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Rep- and Transcription Activator-Like Effector Nuclease-Mediated Gene Transfer to *AAVS1*

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Sequencing of AAV ITRs is challenging because of their high GC content (70%) and palindromic sequence that causes the formation of an extremely stable hairpin structure. LAM PCR in combination with two widely used next generation sequencing platforms, 454 GS FLX (Roche) and MiSeq benchtop sequencer (Illumina), was investigated in regard of its capability to detect AAV ISs with fully conserved ITRs. By comparative sequencing of LAM PCR amplicons, it was demonstrated that both platforms can sequence complete ITRs, yet at different efficiencies (MiSeq: 0.11% of sequence reads; 454: 0.02% of sequence reads), frequencies (MiSeq: 171 full ITRs, 454: 3 full ITRs) and rates of deviation from the derived ITR consensus sequence (MiSeq: 0.8–1.3%; 454: 0.5%). Detection of LAM amplicon reads with fully conserved ITRs demonstrates the accessibility of AAV ISs with complete ITRs to LAM PCR in combination with 454 and MiSeq sequencing.
Subsequently, LAM PCR was used to analyze the IS distribution of wtAAV. Via its endogenous Rep protein, wtAAV primarily integrates into a locus named *AAVS1* on chr19 of the human genome. This study identified 4340 wtAAV ISs in HeLa cells and HDFs. It was revealed that wtAAV integration is not exclusive to *AAVS1* and that RBS motives induce unidirectional wtAAV integration. It was shown that cell type substantially affects CIS distribution, as CIS overlap between cell types is only 8%. The juxtaposition of a *trs*-like motive (GTTGG) to an RBS motive increases the RBS' probability to induce a wtAAV CIS about fourfold. This report is the first high throughput IS analysis demonstrating an influence of a *trs*-like motive on the integration of wtAAV and the first study to functionally validate Rep-nicking at non-*AAVS1* RBSs. These results suggest a replication-based integration mechanism of wtAAV throughout the human genome and enhance the knowledge about the mechanics and potential of Rep-mediated AAV integration.
While the wtAAV IS distribution was extensively analyzed, knowledge about the structure of wtAAV ISs was limited. Viral-viral and viral-chromosomal junctions of integrated wtAAV genomes were analyzed to gauge the propensity of wtAAV to integrate as single copies or as tandem repeats (concatemers). A statistical model to assess the frequency and size of concatemeric integrants was developed. In this study, it was demonstrated that wtAAV primarily integrates as single copy and that concatemeric integration may vary between cell types. Most concatemers were found in head-to-tail and tail-to-tail configurations. The analysis of preferred ITR breakpoints revealed a breakage pattern similar to processed rAAV ITRs. This suggests that, similarly to rAAV, wtAAV ITRs are also processed prior to integration and that concatemer formation in wtAAV also relies on ITR recombination.
While wtAAV can integrate site-specifically into *AAVS1*, this ability is lost in rAAV since the Rep gene is deleted in favor of the therapeutic transgene. However, site-specific integration to *AAVS1* can be restored by *in trans* supplementation of Rep protein. To estimate the therapeutic potential of Rep-mediated rAAV integration, a high throughput IS analysis of Rep-supplemented rAAV was performed and revealed

13,925 viral ISs and 969 CISs. At high doses of Rep, rAAV integration efficiency was increased fourfold and *AAVS1* targeting specificities of up to 4.5% of ISs were achieved. Rep-supplementation induced wtAAV characteristic clustering of rAAV ISs near RBSs. Interestingly, Rep-depleted rAAV exhibited a strong tendency to form CISs. As the first large-scale IS analysis of Rep-supplemented rAAV integration, this study demonstrates that rAAV vectors can successfully be targeted to *AAVS1* by *in trans* supplementation of Rep protein.

Apart from the Rep/AAV system, alternative means exist to target the *AAVS1* locus. The recent advent of TALENs enables to specifically edit genomic loci throughout the human genome. A specificity analysis of six *AAVS1*-directed TALENs revealed three off-target sites in one TALEN. No off-targets were detected in the remaining TALENs. TALEN activity did not correlate with the amount of mismatches at the off-target sites. Accordingly, *in silico* predicted off-targets with high sequence similarity did not show nuclease activity. Additionally, the data indicate that on- and off-target TALEN activity may be increased within regions of open chromatin.

In summary, this report proves that LAM PCR can detect AAV ISs with fully conserved ITRs. wtAAV was found to integrate preferentially as single copies and an additional, previously unknown, factor promoting wtAAV integration, presence of a trs-like motive, was disclosed. Moreover, two approaches for *AAVS1*-targeted gene addition, Rep-mediated rAAV targeting and TALEN-mediated genome editing, were analyzed regarding their efficiency and specificity. Altogether, this study enhances knowledge about wtAAV integration, the reliability of LAM PCR-mediated AAV persistence analyses and the potential of Rep- and TALEN-mediated gene transfer to *AAVS1*. It therefore makes important contributions to the fields of basic virology, biotechnology and gene therapy.