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**PRINCIPAL INVESTIGATOR:** Brandon Cox, PhD

**RECIPIENT:** Southern Illinois University School of Medicine, Springfield, IL 62794

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**6. AUTHOR(S)**
**Brandon C. Cox,** Melissa M. McGovern, Yuanzhao L. Darcy, Michelle R. Randle and Kaley A. Graves

E-Mail: bcox@siumed.edu

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**14. ABSTRACT**

Hearing loss and tinnitus commonly occur after exposure to intense or prolonged levels of loud noise which are inherent to military settings. These noise exposures damage and kill sensory hair cells (HCs) found in the cochlea of the inner ear, resulting in permanent hearing loss and tinnitus. Regeneration of auditory HCs and recovery of hearing function naturally occurs in non-mammalian vertebrates. In contrast, auditory HCs are not replaced after damage in humans and other mature mammals. However, we have recently discovered that the neonatal mouse cochlea is capable of spontaneous HC regeneration. Understanding the molecular mechanism that allows spontaneous HC regeneration to occur in neonatal mice is the first step to stimulating successful HC regeneration in adults and translating these findings to humans who suffer from hearing loss. Our central hypothesis is that following HC damage in the neonatal mouse cochlea, Notch-mediated lateral inhibition is removed which causes a decrease in Notch signaling in supporting cells, allowing them to change cell fate and become HCs. We are also investigating the relationship between individual Notch ligands and target genes in the undamaged, neonatal cochlea to understand which ones control supporting cell to HC conversion. Data collected from year 1 of this award shows changes in Notch signaling following HC damage in the neonatal mouse cochlea using different techniques. Interestingly we observed loss of Notch signaling in three supporting cell subtypes (pillar cells, Deiters' cells and Hensen cells), but not in other subtypes. We also present preliminary data on the effect of deleting the Notch ligand Jagged1 from supporting cells in the neonatal, undamaged cochlea.

**15. SUBJECT TERMS**
Hearing loss, tinnitus, hair cell regeneration, supporting cells, mouse genetics, Notch, Hes5, Jagged1

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## TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Hearing loss and tinnitus commonly occur after exposure to intense or prolonged levels of loud noise which are inherent to military settings. These noise exposures damage and kill sensory hair cells (HCs) found in the cochlea of the inner ear, resulting in permanent hearing loss. High-level noise exposure is also the most common cause of tinnitus. When HCs are killed, the loss of auditory input causes changes in central auditory pathways which generate the phantom sounds that are associated with tinnitus such as ringing in the ears. In addition, the blast wave, which causes Traumatic Brain Injury, also kills HCs and can result in hearing loss and/or tinnitus. Regeneration of auditory HCs and recovery of hearing function naturally occurs in non-mammalian vertebrates such as birds, fish, and amphibians. In contrast, auditory HCs are not replaced after damage in humans and other mature mammals. However, we have recently discovered that the neonatal mouse cochlea is capable of spontaneous HC regeneration. Understanding the molecular mechanism that allows spontaneous HC regeneration to occur in neonatal mice is the first step to stimulating successful HC regeneration in adults and translating these findings to humans who suffer from hearing loss. Our work utilizes sophisticated mouse genetics and *in vivo* approaches to investigate the Notch signaling pathway during spontaneous HC regeneration in the neonatal mouse cochlea. Our central hypothesis is that following HC damage in the neonatal mouse cochlea, Notch-mediated lateral inhibition is removed which causes a decrease in Notch signaling in supporting cells, allowing them to change cell fate and become HCs, but the division of supporting cells is not regulated by the Notch pathway. We are also investigating the relationship between individual Notch ligands and target genes in the undamaged, neonatal cochlea to understand which ones control supporting cell to HC conversion. Data collected from year 1 of this award shows changes in Notch signaling following HC damage in the neonatal mouse cochlea using different techniques. Interestingly we observed loss of Notch signaling in three supporting cell subtypes (pillar cells, Deiters' cells and Hensen cells), but not in other subtypes. We also present preliminary data on the effect of deleting the Notch ligand Jagged1 from supporting cells in the neonatal, undamaged cochlea.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Hearing loss, tinnitus, hair cell regeneration, supporting cells, mouse genetics, Notch, Hes5, Jagged1

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

**What were the major goals of the project?**
*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*

**Specific Aim 1:** To measure changes in Notch signaling after hair cell (HC) damage in the neonatal mouse cochlea. Goals for Y1 were:

1. Task 1: Obtain institutional and DOD approval of the animal protocol. Timeline: months 1-3
    *a.* Completed in Y1,Q1. The Southern Illinois University School of Medicine (SIUSOM) IACUC approved our animal protocol on 30-JUL-2015 and the USAMRMC ACURO approved the protocol on 06-OCT-2015.
2. Task 2: Measure global changes in Notch target genes in the whole cochlea using real-time qPCR. Timeline: months 3-7
    *a.* 75% complete. We tested 36 sets of primers for genes in the Notch pathway and found that only 12 primer sets are specific for cDNA detection. We completed standard curves for these 12 primer sets so that primer efficiency can be incorporated into the data analysis. As presented below, we have data for all 12 genes at the 3 proposed timepoints, however the N value is low and the experiments need to be repeated.
3. Task 3: Measure changes in Notch target genes specifically in supporting cells using immunostaining and *in situ* hybridization. Timeline: months 3-14
    a. 40% complete. We have completed immunostaining for the Notch ligand Jag1 and tested 8 other antibodies for genes in the Notch pathway. Unfortunately none of these 8 antibodies worked for immunostaining, but we are continuing to troubleshoot them. Recently, we have obtained a $Jag2^{LacZ}$ knockin reporter mouse where LacZ expression is controlled by the endogenous Jag2 promoter (another Notch ligand). We are breeding this mouse with our model to measure changes in Jag2 expression during the HC regeneration process.
    b. For *in situ* hybridization, my graduate student traveled to the University of Iowa to learn *in situ* hybridization from Dr. Bernd Fritzsch's lab who are experts in this method using cochlear tissue. We have also obtained probes for 5 genes in the Notch pathway and are in the process of preparing them for the *in situ* experiments.
4. Task 4: Measure changes in the number of Hes5-LacZ+ cells using immunostaining. Timeline: months 3-9
    a. Completed in Y1,Q3. We have measured changes in expression of the Notch target gene Hes5 (using a Hes5-LacZ reporter mouse) in 3 different subsets of supporting cells and at 3 timepoints using immunostaining.

**Specific Aim 2:** To maintain active Notch signaling in supporting cells in the context of HC damage. Goals for Y1 were:

1. Task 1: Obtain the needed mouse lines for Aim 2A & 2B (Sox10-rtTA & TetO-NICD). Timeline: months 3-6
    a. Completed in Y1,Q2. Both mouse lines were received at SIUSOM in DEC-2015 and completed the 8 week quarantine in FEB-2016.
2. Task 2: Perform mouse breeding to obtain mice containing the proposed combination of 5 alleles (Atoh1-$CreER^{TM}$::$ROSA26^{DTA}$::Sox10-rtTA::TetO-NICD::TetO-lacZ). Timeline: months 6-24

a. Ongoing. Mouse breeding is well underway and the 1st pup which contains all 5 alleles was recently born. We have several different breeding strategies and many breeding cages to ensure that enough control and experimental mice are obtained.

**Specific Aim 3A:** To conditionally delete Delta-like1 (Dll1) from neonatal HCs in the normal, undamaged neonatal cochlea. Goals for Y1 were:

1. Task 1: Obtain the needed mouse line for Aim 3A ($Dll1^{loxP}$). Timeline: months 3-6
    a. Completed in Y1,Q4. The cryorecovery process of $Dll1^{loxP}$ mice was successful and mice were received at SIUSOM in AUG-2016.
2. Task 2: Perform mouse breeding to obtain mice containing the desired genotype (Atoh1-CreER::$Dll1^{loxP/loxP}$). Timeline: months 7-12
    a. 50% complete. Despite the fact that shipment of $Dll1^{loxP}$ mice to SIUSOM was delayed, task 2 was started on time. As compensation for the delayed shipment of $Dll1^{loxP}$ mice, the vendor breed the cryorecovered $Dll1^{+/loxP}$ mice (heterozygotes) to generate $Dll1^{loxP/loxP}$ mice (homozygotes). We are currently breeding with the Atoh1-CreER line.

**Specific Aim 3B:** To conditionally delete Jagged2 (Jag2) from neonatal HCs in the normal, undamaged neonatal cochlea. Goals for Y1 were:

1. Task 1: Obtain the needed mouse line for Aim 3B ($Jag2^{loxP}$). Timeline: months 3-6
    a. Completed in Y1, Q3. $Jag2^{loxP}$ mice were received at SIUSOM in JUN-2016 and completed the 8 week quarantine in AUG-2016.
2. Task 2: Perform mouse breeding to obtain mice containing the desired genotype (Atoh1-CreER::$Jag2^{loxP/loxP}$). Timeline: months 7-12
    a. 50% complete. The $Jag2^{loxP}$ mouse line we purchased uses the Knock-out Mouse Project (KOMP) knock-out first strategy (see below), which requires the removal the LacZ and Neomycin resistance cassette to obtain the actual $Jag2^{loxP}$ allele. We have completed the 1st round of breeding to remove this sequence and have obtained the actual $Jag2^{loxP}$ allele. We are currently breeding with the Atoh1-CreER line.

