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REPORT DATE:

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INTRODUCTION:

One of the most common causes of permanent hearing loss – including hearing loss incurred in combat - is the loss of hair cells in the cochlea that are responsible for detecting sound. One of the first genes to be switched on in newly formed hair cells is the transcription factor *Atoh1*. Genetic experiments in mice have shown that *Atoh1* is absolutely necessary for hair cells to develop, and that ectopic expression of *Atoh1* in young mice can cause other cochlear cell types to differentiate into hair cells. However, in the past 5 years, several groups have shown that the efficiency of regeneration evoked by *Atoh1* declines rapidly with age. *At present we do not know why Atoh1 gene therapy becomes less effective with age.* However, recently published data suggests that two other transcription factors expressed in hair cells – *Gfi1* and *Pou4f3* – can cooperate with *Atoh1* and improve its ability to activate hair cell genes in cell lines. Finally, no technologies currently exist to stimulate supporting cell proliferation in the mammalian organ of Corti, despite this being a prerequisite for effective regeneration in other vertebrates. Based on the above published data, we hypothesize that combination gene therapy with three transcription factors: *Atoh1*, *Pou4f3* and *Gfi1* will be significantly better at reprogramming supporting cells into hair cells than *Atoh1* alone. We have engineered a line of transgenic mice that can activate expression of *Atoh1*, *Gfi1* and *Pou4f3*. We have also developed transgenic mice that can drive this activation in different cell populations of the cochlea. We will use these tools to compare the ability of these three factors to convert supporting cells into hair cells after killing of hair cells. We will perform our experiments in transgenic “DTR” mice where we can specifically kill hair cells after the administration of diphtheria toxin. We will validate our experiments by examining the presence of known antigenic markers of hair cells in the cochlea and by performing hearing tests to look for a recovery in auditory function. We will compare the efficacy of this approach in mice that have been acutely deafened versus mice that have been chronically deafened, as it is possible that there exists only a limited therapeutic window after hair cell death for reprogramming to succeed. Although our transgenic mouse system is robust and reproducible, one disadvantage is that once activated, we cannot turn off the expression of the three reprogramming transcription factors. To address this problem, and to move our work in a more translational direction, we will also generate and purify cell-penetrating versions of the three transcription factors by fusing them to known cell-penetrating peptides. We will repeat our experiments in acutely and chronically deafened animals but infuse the cell-penetrating transcription factors acutely to try to regenerate hair cells.

KEYWORDS:

Cochlea	Organ of Corti	Hearing	Hair cells	Supporting Cells
Reprogramming	Transcription Factors	Mouse Models	<i>Atoh1</i>	<i>Pou4f3</i>
<i>Gfi1</i>	Deafness			

ACCOMPLISHMENTS:

What were the major goals of the project?

Task / Goals		Timeline	Completed?
Specific Aim 1A: Can <i>Atoh1</i>, <i>Pou4f3</i> and <i>Gfi1</i> promote hair cell regeneration in the acutely deafened cochlea?			
Task 1	Await IACUC and ACURO animal approval	Year 1, Months 1-3	Yes
Task 2	Breed cohorts of experimental animals: Goal is to generate 28 mice having the genotype <i>Sox9-CreER</i> ; <i>ROSA-GAP</i> ; <i>Pou4f3^{DTR}</i> and 28 control mice having the genotype <i>ROSA-GAP</i> ; <i>Pou4f3^{DTR}</i>	Year 1, Months 4-12	Yes
Task 3:	Expt 1A: Activation of reprogramming factors in 6-week-old animals deafened one week previously; analyze at 9 weeks. The 56 animals described in Aim 1A, Task 2 will be used.	Year 1, Months 9-12 to Year 2,	Yes
Specific Aim 1B: Can <i>Atoh1</i>, <i>Pou4f3</i> and <i>Gfi1</i> promote hair cell regeneration in the chronically deafened cochlea?			
Task 1	Await IACUC and ACURO animal approval	Year 1, Months 1-3	Yes

