

**AWARD NUMBER:** W81XWH-15-1-0475

**TITLE:** Investigation of Notch Signaling during Spontaneous  
Regeneration of Cochlear Hair Cells

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**REPORT DATE:** DECEMBER 2019

**TYPE OF REPORT:** Final

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

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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                         |                            | Form Approved<br>OMB No. 0704-0188           |                                           |
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| 1. REPORT DATE<br>DECEMBER 2019                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              | 2. REPORT TYPE<br>Final |                            | 3. DATES COVERED<br>15 Sep 2015 -14 Sep 2019 |                                           |
| 4. TITLE AND SUBTITLE<br><br>Investigation of Notch Signaling during Spontaneous Regeneration of Cochlear Hair Cells                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |              |                         |                            | 5a. CONTRACT NUMBER<br>W81XWH-15-1-0475      |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              |                         |                            | 5b. GRANT NUMBER<br>MR141238                 |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              |                         |                            | 5c. PROGRAM ELEMENT NUMBER                   |                                           |
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| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>Southern Illinois University School of Medicine<br>Springfield, IL 62702-4910                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |              |                         |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER     |                                           |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |              |                         |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)             |                                           |
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| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |              |                         |                            |                                              |                                           |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |              |                         |                            |                                              |                                           |
| 14. ABSTRACT<br>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 signaling is decreased which causes supporting cells (SCs) 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 ligands control supporting cell to HC conversion. Our data show changes in Notch signaling following HC damage in two SC subtypes (pillar cells and Deiters' cells), but not in other subtypes. We also demonstrate that when Notch signaling is increased in SCs, HC regeneration is reduced by 92%. Therefore we conclude that reduced Notch signaling is required for spontaneous HC regeneration to occur. We also investigated deletion of individual Notch ligands from HCs or SCs and found loss of specific SC subtypes after deletion of one of more Notch ligands. Together, these data suggest that the Notch signaling pathway is a complex network of signaling from different ligands that regulate HC and SC fate in the neonatal cochlea. |              |                         |                            |                                              |                                           |
| 15. SUBJECT TERMS<br>Hearing loss, tinnitus, hair cell regeneration, supporting cells, mouse genetics, Notch, Hes5, Jagged1, Delta-like ligand 1, Jagged2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |                         |                            |                                              |                                           |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              |                         | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                          | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | b. ABSTRACT  | c. THIS PAGE            |                            |                                              | USAMRMC                                   |
| Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Unclassified | Unclassified            | Unclassified               | 107                                          | 19b. TELEPHONE NUMBER (include area code) |

## TABLE OF CONTENTS

|                                                     | <u>Page</u> |
|-----------------------------------------------------|-------------|
| 1. Introduction                                     | 4           |
| 2. Keywords                                         | 4           |
| 3. Accomplishments                                  | 5-62        |
| 4. Impact                                           | 63          |
| 5. Changes/Problems                                 | 63-66       |
| 6. Products                                         | 66-70       |
| 7. Participants & Other Collaborating Organizations | 70-73       |
| 8. Special Reporting Requirements                   | 73          |
| 9. Appendices                                       | 73-107      |

## 10. INTRODUCTION:

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 and others 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. Here, we used sophisticated mouse genetics and *in vivo* approaches to investigate the Notch signaling pathway during spontaneous HC regeneration in the neonatal mouse cochlea. Following HC damage in the neonatal mouse cochlea, we found a decrease in Notch signaling in two supporting cell subtypes (pillar cells and Deiters' cells), but not in other subtypes. To determine whether loss of Notch-mediated lateral inhibition is required for supporting cells to change cell fate and become HCs, we increased Notch signaling in supporting cells by overexpressing the Notch intracellular domain (N1ICD). In this mouse model, HC regeneration was reduced by 92% and therefore we concluded that reduced Notch signaling is required for spontaneous HC regeneration to occur. We also observed that overexpression of N1ICD caused an expansion of the Sox2-expressing region, increased proliferation, and patches of ectopic HCs medial to the organ of Corti in control samples that did not have HC damage. This phenotype was dependent on the level of N1ICD overexpression. Finally, we investigated the function of individual Notch ligands in the undamaged, neonatal cochlea to understand which ligands control supporting cell to HC conversion. When both Delta-like 1 and Jagged2 were deleted from HCs at birth, some SC subtypes (Deiters' cells and Hensen cells) attempted to convert into HCs but died by apoptosis before completing the transition. We saw a similar phenotype when only Jagged2 was deleted from HCs at birth, but fewer SCs were effected. However, there was no phenotype observed when Delta-like 1 or Jagged1 were deleted from HCs individually. When Jagged 1 was deleted from SCs at birth, there was a loss of Hensen cells and preliminary results suggest that Hensen cells may be converting into Claudius cells after Jag1 deletion from supporting cells. We are currently doing fate-mapping experiments to confirm this hypothesis. Together, these data suggest that the Notch signaling pathway is a complex network of signaling from different ligands that regulate HC and supporting cell fate in the neonatal cochlea.

## 11. KEYWORDS:

Hearing loss, tinnitus, hair cell regeneration, supporting cells, mouse genetics, Notch, Hes5, Jagged1, Delta-like1, Jagged2

## 12. ACCOMPLISHMENTS:

### What were the major goals of the project?

**Specific Aim 1:** To measure changes in Notch signaling after hair cell (HC) damage in the neonatal mouse cochlea. Goals 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. Completed in Y2, Q4. 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 with N=10 for all samples.
3. Task 3: Measure changes in Notch target genes specifically in supporting cells (SCs) using immunostaining and *in situ* hybridization. Timeline: months 3-14
  - a. Completed in Y3, Q2. We completed immunostaining for the Notch ligand Jagged1 and completed *in situ* hybridization experiments for the Notch target genes: Hes1, Hes5, Hey1, and HeyL. We also tested 14 other antibodies for genes in the Notch pathway, but unfortunately none of these worked for immunostaining.
4. Task 4: Measure changes in the number of Hes5-LacZ<sup>+</sup> cells using immunostaining. Timeline: months 3-9
  - a. Completed in Y1, Q3. We measured changes in expression of the Notch target gene Hes5 (using a Hes5<sup>LacZ</sup> reporter mouse) in 2 different groups of supporting cells (SCs) and at 3 timepoints using immunostaining.
5. Task 5: Prepare manuscript on Aim 1 and Aim 2A findings for publication. Timeline: months 13-15
  - a. Completed in Y3, Q3. Our manuscript which contains data from Aims 1 and 2A was published in *Frontiers in Cellular Neuroscience* in May 2018.

**Specific Aim 2:** To maintain active Notch signaling in SCs in the context of HC damage. Goals were:

1. Task 1: Obtain the needed mouse lines for Aim 2A & 2B (Sox10<sup>rtTA</sup> & TetO-N1ICD). 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::ROSA26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-N1ICD::TetO-lacZ). Timeline: months 6-24

- a. Completed in Y2, Q2. We obtained multiple breeding pairs that generated experimental samples (which contain all 5 alleles) and different types of control samples.
3. Task 3: Confirm increased Notch signaling in SCs in the proposed mouse model using real time qPCR. Timeline: months 24-27.
  - a. Completed in Y2, Q4. We used the same primer sets that were validated in Aim 1 to measure changes in the Notch1 intracellular domain (N1ICD) and Notch target genes after overexpression of the N1ICD.
4. Task 4: Measure HC regeneration capacity in a mouse model where Notch signaling is increased in SCs and HCs are killed using immunostaining and confocal microscopy. Timeline: months 24-32.
  - a. Completed in Y3, Q2. Using fate-mapping, we quantified the number of spontaneously regenerated HCs when N1ICD was overexpressed in SCs compared to controls where Notch signaling was not manipulated.
5. Task 5: Examine increased proliferation in a mouse model where Notch signaling is increased in SCs and other non-sensory cells, but HCs remain intact. Timeline: months 24-44.
  - a. 95% complete. We used BrdU to investigate whether proliferation caused the expansion of the Sox2-expressing region in control samples where N1ICD was overexpressed in SCs, but HCs remain intact. We used markers of SC subtypes to determine which cell types were proliferating after overexpression of N1ICD and examined the timing of when cells proliferate. We also measured the expression level of Sox2 and Sox10 using real-time qPCR. Finally, we are in the process of examining Sox10<sup>rtTA::TetO-LacZ::TetO-N1ICD</sup> cochlea using cyrosections to determine if other areas of the cochlea outside the organ of Corti have increased proliferation.
6. Task 6: Examine patches of ectopic HCs and markers of prosensory cells in a mouse model where Notch signaling is increased in SCs and other non-sensory cells, but HCs remain intact. Timeline: months 30-44.
  - a. Completed in Y4, Q4. We observed ectopic HCs outside the organ of Corti in samples with N1ICD overexpression and characterized these ectopic cells to determine if they are innervated, have stereocilia bundles, or markers of mature HCs. We also compared the number of ectopic HCs in Sox10<sup>rtTA::TetO-LacZ::TetO-N1ICD</sup> cochlea with various controls to determine what causes the formation of ectopic HCs. Using samples with N1ICD overexpression, we completed immunostaining for markers of prosensory cells (Jagged1, p27<sup>Kip1</sup>, Prox1) and completed *in situ* hybridization experiments for genes that define the organ of Corti boundaries (Fgf10 and BMP4). We also measured Fgf10 and BMP4 expression levels using real-time qPCR.
7. Task 7: Examine the effects of different levels of increased Notch signaling in SCs and other non-sensory cells. Timeline: months 30-44.
  - a. 95% complete. The expanded Sox2 expression and massive proliferation we observed in our model may be caused by the super-

physiological high levels of Notch signaling induced with the high dosage of doxycycline that we used (100mg/kg IP injection plus doxycycline given in the food to the nursing mother). Therefore we tested a lower doses of doxycycline and used expanded Sox2 expression as the read out of the phenotype. To determine if haploinsufficiency of Sox10 is involved in this phenotype, we also performed similar experiments with lower doses of doxycycline using a different rtTA mouse model (Rosa26<sup>rtTA</sup>) that has both copies of Sox10. We completed the histological analysis, but are in the process of measuring the level of NICD overexpression induced by the different doses of doxycycline using real-time qPCR. In addition, the high level of NICD caused death of the pups between postnatal day (P) 9 and P11. We observed that pups with increased Notch signaling were much smaller than their littermates. To further investigate why the pups were dying and based on the role of Notch signaling in the intestine, we measured the length of the small and large intestines and examined the histology of the intestinal cells using sections.

8. **Task 8:** Prepare manuscript on Aim 2B findings for publication. Timeline: months 44-48.
  - a. 50% complete. We have created a draft of the Figures for this manuscript and have a preliminary draft of the methods and results sections. We plan to submit this paper for publication after we complete the last 2 experiments.

**Specific Aim 3A:** To conditionally delete Delta-like1 (Dl1) from neonatal HCs in the normal, undamaged neonatal cochlea. Goals were:

1. **Task 1:** Obtain the needed mouse line for Aim 3A (Dl1<sup>loxP</sup>). Timeline: months 3-6
  - a. Completed in Y1, Q4. The cryorecovery process of Dl1<sup>loxP</sup> 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::Dl1<sup>loxP/loxP</sup>). Timeline: months 7-12
  - a. Completed in Y2, Q1. We obtained multiple breeding pairs that routinely generated Atoh1-CreER::Dl1<sup>loxP/loxP</sup> mice and Cre-negative controls.
3. **Task 3:** Measure the expression level of Notch effector genes after Dl1 deletion using real time qPCR. Timeline: months 12-18
  - a. Completed in Y3, Q2. We performed real-time qPCR to measure the expression level of Notch target genes after deletion of Dl1 from HCs at P0. We used the Hes5<sup>LacZ</sup> knock-in reporter line to measure changes in the number of cells that express Hes5 after deletion of Dl1 from HCs at P0.
4. **Task 4:** Investigate morphological changes to HCs and SCs in the cochlea after Dl1 deletion using immunostaining and confocal microscopy. Timeline: months 14-24
  - a. Completed in Y3, Q3. We quantified the number of HCs or SCs by subtype at P7 or P30 after deletion of Dl1 from HCs at P0. We also

quantified the number of ribbon synapses that connect inner HCs to spiral ganglion neurons and examined the shape and polarity of stereocilia bundles located on the apical surface of HCs after Dll1 deletion.

5. Task 5: Measure hearing after Dll1 deletion at P30 using auditory brainstem response (ABR). Timeline: months 24-27
  - a. Completed in Y3, Q3. We measured hearing loss after Dll1 deletion from HCs at P0 using ABR.

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

1. Task 1: Obtain the needed mouse line for Aim 3B (Jag2<sup>loxP</sup>). Timeline: months 3-6
  - a. Completed in Y1, Q3. Jag2<sup>loxP</sup> 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<sup>loxP/loxP</sup>). Timeline: months 7-12
  - a. Completed in Y2, Q3. We obtained multiple breeding pairs that routinely generated Atoh1-CreER::Jag2<sup>loxP/loxP</sup> mice and Cre-negative controls.
3. Task 3: Measure the expression level of Notch effector genes after Jag2 deletion using real time qPCR. Timeline: months 12-18
  - a. Completed in Y3, Q4. We performed real-time qPCR to measure the expression level of Notch target genes after deletion of Jag2 from HCs at P0. We used the Hes5<sup>LacZ</sup> knock-in reporter line to measure changes in the number of cells that express Hes5 after deletion of Jag2 from HCs at P0.
4. Task 4: Investigate morphological changes to HCs and SCs in the cochlea after Jag2 deletion using immunostaining and confocal microscopy. Timeline: months 14-24
  - a. Completed in Y3, Q4. We quantified the number of HCs or SCs by subtype at P7 or P30 after deletion of Jag2 from HCs at P0. We also quantified the number of ribbon synapses that connect inner HCs to spiral ganglion neurons and examined the shape and polarity of stereocilia bundles located on the apical surface of HCs after Jag2 deletion.
5. Task 5: Measure hearing after Jag2 deletion at P30 using ABR. Timeline: months 24-27
  - a. Completed in Y3, Q4. We measured hearing loss after Jag2 deletion from HCs at P0 using ABR.

**Specific Aim 3B.2:** To conditionally delete both Delta-like1 (Dll1) and Jagged2 (Jag2) from neonatal HCs in the normal, undamaged neonatal cochlea. Goals were:

2. Task 1: Perform mouse breeding to obtain mice containing the desired genotype (Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>). Timeline: months 30-34

- a. Completed in Y3, Q4. We obtained multiple breeding pairs that routinely generated Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice and Cre-negative controls.
3. Task 2: Measure the expression level of Notch effector genes after deletion of both Dll1 and Jag2 using real time qPCR. Timeline: months 34-40
  - a. Completed in Y4, Q4. We performed real-time qPCR to measure the expression level of Notch target genes after deletion of both Dll1 and Jag2 from HCs at P0. We used the Hes5<sup>LacZ</sup> knock-in reporter line to measure changes in the number of cells that express Hes5 after deletion of both Dll1 and Jag2 from HCs at P0. We also examined changes in the expression of Jag1 using immunostaining.
4. Task 3: Investigate morphological changes to HCs and SCs in the cochlea after deletion of both Dll1 and Jag2 using immunostaining and confocal microscopy. Timeline: months 34-42
  - a. Completed in Y4, Q4. We quantified the number of HCs or SCs by subtype at P7 or P30 after deletion of both Dll1 and Jag2 from HCs at P0. To investigate whether the missing cells are dying or attempting to convert into HCs, we performed immunostaining with markers for apoptosis and for early HC-specific genes.
5. Task 4: Measure hearing after deletion of both Dll1 and Jag2 at P30 using ABR. Timeline: months 34-42.
  - a. Completed in Y4, Q4. We measured hearing loss after deletion of both Dll1 and Jag2 from HCs at P0 using ABR.
6. Task 5: Prepare manuscript on Aim 3A, 3B, and 3B.2 findings for publication. Timeline: months 42-46.
  - a. 60% complete. We have finalized the Figures for this manuscript and are in the process of writing the manuscript draft. We plan to submit this paper for publication in January.

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

1. Task 1: Obtain the needed mouse line for Aim 3C (Jag1<sup>loxP</sup>). Timeline: months 3-6
  - a. Completed in Y1, Q1. Jag1<sup>loxP</sup> mice were received at SIUSOM in DEC-2015.
2. Task 2: Perform mouse breeding to obtain mice containing the desired genotype (Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>). Timeline: months 7-12
  - a. Completed in Y1, Q3. We obtained multiple breeding pairs that routinely generated Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> 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. Completed in Y4, Q2. We performed real-time qPCR to measure the expression level of Notch target genes after deletion of Jag1 from SCs at P0. We used the Hes5<sup>LacZ</sup> knock-in reporter line to measure changes

in the number of cells that express Hes5 after deletion of Jag1 from SCs at P0.

4. Task 4: Investigate morphological changes to HCs and SCs in the cochlea after Jag1 deletion using immunostaining and confocal microscopy. Timeline: months 14-24
  - a. 95% complete. We quantified the number of HCs or SCs by subtype at P7, P30, and P60 after deletion of Jag1 from SCs at P0. We confirmed the loss of Hensen cells by staining the samples with markers of different SC subtypes. To investigate whether the missing Hensen cells are dying, we performed immunostaining with markers for apoptosis. To investigate whether the missing Hensen cells are converting into Claudius cells, we have performed immunostaining with Claudius cell markers and are currently using fate-mapping to address this question more directly. To investigate the effect that loss of Hensen cells has on the tectorial membrane, we performed plastic sections and staining. We also quantified the number of HCs and SCs by subtype when Jag1 was deleted from SCs at a later time point (P6/P7).
  - b. Completed in Y4, Q1. All findings in Aim 3C described above have used FGFR3-iCreER:Jag1<sup>loxP/loxP</sup> mice which targets some SC subtypes (pillar and Deiters' cells), but not others. Therefore we used a different CreER line, called GLAST-CreER, to delete Jag1 from other SC subtypes (inner phalangeal cells, border cells, GER cells, and Hensen cells). We quantified the number of HCs or SCs by subtype at P7 and P30 after deletion of Jag1 from SCs at P0 using GLAST-CreER:Jag1<sup>loxP/loxP</sup> mice.
5. Task 5: Measure hearing after Jag1 deletion at P30 using ABR. Timeline: months 24-27
  - a. Completed in Y3, Q4. We measured hearing loss after deletion of Jag1 from SCs at P0 using ABR. Since we found no difference in the number of HCs or SCs in GLAST-CreER:Jag1<sup>loxP/loxP</sup> mice at P30, we discontinued those ABR experiments.
6. Task 6: Prepare manuscript on Aim 3C findings for publication. Timeline: months 27-30.
  - a. 90% complete. We have completed a draft containing the abstract, introduction, figures, figure legends, methods, and results sections that describe the results of Jag1 deletion from neonatal SCs using FGFR3-iCreER:Jag1<sup>loxP/loxP</sup> mice. Since there was no phenotype after deletion of Jag1 from other SC subtypes using GLAST-CreER:Jag1<sup>loxP/loxP</sup> mice, we have decided not to include this data in the paper. The current version of the draft is currently being reviewed by our collaborator Dr. Angelika Doetzlhofer at Johns Hopkins University, who added data from her lab that complements our work. Once we have the fate-mapping experiment completed, we will add that to the paper and draft the discussion section. We plan to submit this manuscript for publication in January.

**Specific Aim 3D:** To conditionally delete Jagged1 (Jag1) from neonatal HCs in the normal, undamaged neonatal cochlea. Goals were:

1. Task 1: Perform mouse breeding to obtain mice containing the desired genotype (Atoh1-CreER::Jag1<sup>loxP/loxP</sup>). Timeline: months 30-34
  - a. Completed in Y3, Q4. We obtained multiple breeding pairs that routinely generated Atoh1-CreER::Jag1<sup>loxP/loxP</sup> mice and Cre-negative controls.
2. Task 2: Measure the expression level of Notch effector genes after deletion of Jag1 from neonatal HCs using real time qPCR. Timeline: months 34-40
  - a. Completed in Y4, Q3. We performed real-time qPCR to measure the expression level of Notch target genes after deletion of Jag1 from HCs at P0. We used the Hes5<sup>LacZ</sup> knock-in reporter line to measure changes in the number of cells that express Hes5 after deletion of Jag1 from HCs at P0.
3. Task 3: Investigate morphological changes to HCs and SCs in the cochlea after deletion of Jag1 from neonatal HCs using immunostaining and confocal microscopy. Timeline: months 34-42
  - a. Completed in Y4, Q3. We quantified the number of HCs or SCs by subtype at P7 or P30 after deletion of Jag1 from HCs at P0.
4. Task 4: Measure hearing at P30 using ABR after deletion of Jag1 from neonatal HCs. Timeline: months 34-42.
  - a. Completed in Y4, Q2. We measured hearing loss after deletion of Jag1 from HCs at P0 using ABR.
5. Task 5: Prepare manuscript on Aim 3D findings for publication. Timeline: months 42-46.
  - a. 60% complete. Since there was no phenotype after deletion of Jag1 from neonatal HCs, we plan to publish the data from this Aim with Aims 3A, 3B, and 3B.2. We have finalized the Figures for this manuscript and are in the process of writing the manuscript draft. We plan to submit this paper for publication in January.

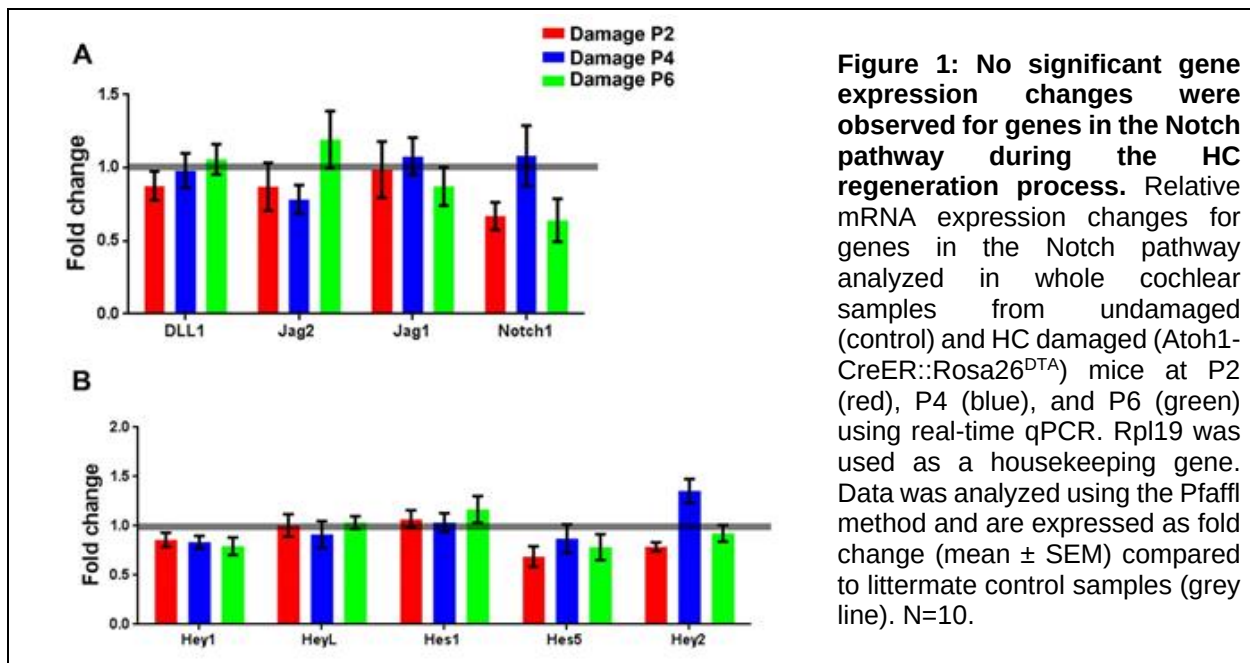
### **What was accomplished under these goals?**

**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 CreER/loxP system to kill HCs because noise and ototoxic drugs are problematic in neonates. Specifically, we used 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<sup>DTA</sup>) mouse line. When tamoxifen is administered at postnatal day (P) 0 and P1 to Atoh1-CreER::Rosa26<sup>DTA</sup> 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).

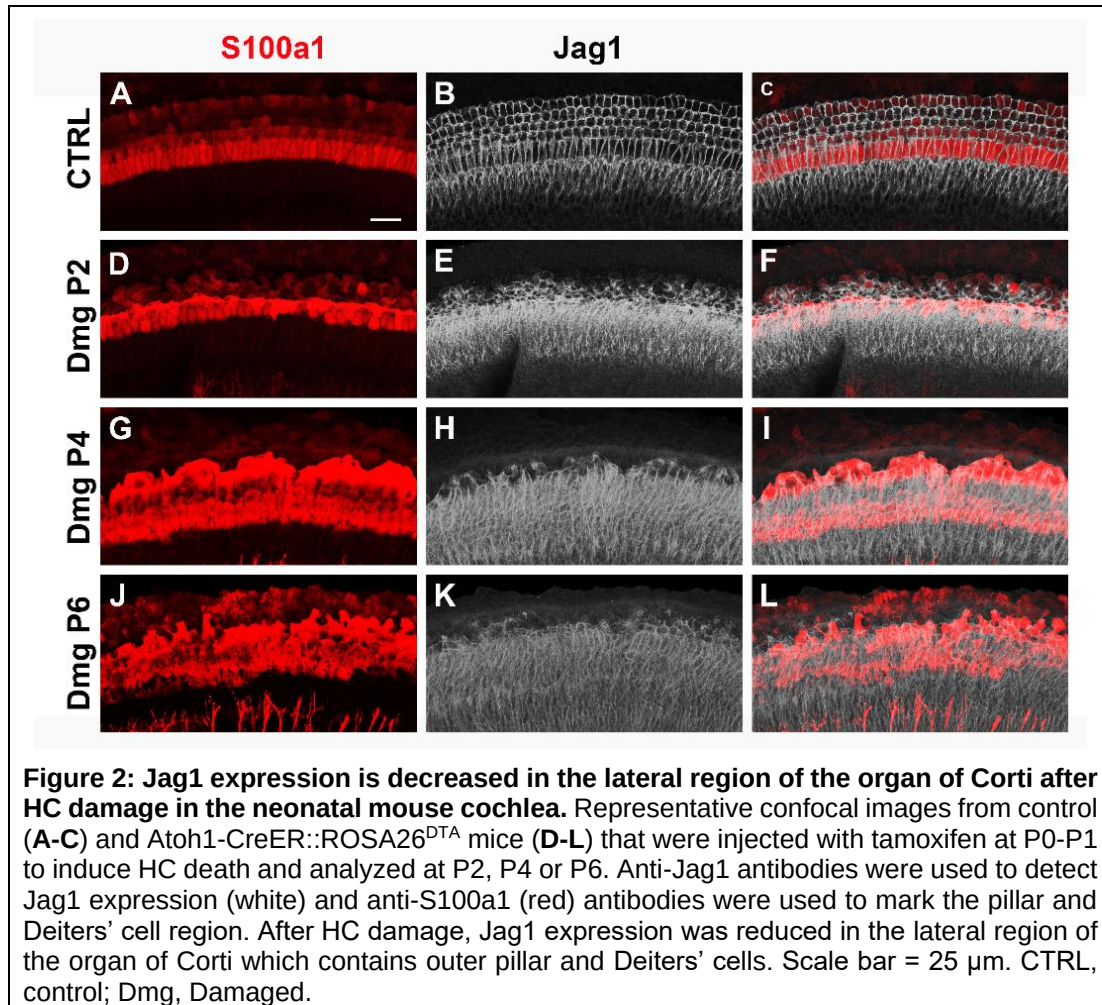
To investigate global changes in the Notch signaling pathway during the spontaneous HC regeneration process, we performed real-time qPCR with whole

cochlear samples obtained from Atoh1-CreER::Rosa26<sup>DTA</sup> mice injected with tamoxifen at P0/P1. 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  $\Delta\Delta C_t$  equation (Pfaffl, 2001), we measured changes in gene expression for Notch ligands [Delta like 1 (DLL1), Jagged1 (Jag1), and Jagged2 (Jag2)], the Notch receptor (Notch1), and Notch target genes (Hey1, Hey2, HeyL, Hes1, and Hes5) at P2, P4, and P6 (Figure 1). There was no statistical difference for any gene at any timepoint analyzed. This result may have occurred because the majority of HC regeneration occurs in the apical turn of the cochlea and whole cochlea samples were used for real-time qPCR experiments. In addition, other studies have suggested that some supporting cell (SC) subtypes have a greater ability to regenerate HCs than others (Bramhall et al., 2014). Furthermore, differential expression of Notch effector genes among SC subtypes have been documented previously (Lanford et al., 2000; Doetzlhofer et al., 2009; Yu et al., 2010; Liu et al., 2012a; Taylor et al., 2012; Maass et al., 2015). Therefore, we continued our investigation of changes in the Notch signaling pathway using methods that could distinguish individual cells and regions of the cochlea.

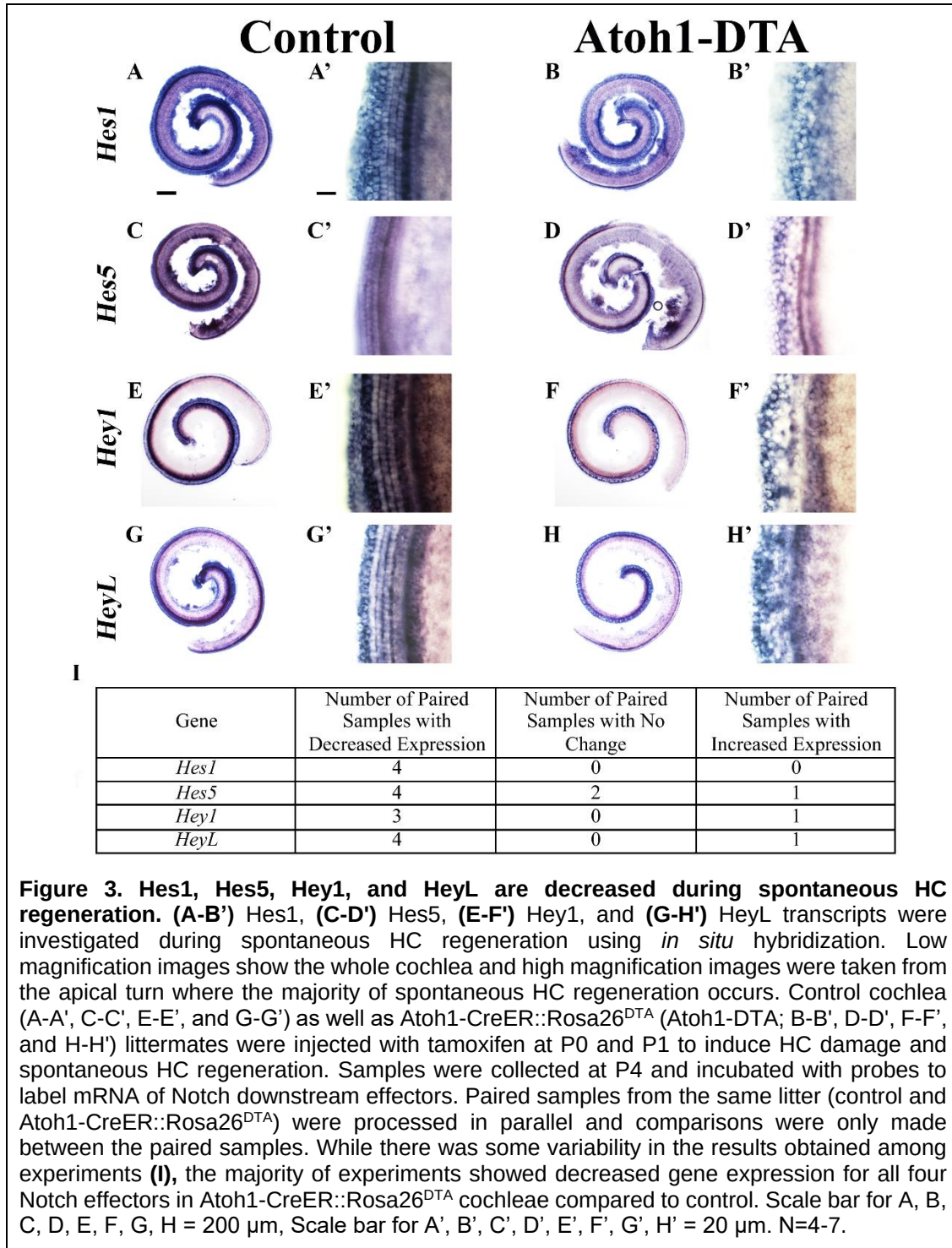


To measure changes in Notch-related genes specifically in SCs, we used immunostaining and *in situ* hybridization. We tested 15 antibodies for various genes in the Notch pathway and the only antibody that worked for immunostaining labels the Notch ligand Jag1. Jag1 is expressed by neonatal SCs and is also a downstream target of Notch activation (Daudet and Lewis, 2005; Murata et al., 2006). Therefore, it can be used as an indicator of active Notch signaling. We investigated changes in Jag1 in the context of HC loss and spontaneous HC regeneration as a way to assess changes in the Notch signaling pathway. In control samples, Jag1 was expressed throughout the organ of Corti from the

cells of the greater epithelial ridge (GER) to the 3<sup>rd</sup> row of Deiters' cells (Figure 2A-C; Oesterle et al., 2008). However after HC damage in Atoh1-CreER::Rosa26<sup>DTA</sup> mice that were injected with tamoxifen at P0/P1, Jag1 labeling was reduced in the lateral region of the organ of Corti, where outer pillar cells and Deiters' cells are located, yet maintained in medial SCs, including inner pillar cells, inner phalangeal/border cells, and the cells of the GER (Figure 2D-L). These data demonstrate that Notch signaling is reduced in some SC subtypes, but not others, during spontaneous HC regeneration.



Because only one antibody raised against genes in the Notch signaling pathway worked for immunofluorescent staining, we used *in situ* hybridization to further investigate changes in Notch signaling during the spontaneous HC regeneration process. Atoh1-CreER::Rosa26<sup>DTA</sup> mice, as well as littermate controls, which lacked the Cre or DTA allele, were injected with tamoxifen on P0 and P1 to induce HC damage and spontaneous HC regeneration. Samples were analyzed at P4 using probes that target Hes1, Hes5, Hey1, and HeyL. Paired samples from the same litter (control and Atoh1-CreER::Rosa26<sup>DTA</sup>) were processed in parallel where each solution, as well as each

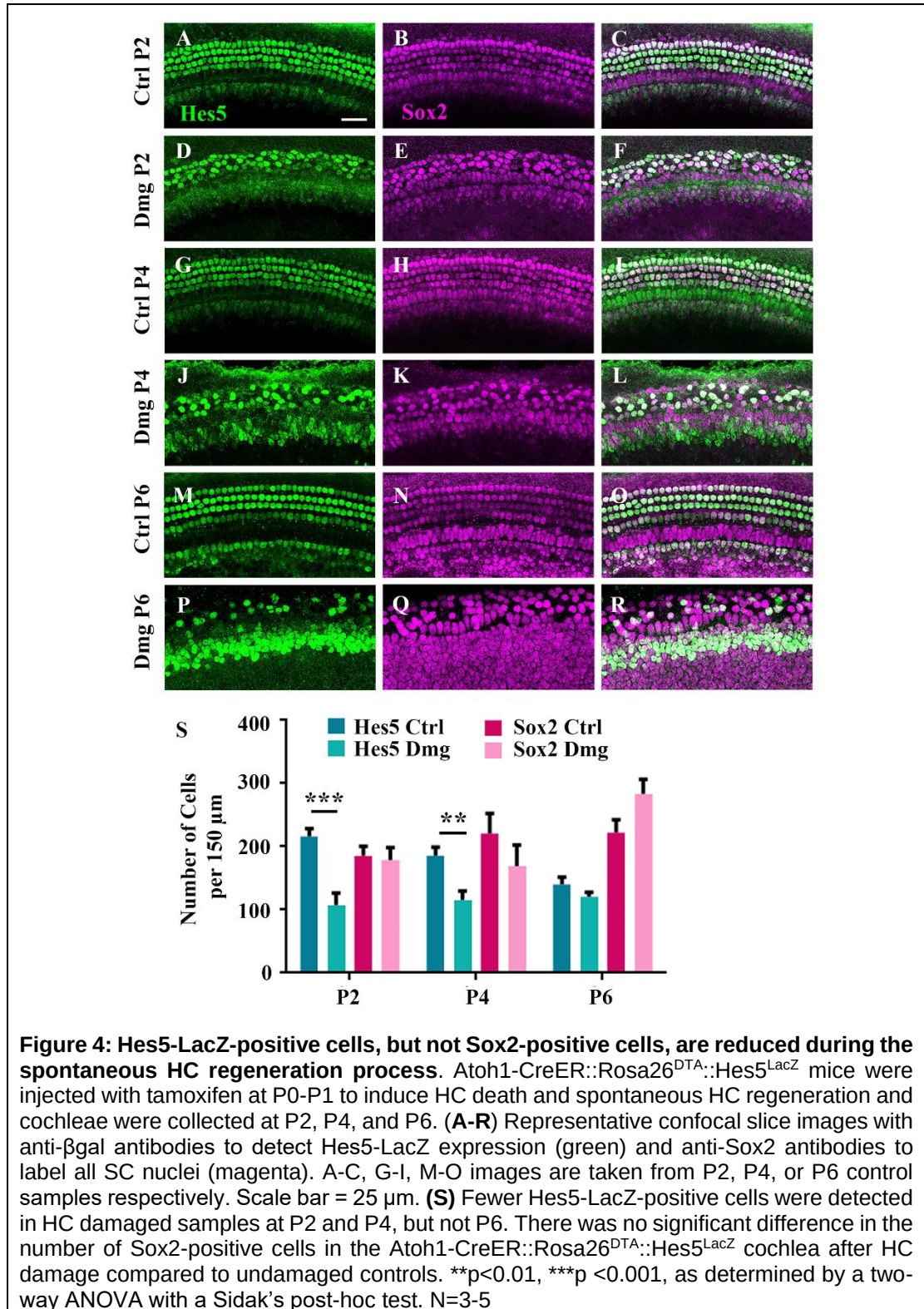


incubation time, was identical. Comparisons were only made between the one experimental and the one control sample that were processed together. Staining intensity in the sensory region (low magnification images in Figure 3A,B,C,D,E,F,G,H) and at the HC level (high magnification images taken in the apical turn, Figure 3A',B',C',D',E',F',G',H') was compared between the paired control and experimental samples to

determine increased or decreased expression. Expression of Hes1 was consistently decreased in Atoh1-CreER::Rosa26<sup>DTA</sup> samples compared to controls (Figure 3A-B', I), while variable results were obtained for the other tested genes. For Hes5, four out of seven samples showed decreased expression, while one showed increased expression and two showed no change in expression (Figure 3C-D', I). Hey1 expression was also variable with three samples showing decreased expression and one sample showing increased expression (Figure 3E-F', I). For HeyL, four samples showed decreased expression, while one showed increased expression (Figure 3G-H', I). SC-to-HC conversion is a dynamic and non-synchronous process that occurs throughout the first postnatal week (Cox et al., 2014); the variability of individual gene changes we observed may depend on the stage of SC conversion into HCs at the time of analysis. Nevertheless, because the majority of the samples showed decreased expression of Notch effectors, we conclude that loss of HCs in the neonatal mouse cochlea caused a modest decrease in Notch signaling in neighboring SCs.

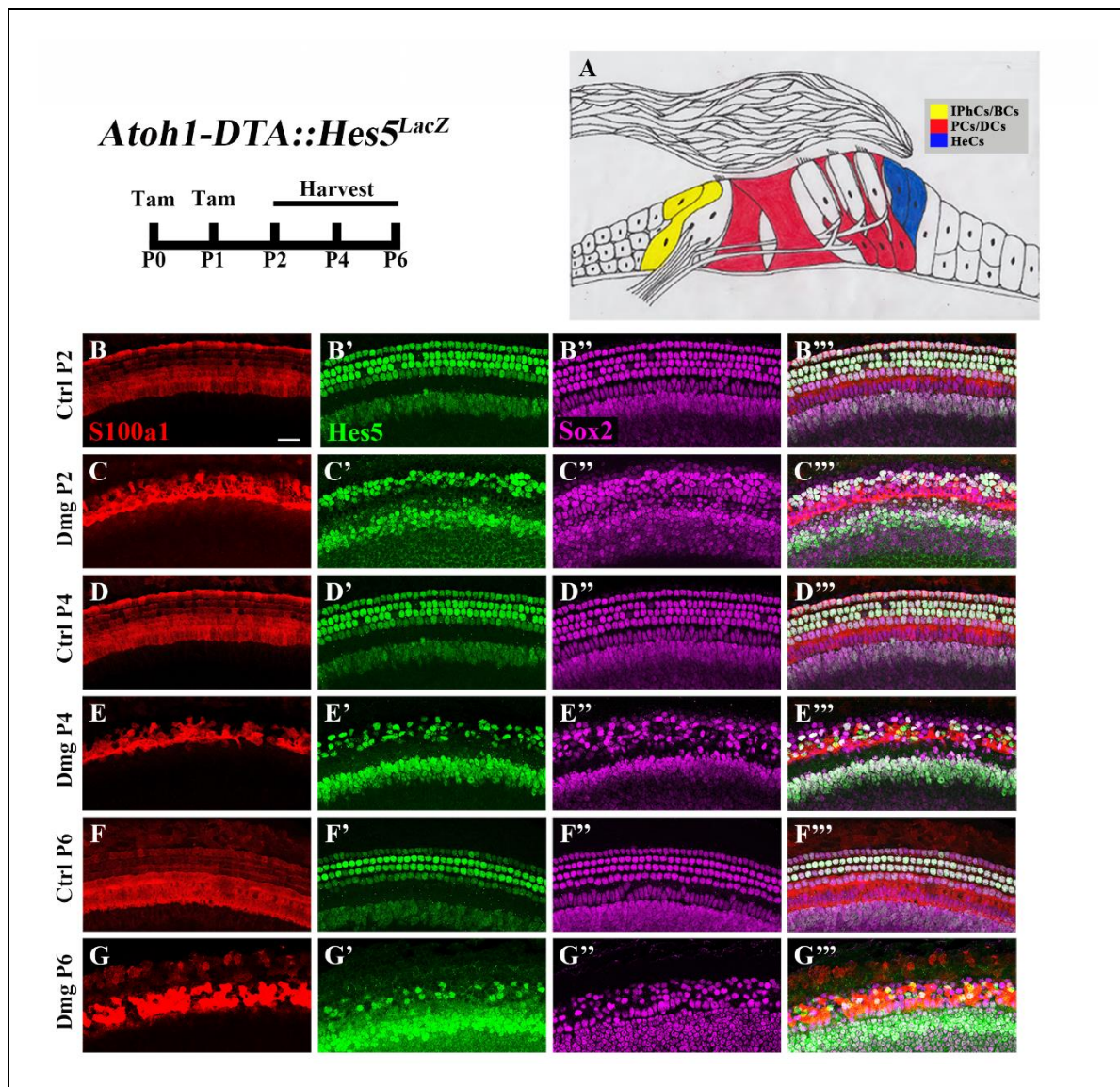
Hes5, a direct inhibitor of HC fate, is a well-known target of active Notch signaling (Mulvaney and Dabdoub, 2012; Abdolazimi et al., 2016). Hes5 is expressed in many SC subtypes during the first postnatal week including Deiters' cells, outer pillar cells, inner phalangeal cells, border cells, and some cells of the GER (Figure 4A-C, G-I, M-O, Figure 5B-B'', D-D'', F-F''; Lanford et al., 2000; Zine et al., 2001; Hartman et al., 2009; Cox et al., 2014; Maass et al., 2015). To quantify the number of cells that express Hes5, we used the Hes5<sup>LacZ</sup> reporter line which has LacZ knocked into the endogenous Hes5 locus and therefore is a faithful representation of endogenous Hes5 expression (Imayoshi et al., 2010). Others have shown that  $\beta$ -galactosidase ( $\beta$ -gal), transcribed by the LacZ gene, continues to be expressed ~1 day after mRNA of the endogenous gene becomes undetectable (Morrison et al., 1999). Therefore, we used Hes5<sup>LacZ</sup> mice to approximate the window when cells lose Hes5 expression. Atoh1-CreER::Rosa26<sup>DTA</sup> mice were bred with Hes5<sup>LacZ</sup> mice and injected with tamoxifen on both P0 and P1 to induce HC ablation and initiate spontaneous HC regeneration. Samples were analyzed at P2, P4, and P6, which was previously established as the window of spontaneous HC regeneration seen in the neonatal mouse cochlea (Cox et al., 2014). We quantified the number of cells that expressed Hes5-LacZ as well as the number of SC and immature HC nuclei using anti-Sox2 antibodies. Significantly fewer Hes5-LacZ-positive cells were detected in the Atoh1-CreER::Rosa26<sup>DTA</sup>::Hes5<sup>LacZ</sup> cochlea at P2 and P4 compared to control samples that lacked either the Cre or DTA allele (Figure 4A-L, S). However, there was no difference observed in Hes5-LacZ expression at P6 (Figure 4M-S). There were also no differences in the number of Sox2-positive cells after HC damage at any age (Figure 4B, E,H,K,N,Q,S) suggesting that as a whole, the Sox2-positive cell population is not reduced.

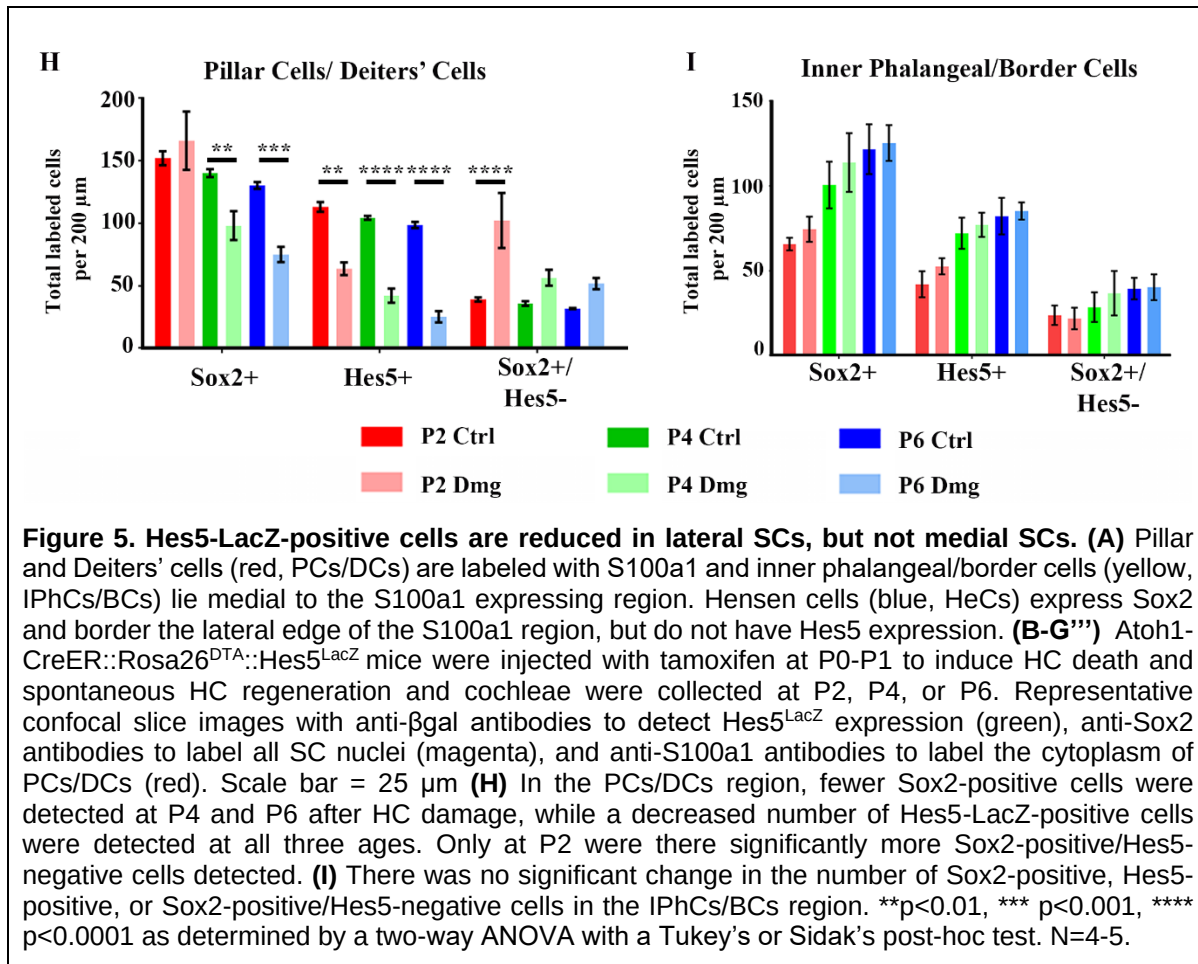
Differences among SC subtypes have previously been documented (Doetzlhofer et al., 2009; Mellado Lagarde et al., 2013; Mellado Lagarde et al., 2014; Lanford et al., 2000; Maass et al., 2016; Taylor et al., 2012; Yu et al., 2010), which may indicate that not all SC subtypes maintain plasticity postnatally. Furthermore, loss of Hes5 at embryonic ages has been implicated in the formation of supernumerary outer HCs, but not inner HCs (Zine et al., 2001). We therefore sought to investigate changes in Hes5 expression within



discrete SC subpopulations. To divide the SC subtypes into two groups, we used pillar and Deiters' cells as a groups, we used pillar and Deiters' cells as a landmark. S100a1 is

a calcium binding protein that is expressed in the cytoplasm and nucleus of pillar and Deiters' cells within the neonatal cochlea (White et al., 2006; Buckiová and Syka, 2009). Therefore, combining anti-S100a1 antibodies with anti-Sox2 and anti- $\beta$ -gal antibodies allowed us to tease apart two groups of SC subpopulations: 1) pillar cells and Deiters' cells which are labeled by S100a1, and 2) inner phalangeal and border cells which are labeled by Hes5<sup>LacZ</sup>-positive/Sox2-positive cells located medial to S100a1 labeling (Figure 5A, B-B''', E-E''', F-F'''). After HC damage and regeneration was induced in *Atoh1-CreER::Rosa26<sup>DTA::Hes5<sup>LacZ</sup></sup>* mice by tamoxifen injection at P0 and P1, there was a significant decrease in the number of Hes5-LacZ-positive pillar and Deiters' cells detected at P2, P4, and P6 compared to control cochlea which lacked either the Cre or DTA allele (Figure 5B-H). Of note, the number of Sox2-positive cells within this population did not decrease until P4 and was also decreased at P6 (Figure 5B-H). Additionally, there was an increase in the number of Sox2-positive/Hes5-LacZ-negative pillar and Deiters' cells at P2 (Figure 5B-H), suggesting that Hes5 is downregulated in these SCs, but the cells are still alive.





