circVec: a versatile circular RNA vector cassette for enhanced and durable intra-cellular protein expression

Poster P803
Presented at ESGCT 2023

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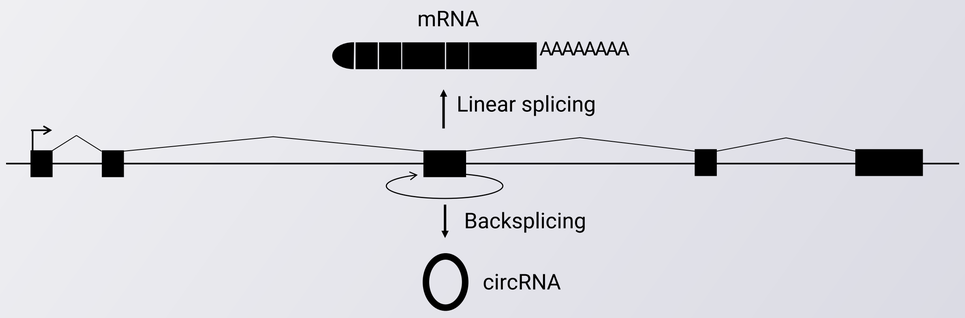
Introduction

Circular RNAs (circRNAs) are naturally occurring non-coding transcripts found in all eukaryotes. CircRNAs typically derive from protein-coding exons prone to backsplicing (non-linear splicing in which an upstream splice acceptor attacks a downstream donor, see schematic below). In contrast to mRNAs, circRNAs are resistant to known RNA degradation pathways, making them highly stable molecules with sustained effects within cells. Understanding the biogenesis, structure and function of circRNA allows repurposing circRNAs into durable protein-coding "circular-mRNA" templates. Here, we describe the development of our highly efficient vector- system, circVec, for intra-cellular circRNA-based protein expression.



CircRNA biogenesis dramatically impacted by choice and design of IR element

Based on endogenous human 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 (**Fig. 1A**). Several natural IR's flanking these abundant circRNAs were incorporated into our circRNA cassette to examine their ability to drive backsplicing (**Fig. 1B-D**). Targeted modifications of these natural IR sequences were found to further enhance the circRNA biogenesis (**Fig. 1E-F**).

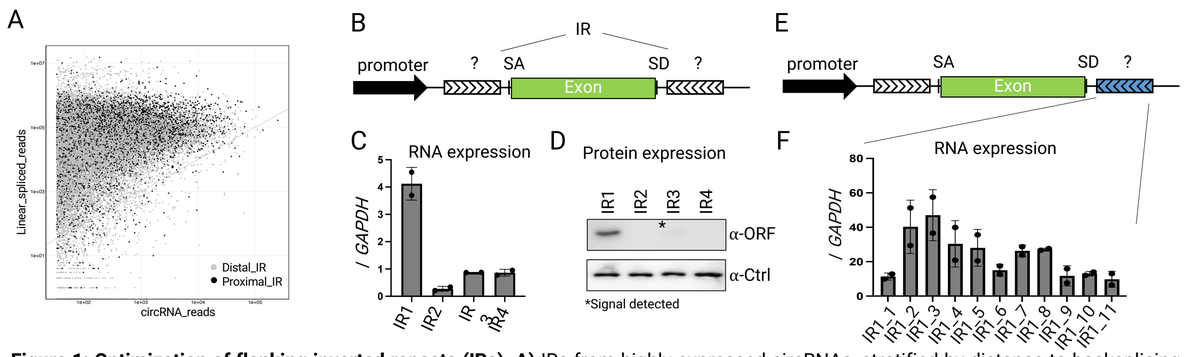


Figure 1: Optimization of flanking inverted repeats (IRs). A) IRs from highly expressed circRNAs, stratified by distance to backsplicing sites, were identified by bioinformatic analysis of publicly available datasets, where circRNA specific reads were compared linear spliced reads. **B)** Schematic representation of circRNA cassette depicting IR position, where different proximal IR elements from highly expressed endogenous circRNAs are incorporated. **C-D)** Biogenesis as measured by qRT-PCR normalized to *GAPDH* mRNA (C) and protein yield measured by western blotting (D) from circRNA cassettes with different IRs. **E)** Schematic representation of circRNA cassette with depicting modification of downstream IR. **F)** The impact of downstream IR sequence composition on circRNA biogenesis as measured by qRT-PCR normalized to *GAPDH* mRNA.

