Optimization and In Vivo Performance of circVec, a Vector-Based Circular RNA Expression Platform

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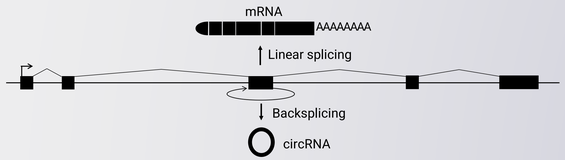
O'Leary ET[1,2], Vikberg S[1,2], Zhang J[1,2], Hjelmqvist D[1,2], Wiklund ED[1], Levitsky V[1], Hansen TB[1]
1. Circio AB, Huddinge, Sweden
2. Karolinska Institutet, CLINTEC, Huddinge, Sweden

Introduction

Circular RNA (circRNA) constitutes a novel class of endogenously expressed RNA. CircRNAs are generated by a non-linear splicing event in which an upstream splice acceptor attacks a downstream donor, known as backsplicing. In contrast to mRNA, circRNAs are resistant to exonucleolytic decay which results in high intra-cellular stability and persistence. Here, we show our development of a circRNA expression platform, termed **circVec**, towards improved vector-based expression of proteins *in vivo*.



1. Choice and composition of IR dramatically impacts circRNA biogenesis

Based on endogenous loci with high-yield circRNA production, the prevalent model for circRNA biogenesis involves flanking inverted repeats (IRs). Here, bioinformatic analysis of publicly available RNA-Seq datasets, was used to identify highly abundant circRNAs. Four IR's flanking the most abundant circRNAs were incorporated into our circRNA cassette to examine their ability to drive backsplicing (**Fig. 1A-B**). We show that modification and optimization of these IR sequences was found to further enhance the biogenesis of circRNAs (**Fig. 1C**), and we have developed a short and effective IR design (IR5) with an improved rate of biogenesis.

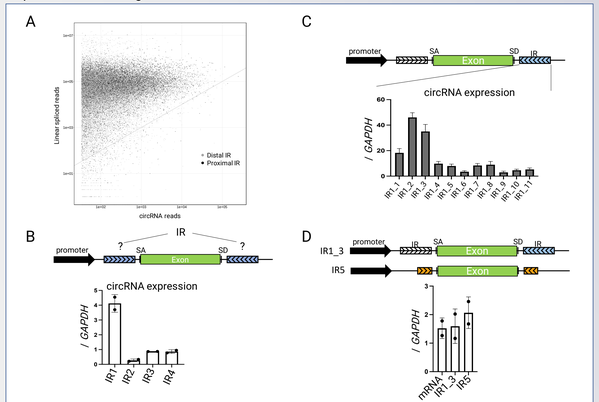


Figure 1: Optimization of flanking inverted repeats (IRs). A) IRs from highly expressed circRNAs, stratified by distance to backsplicing sites, were identified by bioinformatic analysis of publicly available datasets, where circRNA specific reads were compared to linear spliced reads. **B)** Biogenesis as measured by circular RNA-specific qRT-PCR from circRNA cassettes with different naturally occurring IRs. **C)** The impact of downstream IR sequence composition on circRNA biogenesis as measured by qRT-PCR normalized to *GAPDH*. **D)** Comparing circRNA levels from the IR1_3 design with vector-based mRNA expression and the improved, shortened IR5 design by qRT-PCR normalized to *GAPDH*.

2. Cassette design and choice of IRES critical for protein expression level

Protein translation from circular RNA requires an IRES (internal ribosomal entry site) for cap-independent translation (**Fig. 2A**). Here, testing 12 different designs (D1-D12), a dramatic design-dependent effect on protein yield was observed, with superior performance from the D4 and D5 designs (**Fig. 2B**), irrespective of IRES and ORF (data not shown). In addition to exact cassette composition, the choice of IRES significantly affects circRNA biogenesis (**Fig. 2C**). The circVec 2.x generation is based on the best performing IRES across multiple cell types.

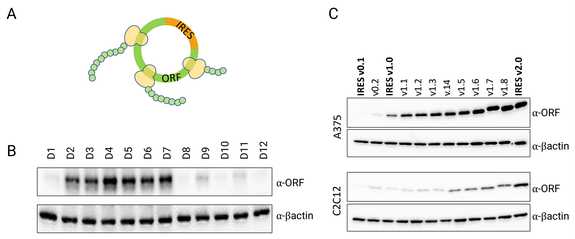


Figure 2: Choice of IRES and IRES/ORF composition impact circRNA expression: A) CircRNA translation depends on cap-independent translation and requires IRES (internal ribosome entry site) and ORF (Open Reading Frame) as shown. **B)** Protein expression from twelve different IRES/ORF designs (D1-D12) was assessed by western blot using antibodies specific to the ORF and β-actin (loading control). **C)** Protein expression from twelve different IRES elements in the D4 cassette design in two cell lines (A375 melanoma cell lines and C2C12 mouse cell line) was assessed by western blotting using antibodies specific to the ORF and β-actin (loading control).

