

AD _____

Award Number: W81XWH-10-2-0116

TITLE: Treating Combat Hearing Loss with Atoh1 Gene Therapy

PRINCIPAL INVESTIGATOR: Andrew K. Groves Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel 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				<i>Form Approved</i> 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 October 2011		2. REPORT TYPE Annual		3. DATES COVERED 1 October 2010 – 30 September 2011	
4. TITLE AND SUBTITLE Treating Combat Hearing Loss with Atoh1 Gene Therapy				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-2-0116	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Andrew K. Groves Ph.D. E-Mail: akgroves@bcm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 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 a validated genetic profile 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 three such models are not suitable for our studies, but we have genetically engineered a fourth line and re-derived a fifth line from our collaborators. Finally, we have established and validated cell culture models to validate these mice and to investigate the molecular changes occurring in the ear following damage and treatment.					
15. SUBJECT TERMS Noise-induced hearing loss, Mouse models, Gene Therapy, Regeneration					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 14	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

Page

Introduction.....	4
Body.....	5
Key Research Accomplishments.....	12
Reportable Outcomes.....	13
Conclusion.....	14
References.....	N/A
Appendices.....	N/A

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 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:

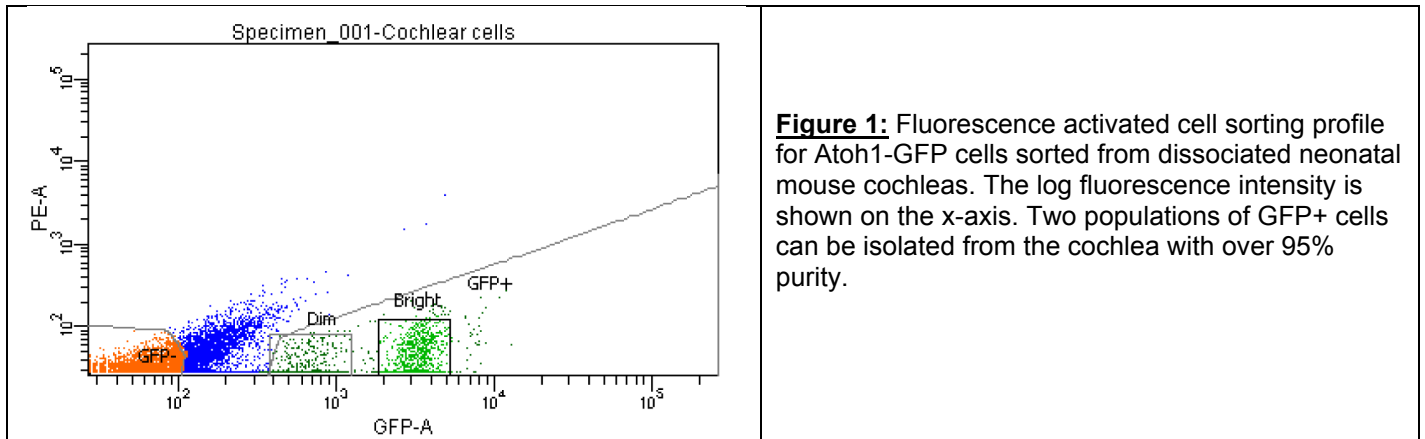
Aim 1: To determine the genetic targets of Atoh1

We proposed the following deliverables for Year 1:

1. We will produce a set of normalized microarray data showing transcripts enriched in Atoh1+ hair cells versus other cochlear cell types. This data will be averaged over triplicate samples.
2. We will produce a set of normalized microarray data showing transcripts enriched in wild type mice versus Atoh1 mutant mice. This data will be averaged over triplicate samples.
3. We will compile a preliminary set of Atoh1-regulated genes based on the data sets produced in (1) and (2) above.
4. We will determine whether chromatin immunoprecipitation and amplification is technically feasible in small amounts of cochlear tissue. *As described in the Research Plan, because this goal is the most technically uncertain, we acknowledge that this may take a further 12-24 months to accomplish and optimize above what has been estimated.*

Results:

Deliverable 1: We have demonstrated over 95% purification of fluorescently labeled Atoh1-GFP cells from the cochlea by fluorescence-activated cell sorting with 30-40% recovery (Figure 1).



We have analyzed the gene expression profiles in the sorted cells by two methods – microarray analysis (“gene chips”) and high throughput deep sequencing (“RNA-Seq”). The two different methods have each yielded a list of expressed genes enriched in hair cells compared to other cell types in the cochlea. An example of the data sets are shown in Table 1 below, with the top five genes enriched in hair cells revealed by the microarray analysis (out of a total of **2,124 genes** enriched at a p value less than 0.05):

RefSeq	Description	Fold change	p-value
AK004064	RIKEN cDNA 1110033I14 gene	90.58	0.000646
BB769641	Transcribed sequence with weak similarity to protein pir:A55119 (H.sapiens) A55119 potassium channel protein romk-1 - human	9.888	0.00058
BB364990	Transcribed sequences	9.861	0.00445
NM_019446	BarH-like 1 (Drosophila)	9.832	0.0173
AV300716	AV300716 RIKEN full-length enriched, 8 days embryo Mus musculus cDNA clone 5730488D07 3', mRNA sequence.	9.824	0.00312

This deliverable has been completed as proposed.

