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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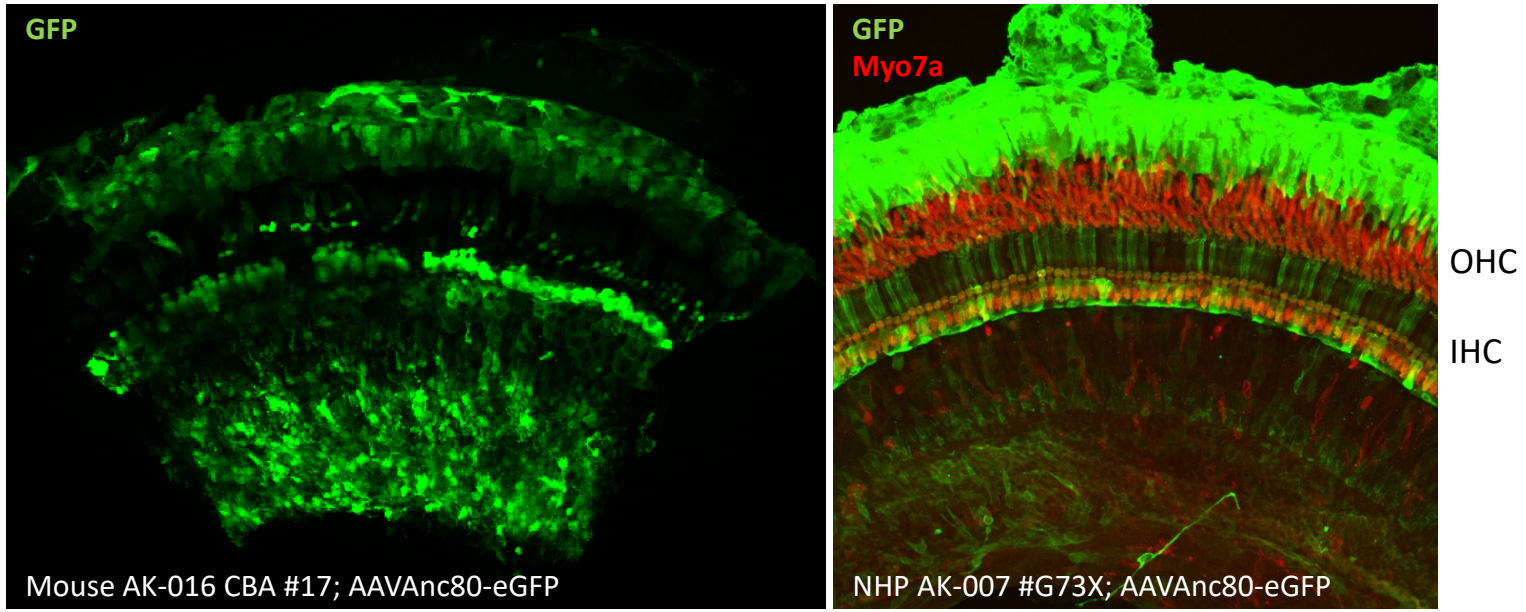
Molecular Biology of Hearing and Deafness Conference | May 24 to 27, 2022

Forward Looking Statements

This presentation includes “forward-looking statements” within the meaning of the Private Securities Litigation Reform Act of 1995, including, but not limited to the ability to use our precision genetic medicine platform to build a product candidate pipeline. These forward-looking statements may be accompanied by such words as “aim,” “anticipate,” “believe,” “continue,” “could,” “estimate,” “expect,” “forecast,” “goal,” “intend,” “may,” “might,” “plan,” “potential,” “possible,” “target,” “will,” “would,” and other words and terms of similar meaning. Akouos may not actually achieve the plans, intentions, or expectations disclosed in these forward-looking statements, and you should not place undue reliance on these forward-looking statements. Actual results or events could differ materially from the plans, intentions, and expectations disclosed in these forward-looking statements as a result of various factors, including: our limited operating history; uncertainties inherent in the development of product candidates, including the initiation and completion of nonclinical studies and clinical trials; the timing of and our ability to submit and obtain regulatory approval; whether results from nonclinical studies will be predictive of results or success of clinical trials; our ability to obtain sufficient cash resources to fund our foreseeable and unforeseeable operating expenses and capital expenditure requirements; our ability to obtain, maintain, and enforce our intellectual property; the impact of the COVID-19 pandemic on our business, results of operations, and financial condition; the potential that our internal manufacturing capabilities and/or external manufacturing supply may experience delays; risks related to competitive programs; and the other risks and uncertainties that are described in the Risk Factors section of our Quarterly Report on Form 10-Q for the quarter ended March 31, 2022, which is on file with the Securities and Exchange Commission, and in other filings that Akouos may make with the U.S. Securities and Exchange Commission. These statements are based on our current beliefs and expectations as of the date of this presentation. We do not undertake any obligation to publicly update any forward-looking statements except as required by law. By attending or receiving this presentation, you acknowledge that: you are cautioned not to place undue reliance on these forward-looking statements; you will be solely responsible for your own assessment of the market and our market position; and you will conduct your own analysis and be solely responsible for forming your own view of the potential future performance of Akouos, Inc.

AAVAnc80 With a Ubiquitous Promoter Transduces Hair Cells and Supporting Cells in Mice and Non-human Primates

- 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



Whole mount immunofluorescent staining from the middle turn of the cochlea.
Myo7a stains inner and outer hair cells (red), eGFP-reporter in green.

Abbreviations: eGFP = Enhanced green fluorescent protein; IHC = inner hair cells; NHP = non-human primate; OHC = outer hair cells.

