AWARD NUMBER: W81XWH-20-1-0715

TITLE: Gene Therapy Strategies for Hearing Restoration

PRINCIPAL INVESTIGATOR: Andrew K. Groves Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine

REPORT DATE: OCTOBER 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*
OCTOBER 2022

2. REPORT TYPE
Annual

3. DATES COVERED *(From - To)*
09/1/2021 - 08/31/2022

4. TITLE AND SUBTITLE

Gene Therapy Strategies for Hearing Restoration

5a. CONTRACT NUMBER
W81XWH-20-1-0715

5b. GRANT NUMBER
RH190053

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

Andrew K. Groves Ph.D.

E-Mail:
akgroves@bcm.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

BAYLOR COLLEGE OF MEDICINE
1 BAYLOR PLAZA
HOUSTON
TX 77030-3411

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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. 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. 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.
The aims of the project seek to answer the following questions:
1: Can Atoh1, Pou4f3 and Gfi1 reprogram supporting cells into hair cells in the acutely and chronically deafened cochlea?
2: Can infusion of cell-penetrating versions of Atoh1, Pou4f3 and Gfi1 reprogram supporting cells into hair cells in the acutely and chronically deafened cochlea?
In the current reporting period, we have generated and bred cohorts of genetically modified mice to complete the first aim. We showed that activation of Atoh1, Pou4f3 and Gfi1 in acutely and chronically deafened mice can indeed generate significant numbers of new hair cells. We have also optimized protein production methods for Aim 2

15. SUBJECT TERMS

Cochlea, Organ of Corti, Hearing, Deafness, Hair cells, Supporting cells, Reprogramming, Transcription Factors, Mouse Models, Atoh1, Pou4f3, Gfi1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	10	

TABLE OF CONTENTS

Page No.

1. **Introduction**
2. **Keywords**
3. **Accomplishments**
4. **Impact**
5. **Changes/Problems**
6. **Products**
7. **Participants & Other Collaborating Organizations**
8. **Special Reporting Requirements**
9. **Appendices**
10. **References**

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, Atoh1, Pou4f3, Gfi1, Deafness

ACCOMPLISHMENTS:

What were the major goals of the project? Goals for the first 12 months from the SOW are shown below.

	Task / Goals	Timeline	Completed?
Specific Aim 1A: Can Atoh1, Pou4f3 and Gfi1 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 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 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,	50%
Specific Aim 1B: Can Atoh1, Pou4f3 and Gfi1 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	50%
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	50%
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	10%
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 reported last year, 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.