In contrast, neither the number of Hes5-LacZ-positive cells, nor the number of Sox2-positive cells within the inner phalangeal and border cells region were significantly altered from control samples (Figure 5B-G''', I), suggesting that weakened Notch signaling is limited to pillar and Deiters' cells during the HC regeneration process.

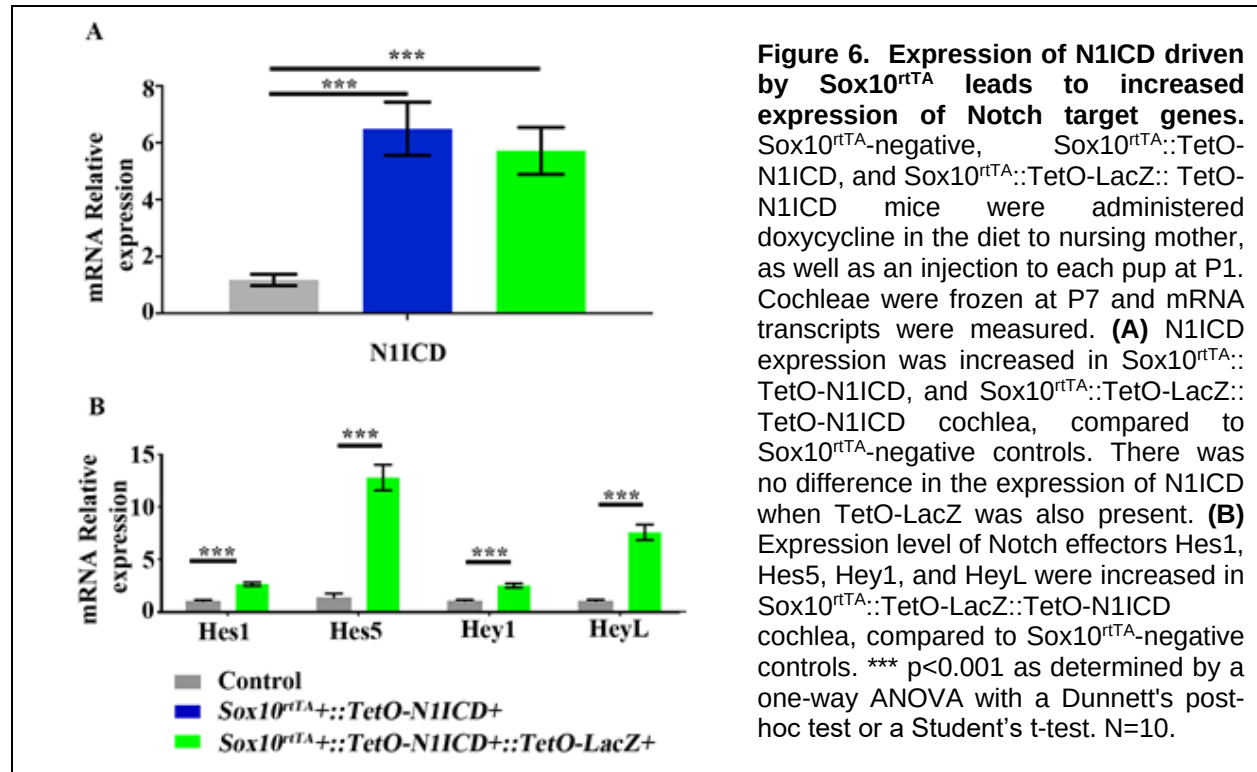
Stated goals not met: None. All tasks planned for Aim 1 have been completed. Data from Aims 1 and 2A were published in *Frontiers in Cellular Neuroscience* in May 2018.

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

While immunostaining and *in situ* hybridization results suggest a modest decrease in Notch signaling during spontaneous HC regeneration, it is currently unknown whether Notch signaling is involved in the mechanism of spontaneous HC regeneration or if it is simply correlated. To investigate this question more directly, we induced overexpression of Notch1 intracellular domain (N1ICD) in SCs and other non-sensory epithelial cells (all other cells within the organ of Corti except for HCs and tympanic border cells, hereinafter referred to as non-HCs) in the context of HC damage and quantified the number of regenerated HCs using fate-mapping. This was achieved by combining CreER/loxP and

Tet-on genetically modified mouse systems to modify gene expression in adjacent cell populations.

To investigate whether downregulation of Notch signaling plays a role in spontaneous HC regeneration, we used Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice to overexpress N1ICD as well as LacZ to fate-map cells. Therefore, in this mouse model, Sox10<sup>rtTA</sup> must activate two Tet-on alleles simultaneously. To verify that active Notch signaling was achieved in our model and that the presence of the TetO-LacZ allele did not affect the level of Notch activation, we measured N1ICD expression levels using real-time qPCR. Specifically, Sox10<sup>rtTA</sup>-negative, Sox10<sup>rtTA</sup>::TetO-N1ICD, and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice were administered doxycycline, both in the diet to the nursing mother, as well as a single injection to the pups at P1. RNA was extracted from samples collected at P7 and N1ICD expression was analyzed with qPCR. Of note, the primers used for N1ICD detection recognize both the endogenous and transgenic transcripts and data is expressed as fold change relative to the Sox10<sup>rtTA</sup>-negative control.

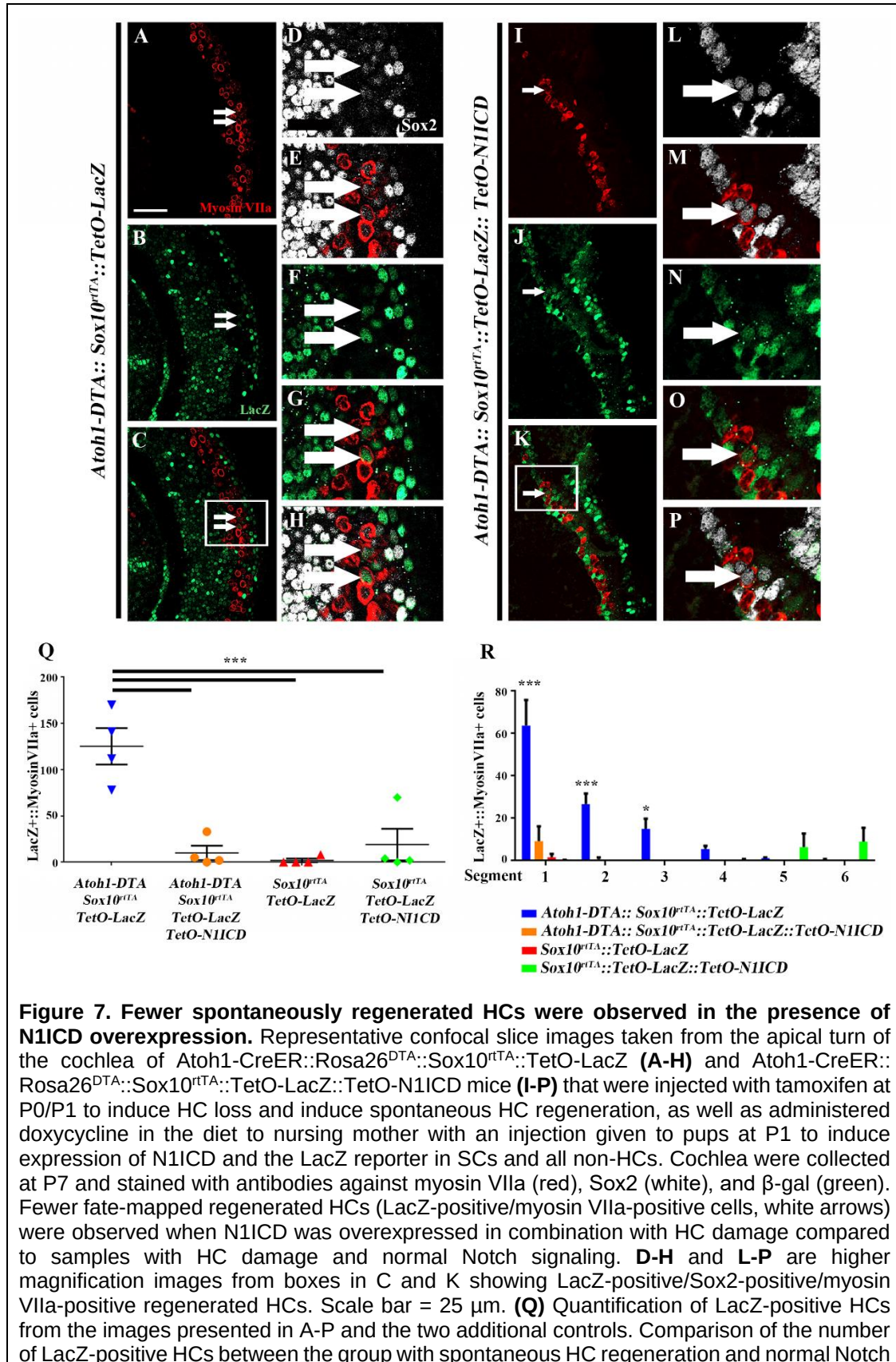


In both Sox10<sup>rtTA</sup>::TetO-N1ICD and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD groups, N1ICD was increased 5 to 6-fold compared to Sox10<sup>rtTA</sup>-negative samples (Figure 6A). There was no difference in the expression level of N1ICD between Sox10<sup>rtTA</sup>::TetO-N1ICD and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD samples, indicating that the addition of the TetO-LacZ allele did not interfere with increased Notch activity induced by the TetO-N1ICD allele. To further confirm increased Notch activity in our model, we used real-time qPCR to investigate the expression level of Notch target genes, Hes1, Hes5, Hey1, and HeyL. All Notch effector genes were increased 2 to 12-fold in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD cochleae compared to Sox10<sup>rtTA</sup>-negative controls (Figure 6B). Therefore, we conclude that Notch signaling was increased by N1ICD overexpression in our model.

To investigate whether spontaneous HC regeneration could be prevented by increased Notch signaling, we overexpressed N1ICD in SCs and other non-HCs in the context of HC damage using Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice. The effect of increased Notch signaling on spontaneous HC regeneration was investigated by quantifying the number of fate-mapped SCs. Specifically, we analyzed the number of LacZ-positive HCs in each of four groups. First, to verify the expression pattern of Sox10<sup>rtTA</sup> in a normal neonatal cochlea, the number of LacZ-positive HCs was quantified in Sox10<sup>rtTA</sup>::TetO-LacZ mice. Second, to determine the number of spontaneously regenerated HCs that occur naturally in the neonatal mouse cochlea after HC loss at birth, we quantified LacZ-positive HCs in Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ mice. Third, to determine the effect of N1ICD overexpression in SCs and other non-HCs in the neonatal mouse cochlea, LacZ-positive HCs were quantified in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice. Finally, to investigate whether overexpression of N1ICD in SCs and other non-HCs will prevent spontaneous HC regeneration from occurring, we quantified LacZ-positive HCs in Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice. Mice from each group were injected with tamoxifen at P0 and P1 to induce HC damage. N1ICD and LacZ expression were induced through administration of doxycycline given both in the food to the nursing mother, as well as one doxycycline injection given to the pups at P1, ~6 hours after tamoxifen injection. Samples were collected at P7 and processed for immunostaining with antibodies against myosin VIIa, Sox2, and  $\beta$ -gal.

As expected from previous work (Walters and Zuo, 2015), LacZ expression in Sox10<sup>rtTA</sup>::TetO-LacZ mice was observed in SCs and other non-HCs within the P7 cochlea. We also observed 2 LacZ-positive HCs in the very apical tip (Figure 7Q-R). There was no significant change in the number of LacZ-positive HCs when N1ICD was overexpressed without HC damage in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice (Figure 7Q). When LacZ was used for fate-mapping during spontaneous HC regeneration, ~125 LacZ-positive HCs were detected in Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ mice which is significantly more than in Sox10<sup>rtTA</sup>::TetO-LacZ controls (Figure 7Q). When N1ICD was overexpressed in the context of HC damage (Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice), there was a 92% reduction in LacZ-positive HCs compared to similar samples without N1ICD overexpression Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ mice) (Figure 7A-Q). Moreover, the number of LacZ-positive HCs when N1ICD was overexpressed and HCs were killed was not significantly different from controls lacking both HC damage and N1ICD overexpression (Sox10<sup>rtTA</sup>::TetO-LacZ samples) or N1ICD overexpression alone (Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD samples) (Figure 7Q). Therefore, we conclude that overexpression of N1ICD prevented the majority of spontaneous HC regeneration from occurring.

We wanted to better understand the location of the fate-mapped, spontaneously regenerated HCs in our samples because the majority of spontaneous HC regeneration is known to occur in the apical third of the neonatal cochlea (Cox et al., 2014) and

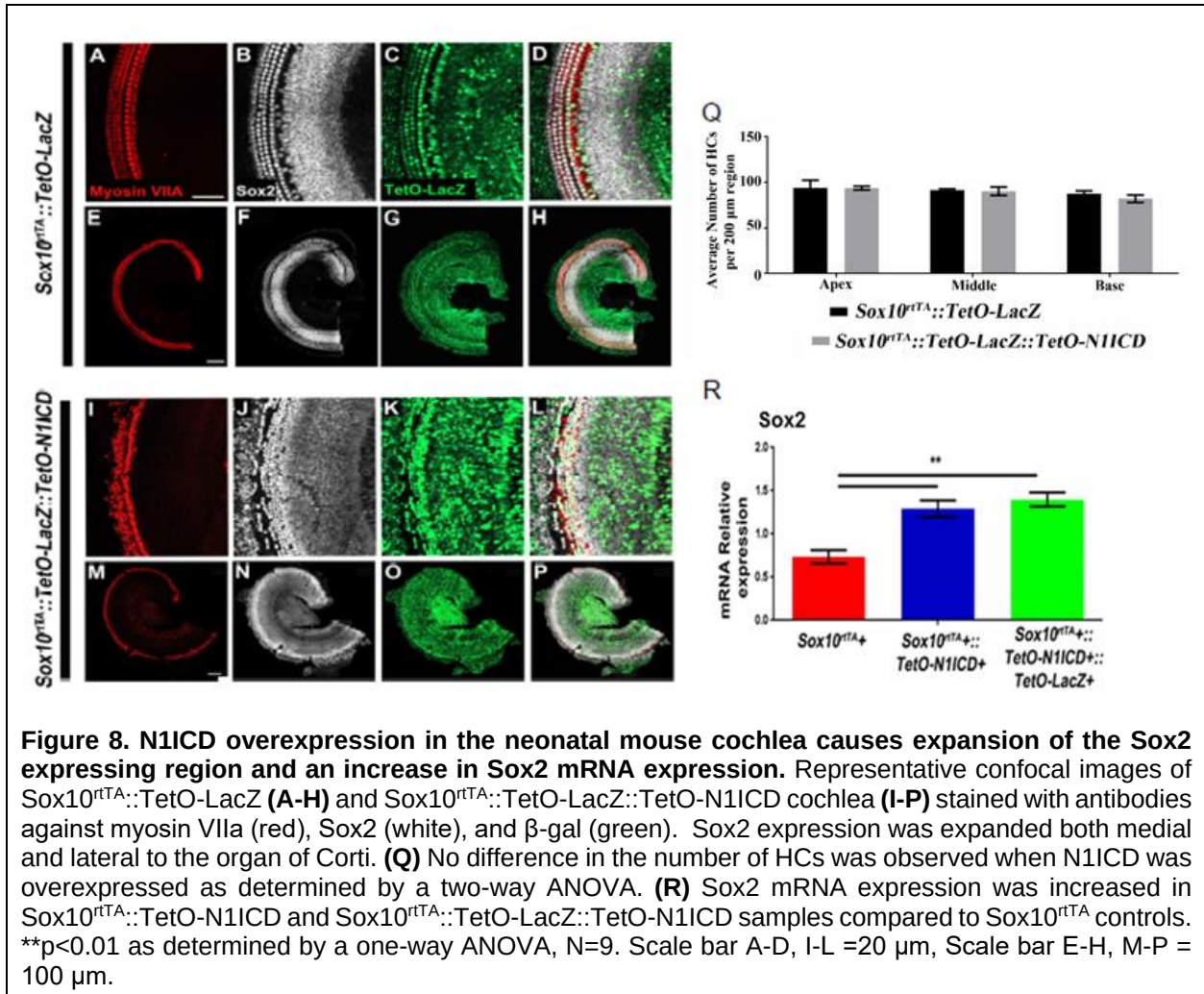


signaling (blue, Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ mice) and the experimental group with N1ICD overexpression as well as HC damage (orange, Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD), showed a ~92% decrease in the number of LacZ-positive, regenerated HCs. **(R)** LacZ-positive HCs after HC damage with physiological Notch signaling (blue, Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ) decreased in an apical to basal gradient while LacZ-positive HCs were only detected in the base when N1ICD was overexpressed without HC damage (green, Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD). Segment 1 is the most apical region and segment 6 is the most basal. N=3-4. \* p<0.05, \*\*\* p<0.001 as determined by a two-way ANOVA with a Tukey's post-hoc test. N=4.

because Notch signaling decreases in a basal to apical gradient during the first postnatal week (Maass et al., 2015). Therefore, the whole cochlea was imaged, measured, and divided into six equal segments from apex to base as previously described (McGovern et al., 2017) and the total number of LacZ-positive/myosin VIIa-positive HCs within each segment was quantified. The majority of fate-mapped, spontaneously regenerated HCs (from Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ mice) were found in the two most apical segments and this declined in an apical to basal gradient (Figure 7R), similar to the previous report (Cox et al., 2014). In one Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD sample that did not have HC damage, we observed 67 LacZ-positive HCs in the two basal segments, but no LacZ-positive HCs were detected in the middle or apical segments of the cochlea where the majority of HC regeneration was observed (Figure 7R). The other three Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD samples had no LacZ-positive HCs. Therefore, the LacZ-positive HCs observed in N1ICD overexpressing controls did not interfere with the quantification of fate-mapped, spontaneously regenerated HCs which were predominantly observed in the apical turn of the cochlea. When N1ICD was overexpressed along with HC damage, the fate-mapped regenerated HCs in the Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice were all located within the first two apical segments (Figure 7R), similar to the control with spontaneous HC regeneration and no manipulation of Notch (Atoh1-CreER::Rosa26<sup>DTA</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ mice). This illustrates that the regenerative capacity of SCs is correlated to the basal to apical changes in the Notch signaling pathway. Our data show weakened Notch signaling during the spontaneous HC regeneration process, as well as the suppression of HC regeneration by increased Notch activity. Taken together, this suggests that Notch signaling plays a role in the ability of SCs to spontaneously convert into, thus regenerate, HCs.

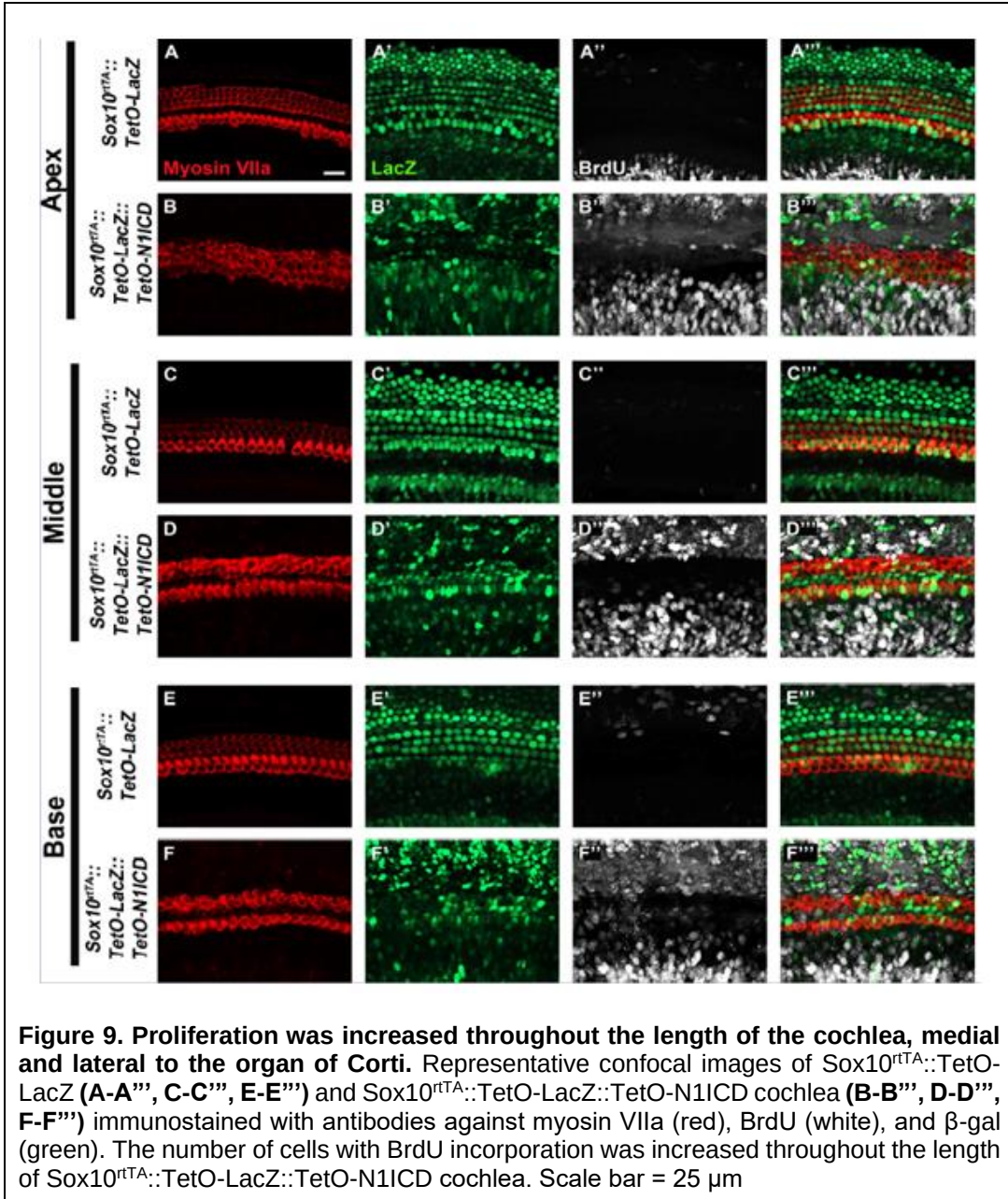
When N1ICD was overexpressed in SCs and other non-HCs (Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice), we also observed an expansion of Sox2 expression both medial and lateral to the organ of Corti and disorganization of HCs (Figure 8A-P), but there was no difference in the number of HCs compared to Sox10<sup>rtTA</sup>::TetO-LacZ mice (Figure 8Q). Therefore, we also measured the expression level of Sox2 using real-time qPCR and found a ~1.5 fold increase in Sox2 expression in Sox10<sup>rtTA</sup>::TetO-N1ICD and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice compared to control Sox10<sup>rtTA</sup>+ mice (Figure 8R).

To investigate whether the expanded Sox2 region was caused by increased proliferation, we injected BrdU twice a day (~6 hours apart) from P3 to P6 to label dividing cells. At P7, Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice had a large increase in the number



of BrdU-positive cells both medial and lateral to the organ of Corti in all turns of the cochlea, which was not observed in Sox10<sup>rtTA</sup>::TetO-LacZ control mice (with normal Notch signaling) (Figure 9A-F''). There were also a small number of BrdU-positive SCs in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice, but no BrdU-positive HCs were observed (Figure 9D'', F''). It also appeared that the number of LacZ-positive cells was increased and disorganized compared to Sox10<sup>rtTA</sup>::TetO-LacZ controls (Figure 8C, K and Figure 9A', B', C', D', E', F').

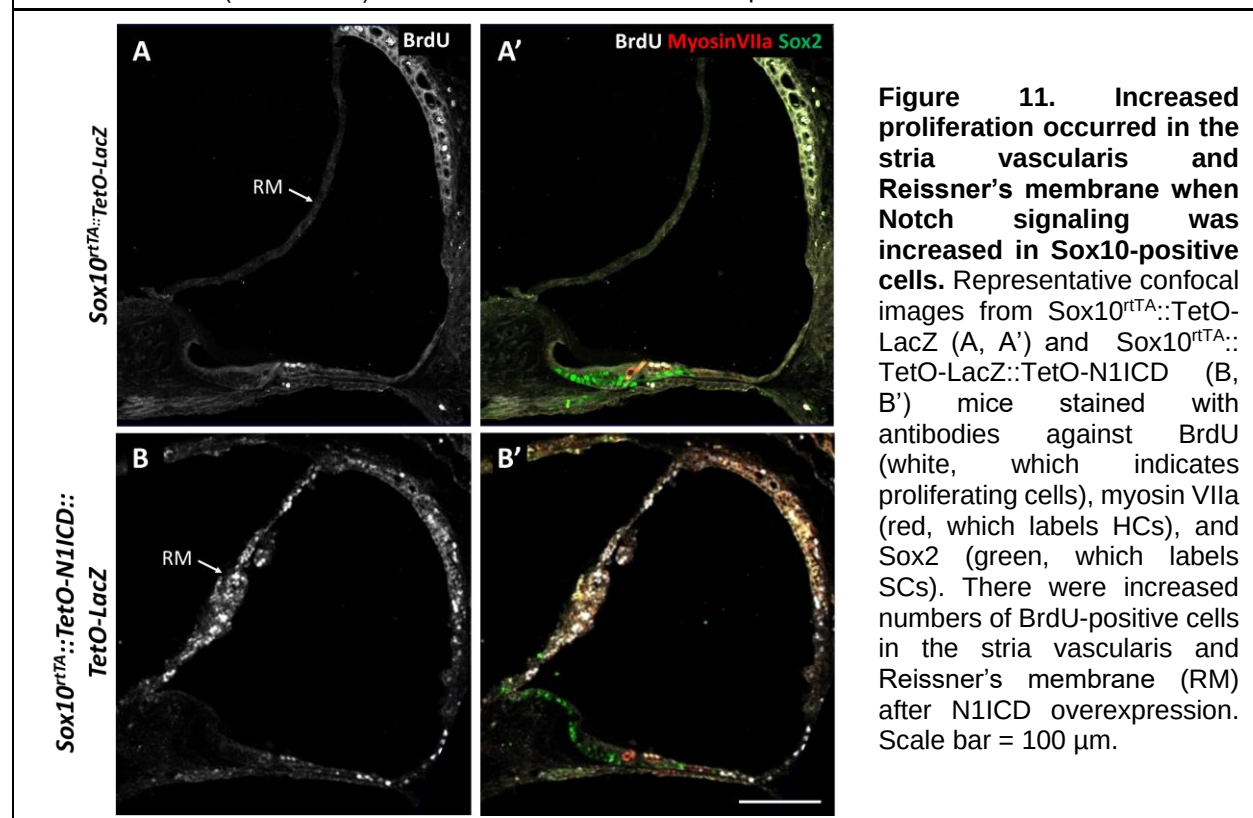
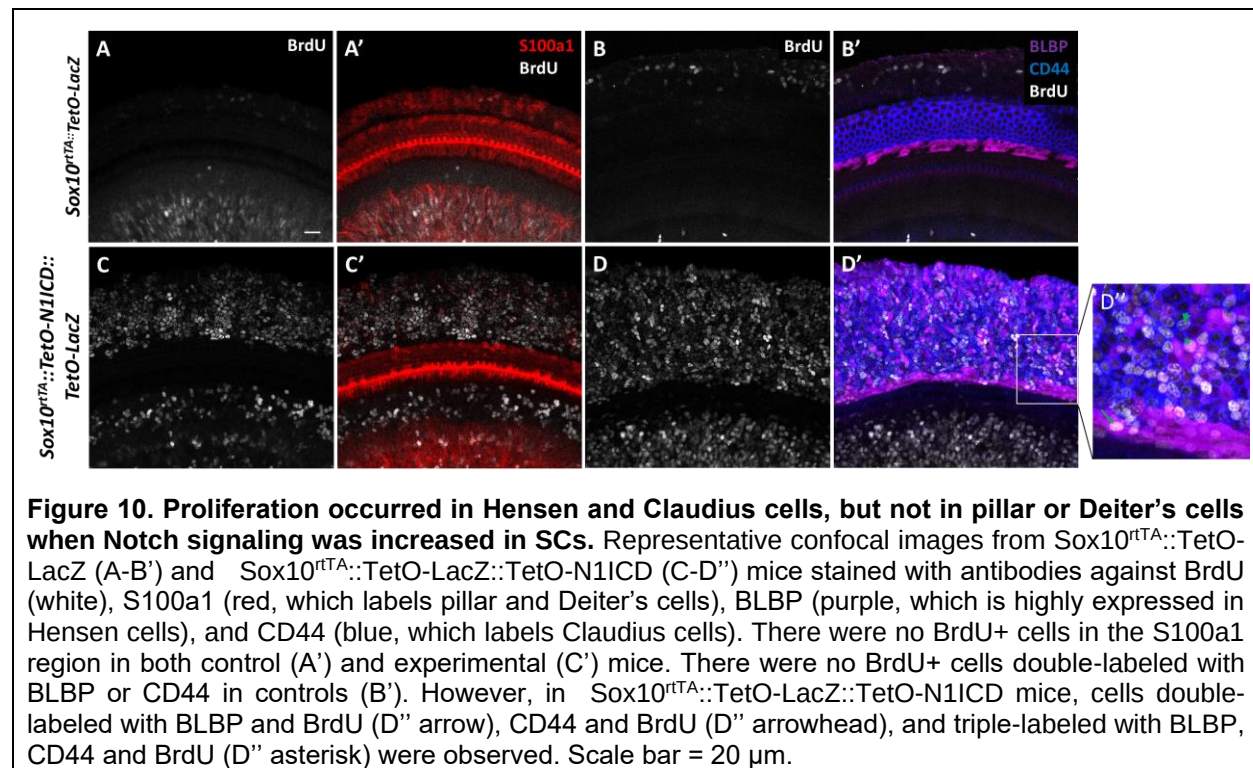
To further investigate the BrdU-positive SCs observed in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice, we used markers of specific SC subtypes to determine which subtypes were proliferating. The SC subtype markers included: S100a1 (which labels pillar and Dieter's cells) (Figure 10A-B'), BLBP (which has high expression in Hensen cells), and CD44 (which labels Claudius cells) (Figure 10C-D'). There was no double labeling of S100a1 with BrdU, indicating that pillar and Dieter's cells were not proliferating (Figure 10C-C'). However, we did observe double labeling of BLBP with BrdU (Figure 10D-D'', arrow) or CD44 with BrdU (Figure 10D-D'', arrowhead), suggesting that Hensen and Claudius cells proliferated. Interestingly, we also observed triple labeled cells with



BLBP, CD44, and BrdU (Figure 10D-D'', asterisk), possibly as a result of expanded BLBP expression into the Claudius cell region. While there were BrdU-positive cells located medial to the S100a1 labeling, the markers of inner phalangeal and border cells did not produce interpretable results and therefore we could not assess whether the medial located BrdU-positive cells were these SC subtypes or were cells in the GER.

To investigate whether increased proliferation occurred in other regions of the cochlea that are not visible using the whole mount dissection method, we performed mid-modiolar cyrosectioning. We observed that in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice, the Reissner's membrane was much thicker than control and there were increased

numbers of BrdU-positive cells in the Reissner's membrane and stria vascularis (Figure 11).



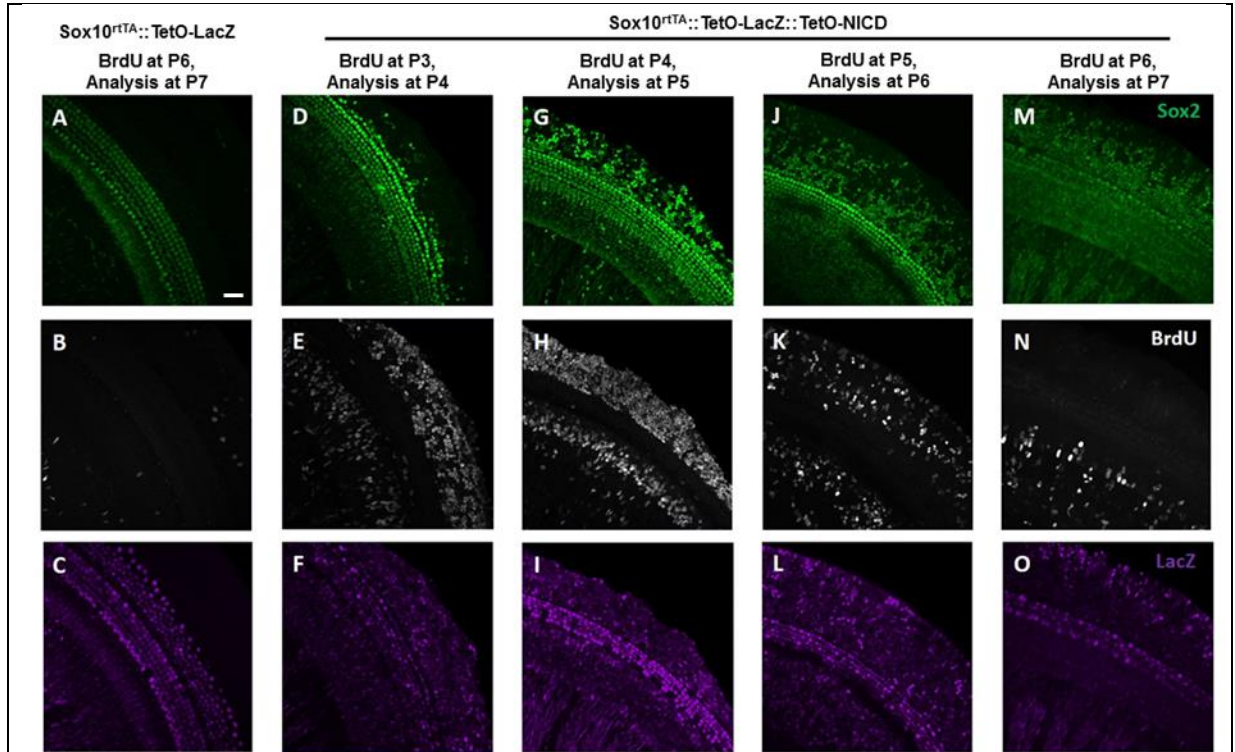
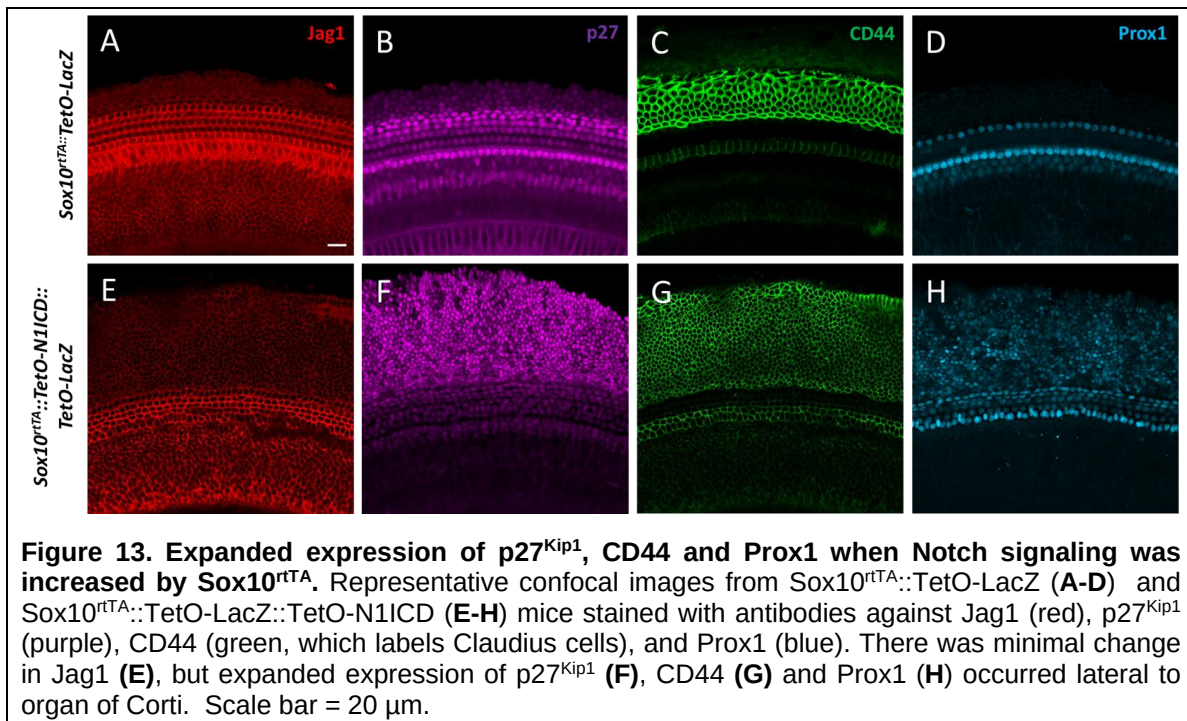


**Figure 12. Proliferation occurs continuously between P3-P6 in cochlea with overexpression of N1ICD.** Representative confocal images from control (Sox10<sup>rtTA</sup>::TetO-LacZ) mice injected with BrdU at P6 (**A-C**) and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice injected with BrdU at P3 (**D-F**), P4 (**G-I**), P5 (**J-L**) or P6 (**M-O**). All mice were given doxycycline from P0-P7 and collected one day after BrdU injection (i.e. on P4, P5, P6 or P7). Cochlea were immunostained with antibodies against Sox2 (green), BrdU (white), and  $\beta$ -galactosidase (to detect LacZ expression, purple). Scale bar = 20  $\mu$ M.

Since these mice were injected with BrdU twice a day from P3 to P6 to label dividing cells, it is possible that cells divided at early ages and then stopped or proliferation may have occurred throughout the entire BrdU injection window. To examine which day(s) cells were proliferating, we injected BrdU for one day only at P3, P4, P5, or P6, with two injections given ~6 hours apart. All mice were given doxycycline from P0-P7 (administered via the food to the nursing mother), as well as a P1 injection to the pups and samples were collected one day after BrdU injection (i.e. P4, P5, P6, or P7). We observed an increased number of BrdU-positive cells both medial and lateral to the organ of Corti in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice regardless of when BrdU was injected, suggesting that proliferation happens continuously between P3-P6 (Figure 12).

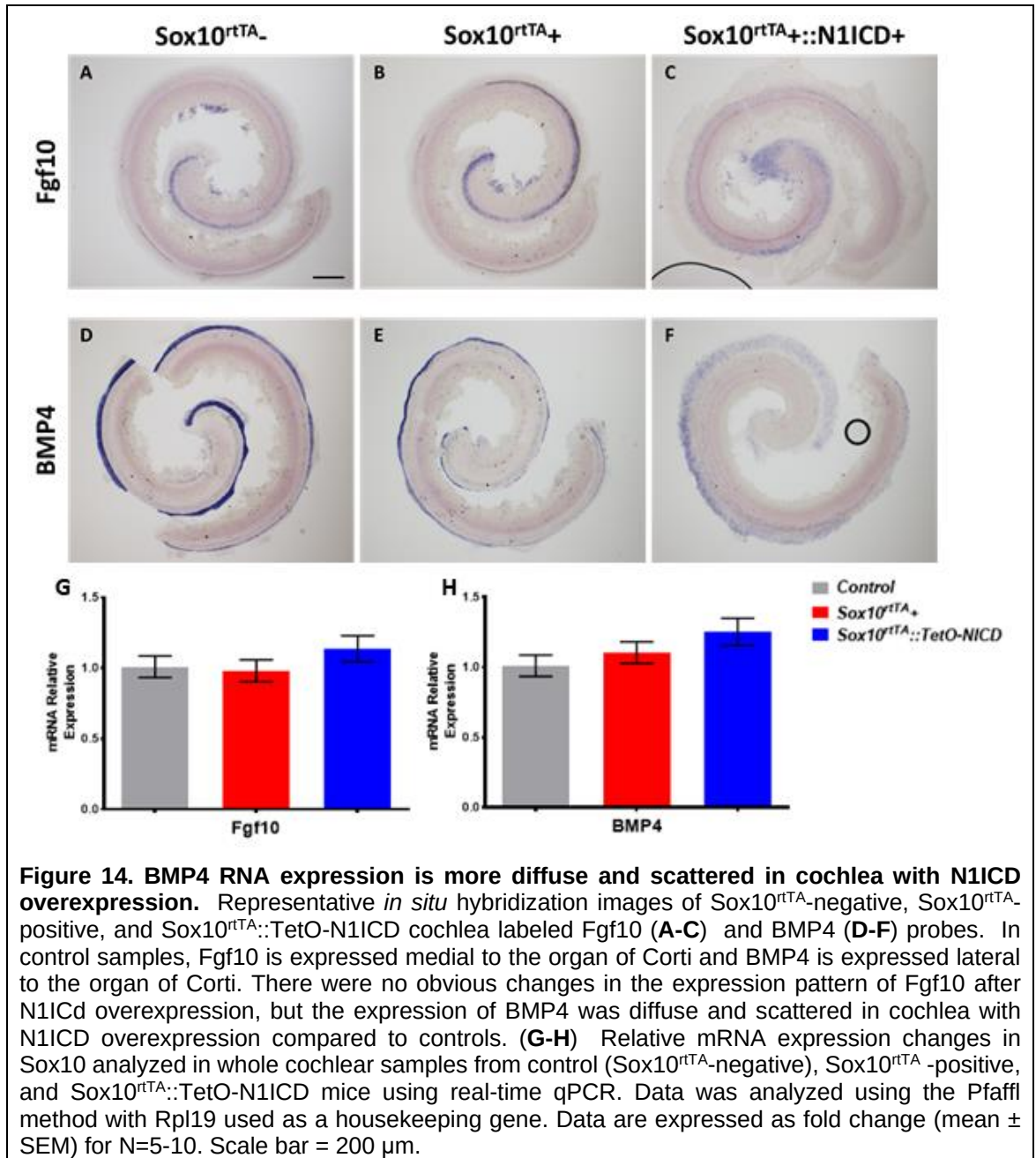
To further investigate the cause of the expanded Sox2 expression and the increased proliferation observed in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice, we performed immunostaining for markers of prosensory cells. Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD and control (Sox10<sup>rtTA</sup>::TetO-LacZ) mice were given doxycycline from P0 to P7 in the food to the nursing mother and were injected with doxycycline at P1. At P7, cochleae were stained with antibodies against Jag1 (Figure 13A, E), p27<sup>Kip1</sup> (Figure 13B, F), or Prox1 (Figure 13D, H). Compared to controls, there was minimal change in Jag1 expression (Figure 13E), but the expression of p27<sup>Kip1</sup> and Prox1 were expanded lateral

to the organ of Corti (Figure 13F,H). The increased p27<sup>Kip1</sup> expression may suggest an expansion of the sensory region since p27<sup>Kip1</sup> is also expressed in prosensory cells during embryonic development (Chen and Segil, 1999; Lee et al., 2006). Alternatively, it may indicate a negative feedback loop to stop or slow down the massive increase in cell division since p27<sup>Kip1</sup> also functions as an inhibitor of the cell cycle (Ezhevsky et al., 1997). While Prox1 is also expressed in prosensory cells (Bermingham-McDonogh et al., 2006; Fritzsche et al., 2010), it is a downstream target of Sox2 (Dabdoub et al., 2008). Therefore the expression pattern observed in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice may be a consequence of the expanded Sox2 expression. In addition, we examined the expression of CD44, a Claudius cell marker, and found expanded CD44 expression lateral to organ of Corti (Figure 13G) similar to what we observed with p27<sup>Kip1</sup> and Prox1.



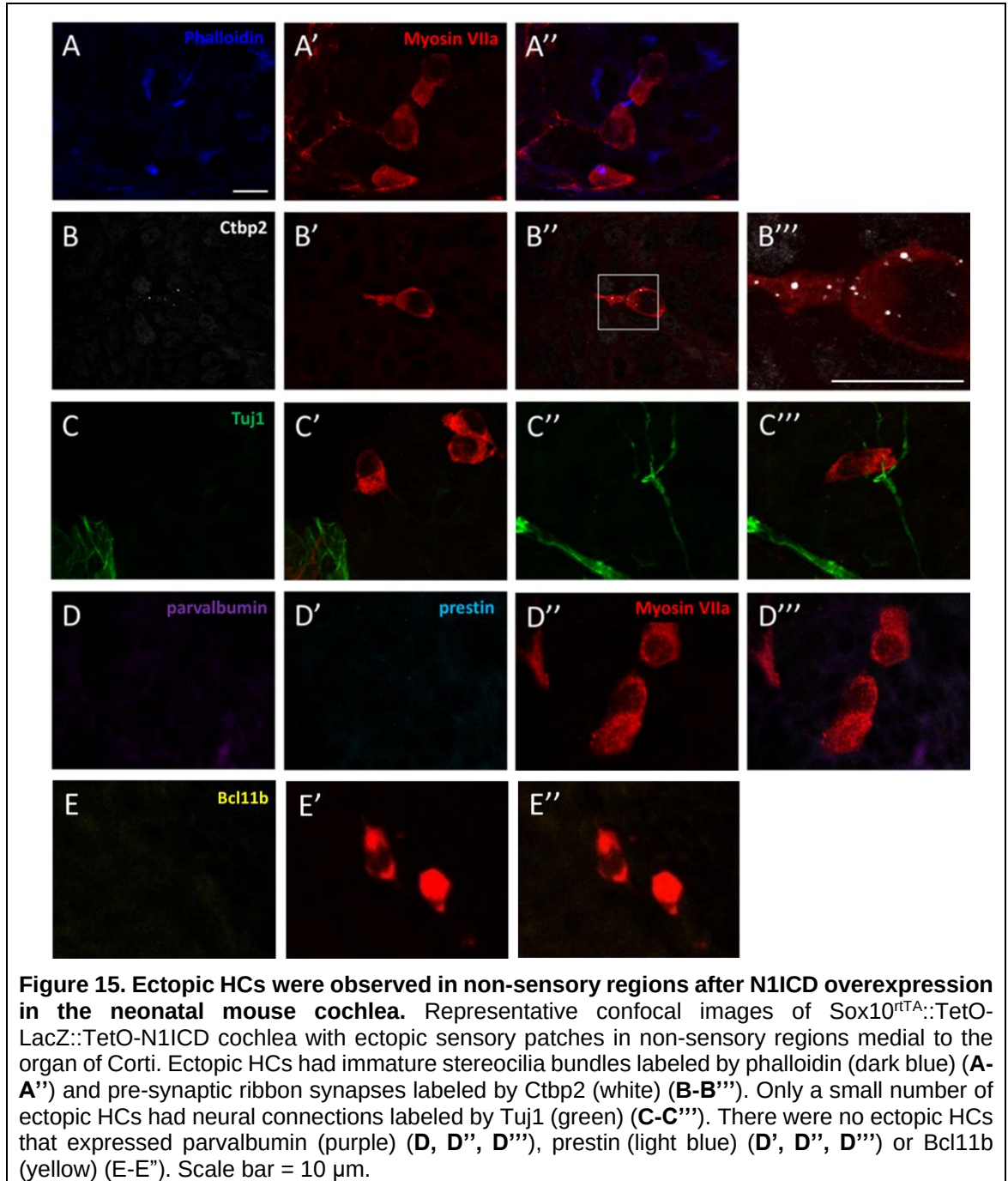
Next we used *in situ* hybridization to examine the expression pattern of Fgf10 and BMP4, two genes that define the boundaries of the organ of Corti during cochlear development (Morsli et al., 1998; Pirvola et al., 2002; Pauley et al., 2003; Hwang et al., 2010; Ohya et al., 2010). There was no obvious changes in the expression pattern of Fgf10 (Figure 14A-C). But the expression of BMP4 became diffuse and scattered in the area lateral to organ of Corti after N1ICD over-expression (Figure 14D-F). We also measured Fgf10 and BMP4 expression levels using real-time qPCR and found no change in the expression level of either gene (Figure 14G-H).