Ubiquitous Promoters Can Drive Safe Expression of Multiple Transgenes, and are Used in Commercially Approved and Development-Stage Gene Therapies

Approved AAV gene therapies use ubiquitous promoters

Commercially approved gene therapy for treatment of patients with confirmed biallelic *RPE65* mutation-associated retinal dystrophy

LUXTURNA® voretigene neparvovec-rzyl for subretinal injection

Illuminating possibilities.

Spark Therapeutics™, a member of the Roche Group

Commercially approved gene therapy for treatment of patients with spinal muscular atrophy with biallelic mutations in the *SMN1* gene

zolgensma® (onasemnogene abeparvovec-xioi)

AveXis, a Novartis company

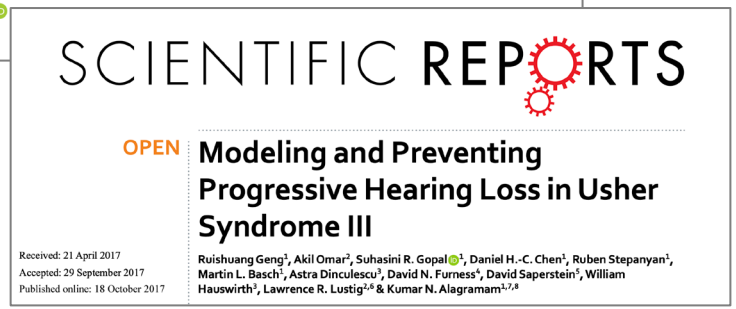
Multiple inner-ear targeted AAV gene therapies in discovery and preclinical development use ubiquitous promoters and do not exhibit tolerability concerns

Article

EMBO Molecular Medicine

Neonatal AAV gene therapy rescues hearing in a mouse model of *SYNE4* deafness

Shahar Taiber[1], Roie Cohen[2], Ofer Yizhar-Barnea[1], David Sprinzak[2], Jeffrey R Holt[3,*] & Karen B Avraham[1,**]



SCIENTIFIC REPORTS

OPEN

Modeling and Preventing Progressive Hearing Loss in Usher Syndrome III

Received: 21 April 2017
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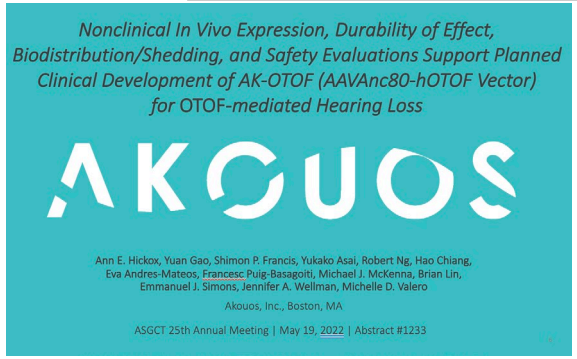
Published online: 18 October 2017

Ruishuang Geng[1], Akil Omar[2], Suhasini R. Gopal[1], Daniel H.-C. Chen[1], Ruben Stepanyan[1], Martin L. Basch[1], Astra Dinculescu[3], David N. Furness[4], David Saperstein[5], William Hauswirth[3], Lawrence R. Lustig[2,6] & Kumar N. Alagramam[1,7,8]



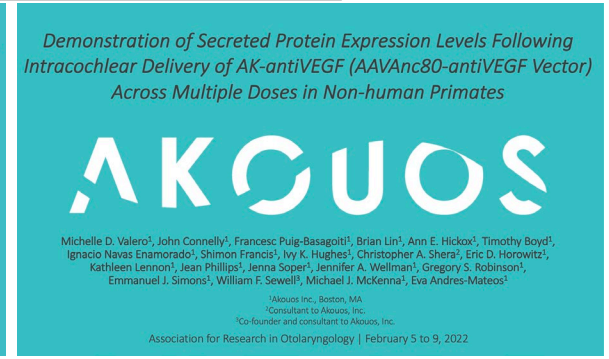
Nonclinical In Vivo Expression, Durability of Effect, Biodistribution/Shedding, and Safety Evaluations Support Planned Clinical Development of AK-OTOF (AAVAnc80-hOTOF Vector) for OTOF-mediated Hearing Loss

AKOUOS

Ann E. Hickox, Yuan Gao, Shimon P. Francis, Yukako Asai, Robert Ng, Hao Chiang, Eva Andres-Mateos, Francesc Puig-Basagoiti, Michael J. McKenna, Brian Lin, Emmanuel J. Simons, Jennifer A. Wellman, Michelle D. Valero

Akouos, Inc., Boston, MA

ASGCT 25th Annual Meeting | May 19, 2022 | Abstract #1233



Demonstration of Secreted Protein Expression Levels Following Intracochlear Delivery of AK-antiVEGF (AAVAnc80-antiVEGF Vector) Across Multiple Doses in Non-human Primates

AKOUOS

Michelle D. Valero[1], John Connelly[1], Francesc Puig-Basagoiti[1], Brian Lin[1], Ann E. Hickox[1], Timothy Boyd[1], Ignacio Navas Enamorado[1], Shimon Francis[1], Ivy K. Hughes[1], Christopher A. Shera[2], Eric D. Horowitz[1], Kathleen Lennon[1], Jean Phillips[1], Jenna Soper[1], Jennifer A. Wellman[1], Gregory S. Robinson[1], Emmanuel J. Simons[1], William F. Sewell[3], Michael J. McKenna[1], Eva Andres-Mateos[1]

