucleotides (ASOs) and miRNA-based gene therapies potentially challenging. High transduction efficiencies and high doses may be required to reach a critical mass since systemic delivery of these targeted therapies is needed for diseases such as FSHD in which many skeletal muscle groups are affected. Despite having a sporadic expression, DUX4 is known to drive disease progression by triggering a gene expression deregulation cascade, causing oxidative stress and chronic inflammation throughout the affected skeletal muscles, leading to muscle atrophy. Mesenchymal stem cell-derived extracellular vesicles (MSC EVs) are known for their regenerative, antioxidant and anti-inflammatory properties. Therefore, we hypothesized that since inflammation and oxidative stress signaling in affected skeletal muscles are not limited to specific myofibers, using MSC EVs as therapeutic agents for FSHD might have a global therapeutic effect. Accordingly, we tested the regenerative properties of human bone marrow MSC EVs *in vivo* using our previously published AAV.DUX4 mouse model. To begin we first ensured that MSC EVs did not prevent AAV transduction of muscle. To do this we co-injected MSC EVs with AAV.eGFP and observed minimal reduction in fluorescence, indicating that MSC EVs did not prevent AAV vectors from being transduced into injected muscles. Next, we co-injected MSC EVs with AAV.DUX4 in the tibialis anterior (TA) of C57BL/6 mice and assessed muscle histology two weeks post-injection. As a result, MSC EVs were very efficient in eliminating DUX4-induced muscle toxicity and mouse *DUX4*-responsive biomarkers, *Wfdc3* and *Trim36*. Future studies will focus on transcriptomic and proteomic analysis of the molecular content of MSC EVs in order to understand the mechanism that led to the reduction of DUX4 expression. Long term studies using multiple dosing regimens will also be explored using the tamoxifen inducible FSHD TIC-DUX4 mouse model. These results support the idea that MSC EVs are an attractive therapeutic agent for FSHD or other disease that could benefit from their anti-inflammatory, antioxidant, and regenerative properties.

993. Synthetic Chromosome for Multiple Gene Induction Systems

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Introduction: The application of next-generation cell-based therapies will be buoyed by the development of portable, multigene delivery systems that are capable of targeting more than one biochemical pathway. However, impediments remain as this approach is developed for precision medicine applications and broad utility in a clinical

setting. These impediments include transgene genomic integration and increased mutagenesis, payload size limitations, and viral tropism. Mammalian synthetic chromosomes (MACs) circumvent many of the limitations associated with plasmid and viral-based gene expression systems and provide an alternative means to introduce large segments of genomic DNA such as fragments containing long-range genetic elements required for appropriate regulation of gene expression, developmentally regulated multi-gene loci or splice variants, or multiple copies of two or more genes in fixed stoichiometry. We illustrate the utility of a portable synthetic chromosome to engineer cells for independently controlled delivery of multiple gene products.

Methods and Materials: A synthetic chromosome-based, dual-inducible (tetracycline and cumate), gene expression system was engineered using phage lambda integrase site-specific recombination. Short half-life fluorescent proteins were utilized for monitoring induction.

Results: The synthetic chromosome-based, dual-inducible system demonstrates the ability to independently control gene expression of two inducible systems in response to increasing inducer, doxycycline and/or cumate, as measured by fluorescent protein expression. The synthetic chromosome, engineered to encode a destabilized green fluorescent protein (ZsGreen1-DR), in response to doxycycline and a destabilized red fluorescent protein (DsRed-Express-DR), in response to cumate. Differential regulation of the two fluorescent proteins within the same cell can be controlled by their respective inducer concentration. This demonstration provides a proof of concept, modular design approach for synthetic chromosome bioengineering.

Discussion: The ability to coordinately or discordantly regulate multiple proteins within a cell is an advance in synthetic biological circuit design and cell engineering. Bioengineered synthetic chromosomes potentially provide a novel cytoreagent system amenable to designing complex genetic circuits for multi-therapeutic biological delivery. Synthetic chromosomes, as engineerable modular platforms, interallied with advances in autologous induced pluripotent stem cell production and next generation cell culture bioreactors could greatly streamline the process and broaden the utility of precision medicine for pharmaceutical manufacturing.

994. Chemically Engineered AAV Vectors to Improve Biodistribution and Gene Transfer to the Central Nervous System

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Introduction. Adeno-associated virus (AAV) vector-mediated gene therapy represents today one of the most promising therapeutic avenues to address rare inherited neurological disorders, as well as acquired neurodegenerative diseases such as Alzheimer's or Parkinson's. Yet, delivery of genetic materials to the most relevant areas to the disease remains a challenge. Systemic approaches, although most desirable because least invasive, have proven to be largely ineffective in reaching nervous system targets and have posed serious safety concerns in recent clinical trials. Local routes of administration, although riskier, have shown better performances. But the limited tissue penetrance of currently available AAV vectors still hinders the therapeutic effect of approaches addressing diseases that affect large and/or deep brain structures, especially those located remotely from the delivery point.

Methods. To address this issue, a chemical engineering approach was used to alter the capsids of existing recombinant AAV vectors. A series of novel conjugated AAV vectors (coAAVs) was produced from various serotypes by covalently grafting chemical ligands to the surface of their capsids. The *in vivo* biodistribution and transduction abilities of these new AAV variants were then investigated in the central nervous system, through different local routes of administration.

Results. One particular AAV2 variant, termed C00AAV2, is presented herein, showing extended biodistribution patterns in both rodent and primate brain tissues. When delivered to the parenchyma, it improved the transduction coverage of basal ganglion structures by a factor 2 to 4 and exhibited increased transgene expression in these same structures, compared to its unconjugated counterpart. Intracisternal delivery of C00AAV2 also resulted in enhanced distribution, not only to surrounding tissues (e.g., brainstem and cerebellum), but also to more distant ones, like the thalamus and the substantia nigra. **Conclusions.** Together, these data indicate that chemical conjugation can effectively transform standard AAVs into more potent vectors with, as in the case of C00AAV2, higher tissue penetrance and transduction efficiency. As they are simple to produce (no genetic engineering, applicable to virtually any existing recombinant AAV), coAAVs thus represent great and unique additions to the toolbox for gene therapy applications.

995. Abstract Withdrawn

996. Lipid Nanoparticle Library for Non-Viral Delivery of mRNA and saRNA Towards Vaccine and Cell & Gene Therapy Applications

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US FDA approval of ONPATPRO[®] (AInlyam), followed by recent vaccine approvals of Comirnaty[®] (BioNTech/Pfizer) and Spikevax[®] (Moderna), and the numerous clinical trials with mRNA-based drugs or vaccines have provided momentum to further develop lipid nanoparticle (LNP)-based genetic medicine candidates. Ionizable amino lipid is a major constituent of the LNPs for delivering nucleic acid therapeutics (e.g., DLin-MC3-DMA in ONPATPRO[®], ALC-0315 in Comirnaty[®], SM-102 in Spikevax[®]). More effective ionizable lipids that are non-toxic, can effectively encapsulate nucleic acids, and can release the nucleic acids from the early endosomes of the target cell are needed for various applications. Scarcity of lipids that are suitable for vaccination, cell therapy, and protein replacement therapies continue to be a problem in advancing many potential therapeutic/vaccine candidates to the clinic. Herein, we describe the development of novel ionizable lipids to be used as functional excipient 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 to showcase the effective translation of several GOI encoded

mRNAs. The protein expression levels and the TE were analyzed by ELISA or by Flow Cytometry. The eGFP and CAR expression in human T cells were found to be significantly higher in comparison to clinically validated DLin-MC3-DMA. LNPs of size 60-120 nm with high encapsulation efficiencies were produced by microfluidic mixing. These lipids were then screened for Influenza, SARS-CoV-2 vaccine applications using self-amplifying RNA (saRNA) encoding H1N1 Influenza antigen or SARS-CoV-2 full-length spike protein. IgG analysis using ELISA indicated high titre values. We evaluated novel ionizable lipids for COVID-19 vaccine using saRNA encoding pre-fusion stabilized SARS-CoV-2 full-length spike protein and found that those novel proprietary lipids showed similar amounts of SARS CoV-2 Spike protein-specific IgG compared to clinically approved SM-102 and ALC-0315. We also assessed the tolerability of the novel lipids in comparison to SM-102 and ALC-0315 in CD-1 mice. We then evaluated various ionizable lipid candidates for protein replacement applications by administering human Erythropoietin (hEPO) encoded mRNA LNPs intravenously at a dose of 0.5 mg/kg in C57BL6 mice. High protein expression and a corresponding increase in hematocrit were observed that were similar or better than clinically approved lipids. In conclusion, we were able to show that physico-chemical profiles of ionizable lipids are going to be different for vaccine, protein replacement, and cell therapy applications. We believe that these studies will pave the path to the advancement in the development of RNA vaccines, cell-based therapy, and targeted delivery of other nucleic acids for finding a cure for many rare diseases, where treatment options rarely exist.

997. A New Benchtop System for Simple and Versatile Introduction of Macromolecules into Human Lymphocytes by Microfluidic Squeezing

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Intracellular delivery of molecules is a key step in biological research that enables the development of emerging generations of cell and gene-based therapies. However, several limitations in existing delivery approaches dramatically restrict the range of deliverable cargos and cell types used to study and treat disease. Furthermore, dysregulated gene expression caused by traditional intracellular delivery methods that can lead to altered cell biology are often overlooked. To address these challenges, we have developed the CellPore™ system, a benchtop device for gentle, yet efficient delivery of cargos to a range of primary cell types at research scales. The CellPore™ system is a vector-free, microfluidic platform that relies on mechanical deformation of the cell membrane (also known as mechanoporation) to facilitate intracellular delivery of target materials, including small molecules, nucleic acids, and proteins. This Cell Squeeze® process creates transient disruptions in the plasma membrane that readily enable cargo diffusion directly into the cytoplasm. We have developed a detailed workflow for use

of the system, including cell isolation and resting, cargo preparation, and delivery optimization parameters. These optimized parameters were subsequently applied to successfully deliver eGFP and mCherry mRNA to unactivated T and NK cells, as well as Cas9 gene editing complexes (ribonucleoproteins) targeting *B2M* and *TRAC* genes to unactivated T cells. Functional studies of edited T cells were also performed in order to assess the impact of the CellPore™ system on overall cell quality. Notably, we demonstrated the ability of unactivated T cells manipulated by the system to undergo similar activation and expansion when compared to unmanipulated samples. Furthermore, gene expression analysis revealed that cells manipulated using the CellPore™ system had minimal transcriptional perturbations compared to a high degree of dysregulation measured in cells that had undergone traditional electroporation. These results underscore the important considerations that need to be made when choosing a suitable delivery method in order to retain appropriate cellular response and function. Efficient yet gentle delivery of cargos to primary cell types by the CellPore™ system offers significant advantages over traditional delivery methods. Particularly, direct cytosolic delivery of macromolecules enabled by the system provides an avenue for multiplexed delivery of heterogeneous cargos. The CellPore™ system provides researchers with a simple and familiar workflow that can be easily integrated as part of their cell therapy research.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases II

998. Development of Chimeric GALC Enzymes with Improved Bioavailability to Refine Gene Therapy Strategies for Globoid Cell Leukodystrophy

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Globoid cell leukodystrophy (GLD) is a lysosomal storage disorder (LSD) caused by the deficiency of β -galactosylceramidase (GALC). The rapid and severe dysfunction of central and peripheral nervous systems (CNS, PNS) of the infantile forms, pose major issues for the development of effective treatments. *Ex vivo* lentiviral vector (LV) hematopoietic stem cell (HSC) gene therapy (GT) is effective in genetic blood diseases and similar neurodegenerative LSDs, metachromatic leukodystrophy (MLD) and mucopolysaccharidosis type I Hurler variant (MPSIH). Indeed, pre-clinical and clinical studies suggest a correlation between enzyme availability, clearance of intracellular storage, and therapeutic benefit. Still, previous work anticipates transgene-specific regulation and potential safety concerns associated to GALC overexpression in HSCs, ultimately resulting in insufficient enzyme secretion by transplanted cells and/or modest enzyme recapture by affected cells (cross-correction). Chimeric lysosomal enzymes with increased secretion

and enhanced capability to cross the blood brain barrier (BBB) boost the therapeutic efficacy of GT in murine models of neurodegenerative LSDs. Therefore, the development of chimeric GALC enzymes with increased bioavailability would be desirable to overcome safety issues and improve efficacy of HSC GT approaches for GLD. We generated lentiviral vectors (LV) encoding for murine GALC enzyme fused to mCherry reporter (LV.GALC-CH). We further engineered GALC-CH construct by:

- replacing GALC signal peptide (sp) with that of highly secreted lysosomal enzymes (such as iduronate-2-sulphatase -IDS- or α -L iduronidase -IDUA-), to increase enzyme secretion;
- adding a tandem repeat of the human apolipoprotein EII (Apo EII), a low density lipoprotein receptor (LDLr)-binding domain, to enhance BBB crossing and favor GALC uptake by GALC-deficient cells through LDL- and related receptors. We showed safe supraphysiological expression and enzymatic activity of chimeric GALC enzymes in GLD murine neural and hematopoietic stem/progenitor cells (NSPCs, HSPCs) and progeny, relevant cell types in the context of GT platforms, with an advantage of IDSsp and even more of IDUAsp in GALC production and secretion compared to the unmodified enzyme. The chimeric enzymes were secreted, recaptured, and delivered to the lysosomes of GALC-deficient neural cells, which were metabolically cross-corrected. Importantly, the expression of LDLr in GLD neurons/glia cells reinforces the use of chimeric GALC enzymes to enhance the GALC supply in CNS cells. These results support the rationale of testing the safety and efficacy of chimeric GALC enzymes in *ex vivo* and *in vivo* GT approaches in GLD animal models, with the final goal of developing novel and more effective GT strategies for this untreatable disease.

999. CRISPR-Based Gene Editing Enhances LDLR Expression and Boosts LDL-C Uptake in Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is a prevalent autosomal dominant disorder characterized by a lifelong elevation of low-density lipoprotein cholesterol (LDL-C), which results in early-onset atherosclerosis and coronary events. About 85% to 90% of genetically confirmed FH is caused by pathogenic mutations in the LDLR gene, haploinsufficiency of which leads to reduced LDL-C uptake. Lifelong lipid-lowering medications, such as Statins and Ezetimibe, are currently available, however, they are often intolerable by some of the patients and fail to attain desired LDL-C levels. A more recent therapeutic approach is based on subcutaneously injected monoclonal antibodies that transiently inhibit proprotein convertase subtilisin/kexin type 9 (PCSK9), a protein that promotes LDLR lysosomal degradation. Such approach, however, is frequently insufficient as a monotherapy, and is thus prescribed in combination with a Statin therapy. Here we present a novel, direct and long-lasting therapeutic strategy for upregulation of LDLR expression by truncating a section of the LDLR 3' UTR, which contains sites that negatively regulate LDLR expression, via CRISPR-based gene editing. Editing strategy was tested in HepG2 cell line, FH patient-derived Lymphoblastoid cell lines (LCLs) and mouse hepatoma cell line Hepa1-6. Excision of the 3'UTR was confirmed by

ddPCR and LDLR mRNA levels were quantified by qRT-PCR. Total and membrane-bound LDLR levels were determined by Western Blot and flow cytometry, respectively, using specific antibodies. Finally, the effect of 3'UTR excision on cholesterol uptake was assessed by measuring the cellular intake of fluorescently labeled LDL-C, using flow cytometry. Excision efficiency in HepG2 cells was about 60%. Excised cells showed a 6-fold upregulation of LDLR mRNA levels and a 3-fold increase in membrane-bound LDLR as compared to non-treated cells. 3'UTR excision resulted in a 2-fold increase in the uptake of cholesterol at all time points tested. Patient-derived LCLs showed similar outcomes, with a 3-4-fold increase in LDL-C uptake (Figure 1). In addition, a comparative analysis showed that our strategy outperforms PCSK9 knockout and Statins in increasing cholesterol uptake. These findings support our CRISPR-based gene editing strategy of truncating regions 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.

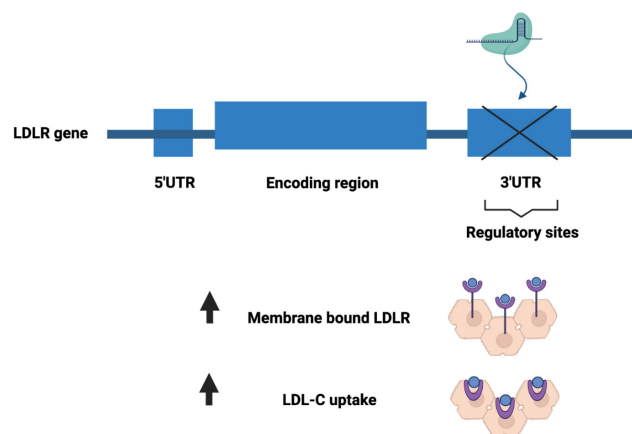


Figure 1: CRISPR-based gene editing for truncating regulatory elements in the 3'UTR of the LDLR gene results in increased expression of membrane-bound LDLR and enhanced LDL-C uptake.

1000. Liver-Directed Homogentisate 1,2-Dioxygenase (HGD) Gene Therapy Blocks Homogentisic Acid (HGA) Accumulation and Ochronosis in a Novel Mouse Model of Alkaptonuria

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Alkaptonuria is a rare recessive metabolic disease with a global prevalence of 1:100,000 - 250,000 and it is caused by a deficiency of the enzyme Homogentisate 1,2-dioxygenase (HGD). HGD is the third enzyme in the phenylalanine/tyrosine metabolism pathway that breaks the aromatic ring in homogentisic acid (HGA). Mutations in the Hgd gene lead to an accumulation of HGA in plasma, despite massive daily

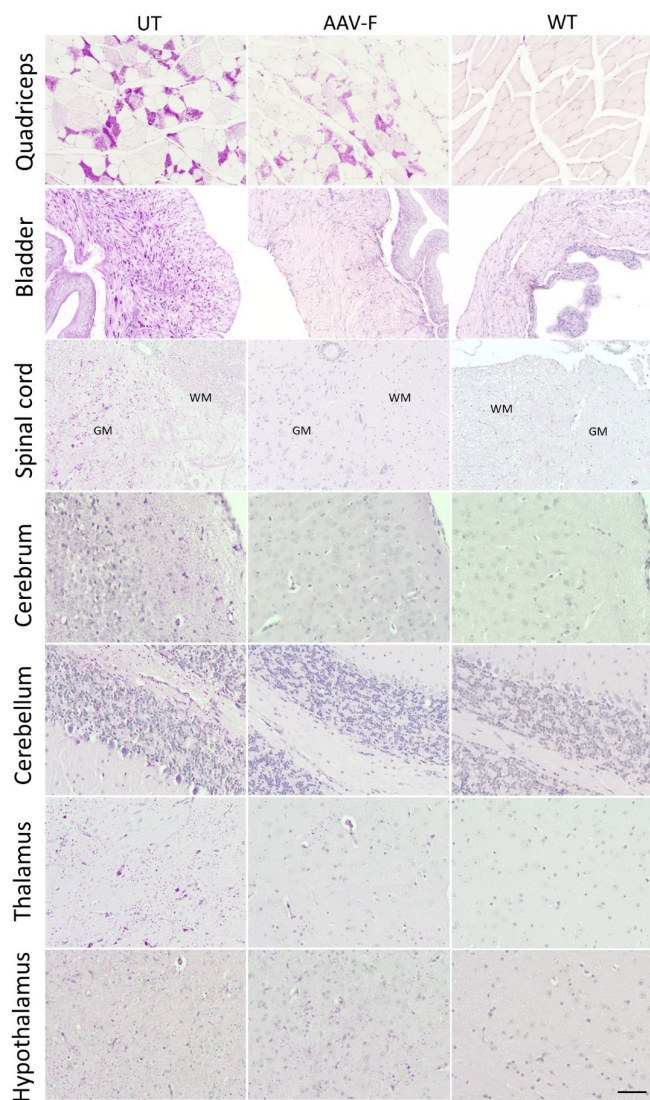
excretion of HGA by the kidneys. HGA enters an alternative metabolic pathway where it oxidizes into benzoquinone acetic acid (BQA) which in turn forms melanin-like polymers that accumulate to cause the pathognomonic darkening of urine and collagenous tissues, especially cartilage. This process is called Ochronosis (as the tissue looks ochre). Ochronotic tissue is stiffened and unusually brittle, leading to early-onset arthritis that necessitates joint replacements usually at an early age. Beyond the pain from the destruction of the connective tissue, the accumulation of HGA can also lead to multiple organ stones, aortic valve disease, and ocular manifestations. Until recently only dietary modifications and palliative care (physiotherapy, pain management and joint/cardiac valve replacement) were the standard of care for AKU and were highly unsatisfactory. Orfadin (Nitisinone) a 4-Hydroxyphenyl pyruvate dioxygenase inhibitor was recently approved in Europe to treat AKU in adult patients. Orfadin reduced circulating HGA, but development of increased levels of tyrosine which can lead to serious health issues. As liver and kidney are the principal organs expressing HGD and normalization of plasma HGA levels has been observed after liver transplantation in AKU patients, we hypothesized that liver-directed gene therapy would restore sufficient HGD expression to reduce plasma HGA levels and prevent ochronosis. A new mouse model of AKU was generated through the deletion of exons 4-7 of the Hgd gene, generating a frameshift from exon 3 to exon 8 and a premature stop codon in exon 8. This deletion resulted in a loss of function of Hgd and recapitulated key clinical features of human disease, including elevated HGA in plasma and urine ([HGA] ~150 μ M and ~200 mM, respectively), and cartilage in stifle joints displaying the characteristic ochronosis upon Schmorl's staining. Liver-directed gene therapy was tested in the AKU model with a single dose of a construct containing a mouse transthyretin liver promoter (mTTR), the wildtype mouse Hgd cDNA (mHGD) and poly-adenylation sequence (PolyA) packaged in an Adeno Associated Virus capsid (AAV.SAN001). Hgd KO mice were dosed with either 3×10^{11} or 1×10^{12} vg/animal via intravenous injection and tested against an empty vector control. Animals were followed for 7 months following administration. Transduction efficiency of Hgd-AAV.SAN001 was measured in the liver tissue of treated animals and found to be >1 vg/cell. Efficacy was initially evaluated by assessing renal clearance of HGA in acidified urine using LC-MS/MS. Both dosages of Hgd-AAV.SAN001 resulted in a reduction of greater than 90% of [HGA] over the course of the study. Plasma HGA levels followed a similar pattern, with both treatment groups maintaining a ~99% reduction over the course of the study. Single-dose treatment with Hgd-AAV.SAN001 also reduced stifle joint ochronosis. These data show that liver-directed gene therapy could be developed into a viable treatment option for AKU patients.

1001. AAV Gene Therapy Effectively Transduces the Central Nervous System and Prevents Polyglucosan Body Formation in Adult Polyglucosan Body Disease Mice

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Background: In patients with adult polyglucosan body disease (APBD), insufficient glycogen branching enzyme (GBE) activity due to pathogenic variants in the *GBE1* gene leads to intracellular formation of less-branched, poorly soluble glycogen aggregates (polyglucosan bodies, PB) across the central nervous system (CNS). Patients with APBD often suffer from progressive neurogenic bladder, spasticity, gait difficulty, paresthesia, fatigue, and cognitive disturbances. Treatment at this time is symptomatic and therapeutic development remains a significant clinical unmet need. In human APBD patients, leukodystrophy, astrogliosis, and spinal cord atrophy are prevalent and debilitating, thus a gene therapy to the CNS is highly desired. Though adeno-associated virus (AAV) vectors are increasingly promising in gene delivery for treatment of genetic diseases, their low efficiency in transducing the CNS when delivered intravenously has been a major hurdle. The recently developed AAV-F capsid, a new AAV-9 variant, was reported to have superior CNS-transduction capability in mice following intravenous administration (Hanlon *et al.*, 2019). In this study, we tested this capsid to evaluate gene delivery and gene expression in the CNS in a mouse model of APBD. This proof of concept study shows the impact of gene replacement therapy for the CNS manifestations of APBD. **Methods:** An AAV vector expressing mouse GBE (mGBE) driven by a ubiquitous promoter was packaged as AAV-F (AAV-F packaging plasmid was obtained from Dr. Casey Maguire of Massachusetts General Hospital) and intravenously (tail vein) injected into 6-week-old APBD mice at a dose of 2.5×10^{13} vg/kg. Mice were euthanized 20 weeks later. Age-matched untreated APBD mice (UT) and wild-type (WT) mice were used as controls. Fresh tissues were immediately frozen for biochemical analyses (AAV copy number, GBE activity, and glycogen content) or fixed in 10% neutral buffered formalin for Periodic acid-Schiff (PAS)-diastase staining. Slides stained with PAS-diastase were evaluated using light microscopy for evidence of PB accumulation. **Results:** AAV genome copy numbers (copies/genome) were 27 ± 6.67 in the brain, 2.77 ± 0.79 in the quadriceps muscle, and 15.29 ± 5.57 in the liver of AAV treated mice. Correspondingly, AAV treatment led to higher GBE activity than UT in the brain (11.3 ± 2.9 vs 0.7 ± 0.2 μ mol/min/g tissue) and quadriceps (11.5 ± 4.9 vs 0.9 ± 0.1 μ mol/min/g tissue). Significant reductions in glycogen content in brain (-53%) and quadriceps muscle (-53%) were observed in AAV-treated mice when compared with UT. PAS-diastase stain (**Figure 1**) demonstrates widespread PB accumulation in the brain, spinal cord, and muscles of UT mice. AAV treatment resulted in no visible PB in the spinal cord (similar to WT) as compared to UT. In the brain, AAV treatment prevented PB formation in cerebrum, cerebellum, and medulla, and remarkably reduced PB accumulation to varying extents in midbrain, thalamus, hypothalamus, and pons. AAV treatment also reduced the amount of PB in both skeletal muscle (quadriceps) and smooth muscle (bladder). **Summary:** This proof of concept study with intravenously administered AAV-F demonstrated the ability of gene therapy to reduce polyglucosan body formation in the brain and spinal cord in APBD mice. **Figure 1. PAS-diastase staining of polyglucosan bodies (purple) in tissues.** Scale bar: 100 μ m.



1002. Oral Delivery of Encapsulated Biosensor Bacteria for Diagnosis of Colon Inflammation in a Rat Model

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Background: The immune system is tolerant of billions of commensal bacteria in the mucosal gastrointestinal (GI) environment. Disruptions in microbiome homeostasis can lead to the development of inflammatory bowel disease. IBD is an incurable chronic disorder characterized by recurrent inflammation of the gastrointestinal tract. The disease's progressive nature necessitates early diagnosis. The recent discovery of IBD-related inflammatory biomarkers has highlighted a potential avenue for obtaining such diagnostic information¹. The

inflamed GI produces various metabolites that can provide important information regarding the degree of homeostatic disruption and disease etiology. Synthetic biology enables engineering of bacteria to function as living diagnostics capable of sensing these metabolites and relaying quantitative information regarding inflamed GI tract². **Methods:** We have combined innovative synthetic biology approaches with hydrogel encapsulation technologies to develop a biomaterial carrier platform that can significantly improve the viability and performance of diagnostic bacteria. Briefly, *E. coli* Nissle 1917 bacteria were genetically engineered to respond to thiosulfate and kynurenine by ligand-induced signaling through a *Shewanella halifaxensis* ThsSR two-component system and KynR one-component system, respectively. To develop hydrogel spheres, these bacteria were encapsulated in alginate, a biocompatible polymer. These spheres were then deployed as living diagnostics in a DSS model of gastrointestinal inflammation, where they sensed fluctuations in levels of each metabolite and produced fluorescent protein reporters GFP and CyPet in response. **Results:** Our results demonstrate that greater than 90% of bacteria remain viable following encapsulation and that there is no significant effect on sensor function or metabolite dose-response characteristics *in vitro*. We also observed the ability of hydrogel encapsulation to regulate the proliferation of the bacteria. A plateau in growth was reached 48 hours after encapsulation at which point the population carrying capacity was measured to be 10^7 CFU. After oral administration, hydrogels were retrieved from fecal samples where a 15-fold increase in CFU was measured, indicating the ability for nutrient exchange throughout digestion. No significant effect on hydrogel integrity was observed, highlighting this platform's potential to mitigate the risk of proliferation in the host. Finally, in the disease model, fluorescent reporter expression linked to the local presence of metabolites correlated linearly with increases in disease activity index. These results were confirmed with histological analysis of the rat colon, where high histological score correlated with DAI and fluorescent reporter expression. **Conclusion:** In summary, we demonstrated the viability of a minimally invasive oral delivery approach to facilitate rapid bacterial retrieval from stool samples, effectively streamlining the diagnostic process. This technology may provide significant advances for the clinical standards of care and the administration and future approval of living bacterial diagnostics as tools to sense and respond to the gut microenvironment.

1003. Hypothalamic BDNF Gene Therapy Ameliorates Abnormal Metabolic Function in the *Magel2*-Null Mouse Model of Prader-Willi Syndrome

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Prader-Willi syndrome (PWS) is a contiguous gene syndrome which occurs in approximately 1 in 15,000 individuals. PWS patients display developmental delays, cognitive impairment, excessive eating, obesity, hypothalamic hypogonadism, obsessive compulsive behavior, anxiety, and temper tantrums. Current treatments to address metabolic dysfunction and behavioral abnormalities are limited to strict supervision of food intake and growth hormone therapy.

PWS management is largely supportive and results in high levels of caregiver burden, highlighting the urgent need for new therapeutic strategies. Loss of function of MAGE Family Member L2 (*MAGEL2*) is thought to contribute to several aspects of PWS pathophysiology, including alterations in the hypothalamic leptin-proopiomelanocortin (POMC) pathway, which interprets peripheral signals of energy needs to drive feeding or fasting. In the PWS-driven absence of *MAGEL2*, the satiety response is blunted and feeding continues, resulting in an inability to maintain energy homeostasis. Brain-derived neurotrophic factor (BDNF) serves as a potential therapeutic target, as it functions downstream in the leptin-POMC pathway and has known roles in energy homeostasis and behavior. PWS patients display reductions in circulating BDNF, and furthermore, PWS-related transcriptomic alterations in the leptin-POMC pathway are associated with reduced BDNF expression. In this preclinical study, we assess the translational potential of hypothalamic AAV-BDNF gene therapy as a novel therapeutic for PWS-related metabolic dysfunction. To facilitate clinical translation, our BDNF vector included an autoregulatory element allowing for therapeutic titration of the transgene in response to the host's physiological needs. Adult female wild-type and *Magel2*-null mice were hypothalamically injected (1.0×10^{10} viral genomes, bilaterally) with either AAV-BDNF or AAV-YFP (yellow fluorescent protein) control. Following injections, mice were subjected to various measures of metabolic and behavioral function over 23 weeks. In adult female *Magel2*-null mice, BDNF gene transfer prevented weight gain, decreased fat mass, and increased lean mass. These changes were accompanied by altered food intake and favorable alterations in energy expenditure as measured by indirect calorimetry. Moreover, BDNF gene therapy improved glucose metabolism, insulin sensitivity, and circulating adipokine levels in *Magel2*-null mice. Improvements in metabolic function were maintained through 23 weeks with limited adverse behavioral effects, indicating high levels of efficacy and safety. In summary, these preclinical data implicate hypothalamic BDNF as a potential target for treating PWS-related metabolic abnormalities and warrant further investigation.

1004. Improvement of Hypertrophic Cardiomyopathy in Two Different Pompe Knock-In Murine Models with Weekly Enzyme Replace Therapy

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Pompe disease (PD) is an autosomal recessive disorder caused by deficient lysosomal acid α -glucosidase (GAA) that in turn leads to reduced degradation and accumulation of intra-lysosomal glycogen in all tissues, especially skeletal and oftentimes cardiac muscle. Enzyme replacement therapy (ERT) with recombinant human GAA enzyme (rhGAA) is the currently FDA approved treatment for PD patients. In order to study the pathogenic mechanism and potential novel therapeutic strategies, we generated two novel PD murine models orthologous to human GAA mutations, c.1826dupA (p.Y609*) and

c.1935C>A (p.D645E) with CRISPR-Cas9 homology-directed repair approach. To elucidate the impact of ERT in these two newly created Gaa mutant mouse strains and establish proof-of-concept of ERT efficacy in the two models, we administered intravenous rhGAA ERT (20 μ g/g weekly) or saline via tail-vein injection for 20 weeks on both strains and unaffected heterozygote controls. Echocardiography was performed at week 19, before the last dose of ERT. One day following final ERT dose at week 20, necropsy was performed, diaphragm, myocardium and gastrocnemius were harvested for GAA enzyme activity assays and glycogen quantification. Biochemical outcomes were compared with untreated *Gaa*^{c.1826dupA}, *Gaa*^{c.1935C>A}, and *Gaa*^{c.1826dupA/+} heterozygous mice. Antibodies against rhGAA were monitored from biweekly plasma collection during the 20-week course of treatment. Comparing with age-matched, untreated mutant mice, echocardiography results showed ERT-treated animals had significantly reduced cardiac wall thickness indices (IVSd, LVPWd, and LVMI) making them comparable to age-matched unaffected mice. Biochemical analyses in diaphragm, myocardium, and gastrocnemius muscle of ERT-treated animals showed significant uptake of the administered enzyme with supra-physiological level of GAA activity. Glycogen levels were reduced to unaffected levels in diaphragm and myocardium, but only partial clearance of glycogen accumulation was observed in gastrocnemius. Anti rhGAA immunoglobulin G (IgG) was observed in all ERT-treated cohorts including the treated heterozygous mice; titers were indistinguishable across the cohorts, suggesting humoral immune responses were generated against recombinant human enzyme. Altogether, preclinical knock-in models of PD recapitulate a response like that observed in human patients which will further accelerate our understanding of how pathogenic GAA mutations result in variable disease onset, progression, and response to current and future therapeutic strategies.

1005. Developing an Improved Understanding for the Neurophenotype of SLC6A8 Deficiency

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SLC6A8 deficiency is an inborn error of creatine metabolism caused by mutations in the X-linked *SLC6A8* gene, coding for a sodium- and chloride-dependent creatine transporter. SLC6A8 enables the uptake of creatine in cells, particularly neurons, that are incapable of endogenous synthesis. Creatine is a high-energy molecule that can come from dietary intake or be endogenously synthesized through the enzymatic activity of the enzymes amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) in the kidneys and liver, respectively, and is subsequently released into the circulation for uptake by other organs. Creatine plays a crucial role in the regeneration of adenosine triphosphate (ATP) in neurons to maintain high energy levels. Symptom onset of SLC6A8 deficiency typically occurs before 3 years of age and is estimated to occur between 0.3% and 3.5% in males with intellectual disability and 2% of all X-linked intellectual disabilities. Abnormalities in SLC6A8 are X-linked recessive; while males are primarily afflicted, females can have differing levels of disease severity. Patients typically have a mild to severe intellectual deficit with expressive speech and language delay; behavioral disorders (i.e. autism,

hyperactivity) are typically present while seizures and hypotonia are also commonly found. Since creatine is unable to enter cells in patients with defective SLC6A8, supplementation with oral creatine is ineffective. At present, there are no FDA-approved treatments for SLC6A8 deficiency. Due to the unmet need for basic studies and improved therapeutics, we plan to improve the field's understanding of the neurophenotype of SLC6A8 deficiency with the goal of developing a gene therapy approach to reestablish the functional creatine transporter *in vivo*. We hypothesize that anatomic and functional abnormalities exist in human neurons deficient in SLC6A8 and that resolution of these abnormalities can be achieved through genomic correction. To test this hypothesis, induced pluripotent stem cells (iPSCs) were reprogrammed from patient fibroblast lines containing unique individual mutations using a plasmid vector containing the 4 Yamanaka factors. Pluripotency was confirmed by immunocytochemistry and teratoma formation in NSG mice. Karyotypic analysis was also performed to confirm that no abnormalities were introduced through reprogramming. *SLC6A8* was corrected in iPSCs using CRISPR/Cas9-mediated homology directed repair after nucleofection of the nuclease and donor templates. On-target editing by Cas9 was confirmed through sequencing. iPSCs are currently being transduced with a lentivirus driving inducible Neurogenin-2 expression to enhance the differentiation of iPSCs into cortical neurons. We predict that SLC6A8-deficient cortical neurons will have abnormal axonal growth, dendritic arboring, functional marker expression, and electrophysiology compared to wildtype controls, and that genomic correction will ameliorate these abnormalities. These studies will provide a foundation leading to the development of novel gene therapies for SLC6A8 deficiency by providing insight on the effectiveness of genomic correction in SLC6A8 deficient human neurons.

1006. Gene Replacement Therapy with JAG101 Reduces Pathogenic Biomarkers in a Mouse Model of Type 1 Galactosemia

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Background: Type 1 galactosemia is a rare genetic disease caused by autosomal recessive mutations in the *GALT* gene that lead to a severe deficiency of the galactose-1-phosphate uridylyltransferase (GALT) enzyme necessary for conversion of galactose to glucose and UDP-galactose. GALT deficiency leads to a toxic accumulation of multiple metabolites including galactose, galactose-1 phosphate (Gal-1p), and galactitol. The build-up of these metabolites is life-threatening in newborns and although such lethality can be prevented by newborn screening programs and a galactose-restricted diet, endogenous production of galactose persists. As a result, despite continued dietary intervention, most patients develop lifelong cognitive, neurological, and speech complications, as well as primary ovarian insufficiency in females. Therefore, there remains a high unmet need to develop disease-modifying therapies. JAG101 is an investigational AAV9 gene therapy in preclinical development intended as a one-time treatment to restore levels of functional GALT enzyme during the critical postnatal to early childhood period. Here, we present data from a pre-proof-of-concept (POC) study characterizing the effects of JAG101 on key biomarkers in a mouse model of Type 1 galactosemia.

Methods: *GALT* gene-trapped mouse pups were treated on postnatal day 9 (P9) with high (1.15E14 vg/kg) or low (3.74E13 vg/kg) dose IV JAG101, or vehicle. Animals were sacrificed at either 4 or 12 weeks after treatment, and organs that have the highest degree of damage in galactosemia patients were assessed in the mice. Postmortem tissue analyses included assessment of biomarkers for galactosemia pathophysiology, GALT enzyme activity and immunohistochemistry. **Results:** Treatment with JAG101 led to a significant reduction of all three major metabolites associated with Type 1 galactosemia. In the brain at 4 weeks post-treatment, high-dose JAG101 led to a 76% reduction ($p=0.0017$) and low-dose JAG101 led to a 55% reduction ($p=0.009$) in galactose levels vs. vehicle. Brain Gal-1p levels were reduced by 59% ($p<0.0001$) and 48% ($p=0.0001$), and brain galactitol levels were reduced 48% ($p=0.0005$) and 27% ($p=0.0178$) by high and low doses of JAG101, respectively, vs. vehicle. Reductions of all metabolites in the brain were still apparent at 12 weeks following treatment and remained statistically significant in the high-dose JAG101 treatment arm vs. vehicle. In muscle at 4 weeks, galactose was reduced by 51% ($p=0.0003$) and 33.6% ($p=0.0068$), and galactitol was reduced 66.8% ($p<0.0001$) and 57.8% ($p<0.0001$) by high and low doses of JAG101, respectively, vs. vehicle. These metabolite changes in muscle were even greater at week 12, when galactose was reduced by 52% ($p<0.0001$) and 45% ($p<0.0001$) and galactitol by 78% ($p<0.0001$) and 74% ($p<0.0001$) by high and low doses of JAG101, respectively. There were no significant changes in Gal-1p in the muscle at week 4 (21% reduction for high-dose JAG101 vs. vehicle, $p=0.3609$), but there was a significant reduction of the biomarker in the high-dose JAG101 treatment arm at week 12 vs. vehicle (38%, $p=0.0185$). This delayed change aligns with clinical experience where intracellular Gal-1p is the slowest metabolite to be impacted by dietary changes. **Conclusion:** The results of this pre-POC pilot study to assess biomarker changes in relevant tissues of interest suggest that a one-time treatment with JAG101 in patients with Type 1 galactosemia may lead to the reduction of metabolites that are known to contribute to disease pathophysiology. Additional preclinical studies are being conducted to evaluate the effect of JAG101 on measures of behavior and fertility.

1007. Defining Minimal Requirements for Sustained Efficacy with Adeno-Associated Viral Vector Based Human Phenylalanine Hydroxylase Gene Transfer to Adult *Pah*-KO Mouse, a Model of Human Phenylketonuria

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Phenylketonuria (PKU) is a genetic deficiency of human phenylalanine hydroxylase (PAH) in liver and results in elevated levels of phenylalanine (Phe) in brain with subsequent neurotoxicity. Standard care consists of Phe restricted diet but compliance among teen and adult patients is poor. A pegylated phenylalanine ammonia lyase ("Palynzig") and

synthetic cofactor (“Kuvan”) are alternative approved therapies and work for some patients but require frequent administration. Hence, strategies providing long-term stable blood Phe control have entered the clinic including recombinant adeno-associated virus (rAAV) based PAH gene transfer to liver. To evaluate the sustained efficacy requirements for the optimized Sanofi rAAV-based gene therapy vector, rAAV.SNY001-WTPAH, we performed a 5-week and 4-month studies in *Pah*-KO mice, a model of human PKU. The vector was delivered one time by IV route into adult male mice at doses of 5×10^{12} , 2×10^{13} and 5×10^{13} vg/kg. The treatment provided significantly improved growth for the animals (increased liver, brain and bodyweights) highlighting a correction of a metabolic dysfunction in previously untreated mice under a normal rodent diet. Transfer of *Pah* gene to liver lowered blood and brain Phe levels comparable to Phe levels in normal mice though variability was observed with the lowest dose cohort. Blood Phe lowering correlated with a dose-dependent detection of vector DNA, vector derived mRNA, PAH positive cells and PAH enzymatic activity in livers of the treated *Pah*-KO mice. Blood Phe lowering restored large neutral amino acid transport into the brain, normalized brain neurotransmitters such as dopamine and serotonin levels and improved brain myelin content. These biochemical changes, in particular the brain serotonin, correlated to improved behavior measured by nest building assay and the effect was already detected by 35 days post treatment. Overall, the study allowed defining the levels of vector DNA, mRNA and PAH positive cells in liver required for blood Phe normalization and hence, roughly 0.1 vg/cell and 20% of PAH positive hepatocytes were sufficient to correct the disease pathology for the study’s duration despite a significant bodyweight increase observed in the 4-month study. In summary, our work demonstrated that the optimized rAAV.SNY001-WTPAH reduced both blood and brain Phe levels, hallmarks of the disease, and the majority of measured PKU related pathologies in adult *Pah*-KO mice in a sustained manner and therefore supports use of the optimized rAAV.SNY001-WTPAH for adolescent and adult PKU patient treatment to provide stable blood Phe control.

1008. Gene Replacement with JAG101 Leads to *GALT* Transgene Expression in Target Organs and Reduces Toxic Metabolites in a Rat Model of Type 1 Galactosemia

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Background: Type 1 galactosemia is an autosomal recessive disease caused by mutations in the *GALT* gene that lead to profound deficiency of galactose-1-phosphate uridylyltransferase (*GALT*), an enzyme required to break down galactose. *GALT* deficiency results in an accumulation of galactose metabolites that can be life-threatening in newborns exposed to galactose from breast milk or dairy milk formula. Immediate restriction of dietary galactose can address acute symptoms of the disease. However, despite continued dietary intervention, most patients develop lifelong cognitive, neurological, and speech complications, ostensibly due to the endogenous production of

galactose. Thus, there remains a high unmet need to develop disease-modifying therapies. JAG101 is an investigational AAV9 gene therapy in preclinical testing intended as a one-time treatment to restore metabolically effective levels of *GALT* activity during the postnatal to early childhood period considered most vulnerable. Here, we present data from a pre-proof-of-concept study characterizing the biodistribution of JAG101 transgene expression and its effects on key biomarkers and cataracts in a rat model of Type 1 galactosemia.

Methods: *GALT*-null rats were treated on postnatal day 2 (P2) with high (1.16×10^{14} vg/kg) or low (3.82×10^{13} vg/kg) dose IV JAG101, or vehicle. All rats were euthanized at either 2 or 5 weeks after treatment, and blood and key organs were assessed for *GALT* transgene expression, activity, and metabolic efficacy.

Results: *GALT* activity in liver and brain was undetectable in vehicle-treated rats but was strikingly elevated in JAG101-treated animals. Both enzyme activity assays and immunohistochemistry (IHC) staining showed that these increases were significant, dose-dependent, and present through 5 weeks. Further, brain samples showed robust *GALT* IHC signal in neurons and glia in both cortex and cerebellum following high-dose JAG101. Finally, strong *GALT* enzyme activity and IHC signals were observed in skeletal muscle at 5 weeks post-dosing in rats treated with JAG101. Consistent with the role of *GALT* in the Leloir pathway, JAG101 not only restored *GALT* activity but also significantly reduced all three major metabolites associated with galactosemia: galactose, galactitol, and galactose 1-phosphate (Gal-1p). In plasma, JAG101 reduced galactose by up to 96% and 97% and galactitol by up to 92% and 50% at 2 and 5 weeks, respectively. In the brain, JAG101 reduced galactose by up to 96% and 95%; galactitol by up to 93% and 86.5%; and Gal-1p by up to 30% and 89% at 2 and 5 weeks, respectively. In the liver, JAG101 reduced galactose by up to 99% and 95%; galactitol by up to 88% and 35%; and Gal-1p by 95% and 97% at 2 and 5 weeks, respectively. Finally, compared to vehicle-treated rats, JAG101 reduced the incidence and severity of cataracts at both timepoints. **Conclusion:** These data provide evidence that JAG101 increases *GALT* enzyme expression and function in both liver and brain; tissues relevant to long-term outcomes in galactosemia. Further, detection in muscle, a relatively stable population of cells, may predict extended durability of transgene expression following a single early dose. In sum, the results reported here documenting *GALT* restoration, metabolic efficacy, and representative phenotypic efficacy in a rat model of *GALT* deficiency support the continued development of JAG101, and suggest that lowering of toxic metabolites associated with Type 1 galactosemia correlates with prevention of disease progression in a target organ.

1009. AAV5-GLA Gene Therapy Results in Sustained Long Term GLA Transgene Expression and Cross Correction of Target Organs in Fabry Disease Mouse Model

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Fabry disease is an X-linked hereditary metabolic disorder caused by mutations in the gene that encodes alpha-galactosidase A (GLA). Deficiency of GLA leads to accumulation of globotriaosylceramide Gb3 and its deacylated derivative globotriaosylsphingosine (lysoGb3) in cells and plasma, resulting in cell abnormalities and organ dysfunction affecting heart, kidney and brain. Current enzyme-replacement therapy has to be administered frequently, has infusion related side-effects and has limited therapeutic efficacy because of poor cross-correction of heart and kidney phenotypes. We developed an AAV5 gene therapy expressing GLA under the control of a strong, proprietary liver-specific promoter. Injection of increasing concentrations of AAV5-GLA in GLA-knockout mice showed a dose-dependent increase in GLA-activity and lowering of (lyso)Gb3 in plasma and liver, kidney, heart and brain. Tissue staining confirmed the presence of GLA-protein and image quantification showed a dose dependent increase of GLA-protein in kidney glomeruli, a key structure for kidney function. Additionally, 10 weeks after AAV5-GLA injection, GLA-knockout mice showed improved nociception approaching that of wild-type animals. Moreover, treatment efficacy and durability was confirmed at 6 months post-IV injections demonstrating sustained long term GLA transgene expression and efficacy of the AAV5-GLA vector in the Fabry disease mouse model. The long-term reduction of substrate in target organs, the phenotypic improvement in GLA-knockout mice, and the favorable profile of AAV5-based gene therapy in humans strongly suggest that AAV5-GLA is an attractive approach for treating human Fabry disease.

1010. AAV-Mediated Gene Therapy for Maturity-Onset Diabetes of the Young Type 3 (MODY3)

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Maturity-onset diabetes of the young (MODY) are a group of monogenic diabetes characterized by onset of hyperglycemia at an early adult age, generally before 25 years. MODYs collectively represent 1-2% of all cases of diabetes. Among them, MODY3, the most common type of MODY, is caused by mutations in the gene encoding for the transcription factor hepatocyte nuclear factor 1A (HNF1A). MODY3 patients are generally misdiagnosed as Type 1 or Type 2 diabetic patients, and treated with lifestyle interventions and drugs that do not address the underlying genetic defect of their disease. The genetic cure of MODY has never been attempted. Here, using a unique novel strategy based on the CRISPR/Cas9 technology, we generated the first MODY3 mouse model that recapitulated the beta-cell dysfunction and diabetes, faithfully mimicking the human clinical phenotype. MODY3 mice treated with AAV vectors encoding HNF1A showed markedly increased HNF1A expression levels and HNF1A protein content in pancreatic islets as well as upregulation of HNF1A target genes, which mediated counteraction of the hyperglycemia under fed and fasted conditions and improvement of glucose tolerance. This study constitutes the first demonstration for the genetic counteraction of a monogenic diabetes and paves the way to the treatment of MODY3 patients in the future. Moreover, these results will also contribute to the development of novel gene therapy approaches for other monogenic forms of diabetes or even for type 2 diabetes.

1011. AAV-Mediated Gene Therapy of Spinal Muscular Atrophy with Progressive Myoclonic Epilepsy (SMA-PME) and Farber Disease

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Farber disease (FD) and spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME) are autosomal recessive disorders caused by mutations in the *ASAHI* gene, which codes for acid ceramidase (ACDase), a lysosomal enzyme that catalyses the bioactive lipid ceramide into sphingosine and fatty acid. These *ASAHI*-related disorders present with a spectrum of clinical manifestations and lead to death in most cases during early infancy (FD) or at teenage (SMA-PME). To date, there is no curative treatment for patients and therefore a clear unmet medical need. In the present study, we evaluated a gene therapy approach in a mouse model of acid ceramidase deficiency. We report that intravenous administration of a recombinant AAV9 vector expressing human ACDase in diseased *Asah1* P361R/P361R mice prolonged survival of all animals until the end of a 6-month study, restored body growth and motor activity, and abolished inflammation in peripheral tissues and the central nervous system. Our findings provide proof-of-concept that systemic AAV-mediated *ASAHI* gene replacement can correct the severe phenotype of a mouse model of acid ceramidase deficiency, paving the way for clinical translation in patients.

1012. AAV-Mediated BMP7 Gene Therapy Counteracts Insulin Resistance and Obesity

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Type 2 diabetes, insulin resistance and obesity are strongly associated and are a major health problem worldwide. Obesity largely results from a sustained imbalance between energy intake and expenditure. Therapeutic approaches targeting metabolic rate may counteract body weight gain and insulin resistance. Bone morphogenic protein 7 (BMP7) has proven to enhance energy expenditure by inducing non-shivering thermogenesis and to improve insulin sensitivity in short-term studies in mice treated with the recombinant protein or adenoviral vectors encoding BMP7. Nevertheless, periodic administrations of recombinant BMP7 protein are required, which in addition could compromise treatment compliance by patients. Likewise, the use of Ad-BMP7 vectors is not able to promote long-term expression of the transgene due to the high immunogenicity of Ad vectors. To achieve long-term BMP7 effects, the use of adeno-associated viral (AAV) vectors would provide sustained production of the protein after a single administration. Here, we demonstrated that treatment of high fat diet-fed mice and ob/ob mice with liver-directed AAV-BMP7 vectors enabled a long-lasting increase in circulating levels of this factor. This rise in BMP7 concentration induced browning of white adipose tissue (WAT) and brown adipose tissue activation, that enhanced energy expenditure, and reversed WAT hypertrophy, hepatic steatosis and WAT and liver inflammation, ultimately resulting in normalization of body weight and insulin resistance. This study underscores the potential of AAV-BMP7-mediated gene therapy for the treatment of insulin resistance, type 2 diabetes and obesity.

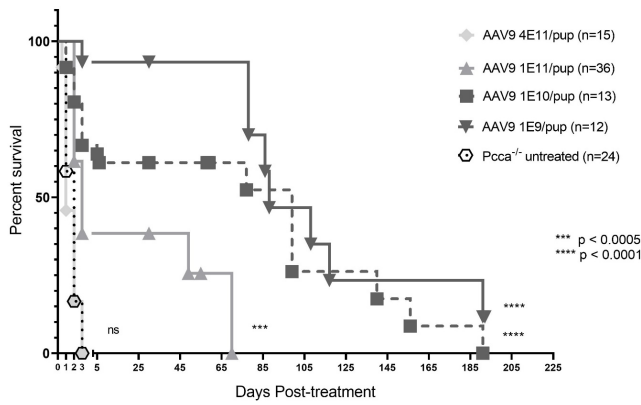
1013. Systemic AAV9 Human Propionyl-CoA Carboxylase, Alpha Subunit (AAV9-hPCCA) Gene Therapy Provides Direct and Sustained Therapeutic Benefits in the Treatment of Propionic Acidemia: From Animal Model to Orphan Drug Designation (ODD)

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Propionic acidemia (PA) is serious and often lethal organic acidemia caused by deleterious mutations in either the *PCCA* or *PCCB* genes, which encode for the α and β subunits, respectively, of the multimeric enzyme, propionyl-CoA carboxylase (PCC). PCC is ubiquitously expressed

and plays a critical role in the terminal metabolism of the branched chain amino acids, odd chain fatty acids and cholesterol. Elective liver transplantation has been offered to severely affected patients because there are no approved therapies for the treatment of PA. Systemic adeno-associated virus 9 (AAV9) gene therapy could enable hepatocardiac correction, provide a viable alternative to liver transplantation that might also ameliorate or prevent cardiomyopathy, a devastating complication of PA, as well as disease progression in other organs. To develop an AAV9 gene therapy to treat PA as an NCATS sponsored Platform Vector Gene Therapy program (PaVe-GT) indication, we first developed improved *Pcca* mouse models and then tested a series of AAV9 vectors expressing a human codon optimized cDNA encoding the propionyl-CoA carboxylase, alpha subunit (*PCCA*) under control of the elongation factor 1 alpha (EF1a) promoter. The most efficacious vector, as assessed by therapeutic effects in PA mice, was further refined for compatibility with manufacturing, and a large-scale vector lot was produced for detailed POC and dose finding studies using *Pcca* knock-out mice (*Pcca*^{-/-}). These mice display uniform lethality by DOL2, and thus can be used to rapidly assess direct therapeutic benefits by analyzing survival-effects, and then longitudinally survey clinical and biochemical improvements in the treated PA mice as compared to untreated mutants and control littermates. The clinical candidate AAV9-hPCCA vector was delivered via retroorbital injection to neonatal mice at birth with doses of 1E9, 1E10, 1E11 and 4E11 GC/pup (equivalent to 7E11, 7E12, 7E13 and 2.8E14 GC/KG, respectively). A dose-dependent increase in *Pcca*^{-/-} survival was observed in the 1E10, 1E11 and 4E11 GC/pup dose range (See below Figure). Treatment with the AAV9-hPCCA vector was accompanied by improvements in clinical (prolonged survival, weight gain), metabolic (reduced plasma 2-methylcitrate levels), and enzymatic (restoration of 1-C¹³ 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. Our results firmly establish that systemic delivery of an AAV9-hPCCA vector rescues *Pcca*^{-/-} mice from neonatal lethality and provides sustained therapeutic benefits, which provides enabling data to support the translation of AAV9 gene therapy in human clinical trials. Based on our aggregate preclinical studies and epidemiological data that confirms PA is an ultrarare metabolic disorder, the FDA has recently granted NCATS an Orphan Drug Designation for AAV9-hPCCA to treat patients with propionic acidemia caused by a deficiency of PCCA.

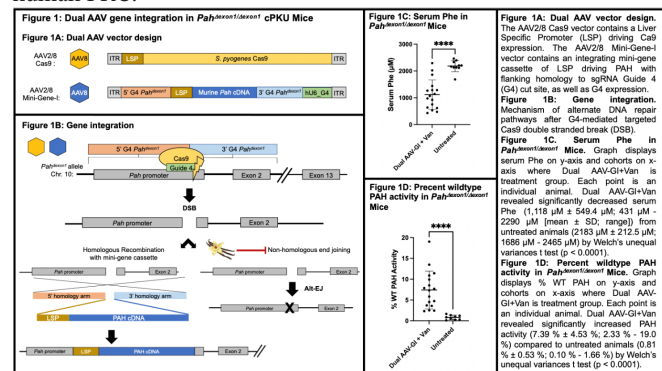


1014. A Broadly Applicable Gene Integration Approach in Murine Phenylketonuria (PKU)

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Phenylketonuria (PKU) due to recessively-inherited phenylalanine hydroxylase (PAH) deficiency, if left untreated, is associated with severe cognitive disability due to the accumulation of L-phenylalanine (Phe), which becomes a neurotoxin at high concentrations. Shortcomings in contemporary therapy support the need for novel treatments including gene correction. We have previously demonstrated lifelong reduction of serum Phe following AAV-delivered CRISPR/Cas9-facilitated homology directed gene editing in neonatal *Pah^{emu2/emu2}* mice, a model of human PKU caused by a missense mutation in the *Pah* gene. The wildtype repair template in this approach was designed only to repair the *Pah^{emu2}* mutation. However, in the human PKU population, there are over 1,000 known pathogenic *PAH* alleles, limiting the translatability of this singular gene correction approach. The current study focused on fusing the benefits of gene addition with gene editing to deliver a functional PAH expression cassette targeted for integration into the *Pah* gene (Figure 1). We utilized a novel murine PKU model, the *Pah^{Δexon1/Δexon1}* mouse, which harbors a *Pah* exon 1 deletion and lacks PAH monomer expression. The necessary reagents for gene targeting were delivered in two recombinant AAV2/8 vectors (Figure 1A). The initial success rate of the desired HDR is low (Figure 1B). To address this, we incorporated vanillin, a potent inhibitor of non-homologous end joining, that we previously showed enhanced HDR frequency. AAV vectors were administered systemically via facial vein (8.2×10^{10} vg AAV2/8 Cas9; 2.0×10^{11} vg AAV2/8 Mini-Gene-I) to post-natal day 3 progeny of *Pah^{+/-Δexon1}* dam X *Pah^{Δexon1/Δexon1}* sire matings, and 100 mg/kg vanillin was administered intraperitoneally daily thereafter for 7 days. Animals were genotyped at 2 weeks revealing N= 17 (N= 7 females, N= 10 males) *Pah^{Δexon1/Δexon1}*10; N=8 females, N=2 males) were evaluated as controls. Animals were weaned at 4 weeks, placed on standard mouse chow, and euthanized at 6-8 weeks of age for tissue harvest. Serum Phe levels of treated *Pah^{Δexon1/Δexon1}* animals were variable but overall significantly reduced from untreated *Pah^{Δexon1/Δexon1}*

animals (Figure 1C). The hepatic PAH enzyme activity similarly revealed a significant increase in % wildtype PAH activity in treated animals (Figure 1D). Further analyses quantifying precise molecular alterations at the genomic level are ongoing. This broadly applicable targeted gene integration approach is enduring and has the potential to provide benefit to all individuals with PKU regardless of their specific inherited *PAH* variant. This approach also provides the additional benefit of disrupting expression from the native *Pah* allele, eliminating any potential dominant negative effects from continued expression of a dysfunctional mutant PAH monomer that could diminish the activity of wildtype PAH. Further studies are needed to evaluate the observed variability in efficacy and any potential for adverse effects of off target integration or genome damage. Despite these challenges, gene integration is a promising platform as a novel therapeutic for human PKU.



1015. Gene Therapy for Complex Disease: In Vivo Pooled Screening Predicts Clinical Outcomes In Steatohepatitis

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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. In vivo testing is essential to determine therapeutic efficacy of candidates, since complex diseases involve the interplay of different cell types and contributions from the systemic environment. To increase the scale at which targets can be validated in vivo, we have developed and validated a screening platform where many AAV gene therapies are injected simultaneously, in a single animal, using single-cell genomics as a phenotypic readout for therapeutic efficacy of each candidate. Here, we present results demonstrating that this platform successfully predicts physiological outcomes in a disease involving multiple biological pathways as well as interactions with the systemic environment, namely non-alcoholic steatohepatitis (NASH). We screened 25 interventions in two mouse NASH models: AMLN diet (8mos) and the FAT model (Tsuchida et al 2018). Therapies were administered by AAV8 to target hepatocytes, and single-nucleus sequencing performed after 4 wks of therapeutic expression. Alongside control and novel interventions, we included six clinical NASH targets:

FXR, FGF19, FGF21, ASK1, PPARA, and PPARD. Our analysis defined 20 features of disease and normal physiology, e.g. de novo lipogenesis, TGFbeta signaling, and bile acid metabolism, and measured effects of each intervention on each feature, relative to controls in the same animal. Features were batched for comparison with clinical readouts, and predictions based on our *in vivo* screen were compared to actual outcomes in Phase 2 and 3 clinical trials. Out of 20 predictions made on the basis of the screen, 14 were consistent with clinical findings, 4 could not be compared due to missing clinical data, and 2 were inconsistent with clinical findings. Stronger predictions based on transcriptomics correlated with stronger clinical results. The majority of interventions had stronger effects on metabolic features than inflammation and fibrotic signaling, and this observation was confirmed in a followup mouse study administering single interventions per cohort. Validating the predictive power of a high-throughput *in vivo* screening platform opens the door to testing more therapeutic targets, and combinations of targets, for NASH and other complex diseases. By testing many interventions in each animal, disease biology can be explored in scarce models, e.g. spontaneous disease models and/or species (such as non-human primates) with human-like anatomy and pathophysiology. This should help enable the power of targeted, long-term gene therapies to be applied to a broader range of diseases.

1016. Haematopoietic Stem Cell Gene Therapy for Mucopolysaccharidosis II Using an IDS.ApoEII Brain Targeted Therapy: Completion of Preclinical Workup and Cell Manufacturing Optimisation

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Haematopoietic stem cell gene therapy (HSCGT) has proved successful in the treatment of a number of neurological paediatric disorders in recent clinical trials. In a proof-of-concept murine study we demonstrated enhanced brain-targeted by using a HSCGT strategy in combination with a lentiviral vector (LV) expressing IDS fused to ApoEII (IDS.ApoEII) for the treatment of Mucopolysaccharidosis type II (MPSII). MPSII is a debilitating paediatric lysosomal disorder caused by mutations in the iduronate-2-sulphatase (*IDS*) gene, with patients typically exhibiting severe neurodegeneration, skeletal abnormalities and cardiorespiratory disease. With our HSCGT system, we were able to normalise brain pathology and behaviour, and the peripheral symptoms of MPSII mice, providing significantly improved correction compared to mice receiving HSCGT with a LV expressing normal IDS or treatment with a conventional allogeneic bone marrow transplant. Here we describe additional preclinical studies conducted in order to accelerate translation of brain-targeted MPSII HSCGT to the clinic and provide additional safety and efficacy data. We performed Luminescence immunophenotyping on a range of organs from the proof-of-concept study including the brain, liver, plasma, spleen, brain, heart and lung using a 23-plex mouse cytokine panel and observed correction of a number of elevated chemokine and cytokine disease markers following HSCGT treatment in support of previous findings. In addition, we have developed an optimised clinical GMP stem cell transduction

protocol for MPSII, with the inclusion of the transduction enhancers protamine sulphate and LentiBOOST™, resulting in a 5-fold reduction in the amount of IDS.ApoEII lentiviral vector required per treatment to achieve a target vector copy number (VCN/dg) in the range of 2-5 copies. This work paves the way for a phase I/II HSCGT clinical trial in MPSII patients anticipated to commence in 2022.

1017. Magnetoliposomes as Carriers for CRISPR/nCas9-Based Gene Therapy on In Vitro Models of Mucopolysaccharidoses IVA and GM2 Gangliosidoses: Proof-of-Concept Experiments

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Introduction. Lysosomal storage diseases (LSD) belong to a group of heterogenic pathologies caused by monogenic mutations that alter several proteins associated with the lysosomal function. Consequently, gene therapy (GT) strategies have emerged as key therapeutic alternatives. In previous works, we tested this premise using CRISPR/nCas9-based gene therapy transfected *in vitro* with the aid of Lipofectamine 3000 (LP) in cellular models of GM2 gangliosidoses and MPS IVA. The treatment led to the recovery of cellular homeostasis after a long-term genome edition. **Methods:** Given that carriers for efficient delivery of GT continue to pose a major challenge, in this study, we evaluated the potential of magnetoliposomes (ML) to deliver a CRISPR/nCas9 system for GM2 gangliosidoses and MPS IVA. Magnetite nanoparticles (MNPs) were prepared by chemical coprecipitation of FeCl₂ and FeCl₃ (2:1 molar ratio) in the presence of 5M NaOH. A silver shell was added to the MNPs by a redox reaction involving 1 mM AgNO₃. A pH-responsive polymer (pDMAEMA) and the membrane translocating peptide Buforin II were conjugated to the core-shell nanoparticles (CS-NPs). Conjugation was confirmed by Fourier transform infrared spectroscopy and the efficiency via thermogravimetric analysis. The plasmids were loaded on the nanovehicle in the presence of PBS 8.0/TAE 8.0 washes. Liposomes were synthesized with soy lecithin as precursor and rotoevaporation to form a lipid film resuspended in buffer. Finally, ML were synthesized by mixing 50µg/mL of CS-NPs with 0,1mg/mL liposomes. Hydrodynamic radii and Zeta-potentials of CS-NPs and LP were characterized by dynamic light scattering. Cytotoxicity, enzyme activity, lysosomal mass, mitochondrial-derived oxidative stress (ROS), and total glycosaminoglycans (GAGs) were determined on human MPS IVA and GM2 gangliosidoses fibroblasts treated with the CRISPR/nCas9 system by standardized assays. Human healthy fibroblasts were used as control. **Results.** Cytotoxicity assays showed no significant reduction in viability (i.e., <10% of cell death for all the primary fibroblast tested) in the presence of ML (25µ/mL/0.1mg/mL magnetite/liposome ratio). We detected between 1.4- and 25.7-fold increase in transfection efficiency in cells treated with ML compared to LP, depending on the disease and the primary fibroblast cell line. Also, we detected an increase in GALNS

activity between 1.1- to 7.8-fold for all treated MPS IVA fibroblasts (n=4). We found a slight recovery in the lysosomal mass, oxidative stress, and total GAGs. Finally, in the case of GM2 gangliosidosis fibroblasts, no apparent differences were detected between ML-treated cells and those treated with LP. **Conclusion.** Our results suggest that depending on the mutation, a differential response might be observed, and consequently, new primary GM2 gangliosidosis cell models will be included in the future. However, our results demonstrate the potential of ML as promising nanovehicles for the efficient delivery of CRISPR/Cas9-based GTs with remarkable cytocompatibility and improved mutation-dependent phenotype recovery in MPS IVA fibroblast when compared with LP.

1018. Preclinical Use of a New scAAV9/SUMF1 Vector for the Treatment of Multiple Sulfatase Deficiency

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Introduction: Multiple Sulfatase Deficiency (MSD) is a rare autosomal recessive disorder characterized by deficient enzymatic activity of all known sulfatases. MSD patients frequently carried two loss of function mutations in the *SUMF1* gene, encoding a formylglycine-generating enzyme (FGE) which activates 17 different sulfatases. MSD patients shown common features of other lysosomal diseases like mucopolysaccharidosis and metachromatic leukodystrophy, including neurologic impairments, developmental delay, and visceromegaly. There are currently no approved therapies for MSD patients. Gene therapy is a promising approach to provide a long-term therapeutic benefit. **Methods:** Using *Sumf1* deficient (KO) mice as a model for MSD, we tested the efficacy of a newly developed self-complementary AAV9/SUMF1 vector as a gene replacement therapy. *Sumf1*-KO mice are characterized by a short life span with a median survival of 10 days. All the *Sumf1*-KO mice were bred in a mixed genetic background B6129S1. As a proof of concept, we injected scAAV9/SUMF1 in *Sumf1*-KO mice at post-natal day 1 (PND1) via an intracerebroventricular (ICV) route of administration. An additional dose-response study was performed by single intrathecal (IT) delivery at PND7, an age that might better model an intervention in human patients. We also tested the benefit of a combined dosing IT and intravenous (IV) at PND7. Mice were genotyped at PND0 and randomized across all experimental groups. **Results:** The ICV treatment resulted in extended life span without signs of neurological impairments at one-year post-treatment, with median survival of the cohort exceeding 320 days. We also tested the efficacy of the scAAV9/SUMF1 in symptomatic mice at PND7 by intrathecal delivery (IT) and a combined treatment targeting CNS and systemic organs by intravenous injection (IV). Both IT and combined IT/IV treatment were able to rescue the mouse phenotype, with more than 50% of the mice surviving beyond 450 days in the highest dose cohorts. Both treatments were similarly effective in correcting the cardiac and vision phenotypes present in *Sumf1*-KO mice. There was not a clearly increased benefit of the combination IT+IV treatment over IT alone. After a year of treatment, *in situ* hybridization analysis

showed a wide biodistribution of scAAV9/SUMF1 expression across the central nervous system. In the brain, aryl sulfatases A, B, C, and E, IDS and SGSH were significantly increased in a dose-dependent manner. In the liver, aryl sulfatases were also increased with treatment. No significant signs of toxicity derived from the viral vector were seen in treated mice after one year. Our preclinical results indicated that the use of scAAV9/SUMF1 for gene replacement therapy could certainly increase the activity of multiple sulfatases and dramatically increase survival, and is thus predicted to provide a benefit for MSD patients.

1019. A Generalizable Approach to Extracting Relevant Biomarkers for AAV Gene Therapy from Natural History Datasets: Application to Propionic Acidemia

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INTRODUCTION: Propionic acidemia (PA) is a metabolic disorder associated with significant multiorgan morbidity and abnormal growth. It has no FDA-qualified surrogate endpoints or FDA-approved genomic therapies. Like many other rare and orphan disorders, natural history data is difficult to collect, and the number of patients is limited. We have developed an analytic approach that uses machine learning to identify candidate biomarkers that correlate with clinical outcomes to support the development of novel genomic PA therapies, specifically systemic AAV gene therapy, a lead indication for the Platform Vector Gene Therapy (PaVe-GT) program. **METHODS:** PA participants were evaluated through a dedicated natural history study (ClinicalTrials.gov ID NCT02890342). The resultant cross-sectional PA dataset, comprised of > 500 clinical, laboratory and imaging parameters, was queried to identify variables associated with PA outcomes. k-Means clustering, supervised machine learning (support vector machine, SVM), and expert knowledge were used to train and test a sparse model of PA and identify stable patterns among categorical and continuous variables. Improvement in the accuracy of SVM models over baseline was used as the performance metric to identify promising novel biomarkers. Highly ranked variables were further validated as response biomarkers in a subset of liver transplanted PA participants. **RESULTS:** 40 PA participants, ages 2-52 years (mean age 16 years, 45% females, 5 individuals status post liver transplants), underwent deep phenotypic evaluation and laboratory sampling at NIH. Clustering and dimensional reduction confirmed the role of classic biomarkers (2-methylcitrate and propionylcarnitine) and identified novel biomarkers (e.g., FGF21, GDF15, and *in vivo* 1-¹³C-propionate oxidation) in defining mild and severe forms of PA. Characteristics of 1-¹³C-propionate oxidation were further explored. In non-transplanted patients, 1-¹³C-propionate oxidation correlated with height z-scores ($R^2 = 0.2$, $P < 0.05$), IQ ($R^2 = 0.5$, $P < 0.0001$), alanine aminotransferase ($R^2 = 0.16$, $P < 0.05$), sensorineural hearing loss ($P < 0.05$), and

incomplete protein intake as % of RDA ($R^2 = 0.16$, $P < 0.05$). 60-min $1\text{-}^{13}\text{C}$ -propionate oxidation was normalized in liver transplant recipients (mean = 32% of the administered label vs. 33% in healthy controls) but markedly depressed in those with biallelic null variants in *PCCA* or *PCCB* (mean = 2.3%) or those that harbored at least one missense variant (mean = 13%, one-way ANOVA $P < 0.0001$). Similar correlative trends were observed for plasma FGF21 and GDF15. CONCLUSIONS: This proof-of-principle study presents and validates a machine learning approach using dense clinical and laboratory data collected via a PA natural history protocol to define outcome parameters and identify correlative biomarkers. Our methods can be extended to other rare disorders where biomarker discovery is required but limited by few available research subjects.

1020. Sustained Correction of a Murine Model of Phenylketonuria and Integration into the Genome Following a Single Administration of an AAVHSC15 Phenylalanine Hydroxylase Gene Editing Vector

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Phenylketonuria (PKU) is a rare autosomal recessive inborn error of metabolism. If left untreated, severe forms of PKU due to phenylalanine hydroxylase (PAH) deficiency result in progressive, irreversible neurological impairment during infancy and early childhood. Neither phenylalanine (Phe)-restricted diet nor the currently available therapeutic treatments address the core biological defect of the disease, which is, the presence of biallelic pathogenic variants in the *PAH* gene. HMI-103 is an investigational gene editing vector designed to deliver functional copies of *PAH* to hepatocytes, integrate into the genome, and has the potential to restore PAH activity and normalize Phe metabolism. HMI-103 contains locus- and species-specific homology arms (HA) flanking the human *PAH* sequence (cDNA) that are designed to guide the cDNA to the target *PAH* locus in the genome and integrate through non-nuclease-based, AAV-mediated homologous recombination. Vectors expressing PAH were packaged in AAVHSC15. Both HMI-103 and a murine surrogate vector were used in preclinical studies; the murine surrogate is different from HMI-103 in that it contains HAs specific to the murine *Pah* locus to enable integration into the murine genome. The mouse-specific vector was tested in a murine model of PKU (*Pah^{enu2}*), while HMI-103 was tested in a humanized-liver murine xenograft model in which the liver is repopulated with human hepatocytes. The vectors were administered as a single injection. Blood Phe was measured by mass spectrometry. Livers were processed to measure vector genome (vg) copy number and mRNA by ddPCR and integration in genome by next generation sequencing (NGS). Following a single administration, the murine surrogate vector resulted in long-term normalization of blood Phe, dose-responsive vg copy number, mRNA expression, and on-target genome integration in *Pah^{enu2}* mice. Administration of HMI-103 in the humanized-liver murine model resulted in stable, dose-responsive vg copy number, mRNA expression, and on-target integration into the

PAH locus with no unwanted changes to the target site in the genome. The mRNA and integration levels achieved were consistent with levels in *Pah^{enu2}* mice that corresponded with correction of the PKU phenotype. Additionally, a long-read, genome-wide assay capable of detecting vector integrations at 0.5% or higher showed no evidence of off-target integration into the human genome. The murine surrogate was also evaluated in a GLP toxicology study in *Pah^{enu2}* mice and a germline transmission study in C57BL/6J mice. In the PKU mouse model, blood Phe was normalized at all doses tested and there were no test-article related clinical pathology or necropsy findings. There was no evidence of germline transmission. These data in mice demonstrate efficacy, integration, fidelity and specificity for the target locus and preclinical safety of HMI-103 and supported clinical trial initiation.

1021. Genome Editing Restores Acid Alpha-Glucosidase Function in Fibroblasts Derived from a Patient with Infantile-Onset Pompe Disease

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Infantile-onset Pompe disease (IOPD) is a fatal autosomal recessive lysosomal disease caused by mutations in *GAA*, which encodes acid alpha-glucosidase (GAA). *GAA* is a critical enzyme in glycogen catabolism. When *GAA* is non-functional, glycogen accumulates within lysosomes. IOPD manifests as cardiac and skeletal myopathy as well as neuronal pathology. Enzyme replacement therapy is the standard of care, but requires regular, life-long, infusions. Genome editing of patient-specific *GAA* mutations *in vivo* may offer a potential one-time therapy for IOPD. Here, we utilized an adenine base editor (ABE) and a single guide RNA (gRNA) targeting the *GAA* c.2227C>T (p.Q743*) nonsense mutation, and a prime editor (PE) and a prime editing gRNA (pegRNA) to target the c.258dupC (p.N87Qfs*9) frameshift mutation in an *in vitro*, non-nuclear, context to establish initial on-targeting efficiency. *GAA* c.2227C>T was base edited with a 47% editing efficiency, and *GAA* c.258dupC was prime edited with up to 29% efficiency. Genome editing efficiency was further confirmed through a restriction enzyme digest that recognizes the presence of wild-type sequence. Given that patients with >8% enzyme activity have an attenuated disease course, these editing efficiencies could translate to mitigation of disease progression for IOPD patients. Next, the base editing strategy was tested in *GAA*^{c.2227C>T/c.258dupC} HDFs and were assessed for genomic editing efficiency by Sanger sequencing, and *GAA* enzyme activity by a fluorometric 4-methylumbelliferone- α -d-glucopyranoside assay at multiple time points over 4 weeks post-nucleofection. Sanger sequencing results revealed that more than 80% of the mutated thymine at c.2227 of *GAA* was restored to the WT cytosine after 4 days post-nucleofection. Intracellular *GAA* activity, which was absent pre-editing, was detected as early as 4 days post-nucleofection, and interestingly peaked at supranormal levels of 165 ± 25 U/mg protein compared to a mock condition with 0.5 ± 0.4 U/mg protein 35 days post-nucleofection. Going forward, next-generation sequencing will be used to determine precise editing efficiency, and further phenotypic characterization of edited HDFs will include assessment of glycogen levels and proteins involved in autophagy and

cellular stress. The abovementioned prime editing strategy for *GAA* c.258dupC in patient-derived HDFs. Additionally, elucidation of the observed supranormal *GAA* enzyme activity is imperative. Future work will assess editing efficiency in iPSC-derived cardiomyocytes and skeletal myoblasts, with a focus on defining optimal packaging and delivery methods for translation of ABE and PE strategies to humanized mouse models of *Gaa*^{c.2227C>T} and *Gaa*^{c.258dupC}. The results presented herein underscore the potential of base and prime editing techniques as personalized therapeutics for not only patients with IOPD, but also patients with other genetic diseases.

1022. Exploring the Natural History of Cobalamin B (*cbIB*) type Methylmalonic Acidemia (MMA) to Guide the Development of Systemic AAV Gene Therapy

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Cobalamin B (*cbIB*) type methylmalonic acidemia (MMA) is an ultra-rare autosomal recessive inborn error caused by pathogenic variants in the *MMAB* gene, which encodes for the enzyme ATP:cob(I)alamin adenosyltransferase (ATR). ATR catalyzes the synthesis and delivery of 5'-deoxyadenosylcobalamin (AdoCbl), an essential cofactor for the methylmalonyl-CoA mutase enzyme (MMUT). Both MMUT and *MMAB* deficiencies present with life-threatening metabolic instability and chronic multisystem injury that are typically non-responsive to B12 supplementation. Supportive care includes dietary management and carnitine supplementation, while elective liver (LT) or liver and kidney transplantation (LKT) is offered to severe patients. Previous preclinical studies have enabled the development of several AAV-based canonical gene therapy and gene editing approaches for MMUT deficiency that have entered phase I/II clinical trials. The efficacy of gene therapy for MMUT has paved the way for future studies on other defects in the propionate oxidation pathway, such as *cbIB* and propionic acidemia (PA). It is unclear if the observations with MMUT will extend to other subtypes, however they are critical in informing animal model construction and proof-of-concept AAV gene therapy experiments and developing translatable laboratory and clinical outcome measures. Through our natural history protocol (ClinicalTrials.gov ID: NCT00078078) we have evaluated the presentation, course, complications, contemporary management, biochemical parameters, and effects of organ transplantation in 17 *cbIB* patients, ranging from 2.5 to 47 years of age, including nine with a LT (35%) or LKT (23%). Five patients were diagnosed through family testing or newborn screening (NBS), while the remainder (70%) had a symptomatic presentation at ages ranging from 24hrs of life to 6 years, followed by confirmatory genetic testing. Half (54%) presented in the neonatal period with hyperammonemic encephalopathy requiring hemodialysis, while 2 patients presented with an acute basal ganglia injury in childhood prior to the implementation of NBS. 63% developed stage 3 chronic kidney disease in the first decade of life. Seven patients were homozygous for p.Arg186Trp in *MMAB*, a common deleterious variant resulting in severe enzyme deficiency, while 6 were compound heterozygotes for

p.Gln234Ter, known to be associated with an attenuated phenotype. 1-¹³C-propionate oxidation breath test was developed for patients with MMA as a safe, non-invasive, *in vivo* indirect enzyme activity measurement that correlated with disease severity, transplant status, and long-term outcomes. The breath test confirmed low enzyme activity in a severe *cbIB* patient homozygous for p.Arg186Trp (16% of the isotope dose oxidized in 120 min) in contrast to near normal enzymatic activity (47%) in a patient compound heterozygous for p.Arg186Trp and p.Gln234Ter variant. Clinically milder and transplanted patients maintained lower levels of methylmalonic acid and mitochondrial dysfunction biomarkers, FGF21 and GDF15, than severe patients pre-transplant. Despite metabolic stability and improved biochemistry, transplanted patients experienced severe complications indicating the need for an alternative treatment for *cbIB* such as gene therapy. Patients with *MMAB* deficiency closely resemble those with vitamin B12 non-responsive *MMUT* in their clinical, laboratory, and biomarker phenotypes. We plan to advance *cbIB* as an indication for systemic AAV gene therapy through the Platform Vector Gene Therapy (PaVe-GT) program, and in parallel, are actively pursuing proof-of-concept studies in *Mmab* animal models to generate supportive pre-clinical data.

