Engineering a Self-inactivating Adeno-associated Virus (AAV) Vector for ARCUS Nuclease Delivery

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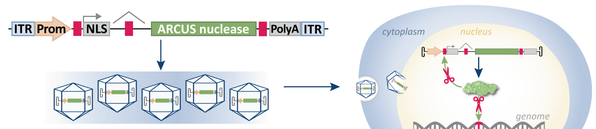
INTRODUCTION

Significant advances in engineering sequence specific nucleases have enabled a broad range of biomedical applications, particularly when combined with AAV, a versatile viral vector for in vivo post-mitotic cell gene delivery. The long-term expression of nuclease mediated by AAV delivery, however, raises concerns about safety and immunogenicity of nuclease-based applications. Persistent expression of the nuclease can increase the likelihood of off-target cleavage which can induce genotoxicity. Additionally, expression of an exogenous nuclease has the potential to elicit an immune response against transduced cells. Thus, it would be advantageous to limit the duration of nuclease expression following delivery.

OBJECTIVE

Self-inactivating AAV

Our self-inactivating AAV system consists of a tissue-specific promoter, a nuclease open reading frame, and adjacent sites targeted by the nuclease. We investigated if target site (TS) and/or PEST insertion in AAV genome would reduce nuclease expression and off-targeting *in vitro* and *in vivo* with the goal of increasing safety of nuclease-based gene therapy.



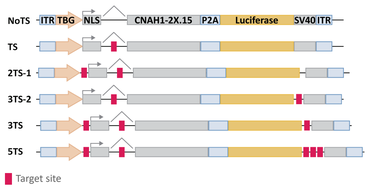
Target site

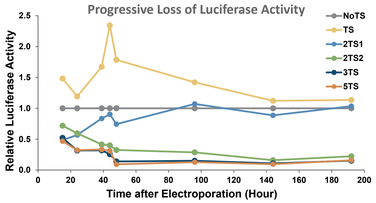
TITR = inverted terminal repeat; Prom = promotor; NLS = nuclear localization sequence

IN VITRO EXPERIMENT

In Vitro Experiment Using HepG2 Cells

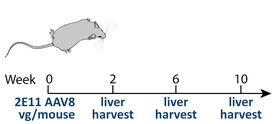
- The nuclease expression levels are determined by measuring luciferase activity which were co-expressed with nuclease in the cells after plasmid DNA electroporation. Incorporation of target sites (TS) in AAV genome led to progressive loss of luciferase signals over time, whose rate can be modulated by the locations and copy numbers of target sites.

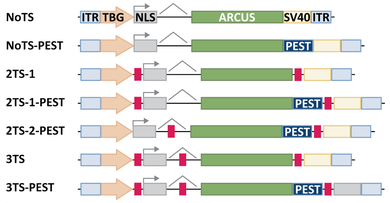




Target site

IN VIVO EXPERIMENTAL DESIGN



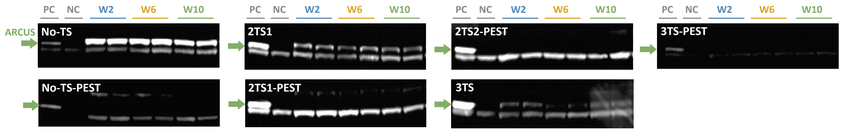


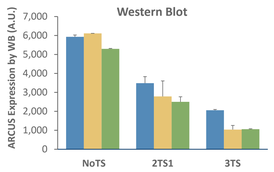
Target site; PEST = Tag for protein degradation

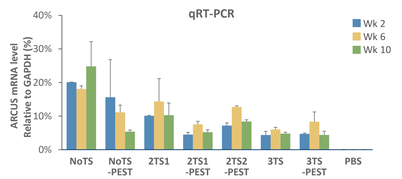
IN VIVO RESULTS

A) ARCUS Nuclease Expression

The amounts of nuclease expressed in mouse liver were determined for protein levels by Western Blot and mRNA levels by qRT-PCR.