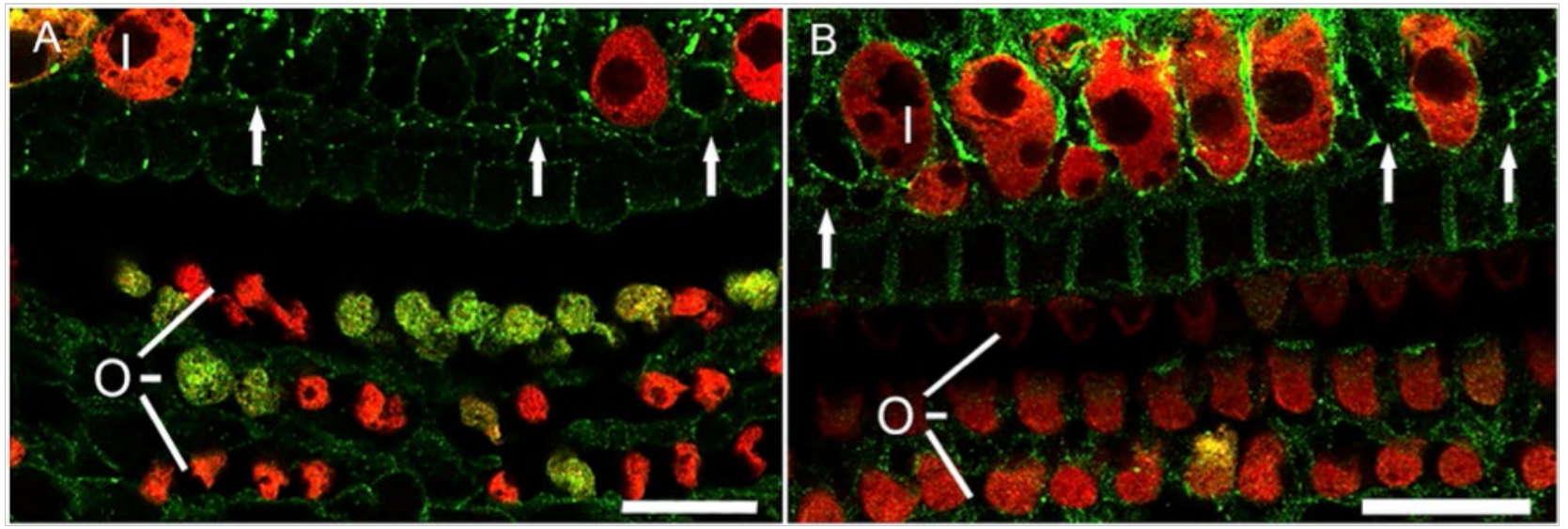
[1]Akouos Inc., Boston, MA
[2]Consultant to Akouos, Inc.
[3]Co-founder and consultant to Akouos, Inc.

Association for Research in Otolaryngology | February 5 to 9, 2022

Abbreviations: AAV = adeno-associated viral; FDA = U.S. Food and Drug Administration.

Expression of Some Transgenes Using a Ubiquitous Promoter May Not Be Well Tolerated

- The *GJB2* gene encodes the connexin 26 (Cx26) protein
- Cx26 is endogenously expressed in supporting cells (SCs) of the inner ear
- Ubiquitous promoter-driven expression of Cx26 in inner hair cells results in cell loss
- A tailored expression pattern may be warranted for this transgene



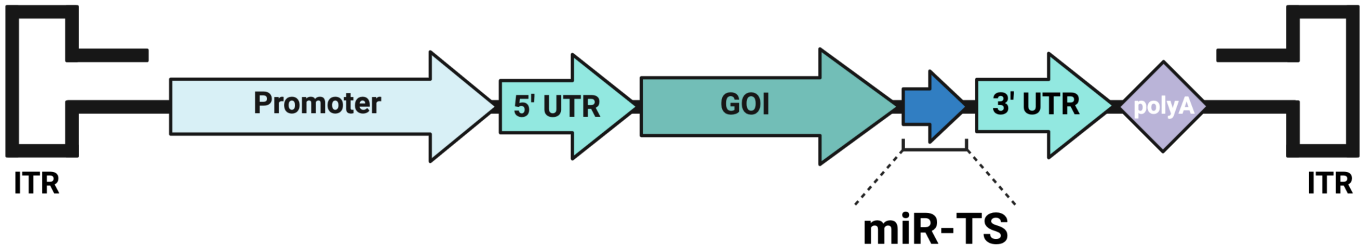
Cx26 Myo7a

Guo J, et al. *Mol Ther Methods Clin Dev.* 2021;23:319-333.

