In vitro and in vivo performance of circVec, a vector-based circular RNA expression platform for enhanced gene therapy

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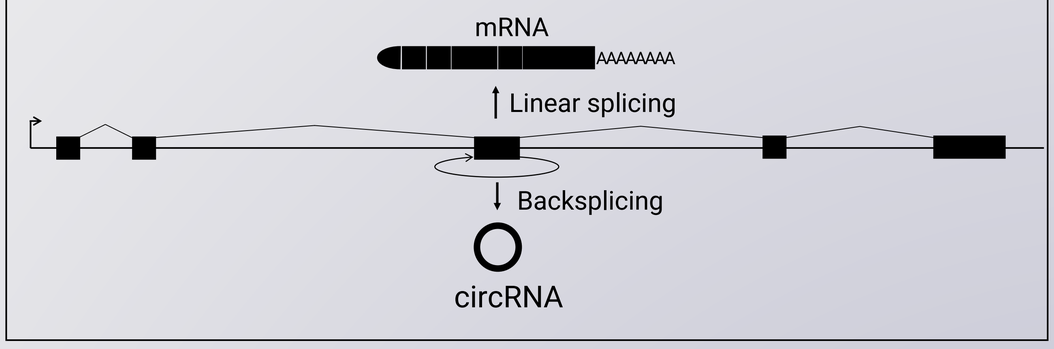
Introduction

Circular RNA (circRNA) is a novel class of endogenously expressed RNA. CircRNAs are generated by a non-linear splicing event, known as backsplicing, where an upstream splice acceptor attacks a downstream splice donor. circRNAs are resistant to exonucleolytic decay, which results in increased intra-cellular stability and persistence compared to mRNA. We have developed a circRNA-based expression platform, **circVec**, which utilizes this natural stability advantage to improve vector-based protein expression.



1. Effective biogenesis through screening and optimization of endogenous circRNA loci

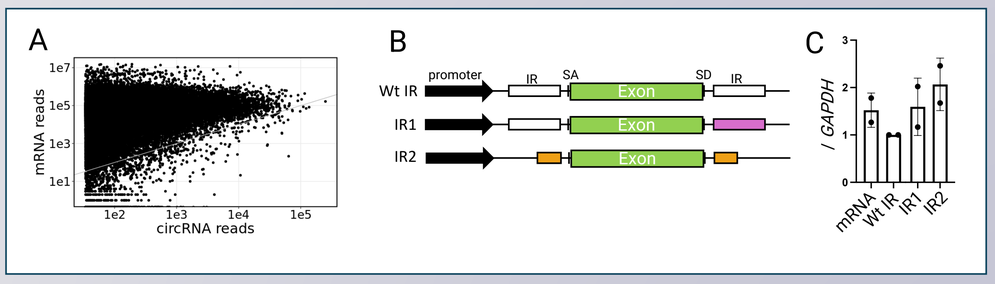


Figure 1: Optimization of flanking inverted repeats (IRs). A) IRs from highly expressed circRNAs, stratified by distance to backsplicing sites, were identified by bioinformatic analysis of publicly available datasets, where circRNA specific reads were compared to linear spliced reads. **B)** Schematic representation of wild-type and optimized (IR1+IR2) circRNA expression cassettes. **C)** Comparing circRNA levels from wt, IR1, and the improved shortened IR2 design relative to the mRNA-based expression 48 hours after transfection.