Task 2	Breed cohorts of experimental animals: Goal is to generate 28 mice having the genotype Sox9-CreER; ROSA-GAP; Pou4f3 ^{DTR} and 28 control mice having the genotype ROSA-GAP; Pou4f3 ^{DTR}	Year 1, Months 4-12	100%
Task 3	Expt 1B: Activation of reprogramming factors in adult animals deafened at birth; activate factors at 6 weeks; analyze at 9 weeks. The 56 animals described in Aim 1B, Task 2 will be used.	Year 2, Months 3-12	Yes
Specific Aim 2A: Can Atoh1, Pou4f3 and Gfi1 proteins promote hair cell regeneration in the acutely deafened cochlea?			
Task 1	Await IACUC and ACURO animal approval	Year 1,	Yes
Task 2	Clone expression constructs for cell-penetrating versions of Atoh1, Pou4f3, Gfi1 and EGFP	Year 1, Months 1-3	Yes
Task 3:	Purify cell-penetrating versions of Atoh1, Pou4f3, Gfi1 and EGFP	Year 1,	50%
Task 4:	Preliminary test of surgical approach and protein concentration: Infuse CPP-EGFP into lateral semicircular canal of 30 neonatal mice, testing 3 different protein concentrations (10 mice each)	Year 1, Months 6-12	No
Task 5:	Preliminary Expt: Breed cohort of experimental animals: Goal is to generate 80 mice having the genotype Sox9-CreER; ROSA-TdTomato	Year 1, Months 6-12	100%
Task 6:	Preliminary Expt: Comparison of reprogramming proteins infused into neonatal animals with regeneration obtained with ROSA-GAP; Sox9-CreER mice. 24 Sox9-CreER; ROSA-TdTomato mice will be used.	Year 1, Months 6-12 to Year 2,	No
Task 7:	Expt 2A: Infusion of reprogramming proteins in 6-week-old animals deafened one week previously; analyze at 9 weeks. 56 animals described in Aim 2A, Task 5 will be used (28 experimental, 28 control).	Year 2, Months 4-12	No
Specific Aim 2B: Can Atoh1, Pou4f3 and Gfi1 proteins promote hair cell regeneration in the chronically deafened cochlea?			
Task 1	Await IACUC and ACURO animal approval	Year 1,	Yes
Task 2	Preliminary Expt: Breed cohort of experimental animals: Goal is to generate 56 mice having the genotype Sox9-CreER; ROSA-TdTomato	Year 1, Months 6-12	50%
Task 3	Expt 2B: Infusion of reprogramming proteins in adult animals deafened at birth; activate factors at 6 weeks; analyze at 9 weeks. 56 animals described in Aim 2B, Task 2 will be used (28 experimental, 28 control).	Year 2, Months 4-12	0%