Abbreviations: GJB2 = Gap-junction beta-2

Gene Therapy Regulation

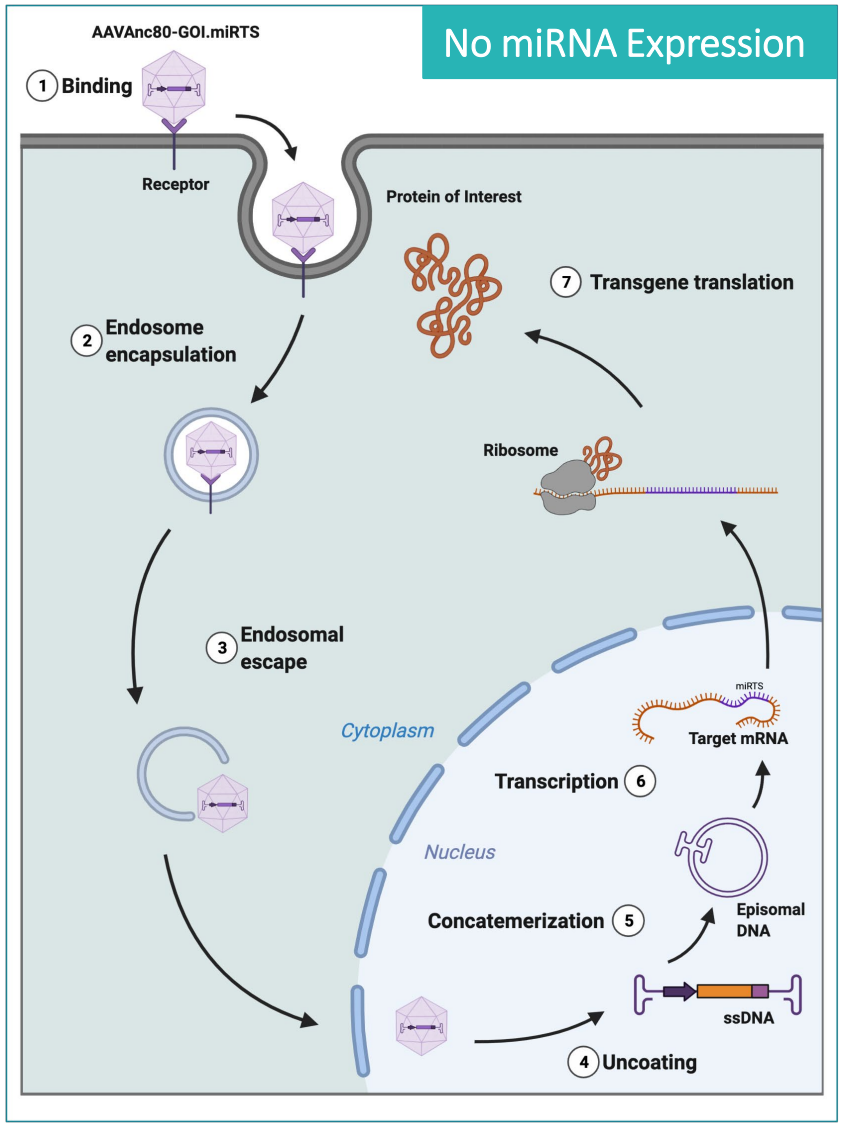
- Customization of regulatory elements induces strong expression in target cells and minimizes expression in nontarget cells



- Multiple regulatory elements can be incorporated to support selective transgene expression:
  - Gene of Interest – Endogenous cell regulation machinery drives reduced expression in nontarget cells to support tolerability
  - Promoter – Drives selective expression in target cells and/or avoid expression in required nontarget cells
  - 5'UTR and 3'UTR - Support endogenous cell regulation machinery to enhance expression in target cells and reduce expression in nontarget cells
  - Enhancer - Enhance transgene expression, primarily in combination with a weaker promoter
  - MicroRNA-target sites (miR-TS) – Endogenous miRNA expression can downregulate transgene expression in a subset of cell types, primarily in combination with a ubiquitous promoter

Abbreviations: GOI = gene of interest; ITR = Inverted terminal repeat; miRNA = microRNA; miR-TS = microRNA target-site ; UTR = untranslated region

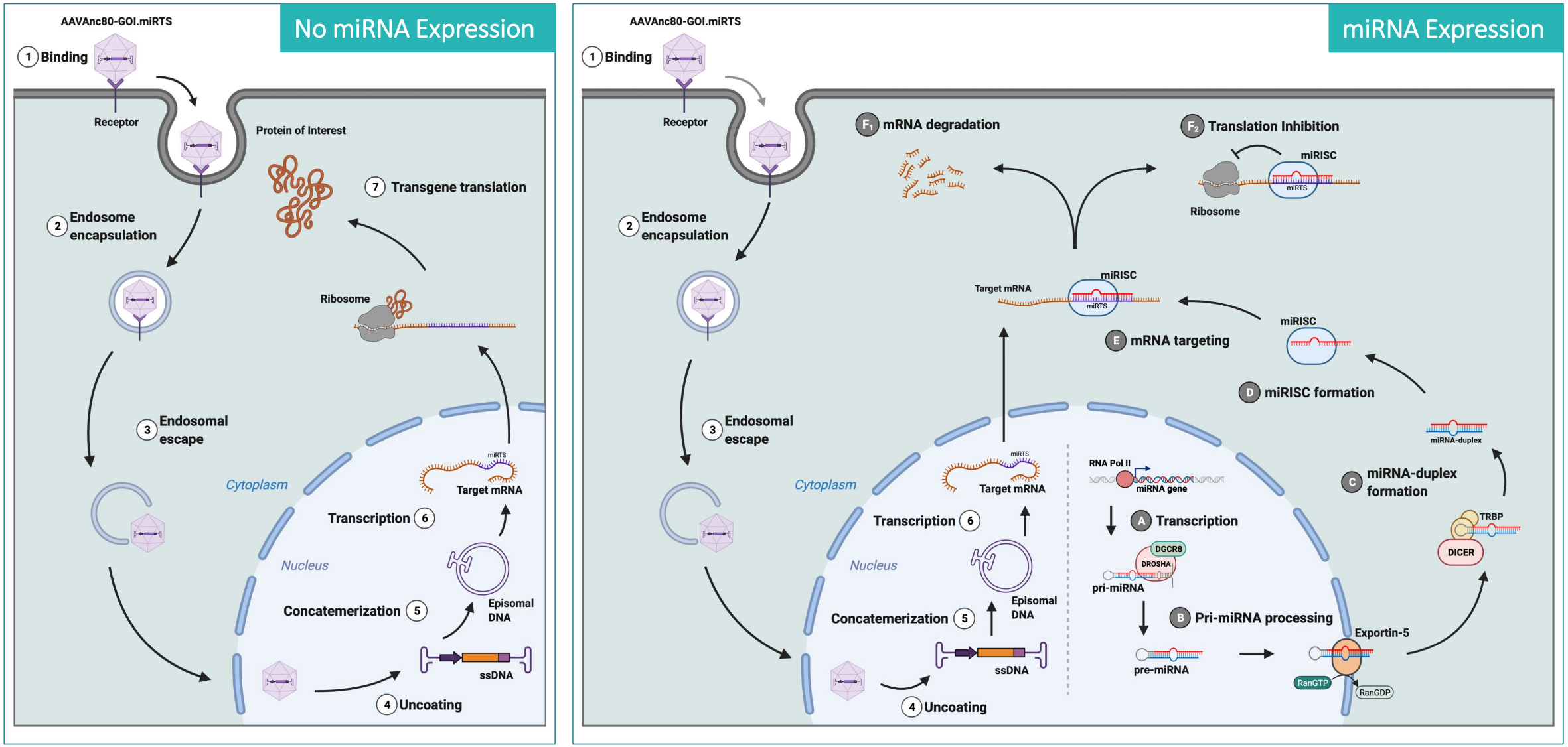
Regulation of Transgene Expression using miRNA-Target Site



