Circular RNA-based AAV gene therapy for cardiomyopathies enhances transgene expression levels, reduces liver off-targeting and minimizes cellular stress.

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Introduction

Circular RNA (circRNAs) is a class of non-coding transcripts with long half-life found in most eukaryotes. Unlike mRNAs, circRNAs resist exonucleolytic degradation, which enhances their stability by up to 75 times depending on the cell and tissue type. Advances in understanding circRNA biogenesis and function have positioned circRNA as a promising next-generation therapeutic RNA, potentially surpassing conventional linear mRNA technologies.

Protein-coding circRNAs can be generated intra-cellularly from expression vectors by incorporating IRES elements to drive translation of an open reading frame of choice. The prolonged half-life of circRNAs leads to RNA accumulation over time, which results in increased protein expression compared to linear mRNA. The Circio technology platform, circVec, introduces a powerful, modular genetic construct that enables potent expression of protein-coding circRNA in vivo. This versatile platform can be effectively deployed in non-viral and viral vector systems to enhance payload expression, enable dose sparing, and reduce toxicity—key challenges in current gene therapy.

In this study, we characterized three versions of circVec (circVec 2.1, 3.0, 3.2) in AAV9 vectors with the cardiomyocyte-specific cTnT promoter to compare heart-specific expression of Firefly Luciferase against a corresponding conventional mRNA-encoding AAV9 (referred to as AAV9-mVec).

Methods

AAV9 vectors (mVec, circVec2.1, circVec3.0, and circVec3.2) were injected I.V. at a dose of 5e12 vg/kg into 8-weeks old BALB/c mice (n=4/group), and luminescence was monitored by IVIS over time. At the end of study, tissue samples were collected and subjected to RNA/DNA quantification, RNAseq, and RNAscope analyses.

Results

Following intravenous injection of AAVs, luminescence from Firefly Luciferase shows 10-fold (AAV9-circVec2.1 and AAV9-circVec3.0) to 40-fold (AAV9-circVec3.2) increase in expression compared to AAV9-mVec. Tissue specific ex vivo analysis confirms this finding, showing 35-fold enhancement in heart expression for circVec3.2. Importantly, in AAV9-circVec-treated mice, over 80% of the total signal emanates from the heart compared to only 50% in AAV9-mVec-treated mice. This enhanced specificity is a consequence of both higher on-target cardiac expression and reduced off-target expression in unwanted tissues, particularly in liver. As such, AAV9-circVec expression in heart is both significantly enhanced in absolute terms, and more tissue specific.

Consistent with these findings, transcriptomic analysis using bulk RNA sequencing and RT-qPCR of heart tissue reveals 35-fold (circVec2.1 and 3.0) to 200-fold (circVec3.2) increased RNA transcript levels relative to mVec, while no significant difference in AAV genome copy numbers was found between the groups.

Notably, circVec-mediated expression shows a reduced activation of the Unfolded Protein Response (UPR) pathway both in heart tissue and *in vitro*, suggesting that circRNA-based gene expression may elicit

less cellular stress than conventional mRNA-based expression vectors. We speculate that this observation is a consequence of steady and gradual build-up of transcripts over time from circVec as well as the bypass of cap-dependent translation.

circVec-AAV expression was further characterized by RNAscope single-molecule detection in heart sections. This analysis showed that 80% of cells were positive for Firefly RNA, with a median of ~60 circRNA transcripts per cell for circVec3.2 compared to just 2 mRNA transcripts for mVec This difference is consistent with the increase in protein expression and indicates that the circVec advantage is largely driven by the reduced circRNA decay leading to higher protein yield *in vivo*.

Conclusion

Ongoing preclinical experiments in Danon disease, dilated and arrhythmic cardiomyopathy, and chronic heart failure aim to further validate the therapeutic potential of the circVec platform. Additionally, the continuous optimization of the circVec cassette is underway to further enhance the efficacy and safety of AAV-circVec-based gene therapy going forward.

Collectively, we propose AAV9-circVec as a next-generation cardiac gene therapy platform that delivers enhanced on-target expression with reduced cellular stress, supporting the development of safer and more effective therapies for rare and chronic heart diseases.