**Specific Aim 3C:** To conditionally delete Jagged1 (Jag1) from neonatal SCs in the normal, undamaged neonatal cochlea. Goals for Y1 were:

1. Task 1: Obtain the needed mouse line for Aim 3C ($Jag1^{loxP}$). Timeline: months 3-6
    a. Completed in Y1, Q1. $Jag1^{loxP}$ mice were received at SIUSOM in DEC-2015.
2. Task 2: Perform mouse breeding to obtain mice containing the desired genotype (Fgfr3-iCreER::$Jag1^{loxP/loxP}$). Timeline: months 7-12
    a. Completed in Y1, Q3. We have multiple breeding pairs that routinely generate Fgfr3-iCreER::$Jag1^{loxP/loxP}$ mice and Cre-negative controls.

3. Task 3: Measure the expression level of Notch effector genes after Jag1 deletion using real time qPCR. Timeline: months 12-18
    a. 20% complete. Since Task 2 was completed ahead of schedule, we have started collecting samples for Task 3 but no experiments have been performed yet. However the qPCR primers for the Notch effector genes have been validated and the standard curves are complete.
4. Task 4: Investigate morphological changes to HCs and supporting cells in the cochlea after Jag1 deletion using immunostaining and confocal microscopy. Timeline: months 14-24
    a. 25% complete. We were also able to begin task 4 early. Our preliminary data shows that after Jag1 deletion in pillar and Deiters' cells at birth, a small number of Sox2+ cells are located in between the supporting cell nuclear layer and the HC layer at postnatal day (P) 7. Some of these cells were also observed in control samples. We are investigating this phenotype further and will also examine morphological changes at P30 (see below).
5. Task 5: Measure hearing after Jag1 deletion at P30 using ABR. Timeline: months 24-27
    a. 40% complete. We were also able to begin task 5 early and preliminary data shows hearing loss at P30 after Jag1 deletion in pillar and Deiters' cells at birth. We collected the cochlea after ABR was complete to examine the morphological changes at P30.

**What was accomplished under these goals?**

*For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

**Specific Aim 1:** To measure changes in Notch signaling after hair cell (HC) damage in the neonatal mouse cochlea.

Our model of spontaneous HC regeneration in the neonatal mouse cochlea uses the Cre/loxP system to kill HCs because noise and ototoxic drugs are problematic in neonates. Specifically, we use a HC-specific inducible Cre line, Atoh1-CreER, to drive expression of diphtheria toxin fragment A (DTA) using the ROSA26-loxP-stop-loxP-DTA ($Rosa26^{DTA}$) mouse line. When tamoxifen is administrated at postnatal day (P) 0 and P1 to Atoh1-CreER::$Rosa26^{DTA}$ mice, CreER is activated in ~80-90% of HCs (Chow et al., 2006; Weber et al., 2008) causing expression of DTA and HC death. We then observed the formation of regenerated HCs between P2-P6 (Cox et al., 2014).

To investigate global changes in the Notch signaling pathway during the spontaneous HC regeneration process, we performed real-time qPCR with whole cochlear samples obtained from Atoh1-CreER::$Rosa26^{DTA}$ mice. Controls without HC

damage were obtained from littermates that lacked either the Cre or DTA allele. Using cDNA and gDNA from wildtype mice, primer sets were first validated to determine their specificity for cDNA detection and then standard curves were completed to measure the efficiency of each primer set. Using SYBR green and the Pfaffl method of analysis which incorporates primer efficiencies into the ΔΔCt equation (Pfaffl, 2001), we measured changes in gene expression for Notch ligands (Delta1 (DLL1), Jagged1 (Jag1), and Jagged2 (Jag2)), Notch receptors (Notch1 and Notch2), Notch target genes (Hey1, Hey2, HeyL, Hes1, Hes5, and RBP-J), and a Notch co-activator (mastermind-like2 (MAML2)) at P2, P4, and P6 (Figure 1). Statistical analysis has not been completed yet since N values are low (ranging from 2-6). However there appears to be an increase in expression of the Notch ligand Jag2 at P2 and the Notch target gene Hes1 at P6 after HC damage. There may also be a decrease in expression of the Notch target gene Hey2 and the Notch co-activator MAML2 at P2, as well as the Notch ligand Dll1 at P6. These results suggest that regulation of the Notch signaling pathway during the HC regeneration process is more complex than just downregulation.

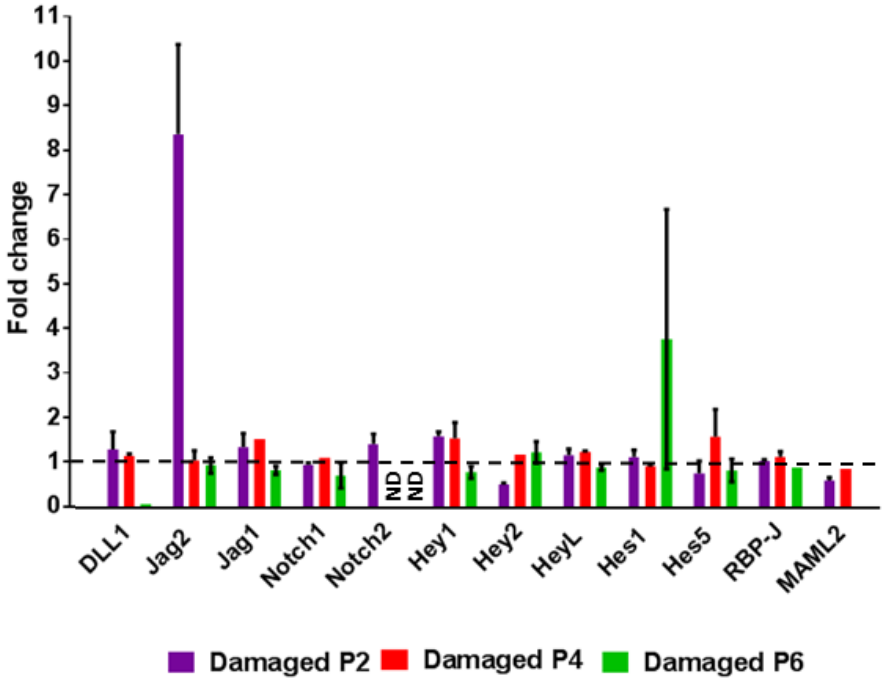



**Figure 1: Gene expression changes in the Notch pathway during the HC regeneration process.** Relative mRNA expression changes for genes in the Notch pathway analyzed in whole cochlear samples from undamaged (control) and HC damaged (Atoh1-CreER::Rosa26$^{DTA}$) mice at P2, P4, and P6 using real-time qPCR. Rpl19 was used as a housekeeping gene, data was analyzed using the Pfaffl method, and data are expressed as fold change (mean ± SEM) compared to littermate control samples (dashed line). For Notch2, the P4 and P6 timepoints were not done (ND). N=2-6.

To measure changes in Notch-related genes specifically in supporting cells we are using immunostaining and *in situ* hybridization. We have tested 9 antibodies for various genes in the Notch pathway and the only antibody that has worked so far for immunostaining is for the Notch ligand Jag1. In control samples (CTRL), Jag1 was expressed in the membranes of the supporting cell subtypes that surround HCs (Deiters' cells, pillar cells, and inner phalangeal cells) (Figure 2A-C). However after HC damage in Atoh1-CreER::Rosa26$^{DTA}$ mice (Dmg), Jag1 expression was lost in the Deiters' cell region between P2-P6 (Figure 2D-L). This suggests that Notch signaling is decreased in Deiters' cells which may allow them to change cell fate and convert into HCs.

