

**Defining *Atoh1* function and regulation in avian supporting cells  
during auditory hair cell regeneration**

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## Abstract

Sensorineural hearing loss is irreversible in all mammals, including humans, since neither hair cells nor neurons are regenerated. In contrast to mammals, non-mammalian vertebrates replace hair cells after damage. In the avian basilar papilla, hair cell injury activates neighboring supporting cells to undergo direct transdifferentiation or mitotic division, both of which contribute to regeneration of hair cells. The molecules that enable hair cell regeneration in birds are not well understood. This dissertation presents a series of experiments to evaluate whether *Atoh1*, a transcription factor required for hair cell development, is sufficient for hair cell differentiation in avian basilar papilla during regeneration after aminoglycoside damage and to determine if the bone morphogenetic factor BMP4, a protein required for development of auditory epithelia, inhibits *Atoh1* mRNA expression and subsequent hair cell differentiation after damage.

In the first study, I tracked the activity of the *Atoh1* enhancer in cultured basilar papillae to determine if it is an accurate predictor of hair cell fate, and I forced expression of mouse *Atoh1* in supporting cells to test the hypothesis that higher levels of *Atoh1* push supporting cells to divide or transdifferentiate. This first study determined that about half of supporting cells with *Atoh1* enhancer activity do not differentiate into hair cells, but relief from notch-mediated lateral inhibition or forced overexpression of *Atoh1* significantly increase the likelihood that a supporting cell will differentiate as a hair cell or proliferate.

In the second study, I used *in situ* hybridization to determine that *Bmp4* mRNA is expressed in hair cells in mature chicken basilar papilla. BMP4 receptors are transcribed in supporting cells and hair cells, while *inhibitor of DNA binding (Id)* mRNA, a downstream effector of BMP4, is enriched in supporting cells in control tissues. Upon hair cell loss, *Bmp4* mRNA expression is lost, while *Atoh1* mRNA is upregulated in supporting cells. Concurrently, downstream *Id* effectors and receptors to BMP4 are upregulated in the area of damage. Given the observation that *Bmp4* and *Atoh1* have opposing expression patterns after hair cell loss, damaged basilar papillae were cultured with BMP4 protein or its inhibitor noggin after hair cell loss to determine if BMP4 antagonizes *Atoh1* expression and subsequent hair cell differentiation. BMP4 eliminated *Atoh1* transcripts along the length of the basilar papilla, while noggin increased *Atoh1* expression. Further, BMP4 application significantly decreased the number of

regenerated hair cells, while noggin application significantly increased them. These findings suggest BMP4 antagonizes hair cell regeneration by reducing *Atoh1* transcripts in supporting cells, preventing them from dividing or transdifferentiating. My results are consistent with other investigators' observations that *Atoh1* is necessary for hair cell differentiation during development but additional factors such as notch ligands and BMP4 limit ATOH1's expression. These are important considerations as investigators examine the potential for ATOH1 to stimulate auditory hair cell regeneration in humans.

## **Dedication**

I dedicate this dissertation to the supporting cells in my life, which have arrived in many forms. My friends and family who have stayed by my side, supportive of my differentiation regardless of the signaling pathway. The several colleagues met at nearby labs or conferences, with whom I have forged a supportive structural system. My graduate mentors who taught me to think critically as a neuroscientist and encouraged judiciously weighing intrinsic and extrinsic signals to evaluate all parts of life: from experimental design to future career decisions. My clinical supervisors, who introduced me to best clinical practices and allowed me to change the lives of patients through excellent, uncompromising clinical care. Of course, I must also dedicate this dissertation to those supporting cells located in the inner ear, which hold potential for restoration of hearing and balance.

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## Chapter 1

### Regulation of Avian Hair Cell Regeneration

#### I. INTRODUCTION

The World Health Organization estimates approximately 360 million people worldwide have a disabling hearing loss, with thresholds of 40dB HL or greater in the better hearing ear (WHO: Deafness and Hearing Loss, 2015). Healthy, functioning hair cells of the inner ear are essential to maintaining normal hearing perception. Hair cells are damaged through hazardous levels of noise (Masterson et al., 2013), exposure to ototoxic drugs to manage health-related issues such as cancer (Schacht et al., 2012), and age-related deterioration of auditory structures (Cruickshanks et al., 1998). Cochlear hair cell damage results in permanent hearing loss, which is a chronic health condition with no cure to restore normal hearing at this time.