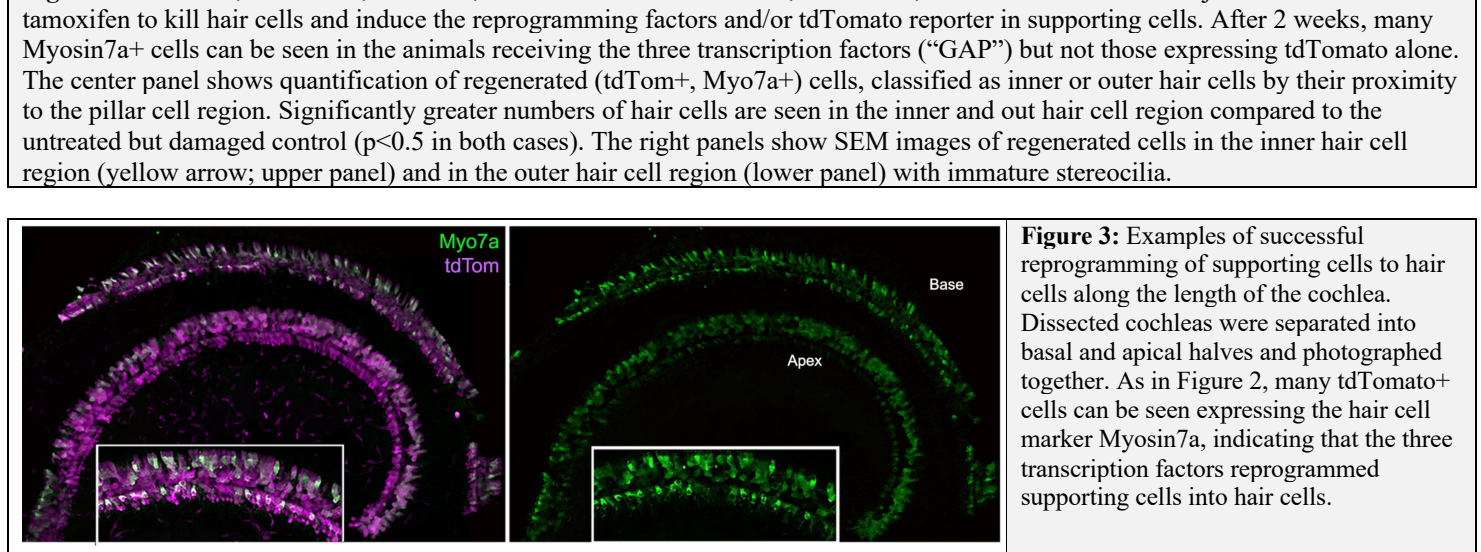
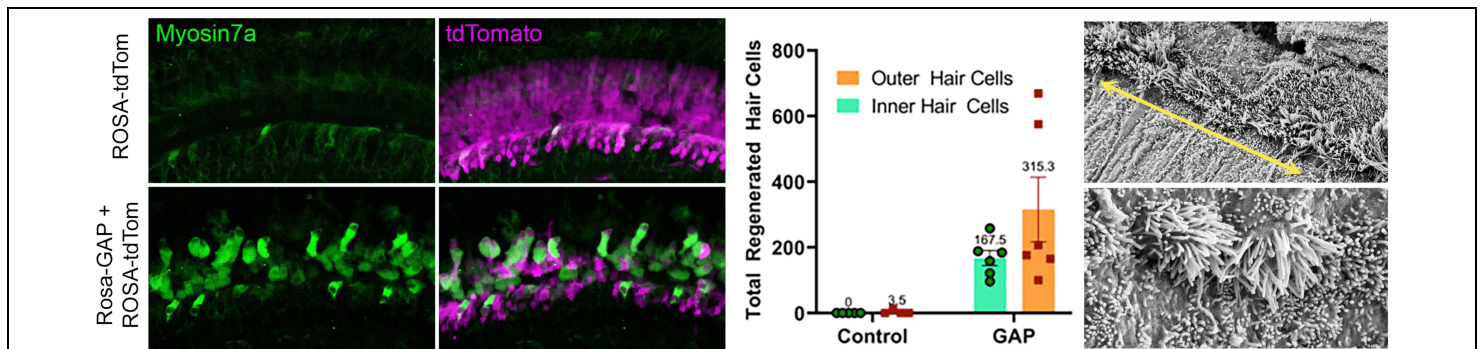
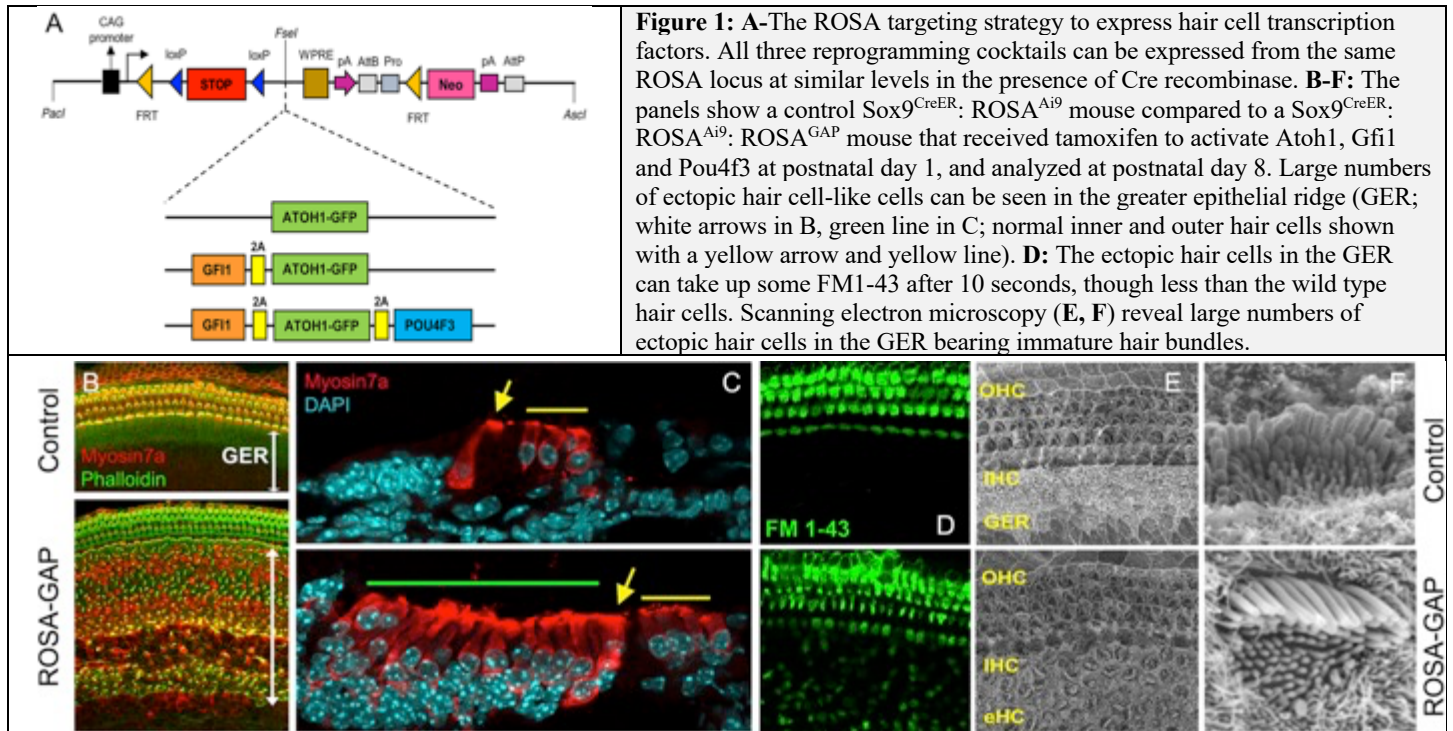
What was accomplished under these goals?