3. circRNA stability confers enhanced protein expression

Exonucleolytic decay is responsible for almost all cellular RNA turnover. Circular RNAs are devoid of 5' and 3' ends and thereby resistant to exonucleases. Consequently, using metabolic labelling, 15x longer half-life was observed for circRNA compared to mRNA (**Fig. 3A**). The extended half-life translates into accumulating signal over time. Here, based on relative luminescence measurements *in vitro*, different generations of the circVec cassette show increasing expression over time and enhanced luminescence compared to the mRNA expressing vector (**Fig. 3B**).

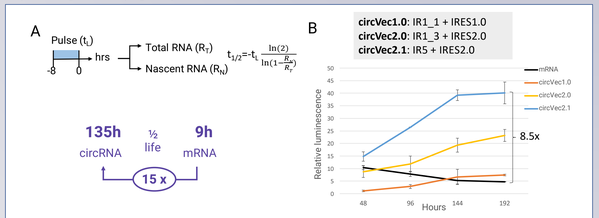


Figure 3: Superior circRNA stability facilitates circRNA accumulation and prolonged protein expression. A) Newly synthesized RNA was labelled with nucleotide analogues for 8 hours and the durability of labelled RNA was quantified over time by qRT-PCR. Half-life RNA estimates were inferred from the nascent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

4. CircVec more than triples protein yield compared to mRNA-based expression vectors *in vivo*

Intra-muscular injection of circVec2.1 and an mRNA-based vector expressing firefly luciferase (FL) in the right and left hindleg, respectively (schematically depicted in **Fig 4A**), shows accumulation and enhanced luminescence over time from circVec in two independent experiments (**Fig. 4B-C**). Raw luminescence was modelled using MCMC on a growth decay formula. This revealed a clear improvement from circVec-based expression (**Fig 4D**). Due to the circRNA stability, the improvement increases over time with 3.3x increased luminescence at day 119 compared to mRNA (**Fig. 4E**). Second experiment still ongoing.

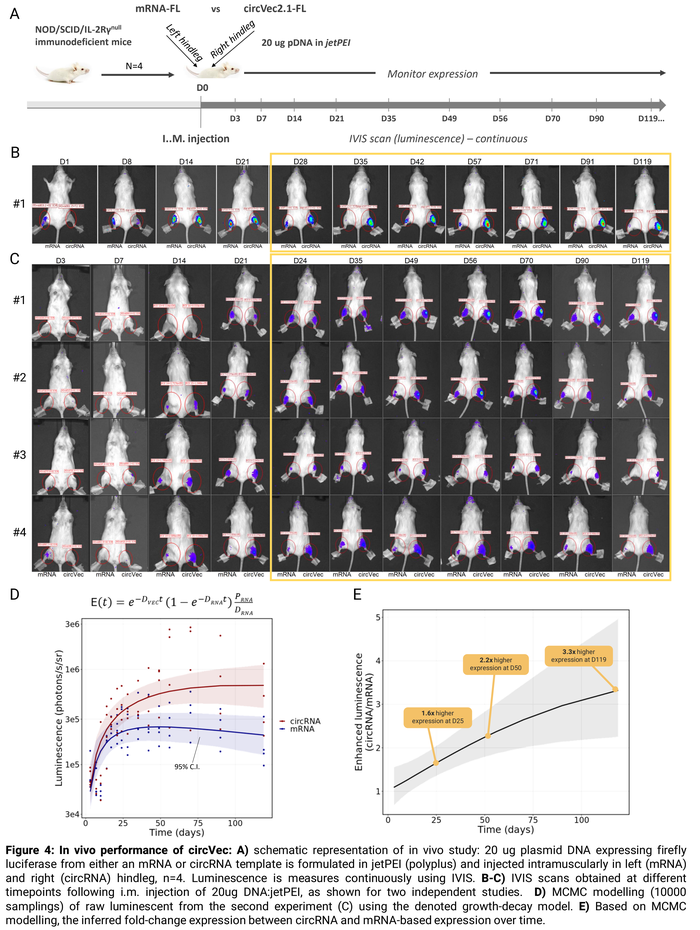


Figure 4: In vivo performance of circVec: A) schematic representation of in vivo study: 20 ug plasmid DNA expressing firefly luciferase from either an mRNA or circRNA template is formulated in jetPEI (polyplus) and injected intramuscularly in left (mRNA) and right (circRNA) hindleg, n=4. Luminescence is measures continuously using IVIS. **B-C)** IVIS scans obtained at different timepoints following i.m. injection of 20ug DNA:jetPEI, as shown for two independent studies. **D)** MCMC modelling (10000 samplings) of raw luminescent from the second experiment (C) using the denoted growth-decay model. **E)** Based on MCMC modelling, the inferred fold-change expression between circRNA and mRNA-based expression over time.

Conclusions

1) Superior circRNA stability facilitates accumulation of circRNA and prolonged protein expression.
2) CircVec shows enhanced and accumulating luminescence *in vivo* compared to vector-based mRNA expression.
3) Choice and composition of IR is crucial for high yield circRNA biogenesis.
4) IRES/ORF design is essential for generation of protein-coding circRNAs.
5) IRES selection is critical for high yield translation from circular RNA

The results support further development of the circVec platform towards therapies where high and prolonged expression of any gene of interest is desired.

Circio is pursuing a lead program in alpha-1-antitrypson deficiency in which circVec-based AAT expression is likely to improve clinical benefit.

See poster #598 for more details on the AATD program

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Contact information

Thomas.hansen@circio.com