Sensorineural hearing loss, the inability to properly discern auditory information, is caused by damage or death of hair cells, the sensory receptors for hearing (Johnsson and Hawkins, 1976). Hair cells are mechanotransducers located in the organ of Corti within the cochlea (Ruben, 1967). They encode the energy in sound and transmit the converted signal via the auditory nerve to the auditory nucleus in the brain. Hair cells respond to vibrations in the fluid that surrounds their stereocilia, which are hair-like structures arranged in a bundle atop each hair cell (Ruben, 1967). Each cochlea has around 15,000 hair cells arranged in several rows along the length of the cochlea. Located medially in the organ of Corti is a row of inner hair cells, which act as the primary sensor and transmitter of auditory input (Ruben, 1967). Lateral to the inner hair cells are outer hair cells, which amplify the movement of the fluid that passes over the inner hair cell stereocilia (Davis, 1983). The membrane upon which inner and outer hair cells sit is tuned to different sound frequencies (Lippe and Rubel, 1983; Cousillas and Rebillard, 1985). Outer hair cell activity helps focus activation of inner hair cells to specific areas of the cochlea to enhance frequency selectivity, making this a finely tuned system (Strelhoff et al., 1985; Dallos and Evans, 1995).

Once sufficient numbers of hair cells are damaged or destroyed by aging, noise, or drugs, the organ of Corti cannot properly encode and transmit auditory information (Ryan and McGee, 1977).

Perceptually, this physical damage results in sensorineural hearing loss (Johnsson and Hawkins, 1976; Hawkins et al., 1976). Mammals cannot restore lost hair cells, so hearing loss is irreversible and can only be remedied through hearing assistive technology (for review, see Sprinzl and Riechelmann, 2010). Hearing assistive technology has its merits and its faults, with none performing as well as the natural anatomy of the cochlea.

The most common approach for managing sensorineural hearing loss is a hearing aid, which isolates and amplifies speech while improving access to environmental surroundings to enhance quality of life (Contrera et al., 2016). Hearing aids require some presence of inner hair cells to transmit the amplified signal from the cochlea to the brain, resulting in a continued reliance on this damaged system to provide access to sound (Turner and Cummings, 1999). Once inner hair cells are lost, a more severe hearing loss may result, preventing amplified sound from being relayed from the cochlea to the central auditory nervous system (Cox et al., 2011; Zhang et al., 2014). In severe-to-profound cases of sensorineural hearing loss, cochlear implants are considered for patients (Gifford et al., 2010). Cochlear implants require surgical implantation of an electrode array into the cochlear space to replace the acoustic function of the hair cell with direct electrical stimulation of the auditory nerve (Sweeney et al., 2016). This allows sound information to bypass damaged hair cells (Leake et al., 1991). Neither of these technologies can restore hearing to normal (Gifford et al., 2012). By contrast, replacement of hair cells with a full set of healthy hair cells would increase the amount of information processed in the tonotopically organized cochlea, which may increase hearing acuity and speech perception, particularly in noisy environments.

## II. AVIAN HAIR CELL REGENERATION

In contrast to mammals, all non-mammalian vertebrates, including birds, reptiles, amphibians, and fish, readily replace auditory hair cells after damage (reviewed in Brignull et al., 2009), and the new hair cells restore hearing (reviewed in Bermingham-McDonogh and Rubel, 2003). Since birds are warm-blooded animals, like mammals, they serve as excellent animal models with which to study the signals

that enable and restrict hair cell regeneration, which might lead to development of regenerative therapies for humans.

In the 1980's, it was discovered that hair cells regenerate after damage in the mature basilar papilla, which is similar in structure and function to the mammalian organ of Corti. While characterizing patterns of damage to the basilar papilla in mature avian species, hair cell damage appeared only transient, as several immature hair cell bundles appeared in the area of damage with increasing time after noise exposure (Cotanche, 1987; Marsh et al., 1990; Ryals and Westbrook, 1990; Cotanche et al., 1991; Adler et al., 1992) and ototoxic drug damage (Cruz et al., 1987; Tucci and Rubel 1990; Duckert and Rubel 1993; Janas et al., 1995). Further studies demonstrated that regenerated hair cells are derived from neighboring supporting cells, which are glia-like cells with an embryonic origin in the otocyst, like hair cells. To identify supporting cells as the source of new hair cells, basilar papillae were damaged and then exposed to a nucleotide analog that allows tracking of mitotic cells *in vivo*. Supporting cells incorporated this nucleotide analog and divided; daughter cells differentiated as hair cells (Girod et al., 1989; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994). Supporting cells also change their phenotype to a hair cell through direct transdifferentiation, during which they phenotypically convert into hair cells (Roberson, 1996; Adler et al., 1996; Roberson et al., 2004; Shang et al., 2010). Mitotic division is required to sustain the progenitor cell population.

Regenerated hair cells acquire mature features (Duckert et al., 1990) and become innervated (Ryals and Westbrook, 1994; Muller and Smolders, 1999; Xiang et al., 2000). As a result, hearing levels are restored to near-normal, as measured through otoacoustic emissions (Froymovich et al., 1995; Trautwein et al., 1996), auditory brainstem response (Adler et al., 1992; Muller and Smolders, 1998; Girod et al., 2000; Irvine et al., 2009), and behavioral measures of pure tone thresholds (Marean et al., 1998; Niemiec et al., 1994; Salvi et al., 1998). While new hair cells are produced only after damage in the basilar papilla, nearby vestibular organs undergo routine cell turnover (Roberson et al., 1992) and also replace hair cells after damage, restoring vestibular function (Dickman and Lim, 2004).