Deliverable 2: As reported in the first quarterly report from this year, we observed that although hair cells do not differentiate in Atoh1 mutant mice, the undifferentiated precursors die shortly after the time at which hair cells will normally differentiate (Figure 2). For this we have been unable to identify Atoh1 targets expressed differentially between Atoh1 mutant mice and normal mice using microarray profiling, as many of the genes up-regulated are involved in cell death, not in hair cells themselves. *This deliverable could not therefore be completed as proposed (but see item 3, below).*

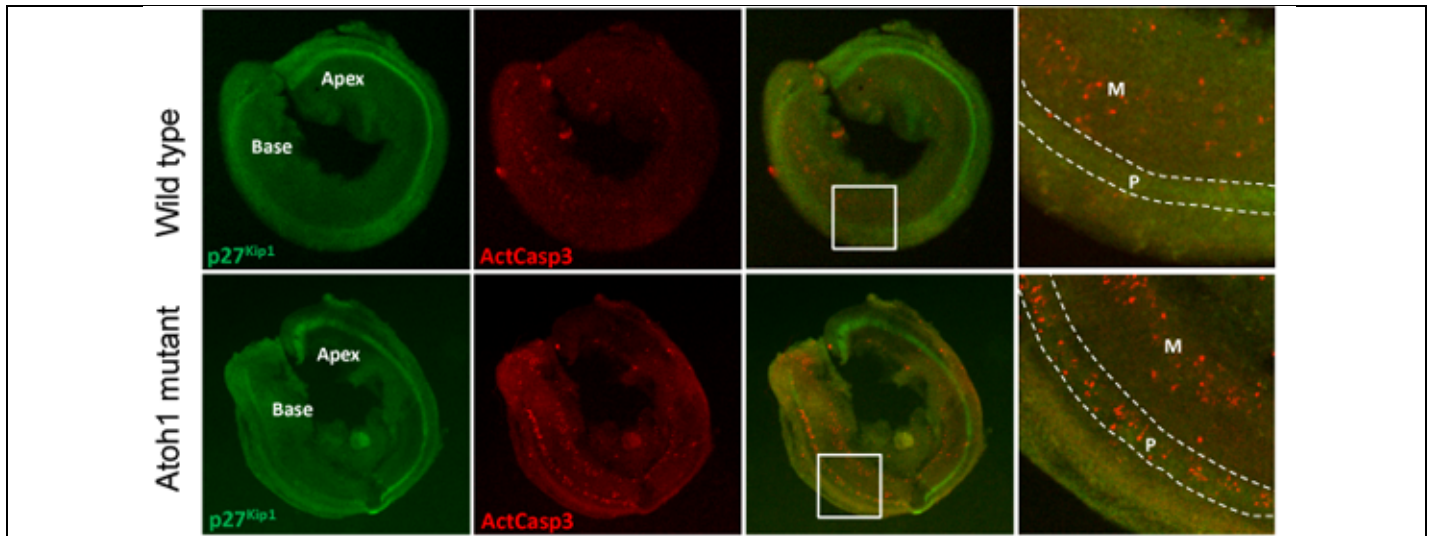


Figure 2: Embryonic cochleas dissected from wild type and Atoh1 mutant mice. A detail of the developing organ of Corti is shown outlined with a square box (third panels) and with dotted lines (fourth panels). Dying cells, revealed by staining for activated caspase-3, are shown in red in the prosensory domain (P). *Significant numbers of differentiating organ of Corti progenitors die in the Atoh1 mutant cochlea.*

Deliverable 3: The data collected from our microarray and deep sequencing work described above gives us a list of all genes expressed preferentially in hair cells. However, it does not tell us which of these genes are *directly* regulated by the Atoh1 transcription factor. To begin to obtain such a list, we have made use of the fact that a 10 nucleotide DNA sequence has been identified to bind Atoh1 specifically by colleagues of ours at Baylor College of Medicine. This Atoh1 E-box Associated Motif or AtEAM occurs in approximately two-thirds of all known Atoh1 binding genes in the cerebellum (Figure 3). We have used this motif to scan regions 5kb upstream and downstream of our hair cell-enriched transcripts to see if those genes contain candidate Atoh1 binding sites. To date, by analyzing the microarray data from purified hair cells, we have identified 79 genes that are enriched in hair cells by two-fold or more and which have at least one Atoh1-binding site.

