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14. ABSTRACT The goal of the project is to test experimental therapies for hearing loss in transgenic mouse models. We have developed molecular methods to identify sensory hair cells in the inner ear, including two different validated genetic profiles of sensory hair cells. We have generated and derived genetically modified mice to serve as models for treating hearing loss with gene therapy. We have shown that five such models are not suitable for our studies, but we have genetically engineered two new mouse lines line and re-derived a third line from our collaborators. We have also generated data to determine the genetic differences in supporting cells of the cochlea at two different ages, and to determine the response of these cells to pharmacological inhibitors that may promote hair cell regeneration.				
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INTRODUCTION:

Hearing and balance dysfunction are frequently seen in military personnel exposed to blast injuries. The most common cause of these disorders is the death of inner ear sensory hair cells. Once hair cells are killed, they are not replaced and combat-induced hearing and balance disorders are therefore permanent. At present, there is no treatment that allows the replacement of these sensory cells. The long term goal of this research is to develop gene or drug-based therapies that allow the regeneration of sensory hair cells and the restoration of hearing and balance in combat personnel. The transcription factor **Atoh1** is one of the first genes to be switched on when hair cells form. Atoh1 has been shown to induce new hair cells when activated in embryonic or neonatal inner ears. We will attempt to re-activate Atoh1 by genetic or pharmacological methods in an animal model to test its ability to promote regeneration of sensory hair cells.

BODY OF REPORT:

Aim 1: To determine the genetic targets of Atoh1

Results: This Aim has been completed as described in the previous Annual report. For completeness, we enclose the list of validated Atoh1 target genes that was presented last year. A manuscript describing these genes is now in preparation.

Accession	Gene	Presence of AtEAM Sites?
NM_029440	4930434E21Rik	GCCATCTGGA:-427;
NM_007500	Atoh1	TACAGATGGC:770;
NM_019446	Barhl1	
NM_029426	Brsk2	CCCATCTGCC:839;
NM_028055	Btbd17	
NM_007583	Cacng2	
NM_015795	Fbxo16	GCCATCTGCT:-495;
NM_019479	Hes6	
NM_018741	Igf1p1	
NM_008595	Mfng	GCCATCTGTG:-1466;
NM_001113199	Naca	GCCACCTGTT:-2207;
NM_009717	Neurod6	
NM_027032	Pacrg	
NM_001099331	R3hdml	
NM_178045	Rassf4	CCCAGCTGCC:-1422;
NM_009162	Scg5	
NM_011352	Sema7a	ACCAGCTGGC:-658;GCCATCTGGC:-2433;TCCATCTGTT:-2302;
NM_019982	Sez6l	ACCAGATGGA:-177;GACAGATGGA:212;AACAGATGGG:-18;GACAGCTGGA:11;
NM_026886	Srrm4	ACCAGATGTC:-4828;
NM_009217	Sstr2	GCCATCTGCC:337;
NM_178874	Tmcc2	
NM_053267	Selm	ACCAGATGGC:1158;GGCATCTGTC:-4211;
NM_134050	Rab15	



Atoh1 E-Box Motif (AtEAM)

Figure 1: List of 23 Atoh1 target genes obtained by cross-relational comparison of hair cell RNA-seq data and cerebellum Atoh1 ChIP-seq data. The AtEAM consensus binding site for Atoh1 is shown on the right; 12 of the candidate genes have AtEAM sites within 5kb of their coding regions. It is likely that some of the 11 other genes may have AtEAM sites located further away from their coding regions.

We have also published a paper this year describing the effects of deleting Atoh1 from the cochlea at different developmental times:

Cai, T, Seymour, M.L., Zhang, H., Pereira, F. A. and **Groves, A.K.** (2013). Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. *J. Neuroscience* **33**, 10110-10122. PMID 23761906; PMC: 3682389

Aim 2: To activate Atoh1 in damaged cochlear organ cultures to promote hair cell regeneration

We proposed the following deliverables for Year 2 and 3. As noted in last year's annual report, we made minor modifications to these deliverables based on our results in year 1:

1. We will determine whether activation of Atoh1 or Atoh1 and Gfi1 in cochlear supporting cells is able to drive them to a hair cell fate.
2. We will demonstrate whether hair cells generated by DAPT treatment and Atoh1 activation (or Atoh1 and Gfi1 activation) resemble *bona fide* hair cells by statistical comparison of these groups with wild type hair cells.