As described in previous reports, we developed several lines of knock-in mice in which different combinations of hair cell transcription factors are targeted to the ROSA locus in a Cre-dependent fashion (Figure 1). Activation of these hair cell transcription factors in the neonatal cochlea induces large numbers of hair cell-like cells in the greater epithelial ridge. These can be labeled with a brief exposure to FM1-43, suggesting they possess rudimentary mechanotransduction abilities. Scanning electron microscopy to show these cells bear immature stereocilia similar to the endogenous hair cells at the time of analysis (8 days).

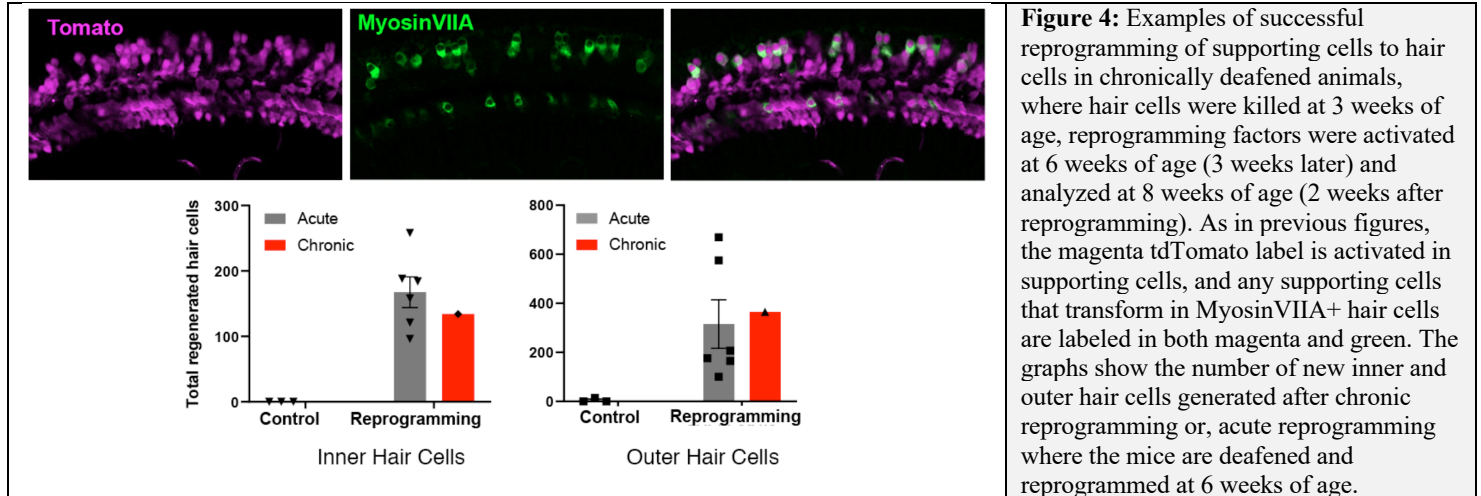
Specific Aim 1:

The goal of Aim 1 was to determine the effectiveness of transcription factor reprogramming in the acutely and chronically deafened cochlea. We combined the Cre-inducible reprogramming system shown in Figure 1 with Pou4f3^{DTR} mice that express a diphtheria toxin receptor in hair cells. This allows specific and efficient killing of hair cells after injection of diphtheria toxin. After hair cell killing in six-week-old animals, we activated Atoh1, Gfi1 and Pou4f3 in the adjacent supporting cells using the Sox9^{CreER} mouse line previously published by our lab and permanently labeled the supporting cells using a Cre-inducible ROSA^{Ai9} fluorescent reporter allele to verify the new hair cells were derived from supporting cells (Figure 2). Activation of Atoh1, Gfi1 and Pou4f3 gave significantly larger numbers of new hair cells compared to untreated controls (Figure 2). This successful reprogramming extends all the way along the cochlea,