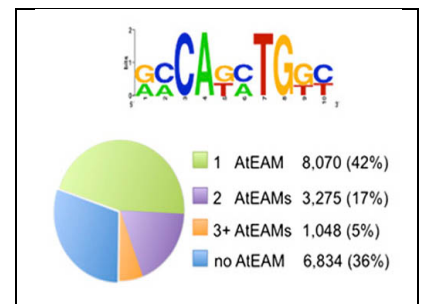


Figure 3: Diagram showing the AtEAM binding sequence and the distribution of AtEAM binding sites among known Atoh1-regulated genes in the cerebellum

The top five genes in this list – enriched between 32 and 92-fold in hair cells, and all at highly significant p-values, are shown in Table 2 below:

Symbol	Fold Change	P.Value	Description	RefSeq
Grik1	92.30278934	6.45E-05	glutamate receptor, ionotropic, kainate 1	NM_146072
Papss2	52.95953347	0.004215384	3'-phosphoadenosine 5'-phosphosulfate synthase 2	NM_011864
Tgfb1	49.90991888	0.00479086	transforming growth factor, beta induced	NM_009369
Met	45.07711248	0.007270847	met proto-oncogene	NM_008591
Rspo2	32.32810472	0.00398084	R-spondin 2 homolog (Xenopus laevis)	NM_172815

This deliverable has been completed with the modifications described.

Deliverable 4: The final and most technically experimental part of this Aim was to perform chromatin immunoprecipitation and sequencing on cochlear tissue to further identify direct targets of the Atoh1 transcription factor. This technique uses a line of Atoh1 knock-in mice in which the endogenous Atoh1 sequence has been tagged with a FLAG epitope to assist with immunoprecipitation. In our first attempt at this experiment, we obtained 200 cochleas from neonatal Atoh1-FLAG mice, and carried out a first run of ChIP-SEQ with this material.

The data we received back was extremely noisy and therefore unusable. To illustrate this, we show in Figure 4 the sequencing reads for the Atoh1 locus itself. It has previously been shown that Atoh1 binds to an enhancer sequence in its own gene – in other words, it positively autoregulates itself. We show a trace from neonatal cerebellum that demonstrates a clear Atoh1 binding site. However, the trace for the same region in our cochlea tissue is barely above background. If our positive control genes such as Atoh1 cannot be detected by this method, we have no hope of detecting other Atoh1 targets in the cochlea.

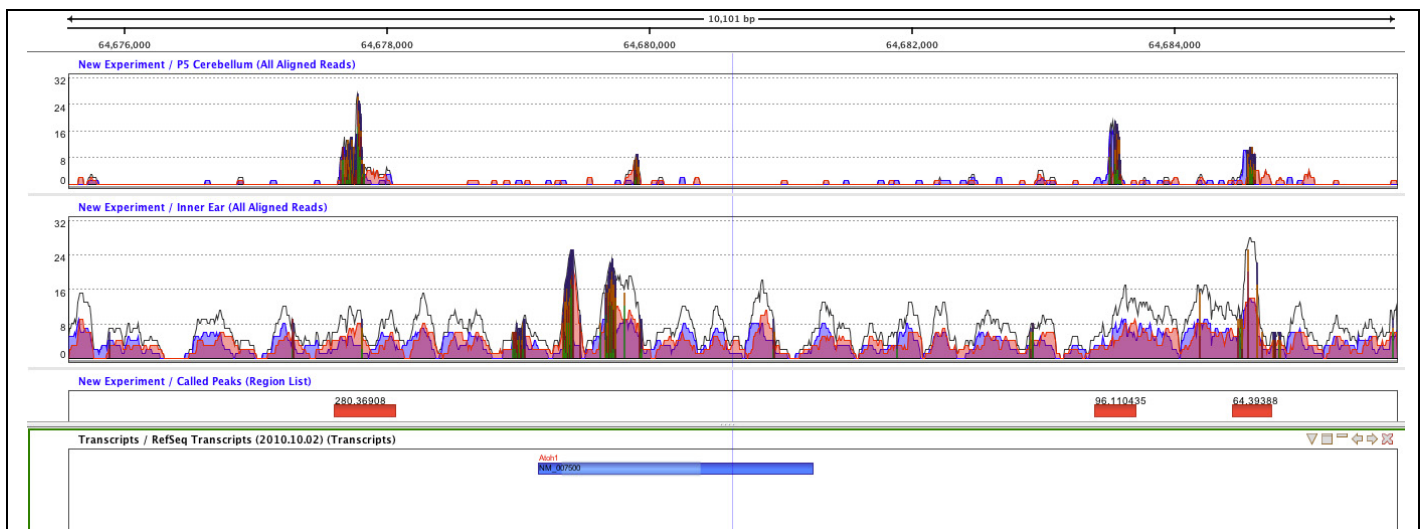


Figure 4: Demonstration of the poor signal-to-noise ratio in a ChIP-SEQ experiment from the cochlea (middle row) compared to the cerebellum (top row). The figure shows sequencing data from a small region of mouse chromosome 6 that contains the Atoh1 gene (blue bar, bottom row). The peaks in the top two rows represent sequenced reads corresponding to Atoh1 binding sites. Note that the cerebellum has three well-defined peaks (summarized by the red bars), but none of these peaks can be clearly identified in the cochlea sample – the background sequence peaks are just too high.

