generation, single molecule, real-time (SMRT) sequencing, this study aims to reveal, 1) the fate of ITRs post-transduction and circularization, and 2) outcomes of recombination between ITRs of different AAV serotypes that yield distinct transgene expression profiles. This work also uncovers concepts that contrasts with common knowledge regarding the retention and conformation of ITRs post-transduction *in vivo*. These results shed light on an unexplored property of vector genome processing and has paradigm-shifting impact for AAV vector biology and the field of gene therapy. In addition, our findings open the door to new avenues of research aimed at improving the transgene expression and stability of rAAV episomes in order to increase the efficacy of rAAV-based gene therapies. G.G. and P.T. are corresponding authors

349. *In Silico* Reconstruction of Genotoxic AAV Integration Events from a Tiling Array Dataset: Proof of Concept and Validation

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Introduction: Prior studies have established that genotoxic vector integrations into the mouse *Rian* locus are associated with hepatocellular carcinoma (HCC) following systemic administration of AAV vectors. The Twitcher (Twi) mouse, a model of Krabbe disease, is deficient in galactosylceramidase (GALC) activity. While CNS-directed AAV2/9-GALC-mediated gene therapy combined with bone marrow transplantation and a substrate reduction drug results in an impressive increase in the lifespan from ~ 110 days to ~ 400 days, >90% of the Twi mice, as well as 100% of the wild type controls, succumb to HCC (PMC7854295). HCC has never been reported in untreated Twi mice. A targeted sequence capture analysis using a tiling array with probes specific for the AAV vector was used to map ITR-containing integrations in or near *Rian*, or other cancer- and cell-growth/death-associated genes, in the HCCs. However, the integrations were not further characterized. Indeed, although several groups have attempted to use targeted PCR and chromosomal walking to recover the full length nucleotide sequence of the genotoxic events in other AAV-associated HCCs, the structure of the integrated vectors has remained elusive. **Methods:** We developed a bioinformatics method designated as AVITA (Assembly of Viral Integrations from Tiling Arrays) to mine tiling array sequence data to reconstruct full length integrations. The first step in AVITA uses the VSeq-Toolkit (PMC7177155) to identify chimeric sequencing reads, i.e., those that span the vector-genome breakpoint at any position in the vector. A pileup of independent reads, aligning to the same vector and genomic coordinates, flags a potential integration junction. Two pileups at nearby genomic regions define the ends of candidate integration sites. The second step assembles overlapping reads from one genome-vector integration junction, through the vector, to the mate vector-genome integration at a nearby genomic position, allowing full length reconstruction of the inserted sequence. One of these complete integrations was selected for validation by locus-specific PCR and Sanger sequencing. **Results:** To test the method, captured sequencing reads from 4 of 10 tumors (PMC7854295) were examined in detail. Each tumor contained two high read count genome-vector breakpoints, which delineated the ends of an integration site. The four integration events, which range in length from 444 - 1326 nt, were fully reconstructed *in silico*. All lack the GALC cDNA, have a single truncated 5' ITR, and contain parts of the enhancer/promoter of the AAV vector transgene. One full-length integration event was confirmed by PCR amplification of genomic DNA, followed by Sanger sequencing. The integration disrupts 12 nt in the mouse *Rian* locus and contains a 444 nt insertion of AAV vector DNA that includes the final 76 nt of the 5' ITR followed by the CMV enhancer. **Conclusions:** Using AVITA, we demonstrate, for the first time, that genotoxic full-length AAV integrations can be fully recovered from a tiling array using an *in silico* approach. Because tiling arrays can capture partial and/or rearranged integrations, hybridization approaches present a less biased method to identify integration events than LM-PCR, which relies upon anchoring amplification in the ITR. Indeed, in 2 of the 4 samples analyzed here, the AAV vector is rearranged and/or the ITR is truncated, both of which would likely preclude capture by LM-PCR. We anticipate applying AVITA to sequence data from all publicly available tiling array datasets to assess global patterns of AAV integration. Enhancements to automate AVITA, coupled with improvements to tiling array performance, might enable AAV integration studies to be conducted in a multiplexed fashion, and amenable to broad implementation.

350. Characterization of AAV Integrations in Preclinical Models of Gene Therapy Using RAAVioli Pipeline with Long and Short Sequencing Reads

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Adeno Associated Viral vectors (AAVs) have been successfully exploited in gene therapy (GT) applications for the treatment of several genetic disorders. Even though they are 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. However, methods and bioinformatic tools that provide a reliable and efficient assessment of AAV integration sites (IS) are required. Here we propose a sonication-based PCR approach combined with short-read sequencing and a bioinformatics pipeline known as RAAVIoli (Recombinant Adeno-Associated Viral Integration analysis) as a new approach to identify and characterize AAV IS and vector rearrangements. The pipeline is based on Python and R scripts that parse the alignments to identify IS and reconstruct vector rearrangements using CIGAR strings. The robustness of our method was evaluated by characterizing AAV IS in a humanized liver mouse model where human primary hepatocytes were transduced with a tomato-expressing AAV. In this model vector insertions were previously characterized using an AAV-specific probe base selection method and