We also observed ectopic sensory patches in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice containing myosin VIIa-positive cells (presumptive HCs) and Sox2-positive cells, that were located medial to the organ of Corti. To further examine the myosin VIIa-positive cells in these patches, we performed immunostaining to determine whether they were innervated, had stereocilia bundles, or expressed markers of mature HCs. Many of the



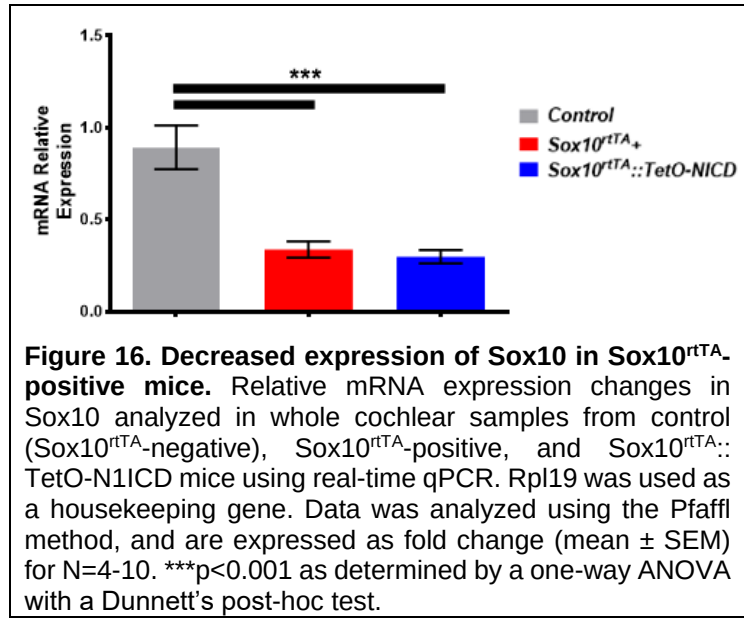
the ectopic HCs had stereocilia bundles (labeled by phalloidin which binds to actin), but the bundle shape appeared immature (Figure 15A-A"). While most ectopic HCs had synapses [labeled by C-terminal binding protein 2 (Ctbp2)] (Figure 15B-B"), only a small number of these cells were innervated by spiral ganglion neurons (labeled with Tuj1) (Figure 15C-C"). We also performed immunostaining with markers of more mature HCs, but did not find evidence that any of the ectopic HCs expressed parvalbumin or prestin (Figure 15D-D"). To investigate whether the ectopic HCs were differentiating as outer HCs, we looked for expression of Bcl11b, a gene that is expressed in immature outer HCs (Wiwatpanit et al., 2018). However, none of the ectopic HCs were observed had Bcl11b

expression (Figure 15E-E''), thus these cells are either developing as inner HCs or vestibular HCs or have not progressed beyond the myosin VIIa expression stage.

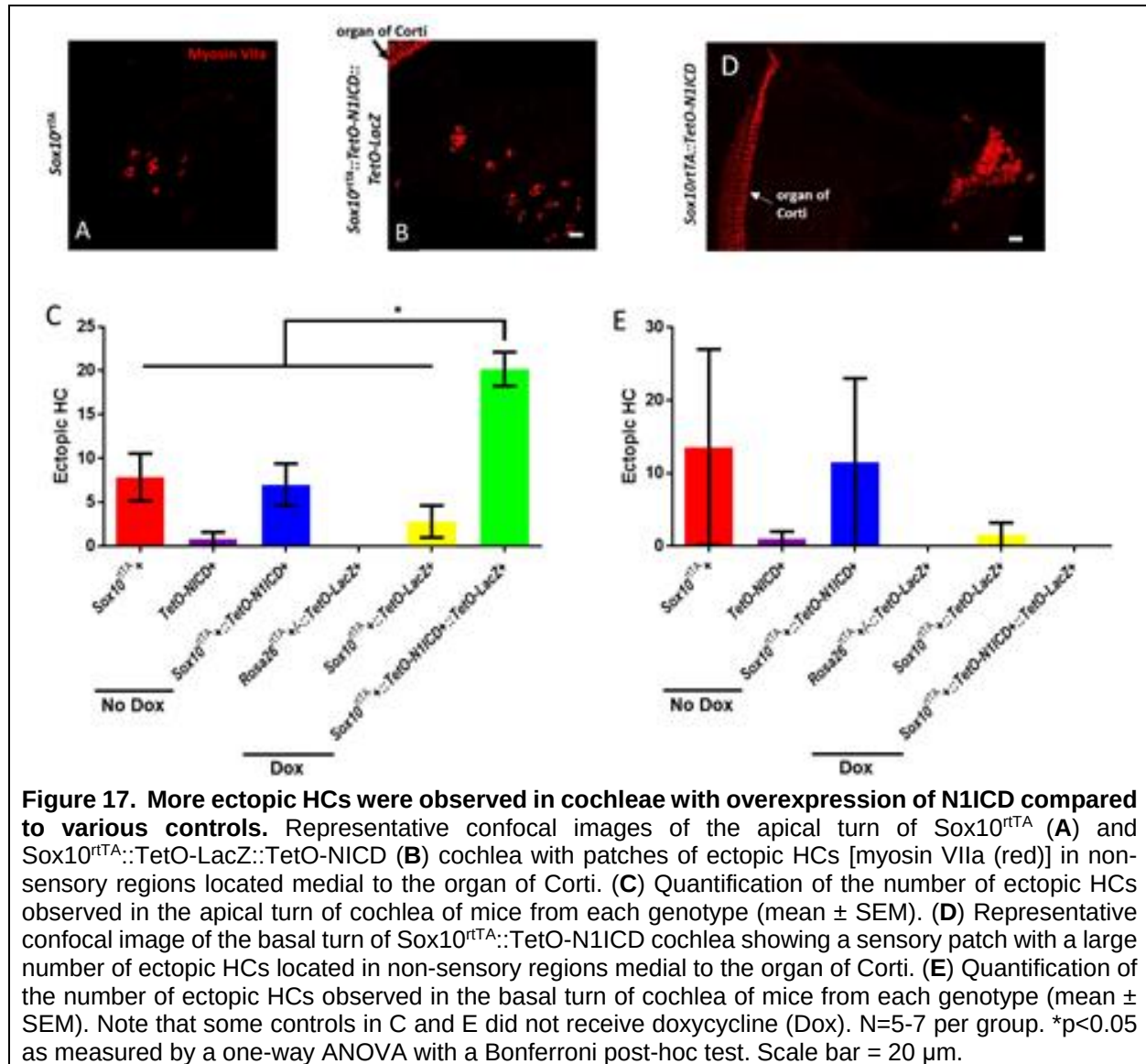


The expanded Sox2 expression, increased number of BrdU-positive cells, and ectopic sensory patches observed in Sox10<sup>rtTA::TetO-LacZ::TetO-N1ICD</sup> mice were unexpected because previous studies have shown that overexpression of N1ICD after embryonic day (E) 14.5 or at postnatal ages results in no effect (Liu et al., 2012b; Pan et al., 2013). Yet a similar phenotype of increased proliferation and ectopic sensory patches

was observed when N1ICD is overexpressed before E14.5 (Hartman et al., 2010; Pan et al., 2010, 2013; Liu et al., 2012b). Since Sox10<sup>rtTA</sup> is a knockin allele, mice carrying this gene have only one copy of Sox10. Therefore we measured the expression level of Sox10 and found that it was decreased by ~60% in Sox10<sup>rtTA</sup>, Sox10<sup>rtTA</sup>::TetO-N1ICD, and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice compared to Sox10<sup>rtTA</sup>-negative mice that have 2 copies of the Sox10 gene (Figure 16).



Since both Sox10 haploinsufficiency and increased N1ICD expression could potentially contribute to the formation of ectopic HCs, we next compared the number of ectopic HCs in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice with various controls (Figure 17). In Rosa26<sup>rtTA</sup>::TetO-LacZ controls, where LacZ was expressed ubiquitously after doxycycline treatment and both copies of Sox10 were present, we observed no ectopic HCs. However, we observed a small number of ectopic HCs in the apical turn of Sox10<sup>rtTA</sup>::TetO-LacZ controls treated with doxycycline. We also observed ectopic HCs in the apical turn of Sox10<sup>rtTA</sup>-positive controls that were not treated with doxycycline. Taken together these data suggest that ectopic HCs may be the result of Sox10 haploinsufficiency, but LacZ expression alone has no effect. We next examined whether the transgenic TetO-N1ICD allele had a role by itself. Although there were few ectopic HCs observed in the apical turn of TetO-N1ICD controls, the number was not significantly different from zero. This indicates that the insertion of the TetO-N1ICD transgene into the genome did not alter expression of another gene that caused the formation of ectopic HCs. In support, we also observed a similar number of ectopic HCs in the apical turn of Sox10<sup>rtTA</sup>::TetO-N1ICD and Sox10<sup>rtTA</sup>-positive controls that were not treated with doxycycline (Figure 17A,C). However, when N1ICD overexpression was induced in Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice by doxycycline treatment, a large increase in the number of ectopic HCs was seen in the apical turn of the cochlea (Figure 17B,C). By contrast in the basal turn of the cochlea, only a few ectopic HCs were observed. Yet one Sox10<sup>rtTA</sup>-positive sample and one Sox10<sup>rtTA</sup>::TetO-N1ICD sample (both of which did not receive doxycycline) had large patches of ectopic HCs (94 and 69 ectopic HCs respectively, Figure 17D,E). This suggests that we may have under-estimated the number of ectopic HCs since the amount of tissue medial to the organ of Corti varies from sample to sample when the whole mount dissection technique is used for cochlear dissection. Taken together, our data suggests that Sox10 haploinsufficiency and N1ICD overexpression have a synergistic effect to stimulate the formation of ectopic HCs.



We hypothesized that the expanded Sox2 expression, massive proliferation, and ectopic HCs may be caused by the super-physiological high levels of N1ICD induced in our model with the high dosage of doxycycline used (100mg/kg IP injection plus doxycycline given in the food to the nursing mother). Therefore, we analyzed the cochlea from Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice that were injected with lower doses of doxycycline (from 5mg/kg to 50mg/kg given only as an injection at P1) and used expanded Sox2 expression as the read out of the phenotype (Figure 18A-B). We also collected the 2<sup>nd</sup> ear from each mouse to measure the N1ICD expression level driven by each dose of doxycycline using real-time qPCR. We observed a clear dose response: mice that received 5 mg/kg doxycycline showed no sign of expanded Sox2 expression; ~57% of mice that received 10 mg/kg doxycycline exhibited expanded Sox2 expression; ~75% of mice that received 25 mg/kg doxycycline had expanded Sox2 expression; and when the dose increased to 50 mg/kg, all mice had expansion of Sox2 expression (Figure 18).

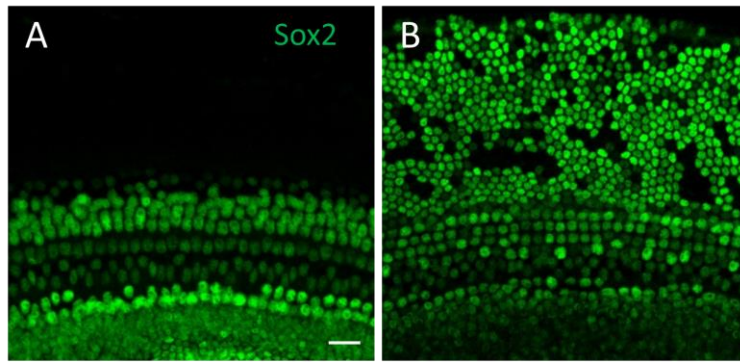


**Figure 18. Expansion of Sox2 expression in cochleae with N1ICD overexpression is dependent on the dose of doxycycline.** Representative confocal images of Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD cochlea treated with 5mg/kg (A) or 10mg/kg (B) doxycycline (Dox). A shows normal Sox2 expression and (B) shows an example of expanded Sox2 expression lateral to the organ of Corti. Table details the number and percentage of cochlea showing Sox2 expansion for each dose of doxycycline. Scale bar = 20  $\mu$ m.

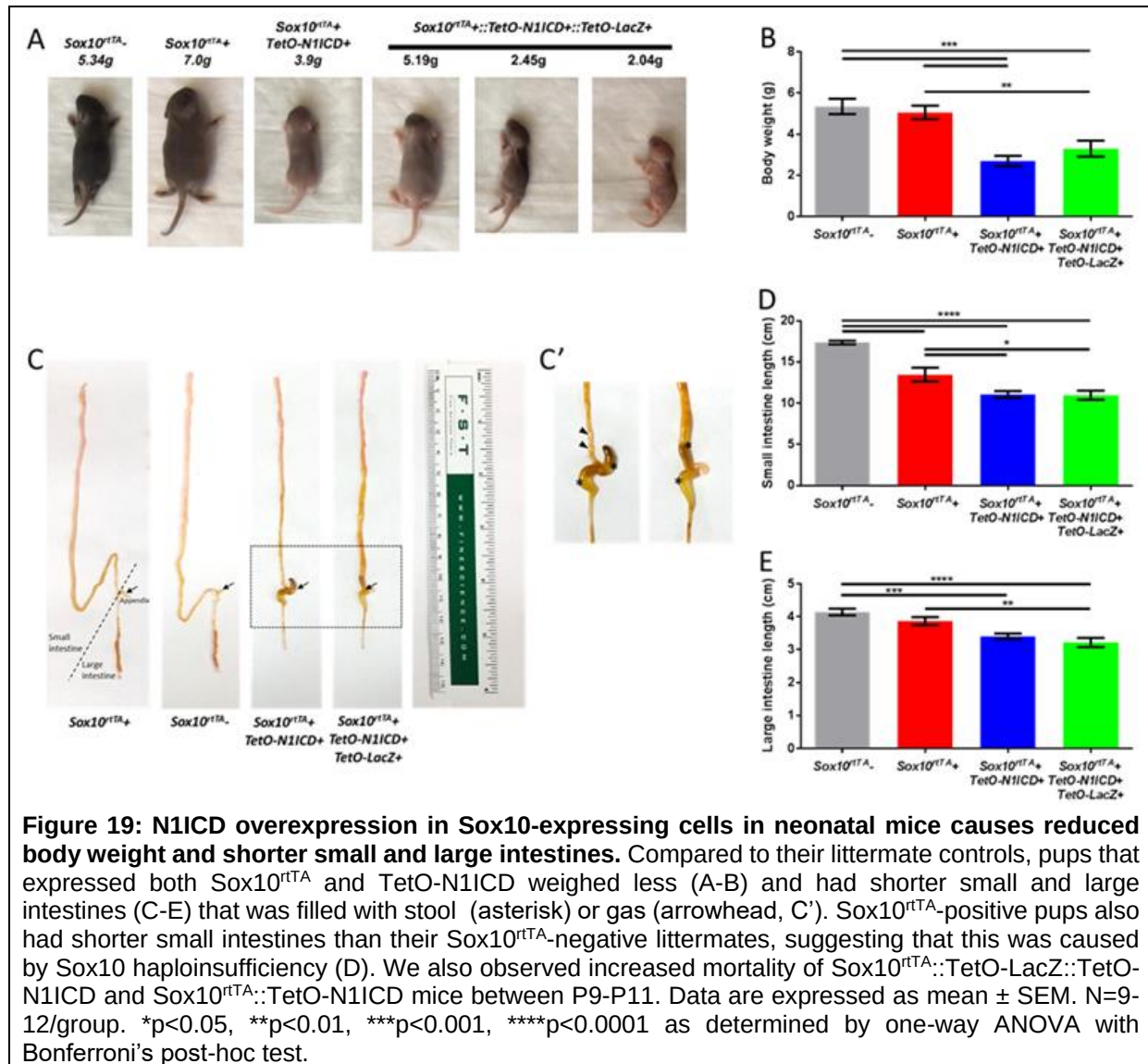
| Dox dose (mg/kg) | # of cochlea with Sox2 expansion | # of cochlea without Sox2 expansion | % of cochlea with Sox expansion |
|------------------|----------------------------------|-------------------------------------|---------------------------------|
| 5                | 0                                | 6                                   | 0%                              |
| 10               | 8                                | 6                                   | 57%                             |
| 25               | 3                                | 1                                   | 75%                             |
| 50               | 7                                | 7                                   | 100%                            |

To determine if haploinsufficiency of Sox10 is involved in this phenotype, we attempted similar doxycycline dose response experiments using a different rtTA mouse model (Rosa26<sup>rtTA</sup>) which has both copies of Sox10 intact. At the dose of 5 mg/kg doxycycline, there was no evidence of expanded Sox2 expression in Rosa26<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice (0 out of 5 cochleae). However, when we increased the dose to 10, 25 or 50 mg/kg, all of the Rosa26<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice died at P2-P3. Although the role of Sox10 haploinsufficiency itself remains inconclusive, we are still investigating the level of N1ICD expression using real-time qPCR experiments performed on the second ear of the same animal where we investigated the Sox2 expression pattern. Comparison of the level of N1ICD overexpression between samples with or without expanded Sox2 expression will help us make better conclusions.

Finally, we attempted to age Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD mice to P30 to assess their hearing using Auditory Brainstem Response (ABR), but unfortunately all pups with N1ICD overexpression died between P9 and P11. We also observed that pups within the same litter consistently varied in size (Figure 19A). To quantify this, we weighed pups at the time of sample collection (at P7). Four groups of mice were compared for weight: Sox10<sup>rtTA</sup>-negative, Sox10<sup>rtTA</sup>-positive, Sox10<sup>rtTA</sup>::TetO-N1ICD, and Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD. All mice were injected with doxycycline (100mg/kg) at P1 as well as doxycycline food given to the nursing mother. Pups that expressed both Sox10<sup>rtTA</sup> and TetO-N1ICD and therefore had N1ICD overexpressed in all Sox10<sup>rtTA</sup>-positive cells throughout the body weighed significantly less than Sox10<sup>rtTA</sup>-negative or Sox10<sup>rtTA</sup>-positive controls (Figure 19B).

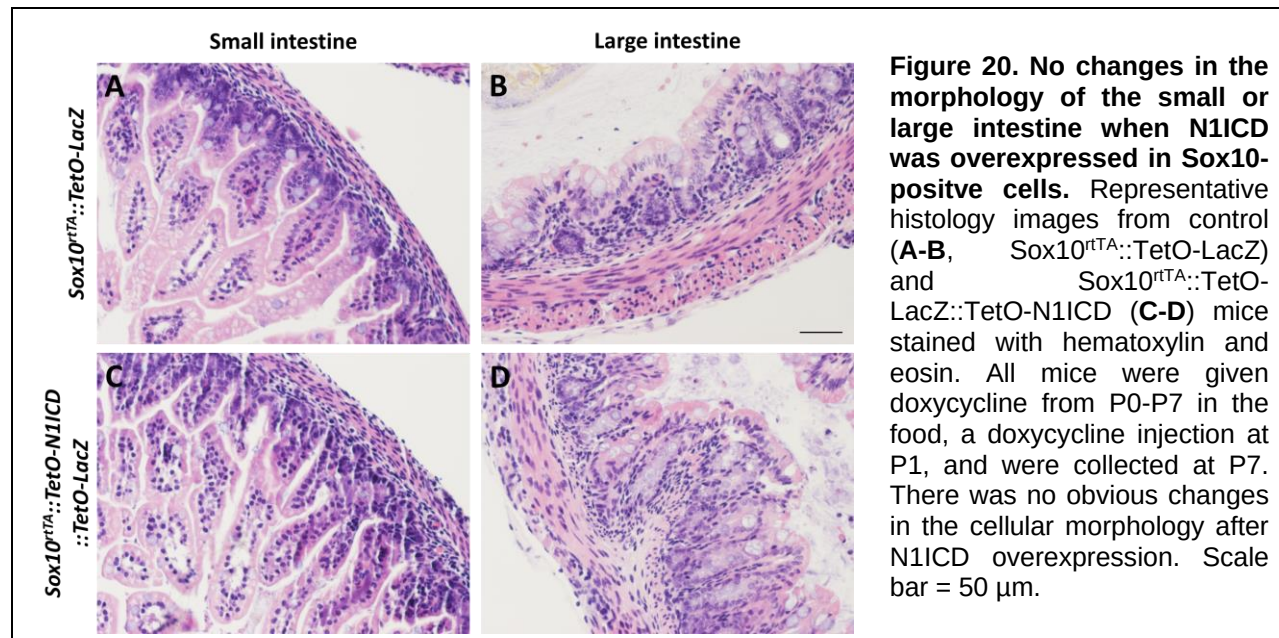
Since mutations in Sox10 have been reported to cause defects in the colon (Herbarth et al., 1998), we measured the length of intestine at P7. Sox10<sup>rtTA</sup>::TetO-N1ICD mice treated with doxycycline as described above had a significantly shorter small and

large intestines than their littermates (Figure 19C-E). This may have caused the increase mortality we observed. In addition, control samples that only expressed Sox10<sup>rtTA</sup> also had a shorter small intestine compared to Sox10<sup>rtTA</sup>-negative controls (Figure 19C-D), but there was no increased mortality of this genotype, possibly because the large intestine was normal length (Figure 19E). Next, we sectioned the small and large intestine and found no differences in the morphology of intestinal crypts and villi (Figure 20).



Stated goals not met: Data from Aim 2A were published in *Frontiers in Cellular Neuroscience* in May 2018. The vast majority of the experiments from Aim 2B are completed and we have created drafts of the figures. While we write the manuscript, we will finish the last experiments which is to examine the Sox10<sup>rtTA</sup>:TetO-LacZ:TetO-N1ICD cochlea using cyrosections to determine if increased proliferation occurs outside the organ of Corti (additional samples need to be analyzed) and to measure the N1ICD

expression level in Sox10<sup>rtTA</sup>:TetO-LacZ:TetO-N1CD mice given different doses of doxycycline.



**Specific Aim 3A:** To conditionally delete Delta-like1 (Dl1) from neonatal HCs in the normal, undamaged neonatal cochlea.

To investigate which Notch target genes are controlled by Dll1, we performed real-time qPCR experiments on P7 samples collected from Atoh1-CreER::Dll1<sup>loxP/loxP</sup> and Cre-negative control mice injected with tamoxifen at P0/P1 (to delete Dll1 from HCs). We also examined the expression of all 3 Notch receptors and the 2 other Notch ligands (Jag1 and Jag2) since there is a known feedback loop where Notch signaling regulates the expression of these Notch ligands as well (Daudet and Lewis, 2005; Murata et al., 2006). After deletion of Dll1 from HCs at P0/P1, we found a significant decrease in the expression of

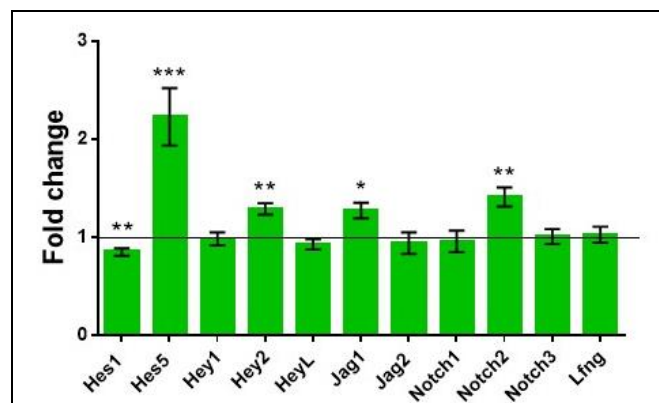


**Figure 21. Decreased expression of Hes1 and increased expression of Hes5, Hey2, Jag1, and the Notch 2 receptor after deletion of Dll1 from HCs.** Relative mRNA expression changes for Notch target genes, Notch ligands, and Notch receptors were analyzed in whole cochlear samples from Atoh1-CreER::Dll1<sup>loxP/loxP</sup> and Cre-negative control mice injected with tamoxifen at P0/P1 using real-time qPCR. Rpl19 was used as a housekeeping gene. Data was analyzed using the Pfaffl method and are expressed as fold change (mean ± SEM) compared to controls (back line). N=10. \*\*p<0.01, \*\*\* p<0.001 as determined by an unpaired Student's t test where each gene was analyzed separately.

Hes1 and a significant increase in expression of Hes5, Hey2, Jag1, and the Notch 2 receptor (Figure 21).

To measure changes in expression of Hes5 at the cellular level after deletion of Dll1 from neonatal HCs, we used the Hes5<sup>LacZ</sup> mouse line where the LacZ gene was knocked into the endogenous Hes5 locus and is thus a reporter of endogenous Hes5 expression (Imayoshi et al., 2010). Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and analyzed at P7 using anti-Sox2 antibodies to label all SC nuclei and anti-βgal antibodies to detect cells with Hes5<sup>LacZ</sup> expression. We then quantified the number of SCs that expressed Hes5 in the apical turn of control and experimental mice at P7. Compared to Cre-negative control mice, we observed no difference in the number of Hes5-positive cells after deletion of Dll1 from neonatal HCs (Figure 22).

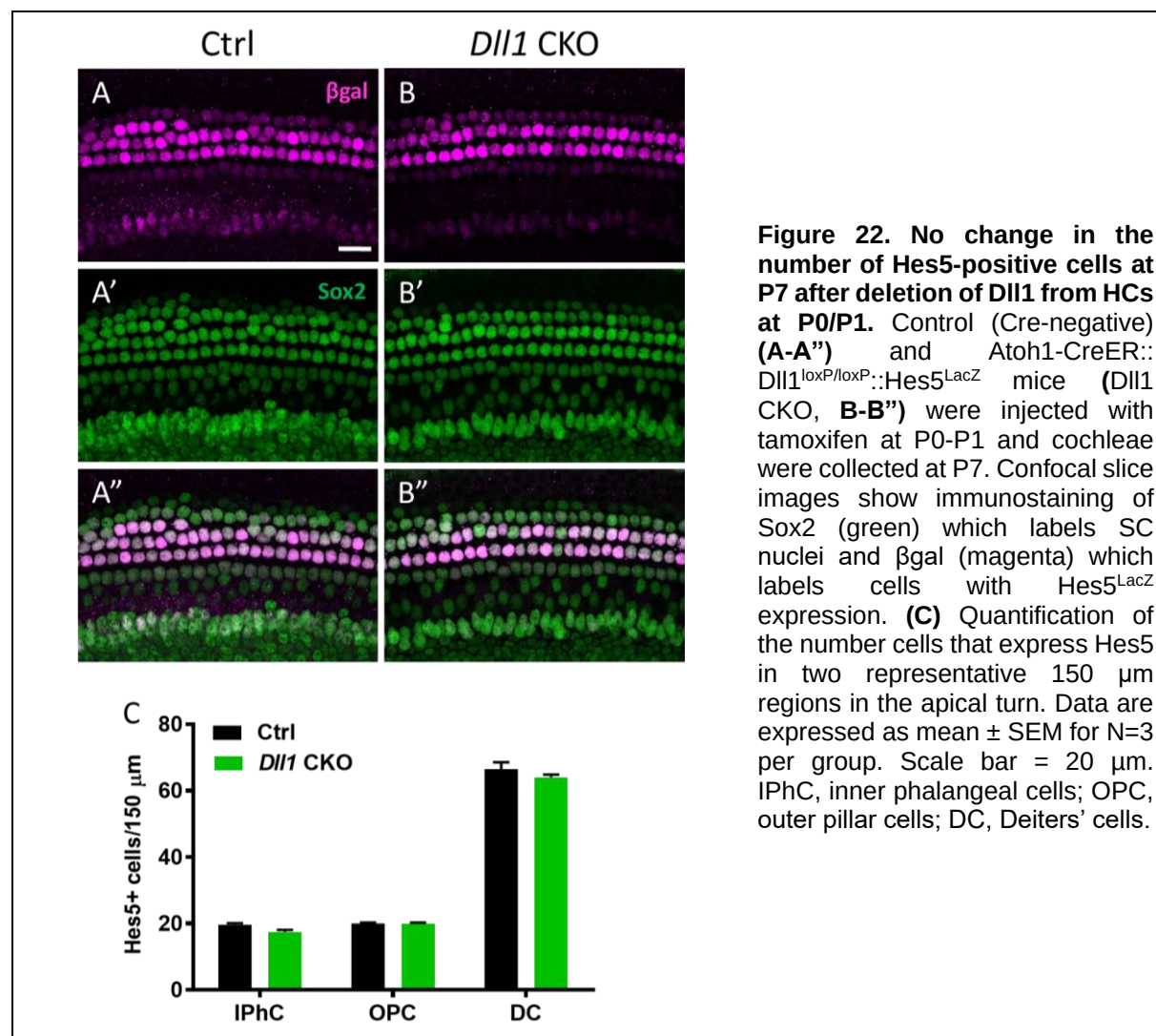
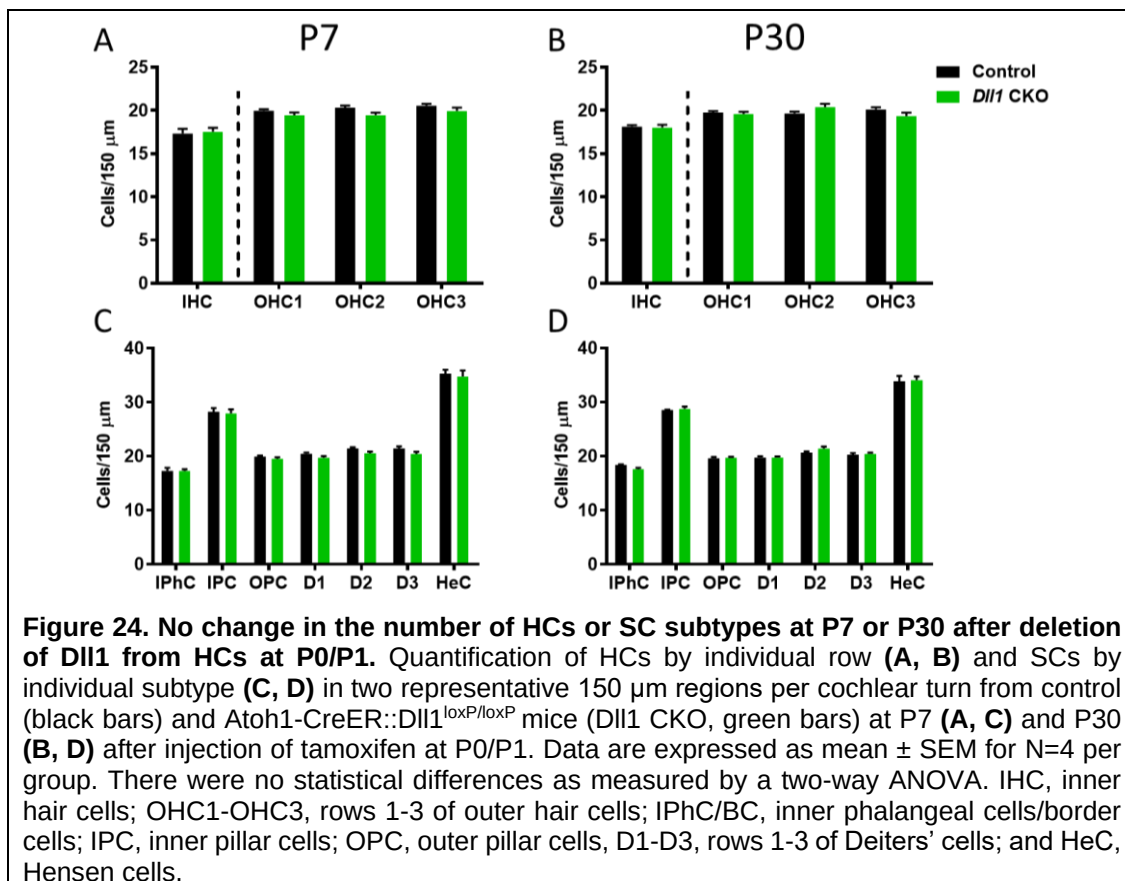
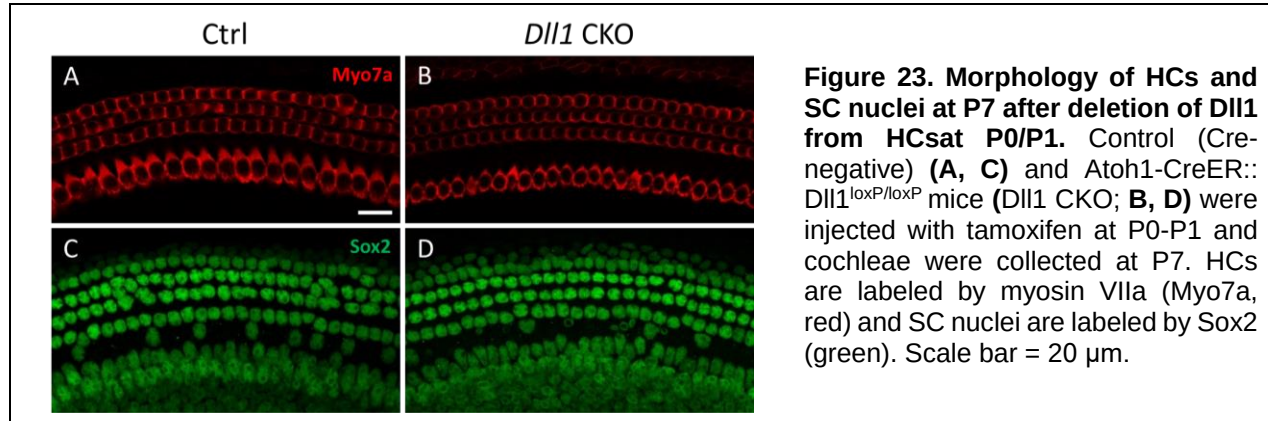


**Figure 22. No change in the number of Hes5-positive cells at P7 after deletion of Dll1 from HCs at P0/P1.** Control (Cre-negative) (A-A'') and Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> mice (Dll1 CKO, B-B'') were injected with tamoxifen at P0-P1 and cochleae were collected at P7. Confocal slice images show immunostaining of Sox2 (green) which labels SC nuclei and βgal (magenta) which labels cells with Hes5<sup>LacZ</sup> expression. (C) Quantification of the number cells that express Hes5 in two representative 150 μm regions in the apical turn. Data are expressed as mean ± SEM for N=3 per group. Scale bar = 20 μm. IPhC, inner phalangeal cells; OPC, outer pillar cells; DC, Deiters' cells.

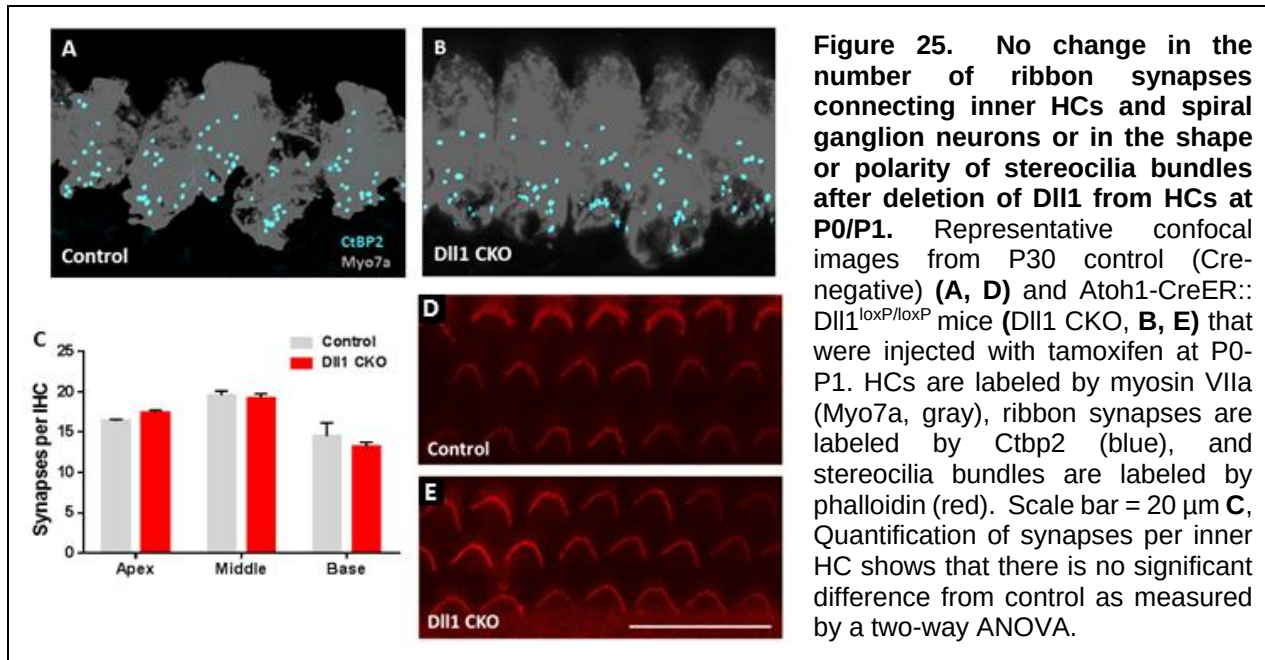
To investigate morphological changes to HCs and SCs after deletion of Dll1 from neonatal HCs, we used immunostaining and confocal microscopy. Atoh1-CreER::

$Dll1^{loxP/loxP}$  and Cre-negative control mice were injected with tamoxifen at P0/P1 and samples were collected at P7 and P30. We observed organized rows of HCs and SC nuclei in both control and  $Atoh1\text{-CreER}::Dll1^{loxP/loxP}$  mice at both ages (P7 shown in Figure 23). Quantification of HCs by row and SCs by individual subtype showed no significant differences from control at either P7 or P30 (Figure 24).



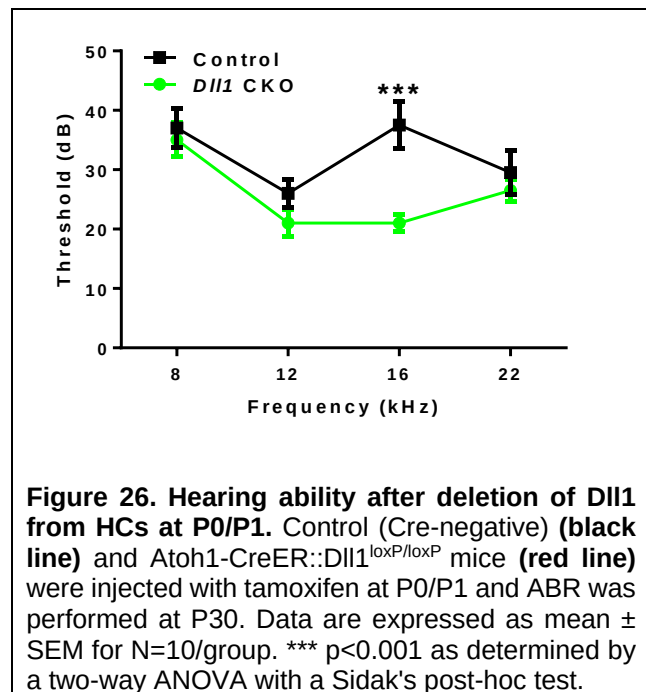
To further investigate more subtle changes to HCs, we examined the synapses that connect inner HCs to spiral ganglion neurons (called ribbon synapses) and the stereocilia bundles located on the apical surface of HCs.  $Atoh1\text{-CreER}::Dll1^{loxP/loxP}$  and Cre-negative control mice were injected with tamoxifen at P0/P1 and samples were

collected at P30 for immunostaining. An anti-C-terminal binding protein 2 (Ctbp2) antibody was used to label the presynaptic structure of the ribbon synapse and stereocilia bundles were labeled with phalloidin which binds to actin. There was no significant difference in the number of ribbon synapses per inner HC between control and Atoh1-CreER::DII1<sup>loxP/loxP</sup> mice (Figure 25A-C). In addition, there were no changes in the shape or polarity of the stereocilia bundles in Atoh1-CreER::DII1<sup>loxP/loxP</sup> mice compared to control samples (Figure 25D-E).



Finally, we examined whether deletion of DII1 from HCs at P0/P1 caused any effect on hearing function. Atoh1-CreER::DII1<sup>loxP/loxP</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and ABR was performed at P30 to measure changes in hearing. There was no hearing loss observed in Atoh1-CreER::DII1<sup>loxP/loxP</sup> mice. However there was an increase in ABR thresholds observed at 16 kHz in control mice that was not detected in Atoh1-CreER::DII1<sup>loxP/loxP</sup> mice (Figure 26). We are not sure why this occurred in the control mice. It may be related to the strain background.

Stated goals not met: Based on our revised Statement of Work (approved in AUG-2018), all tasks in Aim 3A have been completed.



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

To investigate which Notch target genes are controlled by Jag2, we performed real-time qPCR experiments on P7 samples collected from *Atoh1-CreER::Jag2<sup>loxP/loxP</sup>* and Cre-negative control mice injected with tamoxifen at P0/P1 (to delete Jag2 from HCs). We also examined the expression of all 3 Notch receptors and the 2 other Notch ligands (*Dll1* and *Jag1*) since there is a known feedback loop where Notch signaling regulates the expression of these Notch ligands as well (Daudet and Lewis, 2005; Murata et al., 2006). After deletion of Jag2 from HCs at P0/P1, we found a significant decrease in the expression of the Notch 3 receptor and a significant increase in the expression of *Hes5* and the Notch 2 receptor (Figure 27).

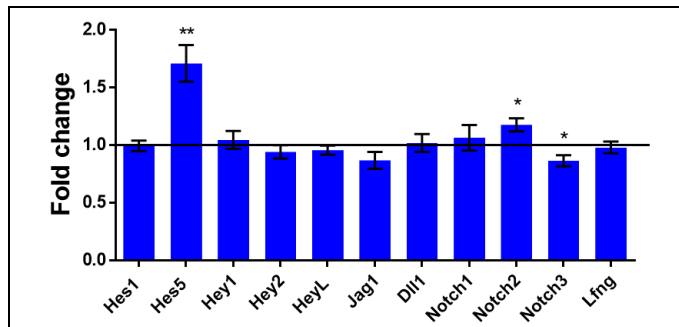
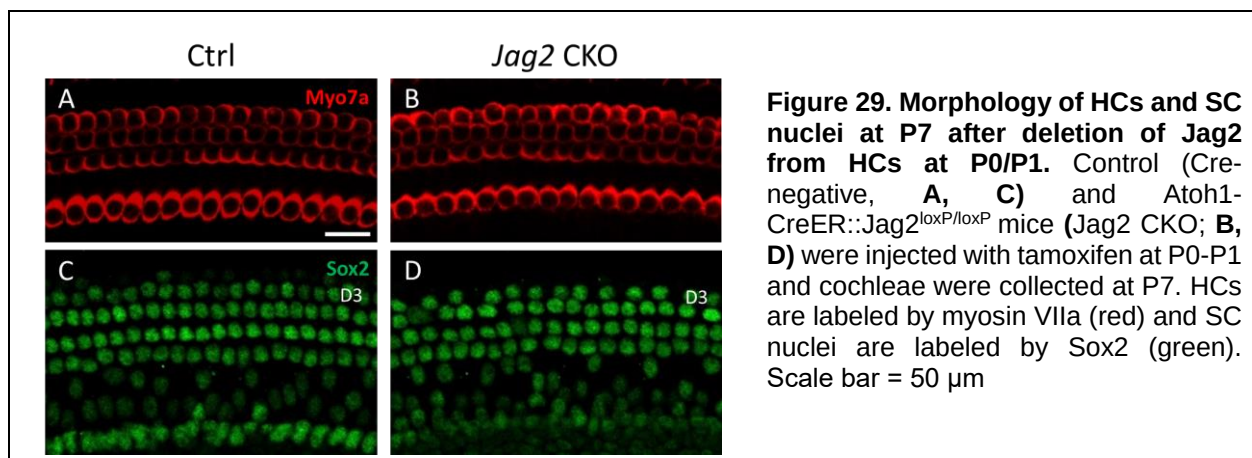
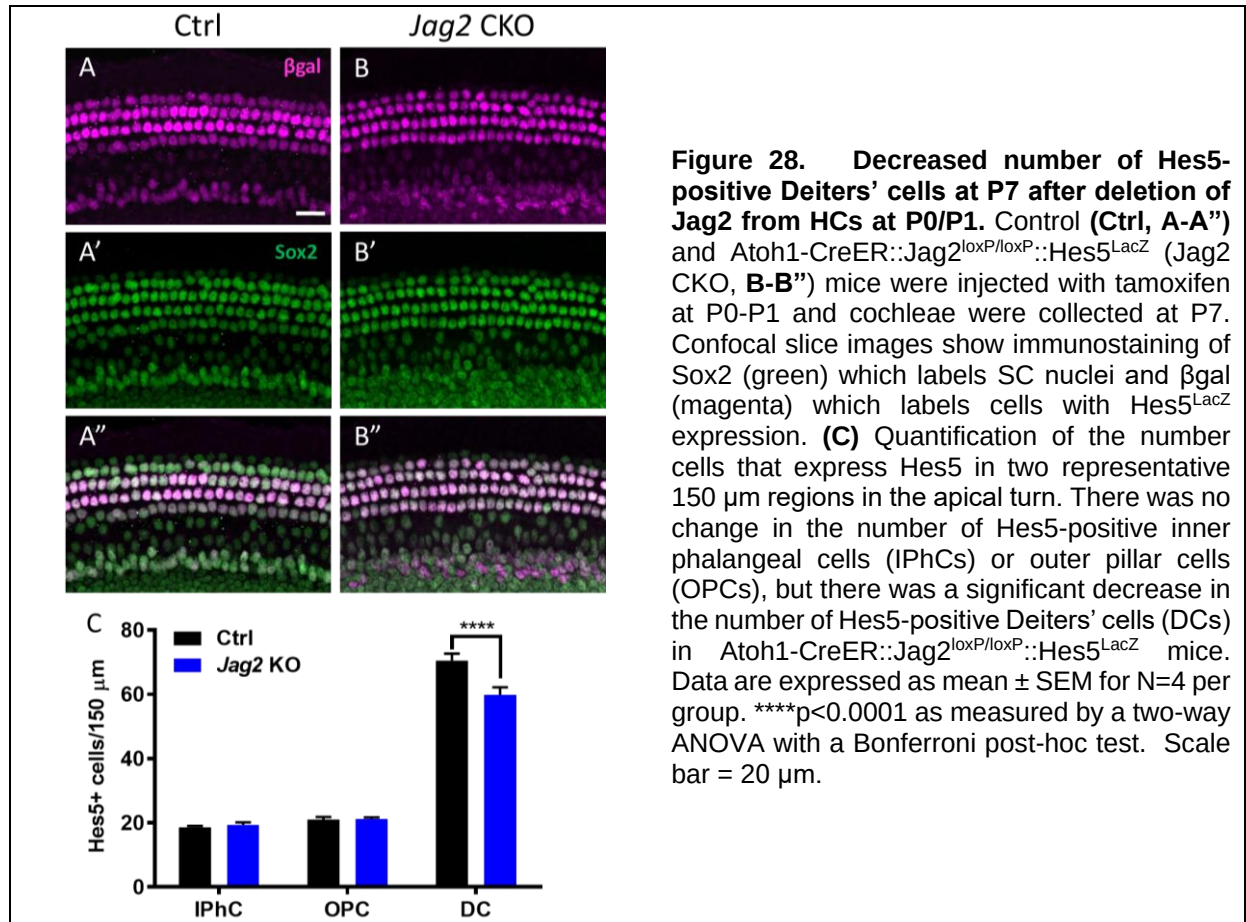


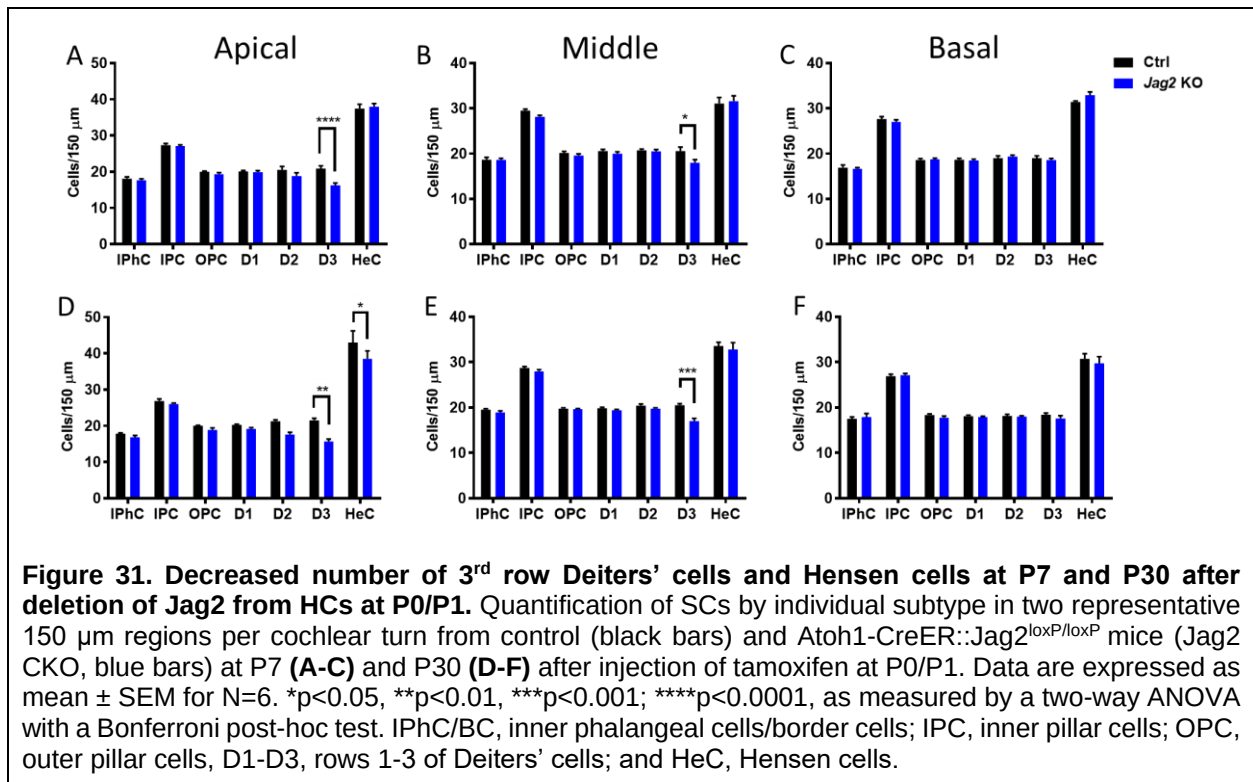
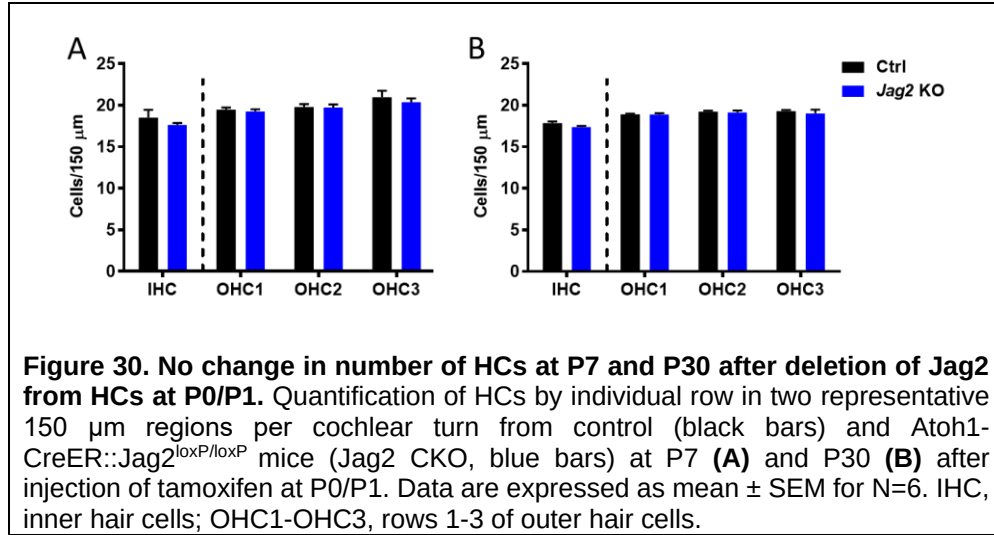
**Figure 27. Decreased expression of the Notch 3 receptor and increased expression of *Hes5* and the Notch 2 receptor after deletion of Jag2 from HCs at P0/P1.** Relative mRNA expression changes for Notch target genes, Notch ligands, and Notch receptors were analyzed in whole cochlear samples from *Atoh1-CreER::Jag2<sup>loxP/loxP</sup>* and Cre-negative control mice injected with tamoxifen at P0/P1 using real-time qPCR. *Rpl19* was used as a housekeeping gene. Data was analyzed using the Pfaffl method and are expressed as fold change (mean  $\pm$  SEM) compared to controls (back line). N=10. \*\*p<0.01 as determined by an unpaired Student's t test where each gene was analyzed separately.