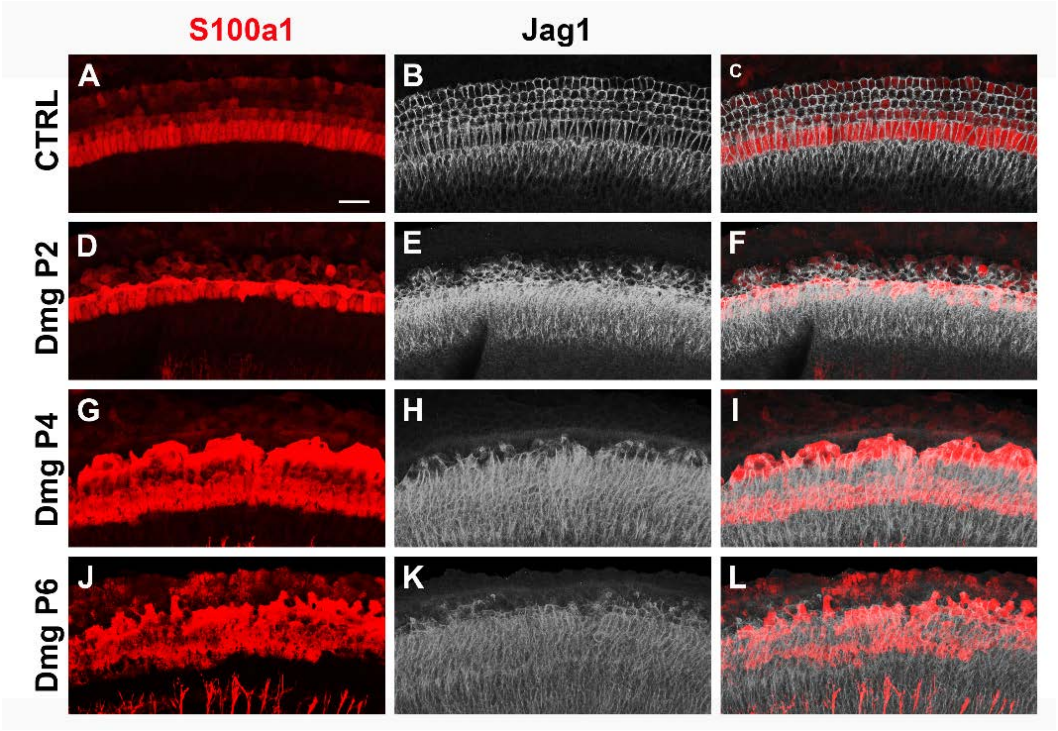


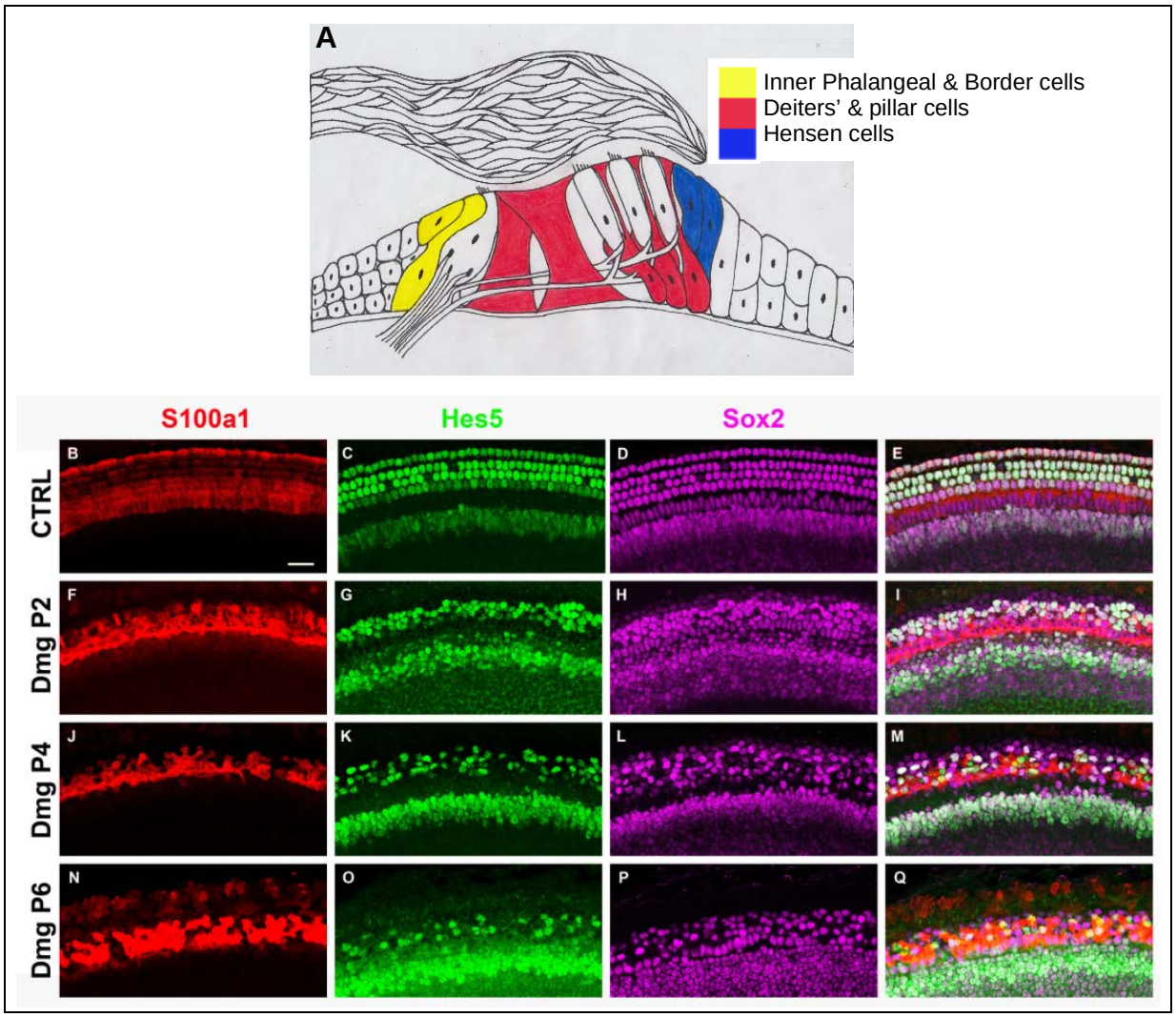

**Figure 2: Jag1 expression is decreased in the Dieters' cell region after HC damage in the neonatal mouse cochlea.** Atoh1-CreER::ROSA26$^{DTA}$ mice were injected with tamoxifen at P0-P1 to induce HC death and cochleae were collected at P2, P4 or P6. Anti-Jag1 antibodies were used to detect Jag1 expression (white) and anti-S100a1 (red) antibodies were used to mark the pillar and Deiters' cell region. After HC damage, Jag1 expression was reduced in the Deiters' cell region. Scale bar = 25 μm. CTRL, control; Dmg, Damaged.

We are continuing to troubleshoot the 8 antibodies that do not work for immunostaining, as well as look for new antibodies to test. Recently, we have obtained a Jag2$^{LacZ}$ knockin reporter mouse where LacZ expression is controlled by the endogenous Jag2 promoter and will report changes in Jag2 expression (another Notch ligand) during the HC regeneration process. We are currently breeding to generate Atoh1-CreER::Rosa26$^{DTA}$::Jag2$^{LacZ+/-}$ mice for this analysis.

For the *in situ* hybridization experiments, my graduate student traveled to the University of Iowa to learn *in situ* hybridization from Dr. Bernd Fritzsch's lab who are experts in this method using cochlear tissue. We have also obtained probes for 5 genes in the Notch pathway from other labs and are in the process of preparing them for the *in situ* experiments. We have also collected samples to use for the *in situ* experiments.

To measure changes in expression of the Notch target gene Hes5 at the cellular level during the HC regeneration process, we used the Hes5$^{nlsLacZ}$ mouse line where the LacZ gene was knocked into the endogenous Hes5 locus and is controlled by the endogenous Hes5 promoter (Imayoshi et al., 2010). We used anti-Sox2 antibodies to label all supporting cells and anti-S100a1 antibodies to label Deiters' and pillar cells, two supporting cell subtypes that are located underneath outer HCs. We then quantified Hes5 and Sox2 expression in 3 subsets of supporting cells; 1) cells within the band of S100a1 labeling (Deiters' and pillar cells); 2) cells medial to the S100a1 band (inner phalangeal

and border cells); and 3) cells lateral to the S100a1 band (Hensen cells) (Figure 3A). There was a decrease in the number of Hes5-positive Deiters' and pillar cells at P2, P4, and P6 in Atoh1-CreER::Rosa26$^{DTA}$::Hes5$^{nlsLacZ+/-}$ (Dmg) mice compared to control (CTRL)(Figure 3B-R). However we only observed a decrease in Sox2-positive cells at P4 and P6 (Figure 3B-R). We also observed an increase in the number Deiters' and pillar cells that were Sox2-positive, but Hes5-negative at P2 in Atoh1-CreER::Rosa26$^{DTA}$:: Hes5$^{nlsLacZ+/-}$ mice compared to control (Figure 3B-R). This suggests that Deiters' and pillar cells are still present, but have lost Hes5 expression and therefore have reduced Notch signaling. However when the same analysis was conducted on the inner phalangeal and border cell population, there was no significant difference in Hes5 or Sox2 labeled cells between experimental and control samples (Figure 3S). For analysis of Hensen cells, only Sox2 expression was quantified because Hes5 is not expressed in these cells. After HC damage, there was a decrease in the number of Sox2-positive Hensen cells at P4 and P6 (Figure 3T). Taken together, our data suggest that the Deiters' cells, pillar cells, and Hensen cells are the source of spontaneously regenerated HCs in the neonatal mouse cochlea and that Deiters' and pillar cells have reduced Notch signaling after HC damage.