Abbreviations: GOI = miRNA = microRNA; miR-TS = microRNA target-site

Created with BioRender.com

Regulation of Transgene Expression using miRNA-Target Site



Abbreviations: GOI = miRNA = microRNA; miR-TS = microRNA target-site

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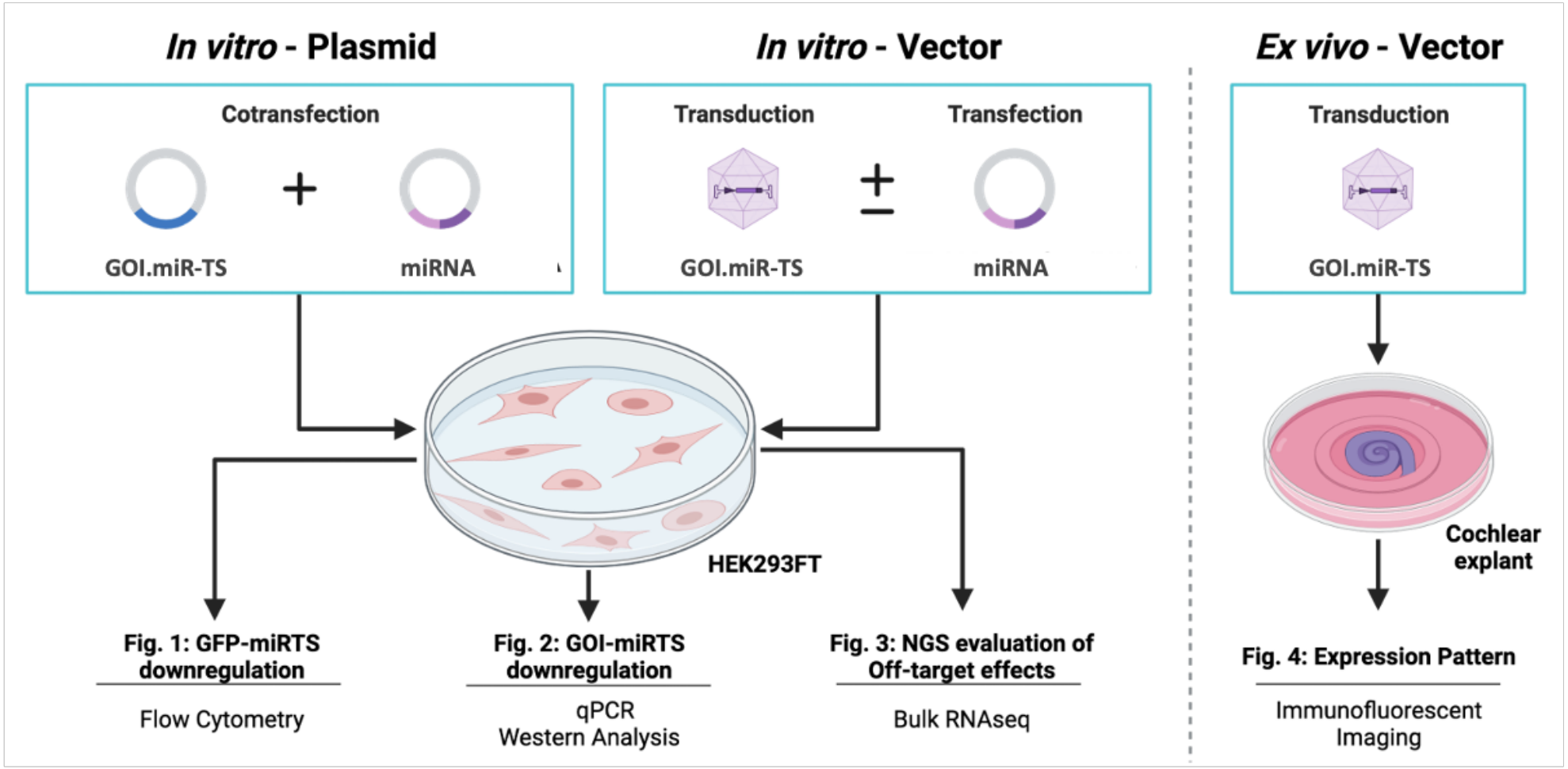
Study Design

