CircVec: a powerful novel circular RNA expression platform to enhance viral and non-viral gene and cell therapies

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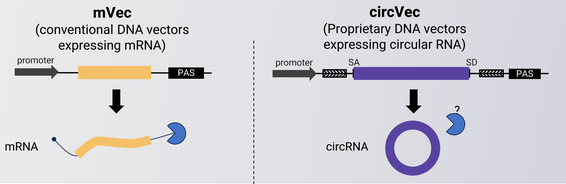
Introduction to circVec

Circular RNA (circRNA) constitutes a novel class of endogenously expressed RNA with substantial therapeutic potential. CircRNAs are generated by an alternative splicing process known as back-splicing, which is guided and stimulated by flanking sequence elements. In contrast to mRNA, circRNAs are resistant to exonucleolytic decay, resulting in high intra-cellular stability and persistence. Therefore, the circRNA is a potentially superior RNA format compared to mRNA in therapeutic situations where prolonged durability and increased expression level is desirable.

Here, we show the development and performance of a novel circRNA vector expression platform, **circVec**, for enhancing gene expression.



1. Superior circRNA stability drives increased protein expression from circVec

The first circVec cassette (circVec1.0) was based on an endogenous locus with high-yield circRNA production (IR1_1). Optimization of flanking elements driving circRNA biogenesis yields ~3x improvement (**Fig. 1A-B**) resulting in a ~35% increase in RNA levels compared to mRNA-based vectors (mVec) 48 hours after transfection (**Fig. 1B**). IRES elements are required for circRNA-templated translation. In an IRES screen, the superior IRES enhances protein yield 3-10x depending on cell type compared to CVB3 (IRES 1.0, **Fig. 1C**). With a 15x enhanced stability of circRNA over mRNA in vitro (**Fig. 1D**), firefly luciferase luminescence from circVec greatly exceeds mVec-based luminescence over time. (**Fig. 1E**).

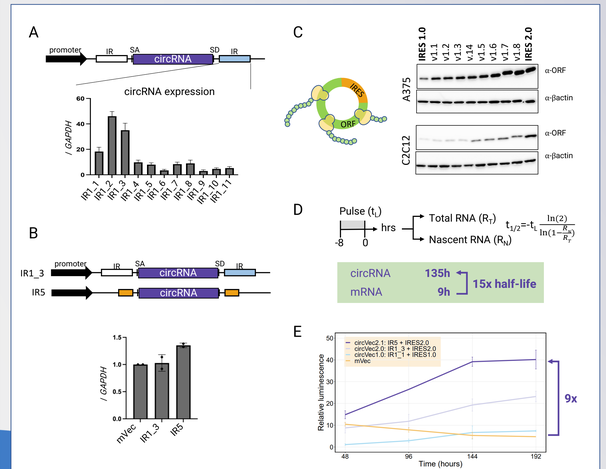


Figure 1: Optimization and characterization of circVec. A-B) The impact of flanking sequence elements on circRNA biogenesis as measured by qRT-PCR and normalized to *GAPDH*. **C)** Protein expression from ten different IRES elements was assessed by western blotting using antibodies specific to the ORF or βactin (loading control) in A375 and C2C12 cells. **D)** circRNA and mRNA half-life estimates derived from nascent RNA (labelled with nucleotide analogues for 8 hours) relative to total RNA assuming steady-state. **E)** Relative luminescence at indicated timepoints after transfection of three different circVec generations and the mVec counterpart, in C2C12 cells.

2. Downstream auxiliary elements serve as general gene expression enhancers

Adding specific DNA elements downstream of the expression cassette enhance protein expression from circVec (**Fig 2A-C**). The enhancing effect also applies to mVec-based expression in two different cell lines (**Fig. 2D-E**). This provides a general approach to enhance the potency of vector-based gene expression and serves as the basis for the circVec 3.x generation of vectors currently being tested *in vivo.*

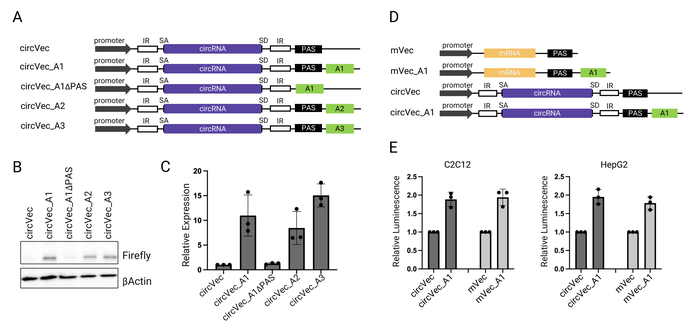


Figure 2: Novel auxiliary element enhances gene expression. A) Schematic representation of vector design with different auxiliary elements positioned downstream. **B)** Western analysis of firefly luciferase expression using the denoted vectors. Bactin was used as loading control. **C)** Quantification of firefly luciferase expression relative to Bactin, n=3. **D)** Schematic representation of mVec and circVec vector with or without the A1 axillary element downstream. **E)** Relative expression of the denoted vectors in C2C12 (left panel) and HepG2 (right panel) cell lines.

3. CircVec results in up to 15x protein yield compared to mVec *in vivo*

Intra-muscular injection of circVec2.1 and mVec expressing firefly luciferase in the right and left hindleg, respectively, shows accumulation and enhanced luminescence over time from circVec (**Fig. 3A-B**). Raw luminescence was modelled with a growth decay formula using a Bayesian approach (Markov chain Monte Carlo, MCMC). This revealed a significant improvement for circVec-based expression with 15x increased luminescence at day 170 compared to mVec in the low-dose group (**Fig. 3C**), and ~70x longer RNA half-life for circRNA compared to mRNA in muscle tissue (**Fig. 3D**).