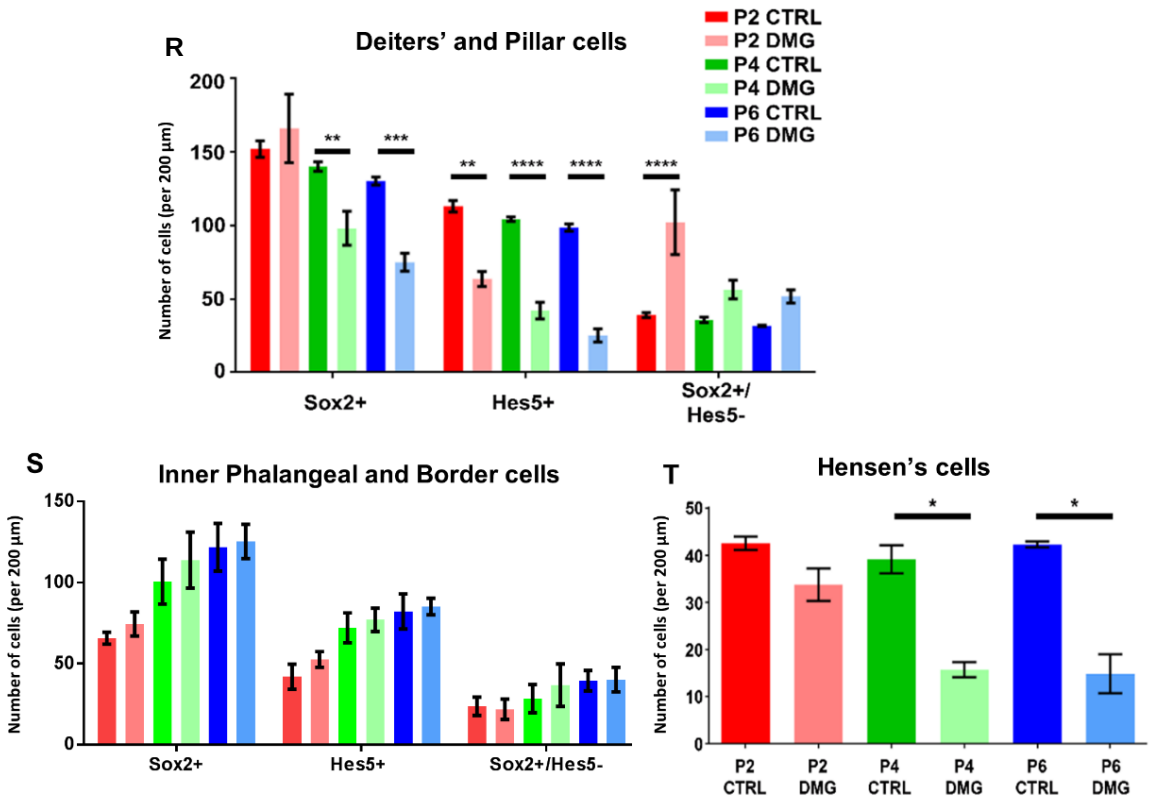



**Figure 3. Hes5 expression is decreased in Deiters' and pillar cells, but maintained in inner phalangeal and border cells after HC damage in the neonatal mouse cochlea. (A)** SCs were divided into three subtypes based on expression of S100a1 (red), which is expressed in Deiters' and pillar cells. The row of Sox2-positive cells lateral to S100a1 are Hensen cells (blue) and the cells medial to S100a1 are inner phalangeal and border cells (yellow). **(B-Q)** Atoh1-CreER::Rosa26$^{DTA}$::Hes5$^{nlsLacZ+/-}$ (Dmg) mice were injected with tamoxifen at P0-P1 to induce HC death and cochleae were collected at P2, P4 or P6. Anti-βgal antibodies were used to detect Hes5-LacZ expression (green), anti-Sox2 antibodies were used to label all SCs (magenta), and anti-S100a1 antibodies were used to mark the Deiters' and pillar cell region (red). Scale bar = 25 µm **(R)** In the Deiters' and pillar cell region, fewer Sox2-positive cells were detected at P4 and P6 after HC damage, while a decreased number of Hes5-positive cells were detected at all three ages. Only at P2 were there significantly more Sox2-positive/Hes5-negative cells detected. **(S)** There was no change in the number of Sox2-positive, Hes5-positive, or Sox2-positive/Hes5-negative cells in the inner phalangeal and border cells region. **(T)** Hes5-LacZ is not expressed in Hensen cells, so only Sox2 quantification was performed. After HC damage, the number of Sox2-positive Hensen cells was reduced at P4 and P6. Data are expressed as mean ± SEM for a N= 4-5 for all. * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Stated goals not met: For Aim1, tasks 2 and 3 are behind schedule. We need to increase the N value for the real-time qPCR experiments for all genes and all timepoints (Task 2). We anticipate that this will be completed in Y2, Q1. For the immunostaining and *in situ* hybridization experiments (Task 3), mouse breeding and troubleshooting of antibodies will take several months, and *in situ* experiments are just starting. We anticipate this task to continue throughout year 2.

**Specific Aim 2:** To maintain active Notch signaling in supporting cells in the context of HC damage**.**

We hypothesize is that following HC damage in the neonatal mouse cochlea, Notch-mediated lateral inhibition is removed which causes a decrease in Notch signaling in supporting cells, allowing them to change cell fate and become HCs, but the division of supporting cells is not regulated by the Notch pathway. To test this hypothesis, we plan to keep Notch signaling active in supporting cells in the context of HC damage by ectopically expressing the Notch intracellular domain (NICD) in supporting cells. If Notch signaling is the molecular mechanism that underlies spontaneous HC regeneration, we predict this gain of function study will prevent supporting cells from converting into HCs. Since spontaneous HC regeneration in the neonatal mouse cochlea can also occur by mitotic regeneration, where supporting cells divide and one or more daughter cells differentiates into a HC (Bramhall et al., 2014; Cox et al., 2014), we will also use BrdU injections to investigate the effects of ectopic NICD expression on cell division.

We are breeding mice to combine our established mouse model, which uses the Cre/loxP system to kill HCs (Atoh1-CreER::Rosa26$^{DTA}$ mice), with the Tet-On system to ectopically express NICD in supporting cells and maintain active Notch signaling (Sox10-rtTA::TetO-NICD mice). We will also breed in the TetO-LacZ reporter to allow fate-mapping of supporting cells that have active Notch signaling. Specifically, we will generate Atoh1-CreER::Rosa26$^{DTA}$::Sox10-rtTA::TetO-NICD::TetO-LacZ mice, give tamoxifen injections at P0/P1 to induce HC damage, and simultaneously give Dox from P0-P6 (administered via the food to the nursing mother) to ectopically express NICD and LacZ in supporting cells. To obtain these 5 alleles in the same mouse, we have performed several rounds of breeding to generate parents which contain 2-4 alleles. The 1$^{st}$ pup which contains all 5 alleles was recently born and we are in the process of analyzing its cochlea. We have several different breeding strategies and many breeding cages to ensure that enough control and experimental mice are obtained.

Stated goals not met: All Aim 2 tasks are on schedule.

**Specific Aim 3A:** To conditionally delete Delta-like1 (Dll1) from neonatal HCs in the normal, undamaged neonatal cochlea.

The cryorecovery process of Dll1$^{loxP}$ mice was successful and mice were received at SIUSOM in AUG-2016. We are currently breeding mice to obtain the needed genotype (Atoh1-CreER::Dll1$^{loxP/loxP}$).

Stated goals not met: For Aim 3A, task 2 is behind schedule. Mice are currently breeding and we anticipate the needed genotype will be obtained in Y2, Q1.

**Specific Aim 3B:** To conditionally delete Jagged2 (Jag2) from neonatal HCs in the normal, undamaged neonatal cochlea.

Jag2$^{loxP}$ mice were received at SIUSOM in JUN-2016 and completed the 8 week quarantine in AUG-2016. However the Jag2$^{loxP}$ mouse line we purchased uses the KOMP knock-out first strategy (explained in Figure 4). We currently have the Tm1a allele which

needs to be bred with a Flp mouse (driven by a ubiquitously expressed promoter) to delete the LacZ and Neomycin resistance cassette (flanked by FRT sites). This will generate the Tm1c allele where loxP sites flank a critical exon of Jag2 (i.e. $Jag2^{loxP}$ -- the allele we need for Aim 3B) allowing Cre-mediated deletion of Jag2. We have completed this 1st round of breeding using a ROSA26-Flp mouse to generate the actual $Jag2^{loxP}$ allele (Tm1c allele). We are currently breeding $Jag2^{loxP}$ with the Atoh1-CreER line to obtain the needed genotype (Atoh1-CreER::$Jag2^{loxP/loxP}$).