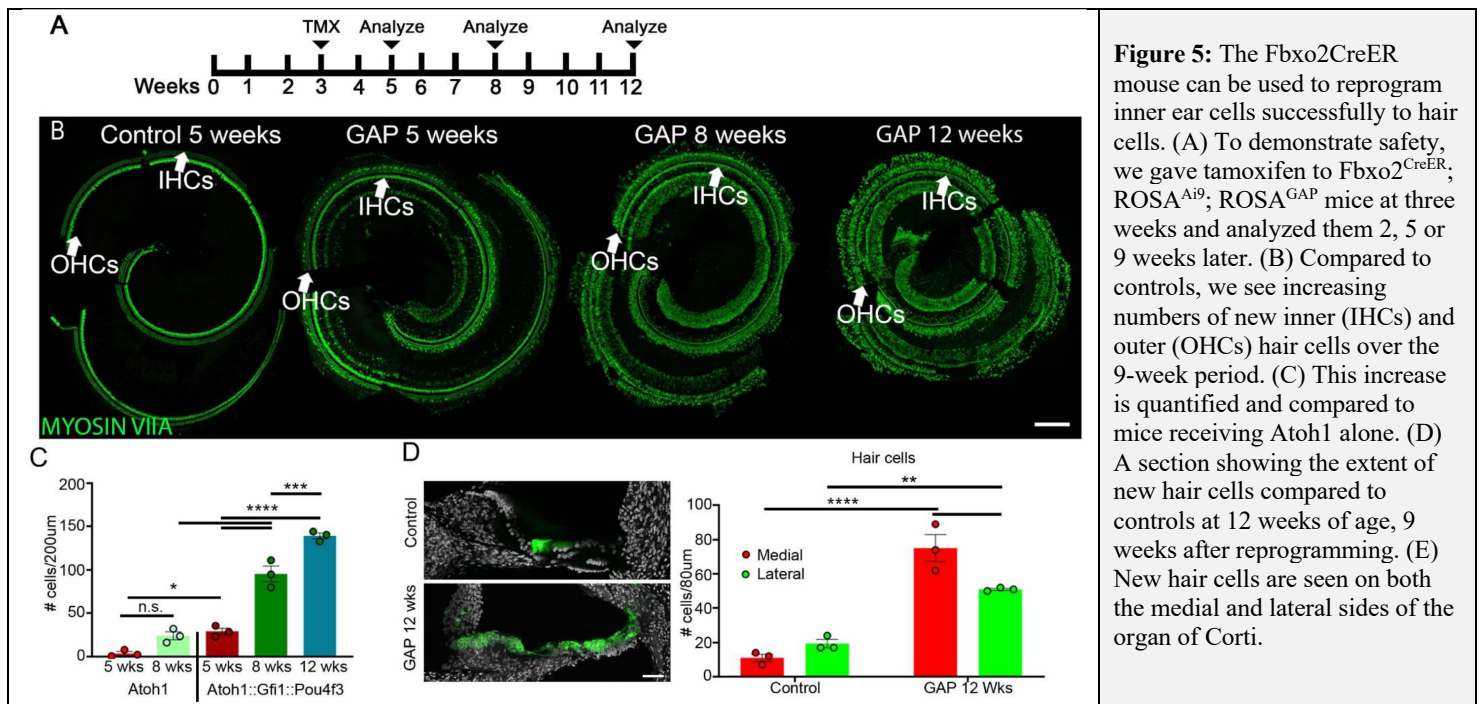
stretching from base (high frequencies) to apex (low frequencies; Figure 3). Again, we were able to confirm that these new hair cells were produced by our reprogramming factors as they expressed the hair cell marker Myosin 7a, together with the tdTomato lineage label, indicating they were derived from supporting cells). In control groups, we saw zero hair cells labeled with tdTomato, **indicating our reprogramming results are highly significant**. The data for these experiments have now all been collected, and the work is being prepared for publication.