2. Genetic cassette design and choice of IRES are critical for circRNA-driven protein expression level

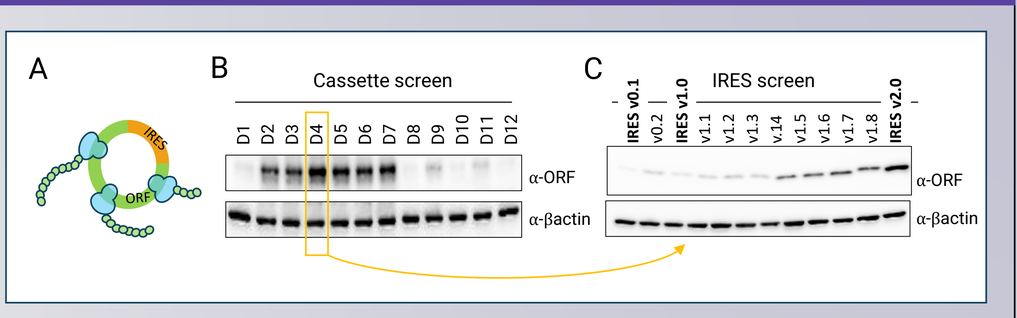


Figure 2: Choice of IRES and IRES/ORF composition impact circRNA expression: A) Schematic representation of circRNA with IRES and ORF. **B)** Protein expression from twelve different IRES/ORF designs (D1-D12) was assessed by western blot. **C**) Protein expression from twelve different IRES elements in the D4 cassette design was assessed by western blotting using antibodies specific to the ORF and β-actin (loading control).

3. circRNA stability confers enhanced protein expression

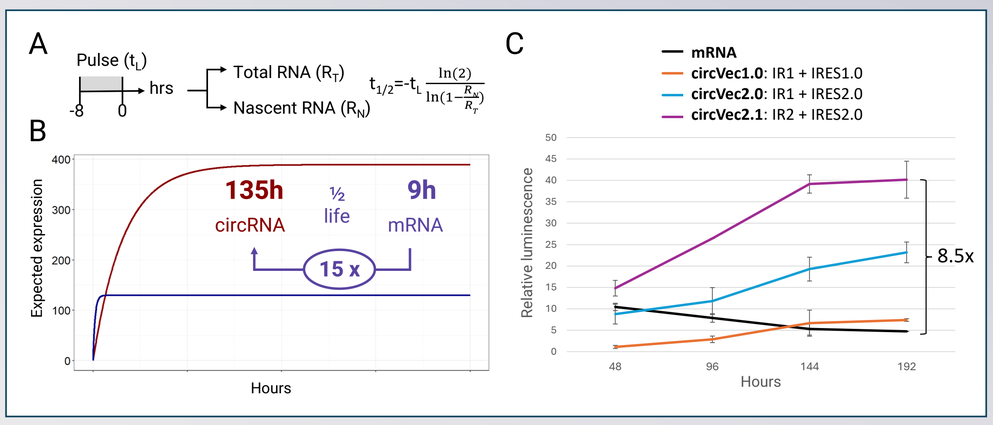


Figure 3: Superior circRNA stability facilitates circRNA accumulation and prolonged protein expression. A) Newly synthesized RNA was labelled with nucleotide analogues for 8 hours and the durability of labelled RNA was quantified over time by qRT-PCR. Half-life RNA estimates were inferred from the nascent fraction (newly synthesised labelled RNA as in(A)) relative to total RNA assuming steady-state**. B)** Simulation of expected expression profile based on empirical half-life estimates from (A). **C)** Protein yield measured by relative luminescence at indicated timepoints after transfection of four different circVec generations and the mRNA counterpart, in C2C12 cells.

4. Bimodal circVec remove-&-replace design successfully depletes pathogenic transcripts while expressing functional proteins