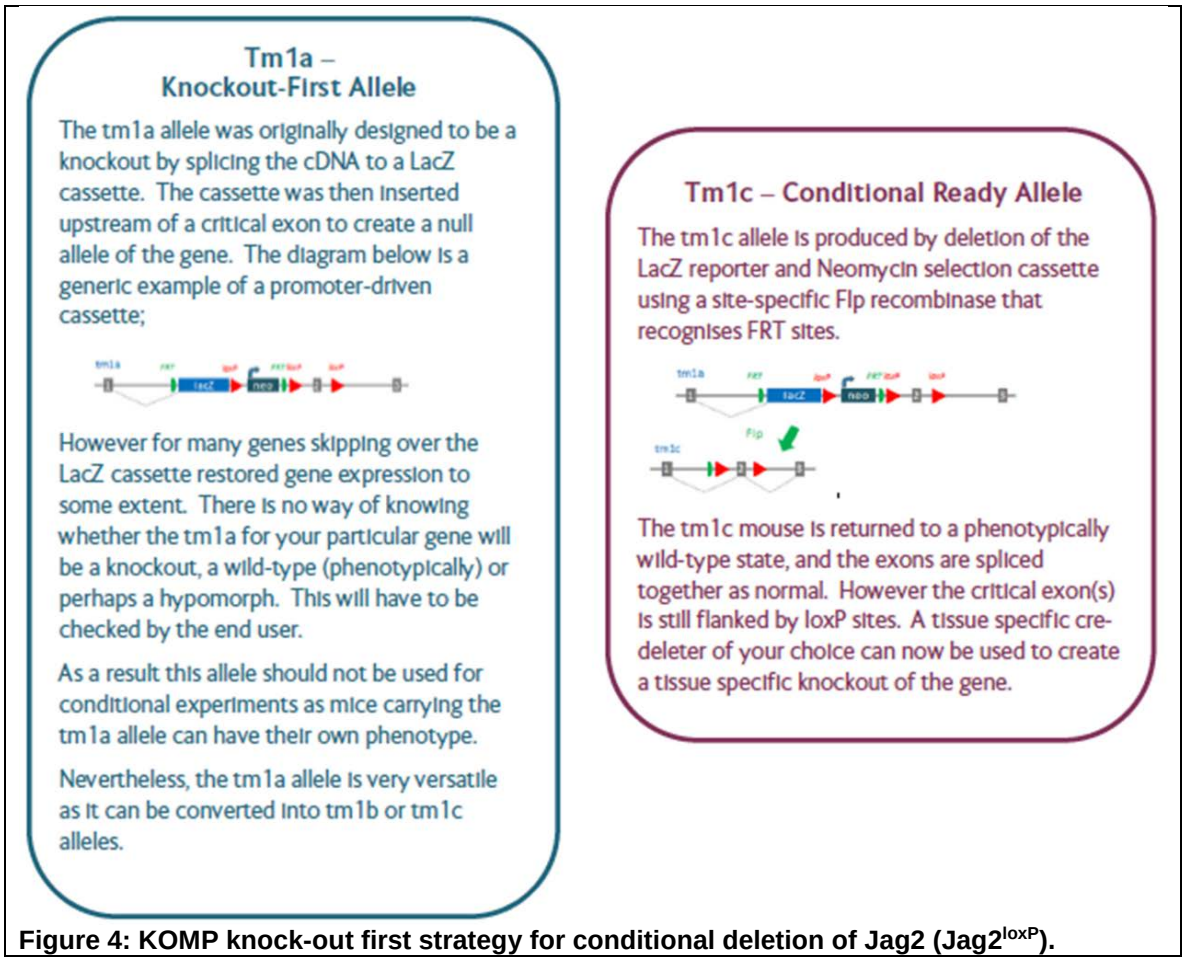



**Figure 4: KOMP knock-out first strategy for conditional deletion of Jag2 ($Jag2^{loxP}$).**

The $Jag2^{loxP}$ Tm1a allele can also be converted into a $Jag2^{LacZ}$ knockin reporter mouse (explained in Figure 5) which would be very useful for our Aim 1 since none of the Jag2 antibodies we have tested work for immunostaining. To generate the Tm1b allele ($Jag2^{LacZ}$), we bred the Tm1a allele with a CMV-Cre mouse (where Cre is driven by a ubiquitously expressed promoter). This will delete the Neomycin resistance cassette and a critical exon of Jag2 (flanked by loxP sites). Therefore LacZ expression will be controlled by the endogenous Jag2 promoter and will report changes in Jag2 expression during the HC regeneration process. We have completed this 1st round of breeding to generate the $Jag2^{LacZ}$ allele (Tm1b allele). We are currently breeding to generate Atoh1-CreER::$Rosa26^{DTA}$::$Jag2^{LacZ+/-}$ mice for Aim 1.

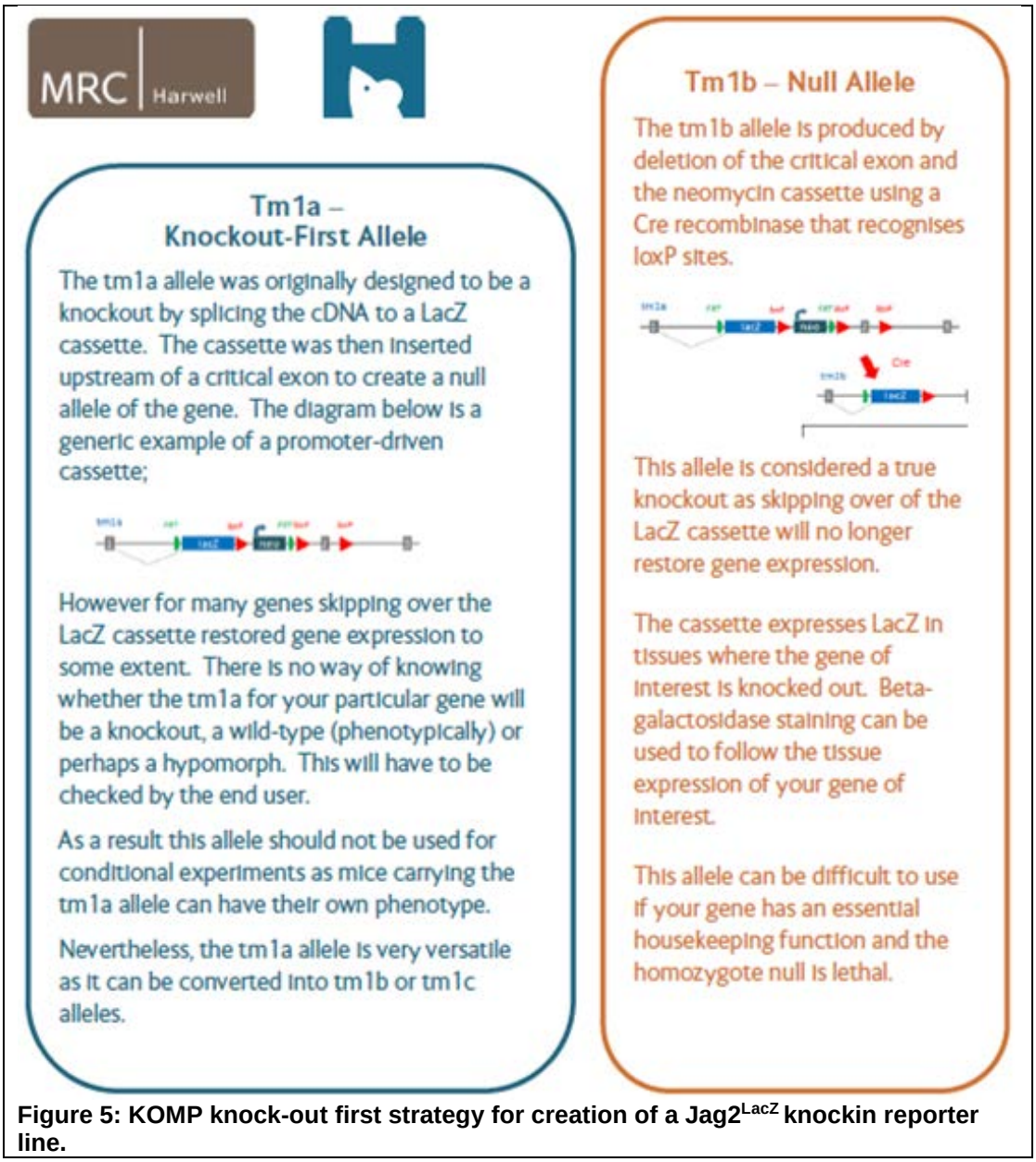



**Figure 5: KOMP knock-out first strategy for creation of a Jag2$^{LacZ}$ knockin reporter line.**

Stated goals not met: For Aim 3B, task 2 is behind schedule. Mice are currently breeding and we anticipate the needed genotype will be obtained in Y2, Q1.

**Specific Aim 3C:** To conditionally delete Jagged1 (Jag1) from neonatal SCs in the normal, undamaged neonatal cochlea.

Jag1$^{loxP}$ mice were received at SIUSOM in DEC-2015. Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice were injected with tamoxifen at P0/P1 (to delete Jag1 in pillar and Deiters' cells) and collected at P7 for analysis of morphological changes to HCs and supporting cells and to confirm deletion of Jag1 using immunostaining and confocal microscopy. Compared to control samples (Cre-negative littermates), cochlea from Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice showed minor changes in Jag1 expression at P7. In controls, Jag1 was located in the

membranes of supporting cells (Figure 6A) and there appeared to be more Jag1 in the cytoplasm of Fgfr3-iCreER::Jag1$^{loxP/loxP}$ samples (Figure 6B). Perhaps the Jag1 protein made prior to tamoxifen injection was being degraded in the cytoplasm. We will have collected samples to measure changes in Jag1 expression at the RNA level using real-time qPCR.

