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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 address the first aim. We have shown that activation of Atoh1, Pou4f3 and Gfi1 in acutely deafened mice can indeed generate significant numbers of new hair cells - on average 870 new hair cells per cochlea compared to zero new hair cells in untreated controls.

15. SUBJECT TERMS

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

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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 Deafness Hair cells Supporting Cells Reprogramming Transcription Factors Mouse Models Atoh1 Pou4f3 Gfi1

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,	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	75%
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,	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	25%

Specific Aim 2A: Can Atoh1, Pou4f3 and Gfi1 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,	No
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
Specific Aim 2B: Can Atoh1, Pou4f3 and Gfi1 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%

What was accomplished under these goals?

We have 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).

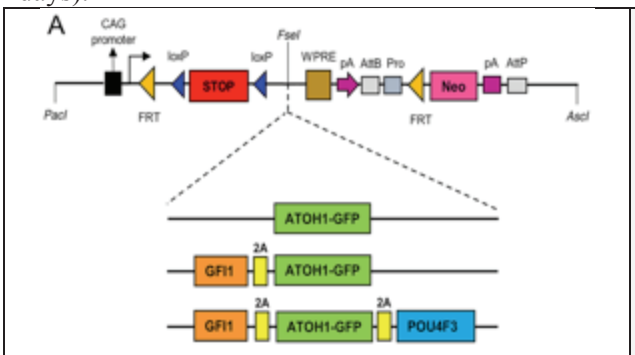
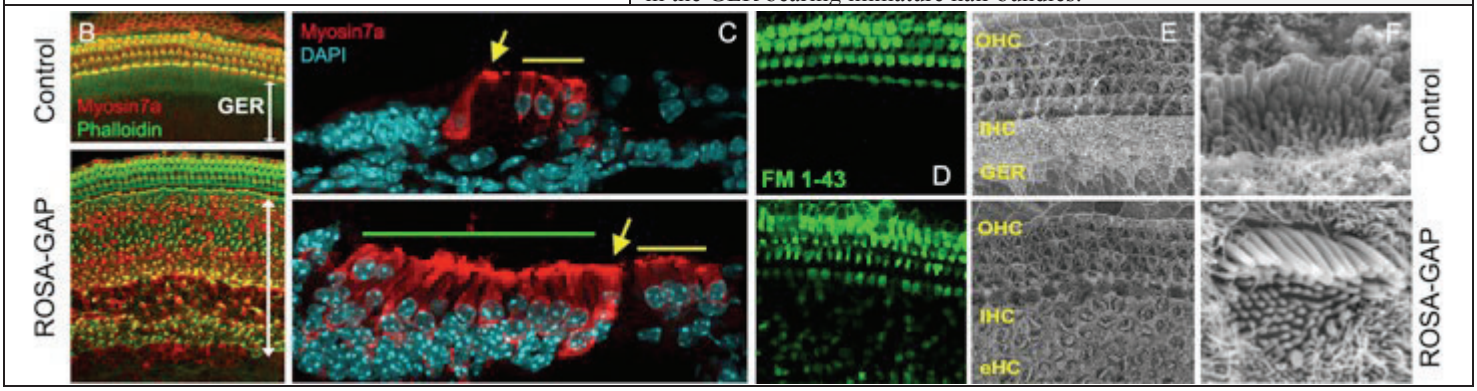


Figure 1: A-The ROSA targeting strategy to express hair cell transcription factors. All three reprogramming cocktails can be expressed from the same ROSA locus at similar levels in the presence of Cre recombinase. **B-F:** The panels show a control Sox9^{CreER}: ROSA^{Ai9} mouse compared to a Sox9^{CreER}: 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.



We combined the Cre-inducible reprogramming system show in in Figure 1 with Pou4f3^{DTR} mice that specifically and efficiently kill 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). Across all animals examined in the first experimental cohort, we observed an average of 869.5 new hair cells (i.e. cells that 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.*** Most of the breeding for Aim 1A has been completed and about 50% of the data in Aim 1B has been collected. We are now getting ready to perform the same experiments in chronically deafened animals (Aim 1B).

