Evaluating miR-Target Sites as a Strategy to Allow AAV Vector-based De-targeting of Gene Expression in the Inner Ear

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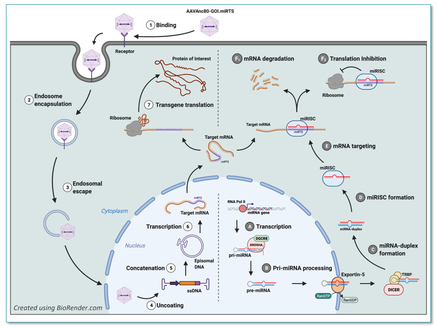
INTRODUCTION

- Gene therapy using adeno-associated viral (AAV) vectors is a promising therapeutic modality for inner ear conditions, enabling delivery of potentially therapeutic genes directly to the cochlea.
- Hearing loss can be a result of mutation(s) in different genes that are expressed in various cells, requiring transduction of multiple cells types in the cochlea for a broad range of conditions.
- The broad cochlear tropism of AAVAnc80 transduction allows for multiple programs with different relevant cell types.
- Ubiquitous promoters can drive safe expression of multiple transgenes, and are used in current commercial gene therapies, such as LUXTURNA, in clinical stage gene therapies, and in Akouos's preclinical development stage gene therapies, AK-OTOF *(ASGCT 2022 Abstract* 1233), and AK-antiVEGF.
- However, expression of some transgenes using a ubiquitous promoter may not be well tolerated. This is the case for *GJB2*, which is endogenously expressed in supporting cells. Expression of *GJB2* in hair cells can result in hair cell loss. In this case, a tailored expression pattern may be warranted.

METHODS

MicroRNA-Target Site (miR-TS) Regulation of Transgene Expression

- 1-6: AAV transduction and transgene expression
- A-E: MicroRNA (miRNA) transcription and processing
- 7: Transgene expression in the absence of the miRNA
- F1-F2: Transgene down-regulation in the presence of the miRNA



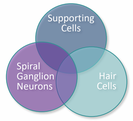
Step 1

Identify key regulatory elements for desired transgene expression profile

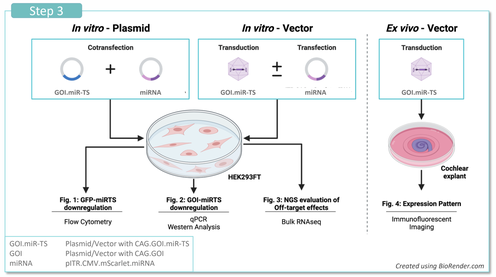
Endogenous miRNA expression can downregulate transgene expression in a subset of cell types, based on added miR-TS, while maintaining the benefits of using a ubiquitous promoter.

Step 2

Select miR-TS based on known miRNA expression in the cells of interest. In this study, miRNA that are expressed in hair cells but not in supporting cells or spiral ganglion neurons were selected.

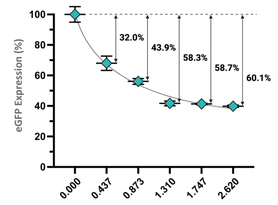


Step 3

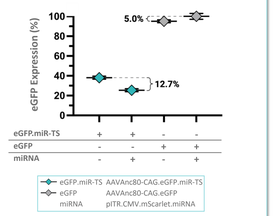


RESULTS: Transgene Expression and Downregulation in HEK293FT Cells

Figure 1: eGFP-miRTS Expression Evaluation using Flow Cytometry

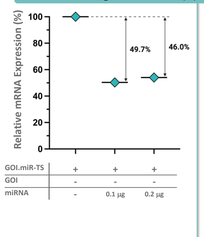


Reduced eGFP-miRTS expression in response to increase in miRNA DNA (average ± SEM)

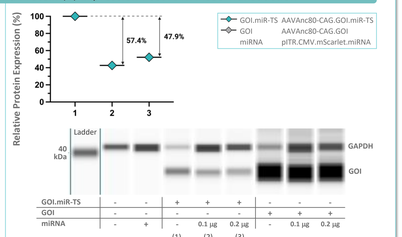


Reduced expression of eGFP-miRTS but not eGFP alone in the presence of miRNA DNA (average ± SEM)