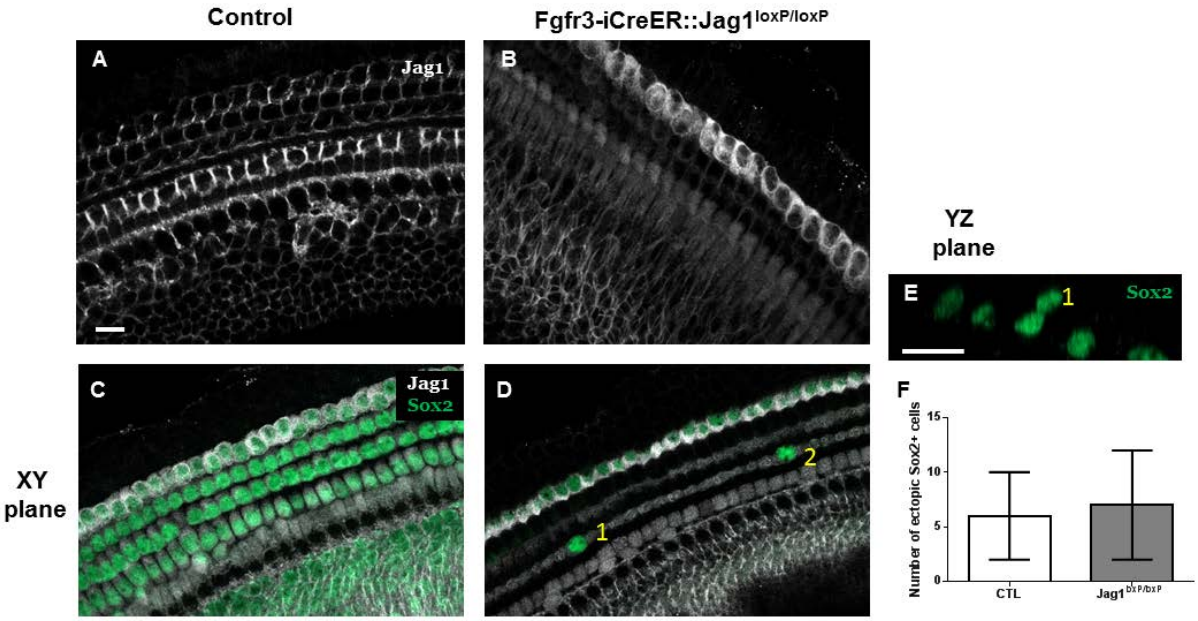



**Figure 6. Mislocalization of some Sox2+ cells after deletion of Jag1 in the neonatal mouse cochlea.** Control (Cre-negative) **(A)** and Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice **(B, D-E)** were injected with tamoxifen at P0-P1 and cochleae were collected at P7. **(A-B)** Jag1 (white) expression pattern. **(C)** XY view of the supporting cell nuclear layer where supporting cell nuclei are labeled by Sox2 (green). **(D)** XY view of area between the supporting cell nuclear layer and the HC layer. 1 & 2 label ectopic Sox2-positive cells (green). **(E)** YZ view of Sox2-positive cells (green) where the ectopic Sox2-positive cell (labeled as 1 in D) is located above the normal supporting cell nuclear layer. Scale bars = 10 μm. **(F)** Quantification of ectopic Sox2-positive cells in control and Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice. Data are expressed as mean ± SEM for a N=2.

Using immunostaining, we observed organized rows of HCs and supporting cell nuclei in both control and Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice (Figure 6C). However we also observed a small number of Sox2-positive nuclei in between the supporting cell nuclear layer and the HC layer (Figure 6D-E). This was seen in both control and Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice (Figure 6F), however it has not been reported previously in the literature. We plan to follow up on this finding by increasing the N value and also looking at older samples.

We also measured hearing after deletion of Jag1 in pillar and Deiters' cells at P30 using auditory brainstem response (ABR). Statistical analysis has not been completed yet since N values are low (ranging from 2-3). However there appears to be an increase in ABR thresholds at all frequencies tested in Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice that were injected with tamoxifen at P0/P1 compared to littermate controls that have intact Jag1 expression (Figure 7). We are repeating these experiments and have also collected the cochlea after ABR was complete to examine the morphological changes at P30.

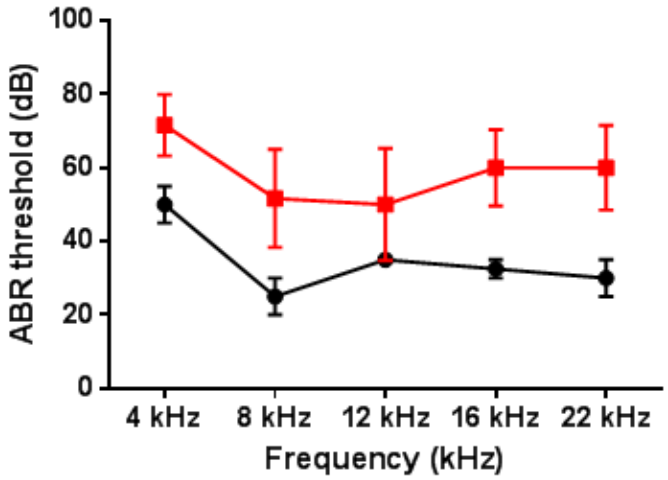



**Figure 7. Hearing ability after deletion of Jag1 in the neonatal mouse cochlea.** Control (Cre-negative) **(black line)** and Fgfr3-iCreER::Jag1$^{loxP/loxP}$ mice **(red line)** were injected with tamoxifen at P0-P1 and ABR was performed at P30. Data are expressed as mean ± SEM for a N=2-3.

Stated goals not met: For Aim 3C, all tasks are ahead of schedule.

## References


Bramhall, N.F., Shi, F., Arnold, K., Hochedlinger, K., and Edge, A.S.B. (2014). Lgr5-positive supporting cells generate new hair cells in the postnatal cochlea. *Stem Cell Reports* 2, 311-322.

Chow, L.M., Tian, Y., Weber, T., Corbett, M., Zuo, J., and Baker, S.J. (2006). Inducible Cre recombinase activity in mouse cerebellar granule cell precursors and inner ear hair cells. *Dev Dyn* 235, 2991-2998.

Cox, B.C., Chai, R., Lenoir, A., Liu, Z., Zhang, L., Nguyen, D., Chalasani, K., Steigelman, K.A., Fang, J., Rubel, E.W., et al. (2014). Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development* 141, 816-829.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. *J Neurosci* 30, 3489-3498.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29, e45.

Weber, T., Corbett, M.K., Chow, L.M., Valentine, M.B., Baker, S.J., and Zuo, J. (2008). Rapid cell-cycle reentry and cell death after acute inactivation of the retinoblastoma gene product in postnatal cochlear hair cells. *Proc Natl Acad Sci U S A* 105, 781-785.

**What opportunities for training and professional development has the project provided?**
*If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

**Training**
1) Melissa McGovern, a graduate student supported by this award, traveled to the University of Iowa to learn *in situ* hybridization from Dr. Bernd Fritzsch's lab who are experts in this method using cochlear tissue.

2) Yunazhao Lv Darcy, a postdoc hired for the project was mentored by Dr. Cox to learn all the techniques needed for the project such as mouse breeding, genotyping, cochlear dissection, immunostaining, confocal microscopy, and ABR.

**Professional Development**
Dr. Cox and all members of the lab supported by this award attended the Association for Research in Otolaryngology 39th annual midwinter meeting held 20-24-FEB-2016 in San Diego, CA.

**How were the results disseminated to communities of interest?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

Nothing to report

**What do you plan to do during the next reporting period to accomplish the goals?**
*If this is the final report, state "Nothing to Report."*

*Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

Our goals for the next reporting period are:
1) Complete the real-time qPCR experiments for genes in the Notch pathway (Aim 1, Task 2).
2) Obtain preliminary data for changes in Notch target genes specifically in supporting cells using *in situ* hybridization (Aim 1, Task 3).
3) Begin writing the manuscript describing the results obtained from Aim 1 (Task 5).
4) Confirm active Notch signaling in supporting cells in the proposed mouse model (Atoh1-CreER$^{TM}$::ROSA26$^{DTA}$::Sox10-rtTA::TetO-NICD::TetO-lacZ) using real time qPCR. (Aim 2, Task 3).
5) Obtain preliminary data for the HC regeneration capacity in the proposed mouse model where Notch signaling is kept active in supporting cells and HC are killed (Aim 2, Task 4).
6) Investigate morphological changes to HCs and supporting cells in the cochlea after Dll1 deletion using immunostaining and confocal microscopy (Aim 3A, Task 4).
7) Investigate morphological changes to HCs and supporting cells in the cochlea after Jag2 deletion using immunostaining and confocal microscopy (Aim 3B, Task 4).
8) Measure the expression level of Notch effector genes after Jag1 deletion using real time qPCR (Aim 3C, Task 3).
9) Continue to investigate morphological changes to HCs and supporting cells in the cochlea after Jag1 deletion using immunostaining and confocal microscopy. (Aim 3C, Task 4).
10) Continue to measure hearing in mice after Jag1 deletion at P30 using ABR. (Aim 3C, Task 5).

**4. IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

**What was the impact on the development of the principal discipline(s) of the project?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

Nothing to Report

**What was the impact on other disciplines?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

Nothing to Report

**What was the impact on technology transfer?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

**What was the impact on society beyond science and technology?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

**5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

1) Unfortunately the power supply of our real-time qPCR machine failed in Y1, Q3 and the machine was sent back to the vendor for repairs. Therefore we were not able to complete Aim 1, Task 2 on time. The repaired machine was returned at the end of Q3 and we have validated that it works properly and resumed the experiments. Due to this setback, the timeline for Aim 1, Task 2 has been extended to include all quarters of Y1 and the 1$^{st}$ quarter of Y2.