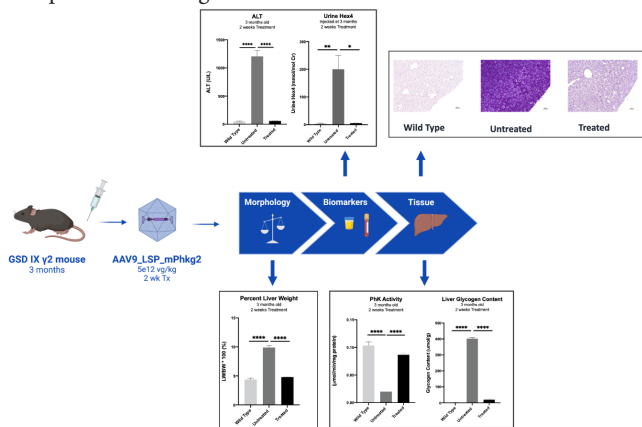
1023. Liver Directed AAV Gene Therapy Reverses Progression of Glycogen Storage Disease Type IX γ 2 in Mice

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Liver Glycogen Storage Disease type IX (GSD IX) is the most common hepatic GSD, accounting for approximately 25% of all GSD cases with an overall estimated prevalence of 1 in 100,000. Liver GSD IX is caused by a deficiency in phosphorylase kinase (PhK), a key enzyme in the breakdown of glycogen to glucose. Liver PhK is a complex, hetero-tetrameric enzyme comprised of four subunits, with α 2, β , and δ subunits regulating the activity of the catalytic γ 2 domain. Mutations in the α 2 subunit are responsible for ~75% of liver PhK deficiencies (GSD IX α 2) and mutations in the γ 2 subunit are responsible for ~25% of liver PhK deficiencies (GSD IX γ 2). All patients with GSD IX present with hepatomegaly, elevated liver enzymes and hypoglycemia. Over 95% of patients diagnosed with GSD IX γ 2 have progressive liver fibrosis and/or cirrhosis, increasing risk for liver failure and death. Despite the life-threatening severity, there is no definitive treatment for Liver GSD IX γ 2. Our group recently described the first mouse model for GSD IX γ 2. Characterization of *Phkg2*^{-/-} mice at age 3 months confirmed that the mouse recapitulates the liver-specific phenotype of GSD IX γ 2 patients, with low PhK enzyme activity, elevated liver glycogen content, elevated ALT, increased urine Hex4 (a product of glycogen breakdown), abnormal hepatocytes, and early perisinusoidal fibrosis on pathology. Here, we describe the long-term natural history of *Phkg2*^{-/-} mice and use liver directed AAV gene therapy to correct *Phkg2*^{-/-} phenotype and prevent further disease progression. The *Phkg2*^{-/-} mice show severe liver disease progression over time. There is an advancement of liver pathology from periportal fibrosis at 9 months

to bridging fibrosis at 12 months, with a concomitant decrease in ALT and urine Hex4. With progressive liver disease characterized, *Phkg2*^{-/-} mice underwent short and long term AAV treatments at ages 3 and 6 months. All *Phkg2*^{-/-} mice were intravenously injected at 5e12 vg/kg with the murine *Phkg2* transgene under the control of a liver specific promoter (AAV9-LSP-mPhkg2). Wild type, untreated, and treated mice at ages 3 and 6 months were evaluated post treatment for PhK enzyme activity, glycogen content, ALT, urine Hex4, and histology. Mice at age 3 months treated for 2 weeks demonstrated restoration of PhK enzyme activity, reduced liver glycogen content, and decreased ALT and urine Hex4 all comparable to wild type levels. Mice at age 3 months treated for 3 months demonstrated restoration of PhK enzyme activity, reduced liver glycogen content, decreased ALT and urine Hex4 comparable to wild type levels. Despite pre-existing liver fibrosis, mice at age 6 months treated for 3 months also demonstrated a restoration of PhK enzyme activity, reduced liver glycogen content, decreased ALT and urine Hex4 comparable to wild type levels, restoration of hepatocyte architecture, and reduction of liver fibrosis. Results of our study suggest that AAV gene therapy may serve as the first definitive treatment for patients with Liver GSD IX γ 2. Additional IND-enabling studies will be performed to evaluate safety and identify the potential therapeutic dose-range for first-in-human clinical trials.



1024. Long-Term Evaluation of the Efficacy of Intracerebroventricularly Injected AAVhu68 Encoding Human Codon Optimized ARSA (hARSA) Transgene in a Mouse Model of Metachromatic Leukodystrophy (MLD)

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Metachromatic leukodystrophy (MLD) is a rare inherited autosomal-recessive disorder seen in children and young adults caused by a defect in the Arylsulfatase-A (ARSA) gene. Patients with MLD lack functional ARSA enzyme and accumulate toxic levels of the enzyme substrate sulfatides in tissues including central and peripheral nervous system. The toxic accumulation of sulfatides in CNS triggers cell death (especially in oligodendrocytes) and inflammation, eventually leading to progressive demyelination. This manifests clinically as severe motor

and gait abnormalities, ataxia, convulsions, and difficulties in breathing, leading to death. A majority of children with the late infantile form of the disease die before the age of 5 years. Currently, the sole approved treatment for MLD, *ex vivo* gene therapy, benefits only pre-symptomatic patients due to the kinetics of hematopoietic engraftment in CNS competing with disease progression. To address the shortcomings of current therapies, we developed a clinical candidate AAVhu68 encoding human codon optimized ARSA (AAV-hARSA) delivered directly into the cerebrospinal fluid for quick enzyme production and diffusion within the central and peripheral nervous systems. In the current IND-enabling studies, we report the efficacy of AAV-hARSA transgene therapy in a novel mouse model of MLD that displays neurobehavioral deficit by 10 months of age. Four-month-old ARSA KO mice were administered an intracerebroventricular injection of AAV-hARSA at three doses (1.3E11, 4.5E10 and 1.3E10 GC/animal). Vehicle-treated ARSA KO, and wildtype mice were used as controls. The animals were monitored for either 180 days or 450 days (survival cohort) using measurements like body weight, neurobehavioral scoring, motor coordination (rotarod) and gait analysis (catwalk). At the end of each study, the animals were euthanized and samples were collected for biochemical and histological analyses. Analysis of the enzyme activity from the interim cohort demonstrated a significant increase in enzyme activity in serum (1.5-fold), brain (1.4-fold), liver (10-fold), and heart (2-fold) over vehicle controls. Results from the long-term cohort showed a complete survival rescue at all doses. Untreated ARSA KO mice had a shortened lifespan with only 50% of mice alive at day 450 (approximately 20 months of age) compared with 100% of WT controls and 100% of AAV-hARSA-treated ARSA KO mice. We also detected significant improvements in the disease phenotype in the AAV-hARSA-treated animals as evidenced by a decrease in generalized tremors, claspings, and postural deficits. AAV-hARSA-treated animals also demonstrated significant rescue of the decline in rotarod performance and gait parameters detected in the untreated ARSA KO mice, further supporting the long-term efficacy of the AAV-hARSA therapy. In summary, the AAV-hARSA-treated MLD mice demonstrated increased enzyme activity, phenotype rescue, and improved motor and gait functions which further benefitted the survival of the animals in a symptomatic mouse model of MLD. Collectively, the results from the current study provides a proof of concept for the efficacy of AAV-hARSA cerebrospinal fluid gene therapy.

1025. Determining the Minimum Effective Dose (MED) of a Clinical Candidate AAV Vector Encoding Engineered Alpha-Galactosidase A Transgene in a Mouse Model of Fabry Disease

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Fabry disease is a rare X-linked lysosomal storage disorder caused by mutations in GLA gene, which provides instructions for making a lysosomal hydrolase, α -galactosidase A (α -Gal A). Deficiency

of α -Gal A results in progressive lysosomal accumulation of globotriaosylceramide (Gb3) or related glycosphingolipids in a variety of tissues with progressive renal, cardiac, sensory, and vascular impairments. An effective and long-lasting approach, using recombinant adeno-associated virus (rAAV), is desirable to address some shortcomings of enzyme replacement therapy. We identified a clinical candidate rAAVhu68 vector encoding an engineered stabilized hGLA gene for gene therapy (GLA-eng). Our candidate demonstrated superior Gb3 clearance compared to AAV encoding non-engineered versions of α -Gal A, and proved to be safe and potent in nonhuman primate pilot pharmacology and toxicology studies. We conducted an IND enabling minimum effective dose (MED) study of this vector in an aggravated murine model of Fabry disease that displays a severe phenotype with shortened lifespan, impaired kidney function, and neurosensory deficits. The aggravated mouse model used in this study was generated by crossing *GLA* KO mice with TgG3S mice, a transgenic line that overexpresses the human alpha 1,4-galactosyltransferase, therefore increasing the substrate load. We treated aggravated Fabry mice at 3 months of age with intravenous administration at one of the four vector doses (1e11 GC/animal or 5e12 GC/kg; 2e11 GC/animal or 1e13 GC/kg, 5e11GC/animal or 2.5e13 GC/kg and 1e12 GC/animal or 5e13 GC/kg) or vehicle (DPBS). Additional wild type (WT) animals and TgG3S-overexpressing mice were also dosed with DPBS as controls. Safety and efficacy endpoints were collected to evaluate the impact of treatment on disease-relevant biomarkers such as hGLA activity, Lyso-Gb3, blood urea nitrogen (BUN) levels, and urine volume and osmolality. Furthermore, thermosensory function was evaluated by measuring nociceptive response to heat stimuli using a hot-plate. Animals were sacrificed and necropsied either on Study Day 120+/-7 (interim necropsy cohort) or at the humane endpoint to evaluate survival. AAVhu68-GLA-eng administration increased the expression and activity of human α -Gal A in the plasma and a variety of tissues in the aggravated Fabry mice and reduced the lyso-Gb3 in the plasma or Gb3 storage in disease-relevant tissues. AAV administration also resulted in improvements in survival, body weight loss, and thermosensory function. In addition, the increased levels of blood urea nitrogen (BUN) and urine volume, and the decreased urine osmolality in the Fabry aggravated model have been significantly ameliorated by AAV administration in a dose-dependent manner. Since the lowest dose tested in this study (1e11 GC/animal or 5e12 GC/kg) led to significant improvements in disease-relevant parameters evaluated, the MED was determined to be 5e12 GC/kg.

Neurologic Diseases III

1026. Ubiquitous ASPA Gene Therapy Changes Neuronal Cell Expression in Region-Specific Manner in Canavan Disease

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Neurodegenerative diseases stem from genetic or structure-function mutations in glial or neuronal cell populations that affect the homeostatic balance of the Central Nervous System (CNS). Left untreated, these CNS diseases can lead to irreversible neuronal loss, resulting in cognitive decline, loss of motor abilities, and even lethality. The use of gene therapy in recent years has shown promise in treating neurodegenerative diseases, however, little is understood about the mechanisms behind how gene therapy corrects neuropathology, particularly in restoring neuronal population and function. One CNS disease which leads to downstream neuronal degeneration is Canavan Disease (CD). CD is a leukodystrophy caused by aspartoacylase (ASPA) gene mutations which prevents oligodendrocytes from catabolizing neuronally derived N-acetylaspartate (NAA), one of the brain's most abundant amino acid derivatives, into acetate and aspartate, both of which are required for neuronal homeostasis. As previously shown, the resulting elevated NAA causes neuropathological changes such as astroglial swelling, decreased axonal density, and neuronal cell death, giving CD brains a disease-characteristic vacuolization pattern. Using CD as a neurodegenerative model, we aimed to explore how gene therapy can repair neuronal populations. Published studies administering rAAV9.CB6.hASPA in CD mice demonstrate restoration of CNS pathology to wildtype levels. In addition, electron microscopy characterization found that in a symptomatic CD mouse model, ASPA gene therapy could restore myelination and neuronal morphology, such as axonal size and density, in up to four weeks after treatment. We hypothesized that affected neurons are thus undergoing cellular repair to restore neuronal morphology and function. To answer this hypothesis we treated Nur7 mice, a CD mouse model with a nonsense mutation in the ASPA gene, at symptomatic juvenile age with rAAV9.CB6.hASPA. One and four weeks post-treatment we used RNAscope to determine brain region-specific changes in neuronal markers. Interestingly, gene therapy treatment increased neuronal stem cell markers, particularly in brain regions with severe disease manifestation, such as the cerebellum, brainstem, and thalamus. Furthermore, this change in stem cell marker expression appeared to correlate with increased markers for neuronal regeneration. For example, the post-mitotic neuronal marker NeuN, which was decreased in untreated Nur7 animals, was observed to increase after treatment, suggesting that neuronal populations in severely symptomatic mice seem to possess the capacity to recover upon gene therapy treatment. Of note, we also found increasing numbers of rAAV genome over time, suggesting that rAAV is stable interstitially and enters cells in a time-

dependent manner. If this is associated with a time-dose correlation and whether this explains the time-dependent shift in neuronal cellular markers observed over the four week period in our study is being actively investigated. Concurrently, this further alludes to the potential therapeutic effect of gene therapy on neurogenesis in juvenile to late-stage neurodegenerative diseases such as Canavan disease. In conclusion, our results suggest that brains of post-symptomatic mice have the capacity to achieve neuronal recovery. The exact mechanism is a continuing measure of this study.

1027. Blocking SNHG14/UBE3A-ATS lncRNA Transcription with Dead Cas9 (CRISPR-GNDM®) Can Un-Silence Paternal UBE3A in an Angelman Syndrome Mouse Model

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Angelman Syndrome is a genetic disorder which results from a lack of functioning UBE3A specifically in neurons. Currently there is no cure and symptoms affecting the nervous system become more and more debilitating for patients. In healthy neurons, maternally inherited UBE3A is expressed while the paternally inherited UBE3A is silenced by the lncRNA, SNHG14/UBE3A-ATS. In Angelman patients, however; the maternally inherited UBE3A is absent or not functional. In these patients, the paternally inherited UBE3A is present but silenced. Several therapeutic strategies have been proposed though each have potential problems. Standard gene therapies to deliver exogenous UBE3A are not preferred because expression cannot be well controlled and overexpression of UBE3A correlates with autism spectrum disorder. Antisense oligonucleotides targeting the lncRNA are effective at un-silencing paternal UBE3A but are a transient therapy. Recent studies have investigated the use of CRISPR/Cas9 to un-silence the paternal UBE3A using an active Cas9. Though effective, active Cas9 results in cleavage of genomic DNA and may not be ideal for use in Angelman patients. We therefore hypothesized that a dead Cas9 (GNDM) could act as a roadblock for the lncRNA and effectively un-silence the paternal UBE3A. Using an Angelman mouse model (UBE3A^{mat-/pat+}) we performed bilateral ICV injections of P1 mice with either AAV9-hSyn1-Control sgRNA-GNDM or AAV9-hSyn1-Targeting sgRNA-GNDM and looked for UBE3A re-expression at P30. Un-injected, age matched, WT mice were used for comparison. 5/5 mice injected with AAV9-hSyn1-Targeting sgRNA-GNDM showed UBE3A staining in the cortex of the brain comparable to WT brains. Conversely, 0/5 of the mice injected with AAV9-hSyn1-Control sgRNA-GNDM showed UBE3A staining in the cortex of the brain. This pattern where only mice injected with AAV9-hSyn1-Targeting sgRNA-GNDM re-express UBE3A was observed throughout the brain, specifically within the cortex, cerebellum, and hippocampus. To further confirm that targeting GNDM to the SNHG14/UBE3A-ATS locus is required for paternal UBE3A re-expression, we stained for GNDM protein using immunohistochemistry and GNDM mRNA using RNA FISH. Both GNDM protein and GNDM mRNA were detected in the cortex and cerebellum of mice injected with either AAV9-hSyn1-Control sgRNA-GNDM or AAV9-hSyn1-Targeting

sgRNA-GNDM. These data indicated that even though GNDM is expressed in the brains of mice injected with both AAV9-hSyn1-Control sgRNA-GNDM and AAV9-hSyn1-Targeting sgRNA-GNDM, the targeting guide is necessary for un-silencing paternal UBE3A. Because the absence of UBE3A mainly affects neurons, we wanted to ensure the re-expression of UBE3A occurred specifically in neurons of the brain. We therefore co-stained for UBE3A and the mature neuron marker NeuN in mice injected with AAV9-hSyn1-Targeting sgRNA-GNDM. We found that UBE3A co-localized with NeuN indicating that UBE3A is specifically being re-expressed in neurons of the brain. Taken together, these data suggest that specifically targeting a dead Cas9 (GNDM) to the SNHG14/UBE3A-ATS locus allows it to act as a roadblock and un-silence paternal UBE3A *in vivo* and further suggests that this therapeutic mechanism of action could be an effective and safer cure for Angelman syndrome in patients.

1028. Development of a Sheep Model for GM3 Synthase Deficiency Through CRISPR-Cas9 Technology

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GM3 Synthase Deficiency is a rare autosomal recessive condition that has an increased frequency in Amish population of the North America (*ST3GAL5* c.862C > T). Patients develop global developmental delay, intellectual disability, motor impairments, intractable epileptic encephalopathy, deafness, visual impairment, failure to thrive, irritability, and insomnia. GM3 synthase deficiency is caused by mutations in the *ST3GAL5* gene that encodes the rate-limiting enzyme for synthesis of GM3 ganglioside and its downstream a-series and b-series derivatives, which constitute >97% of gangliosides in the central nervous system. The *ST3GAL5* knockout mouse does not recapitulate the human disease, except for deafness. Therefore, ongoing gene therapy efforts utilizing this mouse model have limitations for assessing therapeutic efficacy. Using CRISPR-Cas9, we created sheep carrying mutations in *ST3GAL5*. Sheep embryos were edited by electroporation using a 7:1 ratio of ribonucleoproteins (RNPs) of spCas9 and a guide RNA targeting exon 6 near the Amish mutation. Resulting lambs were viable and had normal hearing but exhibited mild neurological features including proprioceptive deficits, ataxia, and urinary incontinence. GM3 ganglioside levels in plasma were reduced by ~50% in edited lambs. Deep sequencing from muscle and skin to determine the distribution of mutations is ongoing. As compared to mice, this ovine model may more accurately represent human GM3 synthase deficiency, thereby facilitating testing of new precision therapies in a large animal model and accelerating preclinical development of treatments for this devastating disease.

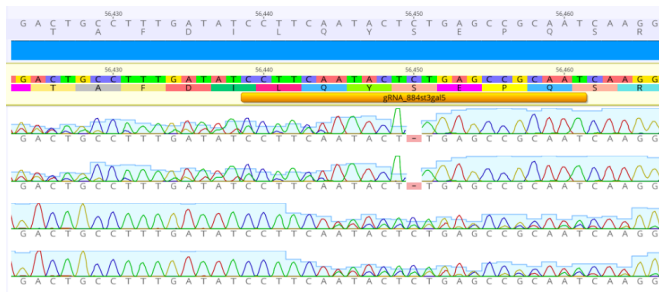


Figure 1: Example of mutations generated in genome edited lambs.

1029. A Human iPSC-Derived Neuron and Cardiomyocyte Platform for Assessing Novel Recombinant Adeno-Associated Viral (AAV) Vectors

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Translating preclinical AAV gene therapy research to successful clinical outcomes has many challenges, in part due to dependence on the differences in AAV biology and predictive potential of available preclinical models. Genetically engineered mouse models have been widely used to study the molecular, physiological, and behavioral impacts of disease-causing mutations, while larger mammals have been utilized to assess vector toxicity and biodistribution. However, while essential, these preclinical models are limited in their ability to recapitulate AAV-host factor interactions in humans. Human-induced pluripotent stem cells (hiPSCs) represent a robust system for bridging human biology with preclinical animal models in evaluating the efficiency of AAV capsids to transduce cell types being targeted for therapeutic intervention in the clinic, as well as assessing the expression of transgene cassettes. We have established a robust platform for expanding, banking and differentiating multiple iPSC lines (normal as well as disease models) into both neurons and cardiomyocytes that have been characterized using specific markers of cell type and maturation. Here, we evaluate the ability of newly evolved AAV strains (our proprietary STRV™ platform) benchmarked against natural AAV serotypes to transduce biologically relevant human cell types. We showcase the improved transduction profiles of a panel of STRV™ capsids evolved through different routes of administration in various animal models in these human cell types. This robust platform allows for understanding and validating the biology of AAV capsids in a clinically relevant setting and the selection of AAV vector candidates with favorable transduction profiles for CNS and cardiac focused clinical trials.

1030. Development of a Sheep Model of Adrenoleukodystrophy (ALD) Using CRISPR-Cas9 Technology

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Adrenoleukodystrophy (ALD) is an X-linked recessive metabolic storage disease caused by mutations in the ABCD1 gene that inhibit the production of adrenoleukodystrophy protein (ALDP). ALDP functions to transport very long-chain fatty acids (VLCFAs) to be metabolized by β -oxidation in peroxisomes. ABCD1 deficiency prevents VLCFAs from being metabolized, resulting in the accumulation of toxic levels of VLCFAs throughout the body. It is hypothesized that the accumulation of VLCFAs leads to the breakdown of myelin in the white matter of the central nervous system and the adrenal cortex, leading to neurological symptoms. There are several types of ALD, but the three major types are the childhood cerebral form (most severe), adrenomyelopathy, and adrenal insufficiency (Addison's Disease). ALD primarily affects young boys, however some forms of the disease affect men later in life. Female carriers of ALD are often asymptomatic but may have mild symptoms due to heightened levels of VLCFAs. Symptoms of childhood cerebral ALD typically occur early in childhood and are often misdiagnosed as ADHD or behavioral issues before more severe neurological symptoms such as vision and hearing loss, loss of coordination, and seizures present. Childhood cerebral ALD typically leads to a vegetative state or death within 2-10 years of symptom onset. ABCD1 mouse models fail to recapitulate the demyelination phenotype of patients, thereby significantly hindering therapeutic development. Our lab has developed a pipeline to generate large animal models of human genetic diseases, therefore we intend to generate a sheep model of ALD as none currently exist. Additionally, we aim to use CRISPR technology to aid in the development of an efficacious AAV gene therapy for ALD. To generate a sheep model of ALD, we will screen guide RNAs targeting exons 1 and 3 of ABCD1 in sheep fibroblasts by electroporation of spCAS9 ribonucleoproteins. The top guide will be used to edit sheep embryos and edited embryos will be reimplanted in surrogate female sheep. ABCD1 edited lambs will be characterized and used to establish a breeding colony to aid in the development of an efficacious therapy for ALD.

1031. Efficacy of a Novel Vectorized Antibody Targeting the C-Terminal Domain of Tau, Antibody 1, using Systemic Dosing of a Blood Brain Barrier Penetrant AAV Capsid in Mouse Models of Tauopathies

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Anti-tau immunotherapy is being pursued as a promising therapy for tauopathies, including Alzheimer's disease (AD), frontotemporal dementia (FTD), and progressive supranuclear palsy (PSP). The success of such an approach relies, in part, on the identification of efficacious antibodies and their delivery to affected or vulnerable brain regions. We have previously demonstrated broad distribution and expression of vectorized anti-tau antibodies in the mouse brain using a blood-brain barrier penetrant capsid administered intravenously (IV). Here we describe the characterization and vectorization of a novel anti-tau antibody, Antibody 1, and efficacy studies in two tauopathy mouse models. We have carried out immunization campaigns with AD patient-derived PHF-tau (paired helical filamentous tau) as an antigen in mice. One of the antibodies discovered, Antibody 1, exhibits strong selectivity for PHF-tau, and potent functional inhibition of PHF seeding and propagation in vitro and in vivo. This antibody contains a novel sequence, recognizes a phospho-specific epitope in the C-terminal region of tau, and shows a significant reduction of tau pathology in an AD-PHF induced P301S hippocampal seeding and propagation model. Furthermore, we have vectorized Antibody 1 into an AAV expression vector and evaluated it in two independent mouse models of tauopathy. This gene therapy-based approach has potential advantages over the traditional passive immunization, including 1) continuous expression of antibody in the central nervous system (CNS) after a single gene therapy administration compared to repetitive administrations of high dose of antibody by passive immunotherapy; 2) increased CNS exposure of tau antibody relative to passive immunotherapy; and 3) the potential to target intracellular tau aggregates which are less effectively accessed by passively delivered antibody; all of which could enhance targeting of pathological tau species in the CNS and resulting efficacy against tau pathology. These results add to accumulating evidence that systemic dosing of a vectorized anti-tau antibody using a BBB-penetrant AAV capsid results in reduced tau pathology and may represent a new single-dose therapeutic strategy for treating various tauopathies.

1032. Minimally Invasive Percutaneous Endovascular Access into the Intracranial Cisterns: A Novel Route of Drug Delivery to the Brain

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Introduction: Delivery of AAV/viral vectors to target brain tissue has remained a difficult challenge due to the blood brain barrier and neutralizing antibodies following intravenous administration, and off-target spinal cord transduction with lumbar intrathecal administration. An intraventricular approach overcomes these obstacles, but requires invasive skull burr hole and brain traversal. We developed a novel percutaneous endovascular catheter-based system that directly accesses the brain for administration of single-dose therapies, without the need for brain surgery. **Methods:** The percutaneous catheter is navigated under fluoroscopic guidance from femoral venous access to the internal jugular vein and then to the inferior petrosal sinus, a fixed redundant vein at the base of the skull. The catheter is then advanced across the dural sinus wall to access the cerebrospinal fluid (CSF) in the adjacent cerebellopontine angle cistern between the anterior surface of the cerebellum and the lateral surface of the pons. This percutaneous transdural catheter access provides a direct fluid path for administration of single-dose therapeutics into a site of high CSF flow potentially enabling diffuse distribution of therapeutic agents throughout the brain (**Figure 1**). The catheter is then withdrawn through the self-sealing dural sinus wall, through the venous system, and out of the patient to complete the procedure. **Results:** A clinical trial relying on the catheter to deploy a permanent implant for the treatment of hydrocephalus is underway and use of the device in the first patient has been recently published. Additional patients have been treated safely and successfully with the catheter. No observations of subarachnoid hemorrhage or injury to adjacent nervous tissue from accessing the cerebrospinal fluid space have occurred thus far. Clinical investigation of the device continues and will help fully characterize safety and effectiveness. **Conclusions:** Initial human clinical data establishes proof of concept of this novel route of administration for single-dose therapeutic agents. The catheter system provides the advantages of a minimally invasive percutaneous approach to the central nervous system- without the need for invasive surgery through the skull and brain tissue. The device may offer significant advantages over conventional routes of administration and consequently could provide increased efficacy of therapies targeting the brain.

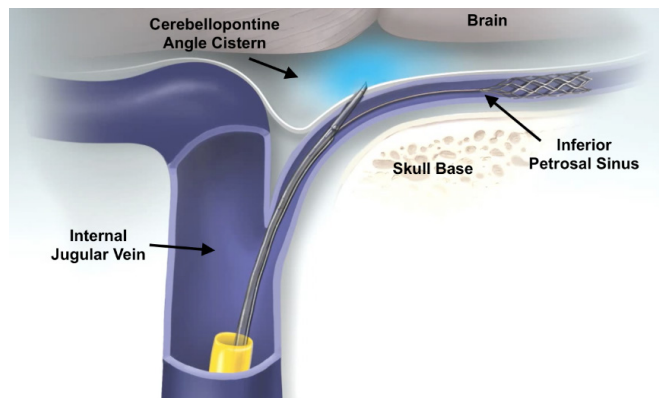


Figure 1: After navigation of the catheter system into the inferior petrosal sinus, the catheter advances through the venous wall to access the adjacent CSF-filled cerebellopontine angle cistern for injection of therapeutics.

1033. Innovative and Regulated Lentiviral Promoter for the Gene Therapy of Neurodegenerative Diseases

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Neuroinflammation is characterized by microglia cell-activation, which plays a major role in the pathogenesis and progression of several neurodegenerative disorders. The clinical effects of *ex vivo* gene therapy (GT), based on the use of genetically modified hematopoietic stem cells (HSCs), for these conditions benefits from functional microglia-equivalent cells that are derived from the transplanted HSCs and engraft into the recipient's Central Nervous System (CNS), where they can exert homeostatic and scavenging functions, normalize CNS homeostasis, reduce neuroinflammation and deliver disease-relevant therapeutic proteins, such as lysosomal enzymes in the case of lysosomal storage diseases. An ideal lentiviral vector (LVV) to be employed in this setting should drive a regulated expression of the therapeutic transgene in the HSC-derived microglia-like progeny: upregulated in the presence of neuroinflammation and downregulated under homeostatic CNS conditions. To this aim, we designed a novel LV promoter, based on the human leukocyte antigen - DR alpha (HLA-DRA) promoter region, able to induce basal levels of gene expression in microglia cells and capable of enhanced expression of a transgene up to 4-5 fold the basal levels upon cell activation. This synthetic HLA-DRA (sHLA) promoter faithfully reproduces the transcriptional pattern of the endogenous promoter *in vitro* and induces a good transgene expression *in vivo* in myeloid/microglia cells derived from HSPCs transplanted in wild type animals. Interestingly, new data are being generated in *in vivo* neuroinflammation models, such as in mice, where experimental autoimmune encephalomyelitis (EAE) was induced to model multiple sclerosis, supporting the capability of the sHLA promoter to maintain its correct regulatory profile in differentiated microglia-like cells, without epigenetic silencing or other forms of undesired regulation.

These results strongly support investigating this new promoter in HSC GT approaches for pathological conditions of the CNS characterized by neuroinflammation.

1034. Systemic Administration of Recombinant AAV9 with a Codon-Optimized, Reduced Size Version of the Human ATP7A Transgene Plus Subcutaneous Copper-Histinate Rescues 100% of Menkes Disease Mice

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Menkes Disease (MD) is an infantile onset, X-linked recessive disorder characterized by rapidly progressive neurodegeneration, seizures and failure to thrive caused by mutations in the ATP7A gene. ATP7A encodes a copper-transporting p-type ATPase responsible for copper (Cu) delivery to the developing brain and for maintaining total body and intracellular Cu homeostasis. In addition to serving up Cu ions to copper-dependent enzymes such as dopamine-beta-hydroxylase and peptidylglycine- α -amidating-monooxygenase in the trans-Golgi compartment and guiding Cu ions across polarized epithelial cell membranes, ATP7A has established roles in axonal outgrowth, synapse integrity, neuronal activation and motor neuron function. The mottled-brindled mouse model (mo/Br) harbors a spontaneously arisen small deletion in *Atp7a*, the murine homolog and recapitulates salient features of MD including low serum Cu levels, severe neurodegeneration and early death (\approx postnatal day 13). We previously documented 22% and 53% rescue of this model with CSF-directed AAV5 and AAV9 transfer of a reduced size (rs) version of human ATP7A trimmed to conform to rAAV packaging limits, in combination with CSF or subcutaneous Copper Histidinate (CuHis). Based on the success of systemic rAAV9 gene therapy in spinal muscular atrophy, an illness with similar age of onset, similar rapid, inexorable neurodegeneration and premature mortality when untreated and similar window of therapeutic opportunity, we recently evaluated a systemic AAV administration approach in the Menkes mouse model. We also developed a codon-optimized version of reduced size ATP7A (*cors-ATP7A*) that is expressed with approximately 20-fold higher efficiency compared to the non-codon optimized version. Using facial vein administration of 2.6×10^{13} vg/kg body weight research grade rAAV9-*corsATP7A* on postpartum day 1 (P1) plus 5 μ g clinical grade CuHis by subcutaneous injection on P4, 5, 6, 10, 20 and 30, we observed 100% (13 out of 13) long term survival of Menkes mice, the only complete rescue of this mouse mutant reported to date. While prior studies in human subjects have shown that early intervention with CuHis alone dramatically improves survival and clinical outcomes, the burden of disease remains considerable. Based on the multi-tasking character of ATP7A, Menkes disease infants identified prior to symptom onset and especially those with complete loss-of-function variants (\approx 75% of Menkes patients) would benefit from complementary gene therapy that provides some working copies of the gene. The synergistic effect of AAV9-*corsATP7A* plus CuHis could further enhance survival and overall quality of life, including attainment of independent ambulation (walking) by 18 months of

age. These latest breakthrough findings in the mo/br mouse model help establish the safety of this combination treatment approach and represents an important step toward a pilot clinical trial for individuals with this illness.

1035. Prime Editing to Repair POLG-Related Epilepsy

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Background and objectives POLG-variants underly a severe inborn error of mitochondrial metabolism called Alpers-Huttenlocher syndrome, causing intractable, therapy-resistant epilepsy, muscle weakness and liver disease. Onset of symptoms is usually before the age of 10 years and most children die within months to years after onset due to status epilepticus or liver failure. The c.1399G>A (p.Ala467Thr) variant is the most common cause of this syndrome. We aim to use base editing and prime editing to genetically repair this variant to develop a curative treatment strategy for POLG-related epilepsy.

Methods We developed guide RNAs against c.1399G>A using in-silico prediction algorithms. We transfected patient-derived fibroblasts with base and prime editor plasmids, gRNAs and our fluoPEER enrichment reporter. We then selected edited cells using fluorescence-activated cell sorting (FACS) and genotyped them to determine editing efficiency. We analyzed various mitochondrial readouts, including mitochondrial DNA levels, mitochondrial density, oxidative phosphorylation, and complex levels.

Results Base and prime editing successfully corrected the c.1399G>A variant in 25% and 46% of patient-derived fibroblasts, respectively, without causing unwanted near-target edits. Repaired cells showed an improvement in mitochondrial readouts.

Conclusion Base and prime editing can be used to genetically correct the most common POLG-variant causing Alpers-Huttenlocher syndrome, and hold promise to form a causal treatment strategy for POLG-related epilepsy.

1036. Analgesic Effect of Recombinant GABAergic Cells Releasing MVIIA in Spinal Cord Injury-Induced Pain Model in Rats

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Chronic pain induced by spinal cord injury represents a clinically unresolved issue. Although the loss of motor function is the most prominent outcome of spinal cord injury, the presence of chronic pain can significantly reduce the quality of life of patients and interfere with rehabilitation strategies. Pharmacological targeting of chronic pain is often unsatisfactory due to multiple pain-signaling pathways and targets involved. Local and simultaneous targeting of specific pathophysiological events might lead to better pain relief in patients.

Recombinant cells engineered to release analgesic substances provide tools to achieve such goals. Key events underlying development of chronic pain are spinal disinhibition and hyperexcitation of nociceptive neurons, causing misinterpretation of incoming low threshold peripheral signals as painful. Reduced GABAergic and enhanced calcium signaling are among the major factors contributing to chronic pain development. Our previous studies showed that intraspinal transplantation of GABAergic neuronal cells may restore the inhibitory potential in the spinal cord and replace dysfunctional interneurons. Grafted cells may also release analgesic peptides by means of genetic engineering to further enhance the benefits of a cell-therapy approach. The omega-conopeptide, MVIIA, an N-type Ca²⁺ channel blocker, was one of the earliest tested treatments for severe pain in animal models and is in clinical use marketed as FDA approved Prialt (Ziconotide). However, due to its poor penetration through the blood brain barrier it must be delivered intrathecally. The goal of this project is to develop transplantable recombinant GABAergic cells releasing MVIIA that can alleviate pain-like behavior following spinal cord injury. Male and female Sprague Dawley rats underwent clip compression spinal cord injury (SCI) and developed hypersensitivity mechanical and thermal stimulation. Rat E15 GABAergic progenitors were used to engineer recombinant GABAergic cells by transfection with p_lenti_MVIIA. Their viability, proneuronal phenotype, and the ability to release recombinant MVIIA were confirmed via immunostaining and FLISA analysis. Recombinant and control cells were intraspinally injected into animals when signs of chronic pain were detected. An analgesic effect of the transplant treatment was observed in both sexes, with a stronger and longer lasting outcome in the recombinant group compared with non-recombinant control neural progenitor cells. Intrathecal injection of MVIIA antibody abolished the effect of recombinant cells. Conditioned place preference with gabapentin revealed significant reduction of ongoing pain in animals with the grafts. Inflammatory biomarkers IL1- β and TNF- α were reduced in treated animals compared to controls. The recombinant cells were detected up to 1 mm distance from the injection site. FLISA analysis confirmed the presence of MVIIA in the spinal homogenates. Our results demonstrate analgesic potential of recombinant cell-based therapy in the management of chronic pain.

1037. Safety Assessment of High-Dose miniMECP2 AAV9 Gene-Replacement Therapy (TSHA-102) for Rett Syndrome in Rats

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Background: Rett syndrome is mainly caused by loss-of-function mutations of the *methyl-CpG-binding-protein 2 (MECP2)* gene. A miniMECP2 gene packaged in a self-complementary AAV9 vector (TSHA-102) and utilizing the miRARE platform designed to regulate MeCP2 expression is in development as an investigational gene-replacement therapy for Rett syndrome. **Methods:** Forty rats (20 male and 20 females, approximately 3-6 weeks old) were assigned to four

groups to receive either vehicle or a total dose of TSHA-102 (4.81×10^{11} vg, 9.62×10^{11} vg, or 3.85×10^{12} vg) via a single intrathecal infusion. These doses correspond to 2.5×10^{14} , 5×10^{14} , and 2×10^{15} vg human equivalent doses, respectively. Scheduled assessments (20 rats/sex/group) were performed at weeks 1, 4, 13, and 26 post TSHA-102 administration. Results: There were no adverse effects on neurobehavioral and macroscopic observations; no adverse effects on clinical pathology findings; and no organ weight changes associated with TSHA-102 administration through week 13. TSHA-102 treatment did not result in significant reduction in the nerve conduction function of any of the sensory nerves tested (caudal and sural). The amplitude and duration of the sensory nerve action potential as well as motor nerve conduction in the tibial nerve remained comparable to age-matched controls. Microscopic evaluations revealed a low treatment-related incidence of minimal to mild severity of axonal degeneration in the sciatic, tibial, and sural nerves not deemed adverse. No obvious TSHA-102 dose relationship was found in the incidence or severity of the microscopic changes in the nerves. Conclusions: Single intrathecal administration of TSHA-102 at equivalent planned clinical doses was well tolerated in Sprague Dawley rats.

1038. Gene Replacement Therapy in a Schwannoma Mouse Model of Neurofibromatosis Type 2

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Loss of function of the neurofibromatosis type 2 (*NF2*) tumor suppressor gene leads to the formation of schwannomas, meningiomas and ependymomas, comprising ~50% of all sporadic cases of these primary nervous system tumors. Although these tumors are typically benign, they compromise neuronal functions and cause significant morbidity. *NF2* syndrome is an autosomal-dominant condition, with bi-allelic inactivation of germline and somatic alleles resulting in loss of function of the encoded protein - merlin (also termed schwannomin) and activation of receptor tyrosine kinases and several signaling pathways including mTOR pathway in *NF2*-deficient cells. Standard treatments include surgery and radiotherapy. However, tumor recurrence, and surgical complications necessitate new therapeutic approaches. Here we describe a "gene replacement" approach through direct intratumoral injection of an adeno-associated virus (AAV) vector

expressing merlin in a novel human schwannoma model in nude mice. In culture, the introduction of an AAV1 vector encoding merlin (AAV-merlin) into CRISPR-modified human *NF2*-null arachnoidal cells (ACs) or Schwann cells (SCs) was associated with decreased cell size in the former, and decreased mTORC1 pathway activation in both, consistent with restored merlin activity. *In vivo*, a single injection of AAV1-merlin directly into human *NF2*-null SC-derived tumors growing in the sciatic nerve of nude mice curtailed growth over a 14-week period in comparison to the vehicle injection (compared to controls). These studies establish that merlin re-expression via gene replacement in *NF2*-null schwannomas is sufficient to suppress tumor growth, thereby potentially providing a more effective treatment for *NF2*.

1039. Non-Invasive Interventions May Synergistically Enhance *MECP2* Gene Therapy

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Background: Mutations in the transcription regulator methyl-CpG binding protein 2 (*MeCP2*) mediate Rett syndrome (RTT), a debilitating neurodevelopmental disorder. The *MECP2* gene therapy literary canon is a multifaceted achievement describing numerous pre-clinical treatment paradigms with strategies to improve efficacy, efficiency, and safety. Yet one facet of *MECP2* gene therapy remains unexplored: combinatorial synergy. Methods: We assessed the relative performance of three monotherapies (gene therapy, environmental enrichment (EE), and voluntary exercise (EX)) and two polytherapies (EX polytherapy and EE polytherapy -- which include gene therapy) in adolescent male and adult female mouse models. Low doses of AAV9/*MeP426-MECP2-myc*-RDH1pA were administered intrathecally to permit detection of synergistic efficacy after add-on treatments. Results: We identified synergy in male and female RTT models. In male KO mice, EX polytherapy extended survival even though the respective monotherapies were ineffective. In female heterozygous mice, gene therapy normalized weight when animals were housed in enriched but not standard housing conditions. Polytherapies improved female initial motor coordination and sustained improved rotarod performance across trials. None of the monotherapies significantly improved initial coordination. Notably, the sustained synergistic motor efficacy observed in heterozygous females was not observed in KO males. This differential efficacy supports previously published observations suggesting an interdependence between endogenous *MECP2* genetic dosage and responsiveness to EE combined with EX. Conclusions: Although these simple EE and EX interventions are not directly translatable to humans, they suggest the positive effect that environmental factors might play clinically that would be missing from standard mouse housing conditions. These results should encourage research further exploring combinatorial synergy at molecular and behavioral levels.

1040. Peptide-Modified AAVs Targeting Brain Endothelial Cells Following Intracerebroventricular or Systemic Administration in Mice

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Brain endothelial cells (BECs) are a major component of the blood-brain barrier. Adeno-associated viruses (AAVs) targeted to BECs have the potential not only to treat diseases affecting these cells directly but also to deliver secreted proteins across the blood brain barrier to the central nervous system (CNS). We and others have previously identified AAV variants that efficiently transduce BECs in mice following systemic administration and shown that these variants can be used to treat mouse models of diseases affecting the CNS, including lysosomal storage diseases. Here, we tested our ability to achieve similar transduction at significantly lower doses after intracerebroventricular (ICV) vs systemic injections. For this, we screened a diverse library of peptide-modified AAVs (PM-AAVs) with two rounds of selection following ICV injection in C57BL/6J and FVB/NJ mice as well as Tpp1 knockout mice, a lysosomal storage disease model of CLN2 deficiency. Amplicon sequencing of AAV capsid sequences revealed variants most enriched in isolated BECs. After sequencing, two PM-AAV variants expressing EGFP were assessed after ICV or retro-orbital injection into C57BL/6J and FVB/NJ mice at a total dose of 2E10 vector genomes. In mice injected unilaterally ICV, we observed bilateral EGFP expression in CD31-positive endothelial cells throughout the brain and spinal cord. EGFP expression was also evident in some neuronal and glial cells, particularly near the injection site, as well as in the liver. Surprisingly, mice injected at the same dose retro-orbitally rather than ICV showed similar or improved transduction of BECs, with minimal EGFP expression observed in other brain cell types. Liver transduction was comparable between the two routes of administration. Together, these results suggest that the PM-AAV variants delivered ICV escaped the CNS into the periphery and transduced BECs from the lumen of brain blood vessels rather than remaining in the CNS, as originally intended. Systemic administration of AAVs is likely to be the preferred route of administration for gene therapies targeting brain blood vessels. However, the PM-AAVs identified here demonstrated highly efficient transduction of BECs at a relatively low total dose, with minimal EGFP expression in other brain cell types. These PM-AAV variants will be valuable tools for studying BEC function and the development of gene therapies for diseases of the CNS.

1041. Efficient Antibody Gene Delivery to CNS by Adeno-Associated Virus Vectors in Mouse

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GWAS data have shown that individuals carrying the rare loss-of-function variants of Gene X, a gene encoding a cell membrane protein in "CNS-specific" myeloid cells, are at increased risk of developing a neurodegenerative disease, suggesting increased Gene X signaling, such as using agonistic antibody, could be beneficial to such patients. To test this hypothesis, we generated a recombinant antibody against mouse Gene X (mAb X) in house. However, it is a major bottleneck to deliver recombinant antibody across the blood-brain barrier. Thus, two recombinant adeno-associated virus vectors (rAAV), rAAV9 and rAAV9PHP.eB, were used to deliver the transgene encoding the mAb X (containing the VH and VL sequences under the control of a CAG promoter) to the mouse central nervous system (CNS). The two rAAV vectors containing mAb X were intravenously administered into 8-week-old C57Bl/6 mice at 1×10^{12} VG/mouse. At 4 weeks post injection (PI), the rAAV9PHP.eB viral genome copies (VG/mouse genome) detected in the brain, spinal cord, liver, and heart were 63, 32, 4.6, and 1.8 times higher than those delivered by rAAV9, respectively, while the mAb X mRNA levels (mAb X/TBP) expressed by rAAV9PHP.eB were 111, 110, 2.9, and 5.9 times higher than those achieved by rAAV9, respectively. Compared to rAAV9, rAAV9PHP.eB resulted in higher CNS/Liver ratios of both VG and mRNA levels due to the combination of lower liver transduction and higher CNS transduction. As a comparator, the recombinant mAb X protein (r-mAb X) was delivered intraperitoneally (ip) at 30mg/kg to the mice and peaked at 72h PI but was barely detected at 4 weeks PI. The level of mAb X in the brain mediated by rAAV9 at 4 weeks PI was comparable to the r-mAb X levels at 72h post ip administration, but the level of mAb X by rAAV9PHP.eB was 9.3 times higher than that of r-mAb X at 72h post ip administration. In addition, the level of mAb X achieved by rAAV9PHP.eB in cerebrospinal fluid (CSF) was 8.3 times higher than that of r-mAb X at 72h PI. Our results demonstrated that a rAAV9PHP.eB vector encoding mAb X could efficiently target the CNS and strongly express mAb X in both brain and CSF at levels higher than those achieved by r-mAb1 ip administration or through a conventional mAb X-encoding rAAV9 vector.

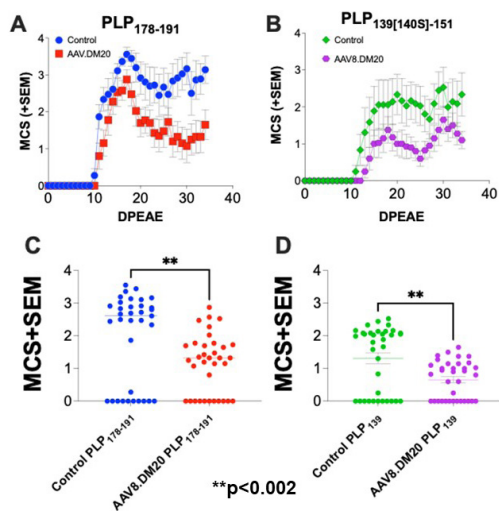
1042. Bystander Suppression from AAV.DM20 Reduced MS-Like Disease

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Multiple sclerosis (MS) is a T cell-mediated autoimmune disorder that results in the destruction of the myelin sheath and affects more than two million people worldwide. Our recent studies using adeno-associated viral (AAV) gene therapy have demonstrated the ability of inducing tolerance against autoantigens to treat autoimmune diseases by successfully generating antigen-specific T regulatory cells against myelin oligodendrocyte glycoprotein (MOG) which prevented and reversed disease in experimental autoimmune encephalomyelitis (EAE), the mouse model of chronic MS, using an AAV.MOG vector. These results were also duplicated in the relapsing-remitting MS model using a AAV vector encoding proteolipid peptide (PLP). Here we wanted to investigate the ability of an AAV vector expressing an

isomer of PLP (DM20) to prevent disease. The DM20 isoform is highly expressed in the thymus in both SJL/J mice and humans but lacks residues 116-150 which encode for the PLP₁₃₉₋₁₅₁ epitope. We hypothesize that since DM20 retains the PLP₁₇₈₋₁₉₁ epitope sequence that it should only prevent onset of PLP₁₇₈₋₁₉₁ induced disease. To test this hypothesis, (C57BL/6 x SJL/J)F1 mice were administered AAV.DM20 or vehicle two weeks prior to disease induction with either PLP₁₃₉₋₁₅₁ or PLP₁₇₈₋₁₉₁ epitopes. Mice were scored for ~35 days post EAE induction. As expected, clinical data demonstrates that intensity of disease onset between those given vector and those without had significant differences. After the initial peak, the mice that received AAV.DM20 and that were immunized with PLP₁₇₈₋₁₉₁ showed a decrease in mean clinical score over time compared to the control group, which displayed a stronger relapsing-remitting disease course (Fig. 1A). However, AAV.DM20 treated mice in the PLP₁₃₉₋₁₅₁ group also showed reduced disease compared to the control group (Figure 1B). This was unexpected considering DM20 lacks the coding sequence for PLP₁₃₉₋₁₅₁ and it should not have initially been able to induce suppressive antigen specific PLP₁₃₉₋₁₅₁ Tregs that would reduce disease severity in mice immunized with PLP₁₃₉₋₁₅₁. Therefore, it is speculated that the reduced disease severity in the PLP₁₃₉₋₁₅₁ induced EAE group occurred through bystander suppression as epitope spreading occurred. Non-Parametric Mann-Whitney tests were used to verify significance among the different groups (Figure 1C-D).



1043. Reduction of Oxidized Phospholipids or Misfolded Protein Aggregates by AAV-VectAbs in ALS Pre-Clinical Models

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It is accepted in the field that protein misfolding underlies multiple neurodegenerative diseases. As the pathogenic mechanisms usually

include both a loss of function (of the wild-type protein) and a gain of toxicity (through the misfolded or aggregated protein), strategies are required which specifically target the toxic (misfolded) protein species, and not the wild-type protein. Misfolded TAR DNA-binding protein-43 (TDP-43) has been associated with the pathology of 97% of sporadic as well as genetic forms of Amyotrophic lateral sclerosis (ALS). Here, misfolded TDP-43 interferes with the translation of mitochondrial proteins at the neuromuscular junction (NMJ) leading to mitochondrial dysfunction. A library of AAV-delivered scFv's (VectAbs) were developed which are expressed as an intrabody, and specifically recognize misfolded TDP-43 species. In addition, these identified candidates show to effectively interfere with TDP43 aggregate formation. This library allows selection of a lead candidate for further development of a Gene Therapy for the treatment of ALS. In addition, this concept forms the basis of similar approaches in other neurodegenerative or neuromuscular diseases with the formation of misfolded protein aggregates that cause cellular toxicity. ALS motor neurons are selectively sensitive to axonal/NMJ mitochondrial dysfunction because of the axon length (up to 60 cm) and the high energy requirement. Mitochondrial dysfunction in ALS motor neurons leads to the formation of oxidized phosphocholine (OxPC) species that are extremely toxic. We have developed a library of secreted OxPC VectAbs that protect motor neurons from OxPC-induced toxicity and cell death. In addition, it was demonstrated that expression levels of AAV-delivered scFv's are at therapeutic levels in ALS-relevant areas within the CNS, including the spinal cord and brainstem. We are currently investigating the efficacy of these approaches in preclinical models.

1044. Safety and Efficacy of Intrathecal Administration of Type 9 AAV Vector Encoding Arylsulfatase A in Adult Metachromatic Leukodystrophy Model Mice

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by an arylsulfatase A (ARSA) deficiency and characterized by severe neurological symptoms resulting from demyelination within the central and peripheral nervous systems. We recently succeeded in treating neonatal ARSA knockout MLD model mice through systemic gene delivery of a single-strand type 9 adeno-associated viral vector encoding human ARSA (AAV9/ARSA). Unfortunately, however, intravenous systemic gene delivery is much less effective in adult mice than neonatal mice due to the established blood brain barrier (BBB). Moreover, the problem with intravenous administration of AAV vectors is the hepatotoxicity that occurs due to their efficient transduction into the liver. To overcome these problems, we investigated the safety and efficacy of intrathecal administration of AAV9/ARSA for the treatment of 6-week-old adult MLD model mice. 13.5 months after intrathecally administration of AAV9/ARSA vectors (4.0×10^{11} vg/body), immunohistochemical analysis showed ARSA expression within the brain, with highest activities in the cerebellum and olfactory bulbs.

Alcian blue staining and quantitative analysis revealed significant decreases in stored sulfatide (Sulf). Sulf/GalC ratios were significantly reduced in the forebrain (0.66 ± 0.05 vs. 0.87 ± 0.03 , $p < 0.01$), hindbrain (0.45 ± 0.06 vs. 0.67 ± 0.04 , $p < 0.01$) and in the cervical spinal cord (0.25 ± 0.02 vs. 0.39 ± 0.02 , $p < 0.001$) of AAV9/ARSA-treated MLD mice compare to untreated mice. Moreover, there was reduction of activated microglia in the both the cerebellum and spinal cord of AAV9/ARSA-treated MLD (cerebellum: 15.5 ± 1.85 % vs. 41.1 ± 7.5 %, $p < 0.01$; spinal cord: 21.57 ± 3.3 % vs. 60.7 ± 7.9 %, $p < 0.0001$). Behaviorally, AAV9/ARSA-treated MLD mice improved ability to traverse narrow balance beams test (latency: 9.4 ± 0.9 s vs. 18.9 ± 2.8 s, $p < 0.03$; number of slips: 6.2 ± 1.3 vs. 11.2 ± 2.0). Biodistribution examined by quantitative PCR analysis showed that the vector genomes were detected in liver but not in muscle and heart. However, the copy number in liver was extremely lower than that of intravenous administration of AAV9/ARSA (0.2 ± 0.1 copy/cell). These findings suggest that intrathecal administration of an AAV9/ARSA vector is a promising approach to treating adult MLD mice without hepatotoxicity.

1045. Correcting Pathogenic Germline Mutations in Neurofibromatosis Type 1 (NF1) Using Novel Gene Therapies

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Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder caused by heterozygous mutations in the *NF1* gene. High degrees of variability and unpredictability are hallmarks of NF1, and patients are at risk of developing multiple symptoms. NF1 predisposes patients to the formation of benign but often disfiguring peripheral nerve associated neurofibromas. Plexiform neurofibromas (PNs) are among the most serious symptoms of NF1, as they can develop into malignant peripheral nerve sheath tumors (MPNSTs), highly aggressive radiation- and chemotherapy-resistant tumors that are often unresectable with a high mortality rate. To date, more than 3,500 pathogenic germline mutations, including specific insertions, deletions, or base substitutions, have been identified in NF1 patients. The *NF1* gene encodes neurofibromin, a 320 kDa protein that functions as 1 of at least 12 human Ras GTPase Activating Proteins (Ras-GAPs). Given the pivotal role of Ras in signal transduction in all cells, treating NF1 tumors by pharmacological blocking of Ras signaling (using MEK inhibitors) is potentially problematic with toxicity and resistance issues.

We are investigating the feasibility of using genome editing as a therapeutic approach to correct *NF1* mutations in PNs. To address the plethora of pathogenic mutations in *NF1*, we are developing personalized therapeutics for individuals with NF1 using both base editing (BE) and prime editing (PE) to precisely correct these lesions in the genome. Further, we are investigating novel advancements in improving these techniques, including improving the stability and prevent degradation of prime editing guide RNAs (pegRNAs) by using engineered pegRNAs (epegRNAs) and utilizing Find and cut-

and-transfer (FiCAT) technology that combines CRISPR-Cas9 and an engineered piggyBac transposase to target integration and repair of large DNA fragments in human cells. In anticipation of moving to *in vivo* testing in the future, we are also testing the feasibility of using split base editing and CASmini systems in the context of our mutations of interest.

Genome editing strategies are being tested in patient-derived cell lines, including both lymphoblastoid cell lines (LCLs) bearing *NF1* germline mutations and immortalized Schwann cells (SC) from PNs with both germline and somatic *NF1* mutations. In addition, we have generated *de novo* SC models with patient-specific *NF1* mutations generated using CRISPR-based editing. Each of these cell models is being used to devise and test BE and PE for efficient and accurate *NF1* mutation correction. We are capitalizing on our continuing efforts to develop novel, engineered Cas variants that improve the efficiency and precision of BEs and PEs. In addition, by increasing the current targeting ranges of these enzymes, we will extend the possibility of precise gene editing to cover the diverse spectrum of *NF1* germline mutations. Our ultimate goal is to develop efficient, personalized, safe, and effective genome editing strategies to treat NF1 tumors.

1047. Abstract Withdrawn

Musculo-skeletal Diseases

1048. DNAJB6 Isoform Specific Knockdown: Mechanistic Insights and Therapeutic Potential for LGMD-D1

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Dominant missense mutations in DNAJB6, a ubiquitously transcribed HSP40 co-chaperone cause limb girdle muscular dystrophy (LGMD) D1, an adult onset myopathy characterized by vacuolar and myofibrillar pathology. No treatments are currently available. DNAJB6 has two isoforms, DNAJB6a and DNAJB6b, each with distinct subcellular localizations in muscle. Disease causing mutations reside within regions shared by both isoforms, yet several lines of evidence suggest DNAJB6b may be solely responsible for disease pathogenesis. Mechanistic data is conflicting regarding a toxic gain of function, a dominant negative mechanism, or even a combination of both. Therapeutic strategies involving global knockdown of both isoforms carries risk as DNAJB6 knockout results in embryonic lethality. Clarifying each isoforms' pathomechanistic role is critical to therapeutic strategy development. We therefore developed an isoform specific knockdown approach using morpholinos. Selective reduction of each isoform in WT or knock-in LGMD-D1 primary mouse myotubes, followed by mass-spectrometry, allowed us to characterize unique proteomic signatures for each condition. Selective reduction of either isoform in WT myotubes did not mimic the proteomic disease signature seen in LGMD-D1

myotubes. However, reducing DNAJB6b levels in LGMD-D1 myotubes corrected much of the proteomic disease signature towards WT levels. These findings support DNAJB6b's involvement in LGMD-D1 pathogenesis, and suggest selective reduction of DNAJB6b may be a viable therapeutic strategy for LGMD-D1.

1049. Pharmacology, Biodistribution and Tolerability 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 pathology caused by a trinucleotide expansion in the *DMPK* gene. Initial studies of our novel delivery technology in transgenic animal models, have demonstrated pharmacological activity of the PATrol™ platform-enabled peptide nucleic acid (PNA) pharmacophore in both brain and muscle after systemic administration. An exploratory radiolabeled biodistribution study of its delivery module administered intravenously in nonhuman primates showed distribution to brain, muscle, and heart, the major organs affected in DM1. To evaluate the capacity of our PATrol™ DM1 lead candidate to correct the DM1 phenotype *in vitro*, we assessed knock down of the human *DMPK* transcript and correction of splicing defects in both fibroblasts and myoblast cultures derived from DM1 patients. Treatment with our DM1 lead candidate rapidly reduced the human *DMPK* transcript and corrected splicing defects at nanomolar concentrations. Molecular and functional pharmacological activity, tissue distribution and tolerability were evaluated in the HSA*LR mouse model following single and repeated subcutaneous, intravenous, and intramuscular administration. As with the *in vitro* results in DM1 patient-derived cell lines, treatment with our lead candidate rapidly reduced mutant transcript levels, corrected splicing defects and rescued myotonia in this mouse model. At well tolerated doses in rodents, the levels of our DM1 lead candidate in key target tissues were above those required to reverse splicing defects *in vitro* in patient cells. These encouraging preclinical data support the potential for our PATrol™ DM1 lead candidate to be developed as a potential therapy for DM1 patients.

1050. Anti-Inflammatory Properties of IL-10-Expressing Multipotent Mesenchymal Stromal Cells for Duchenne Muscular Dystrophy

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[Background] Multipotent mesenchymal stromal cells (MSCs) are potentially therapeutic for muscle disease because they can accumulate at the sites of injury and act as immunosuppressant. MSCs are attractive candidates for cell-based strategies that target diseases with chronic inflammation, such as Duchenne muscular dystrophy (DMD). We focused on the anti-inflammatory properties of IL-10 and hypothesized that IL-10 could improve the potential benefits of IL-10 expressing adeno-associated virus (AAV) vector-transduced MSCs. We designed and examined the strategies for safe and effective *ex vivo* treatment of IL-10 producing MSCs using DMD models for functional recovery of the skeletal muscles, as well as focusing on factors related to the effects of MSC treatment. **[Methods]** IL-10/AAV-transduced MSCs (IL-10-MSCs) or MSCs from dental pulp were intravenously injected into canine X-linked muscular dystrophy in Japan (CXMD_J) in acute phase. To assess the safety and effects of therapeutic interventions, treated CXMD_J was comprehensively analyzed such as blood tests, growth, spontaneous activity, tetanic force, running function, MRI and histological examination. **[Results]** Repeated systemic administration of IL-10-MSCs into the CXMD_J model resulted in long-term engraftment of cells and slightly increased the serum levels of IL-10. IL-10-MSCs showed significantly reduced expression of pro-inflammatory MCP-1 and upregulation of stromal-derived factor-1 (SDF-1). MRI and histopathology of the MSC-treated CXMD_J indicated the regulation of inflammation in the muscles, but not myogenic differentiation from treated cells. IL-10-MSCs-treated CXMD_J showed improved running capability and recovery in tetanic force with concomitant increase in physical activity. While no significant difference was observed in both MSC- and IL-10-MSC-treated CXMD_J on the maintaining apparent function, we confirmed the weight of each muscle obtained from IL-10-MSC-treated CXMD_J increased compared to that in the MSC-treated CXMD_J, indicating morphological improvement. Furthermore, the increased serum creatine kinase levels after running exercise were suppressed in IL-10-MSC-treated CXMD_J, but not in MSC-treated CXMD_J, suggesting a protective effect against dystrophic damage caused by exercise. **[Discussion]** IL-10-MSCs facilitated the long-term retention of the cells in the skeletal muscle and protected muscles from physical damage-induced injury, which improved muscle dysfunction in DMD. We can suggest that the systemic administration of IL-10-producing MSCs offers potential benefits for DMD therapy through the beneficial paracrine effects of IL-10 involving SDF-1. This study would help us understand the mechanisms of MSC function.

1051. Circumventing the Adaptive Immune Response using Lipid Nanoparticles-mRNA for Effective Genome Editing in Skeletal Muscle

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Duchenne muscular dystrophy (DMD) is a genetic disorder caused by mutations in the DMD gene, leading to alterations of dystrophin. In vivo genome editing restores dystrophin expression in mouse and canine models of DMD, but the adaptive immune response to the delivery vector and clustered regularly interspaced short palindromic repeats (CRISPR) - Cas9 remains a primary barrier. Currently, adeno-associated virus (AAV) is the leading gene delivery vehicle for DMD gene therapy clinical trials and preclinical work for AAV-CRISPR. Previously, dystrophin protein restoration has been found to be sustained for at least 1 year after a single administration of AAV-CRISPR intravenously, determined using immunofluorescence staining and western blot. Furthermore, the host immune response to AAV-CRISPR suggests that humoral and cellular immune response profile varies with the age of mice. It was found that irrespective of the injection route (intramuscular, intravenous, and intraperitoneal), newborn mice circumvent the immunogenic response considerably, whereas the adult mice develop a humoral and cellular response to Cas9 and a humoral response to the AAV capsid. Alternatively, the mice injected with CRISPR-Cas9 as newborns do not develop a humoral response when reinjected as adults. This infers that evasion of the immune response can be accomplished through adapting to CRISPR-Cas9 antigenic stimuli. Thus, the goal of this study is to develop a safe and effective method to deliver the gene-editing therapy and compare the adaptive immune response profile of viral (AAV) and non-viral (lipid nanoparticle; LNP) delivery vectors. When compared to the viral vectors, non-viral delivery systems have fewer limitations, such as high delivery efficiency, low immunogenicity, absence of mutations, reduced packaging size limitation, and easier to synthesize in the laboratory. LNPs will be formulated by combining helper lipids, cholesterol, PEG-conjugated lipids, and mRNA encoding Cas9 in a microfluidic chamber. Our antibody-ELISA data showed that the serum samples of mice, when administered with non-viral delivery vehicles, successfully evaded humoral immune response and corrected a target gene in muscle. However, the editing efficiency of the non-viral vector is lower than AAV in skeletal muscle as demonstrated by ELISA and immunohistochemistry data. Taken together, LNP delivery is a potential route for local skeletal muscle editing that avoids an adaptive immune response. However, improvements in non-viral delivery chemistry will be needed to enable systemic correction of skeletal muscle.

1052. Assessment of Efficacy 6 Months After Systemic Injection of an AAV-CRISPR/Cas9 Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disease typically caused by out-of-frame mutations in the *DMD* gene, which leads to lack of the dystrophin protein. One therapeutic strategy for Duchenne is to reframe the *DMD* gene, allowing for production of an at least partially functional dystrophin protein that is associated with the milder, allelic disease Becker muscular dystrophy (BMD). We have developed a CRISPR/Cas9 gene editing therapy to delete exons 45-55, thus reframing the *DMD* gene and generating an in-frame deletion that has been associated with one of the mildest BMD phenotypes, where some Becker patients with a naturally occurring exon 45-55 deletion have been asymptomatic until their 60s. Importantly, this region also encompasses a hotspot of DMD patient mutations meaning this approach would be applicable for ~50% of all Duchenne patients. We previously demonstrated short-term efficacy and restoration of dystrophin after a single systemic injection of AAV9-CRISPR in a humanized DMD mouse model. Here we assess efficacy after 6 months to determine the long-term durability of this therapy. This work will advance preclinical development of our AAV-CRISPR therapy for Duchenne muscular dystrophy.

1053. AAV Gene Transfer of Dlk1-Dio3 miRNAs Indicates That the miRNA Cluster Regulates Mitochondrial Activities in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a severe muscle disease caused by the lack of dystrophin protein. Our recent microRNA profiling in DMD patients and animal models revealed a coordinated upregulation of a miRNA cluster from the *Dlk1-Dio3* locus (DD-miRNAs), in both serum and skeletal muscles. In dystrophic muscle, the DD-miRNA dysregulation correlates with aberrant epigenetic alteration, notably DNA and histone methylation, at a key regulatory genomic region of the locus, namely Intergenic Differentially Methylated Region (IG-DMR). To define the collective role of this cluster in the skeletal muscle, we performed gene transfer experiments using Adeno-associated virus (AAV). In these experiments, we simultaneously overexpressed 14 selected DD-miRNAs in healthy muscle and confirmed that this artificial expression mimicked the elevation seen in DMD. Interestingly, DD-miRNAs overexpression recapitulated many DMD signatures at the transcriptomic level. More importantly, we noted that the most highly down-regulated pathway between the two systems is the mitochondrial oxidative

phosphorylation (OxPhos) system, which is also a top candidate from bioinformatics analysis of DD-miRNAs target prediction. Activities of OxPhos key enzymes were confirmed to be down-regulated globally in the two *in vivo* systems, dystrophic and AAV-injected muscles, where DD-miRNAs are overexpressed. Furthermore, we utilized an *in vitro* myogenic hiPSC-based system to investigate the OxPhos response in the context of lack of DD-miRNA expression, which was achieved by IG-DMR homologous deletion. Knockout hiPSC-derived myotubes showed a global boost in OxPhos transcripts, protein level, and activities. It reinforces that DD-miRNAs can modulate mitochondrial activities in skeletal muscle. Besides, given that mitochondrial defects in DMD are commonly thought to be mediated by membrane instability, the present study elucidated a novel intrinsic mechanism regulating mitochondria via a large number of DD-miRNAs coordinately.

1054. Precise Engineered Prime Editing for Exon Skipping and Deletion in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is an X-linked monogenic progressive muscle wasting disorder caused by mutations in the *DMD* gene producing a non-functional dystrophin protein that results in severe muscle degradation prior to premature death. In most cases of DMD, deletions in one or more of the 79 exons disrupt the open reading frame of dystrophin. Functional restoration of the *DMD* gene can be accomplished by inducing exon skipping to produce frame restoration, generating a sufficiently functional dystrophin protein. Previous strategies have involved pairing classical CRISPR-Cas9 double-stranded breaks (DSBs) to excise whole exons, which has also been demonstrated to result in large off-target and bystander editing byproducts. The newly described prime editor (PE) is composed of a SpCas9 nickase fused to a reverse transcriptase that nicks and transcribes an edit encoded by a 3' extension on a prime editing guide RNA (pegRNA). The 3' extension of a pegRNA is composed of a primer binding site (PBS) that hybridizes to the target region and a reverse transcriptase template (RT-template) transcribing the edit. Improving the PE strategy further, nicking the opposite strand of the pegRNA transcribing the edit (PE3) increases the efficiency as opposed to the single nick method (PE2). Previous applications of PE for resolving DMD mutations involve directly overwriting small mutations or modifying the splice acceptor or donor region, which has been previously demonstrated to have low efficiency but high specificity. The low efficiency of PE edits has been partially attributed to the degradation of the 3' pegRNA extension, which can be reduced through the incorporation of structured 3' motifs connected with a linker. Paired PE-mediated deletions have additionally been demonstrated to decrease off-targets compared with classical Cas9 induced large deletions using dual pegRNAs. We have designed a panel of pegRNAs targeting exon 23 for both paired-end deletions and splice donor or acceptor single nucleotide polymorphisms (SNPs). We have previously shown successful G>C SNPs in the splice acceptor region of exons 45 and 51 in HEK293t cell culture through transfection with Lipofectamine 2000 using both PE2 and PE3 methods. We have additionally transduced mouse myoblasts with PE3 encoding a G>C SNP in the splice acceptor demonstrating a significantly reduced

transfection rate. To further optimize for dystrophin correction in skeletal muscle tissue, a lentiviral library expressing PE and the pegRNAs with varying 3' structural motifs and RT-template and PBS sizes will be used to transduce mouse myoblasts. The resulting genomic DNA and DMD transcript from SNP corrected and paired end deletion cells will be assessed through long-read sequencing (Oxford Nanopore) and short-read next-generation sequencing (Illumina) for an unbiased analysis of the genome editing outcomes. These data will contribute to the assessment of PE as a potential gene therapy for DMD and other similar monogenic genetic diseases.

1055. Long-Term Efficacy and Safety in Preclinical Hematopoietic Stem Cell Gene Therapy in Pompe Mice

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Pompe disease is an inherited neuromuscular disorder caused by acid alpha-glucosidase (GAA) deficiency, leading to lysosomal glycogen buildup in the heart, skeletal muscles, and central nervous system (CNS). Patients with Pompe disease, harboring mutations in the *GAA* gene that lead to the complete absence of functional protein, experience severe and progressive myopathy, leading to cardiorespiratory failure within the first year of life in the absence of treatment. Pompe disease is managed by enzyme replacement therapy (ERT), which slows disease progression, but requires life-long treatment, has substantial risks to develop immunogenicity to the recombinant human *GAA* protein, and fails to address declines in motor function and mortality. In addition, ERT does not cross the blood brain barrier in any appreciable level and cannot address glycogen accumulation-related neuropathology. These unmet needs warrant the development of additional treatment modalities for Pompe disease patients. A lentiviral vector was developed with a clinically proven promoter to test a chimeric human insulin-like growth factor 2 (IGF2)-tagged *GAA* transgene in the hematopoietic system of a Pompe disease mouse model. This vector was used to transduce hematopoietic stem and progenitor cells (HSPCs) that were infused in 7.5 Gy irradiated or busulfan conditioned mice and monitored for 4-8 months. Long-term supraphysiological *GAA* enzyme activity levels were achieved in hematopoietic cells and plasma. *GAA* enzyme penetration was measured in key tissues including heart, skeletal muscles, brain and spinal cord. Complete glycogen clearance and reduction of tissue vacuolation in murine heart, diaphragm, brain, spinal cord, and in most of the skeletal muscles was observed. Consequently, heart pathology was prevented and locomotor function improved. Average vector copy numbers in bone marrow were below four in all the experimental groups at 4 and 8 months, showing high

polyclonal vector integration site patterns with the typical integration preference into genes, without evidence of proto-oncogene selection or clonal dominance. Furthermore, glucose levels in mice undergoing hematopoietic reconstitution of transplanted genetically modified HSPCs were similar to controls during the course of the study, highlighting the safe profile of adding the IGF2 tag. Human CD34+ HSPCs also transduced with high efficiency without altering their colony-forming potential and provided robust production of the transgene product. In conclusion, HSPC gene therapy using the candidate vector was shown to be a potentially effective therapeutic approach in a mouse model of Pompe disease, demonstrating significant preclinical efficacy and safety. We believe this approach could translate into a clinical application of single-dose therapy for Pompe disease patients.

1056. Validation of Novel Safe-Harbor Sites for Targeted Genome Integration for Muscle Disease

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The development of CRISPR-based genome editing is an exciting advance for treating inherited muscle disease. Specifically for Duchenne muscular dystrophy, current leading CRISPR-based approaches are by splice-site disruption, fragment deletions of the mutated gene, or targeted integration of missing portions of a gene. Targeted integration would extend therapeutic options to a broad range of genetic diseases and synthetic biology applications. While splice-site disruption and targeted deletion have been well developed, the integration strategy remains a challenge, especially for translating the CRISPR-based treatment into clinical use. The challenges to developing targeted integration approaches include target-site selection, insert construct design, and efficient delivery. In recent years, several available integration methods have made substantial progress, however, the low targeting efficiency and potential mutagenesis from viral-mediated integration show that there is significant importance in developing a safe-harbor site as a target integration in gene editing. A safe-harbor site is a chromosomal site where transgenes can be stably and reliably expressed in all tissues of interest without adversely affecting endogenous gene structure or expression. At this time, several prospective safe-harbor sites have been used for transgene insertion, but there are very limited data regarding their safety validation and none of them are specific for muscle. Here, we performed a screening and validation of safe-harbor sites in the skeletal muscle and identified two efficient sites for safe integration. We constructed a promoterless donor plasmid containing a reporter gene or a therapeutic gene with an upstream of 43 base pairs (bp) of splice acceptor and a downstream of 21 bp splice donor to be integrated into the intron 1 of the safe-harbor locus. Our *in vitro* validation in mouse myoblast demonstrated sustained expression of the integrated reporter with precise in-frame integration at the DNA and mRNA level of identified sites. To further investigate these findings, our ongoing studies are focusing on the characterization of gene integration safety and efficacy in mouse models and utilizing next-generation sequencing to assess the on-target editing efficiency and global transcriptome profile alterations.

This study synergizes with the emerging developments in CRISPR-transposons and would represent a significant advancement in the development of reliable and stable insertion. Moreover, these results hold a promising roadmap to explore approaches for insertion-based therapy in muscle disease.

1057. DWORF Gene Therapy for Duchenne Muscular Dystrophy Cardiomyopathy

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Duchenne muscular dystrophy (DMD) is a genetic disorder characterized by severe muscle weakness and degeneration. Phenotypes of the disease can be partly attributed to an increased concentration of calcium in the cytosol of muscle cells. This calcium activates proteases and phospholipases that digest cellular proteins and lipids, respectively. The sarcoplasmic reticulum (SR) is the main calcium storage organelle in muscle cells. During muscle contraction, calcium is released from the SR to the cytosol. During relaxation, calcium is pumped back to the SR by the sarcoendoplasmic reticulum calcium ATPase (SERCA). In DMD, SERCA function is reduced, contributing to increased levels of calcium within the cytosol. Improvement of SERCA function is a promising strategy for reducing cytosolic calcium overload and improving muscle function in DMD. Recently, a small peptide named dwarf open reading frame (DWORF) was found to enhance SERCA function. Increasing DWORF expression has been shown to improve cardiac function in mouse models of ischemic heart failure and muscle-specific LIM protein deficiency-associated cardiomyopathy. Since cardiomyopathy is a primary cause of morbidity and mortality in DMD, we tested whether DWORF gene therapy can prevent Duchenne cardiomyopathy in aged mdx mice, a model that recapitulates the cardiac phenotype in patients. We examined DWORF expression in the heart of mdx and normal control BL10 mice and found the level was significantly reduced in mdx mice. To achieve efficient cardiac delivery, we delivered 6 E12 vg/mouse of the AAV9.DWORF vector to 6-week-old mdx mice by tail vein injection. ECG and closed-chest catheterization assay at 18 months of age showed significant improvement in multiple parameters of cardiac electrophysiology and blood flow hemodynamics. Our data suggest DWORF deficiency contributes to SERCA dysfunction in mdx mice and DWORF therapy holds promise to treat Duchenne cardiomyopathy (Supported by the National Institutes of Health R01 AR070517, Jesse's Journey-the Foundation for Gene and Cell Therapy, and Jackson Freil DMD Research Fund).

1058. Comprehensive Evaluation of the Fiber Type and Fiber Size Preference of AAV8 and AAV9 in the Canine DMD Model

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Systemic adeno-associated virus (AAV) gene therapy holds promise to treat Duchenne muscular dystrophy (DMD). A fundamental issue of AAV gene therapy is the fiber type and fiber size preference of the vector. Although this has been investigated extensively in mice, little is known about the fiber type and fiber size tropism of the AAV vector in large mammals. Here, we evaluated fiber type- and size-specific transduction properties of AAV8 and AAV9, two vectors used in clinical trials, in the canine DMD model. We analyzed muscles from 10 and 7 dogs that had received systemic AAV8 and AAV9 injection, respectively. For AAV8, five affected dogs received a therapeutic micro-dystrophin (uDys) vector, three affected and two carrier dogs received an alkaline phosphatase (AP) reporter vector. For AAV9, five affected dogs received the uDys vector, one normal and one affected dog received the AP vector. Fiber type (slow, fast, and hybrid), fiber size and dystrophin expression were evaluated by immunofluorescence staining. AP was evaluated by enzymatic histochemical staining. Correlation between AAV transduction and the fiber type/size was studied in teres major, latissimus dorsi, and biceps femoris, three muscles that showed mosaic transgene expression (ranging from 28 to 52% in AAV.AP transduced muscles and from 31 to 64% in AAV.uDys transduced muscles). There was no statistically significant difference in transduction efficiency between AAV8 and AAV9 in all three muscles. All three muscles were primarily composed of fast fibers (50-75%), followed by slow fibers (21-41%), and then hybrid fibers (2-11%). Overall, AAV transduction showed a similar pattern (fast fiber > slow fiber > hybrid fiber) in most muscles, reflecting the fiber type distribution. However, when transduction was evaluated within each fiber type group, no statistically significant fiber type-specific tropism was detected in most muscles, irrespective of the AAV serotype or the transgene. Surprisingly, the mean myofiber cross-sectional area was significantly altered by uDys, but not AP expression. In summary, our findings provide important information to better understand systemic AAV delivery and uDys gene therapy in large mammals (Supported by NIH, Edgewise Therapeutics, and Jackson Freeland DMD Research Fund).

1059. Development of Administration Protocols for Reduction of the Necessary Dose of rAAV for Treatment

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Backgrounds: AAV vectors are highly safe and allow long-term expression of therapeutic genes. Gene therapy using rAAV has been attempted to treat genetic diseases such as SMA, XLMTM, and DMD. When rAAV is administered intravenously in clinical trials, doses of 3e13 to 1e14v.g./kg BW are common. Systemic administration

of 1e14v.g./kg BW or higher may lead to direct hepatotoxicity, thrombocytopenia, TMA, immune reactions to viral vectors, and germ line transmission. Therefore, it is necessary to reduce the amount of vector administered or to consider a more secure administration protocol. Duchenne muscular dystrophy (DMD) is a congenital disease in which mutations in the dystrophin gene cause gradual loss of skeletal and cardiac muscle. However, in dogs and monkeys, the expression of AAV vector-derived genes decreases with time due to immune response. Here, we investigated the strategy of using somatic stem cells in combination with rAAV to induce immune tolerance to the rAAV9 vector and to express sufficient transgene at a lower dose. Somatic stem cells have been used in various inflammatory diseases, including graft-versus-host disease (GvHD), due to their immunosuppressive effects. In addition, we investigated the immunomodulatory effect of MSCs in rAAV transduction. Methods: Eight-week-old normal or CXMD_J (DMD) dogs were intravenously injected with bone marrow-derived MSCs and rAAV9-luciferase or rAAV9-microdystrophin (2.0e12 v.g./kg BW). Seven days after the injection, MSCs were injected systemically again. Eight days after the first injection, rAAV9-luciferase or rAAV9-microdystrophin was injected intravenously into the same dogs. qRT-PCR was used to analyze IFN- γ expression in purified canine peripheral leukocytes to examine the immune response to rAAV9. luciferase or rAAV9-microdystrophin in the skeletal muscle of transgenic animals was confirmed by immunohistochemistry. Furthermore, MSCs-treated CXMD_J transfected with rAAV9-microdystrophin were compared with non-treated CXMD_J to evaluate gait function and limb lameness. In the present study, we investigated whether we could confirm the function of these MSCs in vitro. Results: MSCs treatment followed by rAAV9 resulted in higher expression of transgenes (luciferase or microdystrophin) in skeletal and cardiac muscle compared to rAAV alone. rAAV9 treatment followed by IFN- γ expression in purified peripheral blood leukocytes was not enhanced by the combination of rAAV9 and MSCs. CXMD_J treated with MSCs and rAAV9-microdystrophin showed superior functional improvement compared to other DMD dogs of the same age. Conclusions: Our results demonstrate that MSCs pretreated rAAV injection improves the expression of rAAV-derived transgene in dogs. This strategy would be a practical approach to analyze the expression and function of transgenes in vivo. These findings also support the future feasibility of rAAV-mediated protein supplementation strategies in the treatment of DMD and various genetic diseases.

1060. Pathophysiological Mechanisms of Bone Damage and Bone Cross Correction in MPSIH Gene Therapy

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Mucopolysaccharidosis type I Hurler (MPSIH) is an autosomal recessive lysosomal storage disorder characterized by the deficit of the ubiquitous lysosomal enzyme α -L-iduronidase (IDUA), involved in the degradation of glycosaminoglycans (GAGs) within the cells. Progressive accumulation of GAGs leads to multi-organ dysfunction and skeletal abnormalities, known as *dysostosis multiplex*. Although allogeneic hematopoietic stem cell transplantation is considered the standard of care, the skeletal disease remains an unmet clinical need, probably due to the insufficient delivery of donor enzyme to bones and/or the irreversibility of the damages at the time of intervention, highly impacting patients quality of life. Results of the phase I/II gene therapy (GT) clinical trial (NCT03488394) at SR-TIGET showed peripheral blood supraphysiologic enzyme levels, near normalization of urinary GAGs, reduced joint stiffness, and growth tracking in-line with baseline percentile. As the pathogenesis of bone damage is still not well defined and there is still limited knowledge of bone correction after GT, we investigated the role and properties of mesenchymal stromal cells (MSCs), osteoblasts (OBs), chondrocytes (CHs) and osteoclasts (OCs) of healthy donors (HDs) and MPSIH patients enrolled in the trial before and after GT. We demonstrated that OC differentiation and their bone resorption activity were not impaired by IDUA absence. Moreover, characterization of patient-derived MSCs before GT was also not altered, although a reduced CD146⁺ cell frequency was assessed, indicating a possible alteration of the osteoprogenitor compartment. Indeed, MPSIH MSCs normally progressed through osteogenic and adipogenic differentiation. With regard to GAG deposit, although patient-derived MSCs did not show GAGs accumulation, when differentiated into OBs and adipocytes, they showed an increase in intracellular GAGs amount at the end of the differentiation. Analyzing cells after GT, we observed that patient-derived OCs and their supernatant showed supraphysiologic enzyme activity. MPSIH-MSCs and OBs were able to uptake the missing enzyme when exposed to the supernatant of gene-corrected OCs, rescuing GAG engulfment found in MPSIH-OBs, demonstrating the feasibility of cross-correction. We will further investigate the interplay between OBs and OCs in a more physiological environment with a 3D vascularized bone remodelling model. Moreover, we are investigating CH differentiation of MPSIH-MSCs together with the endochondral ossification process

by exploiting a 3D model of hypertrophic cartilage in order to mimic the growth plate. We are currently analysing bone biopsies obtained from MPSIH patients before and after GT by immunohistochemistry and transmission electron microscopy. From our preliminary data, we found a reduced expression of type I collagen in untreated MPSIH patients as compared to HD.

1061. Intravenous Delivery of AAV Gene Therapy Provides Broad SOD1 Knockdown in the Spinal Cord and Robust Efficacy in a Mouse Model of SOD1-ALS

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Mutations in superoxide dismutase 1 (SOD1) result in progressive motor neuron loss through toxic gain-of-function properties and are responsible for up to 20% of familial amyotrophic lateral sclerosis (ALS), or 2-4% of all ALS patients in the U.S. Studies using transgenic mice expressing SOD1 mutations have demonstrated reduced neuropathology, improved motor behavior, and extension of survival following several methods of SOD1 reduction. While these approaches have demonstrated varying degrees of efficacy, they often rely on direct spinal cord delivery and fail to achieve the broad SOD1 reduction throughout the CNS thought to be necessary for maximal clinical benefit. Voyager's novel AAV capsids now allow for intravenous delivery that is expected to provide appropriate biodistribution to motor neurons along the entire primate spinal cord, a key translational step for successful therapy. These novel AAV vectors carry transgenes encoding artificial pri-miRNAs using RNA interference (RNAi), a naturally occurring process that mediates gene silencing, to selectively reduce SOD1. Here, we report the results of an AAV gene therapy targeting SOD1 with RNAi, using IV delivery with a BBB-penetrant capsid, in studies in a G93A mouse model of ALS. These studies demonstrated robust SOD1 knockdown throughout the rostral-caudal extent of the spinal cord that correlated with vector genome levels and significant improvements in motor performance and survival extension, beyond what we have previously reported with intraparenchymal, intrathecal, or intracisternal delivery. These results suggest that the combination of potent and tolerable AAV-siRNA mediated knockdown with intravenous dosing of a BBB-penetrant capsid can demonstrate substantial phenotypic rescue in an SOD1-ALS mouse model and support its continued development and translation into the clinic.