In the second part of specific Aim 1, we proposed to repeat these experiments in *chronically* deafened animals. Our original proposed plan was to deafen the animals at birth using Pou4f3^{DTR} mice, let them age for six weeks and then activate the reprogramming factors. In the previous 12 months, we found that deafening at birth gave us very variable amounts of hair cell loss when the animals were analyzed at 6 weeks of age. Such variable hair cell loss is not a suitable basis for reproducible experiments, and so we began to experiment with deafening at later ages. We have now found that administering diphtheria toxin to Pou4f3^{DTR} mice at three weeks of age gives us a consistent lesion and consistent hair cell loss. Our data (Figure 4) suggests that transcription factor reprogramming three weeks after deafening mice can generate new hair cells.

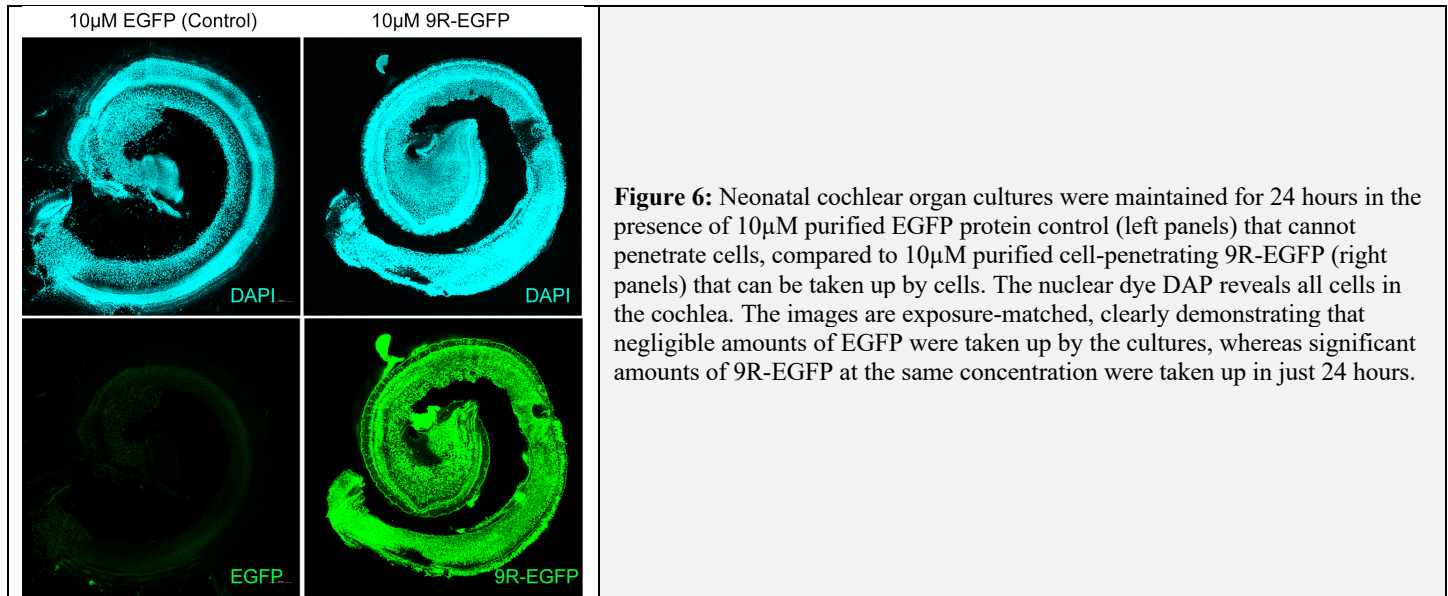


In the past 12 months, we encountered a technical problem with the Cre lines we had been using to activate our reprogramming factors. We found that these lines also drove Cre expression in the brain - this meant that our reprogramming factors would also be expressed in the brain. We found that mice could tolerate this mis-expression for 2-3 weeks, but after this time they began to develop seizures and died. We therefore decided to change the CreER mouse line in our studies to Fbxo2^{CreER} mice recently published by our lab. Our data suggested that we can indeed activate reprogramming factors in the ear for extended periods of time, and indeed that extended periods of reprogramming generate increasing numbers of new hair cells over time (Figure 5):

