American Society of Gene & Cell Therapy (ASGCT) Annual Meeting
Oral Presentation
May 2, 2019
10:45 a.m. ET

Nuclease-Free and Promoter-Less AAVHSC-Mediated Genome Editing *In Vivo* Corrects the Disease Phenotype in a Mouse Model of Phenylketonuria

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A group of Clade F adeno-associated viruses isolated from human CD34+ hematopoietic stem cells (AAVHSCs) mediate homologous recombination (HR)-based and nuclease-free genome editing that is efficient and precise. Here we describe the utility of AAVHSCs for *in vivo* gene insertion at two genomic loci and in two murine models: phenylketonuria (PKU) and humanized liver. Single-stranded DNA in AAVHSC vectors containing a promoter-less luciferase cassette flanked by sequences homologous to mouse F8 gene (AAVHSCmF8-Luc) to drive HR integration to the mF8 locus and express off the endogenous promoter were prepared. A single intravenous (IV) administration of AAVHSC15-mF8-Luc into albino C57BL/6J mice resulted in liver-specific luciferase expression that was dependent on dose the of AAVHSC15-mF8-Luc administered. Genome editing assessed by droplet digital PCR was linear across three doses, reaching a maximum of 20% of alleles edited at the highest dose tested, and was linearly correlated with total body luciferase expression. Luciferase expression within tissues was dependent on the presence of functional transgene splice acceptor and ribosomal skipping sequences (SA2A) as removal of SA2A sequences reduced luciferase expression greater than 95%. Durable editing of the hepatic F8 locus and luciferase expression was seen for 470 days (end of study). AAVHSC-mediated gene insertion to correct a disease phenotype was assessed in Pah^{enu2} mice, the standard PKU murine model. AAVHSC15 single-stranded genome editing vector containing a promoter-less cDNA encoding human *PAH* gene flanked by sequences homologous to the murine *Pah* gene (AAVHSC15-mPAH) was prepared. A single IV dose of AAVHSC15-mPAH produced a significant reduction in serum Phe to below the targeted therapeutic level in humans (≤360 umol/L) one week post-dosing and was sustained to the end of the study at eight weeks. Elevations in serum levels of tyrosine (Tyr), a byproduct of Phe metabolism required for production of neurotransmitters, were noted resulting in normalization of the serum Phe/Tyr ratio. Changes in coat color from brown to black were observed in all treated animals, indicating reconstitution of the Phe-Tyr-melanin metabolic pathway. Molecular characterization of liver DNA from treated animals treated displayed on average 6.4 ±1.9% of *Pah* alleles successfully edited (range 3-9%), resulting in expression of human-specific *PAH* mRNA. Next-generation sequence analysis across the integration site revealed no insertion or deletion mutations. No reduction of serum Phe was observed in

Pah^{enu2} mice using an AAVHSC15 genome editing vector with homology arms targeting the orthologous region of human *PAH* (AAVHSC15-hPAH), supporting selectivity of editing across species. In FRG® knockout mice repopulated with human hepatocytes, animals treated with a single IV dose of AAVHSC15-hPAH showed 5-6% of *PAH* alleles edited in human hepatocytes with no measurable editing in mouse hepatocytes at six weeks post-dosing. Genome editing in human hepatocytes was associated with increases in human specific *PAH* mRNA to levels observed in normal human liver. Thus at two distinct genomic loci, these *in vivo* data show AAVHSCs can mediate promoter-less, nuclease-free and precise gene integration at efficiencies that reach therapeutic levels, demonstrating this platform can be employed for precise *in vivo* gene editing in the liver and correction of disease phenotype.