1062. CRISPR-Cas9 Induced Re-Framing Repairs a Muscular Dystrophy-Causing *DYSF* Founder Mutation in Primary Patient Muscle Stem Cells and a Novel Humanized Mouse Model

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Limb-girdle muscular dystrophy type 2B (LGMD2B) is an autosomal recessive disease caused by loss-of-function mutations in *DYSF*, the gene encoding dysferlin, a protein with an important role in muscle membrane repair. LGMD2B patients suffer from progressive degeneration and atrophy of limb muscles, ultimately leading to loss of ambulation. There is no treatment. CRISPR/Cas9-mediated repair of patient-specific mutations is becoming a realistic scenario to treat rare monogenic disorders by cell-based or *in vivo* gene therapies, provided that sufficient rates of therapeutically relevant edits can be achieved in the target cells or tissues. We obtained pure primary muscle stem cells (MuSC) from two patients carrying a homozygous frameshift founder mutation in *DYSF* exon 44 (c.4872_4876delinsCCCC) and generated induced pluripotent stem cells (iPSC). A screen of mutation-specific sgRNAs in combination with Cas9 in patient-derived iPSC identified one sgRNA with a strong bias (>80% of edits) to induce *DYSF* exon 44 re-framing by insertion of an adenine at a defined position. mRNA-mediated delivery of Cas9 and the sgRNA to primary patient MuSC led to >60% efficient re-framing and a subsequent rescue of dysferlin protein expression. Re-framed dysferlin, albeit with four amino acid exchanges, showed a wild-type localization pattern in gene edited patient myotubes and relocated to the site of membrane injury in response to laser wounding. We then generated a mouse model harboring a humanized exon 44 with the founder mutation on the endogenous *Dysf* locus, and a control mouse model carrying the wild-type human *DYSF* exon 44. Homozygous mice with the wild-type human sequence showed normal levels of dysferlin mRNA and protein, and no pathological phenotype. Homozygous mice carrying the founder mutation completely lacked dysferlin protein and displayed a progressive dystrophic phenotype with hallmarks of LGMD2B. mRNA-mediated Cas9 plus sgRNA delivery to mouse MuSC induced exon 44 re-framing, thereby repairing the founder mutation and rescuing dysferlin protein to almost wild-type levels. Our work sets the stage for cell-based gene therapy to treat individual muscles of patients carrying a founder *DYSF* mutation by autologous transplantation of gene repaired MuSC and introduces a novel mouse model to assess *in vivo* gene editing therapies on the relevant human sequence.

1063. AAV Vectors Consistently Display Higher Transcriptional Activity in MDX Mouse Muscle Versus Normal Mouse Skeletal Muscle

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Introduction: AAV vectors are being developed for gene therapy in the skeletal muscle and the heart. In the course of our studies, we have consistently noted a marked tendency for higher AAV mediated transgene expression in the skeletal muscle of *mdx* mice, a model for Duchenne Muscular Dystrophy, compared to wild type (WT) animals. Here, we present the results of experiments designed to further establish this phenomenon and investigate its underlying causes. Results: 5-week-old *mdx* and WT C57/BL10 mice were injected systemically with an AAV8 vector encoding a microdystrophin (μ Dys) at a dose of 5e13 GC/kg. Tissues were harvested four weeks post injection. The vector DNA biodistribution in skeletal muscle, heart and liver showed identical profiles in WT and *mdx* animals. In contrast, the levels of vector mRNA (ddPCR) and μ Dys protein (Western blot) measured in WT skeletal muscle were around 10 and 20-fold lower than in *mdx* skeletal muscle, respectively. No differences were found in the protein or mRNA levels in the heart and liver between the two strains and the same amount of circularized vector genomes was detected in the gastrocnemius of each strain. The same observation was made in a study using an AAV9 μ Dys vector at the same dose, where marked differences in vector mRNA (31-fold) were detected in the skeletal muscle. These findings were consistent with our previous report (Firnberg et al, 2021 ASGCT) describing muscle transduction in *mdx* vs WT mice with a library of AAV capsids from different clades carrying barcoded CAG-EGFP transgenes. We concluded that transcription from AAV vectors is enhanced in the skeletal muscle of *mdx* mice, independent of the capsid, promoter, and transgene. To investigate this disparate RNA expression in the skeletal muscle, we utilized qPCR arrays in *mdx* and WT samples and identified a group of factors involved in chromatin structure, ubiquitination, and histone methylation that were downregulated in *mdx* skeletal muscle and found to increase AAV transduction when knocked down in differentiated C2C12 cells. Conclusion: Our data indicate an important difference in AAV transduction efficiency in healthy versus dystrophic skeletal muscle. Establishing the role of cellular factors in the transcriptional activity of AAV genomes delivered to the skeletal muscle nuclei may be relevant to the design of efficacious and stable AAV gene therapies targeting the muscle.

1064. The gRNA Vector Level Determines the Outcome of Systemic AAV CRISPR Therapy for Duchenne Muscular Dystrophy

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Adeno-associated virus (AAV)-mediated CRISPR editing holds promise to restore missing dystrophin in Duchenne muscular dystrophy (DMD). Intramuscular co-injection of Cas9 and gRNA vectors resulted in robust dystrophin restoration in short-term studies in the *mdx* mouse model of DMD. Intriguingly, this strategy failed to yield efficient dystrophin rescue in muscle in a long-term (18-month) systemic injection study. In-depth analyses revealed a selective loss of the gRNA vector following long-term systemic, but not short-term local injection. To determine whether preferential gRNA vector depletion is due to the mode of delivery (local versus systemic) or the duration of the study (short-term versus long-term), we conducted a short-term systemic injection study. The gRNA (4e12 vg/mouse in the 1:1 group or 1.2e13 vg/mouse in the 3:1 group) and Cas9 (4e12 vg/mouse) vectors were co-injected intravenously to 4-week-old *mdx* mice. The ratio of the gRNA to Cas9 vector genome copy dropped from 1:1 and 3:1 at injection to 0.4:1 and 1:1 at harvest three months later, suggesting the route of administration, rather than the experimental duration, determines preferential gRNA vector loss. Consistent with our long-term systemic injection study, the vector ratio did not influence Cas9 expression. However, the 3:1 group showed significantly higher dystrophin expression and genome editing, better myofiber size distribution, and a more pronounced improvement in muscle function and electrocardiography. Our data suggest that the gRNA vector dose determines the outcome of systemic AAV CRISPR therapy for DMD.

1065. Stability of Microdystrophin Proteins Measured by Pulse-Chase Assays in Tissue Culture

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Adeno-associated virus (AAV)-mediated gene therapy represents one of the most promising therapeutic strategies for Duchenne Muscular Dystrophy (DMD). Due to limited packaging capacity of the AAV vector, the dystrophin coding sequence must be truncated to produce microdystrophin. Internal or terminal deletions in dystrophin often

leads to unstable proteins, due to either altered folding in rod and hinge repeats junction, or suboptimal interaction with dystrophin associated protein complex (DAPC) resulting in a more labile membrane complex. We have designed microdystrophins with increasing length of carboxyl terminal (CT) domain and sought to measure their stability in tissue culture with two different pulse-chase assays. Three microdystrophin constructs containing different lengths of CT were evaluated: μ Dys-CT194, μ Dys-CT140 and μ Dys-CT48. We previously reported AAV8 encoding μ Dys-CT194 can produce higher microdystrophin level in the skeletal muscle of *mdx* mice compared to μ Dys-CT140 and μ Dys-CT48 (Qiao et al, ASGCT 2021). In the current study, we set to explore microdystrophin stability through transfection of mouse C2C12 myoblasts with plasmid encoding Halo- μ Dys-CT194 (Halo denotes the self-labeling protein tag, HaloTag[®]) and Halo- μ Dys-CT48 fusion protein followed by fluorescent pulse chase study. Kinetic imaging and automatic image analysis were employed to track the decay of Halo- μ Dys fusion protein fluorescent signal over time and calculation of half-life. Our results indicated that the half-life of Halo- μ Dys-CT194 was 1.8-fold higher than Halo- μ Dys-CT48, suggesting CT contribution to microdystrophin stability. In parallel, cycloheximide chase was used to determine the turnover rate of microdystrophin proteins with different length of CT. HEK293 cells were transfected with plasmids encoding μ Dys-CT194, μ Dys-CT140 and μ Dys-CT48, translation was halted with cycloheximide, and microdystrophin level was measured at various timepoints using an anti-dystrophin antibody-based flow cytometric assay. Normalized intensity was plotted as a function of time and the data were fit to an exponential decay curve to calculate half-life. The half-life of μ Dys-CT194 was measured to be 1.5-fold higher than μ Dys-CT140 and 2.1-fold higher than μ Dys-CT48 in HEK293 cells. To decipher factors affecting protein stability, the purified recombinant protein μ Dys-CT194 and μ Dys-CT48 from BV/Sf9 expression system were further characterized by differential scanning fluorimetry (DSF) for melting temperature measurement and serial dilution of proteinase K digestion for protease resistance test. Our data showed the melting temperature and protease resistance were identical for the purified μ Dys-CT194 and μ Dys-CT48. This suggests that the increased stability rendered by longer CT domain in μ Dys-CT194 might occur through interaction with cellular partners, possibly members of the Dystrophin Associated Protein Complex, which are known to assemble at the CT domain. Such interaction was lost in a test tube containing only purified μ Dys proteins in the melting temperature and protease resistance measurement. In conclusion, we were able to successfully measure the half-life of microdystrophin protein in cell culture by fluorescent pulse-chase using HaloTag[®] kinetic imaging and cycloheximide chase flow cytometry. Our data indicate that the extended CT-domain in microdystrophin may increase protein half-life up to 2-fold and have the potential to improve the therapeutic benefit of these novel microdystrophins.

1066. Development of an AAV-Based microRNA Gene Therapy for Myotonic Dystrophy Type 1 (DM1)

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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 dystrophin myotonia 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. DMPK mRNA downregulation (e.g., via siRNA and/or ASOs) has shown promise in animal models of Myotonic Dystrophy type 1 (DM1), and partial DMPK suppression has shown to be well tolerated both in mice and non-human primates; however, those approaches require repeated administrations, thus increasing the treatment burden on the patients. To overcome these limitations, we developed an artificial miRNA to target human DMPK mRNA for constitutive expression following delivery by the recombinant adeno-associated vector (rAAV). Here we showed that AAV-mediated muscle targeted expression of engineered artificial microRNA (amiRDMPK) directed against DMPK mRNA reduces toxic DMPK mRNA and alleviates muscle phenotype in the DMSXL mouse model. To determine the potential of amiRDMPK to correct the DM1 phenotype *in vitro*, we tested its ability to silence the human DMPK transcript and correct the splicing defects in skeletal myoblast cultures from DM1 patients. The treatment of DM1 patient cells with amiRDMPK reduced DMPK mRNA by more than 50% and corrected splicing defect of MBNL1. Next, we evaluated this AAV-amiRDMPK approach in DMSXL mice, a DM1 mouse model carrying a human DMPK gene with toxic CTG repeats. Our results show that a single intravenous injection of AAV vector, expressing the amiRDMPK, resulted in a 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. Overall, our data provide evidence on the efficacy of artificial miRNAs against DMPK as promising AAV-based gene therapy for DM1.

1067. A Novel AAV8-Based Gene Therapy for Duchenne Muscular Dystrophy: Preclinical Studies in the Mdx Mouse

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Duchenne muscular dystrophy is an X-linked developmental disorder caused by mutations in the dystrophin gene leading to progressive muscular weakness and premature death due to respiratory and/or cardiac failure. RGX-202 is a recombinant adeno-associated virus of serotype 8 (AAV8) with an optimized human microdystrophin transgene and a promoter designed to increase expression in muscle (Sp5-12). The RGX-202 microdystrophin transgene is designed to encode key elements of full-length dystrophin including the extended coding region of the C-terminal (CT) domain. The CT domain is known to recruit α -dystrobrevin and α -syntrophin to the dystrophin-associated protein complex (DAPC) that contributes to maintaining membrane integrity and cellular signaling during muscle contraction and relaxation. RGX-202 was administered intravenously to young male *mdx* mice and evaluated over 6 weeks. RGX-202-administered *mdx* mice showed a dose-proportional increase of microdystrophin protein in the skeletal and cardiac muscles. RGX-202-administered *mdx* mice exhibited significant improvements in muscle strength in the diaphragm and Extensor Digitorum Longus (EDL) measured by *in vitro* force (specific force and eccentric contractions). Consistent with these results, the dystrophic pathology (i.e., inflammation, degeneration, and regeneration) was improved in the RGX-202-administered *mdx* mice. Immunofluorescence data revealed that increased RGX-202 microdystrophin expression was correctly localized to the sarcolemma of the skeletal muscles, and DAPC expression also was restored. Our data demonstrate that a single intravenous injection (IV) of RGX-202 in *mdx* mice provided notable improvements in muscle function and dystrophic muscle pathology, and dose-dependently increased RGX-202 microdystrophin protein expression. We are currently developing RGX-202 as a candidate for microdystrophin gene therapy for DMD.

1068. Recruitment of nNOS and Other Dystrophin-Associated Protein Complex Members by Different Microdystrophin Constructs

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Duchenne muscular dystrophy (DMD) is a fatal disease caused by mutations in the gene encoding dystrophin. Dystrophin is a central member of the oligomeric dystrophin-associated protein complex (DAPC) that links intracellular actin to extracellular laminin, thereby stabilizing the highly dynamic plasma membrane (sarcolemma) of skeletal and cardiac muscle. Sustained dystrophin expression

through AAV-mediated gene transfer is an appealing therapeutic strategy for DMD, but the AAV genome is too small to incorporate the entire dystrophin coding sequence. Consequently, engineered “microdystrophins” containing the critical functional domains of dystrophin have been developed for clinical use. Microdystrophins substitute for dystrophin in the DAPC, but the composition of these microdystrophin-containing DAPCs is dependent on the precise domains contained within the microdystrophin construct. For example, binding sites for signaling adaptors like the syntrophins and dystrobrevin are present in the dystrophin C-terminus, while the canonical binding site for neuronal nitric oxide synthase (nNOS) is in spectrin-like repeats 16/17 of the rod domain. We undertook an analysis of microdystrophin constructs in dystrophin-deficient *mdx* mice to determine their abilities to recruit DAPC members. Microdystrophins with or without portions of the dystrophin C-terminus and with or without repeats 16/17 were tested. Surprisingly, we found that nNOS protein and *in situ* activity were detectable by microscopy at the sarcolemma of all treated mice. To follow this result, we evaluated nNOS expression and activity as well as presence of integral and non-integral DAPC proteins in plasma-membrane enriched muscle lysates. Our results contradict accepted doctrine on microdystrophin structure/function relationships and highlight the utility of further investigation into the behavior of clinical microdystrophins. Future work will be directed towards understanding whether specific dystrophin domains are more effective at mobilizing nitric oxide in response to muscle stimulation, irrespective of nNOS localization at the sarcolemma.

1069. Automated Immunofluorescence Analysis of Dystrophin and Other Markers in Mouse Muscle and Heart Sections

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Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease arising due to mutations in the expansive gene encoding the sarcolemmal protein dystrophin. Due to the vast size of the *DMD* gene, a wide variety of mutations have been found in different loci throughout the gene, with varying effects on the resulting protein expression and function. The inability to replace the entire dystrophin coding sequence and the considerable diversity of DMD-causing mutations present major challenges to the development of effective gene therapies for DMD. Preclinical research aiming to develop safe and effective gene therapies for DMD has largely relied on animal models of specific *Dmd* mutations to assess the efficacy, kinetics, and longevity of potential therapeutic approaches for restoring dystrophin expression. Despite the expense and difficulty of *in vivo* research involving new mouse models, methods for quantifying dystrophin expression in these studies remain limited, subjective, and time-consuming, reducing the potential depth and utility of the results from this critical work. To address this gap, we have designed an automated, unbiased, and detailed approach for quantification of dystrophin and other muscle markers in whole tissue sections from mouse muscles and hearts. Images of entire muscle cross-sections undergo automatic segmentation to identify individual muscle fibers and dystrophin-positive segments at their sarcolemma

using automatically and objectively derived thresholds based on untreated control muscles. The precise percentage of dystrophin-positive sarcolemmal coverage and mean sarcolemmal dystrophin intensity are calculated for each muscle fiber, and represented in colorful heatmaps that are automatically generated to reflect analysis results. Muscle fiber size, central nucleation, and sarcolemmal or sarcoplasmic markers other than dystrophin can be automatically and objectively quantified as an integral part or a simple extension of this analysis methodology. Here we present results demonstrating the application of this analysis methodology in a preclinical study of AAV-mediated dystrophin restoration in a mouse model harboring a duplication of *Dmd* exon 2 (Dup2). After systemically treating Dup2 mice with our AAV9.U7.ACCA vector to induce U7snRNA-mediated skipping of exon 2, we show widespread restoration of dystrophin signal at the sarcolemma throughout whole sections from multiple muscles and the heart, accompanied by reduction in central nucleation in treated muscles. We also show inhomogeneity of dystrophin expression following treatment, underscoring the importance of using quantification methods that represent entire tissue sections instead of selecting regions of interest (ROIs). The results of this study reflect the depth, breadth, and precision of quantitative information that can be extracted in an accessible and automated fashion by removing the need for selecting image ROI, subjectively counting fibers by eye, or manually setting signal thresholds. We have published previous work validating that this methodology also shows good repeatability and reproducibility between different operators in human dystrophinopathy biopsies showing a wide range of dystrophin expression levels. We anticipate that making this analysis approach available to more investigators will significantly contribute to progress in preclinical research aiming to develop gene therapies for DMD.

1070. Monocyte Chemoattractant Protein-1 is a Serum Biomarker to Study Muscle Disease in the Duchenne Muscular Dystrophy Canine Model

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Duchenne muscular dystrophy (DMD) is the most common lethal muscle disease caused by mutations in the dystrophin gene. Currently, there is no cure for DMD. A variety of gene, cell, and small molecule drug therapies are being developed to treat DMD. The DMD canine model closely recapitulates human disease and has played a critical role in translating experimental therapeutics to human patients. A prominent feature of DMD is bodywide muscle inflammation. The inflammatory response in DMD can be further complicated by gene or cell therapies, and anti-inflammatory drug therapies. A noninvasive (or minimal invasive) biomarker for muscle inflammation would greatly facilitate preclinical studies in the canine DMD model. Serum samples can be readily and repeatedly obtained from canines. We hypothesize that serum cytokine levels may represent useful biomarkers to monitor inflammatory responses in affected dogs. To test our hypothesis, we collected serum samples from a large cohort of mixed-breed normal

(N) and affected (A) dogs at the age of 0.25 (sample size N=22, A=16), 2 (N=16, A=28), 6 (N=14, A=23), 9 (N=12, A=21), 12 (N=13, A=16), 24 (N=15, A=12), and 36 (N=13, A=12) months. These time points cover the entire course of the disease from newborn (0.25 months) to the pre-symptomatic stage (2 months), clinical disease stage (6, 9, and 12 months), and terminal stage (24 and 36 months). Thirteen serum cytokines/chemokines were quantified using the Luminex system. These include interleukin (IL)-2, 6, 7, 8, 10, 15, and 18, granulocyte-macrophage colony-stimulating factor (GM-CSF), keratinocyte chemoattractant-like (KC-like), monocyte chemoattractant protein-1 (MCP-1; also called C-C motif chemokine ligand 2, CCL2), interferon- γ (IFN γ), IFN γ -induced protein 10 (IP-10), and tumor necrosis factor- α (TNF α). Statistically significant differences between normal and affected dogs were detected for IL-8 at 6, 12, 24, and 36 months, for IL-10 at 6 months, for GM-CSF at 2 months, for KC-like at 6 and 36 months, and for MCP-1 from 2 to 36 months. Our results suggest that serum IL-8 and MCP-1 may represent useful biomarkers of bodywide muscle inflammation in the canine DMD model after the onset of the disease. Further, MCP-1 may represent an early biomarker to study muscle inflammation before the appearance of dystrophic symptoms. The identification of these blood markers paves the way to better use the canine model for novel therapy development. (Supported by NIH R01 AR070517 and R01 NS90634, Jesse's Journey-the Foundation for Gene and Cell Therapy, Jackson Free DMD Research Fund, University of Missouri Life Science Fellowship, and University of Missouri Molecular Biology T32 training grant).

1071. CRISPR Genome Editing in Microphysiological Human Tissue System Models of Duchenne Muscular Dystrophy

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Introduction: Gene editing technologies have shown potential to treat a variety of animal models of devastating human diseases, such as Duchenne muscular dystrophy (DMD). However, there remain significant challenges to evaluating the efficacy and potential adverse consequences of these therapeutics. The ability to model and assess the functional outcomes, cellular toxicity, and off-target editing efficiencies in human tissues, rather than in 2D cell culture or animal models, would enable more accurate preclinical safety studies that would likely better predict outcomes in human clinical trials. To address this unmet need, we have engineered human skeletal muscle microphysiological tissue systems (myobundles) that recapitulate physiological muscle function. We developed these systems from both wild-type (wt) human iPSCs (hiPSCs) and hiPSCs that lack exons 48-50 in the *DMD* gene and thus are unable to produce dystrophin, constituting a model of DMD muscle tissue. In this study, we aim to evaluate the efficacy and possible adverse consequences of an AAV-delivered CRISPR therapeutic targeting exon 51 of *DMD*, which we predict will restore the *DMD* reading frame and rescue the phenotype in treated myobundles.

Methods and Results: We first generated myobundles from three wt hiPSC lines and confirmed that there was little intra-hiPSC line variability in contractile force and calcium transient amplitude. We also generated myobundles from hiPSCs with *DMD* exons 48-50 deleted, however we found no functional contractile differences

between this line and myobundles from the wt hiPSC lines. Next, we sought to optimize AAV transduction by transducing cells at the time of 3D tissue formation with either AAV2, 6, 8, or 9 encoding CMV-GFP at 2E3 vg/cell. By measuring GFP+ cross-sectional area (CSA), we found AAV6 had the highest transduction efficiency at ~40% followed by AAV2 at ~20%, with little to no transduction with AAV8 and 9. AAV6 induced a small but significant 20% decrease in force generation and myobundle CSA while other serotypes had no impact on these parameters compared to control tissues. Ultimately, this led to similar specific forces (force/CSA) in all groups indicating that muscle function is not impaired by AAV transduction. Together, this suggests that successful AAV transduction may result in cell death and/or decreased cell proliferation but has no detrimental effect on muscle function. We then utilized the DMD myobundle platform to evaluate the functional and gene editing outcomes of CRISPR treatment. To assess toxicity, we treated myobundles with low (4e3 vg/cell) or high (4e4 vg/cell) doses of AAV6 expressing either gRNAs targeting exon 51 (Sg51) or scrambled gRNAs (SCR). AAV transduction induced dose-dependent decreases in absolute force and CSA independent of cargo. However, specific force was unchanged between non-transduced, SCR, or Sg51 transduced myobundles. Collectively, this suggests that AAV transduction in myobundles results in dose-dependent and cargo-independent decreases in cell viability and/or proliferation while not impacting final muscle function. Strikingly, myobundles treated with the high dose of Sg51 showed an exon 51 deletion frequency of 20% via ddPCR. This led to a 20% dystrophin restoration compared to the untreated controls, as measured by western blot. **Conclusion:** We have generated human skeletal muscle microphysiological tissue systems that model the muscle of both wt and DMD patients. We have successfully shown that these models can be transduced via AAV and have demonstrated significant editing rates that result in restoration of the target protein. In ongoing studies, we are utilizing these models to evaluate the safety and efficacy of gene editing by measuring off-target editing, rates of AAV integration, and the immune response to bacterial editors.

1072. Evaluation of AAV9 Gene Therapy for SMARD1/CMT2S in Different Mouse Models Reveal Differences in Efficacy Dependent on Promoter Choice

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IGHMBP2 (immunoglobulin mu DNA binding protein 2) is a ubiquitous gene that encodes for an ATPase/helicase of the SF1 superfamily, of unknown exact function. Mutations in IGHMBP2 cause a broad clinical spectrum of symptoms encompassing two diseases, Spinal muscular atrophy with respiratory distress type 1 (SMARD1), characterized by distal muscle weakness with fatal respiratory distress/failure, and Charcot Marie Tooth Disease 2S- CMT2S, with sensory

and milder motor neuropathies and a lesser respiratory involvement. Patients can fall in the middle of the spectrum of both diseases, as there is no evidence of phenotype-genotype correlation. Current treatment strategies are aimed at supportive care, and do not impact disease progression. Thus, there is an urgent need to develop an effective therapy for SMARD1/CMT2S. Previous studies show that intravenously delivered adeno-associated virus 9 (AAV9), carrying a functional IGHMBP2 cDNA, rescue the disease phenotype in an intermediate mouse model. However, prior to moving this therapy to clinic, further optimization was needed for both the delivery method, promoter selection, as well as evaluating the efficacy on the disease spectrum. We designed two transgene cassettes using different promoters driving IGHMBP2 cDNA expression suitable for clinical vector production (AAV9-Promoter1-IGHMBP2; AAV9-Promoter2-IGHMBP2). We have previously presented data on promoter comparison efficacy using a severe mouse model (EM3), as well as preliminary studies on an intermediate (nmd-2J) model. However, to gain confidence that the treatment would work in the whole IGHMBP2 disease spectrum, and ensure reproducibility of the results, the therapeutic constructs were tested in a blind manner in intermediate (nmd-2J) and mild mouse models (EM5). All laboratories included were blinded to the nature of the constructs and received either 2 or 3 viral vectors labelled Virus A, B and C (comprising in random order empty capsid and the 2 vectors). Animals received a single intracerebroventricular injection on postnatal day 1 to spread the viral vector via CSF. All labs independently found that virus A and C improved survival and body weight of the intermediate mouse model. Both virus A and C also improved hindlimb strength (as measured by the hindlimb splay test) and showed a marked improvement in neuromuscular junction innervation and muscle cross sectional area rescue. Moreover, in the intermediate nmd-2J models, electrophysiological measurements previously used in Spinal Muscular Atrophy type 1 pre-clinical and clinical trials showed marked improvement in treated animals. In the mild EM5 model, animals injected with both Virus A and C were indistinguishable from wild type littermates on strength test (measured by hanging wire test) and body weight. In addition, treatment with both virus on EM5 mice significantly improved the axon count in both femoral motor and sensory nerves, as well as improving muscle mass. While the 2 viral vector constructs performed comparable in nmd-2J and EM5 mice, previous testing on the EM3 model (severe) revealed differences in performance. In summary, strong, reproducible, in vivo efficacy data shows CSF delivery is a viable route for AAV9.IGHMBP2-mediated therapy and testing in a more severe animal model revealed differences between the two constructs that are less evident in the intermediate and milder disease form. Clinically applicable outcome measures were evaluated in both animal models and supported the development of further clinical studies.

1073. Development of New Micro-Dystrophins with Enhanced Cardiac Functionality

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Duchenne muscular dystrophy (DMD) is among the most common human genetic disorders, affecting approximately 1 in 5,000 newborn males. It results from genetic mutations in the DMD gene that

prevent expression of functional dystrophin, which is one of the largest proteins made by the human cells. Affected patients develop a progressive skeletal muscle degeneration, weakness, and often die in their late teens or 20s from respiratory insufficiency or cardiomyopathy. Adeno-associated viral (AAV) vector-based gene delivery holds great promise for treating genetic disorder. They are able to efficiently transduce a wide variety of tissues including skeletal and cardiac muscles, making them very attractive for DMD gene therapy. The main limitation of using AAV vectors for DMD gene therapy is their limited cargo capacity (~5 kb) which is three times smaller than the coding sequences of the DMD gene. We previously designed a variety of miniaturized micro-dystrophins (μ Dys) that displayed significant improvement of skeletal muscle function. However, most of them have not been functionally tested in the heart, and the best constructs cloned so far exhibit incomplete rescue of cardiac hemodynamics. Here, we present six novel μ Dys variants which include parts of the rod domain that are hypothesized to be involved in the dystrophin cardiac function, and which are found deleted in some patients with late onset cardiomyopathy. To evaluate their in vivo expression and stability, these new constructs were packaged in AAV6 vectors and delivered locally into tibialis anterior muscles of DMD mouse model. Five weeks following their administration, a strong protein expression was detected in the injected muscles, with a correct localization of μ Dys at the sarcolemma membrane. In addition, analysis of muscle cross-section showed a decrease of abnormal fibers with central nuclei. These data suggest that our new μ Dys are functional and stable in skeletal muscle. Our ongoing study focus on delivering systemically these constructs to evaluate their effects on both skeletal and cardiac function in rodent and large DMD animal models.

1074. Jagged1 Ameliorates Dystrophic Phenotypes in Mouse Model of Duchenne Muscular Dystrophy

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Gene therapy for Duchenne muscular dystrophy (DMD) has been challenged by the large size of the disease causal gene *DMD* and large range of mutation sites along the gene. Genetic modifiers have been shown important in disease progression in preclinical models and clinical data. Targeting key modifiers can be complementary to current therapies and work for a larger patient population. Our lab has previously shown that Jagged1 as the genetic modifier of DMD pathology in a Golden Retriever Muscular Dystrophy (GRMD) dog colony. Two exceptional "escaper" dogs exhibited a drastically milder phenotype than typical GRMD despite being dystrophin-deficient. We have now further assessed and validated the therapeutic potential of Jagged1 to ameliorate DMD pathology using a transgenic mouse model. We created a cardiac and skeletal muscle specific overexpression of Jagged1 (driven by MCK-Cre) in a dystrophic mouse model (mdx5cv). Using behavioral, physiological, histological, and molecular assays, we have evaluated the skeletal and cardiac phenotypes of the mice at

three key time points of lifespan. Compared to the mdx mice, double transgenic mdxJAG1 showed delayed degeneration and increased fiber size in the muscle at early timepoints. At one year, the mdxJAG1 mice had significantly bigger muscles with more force production, as well as smaller hearts with improved cardiac function measured by echocardiogram. This further shows the promise of Jagged1 as a therapeutic target in DMD.

1075. Translating DUX4-Targeted RNAi-Based Gene Therapy for FSHD

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Background: Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder affecting approximately 870,000 people worldwide, with an estimated incidence ranging from 1 in 8,333 to 1 in 20,000. FSHD is a progressive muscle wasting disease with symptoms typically starting in early adulthood. There is currently no treatment for FSHD. Previously, our lab published pre-clinical efficacy and safety for AAV.U6.mi405, a DUX4-targeted RNAi-based gene therapy for FSHD. **Objectives:** 1) To assess the long-term durability of the mi405 product, 2) perform pivotal pre-clinical dosing studies to support clinical trial design, 3) evaluate human biomarkers as potential outcome measures **Results:** We performed multiple long-term studies in our tamoxifen-inducible TIC-DUX4 mice to assess durability and dosing requirements for AAV.mi405. At 1-year post-injection, which is to date the longest timepoint studied, we find sustained mi405 expression and histological muscle protection in TIC-DUX4 mice. For systemic dose finding studies, mice were injected in the tail vein with AAV6.mi405 and AAV9.mi405 vectors at 3×10^{13} vg/kg, 6×10^{13} vg/kg, 9×10^{13} vg/kg, and 3×10^{14} vg/kg, and induced with an aggressive tamoxifen regimen for 10 weeks. Behavioral outcomes were measured weekly for 10 weeks prior to sacrifice. Only high-dose treated animals (3×10^{14} vg/kg) showed significant improvements in activity and rearing. We are currently assessing biodistribution and molecular and histological outcomes in multiple muscle groups of these animals. We also performed a pre-clinical non-GLP toxicology study in wild-type animals treated with systemic doses of AAV6 and AAV9.mi405 vectors (3×10^{14} vg/kg) at 4 wks and 5 mos. No significant adverse events were found, except transient elevations in liver transaminases. Finally, we have determined a panel of mi405-responsive DUX4-activated biomarkers using an FSHD xenograft model. **Ongoing and Future Directions:** We are currently testing two newly developed MyoAAV serotypes to potentially reduce the minimal effective dose necessary for therapeutic effects. **Conclusions:** We expect that these pre-clinical studies will support our planned first-in-human clinical trial of AAV.mi405 gene therapy for FSHD.

1076. Regulation of FKRP Gene Expression and LGMDR9 Therapies

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Limb-girdle muscular dystrophy type R9 (LGMDR9/LGMD2I) is an autosomal recessive muscle disease caused by mutations in the FKRP gene. FKRP encodes for the fukutin-related protein (FKRP), a glycosyltransferase which adds ribitol onto the cell surface proteoglycan alpha-dystroglycan (α -DG). Laminin in the extracellular matrix binds to glycosylated α -DG, which is the extracellular component of the dystrophin-glycoprotein complex (DGC). This intracellular-extracellular connection is essential to stabilize the sarcolemma during muscle contraction. Impaired FKRP activity resulting from missense mutations causes LGMDR9, leading to muscle fiber damage and necrosis. LGMDR9 is characterized by weakness and muscle wasting starting in the upper arms, thighs, and shoulders and slowly progresses until cardiac and respiratory phenotypes develop. No cure for LGMDR9 currently exists, and treatments are limited to palliative care. Preliminary studies suggest that adeno-associated viral vector (AAV)-mediated delivery of FKRP cDNA to rescue disease pathology is promising. However, experimental variability leaves gaps in our understanding of the efficacy of a gene therapy for LGMDR9. Our lab has identified evidence of regulation of gene expression by the FKRP 5' untranslated region (5'UTR), suggesting a tunable instrument for control of gene expression. To examine this phenomena, different iterations of the 5'UTR have been included in a transgene containing the FKRP cDNA behind a muscle-specific expression cassette (MSEC) based on the muscle creatine kinase (MCK) promoter. Three of these constructs are compared here in FKRP448L mutant mice. The differential effects on FKRP expression in vivo will help to identify regulatory sequences that positively or negatively impact expression, thereby allowing us to design a 5'UTR that maximizes efficacious expression. This approach will address the feasibility of gene therapy for treatment of LGMDR9 by optimizing the components of an AAV delivery vector for FKRP.

1077. New Advances in AAV Mediated Gene Therapy for Fibrodysplasia Ossificans Progressiva

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Fibrodysplasia ossificans progressiva (FOP, OMIM 135100) is a rare genetic disorder with an incidence of approximately one per 1 million people and characterized by skeletal malformations and progressive heterotopic ossification (HO). HO develops in childhood and young adulthood during episodic flare-ups, often triggered by trauma, injury, or intramuscular injection, inflammation or without identified triggers, leading to immobility and severe pain through heterotopic endochondral ossification in skeletal muscles, tendons, ligaments, and joints. In FOP, gain-of-function mutations occur in bone morphogenetic protein (BMP) type I receptor *ACVRI* and ~97% FOP patients harbor a classic *Acvr1*^{R206H} mutation (c.617G>A;p.R206H).

While *ACVR1^{R206H}* mutation was thought to induce spontaneous activation of bone-forming BMP signaling or stronger responsiveness to BMPs, recent studies demonstrated that *ACVR1^{R206H}* confers aberrant activation of the BMP signaling in response to Activin A, which normally inhibits BMP signaling through wildtype *ACVR1* receptor. FOP treatment is challenging due to the early onset of systemic skeletal pathology and the difficulty to selectively suppress BMP signaling by *ACVR1^{R206H}* mutation. Currently, there are no effective treatments for FOP except for high dose of corticosteroids for flare-ups, which can reduce the intense pain and edema as a symptomatic relief. Although several new drugs, including a retinoic acid receptor α agonist (palovarotene), anti-Activin A antibody (REGN 2477), an immunosuppressant (rapamycin), and *ACVR1* kinase inhibitors are currently in clinical trials, each can be complicated by either suboptimal efficacy or untoward side effects by long-term use, making this less favorable for chronic treatment of children. Here, we developed three recombinant adeno-associated virus (rAAV)-based gene therapy approaches for FOP, including gene replacement by expression of codon-optimized human *ACVR1* (*ACVR1^{OPT}*), *ACVR1^{R206H}* allele-specific silencing by AAV-compatible artificial miRNA (amiR, amiR-*ACVR1^{R206H}*), and the combination of gene replacement and silencing (*ACVR1^{OPT}*;amiR-*ACVR1^{R206H}*). Moreover, we identified AAV9 capsid as the most effective vector for transduction of major cell populations inducing HO. Compared to gene replacement and silencing, the combined gene therapy is most potent to suppress both traumatic and chronic HO in mice harboring a conditional allele of human *ACVR1^{R206H}*. AAV-mediated gene therapy dampened Activin A signaling and chondrogenic and osteogenic differentiation of human FOP patient-derived induced pluripotent stem cells (iPSCs) and mouse skeletal cells expressing human *ACVR1^{R206H}*. Accordingly, AAV-treated FOP mice displayed a significant decrease in chondrogenic anlagen and heterotopic bone in injured muscle while inflammation and fibroproliferation were largely intact. Direct intradermal injection of AAV vectors into injured muscle at young adulthood and intravenous (i.v.) injection at birth were both effective in suppressing traumatic HO in FOP mice. Moreover, spontaneous chronic HO in juvenile and adult FOP mice was also substantially decreased when i.v. injected at birth and young adulthood, respectively. Collectively, our results identify novel gene therapeutics that can suppress disabling FOP pathology, providing the potential for clinical translation to FOP patients.

1078. Highly Efficient and Precise Correction of a Muscular Dystrophy Causing Founder Mutation in *CAPN3*

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Skeletal muscle stem cells have high regenerative potential and can be used for autologous cell therapies of muscular diseases. However, muscle stem cells from patients with muscular dystrophy carry the disease-causing mutation and cannot be used for autologous transplantation right away. Gene correction with CRISPR/Cas9 evolved as a promising tool to repair these disease-causing mutations. Here we show an efficient and specific gene repair in primary muscle stem cells from patients with the most common founder mutation in *Calpain 3*, *CAPN3c.550delA*, which causes the severe and progressive disease

Limb Girdle Muscular dystrophy Type 2A. We are able to precisely repair the mutation by reframing the ORF with insertion of one base pair which with > 70 % is A:T and positioned exactly at the mutation site. This does not only recover the open-reading frame, but also reverts the DNA-sequence of *CAPN3* back to wild-type which results in re-expression of functional *CAPN3* protein. The repair strategy is not only efficient, but also safe since we do not see any off-target effects. We hereby show the first evidence of precise gene repair of the most common LGMD2A-disease causing mutation in primary human muscle stem cells that can be used for an autologous cell therapy of the more than 4.500 patients worldwide.

1079. Preparing for a Change in Care Delivery: The Impact on the Health Care Team of Live-Online Education on Gene-Based Therapies

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Introduction: Gene therapy has the potential to revolutionize treatment for a broad range of medical conditions, including neurodegenerative and neuromuscular diseases, ophthalmologic disorders, hematologic conditions, and cancers. While several gene therapies have received regulatory approval, the landscape is evolving rapidly with hundreds of others in various stages of investigation. However, patients who may qualify for gene therapy often have limited access to these breakthrough treatments, in part due to multiple knowledge and competence gaps among the clinicians who care for them. **Methods:** We collaborated with leading rare-disease and gene therapy societies to develop a 2-day, 4-hour CE and Maintenance of Certification activity featuring key opinion leaders with experience in developing and using gene-based therapies. Topics focused on: 1) the technologies and vectors (antisense oligonucleotide, adeno-associated virus, plasmid, CRISPR, hematopoietic stem cell), 2) the FDA regulations, and 3) team-based multidisciplinary care post gene therapy with a deep dive into diseases for which gene-based therapies are already available or are in late-stage investigations. Session components included recorded patient vignettes, downloadable resources, live polling, and audience real-time Q&A. Knowledge and competence questions were administered pre- and immediately post-activity. Patient and clinical practice impact questions were also asked at 2-month follow-up. Responses were analyzed to determine engagement and remaining gaps. Chi-square tests were used for statistical analysis. **Results:** As of January 2022, 1,493 clinicians from a variety of specialties had participated in the activities. All 15 test questions reflected improvements in knowledge and competence regarding each of the topics covered (pre 42% vs post 76%, P less than 0.05). At the 2-month follow up, 95% reported improved ability to recognize patients who could benefit from gene-based therapy, and more than 40% reported having already referred patients or discussed it for one of 20 different diseases with their patients (n=60). More than 30 questions and 39 requests for future education on this topic were submitted, indicating that additional education in this area is necessary to continue to support practicing clinicians. Information on long-term safety and efficacy were the most requested topics for future education. **Conclusions:** Following the educational initiative, clinicians

demonstrated large improvements in their knowledge and competence in critical areas related to gene therapies, resulting in enhanced ability to discuss the topic with their patients. Our data also point to a continuing need for education, especially as medical experience with gene therapy grows.

1080. Gene Therapy for the Treatment of Hypophosphatasia

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Hypophosphatasia (HPP) is an inborn error of metabolism caused by loss-of-function mutations within the gene that encodes tissue-nonspecific alkaline phosphatase (TNALP). As a result, substrates for this enzyme accumulate extracellularly including inorganic pyrophosphate (PPi), an inhibitor of bone mineralization, and pyridoxal 5'-phosphate (PLP), a form of Vitamin B6 important for neurotransmitter synthesis. HPP is characterized by skeletal and dental hypo-mineralization with disease severity varying from life-threatening perinatal and infantile forms to a more moderate form that manifests in adulthood. Enzyme replacement via a recombinant bone targeted TNALP (Asfotase Alfa/Strensiq) is the only approved therapy for HPP and its use is limited to the pediatric-onset patients. While ERT markedly improves the lifespan of patients with infantile and juvenile HPP, it has significant limitations including frequent injections (3-6 x/week), injection site reactions and exclusion of the substantial adult-onset HPP population. Here, we evaluate a gene therapy approach as a potential therapeutic strategy for the treatment of HPP. Gene therapy offers the advantage of a one - time treatment obviating the need for repeated protein treatments. We demonstrate the ability of gene replacement of bone targeted alkaline phosphatase (TNALP-D10) to rescue the *Alp*^{-/-} mouse model of severe infantile HPP using an adeno-associated viral vector (AAV) as a gene delivery tool. *Alp*^{-/-} mice exhibit severe hypomineralization and die by 2 weeks of age, if untreated. A single retro-orbital injection of AAV.SAN001-TNALP-D10 (5x10¹⁰ vgs) into PND1 *Alp*^{-/-} mice resulted in 100% of the mice surviving out to 3 months post vector injection (the study end point). *Alp*^{-/-} mice treated with AAV.SAN001-TNALP-D10 grew normally throughout the course of the study and exhibited mature bone mineralization. Quantitative uCT analysis of cortical and trabecular bone parameters revealed the long bone structures of treated *Alp*^{-/-} mice were similar to wildtype age matched controls. This study demonstrates that TNALP-D10 gene replacement can prolong the lifespan and markedly improve the skeletal phenotype of the *Alp*^{-/-} mouse model of severe infantile HPP. These findings suggest that AAV.SAN001-TNALP-D10 mediated neonatal systemic gene therapy is effective, and that AAV gene therapy could be a practical option for treatment of the severe infantile form of HPP, with the possibility of expanding into the juvenile and adult - onset HPP populations.

Cancer - Immunotherapy, Cancer Vaccines III

1081. Retargeted “Fusosomes” for In Vivo Delivery to T Cells

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We have developed a novel gene therapy platform using retargetable viral fusogen proteins - “fusogen platform” - to enable cell-specific delivery of a transgene (or other payloads) via systemic delivery of viral vectors pseudotyped with these fusogens called “fusosomes”. Specifically targeting T cells with fusosomes with gene integration payloads represents a unique opportunity to utilize these vectors to deliver CAR genes and provide *in vivo* CAR T therapy. *In vivo* CAR-T therapies enabled by retargeted fusosomes against T cells would bypass manufacturing challenges with current CAR T therapies and offer greater access and potentially greater efficacy for patients. By engineering viral fusogen proteins from the paramyxovirus family, we can retarget the viral attachment glycoprotein through the addition of single chain, antibody-derived binders in a modular fashion. Hundreds of fusosome candidates against each target can be screened in a high throughput manner to identify candidate vectors with high on-target gene transfer efficiency and specificity. Fusosome vectors have been generated that specifically target CD8+, CD4+, and CD3+ T cells. *In vitro* readouts on human cell lines, primary T cells and PBMCs, and *in vivo* readouts in humanized mouse models demonstrate high gene transfer efficiency and high specificity of these fusosomes. CD3-retargeted fusosomes uniquely confer specific delivery while also activating T cells, as seen by upregulation of activation markers and cell expansion during *in vitro* transduction. *In vivo* delivery of a CD19 CAR transgene payload with either CD8- or CD4-targeting vectors in Nalm-6 tumor bearing mouse models demonstrated robust production and persistence of CAR T cells and tumor eradication.

1082. Differential Response of V δ 1⁺ and V δ 1/V δ 2 $\gamma\delta$ T Cells Contribute to Variable Cytotoxicity of *In-Vitro* Expanded Cord Blood-Derived $\gamma\delta$ T Cell Product Against Human Leukemic Cells

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Gamma-delta T ($\gamma\delta$ T) cells have emerged as an enticing form of adoptive cellular therapy in the past decade. Their ability to provide rapid immune response through mechanisms that include -MHC-independent target recognition, potent cytotoxicity as well as cross presentations of antigens has garnered much research focus in recent years. Current research has however focused mainly on expanding the semi-invariant V γ 9V δ 2 subset, given its predominance in adult human peripheral blood. Its effectiveness on haematological and solid malignancies has however been mixed. Much less is known about the V δ 2⁻ subsets of human $\gamma\delta$ T cells; their expansion ability and their potential role in cancer immunotherapy. Given the preferential enrichment of these subsets of $\gamma\delta$ T cells in cord blood (CB), expansion of CB-derived $\gamma\delta$ T cells provides a means to evaluate the properties and potential of these lesser-known subsets, consequently creating the potential for them to serve as an “off-the-shelf” alternative for cancer immunotherapy. Evaluation of a cohort of 23 units of cryopreserved CB showed that V δ 2⁻ $\gamma\delta$ T cells made up an average of 94.99±0.86% of $\gamma\delta$ T cells. Amongst which, V δ 1⁺ subset constituted 55.49±1.38% while V δ 1/V δ 2⁻ subset constituted 38.56±1.13 %. Flow cytometric analysis showed that the proportion of phenotypically defined naïve (T_n), central memory (T_{cm}) as well as differentiated effector cells (T_{em} and T_{emra}) were similar among the starting, non-manipulated V δ 1⁺ and V δ 1/V δ 2⁻ $\gamma\delta$ T subsets, with majority of the cells in both subsets being CD45RA⁺CD27⁺ T_n cells (V δ 1⁺: 69.97±2.84%; V δ 1/V δ 2⁻: 65.02±3.06, p=0.46). Both subsets expanded to a similar extent after 2-weeks of in vitro culture (fold expansion of 5388±906 and 4554±851 in V δ 1⁺ and V δ 1/V δ 2⁻ respectively, p=0.45). While no significant difference was observed in the proportion of memory cells (T_{cm} and T_{em}) between the 2 subsets at the end of expansion, V δ 1/V δ 2⁻ cells nevertheless produced significantly higher fraction of the terminally differentiated T_{emra} cells compared to V δ 1⁺ cells (21.34 ± 2.28% vs 13.15 ± 2.74%, p=0.007). Consistent to this, the percentage of V δ 1/V δ 2⁻ or T_{emra} cells in week 2-expanded CB-derived $\gamma\delta$ T cells also positively correlated with cytotoxicity against leukemic cell lines. In summary, our experimental data showed that despite achieving similar magnitude of cellular expansion upon in vitro activation, CB derived V δ 1⁺ and V δ 1/V δ 2⁻ cells displayed qualitatively different responses, with the latter having a higher tendency to rapidly differentiate into terminal effector phenotype that correlates with potent cytotoxicity against human leukemic cells. The understanding of individual $\gamma\delta$ T subtype specific activation response is important and can guide the optimisation process for generating potent $\gamma\delta$ T cell therapy products for treatment of cancers.

1083. Off-the-Shelf iPSC-Derived Nature Killer Cells with Enhanced Expression of NK Activating Receptors for the Treatment of Solid Tumors

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CAR-T cells expressing chimeric antigen receptors (CAR) have shown clinical success for treating hematologic malignancies. However, the efficacy of CAR-T therapy in treating solid tumors has been limited, which mainly due to tumor heterogeneity and immunosuppressive tumor microenvironment (TME). Natural Killer (NK) cells derived from genetically engineered human induced Pluripotent Stem Cells (iPSCs) hold great potential to become the next-generation allogeneic cell therapy products. NK cells exert anti-tumor activity through the net outcome of stimulatory and repressive signals from various activating and inhibitory receptors. Some of the activating ligands for NK cell activation are highly expressed on many solid tumors. Thus, overexpression of activating receptors on NK cells might further boost their killing capacity towards those tumor cells. Here we report QN-030 (“Super NK cells”), an investigational, iPSC-derived NK cell product with enhanced expression of a variety of activating receptors (NKG2D and NCRs) intended for the treatment of solid tumors. QN-030 is generated from an allogeneic iPSC clone engineered with three anti-tumor modalities, including a constitutively expressed activating receptor to boost NK killing capacity, a high-affinity, non-cleavable CD16 (hnCD16) to enhance antibody-dependent cell-mediated cytotoxicity (ADCC), and an IL-15 molecule to increase the persistence of allogeneic cells in patients. In this study, we first engineered a small library of human iPSCs, each one carrying 1-3 of engineered NK activating receptors on top of hnCD16 and IL-15. We then differentiated the engineered human iPSCs into NK cells and conducted both in vitro and in vivo screening by using a variety of solid tumor cell lines. We identified QN-030 as the lead, which demonstrated superior anti-tumor activity among the candidates both in vitro and in mouse PDX model. QN-030 cells can be mass-produced in a cGMP process, have phenotype comparable to healthy donor NK cells, and are functionally potent against multiple solid tumor models. When administered in combination with monoclonal antibody, QN-030 demonstrated superior ADCC compared to unmodified iPSC-derived NK cells in mouse PDX models. Close monitoring of NKG2D/NCR expression revealed the potential mechanism of enhanced tumor killing activity. Together, we have engineered iPSC-derived QN-030 NK cells as a promising clinical drug candidate for treatment of solid tumors.

1084. Human Allogenic NK Cells Modified to Express a TGF-B Dominant Negative Receptor and IL-15 Display a Strong Anti-Tumor Activity in an NSG Mouse Model of Human Mesothelioma

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Advanced cancers of the peritoneal cavity such as mesothelioma are difficult to treat with limited treatment options available resulting in a dismal 5-year overall survival rate of only 10% for all stages combined. Cell therapies offer a novel approach for the treatment of these recalcitrant cancers. However, tumor heterogeneity, the immunosuppressive tumor microenvironment (TME), and the dense tumor stroma in solid tumors are challenges that need to be addressed when developing new cell therapeutics. Here, we investigated the anti-mesothelioma activity of PBMC-derived allogeneic NK cells genetically modified to: (i) express a TGF- β dominant negative receptor II (DNR) that confers resistance to TGF- β in the TME and (ii) secrete IL-15 to enhance NK cell survival, activation and cytolytic activity. We engineered a novel DNR construct that includes a CD8a transmembrane domain to increase cell surface expression of the DNR and contains the QBend10 epitope for detection and selection. We showed that the DNR/IL15-expressing NK cells could abrogate TGF- β signaling, as demonstrated by the absence of Smad2/3 phosphorylation after 45min TGF- β treatment. The IL-15 component provided superior anti-tumor activity to the NK cells in 5-day co-culture experiments against the H226 and H2452 mesothelioma cells, with a 70% and 100% reduction in percent live cells remaining in culture that were tumor cells, respectively, compared to the nontransduced conditions at a 1:1 E:T ratio. Killing of H2452 was also achieved at 1:5 and 1:10 E:T ratios, with a 64 and 31% reduction in tumor cells of percent live cells remaining, respectively, compared to the nontransduced conditions. Moreover, tumor cell killing was achieved without the need for exogenous cytokine supplementation and autonomous growth was not observed in NK cells expressing DNR/IL15. Finally, our *in vivo* studies of an H226 intraperitoneal xenograft model in NSG mice demonstrated complete tumor clearance within 2 weeks of DNR/IL15-expressing NK-cell administration with only 1.25E6 NK cells given 7 days after tumor injection. In contrast, non-transduced and DNR only-expressing NK cells had only a negligible effect on tumor progression. Though anti-tumor activity was profound, survival of mice receiving DNR/IL15 NK cells was limited due to acute toxicity. This adverse effect has not consistently been reported by other groups evaluating NK or T cells modified to express IL-15, suggesting that our observations could be model specific. Studies are therefore ongoing to evaluate whether prophylactic treatment can mitigate this toxicity without dampening the anti-tumor effect elicited by the DNR/IL15 NK cell therapy. Taken together, our results demonstrate that genetically modifying allogeneic NK cells to express a novel DNR and IL-15 may present a promising new treatment approach for intraperitoneal, TGF- β -expressing solid tumors.

1085. BAY1688/ATA3271: An Armored, Next-Generation, Off-the-Shelf Mesothelin-CAR T Cell Therapy for Solid Tumors

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Background Mesothelin (MSLN) is a GPI-anchored membrane protein and an attractive CAR T target with high expression levels in an array of malignancies including mesothelioma. Regional administration of autologous, 2nd generation MSLN-targeted CAR-T cells for malignant pleural mesothelioma has shown promise in early clinical evaluation (Adusumilli PS, et al., *Cancer Res* 2019; Adusumilli PS, *Cancer Discov* 2021). A next-generation MSLN-targeted, autologous CAR T therapy leveraging 1XX CAR signaling and PD1DNR is under investigation for advanced mesothelioma [NCT04577326]. Although autologous MSLN CAR-T holds promise, an allogeneic approach may have more widespread application. EBV T cells represent a unique, non-gene edited approach for allogeneic T cell therapy with a favorable safety profile in a currently running phase 3 trial for EBV-positive post-transplant lymphoproliferative disease [NCT03394365]. Clinical proof-of-principle studies for CAR transduced CD19-targeted allogeneic EBV T cell therapies have shown acceptable safety and durable response (Curran KJ, *Biol Blood Marrow Transplant* 2020). Here, we describe updated preclinical data for a MSLN targeting off-the-shelf, allogeneic cell therapy. **Methods** We engineered MSLN CAR+ EBV T-cells (BAY1688/ATA3271) with a novel 1XX signaling domain that is associated with strong effector function and favorable persistence, as well as armored with PD1DNR to provide intrinsic checkpoint blockade (Feucht J, *Nat Med* 2019). Anti-tumor effect of BAY1688/ATA3271 was assessed against a MSTO-211H-derived tumor cell line overexpressing MSLN and PDL1. **Results** Upon MSLN engagement, BAY1688/ATA3271 showed consistent proliferation, efficient tumor cell lysis in the presence of high-level of PD-L1, and secretion of effector cytokines [IL-2, TNF- α , granzyme B]. In a 16-day serial stimulation assay, with PD-L1+ tumor cells added every 2-3 days, BAY1688/ATA3271 expanded 4 to 45-fold without the need for external cytokines and retained comparable antitumor function as CD3/CD28-stimulated 'conventional' CAR-T cells. In an orthotopic mouse model of pleural mesothelioma, BAY1688/ATA3271 administered locally demonstrated potent anti-tumor efficacy and persistence without toxicities. We further optimized the process to reduce donor-to-donor variability in memory characteristics. Potency, both *in vitro* and *in vivo*, and an in-depth phenotypic characterization of the product including single-cell RNA sequencing will be presented, comparing both the original and the optimized process. **Conclusions** These studies reveal potent anti-tumor activity, both *in vitro* and *in vivo*, of BAY1688/ATA3271 allogeneic CAR-T cells generated using an original and an optimized manufacturing process. Overall, BAY1688/ATA3271

may be a promising approach for the treatment of MSLN-positive malignancies, and this data warrants further development of this program towards clinical trials.

1086. Targeted Delivery of a PD-L1-Blocking scFv by B7-H3.CAR-NK Cells Shows Potential as a New Approach to Immunotherapy for Osteosarcoma

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Background: Despite advances in treatment of osteosarcoma (OS), survival rates have remained stagnant for 30 years, highlighting the need for new therapeutic approaches. Natural killer (NK) cells, innate cytotoxic effectors, are showing great potential for cancer immunotherapy.¹ And it is critical to understand more about the NK cells' functional status in OS tumor microenvironment (TME) thus we can specifically develop novel NK cell-based therapies.

Methods: We isolated human peripheral blood NK (pNK) cells from healthy donors. Saos-2 (human primary OS cells) and Saos-2 LM7 (human metastatic OS cells) were used as OS targets. We co-cultured pNK cells with OS cells and then measured PD-L1 expression on these pNK cells. PD-L1 and B7-H3 expression on OS cells were measured. Accordingly, we generated gene-modified NK cells expressing B7-H3.CARs which secrete anti-PD-L1 scFvs locally in the OS TME. These NK cells are being evaluated for their efficacy against OS both *in vitro* and in orthotopic mouse xenografts.

Results: We have found that interactions with both OS cells can significantly upregulate PD-L1 expression on NK cells. And the metastatic Saos-2 LM7 cells present higher effects on the PD-L1 induction (Fig. 1A). Alongside, OS cells also express a decent amount of PD-L1 (Fig. 1B). In addition, the tremendous high amounts of B7-H3 have been detected on the surface of OS cells (Fig. 1C). Accordingly, we have successfully generated genetically-engineered NK cells with a high and stable expression of both B7-H3.CAR and anti-PD-L1 scFv, which are joint together with a cleavable linker (Fig. 1D). And we have confirmed that the linker can be efficiently cut thus releasing the anti-PD-L1 scFvs, which are further able to bind with PD-L1 on both NK and OS cells (Fig. 1E-F). These engineered NK cells enable to dramatically lysis OS cells when compared with wide type NK cells (Fig. 1G).

Conclusions: We have for the first time shown that OS cells can induce PD-L1 expression on NK cells, which may play a critical role in the immunosuppressive TME of OS.² Meanwhile, we have identified the high PD-L1 expression on OS cells, which have been somehow reported to be correlated with OS progression and metastasis.³ Together, it provides us a rational to build a strategy to comprehensively target PD-L1 checkpoint on both NK and OS cells. Besides, high B7-H3 expression has been shown on OS cells, which serves as a potential target. Stimulated by these preliminary results, we have successfully established NK cells that can, at once, secrete the PD-L1-blocking scFv to target PD-L1 both expressed on OS and NK cells, and express the B7-H3.CAR to specifically target B7-H3 on OS cells showing significant *in vitro* anti-tumor effects. We are currently testing the *in vivo* therapeutic efficacy of these engineered NK cells. And we believe our findings will provide an advanced NK cell-based immunotherapy for OS.

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with tumor microenvironment-responsive multifunctional engineered NK cells. *Proc Natl Acad Sci U S A*. 2021;118(45):e2107507118. [2] Sierra JM, et al. Tumor-experienced human NK cells express high levels of PD-L1 and inhibit CD8⁺ T cell proliferation. *Front Immunol*. 2021;12:745939. [3] Koirala P, et al. Immune infiltration and PD-L1 expression in the tumor microenvironment are prognostic in osteosarcoma. *Sci Rep*. 2016;6:30093.

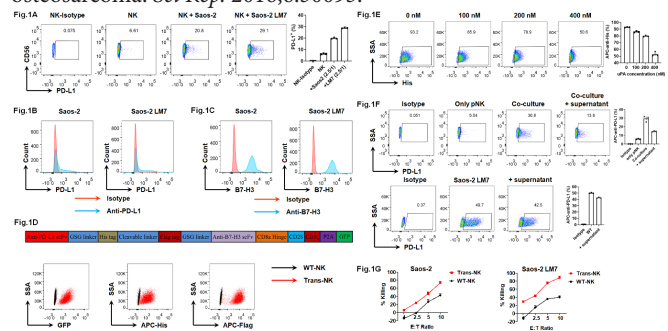


Fig. 1. (A) PD-L1 expression on human primary NK cells after co-incubation with OS target cells, including Saos-2 (primary type) and Saos-2 LM7 (metastatic type) cells at a E:T ratio of 2.5 for 24 h, respectively. (B) PD-L1 expression on both Saos-2 and Saos-2 LM7 cells. (C) B7-H3 expression on both Saos-2 and Saos-2 LM7 cells. (D) Generation of anti-PD-L1 scFv and B7-H3.CAR-NK cells (Upper: transgene structure; Lower: expression characterization). (E) Cleavable properties of the anti-PD-L1 scFv from the engineered NK cells when incubated with different concentration of uPA enzyme (characterized with no-tag expression; uPA concentration: 0, 100, 200 and 400 nM). (F) Binding ability of cleaved anti-PD-L1 scFv (collected from the uPA treatment) with both PD-L1 expressed on NK and OS cells (upper: PD-L1⁺ NK cells; lower: PD-L1⁺ Saos-2 LM7 cells). (G) *In vitro* cytotoxicity of wide type NK and engineered NK cells against both Saos-2 and Saos-2 LM7 cells at indicated E:T ratios over 6 h, respectively. Data are presented as the mean \pm SEM.

1087. Cellular Avidity Between Tumor and Effector Cells, Rather Than Receptor-Ligand Affinity, is a Powerful Predictor of Downstream Function of Cellular Immunotherapies

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With the increasing complexity of cell therapy construct design, novel methodologies to accelerate screening and improve evaluation of lead candidates for clinical benefit are needed. Several methods are currently employed including receptor-ligand affinity measurements. However, affinity between T cell receptors (TCRs) and peptide-MHC complexes (pMHC) has shown to be a poor predictor of T cell functional capacity. The same holds true for the affinity of chimeric antigen receptor (CAR) scFvs to their targets. Recent studies revealed cellular avidity as an emerging biomarker for identifying and developing potent and safe immunotherapies. Unlike affinity, cellular avidity is driven by the overall strength of dynamic surface interactions between effector cells and their targets; this novel biomarker integrates receptor density, the sum of individual affinities, and engagement of the multitude of co-receptors within the immunological synapse to characterize the interaction in a more biologically relevant context. Here, we will demonstrate how increased normalized cellular avidity, *i.e.*, TCRs with the strongest antigen binding and the lowest background, correlated with improved effector function both *in vitro* and *in vivo*. And with CAR-T, how higher avidity has shown a significant correlation with improved tumor control in *in vivo* murine models, but also associated with toxicities in patients, suggesting the need for tuning cell therapies to the desired avidity for ideal function.

1088. Macrophages Cooperatively Phagocytose Solid Tumors in Macrophage Immunotherapy and Initiate an Anti-Tumor Antibody Feedback Loop

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Macrophages reside in large numbers in many solid tumor types, yet typically associate with poor prognosis and immunosuppression. Macrophage immune checkpoint blockade has emerged in clinical trials as a promising therapeutic strategy to promote macrophage-mediated phagocytosis by antibody blockade of the inhibitory CD47-SIRPα in liquid, but efficacy in solid tumors remains challenging. Target cell cohesivity, cancer cell proliferation, and other biophysical barriers limit efficacy of anti-CD47 approaches, even when combined with other therapies such as tumor-specific monoclonal antibodies. We seek to quantify the underlying immune relationships in poorly immunogenic solid tumors in order to engineer more efficacious immunotherapies. Here, we model the tumor microenvironment with cohesive “immuno-tumoroids” of macrophages (bone marrow-derived macrophages - BMDMs, and conditionally immortalized macrophages - CIMs) and B16F10 tumor cells in vitro. Tumoroid cell growth is assessed over several days by confocal and epifluorescent microscopy. Macrophages added to 3D tumoroids are unable to suppress growth of wild-type CD47 B16s unless both (1) anti-CD47 blockade is present and (2) tumor-opsonizing IgG is added as a pro-phagocytic signal. Complete disruption of CD47-SIRPα by CRISPR-Cas9 KO maximizes engulfment by BMDMs when combined with tumor-opsonizing antibody and enables complete elimination of the tumoroids. Varying the ratio of macrophage:tumor cell reveals a dependence on macrophage density in a switch-like fashion, indicative of cooperativity to disrupt solid tumor adhesions in rapidly growing clusters. In vivo, tumor opsonization by therapeutic IgG also fails to control wild-type CD47 tumors in syngeneic, immunocompetent C57 mice, yet completely eliminates ~40% of tumors with CD47 KO. We again show the macrophage dose-dependence and switch-like survival response with IgG opsonization, where adoptive transfer of SIRPα-blocked marrow or engineered SIRPα KO CIMs rescues the non-responding KO tumors and eliminating a fraction of wild-type tumors, respectively. These responses protect against primary and metastatic tumor recurrence and reveal a de novo antibody response that is B16-specific, broadened beyond the original therapeutically targeted antigen, and directly activates phagocytosis through IgG2a subclass binding. Serum IgG from durably cured mice enables suppression of immuno-tumoroid growth in vitro and tumor growth in vivo, providing strengthened signals that feed back to strengthen macrophage phagocytosis. Immunohistochemical staining and flow cytometry of IgG-treated KO tumors show high macrophage infiltration and clustering, further supporting their role in cooperatively initiating an anti-tumor response. Thus, we show that macrophages can potentially eliminate solid tumors when maximally activated and present

in high numbers, while strategies that strengthen pro-phagocytic signals in combination can tilt the balance towards tumor elimination and durable immunity.

1089. Armoring T-Cells with Regulatable, Membrane Tethered Cytokines Using Improved Drug-Responsive Domain Technology (cytoDRIVE™)

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Solid tumors are the next frontier in adoptive cell therapy (ACT) and cytokines such as IL12 and IFNα can amplify its efficacy by modulating the tumor microenvironment. Indeed, combinations of these and other immunomodulators have shown great promise in pre-clinical studies when expressed from genetically modified T-cells, however, they are generally too toxic for systemic exposure. Regulatory systems that allow precise control over the level and timing of their expression are therefore required for their safe use in ACT. To this end we have developed a regulation platform, which enables post-translational control of protein abundance through modulation of protein stability. Proteins of interest are fused to a Drug-Responsive Domain (DRD) which acts as a reversible degron, allowing for dose-dependent increase in protein levels in the presence of a stabilizing drug and decrease in the absence of the drug. DRDs are typically derived from human proteins, e.g. enzymes such as carbonic anhydrase 2, for which FDA approved drugs as the stabilizing agents (e.g. the CA2 inhibitor acetazolamide, a diuretic) are readily available. Advances in our regulation platform based on multimerization of DRDs with homologous or heterologous oligomerization domains to increase their degron effect enable tight regulation with extremely low cell surface abundance in the absence of drug, and robust induction (10-20 fold) in the presence of drug. In addition, membrane tethering of the cytokines further reduces any risk of systemic toxicity, whereas engineering of protease sites allows for controlled shedding, if desirable. Combining these design elements enables full control of the abundance of IL12, IL23, IL18, IL2, and IFNα in genetically modified T-cells using a small molecule drug as the on/off switch for precise control of cytokine activity.

1090. DNA Delivery of Immune-Focused SARS-CoV-2 Nanoparticle Vaccines Drives Rapid and Potent Immunogenicity and Achieves Single-Dose Protection

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Vaccines against SARS-CoV2 may generate antibodies targeting irrelevant epitopes that reduce durability of immunity or increase antigenic escape. Here, we have developed rationally immune-focused SARS-CoV2 vaccines by incorporating glycans into the spike protein receptor binding domain (RBD) of the original Wuhan virus strain to drive antibody responses specifically against neutralizing epitopes (Figure 1A). We then designed DNA-launched nanoparticle vaccines by fusing these immune-focused RBDs to nanoparticle scaffolds for multivalent display (DLNPs). These DLNPs self-assemble *in vivo* and induce rapid seroconversion and potent neutralizing antibodies across multiple animal models (Figure 1B). They also induced superior CD8+ T cell mediated cellular immunity compared to non-focused, non-nanoparticle immunizations. A single, dose-sparing dose protected against lethal SARS-CoV2 challenge (Figure 1C). In addition, these DLNPs are broadly neutralizing against emerging variants and achieved single dose protection against the Delta variant (Figure 1D). While neutralization efficacy was blunted against the Omicron variant, we demonstrate that these DLNPs can be easily modified to accommodate novel variants that may escape previous vaccine constructs. Single-dose immune-focused coronavirus vaccines via DNA-launched nanoparticles provide a potent and versatile platform for rapid clinical translation.

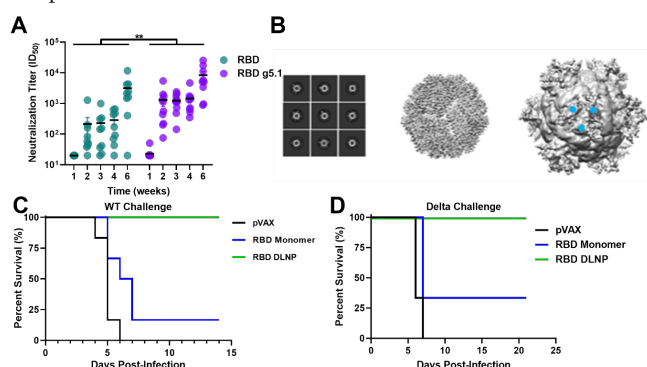


Figure 1. A) Immune-focused RBD induced superior pseudovirus neutralization than wild-type RBD. B) Cryo EM imaging of assembled DLNP. Survival against C) Wuhan and D) Delta live virus challenge.

1091. Overcoming the Challenge of RNA Therapeutics for the Treatment of Cancer, Development of Lipid Nanoparticle-Formulated RNA Viral Immunotherapy

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RNA-based therapeutics delivered using encapsulation in lipid nanoparticles (LNP) have been mostly restricted to applications such as cancer vaccines, ex vivo engineering of cellular therapies, or intratumoral administration. In the absence of effective tumor-targeted LNP, intravenous (IV) administered RNA medicine has largely been

challenged to deliver a significant concentration of RNA payloads outside the liver, the main organ for LNP uptake. Compounding the limited uptake by tumor cells, mRNA, even with modifications to increase stability, are short-lived. To circumvent this limitation, we developed IV-delivered LNP formulated viral RNA (vRNA) immunotherapy for the treatment of cancer. Once the vRNA/LNP is internalized and released in the cytoplasm of a tumor cell, vRNA replicates and generates a burst of infectious virions that spread locally, hence overcoming the initial inefficiencies of delivery to tumors. vRNA amplification and virus formation and infection are self-limiting in healthy tissues by host antiviral responses that are attenuated in tumor cells, thus defining the large therapeutic window for this novel RNA therapy modality. We developed two LNP-encapsulated vRNA encoding potent and clinically validated oncolytic viruses, Coxsackie Virus A21 (CVA21) and Seneca Valley Virus (SVV). For both Synthetic RNA viruses, we demonstrated tolerability in mouse models relevant to viral infection and non-human primates, while reaching exposure above those required for anti-tumor activity. Viral replication in healthy tissues is limited and transient, while robust and durable in tumors. Anti-tumor efficacy for ONCR-021 (based on CVA21) and ONCR-788 (based on SVV) is observed in various tumor models, including CDX, PDX, GEMM-derived, and orthotopic models representing the core indications of interest, NSCLC, and SCLC, respectively for these candidate drugs. In a syngeneic model, ONCR-788 enhances T cell recruitment and activation, PD-L1 expression on tumor cells and myeloid cells, and M2 to M1 macrophage conversion. Combination of ONCR-788 with anti-PD-1 results in improved anti-tumor activity. Interestingly, the *in vivo* efficacy of ONCR-021 and ONCR-788 is maintained even in the presence of neutralizing antibodies against CVA21 and SVV, suggesting that administration of LNP-formulated RNA immunotherapy enables the repeated and systemic exposure of disseminated tumors to potentially oncolytic viruses. These data highlight an innovative RNA therapeutic modality for the IV treatment of tumors which overcomes the limitations of other RNA-based therapeutics and supports ONCR-021 and ONCR-788 into IND-enabling studies.

1092. CRISPR-Mediated T Cell Engineering Against Non-Small Cell Lung Cancer

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Adoptive therapy with T cells engineered to express tumor-specific TCR to redirect antigen specificity toward cancer cells and induce antitumor immunity, represents one of the most promising strategies of

precise cancer therapy against solid tumors. Clinical trials with TCR-engineered T cells are ongoing to treat lung cancers, the leading cause of cancer-related death worldwide. The CRISPR/Cas9 system represents a useful genome-editing tool to safely and effectively edit T cells and enable them to kill tumor cells. In this study, we applied CRISPR/Cas9 system to replace endogenous TCR with tumor-specific TCRs in T cells derived from patients affected by non-small-cell lung cancers (NSCLC). A cohort of HLA-typed NSCLC patients was characterized for the expression of tumor antigens frequently expressed in NSCLC. To simultaneously knock out endogenous TCR and trigger the knock-in of tumor-specific TCRs in the *TRAC* locus, primary T cells were electroporated with ribonucleoproteins (RNPs) of Alt-R SpCas9 in complex to *TRAC* exon1-specific gRNA, and HDR donor template carrying a promoterless TCR sequence. In addition, to completely downregulate the expression of endogenous chains, primary T cells were co-electroporated with Alt-R RNPs targeting *TRBC* loci. Genomic and cytofluorimetric analyses showed highly efficient knock-out of endogenous TCR chains, and precise HDR events leading to TCR replacement. Adverse chromosomal aberrations such as translocation events were investigated in double-edited T cells. Engineered T cells were sorted, expanded and analyzed for their ability to kill tumor cells expressing the cognate tumor peptide. Co-culture experiments of TCR-redirectioned T cells with tumor cell lines demonstrated that edited T cells showed tumor-specific cytotoxic activity. TCR-engineered T cells derived from HLA-A*0201-typed NSCLC patients co-cultured with NSCLC cells and analyzed by flow cytometry for their killing activity, demonstrated that TCR editing enabled T cells to recognize and kill NSCLC cells. These data encourage the application of CRISPR-mediated non-viral TCR editing to generate tumor-specific T cells able to kill lung cancer cells.

1093. Abstract Withdrawn

1094. Targeting B Cell Malignancies with Anti-ROR1 CAR T Cell Therapy

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Anti-CD19 chimeric antigen receptor (CAR) T cell therapy has been shown to induce high complete response rates in the majority of B-cell lymphoma patients. However, over 50% of patients relapse within one year. A major cause of failure appears to be due to loss of CD19 expression on the tumor cell surface. This indicates that novel therapeutic strategies are still needed in clinic. Receptor tyrosine kinase like orphan receptor 1 (ROR1) is an oncofetal receptor for Wnt5 alpha that is expressed in multiple embryonic tissues but absent in virtually all adult tissues. However, ROR1 is expressed at high levels in chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), diffuse Large B-Cell Lymphoma (DLBCL), and several solid tumors. The high expression level of ROR1 in tumor cells is associated with adverse clinical prognosis. In addition, a small percentage of cancer cells with characteristics of undifferentiated leukemia cells or cancer stem cells were found to overexpress ROR1. Using hybridoma technology,

we generated an anti-ROR1 monoclonal antibody that specifically recognized ROR1 protein. The antibody bound to ROR1-expressing MCL cell lines but not to normal donor T cells, B cells, or monocytes, indicating the specificity of the antibody. Lentiviral transduction of a CAR molecule derived from the anti-ROR1 antibody into primary human T cells redirected their specificity against B-cell lymphoma cell lines. We found the anti-ROR1 CAR T cells specifically lysed B cell lymphoma tumor cells at high efficiency but not normal B cells. At an effector: target ratio of 1:1, the anti-ROR1 CAR T cells lysed over 95% of B-cell lymphoma tumor cells during 3-days of coculture. To determine the optimal CAR construct, we evaluated various hinge, transmembrane, and costimulatory domains in the CAR molecule. We found that incorporation of the CD28 hinge and transmembrane domain in the CAR construct dramatically enhanced their *in vitro* lysis efficiency of anti-ROR1 CAR-T cells. In conclusion, we developed a novel and potent anti-ROR1 CAR T-cell therapy product that may be used for treatment of various B-cell malignancies and ROR1-expressing solid tumors.

1095. Multiplexed TCR-T Cell Therapy: A Strategy to Enhance the Efficacy of Engineered T Cell Therapy

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Adoptive Cell Transfer with genetically engineered T cells holds great promise for treating solid tumors. To date, clinical investigations of TCR-engineered T cell therapies have targeted one antigen at a time and have produced encouraging response rates ranging from 30-50%. Unfortunately, complete responses have been rare, and responses are often short-lived. We submit that there are two main challenges associated with single-antigen targeted TCR-T cell therapy. First, expression of most cancer associated antigens is heterogeneous. We performed multiplexed immunohistochemistry with MAGE-C2 and PRAME, two cancer germline antigens, and observed considerable heterogeneity across samples from different solid tumor types. Additionally, heterogenous antigen expression was observed at the single cell level - not every cancer cell within a given tumor expressed each antigen. This indicates that a single TCR would not be sufficient to eliminate all cancer cells within a given tumor, thereby allowing the tumor cells lacking the treated antigen to escape and drive relapse. Second, single agent TCR-T cell therapy targets only a single HLA allele, which is subject to loss through commonly observed HLA loss-of-heterozygosity (LOH) mechanisms. Clonal HLA Class I LOH has been observed in 17% of all solid tumors (Montesion et al, Cancer Discovery, 2021) and sub-clonal HLA LOH occurs in an even larger percentage of tumors. Multiplexed TCR-T cell therapy mimics the natural oligoclonal T cell response to cancer and provides a way to address both challenges associated with treating solid tumors. To test this concept experimentally, we used two distinct TCR:antigen pairs to model multiplexed T cell-mediated cancer killing and heterogeneity *in vitro*. TCR-204-C07 is a naturally occurring, high affinity TCR that recognizes an HLA-C*07:02-restricted epitope of MAGEA1 and exhibits robust killing of cell lines expressing MAGEA1. TCR-LD8-3 is

a low affinity TCR that recognizes an HLA-B*07:02-restricted epitope of MAGEC2. We found that, although TCR LD8-3 effectively kills A101D cells that express MAGEC2 at high levels, it is ineffective at killing SK-MEL-5 cells, which express MAGEC2 at low levels. However, when TCR LD8-3 and SK-MEL-5 cells were co-cultured with TSC-204-C07 and A101D, the cytotoxic activity of TCR LD8-3 was synergistically enhanced. Using a transwell culturing system, we found that cytokines secreted by TSC-204-C07 strongly enhanced T cell activation of TCR LD8-3 upon antigen engagement. These findings support the hypothesis that multiplexed TCR-T has the potential to overcome antigen heterogeneity not only through independent targeting of different cancer cell populations, but also by cytokine-mediated T cell enhancement. We are currently conducting *in vivo* studies to confirm the synergistic effect of multiplexed TCR-T cell therapy and designing trials to test this hypothesis clinically.

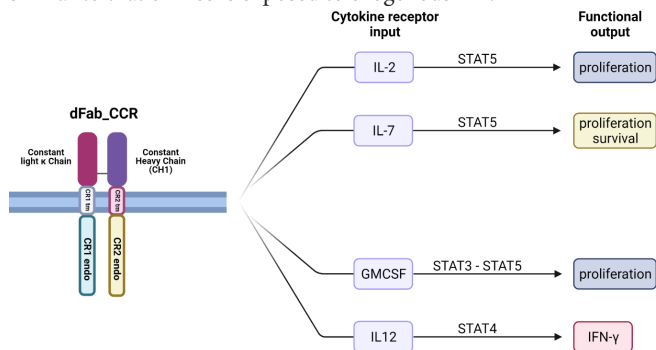
1096. Enhancing CAR T Cell Therapy Using Fab Based Constitutively Heterodimeric Cytokine Receptors

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Introduction. Cytokines are key modulators of adoptively transferred T cell survival and proliferation. Where prolonged trafficking is required to reach the tumour, or in an immunosuppressive microenvironment, paucity of cytokine stimulation can impair T cell engraftment and persistence. Cytokines can be co-administered systemically, or T cells can be engineered to autonomously produce cytokines, however these approaches risk systemic toxicity. An alternative approach is expression of cytokine receptors engineered to be constitutively active. We have developed a versatile constitutive cytokine receptor (CCR) system which recapitulates cytokine signalling by heterodimerization of cytokine receptors. We fully characterize this CCR architecture and identify optimal cytokine signals for use in CAR T cell approaches.

Results. We generated an IL2-CCR by juxtaposing the IL2 receptor chains, using the antibody CH1 and kappa constant region (Fab) to force heterodimerisation. Under cytokine starvation conditions, T cells expressing the IL2-FabCCR expanded and had a transcriptional profile similar to that of T cells exposed to exogenous IL2.



We next sought to determine the universality of this FabCCR architecture. Therefore, we tested the common- γ -chain (IL7, IL4, IL9,

IL21), common- β -chain (GMCSF, IL3, IL5), IL12 family (IL12, IL23, IL27) and IL17 family (IL17A, IL17C) designed FabCCR. Using a combination of functional and biochemical assays, we demonstrated that each specific cytokine signal could be recapitulated in all CCRs tested. GMCSF-CCR was particularly effective resulting in 5-fold expansion under starvation conditions, where IL12 CCR mediated higher basal IFN γ secretion. Following this, we explored the structural constraints of the FabCCR. Substitution of the kappa chain with the lambda chain maintained comparable expansion. We then investigated whether the quantity of intracellular signal affected the FabCCR output. Truncation of the IL2 β chain resulted in an analog tuned response, while truncation of the C γ C generated a digital response. Alternatively, truncation of GMCSF chain did not modify the CCR functionality. We then explored if the FabCCRs could enhance CAR function. We co-expressed a subset of FabCCRs with a GD2 CAR. We revealed that under cytokine starvation or chronic antigen stimulation, GMCSF-CCR improved CAR T cell proliferation and protected CAR effector function. We tested the same subset of FabCCRs with GD2 CAR *in vivo* in two immunocompetent tumour models (B16 F10 and CT26 GD2+ models). Interestingly, we discovered that GMCSF CCR expression boosted CAR T cell expansion (5-fold), persistence and doubled the survival rate of the mice treated. Finally, we examined the transcriptome differences between the CCRs. NanoString analysis revealed a complete overlap between IL2 and IL7 CCR. GMCSF CCR displayed differences, with upregulation of genes associated with chemokines (e.g. CCR5, CCL18), but also genes involved in control of T cell function (e.g. LAIR1).