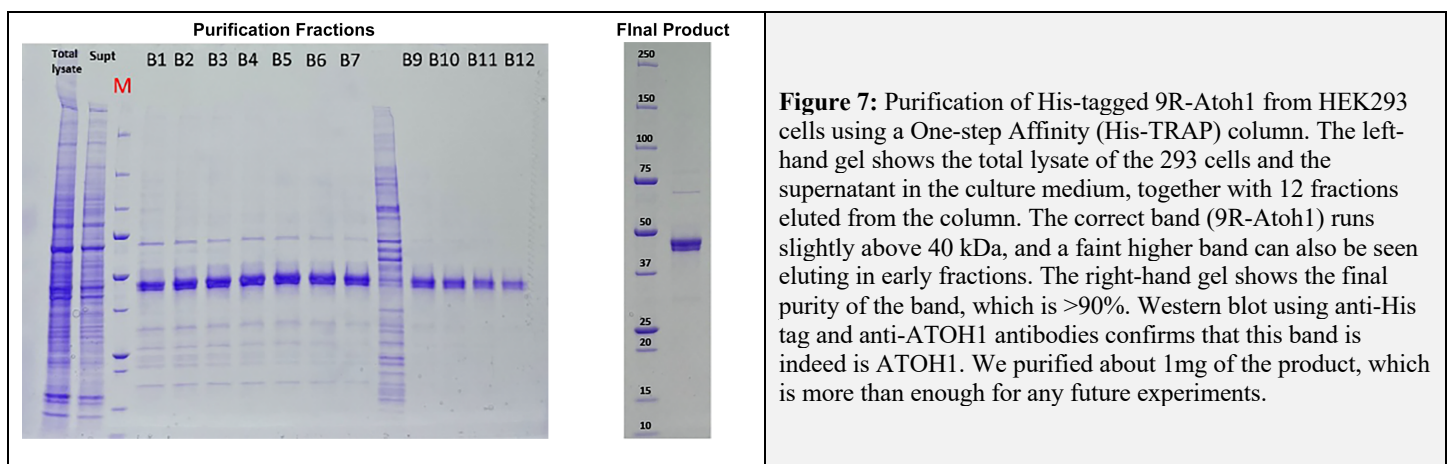


Specific Aim 2:

For Specific Aim 2, we cloned versions of Atoh1, Pou4f3 and Gfi1 fused to a cell-penetrating peptide sequence consisting of 9 arginine residues (9R). As we began to generate the cell-penetrating peptides, we became aware that the 9R peptide was causing problems with bacterial expression. We therefore had to re-design our peptide sequence to be codon-optimized for expression in bacteria rather than mammalian cells. We have been able to produce large amounts of 9R-EGFP as a control demonstrate the feasibility of the cell-penetrating peptide approach. Figure 6 shows this peptide can promote uptake of EGFP by cochlear explants within 24 hours.



We then attempted to express and purify the three transcription factors but encountered problems with aggregation of the protein during expression and purification from bacteria. We therefore switched our expression system from bacteria to mammalian HEK293 cells. After attempting different expression vectors and purification approaches, we were finally able to purify significant amounts (>1mg) of Atoh1 protein, although we did observe some evidence of dimerization (Figure 7).



Our goal in future is to continue with purification of the other transcription factors using the knowledge from our troubleshooting to produce the other transcription factors.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

N/A.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

In 1988, it was shown for the first time that some vertebrates can regenerate their auditory hair cells after deafening, and in subsequent years it was shown that this regeneration led to an impressive restoration of hearing. This regeneration occurs by supporting cells in the hearing organ dividing and differentiating into new hair cells. However, such regeneration does NOT occur in the mammalian hearing organ, the organ of Corti. The past 30 years have seen many attempts to drive mammalian supporting cells to becoming hair cells. The work presented here is some of the first to show efficient production of new hair cells in a deafened mouse. Although this is extremely encouraging, many questions remain unanswered. First, we do not know for how long the new hair cells survive in our reprogrammed mice. Second, we do not know how closely they resemble normal hair cells – do they contain all the cellular and physiological features of hair cells, or are they a “hybrid” cell type somewhere between a hair cell and a supporting cell? Third, are there negative consequences of depleting the existing supporting cell reservoir by driving them to a hair cell fate – in other words, will we ultimately need to create new hair cells AND replace the reprogrammed supporting cells too?

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

None.

Actual or anticipated problems or delays and actions or plans to resolve them

The COVID pandemic has had a significant impact on the progress of this project.

1. In March 2020, our university instructed all investigators to reduce their mouse colonies by 60-70% to preserve PPE and reduce the need for staff in our animal facilities. We were not able to expand our mouse colonies until Fall 2020. Moreover, our university suspended all animal surgeries for much of 2020, again to reduce the need for animal and veterinary staff. These changes caused enormous delays in all mouse projects in our lab. We had already been building up our mouse stocks in advance of the proposal start date, and the measures described above delayed our progress by about 6 months. Our plan to resolve this delay is simply to request a no-cost extension to extend the proposal beyond its original two years. This has now been approved.