Why did the cochlea give us such poor results, whereas the cerebellum gave clear and reproducible results? The first challenge is cell number - a neonatal cerebellum contains over 10^6 Atoh1-expressing granule cells, whereas a neonatal cochlea contains about 2500 Atoh1-expressing hair cells. The second challenge is purity – the Atoh1-expressing granule cells comprise 50% of the neonatal cerebellum, whereas hair cells comprise less than 1% of the cochlea.

As we originally proposed, this method may take up to 24 months to perfect. In the next iteration of these experiments, we will cross the Atoh1-FLAG mice to the Atoh1-GFP mice used in Figure 1. We will use fluorescence-activated cell sorting to purify the hair cells, thereby enriching our starting population from less than 1% to over 95%. *We believe this enrichment will significantly improve the signal-noise ratio that led to the poor quality data shown in Figure 4.* We have completed crossing the two mouse strains together, and these mice will be used for a second attack on the ChIP-SEQ part of this proposal in the next 12 months.

This deliverable has not yet been completed, but a back-up plan has been implemented as detailed in previous quarterly reports.

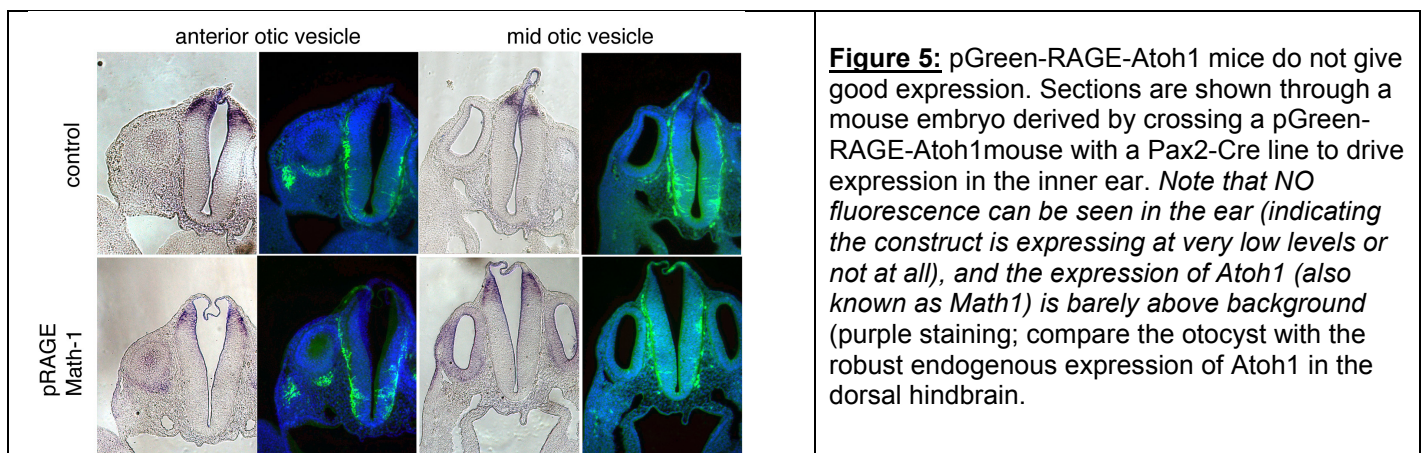
Aim 2: To activate Atoh1 in damaged cochlear organ cultures to promote hair cell regeneration

We proposed the following deliverables for Year 1:

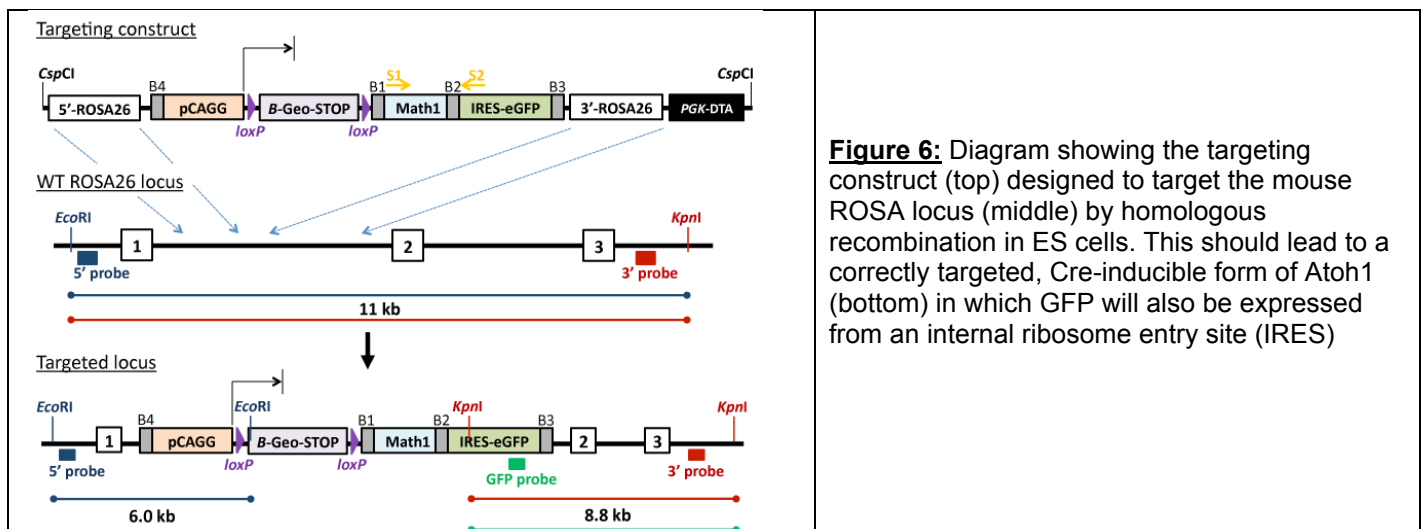
1. We will generate a ROSA26-CAGG-Atoh1 back-up targeting construct.
2. We will verify that the LFn γ -CreER transgenic mice are working in our hands. If the mice do not work as expected, we will acquire either Sox2-CreER or GLAST-CreER mice from other labs and establish colonies in our mouse facility.
3. We will demonstrate optimum conditions for killing hair cells in organ cultures of mouse cochlea.
4. We will show that DAPT treatment of hair cell-depleted cochlear cultures can generate new hair cells.

Results:

Deliverable 1: We verified that our original transgenic mouse, pGreen-RAGE-Atoh1 did not give sufficiently high expression of Atoh1 when crossed with a Cre line. Figure 5 shows sections from such a cross with an ear-specific Cre line developed in our lab. If the mice work successfully, the entire ear should be fluorescent green, with robust purple Atoh1 message staining in the ear. Instead, only weak Atoh1 message can be seen, and the fluorescence in the ear is undetectable.



As proposed, we have now generated a back-up construct (Figure 6) in which a Cre-inducible form of Atoh1 has been targeted to the ubiquitously-expressed ROSA locus in mice.



This construct has been made, validated, and sent to the Baylor ES cell core for gene targeting on September 8, 2011. We will screen and expand any founder animal from this construct in the next 12 months.

This deliverable has been completed as proposed.

Deliverable 2: As described in our first quarterly report, we generated a series of LFn_g-Cre^{Er} transgenic mice to manipulate gene expression in supporting cells. However we were unable to get reproducible transmission of the transgene. Instead, as proposed, we acquired a line of Sox2-Cre^{ER} mice, re-derived them into our facility and tested them for Cre-dependent induction of gene expression in supporting cells. However, as shown in Figure 7, this line did not produce recombination in the organ of Corti (note the absence of green fluorescence in the organ of Corti; red)

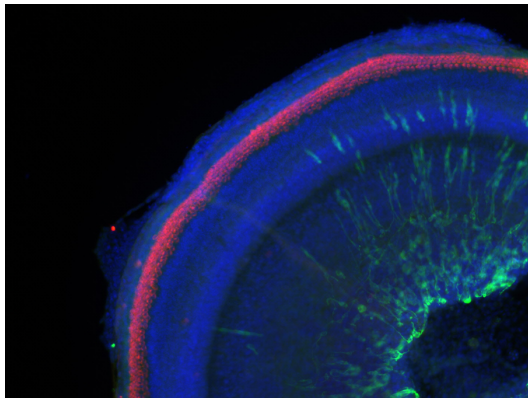


Figure 7: The Sox2-Cre^{ER} line does not produce genetic recombination in supporting cells of the organ of Corti. The image shows an organ of Corti taken from a neonatal mouse derived from a cross between Sox2-Cre^{ER} and GFP Cre reporter parents. The organ was cultured for three days in the presence of tamoxifen to activate the Cre recombinase. Successfully recombined cells are revealed by green fluorescence. The organ of Corti is revealed with red fluorescence, but contains absolutely no green cells. However, the Cre line is working technically, as green fluorescent Schwann cells can be seen located along the processes of spiral ganglion neurons.

Since discovering that this line of mice do not work, we have contacted Konrad Hochedlinger's lab in Boston obtain a second line of Sox2-Cre^{ER} knock-in mice. These will be re-derived into our facility in the next 3 months.