In the 1990's, investigators focused on the basilar papilla as a tool for understanding signals controlling hair cell regeneration in non-mammals. Development of an organotypic culture system for the basilar papilla was instrumental in this process (Oesterle et al., 1993; Warchol and Corwin, 1996). Also

important was the development of molecular markers for regenerated hair cells (e.g., Stone et al., 1996). Investigators were particularly interested in defining molecules that regulate supporting cell division and differentiation of daughter cells into hair cells. To identify candidate molecules, they turned to what was known about factors controlling hair cell development during embryogenesis.

### III. MOLECULAR REGULATION OF HAIR CELL DEVELOPMENT

The inner ear derives from ectoderm that thickens to form the otic placode, then invaginates to become the otocyst (Morsli et al., 1998) through a coordinated series of intrinsic gene expression patterns and extrinsic signaling molecules. The ventral region of the otocyst gives rise to auditory epithelial structures (Wu and Oh, 1996; Rinkwitz et al., 2001) such as the mammalian organ of Corti or the avian basilar papilla. Many different molecules control development of the auditory epithelium. Some of these are signaling molecules, while others are transcription factors that activate or suppress gene expression in response to signaling molecules.

Atonal homologue 1 (*Atoh1*) is a basic-helix-loop-helix protein that promotes differentiation of neural cell types in many systems including hair cells (Bermingham et al., 1999; Zheng and Gao, 2000), cerebellar granule neurons (Ben-Arie et al., 1996; Helms and Johnson, 1998), and dorsal column nuclei (Bermingham et al., 2001). As a transcriptional activator, ATOH1 triggers expression of genes specific for the neural phenotype (reviewed in Bertrand et al., 2002). In the developing organ of Corti, transient *Atoh1* expression occurs among progenitor cells and differentiating hair cells (Chen et al., 2002; Woods et al., 2004; Matei et al., 2005). Young hair cells downregulate *Atoh1* during the early neonatal period, after which *Atoh1* is no longer expressed in the organ of Corti (Lanford et al., 2000; Woods et al., 2004; Batts et al., 2009) or expressed at very low levels (Maass et al., 2015). Forced *Atoh1* overexpression in a nonsensory region of the organ of Corti called the greater epithelial ridge causes ectopic formation of hair cells, demonstrating ATOH1 is sufficient to induce hair cell differentiation (Zheng and Gao, 2000). Similar results are seen upon *Atoh1* misexpression in supporting cells (e.g., Gubbels et al., 2008). However, the capacity of ATOH1 to stimulate auditory hair cell differentiation decreases with age and is completely

absent from mature adult mice (Liu et al., 2012; Kelly et al., 2012). These findings suggest inhibitory factors act to restrict ATOH1's ability to induce hair cell differentiation in mature mammals.

Notch signaling is one mechanism known to modulate ATOH1 activity (reviewed in Lewis, 1996; Daudet et al., 2009; Lin et al., 2011). Notch is a plasma membrane receptor that, when bound by a membrane-localized ligand located on an adjacent cell, transduces a signal to the nucleus that blocks *Atoh1* transcription. Notch1 is broadly expressed in the cochlear duct and the prosensory domain (Lanford et al., 1999). Cells that start to differentiate as hair cells upregulate expression of two notch ligands called delta-like 1 (Dll1) and jagged2 (Jag2; Lanford et al., 1999, Morrison et al., 1999). Activated Notch1 is then observed in adjacent cells that differentiate as supporting cells along with expression of two notch target genes, hairy and enhancer of split homologues 1 and 5 (HES1/HES5; Lanford et al., 2000; Tateya et al., 2011). HES1/HES5 are transcriptional repressors that reduce *Atoh1* transcription (Zine et al., 2001; Du et al., 2013). Through this cell-to-cell mediated lateral inhibition of hair cell differentiation, the notch pathway clearly restricts hair cell differentiation and therefore helps establish proper cellular patterning in the cochlea.

Another signaling protein that restricts ATOH1 function during hair cell development is the secreted, diffusible factor, bone morphogenetic protein 4 (BMP4). BMP4 is part of the transforming growth factor-beta family of cytokine growth factors, known for their effects throughout development of several tissues (reviewed in Murray et al., 2016; Tong et al., 2015). BMP4 is first expressed on the posterior ventral edge of the otocyst before localizing to hair cells in developing sensory patches, including the basilar papilla (Oh et al., 1996; Wu and Oh, 1996). To test the function of BMP4 during hair cell development, Pujades et al. (2006) added exogenous BMP4 to explants of developing otic vesicles. This increased transcripts for the BMP4 effectors *inhibitors of DNA binding* (Kamaid et al., 2010) and decreased *Atoh1* transcripts in the sensory patches. By contrast, treatment of otic vesicles with exogenous noggin, a BMP4 inhibitor, increased *Atoh1* expression and the size of the prosensory patch (Pujades et al., 2006).