Conclusions. Enhanced CAR T proliferation is essential in solid tumours where the access to antigen is restricted. Our FabCCR allowed constitutive signalling of any cytokine receptors. These FabCCRs could improve T cell therapies (TILs, TCR T cells and CAR T cells), but the versatility may also find broader application in cellular engineering.

1097. Evaluation of Implantable Cytokine Factories in Combination with Checkpoint Inhibitors for Eradication of Malignant Pleural Mesothelioma (MPM) Tumors in Mice

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Background: Interleukin-2 (IL-2) has been approved as a cancer immunotherapy for over 30 years (1, 2). Unfortunately, widespread clinical use of IL-2 therapy is dampened by the short serum half-life and the severe toxicities associated with high levels of IL-2 in the blood (3). To overcome these limitations, we developed a hydrogel-based, clinically translatable, cytokine delivery platform (cytokine factories) composed of genetically modified epithelial cells encapsulated in biocompatible polymers. These cytokine factories are able to continuously produce IL-2 from within the polymers and allow for controlled and predictable cytokine dosing *in vivo*. **Results:** *In vivo* administration of cytokine factories created a high local cytokine concentration (IP space) without substantial leakage into the systemic circulation. Local, or tumor adjacent, administration of IL-2-based cytokine factories caused reduction of tumor burden by 70% after

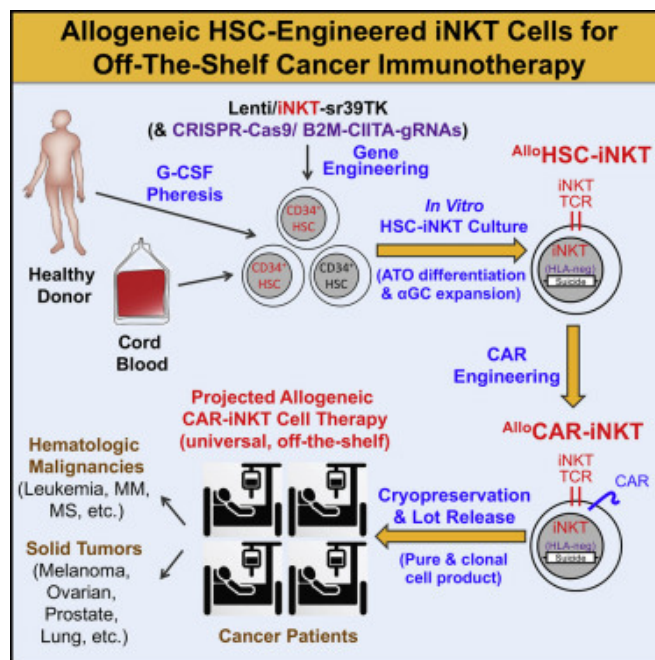
only 1 week of treatment when delivered as a monotherapy to mice with malignant pleural mesothelioma (MPM). Importantly, when administered in combination with local anti-PD1 injections, these cytokine factories lead to eradication of these highly aggressive tumors in 7/7 treated mice and protected 4/4 rechallenged mice from tumor recurrence. In addition, we used mass cytometry, or cytof, to analyze the immunological landscape of tumor-bearing mice treated with our IL-2-based cytokine factories as a monotherapy and in combination with PD1 checkpoint inhibitor. Interestingly, in both groups treated with IL-2, we found increased release of IFN γ from CD4+ T cells which suggests activation of CD4+ effector cells. Finally, we utilized antibodies against CD4 and CD8 to evaluate which T cell subset was required for anti-tumor efficacy with our platform. We found that mice depleted of CD8+ T cells were unable to elicit an anti-tumor response after treatment suggesting that CD8+ T cells are essential for IL-2-based immunotherapy. To validate the translatability of this platform, we evaluated the safety profile and feasibility of dosing in the pleural cavity of rats. Significantly, this platform was well tolerated in the pleural cavity for 30 days without evidence of significant toxicity in 100% of rats tested. **Conclusions:** Our findings demonstrate the safety and efficacy of cytokine factories in preclinical animal models and provide rationale for future clinical testing for the treatment of metastatic peritoneal and pleural cancers in humans. **References** (1) H. Choudhry, N. Helmi, W.H. Abdulaal, M. Zeyadi, M.A. Zamzami, W. Wu, M.M. Mahmoud, M.K. Warsi, M. Rasool, M.S. Jamal, Prospects of IL-2 in Cancer Immunotherapy, *Biomed Res Int*, 2018 (2018) 9056173. (2) D.F. McDermott, M.B. Atkins, Interleukin-2 therapy of metastatic renal cell carcinoma--predictors of response, *Semin Oncol*, 33 (2006) 583-587. (3) P.F. Muhlradt, H.G. Opitz, Clearance of interleukin 2 from the blood of normal and T cell-depleted mice, *Eur J Immunol*, 12 (1982) 983-985.

1098. 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.



1099. Optimization and Validation of the CAR-T Cell Platform Utilizing Cancer Patient Cells

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During the past few years cell therapy treatments have become one of the fastest growing fields in the pharmaceutical industry. Specifically, there has been a focus on the development of therapeutic approaches to use cells as living drugs to achieve better results in hard-to-treat diseases, such as various types of cancers, autoimmune conditions as well as metabolic deficiencies. To facilitate the development of these potential treatments, WuXi Advanced Therapies (WuXi ATU) has designed and released a CAR-T Cell Therapy Platform. The WuXi ATU Platform is a modular program that allows clients to select from a variety of pre-evaluated equipment, technologies, and materials with developed closed processes for different unit operations. Originally the platform technology was developed and optimized by using leukopheresis products obtained from healthy donors. However later we wished to validate the platform utilizing the blood cells obtained from cancer patients. We tested blood samples obtained from patients with melanoma, breast cancer and chronic lymphocyte leukemia to validate the platform and evaluate new available media, activators and cytokines in comparison with the platform reagents for the process performance and final product quality. We have demonstrated that lymphocytes obtained from the cancer patients and healthy donors 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 both for cancer patients and healthy donors. The following unit operations are part of WuXi ATU's platform package: Isolation, Expansion, Wash and Volume Reduction, and Fill. Each individual operation has been evaluated using chemically defined

off-the-shelf GMP grade media and activation reagents. For each unit operation, two or more alternative closed procedures are offered, allowing the client to build their closed process accordingly. Based on the results we obtained above, we will further optimize our CAR-T Cell Therapy Platform for successful manufacturing of cell therapies from cancer patient starting material. Conclusions: 1) Flexible CAR-T Cell Therapy platform is a vital tool to bring cell therapies to patients in efficient and compliant way. 2) Starting materials from healthy donors and cancer patients may perform differently in the course of platform manufacturing. 3) Platform validation and optimization utilizing cancer patient material is important for successful manufacturing of cell therapies from cancer patient starting material.

1100. A Method for Enhanced Lentiviral Transduction and Improved Efficacy of CD19 CAR-T Cells: Clinical Applications for Immunotherapy

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Chimeric antigen receptor (CAR) -modified T-cell therapy against CD19 has shown great promise to treat adults with relapsed/refractory T-cell acute lymphoblastic leukemia or lymphoblastic lymphomas. However, patients typically require a very high dose of T-cell infusion ($\sim 2 \times 10^6 - 2 \times 10^8$ of T-cells/kg) to achieve complete remission. Furthermore, variabilities in the therapeutic efficacies have been reported which could be due to effect of prior treatments on the immune cells, the stage of patient's disease, and the different technology platforms used to prepare CAR-modified T cells. We therefore postulate that scalability and consistency of the T-cell products could be greatly improved through adoption of a common media to support processes from viral production to T-cell transduction and expansion which otherwise would undergo multiple reformulations, thus contributing towards the variability in producing consistent T-cell products. We first compare different serum-free media alongside with fetal bovine serum-supplemented culture media for each of the processes mentioned *i.e.* CD19-CAR lentiviral vectors production, T-cells transduction and T-cells expansion. The results showed that the most optimal media that gave good lentiviral vector yield did not facilitate high levels of T-cell proliferation and vice versa. However, three of the serum-free media were identified, and each one of these was capable of supporting all of the above-mentioned processes. CD19-CAR lentiviral vectors prepared using the representative serum-free media in the presence of retronectin were found to transduce a higher proportion of CD3+ ($\uparrow 14.1\%$); CD4+ ($\uparrow 17.9\%$) and CD8+ ($\uparrow 15.4\%$) expressing CD19+ when compared to CD19-CAR lentiviral vectors prepared in 10% fetal bovine serum media. This led to an increase in tumor specific cell killing to $\sim 27\%$ and $\sim 17\%$ at an effector to target ratio of 10:1 and 5:1 respectively when compared to vectors prepared in 10% fetal bovine serum media. The improved functional efficacy of the specific tumor cell kill could also be replicated using patient-derived PBMCs, suggesting that the new improved method can potentially be used for both allogeneic and autologous therapies. Taken together, these data support the use of a common serum-free media to eliminate the

disparity between different media usage with improved efficacy for T-cell therapies. Notably, it lowers the contamination risks associated with the use of animal origin components, and improve consistency of the T-cell product.

1101. Abstract Withdrawn

1102. Immunostimulatory Photothermal Nanoparticles Engineer Potent and Specific T Cells for Solid Tumor Therapy

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Adoptive T cell therapy (ATCT) is a promising approach that aims to provide durable and persistent anti-tumor immune responses to patients. While ATCT has been successful in treating hematological malignancies, solid tumors have proven more difficult to treat with T cell therapeutics. Many ATCT approaches rely on *a priori* chosen single or known antigen(s) for targeting which, in heterogeneous solid tumors, is often an ineffective strategy. To overcome the limitations of current ATCTs, we have developed a platform to generate personalized and diverse tumor-specific T cells using Prussian blue nanoparticle-based photothermal therapy (PBNP-PTT) to increase the specificity and potency of T cells for solid tumors. We have demonstrated that PBNP-PTT decreases tumor burden and increases long-term survival in mouse models of several solid tumors, including neuroblastoma, melanoma, breast cancer, and glioblastoma multiforme (GBM). These therapeutic benefits were driven by T cell infiltration and specific anti-tumor T cell activation and cytotoxicity. We discovered that at tumor-specific thermal doses, PBNP-PTT elicits immunogenic cell death, characterized by the recruitment and engagement of antigen-presenting cells (e.g. dendritic cells (DCs)) for subsequent T cell activation. Hence, building on this capability of PBNP-PTT to trigger potent endogenous T cell immunity *in vivo*, we utilized a PBNP-PTT strategy *ex vivo* to manufacture tumor-specific T cells for ATCT. This immunoengineered platform offers key advantages to enhance T cell specificity and function by stimulating the processing and presentation of a diverse and personalized set of tumor-specific antigens to T cells *ex vivo*. We found that DCs cultured *ex vivo* with PBNP-PTT-treated tumor cells enabled the expansion of CD4+ and CD8+ tumor-specific T cells. Upon co-culture with target cells, these T cells secreted IFN- γ in a tumor-specific and dose-dependent manner (up to 50-500-fold over controls in models of breast cancer and GBM). Furthermore, T cells developed via PBNP-PTT *ex vivo* expansion were specifically cytotoxic against tumor cells (25-93% killing of GBM cells at an effector to target (E:T) ratio of 20:1), while sparing normal human astrocytes. In summary, tumor-specific T cells engineered using PBNP-PTT demonstrate robust cytolytic activity *in vitro* and we are currently validating their potency in murine xenograft models of solid tumors. Further, our findings suggest that nanoparticle-mediated thermal therapy may stimulate and enhance the development of potent T cell immunity for ATCT of solid tumors.

Cancer - Targeted Gene and Cell Therapy II

1103. Lnc122 - The miR122 Precursor Has an Independent Role as a Tumor Suppressor in Liver

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miR122 is a liver-specific miR122 and makes up 60 -70% of the miRNAs in hepatocytes. The full function of this miR122 is not known but does play a role in cell homeostasis and metabolism. This miRNA has long been studied as a target for treating HCV infection as its presence is required for efficient replication of this virus. We had previously found that reducing the first isoform of miR122 by 10% in the mature liver resulted in severe liver toxicity and in some cases liver failure in mice. In contrast, mice with a germline knockout of miR122 survive but over time develop hepatocellular carcinoma. The miR122 primary transcript is derived from a long non-coding RNA called lnc122 described only as the miR122 precursor RNA. Because the miR122 germline knockouts still produce the multi-kilobase lnc122 RNA (minus the miR122 miRNA sequence), we hypothesized both the lnc122 and miR122 have separate but perhaps coordinated molecular functions. We have constructed a plethora of tools including rAAV vectors expressing CRISPRi and CRISPRa for the lnc122 locus, conditional miR122 knockouts, and AAV-miR122 expression vectors. Using these vectors for functional genetic reconstitution we can independently alter the expression of lnc122 and miR122. Mice conditionally deficient in lnc122 develop large numbers of hepatocellular carcinoma tumors unrelated to miR122 status. We used RNA immunoprecipitation methods and show lnc122 RNA physically binds to the TEAD transcription factor to regulate the activity of the TEAD/YAP/TAZ transcriptional activator. These transcriptional activators are downstream of the Hippo signaling pathway and regulate cell proliferation. We found that in human hepatocellular carcinoma both miR122 and lnc122 are decreased and suppression of lnc122 exaggerates liver tumorigenesis in a myc-driven liver cancer mouse model. Our findings demonstrate that lnc122 RNA plays an important role in the onset of liver cancer as a non-canonical regulator of Hippo signaling. Additional differential roles for miR122 and lnc122 in liver metabolism are currently under study. This work establishes a new regulator pathway that is responsible for hepatocellular carcinoma and provides a potential target for new therapeutics.

1104. Interim Results of Expanded Access for Deltarex-G for an Intermediate Size Population with Advanced Pancreatic Cancer and Sarcoma (NCT04091295) and Single Patient Use INDS for Early Stage Triple Receptor Positive, and Triple Receptor Negative Breast Cancer (IND# 19130)

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Background and Rationale: Innovative therapies that address fundamental drivers of cancer are desperately needed to counter the invariable fatal outcome of metastatic disease. Cell cycle cyclin G1 (CCNG1) inhibitor therapy, exemplified by DeltaRex-G, a tumor targeted retrovector encoding a cytotoxic CCNG1 inhibitor gene, has been tested in over 280 cancer patients worldwide in phase 1, phase 2 and compassionate use studies, inducing long term (10-13 years) survivorship in certain patients with intractable metastatic cancer including pancreatic cancer, osteosarcoma, soft tissue sarcoma, breast cancer, and B-cell lymphoma. Hence, further clinical development and expanded access of DeltaRex-G for cancer patients who have few or no therapeutic options is apropos. **Study Design: Primary objective:** To determine duration of survival. **Secondary objective:** To evaluate disease control, best overall response and incidence of treatment-related adverse events. **Patients and Methods:** Study 1 is entitled "Blessed: Expanded Access for DeltaRex-G for Advanced Pancreatic Cancer and Sarcoma (NCT04091295)". Study 2 is entitled "Compassionate Use of Intravenous DeltaRex-G Tumor-Targeted Retrovector Bearing a Cytotoxic Dominant Negative Cyclin G1 Inhibitor for Advanced Sarcoma, Pancreatic Cancer, Non-small Cell Lung Cancer, Early- Stage Breast Cancer, Cholangiocarcinoma, Basal Cell Carcinoma, Actinic Keratosis and Squamous Cell Carcinoma. In both studies, patients will receive DeltaRex-G at 1-3 x 10¹¹ cfu i.v. over 15-30 minutes, 3 x a week until significant disease progression or unacceptable toxicity occurs. **Results:** Seventeen patients were enrolled, 9 sarcoma, 2 pancreatic adenocarcinoma, 1 non-small cell lung cancer, 2 breast carcinoma, 1 prostate cancer, 1 cholangiocarcinoma and 1 basal cell carcinoma and actinic keratosis. Three patients were enrolled in Study 1 and 14 patients were enrolled in Study 2. Two patients were initially enrolled in Study 1, and later enrolled in Study 2. Eleven of 17 enrolled patients were treated with DeltaRex-G monotherapy or in combination with FDA approved cancer therapies. **Efficacy Analysis:** Of the 11 treated patients, 5 of 11 are alive 6.7- 27 months from DeltaRex-G treatment initiation, with a 6-month Overall Survival rate of 45.4%; 2 patients with early state triple receptor positive or triple receptor negative breast cancer who received DeltaRex-G as adjuvant/ first line therapy are alive in complete remission; 2 patients with chemo-resistant Stage 4 chondrosarcoma and advanced basal cell carcinoma are alive with stable disease, and 1 patient with Stage 4 chordoma is alive

27 months. **Safety Analysis:** There were no treatment-related adverse events reported. **Conclusions:** Taken together, the data suggest that (1) Adjuvant/first line therapy with DeltaRex-G may prevent recurrence of early-stage triple receptor positive invasive carcinoma of breast, (2) DeltaRex-G may evoke tumor growth stabilization after failing standard chemotherapy, (3) DeltaRex-G may prime tumors to respond to standard chemotherapy/targeted therapies, and (4) DeltaRex-G is uniquely safe with minimal, if any, toxicity.

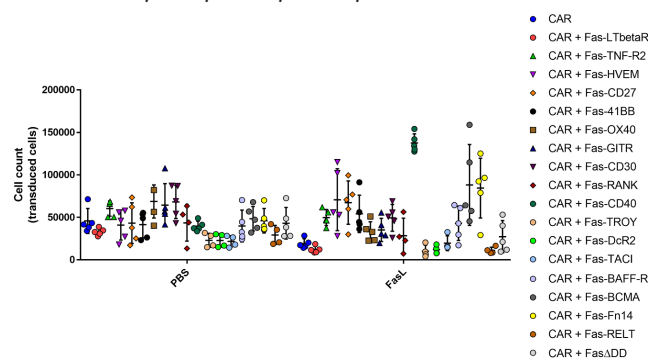
1105. CAR-T Cells Engineered to Express a FAS-CD40 Chimera Display Superior Persistence and Tumour Cytotoxicity

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Introduction Engineered T cells can have remarkable efficacy against haematological cancers, however activity against some tumours, particularly solid cancers, are limited by inhibitory receptors expressed by the tumour or its microenvironment. One key inhibitory receptor is FasL. Upon FasL binding to the death receptor, Fas/CD95, on the T cell, an intracellular cascade is triggered committing that cell to die by apoptosis. FasL is expressed by most cancers, regulatory T cells, endothelial cells, myeloid-derived suppressor cells and cancer-associated fibroblasts. Notably, activated T cells constitutively express Fas and are as such highly susceptible to FasL-mediated apoptosis. We add to the literature of strategies to engineer T cells to be resistant to FasL-mediated killing by testing a set of Fas-TNFR chimeric proteins. **Results** We first investigated over-expression of truncated Fas which lacks an endodomain. This completely rescued FasL-mediated apoptosis. However, truncated Fas significantly reduced CD3/28-induced T-cell proliferation and when co-expressed with a chimeric antigen receptor (CAR) resulted in reduced cytotoxicity. Given that Fas is known to have a dual role, we hypothesized that reduced T cell function was due to loss of Fas activatory signalling. Subsequently, we engineered a set of chimeric proteins that fused the ectodomain and transmembrane domain of Fas to the endodomain of pro-survival tumor necrosis factor receptors (TNFRs). We screened 17 Fas-TNFR chimeric proteins by incubating with immobilised FasL and assessed for their ability to rescue FasL-mediated apoptosis and induce proliferation. All the Fas-TNFRs except the Fas-LTβR, Fas-TROY, Fas-DcR2 and Fas-RELT chimeras rescued FasL-induced T-cell apoptosis, and T-cells expressing Fas-CD40, Fas-Fn14, Fas-BCMA and Fas-CD27 displayed the greatest induction of proliferation with fold-increases relative to truncated Fas of 10.7, 8.2, 6.8 and 5.7 respectively, which also correlated with secretion of IFN-γ. Fas-TNFRs were co-expressed with a GD2 targeting CAR and were subjected to restimulation by target cancer cells expressing either GD2 or GD2/FasL, where Fas-CD40 displayed superior proliferation and cytotoxicity by serially killing targets up to 10 stimulations compared to CAR-T cells alone or CAR-T cells expressing the other Fas-TNFR chimeras, which could only kill targets up to 7 rounds of stimulation. Importantly, CAR-T cells expressing Fas-CD40 did not proliferate autonomously in the absence of target cell stimulation. Furthermore, upon multiple

rounds of target cell stimulation, CAR-T cells expressing Fas-CD40 maintained a less differentiated memory phenotype, evidenced via a reduction in CD45RA+CD62L+ expression, and CD4+ CAR-T cells expressing Fas-CD40 expressed fewer exhaustion markers (PD1, TIM3 and LAG3) compared to CAR-T cells expressing the other Fas-TNFRs. **Conclusions** We have identified a chimeric Fas-CD40 protein that is able to not only rescue FasL-mediated T-cell apoptosis, but also elicit superior proliferation and anti-tumour cytotoxicity in the presence of FasL. **Figure Legend** PBMCs expressing CAR alone or with Fas-TNFRs were cultured with immobilised FasL for five days, at which point cell count was analysed by flow cytometry.



1106. EDIT-202, a Multiplexed CRISPR-Cas12a Gene-Edited iPSC-Derived NK Cell Therapy Has Prolonged Persistence, Promotes High Cytotoxicity, and Enhances *In Vivo* Tumor Killing

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Natural killer (NK) cell-based immunotherapy has emerged as a promising therapeutic approach for solid tumors due to their intrinsic tumor killing capacity, few treatment-related toxicities, and the ability to be given to patients as an off-the shelf therapy. NK cell effector function is diminished by the lack of functional persistence, as well as tumor-intrinsic immunosuppressive mechanisms, such as production of TGF-β, a pleiotropic cytokine that inhibits immune effector function. Furthermore, NK cells' ability to exert antibody-dependent cellular cytotoxicity (ADCC) is impaired when CD16 is cleaved off after NK cell activation. Here, we have established an induced pluripotent stem cell (iPSC) based NK (iNK) platform where these biological limitations can be overcome simultaneously via multiplexed gene editing of iPSCs. Using an engineered AsCas12a and our proprietary SLEEK editing tool, we successfully generated iPSC clones with double knock-out (DKO) of CISH and TGFβR2, and double knock-in (DKI) of CD16 and membrane bound (mb)IL-15. DKO/DKI iPSCs were differentiated into DKO/DKI iNK cells, termed EDIT-202, which were characterized to show edit-mediated enhancement of NK effector functions. Specifically, *in vitro* tumor killing assays showed that EDIT-202 had significantly

increased CISH KO-mediated natural cytotoxicity and were resistant to TGF- β treatment compared to wild type (WT) iNKs. Post-tumor cell challenge, CD16 expression was sustained in EDIT-202 whereas expression was greatly diminished in WT iNKs. Importantly, EDIT-202 demonstrated significantly augmented ADCC against multiple solid tumor cell lines. Constitutive surface expression of mbIL-15 was detected on EDIT-202. Constitutive mbIL-15 expression combined with CISH KO significantly prolonged *in vitro* persistence of EDIT-202 in the absence of exogenous cytokines compared to WT iNKs. EDIT-202 induced significantly enhanced anti-tumor efficacy *in vivo* when evaluated using a SKOV3 HER2+ ovarian cancer model. Tumor bearing mice were treated with an isotype control, trastuzumab alone, WT, or EDIT-202 iNKs in combination with trastuzumab. No exogenous cytokines were administered. EDIT-202 combined with trastuzumab demonstrated the greatest reduction in tumor burden of all cohorts, exceeding treatment with trastuzumab alone or trastuzumab in combination with WT iNKs. Forty percent of mice in the EDIT-202 plus trastuzumab cohort reached complete tumor clearance; no other cohorts had mice that reached complete tumor clearance. Significant tumor reduction in EDIT-202 plus trastuzumab resulted in prolonged survival of these mice compared with other treatment groups. In summary, *in vitro* and *in vivo* studies demonstrated that CISH and TGF β 2 DKO and CD16 and mbIL-15 DK1 resulted in an iNK cell product that is highly potent against multiple solid tumor types, long-lasting, and capable of resisting TGF- β -mediated immune suppression. These data support the development of EDIT-202 as a potential allogeneic cell-based medicine for treating solid tumors.

1107. CD47 is a Critical 'Don't Eat Me Signal' for Human CAR T Cells

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Introduction: CAR T cell therapy holds the promise to improve outcomes for patients with recurrent/refractory solid tumors but there is a limited array of targetable antigens. The CD47 'don't eat me signal' plays a protective role in cancer by preventing macrophage-mediated phagocytosis. Though CD47 expression is ubiquitous, it is overexpressed in multiple cancers and associated with poor clinical outcomes. CD47-specific antibodies exhibit antitumor activity with limited toxicity in early phase clinical trials, making CD47 an attractive candidate for CAR T cell therapy. Here, we aimed to develop CD47-CAR T cells and hypothesized they would have robust anti-solid tumor activity in pre-clinical studies. Instead, we found CD47-CAR T cells lack CD47 surface expression, which was required for CAR T cell persistence *in vivo*. **Methods/Results:** CD47-CARs were constructed with a binding domain derived from the high-affinity SIRP α variant CV1, a CD8 α Hinge/TM domain and CD28 ζ signaling domain (CV1-CAR). CD3/CD28 activated PBMCs were transduced using gamma retroviral particles, and flow cytometry demonstrated ~100% of T cells were CAR positive 7-10 days post transduction. Expansion assays revealed CV1-CAR T cells had limited proliferation up to 4 days post transduction, but thereafter expanded at a similar rate to non-transduced (NT) T cells. Given this delayed expansion,

and the low likelihood of a CAR transducing ~100% of T cells, we postulated they may undergo fratricide. Indeed, while NT T cells expressed high levels of CD47, CV1-CAR T cells were CD47 negative, and coculture assays revealed CV1-CAR T cells kill CD47+ T cells within 24hrs. These findings indicated CV1-CAR T cells initially undergo fratricide and overcome it by downregulating CD47 surface expression. We next evaluated CV1-CAR T cell specificity and cytotoxicity via cytokine ELISA and MTS killing assays. CV1-CAR T cells produced high levels of cytokines (IFN γ and IL-2) and killed in coculture with CD47+ (OV10-CD47) compared to CD47 negative (OV10) cells, demonstrating specificity. CV1-CAR T cells also killed other CD47+ targets including LM7 (osteosarcoma) and A549 (lung cancer). We evaluated antitumor activity *in vivo* by injecting firefly luciferase expressing LM7 (LM7.fluc) intraperitoneally (ip) in NSG mice, followed by CV1-CAR or EphA2-CAR (positive control) T cells ip 7 days later. Surprisingly, while EphA2-CAR T cells rapidly cleared tumors, CV1-CAR T cells failed to contain tumor growth, suggesting limited persistence. To directly compare the persistence of CV1-CAR and EphA2-CAR T cells, we took advantage of the subcutaneous A549 model, and injected intravenously (iv) CV1-CAR or EphA2-CAR T cells modified to express fluc. While EphA2-CAR T cells persisted at tumor sites, CV1-CAR T cells were eliminated within 3 days. Mechanistic studies demonstrated the elimination of CV1-CAR T cells was due to the lack of CD47 expression, since iv injected CD47 KO EphA2-CAR T cells were rapidly eliminated compared to EphA2-CAR T cells in the same A549 model. Live cell imaging revealed CD47 KO T cells were engulfed by two murine macrophage cell lines (RAW264.7, MH-S cells) in contrast to Control KO T cells *in vitro*. Taken together, these data suggest macrophage mediated phagocytosis was most likely responsible for the elimination of CD47 negative CAR T cells *in vivo*. **Conclusion:** While downregulation of CD47 in CV1-CAR T cells renders them functional *in vitro*, CD47 is required for CAR T cell persistence *in vivo*. The lack of cell surface CD47 expression sensitized T cells to macrophage-mediated destruction, highlighting that CD47 is a critical 'don't eat me signal' for CAR T cells.

1108. Engineering Logic-Gated MSLN CAR Architectures to Tune Circuit Fidelity and Function

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CAR T cells have transformed cancer immunotherapy, but more advanced synthetic biology is needed to enable treatment of multiple indications that have no reported targets with sufficient specificity for use of a conventional CAR. While mesothelin (MSLN) is a highly expressed ovarian cancer tumor-associated antigen, it is also expressed on normal mesothelial cells. To address this, we developed a circuit with a prime receptor (PrimeR) binding to ALPP/G that triggers the release of a transcription factor to induce MSLN CAR expression. To examine how modular this system is, we swapped the MSLN binding domain with camelid-derived MSLN-targeting nanobodies. With these binders,

however, there was an undesirable low level of T cell activation and cytotoxicity in the absence of priming antigen both *in vivo* and *in vitro*. In order to reliably engineer cells with tightly gated anti-tumor activity, we created 350 variants of two MSLN VH binders with systematic variation in CAR architecture to find variants that reduce priming-independent activity while maintaining or enhancing on target function. The binding domains were humanized by grafting the CDRs onto a human VH germline acceptor with highest framework homology to the parental sequences. Additional humanized variants were generated by mutating human framework residues back to the parental camelid residue to recapture binding function and stability. The final library used humanized binders that retained MSLN CAR activity upon T cell based *in vitro* screening. We generated a plasmid library of logic gates with a fixed ALPP/G PrimeR, while varying elements of the MSLN CAR. We combinatorially assembled 350 circuit constructs with variation in the humanized binding domains, monovalent vs. bivalent tandem combinations of binders, different hinge and transmembrane pairs, and two different coding sequences. We developed a high-throughput workflow to screen 350 circuits in 4 primary human T cell donors, using CRISPR-mediated non-viral site-specific integration. To assay circuit functionality, we co-cultured engineered T cells with 3 cell lines, K562-MSLN, K562-ALPG/MSLN and K562-ALPG, and read out activation status (upregulation of CD69 and CD25) as well as IL-2 and IFN γ secretion after 72 hours. This high-throughput screen provides a rich dataset to further investigate how different elements can affect CAR sensitivity and fidelity. Our results show circuits with diverse activity, measured by activation status, target cell cytotoxicity and cytokine expression when co-cultured with differing target cells. We studied K562-MSLN co-cultures (no ALPG) to probe the role of the CAR sequence on proper logic gate regulation, and K562-ALPG co-cultures (no MSLN) to identify logic-gated CARs that activated without cytolytic antigen, indicative of tonic signaling. These circuit behaviors were highly correlated across donors ($R=0.92$, $R=0.79$ respectively) validating the reliability of our platform for identifying circuits with desirable properties. We find that CAR sequence affects on-target cytolytic activity and logic gate fidelity of an engineered T cell. We also demonstrate a robust and scalable workflow for screening thousands of circuit, donor, and target cell combinations to select for circuits with desired function and activity. By better understanding how CAR architecture can affect circuit function, along with the ability to robustly screen large libraries of synthetic circuits, we can broaden the solid tumor landscape that can be safely and effectively targeted by cellular therapies.

1109. Oncometabolites in the Tumor Microenvironment Drive Altered Natural Killer Cell Functional Capacity

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Solid tumors have been largely resistant to treatment, owing to unfavorable interactions between immune cells and tumors driven by an immunosuppressive tumor microenvironment (TME). Though immunotherapy using natural killer cells (NK cells) has emerged as a promising treatment modality for various cancers, NK cells experience severe immunosuppression in the TME. Among factors that drive NK cell dysfunction are oncometabolites, substances that

cancer cells generate due to the alterations in their metabolic pathways. Oncometabolites aberrantly accumulate in the TME and mediate changes in signaling pathways in cancer cells to drive oncogenesis, in turn altering immune cell responses. Among tumor-driving oncometabolites, we have found that adenosine (ADO), a critical component of ATP production as part of the TCA metabolic cycle, has the ability to alter the phenotypic profile of NK cells in solid tumors and dampen their activation via LAG-3 expression. Lymphocyte Activation Gene-3 (LAG-3 or CD223), expressed on activated NK and T cells, is downregulated, we have found, on NK cells in response to ADO. This correlates to lower cytolytic capacity of human NK cells, indicating a role for LAG-3 in NK cell activation. We corroborated these findings with the phenotypic characterization of NK cells isolated from fresh blood and tumors of lung cancer patients and revealed a dysfunctional state linked with an inability of NK cells to support proliferative or cytolytic functions. Loss of ADO-producing CD73 from cancer cells reversed LAG-3-related activation changes on NK cells. In addition to ADO, we are investigating a network of related oncometabolites for their role in driving NK cell immunosuppression in solid tumors and understanding how targeting their metabolic pathways can restore NK cell activity.

1110. Enhanced Immunity Against Solid Tumors Through Vaccine-Promoted Co-Engagement of CAR T Cells and Endogenous T Cells

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Introduction: Adoptive cell therapy (ACT) with chimeric antigen receptor (CAR) T cells has shown dramatic clinical responses in hematologic malignancies. However, CAR T therapy in solid tumor shows less promising outcomes due to immunosuppressive microenvironment, antigen loss and tumor heterogeneity. Thus, achieving the full promise of CAR T ACT will require overcoming these hurdles. Here we report a synthetic vaccine to boost CAR T cells and engage endogenous T cells in tandem for enhanced immunity against solid tumors. **Materials and Methods:** To expand CAR T cells *in vivo*, we took advantage of a lymph node (LN)-targeting amphiphile ligand vaccine (amph-vax) platform we recently reported. Amphiphile vaccines were produced by linking a ligand (i.e., pepvIII in this work) to albumin-binding poly (ethylene glycol)-phospholipid (PEG-DSPE) conjugates. CAR T cells were produced by retrovirally transducing an EGFRvIII-targeting CAR into primary mouse T cells *ex vivo*. CAR T cells were infused into recipient mice intravenously. The complete vaccine is formulated with amph-pepvIII and cyclic-di-GMP; T cell expansion was characterized by flow cytometry. We characterized the response of EGFRvIII CAR T and endogenous T cells to amph-pepvIII vaccination in a syngeneic mouse glioblastoma model bearing EGFRvIII⁺CT-2A tumors. **Results:** We previously developed the amph-vax, which following s.c. injection, traffic efficiently to draining lymph nodes and decorate the surfaces of macrophages and dendritic cells with CAR T ligands. CAR T cells encountering ligand-decorated APCs receive stimulation through the CAR in tandem with costimulatory receptor signals and cytokine stimulation, representative of signals delivered during priming of native T cells by dendritic cells. Vaccine

boosting of EGFRvIII-CAR T cells via administration of amph-pepvIII vaccine together with an effective molecular adjuvant was shown to substantially promote CAR T cells expansion, enhance various aspects of CAR T functionality and tumor infiltration, which collectively lead to enhanced tumor rejection. Unexpectedly, amph-vax boosted CAR T therapy was accompanied by the development of endogenous anti-tumor T cell responses (also known as “antigen spreading”). We demonstrated that this endogenous anti-tumor T cell response is required for long-term tumor control during CAR T therapy, presumably by recognizing tumor neoantigens and preventing tumor escape. RNA-seq analysis of vaccine-boosted CAR T cells showed cell-intrinsic enhancements in CAR T cell function that include metabolic reprogramming (i.e., OXPHOS). We identified IFN- γ as a key effector molecule in eliciting the endogenous anti-tumor T cell response and showed that vaccine-boosted CAR T therapy can effectively treat tumors with pre-existing antigenic heterogeneity. **Conclusions:** We have developed a booster vaccine strategy to massively expand CAR T cells and enhance CAR T functionality *in vivo*, and this is accompanied by efficient priming of endogenous anti-tumor T cell immunity that effectively prevents antigen-loss induced tumor escape.

1111. T_{N/SCM} Pre-Selection Generates CAR T Cell Products Driving Superior Anti-Tumor Responses While Curtailing Severe CRS

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Chimeric antigen receptor (CAR) T-cell expansion and persistence represent key factors to achieve complete responses and prevent relapses. These features are typical of early memory T cells, which can be highly enriched through optimized manufacturing protocols. Here, we investigated the efficacy and safety profiles of CAR T-cell products generated from pre-selected naive/stem memory T cells (T_{N/SCM}), as compared to unselected T cells (T_{BULK}). Notwithstanding their reduced effector signature *in vitro*, limiting CAR T_{N/SCM} doses showed superior antitumor activity and the unique ability to counteract leukemia re-challenge in hematopoietic stem/precursor cell-humanized mice, featuring increased expansion rates and persistence, together with an ameliorated exhaustion and memory phenotype. Most relevantly, CAR T_{N/SCM} proved to be intrinsically less prone to induce severe cytokine release syndrome and neurotoxicity, independently of the costimulatory endodomain employed. This safer profile was associated

with milder T-cell activation, which translated in reduced monocyte activation and cytokine release. These data suggest that CAR T_{N/SCM} are endowed with a wider therapeutic index compared to CAR T_{BULK}.

1112. Engineering of Allogeneic T Regulatory Cells Expressing a Chimeric Antigen Receptor (Allo-CAR-Tregs) Using ZF-Nuclease/AAV6-Mediated Editing

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Converting polyclonal regulatory T cells (Tregs) into antigen-specific Tregs by introducing a chimeric antigen receptor (CAR) gained increasing attention as a potential treatment option for autoimmune diseases. Designed to recognize a clinically relevant antigen, it has been shown that CAR-Tregs could be specifically activated through the CAR upon binding to the specific antigen, proliferate and acquire their full immunomodulatory capacities. Therefore, these genetically modified CAR-Tregs are considered to be able to prevent or dampen autoimmune reactions in patients. Autologous cell therapy has several disadvantages such as longer lead-in times for patients due to the manufacturing process. Hence, our team is committed to develop off-the-shelf allogeneic CAR-Treg cell therapies to treat autoimmune diseases. Allogeneic CAR-Tregs were genetically modified using Zinc Finger Nuclease (ZF-nuclease) editing technology and adeno-associated virus type 6 (AAV6) donor DNA to insert the CAR into the Beta 2-Microglobulin (B2M) locus and knock-out the cell-surface expression of major histocompatibility complex class I (MHC-I). MHC-I plays a major role in allogeneic rejection and, by deleting it we aim to limit the elimination of allogeneic CAR-Tregs by the patient's immune system. Furthermore, we describe the development of an AAV6-based toolbox, harboring numerous expression cassettes, to efficiently mediate targeted integration into the B2M locus, testing different expression levels of our CAR candidates. Our results show high MHC-I knock-out efficiency ranging between 70%-90% with no impact on the viability or the phenotype of edited MHC-I minus Tregs. The targeted integration efficiency was overall similar (40% to 50%) between all the AAV6-CAR donor DNA whatever the expression cassette used. However, the level of CAR expression per cell was more variable. Indeed, with the weak FOXP3-derived minimal promoter FXP3.2, we observed higher differences regarding CAR expression levels. To assess the CAR functionality of B2M-edited CAR-Tregs, allogeneic CAR-Tregs were incubated with CAR ligands and the expression of the CD69 activation marker was assessed. The level of activation was comparable between all the constructs except for FXP3.2 expression cassettes, correlating with a lower CAR density per cell. Two endpoints i) the absence of tonic signaling and ii) the intensity of signal to noise ratio were used to identify the construct with optimal CAR functionality in the B2M locus. Overall, our results provide a robust strategy for genome editing in Tregs using ZF-Nucleases in combination with AAV6-CAR donor DNA.

1113. Primary Analysis of ZUMA7: A Phase 3 Randomized Trial of Axicabtagene Ciloleucel (Axi-Cel) versus Standard of Care (SOC) Therapy in Patients (Pts) with Relapsed/Refractory (R/R) Large B-Cell Lymphoma (LBCL)

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The prognosis for pts with R/R LBCL after 1st-line (1L) chemoimmunotherapy (CIT) is poor. In the multicenter, Phase 3 ZUMA-7 study, eligible pts ≥ 18 y with LBCL refractory to or relapsed ≤ 12 mo of 1L CIT were randomized 1:1 to axi-cel (an autologous anti-CD19 chimeric antigen receptor T-cell therapy) or SOC (2-3 cycles of a CIT regimen followed by high-dose chemotherapy with autologous stem cell transplant [HDT-ASCT] if CIT-responsive). The primary endpoint was event-free survival (EFS by blinded central review). Key secondary endpoints were objective response rate (ORR) and overall survival (OS; interim analysis). As of 3/18/21, 359 pts were randomized to axi-cel (N=180) or SOC (N=179). Overall, 74% of pts had primary refractory disease and 46% had high 2L age-adjusted IPI (2-3). While 94% of axi-cel pts were infused, only 36% of SOC pts reached HDT-ASCT. The primary endpoint of EFS was met (HR [hazard ratio]: 0.398; $P < 0.0001$). At 24.9-mo median follow-up, median EFS was significantly longer with axi-cel vs SOC (8.3 vs 2 mo); 24-mo EFS Kaplan-Meier estimates were 41% vs 16%. ORR and complete response (CR) rates were higher with axi-cel vs SOC (ORR: 83% vs 50%; $P < 0.0001$; CR: 65% vs 32%). Median OS, evaluated as an interim analysis, was not reached for axi-cel vs 25.7 mo for SOC (HR: 0.708; $P = 0.0159$). In the SOC cohort, 100 (56%) pts received cellular immunotherapy off protocol as subsequent therapy. Grade ≥ 3 adverse events occurred in 155 (91%) and 140 (83%) pts, and treatment-related deaths occurred in 1 and 2 axi-cel

and SOC pts, respectively. Grade ≥ 3 cytokine release syndrome (CRS) occurred in 11 (6%) axi-cel pts and grade ≥ 3 neurologic events (NEs) in 36 (21%). No grade 5 CRS or NEs occurred. In ZUMA-7, axi-cel demonstrated a statistically significant improvement over SOC, with >4 -fold greater median EFS, double the CR rate, and nearly 3 \times more pts receiving definitive therapy. Safety was manageable and consistent with 3L axi-cel. Axi-cel should replace CIT/HDT-ASCT as the SOC for 2L R/R LBCL.

1114. Novel Epigenetic Targeting of the MYC Oncogene for the Treatment of NSCLC

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The oncogene *MYC* is a master transcription factor that is critical for multiple cell physiologies. Although *MYC* expression is normally tightly controlled in non-transformed cells, its activity is frequently dysregulated in cancers. Given the central role of *MYC* in cancer biology, a direct *MYC*-targeting anti-cancer therapeutic has been a key goal in the field. However, *MYC* has been deemed “undruggable,” largely due to its high degree of downstream autoregulation and the lack of an enzymatic pocket required for effective drug binding. In the genome the *MYC* gene and its regulatory elements are located inside an insulated genomic domain (IGD), a discrete chromatin-looping region within which gene expression is mediated by CTCF and other factors. At Omega Therapeutics, we are developing an epigenomic approach to specifically and durably tune expression of genes to treat or cure diseases, including previously difficult-to-drug targets like *MYC*. The OMEGA platform comprises thousands of DNA sequences that can be epigenetically modified to alter gene expression in associated IGDs; we call these sequence intervention points EpiZips. We have rationally designed and engineered programmable, mRNA-encoded epigenetic medicines, called Omega Epigenomic Controllers (OECs), to target EpiZips within IGDs for precise control of gene expression, including *MYC*. Non-small cell lung cancer (NSCLC) is the leading cause of global cancer-related mortality, making up almost 25% of all cancer deaths. Although several targeted therapies and immunotherapies have been developed for NSCLC, there remains high unmet medical need. NSCLCs often exhibit aberrant *MYC* activity, including genomic amplification and overexpression. In addition, a significant fraction of NSCLCs display amplification of a lung-specific super-enhancer that is located within the *MYC* IGD that acts to upregulate *MYC* expression specifically in this tumor type. Here, we describe a NSCLC-specific therapy that modulates *MYC* expression levels utilizing OECs targeting two sites within the *MYC* IGD. The combined action of these factors, each with a distinct EpiZip and mechanism of action, is to epigenetically downregulate *MYC* expression. The analysis of this novel therapeutic using ChIP-seq shows the on-target binding of each OEC module to its predicted genomic region and demonstrates the corresponding epigenetic modifications in the chromatin at these target sites in NSCLC cells. We additionally demonstrate the efficacy of this therapy for modulating *MYC* mRNA

(up to 98% reduction) and MYC protein levels (down to undetectable) compared to control-treated cells using a panel of NSCLC cell lines and identify multiple MYC-associated transcriptional changes following treatment. Moreover, OEC treatment across a number of NSCLC cell lines rapidly and significantly reduces cell viability by an average of $67 \pm 20\%$ (mean \pm SEM, 3 days post-treatment) with limited effect on normal primary cells. Cell viability continues to decrease beyond 3 days, due in part to induction of apoptosis. Effects on MYC mRNA downregulation are durable for at least 9 days *in vitro*. Furthermore, the combination of OEC treatment with EGFR- or MEK-targeted therapies in NSCLC cells showed enhanced downregulation of MYC protein (by 3-5-fold), corresponding to enhanced inhibition of cell growth. Similarly, *in vivo* studies demonstrate that treatment of NSCLC tumor-bearing mice with mRNA-encoded, MYC IGD-targeting OECs significantly inhibits overall tumor growth. In summary, our findings demonstrate the potential of a novel mRNA-encoded OEC for the treatment of NSCLC via tunable epigenomic modulation of MYC expression. Targeting MYC in this manner may represent a novel and differentiated therapeutic approach in patients with advanced NSCLC.

1115. Epigenetic Reprogramming (Epi-R™) Yields T-Cell Receptor Products with Improved Stemness, Metabolic Fitness, and Functional Activity in the Presence of Persistent Antigen Exposure

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Engineered T-cell Receptor (TCR) therapy targeting NY-ESO-1 is one of the only immunotherapies that shows clinical activity in patients with solid tumor malignancies. Previous studies in several solid tumor models indicate a correlation between the stemness of a T-cell product with antitumor efficacy and durability of response. We employed our Epi-R technology, an optimized expansion process and epigenetic reprogramming media, to manipulate cellular metabolic states and preserve stem-like qualities in LYL132, an NY-ESO-1 TCRT-cell product. We performed a pre-clinical comparison of LYL132 to the first-generation NY-ESO-1 TCR product and a control to determine the effect of Epi-R on overall T-cell functionality. Phenotypic evaluations conducted via multi-color flow cytometry and single-cell sequencing revealed that LYL132 had a higher proportion of stem-like T cells at both large scale (2 healthy donors) and research scale (6 healthy donors and 4 synovial sarcoma patient samples) versus the first-generation NY-ESO-1 TCR product and control. Additionally, upregulation of oxidative phosphorylation and mitochondrial electron transport gene sets was seen in LYL132 compared to the first-generation NY-ESO-1 TCR product, a characteristic suggestive of T cells that are metabolically quiescent and stem-like. Also observed in large-scale products was down-regulation of gene sets often associated with effector-committed T cells, such as glycolysis and hypoxia. Other key measures of cells' metabolic fitness, such as autophagy pathway related proteins (LC3bI and LC3bII), were higher in LYL132 as compared to the first-generation

NY-ESO-1 TCR product in healthy donors. We also evaluated the proliferative capacity and anti-tumor cytotoxicity of LYL132 with persistent exposure to NY-ESO-1 expressing target cells. LYL132 had higher proliferation and superior maintenance of both cytotoxicity and cytokine production in most donors after 7 and 14 days of persistent exposure to antigen versus controls. Additionally, after persistent exposure to antigen, LYL132 preserved a population of T cells with less differentiated phenotype that could potentially give rise to effector cells. Pre-clinical data show LYL132 had a high proportion of stemlike T cells and maintained increased proliferative capacity as well as prolonged functional activity with persistent antigen exposure. Based on these promising pre-clinical data, LYL132 will be evaluated in the clinic to assess the impact of T-cell product persistence and stemness on anti-tumor efficacy and overall clinical outcomes. LYL132 is expected to begin a Phase 1 clinical trial in adult patients with advanced synovial sarcoma or myxoid/round cell liposarcoma in 2022.

1116. High Throughput Screening Enables Selection of Ideal Logic-Gated CARs Built from Hundreds of Novel Binders

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Generating potent clinical responses against solid tumors remains a challenge for CAR T cell therapy. This lack of efficacy is likely due to both reduced on-tumor activity in the tumor microenvironment and the lack of appropriate target antigens that are expressed on tumor cells but not on critical healthy tissues. We addressed such challenges by engineering T cells to target tumors only upon recognition of two antigens through AND Boolean logic. In this case we used a ALPP/G-targeting prime receptor (PrimeR) that induces the expression of a MSLN-targeting CAR upon antigen binding. Logic-gated CARs can be optimized by selecting binders that are sensitive and specific to the cytolytic antigen. Here, we developed methods for generating hundreds of novel and diverse antigen binding domains against MSLN through immunization campaigns and *in vitro* phage display. Novel scFv and VHH binders were generated using transgenic mice immunized with recombinant MSLN - following immunization, plasma cells which secreted MSLN-specific antibodies were isolated, screened, and sequenced by NGS to yield unique binders against MSLN. Additionally, novel VHH binders were isolated from a synthetic phage display library - several rounds of phage panning with recombinant MSLN protein followed by phage ELISA yielded diverse VHH sequences against MSLN. Identifying functional and potent CAR T-cell binders requires rigorous empirical testing of different antigen binding domains, a process which can be stunted in scope due to a variety of factors: limited scalability, resourcing, and binder availability. Here, we demonstrate a high-throughput process for screening hundreds of logic-gated CARs to identify binders with enhanced cytolytic function and specificity. We engineered T cells from 4 donors in multiwell plates

using CRISPR-mediated, non-viral, site-specific integration of circuits bearing a fixed PrimeR and 290 unique MSLN binding domains. These engineered T cells were co-cultured with four target cell lines to evaluate fidelity and on-target functionality. Fidelity was assessed in two ways: first, a lack of T cell activation in response to the priming antigen (K562-ALPG) alone, which indicates tonic activity or off target activity; second, a lack of T cell activation in response to the cytolytic antigen (K562-MSLN) alone, which indicates insufficient regulation of the CAR. On-target activity in the presence of both the priming and cytolytic antigens (K562-ALPG-MSLN) was quantified by T cell activation and cytotoxicity, as well as through IFN γ and IL-2 secretion. We additionally used SKOV3 target cells, which endogenously express low levels of both the priming and cytolytic antigens, to select for logic gates with heightened antigen sensitivity. We combined the above metrics to filter out CARs that were active in the absence of logic gate activation or CAR antigen exposure, and to rank the remaining binders by their activation and cytokine secretion levels in the presence of both antigens. To account for multiple criteria when ranking binders, we used desirability functions, scaling each measurement to a [0-1] range, and using geometric means to combine desirabilities across different criteria. Here we show the utility of a screening process to robustly evaluate hundreds of binder domains as logic-gated CARs to rapidly identify CARs with proper antigen-dependent activation and maximal efficacy. This scalable screening process is now being translated to a fully automated system, which will screen tens of thousands of binder, donor, and target cell combinations to identify specific and sensitive circuits for multiple new solid tumor indications.

1117. Novel Bioluminescent Bioassays for the Discovery and Development of Engineered T Cell Therapies for Cancer

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T cell therapies are a promising approach for the treatment of cancer. For this, autologous or allogeneic T cells are engineered with specific T Cell Receptors (TCR) or Chimeric Antigen Receptors (CAR) that redirect effector responses against tumor cells and then infused into patients. Development of these therapies relies on the ability to rigorously evaluate candidate TCRs and CARs, as well as assays that can precisely and reproducibly measure the potency of cells for development and lot release, which are currently lacking. Therefore, we have developed novel bioluminescent bioassays to address both of these bottlenecks. To facilitate the screening and characterization of transgenic TCRs, we developed two TCR $\alpha\beta$ -null reporter T cell lines, which are CD4+ or CD8+. Reintroduction of peptide-specific TCR α and β chains into TCR $\alpha\beta$ -null reporter T cell lines results in peptide-dependent TCR activation and luciferase reporter expression. The select expression of CD4 or CD8 in the TCR $\alpha\beta$ -null reporter T cell lines can enable the development of transgenic TCRs for both MHC I- and MHC II-restricted tumor antigen targets. To quantitatively measure the functions of engineered T cells, we have used split NanoBiT luciferase technology to develop two

homogenous assays: a HiBiT target cell killing assay and cytokine immunoassays. Incubation of CAR-T cells or similar T cell products with target cells stably expressing HiBiT results in lysis of the target cells and release of HiBiT proteins. These HiBiT proteins then bind to LgBiT in the detection reagent and form functional NanoBiT Luciferase to generate luminescence. Furthermore, cytokine production (e.g IL-2 and IFN- γ) from the engineered T cells can be quantitatively measured in the homogenous NanoBiT Immunoassays. These luminescent assays are homogenous, fast, highly sensitive, and have robust assay windows. They represent a new set of tools for the discovery and development of T cell-based immunotherapies.

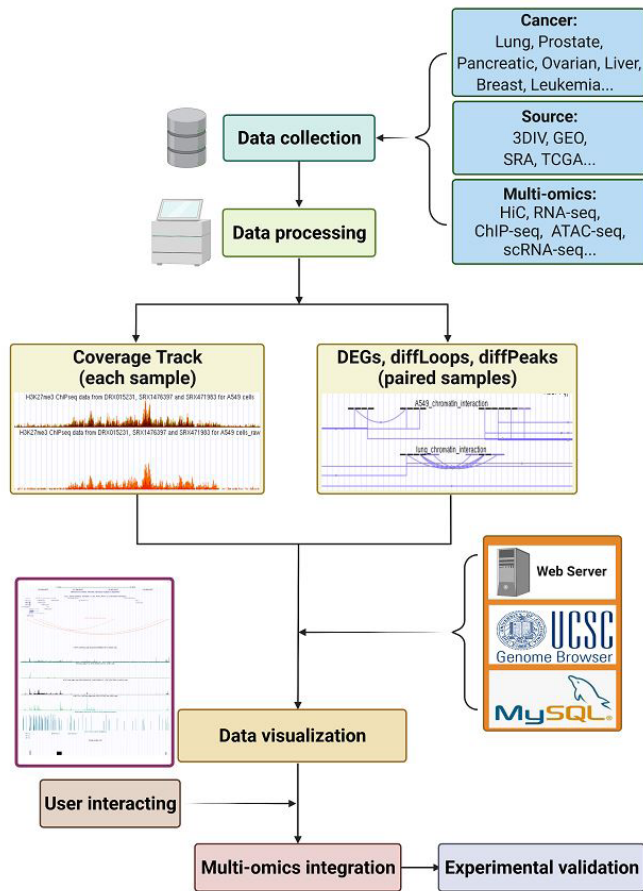
1118. Cancer3D: A Comprehensive Database for Integrating Cancer Chromatin Architecture with Multi-Omics

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With the breakthrough of chromatin conformation capture technologies in recent years, the importance of three-dimensional (3D) genome structure in gene expression, cell function regulation, disease occurrence, and development has been gradually recognized. To provide a comprehensive visualization of chromatin architecture and other multi-omics data for cancer research, we have constructed an extensive database with manual curation of publicly available data, Cancer3D (<http://www.lungcancer3d.net>). This web-based tool focuses on displaying HiC data of major human cancers and controls, such as lung cancer, breast cancer, prostate cancer, liver cancer, along with a variety of multi-omics data, such as bulk RNA-seq, single-cell RNA-seq of COVID-19 patients, (single-cell) ATAC-seq, CTCF/H3K27ac/H3K27me3/Pol II ChIP-seq, DNA methylation, DNA mutation, and copy number variations (Figure 1). Researchers can visualize these cancer orientation multi-omics data directly through the genome browser and discover how gene expression is regulated at diverse levels. For example, we have demonstrated that the high expression level of C-MYC in lung cancer may be caused by the distant enhancer introduced by the *de novo* chromatin loops in lung cancer cells to bind the C-MYC promoter. The integrated analysis identified the variants and somatic mutations of human lung cancer nearby the distant enhancer of C-MYC, suggesting these variants may regulate the C-MYC expression by the chromatin loop. The comprehensive multi-omics analyses of diverse cancers through the Cancer3D website could

reveal cancer development mechanisms and provide potential targets for cancer therapy. (# Xinyu Wu, Anlan Jiang, Jixin Wang, and Shiyang Song contributed equally to this work. # Jian Liu for correspondence: JianL@intl.zju.edu.cn)



1119. Disheveling Tumor's Intrinsic Immunosuppression to Improve IL-12 Therapy in Glioblastoma

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While Immunotherapy has revolutionized the cancer care, glioblastoma (GBM) remains the challenge because of the prominent immunosuppressive microenvironment. Integrated efforts with rational combination of gene therapies represents a promising approach to improve the efficacy of immunotherapy in GBM. Interleukin-12 (IL-12) is considered a promising anti-tumor immunotherapy as it potentiates cytotoxic responses by NK cells and CD8 T cells (CD8+) by stimulating IFN γ production. Phase I clinical trial based on Veledimex (VDX)-regulatable interleukin-12 (IL-12) gene therapy in recurrent high-grade glioma patients showed encouraging results with an overall increase in immune cell infiltration in tumors. However, multiplex analysis of post-therapy patient's tumors also exhibited upregulation

of immunosuppressive molecule such as PDL1. Our previous studies demonstrated the role of Interferon-stimulated non-coding RNA (INCR1) in the regulation of several IFN-regulated immunosuppressive molecules. Thus, INCR1 can be targeted to throw off the shackles of IL-12 mediated immunosuppression. To test this hypothesis, we first analyzed GBM tumor tissues from GBM patients pre- and post-IL-12 therapy and probed for INCR1 and PDL1 transcripts using RNAScope. Data showed that both INCR1 and PDL1 expression was significantly upregulated in post-therapy tumors, in comparison to the pre-therapy tumors. In addition, the expression of INCR1 and PDL1 transcripts positively correlated with each other suggesting that INCR1 could possibly be associated with the upregulation of PDL1 and governing the IL-12-induced immunosuppression. To determine this, we employed control or INCR1 knockdown GBM cell line to study the effects of IL-12 on peripheral blood mononuclear cells (PBMCs) ability to kill tumor cells in a 3D culture system. Our data show that IL-12 in the coculture setting increased INCR1 and PDL1 expression in GBM cells, as compared to the unstimulated control cells. GBM cells with silenced INCR1 had significantly reduced upregulation of PDL1 and other immunosuppressive molecules in response to IL-12 therapy. INCR1 knockdown could remarkably enhance PBMCs-mediated tumor sphere destruction in the presence of IL-12. In conclusion, targeting INCR1 represents a rational therapeutic strategy to improve IL-12 therapy.

1120. Production and Characterization of Virus-Free, CRISPR-CAR T Cells Capable of Inducing Solid Tumor Regression

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Background: Chimeric antigen receptor (CAR) T cells traditionally harbor viral vector-based sequences that encode the CAR transgene in the genome. These T cell products have yet to show consistent anti-tumor activity in patients with solid tumors. Further, viral vector manufacturing is resource intensive, suffers from batch-to-batch variability, and includes several animal components, adding regulatory and supply chain pressures. **Methods:** Anti-GD2 CAR T cells were generated using CRISPR/Cas9 within nine days using recombinant Cas9 protein and nucleic acids, without any viral vectors or animal components. The CAR was specifically targeted to the T Cell Receptor Alpha Constant gene (TRAC). T cell products were characterized at the level of the genome, transcriptome, proteome, and secretome using CHANGE-seq, scRNA-seq, spectral cytometry, and ELISA assays. Functionality was evaluated in vivo in an NSG xenograft neuroblastoma model. **Results:** In comparison to traditional retroviral CAR T cells, virus-free CRISPR CAR (VFC-CAR) T cells exhibit TRAC-targeted genomic integration of the CAR transgene, elevation of transcriptional and protein characteristics associated with a memory phenotype, and low tonic signaling prior to infusion arising in part from the knockout of the

TCR. Upon exposure to the GD2 target antigen, anti-GD2 VFC-CAR T cells exhibited specific cytotoxicity against GD2+ cells in vitro and induced solid tumor regression in vivo, with robust homing, persistence, and low exhaustion against a human neuroblastoma xenograft model.

Conclusions: This proof-of-principle study leveraging virus-free genome editing technology could enable flexible manufacturing of clinically relevant, high-quality CAR T cells to treat cancers, including solid tumors.

1121. Exon Skipping Induced by CRISPR-Directed Gene Editing Influence Genotypic & Phenotypic Outcomes

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We have been developing CRISPR-directed gene editing as an augmentative therapy for the treatment of non-small cell lung carcinoma (NSCLC) by genetic disruption of Nuclear Factor Erythroid 2-Related Factor 2 (NRF2). NRF2 promotes tumor cell survival in response to therapeutic intervention and thus its disablement should restore or enhance effective drug action. Here, we report how NRF2 disruption leads to collateral damage in the form of CRISPR-mediated exon skipping. Heterogeneous populations of transcripts and truncated proteins produce a variable response to chemotherapy, dependent on which functional domain is missing. We identify and characterize predicted and unpredicted transcript populations and discover that several types of transcripts arise through exon skipping; wherein one or two *NRF2* exons are missing. In one specific case, the presence or absence of a single nucleotide determines whether an exon is skipped or not by reorganizing Exonic Splicing Enhancers (ESEs). We isolate and characterize the diversity of clones induced by CRISPR activity in a NSCLC tumor cell population, a critical and often overlooked genetic byproduct of this exciting technology. Finally, gRNAs must be designed with care to avoid altering gene expression patterns that can account for variable responses to solid tumor therapy.

1122. A Novel Approach to Increasing Mutation-Specific CRISPR-Directed Functional Knockout Efficiency

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CRISPR/Cas9 has great potential as a tool for use in gene therapy. One of the key advantages of the technology is its ability to specifically bind to a given genomic sequence with high precision and induce double-stranded breaks, resulting in frame-shifting indels that can lead to a knockout. This advantage can be leveraged in a clinical setting with the intent of disabling a mutated gene that results in a pathological condition, such as a cancer-driving somatic mutation, by designing the gRNA to target the mutant sequence. However, not every CRISPR/Cas9 will act on its intended target sequence with high efficiency. To overcome this limitation, a novel approach using two gRNAs is being developed, with one gRNA specifically targeting the mutated sequence and the second gRNA targeting an intronic sequence in a region of low

consequence. The action of both gRNAs acting in concert will induce exon skipping as a result of deleting the genomic DNA between the two Cas9 cut sites, effectively disabling the production of functional pathogenic protein. In a clinical setting, a systemic delivery of these two gRNAs would allow for the tumor cells harboring the driver mutation to be effectively disabled via exon skipping while healthy cells will be left unaffected as they lack the driver mutation.

1123. Leveraging AAV Gene Delivery to Achieve Long-Term Continuous or Controllable Expression of Cancer Immunotherapies

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T cells redirected to cancer cells either via a chimeric antigen receptor or a bispecific molecule have been breakthrough technologies in cancer immunotherapy. Yet each have significant limitations, the former requiring individual manufacturing and the latter hampered by prolonged continuous intravenous infusion resulting in only relatively short-term use. Here we present an off-the-shelf, single dose solution we dubbed "TransJoin" for achieving prolonged systemic exposure (>1 year) of constitutively secreted protein-based immunotherapeutics via intravenous recombinant adeno-associated virus (rAAV) gene transfer. We demonstrate proof of principle using both rAAV8 and rAAVrh74 expressing a secreted CD19xCD3 bispecific to successfully treat a CD19+ lymphoma model. In addition, we created an inducible version by reverse engineering a defective exon into its coding sequence that was excluded following the intravenous administration of a morpholino designed to induce exon-skipping, restoring expression of the immunotherapeutic. We achieved repeatable, intermittent *in vivo* transgene expression in the bloodstream lasting 2-3 weeks following each morpholino-mediated induction up to >36 weeks after the initial AAV infusion. Our so-called "TransSkip" system could also be considered to achieve transient and/or repeated expression of other transgenes of interest for non-cancer applications.

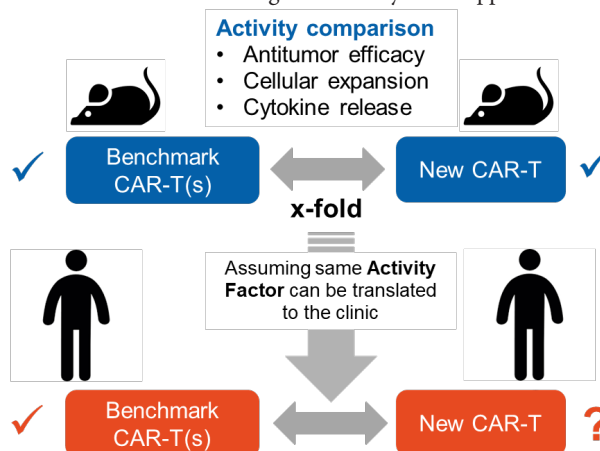
1124. Novel Benchmark Scaling Method for First-in-Human Dose of CAR-T Therapies

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Background Despite several approved CAR-T therapies, the First-in-Human (FIH) dose for new CAR-Ts in clinical development is often determined empirically. Establishing a starting dose in clinical studies for novel CAR-T therapies remains a challenge, due to the lack of preclinical-to-clinical translational methods. **Method** We are proposing a novel method of FIH starting dose selection by scaling from benchmark CAR-T(s) with an activity factor (Figure). The benchmark CAR-T(s) should bind to the same or functionally similar target(s) and have clinical safety or efficacy data available. **Result** The development of a CD19-targeted CAR-T (YTB323) and a BCMA-targeted CAR-T (PHE885) with a new optimized manufacturing method (T-Charge™) are used as examples. The FIH clinical doses were derived based on the method described and both showed acceptable safety profile with some clinical activity at the starting dose. For the development of YTB323, CTL*019 (similar to CTL019) is used as a benchmark CAR-T for preclinical comparison. In a xenograft mouse model of leukemia, YTB323 appeared 40-fold more potent than CTL*019 with greater cellular expansion, higher IFN- γ and IL-2 secretion, and greater tumor-killing potency on a model-estimated per cell basis *in vivo*. In the pivotal trial of CTL019 in *r/r* DLBCL patients (NTC02445248), responses were observed across the dose range of 0.6-6.0 $\times 10^8$ cells with median dose of 3.0 $\times 10^8$ cells in responding patients. Based on the proposed method, the lowest YTB323 effective dose is anticipated to be 1.5 $\times 10^6$ cells (0.6 $\times 10^8$ cells/40) and the median efficacious dose to be 7.5 $\times 10^6$ cells (3.0 $\times 10^8$ cells/40). Assuming a comparable safety profile based on the same mechanism of action as CTL019, an YTB323 dose up to 2.8 $\times 10^7$ cells (highest CTL019 dose with manageable AEs from another study UPCC03712 of 1.1 $\times 10^9$ cells divided by 40) should be tolerable. A starting dose of 2.5 $\times 10^6$ cells was proposed for potential antitumor effects with sufficient safety margin. YTB323 was later tested in patients with B-cell malignancies in a phase I dose-escalation study (NCT03960840). In DLBCL patients, expansion was observed at the starting dose. Strong expansion and durable responses for at least 3 months were observed at the second dose level (12.5 $\times 10^6$ cells). In a second example, the target starting dose for PHE885 was derived based on preclinical comparison and clinical data generated with MCM998, another BCMA-targeted CAR-T previously tested in *r/r* multiple myeloma patients (NCT02546167). Using the proposed approach, the target starting dose of PHE885 at 10 $\times 10^6$ cells with a range of 5-10 $\times 10^6$ cells was proposed. This is based on the clinically active dose of MCM998 (500 $\times 10^6$) divided by a preclinical activity factor of 5 derived from a comparative preclinical study in NSG xenograft mice and an additional safety margin of 10. High cellular expansion and promising clinical activity were observed in the first few patients treated with PHE885 at the lower end of the target dose range of 5 $\times 10^6$ cells in *r/r* multiple myeloma patients (NCT04318327), with higher doses currently being explored.

Conclusion The proposed method to calculate FIH starting dose mitigates the current limitation in preclinical-to-clinical translation in CAR-T therapy. Examples of two CAR-Ts with different targets and indications demonstrated the general utility of the approach.



Immunological Aspects of Gene Therapy and Vaccines II

1125. CpG Content Reduction in Codon-Optimized AAV Transgene Sequences Does Not Reduce hTLR9 Activation in an *In Vitro* Model

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It has been reported that adeno-associated viruses (AAVs) induce an innate immune response. The presence of unmethylated CpG sequences in AAV genomes has been identified as one of the potential causes of the activation of innate immune response in humans via the Toll-like receptor 9 (TLR9) pathway. The ability to adequately control AAV-mediated immune responses is essential for avoiding potential downfalls in gene therapy efficacy, including immunotoxicity and potential loss of transgene expression. In fact, retrospective observations of multiple clinical AAV trials for hemophilia B with different AAV DNA sequences pointed to an association of higher CpG content with higher immunotoxicity and reduced expression duration. To determine whether a decrease in CpG motifs could lower the activation of the innate immune response by AAV genomes, two codon-optimized versions each for two transgene sequences containing either a reduced number of CpG motifs (2 in 3966 nt and 2 in 3840 nt) or a non-reduced number (187 in 3966 nt and 200 in 3840 nt) were designed and cloned into AAV plasmid vectors. We calculated the normalized risk factor (NRF₉) according to J. Wright

(*Mol Ther*, 2020) for the coding sequences of the constructs to be 0 and 0.26 for the CpG-reduced and 21.95 and 26.29 for the non-reduced sequences. To test whether these new sequences induced an innate immune response via TLR9 activation, an *in vitro* assay using a human TLR9 (hTLR9) reporter cell line (HEK-Blue™hTLR9, InvivoGen) was performed. The sequences were tested as plasmid DNA, linearized DNA containing only the AAV genomes (ITR-to-ITR) via lipid nanoparticle transfection, or as an AAV8 infectivity assay. Our results showed very low hTLR9 activation with all constructs, with no significant differences between CpG-reduced vs. CpG non-reduced versions when tested either as DNA or as viral particles. These results suggest that this *in vitro* surrogate assay is not able to assess the impact of CpG motif content of transgenes carried by AAV vectors on the innate immune response. Whether this is due to inadequacies of the assay or because the vectors lack unmethylated CpG motifs is unable to be determined from these results. Thus, further efforts are needed to develop standardized *in vitro* preclinical methods capable of examining the benefits of using sequences with reduced CpG content in AAV-based gene therapy.

1126. Evaluation of Cas9 Mediated Immune Response Effect on Long Term Transgene Expression in WT Mice and NHPs without Immune Suppressant

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Background: CRISPR/Cas9-mediated in-vivo gene editing offers great potential to treat several genetic disorders. Conventional gene editing mediated through DNA strand breaks leads to off-target gene changes and sometimes cause fatal consequences. Our proprietary CRISPR-GNDM¹ (Guide Nucleotide-Mediated Modulation) platform (aka CRISPRa/i), modulates gene expression without DNA strand breaks has great potential to overcome this hurdle. However, Cas9-mediated immunogenicity became a major concern in the field of in-vivo gene editing/modulation. But immunological consequences of Cas9 have not been thoroughly explored thus far. So, to evaluate Cas9 (GNDM¹) mediated immune responses and its effect on transgene-expressing targeted tissue, we performed longitudinal studies in WT mice and NHPs. **Method.** We used two cohorts of WT mice and nonhuman primates (NHPs) to evaluate Cas9-mediated immunogenicity using our therapeutic model AAV-CK8-GNDM¹ without any immune suppressant. In Cohort 1, mice were administered intravenously with 3x10¹⁴vg/kg of AAV9-CK8-GNDM¹. Transgene expression, anti-Cas9 antibody responses and T cell responses were analyzed at different time points from week1 to month 10. In cohort 2, mice were administered with AAV9-CK8-GNDM¹ and immune responses and transgene expression analyzed after 2 years. To further evaluate effect of Cas9-mediated immune responses in larger animals, 1.5-2-year-old NHPs were administered with different dose of AAVrh74-CK8- GNDM¹ and transgene expression and immune responses were evaluated from week1 to month 6. Immune cell infiltration into targeted tissue and CTL mediated toxicity was evaluated by H&E staining of muscle tissue. **Results:** AAV9-CK8- GNDM¹ systemic administration induced high level of GNDM¹ expression within a week in heart and muscles

and sustained through month 10. T cell functional analysis by IFN γ ELISPOT showed moderate response at week2 and these responses declined over the period through month 10. Histological analysis by H&E staining of GC muscles didn't show any immune cell infiltration. Cohort 2 was harvested after 2 years of post-administration, and immunological evaluation showed very mild T cell (IFN γ ELISPOT) and anti-Cas9 antibody responses. The transgene expression was well-maintained even after 2 years of administration in heart, GC, and TA muscles. Moreover, our NHP study with AAVrh74-CK8- GNDM¹ further strengthened our findings in mice. Though we observed moderate level of T cell responses (IFN γ ELISPOT), and anti-Cas9 antibody responses in NHPs, the transgene expression sustained for more than 6 months, and no immune cell infiltration observed into targeted muscle tissues. **Conclusions:** Our proprietary CRISPR-GNDM¹ therapeutic platform efficiently induced transgene expression within a week after systemic delivery in heart and skeletal muscles. Transgene expression was well-maintained up to 2 years in mice and 6 months in NHPs. More importantly, CRISPR-GNDM¹-administered mice mounted very low Cas9-specific T cell responses, and these responses are gradually decreased. On the other hand, anti-Cas9 antibody responses are evident in some of the animals in moderate to low level and some of the animals didn't have any detectable antibody responses. Though our therapeutic model induced moderate Cas9-mediated T cell and antibody responses in NHPs, we didn't observe any infiltration of immune cells into targeted muscle tissue. Overall, sustained expression of transgene and moderate to low level of Cas9-mediated immune responses and modulation of in vivo gene expression without DNA strand breaks will give great advantage to our CRISPR-GNDM¹ therapeutic approach to efficiently treat several genetic diseases that are nearly impossible to treat.

1127. Differential Neutralization of AAV Vectors by F(ab')₂ Fragments

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Vectors based on adeno-associated viruses (AAV) have emerged as a promising approach for treatment of monogenic disorders. However, vector administration can be negatively impacted by presence of preexisting neutralizing antibodies (NAbs). These antibodies arise from natural infection and have been demonstrated to block vector transduction. Presence of even low level NAbs have blocked AAV vector transduction in patients and therefore, patients with preexisting NAbs are often excluded from receiving AAV gene therapy. The F(ab')₂ fragments generated by cleavage of intact IgGs have been used to map potential antigenic epitopes on AAV vectors. While F(ab')₂ fragments bind AAV vectors, their role in neutralization is unclear. Here we evaluated in vivo and in vitro neutralization of AAV vector by F(ab')₂ fragments. Pooled human intravenous immunoglobulin (IVIg) was enzymatically cleaved to provide a source of F(ab')₂ fragments. Purified fragments were ~95-96 % pure and devoid of intact IgGs. F(ab')₂ fragments were first interrogated in a modified ELISA assay to bind vector. F(ab')₂ fragments bound AAV vectors as efficiently

as intact IgG. We next evaluated *in vivo* neutralization by adoptively transferring F(ab')₂ fragments to immunocompromised Rag knockout mice. Groups of mice (N=5/group) were transferred with increasing doses of F(ab')₂ before administering an AAV vector expressing alfafetoprotein (AFP). Vectors were administered shortly after F(ab')₂ infusion to avoid confounding effects of F(ab')₂ clearance. As expected, serum from control animals demonstrated high AFP expression post vector administration. Interestingly, all animals transferred with F(ab')₂ fragments also demonstrated high transgene expression. AFP expression in F(ab')₂ treated animals was similar to control untreated animals. Animals that received lower doses of F(ab')₂ even had transient elevation in AFP transgene levels over their untreated counterparts. Liver vector genome copies confirmed similar gene transfer in both control and F(ab')₂ administered animals. Our studies here demonstrate for the first time a disconnect between *in vitro* and *in vivo* neutralization by F(ab')₂ fragments. These findings observed with cleaved F(ab')₂ fragments, nevertheless, highlight differences in vector neutralization in the *in vitro* transduction inhibition assay that differs from *in vivo* neutralization.

1128. Assessment of Live-Cell Imaging and Flow Cytometry Methods for SARS-CoV-2 Pseudovirus Neutralization Assays Compared to a Surrogate Bead-Based Neutralization Assay

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, the cause of coronavirus disease 2019 (COVID-19), emerged in 2019 and has resulted in a global pandemic. Global efforts to dampen the effects of SARS-CoV-2 infection include mass vaccination, contact tracing, and therapeutic development. Developing broadly protective vaccines and determining titers of neutralizing antibodies (nAbs) against new variants are vital for combating the pandemic. Several methods to quantify nAb titer in patient serum have been established including live virus, pseudovirus, and ELISA-based neutralization assays. While the use of live pathogenic SARS-CoV-2 requires BSL-3 containment, surrogate neutralization assays offer better safety and improve ease-of-use when implemented as an alternative. In this study, we developed pseudovirus neutralization assays that measure neutralization by both live cell imaging and flow cytometry. Briefly, nine serial dilutions of serum samples are incubated with VSV-ΔG pseudotype particles expressing the original SARS-CoV-2 spike protein with a GFP reporter for one hour at 37°C. The mixture then incubates with HEK293-hACE2-TMPPRS2 -mCherry target cells for 16 hours. Live cell imaging is performed using GFP fluorescence to monitor infection, enabling quantification of infection and neutralization dynamics. After imaging, cells are further processed and analyzed via flow cytometry, enabling rapid and high-throughput assessment of neutralization. To evaluate these methods, patient serum samples were processed through the cell-based neutralization assay and compared to a surrogate bead-based assay also developed by NIST. Comparing

the neutralization (NT50) determined for all three assays, advantages and disadvantages of each assay will be discussed. Based on the data generated, the pseudovirus neutralization assays showed reliable performance for detecting varying degrees of neutralization against SARS-CoV-2 in patient serum samples.

1129. ImmTOR Combined with B Cell-Targeted Therapies Provides Synergistic Activity in Mitigating Anti-AAV Capsid Antibody Responses and Enables Repeated Vector Dosing

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ImmTOR tolerogenic nanoparticles encapsulating rapamycin have been demonstrated to mitigate immunogenicity of AAV vector, elevate transgene expression and enable vector redosing in several animal models including mouse model of methylmalonic acidemia, an inborn metabolic disease. While ImmTOR has been shown to directly inhibit germinal center plasmablasts, the primary mechanism of action is thought to be the induction of tolerogenic antigen-presenting cells that induce antigen-specific regulatory T cells. However, in the mouse model ImmTOR only partially inhibits the initial T cell-independent B cell IgM antibody response and blocks subsequent class-switching to IgG. The residual anti-capsid IgM response can have neutralizing activity and affect the efficiency of vector re-administration. Here we evaluated the combination of ImmTOR with currently available B cell targeting agents to mitigate the IgM response and increase the efficiency of re-dosing. ImmTOR combined with a monoclonal antibody (mAb) directed against B cell activation factor (BAFF), a B cell survival factor, synergistically reduced anti-AAV IgM antibodies, provided more durable suppression of anti-AAV IgG antibodies, and enabled multiple re-administrations of an AAV8 vector. Similar, but a weaker effect was observed when ImmTOR was combined with ibrutinib, a Bruton's tyrosine kinase inhibitor. Most advantageous regimens of ImmTOR (monthly) and aBAFF (bi-weekly) led to complete absence of IgG response and minimal IgM response to AAV. This was seen even after four successive AAV administrations over several months at doses up to 5E12 vg/kg and after two AAV administrations at a high 5E13 vg/kg dose, which is similar to therapeutic doses of AAV used in multiple clinical trials. While ImmTOR alone had little or no effect on total splenic B cells or immature pre-B cells, anti-BAFF mAb reduced total B cells by ~50% and increased pre-B cells by ~2-3 fold. The combination of ImmTOR and anti-BAFF mAb showed a synergistic effect in increasing splenic pre-B cells and reducing B cell plasmablasts. These results suggest that ImmTOR could be combined with belimumab, an anti-BAFF mAb to further mitigate anti-AAV antibody responses and enable repeated AAV administration at sufficiently high, but not excessively elevated AAV doses. This approach may lead to stable expression of therapeutic transgene using AAV doses that have been shown to be well-tolerated in humans and thus provide an immense clinical benefit.

1130. Combination of ImmTOR Tolerogenic Nanoparticles and IL-2 Mutein Synergistically Inhibits the Formation of Anti-AAV Antibodies

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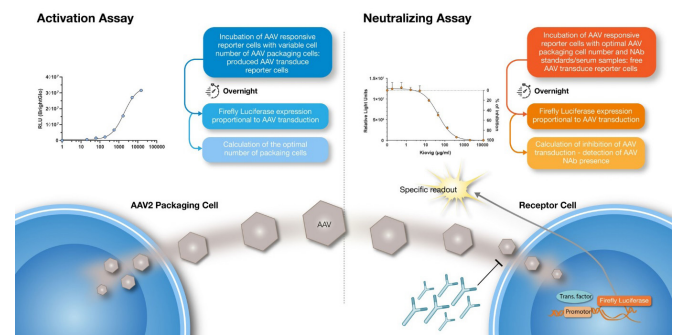
We have previously demonstrated that biodegradable ImmTOR nanoparticles encapsulating rapamycin, an inhibitor of the mTOR pathway, has the ability to mitigate immunogenicity of AAV vectors and enable re-dosing. However, delayed immune responses can result in breakthrough of anti-AAV antibodies in some animals, particularly at higher vector doses. We have investigated the combination of ImmTOR with a regulatory T cell (Treg)-selective interleukin-2 (IL-2) mutant molecule (IL-2 mutein). Treg-selective IL-2 muteins have been shown to expand all pre-existing Tregs, unlike ImmTOR which induces antigen-specific Treg. We hypothesized that ImmTOR could act synergistically with an IL-2 mutein. Here we demonstrate that a single dose of ImmTOR administered the same day as an IL-2 mutein results in increased total Tregs. However, expansion of antigen-specific Treg would be more desirable than expansion of total Treg. We next evaluated the ability of ImmTOR plus antigen combined with IL-2 mutein to induce and/or expand antigen-specific Treg. Ovalbumin-specific OTII T cells were adoptively transferred into mice prior to treatment with ovalbumin and ImmTOR and/or IL-2 mutein. As expected, ImmTOR + ovalbumin did not expand total Treg, but increased the percentage of Foxp3⁺ OTII cells from ~3% to 15%. IL-2 mutein + ovalbumin resulted in more modest increase in ovalbumin-specific that was similar to that observed in mice treated with ovalbumin alone (~6%). However, the combination of ImmTOR + IL-2 mutein + ovalbumin showed a profound synergistic effect, with ~45% of ovalbumin-specific OTII cells expressing Foxp3, the classic marker of Treg. We next tested whether the combination of ImmTOR and IL-2 mutein would enable more durable inhibition of antibody responses to co-administered AAV gene therapy vectors. Mice were treated with two doses of AAV8 vector, on Days 0 and 56, with or without ImmTOR and/or IL-2 mutein administered on Days 0 and 56. Treatment with IL-2 mutein showed a modest reduction in anti-AAV IgG antibodies. Mice treated ImmTOR showed dose-dependent inhibition of anti-AAV antibodies, with a therapeutic dose of ImmTOR (200 µg) inhibiting the formation of antibodies through Day 75, 19 days after the second dose of AAV. However by Day 91, some mice showed delayed development of anti-AAV antibodies. In contrast, the combination of ImmTOR + IL-2 mutein completely inhibited antibody formation through Day 117, even at sub-optimal doses of ImmTOR. These results show that the combination of ImmTOR and IL-2 mutein has the potential to provide more durable antigen-specific immune tolerance to mitigate immunogenicity of AAV gene therapy vectors and enable dose-sparing of ImmTOR.