As described in our previous annual report, we set out to make a line of transgenic mice in which Atoh1 could be expressed in a Cre-inducible fashion. However, we also noted that in 2012, two groups published papers showing that the ability of Atoh1 to induce new hair cells drops dramatically after the onset of hearing, which in mice is 14 days after birth. This suggests that other factors may be required to co-operate with Atoh1 to drive hair cell differentiation in the mature ear. In collaboration with the Raphael

group at the University of Michigan, we have evidence that co-expression of the Atoh1 and a second transcription factor, Gfi1, can generate more hair cells and in older animals than Atoh1 alone. In parallel with the Atoh1-expressing mice described above, we engineered a targeting construct to express *both* Atoh1 and Gfi1 and equal levels in a Cre-inducible fashion. We proposed to directly compare the effects of expressing Gfi1 and Atoh1 together with that of expressing Atoh1 alone.

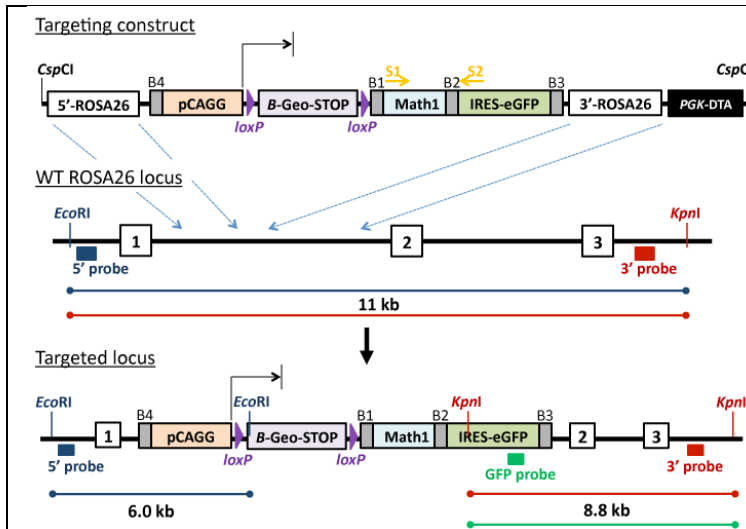


Figure 2: Diagram showing the targeting construct (top) designed to target the mouse ROSA locus (middle) by homologous recombination in ES cells. This generates a correctly targeted, Cre-inducible form of Atoh1 (bottom) in which GFP will also be expressed from an internal ribosome entry site (IRES). The same strategy is used to produce a targeting construct expressing Atoh1 and Gfi1; here the two genes are separated by a picornavirus 2A sequence to allow translation of equal amounts of both genes. This diagram was also provided in the previous Annual Report; we provide it here again for clarification.

As described in quarterly reports in the last year, we successfully targeted the ROSA locus with both constructs and obtained multiple founder mice for each line that gave germline transmission. ***In consultation with the previous GOR, Dr. Michael Holtel, we significantly reduced effort and expenditure on this award in year 3 to allow time for these mouse lines to breed and expand our colonies. As a result, much of our proposed work in the past year has been delayed and will be carried out in a fourth year no-cost extension to the award.***

Results:

1. Our initial tests of the mice are quite promising – Figure 3 shows an example of extra hair cells produced in the cochlea when the Atoh1-Gfi1-expressing mouse line is crossed to a Cre driver line.