Cassette design and choice of IRES critical for circRNA protein expression level

Protein translation from circular RNA requires an IRES (internal ribosomal entry site) for cap-independent translation (**Fig. 2A**). Here, testing 10 different designs using 2 different IRES elements (schematically shown in **Fig. 2B**), a dramatic design-dependent effect on biogenesis was observed by protein expression, with high and consistent yield from the circRNA producing D4 and D5 designs (**Fig. 2C**). In addition to affecting circRNA-derived protein translation, choice of IRES significantly affects circRNA biogenesis (**Fig. 2D**). Here, we show that specific IRES-elements capable of initiating high levels of translation across multiple cell types. IRES_5 consistently shows the greatest level of protein expression across several cell lines tested.

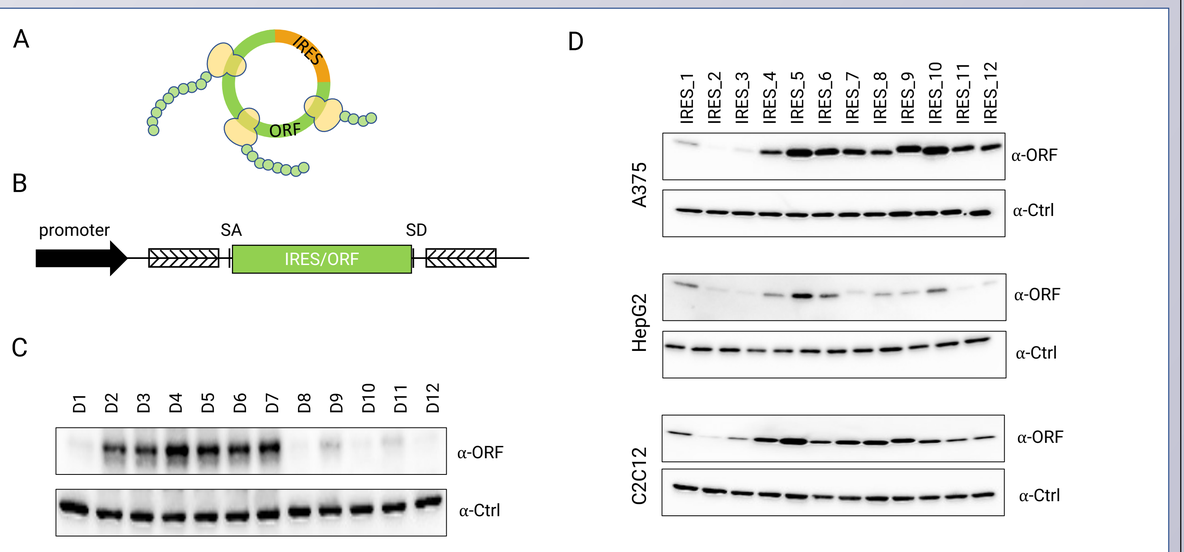


Figure 2: Choice of IRES and IRES/ORF composition impact circRNA expression: A) CircRNA translation depends on cap-independent translation and requires IRES (internal ribosome entry site) and ORF (Open Reading Frame) as shown. **B)** Schematic representation of vector cassette used for circRNA expression. **C)** Protein expression from twelve different IRES/ORF designs (D1-D12) was assessed by western blot using antibodies specific to the ORF and Beta-actin (loading control). **D)** Protein expression from twelve different IRES elements in the circRNA_D4 cassette design in three cell lines A375 melanoma cell line, HepG2 liver carcinoma cell line and C2C12 mouse cell line was assessed by western blot using antibodies specific to the ORF and Beta-actin (loading control).

circVec 2.0 design outcompetes linear mRNA already at early time points

A ~15x improvement in protein expression was achieved in the second-generation vector cassette (circVec 2.0) compared to the first-generation design (circVec 1.0) **(Fig. 3A).** circVec 2.0 outcompetes mRNA-based vector expression at early timepoint following transfection (**Fig 3B-C**) in C2C12 mouse cells. Whereas earlier circVec generations required longer intervals to achieve higher expression levels than mRNA vectors, particularly in C2C12 mouse cells where IRES1 showed suboptimal levels of protein expression.