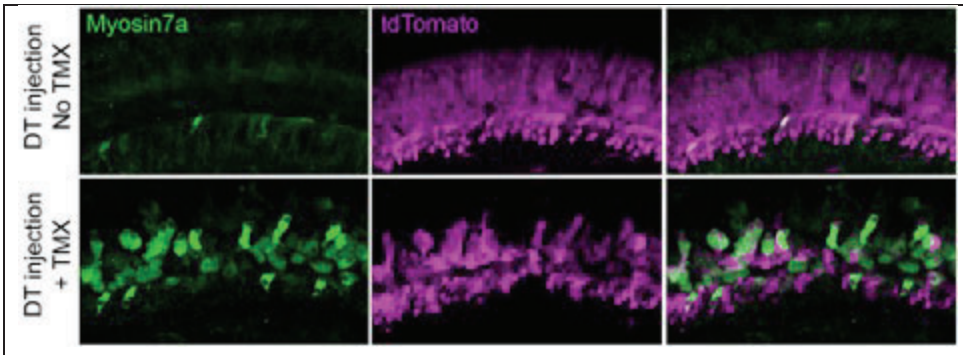


Figure 2: Atoh1, Gfi1 and Pou4f3 can reprogram supporting cells to Myosin7a+ cells following damage. 3 week old Sox9^{CreER}; Pou4f3^{DTR}; ROSA^{Ai9}; ROSA^{GAP} mice were injected with diphtheria toxin. Half the mice received tamoxifen after 24 hours to activate expression of Atoh1, Gfi1 and Pou4f3 and the tdTomato reporter in supporting cells. After two weeks, many Myo7a+ hair cells can be seen in the treated animals. N.B. The image in the lower panel does not capture all tdTom+ cells below the confocal plane of the hair cells.

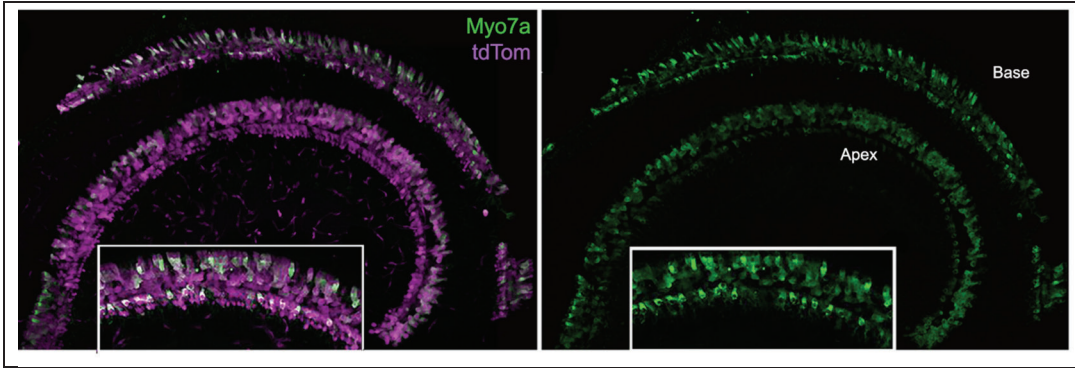


Figure 3: Lower power examples of successful reprogramming of supporting cells to hair cells along the length of the cochlea. Dissected cochleas were separated into basal and apical halves and photographed together. As in Figure 2, many tdTomato+ cells can be seen expressing the hair cell marker Myosin7a,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

For Specific Aim 2, we have cloned versions of Atoh1, Pou4f3 and Gfi1 fused to a cell-penetrating peptide sequence consisting on 9 arginine residues (9R). As described below, issues with availability of personnel and our inability to perform survival surgeries for much of the year due to COVID restrictions at our university meant that the purification and initial testing of these proteins had to be delayed until Year 2.

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 been able to resume breeding, we will complete the breeding of the cohorts for Aim 1A, Aim 2A and Aim 2B and produce the cohorts for Aim 1B. We will then quantify the data from experiments 1A and begin experiment 1B.

2. We will complete the experiments in which reprogramming factors are activated in *chronically* deafened mice (Aim 1B)

3. COVID restrictions meant that survival surgeries were not permitted for much of the previous reporting period. We will therefore initiate 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 decision was made after consultation with our former Science Officer, Colleen LaVinka

2. The postdoctoral fellow working on this project had to reduce her effort as she received a grant to partly fund 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. As above, our plan to resolve this delay is simply to request a no-cost extension to extend the proposal beyond its original two years.

Changes that had a significant impact on expenditures

The delays described above have reduced our expenditures in Year 1. As described above, we will likely request 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

Nothing to report

Journal publications.

Nothing to report

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:	Melissa McGovern
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0003-2254-8782
Nearest person month worked:	4
Contribution to Project:	Carried out experiments in Aim 1
Funding Support:	The Hearing Health Foundation Hearing Restoration Project

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 3.2 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 3.2 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 DC014832-03 (Groves/Raphael) 12/1/2015 – 11/30/2020 0.5 Calendar
NIH/NIDCD (Groves lab)

Enhancing Atoh1 function in hair cell regeneration

The Specific Aims of this grant are to determine the epigenetic changes in mouse supporting cells between birth and the adult animal, to test the effectiveness of the Gfi1 transcription factor to synergize with Atoh1 in hair cell regeneration and to test whether the function of Gfi1 is modulated by SUMOylation and interactions with the Tal1 transcription factor.

Hearing Restoration Project Consortium Grant (Groves) 6/1/2018 – 5/31/2021 0.36 Calendar
Hearing Health Foundation

Comparison of three reprogramming cocktails in the organ of Corti: Cells, transcriptomes and epigenomes

The Specific Aims of this grant are to test the transcriptional and epigenetic effects of the Pou4f3 transcription factor in synergizing with Atoh1 and Gfi1.

5R01 DC013072-06 (Groves): 9/1/2020 – 8/31/2025 3.0 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.

What other organizations were involved as partners?

Nothing to report