1131. Development of an iLite® Reporter Cell Platform for the Quantification of Anti-AAV Neutralizing Antibodies

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The extensive study of AAV vectors as carriers of gene therapy products has advanced the field from a promising new therapeutic concept into today's medical reality. However, several bottlenecks are still present during the development of gene therapy products hence meeting respective regulatory requirements has turned into a challenging process. The use of bioassays facilitates the progression through the different developmental phases as they can be used to address key parameters for regulatory acceptance including vector potency or determination of neutralizing antibodies (NAb). Here we present a novel two-component system for the detection and quantification of NAb directed against recombinant AAV vectors, the *iLite*® AAV Platform. This platform can be used for detecting and optimally quantitating anti-AAV NAb directed against the capsid in a test sample. The availability of reporter cells in a frozen, thaw & use format, obviates the need for cell culture or specialized equipment and provides a means for obtaining highly reproducible results superior to those obtained using the same cells maintained in culture. Furthermore, the time necessary to run the assay is half of the time required in the current alternatives. These features combined allow the advantages of a cell-based assay to quantify neutralizing anti-AAV antibodies, as recommended by regulatory agencies, to be combined with the versatility and ease of an immune-detection assay for use in AAV-based gene therapy trials.



1132. Optimizing the IdeS Treatment Regimen for Enhanced Adeno-Associated Virus Transduction in the Presence of Neutralizing Antibodies

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Pre-existing neutralizing antibodies (NAbs) to adeno-associated viruses (AAVs) remain an impediment for systemic administration of AAV-mediated gene therapy in many patients, and various strategies are under investigation to overcome this limitation. Here, three different IgG-degrading enzymes (Ides) derived from bacteria of the genus *Streptococcus* were tested for their ability to cleave IgG *in vitro*. The selected candidate, IdeS, was found to be highly efficient at cleaving human IgG, less efficient against NHP IgG and inefficient against mouse IgG. Passively immunized mice with human serum or non-human primates (NHP) with naturally occurring anti-AAV NAbs were used to define the optimal IdeS dose and administration window for AAVanc80 and AAV8 vectors in mice and AAV3B in NHPs. Interestingly, we observed differences in how IdeS affected liver transduction in the presence of NAbs depending on the AAV serotype. For AAVanc80 and AAV3B the best transduction levels were achieved when the vector was administered after IgG digestion products were cleared from circulation. However, for AAV8, the presence of IdeS digestion products had a mild inhibitory effect on liver transduction. Therefore, pre-conditioning with IdeS represents a potential treatment opportunity for patients primarily excluded from participation in gene therapy clinical trials due to elevated circulating anti-AAV NAb levels. However, careful determination of the optimal IdeS dose, the timing for administration of each AAV serotype, and NAb levels will be essential for optimal transduction.

1133. Evaluating the Impact of Transgene-Specific CpG Removal on AAV9-Mediated Gene Transfer and Immune Responses in the Balb/C Mouse Strain Provides Novel Insights of CpG Depletion

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Evasion and prevention of inflammatory host immune responses to AAV gene therapy is crucial to ensure successful, long-term transgene expression. A major emergent *cis*-modification strategy to enhance transgene expression is CpG motif depletion in the encoded transgene. Accumulating data from murine studies demonstrate that CpG depletion enacts its beneficial effect by minimizing AAV genome detection by TLR9, thus thwarting innate immune cell activation,

blunting the CTL response and preventing transduced cell loss. In this study, we investigated the impact of transgene-specific CpG depletion in Balb/C mice using two different routes of vector administration. Specifically, adeno-associated virus-9 (AAV-9) encoding an anti-plasma kallikrein antibody sequence (\pm CpG motifs) under control of a universal promoter was administered to mice via intravenous (I.V.) or intramuscular (I.M.) route. We monitored serum transgene concentration over time and found that the I.M. route of administration yielded significantly higher expression levels compared to that of I.V., irrespective of CpG depletion. In accordance to prior studies, CpG depletion dramatically improved transgene expression in mice from both routes of administration. Bio-distribution studies show that peripheral tissues harbored greater AAV genome copies from I.V. administration, while muscle tissue contained greater AAV genome copies from I.M. administration. We then investigated the ensuing adaptive immune response following gene transfer in these mice. Profiling the splenic T cell response mainly showed effects of CpG depletion after I.V. administration, manifested as reduced CD8+ T cell activation and lack of Treg loss. Surprisingly, and in contrast to prior studies, CpG depletion did not affect the anti-transgene B cell response. Mice of both routes of administration with CpG depletion still mounted IgG2a responses comparable to their counterparts. Induced IgG2a responses were evident as early as 7 days post-vector administration, with no induction of anti-transgene IgM antibodies. We are conducting additional studies to better understand this phenomenon. In conclusion, our direct comparison of I.M. versus I.V. administration of transgene-specific, CpG-depleted AAV9 vectors show that transgene expression is highest following I.M. administration of CpG-depleted vector in Balb/C mice compared to other experimental groups. Immunological studies revealed that CpG depletion does not impact B cell antibody production to transgene, despite reports of anti-transgene antibody reduction in C57BL/6 mice. These findings highlight the need for further study into how differences, such as mouse strain and vector routes of administration, may affect the effectiveness of CpG depletion strategies to ameliorate gene therapy.

1134. Dorsal Root Ganglia Single-Nucleus Transcriptomics Reveal Cellular and Molecular Responses to High Dose AAV-Induced Toxicity

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The administration of AAV vectors to NHPs via CSF, especially at high doses, results in adverse-histopathological findings in dorsal root ganglia (DRGs). Though these findings are mostly sub-clinical, histopathologic analysis show infiltration of immune cells into the DRGs, proliferation of satellite glia, neuronal degeneration and secondary axonopathy of central and peripheral axons of DRG neurons. The mechanism driving this inflammation and/or toxicity could be manifold, including but not limited to AAV dose, transgene (over)expression, immune response against the AAV capsid or the transgene protein. We set out to study and characterize the molecular and cellular drivers of AAV-induced DRG toxicity by measuring alterations in

cell content and transcriptome profiles of cells in NHP DRGs. To this end, we compared DRGs from juvenile cynomolgus monkeys dosed with AAV.SAN002-eGFP via a ported intrathecal catheter to a cohort of age-matched monkeys that received formulation buffer. We report the development of a reliable method to isolate nuclei from frozen NHP DRGs, and the classification of neurons, satellite glia, myelinating Schwann cells, vascular endothelial cells, and immune cells in sequenced DRGs. Amongst the immune cells identified we report increased B-cells, naïve and memory T-cells and macrophages, likely supporting important cell homing within the DRG. We also observe increased platelet and NK cell numbers. Differential gene expression within each cluster will help elucidate pathways involved and impact on specific cell population shifts. Gaining insight into the cellular and molecular underpinning of DRG toxicity will be crucial for future AAV-based gene therapies in human patients and will help guide remediation strategies for sensory neuropathies in clinical studies.

1135. Persistent Control of SHIV Infection in Rhesus Macaques by Expressing a Highly Potent HIV Decoy Receptor After *In Vivo* HSC Transduction

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Despite the success achieved by combined anti-retroviral HIV-1 therapies, long-term and repeated drug administration is associated with toxicity, virus evasion, and high cost. We aim to develop a gene therapy approach for persistent control of HIV-1 infection by *in vivo* HSC gene therapy with a secreted decoy protein for HIV receptors CD4 and CCR5 (eCD4-Ig) developed by Mike Farzan's group. In this approach, HSCs are mobilized from the bone marrow into the peripheral blood stream and transduced with intravenously injected virus vectors. We use an integrating, helper-dependent adenovirus (HDAd5/35++) vector system that targets human CD46, a receptor that is abundantly expressed on primitive HSCs. Transgene integration is achieved by a hyperactive *Sleeping Beauty* transposase and transgene marking in peripheral blood cells can be increased by *in vivo* selection. In CD46-transgenic mice, over 100 µg/mL eCD4-Ig in serum was measured after *in vivo* transduction with HDAd-eCD4-Ig and selection, with no obvious adverse events observed. *In vitro* neutralization assays with serum samples showed that the produced eCD4-Ig effectively inhibited the infection with various HIV-1 and SIV strains (HIV-BaL, HIV-NL4-3, SIV_{mac239}, SHIV-D). The IC₅₀ (i.e. the serum eCD4-Ig concentration required for 50% inhibition of S/HIV infection *in vitro*) was ~1.0 µg/ml eCD4-Ig. This implies that serum eCD4-Ig concentrations are ~100x higher than the IC₅₀. We then started a study with HDAd-eCD4-Ig in a rhesus macaque. Both transgenes, eCD4-Ig and mgmt^{P140K} were rhesus. The animal was mobilized with G-CSF/AMD3100. At the peak of mobilization (which is ~8 hours after the AMD3100 injection), HDAd-eCD4-Ig was infused intravenously. Three rounds of O⁶BG/BCNU selections were applied. After *in vivo* HSC transduction/selection, eCD4-Ig serum levels were stable at 15-

30 µg/ml for 45 weeks (ongoing). Serum eCD4-Ig was active in an *in vitro* SIV neutralization assay. High-level eCD4-Ig expression had no clinical or hematological side effects. Intravenous challenge with increasing Simian/Human Immunodeficiency Virus (SHIV-D) doses was started at week 16 after *in vivo* HSC transduction. Viral load in serum was measured by quantitative RT-PCR with a detection limit of 30 viral RNA copies/ml. Six rounds of challenges with doses up to 1600pg (p27) did not establish infection or affect T-lymphocyte subset counts. All hematological parameters were normal. At week 40, the animal was challenged with high-dose SHIV-D (890 nanogram-p27). This led to productive infection with titers greater than 1x10⁶ copies per ml serum two weeks after challenge and a drop in CD3⁺/CD4⁺ cell counts. However, afterwards, serum titers declined and CD4 counts normalized indicating that the infection was suppressed likely by eCD4-Ig. Clearly, we will measure vector and eCD4-Ig mRNA biodistribution at study endpoint. Our challenge study mimicked that of the Farzan group (PMID: 25707797). In that study, a challenge dose of 400pg SHIV-AD8 caused clinical symptoms in 3 out of 4 control animals that required euthanasia. Additional animals are being studied, including an "in-house" SHIV-D challenge study with an animal that received *in vivo* HSC transduction with a control HDAd vector. Our encouraging preliminary data demonstrate that the control of SHIV/HIV can be accomplished with one injection of HDAd-eCD4-Ig vectors and thus a treatment could also be available to people living with HIV in resource-limited settings.

1136. Further Characterizing Immune Responses to AAV Gene Therapy and Evaluation of Inflammatory Cytokines in the Cerebrospinal Fluid of Tay Sachs Patients

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Tay-Sachs disease (TSD) is a rare, autosomal recessive neurodegenerative disease, characterized by the progressive dysfunction of the central nervous system. TSD is caused by the deficiency of an enzyme, called hexosaminidase A (HexA), resulting in excessive accumulation of GM2 gangliosides in the brain and neurons. The infantile TSD is the most common form of the disease, which can set in around six months of age, progressing to loss of motor skills and seizures, and premature death by the age of five. Previously, a first in-human AAV gene therapy for TSD was reported via intrathecal (1x10¹⁴vg) or the combination of bilateral thalamic (1.5x10¹²vg per thalamus) and intrathecal injection (3.9x10¹³vg) of equimolar mix of AAVrh8 HexA and AAVrh8 HexB. TSD patients were immunosuppressed prior to gene delivery (rituximab, sirolimus, methylprednisolone) and post-delivery up to 6 months (rituximab, ~6 months, sirolimus 190 days + 1month taper, prednisone 90 days+1 month taper) showed a moderate increase in the HexA levels in the CSF, with mild anti-capsid IFN-γ immune response and no IFN-γ detectable immune responses against transgenes. To further characterize the cellular immune responses and evaluate the involvement of pro and anti-inflammatory cytokines, we utilized a multiplexed assay platform for the simultaneous analysis of

multiple inflammatory regulators and chemokines. The cytokine bead assay (CBA) was conducted on CSF collected from these patients at pre-dosing and three- and six-month timepoints post AAV infusion. Elevated levels of the IFN- γ inducible chemokines, CXCL10 and CXCL8, were observed in both patients after AAV infusion. CXCL10 and CXCL8, chemokines that mediate T cell-migration and neutrophil transmigration respectively, are both involved in neuroinflammation. The observed increase in these chemokines in the CSF of TSD patients further reinforces their role in inflammatory CNS disorders. Induction of other inflammatory cytokines, like IL-6, IL-1 β , IL-2, IL-17A, etc. post AAV infusion was not observed. Interestingly high levels of MCP-1 were reduced compared to baseline, more obviously in the patient who received both bilateral thalamic and intrathecal delivery. To further delineate gene therapy specific immune responses, as well as local versus systemic responses, similar assays will be performed on serum and peripheral blood mononuclear cells stimulated with capsid or transgene epitopes. These findings will be informative in elucidating immune responses to AAV gene therapy, thereby proving beneficial for evaluating immunomodulatory therapies.

1137. Immunosuppression to Control Neutralizing Antibodies and Transgene Immunity to AAV Liver Gene Transfer

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Systemic AAV delivery for hepatic gene transfer is fast approaching FDA approval. Although AAV is relatively benign, immunogenicity to the vector, its genome, and encoded transgene has resulted in innate and adaptive immunotoxicities as well as loss of the transgene product. These not only limit efficacy, but also raise safety concerns. Importantly, the formation of high-titer neutralizing antibodies (NAb) to the viral capsid following first administration is a major impediment to patient redosing. Here, we tested hepatic gene delivery with AAV serotype 8 in C57BL/6 mice ($n=8-10$), using a dose ($2e9$ vg/animal) that has previously been shown to induce CD8 $^+$ T cells to the model transgene, ovalbumin (OVA). We tested immune suppressive (IS) therapies such as B cell depletion (α CD20) combined with the mTOR inhibitor rapamycin (α CD20+rapa) or an antibody to B cell activating factor, a cytokine that we showed to be crucial for the survival of marginal zone, follicular and germinal center B cells (α CD20+ α BAFF) as indicated (Fig). We observed that IS therapies administered **concurrent** with AAV8 gene therapy caused a high frequency of animals (40 - 75%) to develop transgene specific CD8 $^+$ T cells (H-2 Kb SIINFEKL tetramer+) in the liver and periphery, corresponding with loss of OVA expression in the liver and in circulation. Tetramer+ CD8 $^+$ T cells had a short-lived effector memory phenotype in the blood and spleen, characterized as CD44 $^+$ CD62L $^+$ CCR7 $^+$ KLRG1 $^+$ CX3CR1 $^+$. Interestingly, tetramer+ CD8 $^+$ T cells were present at higher numbers in the liver, even in animals that did not develop detectable tetramer+ CD8 $^+$ T cells in the periphery. CD4 $^+$ T cells and CD4 $^+$ CD25 $^+$ FoxP3 $^+$ regulatory T cell numbers were unaffected. Tetramer+ CD8 $^+$ T cells in the liver expressed multiple exhaustion markers (PD1, 2B4), were negative for KLRG1 and CX3CR1 expression, and their presence did not completely correlate with loss

of OVA expression. Both control and IS treated animals developed functional and binding NAb (IgM and IgG2c, equivalent to IgG1 in human) to the AAV8 vector, which precluded readministration with the same serotype ($1e11$ vg/animal AAV8-hFIX). In contrast, when IS therapies were initiated 3 weeks **prior** to AAV8 gene therapy, only control animals developed transgene immunity and NAb to AAV8-OVA. We were able to readminister AAV8 encoding human factor IX (hFIX) to 0% of control, 70% of α CD20, 75% of α CD20+rapa, and 80% of α CD20+ α BAFF treated animals as determined by circulating levels of hFIX ($n=6-10$). When we tested IS pre-treatment with a more clinically relevant vector dose ($1e11$ vg/animal AAV8-OVA), we were able to readminister AAV8-hFIX to 0% of control, 44% of α CD20, 88% of α CD20+rapa, and 67% of α CD20+ α BAFF treated animals. We observed that mice that received α CD20 and α CD20+ α BAFF had high levels of BAFF in circulation, which correlated with high α AAV8 IgM NAb titers, that in turn precluded redosing with AAV8-hFIX. In confirmation, extending α BAFF injections for an additional 4 weeks in the α CD20+ α BAFF group prevented BAFF and IgM production, allowing for successful redosing in **100%** of animals. We therefore conclude that choice, timing and duration of IS, vector dose and transgene immunogenicity are all important considerations in developing effective immunomodulation to AAV liver gene therapy.

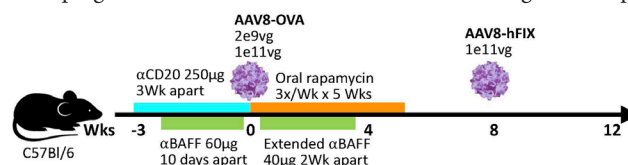


Fig. Schedule of pre-treatment with i) α CD20 ii) α CD20+rapa iii) α CD20+ α BAFF iv) α CD20+ extended α BAFF IS therapies in mice primarily administered with $2e9$ or $1e11$ vg of AAV8-OVA intravenously, and redosed with $1e11$ vg of AAV8-hFIX.

1138. Single-Dose Optimized AAV-Based COVID-19 Vaccine Elicits Durable and Protective Immunity Against Variants of Concern in Nonhuman Primates

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The SARS-CoV-2 pandemic has affected almost 400 million people worldwide resulting in 5.7 million deaths. To contain the pandemic, there is a continued need for safe vaccines that provide durable protection at low and scalable doses that are easily delivered. Previously, we showed that an adeno-associated virus (AAV)-based vaccine candidate (AC1) elicited high humoral and cellular immunogenicity in mice and nonhuman primates (NHP) following a single injection, which provided near-sterilizing immunity against SARS-CoV-2 in NHP. Here, we report 18-month antibody durability data in NHPs vaccinated with this candidate. Additionally, we developed optimized AAVCOVID vaccine candidates for higher potency and

protection against variants of concern (VOC). We have included a more potent minimal CMV promoter in our vaccine candidates, which provides 100-fold higher Spike expression resulting in 40-fold higher antibody responses in mice compared to the prototypic AC1 candidate. In addition, we have engineered our new ACM (miniCMV promoter) platform to express the Beta (ACM-Beta), Delta (ACM-Delta) and Omicron (ACM-Omicron) VOC Spike antigens. We have demonstrated that the four candidates are able to induce equally potent antibody responses against the self-transgene in mice. As expected, cross-reactivity was different for each candidate, in line with published reports. NHPs have also been vaccinated with a single dose of 10^{11} gc total dose of ACM-Beta and ACM-Delta showing a 10-fold greater potency compared to AC1. ACM-Beta triggered protective immunity against Beta SARS-CoV-2 challenge 7 weeks after a single dose administration. Durability of antibody responses is being evaluated in the NHPs vaccinated with ACM-Delta. Finally, immunogenicity of ACM-Omicron is being evaluated in NHPs. This work shows the adaptability and versatility of AAVCOVID vaccine platform to improve potency and protect against SARS-CoV-2 VOC. We also show unique antibody durability in NHP models, with up to 1.5 years peak antibody levels. These observations together with the single, low dose requirement, high yield manufacturability, and 1-month stability for storage at room-temperature may make this technology well-suited to support effective immunization campaigns for emerging pathogens on a global scale.

1139. Select Rapamycin Analogs Promote VSV-G-Mediated Cell Entry by Activating TFEB-Dependent Microautophagy

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Restriction of cellular entry is a major obstacle to gene modification by viral vectors, including lentiviral vectors (LV) that rely upon endocytic membrane fusion mediated by VSV glycoprotein (VSV-G). We previously reported that rapamycin enhances LV delivery to hematopoietic stem cell progenitors and other human cell types by triggering the degradation of interferon-induced transmembrane (IFITM) proteins. IFITM proteins inhibit virus-cell fusion by altering the mechanical properties of membranes. Here, by identifying rapamycin analogs that do or do not enhance LV-mediated gene delivery, we report the molecular mechanism behind this phenomenon. Rapamycin and its analogs everolimus and temsirolimus, but not ridaforolimus, activate the nuclear translocation of transcription factor EB (TFEB) and trigger the induction of an endosomal remodeling pathway known as microautophagy. During this process, antiviral membrane proteins including the IFITM proteins are internalized into intraluminal vesicles of endolysosomes and subsequently degraded. Therefore, by clearing the path for LV fusion in endosomes, rapamycin and some its analogs promote infection mediated by VSV-G and other viral fusion proteins. These findings suggest that the immunosuppressive properties of these drugs may not be limited to effects on adaptive immunity. Our findings suggest that they act at the level of cell-intrinsic immunity to undermine the cell's first line of antiviral defenses. The development of rapamycin analogs that lack this activity may reduce unintended immunosuppression in human subjects.

1140. Defining a Timeline for the Induction of Tolerance Following Liver-Directed AAV Gene Therapy

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Liver-directed AAV gene therapy induces a robust, antigen (Ag)-specific immune tolerance against the AAV transgene products. Tolerance is currently attributed to the continuous production of Ag-specific $CD4^+CD25^+CD25^+Foxp3^+$ Tregs (Tregs). In previous work, we established that liver-directed AAV gene therapy also results in the up-regulation of CD8 regulatory cells (CD8reg), though the role these cells play remains elusive. Further, the timeframe in which tolerance is induced is poorly defined. To date, most AAV studies focus on protein replacement therapies. In protein replacement studies, the long-term effectiveness of treatment relies on high continuous transgene expression levels and long-term Ag-specific Treg production for the life of the patient. Liver-directed AAV gene therapy is not restricted to protein replacement studies. In autoimmunity, a break in tolerance often results in disease onset. Thus, the re-establishment of Ag-specific tolerance would be a powerful treatment option. Previously, we have shown that liver-directed AAV gene therapy can induce tolerance to a neuro-protein resulting in prevention and reversal of Experimental Autoimmune Encephalomyelitis (EAE, an autoimmune disease) in mice. In contrast to protein replacement studies, our model relies solely on tolerance induction for therapeutic effects. Understanding the mechanisms driving tolerance induction and the timeframe in which tolerance induction occurs, will support the development of effective treatments for autoimmune diseases. In the present study, we investigated the role of $Foxp3^+$ Tregs in the induction and maintenance of tolerance to a neuro-protein. From previous protein replacement studies, we hypothesized that ablation of $Foxp3^+$ Tregs, at the time of AAV administration, would prevent tolerance induction. Further, we hypothesized that ablation of $Foxp3^+$ Tregs, after AAV administration, would result in the re-establishment of EAE. When $Foxp3^+$ Tregs were ablated simultaneously with vector administration, we found disease severity to be the same in AAV treated and control animals (Fig 1A). Surprisingly, in mice where $Foxp3^+$ Tregs were ablated 4 days after AAV administration, EAE severity was not significantly different from mice that received AAV only (Fig 1B). Instead, both the AAV-only treated mice and the AAV+DT treated mice, showed decreased disease severity as compared to controls (Fig 1B). Flow cytometry was utilized to verify $Foxp3^+$ Treg ablation (Fig 1C). Based on previous results, we investigated the effects of $Foxp3^+$ Treg ablation on CD8reg populations. Interestingly, $Foxp3^+$ Treg ablation resulted in significant increases in multiple, but not all, CD8reg populations within 1 day of ablation (Fig 1D). This data shows that tolerance induction following liver-directed AAV gene therapy occurs within 4 days of AAV administration. This data also suggests that interplay may exist between CD8regs and $Foxp3^+$ Tregs in the induction and maintenance of tolerance following liver-directed AAV gene therapy, though the role of CD8regs remains unclear.

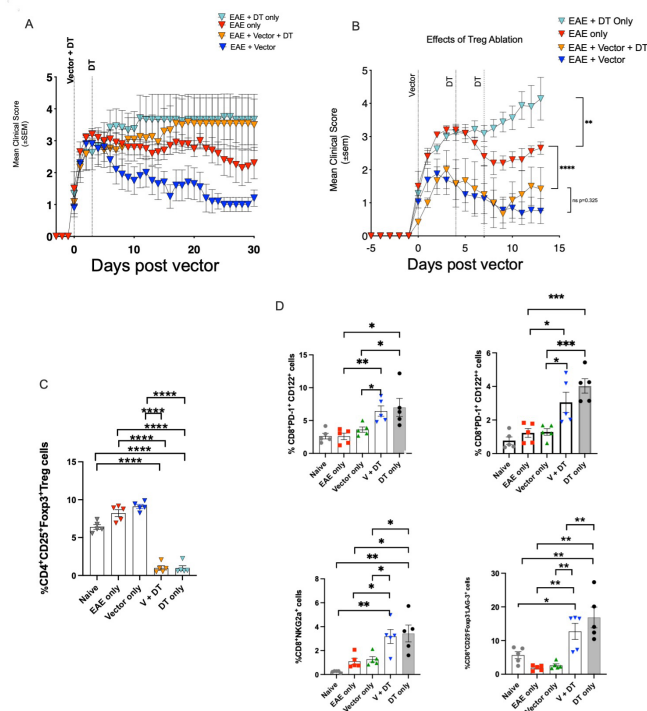


Figure 1: Effects of Foxp3⁺ Treg ablation on tolerance induction (A) and maintenance (B). Treg ablation was confirmed via flow cytometry (C). CD8reg populations were significantly increased in the absence of Foxp3⁺ Tregs (D).

a starting material because the plasmid (code) itself is not considered the active substance. So how is the regulatory guidance evolving for manufacturing of plasmids for cell and gene therapy? The traditional approach is the use of a RUO or GMP-like plasmid. GMP-like would refer to the use of the minimum principles of GMP by the EMA Guidance. This GMP-like material would be used for early phase clinical trials. As a therapeutic transitions to later phase clinical trials and commercial launch, full GMP compliant plasmids would be used. The risk in this approach is timeline delays due to transition of raw material needed for GMP manufacturing; potential for inconsistent batches; and risk to clinical efficacy due to changing the manufacturing process of the starting material. An evolving strategy is to start with GMP plasmid early in clinical development to mitigate risk of changes in the plasmid manufacturing process. This allows for consistency in starting material throughout clinical development. What is not discussed in the EMA 24FEB2021 Question and Answers document is the emerging technology of plasmid as a direct *in vivo* injection (undergoing no mRNA translation to effect the desired cellular change). In this scenario, the plasmid is the active drug substance. This novel therapeutic has the potential to fundamentally shift how plasmid manufacturers are viewed - presently regarded as producers of starting material (and not subject to regulatory inspection) to now being the supplier of the drug substance. Meaning that facilities which are engaged in producing direct inject *in vivo* DS/DP will most likely be subject to regulatory inspection and approval. This is an exciting example where we expect the regulations to adapt to the emergence of a new technology. In this presentation, I will discuss how to the increasing client demand for cGMP-quality plasmids in early-phase cell & gene therapy manufacturing.

1141. Critical Quality Attributes for Supply of Clinical Plasmids for Cell and Gene Therapy Development

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Plasmid DNA is the genetic basis for cell & gene therapy medicines and a sourcing challenge for many innovators. From long-lead times for plasmid manufacturing slots to an evolving regulatory environment, innovators are seeking the highest quality materials that will allow them to meet their timelines. Application of cGMPs to plasmid manufacturing have varied. Fortunately, there have been some recent regulatory publications on this matter - One of the most recent is the EMA 24FEB2021 “Question and Answers document on the principles of GMP for the manufacture of starting materials of biological origin used in to transfer genetic material for the manufacturing of Advanced Therapeutic Medical Products (or ATMPs)”. At the most basic level - regardless of how a plasmid is used (as raw material or as an active substance) - plasmid manufacturing requires the application of cGMPs. Some key differences, however, are that in cell and gene therapies as well as mRNA vaccines the plasmid material is regarded as

1142. Development of a Stable Cell Line and Downstream Process for Improved Titer of GP64-Pseudotyped Lentivirus Particles

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Lentiviral vectors (LVs), derived from human immunodeficiency virus (HIV), have been used to introduce gene(s) of interest in the eukaryotic genome. They offer the advantages of stable transgene expression and ability to infect both dividing and non-dividing cells. Pseudotyping lentiviruses with variable glycoproteins can not only improve their infectivity, but also provide selective tropism. GP64, a Baculovirus envelope protein, is capable of transducing airway epithelia to deliver reporter or cystic fibrosis transmembrane conductance regulator (CFTR) genes in both mouse and pig models respectively. However, unlike the standard vesicular stomatitis virus G (VSV-G) protein, it is difficult to produce high titer GP64 pseudotyped viral vectors (GP64-LV). In this study, we used two approaches to overcome this barrier. First, we have developed a stable GP64-expressing HEK293T cell

line that produces 3-fold more product than parent cells upon GP64 plasmid co-transfection. Second, we performed a transmembrane pressure (TMP) excursion study to determine the optimum parameters for concentrating GP64-LV by tangential flow filtration (TFF) with recoveries ranging between 50-80%. The concentrated vector was further purified by either sucrose step gradient or sucrose cushion and purity, titer, infectivity, and functionality were compared. Overall, we report the development of a stable cell line and an optimized two-step downstream process to produce high titer infectious GP64-LV particles for use in gene therapy applications.

1143. AAV Superproduction: Achieving 10^{16} vg/L Productivity in the High Density Cell Respirator

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Eroom's Law is a playful inversion of the famed Moore's Law and derides the ever-diminishing productivity of the pharmaceutical industry, where the number of drug approvals per dollar spent has halved every 9 years. While silicon technology advances production nodes every 2-3 years, bioproduction remains stuck largely in the stirred-tank reactor node developed 80 years ago. Within gene therapy, production capacity for AAV falls staggeringly short and will need to increase 100-fold to accommodate therapies already in the development pipeline. To this end, we have conceived and operationalized a new bioproduction node that provides intensified, accessible, and cost-effective generation of potent, high-quality AAV vectors. In a process we call "superproduction", we have achieved 10^{15-16} AAV vg/L, a 100-fold improvement over industry standard methods or a leap of 6 "Moore" production nodes. The 1,000 litre STR bioreactor, typically required to make a patient dose of AAV, now fits in inside a standard incubator. This puts pharma-scale production in the hands of patient advocacy groups, academics, rare disease researchers, and gene therapy start-ups who are currently facing 2-3 year waitlists and \$3-5M charges for sufficient vector for Phase I trials. The hope is that access to AAV superproduction will eliminate vector bottlenecks, enabling faster and broader pre-clinical and clinical testing of lead vectors, and accelerate the discovery of blockbuster medicines. Technically, superproduction of AAV is achieved by expanding a bespoke AAV producer cell to very high density (10^8 cells/mL) using an intensified bioreactor and subsequently inducing AAV packaging. As proof-of-concept we engineered an ssAAV2-eGFP producer cell and screened for a highly productive clone that was able to package $>10^5$ vg/cell by qPCR and ELISA. In duplicate runs, this clone was expanded ~100-fold in a high density cell respirator (HDCR) bioreactor to $\sim 10^8$ cells/mL, induced to package AAV, and harvested for subsequent purification. Volumetric productivity of 10^{16} vg/L in crude lysate was achieved by qPCR and ELISA. Purified vector was imaged by negative stain electron microscopy to validate full:empty capsid ratios ($>95\%$) and analyzed by silver stained PAGE for purity. Vector functionality was validated *in vivo* by tail vein injection (10^{11} vg/mouse; $4e12$ vg/kg) and confirmation of eGFP signal by confocal fluorescence microscopy and immunohistochemistry at 2 weeks (n=3) and 4 weeks (n=3) post-injection versus a PBS-injected control (n=3). AAV genome population sequencing by PacBio is ongoing to further characterize

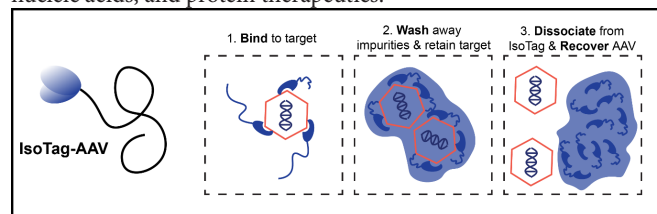
the fidelity of packaged vector. Superproduction offers the potential to significantly reduce AAV production costs and turnaround times. Through partnerships with AAV research labs and start-ups, we are expanding this production service to support additional serotypes and validate across numerous genes-of-interest. Once fully validated this process will be operationalized in a "vector foundry" to support gene therapy programs across the USA.

1144. Non-Chromatographic Purification of Adeno-Associated Viruses Using Phase Separation Biopolymers

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Traditional separation processes - filtration accompanied by affinity capture - are the industry standard for primary contamination clearance. However, affinity purification becomes less efficient and thereby more costly when applied to gene therapy viral vectors due to pore size limitations of traditional resin scaffolds. 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, adeno-associated virus (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 off-the-shelf, 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 greater than 98% capture across a wide range of serotypes (2, 5, 8, 9 & rh10), platform across a 3-log range in titer ($5E7-1E10$ vg/ul) and can be completed in under 2 hours regardless of starting culture volume. Predictable removal of contaminants enables simple process design *a priori*, achieving product specifications by simply adjusting the number of wash diavolumes. Productivity of IsoTag-AAV™, is 2.5-10X more productive than a traditional chromatography column process based on capsids purified per hour. Our process yields highly pure AAV whose *in vitro* infectivity is comparable or exceeds AAV purified by other methods. As we continue to optimize, validate, and scale the technology, we expect IsoTag-AAV™ to offer a true "plug and play" approach to viral vector processing, addressing the current bottleneck that is slowing development and commercialization of these important therapeutics. Furthermore, the technology is a platform, with other applications underway to deliver much needed solutions for other viral vectors, nucleic acids, and protein therapeutics.



1145. Final Formulation and Filtration of Lentivirus

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In recent years, development in the gene therapy industry has grown rapidly, with lentivirus making up one of the largest classes of viral vectors. A critical step in the manufacture of lentivirus is the final formulation and filtration, which processes the vector into its final concentration and buffer matrix and ensures drug substance sterility. In recombinant protein manufacturing, final formulation and filtration has been platformed to use flat sheet tangential flow filtration (TFF) cassettes and 0.2 μm sterilizing-grade filters. However, lentiviral vectors present unique processing challenges at these steps due to their relative instability and large size (approximately 0.12 μm). In this work we demonstrate the flux benefits of flat sheet TFF devices and share data on how molecular weight cutoff and operation conditions can impact processing time, yield, and impurity removal. At the final filtration step several filters were evaluated varying in chemistry and symmetry. We also share insights on how filter filtration performance can be impacted by process parameters (flux, scale), lentivirus concentration, and impurity concentration (aggregates, HCP, DNA). The findings presented here should provide some guidance for which parameters to prioritize when developing these key process steps.

1146. Production of AAV Vectors Using Synthetic, Enzymatically Produced Linear DNA

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The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA and viral vectors for use in gene therapy and vaccines. 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 the manufacturing process 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. Here, we compared the production of AAV vectors using hpDNA™ versus plasmid DNA, and found that viral titres, Full:Empty ratios and infectivity were comparable between the two production methods. AAV vectors were then administered intrathecally to neonatal mice, where GFP expression observed from hpDNA™-derived AAV vectors was equivalent to plasmid-derived vectors. We have demonstrated that functional AAV vectors can be produced using hpDNA™, which could greatly accelerate gene therapy therapeutic development. Moreover, 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.

1147. Manufacturability and Product Quality of Novel AAV Capsids Optimized for Specific Targeting Using Capsid Library Selection in Non-Human Primates

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Capsida Biotherapeutics, Thousand Oaks, CA

Use of recombinant adeno-associated viruses (rAAV) as a delivery tool for gene therapy has significantly increased in the last 10 years. Despite broad application, challenges to the field include ability to efficiently cross the blood brain barrier, cell specific targeting, and productivity and process recoveries sufficient to meet the clinical material demands. For this study, capsid libraries are created by inserting random peptide sequences in the surface of rAAV9 capsids. Following intravenous administration of these libraries in non-human primates, capsid sequences were selected for both productivity and tissue enrichment by screening for high sequence prevalence both in the target tissue and injected virus pools. Multiple capsids demonstrating enhanced brain enrichment were selected from this platform and evaluated for productivity during single variant production by packaging a therapeutic construct in each selected capsid. All capsids tested had similar vector genome titers and ability to bind commercially available purification resins when compared to AAV9, demonstrating that variants with high prevalence in viral libraries maintain high productivity as a single variant. Production of one of Capsida's lead capsids and therapeutic construct were further optimized in a serum-free, chemically defined transfection-based production platform and demonstrated consistent volumetric productivity up to 200L. High product quality of purified novel variants was demonstrated in both development and scaled-up productions through evaluation of overall product purity, aggregation, infectivity, and other process and product related impurities. This data demonstrates that capsid variants selected from Capsida's library selection platform not only show improved brain enrichment but are also capable of generating high titer and high-quality products in a suspension-based scalable production platform.

1148. Bioprocess Development of TILT-123 Oncolytic Adenovirus: From R&D Scale to cGMP Manufacturing

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Oncolytic viruses (OVs) are a promising therapeutic modality harnessing virus biology and host interactions to treat cancer. Adenoviruses are among the earliest OVs to enter clinical trials. Ad5/3-

E2F-d24-hTNFa-IRES-hIL2 is an oncolytic adenovirus constructed to support T-cell therapies and immune checkpoint inhibitors in solid tumors. The adenovirus vector is armed with two potent human immunostimulating cytokines: tumor necrosis factor-alpha (TNF-alpha) and interleukin-2 (IL-2). Viral replication is restricted to cancer cells by the dual control of E2F promoter and D24 base pair deletion in constant region 2 of E1A. The overall success of OV's relies also on the development of robust and consistent up and downstream processes, compatible with the scalability demands that new therapies require. Here we report on the bioprocess development and scale-up activities for producing this oncolytic adenovirus for clinics. Upstream process (USP) development started at a 75 cm² scale to optimize multiplicity of infection, time of harvest and culture media. Production was scaled up to a 10 layer cell factory (CF10). DSP development started with cell lysis and clarification screening studies. Virus capture and polishing were performed using membrane adsorbers followed by formulation with tangential flow filtration and finally sterile filtration. DSP optimization was assessed using TCID₅₀ (infectious titer), qPCR (virus titer), nanoparticle tracking analysis (particle size distribution and quantitation) and TEM (morphology and aggregation). The process developed in R&D (2 L - 1 CF10) was used to perform a proof of concept (6 L - 3 CF10) and was further scaled to an Engineering run (20 L - 10 CF10). Technology transfer to cGMP manufacturing was performed with similar titers and product quality attributes being achieved. Total particle concentration met the dose specification (1E11 TP/ml) and a ratio of total to infectious particles of 30 was achieved, with a global yield of 33%. All the process and product-related impurities were within the specification range required by the regulatory authorities. The technology transfer run was used to supply biodistribution and toxicity studies, the Engineering run is currently being used for quality control development and as reference material, while GMP manufacturing campaigns are being used to supply a clinical trial.

1149. Towards Vector Phagocytosis Evasion: Production of CD47-Coated Lentiviral Vectors by Transient Transfection

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Our group is developing viral vectors for treatment of a range of lung disorders, including cystic fibrosis and lethal surfactant deficiencies. We have previously demonstrated that a recombinant simian immunodeficiency virus (rSIV) vector, pseudotyped with the fusion (F) and hemagglutinin neuraminidase (HN) envelope proteins from Sendai virus (rSIV.F/HN), has high tropism for multiple cell types within the airway and alveolar epithelium of the mouse and human lung. Whilst gene therapies have been able to treat and even cure severe diseases, immune responses to the vectors remain a hurdle to their widespread use. To begin to address this, we aimed to tackle the potential for early clearance of vectors by lung alveolar macrophages following delivery to the lung. We explored the inclusion of the "marker-of-self" CD47 protein into lentiviral vectors. The CD47 protein has previously reported to protect particles from macrophage phagocytosis (Rodriguez et al. 2013, Milani et al. 2019). Specifically, we addressed the question of

whether the CD47 protein could be added to the surface of our vectors using a simple transient transfection protocol. Ultimately, we wish to determine if this approach could reduce vector clearance in the lung. We explored EGFP-tagged-CD47 containing vectors using our rSIV.F/HN vector and a vector pseudotyped with the commonly used vesicular stomatitis virus glycoprotein (VSVG) (rSIV.VSVG) and compared them with their EGFP-CD47+ counterparts. We performed small-scale (25mL), suspension HEK293T vector productions (n=2 for each construct). The presence of EGFP-CD47 on the vector producer cells was confirmed by FACS (71.7-99.6% EGFP+ cells). There were no negative effects of the addition of the EGFP-CD47 to the surface of the vectors. Yields were determined using Droplet Digital (dd)PCR of viral particles (VP/mL) and of transducing units (TU/mL). There was no significant difference between the two rSIV.F/HN constructs (\pm CD47) but the TU/mL value for rSIV.VSVG+CD47 vector was significantly higher than the original rSIV.VSVG ($p=0.0006$). Similarly, transgene (Luciferase reporter) activity in transduced cells showed no difference for the two rSIV.F/HN vectors (\pm CD47), but there was a significant increase for the rSIV.VSVG+CD47 vector compared with the control ($p=0.0016$). The amount of CD47 on the surface of the vectors was also quantified using ELISA; this increased from 0-4 molecules per VP to ~86-1051 molecules per VP for the +CD47 vectors. The incorporation of CD47 into the lentiviral envelope showed no detrimental effects on vector production yield or transgene activity. The +CD47 vectors will now be used in studies of CD47-dependent macrophage evasion to confirm inhibition of macrophage phagocytosis *in vitro* before proceeding to *in vivo* studies in the mouse lung. These data also indicate that transient transfection is a simple method to produce vectors incorporating higher levels of specific proteins into the lentiviral envelopes for further study.

1150. An Innovative Platform for Integrated Continuous Viral Vector Production

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Background and aims: As more advanced therapies reach late stage of clinical trials, it becomes crucial to adopt technologies that enable the manufacture of large quantities of high-quality viral vector. Traditional technologies for viral vector production are divided into 2 main categories: Adherent cell-based technologies (fixed-bed bioreactors, cell factories, roller bottles etc.) and suspension cell-based technologies (stirred tank bioreactors). Both technology approaches have pros and cons as for example suspension-based technologies offer flexibility and enable processing of very high volumes however, external cell retaining devices are required to enable continuous product collection. In the recombinant protein space, continuous processing coupled with single-use technologies have demonstrated to increase productivity at low footprints leading to lower COGs. The advent of stable producer cell lines for viral vector production, provide the first building block for these same manufacturing practices to be translated into the gene therapy field. However, there are specific features of viral vector bioprocessing that must be taken into account during technology selection in order to make continuous processing a reality. As such, this presentation aims at proposing the first purposely built technology for integrated continuous manufacture for viral vectors. This platform is designed to solve the challenges related to both adherent

and suspension-based viral vector manufacturing technologies. Materials & Methods, results and conclusions: This presentation will describe a series of case studies concerning the manufacture of different viral vectors (lentivirus, AAV and Adenovirus) within a structured fixed-bed bioreactor where the cells remain entrapped into the structured fixed-bed design at very high concentrations while media is perfused in and out of the bioreactor enabling continuous product collection while reducing the shear on the cells. The case study will describe the behavior of the cells in a 3D matrix in the presence and absence of FBS that have reached in the past 30x increase in cell densities and 2-4-fold higher specific cell productivities. Finally, this case study will explore the operational and economic benefits of combining this bioreactor with an integrated and continuous platform including additional unit operations resulting in 3-fold reductions in footprint. This presentation will quantify and illustrate the potential for integrated continuous bioprocessing in viral vector bioprocessing.

1151. Establishment of a Robust Serum Free Suspension HEK293 Transient Transfection Platform for Recombinant AAV Production

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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. Successful large-scale manufacturing of AAV vectors is critical to rAAV drug product development. AAV production platforms commonly employed include HEK293 transient transfection, HeLa-based producer cell lines and the Baculovirus/Sf9 platform. Transient transfection of HEK293 cells is the most popular platform due to its flexibility and potential for facilitating fast to clinic timelines. Three separate plasmids that express the Adenoviral helper proteins (pAdhelper), AAV replication and structure proteins (Rep/Cap) and the vector genome (gene of interest flanked by AAV ITRs) are typically used in the transient transfection platform (triple transfection, TTx). We have established a serum-free high titer suspension TTx platform that produces viral vectors that have comparable transgene expression potency as those produced from producer cell line platform. To further improve the transient transfection platform and reduce the raw material cost of plasmid DNA, we compared a two-plasmid system consisting of a triple-play plasmid (containing AAV Rep/Cap genes and the vector genome) and pAdhelper (co-transfection, Co-Tx) to the standard three plasmid-transfection (TTx). We demonstrated that HEK293 Co-Tx using the two-plasmid system generated rAAV at the same or higher yield as TTx. The rAAV vector produced by the Co-Tx method showed equivalent infectivity but a higher full capsid percentage compared to TTx. The two-plasmid transfection system therefore offers the potential to improve quality and reduce COGs, thereby enabling consideration of the TTx platform for disease indications requiring high vector doses.

1152. A Robust and Scalable Platform Process for GMP Manufacturing of Lentiviral Vectors

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Lentiviral vectors (LV) are a potent tool in the growing field of Cell and Gene therapy because they enable efficient delivery of genetic material into cells for therapies such as CAR-T and HSC-based gene therapies. With the increasing number of clinical applications and interest in the field, robust, scalable, and cost-effective platforms for GMP manufacturing of high-quality lentiviral vectors are urgently needed. We describe here a well-established platform process for LV production based on transient transfection of serum-free cells grown in suspension. Cell growth and transfection production parameters were determined using DoE studies to achieve optimal vector yields. The crude LV harvest from upstream operations shows a high infectious titer ($> 1E7$ TU/ml) and a low particle-to-infectious titer ratio (< 1000 particles/TU). More importantly, the robustness and scalability of upstream process has been demonstrated in different reactor configurations (e.g. Ambr 250, bench-top and pilot scale bioreactors). The downstream unit operations have been established and optimized for both small and large-scale production requirements. The purified and highly concentrated LV final product shows efficient and potent transduction of T cells and remarkable reduction in the host cell protein (HCP), host cell DNA and plasmid DNA impurity content. Aiming to provide a robust, scalable and GMP compatible process, this platform also focused on timeline acceleration and seamless transition from PD to GMP manufacturing in the following aspects: ability to produce LVs coding for different genes of interest (GOI) including CAR/TCR constructs, employment of the single-use technologies, employment of non-animal-derived raw materials and alignment of equipment, protocols, and data collection tools between PD labs and GMP manufacturing suites.

1153. Optimization of a Scalable Upstream Process for Novel AAV Capsids

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Recombinant adeno-associated virus (rAAV) has emerged as a promising technology for the delivery of gene therapies. The existing serotypes have limitations in terms of accessing specific tissue and the amounts needed to transduce target cells. The development of novel capsids possessing more efficient gene delivery capabilities is the next evolution in rAAV-based therapeutics. To this end, scalable manufacturing processes are needed to generate material for both novel capsid development and clinical trials. In addition, processes optimized for productivity and quality attributes will reduce the number of

batches needed, provide greater manufacturing flexibility, and lower the risk of producing unrepresentative material. In this study, data are presented from small-scale development runs with multiple novel capsids and large-scale manufacturing batches with one novel capsid. The development and manufacturing runs use a transient transfection process with HEK293 cells in suspension. Overall titer can be improved through screening transfection conditions with shaker flasks and confirming performance in small-scale bioreactors. Selecting more efficient transfection reagents and optimizing transfection parameters led to increased titer. Production of novel capsids, selected from the Capsida platform, is scalable. This study suggests that novel capsids are viable therapeutic options from a productivity and scalability standpoint.

1154. POROS CaptureSelect AAVX Wash Optimization Study for Optimized Recovery and Purity of AAV6 Capsids

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The growing use of viral vectors such as adeno-associated virus (AAV), in the field of gene therapy has emphasized the need for an effective downstream purification process to generate high titer, purity, and recovery of AAV capsids. However, AAV capsids expressed from HEK293 cells typically comprise of less than 1% of the total components, making it a challenge to separate from non-product related impurities such as host cell proteins and host cell DNA. The POROSTM CaptureSelect™ AAVX resin has simplified the AAV purification process by enabling high recovery and purity of the AAV capsids in a single affinity capture step. The purification of AAV capsids can be further optimized by incorporating wash conditions during the AAVX purification to aid in the removal of the host cell proteins and host cell DNA. A wash optimization study for HEK293 AAV6 was performed on the POROSTM CaptureSelect™ AAVX Affinity Resin. The wash buffer conditions include tris buffer at pH 7.5 and 9.0 and citrate at pH 5.5. In addition, sodium chloride and arginine were added to observe the effect of purity and recovery of AAV capsids. The experiments were performed on 1 mL columns and the eluted AAV was analyzed for residual host cell proteins, residual host cell DNA, and recovery. The addition of salt and arginine was shown to have an improvement in the recovery of AAV capsids. The results from this study show optimum wash conditions that can be used to maximum purity and recovery of AAV capsids for a large-scale manufacturing process.

1155. Optimization of Cell Inoculation and Distribution in iCELLis Nano Bioreactors for Improved Viral Vector Production

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Bioreactors provide scalable platforms for the automated and controlled culturing of cells used in the production of viral vectors. Fixed bed

bioreactors can be used to culture adherent cells, which have widely been used in the study and manufacturing of different viral vectors, including lentiviral vectors, adenovirus, and adeno-associated virus (AAV). The iCELLis 500+ bioreactor provides up to 500m² of surface area available for cell growth. This platform has been used in the manufacture of AAV vectors, including in the production of the FDA-approved AAV therapy Zolgensma. The iCELLis Nano bioreactor, a scaled-down version of the iCELLis 500+ bioreactor, is an automated, single-use, fixed-bed bioreactor that provides excellent cell growth conditions for adherent cells. It features a waterfall system for optimal temperature, DO, and pH control. Its fixed-bed design provides a compact, high cell density surface area, thus increasing volumetric productivity when compared to traditional stirred tanks or flatware. The fixed bed in the iCELLis family of bioreactors is composed of hydrophilized fibers of polyethylene terephthalate (PET), and the available surface area depends on the bed height and the level of compaction of the fibers. Homogeneous cell distribution throughout the bioreactor fixed bed and robust cell growth can support reproducible bioreactor performance and viral vector production. The objective of this project is to study and optimize the inoculation of iCELLis Nano bioreactors with the goal of determining optimal conditions for enhanced cell distribution and cell growth. A widely used cell type in viral vector production, HEK293, was used as a model in this study. Multiple iCELLis Nano bioreactor runs were conducted to acquire an understanding of the variables influencing cell growth and distribution within the iCELLis Nano fixed bed bioreactor. These runs consisted of the inoculation of HEK293 cells and the monitoring of temperature, pH, DO, and metabolites over the course of 96 hours. Cell nuclei counts were performed daily by sampling fixed bed carrier material and were compared to capacitance values obtained from a biomass probe to ensure that real-time readings and general biomass probe trends were accurate. When each bioreactor run concluded, fixed-bed carriers were sampled to assess cell distribution radially and axially. The results indicate that linear speed, the presence of pluronic, and different vessel lots do not significantly impact cell growth and distribution for the iCELLis Nano fixed bed bioreactor.

1156. Characterization of AAV Genomic Titer on the QuantStudio Absolute Q dPCR Platform

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Recombinant adeno-associated viruses (rAAV) are widely used for gene and cell therapies due to their low immunogenicity, vast tropism and efficient and persistent gene transfer to treat human diseases. However, optimal concentrations of viral vectors are necessary for clinically effective dosages. Accurate, precise and consistent viral titer measurements are required throughout routine quality control processes for rAAV vector preparations, as developers must measure viral titer during early stages of developing AAV expression systems as well as after purification and concentration steps to ensure that the correct dose of their final product has been reached. Quantification of genomic titer (GC/mL) is important for characterizing rAAV. While qPCR is considered the gold standard method for determining the genomic titer, this approach requires the use of standard curves and is thus more susceptible to variation from run to run. Digital

PCR provides significant advantages for analytical assays necessary for viral vector production and characterization by dividing a single PCR mixture into thousands of 0.5 nL-sized reactions and measuring nucleic acid concentration without the need for a standard curve. dPCR is also less sensitive to contaminants that affect amplification, including those present in solutions used during the development of AAV-mediated gene therapies. The QuantStudio Absolute Q digital PCR platform offers consistent and precise compartmentalization of sample into thousands of microchambers using a simple workflow requiring less than 5 minutes of hands-on time and results in under 90 minutes. In this work, we utilized the Absolute Q to investigate the use of the Absolute Q platform for determining accurate rAAV genome titer. Using commercially available purified AAV particles, we found consistent quantitation of genomic copies across several AAV serotypes when duplexing assays that targeted different regions of the AAV genome: CMV promoter and GFP in the FAM and VIC channels, respectively. Results were obtained without the need for a standard curve nor reference standard material. We also observed that, while qPCR reactions are highly affected by the presence of HEK293 cell lysate, dPCR reactions are more tolerant to the presence of cell lysate, and even less affected when cell lysate is diluted in TE buffer. In summary, we showcase several advantageous features of the QuantStudio Absolute Q digital PCR platform for biopharma and gene therapy research: 1) simple and fast workflow, 2) consistent quantification of AAV genomic titer without the need for standard curves, 3) higher resistance to PCR inhibition due to host cell lysis.

1157. Innovations in AAV Large Scale Manufacturing

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AGC Biologics is a leading global Contract Development and Manufacturing Organization (CDMO), providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA (pDNA), messenger RNA (mRNA), viral vectors and genetically engineered cells. Adeno Associate Vectors (AAV) produced with transient transfection in HEK293 cells are used for gene therapy both in ex-vivo and in-vivo treatments. In the context of clinical studies or commercial phase, an important manufacturing challenge is the availability of an efficient and scalable off-the-shelf AAV vector production process permitting to enter GMP clinical supply with reduced PD efforts. To address this goal, AGC Biologics is developing two different systems for production of AAV at a large scale (in adhesion and in suspension) that is flexible and can be adapted to the different serotypes. The adherent system is based on adherent cell lines, using Pall iCELLis® fixed-bed disposable bioreactors. More than 30 runs in iCellis nano of different size have been performed working on seeding cell density, DNA concentration and lysis protocols. A productivity of $2.6E+09 \pm 7.0E+08$ vg/cm² have been obtained with AAV6. DSP steps, including affinity chromatography, AEX polishing, HF UF/DF and final sterilizing filtration have been studied at small and medium scale leading to a total Yield of 30%. Polishing will be applied where needed to increase full/empty particles ratio, according to serotypes peculiarity. Data obtained in the scale up to iCellis500 333m2 will be presented. The suspension system exploits a cell line that we adapted to grow in suspension in a culture without animal derived reagents, in

stirred tank bioreactor. Several runs of AAV production at scale down (2L STR) have been performed optimizing cell growth, gas consumption and DNA amount obtaining $1E+11$ vg/ml with ratio full/total 63%. DSP will include HF concentration, affinity chromatography, DF and final sterilizing filtration with a total Yield of 50%. Scale up in 50L STR bioreactor is foreseen in 2Q 2022. The system under development is flexible in terms of AAV serotypes and Gene of Interest and suitable to be managed as ready-to-use platform available for clients at scales up to 2.000L. Both systems were shown to be flexible to support several serotypes with comparable results. AAVs have been characterized with an orthogonal strategy including Viral genome, viral particles quantification, infectious viral titer, core protein characterization and process related impurities demonstrating high potency and quality suitable for both ex-vivo and in-vivo applications.

1158. A Novel Gene Delivery Vector with Low Pre-Existing Immunity in Humans

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The *Anelloviridae* family of viruses is extremely diverse and spans a wide range of animal species, including primates, other mammals, and birds. This genetic diversity has been observed in humans, suggesting that anelloviruses could comprise many different serotypes. In addition, there is growing evidence that anelloviruses in humans are only weakly immunogenic and do not cause disease. Therefore, the development of gene therapy vectors that harness anellovirus biology could provide a potential solution to pre-existing anti-vector immunity and facilitate redosing. Chicken anemia virus (CAV) is an avian anellovirus that can serve as a steppingstone to the vectorization of the more complex human anelloviruses. Here we describe the vectorization of CAV and show that the resulting vector, which we name Ring 46 Anellovector, can transduce various genes into avian as well as human lymphoid cells. We found this vector to be stable in a liquid formulation for months at moderate temperatures or at up to 65°C for several minutes. *In vivo*, the vector demonstrated successful delivery of payload in various tissues, including muscle, eye, and heart. Lastly, pre-existing immunity to Ring 46 as measured by neutralization assays using IVIG and human sera, appears to be rare and very weak. Together, these results illustrate how human anelloviruses could be vectorized and indicate that Ring 46 may be useful as a gene therapy agent with unique properties.

1159. Optimization of a Replacement for REACH-Restricted Detergent for Cell Lysis in an AAV2 Manufacturing Process

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Adeno-associated virus (AAV) vectors are becoming a leading gene delivery system for the treatment of various genetic conditions.

Recombinant AAV can be produced in mammalian or insect cells using various chemically mediated transfection methods, virus-mediated transfection, or from cell lines genetically modified to stably produce particles upon induction. While some AAV serotypes are released into the supernatant others have shown a tendency to accumulate within producer cells. To release the cell-associated AAV particles, mechanical or chemical lysis methods are used. Current lysis strategies for large scale vector production rely on the use of detergents such as Triton™ X-100 and Tween® 20 and salts, such as sodium chloride, to drive release and particle dissociation. Digestion of released host-cell genetic material by a nuclease is often used to generate a monodisperse vector suspension. Though many detergents have been shown to lyse producer cells effectively, their impact on human health and the environment has prompted new considerations. Global regulatory agencies have imposed restrictions on certain chemicals and detergents. For example, Triton™ X-100 has been placed on the European Union (EU) REACH list even though it is considered an industry standard. As a result, there is a need to evaluate alternative detergents for cellular lysis without compromising efficacy, vector stability, and impacting downstream manufacturing process performance. Here, the effect of alternative conditions for cellular lysis following AAV2 production in HEK-293 cells was evaluated. The AAV producer cells were lysed using Tween® 20, which is registered as safe on the EU REACH list and lysis efficiency was compared with Triton™ X-100. In addition, several concentrations of lyotropic salt (e.g., sodium chloride) were evaluated to determine the effect on the release of AAV2. Lysis condition efficacy was quantified as the capsid concentration released into suspension as determined with ELISA and viral genomes as determined using ddPCR. The best performing bench-scale condition showed comparable performance to Triton X-100 when scaled-up to 3-L bioreactors and depth filtration.

1160. Development of an Upstream Process and Analytics for AAV Manufacturing

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Development of a robust and scalable manufacturing process early in a product's lifecycle is critical since gene therapy trials are often accelerated. However, establishing such a process often requires a significant amount of time and investment. Although several upstream processes are available for AAV production, choosing the one with most productivity still remains challenging. With the choice between the basic processes of suspension and adherent, a variety of other factors like scalability, yield, use of animal-derived components, quality target product profile and even licensing requirements need to be considered to minimize the target timing to get to the clinic. In addition to the process, it is also crucial to begin analytical development of product specific assays either before or in parallel to process development. Early analytical development is imperative to not only help progress process development, but also to ensure assays will be ready for qualification and timely release of product. We will share updates on our platform upstream process that is scalable and easily transferrable between multiple systems. Additionally, we have been troubleshooting two

critical assays, vg titer quantification and a platform *in vitro* potency, for a challenging self-complementary AAV9 construct - the results of which will be discussed.

1161. Comparative Characterization of AAV Vectors Produced in Insect and Mammalian Systems

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The recombinant adeno-associated viruses (rAAVs) promised a new millennium of gene transfer in various medical needs. As new technologies in medical sciences emerge, the AAV vector has proven itself as a leading delivery tool for both academic and therapeutic research and development. The long-standing safety of this vector in clinical settings and the simplicity of these viruses' productivity make rAAV a selling point among various gene delivery approaches. As the newly engineered rAAV vectors are remarkably expanding, particularly to manipulate gene expression with a therapeutic intent, our company felt an urgent need to expand our product lines based on our proprietary baculovirus (BAC-to-AAV) technology. Among various robust rAAV production methods, we sincerely intend to evaluate these production systems which are already available for unmet needs of medical researchers world-wide. In the present study, we are going to report a comparative analysis of three major production lines. These are (1) triple-transfection of Kidney cell-lines (e.g., HEK293 system, 3rd or 4th generation) by three plasmids, (2) manipulating unique dual baculoviruses for infecting insect cells (e.g., SF9 system, BAC-To-AAV), (3) using Cathepsin gene-deleted baculoviruses (Cath-SF9, modified BAC-To-AAV) for production of rAAVs that are thought to be superior in infecting mammalian cells. We have generated a Luciferase-based reporter transgene in a stipulated pFastBac plasmid in AAV8 serotype-specific Rep-Cap system. We are in a process of generating data towards the investigation of these three systems in a comparative tuning mode with the same constructs, same timing, same assay systems within a GLP environment. The rAAV titers, virus-fitness, infectibility, robustness, and the degree of reporter expression in mammalian cells, will be reported in a comparative manner with analogy. This emerging result will be critical for the researchers & consumers to understand the usefulness of these available AAV-based vector engineering and production technologies for their prudent applicability in gene therapy R&D.

1162. Tetracycline Enabled Self-Silencing Adenovirus (TESSA) Enables Efficient rAAV Manufacture in HEK293 Stable Producer Cells

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Recombinant adeno-associated virus (rAAV) is the vector of choice for *in vivo* gene therapy; however, scalability, yield and quality remain significant issues for rAAV manufacture. We recently described a new helper adenovirus system entitled 'Tetracycline-Enabled Self-Silencing Adenovirus' (TESSA) wherein the adenovirus Major Late Promoter (MLP), primarily responsible for expression of adenovirus structural

proteins, was modified *in situ* to enable self-repression of promoter activity and truncate the adenovirus replication cycle for efficient and contaminant-free manufacture of rAAV (accepted). We previously reported two approaches of using TESSA for rAAV manufacture, either by using two TESSA vectors to deliver adenoviral helper functions, AAV rep and cap genes, and the rAAV genome into HEK293 cells for producing rAAV, or by using one TESSA vector encoding AAV rep and cap genes to propagate existing rAAV. Both approaches are efficient, yielding up to 30-fold more rAAV with improved infectivity compared to the helper-free rAAV production system. To further simplify the rAAV manufacture process, here we developed HEK293-based stable producer cell lines to use with TESSA. We used transposon integration systems to insert up to 20 copies of the rAAV transfer genome in suspension serum-free HEK293 cells. High producer clones were effectively screened using TESSA encoding AAV rep and cap, banked, and readily expanded for the production of a range of rAAV serotypes (including rAAV2, -5, -6, -8 and -9) by simple infection with TESSA expressing the AAV Capsid of interest. We propose this as a high-yielding contaminant-free production system suitable for scalable rAAV manufacture.

1163. Production Media Screening and Harvest Depth Filter Comparison from Different Vendors for AAV Production by Transient Transfection Mediated HEK293 Suspension Cell Culture

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Adeno-associated virus (AAV) has been widely used as the vector for gene therapy on diseases caused by absent or defective genes. Despite the booming development of AAV's medical applications, current AAV manufacturing is still challenged by low expression level and high cost of goods, which limit its use as a viable therapeutic treatment. One commonly used AAV production approach is the HEK293 suspension cell culture with plasmids transient transfection. Many studies have been performed to improve titer, including host cell screening and engineering, helper plasmid optimization, transfection method optimization, and cell culture process optimization. However, few studies have been published comparing production media and depth filters for harvest clarification from different vendors. In this study, we evaluated the effects of several commercially available HEK293 cell culture media and various feeding strategies on AAV titer expression using an Ambr15 microbioreactor system. Cell growth, qPCR titer and full capsid percentage were monitored or analyzed. For AAV harvest clarification by depth filtration, we compared the filter throughput, yield by qPCR and full capsid percentage of selected depth filters from different vendors. A cost and benefit analysis of various options will be presented.

1164. Abstract Withdrawn

1165. Assembling a Comprehensive Potency Assay Matrix for Late-Stage Manufacturing of AAV Viral Vectors

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Understanding of commercial gene therapy products has accelerated rapidly in the last few years, resulting in stringent specifications to maintain a consistent level of potency through process development changes. Viral vectors have a particularly complex mechanism of action and several steps must occur in series for a therapeutic benefit to be observed in patients. As a result, it is critical to demonstrate that various stages in the biological process occur successfully when developing a potency package. SUNRISE is a Phase I/II clinical trial which is exploring the use of hLB-001 to potentially treat pediatric patients with methylmalonic acidemia characterized by methylmalonyl-CoA mutase gene (MMUT) mutations. hLB-001 uses GeneRide technology and incorporates homology arms in the transgene design to precisely integrate a corrected MMUT gene into the albumin locus of targeted cells through homologous recombination. Selective advantage then promotes the proliferation of edited cells that express the functional protein. Here, a combination of mRNA expression and enzymatic activity assays are used to show comparable levels of potency when both genomic integration and functional activity are required to effectively treat the disease. The assays were initially assessed by performing linearity and inter/intra-assay precision experiments, and then were further evaluated using different batches of drug substance and heat-treated samples to demonstrate alignment of the two analytical methods in measuring vector potency.

1166. Development of a Scalable, Suspension Cell Culture-Based Manufacturing Process for VivoVec, a Lentiviral Vector Platform for In Vivo CAR-T Cell Generation

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Ex vivo CAR T-cell therapies have shown significant clinical success in treating hematological cancers. However, access to these lifesaving therapeutics has been severely limited due to the critical challenges in cost, supply chain and manufacturing. To address these challenges, Umoja is developing an in vivo CAR T-cell platform (VivoVec) based on an off-the-shelf, direct injection lentiviral vector platform. CAR T-cell generation in vivo via direct VivoVec vector administration has several significant advantages to the patient, including immediate, on-demand treatment, avoidance of lymphodepleting chemotherapy, and differentiated CAR T-cell biology through expansion of the CAR T-cells in their native milieu. To meet anticipated VivoVec particle production requirements, Umoja is developing a scalable, suspension cell culture-based manufacturing process capable of producing lentiviral vector product with the quality characteristics necessary for direct injection. Our approach to drug substance (DS) and drug product (DP) manufacturing process development is guided by ICH Quality Guidelines (ICH Q11 and

Q8(R2)) and includes the following elements: 1) defining the quality target product profile (QTPP), which provides an understanding of what will ensure the quality, safety, and efficacy of the product for the patient, 2) identifying potential critical quality attributes (CQAs) so they can be studied and controlled by the process to ensure the desired product quality, 3) defining the manufacturing process and identifying critical process parameters (CPPs) and their controls that affect CQAs, and 4) defining a manufacturing control strategy that assures process performance and product quality during all phases of clinical development and commercialization. Umoja has established a QTPP that provides the lentiviral vector product design criteria and forms the basis for development of the CQAs, CPPs, and manufacturing control strategy. Potential lentiviral vector CQAs have been identified and analytical methods are being developed for supporting DS and DP manufacturing process development. The manufacturing process Umoja is developing to control lentiviral vector CQAs and produce product meeting the QTPP uses well-established biopharmaceutical manufacturing methods, including suspension cell culture, filtration, ultrafiltration, and chromatography. The functions of these methods for controlling lentiviral CQAs (e.g., process-related impurities) and vector titer/yield will be described. Process-related impurities include, but are not limited to, host cell DNA and proteins, which form during the upstream cell culture and harvest process steps and must be reduced to acceptable levels by the downstream filtration, ultrafiltration, and chromatography steps. Results will be presented from process development studies to understand the capability of the downstream process steps to reduce host cell DNA and proteins. The process understanding gained from these studies will be used to identify critical process parameters and establish their operating ranges for the clinical and commercial manufacturing control strategy.

1167. Lentiviral Vector Producer Cell Line Generation for Chimeric Antigen Receptor (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 has created a huge demand for the supply of large quantities of high-quality grade lentiviral vectors (LV). Production of LV using producer cell lines (PCLs) is desirable due to reduced costs, ability to scale to large volumes, improved batch consistency and increased streamlined production process when compared to LV 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 can have a detrimental effect on the survival of PCLs. One solution would be to repress the CAR expression in the stable cell line using technology such as the TRiP systemTM. Extensive work has been performed to improve the efficiency of transgene repression in the PCLs using modified LV

genomes and an improved version of the TRiP systemTM. In parallel, Oxford Biomedica has successfully implemented a cell line development platform that enables the generation of PCLs directly in suspension, in accelerated timelines. In combination, these technologies have enabled the successful generation of highly promising suspension PCL LV-CAR clones. In brief, suspension PCL LV-CAR pools of cells were generated and from these more than 200 PCL clones were isolated and evaluated for LV production at high-throughput scale. From these clones the best 60 were expanded and after multiple rounds of screening, the two best suspension clone candidates were selected for further screening and characterisation. These resulting PCL LV-CAR clones were shown to be capable of producing high titre LV-CAR at a larger scale. In summary, the integration of Oxford Biomedica technologies has led to the identification of suspension PCLs that are capable of producing high titre LV-CAR in significantly accelerated cell line development timelines. Given that PCL LV-CARs are more advantageous, for large-scale production than current processes, it is anticipated that this platform technology will support increased yields at reduced cost with the ultimate aim of enabling more patients to be treated with these life changing gene therapies.

1168. A Novel Flow Cytometry Method for Rapid Assessment of Lentiviral Detection

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Background: Lentiviral vectors are a popular gene delivery tool in cell and gene therapy. They are the primary tool for *ex vivo* transduction of T-cells for expression of chimeric antigen receptors and engineered T-cell receptors (TCRs) in T-cell therapies for cancer. To assess lentiviral vector batch-to-batch variability, whether it is in process development, manufacturing, or product characterization, a range of analytical methods are required. Current analytical tools are often low throughput and slow to deliver data. Here, we describe a novel flow cytometry method that offers fast, high-throughput evaluation of in-process vector titers. **Methods:** An intracellular flow cytometry method was developed to evaluate transfection levels in HEK293T cells as a model system. A range of antibodies were assessed to determine their suitability to bind key vector components within the cells. The most successful antibodies were optimized and then used to measure the levels of 3 essential proteins produced by the HEK293T cells, which are VSV-G, Gag-Pol, and TCR, in parallel with harvesting of the vector material. These data were compared with a conventional method of measuring vector titer involving the time-consuming transduction of peripheral blood lymphocytes (PBLs). The comparison was done to determine if there was a correlation between expression of the 3 proteins tested and the titer of the vector harvest. Our new method to evaluate protein vector components can be completed within 3 hours of the harvest. Another advantage is that these proteins can be checked at any stage of the vector manufacturing process. **Results:** We successfully identified antibodies recognizing 3 key proteins marking co-transfection for successful vector manufacture. The results show a strong correlation between each of the 3 proteins and the biological titers produced by the conventional method of transducing PBLs.

VSV-G showed the strongest correlation ($r^2=0.914$) and could be used as a single marker to determine vector titer. This assay can be used on the day of harvest, obviating the need for PBL transduction. We also demonstrated the ability to detect a variety of TCRs used within our workflows. **Conclusions:** We developed a flow cytometric method to allow quick assessment of vector protein levels expressed within HEK293T cells post transfection. We have shown strong correlation between lentiviral vector protein expression and PBL titer. This new method applying flow cytometry can be used as a predictive tool when comparing vector production runs under the same conditions. It also provides valuable insight into the vector manufacturing process and may support continued process improvement.

Cell Therapy Product Engineering, Development or Manufacturing

1170. Enhancement of Polyethylene Glycol-Cell Fusion Efficiency by Novel Application of Transient Pressure Using Pyro-Drive Jet Injector

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Cell fusion is a fusion of somatic cells resulting in somatic cell hybridization. It is not only a physiological process but also an important cell engineering technology applicable to various fields, such as regenerative medicine, antibody engineering, genetic engineering and cancer therapy. There are three major methods of cell fusion: electrical cell fusion, polyethylene glycol-mediated cell fusion (PEG) and Sendai virus-induced cell fusion. PEG-mediated cell fusion method is the most economical approach for cell fusion and does not require expensive instrumentation. Although PEG cell fusion is simple and cost effective, there are still some shortcomings such as high cytotoxicity and low fusion efficiency. To improve the efficiency of the PEG method, here, we used Pyro-drive Jet Injector (PJI) in combination with PEG fusion method. In the PJI-PEG fusion method, the PJI provides transient acute pressure instead of gentle agitation to increase the chance of cell-to-cell contact and shorten the distance between cells in the process of cell fusion. Our results showed that the PJI-PEG fusion method not only decreased cell cytotoxicity during the fusion process, it also increased fusion efficiency in comparison to the traditional PEG method. Using this method, we successfully fused cells for dendritic cell-tumor cell vaccines and hybridoma production which were functionally indistinguishable from those fused using the traditional PEG method. The PJI-PEG fusion method is a fast and efficient method to induce cell fusion and has potential to improve both basic research and clinical protocols that require cell-cell fusion.

1171. A Decentralized and Integrated Manufacturing System for the Rapid and Cost-Effective Production of Cell Therapy Drug Products

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SQZ Biotech, Watertown, MA

The patient impact potential of cell and gene therapies continue to expand as new technological advances promote the development of a widening array of novel therapies. Despite this promising growth, the manufacturing processes and unit operations used to produce clinical batches remain stagnant. Cell therapies are manufactured in clean rooms with multiple open manipulations, require a significant number of trained operators, and are performed at a centralized manufacturing site. Herein we describe the implementation of SQZ® technology to create a closed, automated system for manufacturing cell therapies that integrates cell isolation, washing, delivery and product filling. This system is designed to process patient material within a closed, single use sterile disposable kit. The customized kit has been optimized specifically for aseptic processing and product yield. This enclosed manufacturing system is portable and has the potential to be operated outside of a clean room. This would potentially enable cell therapy manufacturing to be decentralized and located at the point of care - thereby improving patient access and reducing chain of custody logistical issues. The automation of the system is 21 CFR Part 11 compliant which aids in eliminating failure modes, reduces operator introduced variability, captures more process data to aid in further development, and complies with regulatory requirements covering data integrity and storage. The first-generation prototype has demonstrated comparable or improved performance relative to a conventional clean room process. The eventual clinical implementation of this system for cell therapy manufacturing could yield a reduction in manufacturing cost, improve process reproducibility and broaden patient access to cell therapies.

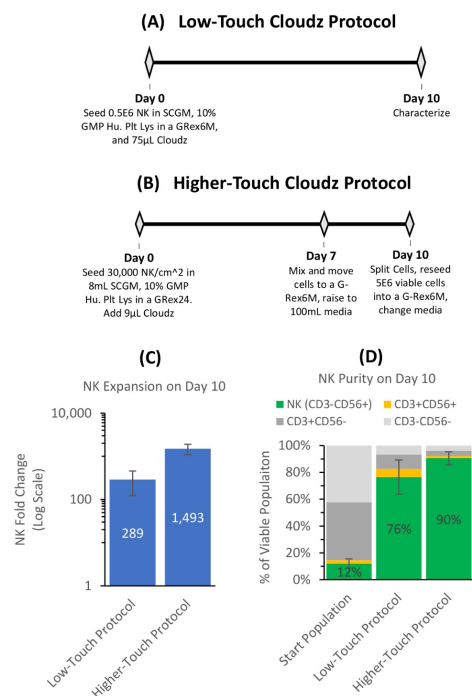
1172. Feeder-Cell-Free Natural Killer Cell Expansion Using NK Cloudz and the G-Rex Culture System

Christopher Johnson, Nicole Zale, Eric Frary, Joseph Lomakin

Bio-Techne, Woburn, MA

Introduction: Generation of clinical scale doses of natural killer (NK) cells is an important challenge for Chimeric Antigen Receptor (CAR)-NK cell therapies. Current manufacturing methods to expand NK cells use irradiated feeder cells and human or bovine serum. However, these methods are restrictive due to high costs, scale-up challenges, licensing restrictions, and clinical translation challenges. A Feeder-free workflow relies on defined reagents to reduce variability and improve consistency for clinical scale manufacturing. In this study we show an effective method to expand NK cells using Cloudz hydrogel microspheres in a G-Rex, feeder-free and FBS-free system. The Cloudz dissolvable microspheres activate and expand NK cells and are easily removed at the end of culture to deliver a feeder-free cell product. We demonstrate a low-touch 10-day protocol for NK

expansion to simplify the manufacturing process of cells that does not require media exchanges for the duration of the culture, along with a higher-touch protocol that provides extended expansion. **Methods:** An NK cell culture expansion method was developed using the Cloudz™ Human NK Cell Expansion Kit. For the low-touch protocol, Human peripheral blood mononuclear cells (PBMCs) were seeded at a density of 50,000 NK/cm² in G-Rex6M flasks in 100mL SCGM media, 10% GMP Human Platelet Lysate, with cytokines: 27 ng/mL IL-2, and 10 ng/mL each of IL-12, IL-18, IL-21. An alternative higher-touch Cloudz protocol was developed that started with 30,000 NK/cm² in an 8mL G-Rex24. The culture was transferred to the larger 100mL G-Rex6M well on day 7, then split on days 10 and 14. All experiments were repeated in at least 3 separate donors. **Results and Discussion:** While standard NK protocols require media exchanges every 2-3 days, the low-touch protocol we developed does not require intervention for the duration of the 10-day culture (Fig 1A). In the low-touch protocol, the NK Cloudz expanded NK cells 289 ± 166-fold in 10 days from a PBMC starting population in 7 donors (Fig 1C). Depending on the donor, the low touch protocol in the G-Rex6M platform produced 105-281 x 10⁶ viable NK cells on day 10. While having fewer touchpoints is beneficial for a manufacturing process, we found that splitting the cultures and changing the media periodically improved the yield and culture viability. We modified the Cloudz protocol to start in a smaller G-Rex24 (8mL), transfer to a G-Rex6M (100mL) on day 7. The culture was further split on days 10 and 14 to simulate transfer to a larger vessel. This higher-touch Cloudz/G-Rex protocol resulted in an average of 1493±410-fold expansion after 10-days in 3 donors. Furthermore, the higher touch protocol could be extended to 20 days, reaching 55,106 ± 24,029-fold-expansion and 96 ± 2% NK Purity. To our knowledge, these Cloudz-based results are the fastest feeder-cell-free expansion to date. Overall, the benefit of the Cloudz/G-Rex protocol is that it is a feeder-free and xeno-free process that can be tuned to either minimize the touchpoints required or to maximize the cell expansion.



1173. CRISPR-Cas9 RNP Editing of Primary T Cells Using the Flowfect® Transfection Technology Platform

Gretchen Lewis, John Zhao, Michael Baladiang, Andrew Hallinan, Jessica Sido, Ross Beighley, Eduardo Latouche, Ta-Chun Hang, Bethany Grant, Cullen R. Buie, Paulo A. Garcia

Kytopen, Cambridge, MA

Cell and gene therapy manufacturing relies on robust and consistent delivery of genomic payloads to cells. Currently, electroporation is a major method of transfection used to deliver various nucleic acid and protein payloads such as DNA, mRNA, and CRISPR-Cas RNPs to target cell types through electrical disruption of cellular membranes. However, these methods can also disrupt normal cell function and often result in poor process efficiency and cell recovery. Kytopen's Flowfect® system is a transfection technology platform that combines continuous fluid flow with electric fields to deliver payloads to cells of interest. The technology provides added benefits over existing payload delivery techniques by seamlessly bridging scaling of high throughput screening used in discovery to large scale manufacturing of cell and gene therapy products. We previously have demonstrated high efficiency and recovery when delivering mRNA payloads to primary T cells; conditions optimal for mRNA delivery are not universal for all payload types. To further expand the possible applications of the Flowfect® system, we explored multiple parameters to modulate waveforms for delivery of CRISPR-Cas9 RNP payloads to primary T cells. Several electromechanical parameter dependencies that significantly influence editing efficiency, viability, and overall primary T cell process recovery were identified. Specifically, our studies identified that while the total amount of

electromechanical energy delivered to cells is important, the overall method and mechanism of energy delivery can significantly alter these results, providing concomitant improvements in both efficiency and recovery. These trends towards increased editing efficiency and process recovery were recapitulated across multiple PBMC donors, cell types (NK, CD34+ HSPCs), and sgRNA targets. When compared with existing transfection technologies, these new parameters resulted in overall better editing efficiency, viability, and total cell recovery. These data provide compelling evidence for the use of the Flowfect[®] system to enable RNP delivery to cells for immune-based therapies at both the small-scale discovery stage and large-scale manufacturing stages of cell therapy development.

1174. Systematic Genomic Safety Assessment of CRISPR-Based Gene Therapy in Muscle Stem Cells

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Limb girdle muscular dystrophies (LGMD) are monogenic diseases that severely reduce the life quality of patients, with potentially fatal outcomes, such as respiratory or cardiac failure. To this date, no medication has been approved for intervention. To lay the groundwork for muscle gene therapy, we are establishing CRISPR-based gene correction in muscle stem cells *ex vivo* for common LGMD-causing mutations. A major concern of the CRISPR-based gene correction, however, is the potential genome-wide introduction of harmful mutations. To detect unintended mutations prior to transplantation and minimize the risk of cell transformation, we here establish, within the scope of a clinical phase 1 study, a systematic evaluation of genome integrity post gene editing, based on experimental and computational off-target analysis, oncogene panels and translocation analysis. Preliminary results suggest that the gene correction by specific sgRNAs and transient mRNA-mediated expression of CRISPR-guided base editors in muscle stem cells is safe and leads to no detectable off-target mutations.

1175. Single-Step Stimulation of CAR-Engineered Natural Killer Cells with Artificial Antigen-Presenting Cells for Ex Vivo Expansion

Mark A. Omobono

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Engineered natural killer (NK) cells expressing therapeutic proteins including chimeric antigen receptors (CARs) are an exciting new therapeutic modality in the cell therapy space, with the potential to be used as off-the-shelf allogeneic therapies for solid and hematologic malignancies. Robust cell expansion during manufacturing is necessary to enable off-the-shelf applications. Artificial antigen presenting cell (aAPC) stimulation is well described in the literature for the

purposes of expanding NK cells and other cell therapy modalities. In particular, aAPCs, such as the K562 immortalized cell line, expressing cell stimulatory ligands are commonly co-cultured with NK cells in multiple rounds to continuously stimulate cell growth during expansion. However, a manufacturing process requiring multiple exposures to aAPCs increases drug product safety risks and complicates allogeneic scalability. Here we describe that a single stimulation of CAR-NK cells with aAPCs is sufficient to theoretically yield more than $1.0E+11$ CAR-NK cells from a single healthy donor apheresis. CAR-NK cells produced with a single round of aAPC stimulation, compared to multiple rounds of aAPC stimulation, result in similar CAR expression, CD56+CD3⁻ purity, viability, and similar performance in *in vitro* phenotypical and functional assessments. In-process measurement of IL-2Ra (CD25) expression reveals that a single aAPC exposure *ex vivo* sufficiently sustains NK cell activation. The process development described here simplifies CMC process operations, reduces cost of goods in manufacturing, and increases product safety by mitigating the risk of aAPC presence in a final CAR-NK drug product.