This deliverable has not yet been completed, but a back-up plan has been implemented as detailed in previous quarterly reports.

Deliverable 3: It is extremely hard to experimentally deafen living mice with ototoxins such as kanamycin or neomycin, as the dose-response curve for hair cell loss is extremely close to the curve for lethality. Although this problem has been ameliorated somewhat by the co-administration of loop diuretics to improve hair cell death, the process can still be quite variable. In contrast, mouse hair cells can be killed very easily if the organ of Corti is exposed to ototoxins when placed in organ culture. Figure 8 shows rapid hair cell killing can be carried out in neonatal cochlear cultures after a 48 hour culture period in gentamicin.

Deliverable 4: We have demonstrated that a short period of culture of the neonatal organ of Corti in the presence of the Notch pathway inhibitor DAPT is sufficient to cause a significant increase in hair cells. This occurs in both undamaged organ of Corti and in damaged (hair cell-depleted) tissue (Figure 8). This process happens in the complete absence of cell division, suggesting that the new hair cells are being generated by trans-differentiation of existing supporting cells. *However, as reported in previous quarterly reports, we have observed a rapid, age-dependent decline in the ability of DAPT to promote hair cells generation* (Figure 9).

This age-dependent decline suggests that supporting cells are becoming unresponsive to Notch signaling with age. We have examined the expression of Notch pathway components in the cochlea with age and find a reduction of many pathway components over this time period (Figure 10). However, in many cases these reductions are in the region of 50%, and with the exception of Jagged1, very few Notch pathway genes display haploinsufficient phenotypes. This suggests that the lack of response to Notch blockade cannot be explained simply by a reduction in signaling machinery. In the coming year we propose to investigate the molecular differences in supporting cells between P1 and P6 to gain more insight into this lack of response. *These experiments are important, as one of the therapeutic approaches we are exploring is the blockade of Notch signaling in adult damaged inner ears.*

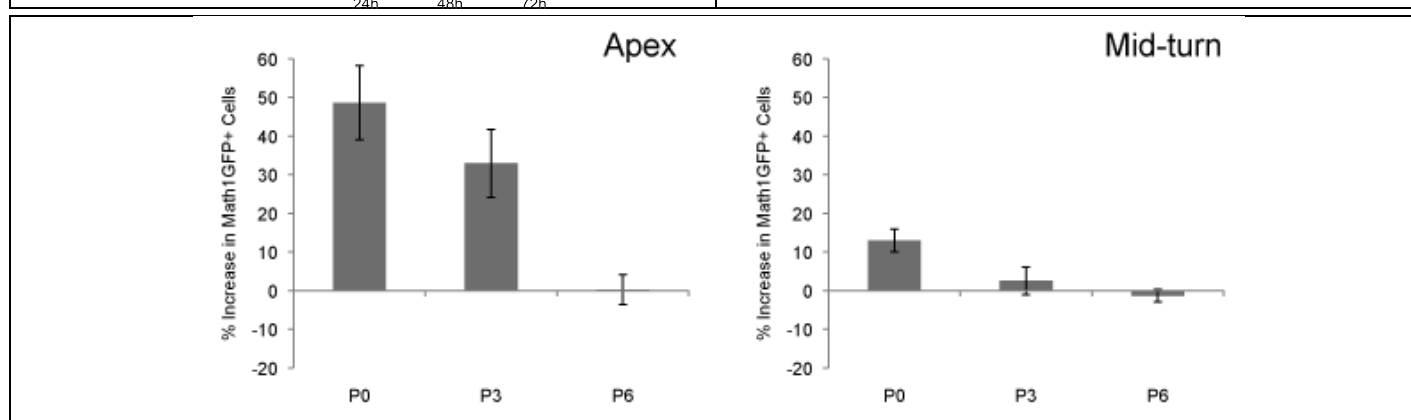
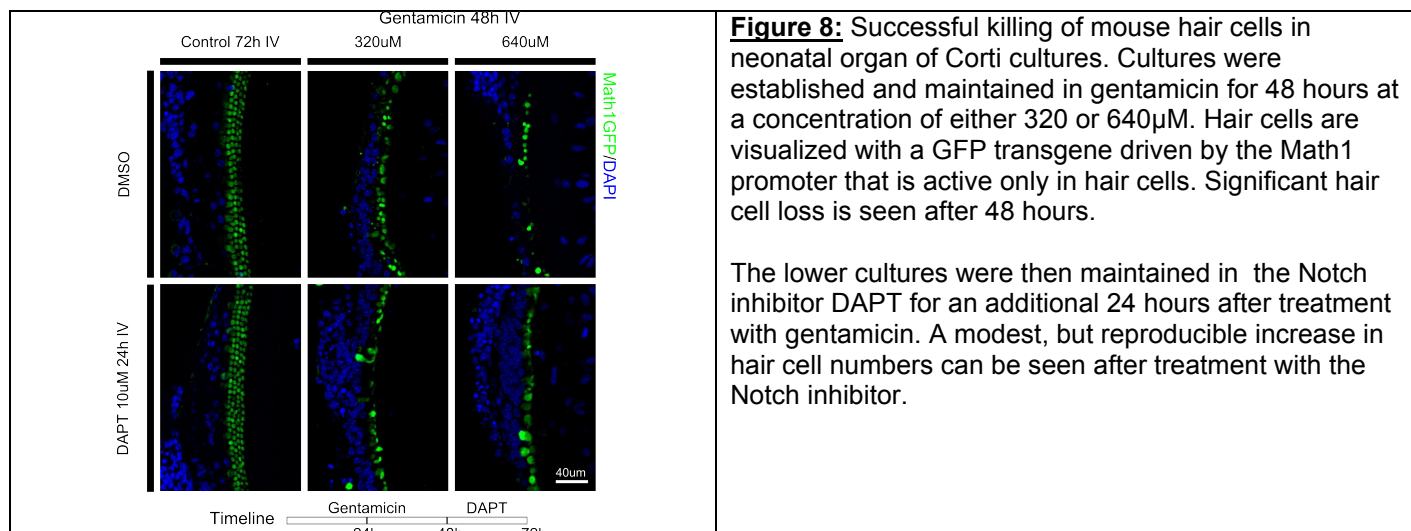