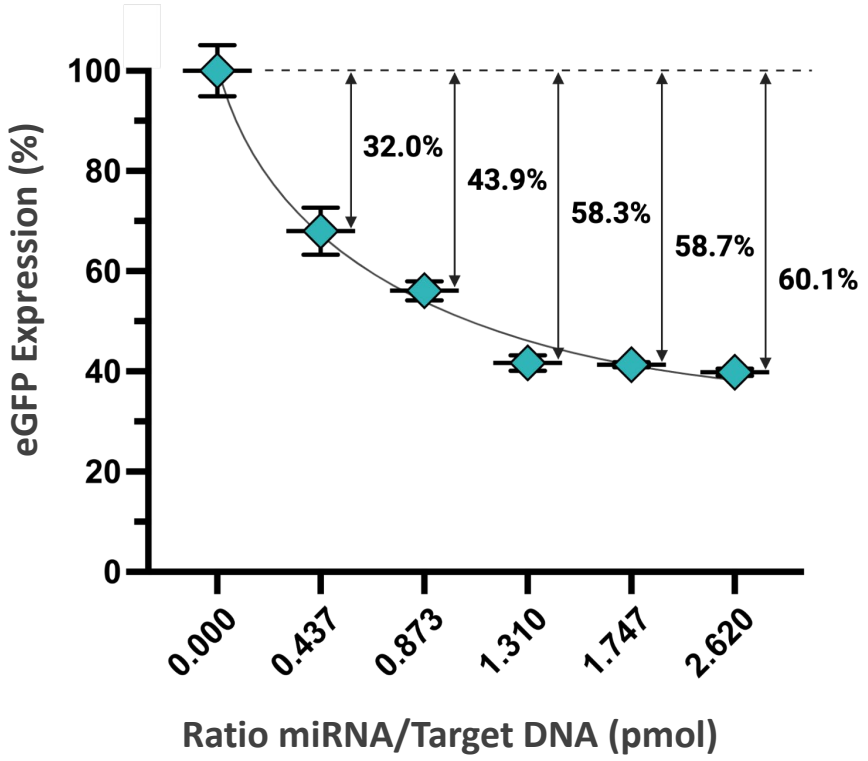


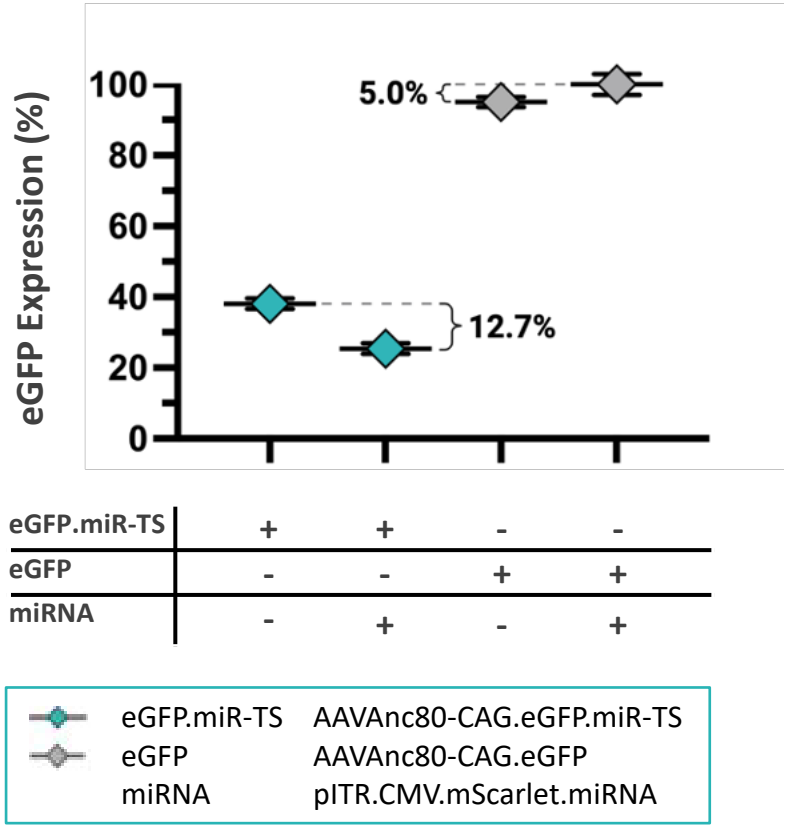
GOI.miR-TS	Plasmid/Vector with CAG.GOI.miR-TS
GOI	Plasmid/Vector with CAG.GOI
miRNA	pITR.CMV.mScarlet.miRNA

Abbreviations: GOI = Gene of interest; eGFP = Enhanced green fluorescent protein; miRNA = microRNA; miR-TS = microRNA target-site; qPCR = Quantitative real-time PCR

Addition of miR-TS to the eGFP Reporter Transgene can Decrease Protein Expression in HEK Cells

Evaluation of miR-TS-mediated downregulation of eGFP plasmid and vector in HEK using flow cytometry