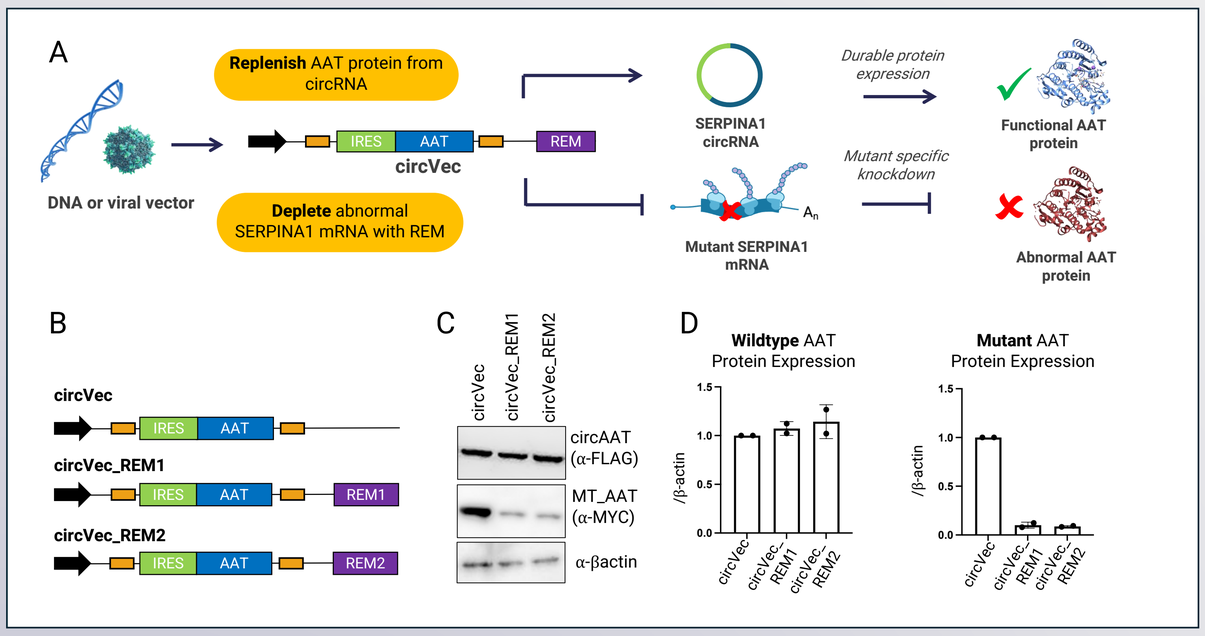


Figure 4: circVec remove'&'replace technology successfully depletes pathogenic AAT variants while replenishing functional AAT. A) Schematic of remove'&'replace circVec concept. **B)** Schematic of circVec cassette designs. **C)** Western blot on protein from HEK293T cells co-transfected with tagged circVec and mutant AAT reporters (MT_AAT), as indicated, using FLAG and MYC antibodies, respectively, and a loading control (β-actin). **D)** Relative wildtype AAT expression from circVec (left side) and mutant AAT (right side); n=2