1176. Rapid, Gentle, and Scalable Engineering of Hematopoietic Stem Cells Using Vector-Free Microfluidic Cell Squeeze[®] Technology

Murillo Silva, Jacquelyn Hanson, Jonathan Gilbert, Devin Bridgen

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Treatment of numerous rare genetic diseases, including hemoglobinopathies, using gene edited autologous hematopoietic stem cells (HSCs) has shown potentially curative impact. However, effective engineering of HSCs, a sensitive cell type, is often limited by toxicity, cargo restrictions, and scalability challenges of current delivery methods. Microfluidic deformation using the Cell Squeeze[®] technology effectively delivers diverse cargo into sensitive cell types and minimally alters the cell state and health of the cells. This is achieved through rapid and transient disruption of the cell membrane, which allows materials in solution around cells to diffuse into the cytosol. The Cell Squeeze[®] platform has been implemented in multiple clinical programs and reliably engineers billions of cells per minute. Here, we characterize the delivery efficiency of multiple material types, including mRNA and gene editing constructs, into HSCs using microfluidic deformation. In primary mobilized CD34+ cells, we see >90% expression of mRNA encoding GFP or CXCR4 mRNAs. When delivering CRISPR/Cas9 RNPs, we see efficient editing as quantified by flow and genomic sequencing. Furthermore, we investigated the impact of squeezing on HSC phenotype, as determined by flow cytometry, gene expression microarray, and engraftment efficiency in NBSGW mice. Gene expression analysis revealed a large degree of perturbations following electroporation, with fewer perturbations observed following squeeze, suggesting a lesser impact on cell phenotype using squeeze compared to electroporation. Further, we demonstrate that Cell Squeeze[®] technology is gentle enough on HSCs to enable immediate use and manipulation, unlike typical electroporation procedures. These data highlight the potential use of our technology for engineering HSCs with both gene editing and mRNA constructs for a wide variety of diseases. In combination with our point-of-care

system under development, the ability to efficiently engineer HSCs as well as rapidly use the cells after manipulation, may enable novel HSC treatment paradigms closer to the patient.

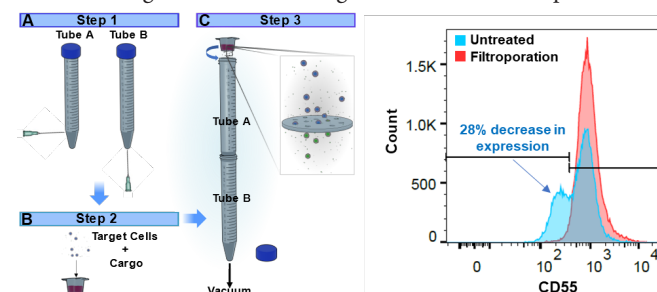
1177. Low-Cost Fluorosilane-Modified Filtration Devices Enabling Gene Knockout in Human Hematopoietic Stem and Progenitor Cells

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¹Bioengineering, UCLA, Los Angeles, CA, ²Pediatrics, David Geffen School of Medicine, UCLA, Los Angeles, CA, ³Chemistry & Biochemistry, UCLA, Los Angeles, CA, ⁴Adaptive Surface Technologies, Inc., Cambridge, MA, ⁵Chemistry and Chemical Biology, Harvard University, Cambridge, MA, ⁶Molecular and Medical Pharmacology, UCLA, Los Angeles, CA, ⁷Microbiology, Immunology and Molecular Genetics, UCLA, Los Angeles, CA, ⁸Eli & Edythe Broad Center of Regenerative Medicine and Stem Cell Research, UCLA, Los Angeles, CA, ⁹Materials Science and Engineering, UCLA, Los Angeles, CA, ¹⁰California Nanosystems Institute, UCLA, Los Angeles, CA, ¹¹Mattel Children's Hospital, Children's Discovery & Innovation Institute, UCLA, Los Angeles, CA

Intracellular delivery technologies that are scalable, cost-effective, and efficient are required to process large populations of cells more effectively into gene and cellular therapy products. Existing commercial non-viral methodologies for intracellular delivery such as electroporation and lipofection are limited by several factors, including high operational and instrumentation costs, low processing throughputs, low efficiencies, and/or cyto- and geno-toxicity. In particular, electroporation has been shown to result in transcriptional abnormalities in both primary human T cells and hematopoietic stem and progenitor cells (HSPCs). More recently, intracellular delivery approaches based on mechanically deforming or squeezing cells to permeabilize the lipid bilayer transiently have been demonstrated by our group and others as promising solutions for delivering biomolecular cargoes to target cells in an efficient manner while preserving cell viabilities. These biophysical membrane manipulation techniques often employ microfluidic devices with constricted channels or porous membranes that deform cells to 30-80% of their resting diameter. However, the requirement for specialized equipment has, to date, limited the broader adoption of these technologies. Here, we report a straightforward and accessible filtration-based solution for permeabilizing cells by pulling them through the pores of a cell culture insert *via* application of vacuum commonly available in biosafety cabinets. These devices cost <\$10 in materials per experiment and employ supplies available in most research laboratories. Applying this technique to Jurkat cells and human CD34⁺ HSPCs, we demonstrated robust and efficient delivery of fluorescently labeled dextrans, expression plasmids, and Cas9 ribonucleoprotein complexes (RNPs) for gene knockout with minimal impact to cell viability. When comparing gene knockout efficiency per micromolar concentration of cargo used in solution, filtration-treated populations outperform nucleofection controls for both Jurkat cells and HSPCs. The current throughput of filtration devices enables processing of 500,000 to 4 million cells per experiment, and if combined with a simple

3D-printed vacuum application chamber, processing rates can be increased up to 6 - 12 times higher *via* parallelization. Altogether, this good manufacturing practice (GMP)-compatible, table-top approach does not require specialized equipment, viral vectors, or reagents, offering a versatile and straightforward solution to democratize the manufacturing of stem cell-based gene and cellular therapies.



1178. A Novel Bioprinted Cell Therapy Platform Normalizes Blood Glucose Control in Diabetic Rats

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Introduction: Transplantation of cadaveric islets can reverse type 1 diabetes (T1D) but requires lifelong immunosuppression. Cell encapsulation has the potential to overcome this challenge by blocking immune cell access to islet grafts while allowing nutrient exchange, glucose sensing and insulin release. In this study, 3D tissues consisting of patterned, multi-shell fibres with an islet-containing core and immunoprotective shell were fabricated using Aspect Biosystems' microfluidic 3D bioprinting technology for implantation in diabetic rats. **Methods:** Our therapeutic development is powered by patented microfluidic 3D bioprinting technology, which has been previously described in more detail (Dickman FASEBJ 2020). Briefly, the printer employs proprietary microfluidic printheads to extrude cell-containing, multi-layered hydrogel fibres and pattern these into 3D structures. This technology allows for custom fibre architecture, multi-material input, precise control over deposition location, and the printing of both single cells and cell spheroids in materials that support their function. It also maximizes cell viability by protecting the cells from shear stress. Blood glucose and animal body weight were monitored for several months following surgery. Upon retrieval, bioprinted tissues were assayed for islet cell viability and function by glucose-stimulated insulin secretion (GSIS), followed by histology and immunohistochemistry (IHC) to analyze foreign body response (FBR) and immune cell infiltration. **Results & Conclusion:** 3D bioprinted implants containing reaggregated allogeneic rat islets re-established normoglycemia for > 120 days in streptozotocin-treated diabetic immunodeficient rats, and > 30 days in diabetic immunocompetent rats. Post-retrieval, tissue implants demonstrate high viability and functionality, with an absence of immune cell penetration through the shell, confirming its immune protective properties. Modification of the outer shell biomaterial to reduce FBR was shown to extend islet tissue implant functionality. This

is the first study describing a 3D bioprinted implant composed of a core/shell micro-fibre used to deliver therapeutic islets into diabetic animals. The promising results obtained in rodent models open the door to further testing in larger animal models and, eventually, T1D patients.

1179. Acoustofluidic Sonoporation Gene Delivery Utilizing DNA- Encapsulated Supramolecular Nanoparticles for Cancer Immunotherapies

Yao Gong

UCLA, Los Angeles, CA

Cancer immunotherapies utilizing gene-modified T cells that express chimeric antigen receptors (CAR) specific for tumor antigens are revolutionizing treatment paradigms across an expanding spectrum of malignancies. The effective manufacturing of CAR-T products from the bench to the clinic, including the current Food and Drug Administration (FDA) products approved for patients with relapsed and refractory leukemia and lymphomas, requires target cell populations to be engineered rapidly, efficiently, safely, and cost effectively. Toward this goal, we report an acoustofluidic strategy to deliver plasmid constructs encoding for CAR transgenes to human primary T cells *in vitro* by exploiting shear forces imposed on the cells as they are directed against the wall of a glass microcapillary. The CAR expression plasmid cargoes include a cassette for the Sleeping Beauty transposon system that enables stable integration of the CAR transgene and are encapsulated in supramolecular nanoparticles (SMNPs) designed to serve as vehicles for intracellular trafficking. Preliminary results indicate ~40% cell viability and ~25% CAR expression on T cell membrane after two weeks, which confirmed the integration of the transgene. Mechanistic investigations of cell membrane permeability post-acoustofluidic treatment reveal that the cell and nuclear membranes are transiently disrupted during processing, which promotes the intracellular entry of DNA and other biomolecular cargoes while causing transient and reversible damage to cells. Overall, this acoustic-based transfection platform offers a promising solution for delivering a wide range of therapeutic payloads to cell populations targeted in the generation of gene therapies, accelerating the research, development, and broader clinical deployment of these exciting medical interventions.

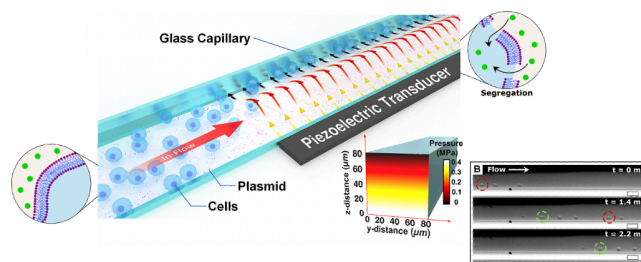


Figure 1. Schematic of the device components and application, where target cells undergo acoustofluidic treatment via flow through a glass capillary over a piezoelectric transducer and are collected at the outlet. Minimum pressure presents at the wall farthest from the piezoelectric transducer at an excitation frequency of 3.3 MHz.

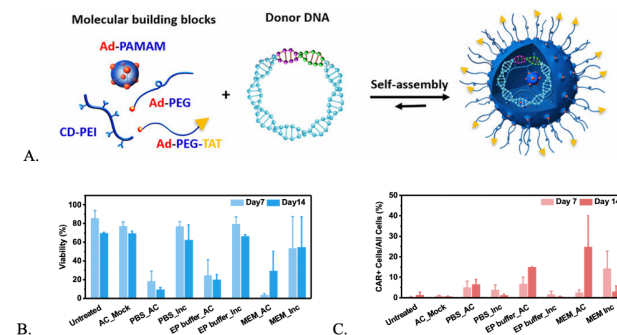


Figure 2. (A) A self-assembled synthetic approach for the preparation of plasmid-encapsulated supramolecular nanoparticles (SMNPs). (B) Cell viability and (C) chimeric antigen receptor (CAR) expression efficiency in human primary T cells in different media 7 days and 14 days post-acoustofluidic treatment.

1180. Corning® Ascent™ Fixed Bed Reactor (FBR), an Automated, Scalable and Closed System for High Efficiency Cell and Gene Therapy Manufacturing

Yulong Hong, Lori E. Romeo, Vasily N. Goral, Yue Zhou, Joel A. Schultes, Daniel Manning, Ann M. Ferrie, Charles P. Harding, Kathleen A. Krebs, Jeffery J. Scibek, Jennifer L. Weber, Todd Upton, Zara Melkounian

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Cell and gene therapies (CGT) are breakthrough medical innovations that are transforming how we treat and potentially cure certain diseases. With more than 2,600 CGT clinical trials underway (based on Alliance of Regenerative Medicine 2021 report), the manufacturing processes for both gene delivery vehicles (such as viral vectors) and therapeutic cells (such as stem cells) continue to be bottlenecks for the pharmaceutical industry. There is an urgent need for automated, scalable, and cost-efficient manufacturing platforms to meet the rapidly increasing clinical demands. The Corning Ascent FBR System is designed to combine the greater yield efficiency, flexibility, and viable cell harvest capability of adherent bioproduction platforms with the scale, automation, and closed system features of suspension manufacturing systems. Key features of the Ascent FBR System include specially treated and packed polymer mesh that enables uniform low-shear fluid flow through the bioreactor bed, uniform high-density HEK 293(T) cell growth, >90% transfection efficiency, and >8-fold higher rAAV2-GFP vector genomes yield/m² compared to other adherent bioreactor

platform. The ability to harvest viable cells from the Ascent FBR System enables its use in seed train for manufacturing scale as well as for other CGT workflows such as lentivirus, MSC and iPSC in addition to viral vaccine and other biologic production applications. The Ascent FBR System is designed to provide a broad range of cell growth surface areas to meet various application and production scale needs: product development size (1-5m²), pilot size (20-100m²) and production size (200-1000m²). The Ascent FBR System consumable set is equipped with disposable sensors for key process parameters (pH, dissolved oxygen, temperature), gamma irradiated and ready-to-use. In conclusion, the Ascent FBR system is purposely designed to address manufacturing bottlenecks by providing automated, high yield, scalable adherent bioreactor platform for CGT workflows.

1181. The ekko™ and ekko™ Select: Acoustic-Based Closed and Automated Systems for Cell and Gene Therapy cGMP Manufacturing

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Acoustic Cell Processing is a unique acousto-fluidics platform technology for minimal manipulation of cells using ultrasonic waves. The platform has broad applications in the field of cell and gene therapy, e.g., cell concentration and washing, acoustic affinity cell selection and label-free cell selection. The acoustic radiation force exerted by the ultrasonic field on the suspended cells in combination with fluid drag forces and gravitational forces is used to manipulate the cells and perform a certain cell processing unit operation, e.g., separate, concentrate, wash or select. The technology is single-use, continuous, and can be scaled up, down or out. It therefore allows for a flexible and modular approach that can be customized to process a desired cell count, cell culture volume or cell concentration within a given required process time. The ekko™ captures cells in the acoustic standing wave, enabling the cells to be concentrated and washed inside the ekko™ consumable. This instrument and consumable work in a closed and automated fashion and can be applied to any of the concentrate and wash unit operations, including cryoprotectant wash out from frozen apheresis products, 200-fold volume reduction and wash pre-electroporation or final harvest and cryoprotectant addition pre-cryopreservation. In this work, we demonstrate high cell recovery, impurity wash out and buffer exchange in different CART T workflow steps using the ekko™. The ekko™ Select uses (non-paramagnetic) affinity beads for positive or negative cell selection. A multi-dimensional acoustic standing wave is then used to separate the affinity bead-cell complexes from the unbound cells, thereby completing the process of cell capture. The instrument, consumable, buffers, antibodies and affinity particles are all included in the same cGMP solution for closed and automated T cell selection from an apheresis product. In this work we show multiple donor T cell selection from apheresis products with a final T cell purity of 95% and better than 70% cell recovery in our closed and automated ekko™ Select system.

1182. Extracellular Vesicles from Physically Confined Mesenchymal Stem Cells Demonstrate Enhanced Angiogenic Bioactivity

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Cell-secreted nanovesicles known as extracellular vesicles (EVs) have emerged as a compelling new therapy for chronic wounds - a complex and deadly phenomenon affecting millions of patients worldwide. Particularly, EVs from mesenchymal stem cells (MSCs) have demonstrated the ability to augment angiogenesis and revascularization in numerous disorders (e.g., myocardial infarction, ischemia-reperfusion injury, and corneal wounds). With therapeutic effects rivaling those of their parental cells as well as a superior safety profile, MSC EVs hold great promise. Yet, fundamental challenges such as inadequate large-scale manufacturing tactics and low levels of endogenous EV cargos ultimately limit the translation of EV therapies. Notably, biophysical cues experienced within the cellular microenvironment, such as confinement, have been shown to enhance the production and therapeutic effects of endothelial cells. Compared with other EV-enhancing strategies (e.g., biochemical priming, exogenous cargo loading), biophysical cues can be manufactured with high reproducibility and scalability. Given the mechanosensitive nature of MSCs, an understanding of how physical confinement can control MSC EV abilities could be instrumental in attempting to simultaneously alleviate issues associated with potency and scalability. To understand the role of physical confinement, bone marrow derived MSCs (passage 3) were cultured within polydimethylsiloxane (PDMS) devices with micropillars of various spacings (5, 10, 20, or 50 μm). EVs secreted from these cells were isolated via ultracentrifugation (Beckman Coulter) or tangential flow filtration (Repligen KrosFlo KR2i; 300 kDa) and characterized using nanoparticle tracking analysis (NanoSight LM10) and western blotting for specific EV markers (e.g., CD63, TSG101). The MSC EVs were applied to human umbilical vein endothelial cells (HUVECs) (5e9 EVs mL⁻¹) in a tube formation assay to assess *in vitro* angiogenesis activity. We found that EVs from MSCs cultured in devices with micropillars spacing of 5, 10, and 20 μm induced a substantial increase in the number of branch points and the total loop count when compared with EVs from cells grown on traditional tissue culture plastic and featureless PDMS devices (i.e., no pillars) (p < 0.05; 3 biological replicates). These results suggest that physical confinement of MSCs can significantly enhance the pro-angiogenic abilities of secreted EVs. Importantly, the observed increase in therapeutic effects was not altered when EVs were isolated using an orthogonal isolation method (TFF), confirming the robustness of the results. Utilization of the physical environment to control MSC EV therapeutic potency is monumental as these are non-invasive tools that can often be precisely controlled and reproduced - a staple for a successful biomanufacturing platform in a highly regulated industry. Further work is currently being completed to understand the underlying molecular mechanisms at work.

1183. Scalable Production of Bioactive Extracellular Vesicles from Induced Pluripotent Stem Cells

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Stem cell-based therapies have been widely investigated as a new frontier in regenerative medicine applications. Recent studies indicate that the therapeutic effects of many of these cell-based therapies, e.g. those involving mesenchymal stem cells (MSCs) can be attributed to their secreted factors such as cytokines, chemokines, and extracellular vesicles (EVs). Of these, EVs - heterogenous, cell-secreted nanovesicles that transport a broad range of proteins, nucleic acids and lipids between cells - stand out for their ability to recapitulate the multifactorial nature of therapeutic stem cells, while also having superior safety profiles due to their small size and lack of replication capability, resulting in reduced blood vessel occlusion potential and lack of tumorigenicity, respectively. However, EVs share a critical shortcoming with cell therapies in the form of limited *ex vivo* expansion of parental cell sources before decreased potency, limiting their scalability and therefore translational potential. One potential solution to circumvent these issues is to utilize multiple donors for therapeutic EV production. However, it has been well documented that donor characteristics including age, sex, and overall genetic differences leads to significant variability in the therapeutic potency of MSCs; in my preliminary work, I have demonstrated that this phenomenon is also observed in their secreted EVs in the form of variable *in vitro* angiogenic (not shown) and anti-inflammatory effects (Figure 1A).

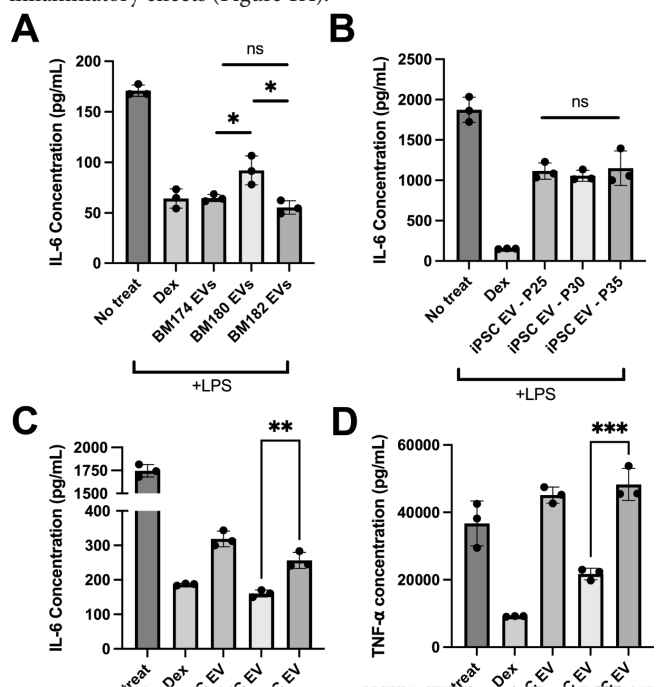


Figure 1: MSC EV anti-inflammatory potency is limited by expansion and donor variability, whereas iPSC EV potency is not (A) In an LPS stimulated mouse macrophage model, bone marrow (BM)-derived MSC EV treatments from different donors (174, 180,

182) decreased the amount of secreted pro-inflammatory IL-6 at varying levels. (B) In the same model, iPSC EVs reduce IL-6 secretion without loss in efficacy with increased passage and (C) at a superior level compared to MSC and iPSCs differentiated into MSCs (iMSCs) (D) while exhibiting the same trend with TNF- α secretion. (* $p < 0.05$, *** $p < 0.001$). Therefore, a self-renewing cell source such as induced pluripotent stem cells (iPSCs) may be a viable option for therapeutic EV production. As iPSCs are self-renewing, a single donor can be utilized as a scalable source for the large-scale production of therapeutic EVs, circumventing issues with donor variability. Differentiation of iPSCs to MSCs is feasible, however, the costly and time-consuming differentiation process may not be necessary for therapeutic EV production. A small number of studies have indicated that iPSC EVs have utility in enhancing tissue repair after myocardial infarction and chronic liver injury; however, no studies have yet directly compared EVs from MSCs and iPSCs nor demonstrated their therapeutic anti-inflammatory effects. In this work, we show that iPSC EVs have enhanced anti-inflammatory properties compared to EVs from other cell types, while also demonstrating that this anti-inflammatory capability is not lost with increased passage (Figure 1B-D). Lastly, we establish that a direct perfusion bioreactor system can be utilized to enhance the production of iPSC EVs, further supporting the use of iPSCs as a scalable manufacturing source for therapeutic EV production.

1184. Rapid and Simplified Process for Manufacturing Multi-Tumor-Associated Antigen Specific T Cells

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MT-401 is a multi-tumor-associated antigen (multiTAA)-specific allogeneic T cell product capable of recognizing multiple targets expressed on the tumor simultaneously, minimizing tumor escape. Currently, MT401 is being used for treatment of AML patients following allogeneic stem-cell transplant in both the adjuvant and active disease settings. **Although MT-401 has shown promising clinical results, the manufacturing process is time prohibitive for cancer patients with rapid disease progression.** Here we demonstrate how additional process improvements streamlined the manufacturing process and resulted in products with superior T cell phenotype and potency, both of which have the potential to enhance clinical responses. The original manufacturing process for multiTAA-specific T cell products is derived from academic studies and begins with the purification of PBMCs from leukapheresis material. Subsequently, dendritic cells (DCs) are matured and pulsed with a pool of exogenous peptides spanning the entire primary sequence of target antigens (Ags). The mature DCs expressing the antigens are co-cultured with T cells in a Gas Permeable Rapid Expansion Device (G-Rex) to stimulate and expand antigen-specific T cells. For MT-401, the following four antigens are used: PRAME, NYESO-1, Survivin and WT1. This 36-day manufacturing process results in products containing an average of 83% CD3+ T cells with a predominantly effector memory T cell phenotype, and an average specificity for 4 tumor Ags of 179 spot-forming units (SFU) per 2e5 cells.

We have now simplified the manufacture of multiTAA-specific T cells and eliminated the need to generate DCs in vitro prior to T cell stimulation. The improved 9-day manufacturing process produces superior T cell products with an average %CD3+ purity of 96%, T cell phenotype showing a uniform distribution of naïve, central memory and effector memory T cells, increased Ag specificity (5-fold), Ag diversity and killing potential. **These process improvements significantly reduced the number of interventions needed during manufacturing, thereby decreasing both the possibility of manufacturing failures and product manufacturing time, which translates to faster patient treatment.** This sophisticated and rapid manufacturing approach has shown to be reproducible regardless of the tumor antigen combination, enabling the extension of this technology to other clinical indications.

1185. Automating Closed System Purification of White Blood Cells for T Cell Therapy Manufacturing

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One of the biggest challenges for cellular immunotherapies is the standardization of cell processing methods, which is crucial for product manufacturing. Here we compared two methods for isolating white blood cells (WBCs) from leukapheresis material in the generation of multi-tumor-associated antigen (multiTAA)-specific T cells. The classical method for separation of WBCs from blood-derived products used by many groups in the field of cell therapy, utilizes centrifugation in density gradient medium such as Ficoll™ and requires careful layering of Ficoll™ and blood product in numerous 50 milliliter conical tubes. **This operator-dependent, time consuming, open process is prone to large losses of valuable starting material, contamination, and variable results which can dramatically impair the manufacturing robustness of cell therapy products.** The Lovo Cell Processing System allows for automated, functionally closed cell processing through spinning membrane filtration without the need for a density gradient medium. The spinning membrane's 4 µm pores enable high WBC recovery, viability, and efficient depletion of platelets. Cell separation using the Lovo resulted in significantly higher yields of WBCs, which was primarily due to greater recovery of lymphocytes, and more specifically CD3+ T cells. The yields of monocytes, B cells and natural killer cells were comparable between the two separation methods. Furthermore, the proportion of CD4+ and CD8+ T cells within the CD3+ T cell population was also comparable. Finally, the quality of multiTAA-specific T cells (in terms of viability, fold expansion, phenotype, and specificity) generated from starting material processed using manual Ficoll™ or the automated Lovo system was comparable. **Implementation of the Lovo allows closing of the manufacturing process, reduction of operator error and variability, as well as increased recovery of valuable starting material, without affecting the quality of the final product.**

1186. Enabling Allogeneic T Cell-Based Therapies - Scalable Stirred-Tank Bioreactor Mediated Manufacturing

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Allogeneic T cells are key immune therapeutic cells to fight cancer and other clinical indications. High T cell dose per patient and increasing patient numbers result in a clinical demand for large number of allogeneic T cells. This necessitates a manufacturing platform that can be scaled-up, while retaining cell quality. Here we present a closed and scalable platform for T cell manufacturing to meet clinical demand. Upstream manufacturing steps of T cell activation and expansion are done in-vessel, in a stirred-tank bioreactor. T cell selection, which is necessary for CAR-T based therapy, is done in the bioreactor itself, thus maintaining optimal culture conditions through the selection step. Platform's attributes of automation and performing the steps of T cell activation, expansion and selection in-vessel, greatly contribute to enhancing process control, cell quality, and to reduction of manual labor and contamination risk. In addition, the viability of integrating a closed, automated, downstream process of cell concentration, is demonstrated. The presented T cell manufacturing platform has scale up capabilities, while preserving key factors of cell quality and process control. This platform could be applied, in principle, not only to donor-derived T cells, but to T cells derived from Pluripotent stem cells (PSCs), facilitating the enabling of "off-the-shelf" PSC-derived cell therapy.

1187. Development of an Optimized Lentiviral Transduction Medium and Process to Manufacture Genetically Modified MSC Working Cell Banks

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Development, RoosterBio Inc., Frederick, MD

Background & Aim Human Mesenchymal stem/stromal cells (hMSCs) are widely used in clinical development and have an excellent safety profile demonstrated in over 1,000 clinical trials, making them ideal candidates for cell-based gene therapies. hMSCs may be genetically engineered to improve their inherent therapeutic properties or to secrete proteins for new applications. A critical challenge, however, is efficiently modifying hMSCs within the context of cGMP manufacturing, especially given the historically low transduction efficiency of primary cells. Here we describe the development of a manufacturing process using a novel medium called RoosterGEM™, optimized for maximum lentiviral transduction to generate working cell bank (WCB) vials of genetically modified hMSCs that could be expanded to a final cell product. **Methods** Various lentiviral promoters, MOI, and transduction processes were investigated for the efficient transduction of human umbilical cord MSCs (hUC-MSCs) and human bone marrow MSCs (hBM-MSCs). Briefly, cells were isolated from fresh bone marrow or cord tissue, expanded in a xeno-free expansion medium (RoosterNourish-XE, RoosterBio) and cryopreserved to create

Master Cell Banks (MCB). Cryopreserved MCBs were thawed and seeded at 3,000 - 5,000 cells/cm². After proliferating for 2 days, hMSCs were transduced with lentiviral vectors expressing Green Fluorescent Protein (GFP) or ZsGreen at MOIs ranging from 2 to 50. 24 hours post-transduction, viral supernatants were aspirated and replaced with RoosterNourish-XF. Transduction efficiencies were assessed by fluorescent microscopy and quantified by flow cytometry. Working Cell Banks (WCBs) of transduced cells were created and further expanded to a final product PDL to monitor protein expression and to characterize MSC Critical Quality Attributes (CQAs) including phenotypic markers (CD90 and CD166), multilineage differentiation, and cytokine secretion (HGF, IL-8, TIMP-1, TIMP-2 and VEGF). **Results & Conclusions** Two hUC-MSC lots and two hBM-MSC lots were efficiently transduced with lentiviral vectors expressing a fluorescent reporter gene using a novel genetic engineering medium, RoosterGEM™. The use of this medium resulted in a 2-5 fold increase in transduction efficiencies above growth medium alone. The technique of Spinoculation in which the virus and cells are centrifuged together did not markedly increase transduction efficiencies. Significant differences were observed in the transduction efficiency of lentivirus from different vendors ranging from 20% to 90%. Although the use of higher MOIs resulted in increased efficiency, MOI as high as 50 showed decreased cell health in some cases. Overall, the use of RoosterGEM™ resulted in WCBs with over 90% transduction efficiency. This novel medium optimizes a critical unit operation of gene modification and provides a useful tool for the generation and expansion of engineered hMSCs for use in clinical therapies.

Clinical Trials Spotlight Symposium

1188. Interim Results from an Ongoing Phase 1/2 Study of Lentiviral-Mediated Ex-Vivo Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I (LAD-I)

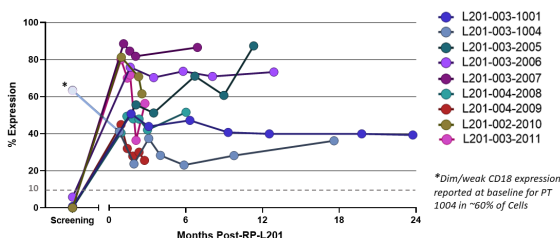
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Background: Leukocyte Adhesion Deficiency-I (LAD-I) is a rare monogenic disorder of neutrophil adhesion resulting from mutations in the ITGB2 gene that encodes for the β 2-integrin component CD18. Severe LAD-I (CD18 on <2% of polymorphonucleocytes [PMNs]) is characterized by severe infections, impaired wound healing, and childhood mortality. Allogeneic hematopoietic stem cell transplant (alloHSCT) is potentially curative; however, efficacy is limited by donor

availability and risk of graft-versus-host disease (GVHD) and graft failure. RP-L201-0318 (NCT03812263), a phase 1/2 open-label trial currently underway, employs autologous CD34+ cells transduced with a lentiviral vector (LV) carrying the ITGB2 gene (cDNA). **Methods:** Pediatric patients \geq 3 months old with severe LAD-I are eligible. HSCs are collected via apheresis after mobilization with granulocyte-colony stimulating factor (G-CSF) and plerixafor and transduced with Chim-CD18-WPRE-LV. Myeloablative therapeutic drug monitoring (TDM) busulfan conditioning precedes RP-L201 infusion. Patients are followed for safety and efficacy (i.e., survival to age 2 and at least 1-year post-infusion, increase in peripheral blood [PB] PMN leukocyte CD18 expression to at least 10%, PB vector copy number [VCN] >0.1 copies/cell, neutrophilia improvement or normalization, decrease in infections/hospitalizations, and resolution of skin or periodontal abnormalities). **Results:** Nine patients (ages 5mos-9yrs) have received RP-L201, with available follow-up of 3 to 24 months. All 9 demonstrated sustained leukocyte CD18 restoration and VCN > 0.1 after infusion. RP-L201 cell doses ranged from 2.8x10⁶ to 10x10⁶ CD34+ cells/kg with a drug product VCN from 1.8-3.8 copies/cell. At 1 year, the OS rate is 100% based on the Kaplan-Meier estimate. In the 4 patients that have been followed up for >1 year after infusion, sustained and stable PMN CD18 expression has been observed. The patient with longest follow-up had ~40% PMN CD18 expression at 24-months (vs. < 1% at baseline), with PB VCN of 1.53 copies/cell. Baseline skin lesions resolved and the patient has not had infections requiring hospitalization despite extensive history of severe infections prior to RP-L201 therapy. The subsequent 8 patients have been followed for 3-18 months, demonstrating PMN CD18 expression of 25.6-86.6%, and stable for each patient. Neutrophilia at baseline has resolved in all subjects after therapy. There have been no new infections characteristic of severe LAD-I post-infusion. The safety profile of RP-L201 has been highly favorable in all subjects with no RP-L201-related serious adverse events. **Conclusion:** RP-L201 confers durable neutrophil CD18 expression and genetic correction and improved clinical course in 9 of 9 patients treated in this phase 1/2 study. Presentation at the meeting will include one-year survival data for the initial 7 of 9 patients.

Figure 1
CD18 Expression in Peripheral Blood Neutrophils after RP-L201 infusion



1189. Safety and Efficacy of SARS-CoV-2-Specific T Cells as Adoptive Immunotherapy for High-Risk COVID-19 Patients: A Phase I/II, Randomized Clinical Trial

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SARS-CoV-2 pandemic poses an urgent need for the development of effective therapies for COVID-19. We here report the feasibility of creating a bank of immediately available off-the-shelf SARS-CoV-2-specific T cells (CoV-2-STs) from convalescent donors and preliminary clinical results of a randomized phase I/II trial (EudraCT 2021-001022-22) using CoV-2-STs in high-risk COVID-19 patients. We prepared ~480 clinical doses of CoV-2-STs from 30 convalescent donors. Peripheral blood mononuclear cells were exposed to pepmixes spanning SARS-CoV-2 antigens (spike/membrane/NCAP) and expanded for 10 days in G-Rex devices supplemented with IL4/7 to produce a median of 6×10^8 T-cells/donor ($2-11 \times 10^8$). The cell products were polyclonal, enriched in CD4+ ($78 \pm 2\%$, CD8+/ $14 \pm 2\%$) cells expressing memory markers and high specificity against SARS-CoV-2 [2428 ± 109 spot forming cells (SFC)/ 2×10^5] and its variants (WT 1873 \pm 481 /alpha 2182 \pm 582 /beta 2177 \pm 624 /delta 1549 \pm 463 SFC/ 2×10^5). At least 1 HLA mediating CoV-2-ST specificity was identified in 29/30 products. Hospitalized COVID-19 patients within 6 days from the symptoms onset with pneumonia, lymphopenia (CD3+ \leq 650/ μ l) and \geq 1 elevated biomarker (D-dimers, ferritin, CRP, LDH) were enrolled and followed for 8 weeks. Patients were evaluated for recovery by the WHO 8-point Ordinal Scale (OS). Safety was demonstrated during phase I where 6 patients received dose escalated (15×10^6 , 2×10^7 /m²) CoV-2-STs sharing at least 1 HLA mediating specificity. In phase II, 90 randomized (2:1) high-risk patients were enrolled; 57 received the standard of care (SoC) plus partially HLA-matched CoV-2-STs and 30 received the SoC (control arm). Three

withdrew consent and 1 from the CoV-2-ST arm was allocated to control arm as referred to ICU prior to receiving CoV-2-STs. On day 60 (d60) the add-on treatment resulted in 51% lower risk of mortality than SoC alone (24.6% [14/57] vs 50.0% [15/30]; risk ratio (RR): 0.49; p=0.016) with crude hazard ratio (HR) 2.42 (1.17, 5.05; p=0.018) in favor of CoV-2-STs. The benefit on survival was confirmed by multiple analysis after adjustment for confounding factors [HR:2.16 (1.02, 4.60); p=0.04]. On d30, 67% had recovered (OS \leq 3) in the CoV-2-ST arm vs 37% in the control arm (RR, 1.82; p=0.02) with HR 0.48 (0.24, 0.94; p=0.03). CoV-2-ST-treated patients were more likely to recover by d30 even after adjustment for confounding factors [HR:0.46 (0.23, 0.92); p=0.03]. Overall, off-the-shelf immunotherapy with CoV-2-STs can serve as a safe and effective treatment in a real world environment for severe COVID-19.

1190. Autologous Cell & Gene Therapy for the Therapeutic Targeting of Immune Payloads to the Solid Tumor Microenvironment: Preliminary Results of the TEM-GBM Study

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The tumor microenvironment (TME) dynamically regulates, in a large variety of cancers, disease progression influencing clinical outcome of many different therapeutic approaches including ICI and CAR-T cells. A shared feature of the TME is the presence of cancer-promoting cells like macrophages. We developed a genetically modified cell-based autologous hematopoietic stem cell platform designed to selectively deliver immunostimulatory cytokines into the TME through Tie-2 expressing monocytes. As a consequence, many tumor-associated macrophages, that are well recognized to foster cancer progression, are reprogrammed toward an immune-stimulating and tumor-suppressive phenotype. This strategy is currently evaluated in a Phase 1/2a clinical trial which delivers IFN α within the TME (NCT03866109). As of Jan 2022, 3 escalating doses of Temferon (from 0.5 up to 2.0×10^6 cells/kg) were tested across 15 pts affected by newly diagnosed unmethylated MGMT Glioblastoma (GBM) assigned to 5 cohorts. In all the pts we observed a rapid engraftment and a fast recovery from the non-

myeloablative conditioning regimens (median engr. Neu D+13, PLT D+14). No DLTs were identified. Temferon-derived differentiated cells, as detected by the presence of VCN, were found at increasing proportions in PB and BM, reaching up to 30% at 1 mth for the highest dose cohorts tested and persisting up to 18 mths (longest time tested), albeit at lower levels. Median OS is 14 mths from surgery with maximum follow-up of 27 mths. SAEs were mostly attributed to conditioning chemotherapy (e.g., infections) or disease progression (e.g., seizures). 1 SUSAR (persistent GGT elevation) has occurred. Of the 15 pts treated so far, 4 pts belonging to low dose cohorts underwent 2nd surgery. Recurrent tumors were analyzed to assess for the presence of BM-derived transduced cells and to capture any potential change in the immune compartment triggered by Temferon. Homing of transduced cells from BM to the tumor site was demonstrated by the significant content of gene-marked cells in the specimens collected from 3 out of the 4 analyzed pts. A preliminary analysis by single cells RNA seq performed on the myeloid compartment (CD45+) purified from the TME of Temferon-treated pts compared to recurrent tumors belonging to GBM pts treated as per the current standard of care (RTx+TMZ), highlighted a clear Temferon signature as defined by the induction of markers of IFN α responses and macrophage repolarization. We believe that our interim results provide initial evidence of Temferon's potential to modulate the TME, as predicted by preclinical studies, which will be confirmed with longer follow up in the higher dose cohorts.

1191. Lentiviral Gene Therapy with Low Dose Busulfan for Infants with X-Linked Severe Combined Immune Deficiency (XSCID) Results in the Development of a Normal and Sustained Immune System: Interim Results of an Ongoing Phase I/II Clinical Study

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XSCID is a rare, life-threatening disorder due to mutations in the gene encoding the cytokine receptor common γ -chain (IL2RG), which is necessary for development of T and NK cells and functional B cells. Treatment options for patients with XSCID, who lack a matched sibling donor for transplant are limited, and early gene therapy trials restored only T cells and were associated with

iatrogenic leukemia. We developed a gene therapy approach utilizing a lentiviral (LV) vector together with reduced exposure busulfan conditioning for newly diagnosed infants with XSCID (NCT01512888). We have treated 23 infants with XSCID at a median age of 3 months (range: 2.4-13.8) with a median follow up of 2.4 years (range: 1.4 months to 5.4 years). To our knowledge, this presents the largest cohort of infants with XSCID, who received LV gene therapy with the longest follow-up. Transduced autologous bone marrow CD34⁺ cells were generated for all patients with a median vector copy number (VCN) of 0.81/cell (range: 0.16-1.81), and a median CD34⁺ cell dose of 9.61x10⁶/kg (range 4.4-18.95). Prior to the infusion of cells, patients received busulfan targeted to a cumulative area-under-the-curve (cAUC) of 22 mg*hr/L. All had hematopoietic recovery. Seventeen of 18 patients with a follow up of >6 months have achieved robust immune reconstitution [median CD3⁺ 2,545/uL (range: 922-4,321), CD4⁺ 1,568/uL (range: 436-3,556), CD4⁺/CCR7⁺/CD45RO⁺ 1,416/uL (range: 298-3,307)]. In these 17 patients, T cells matured appropriately as assessed by normal T cell receptor excision circles (TRECs) and TCR $\nu\beta$ repertoire diversity and were functional as judged by phytohemagglutinin activation. Immunoglobulin replacement has been discontinued in 15 patients, and 12 have been immunized, with 2 more begun immunizations. Substantial multilineage engraftment occurred in all patients and was sustained over time as judged by VCN analysis in T, B, NK, and myeloid cells separated from peripheral blood. This analysis included 55 samples of 14 patients with ≥ 1.5 years of follow (VCN sample range: 1.5 to 5 years). All patients are alive and 20 patients with a follow-up >4 months i) recovered from pre-existing infections, ii) are off protective isolation and prophylactic antimicrobials, and iii) have normal growth velocity. Detailed integration site analysis for the first 10 patients revealed a similar integration site pattern in 9 of them. Identified integration site hotspots were consistent with previous reports for LV vectors, and we observed no evidence of clonal expansion. In conclusion, LV gene therapy for XSCID using low dose busulfan conditioning has been well tolerated and results in the development of a durable, functional immune system without evidence of malignant transformation. Thus, our approach presents a promising alternative to current treatment modalities.

1192. Stable Hemostatic Correction and Improved Hemophilia-Related Quality of Life: Final Analysis from the Pivotal Phase 3 HOPE-B Trial of Etranacogene Dezaparvovec

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Introduction: Etranacogene dezaparvovec (formerly AMT-061), an investigational gene therapy for hemophilia B (HB), comprises an

adeno-associated virus serotype 5 (AAV5) vector, containing a codon-optimized Padua variant human factor IX (FIX) transgene under the control of a liver-specific promoter. **Methods:** Adult participants (n=54) with HB (baseline FIX \leq 2%), with (n=33) or without (n=21) pre-existing AAV5 neutralizing antibodies (NAbs), were treated in the Phase 3, open-label, single-dose, single-arm, HOPE-B trial (NCT03569891) with 2×10^{13} gc/kg etranacogene dezaparvovec, following a \geq 6-month lead-in period of FIX prophylaxis. Adverse events (AEs) were recorded throughout. FIX activity, annualized bleed rate (ABR) and FIX consumption were assessed over a \geq 52-week uniform observation period from the Month 6 study visit to Month 18 post-vector, a period characterized by stable FIX expression without potentially confounding corticosteroid use. Health-related quality of life (HRQoL) was assessed with the hemophilia-specific Hem-A-QoL instrument over the lead-in and 6 and 12 months after etranacogene dezaparvovec. Repeated measures linear mixed models estimated the difference in scores before and after gene therapy. A one-sided p-value \leq 0.025 for the post-treatment - lead-in period was considered statistically significant. **Results:** After dosing, 37 participants experienced 92 treatment-related AEs, of which 74 (80.4%) were mild. A serious AE of hepatocellular carcinoma was determined by independent molecular tumor characterization and vector integration analysis to be unrelated to vector. Mean FIX activity was 39.0 IU/dL (\pm 18.7; 8.2, 97.1) (standard deviation; min, max) at Month 6 and 36.9 IU/dL (\pm 21.4; 4.5, 122.9) at Month 18. All participants who received a full vector dose, with undetectable AAV5 NAbs or with pre-dosing NAbs up to a titer of 678, were able to discontinue FIX prophylaxis, resulting in an overall 97% reduction in mean unadjusted annualized FIX consumption. A single participant with NAb=3212 did not express FIX Padua. Compared with the \geq 6-month lead-in period (ABR 4.19), 52-week adjusted ABR for all bleeds during Months 7-18 (ABR 1.51) was reduced by 64% (p=0.0002), demonstrating statistical superiority over FIX prophylaxis, thereby achieving the pre-determined primary endpoint of the trial. Significant HRQoL improvement was demonstrated by the Total Score (LS means -5.50; p<0.0001; percentage improvement 21.5%) as well as several individual domains of the Hem-A-QoL (TABLE). **Conclusion:** Following a single dose of etranacogene dezaparvovec, participants experienced a stable and durable increase in mean FIX activity into the near-normal range at 18 months and hemostatic protection, achieving the HOPE-B primary efficacy endpoint.

HRQoL Results: Hem-A-QoL						
Dimension/ Domain	Significance	Mean in lead-in FIX pro- phylaxis period (SE)*	Mean in post- gene therapy treat- ment period (SE)*	Mean differ- ence between treat- ment periods (SE)*	p- value**	Percent- age improve- ment
Total	Composite (derived from 10 domains) Hemophilia-specific items assess overall HRQoL	25.56 (2.072)	20.06 (2.054)	-5.50 (0.972)	<0.0001	21.5%
Feelings	Current emotions associated with having hemophilia	20.61 (2.838)	11.19 (2.790)	-9.42 (1.938)	<0.0001	45.7%
Treat- ment	Burden of hemophilia treatments	25.24 (1.857)	10.36 (1.804)	-14.88 (1.789)	<0.0001	59.0%
Work/ School	Impact of hemophilia and pain on participation and focus	17.34 (2.555)	12.35 (2.534)	-4.99 (1.825)	0.0036	28.78%
Future	Concerns about how hemophilia will affect life plans	30.94 (2.753)	25.92 (2.712)	-5.02 (1.736)	0.0023	16.22%

* Scores range from 0 to 100; higher scores indicate lower quality of life.
 ** Endpoints not adjusted for multiplicity.
 FIX, Factor IX; HRQoL, health-related quality of life; SE, standard error.
 Von Mackensen S, et al. Haematologica 2005; 90:115-6 PO290

1193. IGNITE DMD Study of SGT-001 Microdystrophin Gene Therapy for Duchenne Muscular Dystrophy: Long-Term Outcomes and Biomarker Update

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IGNITE DMD is a Phase I/II study of SGT-001, an AAV9-mediated microdystrophin gene therapy in development for the treatment of Duchenne muscular dystrophy (DMD). DMD is a progressive, lethal form of muscular dystrophy caused by the absence of the protein dystrophin, which is responsible for maintaining muscle structure and function through successive bouts of contraction and relaxation. SGT-001 microdystrophin is a unique, rationally designed form of dystrophin that fits within the packaging capacity of an AAV while maintaining critical domains for protein functionality, such as the neuronal nitric oxide synthase (nNOS) binding domain. Localization

of nNOS to the muscle membrane through the nNOS binding domain of dystrophin has been associated with increased circulating nitric oxide levels, and in preclinical studies has been shown to protect against exercise-induced muscle ischemia and preserve muscle function. Patients currently enrolling in the IGNITE DMD study are administered a systemic infusion of SGT-001 at a dose of 2E14 vg/kg. As of a January 2022 data cutoff, 9 total subjects have been administered SGT-001, with 6 of those subjects in the 2E14 vg/kg group. To date the most common treatment emergent adverse events have been nausea, vomiting, fever, thrombocytopenia, and headache. Three subjects experienced SAEs associated with complement activation within the first weeks following dosing, which have all resolved without sequelae. The ninth subject was dosed uneventfully in November of 2021 utilizing an updated risk mitigation strategy. All patients continue to undergo long-term follow up and are doing well up to approximately 4 years post-dosing. We now present updated long-term outcomes data and additional interim biomarker data inclusive of subjects more recently enrolled in the study. At the 2-year post-dosing timepoint, subjects in the 2E14 vg/kg group show stabilization or improvement in motor function (North Star Ambulatory Assessment, 6-Minute Walk Test), pulmonary function (Forced Vital Capacity [FVC] % predicted [%p], peak expiratory flow [PEF %p], and PROMs (Pediatric Outcomes Data Collection Instrument [PODCI]) compared to baseline assessments and against expected natural history declines over this duration. Previously presented biopsy results at 12-24 months post-dosing showed continued expression of microdystrophin, restored membrane localization of β -sarcoglycan and nNOS, and only very mild active dystrophic pathology, compared to interim day 90 biopsy assessments and baseline. Interim results from more recently dosed patients will also be discussed. These preliminary data continue to suggest a positive benefit-risk profile for SGT-001 warranting continued evaluation for the treatment of DMD.

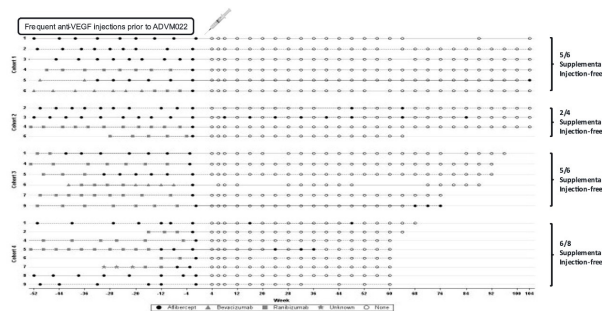
1194. The OPTIC Study of Intravitreal Gene Therapy with ADVM-022 for Neovascular AMD (nAMD): The Role of Neutralizing Antibodies
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Anti-VEGF therapies revolutionized the treatment of nAMD yet real world evidence suggest that long term outcomes decrease over time due to the burden of frequent intravitreal (IVT) injection. ADVM-022, a gene therapy utilizing a novel proprietary AAV.7m8 capsid engineered by directed evolution to transduce retinal cells following IVT administration includes a strong ubiquitous promoter that delivers continuous and durable expression of aflibercept up to 2 years in patients. Previous reports from the ongoing phase 1 OPTIC study (NCT03748784) have demonstrated a single IVT injection of ADVM-022 (AAV.7m8-aflibercept) reduces the need for frequent anti-VEGF injections and maintains visual and anatomical outcomes in treatment experienced nAMD patients. The impact of baseline levels of neutralizing antibodies (NABs) to AAV.7m8 on efficacy and safety outcomes are reported. OPTIC is an open-label, multi-center, dose-ranging study in treatment-experienced nAMD patients receiving a single IVT injection of ADVM-022 at 6E11 vg/eye (Cohort C1 n=6; C4 n=9) or 2E11 vg/eye (C2 n=6; C3 n=9). NABs to AAV-7m8 inclusion criteria were initially set at a titer of <1:5 within 6 months prior to

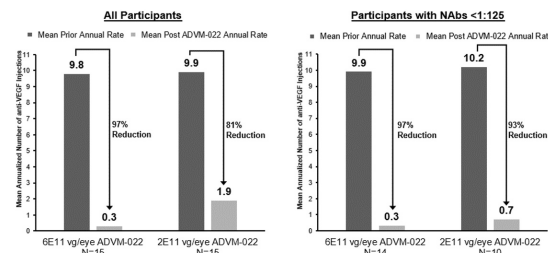
ADVM-022 for C1 and then expanded to $\leq 1:125$ for C2-4. The impact of baseline NABs on efficacy, aflibercept protein expression levels, central subfield thickness (CST) fluctuations and safety outcomes was evaluated. As of August 27, 2021, median follow-up times were 104 weeks (C1, C2), 92 weeks (C3) and 60 weeks (C4). In C2-4 8/9 (89%) of patients receiving 6E11 vg/eye ADVM-022 and 10/15 (67%) of 2E11 vg/eye patients had baseline NABs titer to AAV.7m8 <1:125. Overall, 12/15 patients receiving 6E11 and 8/15 receiving 2E11 vg/eye ADVM-022 remained supplemental anti-VEGF injection free and mean annualized anti-VEGF injection frequency was reduced by 97% (6E11; C1, C4) and 81% (2E11; C2, C3). In patients with baseline NABs <1:125, the reductions in mean annualized anti-VEGF injection frequency were 97% and 93% in the 6E11 and 2E11 vg/eye groups, respectively. Patients with NABs <1:125 at baseline showed more robust aflibercept protein expression, sustained improvements in CST with fewer fluctuations compared with patients with baseline NABs $\geq 1:125$. No correlation between NABs titer and inflammation was observed. ADVM-022 continues to be well tolerated in the nAMD population. While both 6E11 and 2E11 vg/eye doses showed efficacy, patients with baseline NABs <1:125 were more likely to show robust aflibercept protein expression, sustained improvements in CST with minimal fluctuations, and were less likely to require supplemental aflibercept injections. Baseline NABs were not associated with occurrence nor duration of inflammation. Data from OPTIC support the concept that sustained aflibercept expression following a single IVT injection of ADVM-022 may provide a long-term treatment option for patients with nAMD.

Reduction in need for anti-VEGF injections following a single IVT injection of ADVM022 in nAMD patients with baseline NABs <1:125



Data cut: August 27, 2021

93% reduction in annualized anti-VEGF injections in nAMD patients receiving 2E11 vg/eye ADVM-022 with baseline NABs <1:125



Annualized rate (Prior) = (number of IVTs in 12 months prior to ADVM-022) / (days from the first IVT in the past 12 months to ADVM-022 / 365.25)
 Annualized rate (Post) = (numbers of aflibercept IVTs since ADVM-022) / (days from ADVM-022 to the last study follow-up / 365.25)

Data Cut: August 27, 2021

Novel AAV Capsids for the Brain, Eye and Kidney

1195. Machine-Guided Design Reveals AAV Variants Efficient in Transducing NHP Retina After Intravitreal Delivery

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AAVs are the vector of choice for gene therapies in multiple organ systems. However, next-generation capsid variants are needed for increased transduction efficiency and cell type specificity, lower immunogenic responses, NAb evasion, and more scalable manufacturability towards clinical applications. The optimization of AAV capsid properties for improved gene delivery can be accelerated through the use of machine learning models trained on large sequence-property datasets. Efficient discovery relies on selection of exploratory and deep search models, computational and experimental methods that provide high quality property measurements, and the ability to create controlled variation at any region of interest within the capsid sequence. These capabilities were optimized for application to the discovery of capsids with improved transduction of the retina following intravitreal route of administration. A repertoire of computational models capable of designing variants at high edit-distance (>10) from a starting sequence were used to generate a library of capsid variants exploring across the cap gene of a WT AAV serotype. The variants were barcoded to enable removal of DNA synthesis artifacts and quantification of cells transduced per variant separate from overall barcode expression. Positive control WT serotypes and synthetic variant benchmarks identified from the literature were included for comparison, while negative production and transduction controls were included in the library to further assess data quality. Vector libraries were injected intravitreally, intracamerally, and intravenously into cynomolgus macaques. After 4 weeks in-life, transduction efficiencies were measured by NGS of expressed vector genome barcodes and compared to WT variants in the library, with normalization to variant abundance within the vector preparation. To assess efficiency of retinal transduction, measurements were obtained in both eyes of two animals across retinal regions. Data quality was ascertained through the segregation of negative controls and correlation of measurements between tissue replicates and animals. Variants with 20-60x improved retinal transduction rates relative to WT controls were discovered. Interestingly, some improved retina transducers also displayed improved transduction of the trabecular meshwork following intracameral administration, but did not display enhanced transduction of the liver following IV administration. A subset of variants with improvements specifically in peripheral retina or macula transduction were also identified. In addition, the library was assessed for other relevant parameters, such as productivity of individual variants, transduction efficiency in non-ocular tissues,

and biodistribution in ocular and non-ocular tissues. Models used to design the library substantially outperformed random mutagenesis in generating viable variants at all edit-distances. This comprehensive sequence-property dataset was used to train new machine learning models and to design second generation capsid libraries. Further, the improved functional properties were validated in separate lower throughput experiments. In conclusion, these results demonstrate the power of machine learning models in the design of AAV variants with improved clinically relevant properties.

1196. A Newly Evolved AAV Variant Enables Potent Gene Transfer in Kidneys of Multiple Species

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Kidney diseases are estimated to affect 37 million people in the US, with very limited therapeutic options. Many kidney diseases have underlying genetic etiologies that may be amenable by gene therapy, signifying the crucial need for a kidney-targeted gene delivery vehicle. Despite the success of adeno-associated virus (AAV) as a gene delivery vehicle, gene transfer to kidneys has remained an unaddressed challenge. Through cross-species evolution, we recently developed a novel AAV variant, AAV.cc47 that demonstrated broad tissue tropism similar to AAV9, but markedly improved transduction efficiency. Here, we report that AAV.cc47 transduces murine kidneys following IV dosing with high efficiency. Specifically, we observed robust transduction in different segments of the nephron compared to AAV9, in particular, the proximal tubules as corroborated by immunostaining with lotus tetragonolobus lectin (LTL), and the glomerulus by immunostaining with synaptopodin. In contrast, AAV9 only transduced glomerular structures following IV administration. AAV9 and AAV.cc47 packaging either self-complementary or single-stranded genomes encoding Cre recombinase were then evaluated in the Ai9 fluorescent reporter mouse model following IV dosing. Robust Cre-mediated recombination was observed in the kidney interstitium and glomerulus independent of cassette composition for AAV.cc47 treated animals compared to AAV9. Further, preliminary results suggest AAV.cc47 is capable of improved transduction in cortical and medullary regions compared to AAV9 in the kidneys of cynomolgus macaques after IV dosing. Transduction profiles of the two capsids in human kidney organoids are currently being assessed and will be presented as well. The cross-species evolved variant, AAV.cc47 represents a promising capsid for potential application in kidney gene transfer and genome editing applications.

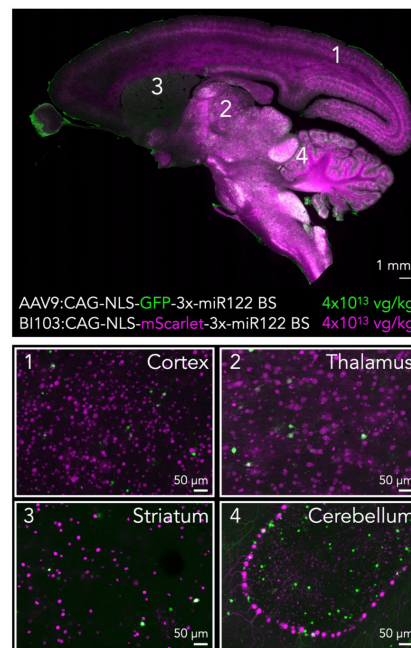
1197. A Promising New Family of Peptide-Modified AAV Capsids for Gene Delivery to the Central Nervous System in Non-Human Primates

Ken Y. Chan¹, Simon Pacouret¹, Qin Huang¹, Qingxia Zheng¹, Charles Vandenburg¹, Jason Wu¹, Pamela Brauer¹, Qiangge Zhang², Isabelle Tobey¹, Carolyn Wu², Albert T. Chen¹, Velina Kozareva¹, Guoping Feng^{1,2}, Benjamin E. Deverman¹

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The development of gene therapies for neurodevelopmental and neurological disorders has been constrained by the inability to efficiently deliver genes throughout the central nervous system (CNS). Several studies have reported engineered AAV9 capsids, such as the AAV-PHP.B family, that are capable of highly effective gene transfer throughout the CNS after intravenous (IV) administration in adult mice. However, these capsids have not shown the same degree of enhanced transduction when tested in other species, including non-human primates. In a screen of 7-mer modified AAV capsids for capsids that more efficiently transduce cells in the CNS of adult marmosets after IV administration, we identified a family of capsids, defined by a shared sequence motif, that was enriched relative to AAV9 in the brain. One of these candidates, AAV-BI103, was chosen for individual validation. Based on biodistribution, AAV-BI103 was >7-fold more efficient at delivering genes to the primate brain than AAV9 when co-administered in the same adult animal. Remarkably, AAV-BI103 mediated transgene expression in approximately half of the cortical neurons in the visual cortex, the majority of neurons in the thalamus, and most of the cerebellar Purkinje cells (Figure 1). In contrast, AAV9 mediated transduction of less than 10% of neurons in the same populations. We also observed efficient transduction by AAV-BI103 in the spinal cord, including within motor neurons. Our data suggest that AAV-BI103 and related capsids represent promising new candidates for CNS gene delivery in primates.

Figure 1. BI103 outperformed AAV9 when administered intravenously to an adult marmoset. AAV9:CAG-NLS-GFP-3x-miR122-BS and BI103:CAG-NLS-mScarlet-3x-miR122-BS were intravenously administered to an adult marmoset at 4×10^{13} vg/kg and reporter expression was assessed after 21 days.



1198. Directed Evolution of AAV9 Peptide Display Libraries Identifies a Family of Cross-Species Variants with Enhanced Brain Tropism in Non-Human Primates and Mice Following Systemic Administration

Tyler Moyer, Jiangyu Li, Dan R. Laks, Matthew A. Child, Katherine Tyson, Rupinderjit Kaur, Xiao-Qin Ren, Ambreen Sayed-Zahid, Michael Grannon, Charlotte Hiu-Yan Chung, Nilesh Pande, Jeff Thompson, Wei Wang, Samuel Hasson, Michael Hefferan, Mathieu Nonnenmacher

Voyager Therapeutics, Cambridge, MA

A challenge in Adeno-Associated Virus (AAV) capsid engineering is the ability to evolve novel capsid variants that display enhanced tropism in clinically relevant tissues across diverse animal models. The central nervous system (CNS), in particular, presents unique difficulties to systemic administration of gene transfer vectors due to inefficient translocation across the Blood-Brain Barrier (BBB). Recent efforts to engineer BBB-crossing AAV variants have mostly focused on modifying the Variable Region VIII (VR-VIII) on the surface of AAV9. In order to diversify our engineering strategies and identify novel regions of the capsid that could better unlock BBB penetration and CNS transduction, we utilized our RNA-driven TRACER capsid evolution platform to generate a pooled, barcoded library to test the viability and transduction of AAV9 capsids harboring random peptide insertions at all possible positions on the capsid surface. When applying this library to cultured cells from diverse origins, several regions of

the capsid were found to tolerate peptide insertions without major detrimental effects on virus viability or infectivity. Among others, the VR-IV surface loop, which forms protruding spikes surrounding the 3-fold symmetry axis, showed a high tolerance to peptide insertion at multiple positions. We generated a series of peptide insertion libraries at multiple positions across VR-IV and performed two rounds of RNA-driven biopanning in cynomolgus macaque brain via intravenous dosing. We then synthesized a focused library of 1500 top candidates for a next generation sequencing (NGS)-based transduction assay in macaques and in C57Bl/6 and BALB/c mouse strains. A family of capsids, defined by a distinct consensus motif, emerged as the top variants capable of increased BBB-penetrance in both primates and rodents. The top variant achieved brain transduction levels 60-fold over AAV9 in macaques and 50-fold over AAV9 in both mouse strains in the NGS-based assay. Furthermore, our analysis suggests that some variants from this capsid family display significant detargeting from the dorsal root ganglia (DRG) and from the liver. When injected intravenously in mice, the top variants showed preferential transduction of cells with a glial morphology, which was further confirmed using single-cell RNA-seq and co-staining experiments. Our work identified a new family of AAV9-derived capsids capable of strong CNS transduction and showing equivalence across primates and rodents, increasing their potential for clinical development. The glial tropism of these vectors may offer a unique potential for treatment of neurologic or neurodegenerative diseases where the use of a non-neurotropic capsid may be advantageous.

1199. Novel AAV Capsids for Intravitreal Delivery: Identifying and Characterizing Novel AAV Variants in Non-Human Primates

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Purpose: Adeno-associated viruses (AAVs) have emerged as the main gene delivery vehicle for the treatment of numerous diseases, including inherited retinal dystrophies. For ocular indications current gene therapy approaches rely largely on the use of subretinal injection to target photoreceptors. While subretinal delivery results in high transduction rate at the site of injection, there is a substantial risk of causing further damage to an already delicate diseased retina. With intravitreal injection (IVT) the AAV is delivered in the vitreous chamber, allowing the vectors to spread to the entire retina. However, to reach the deeper layers of the retina the AAV must first cross the inner limiting membrane (ILM), a known physical barrier for AAV penetration. We completed multiple rounds of directed evolution in canine to discover next-generation capsids with enhanced transduction profile following IVT injection. Here we characterize 4 newly identified capsids with greatly improved tropism for retinal cells. **Methods:** Top performing AAV variants were identified by NGS analysis of fold-enrichment over multiple rounds of directed evolution in dogs. Top variants and AAV2.7m8 were packaged into

AAV encoding CAG-ChrimsonR-GFP and administered IVT to non-human primates at doses ranging from 1.5E+10vg/eye to 5.0E+11vg/eye. ChrimsonR-GFP expression and ocular health were monitored weekly using scanning laser ophthalmoscopy (cSLO) and slit-lamp bio microscopy respectively, for the duration of the study (8 weeks). At the end of the study, eyes were removed and processed for histological examination. 100µm agarose sections of the central and peripheral retina were obtained and direct GFP fluorescence was analyzed by confocal microscopy. In addition, serum was collected before injection and at the end of the study to monitor the immune response to the capsid. **Results:** We identified 4 novel capsids with outstanding retinal transduction profile. Our four AAV2-based capsids are capable of efficiently transducing all retinal layers from IVT injection, resulting in broad expression in ganglion cells, bipolar cells, Muller cells and photoreceptors in 2 NHP species, Cynomolgus Macaques and African Green Monkeys. Strong GFP signal at the foveal ring was visible on cSLO as early as 2 weeks post injection, at all doses tested. Expression continued to increase over the next 4 weeks before plateauing, at which time we observed remarkable GFP signal in the central and peripheral retina. Minimal uveitis and antibody response were observed indicating a favorable immunogenicity profile for all 4 capsids. Histological analysis confirmed the high transduction rate of retinal ganglion cells, with >90% of RGC expressing GFP in the central retina and a more moderate transduction of peripheral cells. Importantly significant transgene expression was also observed in bipolar cells and photoreceptors demonstrating the ability of these capsids to efficiently penetrate the deeper layers of the retina at clinically meaningful doses. The safer approach of intravitreal injections combined with our optimized AAV capsids would significantly increase the ability to treat retinal diseases and will help improve patient's outcome.

1200. Identification and Characterization of an AAV9-Based Engineered Capsid Variant Capable of Mediating Enhanced Transcription in the Central Nervous System of Non-Human Primates and Rodents

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Adeno-associated viral (AAV) vectors represent a promising therapeutic modality for the treatment of many diseases. For a number of these diseases affecting the central nervous system (CNS), variants with improved function following delivery to the CNS are desired. To identify an improved AAV9-based capsid, we administered a 118-member, barcoded library of AAVs ("PAVE118", includes NAV[®] vectors, engineered variants, and reference serotypes) to NHPs (n=3) or mice (n=5) either by direct intraparenchymal (IPa) delivery (intraputamenal delivery in NHPs, intraatrial delivery in mice) or by intracerebroventricular (ICV) delivery. DNA and RNA were extracted from regions of interest (ROIs) and analyzed by next-generation sequencing to determine the relative abundance of each species in the library as well as by qPCR to determine absolute biodistribution.

We identified an AAV9 variant with an engineered homing peptide inserted after residue S454, referred to as BC029, capable of achieving significantly higher transgene expression than AAV9, AAV5, and AAV2, which have been used clinically for direct delivery to the CNS. Following IPa PAVE118 delivery to NHPs, at ROIs at/near the injection site (caudate, putamen), BC029 produced more transcript copies than AAV9 (3-10 fold), AAV5 (13-84 fold), and AAV2 (50-200 fold). This increase was not due to a corresponding increase in GC/ug, as BC029 GC/ug were equivalent to AAV9 and only 2-fold improved over AAV5, indicating that BC029 has an improved RNA/DNA ratio when compared to both AAV9 (4-fold) and AAV5 (10-fold). Additionally, BC029 achieved a 9-45 fold improvement in GC/ug and a 3-10 fold improvement in RNA:DNA over AAV2 in these ROIs. BC029 demonstrated similar properties in ROIs projecting into the striatum (substantia nigra, thalamus, and cortex); in these ROIs, AAV9-P9 produced, on average, 10-fold more RNA than AAV9 from ~50% fewer GCs, again indicating increased transcriptional activity following BC029 transduction. These findings translated to mice, as we found higher BC029 expression in striatum, thalamus, and cortex than AAV9, AAV5, and AAV2. Analysis of RNA following PAVE118 ICV delivery to mice revealed that BC029 produced, on average, 2.5-15 fold more RNA than AAV9 from equivalent GC in cortex, striatum, and hippocampus. While RNA analysis is in progress, analysis of DNA from NHPs receiving PAVE118 ICV similarly indicated that, on average, BC029 and AAV9 GC/ug were equivalent in the cortex, hippocampus, caudate, cerebellum, and hypothalamus. Therefore, evaluation of CNS ROIs in both NHPs and mice by these routes of administration suggests that BC029 has an equivalent tropism to AAV9 within the CNS. However, after ICV delivery in both species, BC029 appears to have altered tropism in the periphery, delivering fewer GCs to liver (4-16 fold in mice, 5-fold in NHPs) and heart (1.5-fold in NHPs) than AAV9, suggesting that BC029 may have an improved off-target peripheral transduction profile. As these data collectively suggest that BC029 may be a more potent vector than AAV9 in the CNS with a more favorable peripheral tropism that translates well across species, we initiated a number of ongoing follow-up studies, including AAV9 vs BC029 single vector comparison by IPa and ICV delivery in mice as well as an AAV9/BC029 multi-fluorophore study in NHPs, administering by IPa delivery to the putamen or to the hippocampus. The upcoming analysis from these studies will further support the use of BC029 as an improved AAV variant for delivery to the CNS.

delivery may suffer from low production yields, poor specificity, or inconsistent tropism enhancements across species, which hinders clinical translation. Here, we used machine learning (ML) to guide the simultaneous engineering of capsids with multiple desirable traits; this is in contrast to the traditional approach of screening massive capsid libraries for one trait at a time and then optimizing screen hits for other traits. We created a novel "Fit4Function" synthetic AAV capsid library that evenly samples the sequence space of capsids with high production fitness. The Fit4Function library has reduced production and amino acid bias, which was instrumental for deriving generalizable ML models that accurately predict multi-trait capsid variants (**Figure 1A**). As a proof-of-concept, we sought to develop capsids with enhanced cross-species hepatocyte transduction. While previous efforts have generated capsids with improved human hepatocyte transduction (e.g., LK03, which is now being evaluated in clinical trials), their species-specific tropisms make in vivo testing in preclinical disease models challenging. We screened the Fit4Function library using five liver tropism assays: binding and transduction of human liver epithelial and hepatocellular carcinoma cell lines and liver biodistribution in C57BL/6J mice. With the ML models, we screened 10 million randomly generated capsid variants in silico and selected 30,000 diverse variants predicted to have enhanced liver phenotypes while maintaining high production fitness. We synthesized and screened these multi-functional variants across the same production fitness and liver function assays. More than 90% of the liver multi-function variants met or exceeded all six of the pre-defined selection criteria (**Figure 1B**). We demonstrate that our ML-driven sequence-to-multi-function approach can be effectively leveraged to predict AAV capsids that exhibit high production fitness alongside multiple traits relevant to gene therapy.

1201. Fit4Function: A Machine Learning-Guided Approach for Systematic Multi-Trait AAV Capsid Engineering

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Systematically identifying AAV capsid variants optimized for multiple desirable traits remains a key challenge for the development of next generation gene therapy vectors. Capsids selected for targeted gene

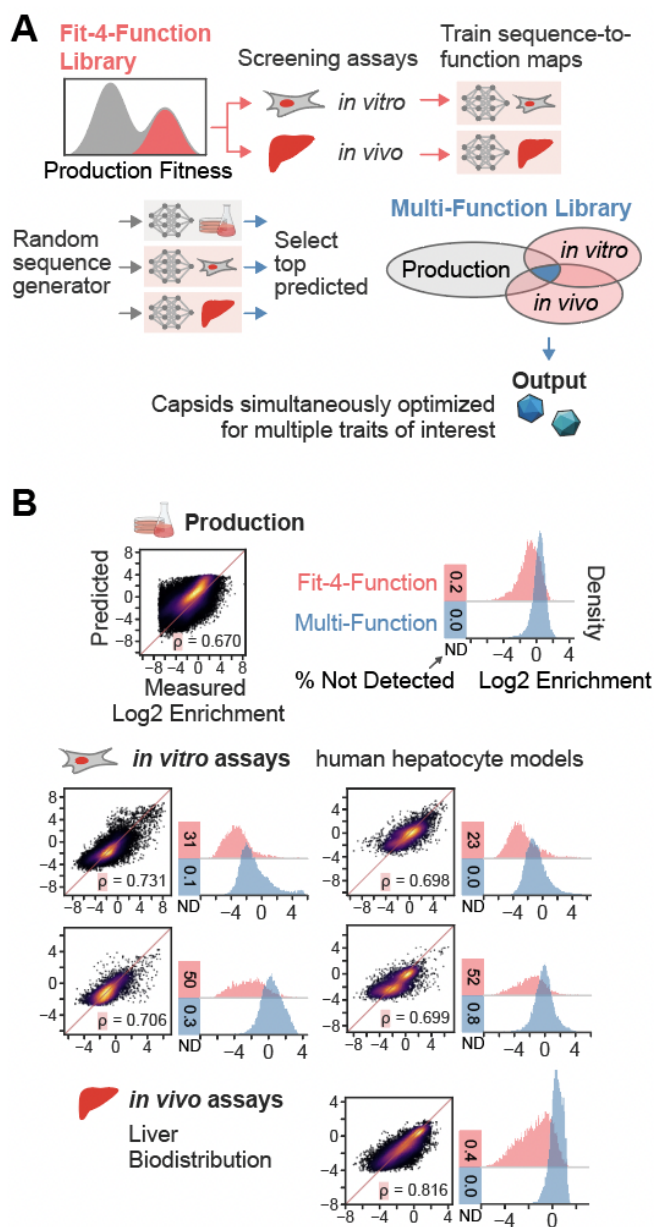


Figure 1. Fit4Function libraries enable ML-guided optimization of AAV capsids with multiple enhanced traits. (A) The Fit4Function library is produced and screened on both *in vitro* and *in vivo* assays. For each assay, data from screens is used to train a corresponding sequence-to-function map. Then, a random pool of millions of sequences is screened *in silico* through all sequence-to-function maps. Sequences which have high predicted values across all assays are selected to comprise the Multi-Function library. Top performing candidates from the Multi-Function libraries are chosen for individual characterization. (B) Scatter plots show the comparison and Pearson correlation of measured log₂ enrichment versus model-predicted log₂ enrichment, for production fitness and five functional liver assays. Histograms show the distribution of assay log₂ enrichment across the Fit4Function (low bias, high production fitness) and Multi-Function

libraries (high production fitness, multi-trait). Histograms are density-normalized and exclude non-detected variants (represented in the ND column for each assay).

New Technologies for AAV Gene Therapy

1202. Effects of Complement Component 1 (C1) Inhibition on AAV-Based Gene Transfer Efficacy and Immunogenicity in Mice

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Recent clinical trials that delivered relatively high (~ 10¹⁴ vg/kg) intravenous dose of the Adeno-Associated Virus (AAV) vector reported adverse reactions, and in particular complement activation (Chand et. al. 2021, Maurya et. al. 2022). It has also been shown that several complement components, including C1, interact with the AAV capsid and may increase humoral immune response to gene therapy in mice (Zaiss, et. al. 2008). Currently, several FDA-approved complement pathway inhibitors are available and could be potentially combined with the AAV-based gene therapy treatments to reduce the immunogenicity of the vector. Herein, we investigate the effects of C1 esterase inhibitor approved for use in hereditary angioedema and effective in mice, on the immune response to AAV *in vitro* and *in vivo*. First, we studied conditions required for complement activation *in vitro*. Complement activation, measured by C3a levels, was observed only with the 5e11 vg/ml or higher dose of AAV in both human and mouse seropositive serum or in whole blood. 1:10 was the minimum NAb titer required for detection of complement activation with the C3a ELISA assay. Complement activation increased proportionally with the increased NAb levels in serum. The C1 inhibitor effectively blocked AAV-induced complement activation in both human and mouse serum and whole blood samples. However, the complement blockade did not result in hampering the pro-inflammatory cytokine response towards AAV. Next, naive or pre-immunized with AAV-seropositive serum mice were intravenously injected with either a low (1e11 vg/kg) or high (4e12 vg/kg) AAV dose. In all experimental groups, there was a trend to 1.6-fold higher complement activation, as measured by C3a ELISA, 90 minutes post AAV injection. This modest increase returned to basal levels for each condition treated with the C1 inhibitor, with no difference between non-immunized and immunized groups. Complement inhibition also slightly lowered humoral immune response against AAV for both anti-AAVspark100 IgG and IgM levels. This limited effect of the C1 inhibitor on the antibody levels may be due to the inhibitor's short half-life of 2.5 hours, and a maximum of two daily doses allowed in the animal protocol. Interestingly, the C1 inhibitor increased liver transduction by ~33% with the high AAV dose in non-immunized mice, and 28% in immunized mice. Transduction was also increased by 55% in the low AAV dose in non-immunized mice. Immunization, on the other hand, as expected, greatly decreased transduction efficacy. Transduction efficiency dropped to 50% in the mice injected with the high AAV dose, and immunized mice in the low AAV dose groups showed no

transgene expression and did not develop IgG or IgM following vector infusion, most likely due to immediate AAV clearance by the NAb. These results indicate the potential impact of C1 inhibition which warrants further investigation to better understand and optimize the potential for lower vector immunogenicity and dose-sparing effect in AAV-based gene transfer.

1203. Inducible Gene Expression for Gene Therapy: Design and Exemplification of Powerful, Small, Modular and Tightly Controlled Regulatable Promoters

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Gene therapy (GT) has been successfully shown to revert diseased phenotypes in both animal models and in clinical settings. There are now 23 FDA approved gene therapies using a variety of modalities, including viral and non-viral based methods. These approvals represent substantial progress in making GT more accepted as a standard of care and have been driven by innovations that have reduced toxicity by improving upon the design of the expression cassette. However, despite these advances a key issue in the widespread implementation of GT is the ability to control gene expression in a tight and consistent manner. This is of paramount importance in the gene editing field where expression of the therapeutic editing construct is only required for a short period of time, enough time to perform targeted editing but preventing off-target effects. Such genetic expressions systems have successfully been demonstrated in the past, most notably the Tet on/off system. However, this system relies on the expression of a bacterial transactivator protein that proved immunogenic which led to a shutdown of gene expression and therefore made this system unsuitable for use in GT. It is therefore desirable the regulation of gene expression is achieved via single component systems, i.e., drug activated with no requirement for the co-expression of a transactivator. In addition, this would allow the control elements to be packaged in a single AAV vector, overcoming the problems observed with 2 vector systems and reducing the costs of manufacture. Here we show, that by using our PromPT platform, the creation of novel, small, potent, and tightly controlled inducible liver promoters. These novel inducible promoters are designed to be used in an AAV and are therefore single component systems and small >250bp. In addition, they are responsive to an FDA approved drug which has a short ½ life with excellent exposure to the target tissue. *In vivo* analysis of these promoters after delivery via AAV8 or AAV9 demonstrated that they were liver specific, with a maximal induction of >5,000-fold in the presence of the drug. Maximal activity of the induced promoters was at least 10-fold higher than CMV demonstrating their potency with peak expression observed a peak expression observed and a return to background upon clearance of the drug. In addition, the promoters were assessed for dose responsiveness and the effect of AAV dose on induction and maximal expression level. Furthermore, there was no difference in induction, strength, or durability of expression whether the drug was administered IP or orally and there were no significant differences between male and female mice. In summary, we believe this

exemplifies a new inducible promoter system that enables high levels of expression, sex agnostic expression and small size. This system is expected to control of the GOI using an FDA approved drug with an exemplary safety record and has the potential to be transformative in the GT field.

1204. Inclusion of AAV Empty Capsids Can Increase Expression in Certain Contexts In Vitro and In Vivo

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Background: Common methods for producing adeno-associated viral vectors (AAV) typically result in 80-90% genome-devoid (or so-called “empty”) capsids that may be reduced or removed from the vector preparation prior to use, depending on purification or enrichment methods. Using AAV6, 8 and 9, we demonstrate that inclusion of empty capsids can result in increased expression following transduction *in vitro* and *in vivo* in certain contexts. **Empty capsids of the same serotype:** Cultures of murine, canine and human myoblasts transduced with AAV6, 8 and 9 showed superior expression with AAV6, and expression was further increased by the inclusion of empty AAV6 capsids in the AAV6 prep. No such increase was seen with AAV9 empty capsids in the AAV9 prep. Intramuscular injections in mice with AAV6, 8 and 9 again showed superior expression with AAV6. Inclusion of AAV6 and 9 empty capsids in their respective preps led to marked improvement in expression after two weeks for both serotypes (AAV8 empty capsids not tested). Results from intravascular delivery of AAV6 and 9 to mice were the same: expression in striated muscle four weeks after injection was best with AAV6 and was increased for both serotypes when empty capsids were not removed from the preps. **Empty capsids of different serotypes:** To test whether the increased expression seen after transduction in the presence of empty capsids is empty-capsid-serotype specific, we transduced fibroblasts with AAV6 mixed 1:4 with AAV1, 2, 6, 8 or no empty capsids. Addition of empty capsids of serotypes AAV1, 2, and—to a lesser extent—8 led to statistically significant increases in expression. Intravascular injections in mice with AAV6 mixed 1:4 with AAV1, 2, 6, 8 or no empty capsids showed expression in striated muscles at two weeks was strikingly increased with addition of AAV1 and 6 empty capsids. Increases seen with AAV2 and 8 empty capsids were not statistically significant. While the inclusion of empty capsids led to increases over AAV6 full vector only, expression did not reach levels seen when the same capsid load using only full AAV6 vector was used (5x AAV6 full capsids). Expression in liver was not positively impacted by the inclusion of empty capsids of any serotype. Rather, there was a negative trend, although no differences were statistically significant. **Dose threshold effect:** Incidentally, we also demonstrated a threshold effect in muscle after systemic AAV6 delivery. Expression two weeks after intravascular injection of AAV6 preps including empty capsids in heart, diaphragm, soleus and tibialis anterior remained at background levels at doses below 6.7x10¹² vg/kg, at which point the dose response increased sharply and non-linearly. For example, between the doses of 1.7x10¹³ and 4.3x10¹³ vg/kg (a 2.6-

fold increase), the expression increased at least 10-fold in all muscles. **Summary:** Here we have demonstrated a systemic threshold effect in IV AAV6 delivery. We have also demonstrated that AAV6 led to superior expression *in vitro* and *in vivo* following IM and IV delivery in mice compared to AAV8 and 9. Finally, we've shown that inclusion of empty capsids—not required to be of the same serotype as the full capsids—can lead to increased expression *in vitro* and *in vivo* following IM and IV delivery. This increase is variable depending on the serotype of the full and empty capsids.