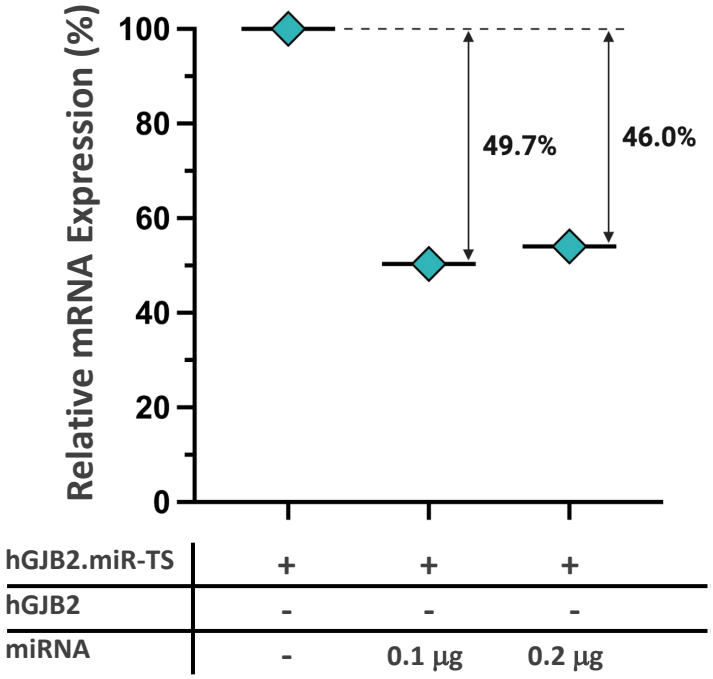




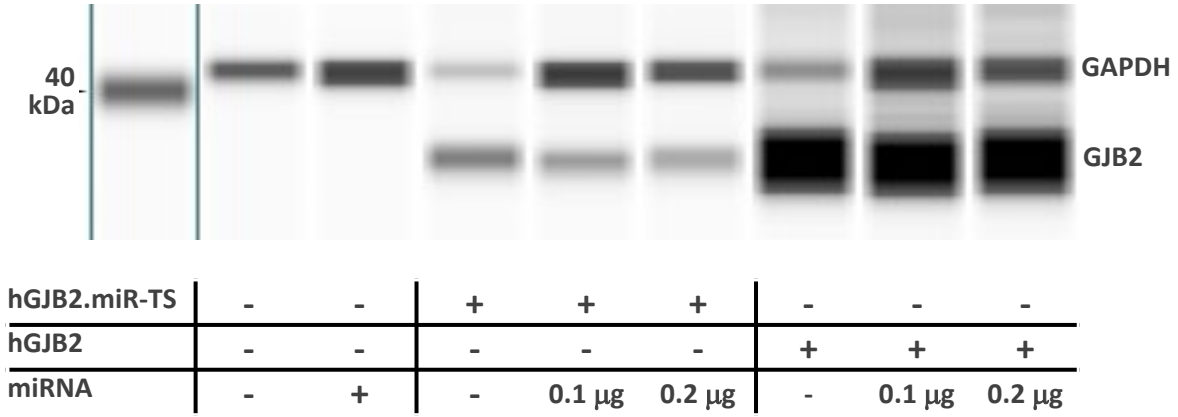
Abbreviations: eGFP = Enhanced green fluorescent protein; miRNA = microRNA; miR-TS = microRNA target-site

Addition of miR-TS to the hGJB2 Transgene can Decrease RNA and Protein Expression in HEK Cells

Evaluation of miR-TS-mediated downregulation of hGJB2 in HEK293FT using qPCR and Wes™