Figure 9: The ability of DAPT to induce supporting cell transdifferentiation into supporting cells declines rapidly with age. The graphs show the percentage increase in hair cell numbers in cochleas cultured from undamaged newborn (P0), and 3 and 6 day old mice (P3, P6) in the apical third of the cochlea and the middle third.

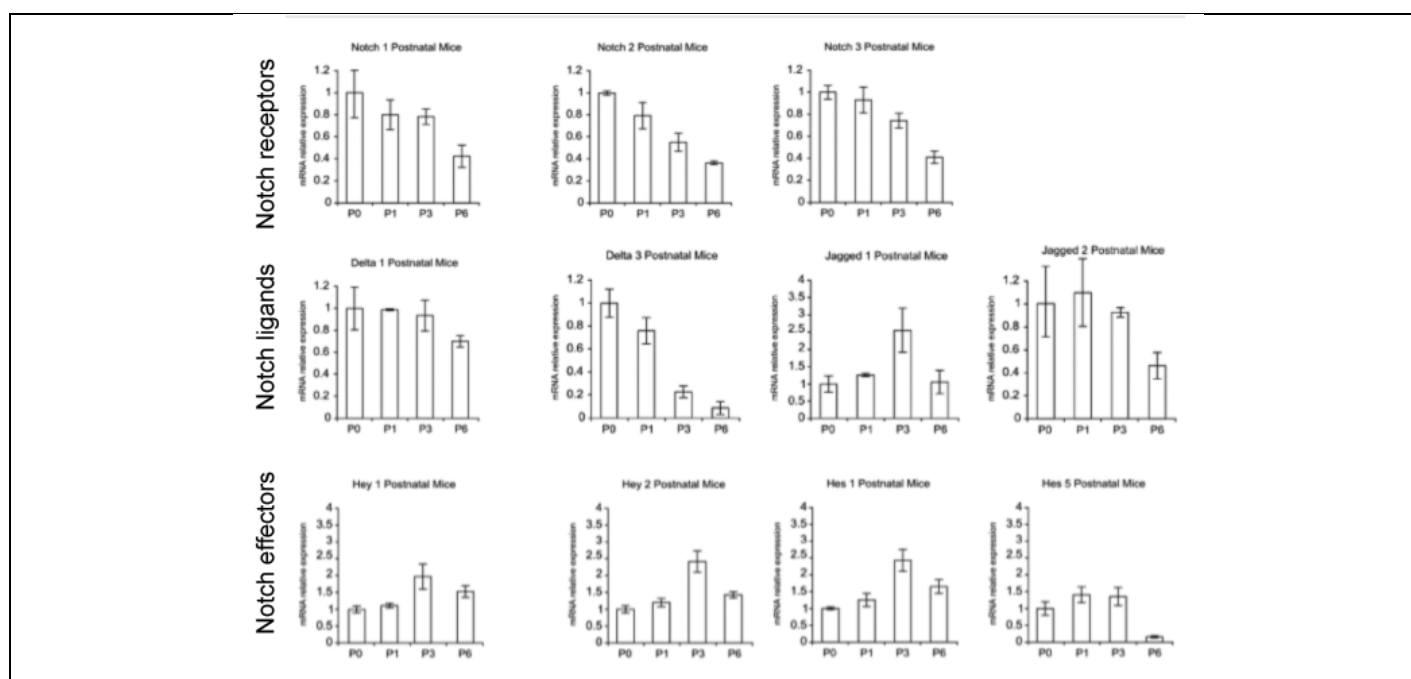


Figure 10: Notch signaling pathway components are down-regulated in the postnatal cochlea with age. mRNA levels are compared by quantitative PCR at postnatal days 0, 1, 3 and 6.