1205. Disease-Responsive Therapy for the Treatment of Myotonic Dystrophy

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Myotonic dystrophy (DM) is the most common muscular dystrophy in adults, affecting approximately 1 in 8000 individuals worldwide, with no currently available disease-modifying therapies. The primary pathomechanism in DM is a toxic RNA gain-of-function caused by non-coding microsatellite repeat expansions in the 3'UTR of *DMPK* (type 1 and congenital) or upstream intron 1 of *CNBP* (type 2). One well-characterized pathomechanism in DM involves the functional sequestration of muscleblind-like (MBNL) proteins by accumulated repeat-expanded (CUG)_n or (CCUG)_n RNA. MBNL proteins are important for pre-mRNA processing, including alternative splicing, cellular localization, and polyadenylation, and their sequestration leads to altered expression and mis-splicing of hundreds of genes. Multiple MBNL-dependent mis-splicing events have been directly linked to specific DM phenotypes and have a quantifiable relationship to disease severity. Here we used well-characterized splicing events to generate DMXon, a disease-responsive platform to control the production of therapeutic protein. Under normal MBNL activity, as in non-disease affected tissues, production of the therapeutic product is inhibited by exclusion of an appropriate initiation sequence. As functional MBNL levels decline, cassette exon inclusion introduces this sequence to enable protein production. DMXon transgenes derived from multiple splicing events show dose-dependent responses to MBNL levels with variable dynamic ranges and therapeutic output. By responding to disease state, DMXon transgenes have the potential to deliver individual, graded responses encountered in each affected patient and allow for adaptation to disease progression when delivered using long-term expression modalities, such as AAV.

1206. Preclinical Gene Reactivation of the X-Linked Cdkl5 Gene Using Dual Adeno-Associated Virus Mediated CRISPR/dCas9 Epigenetic Editing

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CDKL5 deficiency disorder (CDD) is an 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 cells using a dCas9 epigenetic editor. In this study, we demonstrate that reactivation requires the presence of a DNA methylation eraser targeted to a specific site within the gene promoter. For further preclinical testing of *in vivo* gene correction, we have designed an adeno-associated virus (AAV)-mediated intein split dCas9 epi-editor for mouse *Cdkl5* gene reactivation. We have further optimized AAV vector packaging efficiency by using a gRNA-tRNA array, in which tRNAs are interspersed in the transcript, allowing for multiplex gRNA expression from a single U6 promoter post tRNA processing. Following direct intracranial injection of AAV9 we detect widespread split dCas9 expression and virus biodistribution in the brain, expression in neurons, *in vivo* trans-splicing of the split dCas9 construct, and significant upregulation of *Cdkl5*. We also assessed the on-target mechanism of action by DNA methylation removal from the inactive X chromosome via bisulfite sequencing of the *Cdkl5* CpG promoter and genome-wide off-target demethylation using reduced representation bisulfite sequencing. In addition, we demonstrate promoter and polyA optimization to improve AAV size to further increase capsid ratios and *in vivo* bioactivity. Future studies will address molecular and behavioral rescue in animal models of CDD. This approach holds great promise for those affected by CDD.

1207. Characterization of Optimized Dual AAV-MYO7A Vectors for the Treatment of Usher Syndrome (USH1B) in *Myo7a*^{-/-} Mice and NHP

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PURPOSE: Patients with Usher Syndrome 1B (USH1B) are born profoundly deaf, have vestibular defects and begin losing vision in their first decade. The causative gene, *MYO7A*, is too large to fit in a standard AAV vector. We previously showed that “hybrid” (HY) and “overlap” (OV) dual vectors drive full-length *MYO7A* expression in subretinally injected (SRI) mice. However, first-generation (v1) OV vectors produced relatively low levels of full-length *MYO7A*, and v1 HY vectors, while capable of driving high levels of *MYO7A*, also produced a truncated protein (TRP) emanating from the front half vector which may have been responsible for reductions in rod-mediated function in injected mice. The purpose of this study was to optimize the efficiency and safety of our v1 dual AAV vectors and evaluate *MYO7A* expression in non-human primate (NHP) retina. **APPROACH:** New front and back half OV vectors were generated (v2 and v3) by altering the shared sequence region. Nine different vector pairs were tested, with overlap lengths ranging from 1365 to 20 bp. HY dual AAV-*MYO7A* vectors were optimized by altering the split point of *MYO7A* coding sequence between the two vectors (HY-v2 dual vectors). HY-v2 vectors were further altered by eliminating potential in-frame stop codons from the front half sequence. Three codon modified HY front half vectors (CM.v1, CM.v2, CM.v3) were created. All back half vectors contained a C-terminal HA tag. *Myo7a*^{-/-} mice were SRI with 5e11 vg/mL of dual vectors packaged in AAV8(Y733F) or AAV.SPR. Retinal structure/function were analyzed using optical coherence tomography and electroretinogram at 6 weeks post-injection (p.i.). *MYO7A* expression was interrogated using western blot (WB) and IHC. The best performing HY and OV dual vectors were then tested in NHP. Six macaques received bilateral SRI of either HY or OV dual vectors, driven by the smCBA promoter and packaged in AAV8(Y733F) or AAV.SPR. Vectors were delivered at a total concentration of 6.6e12 vg/mL. Two blebs (75µL each) were peripherally placed outside the macula in each eye. At 6 weeks p.i., retinas were analyzed using WB and IHC. **RESULTS:** OV vectors with minimized shared sequence (945 and 687 bp) produced higher levels of full length *MYO7A* than v1 vectors (1365 bp). Codon modification of front-half HY vectors significantly reduced production of TRP in mice. Retinal structure/function were well preserved post-injection. Optimized dual vectors produced heterozygous levels of full length *MYO7A* that localized to both photoreceptors (PRs) and RPE. HY-v2-CM.v3 dual vectors and OV-v3/v2 dual vectors were selected for testing in NHP (*M. fascicularis*). HY AAV.SPR-smCBA-mediated *MYO7A* (HA) expression was seen in peripheral retina (within the bleb) and in the macula/fovea (outside the bleb). Robust signal was detected in NHP photoreceptors and no gross structural changes/signs of toxicity were noted. Supraphysiological levels of full length *MYO7A* were

achieved with AAV.SPR HY dual vectors. **CONCLUSIONS:** Altering the length of shared sequence increased *MYO7A* expression relative to v1 OV vectors. Altering the split point in HY dual vectors, and codon modifying the front-half sequence reduced/eliminated TRP expression and functional declines seen in v1 vectors. In NHP, dual AAV-*MYO7A* vectors were well-tolerated, producing supraphysiological levels of properly localized *MYO7A*. Finally, due to the spreading ability of AAV.SPR, efficient *MYO7A* expression was achieved in the macula without detachment. Taken together, our optimized dual AAV-*MYO7A* vectors have increased safety and efficiency, and their performance in NHP provides strong support for clinical application.

1208. Differential Histopathological and Proteomics Changes in Dorsal Root Ganglia (DRG) of *Cynomolgus Macaques* Following Intrathecal (IT) Delivery of “Empty” AAV9 Capsids, and AAV9 Carrying Transcriptionally Active or Inert Cargo

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DRGs are constituted of several cell types that play a role not only in sensory circuitry but also in immune surveillance. Recent publications have reported that AAV treatment may lead to DRG-associated lesions in non-human primates (NHP), especially when administered via the intra-cerebrospinal fluid (CSF) intrathecal (IT) routes. The etiology of these DRG lesions is still not fully understood, but is believed to be related to high transduction of DRGs and resulting in supra-physiological level of transgene within the sensory neurons. This results in mononuclear cell infiltration that subsequently culminates in neuronal degeneration. In order to assess the contributions of the viral capsid, viral transgene/cargo DNA, and transcriptional activity as well as transgene expression in the etiology of DRG lesions, we carried out a study with NHP in which animals were dosed via IT lumbar puncture delivery route with either control vehicle or three different configurations of the AAV9 : i) “empty” AAV9 capsid, ii) full AAV9 capsid with active transcription of the GFP transgene, iii) full capsid with promoter-less/transcriptionally inert cassette. The histopathology data showed absence of DRG microscopic lesions in animals dosed with empty capsid or transcriptionally inert vectors. Targeted proteomic characterization of CSF samples across the groups identified elevated IL-12 levels to be associated with animals displaying DRG lesions. Furthermore, an orthogonal methodology using MSD ELISA confirmed 35-fold increase in average of IL-12 in the CSF samples of these same animals. Finally, a retrospective analysis of IL-12 in CSF samples previously collected from NHP with documented evidence of DRG lesions following dosing with AAV9 vectors were also added for further confirmation. Overall, the data point to the central role that AAV-encoded transgene products may play in DRG toxicity, and suggest that IL-12 could be a potential candidate biomarker for AAV-induced DRG toxicity.

AAV Vectors - Clinical Studies

1209. A Phase 2 Trial Evaluating Safety and Efficacy of Delandistrogene Moxeparvec in Duchenne Muscular Dystrophy

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Delandistrogene moxeparvec (SRP-9001) is an investigational gene transfer therapy developed for targeted skeletal and cardiac muscle expression of micro-dystrophin, a shortened, functional, dystrophin protein. This three-part, Phase 2 clinical trial (NCT03769116) evaluates the safety and efficacy of systemic delivery of delandistrogene moxeparvec in patients with Duchenne muscular dystrophy (DMD). Part 1 is a 48-week, randomized, double-blind, placebo-controlled period. Part 2 is a 48-week period in which patients randomized to placebo in Part 1 receive delandistrogene moxeparvec. Part 3 is an open-label follow-up period (≤ 212 weeks). Participants from Part 1 (N=41) were 4-7-year-old ambulatory boys with a confirmed DMD mutation between exons 18-58 and stable steroid dosing. The intended dose was 2.0×10^{14} vg/kg (supercoiled qPCR, linear plasmid standard equivalent of 1.33×10^{14} vg/kg). Randomization was stratified by age (4-5 and 6-7 years). Primary endpoints were change from baseline in micro-dystrophin expression (western blot; baseline to Week 12) and North Star Ambulatory Assessment (baseline to Week 48). Safety endpoints included serious adverse events and treatment-emergent adverse events. One-year functional results from Part 1 have been presented previously. Full analyses from Parts 1 and 2 will be presented, including 2-year functional and safety data from patients who received delandistrogene moxeparvec in Part 1; 1-year functional and safety data from patients who received delandistrogene moxeparvec in Part 2; and expression data for all patients 12 weeks post-delandistrogene moxeparvec infusion and 60 weeks post-infusion for those dosed in Part 1. Results suggest that delandistrogene moxeparvec has a biological effect that may be clinically relevant in people with DMD. Results reinforce a potentially favorable benefit-risk profile.

1210. Evaluation of Total Binding Antibodies Against rAAVrh74 in Patients with Duchenne Muscular Dystrophy

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Background: Adeno-associated virus (AAV) vectors have become the vehicle of choice in studies of investigational gene transfer therapies for Duchenne muscular dystrophy (DMD). The rAAVrh74 serotype, which efficiently transduces tissues impacted by DMD, including skeletal and cardiac muscle, was derived from Rhesus monkey, which may confer a lower risk of pre-existing antibodies in humans. It was therefore selected as the vector for development of a DMD gene therapy that delivers a transgene encoding micro-dystrophin, a shortened, but functional, dystrophin protein, as pre-existing antibodies to AAVs can limit therapeutic potential and pose safety concerns. **Objective:** Here we sought to evaluate total binding antibodies (neutralizing and non-neutralizing) against rAAVrh74 in patients with DMD. Measuring total binding antibodies is more a comprehensive approach to assessing pre-existing immune response versus measuring neutralizing antibodies alone. **Methods:** Eligible individuals were ≥ 4 to < 18 years old with genetically confirmed DMD and were excluded from the study if they lived with a person who had exposure to rAAVrh74 or other gene transfer therapy, or if they received prior treatment with gene transfer therapy. A single blood sample was obtained from each participant and anti-rAAVrh74 total binding antibodies were measured by enzyme-linked immunosorbent assay. Total binding antibody level $< 1:400$ was defined as 'not elevated'. Primary endpoint was the percentage of subjects with elevated total antibody titers to rAAVrh74. **Results:** Overall, 86.1% (87/101) of study participants were seronegative, with pre-existing total antibody titers to rAAVrh74 $< 1:400$. There was no association between seroprevalence and age observed in this limited study of individuals with DMD, aged ≥ 4 to < 18 years. In the 14 seropositive participants, anti-rAAVrh74 antibody titers ranged from 1:400 to 1:3200. **Conclusions:** The comprehensive approach of measuring total binding antibodies (both neutralizing and non-neutralizing) demonstrates that the majority of patients (86.1%) with DMD in this final data set were seronegative ($< 1:400$) for anti-rAAVrh74 total binding antibodies.

1211. Phase 1 Study of Gene Therapy in Late-Onset Pompe Disease: Initial 104-Week Experience

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Background: Gene therapy with an adeno-associated virus serotype 8 (AAV8) vector (AAV8-LSPHGAA) could eliminate the need for enzyme replacement therapy (ERT) by creating a liver depot for acid-alpha glucosidase (GAA) production. **Objectives:** We report initial analyses of safety and bioactivity of the first (low-dose) cohort (n=3) in a 52-week open-label, single-dose, dose-escalation study (NCT03533673) in patients with late-onset Pompe disease (LOPD). The primary study objective is to evaluate the safety of AAV8-LSPHGAA in adult subjects as assessed by the incidence of adverse events (AEs), serious AEs (SAEs), and clinical

laboratory abnormalities. Secondary objectives include six-minute walk test distance (6MWT), forced vital capacity (FVC), serum GAA activity, and muscle GAA activity and glycogen content. **Methods:** Eligible subjects had LOPD, no detectable anti-AAV8 neutralizing antibodies, >12 months stable ERT dosing, and ability to walk at least 100 meters on the 6MWT. Subjects continued bi-weekly ERT until ERT withdrawal at Week 26 based on the detection of quantifiable serum GAA activity from AAV8-LSPH-GAA and the absence of clinically significant declines in FVC or 6MWT performance. Prednisone (60 mg/day) was administered as immunoprophylaxis through Week 4, followed by an 11-week taper. **Results:** All subjects demonstrated sustained serum GAA levels from 101% to 235% of baseline trough activity measured two weeks following the preceding ERT dose, which confirmed bioactivity as GAA is not normally secreted into the blood. There were no treatment-related serious adverse events (SAEs) and laboratory assessments supported the safety of AAV8-LSPH-GAA. No subject had elevated serum alanine aminotransferase or ELISPOT signals, demonstrating an absence of anti-AAV8 T-cell responses encountered in other, similarly designed studies. Although there were no treatment-related SAEs, one subject experienced two moderate severity treatment-related AEs (headache). At Week 24 all subjects met criteria for ERT withdrawal. Subsequently, all subjects met criteria to remain off ERT at Weeks 52 and 104, although one subject opted to resume ERT at Week 97. Muscle biopsy at Week 24 revealed a large decrease (-40%) in muscle glycogen content for one subject. At Week 52 muscle GAA activity for the cohort was significantly increased in comparison with baseline ($p < 0.05$). One subject had a 10-fold increase from 2.8% to 28% of normal, the second increased from 19% to 44% of normal, and the third increased from 56% to 94% of normal. **Conclusions:** Overall, these initial data support the safety and bioactivity of AAV8-LSPH-GAA, the safety of withdrawing ERT, successful immunoprophylaxis, and justify continued clinical development of AAV8-LSPH-GAA therapy in Pompe disease.

1212. Sustained Efficacy and Safety at Week 52 and Up to Three Years in Adults with Glycogen Storage Disease Type Ia (GSDIa): Results from a Phase 1/2 Clinical Trial 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:** This global, open-label, phase 1/2, dose escalation gene therapy trial (NCT03517085) is evaluating the safety and efficacy of a single DTX401 intravenous infusion in adults with GSDIa. 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. Cohort 4 includes a prophylactic steroid regimen to prevent transaminase elevation. **Efficacy Results:** In the twelve patients enrolled in Cohorts 1 through 4, mean (SD [range]) total daily cornstarch intake reduction from baseline to Week 52 was 66.4% (24.8 [28-100%]), $p < 0.0001$. From baseline to last available timepoint (~3 years for three patients), mean (SD [range]) total daily cornstarch intake reduction was 70.8% (22.8 [30-100%]), $p < 0.0001$. From Cohort 3 onward, continuous glucose monitoring was implemented. In Cohort 3 and Cohort 4, the average percentage of time in euglycemia (blood glucose 60 mg/dL to 120 mg/dL) from Baseline to Weeks 49 to 52 increased 14.2% and 0.5%, despite reductions in average cornstarch intake of 63% and 50%, respectively. At Week 52 exit interviews, patients reported more energy and stamina, better mental clarity, improved glycemic control independent from cornstarch, improved sleep quality, and improvements in health-related quality of life related to cornstarch intake reduction. Patient Global Impression of Change (PGIC) scores at the Week 52 visit indicated that 67% of patients ($n=9$) felt their disease was moderately or much improved since the start of the study. **Safety Results:** No infusion-related or treatment-related serious adverse events (SAEs) were reported. 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 three 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. Additional glycemic control, fasting time, and health-related quality of life data will be reported.

1213. Rationally Designed Cardiotropic AAV Capsid Demonstrates 30 Fold Higher Efficiency in Human vs Porcine Heart

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Earlier clinical trials targeting chronic heart disease failed to show clinical efficacy and biopsy samples obtained at the time of cardiac transplant or left ventricular assist device (LVAD) placement showed gene copy numbers < 0.012 vector genomes per diploid genome (vg/dg) [1]. To resolve this problem novel AAV serotypes, capsid library, and capsid rationally designed have all been employed. Using rational design of AAV2 specific loop sequences [2], we developed a novel cardiotropic capsid that de-targeted liver to support our clinical trial for the treatment of non-ischemic cardiomyopathy. This engineered AAV2i8 capsid with the CMV promoter delivers constitutively active

truncated form of the protein phosphatase 1 inhibitor 1 (I-1c). Here we report performance characteristics of the vector in preclinical porcine studies and human tissue from the ongoing clinical trial. Intracoronary infusion of increasing doses of AAV2i8.I-1c (1x10E13 vg, 1x10E14 vg, and 1x10E15 vg/pig) in normal pigs weighing ~35-50 kg, resulted in a dose-dependent increase in myocardial uptake [3]. In the high dose toxicology study, we infused 1x10E15 vg of AAV2i8.I-1c in pigs by intracoronary route since heart size approximates that of human. Biodistribution studies performed 3 months after injection showed 0.01-0.075 vg/dg in the left ventricle anterior wall as determined by digital droplet PCR (ddPCR). Supporting preclinical studies enabled this novel capsid to be administered to eight trial participants with NYHA Class III non-ischemic heart failure all displaying reduced ejection fraction (<35%). Three participants received a dose of 3E13 vg total, and 5 have received a dose of 1E14 vg total, infused via the intracoronary route. Clinical results of the study are reported elsewhere at this meeting. One participant in the 1E14 vg dose cohort was referred for LVAD placement 13 months post vector infusion. Tissue biopsy sample obtained at LVAD placement was analyzed for vector genome copy number. Briefly, from a 3g piece of tissue, 5 samples across the tissue were harvested for DNA analysis. DNA from control non-failing and non-injected human and pig hearts was extracted from the same anatomical location as the LVAD tissue biopsy sample, and used for matrix interference studies as well as negative controls. Primers targeting SV40 poly A present in the transgene cassette were used to quantify the vector genomes using ddPCR. Samples showed 0.57, 0.67, 0.9, 1.2, and 2.6 vg/dg. Compared to transduction efficiency in the porcine model, AAV2i8 demonstrated a 30-fold higher transduction in human heart (1e15vg/pig vs 1e14vg/patient respectively). We conclude that rational design of an AAV capsid has led to the development of a capsid with superior transduction efficiency in human vs. porcine heart. These data underscore the importance of obtaining quantitation of vector performance for novel capsids in human tissues, and also support the validity of rational design strategies. 1. Greenberg et al., JACC Heart Fail, 2014, 2(1): p. 84-92. 2. Asokan et al., Nat Biotechnol. 2010 Jan;28(1):p. 79-82. 3. Watanabe et al., J Am Coll Cardiol, 2017, 70(14): p. 1744-1756.

1214. Safety, β -Sarcoglycan Expression, and Functional Outcomes from Systemic Gene Transfer of rAAVrh74.MHCK7.hSGCB in LGMD2E/R4

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Background: Limb-girdle muscular dystrophy type 2E/R4 (LGMD2E/R4) is caused by mutations in the β -sarcoglycan gene (SGCB), resulting in loss of SGCB protein and, subsequently, an absence of the

dystrophin-associated protein complex (DAPC) at the sarcolemma. LGMD 2E/R4 manifests as progressive hip/shoulder muscle weakness. **Objective:** This first-in-human, phase 1/2 trial (NCT03652259) evaluated SRP-9003, a self-complementary rAAVrh74.MHCK7.hSGCB construct designed to restore SGCB protein production. **Methods:** Patients aged 4-15 years with SGCB mutation (both alleles) received 1 SRP-9003 IV infusion: Cohort 1 (n=3), 1.85x10¹³ vg/kg; Cohort 2 (n=3), 7.41x10¹³ vg/kg. Endpoints included safety (primary), SGCB protein expression (secondary), and function (North Star Assessment for Limb-girdle Type Muscular Dystrophies [NSAD], time to rise [TTR], 4-stair climb [4-sc], 100-meter timed test [100m], 10-meter timed test [10m]). **Results:** Previously reported results: Year 1 (Y1) for Cohort 2 and Year 2 (Y2) for Cohort 1 showed that as of January 2021, SRP-9003 was well tolerated; adverse events occurred early and were manageable. Immunofluorescence showed robust SGCB expression and correct sarcolemmal localization post treatment, leading to DAPC reconstitution, maintained to Y2 (Cohort 1). SRP-9003-treated patients showed functional improvements, maintained at Y2 in Cohort 1 (NSAD, +5.7 points; TTR, -0.6 s; 4-sc, -0.3 s; 100m, -2.8 s; 10m, -0.2 s) and Y1 in Cohort 2 (NSAD, +4 points; TTR, -1.1 s; 4-sc, -0.4 s; 100m, -7.9 s; 10m, -0.6 s). Post hoc analysis showed improved NSAD outcomes versus an untreated natural history cohort (9.2-point difference, Y2; 95% CI, 3.2–15.1). An update with 3-year functional data for Cohort 1 and 2-year protein expression and functional data for Cohort 2 will be presented. **Conclusion:** These data suggest long-term efficacy of SRP-9003 therapy, supporting advancement of the clinical development program.

Delivery Technologies and In Vivo Gene Editing

1215. Durable Silencing of Pcsk9 by In Vivo Hit-and-Run Epigenome Editing

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Epigenome editing is emerging as a powerful new strategy to silence genes without altering the primary DNA sequence. In this regard, we and others have previously developed Engineered Transcriptional Repressors (ETRs), chimeric proteins containing a programmable DNA Binding Domain (DBD) fused to either one or more of the following epigenetic repressive effectors: KRAB, the catalytic domain of DNMT3A and DNMT3L. Of note, transient delivery of such ETRs, either as a triple combination or as all-in-one fusion proteins, was shown to induce efficient, long-term stable and specific epigenetic silencing of endogenous genes in both human and mouse cell lines, and primary human cells, including T lymphocytes and iPSCs. Here, we explored for the first time the ETR technology for silencing genes *in vivo*. To this end, we focused on *Pcsk9*, a gene involved in cholesterol homeostasis whose hepatic inactivation is under clinical testing for the treatment of hypercholesterolemia. First, we used an *ad hoc* developed

Pcsk9 murine reporter cell line to select the best performing triple ETR combination for each of the following DBD platforms: catalytically deactivated Cas9 (dCas9; 8 target sites), Transcription Activator-Like Effectors (TALEs; 16 target sites) and Zinc Finger Proteins (ZFPs; 16 target sites). These *in vitro* studies identified ZFP-based reagents capable of inducing >90% long-term stable (up to 50 days) epigenetic silencing of *Pcsk9*. In parallel, we set-out to deliver the ETRs to the liver of mice. As the ETR technology entails the use of transient gene delivery modalities, we performed an *in vivo* screening to identify Lipid Nanoparticles (LNPs) compatible with efficient transfer of editing machinery to the liver, using here CRISPR-Cas9-mediated inactivation of *Pcsk9* as a surrogate readout for LNP-mediated gene delivery efficiency. Among the 11 LNPs tested, 8 consistently induced robust reduction in circulating levels of *Pcsk9* (up to 90%). Of these 8 candidates, one was selected for further studies, given its favourable toxicity and biodistribution profiles. We thus packaged the *Pcsk9* ZFP-based ETRs into this LNP and first tested its performance on cultured primary murine hepatocytes. In this setting, ZFP-based ETRs nearly abrogated *Pcsk9* expression, at levels comparable to those obtained with conventional gene editing. Prompted by these results, we then administered intravenously the LNP-ETRs in adult mice and followed the circulating levels of *Pcsk9* and Low-Density Lipoproteins (LDLs) for up to 100 days. Early analyses of mice showed a rapid reduction of *Pcsk9* which stabilized at ~50% of mock-untreated levels until the last time point analyzed. In line with these data, at day 30 post-LNP injection, LDL levels were reduced as compared to mock-treated mice (~35%). Comparable efficiencies and kinetics of *Pcsk9* reduction were observed in mice treated with LNPs loaded with the catalytically active CRISPR-Cas9 programmed against *Pcsk9*. We are currently monitoring *Pcsk9* in LNP-ETR treated mice as well as further optimizing ETR architecture. Overall, these data provide the first demonstration that transient ETR delivery can mediate durable epigenetic silencing *in vivo*, opening exciting new possibilities in the field of therapeutic gene silencing.

1216. AAV-CRISPR/Cas9 Gene Editing is Therapeutic in a Novel, Humanized Mouse Model of *GUCY2D*-Associated Cone Rod Dystrophy (CORD6)

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Purpose: Autosomal dominant mutations in *GUCY2D*, the gene encoding retinal guanylate cyclase-1 (retGC1), are the leading cause of cone-rod dystrophy (CORD6). Given our proven ability to deliver therapeutic *GUCY2D* to photoreceptors (PR) via AAV to treat recessive disease, an attractive treatment approach for CORD6 is to combine AAV-CRISPR/Cas9-based knockout of *GUCY2D* with complementation of a CRISPR/Cas9-resistant ('hardened') cDNA copy of *GUCY2D in trans*. Preliminary data shows that this 'edit and complement *in trans*' approach is capable of maintaining photoreceptor structure and function compared to editing alone in a healthy mouse

model. We have also established the optimal dose of both the gene editing and complementation reagents that allow for robust editing while avoiding toxic overexpression of transgene products. Recently, we developed a novel, humanized model of CORD6, the human R838S knock-in (hR838S KI) mouse, that recapitulates the genotype and retinal degenerative phenotype seen in CORD6 patients. The purpose of this study is to establish whether the 'edit and complement *in trans*' approach is successful in treating retinal dystrophy in the hR838S KI mouse. **Methods:** On one allele, hR838S KI mice have murine *Gucy2e* exon 13 replaced with *GUCY2D* exon 13 sequence carrying a transversion mutation, leading to an amino acid change of arginine to serine at residue 838. The R838S mutation in humans causes the most severe form of CORD6. The second allele has *Gucy2e* exon 13 replaced with WT *GUCY2D* exon 13. We performed a natural history study to evaluate retinal structure and function in hR838S KI mice using optical coherence topography (OCT) and electroretinogram (ERG) analysis, respectively at 4, 8, 12, 24, 28, 32, and 52 weeks of age. Results were compared to those in control mice homozygous for the WT human exon 13 (hWT KI). At 52 weeks of age, a cone survivability assay was performed to determine the extent of cone cell death. The *S. aureus* Cas9 system was designed to target exon 2 of *Gucy2e*. The 'hardened' *Gucy2e* contains five silent mutations in the gRNA-recognition sequence and two silent mutations in the PAM sequence. Relevant constructs were packaged in AAV44.9(E531D), a capsid with high PR transduction efficiency. hR838S KI mice were injected at postnatal day 21 with AAV-hGRK1-Cas9 and AAV44.9(E531D)-*Gucy2e*-gRNA-hGRK1-'hardened' *Gucy2e* and contralateral eyes were injected with buffer. hWT KI mice were injected bilaterally with buffer. ERG and OCT analyses were performed at 4, 8, 12, 16, and 24 weeks post-injection (p.i.). Visually guided behavior was measured at 20 weeks p.i. **Results:** The hR838S KI mouse model showed significant rod cell loss in addition to a loss of rod-mediated (scotopic) function beginning at four weeks of age and continuing over the lifetime of the mouse. While there was reduced cone-mediated (photopic) function compared to the hWT KI mouse, there was no evidence of cone cell death. The 'edit and complement *in trans*' system was capable of significantly preserving photoreceptor structure and function in the hR838S KI mouse model. Scotopic function and photoreceptor survivability were stably preserved through at least 24 weeks of age. There was also some evidence of increased photopic function in treated eyes. **Conclusions:** The hR838S KI mouse recapitulates aspects the retinal degenerative phenotype of human CORD6. Preservation of mouse photoreceptor structure and function was achieved in the R838S KI model via an AAV-CRISPR/ Cas9 approach. This provides support for the use of an 'edit and complement *in trans*' approach for the clinical treatment of CORD6.

1217. A Large-Scale Exon Editing Solution for Treating Genetic and Complex Disorders

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Here we describe the history and natural biological mechanisms underpinning exon editing and replacement via RNA trans-splicing. We further describe a designer RNA discovery screening engine which enables high-throughput and non-biased lead optimization of RNA editing molecules. Proof-of-concept preclinical *in vitro* and *in vivo* demonstration of this large-scale RNA editing approach is applied to Stargardt Disease 1, which is an autosomal recessively inherited retinal disease caused by mutations in the ATP-Binding Cassette sub-family A type 4 (ABCA4) gene. Loss of ABCA4 results in the build-up of bisretinoids and other fatty byproducts (lipofuscin) in the retina leading to cellular toxicity and a progressive loss of vision. ABCA4 genotype-phenotype relationships have been linked to additional retinal diseases, including retinitis pigmentosa, cone-rod dystrophy and age-related macular degeneration. The full-length ABCA4 gene is too large to package inside an AAV capsid, precluding conventional gene therapy; and hundreds of disease-causing mutations have been observed, rendering single base editing approaches insufficient. There are currently no effective treatments to address ABCA4-related retinopathies. Therefore, we set out to develop a large-scale exon editing solution by delivering a therapeutic RNA construct, which induces trans-splicing of multiple functional ABCA4 exons, via subretinal AAV injection. This approach allows for durability of gene therapy and correction of multiple mutations without directly editing DNA. Following *in vitro* optimization, we show successful and well-tolerated *in vivo* trans-splicing of ABCA4 in non-human primate photoreceptors following subretinal delivery of several AAV-based exon editing molecules. To our knowledge this is the first reported demonstration of potentially therapeutically relevant RNA trans-splicing for any gene target in non-human primates. Finally, we describe ongoing work towards further long-term durability and safety assessment of RNA based exon editing strategies with a goal to address the therapeutic needs of patients living with ABCA4-related retinopathies, and other diseases for which exon editing may provide a novel treatment strategy.

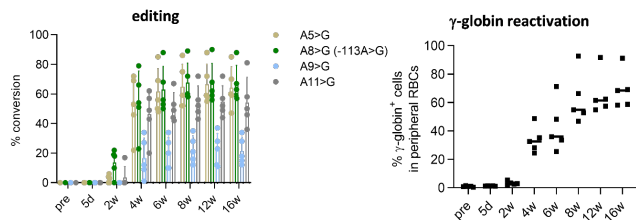
1218. Highly Efficient and Safe *In Vivo* HSC Base Editing for the Treatment of Hemoglobinopathies

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Base editors can install precise nucleotide mutations at targeted genomic loci independent of double-stranded DNA breaks. We previously used an early version of base editor (ABEmax) under the control of a human PGK promoter to target critical motifs regulating γ -globin reactivation. In CD46/ β -YAC mice that carry 248kb of the human β -globin locus (β -YAC mice), we showed that an average of 20% editing (-113A>G in the *HBG1/2* promoter) was generated after *in vivo* hematopoietic stem cell (HSC) transduction with an integrating, helper-dependent HDAd5/35++ vector system consisting of HDAd-ABEmax/mgmt^{P140K} + HDAd-SB100x-transposase and 4 rounds of *in vivo* selection with O⁶BG/BCNU. Here, we report a highly efficient, vectorized base editor system using ABE8e and an optimized *in vivo* HSC genome editing strategy that requires only one intravenously injected HDAd5/35++ vector without transgene integration. The ABE8e gene is placed under the control of a human EF1 α promoter which is stronger than the PGK promoter. We successfully produced an HDAd.EF1 α -ABE8e/mgmt^{P140K} vector using microRNA and virus-associated RNA-suppression of ABE8e in HDAd producer cells. Transduction studies with this vector in cell lines, including erythroid progenitor HUDEP-2 cells, resulted in near saturating editing at the -113A site. In human CD34⁺ cells, ~5-fold higher -113A>G conversion rates were measured in comparison to HDAd-ABEmax/mgmt^{P140K} vector transduction. In an *ex vivo* setting, lineage minus cells (Lin⁻) of CD46/ β -YAC mice were transduced with HDAd-ABE8e followed by transplantation into lethally irradiated recipient mice. Over 90% stable -113A>G editing in blood cells of the recipient mice was achieved. We then performed *in vivo* HSC transduction studies in CD46/ β -YAC mice with a non-integrating HDAd-ABE8/mgmt^{P140K} vector. Three modifications were made to the protocol: *i*) The HDAd-SB100x vector was excluded. *ii*) The selection was started 3 days after *in vivo* transduction to expand HSPCs containing episomal mgmt^{P140K} DNA and RNA. *iii*) Only three rounds of O⁶BG/BCNU were administered within 4 weeks. With this new protocol, we achieved 60% -113A>G editing in bone marrow mononuclear cells, PBMCs, and splenocytes after *in vivo* HSC transduction of CD46/ β -YAC mice. *In vivo* HBG1/2 promoter editing resulted in the reactivation of fetal globin reflected by γ -globin expression in 65% peripheral red blood cells. Single-cell derived CFU assays with week 16 Lin⁻ cells showed that 66% of progenitor colonies have the -113A>G conversion, with bi-allelic editing present in the promoters of both *HBG1* and *HBG2* genes at a rate of 46%. No obvious alterations in hematological parameters, erythropoiesis, and bone marrow cellular composition were observed after treatment, demonstrating a good safety profile of our approach. Lin⁻ cell transplant recipients had stable rates of target site editing and γ -globin expression (study ongoing). This highly efficient and safe vectorized base editor system represents a promising strategy for precise *in vivo* genome engineering for the treatment of β -hemoglobinopathies. Because our *in vivo* approach involves only subcutaneous and intravenous injections

and does not require myeloablation and HSC transplantation, it has the potential for being used to treat hemoglobinopathies in developing countries where these diseases are prevalent.



1219. Dual Editing and *In Vivo* Selection at the *HBG* Promoter Using *MGMT-P140K* Cassette

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Strategies using lentiviral gene therapy or designer nuclease-based gene editing of autologous hematopoietic stem cells have demonstrated therapeutic promise in treatment of hemoglobin disorders including sickle cell disease (SCD) and β -thalassemia. In an effort to further propel progress in this arena, we devised a dual editing approach that: a) targets the BCL11A binding site in the promoter of *HBG1/2* for Crispr/Cas9-mediated disruption and, in parallel, b) utilizes AAV-based co-delivery to introduce an anti-sickling globin β^{T87Q} expression cassette into the *HBG1/2* cleavage site by HDR. This strategy is designed to provide benefit mediated both by non-homologous end joining (NHEJ) and homology directed repair (HDR) editing outcomes leading to re-activation of HbF (*HBG1* or *HBG2* alleles with NHEJ) and expression of anti-sickling globin β^{T87Q} (in *HBG1* or *HBG2* alleles with successful HDR), to drive combined therapeutic benefit in SCD or β -thalassemia. In parallel, to allow for *in vivo* selection of cells with successful HDR, an MND-driven *MGMT-P140K* cassette was included within the AAV donor, providing selective resistance to chemo-selection using O⁶ Benzyl Guanine (O⁶BG) and 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU). This dual editing approach was first tested *in vitro* in human CD34⁺ peripheral blood stem cells (PBSCs). PBSCs were mock edited or edited with RNP alone, or both RNP and AAV donor, then cultured in erythroid differentiation media and subsequently exposed to chemo-selection. NHEJ edited alleles ranged from 60-90% across all RNP conditions and HDR edited alleles averaged 7%. With chemo-selection, there was a 4.5-fold increase in HDR-modified cells, that coincided with a 2-fold increase in expression of β^{T87Q} protein (n = 8 experiments, using 4 independent CD34⁺ donors). To assess the impact of gene editing on engraftment and hemoglobin expression *in vivo*, we next transplanted gene edited PBSCs into NBSGW recipient mice, followed by treatment (or no treatment) with a single dose of chemo-selection at 4 weeks post-transplant. After 16-18 weeks, similar rates of human cell engraftment were present in bone marrow (BM) of all cohorts that did not receive chemo-selection. Although total

human cell engraftment was decreased in mice receiving chemo-selection, this cohort exhibited a 15-fold increase in HDR-modified cells and 23-fold increase in β^{T87Q} RNA transcripts (n = 4 transplants, using 2 independent CD34⁺ donors). Interestingly, there was also a modest increase in NHEJ events after chemo-selection in RNP AAV gene edited groups. To determine the effect of gene editing on HbF expression, BM cells were cultured in erythroid differentiation media and subsequently evaluated. A 1.4-fold increase in CD235 HbF double positive cells was observed in recipients of RNP AAV edited cells that had undergone *in vivo* chemo-selection. Taken together, these findings demonstrate the potential therapeutic benefit of a single gene editing procedure leading to both HbF and β^{T87Q} expression in parallel with a robust mechanism to reach the threshold for clinical benefit via single or iterative chemo-selection.

1220. *In Vivo* Expansion of Gene-Targeted Hepatocytes Through Inhibition of an Essential Gene

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Targeted integration of therapeutic transgenes into a common genomic site could be used to treat a broad range of liver diseases. Recently, we showed that the Apolipoprotein A1 (*Apoa1*) locus could be targeted with adeno-associated viral (AAV) delivery of CRISPR-Cas9 for transgene expression in the liver. We achieved a rate of ~6% of targeted hepatocytes through homology-directed repair. However, a significant number of liver diseases will require higher targeting efficiencies for therapeutic benefit. The goal of this work is to develop a robust and modular system for selective expansion of gene-targeted hepatocytes using essential genes. We developed an expansion strategy consisting of: (1) transient conditioning of the liver by knocking down an essential gene and (2) delivery of an untargetable version of the essential gene (selectable marker) in *cis* with the therapeutic gene of interest. To test this approach, we generated AAV-based targeting vectors to insert an essential gene - fumarylacetoacetate hydrolase (*FAH*) - in tandem with a fluorescent marker (TdTomato) into the *Apoa1* locus. We screened siRNAs to identify sequences capable of mediating highly efficient knockdown of endogenous murine *Fah* *in vivo* using N-acetylgalactosamine (GalNac)-modification. Mice were injected with AAV vectors encoding CRISPR/Cas9 (AAV-CRISPR) and a Donor template (AAV-Donor) to insert the TdTomato reporter cassette at the *Apoa1* locus. Following genome editing, mice were injected with siRNA targeting *Fah* on a monthly basis for four months. Mice receiving only AAV-CRISPR and AAV-Donor showed ~3% of targeted hepatocytes at the endpoint. This was dramatically increased 5-fold in animals subjected to selective expansion with the conditioning siRNA, reaching ~16% of all hepatocytes. Expansion of correctly targeted *Apoa1* alleles was confirmed by sequencing, droplet digital PCR, and western blotting for secreted apoA1 with a 2A epitope tag. We also show that selective

pressure can be further increased using a high protein diet, where up to 25% of hepatocytes express TdTomato after three months. This system achieves efficient *in vivo* expansion of gene-targeted hepatocytes, through transient conditioning of the liver with siRNA, without the use of exogenous drug resistance genes. Importantly, correctly targeted cells do not contain permanent disruption of other genes. This approach should broaden the range of liver diseases correctable with gene therapy using targeted transgene insertion.

1221. Intravitreal Delivery of AAV2 - Exon-Specific U1snRNA Corrects *ELP1* Splicing and Rescues Retinal Degeneration in a Mouse Model of Familial Dysautonomia

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Familial dysautonomia (FD) is an autosomal recessive neurodegenerative disease caused by a splicing mutation in the gene encoding Elongator complex protein 1 (*ELP1*, also known as *IKBKAP*). A T-to-C base change in the 5' splice site of *ELP1* exon 20 results in exon 20 skipping with tissue specific reduction of *ELP1* protein predominantly in central and peripheral nervous system. In addition to a complex neurological phenotype, FD patients exhibit progressive retinal degeneration that severely affects their quality of life. Retinal ganglion cells (RGCs) are major retinal cell population affected in FD patients. The major ocular defects observed in FD include, loss of visual acuity, reduction of maculo-papillary retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL). To restore *ELP1* splicing defect and prevent RGC loss in FD, we have designed a novel splice targeted therapy using modified version of the spliceosomal U1 small nuclear RNA (ExspeU1) that permit targeted binding to intronic sequences downstream of the mutant 5' splice site enabling efficient recruitment of spliceosomal machinery. Using the FD phenotypic mouse model, *TgFD9; Ikbkap^{Δ20/flox}*, we have tested the therapeutic efficacy of ExspeU1 in the correction of *ELP1* mis-splicing and rescue of retinal degeneration. Delivery of a self-complementary adeno associated vector capsid 2 expressing ExspeU1 to the retina (scAAV2-ExspeU1) was performed through Intravitreal injection. Transduction efficiency of scAAV2- ExspeU1 and *ELP1* splicing correction was evaluated by immunohistochemistry and RT-PCR respectively. Phenotypic rescue of retinal degeneration in FD mice was evaluated by measuring the thickness of the retinal layers using Optical Coherence Tomography (OCT). Intravitreal injection of AAV2-ExspeU1 (1x10¹² vg) led to high transduction in RGCs, 40 days post injection. Our findings indicated a significant improvement in *ELP1* splicing correction in the retina in AAV2-ExspeU1 injected mice (n=6) compared to sham injected mice (p<0.001). We were able to demonstrate that AAV2- ExspeU1 correct *ELP1* splicing defect and has the potential to rescue RNFL and GCL loss in FD mice. AAV2 has been previously used in several clinical trials which lead to the development of first FDA approved drug, Luxturna, for treatment of Leber Congenital Amaurosis (LCA). AAV2 mediated delivery of

U1snRNA to the retina has tremendous therapeutic value and has the potential to become a valuable therapeutic intervention to stop retinal degeneration in FD patients.

Breakthroughs in Neuromuscular and Hearing Disorders

1222. CYP46A1 as a Relevant Target to Treat ALS Pathology Independent from its Origin

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Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron disease and is characterized by the progressive loss of upper and lower motor neurons, leading to paralysis and death. Accumulation of cholesterol in the central nervous system (CNS) has been reported to actively contribute to the disease progression in Alzheimer's disease, Huntington's disease, Spinocerebellar ataxia and more recently ALS. Cholesterol is essential for myelin compartment, but also for its functional and structural role in plasmatic membrane. However, in the CNS, cholesterol is synthesized *in situ* and is not able to freely cross the blood brain barrier (BBB). Cholesterol-24-hydroxylase (CYP46A1) allows the conversion of cholesterol to 24-hydroxycholesterol, able to cross the BBB, thus regulating cholesterol homeostasis. Furthermore, this enzyme is a key neuronal stress response such as oxidative stress or protein aggregation. Therefore, we hypothesized that CYP46A1 could be relevant for a therapy in ALS to target both familial and sporadic forms of ALS independently from their genetic origin. In the severe SOD1G93A model, we overexpressed CYP46A1 using an AAVPHP.eB As a first step, we confirmed that the AAV has a specific tropism for the CNS and especially motoneurons. Secondary, we demonstrated a significant and prolonged motor rescue of animals treated pre or post-symptomatically, but also a preventive effect on motoneuron degeneration, myelin loss, compared to untreated animals but also a significant rescue of muscle and neuromuscular junction phenotype as well as a complete rescue of misfolded SOD1 aggregation. Moreover, our therapy is also efficient in another model of the ALS: the C9ORF72 expansion model with a prevention/correction of the behavior abnormalities. Results on both model will be presented.

1223. Gene Therapy for ALS by Specifically Overexpressing a Pleiotropic Chronokine, Secreted α -Klotho, in Skeletal Muscles

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by the loss of cortical and spinal motoneurons (MNs). Denervation of endplates and axonal retraction is thought to lead, in a “dying-back” pattern, to the death of MNs and subsequent muscle atrophy. ALS neuropathology is mainly associated with oxidative stress, inflammation, excitotoxicity, and mitochondrial dysfunction while chronokines like α -Klotho (α -KL) may counteract some of these pathways. Indeed, recent studies indicate that muscle and myofiber regeneration highly depend on klotho expression. Moreover, klotho-deficient mice show altered gait and have less MNs in the spinal cord, with astrogliosis and neurofilament accumulation, resembling an ALS phenotype, and they develop extensive fibrosis and scarring after acute muscle injury. In the SOD1^{G93A} mouse model, which recapitulates most of ALS abnormalities, we have found decreased mRNA levels of α -KL in skeletal muscles, motor cortex and lumbar spinal cord. Furthermore, in rat spinal cord organotypic cultures, the overexpression of α -KL protects spinal MNs from glutamate-induced excitotoxicity. Given the pleiotropic beneficial properties of α -KL, we hypothesized that boosting the expression and secretion in skeletal muscles through a one-time gene therapy treatment would protect muscles from atrophy and prevent axonal retraction and neuronal loss in SOD1^{G93A} mice. Our results show that the overexpression of secreted α -KL in muscles, mediated by an AAV vector, enhances motor function and delays disease onset as evidenced by rotarod and grip strength tests. Improvement of the functional outcome is corroborated by increased compound muscle action potential amplitudes of the tibialis anterior and the plantar interossei muscles compared to non-treated controls. Increased amplitude of motor evoked potentials also reflects the preservation of central connectivity between upper and lower MNs. All this correlates with a higher number of innervated neuromuscular junctions and a heavier gastrocnemius muscle. These findings indicate that increasing the expression and concentration of the pleiotropic chronokine, secreted α -klotho, by muscles is a promising approach for promoting functional improvement in ALS.

1224. AAV-9 Mediated Delivery of RNA Targeting Systems Eliminates Hexanucleotide Repeat Expansions in C9ORF72 ALS/FTD Models

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A GGGGCC (G_4C_2) hexanucleotide repeat expansion (HRE) in the first intron of *C9ORF72* gene is the most common genetic cause of frontotemporal dementia and amyotrophic lateral sclerosis (c9ALS/FTD). Bidirectional transcription at the *C9ORF72* repeat locus produces both sense G_4C_2 and antisense C_4G_2 containing transcripts. Studies to date have elucidated multiple pathogenic mechanisms including RNA gain-of-function of both sense and antisense HREs transcripts leading to formation of nuclear RNA foci and aggregating dipeptide repeat proteins generated by repeat-associated non-AUG (RAN) translation of both sense and antisense HREs, and a loss-of-function due to haploinsufficiency of the C9ORF72 protein. However, it remains unclear how much C9ORF72 protein loss contributes to neuronal death. To explore therapeutic targeting of both sense and antisense HREs, we engineered novel CRISPR/Cas13d based RNA targeting systems with 2 guide RNAs targeting G_4C_2 and C_4G_2 -containing-transcripts that can be packaged in a single AAV genome. Notably, qPCR and RNA-FISH analyses of cells transfected with G_4C_2 and C_4G_2 reporters showed efficient elimination of HREs. Furthermore, we successfully packaged a G_4C_2 targeting guide RNA with two different orthologues of CRISPR/Cas13d into AAV9 with high yields. These AAV9-packaged G_4C_2 targeting CRISPR/Cas13ds showed no overt safety concerns in wildtype mice at 8 weeks post-subpial delivery. Importantly, these constructs reduced the G_4C_2 -HRE containing isoforms of *C9ORF72* in both cultured neonatal cortical neurons and in the spinal cord of transgenic *C9ORF72* mice containing 500- G_4C_2 repeats following subpial delivery, while largely preserving total *C9ORF72* transcript (transcripts from both alleles and including normal non-expanded isoforms) levels. In summary, we show a novel approach that can effectively target both sense and antisense HREs in c9ALS/FTD with a single product.

1225. TFRC-Targeted GAA Delivered as Gene Therapy Treats CNS and Muscle in Pompe Disease Model Mice

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Pompe disease is a Lysosomal Disease (LD) caused by mutations in the acid alpha-glucosidase (*GAA*) gene. Loss of *GAA* results in toxic accumulation of lysosomal glycogen in tissues. Pompe patients present with muscle weakness, respiratory failure, increased risk of aneurysm and stroke, and in severe cases intellectual disability. The current standard-of-care enzyme replacement therapy treats the peripheral pathologies in Pompe patients, but this therapeutic requires lengthy biweekly infusions and does not cross the blood-brain barrier (BBB) to treat the central nervous system (CNS). We have developed a BBB-

crossing antibody-guided therapeutic by fusing the GAA enzyme with a single chain variable fragment (scfv) that binds Transferrin Receptor C (TFRC). We have delivered this anti-TFRCscfv:GAA in Pompe disease model mice as an AAV8 liver-depot gene therapy, allowing for single-dose treatment. Expression is restricted to the hepatocytes by the TTR promoter, and the anti-TFRCscfv:GAA is secreted into the blood where it can bind TFRC in target tissues, facilitating uptake. Anti-TFRCscfv:GAA crosses the BBB and completely rescues the glycogen storage phenotype in brain and spinal cord of *Gaa*^{-/-} mice, while untargeted GAA does not. We also show that AAV8 anti-TFRCscfv:GAA gene therapy clears glycogen in the heart and skeletal muscle of *Gaa*^{-/-} mice more effectively than AAV8 expressing untargeted GAA. This gene therapy eliminates the need for frequent dosing of purified GAA protein and targets both CNS and muscle with a single therapeutic in the Pompe disease mouse model.

1226. Preclinical Development of an AAV-Based Gene Therapy (OTO-825) for Congenital Hearing Loss Due to GJB2 Deficiency

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Introduction: *GJB2* mutations account for 50% of cases of autosomal recessive nonsyndromic hearing loss, making it the most common cause of genetic hearing loss in humans. *GJB2* encodes connexin 26 (CX26), a gap junction protein that is natively expressed in cochlear support cells. CX26 containing gap junctions are thought to be required for maintaining K⁺ homeostasis, an essential component of auditory transduction. Individuals with biallelic *GJB2* mutations present with hearing loss ranging from mild to profound depending on mutational severity. To rescue this *GJB2* deficient phenotype we sought to develop an AAV therapy to deliver functional copies of *GJB2* locally to the cochlea. The cochlea represents a relatively isolated compartment and thus offers a unique opportunity for localized gene therapy with the potential for limited systemic exposure. We previously presented a novel class of AAV capsids that efficiently transduces target cochlear cell types that natively express CX26. Here, we further refined the design of the AAV construct by packaging the novel capsid, promoter, and optimized human *GJB2* gene elements with and without a FLAG tag (OTO-825-FLAG and OTO-825, respectively). Cell-based dye uptake studies were conducted to confirm the ability of OTO-825 to drive expression of CX26 capable of forming functional gap junctions. Further, the therapeutic potential of OTO-825 to restore hearing in vivo was evaluated using two different *Cre* mouse models that exhibit moderate to severe *GJB2* hearing loss. **Methods:** To confirm tropism we injected OTO-825-FLAG intracochlearly into either mice or nonhuman primate ears and visualized expression via immunohistochemistry and confocal microscopy. To assay efficacy, we utilized two different conditional knockout strains (*Cx26* cKO) generated by crossing *Cx26*^{loxP/loxP} mice

with either an inducible *Cre* mouse line or with a constitutive *Cre* mouse line. By postnatal day 30, both mouse lines exhibit severe to profound hearing loss when assessed by auditory brainstem response, reduction in CX26 expression, and a flat epithelium phenotype characterized by regions of mechanosensory hair cell and support cell loss that is consistent with clinical findings. Mice from both *Cx26* cKO knockout strains received a single intracochlear administration of OTO-825 or vehicle and were assessed for auditory function, CX26 expression, and cochlear morphology at postnatal days 30-60. **Results:** Intracochlear injection of OTO-825-FLAG provides a high level of expression in target cochlear cell types in both mice and nonhuman primate ears. Additionally, FLAG-tagged CX26 formed plaques that were morphologically consistent with the gap junctional plaques of endogenous CX26. Compared with a vehicle control, intracochlear administration of OTO-825 into postnatal mice of both *Cx26* cKO strains significantly restored CX26 expression, reduced the prevalence of flat epithelium, and provided a marked improvement in hearing across multiple frequencies. **Conclusions:** We demonstrate that a single intracochlear injection of OTO-825 can transduce the desired cell types in both mice and nonhuman primates, significantly rescuing CX26 deficient hearing loss and cochlear pathologies in two clinically relevant models of *GJB2* hearing loss. These encouraging preclinical results support the advancement of OTO-825 into clinical development for the treatment of congenital hearing loss caused by *GJB2* deficiency.

1227. Adult AAV Gene Therapy Rescues Auditory Function in a Mouse Model of Human TMPRSS3 Recessive Deafness DFNB8/10

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Patients with mutations in the TMPRSS3 gene suffer from recessive deafness DFNB8/10 as the result of deficits in hair cells and auditory ganglion neurons for which cochlear implantation (CI) is the only treatment option. However, in a high percentage of TMPRSS3 patients with CI, the treatment effect diminishes over time, leaving the patients with no alternative treatment option. To develop sustained biological treatment for DFNB 8/10 patients, we generated a knock-in (KI) mouse model with the most frequent human TMPRSS3 mutation. The *Tmprss3* mice displayed late onset progressive hearing loss similar to human TMPRSS3 patients. We used AAV2 to carry a human TMPRSS3 gene to inject into adult KI mouse inner ear. We showed that AAV2-TMPRSS3 delivery resulted in the re-expression of TMPRSS3 gene in the auditory hair cells and the modiolus region. AAV2-TMPRSS3 injection rescued auditory function in the KI mouse model to a level of the wildtype mice of the same age, with hearing maintained by 24 months. Inner ear study showed both hair cells and spiral ganglions were rescued as the result of AAV2-TMPRSS3 delivery in the KI mouse. This is the first time that gene therapy was successfully implemented by a late-stage intervention in a mouse model of human genetic deafness. Our study strongly supports the development of AAV2-TMPRSS3 gene delivery as a standalone therapy or in combination with CI to treat DFNB8/10 patients.

1228. A Knockdown and Replacement Strategy for the Treatment of Charcot Marie Tooth Type 2A

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Introduction: Charcot-Marie-Tooth Type 2A (CMT2A) is an inherited motor and sensory neuropathy. Patients develop progressive muscle weakness and atrophy beginning in the feet, ankles, and legs. CMT2A is caused by mutation of the Mitofusin 2 (*Mfn2*) gene. *Mfn2* is a GTPase protein that forms homomers and heteromers with Mitofusin 1 (*Mfn1*) and functions in mitochondrial fusion, metabolism, and transport. Mutation of *Mfn2* disrupts fusion of mitochondria which leads to toxic aggregation. Complete loss of *Mfn2* is known to be lethal, therefore, we have chosen a gene therapy strategy to both knockdown mutant *Mfn2* and add back functional *Mfn2* to restore function and rescue the disease phenotype. **Results:** Candidate miRNA sequences targeting *Mfn2* were screened and selected *in vitro* in HEK293 cells. miRNA A resulted in approximately 60% knockdown of human *Mfn2* protein in HEK293 cells. miRNA A was then cloned into a vector expressing a codon optimized and miRNA-resistant *Mfn2* transgene. Vectors containing different arrangements of sequences and promoters were tested for expression and activity of both miRNA and transgene in HEK293 cells and *Mfn2*-knockout cell lines. We found that *Mfn2* transgene expression is highly regulated in cell types already expressing endogenous *Mfn2*, therefore, we used *Mfn2*-null mouse embryonic fibroblasts (MEFs) to screen for expression of our transgene-containing constructs. miRNA A in combination with the *Mfn2* transgene expressed 40% of wildtype levels of the *Mfn2* as measured in *Mfn2*-null MEFs and the miRNA achieved knockdown of 34% of endogenous *Mfn2* in HEK293 cells. We chose to move forward with three vectors: one containing the *Mfn2* transgene alone and two with both *Mfn2* transgene and miRNA, but with 2 different promoters, CB7 and CAG. All selected vectors were then packaged in AAV for *in vivo* testing in both wildtype C57BL/6J mice and the *Mfn2*(R94Q) mouse model. Wildtype mice were injected intravenously with PHP.eB vectors via tail vein. We confirmed expression of the *Mfn2* transgene and miRNA *in vivo* in the spinal cords of these mice via qPCR and Western blot. After confirmation, vectors were packaged into AAVhu68 for testing in the *Mfn2*(R94Q) mouse model. The *Mfn2*(R94Q) mouse model displays hindlimb weakness that can be measured using several different behavioral methods including grip strength. In addition, these mice show increased degeneration of motor neurons in the spinal cord and tibial nerve. *Mfn2*(R94Q) mice received intracerebroventricular dosing with vehicle or one of three different vectors. Grip strength data indicated that both combination vectors had statistically significant restoration of muscle strength at 6 weeks and 4 months and performed better than the vector containing only the *Mfn2* transgene. In addition, one combination vector showed significant improvement in body weight at the same time points. **Conclusions:** We have identified a potential candidate vector for the treatment of CMT2A. This vector restores wildtype *Mfn2* while simultaneously knocking down endogenous mutant and wildtype *Mfn2*. *Mfn2*(R94Q) mice dosed with a combination vector had increased muscle strength and body weight compared to other vectors and untreated littermates. Ongoing studies in these mice will include axon counts from spinal

cord and tibial nerves. In addition, the vector is currently being tested in nonhuman primates to determine vector toxicity to the CNS and peripheral nerves. Expression and knockdown of *Mfn2* will also be determined via laser capture microdissection of motor neurons from the spinal cord of the nonhuman primates. Together, these data confirm that a combination vector strategy may be an important route to gene therapy for CMT2A.

Ophthalmic and Auditory Diseases

1229. A Mutation-Independent CRISPR/Cas9-Based 'Knockout and Replace' Strategy to Treat Rhodopsin-Associated Autosomal Dominant Retinitis Pigmentosa

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Rhodopsin-associated autosomal dominant retinitis pigmentosa (RHO-adRP) is an inherited retinal disease that results in blindness due to photoreceptor degeneration. Over 150 mutations in the RHO gene are known to cause RHO-adRP. Using a dual AAV system, Editas Medicine is exploring potential therapy with a highly efficient CRISPR/Cas9 to knockout aberrant endogenous rhodopsin and replace it with an exogenous functional rhodopsin (KO&R). The specificity of a human and non-human primate (NHP) cross-reactive Cas9 guide was tested using human retina explants transduced with the KO&R. Pharmacokinetics and pharmacodynamics of the KO&R were assessed in humanized *mRho*^{hRHO/+} mice. Efficacy was measured in NHPs by comparing knockout (KO) only and KO&R versions of the dual AAV system delivered via subretinal injection. A minimal RHO promoter was used to drive Cas9 as well as RHO expression while restricting editing and replacement of RHO expression to the rod photoreceptors. On- and off-target editing was assessed using Next Generation Sequencing. Endogenous and replacement RHO levels were quantified using NanoString and tandem mass spectrometry. Morphological and functional readouts were assessed by histopathology and electroretinography. No off-target editing was observed in human retina explants after transduction with the KO&R. The KO&R achieved maximal levels approximately 6 weeks post-dose and remained stable for at least 13 weeks post-dose in *mRho*^{hRHO/+} mice. The KO&R levels displayed a dose response reaching a plateau at a dose of 6E12 vg/ml. Studies in NHP demonstrate nearly 100% knockout of endogenous RHO, and replacement RHO produced over 30% of normal RHO protein. The KO&R-injected eyes showed restoration of RHO expression in the outer segments and retention of normal photoreceptor structure and function compared to the KO-injected eye. In conclusion, Editas characterized and demonstrated high efficacy of a CRISPR/Cas9-based KO&R therapeutic strategy for RHO-adRP. The experimental therapy is mutation-agnostic and could be a potential one-time treatment to permanently suppress the toxic gain of function

associated with mutated RHO. The efficacy of our KO&R gene editing therapy in NHP supports continued advancement of this approach toward treating patients.

1230. Development of an AAV-Based Gene Therapy for the Ocular Phenotype of Friedreich's Ataxia

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Purpose: *FXN* mutations are associated with Friedreich's Ataxia (FA), an autosomal recessive neuromuscular disorder characterized by cardiomyopathy, ataxia, kyphoscoliosis, auditory, and retinal defects. Ocular symptoms include nystagmus, oculomotor apraxia, and loss of retinal nerve fiber layer (RNFL) thickness. AAV-based gene therapies for the cardiac and neuronal symptoms of FA delivered systemically or directly to the CNS are being developed, but neither are expected to treat the retina. Our goal is to develop a gene therapy to prevent vision loss in FA. Complete knockout of frataxin is embryonically lethal and existing conditional knockout models do not focus on retina. The purpose of our study was to characterize the impact of retina-only *FXN* deficiency in a novel conditional KO mouse, and to use this model to evaluate retinal-targeted AAV-*FXN*. **Approach:** The mRx-Cre driver line carries a single Cre allele under the control of the retina-specific mRx promoter (mRx-Cre Het). mRx-Cre Het mice were crossed with *Fxn* heterozygous mice (*Fxn*^{+/-}) to create offspring heterozygous for both frataxin KO and Cre recombinase (*Fxn*^{+/-}; Cre^{+/-}). These were bred with homozygous floxed frataxin mice (fl*Fxn*^{+/+}), giving rise to the experimental mice (fl*Fxn*^{+/-}; Cre^{+/-}), referred to as "mRx-*FXN* KO". fl*Fxn*^{+/-}; Cre^{-/-} animals were used as controls. In life assessments occurred over 2 months and thickness of the RNFL, ganglion cell layer (GCL) and outer nuclear layer (ONL) were measured via optical coherence tomography (OCT). Electroretinogram (ERG) was used to assess retinal function. *FXN* expression was evaluated by immunohistochemistry (IHC) and immunoblotting. Utilizing an AAV capsid variant P2-V1(trpY-F+T-V) identified through directed evolution and further enhanced by rational design, two AAV-*FXN*-HA vectors using CBA or hSyn1 promoters were produced and intravitreally injected into one eye of mRx-*FXN* KO and control mice. Control GFP vectors were injected in the contralateral eyes. Retinal structure and function were assessed in life and post mortem as described above. **Results:** mRx-*FXN* KO mice presented no ataxia or other symptoms of broad *FXN* deficiency, and survived without any health issues through the study. OCT and ERG revealed aggressive, progressive and early onset loss of retinal structure and function. Significant retinal thinning, especially in the GCL and ONL, was observed via OCT and IHC of retinal cross sections at all time points evaluated. At post-natal day 14, the thickness of each layer was reduced up to 30%. and further reduced up to 79% by day 60. *FXN* expression was absent from mRx-*FXN* KO retinas. Treatment with either CBA or hSyn1- containing AAV-*FXN*-HA vectors at post-natal day 15, led to significant protection (12-30%) of all retinal layers, 1- and 2-months post injection versus GFP injected

eyes. However, no improvements in retinal function were observed by ERG. IHC revealed high levels of *FXN* in eyes injected with AAV-*FXN*-HA localized the retinal ganglion cells. **Conclusions:** We successfully characterized a novel, retina-only *FXN* KO mouse that exhibits early onset and progressive retinal dystrophy. AAV-*FXN*-HA mediated gene delivery conferred significant protection of retinal structure but not retinal function. We attribute the more aggressive phenotype in mice vs. FA patients to the complete lack of retinal *FXN* in these mice vs. FA patients who retain up to 30% of *FXN* expression. Taken together, our data supports continued development of a gene therapy to target FA-associated vision loss. Efforts are underway to develop a more refined retinal mouse model of FA.

1231. SPVN06, a Novel Mutation-Independent AAV-Based Gene Therapy, Dramatically Reduces Vision Loss in the rd10 Mouse Model of Rod-Cone Dystrophy and is Well Tolerated in a 1-Month Pilot Safety Monkey Study

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SPVN06 is a proposed gene therapy for patients experiencing vision loss due to rod-cone dystrophy (RCD), regardless of the causative mutation. As a consistent pattern in RCD, photoreceptor degeneration is first observed in rods, and subsequently in cones, in large part due to a lack of trophic support. SPVN06 encodes, in a single AAV, the cDNAs for trophic factor Rod-derived Cone Viability Factor (RdCVF) and thioredoxin enzyme RdCVF-Long (RdCVFL), both isoforms encoded by the *NXNL1* gene. RdCVF, secreted by rods, protects cones from degeneration by stimulating aerobic glycolysis. RdCVFL is a potent antioxidant protecting photoreceptors from oxidative stress. SPVN06 early nonclinical development included several pharmacology studies in *rd10* mice, a fast-progressing model of RCD and a 1-month pilot safety study in non-human primates. *rd10* mice were born and raised in darkness until transferred to regular cyclic light on postnatal day (P) 31 up to study termination at P48. At P15 or P18, the animals received a bilateral subretinal administration of 1 μ L of vehicle or SPVN06 at 1E8 vg/eye. Effect of SPVN06 on retinal function and structure were evaluated by optokinetic (OKT), electroretinography (ffERG), optical coherence tomography (OCT) and histology. Four female cynomolgus monkeys were dosed in the 1-month pilot safety study. The animals received a bilateral subretinal administration (100 μ L) of vehicle (n=1) or SPVN06 7E10 vg/eye (n=3). The standard safety assessment included ophthalmic examinations, intraocular pressure measurements, wide-field color fundus imaging, OCT and ffERG. A highly significant protection of the visual function (OKT) was noted at P32 (p<0.0001) when the animals received SPVN06 1E8 vg/eye at P18. The level of visual function in SPVN06-treated eyes was similar to wild-type (WT) level versus only ~50% of WT in vehicle-injected eyes. At the later timepoints (P38 and P45), the difference between the

2 groups remained significant, although visual loss continued in both vehicle and SPVN06 treated eyes. H&E stained sections of the retina at P48 didn't reveal a protection of the ONL, whereas PNA staining at the same timepoint revealed greater density of the cone outer segments and/or their membrane remnants in SPVN06-treated eyes. When injection was conducted at P15, no protection of the visual function was noted suggesting an optimal timing (P18) for injection/treatment in this model. There were no significant SPVN06-related changes in fERG or OCT parameters at any timepoints. SPVN06 at 7E10 vg/eye was well tolerated in the monkeys with minimal posterior uveitis and RPE hypertrophy confined to the dose site of SPVN06-treated animals that was not observed in the control. There were no retinal functional effects due to SPVN06 based on fERG analysis and no test article-related systemic effects. In conclusion, SPVN06 was well tolerated in monkeys at 7E10 vg/eye and dramatically protected retinal degeneration in *rd10* mice at 1E8 vg/eye, a fast-progressing model of RCD. These data support SPVN06 clinical development.

1232. Reduced Ocular Inflammation and Improved GFP Expression in Rabbits with Controlled Release of Adeno-Associated Virus from Degradable Hydrogel Implants

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Purpose: Inflammation often arises during ocular gene therapy with viral vectors and the severity is generally related to vector dose. Severe inflammation can be associated with reduced gene therapy efficacy. Here we investigated how release kinetics of adeno-associated virus (AAV) mediated gene therapy from a hydrogel implant affects ocular inflammation and GFP expression. **Methods:** In our first rabbit study, low dose ($1.2E+10$ GC/eye) AAV2-CMV-GFP loaded hydrogel implants were formulated to release AAVs over 4 days. A single implant was injected intravitreally (IVT) in both eyes. Bilateral injections of a bolus 50 μ L AAV solution at the same dose were performed as a positive control. In the second rabbit study, we prepared AAV2.7m8-CMV-GFP loaded hydrogel implants at a higher dose ($3.6E+10$ GC/eye) with two different release formulations (4 and 14 days). We chose a modified AAV2 vector- AAV2.7m8 due to its enhanced retinal transduction properties over its parental serotype. No immunosuppressants were used in either study. The impact of sustained AAV delivery on inflammation and green fluorescent protein (GFP) expression was investigated through ocular examinations (SPOT scoring system) and fundus autofluorescence (FAF) imaging in both studies (n=6 eyes per group). **Results:** Implants could be tuned to control the release of AAVs through degradation of the hydrogel (Figure 1). Both rabbit studies showed that a single 50 μ L injection of AAV, without immunosuppressants, resulted in mild to moderate inflammation and loss of GFP expression over time (Figure 2). In contrast, AAV implants exhibited a different response. When AAV dose was low (1st study), the AAV release within 4 days reduced inflammation to none-trace and resulted in steady GFP expression compared to the bolus dose. However, the AAV release within 4 days in the 2nd study was not enough to manage inflammation since AAV dose was 3x higher

than the first study. The medium release formulation had the lowest inflammation scores and strongest GFP expression among all groups in the 2nd rabbit study due to release of higher titer AAVs over 2 weeks. **Conclusions:** In this study, we demonstrated that controlled release of AAVs from degradable hydrogel implants produced less severe inflammation and greater in vivo transduction compared to a single bolus administration. These data show a potential dose-rate response for AAV therapy and demonstrate the promise of sustained-release hydrogel implants as a therapeutic and safety benefit for ocular AAV gene therapy.

Figure 1: Total AAV capsid released via ELISA

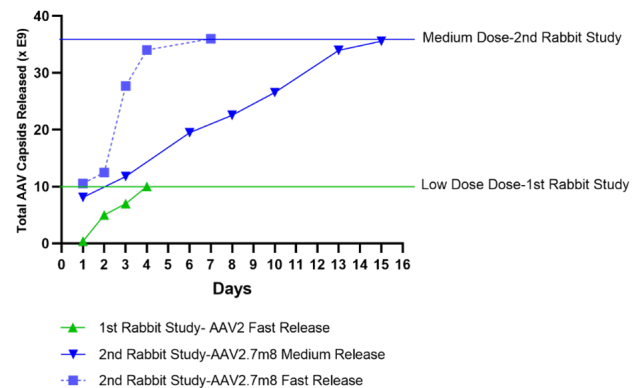
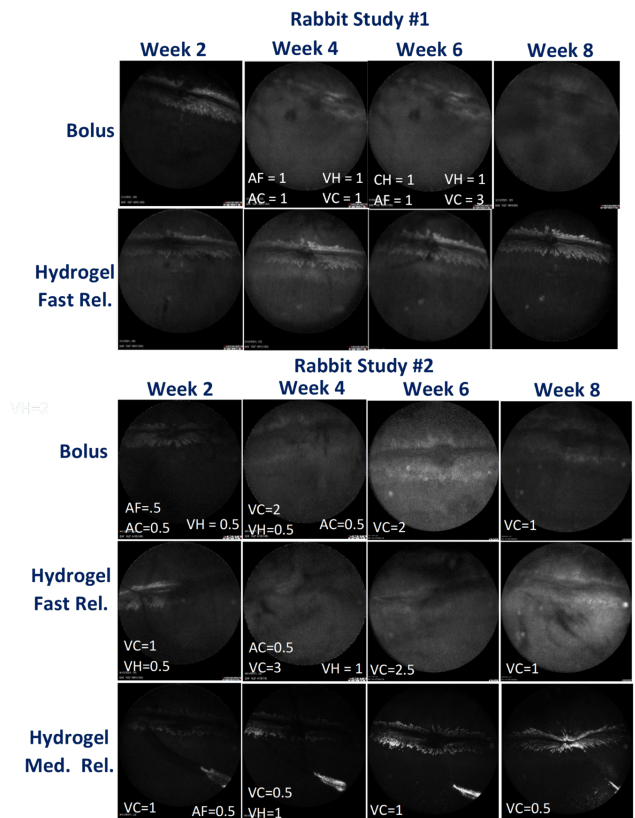


Figure 2: Fluorescent images of GFP signal at each time from each group.



1233. Nonclinical *In Vivo* Expression, Durability of Effect, Biodistribution/Shedding, and Safety Evaluations Support Clinical Development of AK-OTOF (AAVAnc80-hOTOF Vector) for *OTOF*-Mediated Hearing Loss

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Millions of people worldwide have disabling hearing loss because mutations in one of their genes result in loss of expression or generation of an incorrect version of a protein required for hearing. The *OTOF* gene encodes the otoferlin protein, which is critical for afferent signaling at the cochlear inner hair cell (IHC) synapse; thus, individuals with biallelic mutations in *OTOF* typically present with congenital, Severe to Profound sensorineural hearing loss. Gene therapy using adeno-associated viral (AAV) vectors is a promising therapeutic modality for individuals with *OTOF*-mediated hearing loss. Because the otoferlin coding sequence is approximately 6 kb, exceeding the packaging capacity of a single AAV vector, a dual vector approach is utilized. AK-OTOF (AAVAnc80-hOTOF) is a dual vector that consists of two component AAVAnc80 vectors, one that encodes a ubiquitous promoter and the upstream (5') hOTOF transcript and one that encodes the downstream (3') hOTOF transcript; the product candidate is intended for the treatment of individuals with *OTOF*-mediated hearing loss. Following intracochlear delivery and subsequent co-transduction of the target cells (IHCs) by each component vector, the two transgene products recombine to generate a full-length otoferlin mRNA transcript and subsequently a full-length otoferlin protein. Nonclinical studies to support the clinical development of AK-OTOF have evaluated localization of expression, durability of effect at a range of doses, biodistribution/shedding, and safety. One month following a single intracochlear administration of AAVAnc80-hOTOF to otoferlin knockout (*Otof*^{-/-}) mice or an AAVAnc80 vector encoding a 3'-FLAG-tagged human otoferlin protein (AAVAnc80-FLAG.hOTOF) to macaques, robust *de novo* expression of full-length human otoferlin protein was observed only in IHCs, and not in other cochlear cell types. Expression of human otoferlin protein in the IHCs of *Otof*^{-/-} mice was sufficient to restore auditory function as early as 2 weeks post-administration, and restoration was durable through at least 6 months (the longest survival duration evaluated). Biodistribution/shedding, evaluated with validated qPCR assays, showed persistent presence of both component vector sequences, through at least 6 months post-administration, in the inner ear of non-human primates (NHPs); detection of vector sequences in non-cochlear tissues or fluids (i.e., saliva, blood, and serum) occurred less frequently and the number of copies decreased, often rapidly, over time. Evaluations from NHPs that received bilateral intracochlear administration of AAVAnc80-FLAG.hOTOF, by RT-qPCR, showed low levels of human otoferlin-FLAG mRNA in some liver and spleen samples at one-month post-administration, but no human otoferlin-FLAG protein was detected in these tissues by Wes™. AAVAnc80-hOTOF was systemically and locally well tolerated in both mice and NHPs, and no adverse effects were observed in clinical

pathology, otic pathology, systemic histopathology, and/or auditory function. Together, these IND-enabling nonclinical studies support the clinical development of AK-OTOF for the treatment of *OTOF*-mediated hearing loss.

1234. Novel Capsid LSV1 Has a Unique 3D Structure at the Loop Substitution Area - Confers Superior Retinal Transduction from Intravitreal Injection

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Recombinant adeno-associated viruses (AAV) are among the most used gene therapy vectors, with efficacy and safety already proved in humans and several animal models. Nevertheless, there are still many challenges that need to be overcome to successfully expand its use for more diseases. One of the largest hurdles for AAV-based retinal gene therapy to overcome is the delivery of the gene therapy itself. The ocular space being small, enclosed, and external makes it an ideal gene therapy target area, but the retina itself remains difficult to transduce. The two most common delivery techniques are subretinal injections (SRI) and intravitreal injections (IVT). The SRI is a surgical procedure, involving the formation of a bleb to deposit the AAV, thus with a high potential for retinal and general ocular damage. Further, AAV has difficulty escaping the bleb area restricting expression to certain areas. IVT is far less invasive and has the potential to transduce cells panretinally. However, most AAVs are impeded by the inner limiting membrane (ILM) and fail to transduce upstream retinal cells like photoreceptors and retinal pigmented epithelium (RPE). To overcome the ILM, we generated a library of over 10⁶ AAV variants by inserting or substituting 9-10 amino acid loops into AAV2.5T and screened the fitness of these variants iteratively in non-human primate (NHP) eyes. After three rounds of screening, Loop Swap Variant I (LSV1) became the most enriched variant in the library. The 3D structure of LSV1 by cryoEM reveals that it has a unique structure among all AAV. The LSV1 loop substitution, located in variable loop VIII, has changed the overall topology of the 3-fold protrusions by shifting and essentially merging the three together. This new formation interacts with heparin, which is thought to enable capsid passage through the ILM. When injected by IVT into NHP, LSV1 carrying a GFP transgene generates pan-retinal fluorescence as observed by fundus and flat-mount. Retinal cross sections reveal deep retinal penetration by LSV1, with GFP expression found in outer retinal cells like rods, cones, and RPE. The ability to transduce retinal cells widely and thoroughly makes LSV1 an excellent capsid for clinical retinal gene therapy, especially for disorders affecting outer retinal cells like retinitis pigmentosa, cone dystrophies, macular degeneration (dry & wet), and geographic atrophy.

1235. Development of Dual-PCDH15 AAV Gene Therapy for Usher Syndrome Type 1F Deafness and Blindness

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Usher syndrome type 1F is caused by mutations in the *PCDH15* gene, which encodes the tip-link protein PCDH15. It is a recessively inherited syndrome characterized by profound congenital deafness and absence of vestibular function, and by progressive blindness beginning in the second decade. Gene addition therapy could be an attractive treatment; however, the *PCDH15* coding sequence of ~5.8 kb is too large to fit into a single AAV capsid. We used a dual-AAV strategy to circumvent the size limitation and to treat the deafness in an Usher 1F mouse model.

Methods: We engineered two vectors that each encode part of the full-length protein. In a cell, AAV genomes can recombine to create a full-length coding sequence. We first assessed function in vitro. We treated HEK cells with dual AAVs and assessed recombination and translation using RT-PCR and immunofluorescence microscopy. To evaluate function in cochlea in vivo, *Pcdh15* conditional knockout mice were injected with dual AAVs at P1 through the round window membrane. Hearing tests and histological analyses were performed at P30. To evaluate rescue of vestibular function in mice we injected constitutive knockout mice with dual AAV and perform vestibular tests at P30. To assess the potential of dual-AAV-mediated gene expression in human retina in vitro, we transduced retinal organoids from human iPSCs with dual AAVs and five weeks later evaluated HA-tagged PCDH15 expression and localization with immunofluorescence and immunogold SEM imaging.

Results: In HEK cells in vitro, full-length PCDH15 was successfully produced using dual-AAV delivery. Proper recombination and splicing were confirmed with RT-PCR and Sanger sequencing. With an anti-PCDH15 antibody, strong labeling at HEK cell membranes was observed. We found that *Pcdh15* conditional knockout mice, injected at P1 through the round window membrane with dual AAVs encoding HA-tagged PCDH15, displayed HA immunoreactivity at the tips of stereocilia, four weeks after injection. They also showed robust rescue of hair bundle morphology observed by both actin labeling and SEM, and rescue of mechanotransduction assessed with FM1-43 loading. While uninjected *Pcdh15* conditional knockout mice were deaf and had degenerated hair bundles, mice treated with dual AAVs encoding PCDH15 demonstrated good hearing rescue at low and middle frequencies. Constitutive knockout mice treated with dual AAVs encoding PCDH15 showed restoration of vestibular function to wild-type levels while uninjected *Pcdh15* null mice showed a severe vestibular phenotype: intensive head bobbing, circling behavior and hyperactivity, swimming difficulties, inability to stay on the rotarod. We also characterized the ultrastructure of photoreceptors in retinal organoids. SEM showed that a majority of photoreceptors developed inner segments, while some formed outer segments and connecting cilia. We observed nascent calyceal processes at the apical ends of inner segments. Retinal organoids from human iPSCs transduced with dual AAVs encoding HA-tagged PCDH15 showed antibody labeling of HA-tag in photoreceptors,

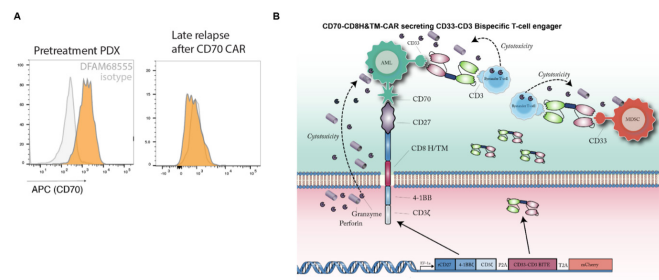
which was localized on the surface of the inner segments and at the inner/outer-segment junction where calyceal processes develop. **Conclusions:** Dual-AAV delivery of PCDH15 restores hearing and balance in a mouse model of Usher 1F, and mediates expression and normal localization of PCDH15 in human photoreceptors in vitro. It holds promise for treatment of Usher 1F.

Cell-Based Cancer Immunotherapies III

1236. Optimized CD70-Targeted CAR Secreting a CD33-Targeted Bispecific T-cell Engager Overcomes Antigen Heterogeneity for Acute Myeloid Leukemia

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Chimeric Antigen Receptor T cells (CAR-T) have been highly effective for B-cell malignancies, but not yet for acute myeloid leukemia (AML). The TNF-alpha family member CD70 has emerged as a promising surface target antigen in AML. We have previously optimized a CD70-targeted CAR for AML via modifications to the hinge and transmembrane regions (Cancer Cell, in revision), which resulted in substantially improved potency over prior generations of CD70-targeted CARs. After long-term follow-up in NOD-SCID IL2Ry^{null} (NSG) mice engrafted with patient derived xenografts of AML, a subset of animals developed late CD70 antigen-negative relapses (Fig. 1A). To overcome such antigen-escape, we designed a dual targeting strategy in which the optimized CD70 CAR vector had a second transgene to drive constitutive secretion of a bispecific T-cell engager antibody ("TEAM") (Fig. 1B). Despite the larger size of the bicistronic vector, the CD70-CAR-CD33-TEAM had comparable transduction efficiency to monotargeted constructs with greater than 60% across three healthy donors' T-cells. We show that TEAMs from the supernatant from the CD70-CAR-CD33-TEAM bind specifically to target-expressing cells. Next, in a simple real-time cell analysis (RTCA) transwell assay with the CD70-CAR-CD33-TEAM on top and untransduced T-cells on bottom, the CD33-TEAM (but not a control CD19-TEAM) mediated AML-target cell (Kasumi-1) eradication. Also, in a RTCA we demonstrate elimination of GFP-expressing molm13 wild type, CD33 knockout, and CD70 knockout tumors using the dual targeting CD70-CAR-CD33-TEAM. Next using AML cell lines with a variety of native CD70 and CD33 expression levels, we demonstrate CD70-CAR-CD33-TEAM killing of all targets along with superior expansion compared to the CD33 and optimized CD70 CARs alone, or with an irrelevant CD19 TEAM. Our findings demonstrate the potency of a CD70-targeted CAR which secretes a CD33-targeted bispecific T-cell engager against tumor targets with variable antigen expression. Ongoing safety and efficacy testing using in vivo mixed tumor and PDX models is underway.