mRNA downregulation was evaluated using qPCR

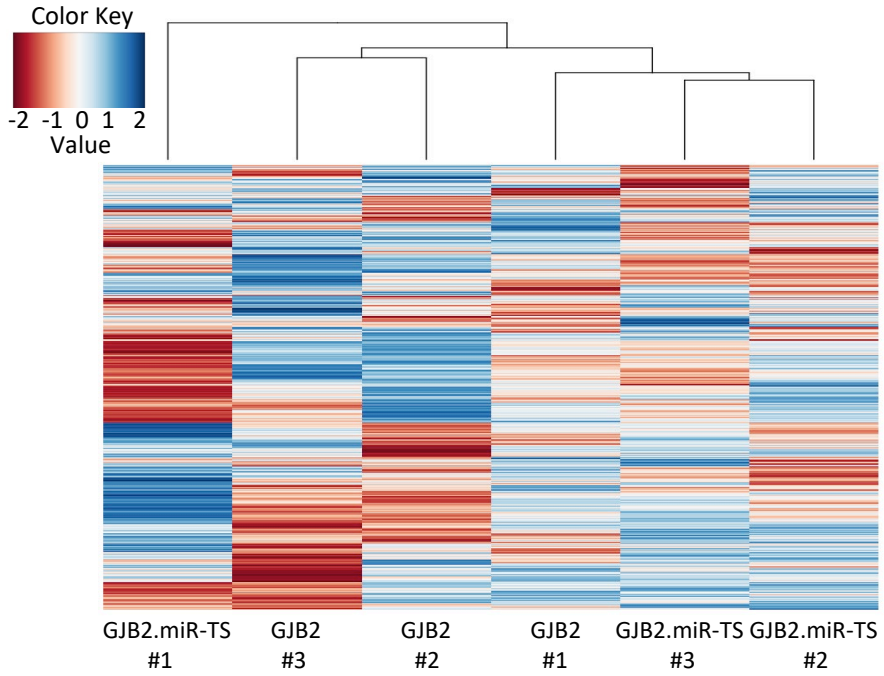


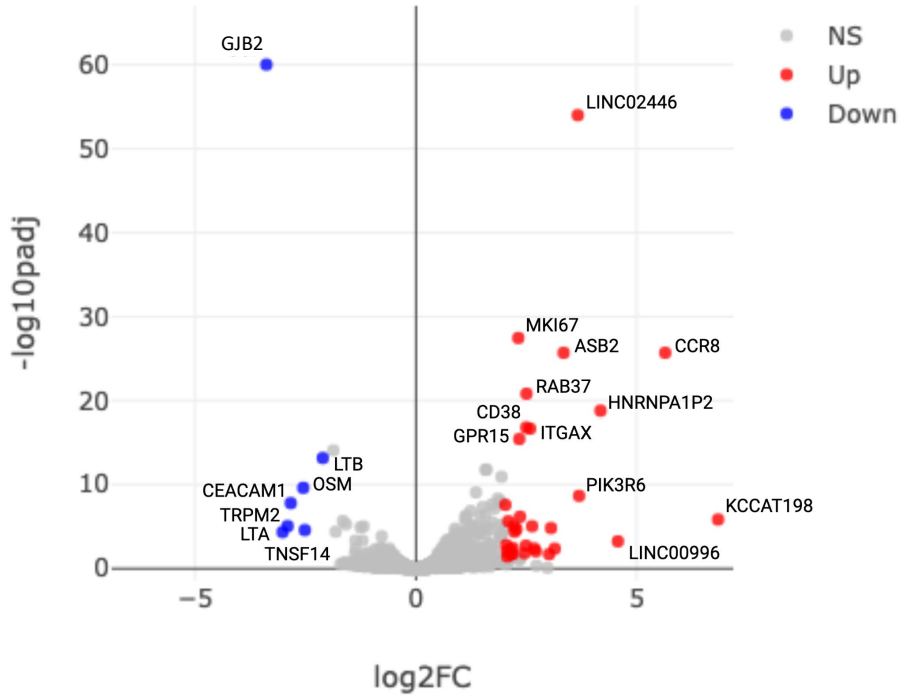
Protein downregulation was evaluated using Wes™ protein assay

Abbreviations: GJB2 = Gap-junction beta-2; miRNA = microRNA; miR-TS = microRNA target-site; qPCR = Quantitative real-time PCR

Expression of hGJB2 with miR-TS Results in Minimal Off-Target Effects *In Vitro*

RNA-sequencing analysis was conducted to evaluate off-target effects after transduction of AAVAnc80-hGJB2 compared to AAVAnc80-hGJB2 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).

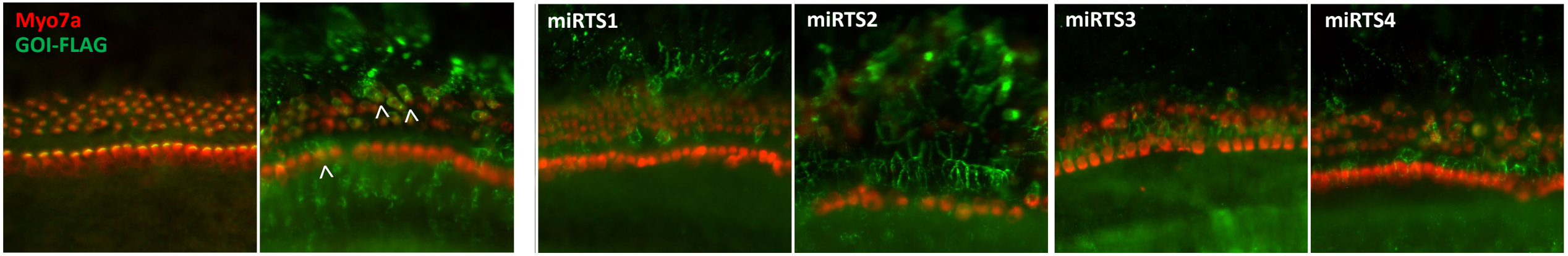




Abbreviations: GJB2 = Gap-junction beta-2; miR-TS = microRNA target-site; Log2FC = Log2 fold-change; -log10padj = -Log10 adjusted p-value

AAVAnc80 with miR-TS can Drive Various Transgene Expression Patterns in Cochlear Explants

- Explant screening for miR-TS enabled prioritization prior to in vivo studies
- Explants were generated from P2 neonate mice and incubated for 5 days with vector at 1E10 vector genomes (vg)/explant



Strong ubiquitous promoter without any miR-TS

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

Strong ubiquitous promoter with various miR-TS sequences

- FLAG expression in medial and lateral SCs
- No apparent hair cell expression except in miRTS4

Abbreviations: GJB2 = Gap-junction beta-2; IHC = inner hair cells; miR-TS = microRNA target-site; OHC = outer hair cells; SCs = supporting cells; vg = vector genome
Data Cutoff: 3Nov2021

Summary and Conclusions

1. Ubiquitous promoters drive strong widespread expression in the inner ear in mice and NHP.
2. 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.
3. Akouos identified multiple microRNA-target sites (miR-TS) to drive various differential expression patterns.
4. miR-TS evaluation in vitro confirmed successful downregulation of eGFP and GJB2 and minimal off-target effects.
5. Several miR-TS were evaluated in cochlear explants, demonstrating differential expression in various cell types.
6. A combination of AAVAnc80 and miR-TS can drive expression in supporting cells, while limiting expression in hair cells in cochlear explants.
7. Future work will focus on evaluating miR-TS regulation in vivo and identifying combination of different miR-TSs to enhance de-targeting in specific cell types, where expression driven by ubiquitous promoters is not well tolerated.

Thank you!