5. CircVec achieves up to 15-fold higher protein expression than mRNA-based vectors *in vivo*

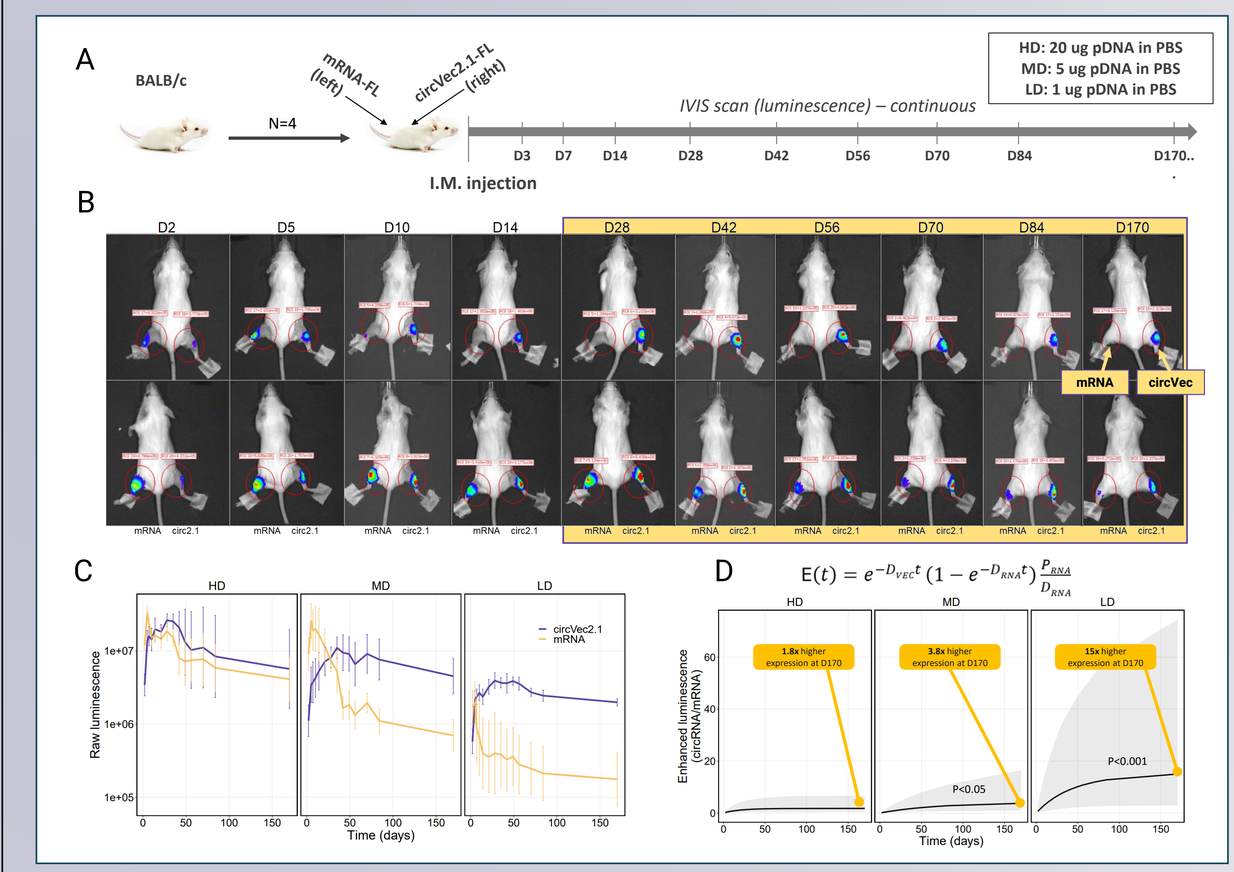


Figure 5: In vivo performance of circVec: A) schematic representation of in vivo study: Intramuscular injection of either circRNA (right hindleg) or mRNA (left hindleg)-encoding plasmids expressing firefly luciferase using three different doses: 20 (HD), 5 (MD), or 1 ug (LD) followed by continuous measurement of bioluminescence with IVIS. **B)** IVIS scans obtained at different timepoints after intramuscular injection of 1ug DNA shown for two independent studies. **C)** Quantified bioluminescence for the three dose groups over time. **D)** Inferred fold-change between circRNA and mRNA-based luminescence over time by MCMC modelling using the denoted growth-decay formula

6. 2-4x improvement of circVec performance by proprietary codon optimization approach

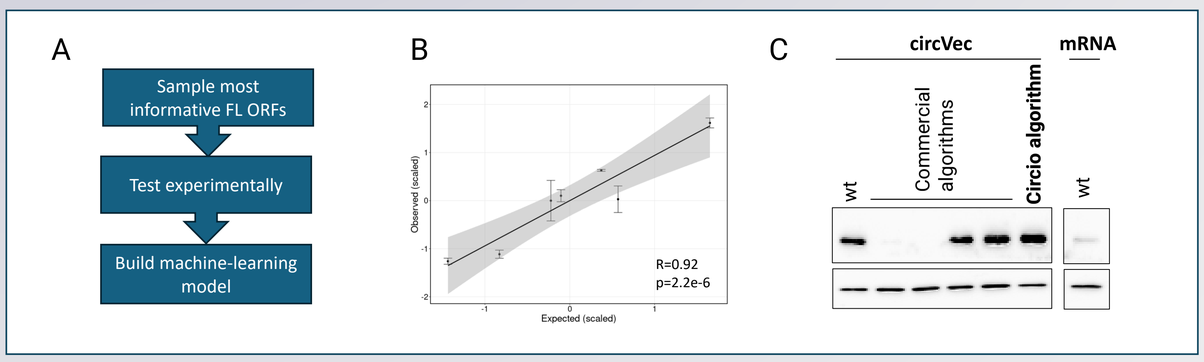


Figure 6: Codon optimization of circVec ORF: A) Schematic flow of algorithm development based on Firefly luciferase expression. **B-C)** Validation of codon optimization model using AAT ORF optimized by different algorithms showing superior performance of Circio algorithm improving yield 2-4x over the wild-type codon composition.

Conclusions

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- Superior stability leads to accumulation of circRNA resulting in higher and prolonged protein expression vs. mRNA
- circVec achieves up to 15x enhanced reporter signal *in vivo* compared to standard vector-based mRNA expression
- Choice and composition of IR and IRES/ORF design is critical for high yield expression
- circVec remove-&-replace design effectively depletes pathogenic mRNA and rescues wt protein expression
- Further circVec performance enhancement achieved by machine learning codon optimization approach