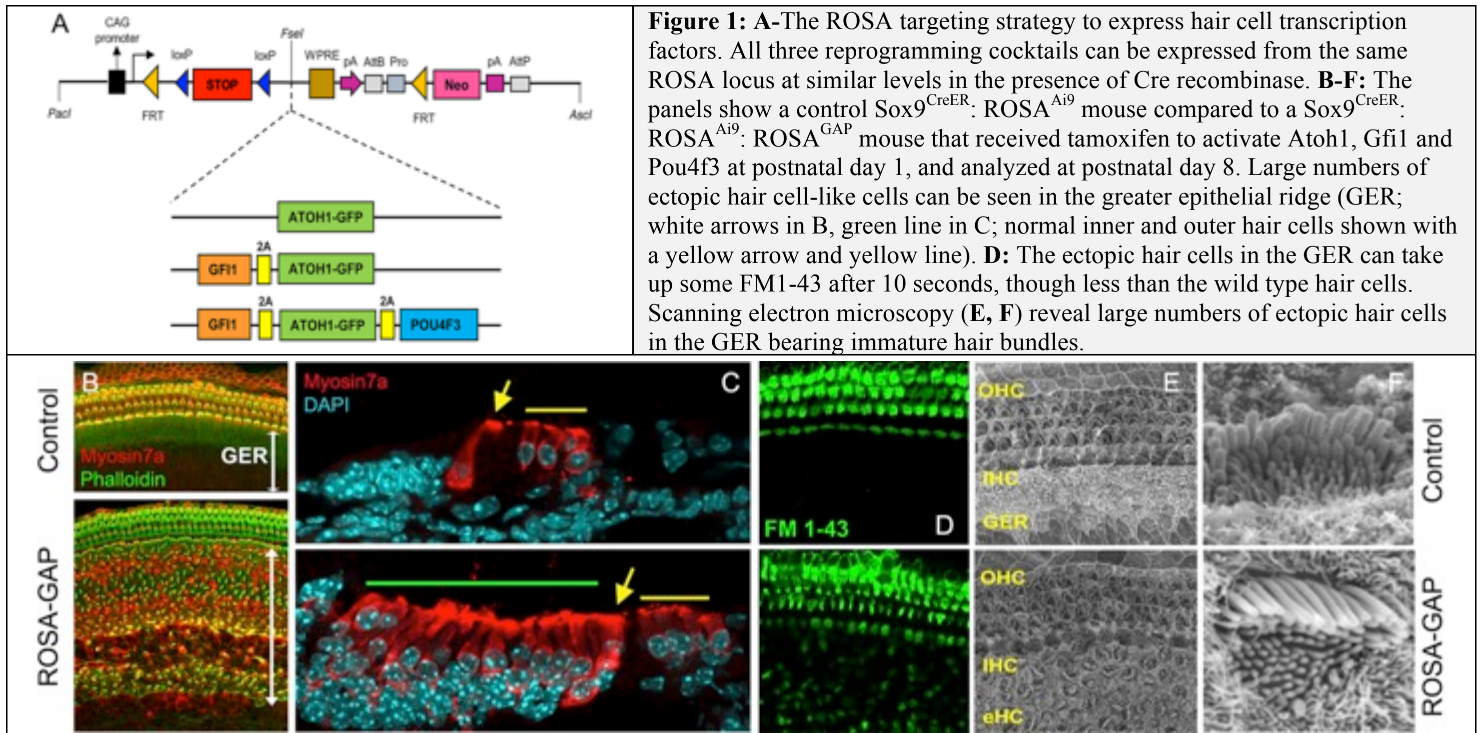

Figure 1: **A**-The ROSA targeting strategy to express hair cell transcription factors. All three reprogramming cocktails can be expressed from the same ROSA locus at similar levels in the presence of Cre recombinase. **B-F:** The panels show a control $Sox9^{CreER}$: $ROSA^{Ai9}$ mouse compared to a $Sox9^{CreER}$: $ROSA^{Ai9}$: $ROSA^{GAP}$ mouse that received tamoxifen to activate Atoh1, Gfi1 and Pou4f3 at postnatal day 1, and analyzed at postnatal day 8. Large numbers of ectopic hair cell-like cells can be seen in the greater epithelial ridge (GER; white arrows in B, green line in C; normal inner and outer hair cells shown with a yellow arrow and yellow line). **D:** The ectopic hair cells in the GER can take up some FM1-43 after 10 seconds, though less than the wild type hair cells. Scanning electron microscopy (**E, F**) reveal large numbers of ectopic hair cells in the GER bearing immature hair bundles.