2) The cryorecovery process of Dll1$^{loxP}$ mice was successful however shipment of mice to SIUSOM was delayed by the vendor. We received the mice AUG-2016. However Aim 3A, Task 2 began on time. As compensation for the delayed shipment of Dll1$^{loxP}$ mice, the vendor breed the cryorecovered Dll1$^{+/loxP}$ mice (heterozygotes) to generate Dll1$^{loxP/loxP}$ mice (homozygotes).

**Actual or anticipated problems or delays and actions or plans to resolve them**
*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

Yunazhao Lv Darcy, a postdoc who works on Aim 3 of the project, will go on maternity leave next year which will slow progress in Y2.

**Changes that had a significant impact on expenditures**
*Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

Two of the mouse lines needed for the study were found to be available commercially, but required cyrorecovery of frozen sperm (costing ~$6,000 each which includes shipping fees). Thus we will used an additional ~$12,000 for mouse procurement in Y1. In addition one of the needed mouse lines has a $2,500 yearly licensing fees that will need to be paid in Y2 and Y3. Despite these unexpected expenditures, we are financially on track, spending less that the budgeted amount in Y1.

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
*Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

**Significant changes in use or care of human subjects**

1) Amendment A1 to the vertebrate animal protocol was approved by SIUSOM IACUC on 18-APR-2016 and by ACURO on 11-MAY-2016. This amendment proposed the use of $Hes5^{LacZ}$ reporter mice to investigate changes in Hes5 expression (a Notch target gene) after deletion of the Notch ligands, Dll1 (Aim 3A), Jag2 (Aim 3B), and Jag1 (Aim 3C) as an addition to the planned experiments using real-time qPCR.

2) Amendment A2 to the vertebrate animal protocol was approved by SIUSOM IACUC on 7-JUL-2016 and by ACURO on 11-AUG-2016. This amendment proposed the use of CMV-Cre and ROSA26-Flp mice to convert the Jag2 Tma1 allele to the Tm1b allele ($Jag2^{loxP}$ needed for Aim 3B) and the Tm1c allele ($Jag2^{LacZ}$ reporter). The amendment also added $Jag2^{LacZ}$ reporter to Aim 1 to investigate changes in Jag2 expression (a Notch ligand) during the HC regeneration process.

No human subjects were used in research supported by this award.

**Significant changes in use of biohazards and/or select agents**

| |
|---|
| Nothing to report |

**6. PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  | |
  |---|
  | Nothing to report |

  **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  | |
  |---|
  | Nothing to report |

  **Other publications, conference papers and presentations**. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1) Poster presentation at an international conference:
McGovern MM, Randle MR, Graves KA, Darcy YL and Cox, BC (2016) Differential ability of supporting cell subtypes to regenerate hair cells in the neonatal mouse cochlea. *Association for Research in Otolaryngology:39th annual midwinter meeting*, 2016 February 20-24, San Diego, CA
2) Oral presentation at a local symposium:
McGovern MM, Randle MR, Graves KA, Darcy YL, and Cox, BC (2016) Differential ability of supporting cell subtypes to regenerate hair cells in the neonatal mouse cochlea. *26th Annual Graduate Student Research Symposium, Southern Illinois University School of Medicine,* 2016 April 29, Springfield, IL
3) Oral presentation at a local symposium:
McGovern MM, Randle MR, Graves KA, Darcy YL, and Cox, BC (2016) Differential ability of supporting cell subtypes to regenerate hair cells in the neonatal mouse cochlea. *14th Horst R. Konrad Visiting Professor & ENT Resident Research Day, Southern Illinois University School of Medicine,* 2016 June 03, Springfield, IL
4) Invited oral presentation at an international conference:
*Gordon Research Conference on the Auditory System.* Cellular and molecular mechanisms that regulate spontaneous hair cell regeneration in the neonatal mouse cochlea. July 10-15, 2016, Lewiston, ME.
5) Invited oral presentation at a University:
*Department of Otolaryngology, Washington University School of Medicine*. Cell source and mechanism of spontaneously regenerated hair cells in the neonatal mouse cochlea. February 10, 2016, St. Louis, MO.
6) Invited oral presentation at a University:
*Department of Otolaryngology, University of Washington*. Cell source and mechanism of spontaneously regenerated hair cells in the neonatal mouse cochlea. June 30, 2016, Seattle, WA.
7) Poster presentation at a ONR program review:
McGovern MM, Randle MR, Graves KA, Darcy YL and Cox, BC (2016) Differential ability of supporting cell subtypes to regenerate hair cells in the neonatal mouse cochlea. *Noise-Induced Hearing Loss Program Review, Office of Naval Research,* 2016 September 13-15, Memphis, TN.

- **Website(s) or other Internet site(s)**
  *List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*

Nothing to report

- **Technologies or techniques**
  *Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.*

| |
|---|
| Nothing to report |

- **Inventions, patent applications, and/or licenses**
  *Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.*

| |
|---|
| Nothing to report |

- **Other Products**
  *Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:*
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

| |
|---|
| Nothing to report |

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

*Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".*

*Example:*

| | |
|---|---|
| *Name:* | *Mary Smith* |
| *Project Role:* | *Graduate Student* |
| *Researcher Identifier (e.g. ORCID ID):* | *1234567* |
| *Nearest person month worked:* | *5* |
| *Contribution to Project:* | *Ms. Smith has performed work in the area of combined error-control and constrained coding.* |
| *Funding Support:* | *The Ford Foundation (Complete only if the funding support is provided from other than this award.)* |

| | |
|---|---|
| Name: | **Brandon C. Cox** |
| Project Role: | PI |
| Nearest person month worked: | 4 |
| Contribution to Project: | Responsible for all aspects of the research conducted under this award including hiring and training personnel, experimental design, data analysis and interpretation, administrative duties, managing the budget, and reporting to the DOD |
| Funding Support: | NIH R01DC01441, NIH R01DC00151, NIH R01DC13771, & ONR N00014-16-1-2306 |

| | |
|---|---|
| Name: | **Michelle R. Randle** |
| Project Role: | Researcher III (Technician) |
| Nearest person month worked: | 8 |
| Contribution to Project: | Assisted in training personnel, assisted in mouse colony maintenance, performed real-time qPCR, quantified confocal images, administrative duties, and ordered supplies |
| Funding Support: | NIH R01DC01441 & NIH R01DC13771 |

| | |
|---|---|
| Name: | **Kaley A. Graves** |
| Project Role: | Researcher I (Technician) |
| Nearest person month worked: | 9 |
| Contribution to Project: | Managed mouse colony, collected tissue samples, performed immunostaining, quantified confocal images, and assisted in training personnel |
| Funding Support: | NIH R01DC01441 |

| | |
|---|---|
| Name: | **Melissa M. McGovern** |
| Project Role: | Graduate Student |
| Nearest person month worked: | 12 |
| Contribution to Project: | Collected tissue samples, performed immunostaining and real-time qPCR, quantified confocal images, performed data analysis, and assisted in mouse colony maintenance |
| Funding Support: | None |

| | |
|---|---|
| Name: | **Yuanzhao L. Darcy** |
| Project Role: | Postdoc |
| Nearest person month worked: | 10 |
| Contribution to Project: | Collected tissue samples, performed immunostaining and ABR, quantified confocal images, performed data analysis, and assisted in mouse colony maintenance |
| Funding Support: | NIH R01DC01441 |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

I was awarded the NIDCD/NIH grant (R01 DC014441) listed as pending during the award negotiation starting 01-JUN-2016. The following changes were made:

Michelle Randle, Researcher III -- reduced to 25% effort
Kaley Ramsey, Researcher I – reduced to 50% effort
TBN, Postdoc – added at 20% (to be hired in Fall 2016)
TBN, Postdoc – added at 70% (to be hired in Year 2)

**What other organizations were involved as partners?**
*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

*Provide the following information for each partnership:*
*Organization Name:*
*Location of Organization: (if foreign location list country)*
*Partner's contribution to the project (identify one or more)*

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

1) University of Washington, Seattle, WA, in-kind support: supplied probe for *in situ* hybridization
2) University of Iowa, Iowa City, IA, in-kind support: supplied probes for *in situ* hybridization and trained graduate student on this technique
3) St. Jude Children's Research Hospital, Memphis, TN, in-kind support: supplied Sox10-rtTA mice (with permission from Dr. Wegner who made the mice)
4) University of Pennsylvania, Philadelphia, PA, in-kind support: supplied TetO-NICD mice

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

**QUAD CHARTS:** If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

## 9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

# Investigation of Notch signaling during spontaneous regeneration of cochlear hair cells

**PI:** Brandon Cox, PhD **Org:** Southern Illinois University, School of Medicine

## Study/Product Aim(s)

**Aim 1: To measure changes in Notch signaling after hair cell damage in the neonatal mouse cochlea.**
**Aim 2: To maintain active Notch signaling in supporting cells in the context of hair cell damage.**
**Aim 3: To delete Notch ligands from the organ of Corti in the undamaged cochlea.**

## Approach

The neonatal mouse cochlea provides a unique environment that is conducive for regenerated hair cells to form spontaneously and offers the opportunity to study auditory hair cell regeneration in a postnatal mammalian model. Proposed studies will utilize sophisticated mouse genetics and *in vivo* approaches to investigate the Notch signaling pathway during spontaneous hair cell regeneration in the neonatal mouse cochlea.