To measure changes in expression of *Hes5* at the cellular level after deletion of Jag2 from neonatal HCs, we used the *Hes5<sup>LacZ</sup>* mouse line where the *LacZ* gene was knocked into the endogenous *Hes5* locus and is thus a reporter of endogenous *Hes5* expression (Imayoshi et al., 2010). *Atoh1-CreER::Jag2<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup>* and Cre-negative control mice were injected with tamoxifen at P0/P1 and analyzed at P7 using anti-Sox2 antibodies to label all SC nuclei and anti- $\beta$ gal antibodies to detect cells with *Hes5<sup>LacZ</sup>* expression. We then quantified the number of SCs that expressed *Hes5* in the apical turn of control and experimental mice at P7. Compared to Cre-negative control mice, there was no change in the number of *Hes5*-positive inner phalangeal cells or outer pillar cells, but there was a significant decrease in the number of *Hes5*-positive Deiters' cells after deletion of Jag2 from neonatal HCs (Figure 28). Taken together with the qPCR data, our results suggest that *Hes5*-positive cells have a higher level of *Hes5* expression than in control samples.

To investigate morphological changes to HCs and SCs after deletion of Jag2 from neonatal HCs, we used immunostaining and confocal microscopy. *Atoh1-CreER::Jag2<sup>loxP/loxP</sup>* and Cre-negative control mice were injected with tamoxifen at P0/P1 and samples were collected at P7 and P30. We observed organized rows of HCs and SC nuclei in both control and *Atoh1-CreER::Jag2<sup>loxP/loxP</sup>* mice at both ages (P7 shown in Figure 29). Quantification of HCs by row showed no change across all 3 cochlear turns at P7 and P30 (Figure 30). Quantification of SCs by individual subtype showed that there

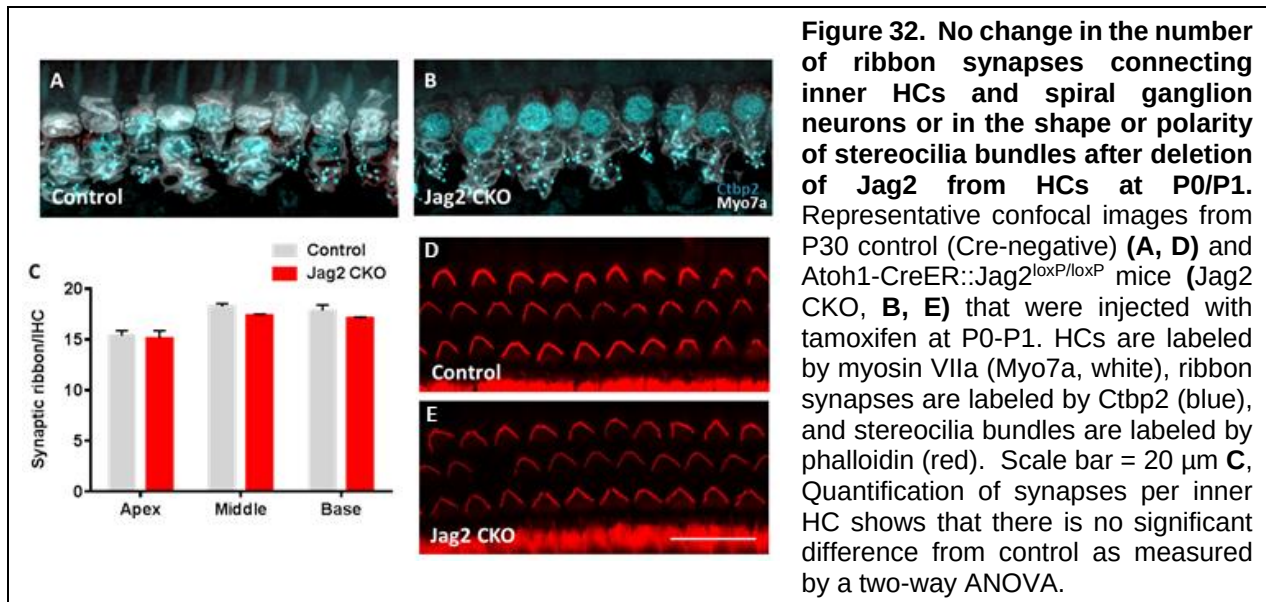
was a decrease in the number of 3<sup>rd</sup> row Deiters' cells in the apical and middle turns at P7 and P30, and a decrease in the number of Hensen cells in the apical turn at P30 compared to control samples, but there was no change observed in the number of any other SC subtype (Figure 31).





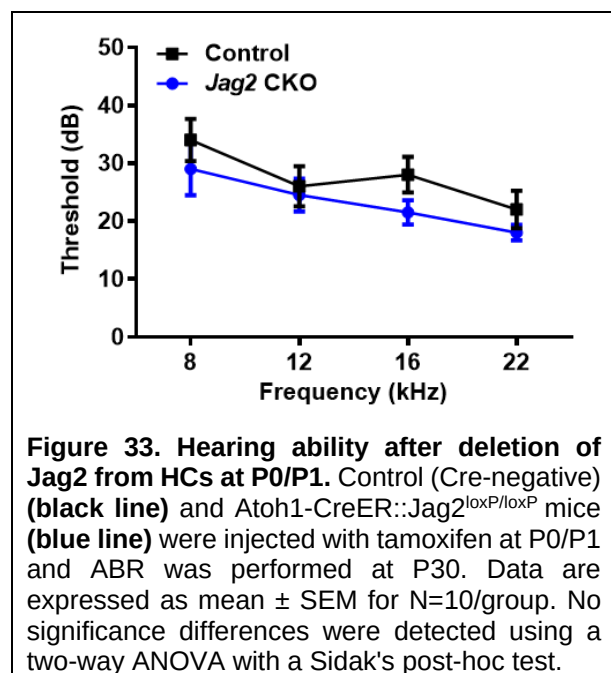
To further investigate more subtle changes to HCs, we examined the synapses that connect inner HCs to spiral ganglion neurons (called ribbon synapses) and the stereocilia bundles located on the apical surface of HCs. Atoh1-CreER::Jag2<sup>loxP/loxP</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and samples were collected at P30 for immunostaining. An anti-Ctbp2 antibody was used to label the presynaptic structure of the ribbon synapse and stereocilia bundles were labeled with phalloidin which binds to actin. There was no significant difference in the number of ribbon synapses per inner HC between control and Atoh1-CreER::Jag2<sup>loxP/loxP</sup> mice (Figure 32A-

C). In addition, there were no changes in the shape or polarity of the stereocilia bundles in Atoh1-CreER::Jag2<sup>loxP/loxP</sup> mice compared to control samples (Figure 32D-E).



Finally, we examined whether deletion of Jag2 from HCs at birth caused any effect on hearing function. Atoh1-CreER::Jag2<sup>loxP/loxP</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and ABR was performed at P30 to measure changes in hearing. There was no hearing loss observed in Atoh1-CreER::Jag2<sup>loxP/loxP</sup> mice at any frequency tested (Figure 33).

Stated goals not met: Based on our revised Statement of Work (approved in AUG-2018), all tasks in Aim 3B have been completed.



**Specific Aim 3B.2:** To conditionally delete both Dll1 and Jag2 from neonatal HCs in the normal, undamaged neonatal cochlea.

To investigate which Notch target genes are controlled by deletion of both Dll1 and Jag2 from HCs, we performed real-time qPCR experiments on P7 samples collected from Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> and Cre-negative control mice injected with tamoxifen at P0/P1 (to delete both ligands from HCs). We also examined the expression of all 3 Notch receptors and the other Notch ligand (Jag1) since there is a known feedback loop where Notch signaling regulates the expression of these Notch ligands (Daudet and Lewis, 2005; Murata et al., 2006). After deletion of both ligands from HCs at P0/P1, we found a significant decrease in the expression of Hes5, Hey1, Jag1, and the Notch 1 receptor and a significant increase in the expression of Lfng, a gene that modulates Notch signaling (Figure 34).

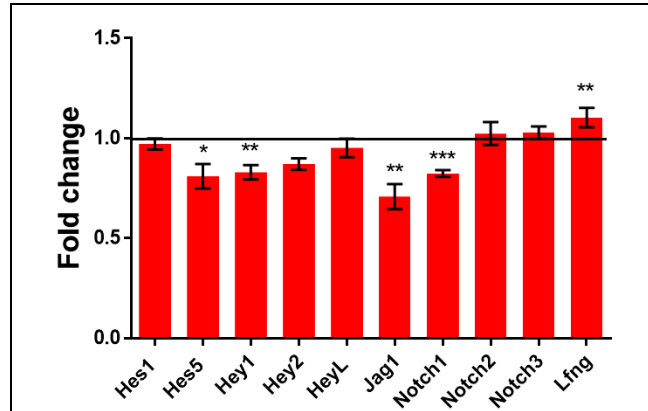


**Figure 34. Decreased expression of Hes5, Hey1, Jag1 and the Notch 1 receptor and increased expression of Lfng after deletion of both ligands from HCs.** Relative mRNA expression changes for Notch target genes, Notch ligands, and Notch receptors were analyzed in whole cochlear samples from Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> and Cre-negative control mice injected with tamoxifen at P0/P1 using real-time qPCR. Rpl19 was used as a housekeeping gene. Data was analyzed using the Pfaffl method and are expressed as fold change (mean  $\pm$  SEM) compared to controls (back line). N=10. \*\*p<0.01 as determined by an unpaired Student's t test where each gene was analyzed separately.

To measure changes in the expression of Hes5 at the cellular level after deletion of Dll1 and Jag2 from neonatal HCs, we used the Hes5<sup>LacZ</sup> mouse line where the LacZ gene was knocked into the endogenous Hes5 locus and is thus a reporter of endogenous Hes5 expression (Imayoshi et al., 2010). Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and examined at P7 using anti-Sox2 antibodies to label all SC nuclei and anti- $\beta$ gal antibodies to detect cells with Hes5<sup>LacZ</sup> expression. There was no change in the number of Hes5-positive inner phalangeal cells or outer pillar cells, but we observed a decrease in the number of Hes5-positive Deiters' cells in the cochlea of Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> mice compared to Cre-negative littermate controls (Figure 35C), which confirms our qPCR results.

Since Jag1 expression was also decreased and we had an antibody that works well for immunostaining, we measured changes in the expression of Jag1 at the cellular level after deletion of Dll1 and Jag2 from HCs. Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> and Cre-negative control mice received tamoxifen injections at P0/P1 and their cochleae were analyzed at P7 and P30. We observed a similar Jag1 expression pattern in control and Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice at P7, except for slight disorganization in the 2<sup>nd</sup> and 3<sup>rd</sup> row of Deiters' cells (Figure 36A-B). However, at P30, there was decreased

Jag1 staining in the 2<sup>nd</sup> and 3<sup>rd</sup> row of Deiters' cells in Atoh1-CreER::Dl1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice compared to Cre-negative littermate controls (Figure 36C-D).

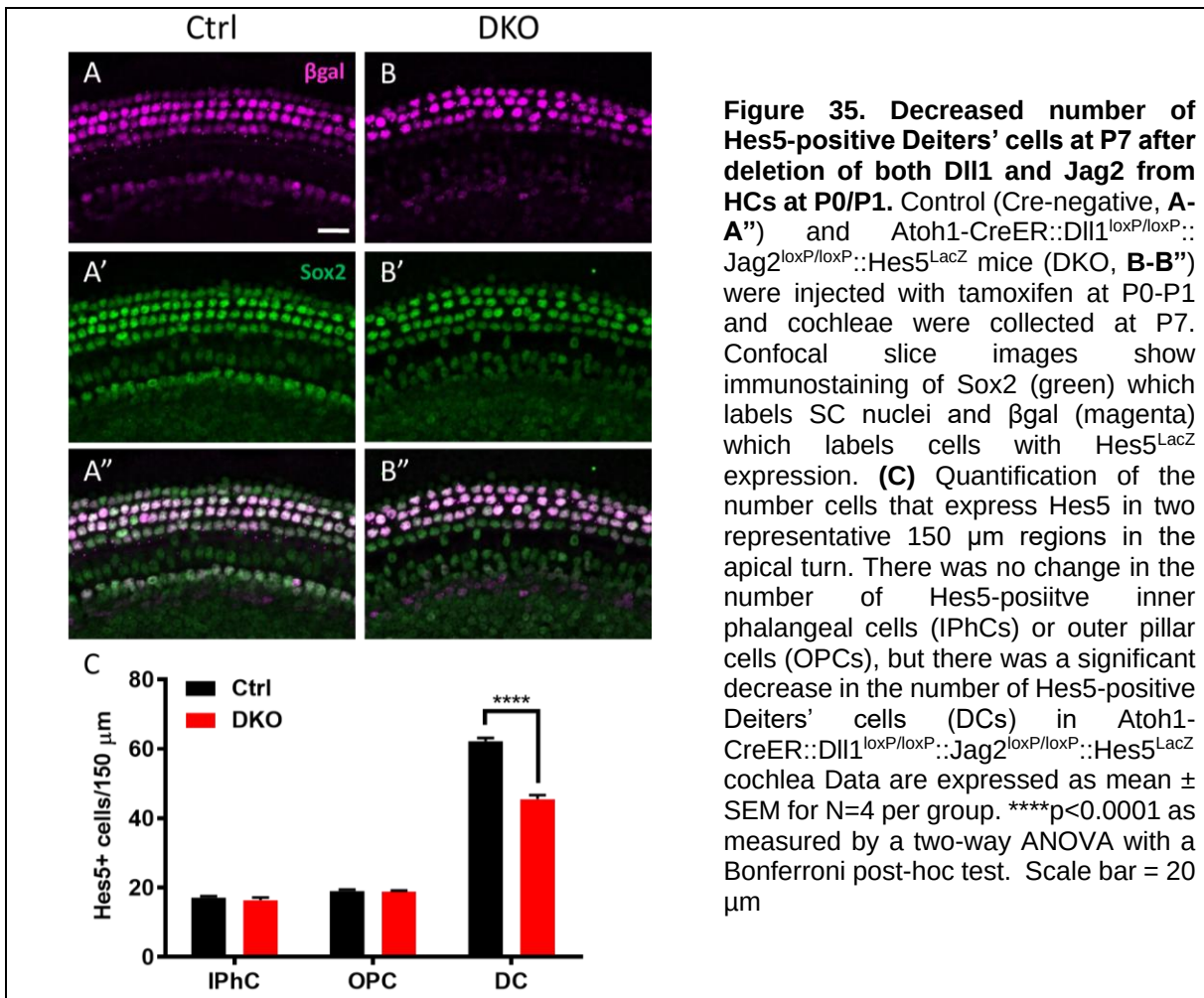


**Figure 35. Decreased number of Hes5-positive Deiters' cells at P7 after deletion of both Dll1 and Jag2 from HCs at P0/P1.** Control (Cre-negative, A-A'') and Atoh1-CreER::Dl1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> mice (DKO, B-B'') were injected with tamoxifen at P0-P1 and cochleae were collected at P7. Confocal slice images show immunostaining of Sox2 (green) which labels SC nuclei and  $\beta$ gal (magenta) which labels cells with Hes5<sup>LacZ</sup> expression. (C) Quantification of the number cells that express Hes5 in two representative 150  $\mu$ m regions in the apical turn. There was no change in the number of Hes5-positive inner phalangeal cells (IPhCs) or outer pillar cells (OPCs), but there was a significant decrease in the number of Hes5-positive Deiters' cells (DCs) in Atoh1-CreER::Dl1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> cochlea. Data are expressed as mean  $\pm$  SEM for N=4 per group. \*\*\*\*p<0.0001 as measured by a two-way ANOVA with a Bonferroni post-hoc test. Scale bar = 20  $\mu$ m

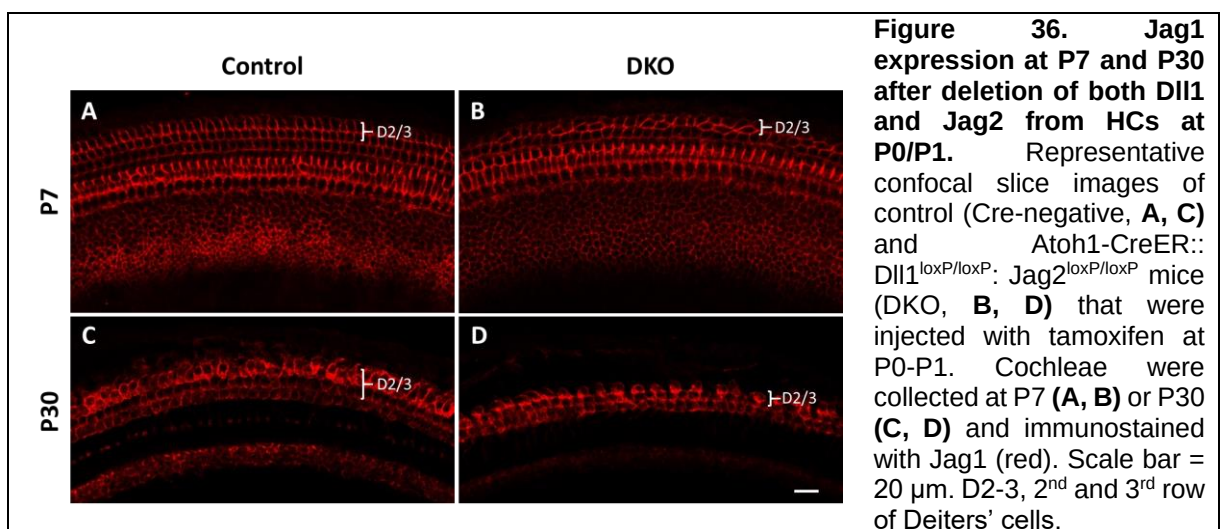
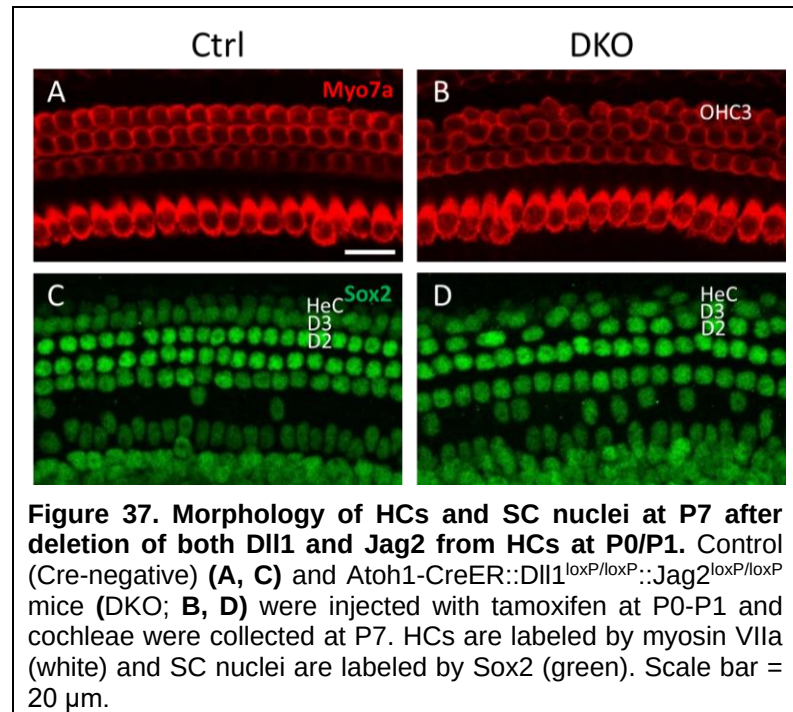
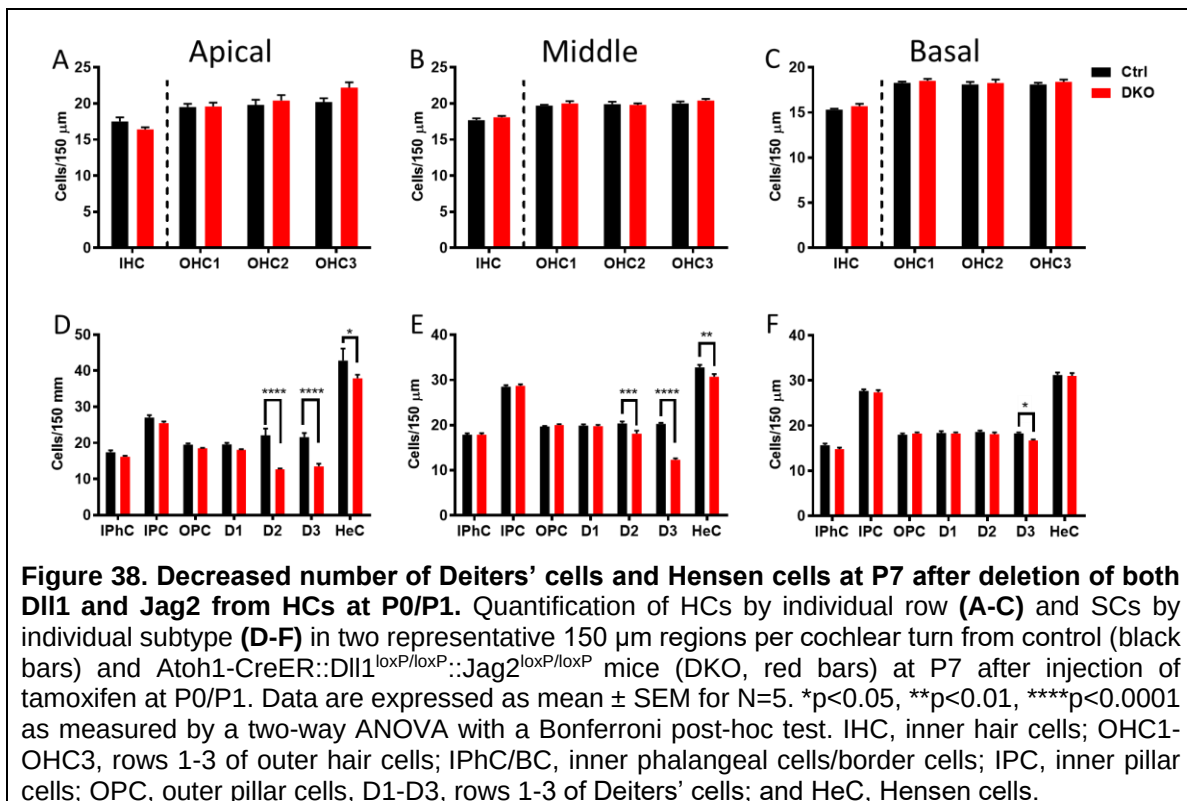


**Figure 36. Jag1 expression at P7 and P30 after deletion of both Dll1 and Jag2 from HCs at P0/P1.** Representative confocal slice images of control (Cre-negative, A, C) and Atoh1-CreER::Dl1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice (DKO, B, D) that were injected with tamoxifen at P0-P1. Cochleae were collected at P7 (A, B) or P30 (C, D) and immunostained with Jag1 (red). Scale bar = 20  $\mu$ m. D2-3, 2<sup>nd</sup> and 3<sup>rd</sup> row of Deiters' cells.

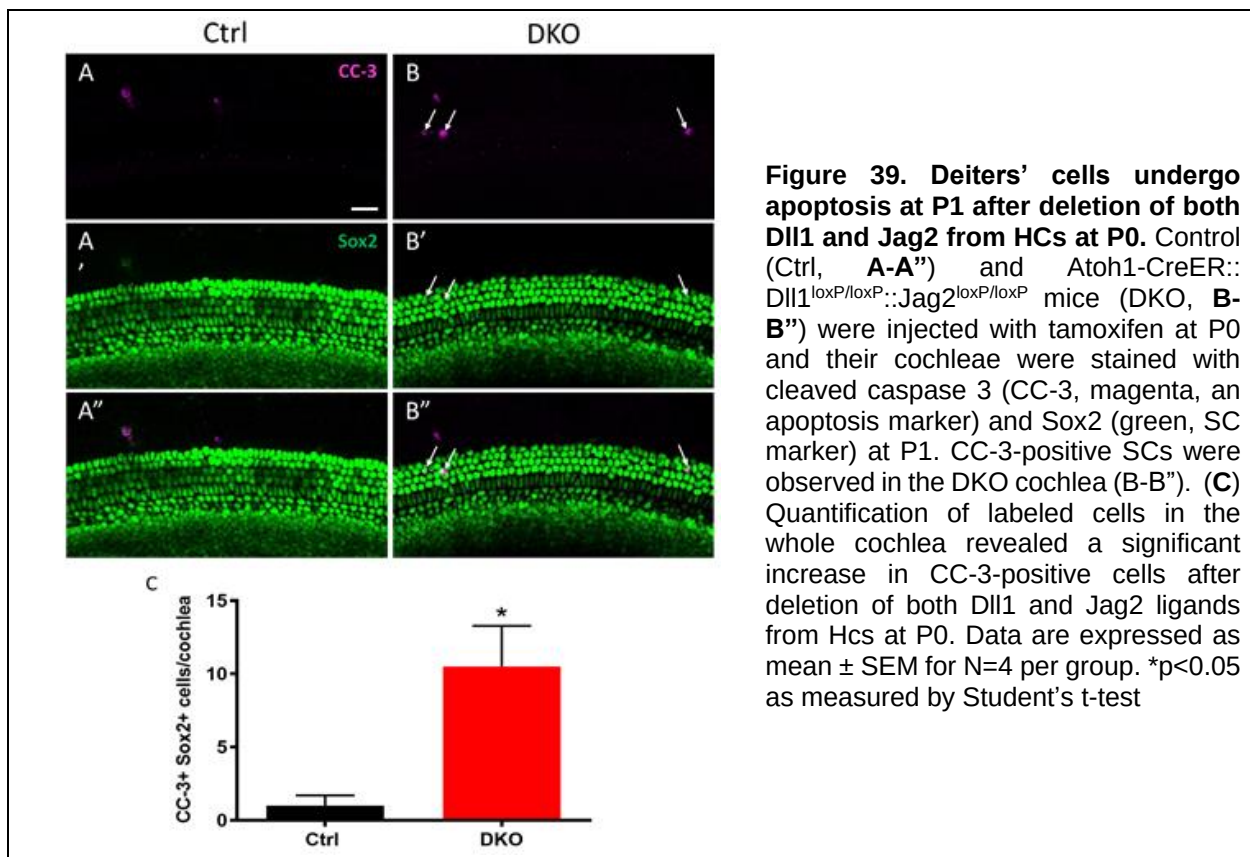
To investigate morphological changes to HCs and SCs after deletion of both *Dll1* and *Jag2* from HCs, we used immunostaining and confocal microscopy. *Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>* and Cre-negative control mice were injected with tamoxifen at P0/P1 and samples were collected at P7 and P30. At P7, we observed organized rows of HCs and SC nuclei in both control and *Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>* mice (Figure 37A-D, although there was some disorganization in the 3<sup>rd</sup> row of OHCs (Figure 37C) and perhaps some loss of Deiter's cells (Figure 37D) in *Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>* mice. Quantification of HCs and SCs by individual

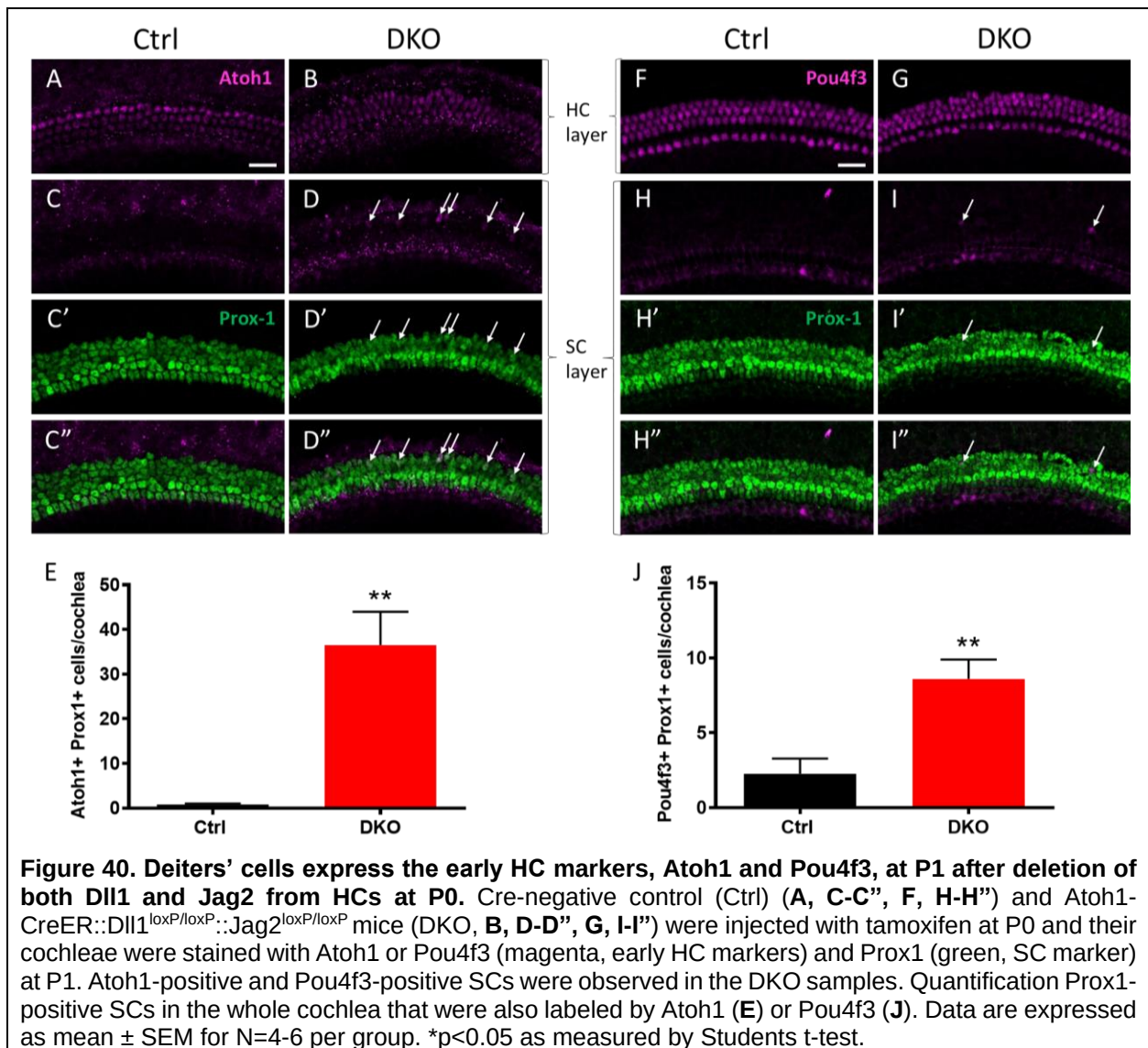


subtype at P7 showed a decrease in the number of Hensen cells and 2<sup>nd</sup> and 3<sup>rd</sup> row of Deiter's cells in the apical and middle turns of the cochlea (Figure 38D-E), but there was only a decrease in the 3<sup>rd</sup> row of Deiter's cells in the basal turn (Figure 38F). There was no change in the number of other SC subtypes or HCs (Figure 38A-F).



To determine whether SCs are dying after deletion of both Dll1 and Jag2 from HCs at P0/P1, we used cleaved caspase 3 (CC-3) staining to label cells undergoing apoptosis. At P1, we observed many CC-3-positive Deiters' cells in the cochlea of Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice that were injected with tamoxifen at P0/P1, while only 1-2 CC-3-positive Deiters' cells were observed in control samples (Figure 39). Since previous studies have shown that inhibition of Notch signaling using gamma secretase inhibitors or deletion of the Notch 1 receptor caused SCs to convert into HCs (Yamamoto et al., 2006; Doetzlhofer et al., 2009; Li et al., 2015; Maass et al., 2015), we hypothesized that SCs attempted to convert into HCs after deletion of Dll1 and Jag2 from HCs at P0/P1, but this process triggered cell death. To investigate this, we performed immunostaining with Atoh1 and Pou4f3 antibodies, two transcription factors that are the first genes expressed in developing HCs (Xiang et al., 1997, 1998; Bermingham et al., 1999; Chen et al., 2002; Woods et al., 2004). At P1, we observed significantly more Deiters' cells or Hensen cells that expressed Atoh1 or Pou4f3 in the cochlea of Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice than in controls (Figure 40). Together, our data suggest that after deletion of Dll1 and Jag2 from HCs at birth, Deiters' cells attempt to convert into HCs, but undergo apoptosis before the conversion is completed.





Next we examined the morphology of the cochlea at an older age to see if there was evidence of progressive cell loss. At P30, there was massive loss of outer HCs (Figure 41B) and disorganization of SC nuclei in the lateral compartment (underneath outer HCs) (Figure 41F) in the apical turn of Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> cochlea. In all turns of the cochlea Deiters' and Hensen cells were not organized in distinct rows, thus we counted both of these cell types together as one group. Similar to the results at P7, there was a decrease in the number of Deiters' and Hensen cells in the apical and middle turns of the cochlea at P30 (Figure 42D-E), but no change the number of these cells in the basal turn (Figure 42F). There was also no change in the number of other SC subtypes compared to controls (Figure 42D-F). However, unlike P7, we observed a massive loss of outer HCs in the apical turn, with the largest decrease found in the 2<sup>nd</sup> and 3<sup>rd</sup> row of outer HCs (Figure 42A). This was likely caused by the decreased number of Deiters' cells that we observed at P7, since a previous study has shown that when pillar and Deiters' cells are killed there is a delayed loss of outer HCs (Mellado Lagarde et al., 2013).

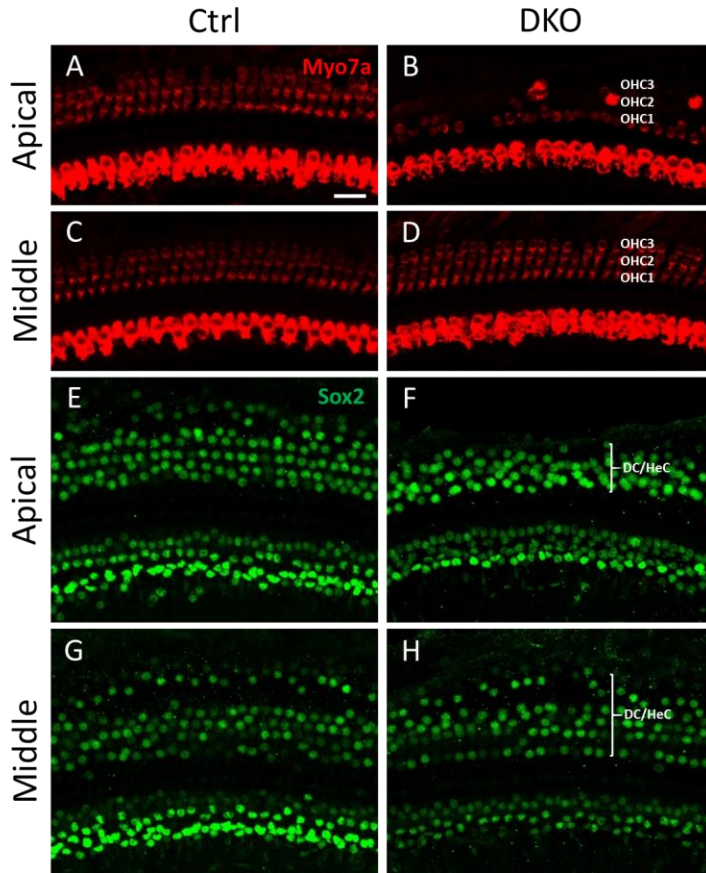


**Figure 41. Morphology of HCs and SC nuclei at P30 after deletion of both DII1 and Jag2 from HCs at P0/P1.** Control (Cre-negative) (A, C, E, G) and Atoh1-CreER::DII1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice (DKO; B, D, F, H) were injected with tamoxifen at P0-P1 and cochleae were collected at P30. HCs are labeled by myosin VIIa (red) and SC nuclei are labeled by Sox2 (green). Scale bar = 20 μm.

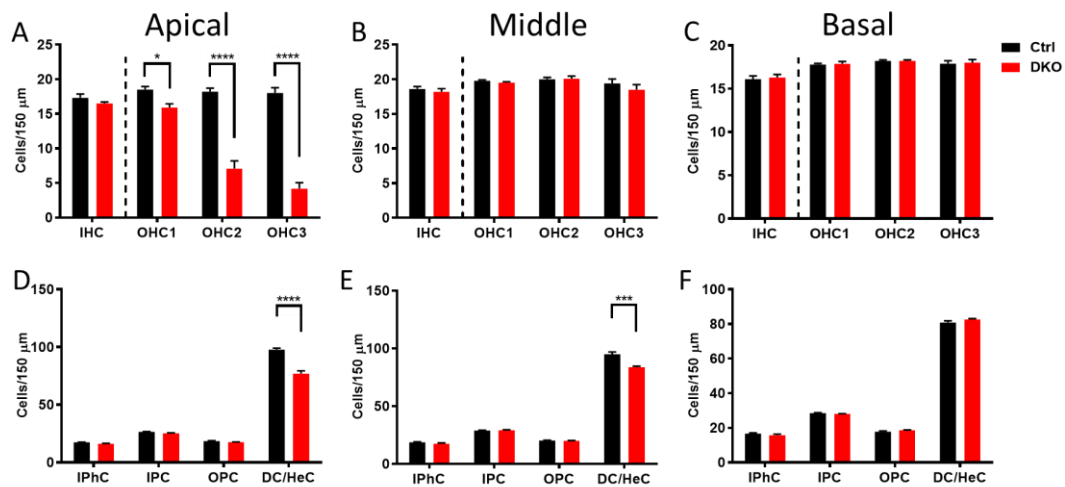
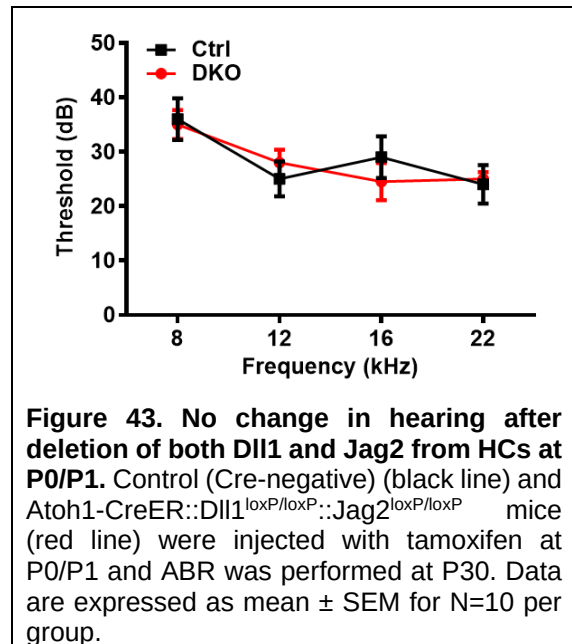


**Figure 42. Loss of Deiters' cells, Hensen cells, and outer HCs at P30 after deletion of both DII1 and Jag2 from HCs at P0/P1.** Quantification of HCs by individual row (A-C) and SCs by individual subtype (D-F) in two representative 150 μm regions per cochlear turn from control (grey bars) and Atoh1-CreER::DII1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup> mice (DKO, red bars) at P30 after injection of tamoxifen at P0/P1. Data are expressed as mean ± SEM for N=5 per group. \*p<0.05, \*\*\*p<0.001, \*\*\*\*p<0.0001 as measured by a two-way ANOVA with a Bonferroni post-hoc test. IHC, inner hair cells; OHC1-OHC3, rows 1-3 of outer hair cells; IPhC/BC, inner phalangeal cells/border cells; IPC, inner pillar cells; OPC, outer pillar cells; DC/HeC, Deiters' cells/Hensen cells.

Finally, we examined whether deletion of both Dll1 and Jag2 from HCs at birth caused any effect on hearing function. *Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup>* and Cre-negative control mice were injected with tamoxifen at P0/P1 and ABR was performed at P30 to measure changes in hearing. Our results indicate that there is no change in hearing after deletion of both Dll1 and Jag2 from neonatal HCs (Figure 43), despite the outer HC loss observed in the apical turn at P30 (Figure 42A). Other studies have reported a similar disconnect between HC loss and changes in ABR thresholds. This may be caused of the lack of sensitivity in the ABR method.



Stated goals not met: Aim 3B.2 was added in our revised Statement of Work that was approved in AUG-2018. All experiments are completed and we have created a draft of the Figures for data obtained in Aims 3A, 3B, and 3B.2. We are writing the manuscript now and plan to submit for publication by the end of January.

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

To investigate which Notch target genes are controlled by Jag1, we performed real-time qPCR experiments on P7 samples collected from *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* and iCre-negative control mice injected with tamoxifen at P0/P1 (to delete Jag1 from SCs). We also examined the expression of the other 2 Notch ligands (Jag1 and Jag2) since there is a known feedback loop where Notch signaling regulates the expression of the Notch ligands as well (Daudet and Lewis, 2005; Murata et al., 2006). We observed an increase in the expression of Hes5, Dll1, and the Notch1 receptor after deletion of Jag1 from SCs at P0/P1 (Figure 44).

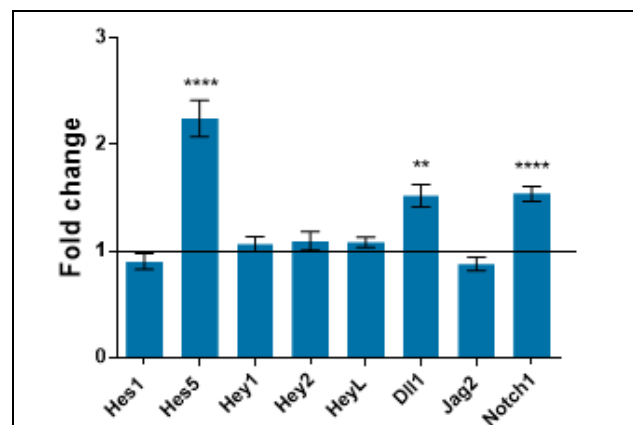
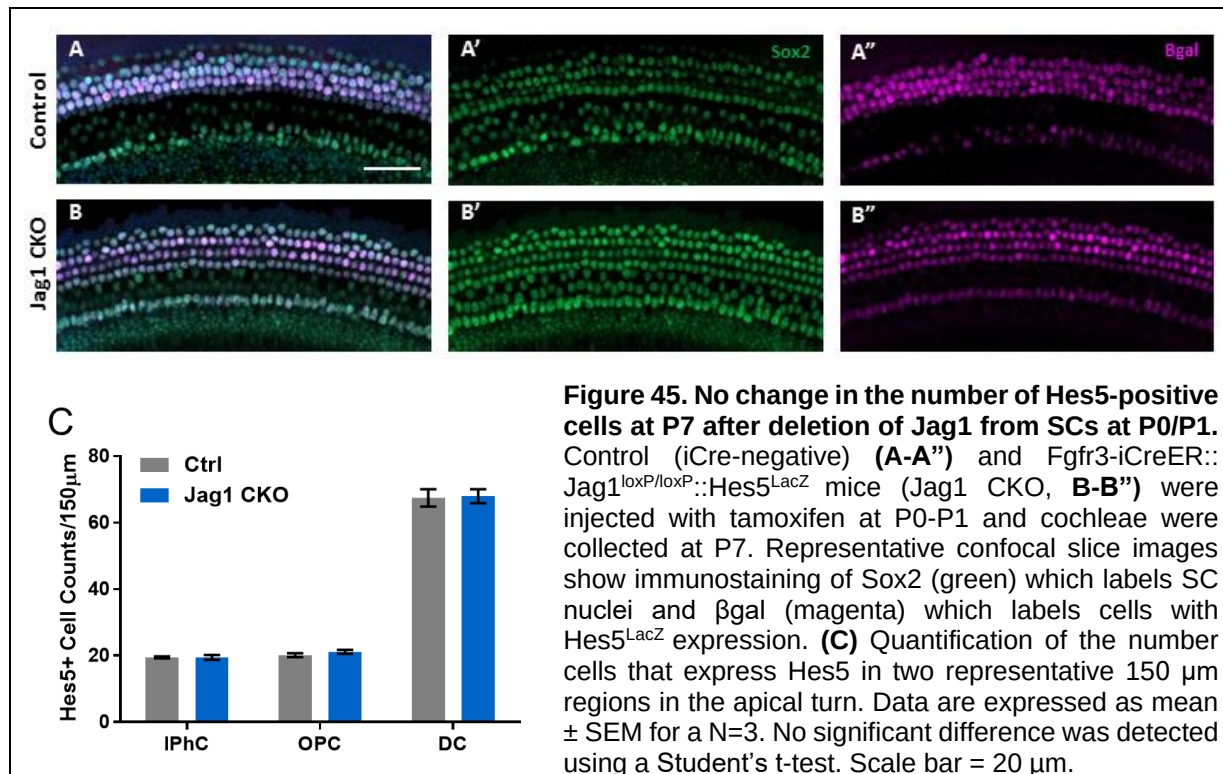


**Figure 44. Increased expression of Hes5, Dll1 and the Notch1 receptor after deletion of Jag1 from SCs at P0/P1.** Relative mRNA expression changes for Notch target genes, Notch ligands, and the Notch1 receptor analyzed in whole cochlear samples from *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* and iCre-negative control mice injected with tamoxifen at P0/P1 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  $\pm$  SEM) compared to controls (back line). N=8-9.