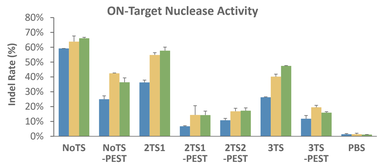


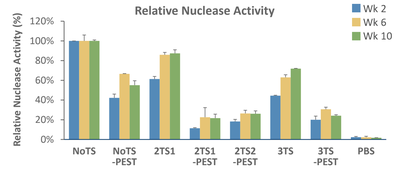


- Western blot results show that PEST tag insertion reduced nuclease expression to undetectable levels. TS insertion led to progressive reduction of nuclease over time (~50% for 2TS1 and ~80% for 3TS).
- qRT-PCR results show that PEST tag or TS insertion reduced nuclease expression on mRNA levels.

B) Nuclease Cleavage Activity

The nuclease activity in mouse liver was determined by measuring the insertion and deletion (Indel) rates generated by the nuclease at the target site on the mouse genome by Amplicon-Seq and ddPCR.



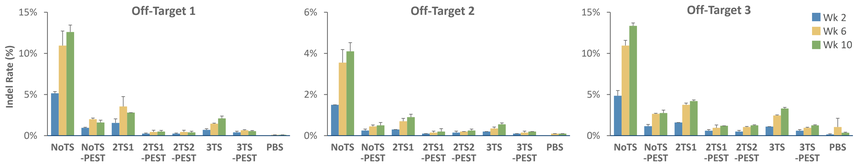


- The lower nuclease levels (<50%) resulted from TS and/or PEST tag insertion in AAV genome are able to generate indel levels up to 90% of NoTS.
- TS and PEST tag insertion effects to reduce indel formation are additive.

C) Off-targeting

The Indel rates of ARCUS nuclease at three different off-target sites in mouse genome were determined by Amplicon-Seq. The lower Indel rates at off-targets suggest higher safety.

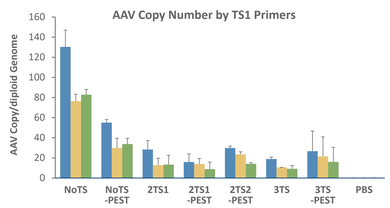
		Chr	BP
ON-Target	AAGACATTGGTGAGGAAAAATC	chr2	134324016
Off-Target1	TATACATCTGTAAAGAAAAATA	chr10	121990375
Off-Target2	TGGACATGATTAAGGAAACATC	chr11	64902548
Off-Target3	TAGACATTGGTAAAGACAAATA	chr1	193578707

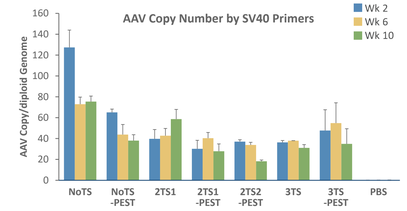


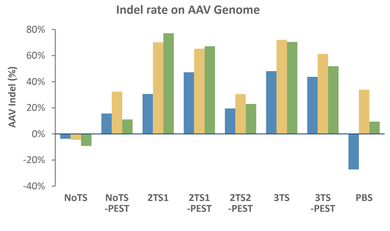
- Amplicon-Seq results showed that cleavage of three off-target sites decreased by 5-8 fold due to TS or PEST tag insertion.
- TS and PEST tag reductions in off-targeting appear additive.

D) AAV Copy Number

Nuclease cleavage of the AAV genome was determined by comparing the AAV titers of two different qPCR primer pairs, a TS1 primer pair flanking the TS and an SV40 primer pair targeting SV40 poly A region in AAV genome.







- ARCUS nucleases cleaved the TS inserted in the AAV genome, which led to significant reduction of AAV copy number.

CONCLUSIONS

- In vitro, our system can progressively reduce nuclease expression over time, and the decrease in nuclease expression can be impacted by the locations and copy numbers of the target site insertions.
- In vivo, our self-inactivating AAV system enabled sufficient on-target cleavage (20-90%).
- In addition, this system significantly reduced nuclease expression, AAV copy number and off-targeting by 50-90%.
- Our self-inactivating system has the potential to improve the safety of therapeutic applications using ARCUS nuclease with its ability to reduce both genotoxicity and nuclease immunogenicity through tunable control of nuclease levels over time.

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