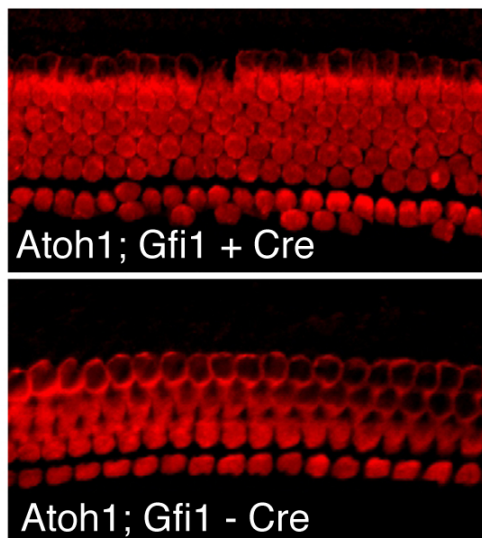


Figure 3: Activation of Atoh1 and Gfi1 in our transgenic mice generates significant numbers of extra inner and outer hair cells in the presence of Cre recombinase, but not in negative control mice that lack Cre.

Although these results are promising, we are mindful of the possibility that the levels of ectopic Atoh1 and/or Gfi1 expressed by the transgene may vary from founder to founder, and so we are still the process of screening through all founder lines to test for a) expression of the Atoh1 and Gfi1 transgenes and b) the ability of the transgenes to induce extra hair cells when crossed to an appropriate Cre line.

2. As discussed in previous reports, we showed that although blocking the Notch signaling pathway can cause supporting cells to transdifferentiate into hair cells, this effect is only seen in neonatal mice, with virtually no response occurring in one week old mice. We proposed to analyze the transcriptional changes in supporting cells from neonatal and one week old animals exposed to inhibitors of Notch signaling.

As described in previous quarterly reports in the past year, we have now completed our transcriptome analysis of supporting cells purified from newborn and one week old mice. Throughout the year we have applied different forms of statistical analysis to our data to produce a consensus

list of genes enriched in the two ages of supporting cells. Our most stringent analysis has identified 79 transcripts significantly enriched in newborn supporting cells versus seven day old supporting cells, and 260 transcripts that are significantly enriched in one week old supporting cells compared to newborn cells. Our gene ontology and pathway analysis suggests that the broad changes in supporting cell gene expression during this period include changes in the extracellular matrix, lipid metabolism and increases in chromatin remodeling complexes associated with gene repression.

In a second series of experiments, we analyzed the gene expression profiles of cochlear cultures grown in the presence of Notch inhibitors from new born animals (where the inhibitors generate significant new numbers of supporting cells) and one week old animals (where the inhibitors no longer work and no new hair cells are generated). As reported last quarter, these libraries were sent for sequencing at Baylor's Human Genome Sequencing Center. Unfortunately, they experienced unexpected technical difficulties with the library preparation due to a faulty batch of the library reagents from the sequencing company. *As a result, these data were of extremely low quality and this experiment will have to be repeated.* We expanded the line of transgenic mice used to purify these cells, and have enough mice ready to repeat these experiments in November 2013. Once positive piece of news to come from our sequencing work is that we have verified that in our lab we can generate good quality, reproducible RNA-seq libraries from as few as 10,000 cells – five times fewer than we have previously used. Thus, although these technical problems are a setback, we hope to repeat the experiment much more quickly than before, as less tissue will be required.