To measure changes in expression of Hes5 at the cellular level after deletion of Jag1 from neonatal SCs, we used the Hes5<sup>LacZ</sup> mouse line where the LacZ gene was knocked into the endogenous Hes5 locus and is thus a reporter of endogenous Hes5 expression (Imayoshi et al., 2010). Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> and iCre-negative control mice were injected with tamoxifen at P0/P1 and analyzed at P7 using anti-Sox2 antibodies to label all SC nuclei and anti-βgal antibodies to detect cells with Hes5<sup>LacZ</sup> expression. We then quantified the number of SCs that expressed Hes5 in the apical turn of control and experimental mice at P7. Compared to iCre-negative control mice, there was no significant difference in the number of Hes5-positive cells after deletion of Jag1 from neonatal SCs (Figure 45).



To investigate morphological changes to HCs and SCs after deletion of Jag1 from neonatal SCs, we used immunostaining and confocal microscopy. Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice and iCre-negative controls were injected with tamoxifen at P0/P1 and collected at P7, P30, or P60. Compared to control samples, cochlea from Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice showed normal overall morphology and organization of HCs and SC nuclei after Jag1 deletion at birth (Figure 46). Quantification of cells showed no change in the number of inner or outer HCs when quantified in rows at P7, P30 or P60 (Figure 47A-C). Next, we quantified SCs by individual row and found that Hensen cells were missing at P7 (Figure 47D). Similar results were observed at P30 (Figure 47E) and at P60 (Figure 47F).

To confirm the loss Hensen cells, we performed immunostaining with S100a1, which is expressed in pillar and Deiters' cells, along with Sox2 which labels the nuclei of

all SCs, including Hensen cells. iCre-negative control samples contained a row of Sox2-positive Hensen cells that were located lateral to the last row of S100a1-positive Deiters' cells. However after Jag1 deletion in Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice, there were no Sox2-positive cells lateral to S100a1 staining (Figure 48 C-D'). We next stained for BLBP, a Hensen cell marker, and found that there was reduced BLBP expression in the cochlea of Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice (Figure 48E-E',F-F'). We also performed immunostaining with CD44, to label Claudius cells, which are located lateral to Hensen cells. In Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice, the CD44 expression pattern appeared similar to the control, however the CD44 staining was immediately adjacent to the Sox2-positive 3<sup>rd</sup> row of Deiters' cell nuclei in Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice (Figure 48F'). Together these results confirm that the missing Sox2-positive cells are Hensen cells.

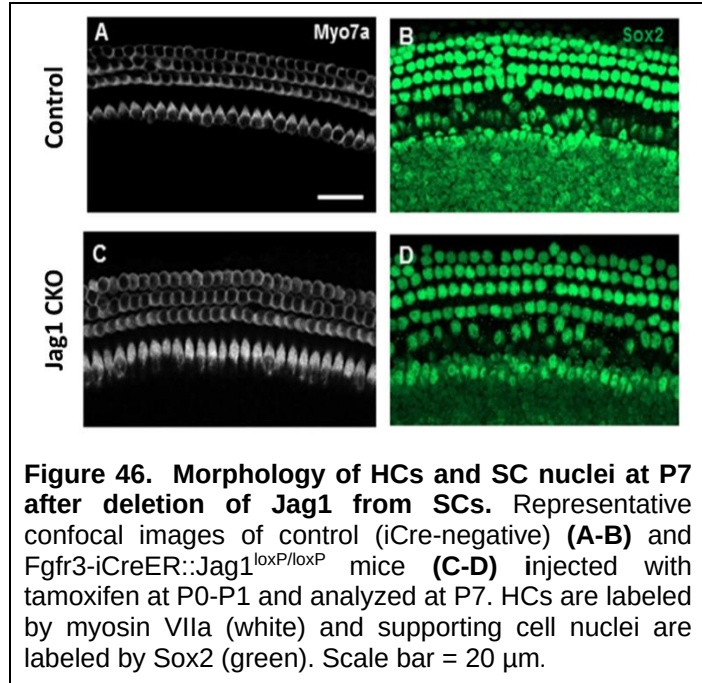


**Figure 46. Morphology of HCs and SC nuclei at P7 after deletion of Jag1 from SCs.** Representative confocal images of control (iCre-negative) (A-B) and Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice (C-D) injected with tamoxifen at P0-P1 and analyzed at P7. HCs are labeled by myosin VIIa (white) and supporting cell nuclei are labeled by Sox2 (green). Scale bar = 20  $\mu$ m.

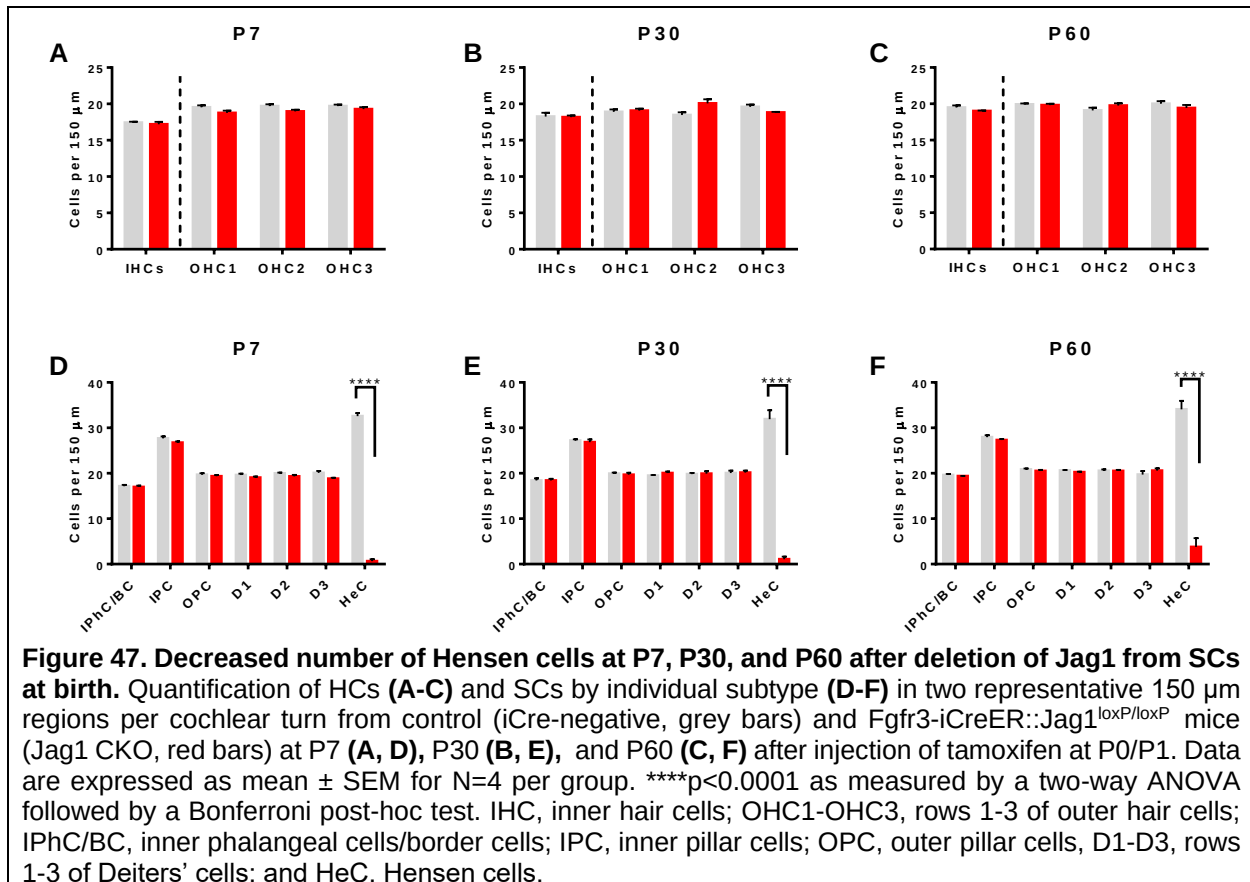


**Figure 47. Decreased number of Hensen cells at P7, P30, and P60 after deletion of Jag1 from SCs at birth.** Quantification of HCs (A-C) and SCs by individual subtype (D-F) in two representative 150  $\mu$ m regions per cochlear turn from control (iCre-negative, grey bars) and Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice (Jag1 CKO, red bars) at P7 (A, D), P30 (B, E), and P60 (C, F) after injection of tamoxifen at P0/P1. Data are expressed as mean  $\pm$  SEM for N=4 per group. \*\*\*\*p<0.0001 as measured by a two-way ANOVA followed by a Bonferroni post-hoc test. IHC, inner hair cells; OHC1-OHC3, rows 1-3 of outer hair cells; IPhC/BC, inner phalangeal cells/border cells; IPC, inner pillar cells; OPC, outer pillar cells, D1-D3, rows 1-3 of Deiters' cells; and HeC, Hensen cells.

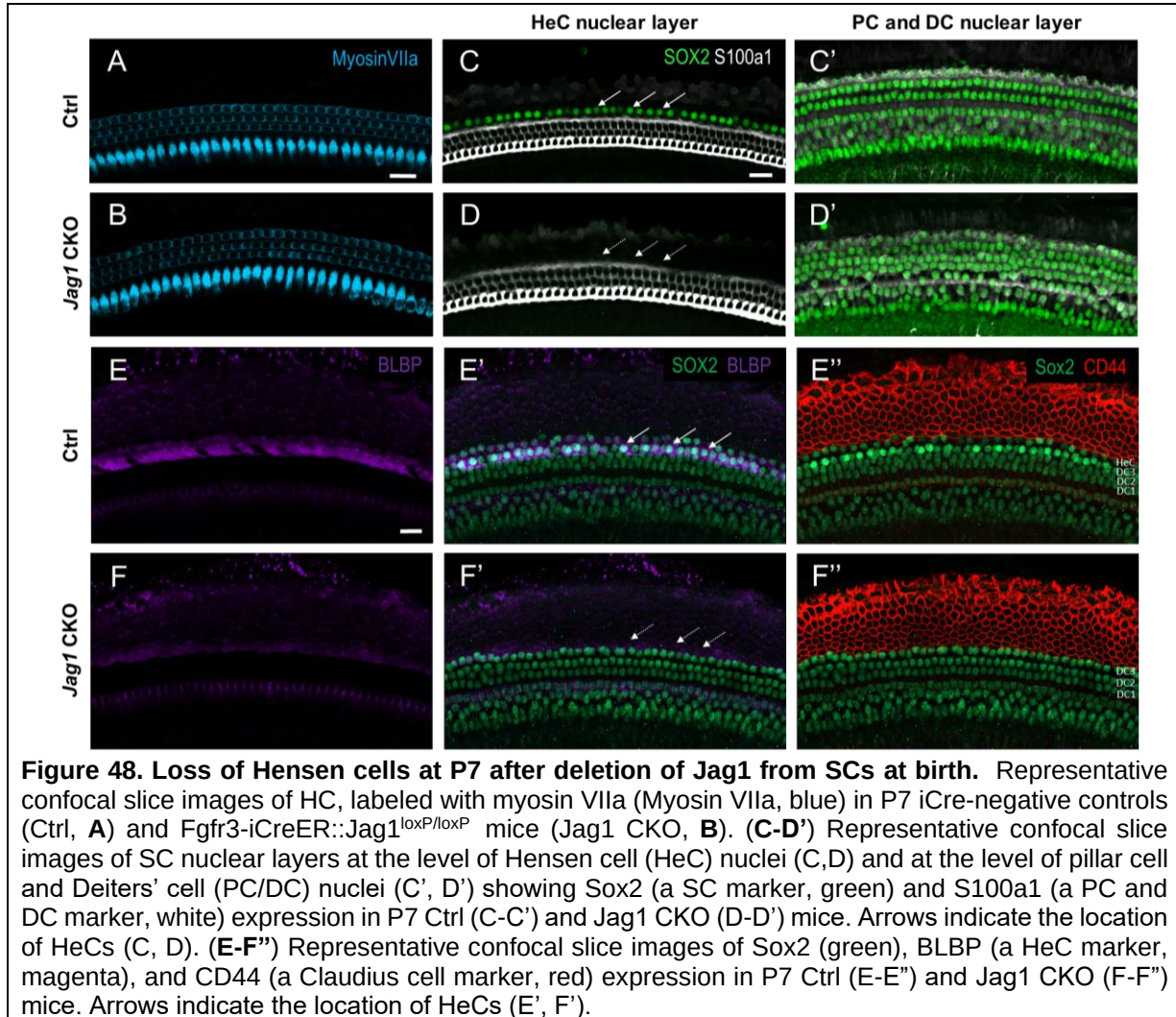


**Figure 48. Loss of Hensen cells at P7 after deletion of *Jag1* from SCs at birth.** Representative confocal slice images of HC, labeled with myosin VIIa (Myosin VIIa, blue) in P7 iCre-negative controls (Ctrl, **A**) and *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* mice (*Jag1* CKO, **B**). (**C-D'**) Representative confocal slice images of SC nuclear layers at the level of Hensen cell (HeC) nuclei (C,D) and at the level of pillar cell and Deiters' cell (PC/DC) nuclei (C', D') showing Sox2 (a SC marker, green) and S100a1 (a PC and DC marker, white) expression in P7 Ctrl (C-C') and *Jag1* CKO (D-D') mice. Arrows indicate the location of HeCs (C, D). (**E-F''**) Representative confocal slice images of Sox2 (green), BLBP (a HeC marker, magenta), and CD44 (a Claudius cell marker, red) expression in P7 Ctrl (E-E'') and *Jag1* CKO (F-F'') mice. Arrows indicate the location of HeCs (E', F').

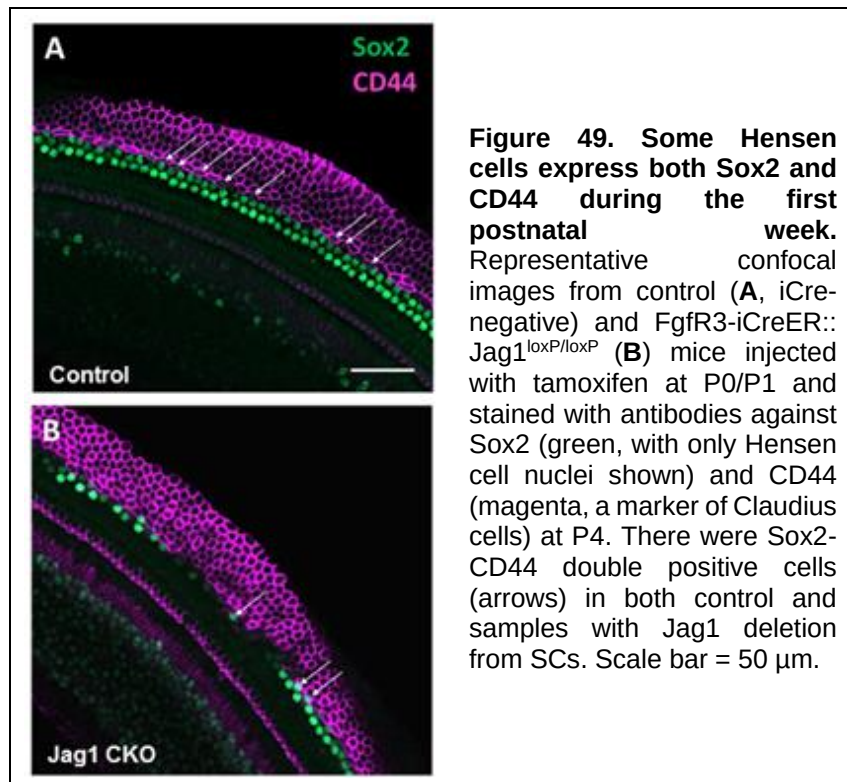
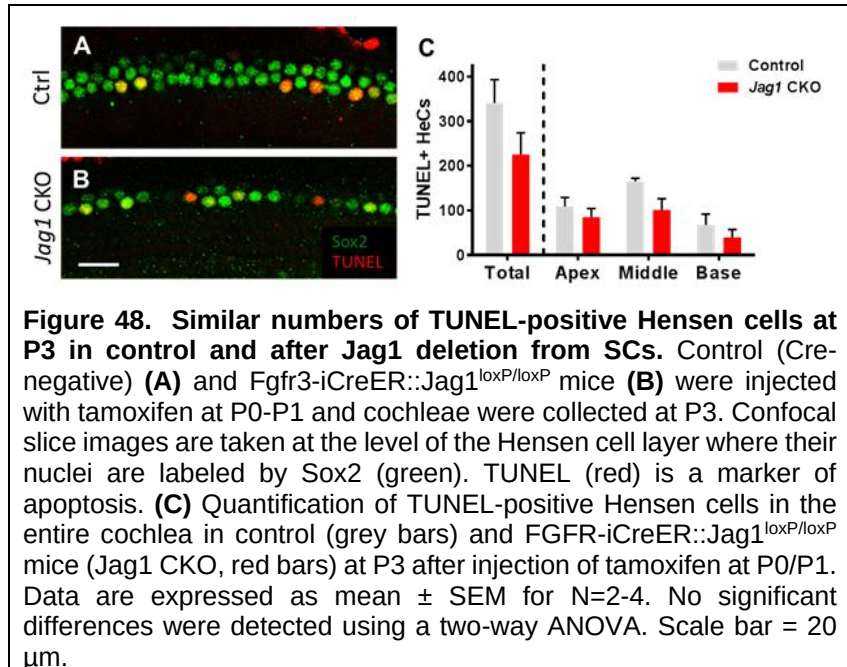
To determine whether Hensen cells are dying, we performed immunostaining for TUNEL, which is a marker of apoptosis. *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* mice and iCre-negative controls were injected with tamoxifen at P0/P1 and cochleae were collected at P3. We observed a similar number of TUNEL-positive Hensen cells in both control samples and those with *Jag1* deletion from neonatal SCs (Figure 48A-C). We do not currently understand why Hensen cells in the control samples are TUNEL-positive. While there are some reports that TUNEL staining is present in cells with high metabolic activity, we cannot rule out that some Hensen cells die in control samples during the first postnatal week.

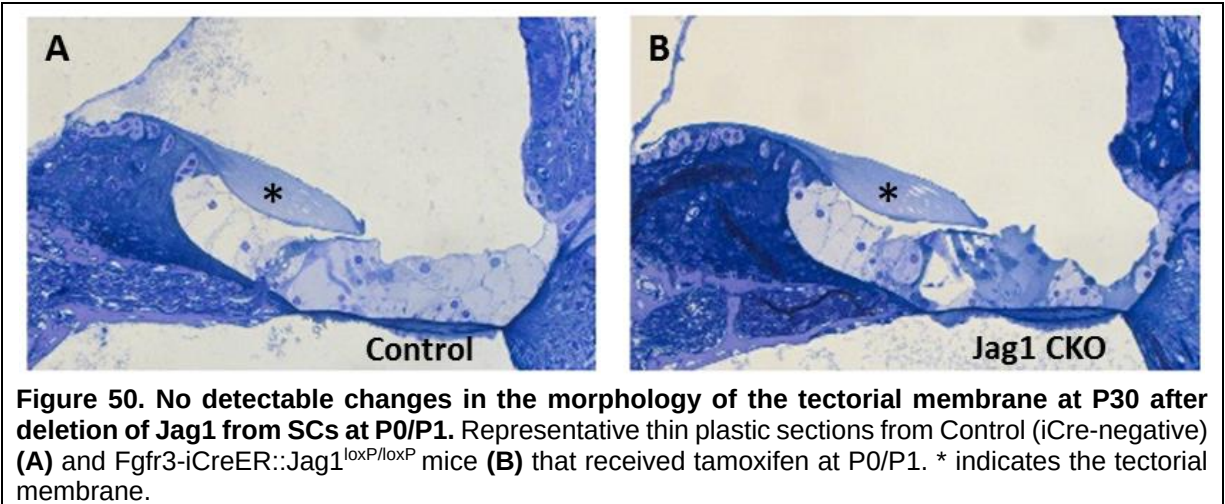
Another possible explanation for the loss of Hensen cells after *Jag1* deletion from SCs at birth is that these cells may lose Sox2 expression and convert into Claudius cells. To investigate this we performed double staining of Sox2 and CD44 (a Claudius cell marker) and found some double-labeled cells at P2-P4 in both iCre-negative control samples (Figure 49A) and in samples with *Jag1* deletion from SCs (Figure 49B). This may

suggest that some Hensen cells (the most lateral ones) convert into Claudius cells during the first postnatal week as part of their normal maturation process. Deletion of Jag1 from SCs may increase the number of cells that convert, resulting in an apparent loss of Sox2-positive Hensen cells. To confirm this hypothesis, we are currently performing experiments to fate-map Hensen cells after Jag1 deletion.

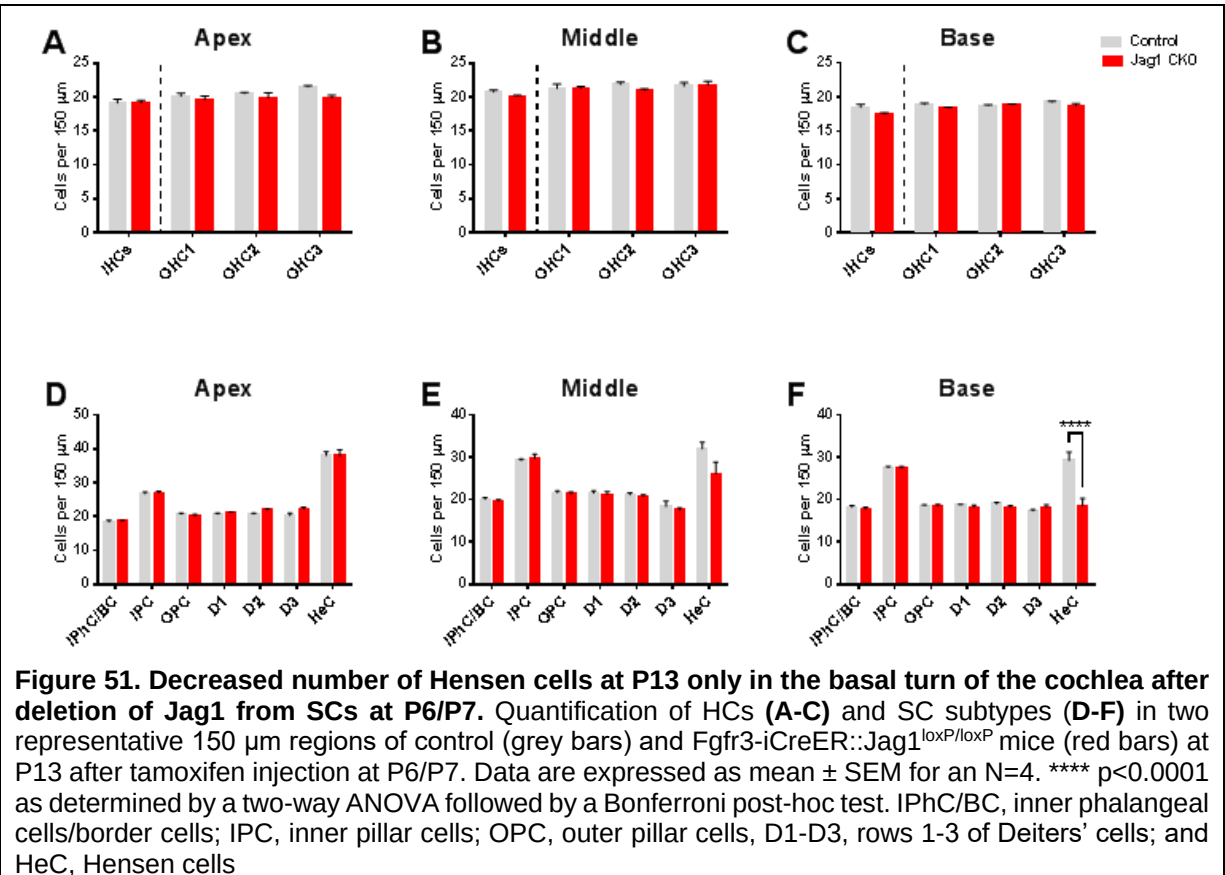
Hensen cells are involved in the secretion of the proteins that form the tectorial membrane (Richardson et al., 2008). Thus loss of Hensen cells caused by Jag1 deletion from SCs may cause morphological changes in the tectorial membrane, which in turn would negatively impact hearing. To test this hypothesis, we collaborated with Dr. Guy Richardson at the University of Sussex to investigate the morphology of the tectorial membrane after Jag1 deletion from SCs at P0/P1. Dr. Richardson's lab used thin plastic sections to examine the morphology of the

tectorial membrane in P30 FGFR3-iCreER::Jag1<sup>loxP/loxP</sup> and iCre-negative control mice that were injected with tamoxifen at P0/P1. No obvious changes were observed in the morphology of the tectorial membrane (Figure 50), which indicates that Jag1 deletion from SCs does not affect the development of tectorial membrane.





Next, we investigated the effect of Jag1 deletion at a later age when the cochlea is more mature. *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* mice and iCre-negative controls were injected with tamoxifen at P6/P7 and cochleae were collected at P13. We observed a ~35% loss of Hensen cells only in the basal turn of the cochlea in *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* mice (Figure 51), which was less severe than what we observed when Jag1 was deleted at P0/P1 (Figure 47). This suggests that the effect of Jag1 deletion on Hensen cell fate is time dependent.



Finally, we examined whether deletion of Jag1 from SCs at birth caused any effect on hearing function. Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> and iCre-negative control mice were injected with tamoxifen at P0/P1 and ABR was performed at P30 to measure changes in hearing. While the ABR thresholds for all frequencies appeared higher in Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice compared to controls, there was only a significant difference at 4 kHz (Figure 52). Since there were no differences in the quantification of HCs and SCs between P30 and P60, we decided to discontinue the ABR experiments at P60.

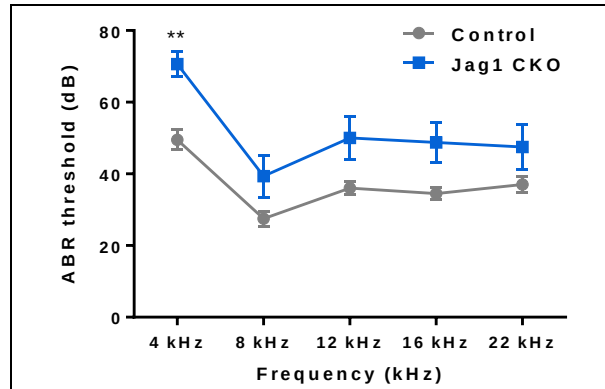


**Figure 52. Low frequency hearing loss after deletion of Jag1 from SCs at P0/P1.** Control (iCre-negative) (black line) and Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice (blue line) were injected with tamoxifen at P0-P1 and ABR was performed at P30. Data are expressed as mean  $\pm$  SEM for a N=8-10. \*\*p < 0.01 as determined by a two-way ANOVA followed by a Sidak's post-hoc test.

All result described thus far in Aim 3C have used Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup> mice which target some SC subtypes (pillar and Deiters' cells), but not others. Therefore we used a different CreER line, called GLAST-CreER, to delete Jag1 from other SC subtypes (inner phalangeal cells, border cells, GER cells, and Hensen cells) by injecting them with tamoxifen at P0/P1. Compared to Cre-negative controls, there was no change in the number of HCs or any SC subtype in GLAST-CreER::Jag1<sup>loxP/loxP</sup> samples at P7 or P30 (Figure 53). Since there were no differences in the number of HCs or SCs in GLAST-CreER::Jag1<sup>loxP/loxP</sup> mice at P30, we did not complete ABR experiments for these mice.

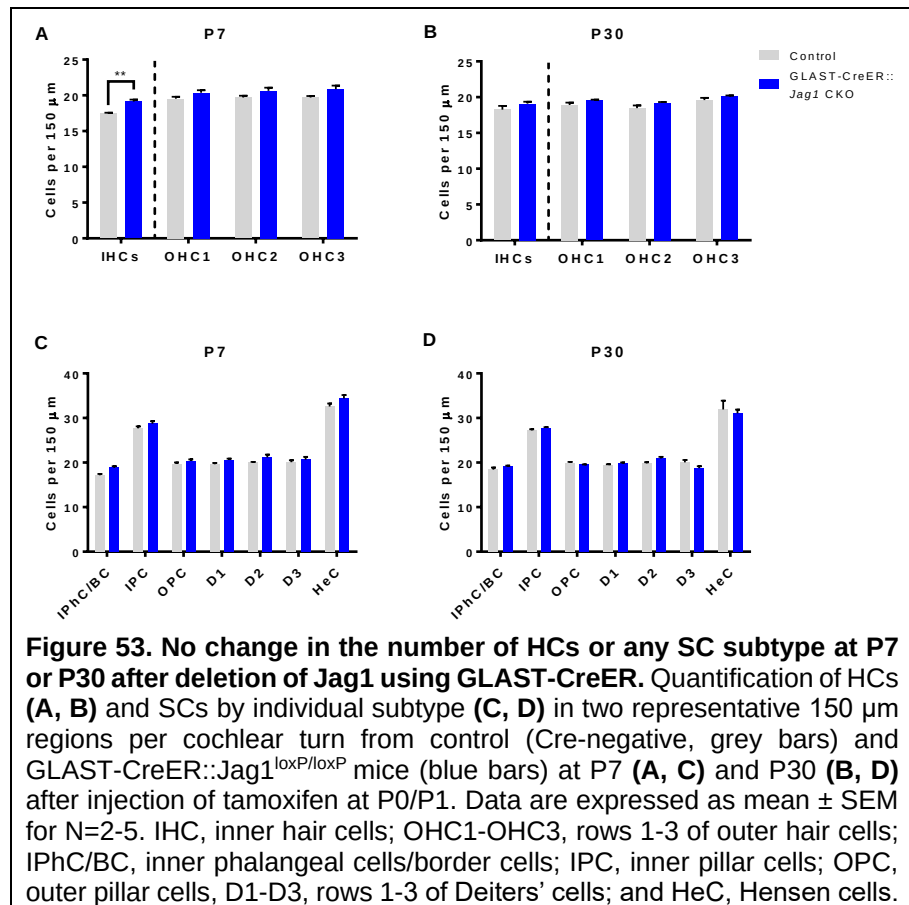


**Figure 53. No change in the number of HCs or any SC subtype at P7 or P30 after deletion of Jag1 using GLAST-CreER.** Quantification of HCs (A, B) and SCs by individual subtype (C, D) in two representative 150 μm regions per cochlear turn from control (Cre-negative, grey bars) and GLAST-CreER::Jag1<sup>loxP/loxP</sup> mice (blue bars) at P7 (A, C) and P30 (B, D) after injection of tamoxifen at P0/P1. Data are expressed as mean  $\pm$  SEM for N=2-5. IHC, inner hair cells; OHC1-OHC3, rows 1-3 of outer hair cells; IPHC/BC, inner phalangeal cells/border cells; IPC, inner pillar cells; OPC, outer pillar cells, D1-D3, rows 1-3 of Deiters' cells; and HeC, Hensen cells.

Stated goals not met: For Aim 3C, all experiments in the revised Statement of Work (approved in AUG-2018) have been completed. We have completed a draft containing the abstract, introduction, figures, figure legends, methods, and results sections that describe the results of Jag1 deletion from neonatal SCs using FGFR3-iCreER:Jag1<sup>loxP/loxP</sup> mice. Since there was no phenotype after deletion of Jag1 from other SC subtypes using GLAST-CreER:Jag1<sup>loxP/loxP</sup> mice, we have decided not to include this data in the paper. The current version of the draft is currently being reviewed by our collaborator Dr. Angelika Doetzlhofer at Johns Hopkins University, who added data from her lab that complements our work. Based on our unexpected results from task 4, we are performing additional experiments to investigate why Hensen cells are disappearing after Jag1 deletion from SCs. These fate-mapping experiments are underway and once completed, we will add them to the paper and draft the discussion section. We plan to submit this manuscript for publication in January.

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

To investigate which Notch target genes are controlled by deletion of Jag1 from HCs, we performed real-time qPCR experiments on P7 samples collected from Atoh1-CreER::Jag1<sup>loxP/loxP</sup> and Cre-negative control mice that were injected with tamoxifen at P0/P1. We also examined the expression level of the other 2 Notch ligands (Dll1 and Jag2) and the three Notch receptors since there is a known feedback loop where Notch signaling regulates the expression of Notch ligands (Daudet and Lewis, 2005; Murata et al., 2006). We observed an increase in the expression of Hes5, Dll1, the Notch1 receptor, the Notch2 receptor, and Lfng after Jag1 deletion from HCs at P0/P1 (Figure 54).

To measure changes in the expression of Hes5 at the cellular level after deletion of Jag1 from neonatal HCs, we used the Hes5<sup>LacZ</sup> mouse line where the LacZ gene was knocked into the endogenous Hes5 locus and is thus a reporter of endogenous Hes5 expression (Imayoshi et al., 2010). Atoh1-CreER::Jag1<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and analyzed at P7 using anti-Sox2 antibodies to label all SC nuclei and anti-βgal antibodies to detect cells with Hes5 expression. There was no change in the number of

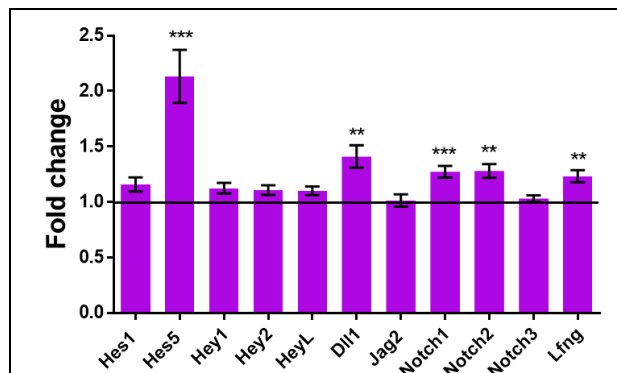


**Figure 54. Increased expression of Hes5, Dll1, the Notch1 receptor, the Notch2 receptor, and Lfng after deletion of Jag1 from neonatal HCs.** Relative mRNA expression changes for Notch target genes, Notch ligands, and Notch receptors were analyzed in whole cochlear samples from Atoh1-CreER::Jag1<sup>loxP/loxP</sup> and Cre-negative control mice injected with tamoxifen at P0/P1 using real-time qPCR. Rpl19 was used as a housekeeping gene. Data was analyzed using the Pfaffl method and are expressed as fold change (mean ± SEM) compared to controls (back line). N=10. \*\*p<0.01, \*\*\*p<0.001 as measured by a Student's t test for each gene.

Hes5-positive inner phalangeal cells, outer pillar cells, or Deiters' cells after Jag1 deletion from HCs at P0/P1 (Figure 55).

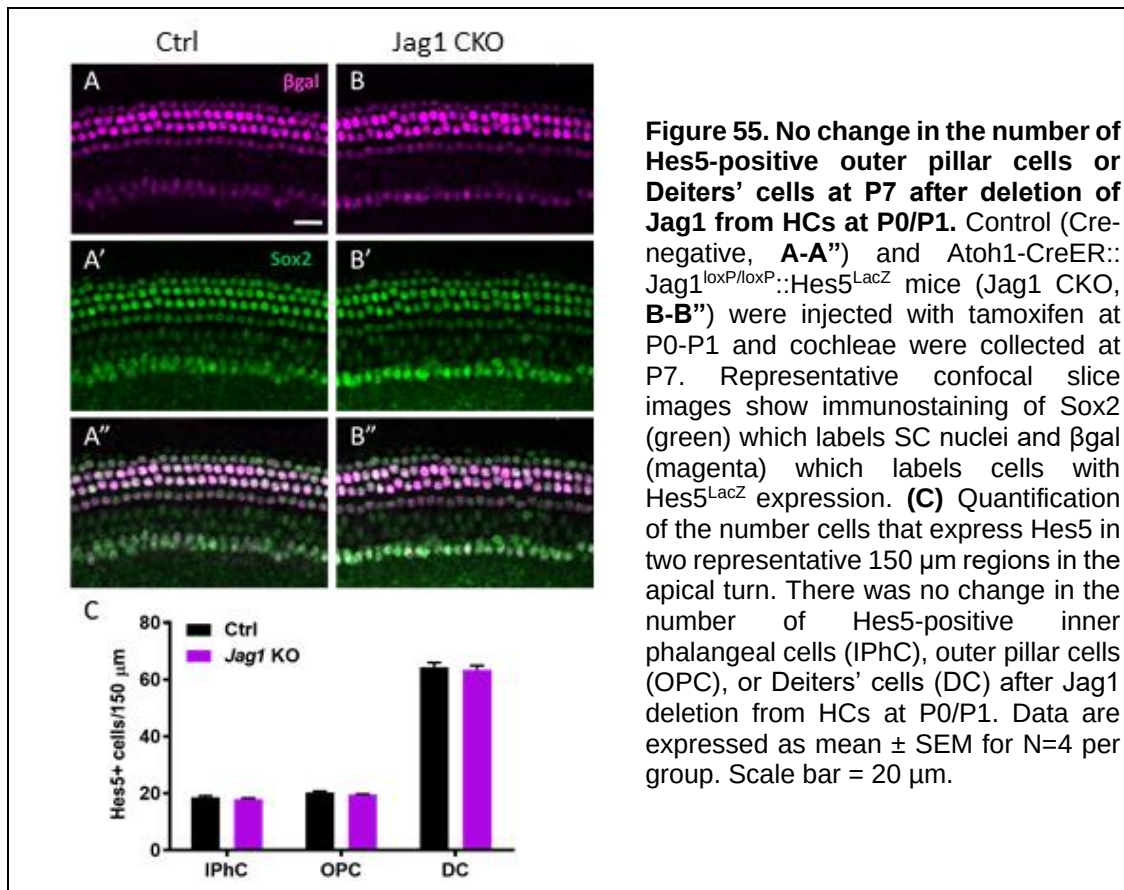


**Figure 55. No change in the number of Hes5-positive outer pillar cells or Deiters' cells at P7 after deletion of Jag1 from HCs at P0/P1.** Control (Cre-negative, A-A'') and Atoh1-CreER::Jag1<sup>loxP/loxP</sup>::Hes5<sup>LacZ</sup> mice (Jag1 CKO, B-B'') were injected with tamoxifen at P0-P1 and cochleae were collected at P7. Representative confocal slice images show immunostaining of Sox2 (green) which labels SC nuclei and  $\beta$ gal (magenta) which labels cells with Hes5<sup>LacZ</sup> expression. (C) Quantification of the number cells that express Hes5 in two representative 150  $\mu$ m regions in the apical turn. There was no change in the number of Hes5-positive inner phalangeal cells (IPhC), outer pillar cells (OPC), or Deiters' cells (DC) after Jag1 deletion from HCs at P0/P1. Data are expressed as mean  $\pm$  SEM for N=4 per group. Scale bar = 20  $\mu$ m.

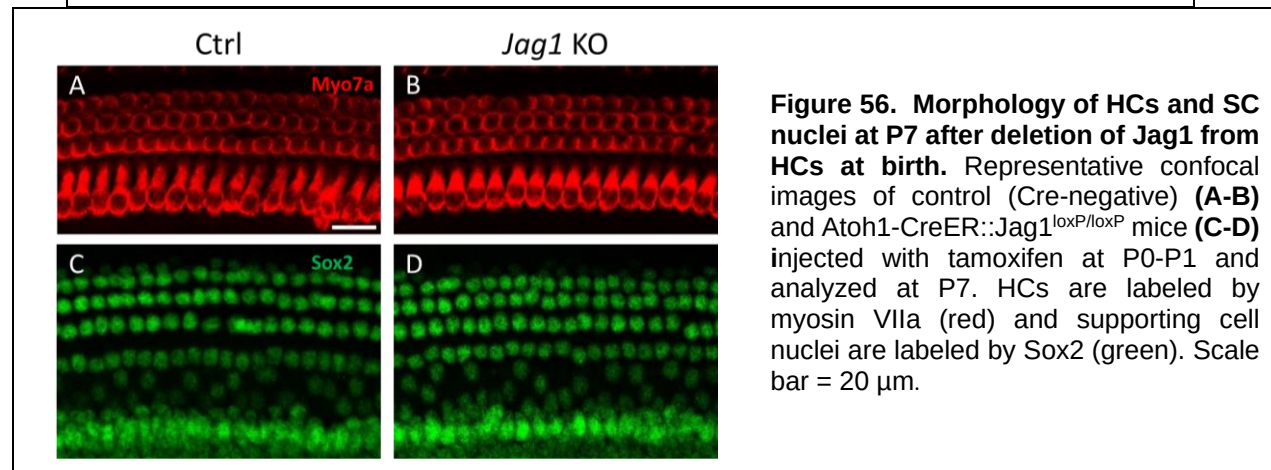
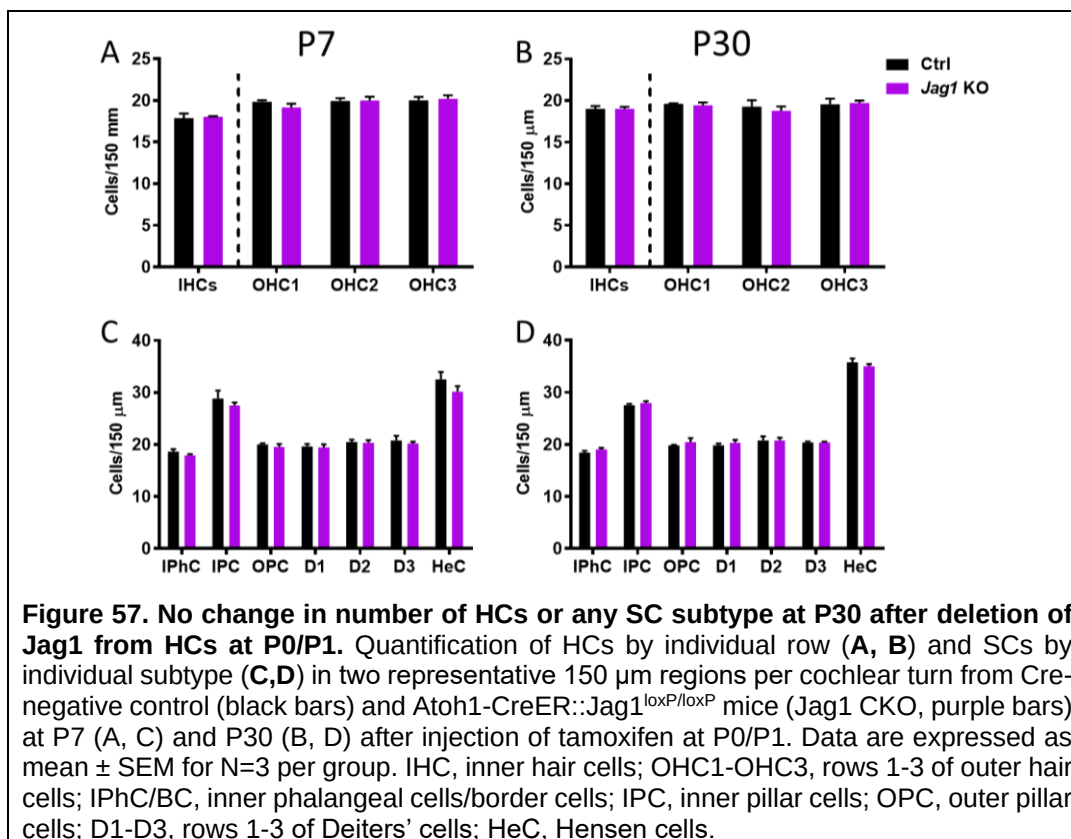


**Figure 56. Morphology of HCs and SC nuclei at P7 after deletion of Jag1 from HCs at birth.** Representative confocal images of control (Cre-negative) (A-B) and Atoh1-CreER::Jag1<sup>loxP/loxP</sup> mice (C-D) injected with tamoxifen at P0-P1 and analyzed at P7. HCs are labeled by myosin VIIa (red) and supporting cell nuclei are labeled by Sox2 (green). Scale bar = 20  $\mu$ m.

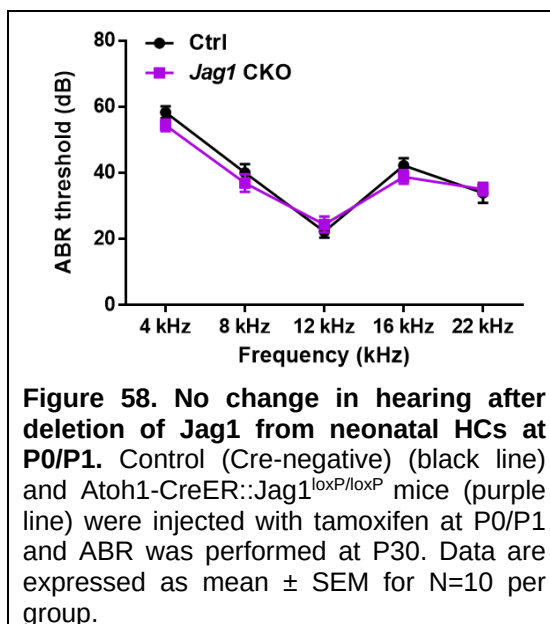
Next, we investigated morphological changes to HCs and SC subtypes after deletion of Jag1 from neonatal HCs using immunostaining and confocal microscopy. Atoh1-CreER::Jag1<sup>loxP/loxP</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and quantification was performed at P7 and P30. Compared to control samples, cochlea from Atoh1-CreER::Jag1<sup>loxP/loxP</sup> mice showed normal overall morphology and organization of HCs and SC nuclei after Jag1 deletion from HCs at birth (Figure 56).

Quantification of HCs by row and SCs by individual subtype showed no change in the number of HCs or any of the SC subtypes after deletion of Jag1 from neonatal HCs (Figure 57).



Finally, we examined whether deletion of Jag1 from HCs at birth caused any effect on hearing function. Atoh1-CreER::Jag1<sup>loxP/loxP</sup> and Cre-negative control mice were injected with tamoxifen at P0/P1 and ABR was performed at P30 to measure changes in hearing. Our results indicate that there is no change in hearing after deletion of Jag1 from neonatal HCs (Figure 58).

Stated goals not met: Aim 3D was added in our revised Statement of Work that was approved in AUG-2018. All experiments are completed and we plan to include data from this aim in the paper with data obtained in Aims 3A, 3B, and 3B.2. We have completed drafts of the figures and are writing the manuscript now. We plan to submit for publication by the end of January.



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### **What opportunities for training and professional development has the project provided?**

#### **Training**

- 1) Melissa McGovern, a graduate student supported by this award, traveled to the Dr. Bernd Fritzsch's lab at the University of Iowa and to Dr. Andy Groves's lab at Baylor College of Medicine to learn *in situ* hybridization. She was also selected to attend the Biology of the Inner Ear course, which is a three week course held at Woods Hole, MA that has a very competitive application process. During this course Melissa attend lectures given by experts in the hearing field and participated in hands on labs where she learned a variety of new techniques for auditory research. When she returned to SIUSOM, she shared this new knowledge with the rest of the lab.
- 2) Yunazhao Lv Darcy, PhD, a postdoc hired for the project in Y1, 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.
- 3) Luyi Zhou, PhD, a postdoc hired for the project in Y2 to replace Dr. Darcy, was mentored by Dr. Cox to learn all of the techniques needed for the project such as cochlear dissection, immunostaining, confocal microscopy, *in situ* hybridization, and ABR.
- 4) Candice Cuppini, a medical student, who was mentored by graduate student Melissa McGovern, learned how to quantify confocal images and assisted with data analysis.
- 5) Bridget Brian, an undergraduate student, who was mentored by postdoc Luyi Zhou, learned immunostaining and how to quantify confocal images to assist with data analysis.
- 6) Ariel Tegler, an undergraduate student, who was mentored by postdoc Luyi Zhou, learned immunostaining and how to quantify confocal images to assist with data analysis.

**Professional Development**

1) Dr. Cox and all members of the lab supported by this award attended the Association for Research in Otolaryngology 39<sup>th</sup> annual midwinter meeting held 20-24-FEB-2016 in San Diego, CA, the Association for Research in Otolaryngology 40<sup>th</sup> annual midwinter meeting held 11-15-FEB-2017 in Baltimore, MD, the Association for Research in Otolaryngology 41<sup>st</sup> annual midwinter meeting held 10-14-FEB-2018 in San Diego, CA, and the Association for Research in Otolaryngology 42<sup>nd</sup> annual midwinter meeting held 9-13-FEB-2019 in Baltimore, MD.

2) Dr. Cox also attended the Society for Neuroscience 47<sup>th</sup> Annual Meeting held 11-15-NOV-2017 in Washington, DC.