Figure 2: mRNA (A) and Protein (B) Expression Post AAVAnc80 Transduction

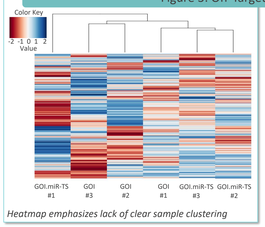


(A) mRNA expression using qPCR

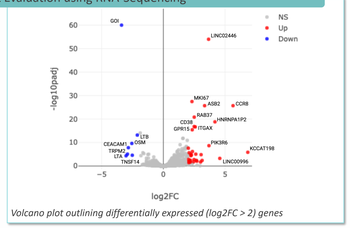


(B) Protein expression using Wes

Figure 3: Off-Target Evaluation using RNA-Sequencing



Heatmap emphasizes lack of clear sample clustering



Volcano plot outlining differentially expressed (log2FC > 2) genes

RNA-sequencing analysis was conducted to evaluate off-target effects after transduction of GOI compared to GOI with miR-TS. Only 40 genes were detected as significantly (adjusted p-value < 0.05) differentially expressed with log2FoldChange > 2 or < -2, which are primarily associated with immune response (gProfiler: biit.cs.ut.ee).

RESULTS: AAVAnc80 with miR-TS can drive various transgene expression patterns in cochlear explants

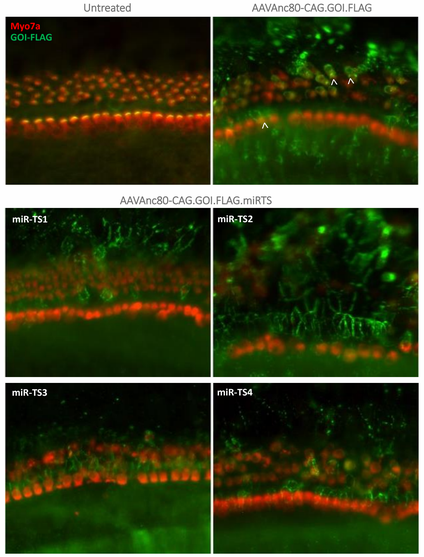
Figure 4: Explant screening for miR-TS enables prioritization prior to in vivo studies

Strong ubiquitous promoter without any miR-TS

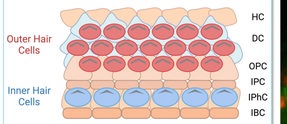
- FLAG expression in medial and lateral supporting cells
- Expression is also detected in hair cells (arrowhead)

Strong ubiquitous promoter with various miR-TS sequences

- FLAG expression in medial and lateral supporting cells
- No apparent hair cell expression except in miR-TS4



Schematic representation of the sensory epithelium of the cochlea



HC: Hensen cells, DC: Dieter's cells, OPC: Outer pillar cells, IPC: Inner pillar cells, IPhC: Inner phalangeal cells, IBC: Inner boarder cells

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SUMMARY & CONCLUSIONS

- Ubiquitous promoters can drive strong widespread expression in the inner ear in mice and NHP. This expression can be well tolerated across the inner ear, as is the case for Akouos's first two programs, AK-OTOF and AK-antiVEGF.
- Addition of selective cis-regulatory elements may be needed for some transgenes, such as *GJB2,* where expression in a portion of nontarget cells is not well tolerated.
- Akouos identified multiple microRNA target sites to drive various differential expression patterns.
- miR-TS evaluation *in vitro* confirmed successful downregulation of GOI and minimal off-target effects .
- Several microRNA target sites were evaluated in cochlear explants, demonstrating differential expression in various cell types.
- A combination of AAVAnc80 and miR-TS can drive expression in supporting cells, while limiting expression in hair cells in cochlear explants.
- Future work will focus on evaluating miR-TS regulation *in vivo* and identifying combinations of different miR-TSs to enhance de-targeting in specific cell types, where expression driven by ubiquitous promoters is not well tolerated.

Abbreviations: GFP = Green fluorescent protein; GJB2 = Gap junction beta-2 protein; GOI = Gene of interest; Log2FC = Log2FoldChange; Log10padj = Log10 of adjusted p-value; miR = microRNA; miR-TS = microRNA target site; NGS = Next generation sequencing; NHP = Non human primates; OTOF = Otoferlin; UTR = Untranslated region; qPCR = Quantitative PCR;