An alternative approach to decrease BMP signaling uses conditional inactivation of the BMP4 receptors *Bmpr1a* and *Bmpr1b* in the developing mouse inner ear. Oddly, this resulted in reduced development of the organ of Corti and expanded development of non-sensory regions of the cochlea

(Ohyama et al., 2010). This finding, which is contradictory to what was seen upon BMP4 inhibition in the chick otocyst, may be due to the concentration-dependent effects of the morphogenetic function of BMP4.

#### IV. MOLECULAR REGULATION DURING REGENERATION

Although there is substantial evidence to support the inhibitory function of Notch and BMP4 upon *Atoh1* expression and hair cell differentiation during embryogenesis, there has been limited investigation of how these factors influence *Atoh1* expression during hair cell regeneration. For my dissertation, I tested the hypothesis that these pathways have similar roles during hair cell regeneration in organotypic cultures of basilar papillae from post-hatch chickens.

At the time I embarked on my thesis, much was already known about *Atoh1* expression during avian hair cell regeneration. As discussed above, supporting cells are quiescent in the normal basilar papilla; they are neither dividing nor transdifferentiating. Increased ATOH1 protein expression is one of the earliest signs after hair cell damage that supporting cells are initiating either direct transdifferentiation or mitosis (Cafaro et al., 2007). ATOH1 protein is elevated among many supporting cells immediately after hair cells have been damaged, and similar to development, it becomes highly expressed in early differentiating hair cells. However, we know that not all supporting cells will go on to form hair cells. The first goal of my dissertation was to define how *Atoh1* expression is regulated such that the appropriate numbers and patterns of hair cells are established during hair cell regeneration. In Chapter 2, I performed several experiments in cultured chicken basilar papillae to address this goal. I used a reporter of *Atoh1* to determine what proportion of supporting cells that upregulate *Atoh1* after hair cell damage go on to form hair cells. I tested if inhibition of notch signaling altered this proportion. I also asked whether forced expression of *Atoh1* in supporting cells guarantees they will transdifferentiate into hair cells. I found that more than half of supporting cells that upregulate *Atoh1* will go on to differentiate as hair cells. Despite inhibition of notch signaling and overexpression of *Atoh1*, not all supporting cells with *Atoh1* will differentiate as hair cells, suggesting additional factors influence ATOH1 accumulation and hair cell differentiation.

The second goal of my dissertation, discussed in Chapter 3, was to examine the role of BMP4 in regulating *Atoh1* expression and hair cell differentiation in the regenerating basilar papilla. I determined

that hair cell destruction results in loss of *Bmp4* transcripts from the basilar papilla and reduction in transcripts for BMP4 effectors *Id2* and *Id3*. In addition, hair cell loss resulted in an increase in *Atoh1* transcripts, suggesting reduction of BMP4 signaling enables upregulation of *Atoh1* in supporting cells and their subsequent transdifferentiation into hair cells. Consistent with this, addition of exogenous BMP4 to damaged basilar papilla resulted in downregulation of *Atoh1* mRNA and decreased hair cell regeneration while inhibition of BMP4 in damaged basilar papilla produced opposing results.

Together, these data demonstrate BMP4 is an additional factor besides notch that antagonizes *Atoh1* and regulates numbers of hair cells that are regenerated in the chicken basilar papilla after hair cell damage.

**Chapter 2**  
***Atoh1* Expression and Function During**  
**Auditory Hair Cell Regeneration in Post-Hatch Chickens**

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**Abstract**

Loss of hair cells in humans leads to irreversible hearing deficits, since auditory hair cells are not replaced. In contrast, hair cells are regenerated in the auditory epithelium of mature birds after damage by non-sensory supporting cells that transdifferentiate into hair cells by mitotic and/or non-mitotic mechanisms. Factors controlling these processes are poorly understood. The basic helix-loop-helix transcription factor ATOH1 is both necessary and sufficient for developmental hair cell differentiation, but it is unclear if it plays the same role in the mitotic and non-mitotic pathways in hair cell regeneration. We examined *Atoh1* expression and function during hair cell regeneration in chickens. *Atoh1* transcripts were increased in many supporting cells in the damaged auditory epithelium shortly after ototoxin administration and later became restricted to differentiating hair cells. Fate-mapping *in vitro* using an *Atoh1* enhancer reporter demonstrated that only 56% of the supporting cells that spontaneously upregulate *Atoh1* enhancer activity after damage acquired the hair cell fate. Inhibition of notch signaling using a gamma secretase antagonist stimulated an increase in *Atoh1* reporter activity and induced a higher proportion of supporting cells with *Atoh1* activity (73%) to differentiate as hair cells. Forced overexpression of *Atoh1* in supporting cells triggered 66% of them to acquire the hair cell fate and nearly tripled their likelihood of cell cycle entry. These findings demonstrate that *Atoh1* is broadly upregulated in supporting cells after damage, but a substantial proportion of supporting cells with *Atoh1* activation fails to acquire hair cell features, in part due to gamma secretase-dependent activities.