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

We proposed the following deliverable for Year 1:

1. We will present data showing that deafening of adult mice is feasible in our hands.

Results:

We are experimenting with a number of different ways of deafening adult mice. Below in Figure **, we show pilot data generated with our collaborator John Oghalai, in which adult mice were exposed to 4 hours of broad band (4-22kHz) noise at a pressure of 98 ± 3 dB SPL. Auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE) are shown across a range of frequencies. Note the elevated thresholds in noise-exposed animals increasing significantly after 1 day, and reducing (but remaining elevated) after 7 days.

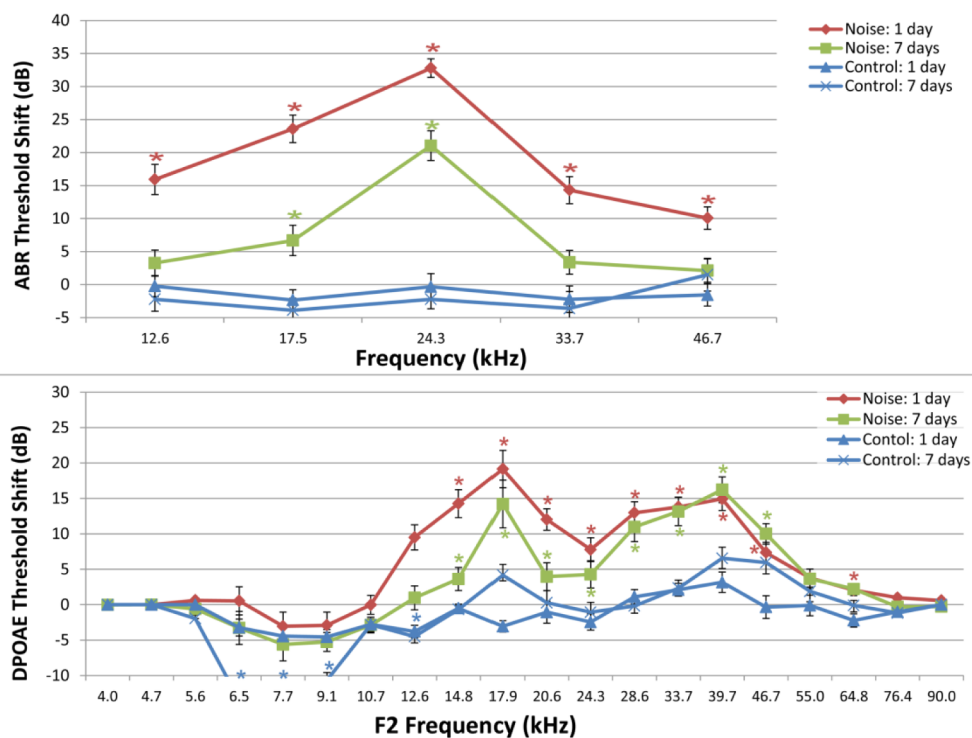


Figure **: Demonstration of noise-induced hearing loss in mice, measured by both ABR and DPOAE at 1 and 7 days post-exposure.

This deliverable has been completed as proposed.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed techniques to purify hair cells to >95% purity
- Generated a validated list of genes expressed in hair cells using microarray analysis
- Generated a high-quality RNA-Seq library from hair cells.
- Generated a targeting construct to conditionally activate Atoh1 in any cell or tissue type in the mouse.
- Established a culture and killing system for the neonatal organ of Corti
- Demonstrated that inhibition of Notch signaling is able to generate new hair cells in the mouse cochlea, BUT that this ability rapidly declines after birth.

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 also 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.

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. Year 1 of the project has been spent in optimizing the techniques and reagents used to address these Aims.

We have established expression databases of genes expressed in hair cells using two different methods. Our first attempts to determine which of these genes are direct targets of Atoh1 were confounded by a poor signal-to-noise ratios in the ChIP-sequencing data. However, we have initiated a modified strategy to significantly improve our signal-to-noise ratio. We have established methods for culturing and damaging the postnatal organ of Corti and for deafening adult mice. Significantly, we have shown that one approach to activating Atoh1 in the organ of Corti by inhibition of the Notch signaling pathway is extremely dependent on the age of the animal used. In the coming year, we propose to modify our approach to understand the molecular changes that underlie this age-dependent decrease in the effectiveness of this potentially therapeutic approach.

Finally, we have begun to assemble transgenic mice that we will use to genetically activate Atoh1 in damaged adult mice. Some of our first mouse lines proved to be unsuccessful, but we have now selected different mouse lines to use and are also in the process of generating alternative lines in our laboratory as laid out in our original proposal.