**How were the results disseminated to communities of interest?**

Dr. Cox has given lab tours to a variety of different groups in the community where she talked about her current research, including data obtained under this award. In 2016, tours were given to Illinois EPA staff and IEPA Governor's Environmental Corps Interns, University of Illinois Springfield clinical lab science students, employees of Transnetyx, Inc, Cordova, TN, Illinois State Representative, Sara Jimenez, and the Western Illinois University pre-health club. In 2017, tours were given to Illinois EPA staff and IEPA Governor's Environmental Corps Interns, University of Illinois Springfield clinical lab science students, and Leadership Springfield (a local professional development and leadership training program). In 2018, tours were given to Franklin high school biology students, and a delegation of the American Council of Young Political Leaders from China. In 2019, tours were given to Illinois EPA staff and IEPA Governor's Environmental Corps Interns, University of Illinois Springfield clinical lab science students, and Western Illinois University pre-health club students.

**What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to Report

#### 4. IMPACT:

##### **What was the impact on the development of the principal discipline(s) of the project?**

The findings from Aims 1 & 2 demonstrate that the Notch signaling pathway plays a critical role in the spontaneous hair cell regeneration process. While this was previously hypothesized, our studies are the first to demonstrate Notch's role and have implications for the development of therapies to stimulate hair cell regeneration in the adult cochlea, and eventually in humans who suffer from hearing loss. Results from Aim 3 suggest that the Notch pathway is a complex signaling network where different ligands and different levels of Notch activity play critical roles in specifying the fate of hair cells and supporting cells. Further investigation of the function of each ligand at different stages of cochlear development is warranted.

##### **What was the impact on other disciplines?**

Results from Aim 3 which suggest that different Notch ligands and different levels of Notch activity play critical roles in specifying cell fate will have an impact on other fields. These functions will likely be conserved in other sensory systems and in organs such as the intestine which is dependant on Notch signaling for the development of different cell types.

##### **What was the impact on technology transfer?**

Nothing to Report

##### **What was the impact on society beyond science and technology?**

Nothing to Report

#### 5. CHANGES/PROBLEMS:

##### **Changes in approach and reasons for change**

Based on our results from Aim 2A, we changed the focus of Aim 2B. Specifically, we found a large increase in the number of proliferating cells in control samples where Notch signaling is increased in supporting cells, but hair cells remain intact (Sox10<sup>rtTA</sup>:TetO-LacZ:TetO-NICD mice). This was unexpected based on published literature and required further investigation. Also because there is so much proliferation in these control samples, we could not measure supporting cell proliferation in the experimental samples (where Notch signaling is increased in supporting cells and hair cells are killed). We also added additional sub-Aims to Aim 3 to further investigate our finding from the original Aim 3. All of these changes were submitted as a revised Statement of Work which was approved in AUG-2018.

### **Actual or anticipated problems or delays and actions or plans to resolve them**

- 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. The repaired machine was returned at the end of Q3. We validated that it worked properly and resumed the experiments. Due to this setback, the experiments using qPCR were delayed.
- 2) The cryorecovery process of Dll1<sup>loxP</sup> 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<sup>loxP</sup> mice, the vendor breed the cryorecovered Dll1<sup>+/-loxP</sup> mice (heterozygotes) to generate Dll1<sup>loxP/loxP</sup> mice (homozygotes).
- 3) Yunazhao Lv Darcy. PhD, a postdoc who was in charge of Aim 3 of the project, went on maternity leave in Y2Q3 and a few weeks after her return, Dr. Darcy left the lab. In addition, Melissa McGovern, the graduate student who led the experiments performed in Aims 1 and 2 completed her PhD in Y2Q4 and left the lab. However, we were able to hire a new postdoc, Luyi Zho, PhD who joined the lab in AUG-2107. Dr. Zuo took over as the lead researcher for all experiments remaining in all Aims. She was assisted by the two technicians, Michelle Randle and Kaley Graves.

### **Changes that had a significant impact on expenditures**

Two of the mouse lines needed for the study were found to be available commercially, but required cryorecovery of frozen sperm (costing ~\$6,000 each which includes shipping fees). Thus we 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 was paid in Y2-Y4. Despite these unexpected expenditures, we remained financially on track, spending less than the budgeted amount in Y1-Y3, and completed a one year no cost extension of the project to complete the experiments.

### **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

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

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

#### **Significant changes in use or care of vertebrate animals**

The animal protocol was approved by the Southern Illinois University School of Medicine (SIUSOM) IACUC on 30-JUL-2015 and approved by USAMRMC ACURO on 06-OCT-2015.

- 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<sup>LacZ</sup> 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<sup>loxP</sup> needed for Aim 3B) and the Tm1c allele (Jag2<sup>LacZ</sup> reporter). The amendment also added Jag2<sup>LacZ</sup> reporter to Aim 1 to investigate changes in Jag2 expression (a Notch ligand) during the HC regeneration process.
- 3) Amendment A3 to the vertebrate animal protocol was approved by SIUSOM IACUC on 9-NOV-2016 and by ACURO on 17-NOV-2016. This amendment proposed additional, later timepoints for Aim 3C since there was a mild hearing loss at P30 after deletion of Jag1 at P0/P1.
- 4) Amendment A4 to the vertebrate animal protocol was approved by SIUSOM IACUC on 7-APR-2017 and by ACURO on 4-MAY-2017. This amendment proposed to use a different mouse model (combination of CreER and iCreER alleles) to delete Jag1 in as many cell types as possible in the neonatal mouse cochlea.
- 5) Amendment A5 to the vertebrate animal protocol was approved by SIUSOM IACUC on 24-MAR-2017 and by ACURO on 4-MAY-2017. This amendment proposed additional experiments to further investigate the phenotype of control samples where Notch signaling is kept active in SCs, but HCs remain intact (Sox10<sup>rtTA</sup>::TetO-N1ICD::TetO-LacZ mice).
- 6) Amendment A6 to the vertebrate animal protocol was approved by SIUSOM IACUC on 21-AUG-2017 and by ACURO on 15-SEP-2017. This amendment proposed additional experiments to further investigate the phenotype of control samples where Notch signaling is kept active in SCs, but HCs remain intact (Sox10<sup>rtTA</sup>::TetO-N1ICD::TetO-LacZ mice).
- 7) Amendment A7 to the vertebrate animal protocol was approved by SIUSOM IACUC on 27-NOV-2017 and by ACURO on 7-DEC-2017. This amendment proposed additional experiments to determine whether the reduced number of Hensen cells after deletion of Jag1 from SCs was caused by cell death.
- 8) Amendment A8 to the vertebrate animal protocol was approved by SIUSOM IACUC on 18-DEC-2017 and by ACURO on 24-JAN-2018. This amendment proposed 1) additional experiments to further investigate the phenotype of control samples where Notch signaling is kept active in SCs, but HCs remain intact (Sox10<sup>rtTA</sup>::TetO-N1ICD::TetO-LacZ mice); 2) to investigate the deletion of both Dll1 and Jag2 from HCs since preliminary results showed very little changes after deletion of each ligand individually, and 3) to investigate the effects of Jag1 deletion from HCs.

9) Amendment A9 to the vertebrate animal protocol was approved by SIUSOM IACUC on 6-FEB-2018 and by ACURO on 9-MAY-2018. This amendment proposed additional experiments to determine investigate 1) the cause of Hensen cells after deletion of Jag1 from SCs, 2) The effect of deleting Jag1 from SCs at a later age, and 3) the effect of deleting Jag1 from other SC subtypes.

10) Amendment A10 to the vertebrate animal protocol was approved by SIUSOM IACUC on 4-APR-2018 and by ACURO on 9-MAY-2018. This amendment added new personnel.

11) The 3 year rewrite of the vertebrate animal protocol was approved by the SIUSOM IACUC on 6-JUN-2018 and approved by USAMRMC ACURO on 02-AUG-2018.

12) Amendment B1 to the vertebrate animal protocol was approved by SIUSOM IACUC on 17-SEP-2018 and by ACURO on 4-OCT-2018. This amendment proposed additional experiments to understand the cause of the phenotype of control samples where Notch signaling is kept active in SCs, but HCs remain intact (Sox10<sup>rtTA</sup>::TetO-N1ICD::TetO-LacZ mice).

13) Amendment B2 to the vertebrate animal protocol was approved by SIUSOM IACUC on 5-FEB-2019 and by ACURO on 27-MAR-2019. This amendment added new personnel.

14) Amendment B3 to the vertebrate animal protocol was approved by SIUSOM IACUC on 30-MAY-2019 and was sent to ACURO on 6-JUN-2019. However, based on the new ACURO guidelines, it was not considered significant and did not require ACURO approval. This amendment added new personnel.

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

Nothing to report

## **6. PRODUCTS:**

### **Journal publications.**

1) Published: McGovern MM, Zhou L, Randle MR, and Cox BC (2018) Spontaneous hair cell regeneration is prevented by increased Notch signaling in supporting cells. *Front Cell Neurosci* 12:120. Federal support was acknowledged.

2) In preparation (will be submitted in a few months): McGovern MM\*, Zhou L\*, Randle MR, and Cox BC (*in preparation*) Increased Notch signaling at birth induces widespread proliferation and ectopic hair cells in non-sensory tissue of the neonatal mouse cochlea

Federal support will be acknowledged. (\*denotes co-first authors)

3) In preparation (will be submitted in January): Chrysostomou E\*, Zhou L\*, Darcy YL,

Graves, KA, Doetzlhofer A, and Cox BC (*in preparation*) The Notch ligand Jagged1 is required for the formation and maintenance of Hensen cells in the mouse cochlea. Federal support will be acknowledged. (\*denotes co-first authors)

4) In preparation (will be submitted in January): Zhou L, Randle MR, Telger A, Graves KA, Darcy YL, and Cox BC (*in preparation*) Postnatal deletion of the Notch ligands Jagged 2 and Delta-like 1 results in death of supporting cells in the neonatal mouse cochlea. Federal support will be acknowledged.

#### **Books or other non-periodical, one-time publications.**

Nothing to report

#### **Other publications, conference papers and presentations.**

Poster presentations at international conferences:

- 1) 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:39<sup>th</sup> annual midwinter meeting*, 2016 February 20-24, San Diego, CA
- 2) McGovern MM and Cox, BC (2017) Fate-mapping identifies supporting cell subtypes capable of spontaneously regenerating cochlear hair cells. *Association for Research in Otolaryngology:40<sup>th</sup> annual midwinter meeting*, 2017 February 11-15, Baltimore, MD
- 3) \*McGovern MM, Randle MR, Zhou L, and Cox BC (2018) Maintaining active Notch signaling in supporting cells prevents spontaneous hair cell regeneration *in vivo*. *Association for Research in Otolaryngology:41<sup>st</sup> annual midwinter meeting*, 2018 February 10-14, San Diego, CA
- 4) \*Chrysostomou E, Zhou L, Graves KA, Darcy YL, Randle MR, Doetzlhofer A, and Cox BC (2018) The Notch ligand Jagged1 is required for the survival of supporting cells in the mouse cochlea. *Association for Research in Otolaryngology:41<sup>st</sup> annual midwinter meeting*, 2018 February 10-14, San Diego, CA

- 5)\* Zhou L, Darcy YL, Graves KA, Randle MR, and Cox BC (2018) The role of the Notch ligand Jagged1 in postnatal development of the mouse cochlea. *Gordon Research Conference on the Auditory System*, 2018 July 8-12, Bryant University, Smithfield, RI
- 6) \*McGovern MM, Zhou L, Randle MR, and Cox BC (2018) Increased Notch signaling at birth induces widespread proliferation and ectopic hair cells in the non-sensory tissue of the neonatal mouse cochlea. *Gordon Research Conference on the Auditory System*, 2018 July 8-12, Bryant University, Smithfield, RI
- 7) \*Zhou L, McGovern MM, Randle MR, and Cox BC (2019) Increased Notch signaling induces proliferation in the neonatal mouse cochlea. *Association for Research in Otolaryngology:41<sup>st</sup> annual midwinter meeting*, 2019 February 9-13, Baltimore, MD

Oral presentation at regional conference:

- 1) \*Zhou L, Randle MR, Graves KG, and Cox BC (2019) Deletion of the Notch ligands Jagged2 and Delta-like 1 results in loss of supporting cells in the neonatal mouse cochlea. *Midwest Auditory Research Conference*, 2019 July 11-13, Springfield, IL

Poster presentation at regional conference:

- 1) \*Zhou L, McGovern MM, Randle MR, and Cox BC (2019) Increased Notch signaling induces proliferation in the mouse cochlea. *1<sup>st</sup> Annual Bellucci Symposium on Hearing Research*, 2019 May 10, Omaha, NE

Oral presentations at local symposia:

- 1) 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
- 2) 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
- 3) \*Zhou L, Darcy YL, Graves KA, Randle MR, and Cox, BC (2018) The Notch ligand Jagged1 is required for the survival of Hensen cells in the mouse cochlea. *16th Annual Horst R. Konrad Visiting Professor & ENT Resident Research Day, Southern Illinois University School of Medicine*, 2018 June 08, Springfield, IL

Poster presentations at local symposia:

- 1) McGovern MM, Cuppini CL, Graves KA, Darcy YL, and Cox BC (2017) The majority of spontaneously regenerated hair cells come from pillar and Deiters' cells compared to other supporting cell subtypes. *27th Annual Graduate Student Research Symposium, Southern Illinois University*

*School of Medicine, 2017 April 21, Carbondale, IL*

- 2) \*Zhou L, Darcy YL, Graves KA, Randle MR, and Cox, BC (2018) The Notch ligand Jagged1 is required for the survival of Hensen cells in the mouse cochlea. *28th Annual Graduate Student Research Symposium, Southern Illinois University School of Medicine, 2018 April 26, Springfield, IL*

Invited oral presentations at international conferences:

- 1) *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.
- 2) \**Society for Neuroscience 47<sup>th</sup> Annual Meeting*. Nanosymposium 548: Hair cells, The Notch ligand Jagged1 is required for the survival of supporting cells in the postnatal mouse cochlea. 2017 November 11-15, Washington, DC.

Invited oral presentations at Universities:

- 1) \**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.
- 2) \**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.
- 3) \**Department of Biomedical Sciences, Creighton University*. Cell source and mechanism of spontaneously regenerated hair cells in the neonatal mouse cochlea. October 24-25, 2016, Omaha, NE.
- 4) \**Southern Illinois University, Edwardsville School of Pharmacy*. Plasticity in the auditory and vestibular systems. April 14, 2017, Edwardsville, IL.
- 5) \**Department of Neurobiology and Anatomical Sciences, University of Mississippi Medical Center*. Plasticity in the auditory and vestibular systems. October 10, 2017, Jackson, MS.
- 6) \**Department of Physiology, Southern Illinois University*. Plasticity in the auditory and vestibular systems. September 14, 2018, Carbondale, IL
- 7) \**Department of Neuroscience, University of Virginia*. Unique role of Notch signaling in the neonatal cochlea. August 28, 2019, Charlottesville, VA

Poster presentation at an 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.*

Invited oral presentation at a USAMRMC program review:

Cox, BC (2017) Investigation of Notch signaling in during spontaneous regeneration of cochlear hair cells. *US Army Medical Research and Materiel Command Clinical and Rehabilitative Medicine Hearing and Balance In-Progress Review Meeting. 2017 August 3, Fort Detrick, MD.*

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

|                              |                                                                                                                                                                                                                                             |
|------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Name:                        | <b>Brandon C. Cox</b>                                                                                                                                                                                                                       |
| Project Role:                | PI                                                                                                                                                                                                                                          |
| Researcher Identifier        | ORCID ID# 0000-0002-6989-161X                                                                                                                                                                                                               |
| 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, NIH R13DC018245, NIH R03HD097543, ONR N00014-16-1-2306, & DOD W81XWH-19-1-0017                                                                                                              |

|                              |                                                                                                                                                                                                                                                                  |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Name:                        | <b>Michelle R. Randle</b>                                                                                                                                                                                                                                        |
| Project Role:                | Researcher IV (Technician)                                                                                                                                                                                                                                       |
| Researcher Identifier        | N/A                                                                                                                                                                                                                                                              |
| Nearest person month worked: | 3                                                                                                                                                                                                                                                                |
| Contribution to Project:     | Assisted in training personnel, assisted in mouse colony maintenance, performed real-time qPCR, performed immunostaining, quantified confocal images, performed ABR, administrative duties, and ordered supplies                                                 |
| Funding Support:             | NIH R01DC01441 & NIH R01DC13771                                                                                                                                                                                                                                  |
|                              |                                                                                                                                                                                                                                                                  |
| Name:                        | <b>Kaley A. Graves</b>                                                                                                                                                                                                                                           |
| Project Role:                | Researcher II (Technician)                                                                                                                                                                                                                                       |
| Researcher Identifier        | N/A                                                                                                                                                                                                                                                              |
| Nearest person month worked: | 6                                                                                                                                                                                                                                                                |
| Contribution to Project:     | Managed mouse colony, collected tissue samples, performed immunostaining, quantified confocal images, performed ABR, and assisted in training personnel                                                                                                          |
| Funding Support:             | NIH R01DC01441& NIH R01DC13771                                                                                                                                                                                                                                   |
|                              |                                                                                                                                                                                                                                                                  |
| Name:                        | <b>Melissa M. McGovern</b>                                                                                                                                                                                                                                       |
| Project Role:                | Graduate Student                                                                                                                                                                                                                                                 |
| Researcher Identifier        | ORCID ID# 0000-0003-2254-8782                                                                                                                                                                                                                                    |
| Nearest person month worked: | 6                                                                                                                                                                                                                                                                |
| Contribution to Project:     | Assisted in mouse colony maintenance, collected tissue samples, performed immunostaining, real-time qPCR, and <i>in situ</i> hybridization, quantified confocal images, performed data analysis, and assisted in the writing of the manuscripts for publication. |
| Funding Support:             | None                                                                                                                                                                                                                                                             |
|                              |                                                                                                                                                                                                                                                                  |
| Name:                        | <b>Yuanzhao L. Darcy, PhD</b>                                                                                                                                                                                                                                    |
| Project Role:                | Postdoc                                                                                                                                                                                                                                                          |
| Researcher Identifier        | N/A                                                                                                                                                                                                                                                              |
| Nearest person month worked: | 6.3                                                                                                                                                                                                                                                              |
| Contribution to Project:     | Assisted in mouse colony maintenance, collected tissue samples, performed immunostaining and ABR, quantified confocal images, and performed data analysis.                                                                                                       |
| Funding Support:             | NIH R01DC01441                                                                                                                                                                                                                                                   |

|                              |                                                                                                                                               |
|------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| Name:                        | <b>Luyi Zhou, PhD</b>                                                                                                                         |
| Project Role:                | Postdoc                                                                                                                                       |
| Researcher Identifier        | N/A                                                                                                                                           |
| Nearest person month worked: | 12                                                                                                                                            |
| Contribution to Project:     | Collected tissue samples, performed immunostaining and <i>in situ</i> hybridization, quantified confocal images, and performed data analysis. |
| Funding Support:             | None                                                                                                                                          |

|                              |                                                                                |
|------------------------------|--------------------------------------------------------------------------------|
| Name:                        | <b>Chantz Pinder</b>                                                           |
| Project Role:                | Researcher I (Technician)                                                      |
| Researcher Identifier        | N/A                                                                            |
| Nearest person month worked: | 5                                                                              |
| Contribution to Project:     | Managed mouse colony, collected tissue samples, and quantified confocal images |
| Funding Support:             | NIH R01DC01441& NIH R01DC13771                                                 |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

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 (Technician) -- reduced to 25% effort  
 Kaley Ramsey, Researcher I (Technician) – reduced to 50% effort  
 Yuanzhao Darcy, Postdoc – added at 90%

When Melissa McGovern (graduate student) and Yuanzhao Darcy (postdoc) left the lab, Luyi Zhou (postdoc) was hired as their replacement.

When Kaley Graves (Technician) left the lab, Chantz Pinder (Technician) was hired as her replacement.

**What other organizations were involved as partners?**

- 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<sup>rtTA</sup> mice (with permission from Dr. Wegner who made the mice)
- 4) University of Pennsylvania, Philadelphia, PA, in-kind support: supplied TetO-N1ICD mice

- 5) Baylor College School of Medicine, Houston, TX, in-kind support: trained graduate student on *in situ* hybridization technique
- 6) Johns Hopkins University, Baltimore, MD, collaboration: performed complimentary experiments for Aim 3C.
- 7) University of Sussex, Brighton, UK, collaboration: performed analysis of the tectorial membrane for Aim 3C.

## 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

Award Amount: \$1,499,960

## 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.

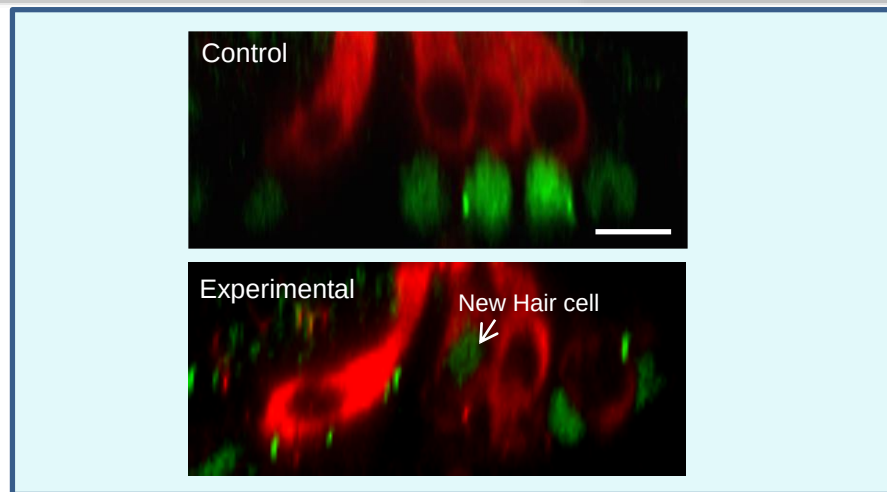


Figure Legend: Fate-mapping of supporting cells (green nuclear label). After hair cell damage at birth, new regenerated hair cells (red with green nucleus) are formed spontaneously in the neonatal mouse cochlea. Scale Bar: 10  $\mu$ m

## Timeline and Cost

| Activities             | CY | 15-16  | 16-17  | 17-18  | 18-19  |
|------------------------|----|--------|--------|--------|--------|
| Aim 1                  |    |        |        |        |        |
| Aim 2                  |    |        |        |        |        |
| Aim 3                  |    |        |        |        |        |
|                        |    |        |        |        |        |
| Estimated Budget (\$K) |    | \$500K | \$333K | \$333K | \$333K |

## 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 1, 2, & 3
- ☐ Finish preparing manuscripts for publication & submit

### Budget Expenditure to Date

Projected Expenditure: \$500K for Y1, \$333K for Y2-Y4

Actual Expenditure: \$1,499,960

Updated: (12/13/2019)

*This generic Statement of Work document is intended to assist applicants with the format preferred by CDMRP. This particular SOW does not contain any specific scientific information and is intended to be easily modifiable for any project. Not all components will be applicable for every project; please consult your Program Announcement for specific award requirements.*

**REVISED STATEMENT OF WORK – July 5, 2018**  
**ORIGINAL AWARD START DATE September 15, 2015**  
**One year no cost extension requested**

Site 1: Southern Illinois University School of Medicine  
801 N. Rutledge Street, Springfield, IL 62702  
PI: Brandon Cox, PhD

Additions to the previous SOW are highlighted in yellow.  
Deleted tasks are marked with strikethrough text.

|                                                                                                                                                                                                                        | Timeline<br>(months) | Site 1  | Percent<br>complete |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------|---------|---------------------|
| <b>Specific Aim 1: To measure changes in Notch signaling after hair cell (HC) damage in the neonatal mouse cochlea</b>                                                                                                 | 1-15                 | Dr. Cox |                     |
| Task 1: Obtain institutional and DOD approval of animal protocol for all Aims                                                                                                                                          | 1-3                  | Dr. Cox | 100%                |
| Task 2: Measure global changes in Notch target genes in the cochlea using real-time qPCR. Number of mice needed: 72                                                                                                    | 3-7                  | Dr. Cox | 100%                |
| Task 3: Measure changes in Notch target genes specifically in supporting cells using immunostaining and <i>in situ</i> hybridization. Number of mice needed: 108                                                       | 3-14                 | Dr. Cox | 100%                |
| Task 4: Measure changes in the number of Hes5-LacZ <sup>+</sup> cells using immunostaining. Number of mice needed: 72                                                                                                  | 3-9                  | Dr. Cox | 100%                |
| Task 5: Prepare manuscript on Aim1 and Aim 2A findings for publication                                                                                                                                                 | 13-15                | Dr. Cox | 100%                |
| <b>Specific Aim 2: To maintain active Notch signaling in supporting cells (SCs) in the context of HC damage.</b>                                                                                                       | 3-36                 | Dr. Cox |                     |
| Task 1: Obtain needed mouse lines for Aim 2A& B ( <i>Sox10-rtTA</i> & <i>TetO-NICD</i> )                                                                                                                               | 3-6                  | Dr. Cox | 100%                |
| Task 2: Perform mouse breeding to obtain mice containing the proposed combination of 5 alleles ( <i>Atoh1-CreER<sup>TM</sup>::ROSA26<sup>DTA</sup>::Sox10-rtTA::TetO-NICD::TetO-lacZ</i> ). Number of mice needed: 60. | 6-24                 | Dr. Cox | 100%                |
| Task 3: Confirm active Notch signaling in SCs in the proposed mouse model using real time qPCR. Number of mice needed: 53                                                                                              | 24-27                | Dr. Cox | 100%                |
| <b>Specific Aim 2A:</b> To investigate the ability of SCs to directly transdifferentiate into HCs when active Notch signaling is maintained.                                                                           |                      |         |                     |
| Task 4: Measure HC regeneration capacity in mouse model where Notch signaling is kept active in SCs and HC are killed using immunostaining and confocal microscopy. Number of mice needed: 53                          | 24-32                | Dr. Cox | 100%                |
| <b>Specific Aim 2B:</b> To investigate the phenotype of increased Notch signaling in SCs and other non-sensory cells in control samples where HCs remain intact.                                                       |                      |         |                     |

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|                                                                                                                                                                                           |       |         |      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|---------|------|
| Task 5: Examine increased proliferation in a mouse model where Notch signaling is increased in SCs and other non-sensory cells. Number of mice needed:120                                 | 24-44 | Dr. Cox | 95%  |
| Task 6: Examine patches of ectopic HCs and markers of prosensory cells in a mouse model where Notch signaling is increased in SCs and other non-sensory cells. Number of mice needed: 240 | 30-44 | Dr. Cox | 100% |
| Task 7: Examine the effects of different levels of increased Notch signaling in SCs and other non-sensory cells. Number of mice needed: 240                                               | 33-44 | Dr. Cox | 95%  |
| Task 8: Prepare manuscript on Aim 2B findings for publication                                                                                                                             | 44-48 | Dr. Cox | 50%  |
| <b>Specific Aim 3A: To conditionally delete <i>Delta-like1 (Dll1)</i> from neonatal HCs in the normal, undamaged neonatal cochlea.</b>                                                    | 3-30  | Dr. Cox |      |
| Task 1: Obtain needed mouse line for Aim 3A ( <i>Dll1<sup>loxP/loxP</sup></i> )                                                                                                           | 3-6   | Dr. Cox | 100% |
| Task 2: Perform mouse breeding to obtain mice containing the desired genotype ( <i>Atoh1-CreER::Dll1<sup>loxP/loxP</sup></i> ). Number of mice needed: 10.                                | 7-12  | Dr. Cox | 100% |
| Task 3: Measure the expression level of Notch effector genes after <i>Dll1</i> deletion using real time qPCR. Number of mice needed: 24.                                                  | 12-18 | Dr. Cox | 100% |
| Task 4: Investigate morphological changes to HCs and SCs in the cochlea after <i>Dll1</i> deletion using immunostaining and confocal microscopy. Number of mice needed: 24                | 14-24 | Dr. Cox | 100% |
| Task 5: Measure hearing in mice after <i>Dll1</i> deletion at P30 using ABR. Number of mice needed: 24                                                                                    | 24-27 | Dr. Cox | 100% |
| Task 6: Prepare manuscript on Aim 3A findings for publication                                                                                                                             | 27-30 | Dr. Cox |      |
| <b>Specific Aim 3B: To conditionally delete <i>Jagged2 (Jag2)</i> from neonatal HCs in the normal, undamaged neonatal cochlea.</b>                                                        | 3-30  | Dr. Cox |      |
| Task 1: Obtain needed mouse line for Aim 3B ( <i>Jag2<sup>loxP/loxP</sup></i> )                                                                                                           | 3-6   | Dr. Cox | 100% |
| Task 2: Perform mouse breeding to obtain mice containing the desired genotype ( <i>Atoh1-CreER::Jag2<sup>loxP/loxP</sup></i> ). Number of mice needed: 10.                                | 7-12  | Dr. Cox | 100% |
| Task 3: Measure the expression level of Notch effector genes after <i>Jag2</i> deletion using real time qPCR. Number of mice needed: 24.                                                  | 12-18 | Dr. Cox | 100% |
| Task 4: Investigate morphological changes to HCs and SCs in the cochlea after <i>Jag2</i> deletion using immunostaining and confocal microscopy. Number of mice needed: 24                | 14-24 | Dr. Cox | 100% |
| Task 5: Measure hearing in mice after <i>Jag2</i> deletion at P30 using ABR. Number of mice needed: 24                                                                                    | 24-27 | Dr. Cox | 100% |
| Task 6: Prepare manuscript on Aim 3B findings for publication                                                                                                                             | 27-30 | Dr. Cox |      |
| <b>Specific Aim 3B.2: To conditionally delete both <i>Delta-like1 (Dll1)</i> and <i>Jagged2 (Jag2)</i> from neonatal HCs in the normal, undamaged neonatal cochlea.</b>                   | 30-44 | Dr. Cox |      |

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|                                                                                                                                                                                                    |       |         |      |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|---------|------|
| Task 1: Perform mouse breeding to obtain mice containing the desired genotype ( <i>Atoh1-CreER::Dll1<sup>loxP/loxP</sup>::Jag2<sup>loxP/loxP</sup></i> ). Number of mice needed: 10.               | 30-34 | Dr. Cox | 100% |
| Task 2: Measure the expression level of Notch effector genes after deletion of both <i>Dll1</i> and <i>Jag2</i> using real time qPCR. Number of mice needed: 24.                                   | 34-40 | Dr. Cox | 100% |
| Task 3: Investigate morphological changes to HCs and SCs in the cochlea after deletion of both <i>Dll1</i> and <i>Jag2</i> using immunostaining and confocal microscopy. Number of mice needed: 24 | 34-42 | Dr. Cox | 100% |
| Task 4: Measure hearing in mice after deletion of both <i>Dll1</i> and <i>Jag2</i> deletion at P30 using ABR. Number of mice needed: 24                                                            | 34-42 | Dr. Cox | 100% |
| Task 5: Prepare manuscript on Aims 3A, 3B, and 3B.2 findings for publication                                                                                                                       | 42-46 | Dr. Cox | 60%  |
| <b>Specific Aim 3C: To conditionally delete <i>Jagged1</i> (<i>Jag1</i>) from neonatal SCs in the normal, undamaged neonatal cochlea.</b>                                                          | 3-30  | Dr. Cox |      |
| Task 1: Obtain needed mouse line for Aim 3C ( <i>Jag1<sup>loxP/loxP</sup></i> )                                                                                                                    | 3-6   | Dr. Cox | 100% |
| Task 2: Perform mouse breeding to obtain mice containing the desired genotype ( <i>FgfR3-iCreER::Jag1<sup>loxP/loxP</sup></i> ). Number of mice needed: 10.                                        | 7-12  | Dr. Cox | 100% |
| Task 3: Measure the expression level of Notch effector genes after <i>Jag1</i> deletion using real time qPCR. Number of mice needed: 24.                                                           | 12-18 | Dr. Cox | 100% |
| Task 4: Investigate morphological changes to HCs and SCs in the cochlea after <i>Jag1</i> deletion using immunostaining and confocal microscopy. Number of mice needed: 24                         | 14-24 | Dr. Cox | 95%  |
| Task 5: Measure hearing in mice after <i>Jag1</i> deletion at P30 using ABR. Number of mice needed: 24                                                                                             | 24-27 | Dr. Cox | 100% |
| Task 6: Prepare manuscript on Aim 3C findings for publication                                                                                                                                      | 27-30 | Dr. Cox | 90%  |
| <b>Specific Aim 3D: To conditionally delete <i>Jagged1</i> (<i>Jag1</i>) from neonatal HCs in the normal, undamaged neonatal cochlea.</b>                                                          | 30-44 | Dr. Cox |      |
| Task 1: Perform mouse breeding to obtain mice containing the desired genotype ( <i>Atoh1-CreER::Jag1<sup>loxP/loxP</sup></i> ). Number of mice needed: 10.                                         | 30-34 | Dr. Cox | 100% |
| Task 2: Measure the expression level of Notch effector genes after <i>Jag1</i> deletion in HCs using real time qPCR. Number of mice needed: 24.                                                    | 34-40 | Dr. Cox | 100% |
| Task 3: Investigate morphological changes to HCs and SCs in the cochlea after <i>Jag1</i> deletion in HCs using immunostaining and confocal microscopy. Number of mice needed: 24                  | 34-42 | Dr. Cox | 100% |
| Task 4: Measure hearing in mice after <i>Jag1</i> deletion in HCs at P30 using ABR. Number of mice needed: 24                                                                                      | 34-42 | Dr. Cox | 100% |
| Task 5: Prepare manuscript on Aim 3D findings for publication                                                                                                                                      | 42-46 | Dr. Cox | 60%  |



# Spontaneous Hair Cell Regeneration Is Prevented by Increased Notch Signaling in Supporting Cells

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During embryonic development, differentiation of cochlear progenitor cells into hair cells (HCs) or supporting cells (SCs) is partially controlled through Notch signaling. Many studies have shown that inhibition of Notch signaling allows SCs to convert into HCs in both normal and drug damaged neonatal mouse cochleae. This mechanism is also implicated during HC regeneration in non-mammalian vertebrates; however, the mechanism of spontaneous HC regeneration in the neonatal mouse cochlea is less understood. While inhibition of Notch signaling can force SCs to convert into HCs and increase the number of regenerated HCs, it is currently unknown whether this pathway is involved in spontaneous HC regeneration observed *in vivo*. Therefore, we investigated the role of Notch signaling during the spontaneous HC regeneration process using *Atoh1-CreER<sup>TM</sup>::Rosa26<sup>loxP-stop-loxP-DTA/+</sup>* mice injected with tamoxifen at postnatal day (P) 0 and P1 to ablate HCs and stimulate spontaneous HC regeneration. Expression changes of genes in the Notch pathway were measured using immunostaining and *in situ* hybridization, with most changes observed in the apical one-third of the cochlea where the majority of HC regeneration occurs. Expression of the Notch target genes *Hes1*, *Hes5*, *Hey1*, *HeyL*, and *Jagged1* were decreased. To investigate whether reduction of Notch signaling is involved in the spontaneous HC regeneration process, we overexpressed the *Notch1* intracellular fragment (*N1ICD*) in cochlear SCs and other non-sensory epithelial cells in the context of HC damage. Specifically, *Atoh1-CreER<sup>TM</sup>::Rosa26<sup>loxP-stop-loxP-DTA/+</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* mice were injected with tamoxifen at P0/P1 to stimulate spontaneous HC regeneration and given doxycycline from P0–P7 to induce expression of *N1ICD* as well as *LacZ* for fate-mapping. We observed a 92% reduction in the number of fate-mapped regenerated HCs in mice with *N1ICD* overexpression compared to controls with HC damage but no manipulation of Notch signaling. Therefore, we conclude that increased Notch signaling prevents spontaneous HC regeneration from occurring in the neonatal mouse cochlea. Understanding which components of the Notch pathway regulates regenerative plasticity in the neonatal mouse cochlea will inform investigations focused on stimulating HC regeneration in mature cochlea and eventually in humans to treat hearing loss.

**Keywords:** hair cell regeneration, cochlea, Notch, CreER, Tet-On, *N1ICD*

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### Edited by:

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**Received:** 30 January 2018

**Accepted:** 13 April 2018

**Published:** 04 May 2018

### Citation:

McGovern MM, Zhou L, Randle MR  
and Cox BC (2018) Spontaneous  
Hair Cell Regeneration Is Prevented  
by Increased Notch Signaling  
in Supporting Cells.  
*Front. Cell. Neurosci.* 12:120.  
doi: 10.3389/fncel.2018.00120

## INTRODUCTION

Hearing loss, one of the most prevalent conditions in the world, can result from multiple etiologies such as aging, viral infection, or exposure to noise or ototoxic drugs. In those born with normal hearing, death of sound-sensing hair cells (HCs) located within the cochlea is the most common source of hearing impairment. The mature mammalian cochlea does not regenerate HCs after damage, thus hearing loss is permanent (Bohne, 1976; Hawkins et al., 1976; Oesterle et al., 2008). However, in non-mammalian vertebrates, including birds, fish, and amphibians, the inner ear responds to damage by regenerating lost HCs throughout the life of the animal (reviewed in Bermingham-McDonogh and Rubel, 2003; Stone and Cotanche, 2007). Several studies have suggested that the Notch signaling pathway is involved in the HC regeneration process in non-mammals (reviewed in Stone and Cotanche, 2007; Brignull et al., 2009).

The Notch pathway uses membrane bound ligands and receptors expressed on adjacent cells to generate a mosaic pattern of cell types. In the mammalian cochlea, HCs and neighboring supporting cells (SCs) develop from the same pool of progenitor cells through a Notch-mediated process. As progenitor cells take a HC fate, they begin to express the Notch ligands Delta-like ligand 1 (Dll1), Delta-like ligand 3 (Dll3), and Jagged2 (Jag2) (Lanford et al., 1999, 2000; Morrison et al., 1999; Kiernan et al., 2005; Brooker et al., 2006; Hartman et al., 2007). These ligands signal to neighboring progenitor cells through Notch receptors which inhibits them from becoming HCs and instructs a SC fate (Lanford et al., 2000; Zheng et al., 2000; Zine et al., 2001; Murata et al., 2006; Hayashi et al., 2008; Doetzlhofer et al., 2009). A recent study reported limited down-regulation of Notch target genes after HC damage in the neonatal mouse cochlea (Korrapati et al., 2013). However, inhibition of the Notch signaling pathway, using pharmacological agents or genetically modified mouse models, in the late embryonic and early postnatal cochlea has the consistent effect of inducing the conversion of SCs into HCs either in the undamaged cochlea (Yamamoto et al., 2006; Doetzlhofer et al., 2009; Li et al., 2015; Maass et al., 2015) or after HC loss (Korrapati et al., 2013; Mizutani et al., 2013; Bramhall et al., 2014; Hu et al., 2016). Therefore, active Notch signaling in SCs plays an important role in maintaining their cell fate in the neonatal cochlea.

Recently, several groups have demonstrated that the immature murine cochlea has the capacity to spontaneously regenerate HCs during the first postnatal week (Bramhall et al., 2014; Cox et al., 2014; Hu et al., 2016) and loss of Notch signaling has been shown to increase the degree of HC regeneration over that which occurs spontaneously in HC damaged cochlear explants (Korrapati et al., 2013; Bramhall et al., 2014; Hu et al., 2016). While these results demonstrate that SCs can be forced to convert into HCs through inhibition of the Notch pathway, it is unclear whether the Notch signaling pathway is involved in the mechanism of spontaneous HC regeneration. To address this question, we first investigated changes in Notch signaling during the spontaneous HC regeneration process in the neonatal mouse cochlea and found that the expression of the Notch target genes *Hes1*, *Hes5*, *Hey1*, *HeyL*, and *Jagged1* (*Jag1*) were decreased.

Next, to determine whether the spontaneous HC regeneration process is inhibited by sustained Notch signaling, we combined HC damage with overexpression of the *Notch1 intracellular domain* (*NIICD*) in SCs and other non-sensory epithelial cells and measured the effect on spontaneous HC regeneration. This model showed a 92% reduction in the number of spontaneously regenerated HCs and suggests that weakened Notch signaling contributes to SC plasticity which underlies spontaneous HC regeneration.

## MATERIALS AND METHODS

### Animals

*Plp-CreERT<sup>2</sup>* mice (Jax stock #5975, Doerflinger et al., 2003), *Rosa26<sup>loxP-stop-loxP-DTA</sup>* mice (Jax stock #6331, Ivanova et al., 2005), *Rosa26<sup>loxP-stop-loxP-tdTomato</sup>* mice (also called Ai14, Jax stock #7914, Madisen et al., 2010), and *TetO-LacZ* mice (Jax stock #2621, Furth et al., 1994), were obtained from The Jackson Laboratory (Bar Harbor, ME). *Atoh1-CreER<sup>TM</sup>* mice (Chow et al., 2006) were provided by Dr. Suzanne Baker (St. Jude Children's Hospital, Memphis, TN, United States); *Hes5<sup>LacZ</sup>* mice (Imayoshi et al., 2010) were provided by Dr. Ryoichiro Kageyama (Kyoto University, Kyoto, Japan); *Sox10<sup>rtTA</sup>* mice (Ludwig et al., 2004; Walters and Zuo, 2015) were provided by Dr. Michael Wegner (Erlangen University, Erlangen, Germany); and *TetO-NIICD* mice (Stanger et al., 2005) were provided by Dr. Ben Stranger (University of Pennsylvania, Philadelphia, PA, United States). Genotyping for all mouse lines was performed by Transnetyx, Inc. (Cordova, TN, United States). Mice of both genders were used in all studies and all animal work was performed in accordance with approved animal protocols from the Institutional Animal Care and Use Committee at Southern Illinois University School of Medicine.

### Substances Given to Animals

To induce CreER recombination for either HC death or tdTomato expression, tamoxifen (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in 100% corn oil and injected intraperitoneally (IP) at 3 mg/40 g on either postnatal day (P) 0, or both P0 and P1. To induce *LacZ* and *NIICD* overexpression in cochlear SCs and other non-sensory epithelial cells, doxycycline (100 mg/kg, IP, Fisher, Hampton, NH, United States) was injected at P1 (~6 h after the second tamoxifen injection) as well as administered in the food (2000 mg/kg, Envigo, Huntingdon, United Kingdom) to the nursing mother for the duration of the experiment.

### Real-Time qPCR

Quantification of gene expression was conducted using real time quantitative polymerase chain reaction (qPCR). Neonatal pups were euthanized under isoflurane anesthesia (Henry Schein, Melville, NY, United States) and cochleae were collected from P7 animals, flash frozen in either liquid nitrogen or a dry ice slurry, and then stored at  $-80^{\circ}\text{C}$ . Isolation of total RNA was conducted using tri-reagent (Fisher, Hampton, NH, United States), and precipitated with isopropanol (Acros Organics, Geel, Belgium).

at  $-20^{\circ}\text{C}$  overnight followed by purification with DNase treatment (cat #AM1906, Fisher, Hampton, NH, United States) and reprecipitated using sodium acetate (Fisher, Hampton, NH, United States). Once total RNA was isolated, the concentration was measured using a Nanodrop 2000 (Fisher, Hampton, NH, United States) followed by reverse transcription into cDNA using Thermo Scientific Maxima First Strand cDNA synthesis kit (cat #K1641, Fisher, Hampton, NH, United States) and stored at  $-20^{\circ}\text{C}$ . The expression level of selected genes was measured from cDNA by qPCR using Sybr Green (cat #K0391, Fisher, Hampton, NH, United States) and a CFX Connect Optics Module (Bio-Rad, Hercules, CA, United States). Comparison of gene expression among samples was conducted using the Pfaffl method which is a modified version of the delta-delta Ct equation that takes into account the efficiency of each primer set (Pfaffl, 2001). Gene expression was normalized to a housekeeping gene (Rpl19), which has been used previously for qPCR experiments in cochlear tissue (Basch et al., 2011; Korrapati et al., 2013; Wan et al., 2014; Maass et al., 2016). Data are expressed as fold change from control. **Table 1** lists the primer sets used for each gene that was investigated and each primer set was tested for specificity with a reverse transcriptase-negative control to ensure they did not detect genomic DNA.

## Immunostaining

Neonatal pups were euthanized under isoflurane anesthesia and cochleae were collected and post-fixed in 4% paraformaldehyde (Polysciences, Inc., Warrington, PA, United States) for  $\sim 2$  h at room temperature (RT). The samples were then transferred to 10 mM Phosphate Buffered Saline (PBS, Sigma, St. Louis, MO, United States) and stored at  $4^{\circ}\text{C}$ . Following whole mount dissection, cochleae were cut into 3 turns of equal length and placed into a 48 well plate for immunostaining as free floating tissue. Samples were blocked and permeabilized at RT for 1 h with a solution of Normal Horse Serum or Normal Goat Serum (10%, NHS, or NGS, Vector Labs, Burlingame, CA, United States), Bovine Serum Albumin (1%, BSA, Fisher, Hampton, NH, United States), and Triton-X-100 (1%, Sigma, St. Louis, MO, United States) in 10 mM PBS prior to the application of the primary antibodies. Primary antibodies were diluted in a solution of 10 mM PBS containing NHS or NGS (5%), BSA (1%), and Triton-X-100 (0.1%) and incubated overnight at  $4^{\circ}\text{C}$  or  $37^{\circ}\text{C}$ . The following primary antibodies were used: chicken anti- $\beta$ -galactosidase ( $\beta$ -gal, 1:500, cat#ab9361, Abcam, Cambridge, United Kingdom), goat anti-Jag1 (1:500, cat#sc6011, Santa Cruz Biotechnology, Dallas, TX, United States), rabbit anti-myosin VIIa (1:200, cat#25-6790, Proteus Biosciences, Ramona, CA,

United States), rabbit anti-S100a1 (1:1000, cat#ab868, Abcam, Cambridge, United Kingdom), and goat anti-Sox2 (1:400, cat#sc-17320, Santa Cruz Biotechnology, Dallas, TX, United States). The next day, samples were washed three times with 10 mM PBS for 5 min each at RT. Alexa fluor-conjugated secondary antibodies (Life Technologies, Waltham, MA, United States) were diluted 1:1000 in a solution of NHS or NGS (5%), BSA (1%), and Triton-X-100 (0.1%) in 10 mM PBS and incubated for  $\sim 3$  h in the dark at RT. Following the secondary antibodies, samples were washed three times for 5 min each in 10 mM PBS followed by a 20-min incubation with Hoechst (1:2000, Fisher, Hampton, NH, United States) and three more, 5-min washes in 10 mM PBS at RT. Following immunofluorescent labeling, samples were mounted on slides in Prolong Gold (Fisher, Hampton, NH, United States).

Additionally, chicken anti- $\beta$ -gal antibodies produced excessive background and therefore to increase the signal to noise ratio, signal enhancer (enough volume to cover each sample, cat#R37107, Thermo Fisher Scientific, Hampton, NH, United States) was applied at RT for 30 min followed by three 5-min PBS washes prior to the blocking/permeabilization step. A low pH antigen unmasking solution (AUM, cat#H-3300, Vector Labs, Burlingame, CA, United States) was beneficial to increase antibody binding to the epitope for the anti-Jag1 antibodies. For this step, samples were incubated in a hybridization oven at  $95^{\circ}\text{C}$  for 45 min with a 1:100 dilution of the AUM solution in ddH<sub>2</sub>O followed by three 5-min washes in 10 mM PBS prior to blocking/permeabilization.

Imaging was conducted using a Leica SP5 or a Zeiss LSM 800 confocal microscope and images were processed with LAS-lite (Leica, Wetzlar, Germany) or Zen Blue lite (Zeiss, Oberkochen, Germany) software.

## In Situ Hybridization

Probes were prepared by transfecting plasmids containing the full-length mouse cDNA sequences for *Hes1* (Accession: BC051428, Clone ID: 6478994, Dharmacon, Inc., Lafayette, CO, United States), *Hes5* (Accession: BC103539, Clone ID: 40039948, Dharmacon, Inc., Lafayette, CO, United States), *Hey1* (Accession: BC086635, Clone ID: 6809680, Dharmacon, Inc., Lafayette, CO, United States), and *HeyL* (Accession: BC130263, Clone ID: 40142873, Dharmacon, Inc., Lafayette, CO, United States) into competent *Escherichia coli* cells (Fisher, Hampton, NH, United States) and incubating for 1 h at  $37^{\circ}\text{C}$  before spreading the cells on an agar plate containing the appropriate selection marker and incubating overnight. Once a clone was selected, it was inoculated with the appropriate selection antibiotic

**TABLE 1** | Primer sets used for qPCR of Notch effector genes.

| Gene         | Forward primer            | Reverse primer           | Reference              |
|--------------|---------------------------|--------------------------|------------------------|
| <i>Hes1</i>  | TCAACACGACACCGGACAAAC     | ATGCCGGGAGCTATCTTTCTT    | Chonko et al., 2013    |
| <i>Hes5</i>  | GCACCAGCCCAACTCCAA        | GGCGAAGGCTTTGCTGTGT      | White et al., 2006     |
| <i>Hey1</i>  | CACTGCAGGAGGAAAGGTTAT     | CCCCAACTCCGATAGTCCAT     | Korrapati et al., 2013 |
| <i>HeyL</i>  | GCGCAGAGGGATCATAGAGAA     | TCGCAATTGAGAAAGGCTACTG   | Korrapati et al., 2013 |
| <i>N11CD</i> | GACAACTCCTACCTCTGCTTATGCC | TTACTGTTGCACTCGTTGACCTCG | Yamamoto et al., 2006  |

and incubated overnight in 250 ml of media containing the selection marker at 37°C, after which cells were centrifuged and the supernatant was decanted. The cells were processed with a ZymoPURE plasmid maxiprep kit (cat#D4202, Zymo Research, Irvine, CA, United States) to isolate the plasmid DNA. Concentration was measured on a Nanodrop 2000 (Fisher, Hampton, NH, United States) and the plasmids were linearized and cleaned up with phenol::chloroform::isoamyl alcohol (Fisher, Hampton, NH, United States). Plasmids were sequenced by GenScript (Piscataway, NJ, United States) and each sequence was entered into BLAST (NCBI, Bethesda, MD, United States) to determine their correspondence to the endogenous mouse mRNA sequence (*Hes1* = 97.4% homology, *Hes5* = 100% homology, *Hey1* = 99.9% homology, and *HeyL* = 99.5% homology). Then the probes were digoxigenin-labeled (DIG; cat# 286 036 910, Roche, Indianapolis, IN, United States) through *in vitro* transcription and cut into ~300 bp pieces through a mild alkaline bath to facilitate interaction with the endogenous RNA.