2. The postdoctoral fellow working on this project switched to a second project in the lab unrelated to this proposal. We therefore decided to hire a new team member to carry out the experiments in Aim 2. Once again, COVID restrictions on

hiring implemented by our university, and the difficulty in being able to recruit new personnel once these restrictions were lifted have both delayed work on Aim 2. We have now recruited a graduate student, Juwan Copeland, who is now working full time on the proposal. As above, our plan to resolve this delay has been to request a no-cost extension to extend the proposal beyond its original two years. This extension was approved.

Changes that had a significant impact on expenditures

The delays described above have reduced our expenditures in Year 1. As described above, we requested a no-cost extension to the project at the end of Year 2.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

No significant changes

Significant changes in use of biohazards and/or select agents

Not applicable

PRODUCTS:

Publications, conference papers, and presentations

Melissa McGovern, a postdoctoral fellow who had been working on Aim 1, presented some of her data at the Association for Research in Otolaryngology Midwinter meeting held virtually in February 2022, and at the Molecular Biology of Hearing and Deafness meeting held in Iowa City in May 2022.

Journal publications.

A manuscript describing the experiments in Aim 1A is being prepared for publication.

Books or other non-periodical, one-time publications.

We have submitted one book chapter “Specification and Plasticity of Cochlear Hair Cell Progenitors” for publication in a volume of the Springer Handbook of Auditory Research series, entitled “Hair Cell Regeneration”.

Other publications, conference papers, and presentations.

Nothing to report.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Andrew Groves
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-0784-7998
Nearest person month worked:	1
Contribution to Project:	Oversees project design, execution, data analysis and reporting
Funding Support:	R01 DC017689 (3 person months) R01 DC014832 (2 person months) R01 DC013072 (2 person months) R01 DE032244 (2 person months) R21 DC021041 (1 person month) The Hearing Health Foundation Hearing Restoration Project (1 person month)

Name:	Juwan Copeland
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	7
Contribution to Project:	Carried out experiments in Aim 2
Funding Support:	Current award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Changes to current support in the past 12 months are indicated in red

5R01 DC017689-01 (Groves) 12/1/2018 – 11/30/2023 **3.19 Calendar**
NIH/NIDCD (Groves lab)

Genetic Regulation of Cochlear Development

The Specific Aims of this grant are to understand the role of the Notch and BMP signaling pathways in inducing and patterning the cochlea, and to screen new mice from the KOMP project to identify genes with roles in ear development.

5R01 DE032244 (Groves) 3/1/2023 – 1/1/2028 **2.4 Calendar**
NIH/NIDCR

The role of the Foxi3 transcription factor in craniofacial microsomia

The Specific Aims of this grant are to determine the mechanisms by which pathogenic variants of Foxi3 cause CFM, and whether Foxi3 interacts genetically with the CFM candidate Gata3; to test if Foxi3 is required in ectoderm or endoderm to regulate the development of the pharyngeal arches, and to identify the gene networks regulated by Foxi3 during craniofacial development

5R01 DC013072-06 (Groves): 9/1/2020 – 8/31/2025 **2.40 Calendar**
NIH/NIDCD

Genetic Regulation of Inner, Middle and Outer Ear Development

The Specific Aims of this grant are to determine the role of the Foxi3 transcription factor in the development of the inner ear; to test whether Foxi3 is acting as a pioneer transcription factor in a mouse ES cell model of inner ear differentiation, and to investigate the role of Foxi3 in branchial arch development, with particular focus on the middle ear ossicles.

5R21 DC021041-01(Groves):
NIH/NIDCD

4/1/2023 – 3/31/2025 0.96 Calendar

Optimizing MERFISH to allow multiplexed measurement of developmental and tonotopic gene expression gradients in the cochlea

It has historically been very hard to perform measurements and experiments on the adult cochlea, as it is a structure of extreme mechanical sensitivity and delicacy, contains relatively small numbers of cells, and is encased in the strongest bone in the body. This proposal seeks to overcome these limitations by using a new technique, MERFISH to directly measure the expression of genes in the different cell types of the cochlea. This technique may shed light on how the cochlea is able to respond to different sound frequencies, and how different regions of the cochlea respond to injury and age-related degeneration.

Hearing Restoration Project Consortium Grant (Groves)
Hearing Health Foundation

10/1/2022 – 9/30/2023

0.6 Calendar

Cross-Species Epigenetics Working Group & Reprogramming and Gene Delivery Working Group

The Specific Aims of this grant are to obtain transcriptional and epigenetic data on the responses of mature cochlear supporting cells to hair cell loss

What other organizations were involved as partners?

Nothing to report.