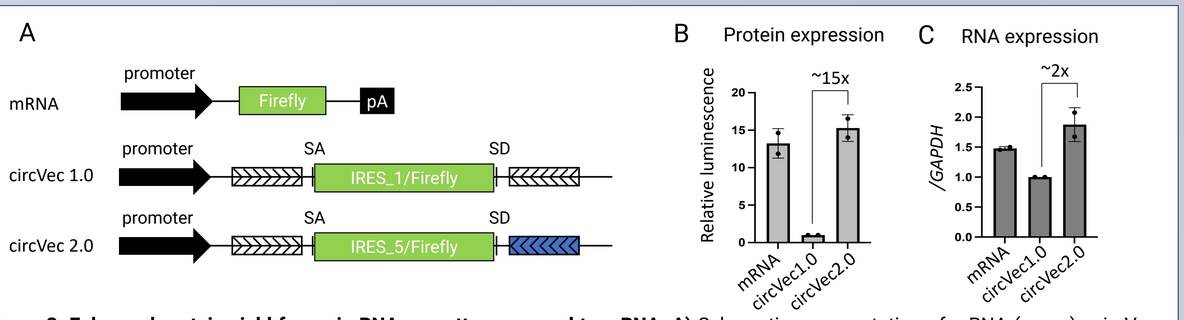


Figure 3: Enhanced protein yield from circRNA cassette compared to mRNA. A) Schematic representation of mRNA (upper), circVec 1.0 (middle) and circVec 2.0 (lower) expression cassettes. **B)**) Luciferase reporter assay from C2C12 cells measuring Firefly luminescence from mRNA, circVec 1.0 and circVec 2.0 as denoted. Firefly expression was normalized to Renilla luminescence. **C)** Quantification of Firefly RNA expression from transient transfection as in (B) by qRT-PCR normalized to *GAPDH* mRNA.

Enhanced circRNA stability confers enhanced and prolonged protein expression

Cellular RNA turnover is generally driven by exonucleolytic decay. CircRNAs lack free 5' and 3' ends and are therefore resistant to exonucleases. Consequently, circRNA intrinsically has vastly prolonged stability and half-live compared to mRNA (**Fig. 4A-B**). Based on experimental estimates of circRNA half-life, long-term expression profiles of circRNA vs. mRNA vectors were computationally simulated, showing circRNA accumulation to significantly higher and more durable intra-cellular steady-state RNA levels (**Fig. 4C**). The relative expression ratio between circRNA and mRNA was confirmed in a 6-day time-course using circRNA- and mRNA- based luciferase reporter vectors (**Fig. 4D**).