Aim 3: To activate Atoh1 in deafened mice to promote hair cell regeneration

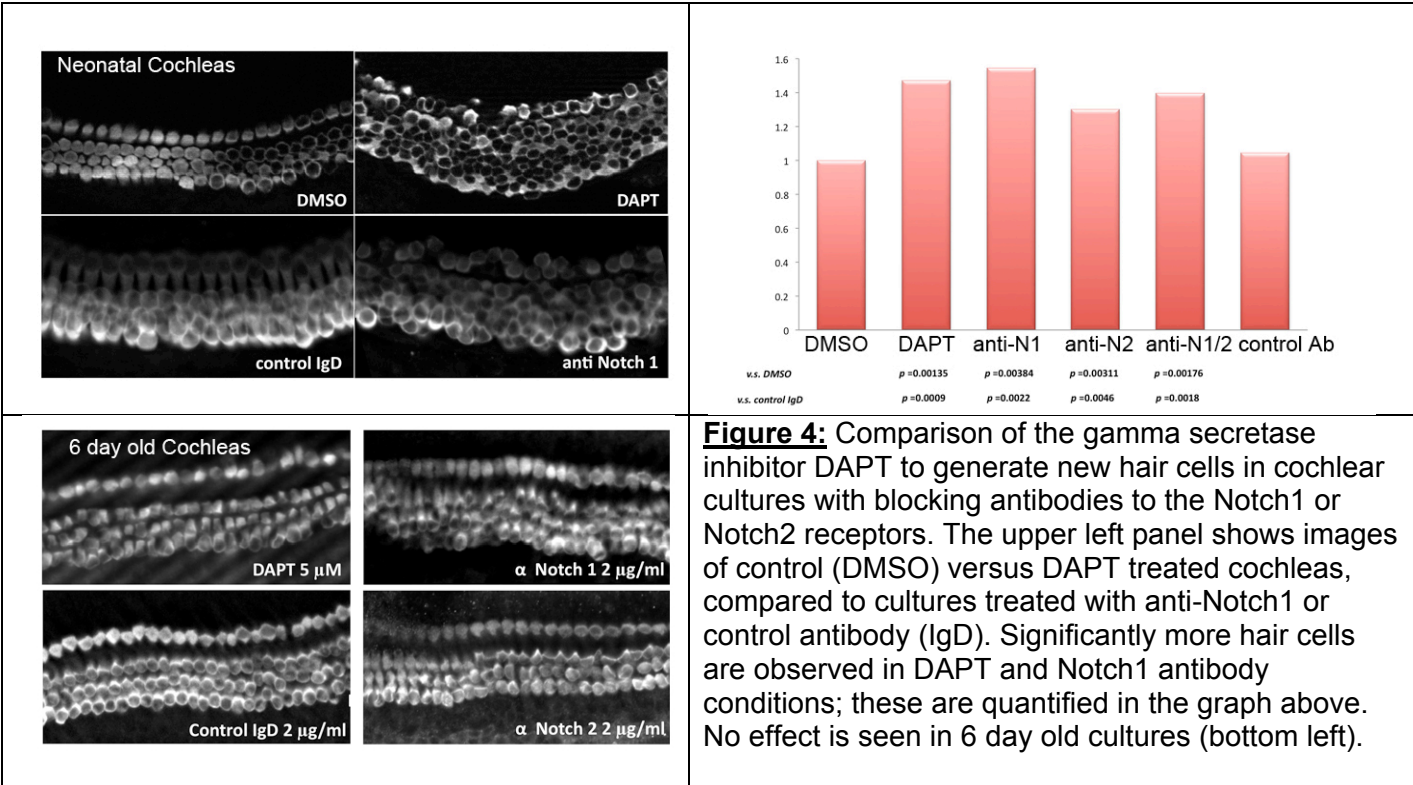
We proposed the following deliverables for Years 2 and 3:

Year 2: We will verify that the Sox2-CreER/dnMAML system is able to generate new hair cells in our hands.

Year 3: We will present audiological and morphological data to determine whether activation of Atoh1 in deafened mice can promote hair cell regeneration and restore some aspects of functional recovery.