Cochleae were fixed in RNase free 4% paraformaldehyde (PFA; Polysciences, Inc., Warrington, PA, United States) for ~2 h at RT or overnight at 4°C. Following fixation, cochleae were dissected and dehydrated in a graded methanol series (Fisher, Hampton, NH, United States) for 5 min in each concentration [25, 50, 75, and 100% diluted in RNase free 10 mM PBS with 0.1% Tween-20 (PTw) (Sigma, St. Louis, MO, United States)]. Samples were rehydrated through a reverse graded methanol series and transferred to RNase free 2 ml tubes. Samples were washed three times for 5 min each in RNase free PTw at RT. Next, samples were digested for 15 min at RT with 10 µg/ml proteinase K (Fisher, Hampton, NH, United States) in PTw then incubated with 4% PFA containing 0.2% glutaraldehyde (Sigma, St. Louis, MO, United States) for 20 min to halt the digestion. Then samples were again washed twice with RNase free PTw for 5 min each. Next, hybridization buffer [50% formamide (Sigma St. Louis, MO, United States), 1.5× saline-sodium citrate (SSC, Sigma, St. Louis, MO, United States), pH adjusted to 4.5 with citric acid (Sigma, St. Louis, MO, United States), 50 µg/ml yeast tRNA (Sigma, St. Louis MO, United States), 100 µg/ml heparin (Sigma, St. Louis, MO, United States), 0.2% Tween-20 (Sigma, St. Louis, MO, United States), 0.5% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate Sigma, St. Louis, MO, United States), and 5mM EDTA pH 8.0, (Millipore, Billerica, MA, United States)] was added to each sample and incubated at 60°C for 3 h followed by the addition of the DIG-labeled riboprobe to the hybridization buffer at a concentration of 0.5 µg/ml (*Hes5*) or 1 µg/ml (*Hes1*, *Hey1*, and *HeyL*) and incubated at 60°C overnight.

The following day, samples were rinsed twice (~1 min each) with hybridization buffer and washed twice (30 min each) with hybridization buffer at 60°C. Samples were then washed with a 1:1 mixture of hybridization buffer and 1X MABT [maleic acid buffer containing Tween-20, 5X stock concentration of 500 mM maleic acid (Sigma, St. Louis, MO, United States), 744 mM NaCl (Sigma, St. Louis, MO, United States), 44 mM Tween-20 (Sigma, St. Louis, MO, United States) and ddH<sub>2</sub>O and pH adjusted with Tris Base (Roche, Indianapolis, IN,

United States) to ~7.5] for 20 min at 60°C. Samples were then rinsed three times (~1 min each) with 1X MABT and washed twice (30 min each) with 1X MABT at RT. Samples were blocked with 1X MABT containing 20% sheep serum (Sigma, St. Louis, MO, United States) and 2% Boehringer blocking reagent (Sigma, St. Louis, MO, United States) for 3 h at RT. Next the samples were incubated overnight at 4°C with anti-digoxigenin antibodies (cat#11093274910, Sigma, St. Louis, MO, United States) in 1X MABT at a final concentration of 1:2000.

The following day, samples were rinsed three times (~1 min each) with 1X MABT and then washed three times (1 h each) with 1X MABT at RT followed by an overnight wash at 4°C. Samples were then washed twice with alkaline phosphatase buffer [100 mM Tris (Sigma, St. Louis, MO, United States) pH 9.5, 50 mM MgCl<sub>2</sub> (Fisher, Hampton, NH, United States), 100 mM NaCl (Sigma, St. Louis, MO, United States), and 0.1% Tween-20 (Sigma, St. Louis, MO, United States)] for 1 h each and then processed with 4.5 µl/ml nitro-blue tetrazolium (Sigma, St. Louis, MO, United States) and 3.5 µl/ml 5-bromo-4-chloro-3'-indolylphosphate (Sigma, St. Louis, MO, United States) in alkaline phosphatase buffer in the dark between 2 and 20 h. Samples were then washed twice in alkaline phosphatase buffer (5 min each), washed three times in PTw (1 h each), and fixed in 4% PFA for 30 min. Samples were then cleared in 60% glycerol overnight and mounted on slides in 100% glycerol (Fisher, Hampton, NH, United States) and imaged with a Neurolucida system (MicroBrightField, Inc., United States) integrated with a Zeiss AxioImager M2 microscope (Carl Zeiss Microscopy, Germany) or an Olympus IX70 microscope (Olympus Corporation, Waltham, MA, United States). All *in situ* experiments were performed with matched experimental and control samples from the same litter run in parallel.

## Quantification and Statistical Analysis

For quantification of Sox2-positive and Hes5-LacZ-positive cells, images were taken from two representative regions in the apical turn of the cochlea and cells in either a 150 µm or 200 µm region per image were quantified and averaged for each sample. Positive expression of LacZ was determined by the overlap of LacZ labeling and nuclear staining with Hoechst once antibody background was reduced using the imaging software. The same trained examiner counted images for both control and experimental samples to make sure that the same threshold for positive staining was maintained. To quantify the amount of HC regeneration (LacZ-positive HCs) in the model where *NIICD* was overexpressed, images of the entire cochlea were taken using tile scan with z-series. Each cochlea was measured and divided into six equal sections as previously described (McGovern et al., 2017). For quantification of HCs in cochlea overexpressing *NIICD*, 200 µm regions were selected from two representative images per cochlear turn and total HCs were quantified and averaged. All data are presented as mean ± standard error of the mean (SEM). Data was analyzed using a one- or two-way ANOVA or a Student's *t*-test with Graphpad Prism 6.02 (Graphpad Software Inc., La Jolla, CA, United States).

## RESULTS

### Hes5 Expression Is Reduced During Spontaneous HC Regeneration

Because the majority of spontaneously regenerated HCs are formed in the apical turn of the cochlea (Cox et al., 2014), we investigated gene changes in this region at the cellular level. We first measured changes in the expression of *Hes5*, a direct inhibitor of HC fate that is a well-known target of active Notch signaling (Mulvaney and Dabdoub, 2012; Abdolazimi et al., 2016). *Hes5* is expressed in many SC subtypes during the first postnatal week including Deiters' cells, outer pillar cells, inner phalangeal cells, border cells, and some cells of the greater epithelial ridge (GER) (Figures 1A–C, G–I, M–O, 2B–B", D–D", F–F"; Lanford et al., 2000; Zine et al., 2001; Hartman et al., 2009; Cox et al., 2014; Maass et al., 2015). To quantify the number of cells that express *Hes5*, we used the *Hes5<sup>LacZ</sup>* reporter line which has *LacZ* knocked into the endogenous *Hes5* locus and therefore is a faithful representation of endogenous *Hes5* expression (Imayoshi et al., 2010). Others have shown that  $\beta$ -galactosidase ( $\beta$ -gal), transcribed by the *LacZ* gene, continues to be expressed ~1 day after mRNA of the endogenous gene becomes undetectable (Morrison et al., 1999). Therefore, we used *Hes5<sup>LacZ</sup>* mice to approximate the window when cells lose *Hes5* expression. *Atoh1-CreER<sup>TM</sup>::Rosa26<sup>loxP-stop-loxP-DTA</sup>* mice (hereinafter referred to as *Atoh1-DTA* mice) were bred with *Hes5<sup>LacZ</sup>* mice and injected with tamoxifen (3 mg/40 g, IP) on both P0 and P1 to induce HC ablation and initiate spontaneous HC regeneration. Samples were analyzed at P2, P4, and P6, which was previously established as the window of spontaneous HC regeneration seen in the neonatal mouse cochlea (Cox et al., 2014). We quantified the number of cells that expressed Hes5-LacZ as well as the number of SC and immature HC nuclei using anti-Sox2 antibodies. Significantly fewer Hes5-LacZ-positive cells were detected in the *Atoh1-DTA::Hes5<sup>LacZ</sup>* cochlea at P2 ( $216.1 \pm 12.3$  vs.  $107.3 \pm 19.1$ ;  $p < 0.001$ ;  $N = 3$ ) and P4 ( $185.6 \pm 13.3$  vs.  $115.5 \pm 14.1$ ;  $p < 0.01$  as determined by a two-way ANOVA with a Tukey's *post hoc* test;  $N = 3$ ) compared to control samples that lacked either the *Atoh1-CreER<sup>TM</sup>* or *Rosa26<sup>DTA</sup>* allele (Figures 1A–L, S). However, there was no difference observed in Hes5-LacZ expression at P6 ( $140.1 \pm 11.6$  vs.  $120.3 \pm 7.3$ ;  $N = 4$ ; Figures 1M–S). There were also no differences in the number of Sox2-positive cells after HC damage at any age (Figures 1B, E, H, K, N, Q, S) suggesting that as a whole, the Sox2-positive cell population is not reduced.

Differences among SC subtypes have previously been documented (Lanford et al., 2000; Doetzlhofer et al., 2009; Yu et al., 2010; Taylor et al., 2012; Lagarde et al., 2013; Maass et al., 2016), which may indicate that not all SC subtypes maintain plasticity postnatally. Furthermore, loss of *Hes5* at embryonic ages has been implicated in the formation of supernumerary outer HCs, but not inner HCs (Zine et al., 2001). We therefore sought to investigate changes in *Hes5* expression within discrete SC subpopulations. To divide the SC subtypes into two groups, we used pillar and Deiters' cells as a landmark. *S100a1* is a calcium binding protein that is expressed in the cytoplasm

and nucleus of pillar and Deiters' cells within the neonatal cochlea (White et al., 2006; Buckiová and Syka, 2009). Therefore, combining anti-S100a1 antibodies with anti-Sox2 and anti- $\beta$ -gal antibodies allowed us to tease apart two groups of SC subpopulations: (1) pillar cells and Deiters' cells which are labeled by S100a1, and (2) inner phalangeal and border cells which are *Hes5<sup>LacZ</sup>*-positive/Sox2-positive cells located medial to S100a1 labeling (Figures 2A–B", D–D", F–F"). Because *Hes5<sup>LacZ</sup>* is also expressed in the cells of the GER, the medial border of inner phalangeal and border cells was established for quantification by measuring the width of tdTomato expression medial to inner pillar cells in *Plp-CreER<sup>2</sup>::Rosa26<sup>loxP-stop-loxP-tdTomato</sup>* mice injected with tamoxifen (3 mg/40 g, IP) at P0, which has previously been shown to target inner phalangeal and border cells, but not the GER (Supplementary Figures S1A–C; Doerflinger et al., 2003; Gómez-Casati et al., 2010; Cox et al., 2012; Mellado Lagarde et al., 2014; McGovern et al., 2017).

After HC damage and regeneration was induced in *Atoh1-DTA::Hes5<sup>LacZ</sup>* mice by tamoxifen injection at P0 and P1, there was a significant decrease in the number of Hes5-LacZ-positive pillar and Deiters' cells detected at P2 ( $113.1 \pm 3.8$  vs.  $63.6 \pm 5.1$ ,  $p < 0.01$ ,  $N = 4$ ), P4 ( $104.4 \pm 1.5$  vs.  $42.1 \pm 5.7$ ,  $p < 0.001$ ,  $N = 4$ –5), and P6 ( $98.6 \pm 2.4$  vs.  $25.3 \pm 4.4$ ,  $p < 0.001$ ,  $N = 4$ ) compared to control cochleae which lacked either the *Atoh1-CreER<sup>TM</sup>* or *Rosa26<sup>DTA</sup>* allele (Figures 2B–H). Of note, the number of Sox2-positive cells within this population did not decrease until P4 ( $140.2 \pm 3.1$  vs.  $98.1 \pm 11.5$ ,  $p < 0.001$ ;  $N = 4$ –5) and was also decreased at P6 ( $130.3 \pm 2.7$  vs.  $75.0 \pm 6.0$ ,  $p < 0.0001$ ;  $N = 4$ ; Figures 2B–H). Additionally, there was an increase in the number of Sox2-positive/Hes5-LacZ-negative pillar and Deiters' cells at P2 ( $39.0 \pm 1.6$  vs.  $102.2 \pm 21.9$ ,  $p < 0.001$ ;  $N = 4$ ; Figures 2B–H), suggesting that *Hes5* is downregulated in these SCs, but the cells are still alive. In contrast, neither the number of *Hes5*-LacZ-positive cells, nor the number of Sox2-positive cells within the inner phalangeal and border cells region were significantly altered from control samples (Figures 2B–G", I), suggesting that weakened Notch signaling is limited to pillar and Deiters' cells during the HC regeneration process.

### Reduced Expression of Notch Effectors *Hes1*, *Hes5*, *Hey1*, and *HeyL* Was Observed During Spontaneous HC Regeneration

Further changes in the expression of Notch target genes were investigated using *in situ* hybridization. *Atoh1-DTA* mice as well as littermate controls, which lacked the *Atoh1-CreER<sup>TM</sup>* or *Rosa26<sup>DTA</sup>* allele, were injected with tamoxifen (3 mg/40 g, IP) on P0 and P1 to induce HC damage as well as spontaneous HC regeneration. Samples were collected and analyzed at P4 using probes that target *Hes1*, *Hes5*, *Hey1*, and *HeyL*. Paired samples from the same litter (control and *Atoh1-DTA*) were processed in parallel where each solution, as well as each incubation time, was identical. Comparisons were only made between the one experimental and the one control sample that were processed together. Staining intensity in the sensory

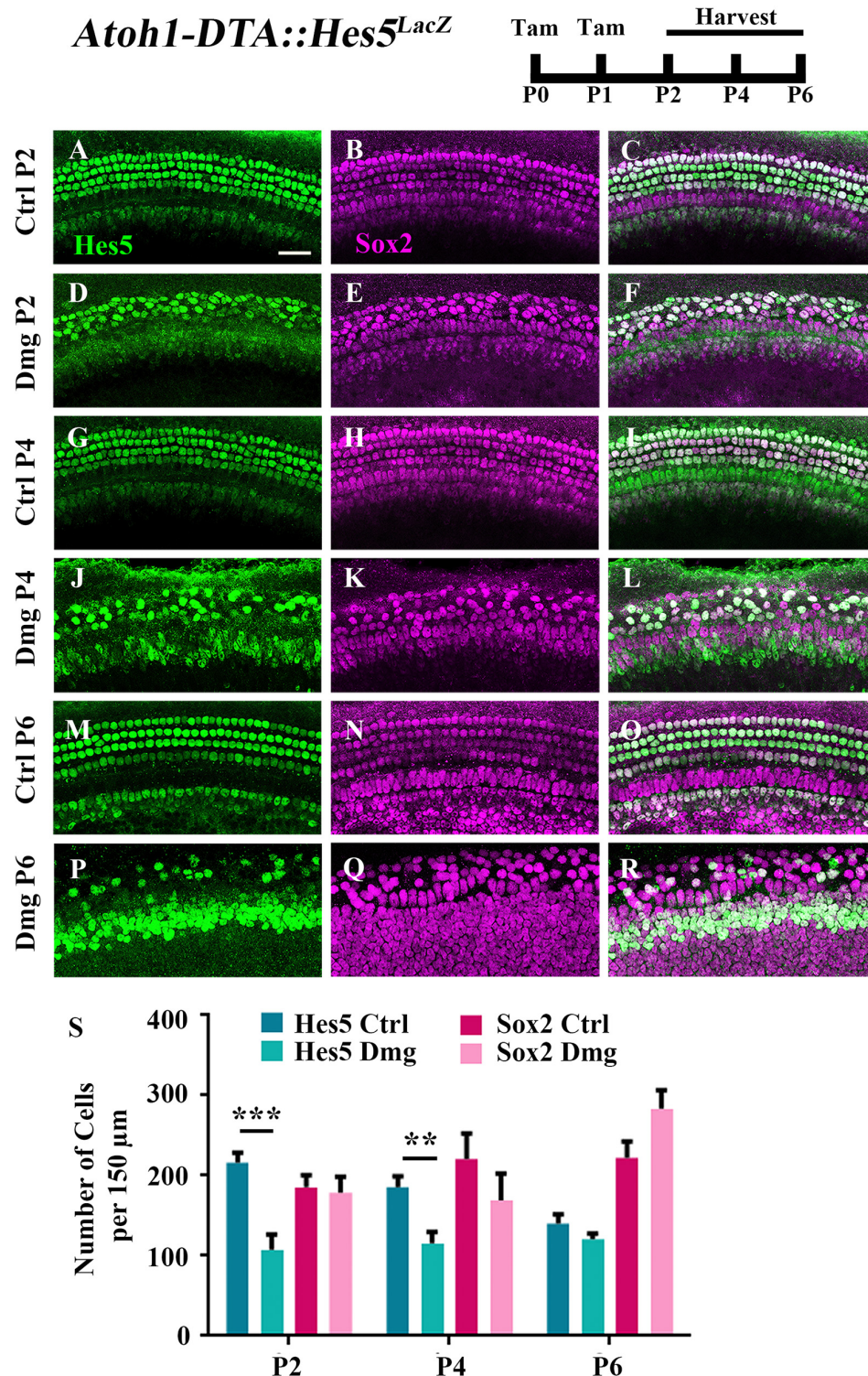


**FIGURE 1** | Hes5-LacZ-positive cells, but not Sox2-positive cells, are reduced during the spontaneous HC regeneration process. *Atoh1-DTA::Hes5<sup>LacZ</sup>* mice were injected with tamoxifen (Tam) at P0–P1 to induce HC death and spontaneous HC regeneration and cochleae were collected at P2, P4, and P6. **(A–R)** Representative confocal slice images with anti-β-gal antibodies to detect Hes5-LacZ expression (green) and anti-Sox2 antibodies to label all SC nuclei (magenta). **(A–C, G–I, M–O)** Images are taken from P2, P4, or P6 control samples, respectively. Scale bar = 25 μm. **(S)** Fewer Hes5-LacZ-positive cells were detected in HC damaged samples at P2 and P4, but not P6. There was no significant difference in the number of Sox2-positive cells in the *Atoh1-DTA::Hes5<sup>LacZ</sup>* cochlea after HC damage compared to undamaged controls. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , as determined by a two-way ANOVA with a Sidak's *post hoc* test.  $N = 3–5$ .

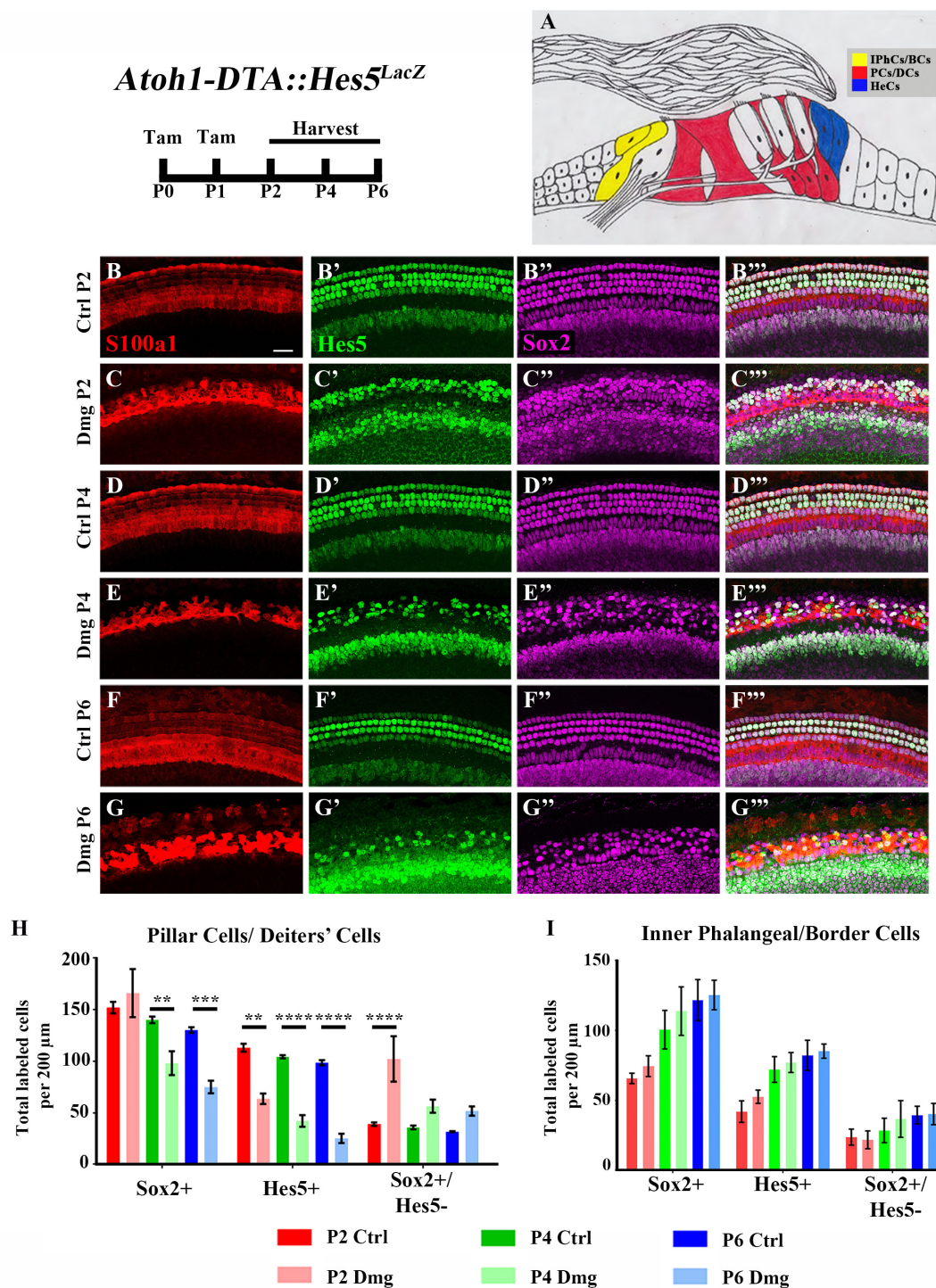
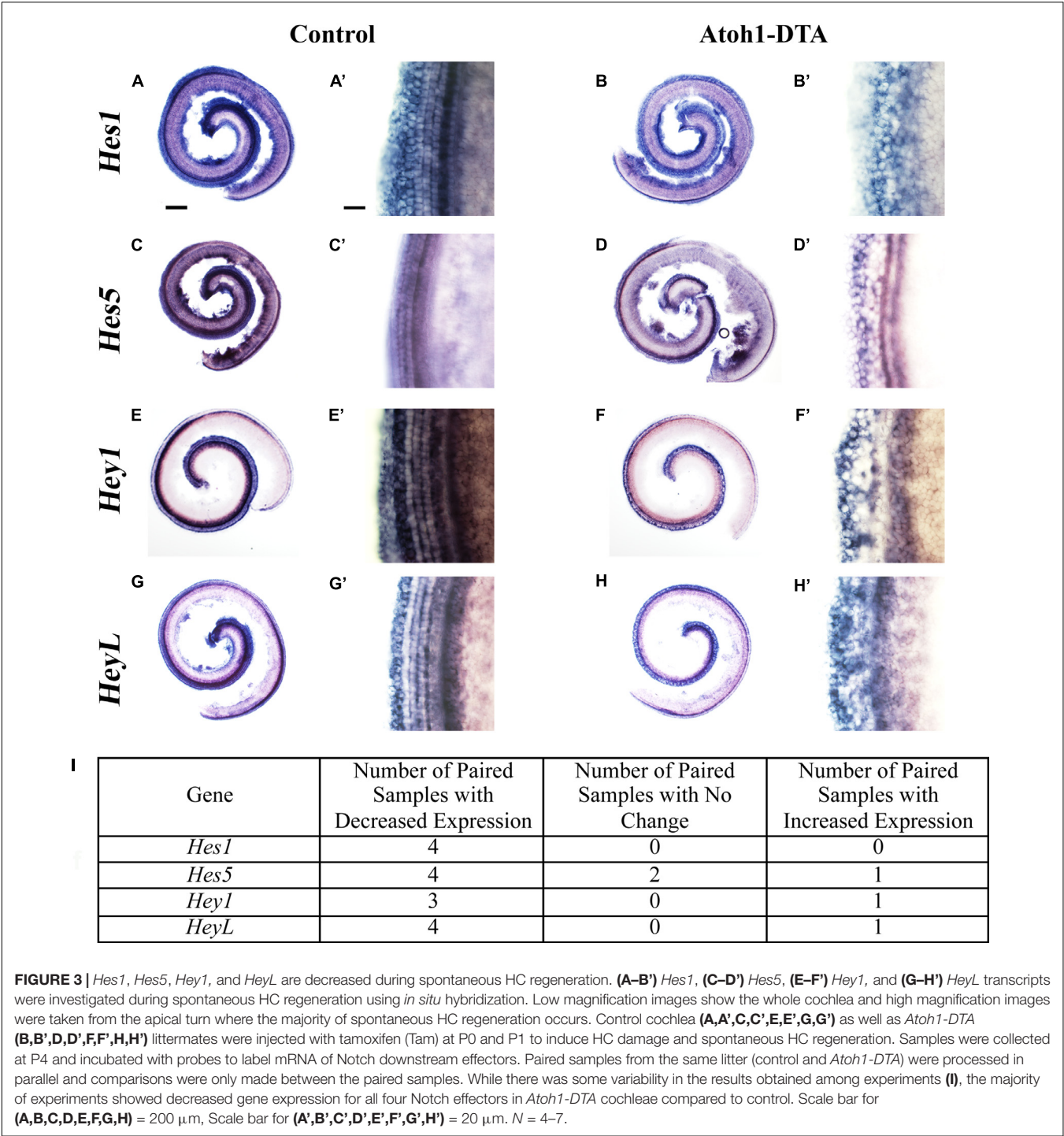


**FIGURE 2 |** Hes5-LacZ-positive cells are reduced in lateral SCs, but not medial SCs. **(A)** Pillar and Deiters' cells (red, PCs/DCs) are labeled with S100a1 and inner phalangeal/border cells (yellow, IPHs/BCs) lie medial to the S100a1 expressing region. Hensen cells (blue, HeCs) express Sox2 and border the lateral edge of the S100a1 region, but do not have Hes5 expression. **(B–G'')** *Atoh1-DTA::Hes5<sup>LacZ</sup>* mice were injected with tamoxifen (Tam) at P0–P1 to induce HC death and spontaneous HC regeneration and cochleae were collected at P2, P4, or P6. Representative confocal slice images with anti-βgal antibodies to detect Hes5-LacZ expression (green), anti-Sox2 antibodies to label all SC nuclei (magenta), and anti-S100a1 antibodies to label the cytoplasm of PCs/DCs (red). Scale bar = 25 μm **(H)** In the PCs/DCs region, fewer Sox2-positive cells were detected at P4 and P6 after HC damage, while a decreased number of Hes5-LacZ-positive cells were detected at all three ages. Only at P2 were there significantly more Sox2-positive/Hes5-LacZ-negative cells detected. **(I)** There was no significant change in the number of Sox2-positive, Hes5-LacZ-positive, or Sox2-positive/Hes5-LacZ-negative cells in the IPHs/BCs region. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  as determined by a two-way ANOVA with a Tukey's or Sidak's *post hoc* test.  $N = 4–5$ .



postnatal week (Cox et al., 2014); the variability of individual gene changes we observed may depend on the stage of SC conversion into HCs at the time of analysis. Nevertheless, because the majority of the samples showed decreased expression of Notch effectors, we conclude that loss of HCs in the neonatal mouse cochlea caused a modest decrease in Notch signaling in neighboring SCs.

## The Notch Ligand and Target Gene *Jag1* Is Reduced During Spontaneous HC Regeneration

The Notch ligand *Jag1* is expressed by neonatal SCs and is also a downstream target of Notch activation (Daudet and Lewis, 2005; Murata et al., 2009). Therefore, it can be used as an indicator of active Notch signaling. Early in development *Jag1* mediates lateral induction, establishing the progenitor pool from which all HCs and SCs differentiate (Brooker et al., 2006; Kiernan et al., 2006; Murata et al., 2009). However, its function during and after HC differentiation is not well understood, yet it is expressed throughout the organ of Corti from the cells of the GER to the 3rd row of Deiters' cells (Figures 4A–C, G–I, M–O; Oesterle et al., 2008). We investigated changes in *Jag1* in the context of HC loss and spontaneous HC regeneration as an additional way to assess changes in the Notch signaling pathway. Specifically, *Atoh1-DTA* mice were injected with tamoxifen (3 mg/40 g, IP) at P0 and P1 to induce HC ablation thus initiating spontaneous HC regeneration. Samples were collected at P2, P4, and P6 and immunolabeled with antibodies against S100a1 and *Jag1*. Because *Jag1* is a membrane bound protein, we could not determine which neighboring cells expressed *Jag1* and therefore quantitative analysis was not performed. However, qualitative analysis of *Atoh1-DTA* cochleae showed that *Jag1* labeling was reduced in the lateral region of the organ of Corti, where outer pillar cells and Deiters' cells are located, yet maintained in medial SCs, including inner pillar cells, inner phalangeal/border cells, and the cells of the GER (Figures 4D–F, J–L, P–R). In contrast, *Jag1* labeling was consistent in all SCs throughout the width of organ of Corti in control mice (Figures 4A–C, G–I, M–O). These data confirm the results obtained using the *Hes5<sup>LacZ</sup>* reporter mice and demonstrate that Notch signaling is reduced in some SC subtypes, but not others, during spontaneous HC regeneration.

## Overexpression of *NIICD* Caused Increased Notch Activity in the Neonatal Cochlea

While immunostaining and *in situ* hybridization results suggest a modest decrease in Notch signaling during spontaneous HC regeneration, it is currently unknown whether Notch signaling is involved in the mechanism of spontaneous HC regeneration or if it is simply correlated. To investigate this question more directly, we induced overexpression of *NIICD* in SCs and other non-sensory epithelial cells (all other cells within the organ of Corti except for HCs and tympanic border cells, hereinafter referred to as non-HCs) in the context of HC damage and quantified the number of regenerated HCs using fate-mapping. This was achieved by combining CreER/loxP and Tet-on genetically

modified mouse systems to modify gene expression in adjacent cell populations.

To ensure feasibility of using these two inducible systems in adjacent groups of cells without cross-reaction, dual reporter expression was investigated in *Atoh1-CreER<sup>TM</sup>::Rosa26<sup>loxP-stop-loxP</sup>-tdTomato::Sox10<sup>rtTA</sup>::TetO-LacZ* mice. tdTomato expression was induced with tamoxifen injection (3 mg/40 g, IP) at P0 and P1, while LacZ expression was induced by administering doxycycline to the nursing mother in the food (2000 mg/kg) from P0–P7, as well as a single injection (100 mg/kg) to each pup at P1. Samples were analyzed at P7. As expected, tdTomato expression was only detected in HCs, while LacZ expression was observed in SCs and non-HCs (Figures 5A–G). We also observed variability in the intensity of LacZ expression cell to cell, which was similar to the previously described pattern using the same doxycycline induction paradigm in *Sox10<sup>rtTA</sup>::TetO-LacZ* mice (Walters and Zuo, 2015).

To investigate whether downregulation of Notch signaling plays a role in spontaneous HC regeneration, we used *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice to overexpress *NIICD* as well as LacZ to fate-map cells. Therefore, in this mouse model, *Sox10<sup>rtTA</sup>* must activate two Tet-on alleles simultaneously. To verify that active Notch signaling was achieved in our model and that the presence of the *TetO-LacZ* allele did not affect the level of Notch activation, we measured *NIICD* expression levels using qPCR. Specifically, *Sox10<sup>rtTA</sup>*-negative, *Sox10<sup>rtTA</sup>::TetO-NIICD*, and *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice were administered doxycycline, both in the diet to the nursing mother (2000 mg/kg), as well as a single injection to the pups (100 mg/kg, IP) at P1. RNA was extracted from samples collected at P7 and *NIICD* expression was analyzed with qPCR. Of note, the primers used for *NIICD* detection recognize both the endogenous and transgenic transcripts and data is expressed as fold change relative to the *Sox10<sup>rtTA</sup>*-negative control. In both *Sox10<sup>rtTA</sup>::TetO-NIICD* and *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* groups, *NIICD* was increased 5 to 6-fold ( $6.4 \pm 0.9$ -fold for *Sox10<sup>rtTA</sup>::TetO-NIICD*,  $p < 0.001$ ,  $N = 10$  and  $5.7 \pm 0.8$ -fold for *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD*,  $p < 0.001$ ,  $N = 10$  compared to *Sox10<sup>rtTA</sup>*-negative samples; as determined by a one-way ANOVA with a Tukey's *post hoc* test, Figure 6A). There was no difference in the expression level of *NIICD* between *Sox10<sup>rtTA</sup>::TetO-NIICD* and *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* samples, indicating that the addition of the *TetO-LacZ* allele did not interfere with increased Notch activity induced by the *TetO-NIICD* allele.

To further confirm increased Notch activity in our model, we used qPCR to investigate the expression level of Notch target genes, *Hes1*, *Hes5*, *Hey1*, and *HeyL*. All Notch effector genes were increased 2 to 13-fold in *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* cochleae compared to *Sox10<sup>rtTA</sup>*-negative controls (*Hes1* increased  $2.6 \pm 0.1$ -fold,  $p < 0.0001$ ,  $N = 10$ ; *Hes5* increased  $12.7 \pm 1.2$ -fold,  $p < 0.0001$ ,  $N = 10$ ; *Hey1* increased  $2.4 \pm 0.2$ -fold,  $p < 0.0001$ ,  $N = 10$ ; and *HeyL* increased  $7.5 \pm 0.7$ -fold,  $p < 0.0001$ ,  $N = 10$  compared to *Sox10<sup>rtTA</sup>*-negative controls as determined by a Student's *t*-test for each gene; Figure 6B). Therefore, we conclude that Notch signaling was increased by *NIICD* overexpression in our model.

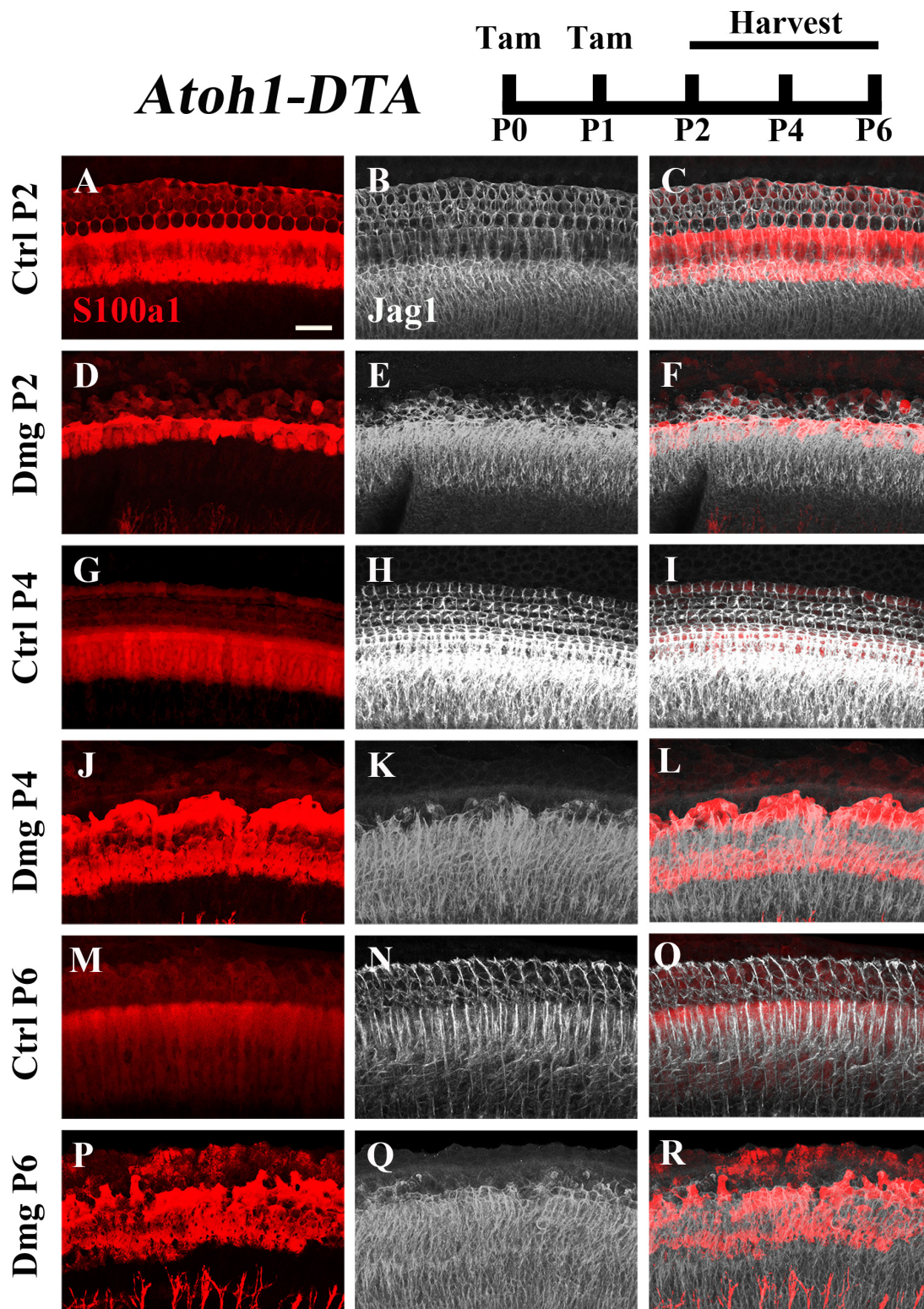
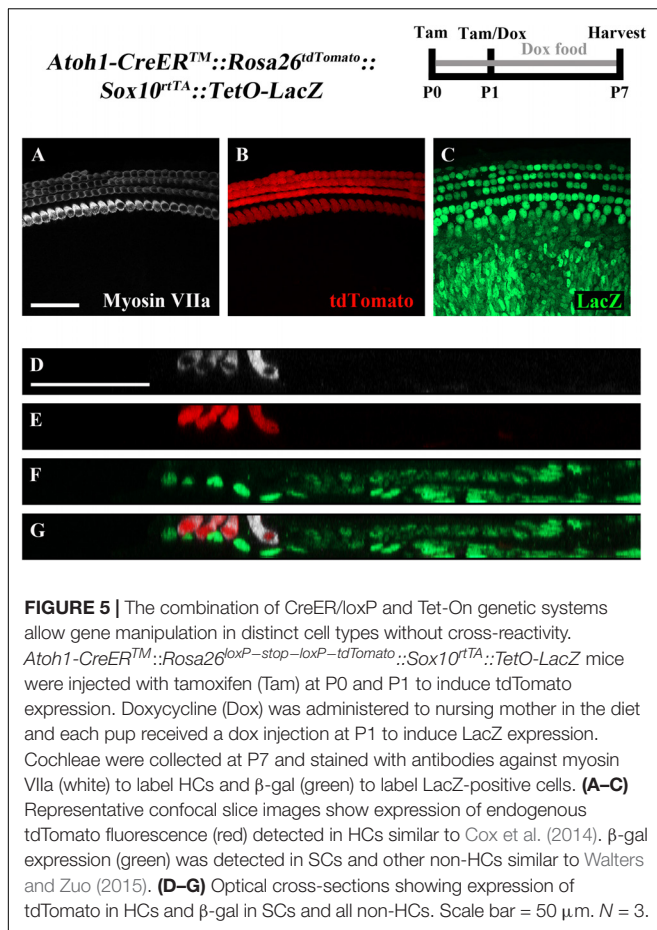
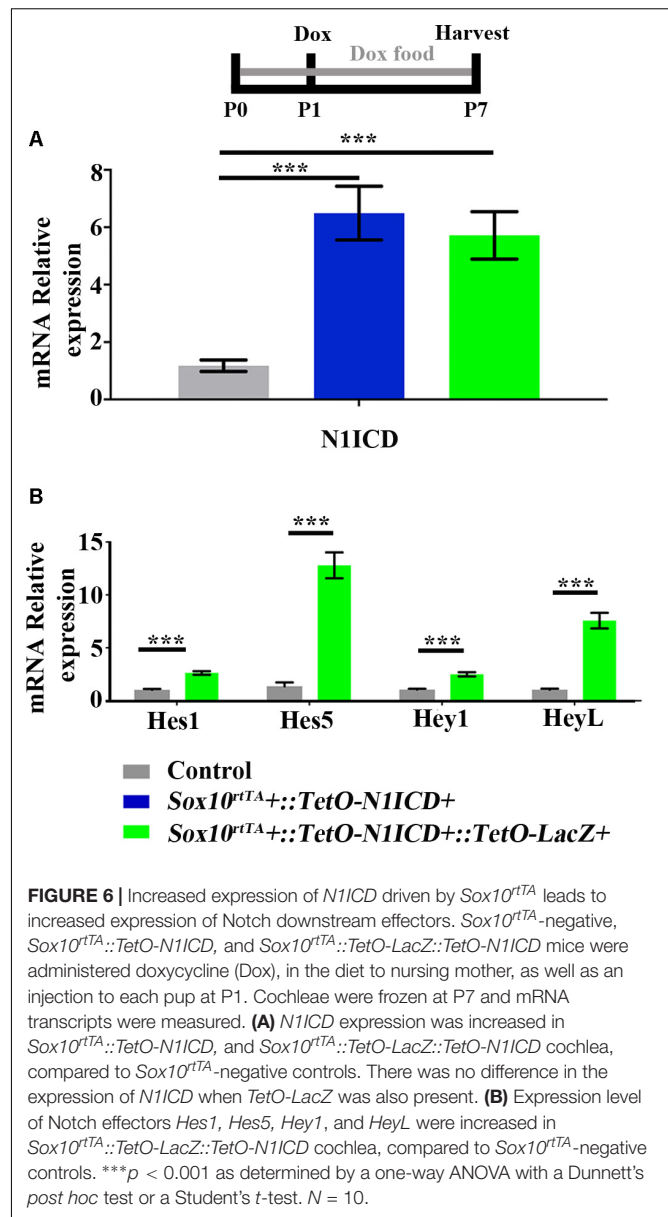


**FIGURE 4 |** Jag1 labeling is reduced in lateral, but not medial, SCs during spontaneous HC regeneration. *Atoh1-DTA* mice were injected with tamoxifen (Tam) at P0–P1 to induce HC death and spontaneous HC regeneration and cochleae were collected at P2, P4, or P6. **(A,R)** Representative maximum projection images with anti-Jag1 antibodies to detect Jag1 expression (white) and anti-S100a1 (red) antibodies to mark the pillar and Deiters' cells region. After HC damage, both S100a1 and Jag1 labeling was reduced in the outer pillar and Deiters' cell region, while expression is maintained in medial SCs **(D–F,J–L,P–R)** and in all SCs in control samples **(A–C,G–I,M–O)**. Scale bar = 25  $\mu$ m.  $N = 3$ .



## Spontaneous HC Regeneration Is Reduced When the *N1ICD* Is Overexpressed in SCs and Other Non-HCs

To investigate whether spontaneous HC regeneration could be prevented by increased Notch signaling, we overexpressed *N1ICD* in SCs and other non-HCs in the context of HC damage using *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* mice. The effect of increased Notch signaling on spontaneous HC regeneration was investigated by quantifying the number of fate-mapped SCs. Specifically, we analyzed the number of LacZ-positive HCs in each of four groups. First, to verify the expression pattern of *Sox10<sup>rtTA</sup>* in a normal neonatal cochlea, the number of LacZ-positive HCs was quantified in *Sox10<sup>rtTA</sup>::TetO-LacZ* mice. Second, to determine the number of spontaneously regenerated HCs that occur naturally in the neonatal mouse cochlea after HC loss at birth, we quantified LacZ-positive HCs in *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* mice. Third, to determine the effect of *N1ICD* overexpression in SCs and other non-HCs in the neonatal mouse cochlea, LacZ-positive HCs were quantified in *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* mice. Finally, to investigate whether overexpression of *N1ICD* in SCs and other non-HCs will prevent spontaneous HC regeneration from occurring, we quantified LacZ-positive HCs



in *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* mice. Mice from each group were injected with tamoxifen (3 mg/kg, IP) at P0 and P1 to induce HC damage. *N1ICD* and *LacZ* expression were induced through administration of doxycycline given both in the food (2000 mg/kg) to the nursing mother, as well as one doxycycline injection (100 mg/kg, IP) given to the pups at P1, ~6 h after tamoxifen injection. Samples were collected at P7 and processed for immunostaining with antibodies against myosin VIIa, Sox2, and  $\beta$ -gal.

As expected from previous work (Walters and Zuo, 2015), *LacZ* expression in *Sox10<sup>rtTA</sup>::TetO-LacZ* mice was observed in SCs and other non-HCs within the P7 cochlea. We also observed  $2.0 \pm 2.0$  LacZ-positive HCs in the very apical tip (Figures 7Q,R, *N* = 4). There was no significant change in LacZ-positive HCs when *N1ICD* was overexpressed without HC damage in

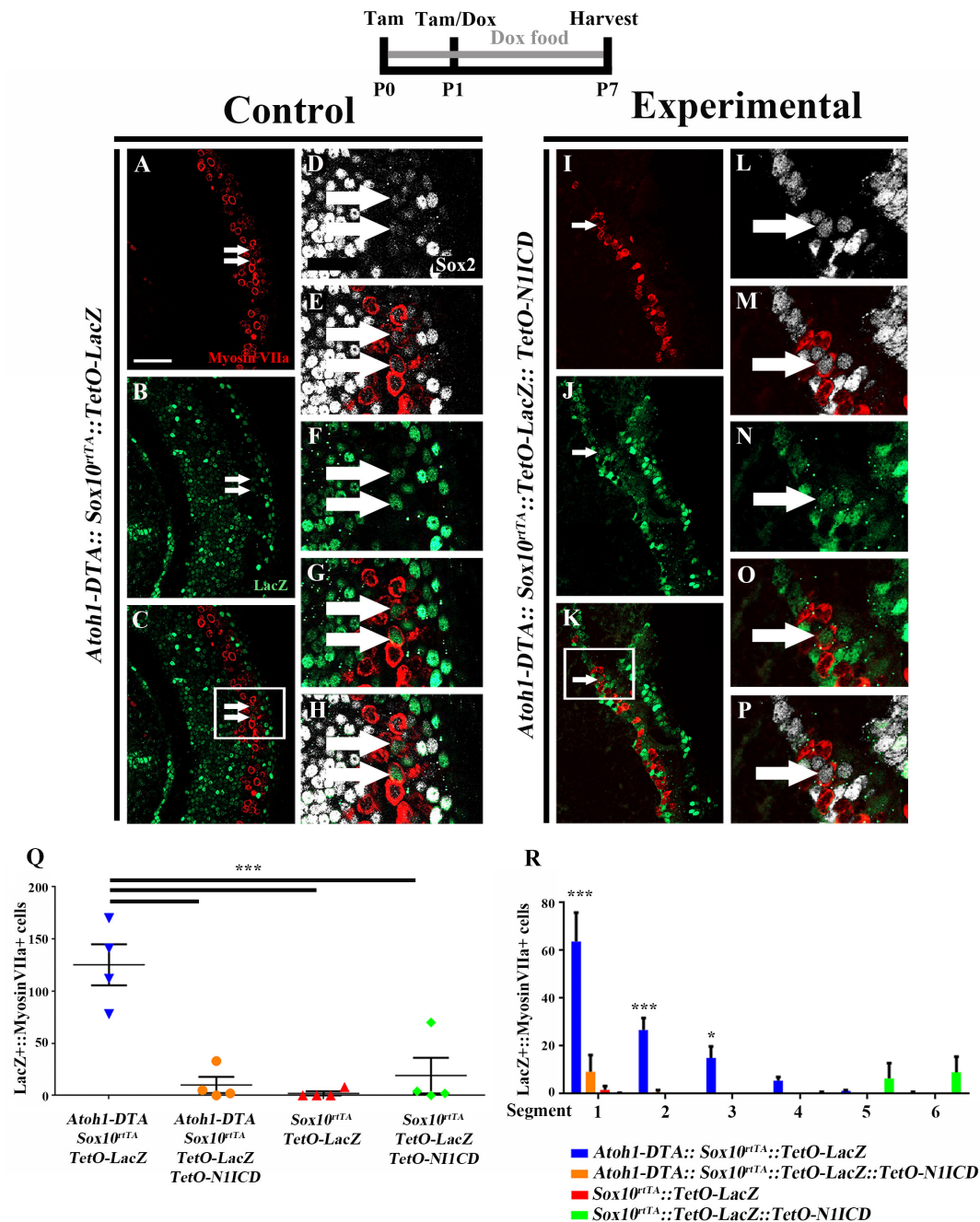


**FIGURE 7 |** Fewer spontaneously regenerated HCs were observed in the presence of *N1ICD* overexpression. Representative confocal slice images taken from the apical turn of the cochlea of (A–H) *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* and (I–P) *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* mice that were injected with tamoxifen (Tam) at P0/P1 to induce CreER-mediated HC loss and induce spontaneous HC regeneration, as well as administered doxycycline (Dox) in the diet to nursing mother with a dox injection given to pups at P1 to induce expression of *N1ICD* and the LacZ reporter in SCs and all non-HCs. Cochlea were collected at P7 and stained with antibodies against myosin VIIa (red), Sox2 (white), and  $\beta$ -gal (green). Fewer fate-mapped regenerated HCs (LacZ-positive/myosin VIIa-positive cells, white arrows) were observed when *N1ICD* was overexpressed in combination with HC damage compared to samples with HC damage and normal Notch signaling. (D–H, L–P) High magnification images from boxes in (C, K) showing LacZ-positive/Sox2-positive/myosin VIIa-positive regenerated HCs. Scale bar = 25  $\mu$ m. (Q) Quantification of LacZ-positive HCs from the images presented in (A–P) and the two additional controls. Comparison of the number of LacZ-positive HCs between the group with spontaneous HC regeneration and normal Notch signaling (blue, *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* mice) and the experimental group with *N1ICD* overexpression as well as HC damage (orange, *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD*), showed a ~92% decrease in the number of LacZ-positive, regenerated HCs. (R) LacZ-positive HCs after HC damage with physiological Notch signaling (blue, *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ*) decreased in an apical to basal gradient while LacZ-positive HCs were only detected in the base when *N1ICD* was overexpressed without HC damage (green, *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD*) mice. Segment 1 is the most apical region and segment 6 is the most basal.  $N = 3-4$ . \* $p < 0.05$ , \*\*\* $p < 0.001$  as determined by a two-way ANOVA with a Tukey's *post hoc* test.  $N = 4$ .

*Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice ( $19.0 \pm 17.2$ , compared to *Sox10<sup>rtTA</sup>::TetO-LacZ* controls,  $N = 4$ ,  $p = 0.8$  as determined by a one-way ANOVA with a Tukey's *post hoc* test; **Figure 7Q**). However, we observed disorganization of HCs (**Supplementary Figures S2A,G**) as well as an expansion of Sox2 expression both medial and lateral to the organ of Corti, (**Supplementary Figures S2A–D,F–I**). Quantification of HCs in *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice showed that there was no difference in the number of HCs compared to *Sox10<sup>rtTA</sup>::TetO-LacZ* mice ( $91.0 \pm 2.5$  compared to  $88.7 \pm 5.9$  per 200  $\mu\text{m}$ ,  $p = 0.5$  as determined by a Student's *t*-test, **Supplementary Figure S2E**) and therefore this phenotype likely did not affect spontaneous HC regeneration observed in our study.

When LacZ was used for fate-mapping during spontaneous HC regeneration, significantly more LacZ-positive HCs were detected in *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* mice than in *Sox10<sup>rtTA</sup>::TetO-LacZ* controls ( $125.3 \pm 19.7$  compared to  $2.0 \pm 2.0$ ,  $p = 0.0002$ ,  $N = 4$ , **Figure 7Q**). When *NIICD* was overexpressed in the context of HC damage (*Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice), there was a 92% reduction in LacZ-positive HCs compared to similar samples without *NIICD* overexpression (*Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* mice) ( $10.0 \pm 7.7$  compared to  $125.3 \pm 19.7$ ,  $p = 0.0003$ ,  $N = 4$ , **Figures 7A–Q**). Moreover, the number of LacZ-positive HCs when *NIICD* was overexpressed and HCs were killed was not significantly different from controls lacking both HC damage and *NIICD* overexpression (*Sox10<sup>rtTA</sup>::TetO-LacZ* samples) ( $10.0 \pm 7.7$  compared to  $2.0 \pm 2.0$ ,  $p = 0.9$ ) or *NIICD* overexpression alone (*Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* samples) ( $10.0 \pm 7.7$  compared to  $19.0 \pm 17.0$ ,  $p = 0.9$  as determined by a one-way ANOVA with a Tukey's *post hoc* test; **Figure 7Q**). Therefore, we conclude that overexpression of *NIICD* prevented the majority of spontaneous HC regeneration from occurring. Of note, we observed an expansion of Sox2 expressing cells in *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice, similar to what we observed in *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* controls (**Supplementary Figures S2F–M**).

We wanted to better understand the location of fate-mapped, spontaneously regenerated HCs in our samples because the majority of spontaneous HC regeneration is known to occur in the apical third of the neonatal cochlea (Cox et al., 2014) and because Notch signaling decreases in a basal to apical gradient during the first postnatal week (Maass et al., 2015). Therefore, the whole cochlea was imaged, measured, and divided into six equal segments from apex to base as previously described (McGovern et al., 2017) and the total number of LacZ-positive/myosin VIIa-positive HCs within each segment was quantified. The majority of fate-mapped, spontaneously regenerated HCs (from *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* mice) were found in the two most apical segments ( $63.8 \pm 12.0$  in segment 1 and  $26.8 \pm 4.9$  in segment 2) and this declined in an apical to basal gradient ( $15.4 \pm 4.8$  in segment 3,  $5.6 \pm 1.4$  in segment 4,  $1.2 \pm 0.3$  in segment 5, and  $0.4 \pm 0.4$  in segment 6,  $r^2 = 0.66$ ,  $p < 0.0001$  **Figure 7R**), similar to the previous report (Cox et al., 2014). In one *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* sample that did not have HC damage, we observed 67 LacZ-positive HCs in the two basal segments, but no LacZ-positive HCs were detected

in the middle or apical segments of the cochlea where the majority of HC regeneration was observed (**Figure 7R**). The other three *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* samples had no LacZ-positive HCs. Therefore, the LacZ-positive HCs observed in *NIICD* overexpressing controls did not interfere with the quantification of fate-mapped, spontaneously regenerated HCs predominantly observed in the apical turn of the cochlea. When *NIICD* was overexpressed along with HC damage, the fate-mapped regenerated HCs in the *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice were all located within the first two apical segments (**Figure 7R**), similar to the control with spontaneous HC regeneration and no manipulation of Notch (*Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ* mice). This illustrates that the regenerative capacity of SCs is correlated to the basal to apical changes in the Notch signaling pathway. Our data show weakened Notch signaling during the spontaneous HC regeneration process, as well as the suppression of HC regeneration by increased Notch activity. Taken together, this suggests that Notch signaling plays a role in the ability of SCs to spontaneously convert into, thus regenerate, HCs.

## DISCUSSION

This study investigated the role of Notch signaling during spontaneous HC regeneration within the neonatal mouse cochlea *in vivo*. After HC damage and during the window of spontaneous HC regeneration, we observed a modest decrease in Notch signaling, as evidenced by decreased expression of the Notch target genes *Hes1*, *Hes5*, *Hey1*, *HeyL*, and *Jag1* using immunostaining or *in situ* hybridization. These changes suggest that Notch signaling is weakened in SCs after HC loss occurs and may allow their conversion into HCs. To investigate this further, we increased Notch signaling in SCs and other non-HCs by overexpressing *NIICD* during the window of spontaneous HC regeneration. In samples with increased Notch signaling, there was a 92% reduction in the number of spontaneously regenerated HCs, suggesting that Notch signaling plays a role in spontaneous HC regeneration.

### The Expression of *Hes1*, *Hes5*, *Hey1*, and *HeyL* Were Reduced During the Window of Spontaneous HC Regeneration

Using *in situ* hybridization and a *Hes5<sup>LacZ</sup>* knockin reporter line, we discovered that the expression of *Hes1*, *Hes5*, *Hey1*, and *HeyL* was reduced after HC loss and during the spontaneous HC regeneration process. Both *Hes1* and *Hes5* directly inhibit a HC fate by inhibiting the expression of *Atoh1*, a transcription factor that is necessary for HC differentiation (Zheng et al., 2000; Mulvaney and Dabdoub, 2012; Abdolazimi et al., 2016). Previously, Korrapati et al. (2013) found that HC damage after gentamicin treatment *in vitro* leads to downregulation of *Hes5*, but no other Notch-related genes expressed in SCs. However, no HC regeneration was observed in this model until Notch signaling was inhibited with a  $\gamma$ -secretase inhibitor, which caused decreased expression of all Notch downstream effectors. This suggests that reduction of *Hes5* signaling alone

is insufficient to induce spontaneous HC regeneration, however, when reduced expression is expanded to additional Notch effectors, regeneration is possible. In support, overexpression of *Hes1* is able to inhibit the production of ectopic HCs in the GER that were created by ectopic expression of *Atoh1*. This indicates that *Hes1* is also a direct inhibitor of HC fate (Zheng et al., 2000). Furthermore, combined loss of *Hes1*, *Hes5*, and *Hey1* during late embryonic development [embryonic day (E) 18.5] caused increased outer HCs with disorganized pillar and Deiters' cells (Tateya et al., 2011). Unfortunately, less is known about the function of the Notch effector *HeyL* in cochlear development, however, it is expressed around the time of HC differentiation and is linked to neuronal differentiation in other systems (Doetzlhofer et al., 2009; Jalali et al., 2011). Therefore, combined reduction of *Hes1*, *Hes5*, and *Hey1*, and possibly *HeyL*, observed in our study are correlated with the ability of SCs to spontaneously regenerate HCs in the neonatal mouse cochlea, thus supporting our conclusion that weakened Notch signaling allows SC-to-HC conversion and plays a role in spontaneous HC regeneration.

### Jag1 Is Reduced During the Spontaneous HC Regeneration Process

In the developing cochlea, *Jag1* is initially expressed throughout the sensory epithelium, then restricted to SCs, with expression in all SC subtypes from the GER to the 3rd row of Deiters' cells at neonatal ages and in adulthood (Morrison et al., 1999; Daudet and Lewis, 2005; Oesterle et al., 2008; Murata et al., 2009). However, its function during and after HC differentiation is not well understood. Furthermore, *Jag1* is both a Notch ligand as well as a Notch target gene (Daudet and Lewis, 2005; Murata et al., 2006) and as such, reduced expression of this gene can indicate loss of Notch signaling. Consistent with results from the *Hes5<sup>LacZ</sup>* reporter and *in situ* hybridization, *Jag1* expression was decreased in *Atoh1-DTA* samples in outer pillar and Deiters' cells, the SC subtypes located underneath outer HCs in the lateral compartment of the cochlea. This finding further supports our conclusion that there is a modest decrease in Notch activity in SCs after HC damage and during HC regeneration in the neonatal cochlea.

### The Majority of Spontaneous HC Regeneration Is Prevented When *NIICD* Is Overexpressed in SCs and Other Non-HCs in the Neonatal Mouse Cochlea

Previous work has focused on the effect of induced Notch inhibition via pharmacologic manipulation with  $\gamma$ -secretase inhibitors (Yamamoto et al., 2006; Korrapati et al., 2013; Li et al., 2015) or deletion of Notch receptors or *RBP-J* (Yamamoto et al., 2006; Bramhall et al., 2014; Li et al., 2015). From these studies we know that loss of Notch signaling triggers SC-to-HC conversion, however, while this approach produces new HCs, it applies a blanket reduction in all Notch related genes, as well as potentially affects other signaling pathways that  $\gamma$ -secretase interacts with such as ErbB-4, E-Cadherin, N-Cadherin, EphrinB, and CD44 (Ni et al., 2001; Lammich et al., 2002; Marambaud

et al., 2002, 2003; Georgakopoulos et al., 2006). Therefore, it is unclear whether decreased Notch signaling in SCs is part of the molecular mechanism for spontaneous HC regeneration. Our study attempts to address this gap in knowledge by showing that overexpression of *NIICD* in SCs and other non-HCs causes an increase in Notch signaling and results in a 92% reduction in the number of regenerated HCs. However, the mouse model we used generated supraphysiological levels of Notch activity with *NIICD* increased 5 to 6-fold and *Hes/Hey* genes increased 2 to 13-fold over controls. It is possible that the level of Notch signaling is important in controlling HC and SC fate. In support of this notion, there was one *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* sample with 33 fate-mapped regenerated HCs, while the other three samples that had 0-5 fate-mapped regenerated HCs. While not directly tested, the sample with more regenerated HCs may have had lower expression of *NIICD* compared to the other three samples, which could have been caused by decreased exposure to doxycycline. Despite the limitation of supraphysiological *NIICD* and Notch effector levels in our model, data from this study support a role for Notch signaling during the spontaneous HC regeneration process.

*Sox10<sup>rtTA</sup>* is a knockin allele that may produce decreased expression of *Sox10* in the neonatal cochlea. It is possible that decreased *Sox10* expression may alter normal spontaneous HC regeneration making it more dependent on Notch signaling and therefore making *NIICD* overexpression more effective at preventing regeneration. While we cannot rule out this possibility, a previous study showed that *Sox10<sup>rtTA</sup>* mice do not have a haploinsufficient phenotype in the cochlea (Walters and Zuo, 2015). Yet little is known about the relationship between *Sox10* and Notch signaling, and further research is needed.

Unexpectedly, in the control samples where *NIICD* was overexpressed without HC damage (*Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice) and in experimental samples (*Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* mice), we observed an expansion of the region with Sox2-positive cells both medial and lateral to the organ of Corti. *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-NIICD* controls also had disorganized HCs, but no decrease in HC number. Similar studies were done previously in the embryonic and early neonatal cochlea where *NIICD* overexpression led to ectopic sensory patches containing both Sox2-positive cells and HCs in non-sensory regions. Yet this was only observed when *NIICD* was overexpressed prior to E14.5 (Hartman et al., 2010; Pan et al., 2010, 2013; Liu et al., 2012; Campbell et al., 2015). Studies where *NIICD* overexpression was induced after E14.5 showed no ectopic HCs or Sox2-positive cells (Liu et al., 2012; Pan et al., 2013). These studies demonstrate that *NIICD* overexpression can induce Sox2 expression and sensory progenitor cells in non-sensory regions, yet there is a temporal limitation for when these cells can respond. Therefore, the increased numbers of Sox2-positive cells we observed when *NIICD* was overexpressed at P0 was unexpected. We may have overcome temporal limitations by producing supraphysiological levels of *NIICD* and Notch effectors, but further experiments are needed to confirm this hypothesis. Regardless of the cause, we do

not believe expanded Sox2 expression altered the spontaneous HC regeneration process or affected the conclusions of our study since all abnormalities, except HC disorganization, were observed outside the organ of Corti.

Notch signaling changes dynamically in the first week after birth where ligands, receptors, and effectors are downregulated in a basal-to-apical gradient between P0 and P6 (Murata et al., 2006; Hartman et al., 2007; Maass et al., 2015). However, Jag1 is the exception and its expression in SCs continues throughout adulthood (Oesterle et al., 2008). During the first postnatal week, inhibition of Notch signaling using  $\gamma$ -secretase inhibitors has differential effects on SC-to-HC conversion along the basal to apical axis. Notch inhibition at P0 produces a  $\sim 200\%$  increase in HCs in the apex compared to only a  $\sim 30\%$  increase in the base, while there is no evidence of HC production in any cochlear turn when  $\gamma$ -secretase inhibitors are used at P3 or later (Maass et al., 2015). There are conflicting reports for whether SCs can produce HCs in response to Notch inhibition in the adult cochlea (Batts et al., 2009; Doetzlhofer et al., 2009; Hartman et al., 2009; Mizutani et al., 2013). These data taken together with our results suggest that spontaneous HC regeneration likely occurs when HCs are killed at P0–P1 because SCs, especially those in the apical turn, are still dependent on Notch signaling to maintain their cell fate. As the cochlea matures, it appears that SC fate maintenance becomes uncoupled from Notch signaling, yet the upstream regulators or epigenetic changes that cause downregulation of the Notch pathway, as well as the mechanisms that maintain SC fate after the first postnatal week are unknown.

Nevertheless, while previous studies have shown that increased numbers of regenerated HCs can form when Notch signaling is inhibited (Korrapati et al., 2013; Bramhall et al., 2014; Hu et al., 2016), the findings presented here are the first to demonstrate that the spontaneous HC regeneration process is prevented by increased Notch signaling. This suggests that weakened Notch signaling plays a role in the spontaneous HC regeneration process. Understanding the molecular mechanism of spontaneous HC regeneration is a critical step to understanding why HC regeneration does not occur in the mature cochlea. Additionally, while some Notch effectors, such as *Hes1* and *Hes5*, are well established as inhibitors of HC fate, less is known about the contribution of other effectors on SC-to-HC conversion. The findings presented here will hopefully drive research to identify cellular and molecular changes that occur within the first postnatal week to uncouple Notch signaling and SC fate maintenance. Understanding what assumes control over SC fate in the adult cochlea will potentially lead investigations into therapeutic targets to stimulate HC regeneration in the mature cochlea.

## AUTHOR CONTRIBUTIONS

MM designed and performed the experiments, analyzed the data, and wrote the manuscript. LZ and MR performed the experiments, analyzed the data, and contributed to writing the

manuscript. BC designed the experiments, analyzed the data, and wrote the manuscript.

## FUNDING

This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs (W81XWH-15-1-0475), the Office of Naval Research (N00014-13-1-0569), and the National Institutes of Health (R01 DC014441). The SIUSOM Research Imaging Facility is supported by a grant from the Office of Naval Research (N00014-15-1-2866) and the National Center for Research Resources-Health (S10RR027716).

## ACKNOWLEDGMENTS

We thank Kaley Graves from Southern Illinois University School of Medicine for technical assistance, Dr. Bernd Fritzsch from the University of Iowa, and Dr. Andy Wilber from Southern Illinois University School of Medicine for their assistance with *in situ* hybridization experiments. We thank Dr. Andy Groves from Baylor College of Medicine for the critical discussion and assistance with *in situ* hybridization experiments. We are grateful to Dr. Suzanne Baker (St. Jude Children's Research Hospital) for sharing *Atoh1-CreER<sup>TM</sup>* mice, Dr. Ryoichiro Kageyama (Kyoto University) for sharing *Hes5<sup>LacZ</sup>* mice, Dr. Michael Wegner (Erlangen University) for sharing *Sox10<sup>rtTA</sup>* mice, and Dr. Ben Stranger (University of Pennsylvania) for sharing *TetO-N1ICD* mice.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fncel.2018.00120/full#supplementary-material>

**FIGURE S1 |** Inner phalangeal and border cells labeling by *Pip-CreER<sup>T2</sup>::Rosa26<sup>tdTomato</sup>*. **(A–C)** Representative confocal images of *Pip-CreER<sup>T2</sup>::Rosa26<sup>tdTomato</sup>* samples injected with tamoxifen (Tam) at P0. *tdTomato* labeling allowed the measurement of the inner phalangeal and border cell region for quantification of *Hes5-LacZ* and Sox2 expressing cells in **Figure 2**. This width was 9.6  $\mu\text{m}$  at P2, 13.2  $\mu\text{m}$  at P4, and 16.8  $\mu\text{m}$  at P6. Scale bar = 25  $\mu\text{m}$ .

**FIGURE S2 |** Expansion of the Sox2-positive region and HC disorganization when *N1ICD* is overexpressed. **(A–D)** Representative confocal images taken from the apical turn of the cochlea from *Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* mice that were injected with tamoxifen (Tam) at P0/P1 as well as administered doxycycline (Dox) in the diet to nursing mother with a doxycycline injection given to pups at P1 to induce expression of *N1ICD* and the *LacZ* reporter in SCs and other non-HCs. Cochlea were collected at P7 and stained with antibodies against myosin VIIa (red), Sox2 (white), and  $\beta$ -gal (green). While there was disorganization of HCs **(A)**, there was no change in the number of HCs in any of the cochlear turns. **(E)**, quantified in a 200  $\mu\text{m}$  region). Scale bar = 25  $\mu\text{m}$ . *N* = 4. **(F–I)** Maximum projection images of *Sox10<sup>rtTA</sup>::TetO-LacZ* and **(J–M)** *Atoh1-DTA::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD* apical turns showing expanded Sox2 expression medial and lateral to the organ of Corti. Scale bar = 20  $\mu\text{m}$ .

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**Conflict of Interest Statement:** BC is a consultant and MR is a part-time employee of Turner Scientific, LLC.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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*Abstract for poster presentation at the Association for Research in Otolaryngology: 39<sup>th</sup> 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**

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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<sup>+</sup>::Rosa26-loxP-Stop-loxP-DTA<sup>+/f</sup> 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<sup>nlsLacZ</sup> reporter mouse. When analyzing the total SC population, there was no significant difference in the number of Hes5-LacZ<sup>+</sup> cells after HC damage. However, when only Deiters' cells (DCs) and pillar cells (PCs) were quantified, the number of Hes5-LacZ<sup>+</sup> cells was significantly reduced in Atoh1-DTA::Hes5<sup>nlsLacZ</sup> cochleae compared to controls without HC damage. In addition the number of Sox2<sup>+</sup> cells in the DC/PC population increased in Atoh1-DTA::Hes5<sup>nlsLacZ</sup> 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<sup>DTR</sup> 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.

**Funding:** Supported by the Office of Naval Research (N000141310569), Office of the Assistant Secretary of Defense for Health Affairs (W81XWH-15-1-0475), and the National Center for Research Resources-Health (S10RR027716).

*Abstract for poster presentation at the Association for Research in Otolaryngology:  
40th annual midwinter meeting, 2017 February 11-15, Baltimore, MD*

## **Fate-mapping identifies supporting cell subtypes capable of spontaneously regenerating cochlear hair cells.**

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The mammalian organ of Corti contains distinct subtypes of supporting cells (SCs) that surround and aide sensory hair cells (HCs). Little is known about the differences among subtypes and their plasticity. Recently, our lab has identified pillar cells (PCs), Deiters' cells (DCs), and Hensen cells (HeCs) as potential sources of spontaneous HC regeneration in the neonatal mouse cochlea. Specifically, our preliminary data showed that PCs and DCs downregulate the Notch effector Hes5, a direct inhibitor of HC fate, during the window of spontaneous HC regeneration observed in the neonatal mouse cochlea. No changes in Hes5 expression was observed in other SC subtypes. Importantly, some PCs/DCs downregulated the cell cycle inhibitor p27<sup>Kip1</sup> after HC damage occurred suggesting these cells retain mitotic potential. Additionally, we observed a decrease in the number of Sox2<sup>+</sup> HeCs after HC damage suggesting HeCs may also play a role in regeneration. To investigate which SC subtypes can act as the source for spontaneously regenerated HCs, we have fate-mapped three distinct groups of SC subtypes. Using the *Pou4f3*<sup>DTR/+</sup> mouse to induce HC damage at postnatal day (P)1 and stimulate the spontaneous HC regeneration process, we fate-mapped SC subtypes using three CreER lines paired with the *ROSA26*<sup>tdTomato</sup> reporter. To target PCs and DCs, *Prox1-CreER*<sup>T2</sup> was used; for inner phalangeal and border cells, *Plp-CreER*<sup>T2</sup> was used; and to investigate cells of the greater epithelial ridge as well as Hensen cells, *GLAST-CreER*<sup>TM</sup> was used. For all three groups, tamoxifen was administered at P0 to induce tdTomato expression in SCs prior to HC damage induced at P1. Preliminary data confirm our hypothesis and suggest that PCs and DCs are capable of spontaneously regenerating HCs in the neonatal mouse cochlea. Further studies will investigate the ability of each group to regenerate HCs via mitotic regeneration vs direct transdifferentiation by incorporating a mitotic tracer in the study. Identifying the SC subtypes capable of spontaneously regenerating HCs will aid future research by identifying which SCs are plastic and therefore narrowing potential therapeutic targets to initiate regeneration in adult animals.

**Funding:** Supported by the Office of Naval Research (N00014-13-1-0569) and (N00014-15-1-2866), the Office of the Assistant Secretary of Defense for Health Affairs (W81XWH-15-1-0475), and NIDCD (R01DC014441-01A1).

*Abstract for poster presentation at the Association for Research in Otolaryngology: 41<sup>st</sup> annual midwinter meeting, 2018 February 10-14, San Diego, CA*

## **Maintaining Active Notch Signaling in Supporting Cells Prevents Spontaneous Hair Cell Regeneration *in vivo***

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During embryonic development, progenitor cells differentiate into hair cells (HCs) or supporting cells (SCs) by Notch-mediated lateral inhibition. Many studies have shown that inhibition of Notch signaling allows SCs to convert into HCs in both normal and drug damaged neonatal mouse cochleae. This mechanism is also implicated during spontaneous HC regeneration in non-mammalian vertebrates. We and others have observed that spontaneous HC regeneration can occur in the neonatal mouse cochlea. While the inhibition of Notch signaling can force SCs to convert into HCs, it is currently unknown whether loss of Notch signaling is the mechanism underlying spontaneous HC regeneration observed *in vivo*. Therefore, to investigate the role of Notch signaling during the spontaneous HC regeneration process, we used *Atoh1-CreER<sup>TM</sup>::Rosa26<sup>loxP-stop-loxP-DTA/+</sup>* mice with tamoxifen administered at postnatal day (P) 0/P1 to ablate HCs and stimulate the spontaneous HC regeneration process. Changes in the expression of genes in the Notch pathway were measured using real time qPCR, immunostaining, and *in situ* hybridization, with most changes limited to the apical one-third of the cochlea. Expression of the Notch effector *HeyL* was increased, while the ligand *Jagged 1* and the effector *Hes5*, which is directly responsible for inhibiting HC fate, were decreased. We also investigated whether inhibition of Notch signaling is required for spontaneous HC regeneration to occur by maintaining active Notch signaling in all SCs in the context of HC damage. The mouse model, *Atoh1-CreER<sup>TM</sup>::Rosa26<sup>loxP-stop-loxP-DTA/+</sup>::Sox10<sup>rtTA</sup>::TetO-LacZ::TetO-N1ICD*, was injected with tamoxifen at P0-P1 to stimulate spontaneous HC regeneration and given doxycycline, in the diet to nursing mothers from P0-P7 and injected in pups on P1, to induce expression of the Notch1 intracellular domain (N1ICD) and LacZ in all non-HCs. Fewer fate-mapped regenerated HCs were detected after HC loss with N1ICD overexpression compared to HC damage alone. Therefore we conclude that loss of Notch-mediated lateral inhibition is required for the majority of spontaneous HC regeneration to occur. Understanding how Notch signaling regulates this regenerative plasticity will allow more directed investigation into why HC regeneration does not occur in the mature cochlea. This understanding will help identify therapeutic targets to treat hearing loss due to HC loss.

Funding: DOD CDMRP W81XWH-15-1-0475 (to BCC)

*Abstract for poster presentation at the Association for Research in Otolaryngology: 41<sup>st</sup> annual midwinter meeting, 2018 February 10-14, San Diego, CA*

## **The Notch Ligand Jagged1 is Required for the Survival of Supporting Cells in the Mouse Cochlea.**

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The highly organized, mosaic pattern of hair cells (HCs) and supporting cells (SCs) in the mammalian cochlea is thought to be the result of Notch-mediated lateral inhibition which occurs during embryonic development. Progenitor cells destined to become HCs express Notch ligands which bind to the Notch receptor expressed on neighboring cells to inhibit a HC fate and promote a SC fate. In addition to this HC repressive function, a recent study by Campbell et al., suggests that Notch signaling may play an instructive role in SC development and survival. A potential candidate for mediating this function is the Notch ligand, *Jagged 1* (*Jag1*). In the differentiating cochlea *Jag1* is expressed by SCs. While other Notch ligands are downregulated during the first postnatal week, SC-specific *Jag1* expression continues throughout adulthood where its function is unknown. To investigate the role of *Jag1* in differentiating SCs, we generated *Sox2-CreER<sup>+</sup>::Jag1<sup>loxP/loxP</sup>* mice which received tamoxifen at embryonic day (E) 14.5 and E15.5 and were analyzed at E18.5. Controls included Cre-negative littermates, as well as *Sox2-CreER<sup>+</sup>::Jag1<sup>loxP/loxP</sup>* and *Sox2-CreER<sup>+</sup>* mice that did not receive tamoxifen to control for *Sox2* haploinsufficiency. In the absence of *Jag1*, we found that the density of SCs that surround outer HCs, particularly the Deiters' cells and Hensen cells, was significantly reduced compared to controls. Similar changes were also observed in the postnatal cochlea, when *Jag1* was deleted from HCs, pillar, and Deiters' cells using *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* mice that received tamoxifen at postnatal day (P) 0 and P1. In the samples with P0 deletion of *Jag1*, we observed a loss of Hensen cells at P7, and in contrast to embryonic deletion of *Jag1*, Deiters' cells remained intact. However, loss of *Jag1* did not alter HC density in either model, suggesting that the reduction in SCs was not due to SC-to-HC conversion, but likely due to defects in SC formation and survival. At P30, mice lacking *Jag1* expression exhibited hearing loss at 4 kHz and besides the loss of Hensen cells, there were no further morphological changes. To identify pathways/genes that may be deregulated in the absence of *Jag1*, we conducted a series of microarray experiments using *Sox2-CreER<sup>+</sup>::Jag1<sup>loxP/loxP</sup>* mice with tamoxifen given at E14.5/15.5. These experiments revealed mitochondrial dysfunction as a potential mechanism for the observed defects in SC formation/survival. Taken together, our preliminary data suggests that *Jag1* is required for the survival/formation of SCs and that loss of these cells leads to hearing loss.

Funding: NIH/NIDCD R01DC011571 (AD and EC) and DOD CDMRP W81XWH-15-1-0475 (BC)

## The role of the Notch Ligand Jagged1 in Postnatal Development of the Mouse Cochlea

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**Background:** The highly organized, mosaic pattern of hair cells (HCs) and supporting cells (SCs) in the mammalian cochlea is thought to be the result of Notch-mediated lateral inhibition which occurs during embryonic development. Progenitor cells destined to become HCs express Notch ligands which bind to the Notch receptor expressed on neighboring cells to inhibit a HC fate and promote a SC fate. In addition to this HC repressive function, a recent study by Campbell et al. (2016, *Sci Rep*), suggests that Notch signaling may play an instructive role in SC development and survival. A potential candidate for mediating this function is the Notch ligand, *Jagged 1* (*Jag1*). In the differentiating cochlea *Jag1* is expressed by SCs. While other Notch ligands are downregulated during the first postnatal week, SC-specific *Jag1* expression continues throughout adulthood where its function is unknown. Here, we investigate the role of *Jag1* in differentiating SCs using *Jag1* conditional knockout mouse lines.

**Methods:** To specifically delete *Jag1* from outer HCs and subset of SCs (pillar and Deiters' cells), we used the CreER-LoxP mouse genetic system by breeding a cell type-specific CreER mouse line (*Fgfr3-iCreER*<sup>T2/+</sup>) with *Jag1*<sup>loxP/loxP</sup> mice. To specifically delete *Jag1* from a different subset of SCs (cells of the greater epithelial ridge, inner phalangeal cells, border cells and Hensen cells), *Jag1*<sup>loxP/loxP</sup> mice were bred with the *GLAST-CreER*<sup>T2/+</sup> mouse line. Tamoxifen (3mg/40g) was injected at postnatal day (P) 0 and P1 to induce *Jag1* deletion in both models. We then analyzed the morphology of the cochlea of both models at P7 and P30 using immunofluorescence. Controls were iCre- or Cre-negative littermates. Standard immunostaining was performed using anti-Sox2 (a SC marker) and anti-myosin VIIa (a HC marker) primary antibodies. To access hearing after *Jag1* deletion, auditory brainstem response (ABR) was measured in *Fgfr3-iCreER*<sup>T2/+</sup>::*Jag1*<sup>loxP/loxP</sup> mice and controls at P30 after tamoxifen was injected at P0 and P1.

**Results:** When *Jag1* was deleted from terminally differentiated outer HCs, pillar cells, and Deiters' cells, we observed a significant loss of Hensen cells in *Fgfr3-iCreER*<sup>T2/+</sup>::*Jag1*<sup>loxP/loxP</sup> mice at P7, but no change in the density of HCs and other SC subtypes, suggesting that the reduction in Hensen cells was not due to SC-to-HC conversion. Similar loss of Hensen cells were also observed at P30 with no further morphological changes. In addition, analysis of ABR recordings in *Fgfr3-iCreER*<sup>T2/+</sup>::*Jag1*<sup>loxP/loxP</sup> mice at P30 revealed a mild hearing deficit with increased thresholds at the 4 kHz low frequency region. However, when *Jag1* was deleted from cells of the greater epithelial ridge, inner phalangeal cells, border cells and Hensen cells, we observed an increased number of outer HCs and multiple SC subtypes (inner phalangeal cells/border cells, inner pillar cells, Deiters' cells and Hensen cells) in the cochlea of *GLAST-CreER*<sup>T2/+</sup>::*Jag1*<sup>loxP/loxP</sup> mice at P7, with less changes observed at P30.

**Conclusions:** Taken together, our preliminary data suggests that *Jag1* plays different role in different cells in the development of cochlear mosaic pattern. Ongoing experiments are exploring the mechanisms of Hensen cell loss in *Fgfr3-iCreER*<sup>T2/+</sup>::*Jag1*<sup>loxP/loxP</sup> mice, as well as the increased cell density in *GLAST-CreER*<sup>T2/+</sup>::*Jag1*<sup>loxP/loxP</sup> mice.

Funding: DOD CDMRP W81XWH-15-1-0475 (BC)

## **Increased Notch Signaling at Birth Induces Widespread Proliferation and Ectopic Hair Cells in Non-Sensory Tissue of the Neonatal Mouse Cochlea**

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During development of the mammalian cochlea, Notch signaling promotes sensory progenitor formation by acting through the Notch ligand Jagged1. These progenitor cells will further differentiate into the hair cells and supporting cells of the cochlea through a Notch-mediated process involving the ligands Delta-like ligand 1 and Jagged2. Previous studies have demonstrated that overexpression of the *Notch1 intracellular domain (N1/CD)* in the early embryonic cochlea results in the formation of ectopic sensory patches in non-sensory regions; however this effect declines with age and no ectopic patches or other abnormal phenotype in the organ of Corti are seen when *N1/CD* is overexpressed after embryonic day (E) 14.5. Using a different mouse model, our lab has recently observed that increased *N1/CD* expression at birth in all non-HCs within the cochlea produces increased Sox2 expression and a disorganized organ of Corti. To further investigate this phenotype, *Sox10<sup>rtTA</sup>::TetO-N1/CD::TetO-LacZ* mice, as well as littermate controls lacking the *TetO-N1/CD* allele, were administered doxycycline in the diet to nursing mothers from postnatal day (P) 0 to P7 as well as a doxycycline injection at P1. Cochlea with *N1/CD* overexpression had a disorganized organ of Corti with inner and outer hair cells indistinguishable from each other, yet there was no change in total hair cell numbers. Additionally, there was expanded expression of Sox2 medial and lateral to the organ of Corti, as well as increased Sox2 expression measured by qPCR. In addition, the non-sensory epithelium medial to the organ of Corti contained ectopic growths of cells, many of which had ectopic hair cells. Investigation with the mitotic tracer BrdU showed wide spread proliferation occurring in the medial and lateral compartments; however, no proliferation was observed in the organ of Corti. Previously, the cochlea of *Sox10<sup>rtTA</sup>::TetO-LacZ* mice was characterized and no changes were observed in cochlear morphology or function. However, because *Sox10<sup>rtTA</sup>* is a knock-in allele we measured the level of Sox10 expression in the cochleae of our mice. qPCR analysis revealed that *Sox10<sup>rtTA</sup>* cochlea had a ~60% reduction in Sox10 expression. Therefore, it is possible that the combination of Sox10 haploinsufficiency combined with elevated Notch signaling induced the phenotype we observed. Additionally, there was a 5-6 fold increase in *N1/CD* expression compared to control cochleae. Therefore, it is also possible that the supraphysiological level of Notch activity we induced was increased over the level produced in previous studies, which prolonged the competence of non-sensory regions of the cochlea to differentiate into prosensory tissue. Further investigation is underway to understand the impact this mouse model has on the hearing organ. These studies may further elucidate the role of Notch signaling to induce progenitor cell populations within the neonatal organ of Corti.

**Funding:** Research was supported by the Office of the Assistant Secretary of Defense for Health Affairs (W81XWH-15-1-0475) and the NIH (R01 DC014441). The Southern Illinois University School of Medicine Research Imaging facility equipment was supported by the Office of Naval Research (N00014-15-1-2866).

*Abstract for the oral presentation at the Midwest Auditory Research Conference, 2019  
July 11-13, Springfield, IL*

**Deletion of the Notch ligands Jagged 2 and Delta-like 1 results in loss of supporting cells in the neonatal mouse cochlea**

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**Background:** Previous studies have shown that the Notch ligands Jagged 2 (Jag2) and Delta-like 1 (Dll1) function synergistically to regulate hair cell (HC) differentiation in the developing cochlea. Embryonic deletion of *Dll1* and/or *Jag2* results in supernumerary HCs and loss of supporting cells (SCs). However, the role of these Notch ligands in postnatal HCs is unknown. In this study, we investigated this question by conditionally deletion of *Jag2* and/or *Dll1* from neonatal HCs.

**Methods:** *Atoh1-CreER<sup>TM</sup>::Jag2<sup>loxP/loxP</sup>*, *Atoh1-CreER<sup>TM</sup>::Dll1<sup>loxP/loxP</sup>*, and *Atoh1-CreER<sup>TM</sup>::Jag2<sup>loxP/loxP</sup>::Dll1<sup>loxP/loxP</sup>* mice, as well as littermate controls lacking the *Atoh1-CreER<sup>TM</sup>* allele, were administered tamoxifen at postnatal day (P) 0 and P1. Cochleae were collected at P7 and P30 and immunostained with myosin VIIa (a HC marker) and Sox2 (a SC marker).

**Results:** Postnatal *Jag2* deletion from HCs resulted in a mild loss of the third row of Dieters' cells (DC3) in the apical and middle turns of the cochlea at P7. At P30, in addition to the loss of DC3, we also observed loss of Hensen's cells (HeCs) in the apex. No changes in other SC subtypes or HCs were found. Cochlea with postnatal *Dll1* deletion had no changes in the number of HCs or SCs at both time points. However, when both *Jag2* and *Dll1* were deleted, there was a larger decrease in the number of DCs and HeCs at P7 (~20% loss), compared to the *Jag2* single deletion. In addition, DC loss extended into the second row (DC2). No changes in other SC subtypes or HCs were observed at P7. At P30, the loss of DCs and HeCs did not progress, but we observed significant decrease in all three rows of outer HCs with a decreasing gradient from the 3<sup>rd</sup> row to 1<sup>st</sup> row. We predict that this gradient is the result of SC loss.

**Conclusions:** Our results indicate that Jag2 and Dll1 act synergistically in the neonatal cochlea to regulate the survival of SCs. Current investigation is underway to examine the mechanism of cell death after *Jag2* and *Dll1* deletion, as well as their potential genetic interaction. The effect of *Jag2* and *Dll1* deletion on hearing is also under investigation.

**Funding:** DOD W81XWH-15-1-0475.

*Abstract for the oral presentation at the 26th Annual Graduate Student Research Symposium, Southern Illinois University School of Medicine, 2016 April 29, Springfield, IL*

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

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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<sup>+</sup>::Rosa26-loxP-Stop-loxP-DTA<sup>+/f</sup> 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<sup>nlsLacZ</sup>* reporter mouse. When analyzing the total SC population, there was no significant difference in the number of *Hes5-LacZ*<sup>+</sup> cells after HC damage. However, when SCs were quantified based on subtype, the number of *Hes5-LacZ*<sup>+</sup> cells was significantly reduced in Deiters' cells and pillar cells of Atoh1-DTA::Hes5<sup>nlsLacZ</sup> cochleae compared to controls without HC damage. In addition, the number of Sox2<sup>+</sup> cells in the pillar cell/Deiters' cell population was maintained in Atoh1-DTA::Hes5<sup>nlsLacZ</sup> cochleae until P6 at which point the number of Sox2<sup>+</sup> pillar cells and Deiters' cells was reduced. Interestingly, there was no change in the number of *Hes5-LacZ*<sup>+</sup> cells or Sox2<sup>+</sup> 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.

**Funding:** Supported by the Office of Naval Research (N000141310569), Office of the Assistant Secretary of Defense for Health Affairs (W81XWH-15-1-0475), and the National Center for Research Resources-Health (S10RR027716).

## **The majority of spontaneously regenerated HCs come from Pillar and Deiters' cells compared to other supporting cell subtypes**

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**Background:** The mammalian organ of Corti contains distinct subtypes of supporting cells (SCs) that surround and aide sensory hair cells (HCs). Little is known about the differences among subtypes and their plasticity. Recently, our lab has identified pillar cells (PCs), Deiters' cells (DCs), and Hensen cells (HeCs) as potential sources of spontaneous HC regeneration in the neonatal mouse cochlea. Specifically, our preliminary data showed that PCs and DCs downregulate the Notch effector *Hes5*, a direct inhibitor of HC fate, during the window of spontaneous HC regeneration observed in the neonatal mouse cochlea. No change in *Hes5* expression was observed in other SC subtypes. In addition, some PCs/DCs downregulated the cell cycle inhibitor *p27<sup>Kip1</sup>* after HC damage occurred suggesting these cells retain mitotic potential. Additionally, we observed a decrease in the number of *Sox2+* HeCs after HC damage suggesting HeCs may also play a role in regeneration.

**Methods:** To investigate which SC subtypes can act as the source for spontaneously regenerated HCs, we have fate-mapped three distinct groups of SC subtypes. Using the *Pou4f3<sup>DTR/+</sup>* mouse to induce HC damage at postnatal day (P)1 and stimulate the spontaneous HC regeneration process, we fate-mapped SC subtypes using three CreER lines paired with the *ROSA26<sup>tdTomato</sup>* reporter. To target PCs and DCs, *Prox1-CreER<sup>T2</sup>* was used; for inner phalangeal and border cells, *Plp-CreER<sup>T2</sup>* was used; and to investigate cells of the greater epithelial ridge as well as HeCs, *GLAST-CreER<sup>TM</sup>* was used. For all three groups, tamoxifen was administered at P0 to induce tdTomato expression in SCs prior to HC damage induced at P1.

**Results:** Significantly more tdTomato+ HCs were observed after damage when PCs and DCs were fate mapped. While preliminary data suggests that there were more tdTomato+ HCs compared to controls when IPhCs/BCs, HeCs, and GER were fatemapped, there were fewer regenerated HCs from these populations compared to PCs and DCs.

**Conclusions:** Preliminary data confirm our hypothesis and suggest that more PCs and DCs spontaneously regenerate HCs than other SC subtypes in the neonatal mouse cochlea. Further studies will investigate the ability of each group to regenerate HCs via mitotic regeneration by incorporating the mitotic tracer BrdU in the study. Identifying the SC subtypes capable of spontaneously regenerating HCs will aid future research by identifying which SCs are plastic and therefore narrow potential therapeutic targets to initiate regeneration in adult animals.

**Funding:** Supported by the Office of Naval Research (N00014-13-1-0569) and (N00014-15-1-2866), the Office of the Assistant Secretary of Defense for Health Affairs (W81XWH-15-1-0475), and NIDCD (R01DC014441).

## The Notch Ligand Jagged1 is Required for the Survival of Hensen Cells in the Mouse Cochlea

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**Background:** The highly organized, mosaic pattern of hair cells (HCs) and supporting cells (SCs) in the mammalian cochlea is thought to be the result of Notch-mediated lateral inhibition which occurs during embryonic development. Progenitor cells destined to become HCs express Notch ligands which bind to the Notch receptor expressed on neighboring cells to inhibit a HC fate and promote a SC fate. In addition to this HC repressive function, a recent study by Campbell et al. (2016, *Sci Rep*), suggests that Notch signaling may play an instructive role in SC development and survival. A potential candidate for mediating this function is the Notch ligand, *Jagged 1* (*Jag1*). In the differentiating cochlea *Jag1* is expressed by SCs. While other Notch ligands are downregulated during the first postnatal week, SC-specific *Jag1* expression continues throughout adulthood where its function is unknown. Here, we investigate the role of *Jag1* in differentiating SCs using *Jag1* conditional knockout mouse lines.

**Methods:** To specifically delete *Jag1* from outer HCs and subset of SCs (pillar and Deiters' cells), we used the CreER-LoxP mouse genetic system by breeding a cell type-specific CreER mouse line (*Fgfr3-iCreER*<sup>T2/+</sup>) with *Jag1*<sup>loxP/loxP</sup> mice. Injection of tamoxifen (3mg/40g) at postnatal day (P) 0 and P1 in *Fgfr3-iCreER*<sup>T2/+</sup> :: *Jag1*<sup>loxP/loxP</sup> mice will delete *Jag1* in outer HCs, pillar cells, and Deiters' cells. We then analyzed the morphology of the cochlea at P7, P30, and P60 using immunohistochemistry. Controls were iCre-negative littermates. Standard immunostaining was performed using the following primary antibodies: Sox2 (a SC marker) and myosin VIIa (a HC marker). To access hearing after *Jag1* deletion, auditory brainstem response (ABR) was measured in *Fgfr3-iCreER*<sup>T2/+</sup> :: *Jag1*<sup>loxP/loxP</sup> mice and controls at P30 after tamoxifen was injected at P0 and P1.

**Results:** When *Jag1* was deleted from terminally differentiated outer HCs, pillar cells, and Deiters' cells, we observed a loss of Hensen cells at P7, but no change in the density of other SC subtypes. Loss of *Jag1* did not alter HC density, suggesting that the reduction in Hensen cells was not due to SC-to-HC conversion, but likely due to defects in Hensen cell formation or survival. Similar loss of Hensen cells were also observed at P30 and P60 with no further morphological changes. Analysis of ABR recordings in *Fgfr3-iCreER*<sup>T2/+</sup> :: *Jag1*<sup>loxP/loxP</sup> mice at P30 revealed a mild hearing deficit with increased thresholds most pronounced at the 4 kHz low frequency region.

**Conclusions:** Taken together, our preliminary data suggests that *Jag1* is required for the survival of Hensen cells and that loss of these cells leads to a mild hearing loss.

Funding: DOD CDMRP W81XWH-15-1-0475 (BC)

*Abstract for the oral presentation at the Society for Neuroscience 47<sup>th</sup> Annual Meeting, 2017  
November 11-15, Washington, DC.*

## **The Notch ligand Jagged1 is required for the survival of supporting cells in the postnatal mouse cochlea.**

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The highly organized, mosaic pattern of hair cells (HCs) and supporting cells (SCs) in the mammalian cochlea is thought to be the result of Notch-mediated lateral inhibition which occurs during embryonic development. Progenitor cells destined to become HCs express the Notch ligands *Jagged2* and *Delta-like1* which bind to the Notch receptor expressed on neighboring cells to inhibit a HC fate and instead promote a SC fate. Another Notch ligand, *Jagged 1* (*Jag1*), is expressed by both HCs and SCs, but its role in HC differentiation is not well understood. Many studies have shown that inhibition of the Notch signaling pathway, using either pharmacological agents or genetically altered mice, can produce supernumerary HCs in the intact mammalian cochlea or form new HCs in the damaged cochlea. While *Jagged2* and *Delta-like1* are downregulated in the cochlea during the first postnatal week, *Jag1* expression continues throughout adulthood where its function is not clear. To investigate the role of *Jag1* in the postnatal cochlea, we generated conditional knockout mice using the CreER/loxP system. Specifically, *Jag1* was deleted in HCs and the SC subtypes called pillar cells and Deiters' cells using *FGFR3-iCreER::Jag1<sup>loxP/loxP</sup>* mice given tamoxifen at postnatal day (P) 0 and P1. Using immunostaining, we observed organized rows of HCs and SC nuclei in both Cre-negative controls and *Fgfr3-iCreER::Jag1<sup>loxP/loxP</sup>* mice at P7. However quantification of cells showed a significant decrease in the number of SCs in the lateral compartment of the cochlea (pillar cells, Deiters' cells, and Hensen cells), but no change in the number of inner or outer HCs. Further investigation using markers of pillar cells and Deiters' cells show that Hensen cells are missing in *FGFR3-iCreER::Jag1<sup>loxP/loxP</sup>* mice. At P30, auditory brainstem response (ABR) showed a mild hearing loss in mice lacking *Jag1* expression, with a significant increase in ABR thresholds at 4 kHz, but not at other frequencies. We are currently investigating the morphology of the cochlea at P30, as well as measuring changes in the various Notch target genes after *Jag1* deletion. We also plan to perform ABR in older animals (at P60) to see if the hearing loss is progressive. Taken together our preliminary data suggest that *Jag1* is required for the survival of Hensen cells and loss of these cells leads to hearing loss.

Funding: DOD CDMRP W81XWH-15-1-0475