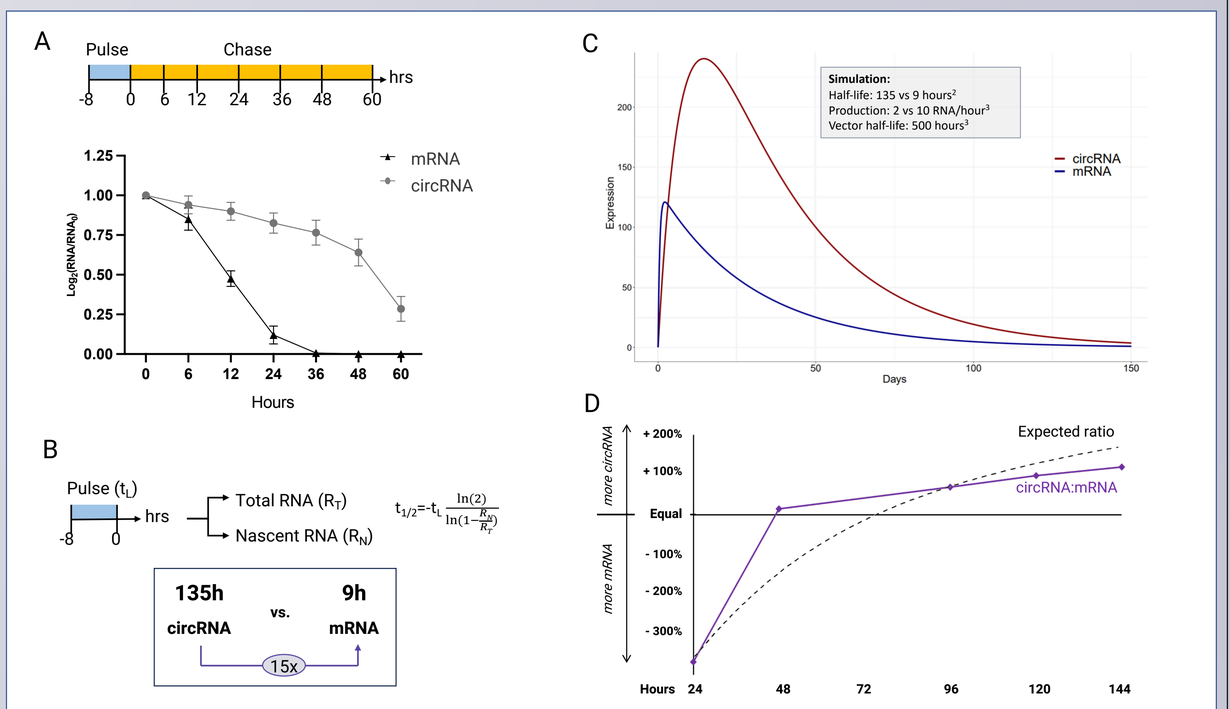


Figure 4: Superior circRNA stability facilitates circRNA accumulation and prolonged protein expression. Enhanced stability of circRNA compared to mRNA was demonstrated using a pulse-chase experiments (A) and half-life estimations (B). **A)** Newly synthesized RNA was labelled with nucleotide analogues for 8 hours and the durability of labelled RNA was quantified over time by qRT-PCR. **B)** Half-life RNA estimates were inferred from the nascent fraction (newly synthesised labelled RNA as in(A)) relative to total RNA assuming steady-state**. C)** Simulation of circRNA and mRNA expression profile over time based on half-life measurements and assuming a 5x enhanced biogenesis of mRNA over circRNA as denoted. **D)** Time course experiment measuring the relative luminescence (Firefly / Renilla) from circRNA (circVec 1.0) and mRNA-encoding vectors. Dashed line represents expected ratio based on simulation in C.

CircVac, a viral circRNA expression vector for durable antigen production

The enhanced stability of circRNA over mRNA has led to significant interest and investment into development of novel circRNA therapeutics for disorders where durable protein expression may lead to favourable outcomes, such as vaccination and gene therapy. Viruses are effective vehicles for delivery of transgenes and have been engineered into vaccine vectors capable of stimulating immune responses against the antigens from the encoded transgenes. Here, we describe the generation of our proprietary circVac vector system, a viral vector for delivery of circRNA-encoded antigens (**Fig. 5A)**. We demonstrate successful expression of a model antigen from our circVac vector and observe superior expression of the antigen at later timepoints (**Fig. 5B-C)**. We hypothesize that the combination of intrinsic immunostimulatory features of the circVac vector and prolonged antigen expression from the circRNA template, could improve vaccine efficacy.

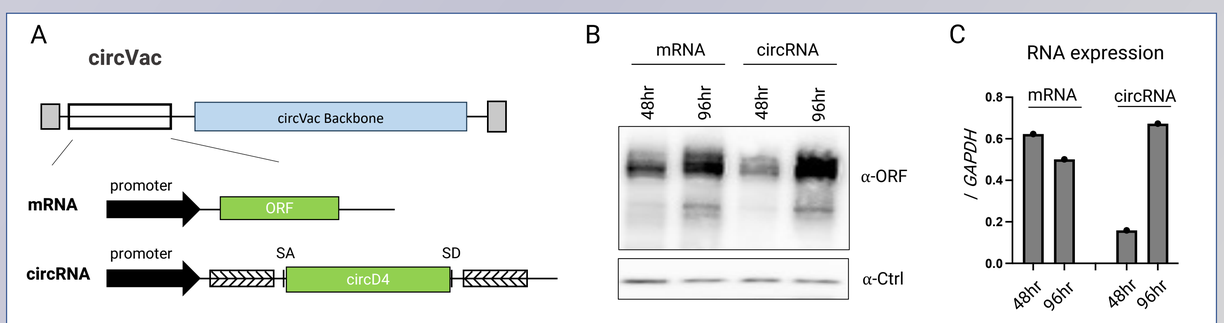


Figure 5: circVac, a viral vector-based system for durable antigen expression. A) Schematic representation of circVac vector backbone (upper). Schematics of mRNA and circRNA expression cassettes, as denoted, inserted within circVac backbone at denoted position (lower). **B)** Antigen protein expression of antigen payload at early (48hr) and late (96hr) timepoints was assessed by western blot. Beta-actin was used as a loading control. **C)** Quantification of antigen RNA expression from circVac and mRNA encoding viral vector, was assessed by RT-qPCR and normalised to *GAPDH* mRNA.

Conclusions

1) Targeted modifications of flanking IR elements for improved circRNA biogenesis identified and implemented into circVec design.
2) Fundamental IRES/ORF design feature established for maximum circVec protein expression.
3) Potent, promiscuous IRES identified for high-yield protein expression across multiple cell types.
4) Superior circRNA biogenesis, stability and prolonged protein expression from circVec circRNA vector designs tested and validated.
5) Optimized second-generation circVec 2.0 design outcompetes mRNA-based vector systems.
6) circVac viral vector design established for durable antigen expression.

These encouraging results support further development of the circRNA expression system with the aim to replace traditional mRNA-based DNA and viral therapeutics in the future.

Acknowledgements

This study was funded by Circio Holding ASA Norway. The authors thank everyone in the company for a great atmosphere and working environment. The authors also thank the MUK group at the Karolinska Institutet for their collaboration.

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