American Society for Gene & Cell Therapy (ASGCT) 25th Annual Meeting
Poster Session
May 16, 2022

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.