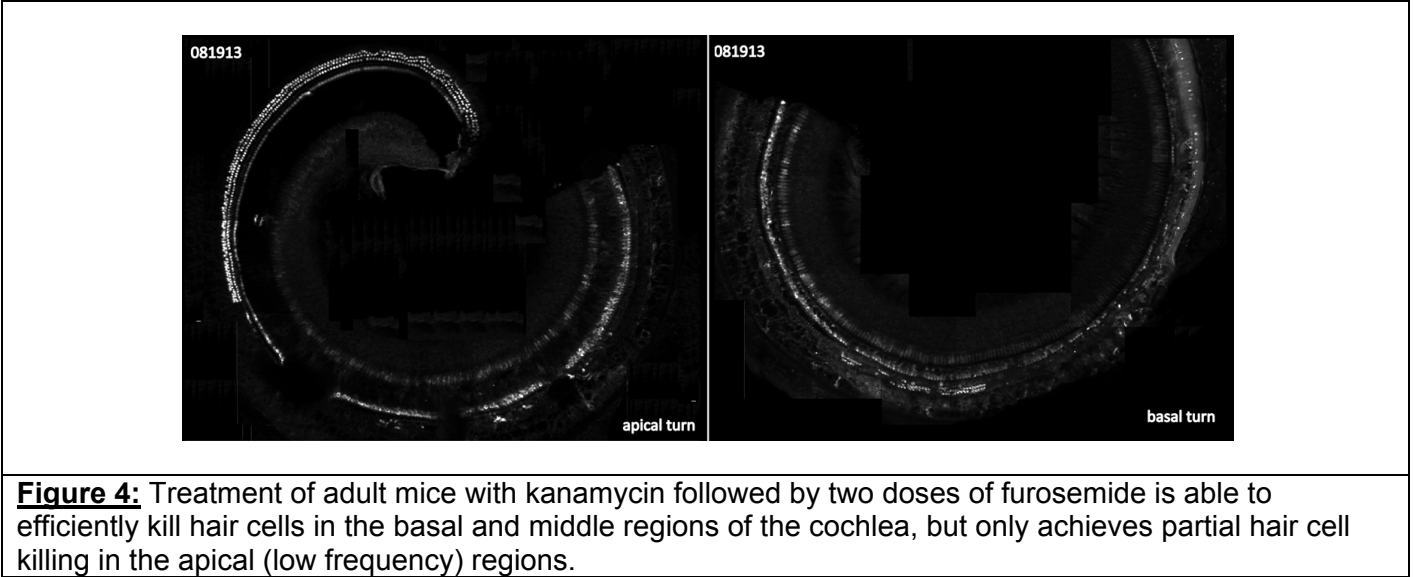
1. As described in last year's report, we have shown that the Sox2-CreEr mice work well in our hands. However, we were not able to generate new hair cells by inhibiting Notch signaling with the dnMAML transgenic mice, despite the fact that the gamma secretase inhibitor DAPT is capable of generating many new hair cells in neonatal mice. Since DAPT can inhibit cleavage of a number of proteins in addition to the Notch receptors, we were concerned to test whether this was a specific effect on the Notch pathway. To this end, we tested a number of blocking antibodies to the Notch1 and Notch2 receptors that are currently being developed by Genentech for treatment of T-cell leukemia. By directly comparing DAPT treatment of neonatal cochlear cultures with antibodies to Notch1, Notch2 or to both antibodies simultaneously, we were able to show that these antibodies gave similar results to treating the cultures with saturating concentrations of the gamma secretase inhibitor DAPT (Figure 4). This suggests that the bulk of the inhibitory activity of DAPT is mediated mainly through the Notch receptors. As described in previous reports, we also find that like DAPT, the hair cell generating activity of Notch antibody treatment declines precipitously, being significantly lower in cochleas taken from 3 day old mice, and completely gone by six days (Figure 4).

While these results suggest that the Notch pathway is indeed being inhibited in our studies, they leave open the question of which dominant-negative mutant forms of MAML1, the transcriptional co-activator of the Notch pathway, do *not* generate ectopic hair cells as we reported last year. We have repeated these experiments this year to confirm our results. We are now proposing to compare the effect of DAPT, anti-N1 or 2 and the dnMAML1 transgenic mice on downstream effectors of the Notch pathway to determine if hair cell generation is proceeding through canonical effectors of the Notch pathway.



2. Due to the delay in generating transgenic mice for Aims 2 and 3, we spent some months this year optimizing the deafening paradigm for these experiments. Mice have traditionally been hard to deafen with ototoxic drugs such as neomycin or kanamycin, as the dose at which hair cell death is observed with these drugs is close to the toxic dose for the mouse. Recent work has suggested that survival may be improved by treatment with a lower dose of aminoglycoside antibiotic, followed by the loop diuretic furosemide 45 minutes later.