**Abbreviations:**

Atoh1 = atonal homolog 1

Myo6 = myosin 6

$\beta$ III tub =  $\beta$ III tubulin

SCA = supporting cell antigen

$\beta$ tect =  $\beta$  tectorin

**1. Introduction**

Auditory hair cells are mechanotransducers that convert energy in the form of sound waves into chemical signals that are received and processed by the auditory nerve. Degeneration of hair cells is the leading cause of sensorineural hearing disorders in humans. This type of hearing loss is irreversible, because auditory hair cells cannot be replaced in any mammal (Hawkins, 1973).

The signals that block hair cell regeneration from occurring in mammals are poorly understood. Proneural transcription factors are essential for neuronal differentiation of the central and peripheral nervous system (Jarman et al., 1993a; Bertrand et al., 2002). Atonal homolog 1 (ATOH1), a proneural transcription factor in the basic helix-loop-helix (bHLH) family (Jarman et al., 1993b; Ben-Arie et al., 1996) is both necessary and sufficient for hair cell development in the mammalian inner ear (Bermingham et al., 1999; Zheng and Gao, 2000). *Atoh1* is not expressed in mature hair cells (Shailam et al., 1999), and it is not reactivated in the mature organ of Corti after injury (Batts et al., 2009). However, under appropriate conditions, forced expression of *Atoh1* in adult mammalian supporting cells, in either the auditory or vestibular sensory epithelia, causes them to convert into hair cells (Kawamoto et al., 2003; Shou et al., 2003; Staecker et al., 2007). Importantly, *Atoh1* misexpression is not sufficient to drive all cells in the developing organ of Corti to acquire the hair cell fate (Zhao et al., 2011), and it also fails to trigger long-injured supporting cells to transdifferentiate into hair cells (Izumikawa et al., 2008). There is evidence that the notch pathway is at least partially responsible for this limitation. Inhibition of the notch receptor (reviewed in Lewis, 1996), which antagonizes *Atoh1* transcription (Lanford et al., 2000), significantly increases the number of hair cell-like cells that are regenerated in the adult mouse utricle (Lin et al.,

2011) over baseline levels (Forge et al., 1993, 1998; Kawamoto et al., 2009). In order to identify strategies to promote hair cell regeneration in humans, it is essential that we understand how *Atoh1* is regulated after hair cell damage and identify additional ways to enhance its expression and function in adult mammals.

In contrast to mammals, mature birds robustly regenerate hair cells after damage, and new hair cells restore hearing and balance function (reviewed in Bermingham-McDonogh and Rubel, 2003; Brignull et al., 2009). When hair cells die in the chicken auditory epithelium (called the basilar papilla), supporting cells regenerate hair cells using two mechanisms (reviewed in Stone and Cotanche, 2007). Initially, some supporting cells phenotypically convert into hair cells without dividing, via a process called direct transdifferentiation (Adler and Raphael, 1996; Baird et al., 1996; Roberson, 1996; Adler et al., 1997; Roberson et al., 2004; Cafaro et al., 2007). Then, additional supporting cells divide and their progeny differentiate into hair cells (Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994).

In the undamaged chicken inner ear, ATOH1-immunoreactive cells are detected only in vestibular epithelia (Cafaro et al., 2007), where new hair cells are continually formed (Jorgensen and Mathiesen, 1988; Roberson et al., 1992). In the basilar papilla, hair cells are not normally turned over (Corwin and Cotanche, 1988; Oesterle et al., 1993) and ATOH1-positive cells are not detected (Cafaro et al., 2007). However, shortly after the induction of hair cell damage, ATOH1 is upregulated in supporting cells, some of which are actively dividing (Cafaro et al., 2007). Later, ATOH1 is also found in differentiating hair cells, suggesting that its reactivation in supporting cells promotes transdifferentiation of supporting cells into hair cells. This idea is supported by Daudet et al. (2009), who showed that inhibition of notch signaling causes increased ATOH1 levels and hair cell production in the damaged basilar papilla. Based on the important role of ATOH1 in mammals and its dynamic expression patterns during avian hair cell regeneration, ATOH1 likely has a central role in supporting cell transdifferentiation in non-mammals.

In this study, we tracked the fate of supporting cells with *Atoh1* transcriptional activity, and we examined effects of *Atoh1* overexpression in supporting cells following streptomycin-induced hair cell damage in the post-hatch chicken basilar papilla in culture. A little more than half of supporting cells that transcriptionally activate *Atoh1* after hair cell damage had acquired hair cell features one week later.