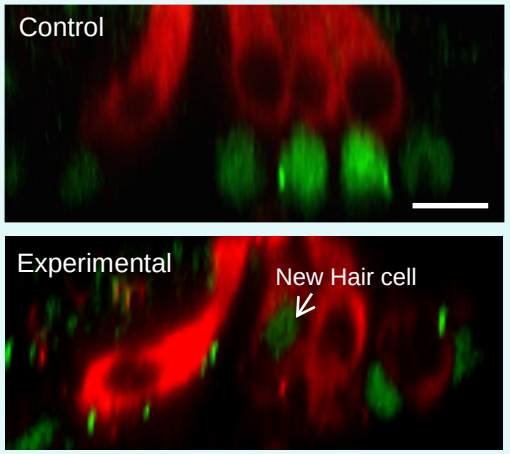



Figure Legend: Fate-mapping of supporting cells (green nuclear label). After hair cell damage at birth, new regenerated hair cells (red with green nucleus) are formed spontaneously in the neonatal mouse cochlea. Scale Bar: 10 µm

## Timeline and Cost

| Activities CY | 15-16 | 16-17 | 17-18 | |
|---|---|---|---|---|
| Aim 1 | | | | |
| Aim 2 | | | | |
| Aim 3 | | | | |
| | | | | |
| **Estimated Budget ($K)** | **$500K** | **$500K** | **$500K** | |

**Updated:** (10/13/2016)

## Goals/Milestones

**CY15-16 Goals**

☑ Perform experiments for Aim 1 where changes in Notch signaling are measured after hair cell damage – In progress
☑ Order mice & begin breeding for Aims 2 & 3

**CY16-17 Goal**

☐ Start experiments for Aim 2 where Notch signaling is maintained in supporting cells
☑ Start experiments for Aim 3 where Notch ligands are deleted from the organ of Corti
☐ Begin writing manuscripts for publication

**CY17-18 Goal**

☐ Finish experiments for Aims 2 & 3
☐ Finish preparing manuscripts for publication & submit

**Budget Expenditure to Date**

Projected Expenditure: $500K for each year
Actual Expenditure: $458,037 (Y1)

*Abstract for poster presentation at the Association for Research in Otolaryngology:39th annual midwinter meeting, 2016 February 20-24, San Diego, CA*

# Differential Ability of Supporting Cell Subtypes to Regenerate Hair Cells in the Neonatal Mouse Cochlea

Melissa M. McGovern[1], Michelle R. Randle[1], Kaley A. Graves[1], Yuanzhao L. Darcy[1], and Brandon C. Cox[1,2]
1. Department of Pharmacology,
2. Department of Surgery, Division of Otolaryngology
Southern Illinois University, School of Medicine, Springfield, Illinois

In the mammalian cochlea, five major groups of supporting cell (SC) subtypes reside in close proximity to hair cells (HCs) and may have the potential to regenerate HC after damage. These subtypes include the greater epithelial ridge, inner phalangeal/border cells, pillar cells, Deiters' cells, and Hensen/Claudius cells. During embryonic development, progenitor cells differentiate into HCs or one of the SC subtypes by Notch-mediated lateral inhibition. In the neonatal mouse cochlea, many studies have shown that inhibition of Notch signaling allows SCs to convert into HCs in both normal undamaged cochleae, as well as in drug-damaged cochlear explants. This mechanism is also implicated during spontaneous HC regeneration that occurs in non-mammalian vertebrates. We and others have recently observed that spontaneous HC regeneration can also occur in the neonatal mouse cochlea. However, little is known about the molecular mechanism or the SC subtypes which act as the source of regenerated HCs. In the neonatal mouse cochlea, HCs were killed *in vivo* by Cre-mediated expression of diphtheria toxin fragment A (DTA) using Atoh1-CreER+::Rosa26-loxP-Stop-loxP-DTA$^{+/f}$ mice (Atoh1-DTA) and tamoxifen administration at birth. Subsequently, SCs formed new HCs by either direct transdifferentiation or mitotic regeneration. To investigate whether the Notch signaling pathway was involved in HC regeneration in the neonatal mouse cochlea, we measured the expression of the Notch target gene, Hes5, using a knock-in Hes5$^{nlsLacZ}$ reporter mouse. When analyzing the total SC population, there was no significant difference in the number of Hes5-LacZ+ cells after HC damage. However, when only Deiters' cells (DCs) and pillar cells (PCs) were quantified, the number of Hes5-LacZ+ cells was significantly reduced in Atoh1-DTA:: Hes5$^{nlsLacZ}$ cochleae compared to controls without HC damage. In addition the number of Sox2+ cells in the DC/PC population increased in Atoh1-DTA::Hes5$^{nlsLacZ}$ cochleae. Therefore, we hypothesize that SC subtypes have differential abilities to regenerate HC after damage. To investigate which subtypes are capable of regenerating HCs, we are currently fate-mapping SC subtypes by combining Pou4f3$^{DTR}$ mice, where injection of diphtheria toxin will induce HC-specific damage, with several CreER lines that target different SC subtypes. Based on our Hes5 results, we predict that DC and PCs will be the main progenitor cells for HC regeneration.

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**Differential Ability of Supporting Cell Subtypes to Regenerate Hair Cells in the Neonatal Mouse Cochlea**

Melissa M. McGovern[1], Michelle R. Randle[1], Kaley A. Graves[1], Yuanzhao L. Darcy[1], and Brandon C. Cox[1,2]
1. Department of Pharmacology,
2. Department of Surgery, Division of Otolaryngology
Southern Illinois University, School of Medicine, Springfield, Illinois

One of the most common disabilities in the US, hearing loss is reported by The National Institutes of Health to affect approximately 36 million Americans. One of the major contributing factors to this loss in hearing is the loss of the sensory hair cells within the cochlea. Also in the mammalian cochlea, six major groups of supporting cell (SC) subtypes reside in close proximity to hair cells (HCs) and may have the potential to regenerate HCs after damage. These subtypes include the greater epithelial ridge, inner phalangeal/border cells, pillar cells, Deiters' cells, Hensen cells, and Claudius cells. During embryonic development, progenitor cells differentiate into HCs or one of the SC subtypes by Notch-mediated lateral inhibition. In the neonatal mouse cochlea, many studies have shown that inhibition of Notch signaling allows SCs to convert into HCs in both normal undamaged cochleae, as well as in drug-damaged cochlear explants. This mechanism is also implicated during spontaneous HC regeneration that occurs in non-mammalian vertebrates. We and others have recently observed that spontaneous HC regeneration can also occur in the neonatal mouse cochlea. However, little is known about the molecular mechanism or the SC subtypes which act as the source of regenerated HCs. In the neonatal mouse cochlea, HCs were killed *in vivo* by Cre-mediated expression of diphtheria toxin fragment A (DTA) using Atoh1-CreER+::Rosa26-loxP-Stop-loxP-DTA$^{+/f}$ mice (Atoh1-DTA) and tamoxifen administration at birth. Subsequently, SCs formed new HCs by either direct transdifferentiation or mitotic regeneration. To investigate whether the Notch signaling pathway was involved in HC regeneration in the neonatal mouse cochlea, we measured the expression of the Notch target gene, Hes5, using a knock-in Hes5$^{nlsLacZ}$ reporter mouse. When analyzing the total SC population, there was no significant difference in the number of Hes5-LacZ+ cells after HC damage. However, when SCs were quantified based on subtype, the number of Hes5-LacZ+ cells was significantly reduced in Deiters' cells and pillar cells of Atoh1-DTA::Hes5$^{nlsLacZ}$ cochleae compared to controls without HC damage. In addition, the number of Sox2+ cells in the pillar cell/Deiters' cell population was maintained in Atoh1-DTA::Hes5$^{nlsLacZ}$ cochleae until P6 at which point the number of Sox2+ pillar cells and Deiters' cells was reduced. Interestingly, there was no change in the number of Hes5-LacZ+ cells or Sox2+ cells in the inner phalangeal and border cell region. Additionally, Hensen cells were quantified based on Sox2 expression and preliminary data shows a trend towards reduction of this cell type at P4 and P6 after HC damage. Similarly, Jagged1 a SC specific Notch ligand was reduced in the lateral compartment of the cochlea, while it was maintained in the medial compartment. We conclude that Hes5, the major Notch target gene that mediates the inhibition of HC fate, as well as Jagged1, a Notch ligand, are differentially reduced among SC subtypes. Taken together, our data suggest that pillar cells and Deiters' cells, which are located in the lateral compartment of the cochlea, have decreased Notch-mediated lateral inhibition after HC damage in the Atoh1-DTA mouse, while no change was seen in inner phalangeal and border cells, which reside in the medial compartment of the cochlea. Therefore, we hypothesize that pillar cells and Deiters' cells are

the source of regenerated HCs in the neonatal mouse cochlea. To directly test this hypothesis, we will fate-map different SC subtypes during the HC regeneration process.

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