After considerable optimization, we have shown that we can obtain very effective killing of hair cells in the basal and middle turns of the mouse cochlea by administration of 1g/kg kanamycin, followed by 200mg/kg furosemide given at 30 minutes and 4 hours after kanamycin application (See Figure 5).



However, the seven day viability of these animals was extremely poor – typically less than 20% surviving after 7 days – likely due to kidney damage. Despite using different strategies to preserve kidney function and hydration, we were not able to improve survival rates. We are therefore proposing to acquire a line of transgenic mice from the Rubel lab in Seattle that express a diphtheria toxin receptor specifically in hair cells. Recent published work from this group suggests that impressive hair cell killing can be achieved with almost no toxicity by a single dose of diphtheria toxin. We will test these animals and then cross them to our Atoh1 and Atoh1/Gfi1-expressing transgenic mice to complete this part of the project in the next two quarters.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed techniques to purify supporting cells from cultured organ of Corti
- Generated a validated list of genes expressed in hair cells using RNA-seq
- Generated a validated list of genes expressed in neonatal and six day old supporting cells.
- Completed material collection to compare the effects of DAPT on neonatal and six day old supporting cells
- Validated RNA-Seq library construction on small numbers of cells
- Generated targeted founder mouse lines to conditionally activate Atoh1 and Gfi1 in any cell or tissue type in the mouse.

REPORTABLE OUTCOMES:

Informatics: We have compiled two databases of genes whose expression is enriched in hair cells by both microarray and RNA-seq. We have cross-referenced these databases to extract genes in these lists that contain Atoh1-binding sites within 5kb upstream or downstream by bioinformatic interrogation with a consensus AtEAM site. We have established that the two different methods give distinct but overlapping results. We have now compiled an additional database of genes significantly enriched in neonatal and six day old mouse supporting cells.

ES cell production: We have generated successfully targeted ES cell lines to generate Cre-inducible forms of Atoh1 and both Atoh1 and Gfi1 simultaneously. We have also generated adenoviral expression constructs to express these genes in the organ of Corti if required.

Transgenic mouse production: We have generated 14 founder mice to activate Atoh1 alone and both Gfi1 and Atoh1 in supporting cells. These mice have now been expanded and are being screened to reveal lines that give good hair cell production.

Publication, supported in part by this proposal:

Cai, T, Seymour, M.L., Zhang, H., Pereira, F. A. and **Groves, A.K.** (2013). Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. *J. Neuroscience* **33**, 10110-10122.

CONCLUSION:

The long-term goal of this project is to use activation of the Atoh1 gene by pharmacological or genetic means to promote hair cell production in the damaged cochlea as a means of hearing restoration.

The three goals of the proposal are to identify the genetic targets of Atoh1 and to demonstrate as a proof of principle that activation of Atoh1 can generate hair cells in organ culture and transgenic mouse models. Much of our efforts in Year 2 and 3 were devoted to collecting material for and generating RNA-Seq libraries from neonatal and six day old supporting cells, and from supporting cells that had been treated with DAPT in culture for 24 hours. These experiments will allow us to identify genes that change in supporting cells with age and after blockade of the Notch pathway.

The second goal for Year 2 was to complete the generation of transgenic mice for our in vivo studies. We have now completed the generation of targeted founder mice and the establishment of the Sox2-CreER mouse line. The delay in generating these mice and breeding their founders has necessitated extension of the proposal period to complete these experiments in a 4th year.

Finally, we are exploring the possibility that was raised throughout year 2's work that inhibition of the Notch signaling pathway may not promote hair cell generation from supporting cells, but rather, that another gamma secretase-dependent pathway is responsible for the effects of DAPT. We have now shown that the effect of DAPT can be mimicked by blockade of Notch receptors, but not of the conventional/canonical Notch transducer, MAML. This has the potential to reveal a novel pathway for hair cell regeneration, and so we are very interested in pursuing this avenue of research.