Inhibition of notch promoted more cells with *Atoh1* activation to acquire the hair cell fate. Overexpression of *Atoh1* in supporting cells triggered only 66% of them to transdifferentiate into hair cells and nearly tripled the rate of their entry into the cell cycle. These findings demonstrate that, in some supporting cells, elevated *Atoh1* is insufficient to drive transdifferentiation. They suggest a potential role for *Atoh1* in controlling cell cycle entry in supporting cells. Further, our results suggest that unidentified notch-independent mechanisms may act to block hair cell differentiation. These molecular barriers may be similar to those that prevent hair cell regeneration in mature mammals.

## **2. Materials and Methods**

### **2.1. Animal Care**

Fertile White Leghorn chicken eggs were purchased from Featherland Farms (East Pearl Coburg, OR) and stored in a refrigerator until setting. Eggs were placed in a humidified incubator at 37°C and maintained until hatching. Hatchlings were housed in heated brooders with freely available food and water at the Virginia Merrill Bloedel Hearing Research Vivarium. All procedures were approved by the University of Washington Animal Care Committee.

### **2.2. Organ Culture Techniques**

7- to 10-day-old post-hatch chicks were decapitated. Auditory end organs, which are mature at this point of development, were explanted and cultured using methods developed by Oesterle et al. (1993) and modified by Shang et al. (2010). Streptomycin (Sigma-Aldrich; 78 µM) was present in culture media for the first two days after explantation, which caused complete hair cell loss (Shang et al., 2010). Supporting cells were electroporated (see Section 2.5), and organs were maintained in culture without streptomycin for various periods (2-11 days; **Fig. 1**). The media of some cultures contained bromodeoxyuridine BrdU (Sigma-Aldrich; 1 µM) to monitor supporting cell division. In other cultures, media contained gamma secretase inhibitor (DAPT; EMB Biosciences, 10 µM), which was sufficient to drive upregulation of *Atoh1* in chicken basilar papilla (Daudet et al., 2009), or its vehicle DMSO. *In vitro*, hair cell regeneration

followed a similar temporal progression as *in vivo*, and both mitotic and non-mitotic forms of regeneration are preserved (Shang et al., 2010).

### **2.3. *In Vivo* Drug-Damaged Basilar Papillae**

All electroporated basilar papillae required *in vitro* drug damage to occur, as explained above. For experiments using *in situ* hybridization (see below) chicks received subcutaneous injections of gentamicin (Sigma-Aldrich) at 100 mg/kg body weight on two consecutive days, then recovered in the animal care facility after injections for 1, 2 or 4 days post-gentamicin. All injected chicks exhibited transient systemic toxic effects of the gentamicin injections.

### **2.4. *In situ* hybridization**

*In situ* hybridization on whole-mount basilar papillae was conducted using digoxigenin-labeled cRNA probes on control (undamaged tissue) and on tissue at various times post-gentamicin treatment *in vivo*. Methods closely followed those described in Henrique et al. (1995) and Stone and Rubel (1999). Constructs for generating antisense probes to chicken *Atoh1* were obtained from Fernando Giraldez (University Pompeu Fabra, Barcelona).

### **2.5. Gene Delivery**

**Fig. 1** outlines the experimental timelines used in this study. Electroporation was used to deliver plasmid DNA into supporting cells using methods similar to those published in Daudet et al. (2009). Briefly, explanted auditory end organs were cultured with streptomycin for two days. Electroporation was performed on day two of culture, which ensured that the plasmid transfected supporting cells only. Electrodes were placed on either side of the cartilaginous plates flanking the basilar papilla, in proximal, middle, and apical regions. Several plasmids were used independently for each experimental group. Electroporation parameters were established using *pMES-IRES-GFP* (a gift from Catherine Krull, University of Michigan), which encoded GFP under the control of the CMV promoter,  $\beta$ -actin enhancer, and an internal ribosomal entry site (IRES).

In subsequent experiments, *pShuttle plasmid* (Stratagene) was used to engineer three constructs. First, *pShuttle-J2Xn-GFP* encoded GFP under the control of two mouse *Atoh1* enhancer elements (Lumpkin et al., 2003), the  $\beta$ -globin promoter, and a nuclear localization signal (Helms et al., 2000). We used this plasmid (a gift from Jane Johnson; University of Texas Southwestern Medical School) to identify cells with current or past endogenous *Atoh1* enhancer activity. The two mouse enhancer sequences, A and B, were 75% and 62% homologous to corresponding chicken sequences (Ebert et al., 2003). An identical reporter construct was used to generate transgenic mice for studies of hair cell development (Chen et al., 2002; Lumpkin et al., 2003) and faithfully reflected patterns of endogenous *Atoh1* transcription. Further, activity of this reporter was driven in regions of the chicken spinal cord where *Atoh1* is normally transcribed, suggesting cross-species fidelity (Ebert et al., 2003).