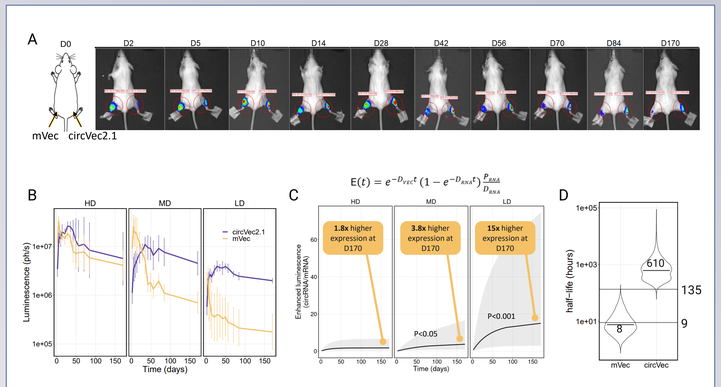


Figure 3: In vivo performance of circVec: A) DNA vectors expressing firefly luciferase from either mVec or circVec were injected intra-muscularly in left (mVec) and right (circVec) hindleg using three different doses: 20, 5, 1 ug. Representative IVIS scans from different timepoints following i.m. injection of 1ug DNA shown. **B)** Quantified luminescence for the three dose groups over time. **C-D)** Inferred fold-change between circVec and mVec-based expression over time (C) and RNA half-life (D) using MCMC modelling (10000 samplings) of raw luminescent using the denoted growth-decay model.

4. LNP:circVec accumulates in the spleen and drives payload expression for at least 12 weeks

Intravenous injection of LNP-packaged DNA vectors shows early and transient expression in the liver from mVec (**Fig.4A**, upper panel), whereas signal from circVec accumulates in the spleen from week 3 onwards (**Fig 4A,** upper panel, **Fig. 4B**). Distinct spleen-derived signal was confirmed using 3D bioluminescence and CT scanning (**Fig. 4D**).

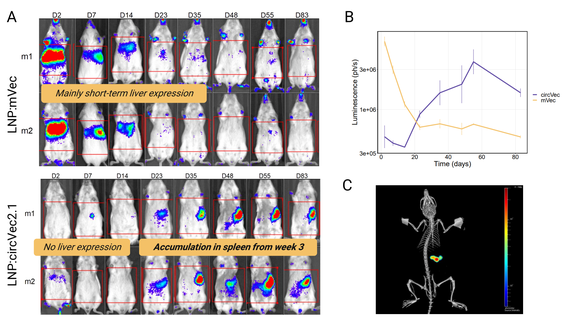


Figure 4: Durable spleen-specific signal from LNP:circVec injected i.v. A) IVIS scans measuring luminescence from mice injected with 1mg/kg LNP:mVec (upper panel) and LNP:circVec2.1 (lower panel). **B)** Whole-body quantification of signal from the two groups over time. **C)** 3D bioluminescence overlayed CT scans from an LNP:circVec2.1-treated mouse at day 35.

5. CircVec increases AAV gene expression with distinct tissue distribution

Intra-venous injection of AAV9 expressing firefly luciferase from mVec or circVec2.0 cassettes controlled by a muscle/heart specific promoter. The two vector types show similar expression levels in the high dose group (HD), whereas a circVec increases expression by 50% in the low-dose (LD) group (**Fig. 5A-B**). Notably, ex vivo analysis at D170 shows a distinct tissue expression profile for circVec, with low signal in liver and higher expression levels in both heart and diaphragm (**Fig. 5C**).

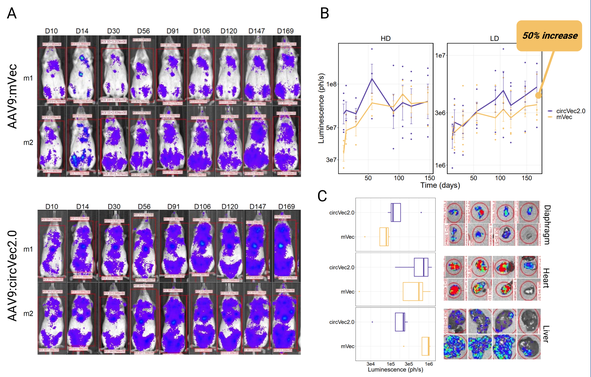


Figure 5: circVec2.0 expression from AAV9 shows higher muscle/heart specific expression. A) IVIS scans measuring luminescence from mice injected with AAV9-mVec (upper panel) and AAV9:circVec2.0 (lower panel) using 5e11 gc/kg (LD). **B)** Whole-body quantification of signal from high-dose group (HD: 5e12 gc/kg) and low-dose group (LD, 5e11 gc/kg) over time. **C)** Ex vivo analysis of diaphragm, heart and liver (as denoted) on day 170 after injection. The raw IVIS scans are depicted to the right with the corresponding quantification to the left.

Conclusions

- circVec drives the biogenesis of **engineered circular RNA** leading to **increased payload expression** compared to conventional mRNA-based vectors
- The **superior performance** of **circVec** has been **validated *in vitro*** and ***in vivo*** for both **viral and non-viral vectors**
- circVec shows the **strongest impact** on payload expression levels and durability at **lower dose-levels**
- Early data indicate that circVec drives **distinct and sustained tissue-specific expression** with reduced liver accumulation and **unlocks new gene and cell therapy opportunities**

circVec is a promising platform for the development of novel therapeutics where high and prolonged expression of a protein of interest is desired.

Based on promising data in spleen, muscle and heart, Circio is pursuing pre-clinical development in muscular dystrophies, cardiomyopathies, and different forms of cell- and immuno-therapies.

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