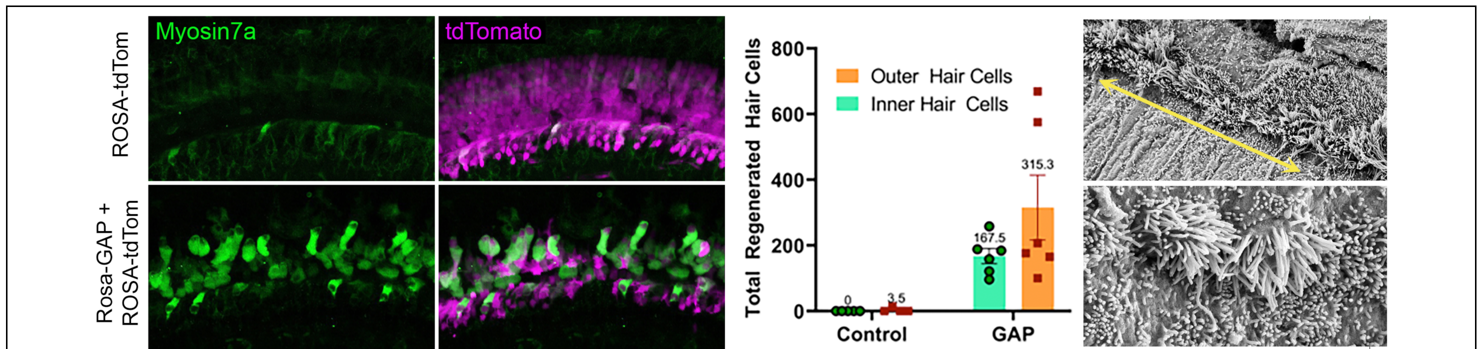

Figure 2: $Sox9^{CreER}$; $Pou4f3^{DTR}$; $ROSA^{Ai9}$; $ROSA^{GAP}$ mice or $Sox9^{CreER}$; $Pou4f3^{DTR}$; $ROSA^{Ai9}$ controls were injected with DT and tamoxifen to kill hair cells and induce the reprogramming factors and/or tdTomato reporter in supporting cells. After 2 weeks, many Myosin7a+ cells can be seen in the animals receiving the three transcription factors ("GAP") but not those expressing tdTomato alone. The center panel shows quantification of regenerated (tdTom+, Myo7a+) cells, classified as inner or outer hair cells by their proximity to the pillar cell region. Significantly greater numbers of hair cells are seen in the inner and out hair cell region compared to the untreated but damaged control ($p<0.5$ in both cases). The right panels show SEM images of regenerated cells in the inner hair cell region (yellow arrow; upper panel) and in the outer hair cell region (lower panel) with immature stereocilia.

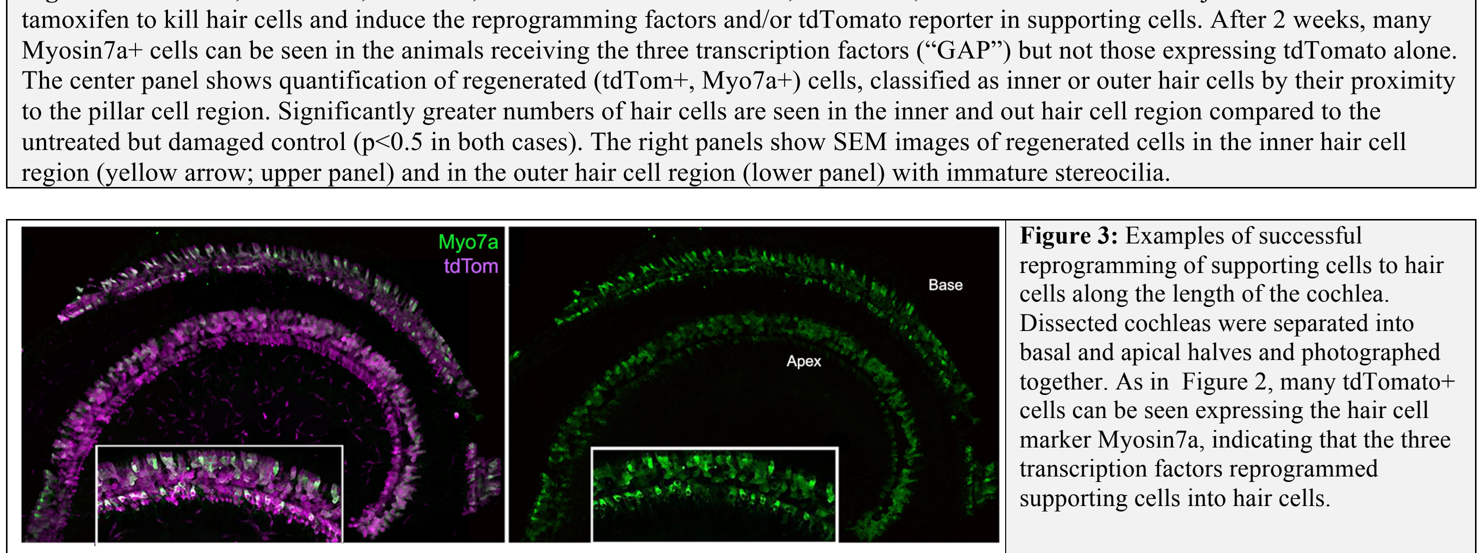

Figure 3: Examples of successful reprogramming of supporting cells to hair cells along the length of the cochlea. Dissected cochleas were separated into basal and apical halves and photographed together. As in Figure 2, many tdTomato+ cells can be seen expressing the hair cell marker Myosin7a, indicating that the three transcription factors reprogrammed supporting cells into hair cells.

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 last 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 preliminary data (Figure 4) suggests that transcription factor reprogramming three weeks after deafening mice is able to generate new hair cells.