*pShuttle-Math1-IRES-GFP* encoded full-length mouse *Atoh1* homologue (*Math1*) and GFP under control of the CMV promoter, with GFP expression enabled by an IRES. We used this plasmid to induce *Atoh1* overexpression. A similar construct was used to drive *Math1* overexpression in mouse tissues (Farah et al., 2000; Gubbels et al., 2008). The bHLH domain of ATOH1 is highly conserved across species; proteins from mice and chicken share 95% identity (Ben-Arie et al., 1996). It is not surprising, therefore, that overexpression of *Atoh1* from one species readily drives ectopic sensory differentiation in another species (e.g., human or mouse *Atoh1* drives hair cell differentiation in rat utricles (Shou et al., 2003) or organ of Corti (Zheng and Gao, 2000), respectively), or that misexpression of mouse *Atoh1* can partially rescue the chordotonal organ phenotype in the *Drosophila Atoh1* mutant (Ben-Arie et al., 2000). The unmodified vector, *pShuttle-IRES-GFP*, served as a negative control for overexpression (*pShuttle-Math1-IRES-GFP*) and reporter (*pShuttle-J2Xn-GFP*) experiments.

Between two and five culture runs were performed for each experiment, and 8-10 basilar papillae were electroporated during each run.

## **2.6. Immunolabeling and Microscopic Imaging**

At the end of the culture period, auditory end organs were fixed with buffered 4% paraformaldehyde for 30 minutes, rinsed with phosphate buffered saline (PBS), and stored at 4°C. Organs were immunolabeled to detect the following antigens using standard immunofluorescence methods (Cafaro et al., 2007;

Daudet et al., 2009). Briefly, organs were immersed in blocking solution (10% normal goat serum) for 30 minutes and then placed in primary antibody solution where the antibody was diluted in normal goat serum. The following primary antibodies were used: rabbit polyclonal anti-myosin 6 (diluted 1/300; Proteus Biosciences), rabbit polyclonal anti-GFP (diluted 1/400, Invitrogen), mouse monoclonal anti-GFP (diluted 1/400; Invitrogen), rabbit polyclonal anti- $\beta$ III tubulin (diluted 1/1000; gift of Anthony Frankfurter, University of Virginia, Charlottesville, VA), mouse monoclonal anti-supporting cell antigen (SCA) and mouse monoclonal anti- $\beta$  tectorin precursor (diluted 1/1000, gift of Guy Richardson, University of Sussex, Brighton, United Kingdom; Goodyear et al., 1996), rat monoclonal anti-BrdU (diluted 1/500; SeraLabs), mouse anti-ATOH1 (diluted 1/300, from Developmental Studies Hybridoma Bank, Iowa City, IA), and rabbit polyclonal anti-ATOH1 (diluted 1/1-300; from Dr. Jane Johnson, University of Texas Southwestern Medical Center). The following secondary antibodies (all from Invitrogen) were used: goat anti-rabbit Alexa 594, goat anti-mouse 488, goat anti-rabbit 488, and mouse anti-rat 594, all diluted 1/300. Additionally, nickel chloride ( $\text{NiCl}_2$ ) enhancement of diaminobenzidine precipitation was implemented to allow for signal amplification for ATOH1 (Cafaro et al., 2007). Briefly, endogenous peroxidases were blocked using a 30-minute incubation in 1.5%  $\text{H}_2\text{O}_2$ . Tissue was permeabilized, and nonspecific antibody binding was inhibited using blocking solution (10% normal horse serum and 0.1% Triton X-100 dissolved in PBS, pH 7.4). Organs were counter-labeled for DAPI (0.1mg/ml 4,6-diamidino-2-phenylindole) to detect nuclei. Tissue samples labeled for ATOH1 with  $\text{NiCl}_2$  were temporarily mounted in PBS for imaging. All other organs were mounted on glass slides and coverslipped using Vectashield (Vector Laboratories). Organs were examined using an inverted Olympus Fluoview-1000 confocal microscope. Z-series 16-bit images of regions of the basilar papilla containing GFP-positive cells were taken at 20-60x at 512 pixel x 512 pixel resolution. Olympus files were converted to TIF formats using ImageJ. Some files were imported into Photoshop for image enhancement.

## **2.7. TUNEL labeling**

To detect apoptotic cells, terminal dUTP nick-end labeling (TUNEL) was performed using a TUNEL kit (*In situ* Apoptosis Detection kit; Millipore). The manufacturer's protocol was followed with minor modifications. TdT enzyme solution was diluted 3:10 with TUNEL reaction buffer (Millipore), and

incubated for 1.5h. Anti-digoxigenin conjugate label solution was diluted 1:1 with TUNEL blocking solution (Millipore), and incubation time was extended to 1 hour.