long-reads PacBio sequencing. Sequencing reads were analyzed in both cases using the same RAAVioli pipeline. A greater number of AAV IS were identified by PCR/short-read sequencing (N=811) as compared to the long-read sequence approach (N=370). AAV IS distributed similarly within the human genome showing the typical preference of targeting CpG islands and transcriptional start sites. Moreover, 32 IS were shared among the two datasets, demonstrating the consistency of the results obtained independently from the platform adopted. A comparable number of AAV IS (~25%) were composed of rearranged AAV genomes, although, a higher number of vector re-arrangements (up to 7 AAV fragments) were identified by long-read compared to short-read sequences. Therefore, our PCR-based method produced comparable findings to long-read sequencing approaches regarding the pattern and structure of AAV integration and rearrangements. Nevertheless, it demonstrates significantly greater efficiency in retrieving AAV insertion sites. Thanks to this platform, we observed that in functionally corrected T cells of immune-deficient ZAP70 knock-out mice intra-thymically injected with an AAV expressing the therapeutic transgene, AAV Inverted Terminal Repeats (ITRs) were enriched at DNA breaks created by the enzymatic activity of recombination activating genes (RAG) during V(D)J recombination. Furthermore, AAV IS were successfully identified in the liver and cell-free DNA of Non-Human Primates systemically injected with therapeutic AAVs, underlining the feasibility of the methods as a potential minimally invasive approach for monitoring AAV IS in in-vivo GT applications. In summary, our work indicates that the development of reliable methods for the characterization of AAV integrations is fundamental to providing insights on the safety and efficacy of these vectors in gene therapy applications.

351. A Highly Potent Engineered AAV Capsid, STAC-150, Enables High-Throughput AAV Production and Arrayed Epigenetic Regulator Screening Directly in Cultured Neurons

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Epigenetic regulators have the potential to transform the treatment of neurodegenerative and neurodevelopmental disorders by specific and tunable modulation of disease-associated genes. Identifying potent and highly specific epigenetic regulators, however, can require extensive empirical testing with iterative rounds of on- and off-target screening. Candidate epigenetic regulators can be evaluated in immortalized cell lines that are well-suited for high-throughput screening, but these cells often fail to recapitulate the gene expression levels, disease biology or native epigenetic signature present in more biologically relevant cells. Alternatively, screening can take place directly in the target cell type of interest, such as primary neurons, but these tend to be less experimentally tractable and can require production of viral vectors for each transgene - a step that increases cost and significantly lowers throughput. We sought to circumvent these challenges by building a robust platform for screening epigenetic regulators directly in primary- or iPSC-derived neurons. We accomplished this by engineering a novel AAV capsid, STAC-150, with two important properties: (1) the ability to transduce neurons *in vitro* at substantially lower MOIs than conventional serotypes, and (2) significant partitioning of total production yield into the cell culture medium during HEK293 capsid production. We leveraged these capsid properties in a microliter-scale, automation-compatible AAV production and purification process capable of efficient and cost-effective generation of thousands of arrayed AAVs directly from crude AAV production media. In the first step of this workflow, we transiently transfected plasmids encoding the viral genome, helper genes and epigenetic regulator transgenes into 1000 µL HEK293 suspension cell cultures in 96-well deep well plates. After 48 hours, we centrifuged the culture plates and purified secreted virus from the production medium supernatants using AAVX magnetic beads. We assessed viral protein ratios and capsid purity by total protein stain and western blot. Purified virus showed the expected viral protein ratios and minimal contaminating proteins. DNAse-resistant viral particles were quantified by qPCR, where we observed yields exceeding 1e10 viral genome copies with corresponding titers up to 5e11 vg/ml. We evaluated the functionality of these purified STAC-150 AAVs by assessing the on-target activity of their epigenetic regulator payloads in primary mouse cortical neurons by RT-qPCR. We observed potent and dose-dependent target gene regulation with strong epigenetic regulators showing EC_{50} values as low as MOI 10. In summary, we identified a secreted, neurotropic AAV capsid, STAC-150, with high potency *in vitro* and built a high-throughput, scalable production workflow to enable epigenetic regulator arrayed library screening in primary- or iPSC-derived neurons. Together, these advancements establish a platform with the potential to accelerate the development of potent and highly specific epigenetic regulators for the treatment of neurological disorders.

AAV Vectors - Immune Modulation

352. C3 Complement Inhibitor Limits Transaminitis and Immune Response Following Liver Targeted Adeno-Associated Virus (AAV) Gene Transfer (GT) in Non-Human Primates (NHPs)

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AAV vector-based gene therapies are shifting the medical paradigm by offering a potentially curative treatment for genetic diseases that previously lacked effective treatments. Although systemic AAV gene therapies are very promising, durability may be limited by liver transaminitis that usually subsides with corticosteroid treatment. Additionally, in clinical trials that use high systemic AAV doses (≥ 1×10^{13} vector genome [vg/kg]), complement activation accompanied by a form of thrombotic microangiopathy (TMA) was reported. TMA is defined by the presence of hemolytic anemia, thrombocytopenia and subsequent organ damage. Excessive complement activation has been observed in multiple trials across a range of disease indications and capsid serotypes. The emergence of severe adverse events at high doses of AAV presents a clear need for more effective immune suppression protocols to address the impact of complement