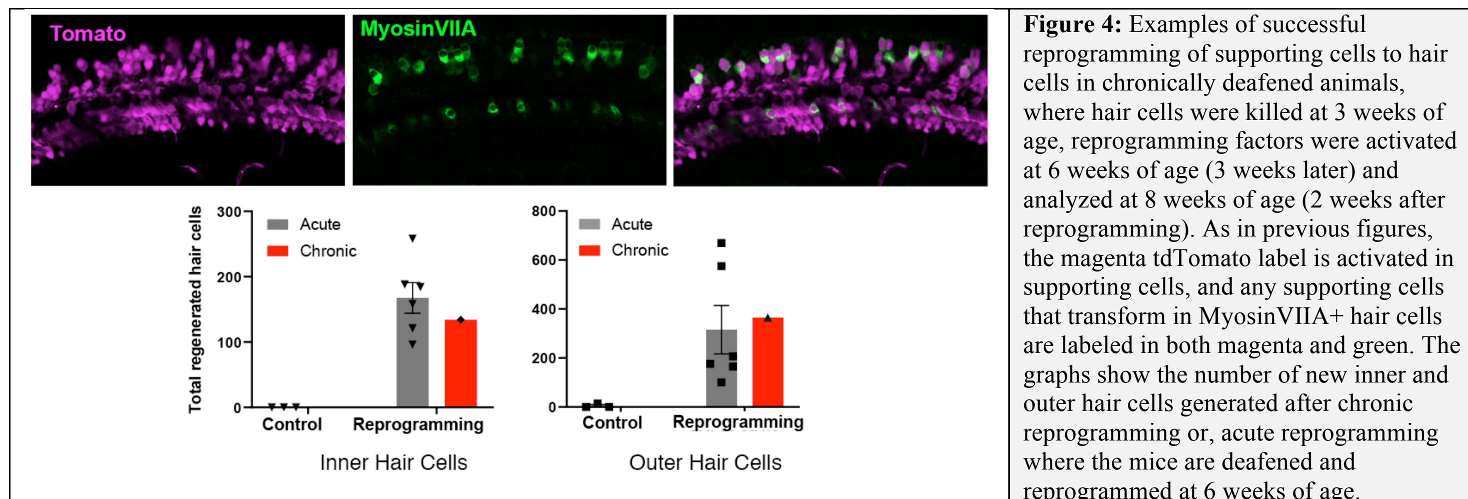

Figure 4: Examples of successful reprogramming of supporting cells to hair cells in chronically deafened animals, where hair cells were killed at 3 weeks of age, reprogramming factors were activated at 6 weeks of age (3 weeks later) and analyzed at 8 weeks of age (2 weeks after reprogramming). As in previous figures, the magenta tdTomato label is activated in supporting cells, and any supporting cells that transform in MyosinVIIA+ hair cells are labeled in both magenta and green. The graphs show the number of new inner and outer hair cells generated after chronic reprogramming or, acute reprogramming where the mice are deafened and reprogrammed at 6 weeks of age.

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 described below, issues with availability of personnel and our inability to perform survival surgeries for much of Year 1 due to COVID restrictions at our university meant that the purification and initial testing of these proteins was delayed until Year 2. 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 are now obtaining excellent induction and production of the cell-penetrant versions of the transcription factors and have begun a preliminary characterization of these peptides in cochlear orgwan cultures before attempting in vivo experiments later this year as part of the approved no-cost extension.

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?

1. As described below, the COVID pandemic required us to eliminate 65% of our mouse colony, which negatively impacted our entire mouse breeding and production program. Now that we have optimized our chronic deafening model, we will complete the experiments in Aim 1B. A paper describing the experiments in Aim 1A is currently being prepared for submission

2. COVID restrictions meant that survival surgeries were not permitted for much of the previous reporting period. We have now initiated the experiments proposed in Specific Aim 2A, tasks 3, 4 and 6 (purification of cell-penetrating peptides and infusion into neonatal mice as a proof of principle).

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 are capable of regenerating 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 has been approved.

Changes that had a significant impact on expenditures

The delays described above have reduced our expenditures in Year 1. As described above, we have 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 Aim1A 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:	RO1 DC017689 (3 person months) RO1 DC014932 (3 person months) RO1 DC014832 (0 person months; no cost extension) RO1 DC013072 (2 person months) 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 4.2 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 DC014932-02 (Groves) 12/1/2016 – 11/30/2021 0.6 Calendar
NIH/NIDCD

A multi-species approach to find regulators of deafness genes

The Specific Aims of this grant are to understand the role of the Ubr3 ubiquitin ligase in mouse and Drosophila hearing organ development, to understand the regulation of MyosinIIA by Ubr3 in mouse hair cells and to carry out a new forward genetic screen for genes expressed in the Drosophila hearing organ.

5R01 DC013072-06 (Groves): 9/1/2020 – 8/31/2025 2.28 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.

Hearing Restoration Project Consortium Grant (Groves) 10/1/2021 – 9/30/2022 0.6 Calendar
Hearing Health Foundation

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