1237. Safety Lead-In of Ph2 AML Study Results Using Zedenoleucel

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Background: Measurable residual disease (MRD) testing has become more prevalent in AML. MRD positivity is associated with increased relapse risk and shorter survival in AML, and currently, there are no approved therapies for these patients. Zedenoleucel (also known as MT-401) is a non-genetically modified allogeneic multi-tumor associated antigen (mTAA)-specific T cell therapy with selectivity to multiple tumor antigens, PRAME, WT1, NY-ESO-1 and Survivin. There are promising preliminary clinical results with mTAA-specific T cell therapy targeting these same tumor antigens (Lulla et al., 2021). **Methods:** The Safety Lead-in portion of a multicenter Phase 2 study (ARTEMIS) evaluating the safety, tolerability and efficacy of zedenoleucel in patients with AML post-HSCT, has been completed. The Safety Lead-in includes patients with active disease (frank relapse or MRD⁺) in two cohorts with a primary objective to evaluate the safety and tolerability of zedenoleucel manufactured using reagents obtained from two different vendors. Patients may receive up to 3 consecutive infusions of zedenoleucel as a monotherapy (50×10^6 cells every 2 weeks). Primary endpoints include various safety measurements and incidence of dose-limiting toxicities (DLTs). Efficacy evaluations occur using ELN recommendations for standard AML response criteria. **Results:** Six patients were enrolled and treated in the Safety Lead-in portion of ARTEMIS (4 patients with frank relapse, 1 patient with morphologic leukemia-free state [MLFS] and 1 MRD⁺ patient). All patients received at least 1 infusion, and 5 patients were able to complete all 3 infusions. No DLTs were observed. Regarding efficacy, one patient had MLFS at baseline but developed increased blast count at week 8 disease assessment. The other 4 patients had frank relapse with blast counts at baseline ranging from 5-40% and their disease worsened. However, the MRD⁺ patient with t(8/21)(q22;q22.1) [RUNX1-RUNX1T1] genetic abnormality showed a decrease in MRD by PCR from a starting baseline of 0.8093% to resolution via peripheral blood at approximately 32 weeks post-treatment with zedenoleucel.

T cell composition of the product consisted of 71% CD4⁺ and 24% CD8⁺ T cells. T cell receptor (TCR) analysis identified 3,117 antigen-specific clones (881 Survivin, 783 NY-ESO-1, 750 PRAME, 709 WT1). Immune monitoring of this patient using biomarker analysis showed T cell specificity not only for the targeted antigens but also for non-targeted antigens over time, thereby demonstrating epitope spreading. Interestingly, the tumor antigen composition by RNASeq identified an antigen expression profile that changes over time and inversely correlates with the presence of antigen-specific T cells, demonstrating the interplay of tumor cell immunogenicity and antigen-specific T cells. **Conclusions:** Zedenoleucel was successfully manufactured using two different reagent vendors and was safely administered to 6 post-transplant AML patients with no DLTs noted. Preliminary evidence of anti-tumor activity with zedenoleucel was observed in the Safety Lead-in portion of the Phase 2 ARTEMIS study which further supports the promising Phase 1 ADSPAM data using mTAA-specific T cells. Specifically, the ARTEMIS results showed that administration of zedenoleucel converted an MRD⁺ patient to MRD⁻ and suggests that AML patients could potentially benefit from administration of zedenoleucel.

1238. N-Glycosylation Inhibition Hinders Immunosuppressive Activity of Tumor Microenvironment Cells and Improves CAR T Cell Efficacy

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CAR T cells have shown considerable results against B-cell malignancies, but still limited efficacy against solid tumors, where there are multiple challenges, e.g., an immunosuppressive tumor microenvironment (TME). Our Unit has recently reported that surface N-glycans protect tumor cells from CAR T cells targeting and has described a pharmacological strategy based on the glucose/mannose analogue 2DG to offset this barrier. In this project, we investigated the role of N-glycosylation blockade on immunosuppressive TME cells in the context of CRC/PDAC-derived liver metastases and CEA-specific CAR T cell therapy. To understand the effect of 2DG on the immunosuppressive function of M2 macrophages (M2-M) and hepatic stellate cells (HepSCs), we performed tripartite co-cultures and suppressive assays in the presence or absence of glycosylation inhibitors. Moreover, we analyzed how glycosylation blockade impacts lineage marker expression, gene expression profile, cytokine release and PD-1/PD-L1 interaction. For the *in vivo* part, we are exploiting a mouse model where SGM3 mice are reconstituted with human HSPCs (huSGM3), engrafted intra-liver with tumor cells (either BxPC3, LoVo or patient-derived organoids, PDOs) and treated with CEA CAR T cells. We have analyzed the ability of tumor cells and 2DG to modulate the human TME in the liver of huSGM3 mice and we are setting up the conditions for treatment with CEA CAR T cells. We observed that M2-M and HepSCs suppress T cells proliferation, but in the presence of 2DG and also tunicamycin, a specific N-glycosylation inhibitor, the immunosuppressive activity of TME cells was abolished.

Moreover, adding exogenous mannose we could revert 2DG effect, suggesting that this was due to N-glycosylation blockade, since mannose inhibits 2DG in this pathway. Interestingly, we observed that 2DG inhibits M2-polarization in macrophages, in terms of phenotype (CD206 and PD-L2 downregulation), gene expression profile and IL-10 secretion. Strikingly, it seems that N-glycosylation blockade modify the polarization of M2 macrophages, becoming similar to M1 like (upregulation of M1 genes and secretion of TNF-alpha). Trying to recapitulate the human TME *in vivo*, we observed that in the liver of tumor-bearing huSGM3 mice treated with 2DG, the frequency of M2-M was significantly reduced, in line with our *in vitro* data. On the other hand, N-glycosylation inhibition proved able to block the PD-1/PD-L1 axis (a binding that requires N-glycans) and reduced the expression of PDGFR- β in HepSCs. Interestingly, *in vitro* co-cultures show that 2DG is able to increase CEA CAR T cell killing capacity despite the presence of TME cells, against cell lines (BxPC3 and LoVo) and CRC-liver metastases PDOs. While setting up the *in vivo* model with CEA CAR T cells, we observed that intra-liver injection of CAR T cells is more potent than intravenous infusion and that humanization can boost antitumor efficacy, indicating that immune cells and cytokines contribute to CAR T cell efficacy, as already observed with B-cell malignancies. We are currently investigating to combine CAR T cell therapy with 2DG in these mice. N-glycosylation plays an important role not only on tumor cells but also on TME cells. Blocking N-glycosylation with 2DG reduces the immunosuppressive function of TME cells, leading to increased CEA CAR T cells activity not only against cell lines, but also against primary PDOs.

1239. CAR T That Targets MUC1 Transmembrane Cleavage Product Has Increased Persistence and Kills Low Antigen Cells

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Background: At ASGCT 2019, we presented data on a CAR T that targets the MUC1 transmembrane cleavage product called MUC1* (muk 1 star). MUC1* is a growth factor receptor that is activated when onco-embryonic growth factor NME7AB dimerizes its truncated extra cellular domain. The binding site for NME7AB is ectopic. It is masked in full-length MUC1, as is the binding site for our anti-MUC1* antibody, huMNC2. Thus, huMNC2 does not bind to full-length MUC1, which is expressed on healthy epithelium. Previous attempts at a MUC1 targeting therapeutic failed, presumably because they targeted the tandem repeat domain of full-length MUC1, which is shed after cleavage to MUC1*. Antibody huMNC2 is highly selective for cancerous tissues, first because it binds MUC1*, not the healthy full-length form. Secondly, huMNC2 antibody recognizes a specific conformation of the MUC1* extra cellular domain that is created when MUC1 is cleaved by an enzyme that is overexpressed in many cancers, especially breast cancers. **Update:** Our 1st-in-human clinical trial of anti-MUC1* CAR T, huMNC2-CAR44, for the treatment of metastatic breast cancers has validated the target, MUC1*, with early evidence

of efficacy and safety, but has also highlighted the problem of CAR T cell persistence for the treatment of solid tumor cancers. **huMNC2-CAR44-1XX:** We are now preparing to open a clinical trial of an improved CAR T, to be studied in parallel with huMNC2-CAR44. The improved CAR incorporates the "1XX" mutations in the CD3z signaling domain, intended to slow signaling (1XX technology in-licensed). Head-to-head experiments compared the current huMNC2-CAR44 to huMNC2-CAR44-1XX. Experiments were performed in an animal model we developed in which the killing of high antigen versus low antigen cells can be *separately* tracked in *live* animals by implanting heterogeneous tumors, wherein the two different cell populations emit light at very different wavelengths. In addition, overall tumor volume was tracked by luciferase/luciferin emission on an IVIS instrument. These experiments showed that at high dose, standard CAR T cells efficiently killed high antigen expressing cells, but did not kill the low antigen expressing cells, which survived and drove tumor recurrence after about Day 45. At low CAR T cell dose, standard CAR T cells rapidly got exhausted and tumor growth was driven by both high and low antigen expressing cells. huMNC2-CAR44 T cells could not be recovered from animals after sacrifice at Day 93. In stark contrast, huMNC2-CAR44-1XX, at both high and low dose, killed both high and low antigen expressing cells. There was virtually no tumor recurrence at either dose level and huMNC2-CAR44-1XX T cells were readily recovered from tumor, spleen and blood post-sacrifice Day 93. IHC studies of thousands of normal vs. cancerous human tissue specimens show that huMNC2-scFv almost exclusively binds to tumor tissues, hitting over 90% of breast, 83% ovarian, 78% pancreatic and 71% of lung cancers. Recognition of breast cancer specimens appears not to be limited by cancer sub-type. Screen-fail rate in patients, at this point, mirrors the percent positivity for MUC1* expression seen in the *in vitro* IHC studies.

1240. CD4 CAR T Cells Drive Extensive CD8 CAR T Cell Expansion, Leading to Severe Cytokine Release Syndrome

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So far, CD19-redirected Chimeric Antigen Receptor (CAR) T cells have gained impressive clinical success for the treatment of B-cell malignancies. However, treatment failures are still common and the occurrence of severe toxicities, such as Cytokine Release Syndrome (CRS) and neurotoxicity, still limits the full exploitation of this therapeutic strategy. Therefore, the development of T-cell products with improved therapeutic indexes is warranted. CAR T cell responses are influenced by multiple factors, including the composition of the infused product, both in terms of memory differentiation and CD4/CD8 ratio. In this project, we investigated how CD4 and CD8 CAR T

populations cooperate during CAR T cell responses and what is their specific role in CRS development. Interestingly, CD4 CAR T cells *in vitro* signature showed superior proliferation and degranulation activity, notwithstanding a reduced killing activity compared to CD8. Moreover, CD4 CAR T cells displayed a higher activation profile that translates in a higher activation of myeloid cells, the main trigger of toxic events. Accordingly, toxicity assessment in a HSPC-humanized mouse model identified CD4 CAR T cells as key contributors to CRS development, revealing moreover a safer profile when CD4 CAR are embedded with the 4-1BB rather than CD28 co-stimulatory domain. When we evaluated the *in vivo* performances of differentially costimulated CD4:CD8 1:1 CAR T cell products, we observed that CD4 CAR T cells shape the expansion kinetics of CD8. Moreover, the combination of CD4.BBz with CD8.28z resulted in lower toxicity compared to all the other conditions, without impacting on antitumor efficacy. Taken together, these data point out that CD4 CAR T cells have a key role in shaping CAR T cell responses and are active players in toxicity development. Therefore, the specific engineering of these cell subsets may result in the generation of CAR T cell products with improved therapeutic indexes.

1241. Targeting Tumors and the Tumor Microenvironment with Banana Lectin Expressing T Cells

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In contrast to successful treatment of hematological malignancies, cell therapies for solid tumors have been thwarted by the hostile tumor microenvironment (TME), and by heterogeneous and dynamic expression of potential target antigens by tumor cells. We are tackling both limitations by combining conventional, antibody-based CARs with a novel class of chimeric receptors based on plant lectins, which recognize the increased or aberrant sugar residues present both on malignant cells and their associated supportive stroma in the TME. We have expressed a modified lectin from banana, H84T BanLec, attached to a chimeric antigen receptor (H84T-CAR) that recognizes high-mannose (molecules with 5-9 mannoses), a glycan that is largely absent from normal cells, allowing selective binding to tumors whilst sparing essential normal tissues. We have tested the efficacy of H84T-CAR against pancreatic ductal adenocarcinoma (PDAC), an intractable tumor with aberrant glycosylation and highly characterized by desmoplastic stroma. In our 3D tumor culture models, which recapitulate the solid tumor structure, H84T-CAR T cells disrupt the stromal architecture provided by pancreatic stellate cells (PSCs) and allow substantial additional infiltration of CAR T cells (**Figure 1**). We did not observe this same effect with clinically validated HER.2 specific CAR-T cells suggesting a unique stromal targeting by H84T CAR. Co-expression of both H84T and HER.2 CAR on the same T cell suggests better anti-tumor activity in a xenograft model of heterogeneous PDAC composed of both CFPAC tumor cells and PSCs (**Figure 2**: HER.2 CAR vs H84T+HER.2 CAR $p=0.09$). Our approach therefore can limit the

consequences of both tumor heterogeneity and plasticity and of the shelter provided by the components of the tumor microenvironment. This and related CARs targeting altered sugar structures in the solid tumor microenvironment may provide a targeting platform that can be extended to multiple solid tumors.

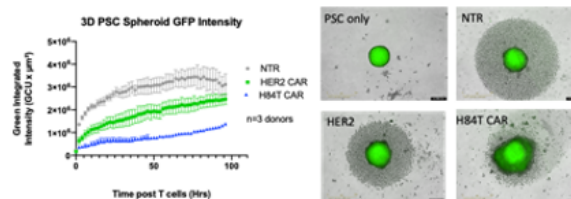


Figure 1: H84T CAR T cells disrupt PSC spheroids. 8×10^3 CSFE labeled PSCs were seeded in 96well agarose coated plate. 48hrs later, 1×10^4 non-transduced (NTR), HER.2 or H84T CAR expressing T cells were added. Microscope images were acquired 96hrs post T cell addition and green fluorescence integrated intensity was determined by Incucyte image analysis.

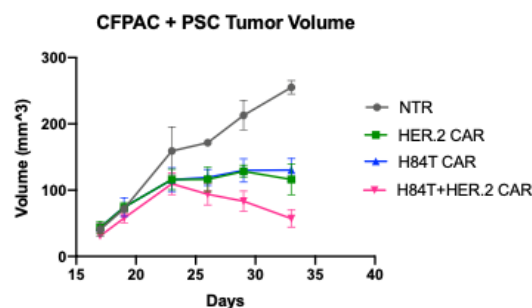


Figure 2: 2×10^6 CFPAC tumor cells and 2×10^6 PSCs were engrafted subcutaneously in NGS mice and established for two weeks. 1×10^6 T cells were then infused intravenously and tumor growth was quantified by caliber volume measurement. $n=4-5$ per group.

1242. Preclinical Activity and Safety of UB-VV100, A Novel Lentiviral Vector Product Designed for Selective and Effective In Vivo Engineering of Therapeutic Anti-CD19 CART Cells for B cell Malignancies

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Introduction: Chimeric antigen receptor (CAR) T cell therapy has demonstrated transformative outcomes in hematologic malignancies; however, many challenges remain associated with manufacturing complexity, high cost, requirements for lymphodepletion, and poor product persistence. We are developing a scalable, off-the-shelf in

vivo lentiviral vector-based platform, termed VivoVec, with the potential to achieve efficient and selective T cell transduction upon direct administration to patients. VivoVec lentiviral particles are surface engineered to facilitate T cell activation and transduction, and incorporate the Cocal glycoprotein to enable cellular entry. VivoVec particles will be delivered directly to patients' lymph nodes to maximize exposure to target T cells and minimize exposure of non-immune cells. UB-VV100, a clinical candidate for the treatment of B cell malignancies, contains a payload encoding an anti-CD19 4-1BBz CAR and a novel synthetic receptor, rapamycin-activated cytokine receptor (RACR), designed to drive *in vivo* CAR T cell survival and persistence without requirements for lymphodepletion through administration of rapamycin. Here we present preclinical studies demonstrating the anti-tumor activity and preliminary safety of UB-VV100.

Results: UB-VV100 particles cultured with unprimed PBMCs activate and efficiently transduce CD3⁺ T cells with minimal transduction of CD3-negative cells. The resulting CAR T cells expand in response to antigen and RACR activation and exhibit polyfunctional antigen-specific function including cytotoxic activity in the presence of CD19⁺ tumor cells. Studies in a humanized mouse model of B cell malignancies demonstrate selective transduction of T cells following UB-VV100 administration, resulting in CAR expression, CAR T cell expansion augmented by RACR activation, and anti-tumor activity. UB-VV100 preliminary toxicology studies demonstrate a favorable safety and biodistribution profile using two preclinical animal models: intranodal administration to canines and systemic administration to humanized mice. Intranodal administration of VivoVec particles to canines was well tolerated and resulted in transduction that was largely restricted to the injected lymph nodes, with ~90% lower transduction in the downstream draining lymph nodes, and no transduction of non-immune tissues. Evaluation of UB-VV100 safety and biodistribution in the presence of human CD3⁺ T cells was performed in CD34-humanized NSG mice. Intraperitoneal administration was used since NSG mice lack a developed lymphatic system, thus, biodistribution profiles in this model represent a systemic particle distribution scenario with maximum potential for off-target tissue transduction. Intraperitoneal administration of up to 10⁸ transducing units of UB-VV100 was well tolerated, without any evidence of systemic toxicity. Molecular analysis of transduction events at 1-12 weeks post treatment demonstrated the presence of vector genomes primarily in the liver and spleen, and RNA *in situ* hybridization analysis demonstrated that the predominant transduced cell types were human T cells and murine macrophages. **Conclusion:** These findings support the potential of the VivoVec platform for generating safe and effective CAR T cells *in vivo*, which could expand patient access to CAR T technology in both hematologic and solid tumors without the need for *ex vivo* cell manufacturing or lymphodepletion.

Cell Therapies for Hematological Disorders

1243. *Ex Vivo* Generated ProTcell™ Product Exhibits Fate Plasticity Between T and NK Lineages: An Opportunity for Innate and Adaptive Cell Therapy Strategies

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Developed as an add-on of Hematopoietic Stem Cell Transplantation (HSCT), ProTcell™ therapy consists in the injection of *ex vivo* generated lymphoid progenitors able to seed patient thymus rapidly, enabling to achieve a polyclonal T cell repertoire reconstitution faster and more efficiently than classical CD34⁺ hematopoietic stem and progenitor cell (HSPC) treatment. Currently under investigation in two clinical trials in Europe and in the US, ProTcell™ aims to improve significantly patient prognosis after HSCT by shortening the immunodeficiency window following treatment, while preventing GvHD thanks to cell graft education in patient thymus. ProTcell™ culture process consists in CD34⁺ HSPC exposure to Delta-like 4 recombinant protein and a cocktail of cytokines, supporting Notch pathway triggering and early T lymphoid cell differentiation up to the acquisition of CD34⁺CD7⁺ phenotype. Flow cytometry analysis revealed that CD34⁺CD7⁺ cells express BCL11B, a transcription factor essential for T-cell development and for maintenance of T-cell identity, but only a small fraction express CD5 (<10%) and CD1a (<2%). They are able to seed NSG mice thymus where they give rise to CD4⁺/CD8⁺ double positive and CD3⁺ single positive T cells within one month. Here, we describe a complementary single-cell RNA sequencing and mass cytometry approach to provide in-depth characterization of ProTcell™ phenotype and potential. Through our analysis, we identified cell subsets with innate lymphoid cell potential (CD7⁺CD161⁺). Gradient of expression of CD161 marker was associated with occurrence of CD56 marker, highlighting a T/NK potential existing in ProTcell™ product. Taking advantage of this observation, we developed a scalable, feeder-free and clinically compatible method for generating large numbers of immunotherapeutic NK from ProTcell™. These specific culture conditions enable efficient differentiation of ProTcell™ product into high number (~10 000 fold expansion) of fully potent, highly pure (up to >90% and no T cell contamination) NK cells. Characterization of these NK cells demonstrated expression of activation receptors (NKG2D, NKp46, NKp30) and inhibitory receptor CD94/NKG2A heterodimer comparable to activated peripheral blood NK cells. Lack of expression of inhibitory receptors KLRG1 as well as inhibitory KIRs was observed. Cells exhibited good transduction potential. We then challenged the plasticity of ProTcell™ cells both *in vitro*

and *in vivo*, using NSG murine model. CD161⁺ and CD161⁻ fractions as well as CD5⁺ and CD5⁻ fractions were sorted to delineate a biological boundary between NK and T lineage differentiation. Results obtained from these different experiments demonstrated a prominent potential plasticity of these lymphoid progenitors. In-depth characterization of our cell therapy product underlines diversity of cell phenotype and provides an interesting insight on lymphoid cell expansion and differentiation upon Notch ligand exposure. Monitoring of hematopoietic cell populations during patient's follow-up in ongoing clinical trials may confront our observations of a multi-lymphoid potential of cell products. Overall, these results triggered additional orientations for our ProTcell™ platform and reinforced our ambition to address unmet medical needs.

1244. Hypoimmunemouse Primary Pancreatic Islet Cells Survive and Functionally Rescue Allogeneic Diabetic Mice

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Allogeneic donor islet transplantation as a treatment for Type 1 Diabetes has limited success due to the morbidity related to the use of potent immunosuppression (IS). Very few patients remain insulin independent 4 years beyond transplantation due to gradual loss of islet function caused by general immune rejection and local factors in the hepatic microenvironment following portal vein injection. We sought to engineer mouse primary islet cells (mPI) that avoid host immune detection and rejection without immunosuppression, and that could be transplanted intramuscularly. We built on our previously described hypoimmune engineering strategy knocking-out function of MHC class I and II and overexpressing CD47 to evade both adaptive and innate immune cell killing. Mouse islets isolated from B2M-KO (002087 Jackson) mice on the C57/Bl6 background do not express MHC class I or class II. Islet cells from these mice were engineered to overexpress CD47 (HIP mPI). Control islet cells (Wt mPI) from C57/Bl6 mice were left unmanipulated but cultured in the same way. Allogeneic Balb/C mice were made diabetic using streptozotocin and blood glucose levels were monitored every 4 days. A total of 600 firefly luciferase+ HIP mPI or Wt mPI were transplanted intramuscularly. After 6 days, splenocytes and serum were obtained for immune analyses. HIP mPI showed no measurable T cell recognition in ELISpot assays nor T cell killing in xCELLigence, showed no induction of graft-specific antibodies as assessed by flow cytometry and xCELLigence, and were protected from NK cell and macrophage killing by xCELLigence. Transplanted Wt mPI elicited a strong adaptive immune response with high ELISpot frequencies and T cell killing as well as a surge in graft-directed antibodies resulting in complement-dependent cytotoxicity (CDC). Serial bioluminescence imaging showed survival of all HIP mPI but rapid rejection of Wt mPI. After 4 weeks, histopathology revealed transplanted HIP mPI between muscle fibers without any signs of local immune reaction indicating the suitability of the intramuscular site. Indeed, survival and function of intramuscular HIP mPI was consistent with the "standard preclinical" kidney capsule location. Elimination of HIP mPI could be achieved by blocking CD47, highlighting the importance of CD47 to prevent innate immune cell killing. Glucose levels (measured 4h after fasting) gradually decreased

after HIP mPI transplantation and remained stable around 200 mg/dl after 3 days and beyond. No effect on glucose levels were seen with Wt mPI transplantation. To assess whether hypoimmune engineering impacts survival or function of islet cells, we compared the survival of HIP mPI and Wt mPI in immunodeficient NSG mice and found no difference. These pre-clinical findings suggest that HIP islet cells transplanted intramuscularly may be capable of persisting and functioning in diabetic patients without IS. Furthermore, this strategy could be expanded into pluripotent stem cell-derived islet cell therapies.

1245. FVIII-Expressing Human Placental Cells Engraft in Multiple Organs and Provide FVIII Protein While Evading Induction of FVIII Inhibitors When Administered to Juvenile Sheep

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Previously, we have shown that direct intravenous (IV) infusions of 20IU/kg of human (n=3) or a bioengineered hybrid (ET3) FVIII protein (n=3) to normal juvenile sheep for 5 weeks results in the development of IgG antibodies with a titer of 1:20-1:245 that inhibit FVIII with 10-116 BU. However, the intraperitoneal (IP) or IV administration of a cell therapy consisting of human placental mesenchymal cells (PLC) transduced to produce 4.9-6IU/10⁶ cells/24h of mcoET3, a modified FVIII transgene, provided FVIII protein to these animals without causing an antibody response. PLC-mcoET3 doses calculated to provide ~20-60 IU/kg of ET3 each 24h to mirror the quantity of FVIII protein that was infused. Both IV and IP routes of administration resulted in similar mean increases in FVIII activity of 30.9% and 34.2% respectively 15 weeks after the first administration of the cell therapy. Here we examined the engraftment of PLC-mcoET3 in tissues and a sheep-specific multiplexed analysis (NanoString) was used to determine what immune signaling pathways are involved in preventing a FVIII/ET3 protein immune response when the protein is delivered through the cell therapy. Tissues from various organs were collected at euthanasia and RT-qPCR as well as immunohistochemistry (IHC) were used to quantify PLC-mcoET3 engraftment in tissues. RT-qPCR using primers specific to an mcoET3 transgene and normalized with sheep GAPDH demonstrated that PLC-mcoET3 engrafted in the liver, lung, lymph nodes, thymus, and spleen in both the IP IV groups. The spleen had the highest levels of engraftment with 2.41% engraftment in the IP group and 0.64% in the IV group. The IP group showed significantly higher engraftment in the left lobe of the liver compared to the IV group with engraftment of 1.36% and 0.041% respectively. This was confirmed with IHC staining using an antibody to the human nuclear Ku80 and ImageJ analysis which showed 5.24% engraftment in

the left lobe of the liver for IP recipients but 0% in IV recipients. The IP route of administration also showed higher levels of engraftment in the thymus, while IV infusion led to higher engraftment in the lymph nodes. H&E staining was used to examine the morphology of tissues showing no abnormal changes and no evidence of hyperplasia or neoplasia, supporting the safety of both routes of administration for this treatment. NanoString analysis at week 1 and 5 compared to day 0 demonstrated that animals that received protein had upregulated BATF and UBA5, which are involved in Th17 signaling pathways and antigen presentation respectively. While both IV and IP groups also showed increased BATE, the IV group had upregulated BTLA which is involved in immune tolerance. Both IP and IV groups showed downregulation of NOLCH and DLL1 which are involved in T cell differentiation. Since ET3-reactive Th₁ were not present in any of the animals regardless of treatment, it is possible that Th17 cells are responsible for the development of inhibitory antibodies that is seen when sheep are treated with FVIII or ET3 protein, while receiving PLC-mcoET3 downregulates genes involved in T cell differentiation to suppress this immune response.

1246. Modeling Clinical Scale, Efficacious CRISPR-Edited HSPC Therapies in Nonhuman Primates

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Introduction Although hematopoietic stem and progenitor cell (HSPC) gene therapies provide lifelong benefit in numerous disease settings, safety and toxicity remain a critical barrier. Especially for diseases with a DNA damage repair defect like Fanconi anemia (FA) or immunodeficiencies like severe combined immunodeficiency (SCID) and human immunodeficiency virus (HIV) infection, high-dose conditioning regimens with alkylating agents or total body irradiation would be too toxic. In these and potentially other disease settings, driving the engraftment and persistence of modified HSPC therapy may actually exacerbate the underlying disease. To address this, we have optimized a nonhuman primate (NHP) model of CRISPR-edited HSPC gene therapy to closely match and augment each stage of clinical HSPC gene therapy processes. Our primary goals in the present study are to understand how distinct HSPC sources and mobilization regimens impact the efficiency of CRISPR editing, and to identify conditioning regimens that maximize engraftment of CRISPR-modified HSPC while minimizing conditioning-related toxicities. **Methods** Rhesus and pigtail macaques were studied in parallel, to broaden the applicability of our model. We compared yields of bulk HSPC and HSC (CD34⁺CD90⁺CD45RA⁻) from G-CSF/AMD3100 mobilized apheresis and G-CSF/SCF primed bone marrow. Conditioning regimens including total body irradiation (historical controls), busulfan and antibody-drug conjugates were analyzed based on engraftment of autologous, CRISPR-edited HSPC, extent of myeloablation/ immunosuppression, and time to hematopoietic recovery. Bulk CD34⁺ HSPC were edited with NHP CCR5-specific CRISPR ribonucleoprotein (RNP) complexes, then cryopreserved to mimic clinical processes. **Results** In each animal, concentrations of CD34⁺ cells per microliter exceeded the clinical threshold for HSPC yield up to 15 hours after

mobilization. We have successfully remobilized rhesus donors a second time. Yields of CD34⁺ cells after apheresis aligned favorably with clinical practice and contained more true HSCs per kilogram than bone marrow mobilized with G-CSF and SCF. After electroporation with NHP CCR5 CRISPR RNP, G-CSF/AMD3100 cells retained colony forming capacity with CCR5 editing levels often greater than 80%. Importantly, these cells maintained proliferative capacity and gene editing after freeze/thaw, an important step in the manufacturing process to facilitate multi-day conditioning regimens. Preliminary results show high efficiency engraftment of CCR5-edited cells in peripheral blood (up to 60%) following busulfan conditioning. We are continuing to monitor these animals post-transplant. **Conclusions** We have advanced our NHP model of disease-specific HSPC gene therapy to match or exceed clinical standards at each key stage, including i) HSPC yield following mobilized apheresis, ii) successful application of next generation conditioning regimens, and iii) manufacturing of CRISPR-edited HSPC including post-editing cryopreservation and bedside thaw. Most importantly, we observe long-term persistence of CCR5-edited HSPC and progeny *in vivo*. Currently, we are building on this platform to compare strategies to engraft cells that carry large therapeutic transgenes targeted to the edited CCR5 locus, relevant both for HIV cure approaches and as a safe harbor for FA, SCID, and other diseases.

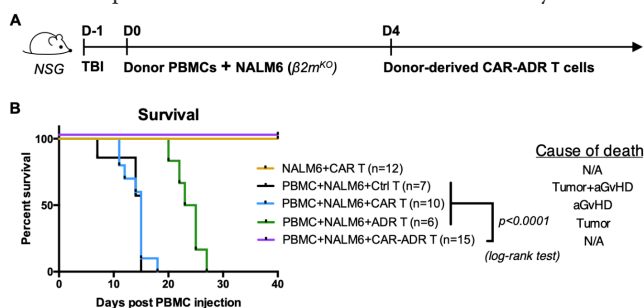
1247. Engineering T Cells to Prevent Acute Graft-versus-Host Disease and Leukemia Relapse Following Allogeneic Stem Cell Transplantation

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Acute graft-versus-host disease (aGvHD) and leukemia relapse remain leading causes of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (alloHSCT). Prophylaxis and treatment of aGvHD rely on generalized immunosuppression, increasing the risk of opportunistic infections and attenuating the graft-versus-leukemia effect. Targeted therapies selectively inhibiting aGvHD while preserving anti-tumor and anti-viral immunity would improve clinical outcomes after alloHSCT. As alloreactive donor CD4⁺ T cells play a critical role in aGvHD pathogenesis, we hypothesized that targeted ablation of activated donor CD4⁺ T cells with engineered donor-derived T cells following alloHSCT would attenuate aGvHD while preserving protective CD8⁺ T cell immunity. We developed an alloimmune defense receptor (ADR) targeting OX40, a surface marker that is expressed on subsets of activated CD4⁺ (84.4±1.5%) and CD8⁺ (29.4±4.4%) T cells (n=6; mean±SEM) but not in resting T cells. OX40 ADR-expressing T cells eliminated predominantly activated autologous CD4⁺ T cells during coculture (mean % count reduction vs unmodified control T cells: CD4⁺, 81%; CD8⁺, 29%), but had no discernible activity against resting T cells (n=6). Notably, OX40 ADR T cells did not inhibit anti-viral activity of autologous memory T cells against EBV, CMV, and adenoviral antigens (n=4). In contrast, OX40 ADR T cells fully suppressed the expansion and function of alloreactive T cells in mixed lymphocyte reaction assays

(n=5). In a mouse model of xenogenic aGvHD induced by intravenously infused human PBMCs, a single dose of PBMC donor-derived OX40 ADR T cells significantly reduced the expansion of CD3⁺CD4⁺ PBMCs compared to mice receiving unmodified control T cells ($p=0.003$). While control mice rapidly developed fatal aGvHD with a median survival of 16 days, mice treated with OX40 ADR T cells all remained free of aGvHD signs for over 40 days (n=10 per group; $p<0.0001$). To enable simultaneous protection against aGvHD and leukemia relapse, we armed ADR T cells with a CD19 CAR. The resulting CAR-ADR T cells exhibited uninhibited activity through both receptors upon coculture with respective targets (n=6). In a mouse model of residual leukemia post-transplant, administration of donor-derived CAR-ADR T cells protected against both relapse and aGvHD and prolonged animal survival compared to mice receiving T cells expressing either CAR or ADR alone (see Figure). In conclusion, OX40-targeting ablated activated alloreactive T cells but did not impair specific antiviral T cell responses *in vitro*. Administration of OX40 ADR T cells protected mice from fatal aGvHD driven by xenoreactive human PBMCs. Finally, OX40 ADR-armed CD19 CAR T cells mediated dual protection against leukemia relapse and aGvHD, thus supporting the feasibility of a bi-functional CAR-ADR T cell product to reduce alloHSCT-related mortality.



1248. Postnatal Boosting with FVIII-Expressing Human Placental Cells Supports Immune Tolerance was Induced During Prenatal Exposure, and is BTLA-Mediated

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We have previously reported that in utero transplantation (IUTx) of sheep fetuses (n=14) with human placental cells (PLC) transduced with a lentiviral vector encoding mcoET3, an expression/secretion-optimized, bioengineered fVIII transgene (PLC-mcoET3) increased plasma FVIII activity levels by 57%, 42%, and 35% at 1, 2, and 3 years post-IUTx, respectively. We also demonstrated that immune tolerance to the cell/gene product was maintained after postnatal administration of PLC-mcoET3 (cells producing 20 IU/kg/24h). However, when

IUTx-treated animals received weekly i.v. infusions of purified ET3 protein (20IU/kg) for 5 weeks, all recipients developed a robust ET3-specific IgG response. Here, we investigated differences in the immune responses of animals that received IUTx with PLC-mcoET3 and were boosted postnatally with PLC-mcoET3 (IUTx-PLC-mcoET3) vs. ET3 protein (IUTx-ET3) to define the pathways by which the immune system differentially responds to protein vs. cell-secreted ET3. A sheep-specific multiplex gene expression analysis with 165 genes involved in immune cell signaling pathways (NanoString) was used to evaluate mRNA isolated from peripheral blood mononuclear cells collected at Weeks (W) 0, 1, and 5 of postnatal infusions. Significant fold-change expression in these mRNA targets was determined using NanoString nSolver 4.0 software. Animals in the IUTx-PLC-mcoET3 group (known to be devoid of inhibitors to ET3 post-boosting) showed that immunoregulation and immune tolerance gene clusters were among the top three clusters that increased expression from W0 to W5 (adj. p-value<0.01). Differential expression of genes in pathways involved in Th1, Th2, and Th17 responses was also found, at differing levels, in the IUTx-PLC group, suggesting a balance between immunity and tolerance was maintained. Surprisingly, the IUTx-ET3 group, which developed inhibitory antibodies after ET3 boosting, also showed significantly increased expression of immune tolerance genes, and downregulation of Th1 and Th17 cell signaling. To determine if the increase in expression of immune tolerance genes was due to the IUTx treatment, we also evaluated a group of aged-matched, non-transplanted sheep that received ET3 protein under the same dose and schedule. Results from Gene Set Analysis (GSA) demonstrated significant upregulation of genes involved in interferon signaling, class I MHC antigen processing, and Th17 signaling in these animals, suggesting the potential involvement of Th17 cells in the immune response in this group. In conclusion, IUTx with PLC-mcoET3 induces the upregulation of genes associated with immune tolerance, providing an explanation for the long-lasting elevation in plasma FVIII levels in these animals in the absence of inhibitors. Nevertheless, despite the continued expression of tolerogenic genes, administration of ET3 protein to these IUTx recipients induced upregulation of Th2 signaling, a pathway that was not observed in animals that only received ET3 protein, demonstrating that the mechanism by which immune tolerance is broken in IUTx recipients differs from that by which an immune response to ET3 occurs in animals with no prior exposure. These studies underscore the need for a more complete understanding of the mechanisms by which immune tolerance to FVIII develops during ontogeny.

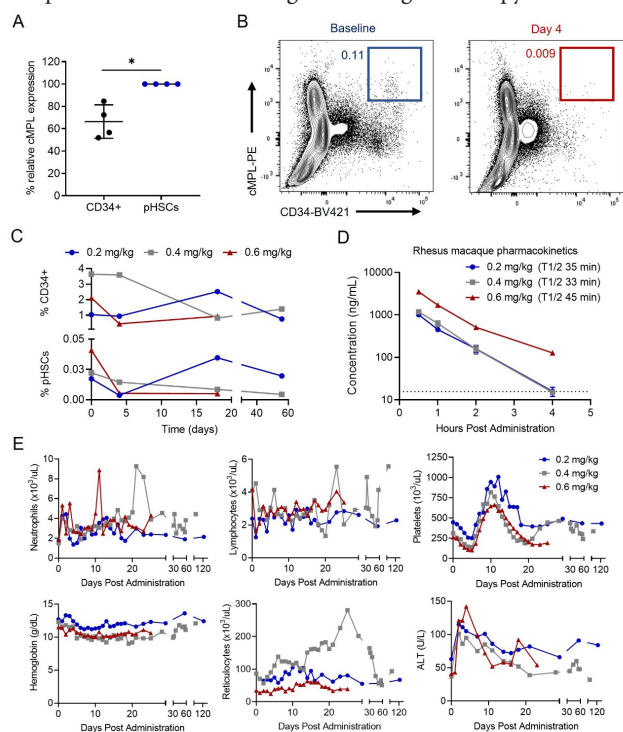
1249. Diphtheria Toxin Based Bivalent Anti-cMPL Immunotoxin Effectively and Safely Depletes Rhesus Hematopoietic Stem and Progenitor Cells

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Autologous hematopoietic stem/progenitor cell (HSPC) gene therapy holds a curative potential for patients with inherited blood disorders. However, conditioning regimens have traditionally been achieved by cytotoxic chemotherapies and radiation that impair host immune function and are associated with morbidity and mortality. Thrombopoietin (TPO) and its receptor cMPL act as primary regulators of HSPC self-renewal and survival. The TPO:cMPL axis also regulates megakaryopoiesis and platelet production but cMPL is otherwise not highly expressed in other blood cell types or in non-hematopoietic tissues. Thus, we hypothesized that immunotoxins targeting cMPL antigen may allow specific depletion of host hematopoietic stem cells (HSCs) and provide the basis for a novel pre-transplant conditioning regimen. We previously reported that diphtheria toxin based recombinant bivalent anti-cMPL immunotoxin (DT390-biscFV(cMPL)) effectively depletes human HSPCs (Araki, ASH 2021). In this study, we evaluated its safety, efficacy and pharmacokinetic (PK) profile in the rhesus macaque, a preclinical model highly predictive of outcomes in human gene therapy studies. To determine the potential of DT390-biscFV(cMPL) to target HSPCs in rhesus macaques, we first measured surface expression of cMPL in rhesus HSPCs by flow cytometry using clones of human anti-cMPL antibodies shown to cross-react with cells of both species. We confirmed surface expression of cMPL on >80% of rhesus CD34+ cells, with higher densities of cMPL receptors in a phenotypic subset (CD34+CD38-CD90+CD45RA-CD49f+) highly enriched in cells with long-term repopulating activity (pHSCs) (Fig. A). To evaluate safety and efficacy *in vivo*, we administered a single dose of 0.2, 0.4 or 0.6 mg/kg DT390-biscFV(cMPL) by IV infusion into three independent rhesus macaques. HSPC depletion was assessed by immunophenotyping of bone marrow aspirates collected pre- and post-treatment. Notably, we depleted >90% of cMPL+CD34+ cells at all doses tested, resulting in preferential depletion of the pHSC subset relative to total CD34+ cells (Fig. B, C). HSPC depletion was transient at lower doses but persistent and robust at higher doses (Fig. C). Next, we evaluated the PK profile by ELISA assay. Remarkably, the half-life was short (33-45 minutes) and the drug was undetectable at 8 hours, conferring a distinct advantage for pre-transplant conditioning applications (Fig. D). The safety profile was favorable. All animals remained well and active. We observed a transient 2 to 4-fold elevation in liver transaminases and mild-to-moderate thrombocytopenia that required no transfusion, but other hematopoietic lineages were largely unaffected (Fig. E). In conclusion, we have developed a cMPL-specific immunotoxin that

displays a favorable safety profile, is effective at depleting primitive HSPCs, spares most progenitors and mature blood cells, and is rapidly cleared within 8 hours. Ongoing experiments will confirm the utility of DT390-biscFV(cMPL) as a safe and effective preparative regimen to improve outcomes of autologous HSPC gene therapy.



Figures. (A) Relative cMPL expression in CD34+ cells and phenotypically defined CD34+CD38-CD90+CD45RA-CD49f+ hematopoietic stem cells (pHSCs). Mean fluorescence intensity (MFI) was used to compare expression in target populations by flow cytometry. Results were normalized to the pHSCs group. (B) Representative flow cytometry plots showing cMPL+CD34+ cell population in bone marrow (BM) aspirates collected at baseline and 4 days post-treatment of DT390-biscFV(cMPL) (0.6 mg/kg). (C) Frequency of CD34+ cells and pHSCs in BM aspirates from each animal. BM aspirates were collected at baseline and on days 4, 18 and 56. (D) Pharmacokinetic profile of DT390-biscFV(cMPL). Levels measured at 8 hours post-treatment fell below the assay detection limit of 15 ng/mL (dotted line) at all doses tested. (E) Peripheral blood counts and liver transaminase post-treatment of DT390-biscFV(cMPL).

Cell Therapy Product Engineering, Development or Manufacturing

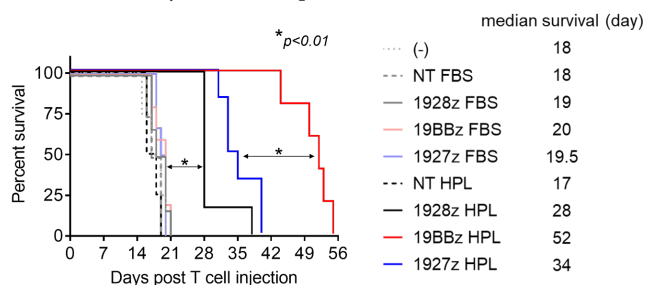
1250. Improved Anti-Tumor Potency of CAR Gamma Delta T Cells Expanded with Human Platelet Lyse

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The V γ 9V δ 2 subset of $\gamma\delta$ T cells (hereafter V δ 2 T cells) is an attractive cell platform for off-the-shelf cancer therapy due to its lack of alloreactivity, high cytotoxicity, and ease of *ex vivo* expansion. However, compared to conventional CAR-modified $\alpha\beta$ T cells, CAR V δ 2 T cells have inferior *in vivo* expansion and persistence in mouse xenograft models of human malignancies. In this study, we optimized the engineering and manufacturing method of CAR V δ 2 T cells by investigating the effect of CAR costimulation and medium composition on the phenotype

and anti-tumor function of the resultant T cells *in vitro* and *in vivo*. Using CD19.CAR as a model, we generated CD19.CAR V δ 2 T cells by first stimulating PBMCs with Zoledronate (1 μ M) in the presence of IL2 (100 U/mL) in either 10% FBS- or 10% human platelet lysate (HPL)-supplemented medium. On day 5 after activation, we isolated $\gamma\delta$ T cells and transduced them with second-generation CD19 CARs containing either CD28, 41BB, or CD27 costimulation (1928z, 19BBz, and 1927z, respectively) using gammaretroviral vectors. After 14 days of culture, control non-transduced (NT) and all CAR-transduced V δ 2 T cells demonstrated higher expansion in HPL-supplemented medium compared to FBS-supplemented medium (FBS vs HPL fold expansion $mean \pm S.E.$ n=7: NT; 73.1 ± 10.9 vs 138.7 ± 41 , 1928z; 57.3 ± 13 vs 149.2 ± 40.7 , 19BBz; 56.4 ± 14.0 vs 137.7 ± 33.1 , 1927z; 58.8 ± 12.9 vs 141.6 ± 36.3). We observed no difference in memory phenotype measured by CD27/CD45RA expression in neither NT vs CAR V δ 2 T cells nor FBS vs HPL conditions at the end of the expansion. However, HPL-expanded CAR V δ 2 T cells showed higher expression of activation markers CD25 and CD71 and lower levels of senescence markers CD57 and KLRG1. In addition, HPL-expanded CAR V δ 2 T cells exhibited higher telomerase activity measured by the TRAP assay, consistent with their higher proliferative potential *ex vivo*. Upon coculture with CD19+ NALM6 leukemic cells, HPL-expanded CAR V δ 2 T cells showed enhanced tumor killing and increased expansion compared to FBS-expanded CAR V δ 2 T cells in the presence of IL2, regardless of the CAR costimulation. To evaluate the anti-tumor effect of CAR V δ 2 T cells, we administered a single dose of freshly thawed CAR V δ 2 T cells intravenously (10^7 CAR+ cells) in mice pre-engrafted with systemic NALM6 leukemia. We observed that FBS-expanded CAR V δ 2 T cells showed limited persistence in peripheral blood and minimal anti-tumor effect, even with the administration of exogenous IL2 (twice per week). In contrast, HPL-expanded CAR V δ 2 T cells exhibited better anti-tumor effect especially with the 41BB endodomain, showing the longest T cell persistence and highest anti-leukemic activity resulting in a significant prolongation of animal survival (Figure). These results indicate that HPL-expanded CAR V δ 2 T cells show superior anti-tumor effect, which can be further enhanced with 41BB CAR costimulatory domain in a preclinical model of human leukemia.



1251. Base Editors as a Singular Platform for Polyfunctional Multiplex Engineering of T Cells for Cancer Immunotherapy

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Effective adoptive cell therapies remain limited to a subset of hematological malignancies. As the circuitry of immune function becomes more clear, increasingly complex engineering is being pursued to improve outcomes. We previously implemented base editors for multiplex gene knockout (KO) in T cells, which enhanced gene KO and reduced genotoxicity over Cas9 nuclease1,2. However, the introduction of tumor-specific receptors using randomly integrating vectors poses safety risks. To implement an all-in-one tool for multiplex KO and knockin (KI), we expand the ability of SpCas9-base editor (BE) 4 and adenine base editor (ABE) 8e nickase activity to stimulate homology-directed repair (HDR). Although both editors stimulated HDR (BE4=11.4%±2.7, ABE8e=25.3%±3.2), efficiencies were significantly lower than SpCas9 nickase (74.2%). Through a novel gRNA design strategy and an rAAV template, we enhanced the efficiency of ABE8e-stimulated HDR by >2-fold, achieving transgene KI rates of >60% in human T cells. By combining KI of CD19, CD33 or mesothelin chimeric antigen receptors (CARs) with >90% quadplex gene KO (B2M/TRAC/PD1/CISH), we generated effector T cells from multiple donors that enhanced killing of tumor lines. In serial co-culture challenge assays, multiplex engineered CAR-T cells resisted exhaustion and sustained tumor cell killing compared to controls. This efficient ABE8e-mediated KI and BE-mediated KO of 4 genes establishes a safe, scalable platform for complex therapeutic cellular manufacturing. 1. Webber, Lonetree, Kluesner, et al. (2019). Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. *Nat. Commun.* 10, 5222 (PMID: 31745080) 2. Kluesner, Lahr, Lonetree, et al. (2020). CRISPR-Cas9 cytidine and adenosine base editing of splice-sites mediates highly-efficient disruption of proteins in primary cells. *Nat. Commun.* 12: 2437

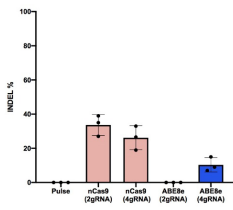
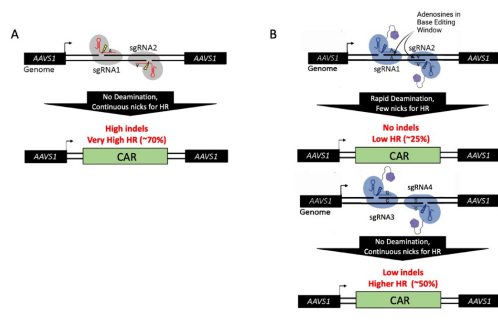


Figure 1. Cas9 nickase and ABE8e nickase are capable of high rates of homologous recombination (HR) in combination with rAAV delivered DNA donors. **A.** Cas9 nickase generates high indel rates and high rates of HR. **B.** ABE8e mediates low-level nicking and HR when target adenosines are in the protospacer (top). The addition of 'retargeting' gRNAs complementary to the base edited genomic sequence allows continued nicking that leads to high HR with low level indels (bottom).

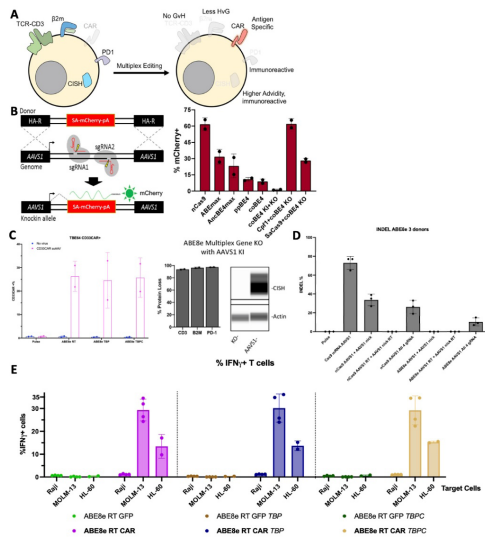


Figure 2. Multiplex KO and KI in primary human T cells. **(A)** Cartoon for multiplex gene KO + KI. **(B)** Strategy and proof of concept using mCherry KI. **(C)** CD33 CAR KI with TRAC, B2M, PDCD1, and CISH protein KO. **(D)** INDELs generated when performing this dual nicking strategy **(E)** Demonstration of T cell activation following CAR KI and co-culture with MOLM13.

Autologous chimeric antigen receptor (CAR) T cell therapy has shown promising efficacy in treating relapsed/refractory B cell malignancies. Despite clinical success, autologous CAR T cell therapy has disadvantages including delays in treating patients and the inability to treat all patients due to manufacturing failures stemming from dysfunctional T cells present in this patient population. In contrast, investigational allogeneic CAR T cell therapy uses T cells from healthy individuals as starting material, simplifying supply, and providing off-the-shelf product convenience. Using healthy donors T cells also opens the possibility to optimize therapeutic efficacy by using donor T cells that are immunologically fit and provide a more homogeneous product. Given that most CAR T cell performance assessments have been conducted with patient-derived CAR T cells, what constitutes an unfit donor for allogeneic T cell therapy is still unclear. To understand factors influencing donor suitability, this study evaluated T cells from 19 healthy donors. The healthy donors chosen were diverse in age (range: 18 - 62) and body mass index (BMI) (range: 19 - 52) to capture a broad spectrum of physical fitness and evaluate the impact of age and BMI on CAR T cell phenotype and function. In addition, T cells from 11 donors with relapsed/refractory heme malignancies, referred to here as patient-derived T cells, were also included in the donor pool as a control for dysfunctional T cells, emulating autologous CAR T cell therapies. T cells were isolated from the peripheral blood mononuclear cells (PBMC) of these 30 donors and used as starting material to generate CAR T cells. During the CAR T cell production in the lab, 6 out of 11 patient-derived T cell preparations failed to expand due to the limited number of viable and fit T cells in the starting material. CAR T cells were successfully generated, however, from the remaining 24 donors (19 healthy-donor and 5 patient-derived T cell preparations). These CAR T cell batches were characterized further with an array of *in vitro* assays, including deep immunophenotyping by flow cytometry and cytotoxicity assays. Correlational analyses revealed a negative correlation between *in vitro* anti-tumor activity and increased age of the donor. Furthermore, there was a negative correlation between the percentage of less differentiated T cells in both the starting material and the CAR T cell product and age, with older donors having less stem/central memory T cells than younger donors. Despite having a similar percentage of stem cell memory T cells (T_{SCM}) in the starting material as age-matched healthy donors, patient-derived CAR T cells also tended to have a lower percentage of T_{SCM} at the end of culture compared to CAR T cells generated from healthy donor material, highlighting the limited fitness of disease donor T cells. Statistically significant associations between expression of specific T cell activation markers and inhibitory markers, and worse *in vitro* anti-tumor activity, were also observed. Expression of these specific T cell markers positively correlated with increased age. The findings in this study demonstrate the opportunity of using young healthy donor material for allogeneic CAR T products, potentially eliminating manufacturing failures and improving patient outcomes.

1252. Allogeneic CAR T Cells Derived from Younger Donor T Cells Have More Desirable T Cell Phenotype and Better *In Vitro* Functionality

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1253. Enhanced CAR T Cell Generation by CD8-LV Through Alleviating Antiviral Mechanisms with mTOR Inhibitors

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Lentiviral vectors (LV) have become the most prominent delivery tool for the genetic manipulation and stable transduction of lymphocytes. LV-generated chimeric antigen receptor (CAR) T cells comprise one of the breakthroughs in cancer therapy, exploiting potent and cell-specific cytotoxic activity against tumor cells. Hence, there are numerous efforts and studies ongoing for better understanding and further development of this prominent tool. We have started to characterize early events in CAR T cell generation by single-cell RNA sequencing aiming at identifying molecular mechanisms that prevent particular vector-exposed cells from being properly transduced. We have conducted single-cell RNA sequencing in CAR T cell products generated with VSV-LV or CD8 α -targeted LV. CD8-LV is highly selective for CD8 $^+$ T lymphocytes and relies on engineered Nipah virus glycoproteins. Differential gene expression analysis revealed significant differences not only between untransduced control cells and CAR $^+$ T cells, but also between cells that were exposed to CD8-LV or VSV-LV. Even more importantly, distinct expression patterns for transduced and non-transduced cells within each vector-exposed group were observed. While the CAR $^+$ cells exhibited a more activated and proliferative profile, possibly due to CAR tonic signaling, CAR $^-$ cells showed upregulated restriction factors, which could have possibly prevented the proper transduction of these cells. Among these were the interferon-induced transmembrane proteins *IFITM2* and *IFITM3*. The mTOR inhibitor, rapamycin (RAP), has been previously shown to alleviate the activities of IFITM proteins in human hematopoietic stem and progenitor cells (HSPC) and make them prone to VSV-LV mediated transduction. However, its use with human PBMC accompanied by VSV-LV inoculation resulted in no effects at all. To our surprise, we observed 2- to 3-fold enhanced transduction of human PBMC in presence of RAP when incubated with receptor-targeted CD8-LV or CD4-LV, bearing either Nipah or measles virus envelope glycoproteins. A dose of 30 μ M RAP turned out to be optimal. Notably, it had no effect on VSV-LV. CAR T cells generated in presence of 30 μ M RAP and CD8-LV exhibited the same killing capacity as the conventional product. Even though 1.5 hours incubation with the immunosuppressive drug resulted in a slightly reduced proliferation detectable 3 days later, it was resolved on day 7. In addition, RAP did not affect the memory and exhaustion phenotype of the CAR T cells. Our data show that overexpressed restriction factors like IFITMs prevent transduction of some T cells during CAR T cell production. This can be overcome with RAP when using T cell targeted LVs pseudotyped with engineered paramyxoviral envelope proteins. Intriguingly, opposite to VSV-LV, these pseudotypes function in a pH-independent manner, bypassing the known endosomal antiviral restriction factors. This suggests that other mTOR-regulated antiviral restriction mechanisms might also be implicated in PBMC transduction by LVs.

1254. Generation of Off-the-Shelf Allogeneic Hypoimmune Tregs

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Developments in gene editing techniques have allowed for the precise engineering of cells and the potential to generate universally transplantable therapeutic cell products. Here we report a method to genetically engineer immune evasive “hypoimmune” regulatory T cells (Tregs) *ex vivo* as a proof-of-concept study building on our previously described hypoimmune work (Deuse *et al.*, 2019 & 2021; Schrepfer, ISSCR 2021). Tregs are crucial for the induction and maintenance of peripheral tolerance and are key in preventing excessive immune responses and autoimmunity. Notably, either a deficiency or dysfunction of Tregs results in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) indicating the critical biological role of these cells in immune homeostasis. This highlights the potential benefit in generating immune evasive Tregs and adds to our existing dataset- showing the applicability of the hypoimmune technology to another cell type. For the purpose, human Tregs (CD4 $^+$ CD25 $^+$ CD127 low) were obtained by fluorescent activated cell sorting (FACS) of peripheral blood mononuclear cells (PBMC) from healthy donors. Sorted Tregs were engineered to knockout function of human leukocyte antigen (HLA) class I and II and overexpress CD47 (HIP Treg). Unedited Tregs (WT) and HLA class I and II knock-out (dKO) Tregs were used as controls and cultured identically as HIP Tregs. Phenotypic characterization revealed preservation of the canonical Foxp3 Treg marker in HIP Tregs. Additionally, secretion of suppressive cytokines, such as IL-10 and TGF- β , was preserved in HIP Tregs when compared to WT Tregs. Indeed, HIP edits did not interfere with the capacity of HIP Tregs to suppress T responder cells in an *in vitro* suppression assay. Moreover, overexpression of CD47 protected HLA class I and class II knock-out cells from innate immune cell killing. In particular, when CD47/SIRP α binding is blocked (anti-CD47) protection from innate killing is lost, thus further highlighting the importance of CD47 in immune evasion (Figure 1). Our findings demonstrate generation of HIP Tregs that are immune evasive, functional in the assays tested, and protected from innate immune reactivity. This proof-of-concept study supports the development of hypoimmune cell therapeutics that may increase feasibility of off-the-shelf allogeneic treatments.

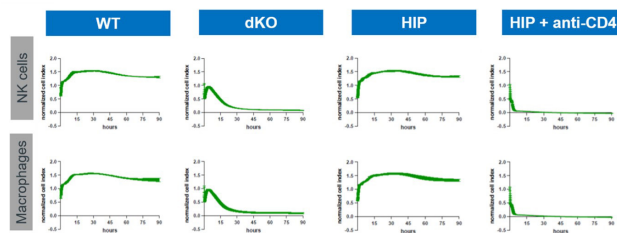


Figure 1. HIP Treg cells overexpressing CD47 are protected from innate cell-mediated killing. WT Treg, HLA class I and II knock-out (dKO) Treg, and HIP Treg cells were plated in the presence of Natural Killer (NK) cells or macrophages. XCELLigence platform was used to

measure innate killing. When anti-CD47 strategy is used, HIP Tregs are killed by innate immune cells confirming the relevance of CD47 overexpression.

1255. Dysfunctional Immune Synapses Restrain Anti DIPG Activity of CAR T Cells

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Introduction: Diffuse intrinsic pontine gliomas (DIPGs) are highly lethal pediatric brain tumors. Thus, there is an urgent need for novel therapeutics. While chimeric antigen receptor (CAR) T-cell therapy has the potential to meet this need, early phase clinical studies with CAR T cells have shown limited antitumor activity for brain tumors. T cells require three signals for optimal activity: 1) T cell activation, 2) costimulation, and 3) cytokines. While numerous investigators have focused on improving signals 2 and 3, the goal of this study was to investigate the role of immune synapse formation, which is critical for proper T cell activation, in the context of DIPG-targeted CAR T cell therapy.

Methods and Results: To study CAR T cell activation we focused on targeting GRP78, a key regulator of the unfolded protein response that is broadly expressed on the cell surface of DIPGs but not normal brain tissue. We generated GRP78-CAR T cells by expressing a 2nd generation CAR with a CD28.z signaling domain, and compared the effector function of GRP78-CAR T cells against U87 glioma and the patient-derived DIPG007 cell line, both of which express GRP78 on the cell surface. We focused on these two models since in our initial in vivo experiments we demonstrated that GRP78-CAR T cells eradicated orthotopic U87 brain tumors, but not DIPG007. GRP78-CAR T cells efficiently killed U87 and DIPG007 cells in vitro in MTS-based cytotoxicity assays. The total cytokine production by GRP78-CAR T cells was significantly lower (28-fold) after exposure to DIPG007 than when exposed to U87. Notably, IFN γ , GM-CSF, TNF α , IL-2 and IL-13 were significantly suppressed in cocultures with DIPG007. The differences in effector function of U87- or DIP007-activated CAR T cells became even more pronounced in repeat stimulation assays in which CAR T cells were only able to expand and retain their cytolytic activity in the presence of U87 cells. To gain mechanistic insight into the limited ability of DIPG007 to activate T cells, we analyzed immune synapse formation by confocal microscopy. CART:DIPG007 immune synapses resulted in a significantly lower calcium flux quantity (2-fold) and duration (30min vs 50min) in comparison to CART:U87 synapses as determined by live imaging. This difference also applied to lysosome recruitment, with significant decrease in CART:DIPG007 immune synapses. Importantly, we observe the same dysfunctional IS formation regardless of CAR T cell specificity and targeted antigen expression level indicating that the suppressive effect is DIPG-tumor mediated.

Conclusion: Our study demonstrates that DIPG tumors suppress CAR T cell effector function, potentially arising from the formation of dysfunctional immune synapses. We are extending our studies to

other CARs and testing genetic engineering approaches directed at improving immune synapse formation to overcome the suppressive effects of DIPGs.

1256. mRNA-Based Gene Editing in Primary Human Muscle Stem Cells

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Muscular dystrophies (MD) are a large group of monogenic muscle wasting disorders. The progressive decline of muscle strength and potential involvement of cardiac or respiratory musculature causes severe impairment of the patients' autonomy, quality of life, and premature death. Long-term regeneration of skeletal muscle is only attainable via its stem cell population, the satellite cells. These muscle stem cells (MuSC) can be isolated from human biopsy material and expanded *ex vivo*. Gene-corrected human primary MuSC are therefore an ideal candidate for autologous cell therapy to treat MD. Preferably, gene-correcting tools, such as CRISPR/Cas9-based nucleases or gene-modifying enzymes, are delivered transiently to reduce genotoxic risks. Thus, we harnessed *NCAM1* as an endogenous reporter locus to establish highly efficient mRNA-mediated gene editing in human primary MuSC from a wide range of donors. The *NCAM1* gene encodes a membrane receptor expressed by all MuSC with an extracellular domain that allows for an easy immunolabelling-based readout. We achieved gene editing efficiencies of >90% targeting *NCAM1* with both SpCas9 and adenine base editor (ABE) mRNA and suitable sgRNAs in a single selection-free step. The knock-out of *NCAM1* was quantified by flow cytometry and did not affect the cells' fitness. Following RNA-mediated gene editing, the myogenic and proliferative properties of MuSC remained unaltered, as assessed by immunofluorescence staining for the myogenic markers Desmin, PAX7, MYF5, MYOD, and the proliferation marker KI-67. Gene-edited MuSC also preserved their ability to fuse into large multinucleated myotubes expressing myosin heavy chain. Furthermore, we validated our approach, by correcting the MD-causing mutation *SGCA* c.157 G>A with >80% efficiency in human carrier-derived MuSC via mRNA-mediated delivery of an ABE. In sum, we established a universal read-out system to optimize gene editing strategies in human MuSC in a donor- and mutation-independent manner, thus maximizing the number of patient-derived primary cells that remain available for clinical application. Our mRNA-based gene editing pipeline has immediate implications for the repair of disease-causing mutations in the context of autologous cell replacement therapies for MD. A thorough off-target analysis in human primary MuSC derived from MD patients and a range of healthy donors to assess off-target risks and inter-individual variations will be a future perspective.

Pharmacology/Toxicology Studies or Assay Development II

1257. A GLP-Compliant Toxicology and Biodistribution Study of ADV-062 (AAV.7m8-L-opsin), a Novel Gene Therapy Product Being Developed as a Potential Single Intravitreal Administration for the Treatment of Blue Cone Monochromacy

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Blue cone monochromacy (BCM) is a debilitating, rare X-linked retinal disease resulting from the congenital absence of both L- and M-opsins. BCM causes severely impaired color discrimination, low vision, nystagmus, and photosensitivity. Previous studies utilizing sub-retinal injection have documented loss of foveal cone outer segments and cone cells following the procedure thus underscoring the unsuitability of sub-retinal injection for BCM patients. This warrants development of gene therapy products that could be efficiently delivered via less invasive methods such as intravitreal injection. We developed ADV-062, a vector optimized for intravitreal (IVT) delivery aimed at restoring the cone-specific expression of human L-opsin (hL-opsin). ADV-062 utilizes the AAV.7m8 capsid to provide efficient transduction of foveal cones in primate retina when delivered intravitreally and utilizes the opsin LCR (locus control region) and a minimal M-opsin promoter to express hL-opsin specifically in cones. Tolerability of IVT-administered ADV-062 was assessed in an IND-enabling GLP-toxicology study in cynomolgus monkeys. Measuring the expression, subcellular localization, and function of hL-opsin on the background of the trichromatic non-human primate retina is challenging due to the presence of endogenous L- and M-opsins, which are highly homologous to hL-opsin. Thus, doses in the current study were selected based on a previous non-GLP study using an ADV-062.myc surrogate, a vector similar to ADV-062 engineered to express hL-opsin with a C-terminal myc tag for enumeration of transduced foveal cones. The results of the non-GLP study suggested that IVT of ADV-062 at similar doses may effectively transduce sufficient numbers of foveal cones to potentially achieve clinical efficacy. In the current GLP study, single bilateral intravitreal injection of ADV-062 was administered to 2-year-old male cynomolgus monkeys (n=3/group) at 5E10, 1E11, or 3E11 vector genomes (vg)/eye. Ophthalmic examinations (slit lamp biomicroscopy and indirect ophthalmoscopy) and tonometry were performed throughout the course of the study. Electroretinography and optical coherence tomography were performed prior to injection, and during weeks 4 and 12. ADV-062 was well-tolerated throughout the course of the 3-month GLP study with no adverse clinical signs observed. One monkey was euthanized early due to incidental aspiration of gastric contents during a procedure and the death was not related to ADV-062. Administration of ADV-062 at 5E10 vg/eye resulted in no ophthalmic,

macroscopic, or microscopic findings. Doses of 1E11 vg/eye and 3E11 vg/eye resulted in slight to mild inflammation characterized by pigment and cells in the vitreous. There were no adverse tonometry findings in any treatment groups. ADV-062-related microscopic findings were limited to minimal mononuclear infiltrates. Other microscopic findings observed were considered incidental, of the nature commonly observed in cynomolgus monkeys, and/or were of similar incidence and severity in control and dosed animals and, therefore, were considered unrelated to administration of ADV-062. In summary, IVT administration of ADV-062 at doses of 5E10, 1E11 and 3E11 vg/eye were well tolerated supporting the selection of clinical doses for the first in human clinical study in BCM patients.

1258. Visium CytAssist: A Novel Platform for Spatial Transcriptomic Analysis of FFPE Sections Mounted on Standard Glass Slides

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Background: Recent advancements in spatial technologies have allowed investigation of the molecular landscape of healthy and disease associated tissues. The current Visium platform allows transcriptomic analysis on tissue sections mounted directly on the capture arrays of the Visium Spatial Gene Expression slides. Here, we utilize the Visium CytAssist instrument to perform spatial transcriptomic analysis of formalin fixed and paraffin-embedded (FFPE) tissue sections either mounted recently or mounted and archived for 6 months on standard glass slides. **Methods:** FFPE specimens were mounted on superfrost glass slides and used within 1 day or archived before being processed through the Visium assay workflow. Archived sections were H&E stained, coverslipped using Cytoseal, a hardset mounting media, and stored at room temperature for 4-6 months. The RNA quality of the tissue sections was evaluated using a DV200 assay. Sections were processed according to a modified Visium Spatial Gene Expression FFPE workflow. Probe pairs hybridized to mRNA were transferred from the tissue sections on standard glass slides to the Visium Gene Expression slides using Visium CytAssist, followed by Spatial Gene Expression library construction. The libraries were sequenced on NovaSeq and the data were analyzed and visualized using Space Ranger and Loupe Browser. **Results:** FFPE sections (n=2) from the human lymph nodes with reactive follicular hyperplasia, H&E stained ~6 months ago, and stored at room temperature showed clusters of Ki67 antigen+ cells in the lymph follicles. In the same section, ligated paired probes transferred using Visium CytAssist detected on average 2,718 genes/spot at the sequencing depth of 10k reads per spot. Ki67 RNA expression correlated with the Ki67 protein expression. Unsupervised clustering using transcriptomes segregated Ki67+ cells in the lymph follicles into a distinct cluster, suggesting that Visium CytAssist enabled accurate capture of the biological cell states in the archived sections. In another scenario, transcriptome analysis from recently placed FFPE sections (n=2) from a glioblastoma sample identified a large mass of cells expressing high levels of oncogenes MYCC, MYCN and cell cycle related genes Ki67 and CCNB1. In addition, Visium CytAssist enabled identification of a smaller mass of cells which expressed high levels of MYCC and weak Ki67. Both tumors segregated as distinct clusters, however, the bigger tumor contained

a small population of cells that clustered with the cells of the smaller tumor, indicative of cells which are precursors to the smaller tumor. **Conclusions:** Our data highlights that Visium CytAssist can retrieve transcriptome information from archived FFPE sections and accurately identify molecular changes in a spatial context. Visium CytAssist will be a valuable platform to mine molecular data from archived FFPE sections that can potentially lead to novel discoveries.

1259. Development of an AAV-Based Gene Therapy for Children with Congenital Hearing Loss Due to Otoferlin Deficiency (DB-OTO)

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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 leads to congenital severe-to-profound auditory neuropathy in both humans and in mice. These mutations are believed to be causal in 2-3% of individuals born with hearing loss. Infants with biallelic OTOF mutations are currently managed with assistive devices, but several groups are developing AAV-based gene therapies to address this population. **Methods** We have developed DB-OTO, an Adeno-Associated Virus (AAV)-based gene transfer therapy for the rescue of hearing in Otoferlin deficiency. DB-OTO expresses a corrected OTOF cDNA from a hair cell-specific Myo15 promoter and is delivered using an AAV1-based capsid. Because human OTOF cDNA exceeds the packaging capacity of a standard AAV, DB-OTO uses a dual AAV system to reconstitute the full-length OTOF coding sequence. **Results** We have previously shown that DB-OTO can rescue hearing function in OTOF^{Q828X/Q828X} mutant mice as measured by ABR, that it can be successfully delivered to the primate ear via RW infusion with vestibular fenestration, and that using a cell-specific promoter is key to its function. Here, in preparation for the initiation of clinical studies, we further characterized the dose translatability of DB-OTO between mice and non-human primates. To better characterize the dose-response of DB-OTO, we dosed mice over a >10X dose range and evaluated their hearing recovery over several months. We found that at higher doses, tone-burst response in previously deaf OTOF^{Q828X/Q828X} mice extends to the apex of the cochlea, whereas with low doses, the best recovery is seen around 22 kHz. We also treated OTOF^{wt/wt} mice to characterize the tolerability of DB-OTO over the same dose range. Using RT-PCR, we showed that DB-OTO expression increases continuously for the first several weeks after dosing in mice, consistent with functional recovery. In parallel, we evaluated the expression timing in non-human primates using the same assay. There, the expression appeared to plateau in a comparable timeframe following dosing. Using this assay, DB-OTO expression levels at plateau in mice and primates were compared and used to confirm prior assumptions about dose translation based on

expression from surrogate vectors. Together, these data support and inform our plans for clinical translation of DB-OTO. **Conclusions** DB-OTO is a promising emerging therapy for genetic hearing loss and has the potential to provide the first clinical proof-of-concept for gene therapy in the inner ear.

1260. Long-Read Sequencing and Multiplex ddPCR for Viral Vector Genome Integrity Identification

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In the production of therapeutic viruses, such as AAVs, the harvest always contains a mixture of full, empty, and partially filled particles. Downstream purification then focuses on the fraction of full virus particles and the removal of impurities. However, the overall population of different particles in the harvest can be relatively diverse, leading to problems in purifying and separating the desired virus particles for the final drug product. Moreover, at present, even the purified drug products always contain at least some unwanted particles. The actual content of the virus particles is another mystery - theoretically, they should contain the expected genome construct, but usually the particles may contain various nucleic acid impurities or fragmented genomes in addition to the desired genome. Another problem is the uncertainty about the integrity of the genome - most analytical methods do not provide information about whether complete, intact vector genomes are actually present in all full vector particles. We have addressed this problem by using long read sequencing (Oxford Nanopore Technologies MinION) and by developing an advanced dPCR multiplex approach to assess viral vector genome integrity. MinION sequencing has been shown to favor shorter reads, resulting in a biased picture of the actual presence of full-length genomes in samples. Nevertheless, the sequencing results showed that full-length antibiotic resistance genes from plasmids were encapsidated in the viral capsids. To quantify the integrity of the vector genome, we started to develop a 4-plex dPCR. We first tested the multiplex assay on synthetic DNA sequences where we could intentionally introduce controlled fragmentation. The results showed that the majority of fragments produced the expected signal, but we were still able to detect some unwanted fragments. At the same time, we were able to identify interactions between assays that were not revealed by *in silico* analysis. We have shown that the use and development of assays to identify genome integrity is not trivial and that the results must be interpreted carefully. However, the assays developed are an important step toward a better understanding of actual capsid content.

1261. Toxicity of Frataxin Overexpression in Nonhuman Primates Treated with Intravenous and MRI-Guided Intracerebellar Infusion of an AAV Vector

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Background and Objective: Friedreich's ataxia (FA) is a mitochondrial disorder in which the deficient expression of frataxin leads to cardiomyopathy and the degeneration of neurons in the cerebellar dentate nuclei. We developed a gene therapy approach to address cardiac and cerebellar pathology involving administration of an AAV vector expressing frataxin via two routes; intravenous injection to target the heart and MRI-guided, convection-enhanced infusion to target the deep cerebellar nuclei. **Methods:** We conducted pharmacology studies in two mouse models and evaluated the safety and transduction efficiency of the dual route-of-administration approach in a nonhuman primate toxicology study. **Results:** Intravenous vector administration in murine models improved both cardiac and neurological disease manifestations. In nonhuman primates, combined intravenous and intracerebellar infusion achieved extensive transduction of the cerebellum and heart across a wide dose range. Although the safety profile appeared acceptable in a cohort of animals observed for 28 days post injection, longer follow up revealed severe neurological and cardiac toxicities apparently associated with frataxin overexpression in animals that received higher vector doses. Intravenous vector doses of 3×10^{13} GC/kg or greater resulted in troponin I elevations in all animals by 90 days post injection. One animal treated with 3×10^{13} GC/kg developed a fatal arrhythmia 119 days after vector administration. Histopathology demonstrated cardiomyocyte degeneration in animals treated with doses of 3×10^{13} GC/kg or greater. Two animals treated with a lower dose of 1×10^{13} GC/kg did not exhibit cardiomyocyte degeneration 6 months after vector administration, despite broad cardiac transduction, with approximately half of ventricular cardiomyocytes transduced. High-dose vector administration to the dentate nuclei was associated with marked neurological deficits correlating with focal hyperintensities on T2 MRI six weeks after injection. Lower doses resulted in only transient MRI signal changes without clinical sequelae. **Discussion and Conclusion:** Combined intravenous and direct cerebellar AAV administration readily achieved potentially therapeutic levels of frataxin expression in nonhuman primates. However, toxicity emerging at later timepoints revealed a narrower therapeutic index than initially anticipated. These studies illustrate the promise of gene therapy for FA, as well as the critical need to perform long-term NHP studies to evaluate toxicity associated with frataxin overexpression. The kinetics of the observed toxicity will also have important implications for clinical trial design.

1262. ASC618, a Second Generation of FVIII Gene Therapy for Hemophilia A, Exhibits Major Transduction and Transgene Expression in the Target Liver Tissues: Results of IND-Enabling Pharmacokinetics Studies in Mice and Non-Human Primates

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ASC Therapeutics, Inc has developed a second generation of AAV-based Gene Therapy for Hemophilia A, called ASC618. ASC618 (AAV8-HCB-ET3-LCO) is an adenoviral-associated, recombinant gene therapy vector containing a bioengineered human Factor VIII

transgene and intended for the treatment of patients with severe and moderately severe hemophilia A. It is designed using both rational and empirical design approaches for the development of a minimally-sized, highly potent AAV-FVIII vector that incorporates three unique elements: a chimeric human/porcine FVIII molecule designated ET3, a liver-directed 146 nucleotide synthetic Hepatic Combinatorial Bundle (HCB promoter) and a liver-specific codon optimization algorithm (LCO). The IND-enabling pharmacokinetics studies consisted of the vector and mRNA biodistribution analysis in mice and Non-Human Primates (NHPs). These analyses were incorporated in the pharmacology and toxicology studies and the results presented here. A qPCR Assay was developed to explore the viral biodistribution of ASC618 vector in both mouse (C57BL/6J) and NHP (Cynomolgus Macaques) models. This qPCR-based assay was either validated or qualified to be accurate and precise to quantitate the ASC618 viral vector DNA copies in liver (the target tissue), testis, brain, heart, lung, spleen, kidney and blood in these two species with quantitative range of $4\text{E}+02$ - $4\text{E}+08$ copies/ μg gDNA and sensitivity of 50 copies/ μg gDNA. The viral biodistribution results clearly demonstrated that ASC618 primarily transduces the target liver tissue in both mouse and NHP models. ASC618 DNA levels in liver gDNA were about hundred to thousand folds of those in brain, spleen, lung, kidney, testis, heart and blood cells. Also, the presence of ASC618 sequence in C57BL/6J mice was stable in target liver tissue and non-target (testis, brain, kidney, lung, and heart) tissues for at least 26 weeks following direct in vivo administration of the GT product ASC618 at the dose of $2\text{E}+13$ vg/kg. To evaluate ET3-FVIII mRNA biodistribution, a ddPCR-based assay was developed and qualified to precisely and accurately quantify ET3-FVIII mRNA in tissues from ASC618-treated mice at the dose of $2\text{E}+13$ vg/kg, with the quantitative range of $5\text{E}+03$ - $3.4\text{E}+06$ copies/ μg RNA and sensitivity of $5\text{E}+03$ copies/ μg RNA for tissues including liver, brain, heart, kidney, spleen, lung and testis, and the quantitative range of $1\text{E}+04$ - $6.8\text{E}+06$ copies/ μg RNA and sensitivity of $1\text{E}+04$ copies/ μg RNA for blood cells. The ET3-FVIII mRNA biodistribution study showed that the distribution of ET3-F8 mRNA is limited to mouse liver tissue. ET3-FVIII mRNA was not observed in tested non-target tissues (testis, brain, kidney, lung, heart, spleen and blood cells) from Week 4 to Week 26, with some exceptions that low expression ($< 1\%$ of ET3-FVIII mRNA in liver tissue) detected in testis (1 out of 5 animals tested) and kidney (3 out of 5 animals tested) tissues at time point Week 4. In NHP, biodistribution of ET3-F8 mRNA is also limited to target liver tissue and was not observed in any tested non-target tissues, even at the highest tested dose of $5\text{E}+12$ vg/kg. In summary, ASC618 viral vector showed dominant transduction in target liver tissue and the expression of ET3-FVIII transgene is majorly limited to liver, both in mice and NHPs. These results were included in the ASC618 IND submission and the program received IND clearance from the U.S. FDA in July 2021. A phase 1/2 clinical trial to evaluate the safety, tolerability, and preliminary efficacy of ASC618 (NCT04676048) will be conducted.

1263. Safety and Biodistribution of VTX-801, an AAV3B Gene Therapy Vector, in Healthy Cynomolgus Monkeys

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INTRODUCTION: Adeno-associated viral (AAV) vectors have demonstrated safety and clinical benefits for the treatment of inherited metabolic liver diseases. Some of the naturally occurring AAV serotypes along with newly engineered capsids have shown promising enhanced liver tropism to date. Here, we conducted a good-laboratory practice-compliant investigational new drug-enabling study to assess the safety of a single intravenous injection of VTX-801, a liver-tropic AAV3B gene therapy product developed for the treatment of Wilson's Disease. **METHODS:** Juvenile male and female cynomolgus monkeys received intravenously vehicle (control subjects) or a low or high dose of VTX-801 vector (1.8×10^{13} and 9×10^{13} vector genomes (vg)/kg, respectively) and were monitored for 26 weeks for *in-life* safety with interim sacrifices at 4 and 13 weeks post-vector administration. Upon completion of monitoring period, animals were euthanized to study vector biodistribution in tissues and body fluids, cellular and humoral immune responses, and histopathology. **RESULTS:** A mild and transient increase in liver enzymes (ALT and/or AST) activity was observed in animals administered with the highest dose at Day 3 and/or 8 post-vector injection. All other safety parameters evaluated in this study including clinical condition, body weight, cardiovascular and respiration, hematology, complement assay, anti-transgene, cellular response (ELISpot), were unaffected by the treatment. Vector DNA shedding in faeces, plasma, urine, saliva and nasal fluid was evaluated. Viral DNA was quantifiable in a dose-dependent manner from a maximum peak at Day 1 following vector administration then the DNA signal gradually declined to undetectable levels in all treated animals by Day 120 of the treatment schedule. Biodistribution analysis was also performed on a broad range of tissues and confirmed results published by other groups for AAV3 vectors. VTX-801 exhibited a remarkable liver tropism, with only partial biodistribution to other tissues like gallbladder and spleen. No signal was detected in the central nervous system and no or very low transduction was observed in the reproduction system. In agreement with the PCR assessment, the highest expression levels of transgene mRNA were observed in the liver with no significant topological differences. There was little to no expression in the other tissues. Finally, there were no adverse events or abnormal histological lesions observed at the doses administered (higher than those intended for clinical use), supporting safety of this vector in juvenile animals. **CONCLUSION:** This study of a single intravenous administration of VTX-801 in juvenile cynomolgus monkeys demonstrated safety of the doses evaluated with no toxicity, an excellent liver tropism with limited off-target biodistribution, no immune response, and sustained expression of the gene therapy product in vector-treated animals. This study has supported the clinical development of VTX-801 for a first-in-human clinical trial in Wilson's patients (GATEWAY).