## 2.8. Cell Counts

Cell counts were performed offline. Basilar papillae were scanned to identify regions with GFP-positive (+) cells. GFP+ cells were only included in counts when they appeared healthy (i.e., had normal epithelial shapes and even GFP distribution) and when strong myosin 6- or BrdU-immunolabeling had occurred in the same region, indicating that hair cell differentiation and supporting cell division had proceeded *in vitro*. Each GFP+ cell was scored as “myosin 6+” or “myosin 6-negative (-)” or “BrdU+” or “BrdU-”. In addition, the shape of each GFP+ cell was noted. Between 5 and 15 basilar papillae per experimental group met these criteria and were included for counts (see **Table 1**). To account for the variability in the number of GFP+ cells across samples, we first determined the percent of GFP+ cells that developed immunoreactivity for myosin 6 or BrdU. Once the percentage was calculated for each basilar papilla, percentages were averaged across basilar papillae for the entire experimental group. A one-way analysis of variance (ANOVA) was performed to assess a difference in cell numbers across groups, and a Neuman-Keuls multiple comparison test was performed to assess between-group differences. Experiments that measured proliferation were examined using a separate analysis, with a two-tailed t-test to assess effects of *Atoh1* overexpression on cell proliferation. Differences were considered statistically significant if  $p \leq 0.05$ . All data are expressed as mean  $\pm$  standard deviation.

## 3. Results

### 3.1. *Atoh1* transcripts are upregulated in many supporting cells after hair cell damage

Undamaged, control basilar papillae had very low expression of *Atoh1* mRNA (**Fig. 2A,A'**). One day after *in vivo* gentamicin injection, there was a small increase in *Atoh1* mRNA in the proximal, damaged region, but there was no change in the apical, undamaged region (**Fig. 2B**). *Atoh1* was further upregulated in the damaged region at 2 and 4 days post-gentamicin (**Fig. 2C,D**). The pattern of *Atoh1* expression in the damaged region changed over time. At 1 and 2 days post-gentamicin, *Atoh1* mRNA was detected in

supporting cells (**Fig. 2B',C'**). At 4 days post-gentamicin treatment, *Atoh1* mRNA expression was lost from some cells and became elevated in others (**Fig. 2D'**). Observations by Cafaro et al. (2007) and others suggest that cells with elevated *Atoh1* expression are differentiating hair cells, while cells with low levels of *Atoh1* expression are supporting cells.

### **3.2. *Atoh1* transcriptional activation in supporting cells biases them toward transdifferentiation but does not guarantee they will acquire the hair cell fate**

Although *Atoh1* upregulation occurs broadly throughout the supporting cell population following hair cell damage, not every supporting cell will transdifferentiate into a hair cell during regeneration (e.g., Roberson et al., 1996). Therefore, we sought to examine the degree to which *Atoh1* transcriptional upregulation predicts hair cell fate using a basilar papilla culture system and an *Atoh1* enhancer reporter plasmid (pShuttle-J2Xn-GFP). pShuttle-J2Xn-GFP encodes GFP under control of the *Atoh1* enhancer (see Materials and methods for details). We eliminated mature hair cells using streptomycin *in vitro* (Daudet et al., 2009; Shang et al., 2010) and then delivered plasmids into supporting cells using electroporation. Organs were cultured for various periods, and the level and distribution of GFP expression were evaluated by fluorescence microscopy.

First, we determined the general pattern and efficiency of electroporation in basilar papilla cultures. We electroporated supporting cells at 2 days post-streptomycin with pMES-IRES-GFP, a plasmid driving GFP under control of the  $\beta$ -actin enhancer and the CMV promoter. This control reporter was expected to be active in all cells, irrespective of *Atoh1* expression. Basilar papillae were cultured for an additional 2 days without streptomycin, at which point they were fixed and immunolabeled to detect GFP. GFP+ cells were present in the basilar papilla in one quarter to one half of all electroporated organs. Using the pMES-IRES-GFP construct, between 20 and 180 supporting cells per basilar papilla were GFP+, and most cells were located in the neural half of the epithelium (**Fig. 3**).

Next, we electroporated drug-treated basilar papillae with pShuttle-J2Xn-GFP, fixed them two days later, and immunolabeled them with polyclonal antibodies to ATOH1. Many GFP+ cells had high levels of ATOH1 immunoreactivity (**Fig. 4A-A''**), consistent with its efficacy at reporting *Atoh1* expression. Next, additional drug-treated basilar papillae were electroporated with pShuttle-J2Xn-GFP and then

cultured for 7 or 11 days, when regenerated hair cells would be seen throughout the epithelium (Daudet et al., 2009; Shang et al., 2010). At both times, many GFP+ cells were immunoreactive for myosin 6 (**Fig. 4C–D'**) or  $\beta$ III tubulin (**Fig. 4E,E'**), which are two hair cell-specific markers (Hasson et al., 1997; Stone and Rubel, 2000). Many GFP+/myosin 6+ cells had round or oval cell bodies (**Fig. 4C–E'**) and small stereociliary bundles (**Fig. 4F,F'**), characteristic of immature hair cells (Cotanche, 1987; Duckert and Rubel, 1990). Additional GFP+ cells lacked myosin 6 labeling and they had elongated cell bodies typical of supporting cells (**Fig. 4G**), and they demonstrated immunoreactivity to a mixture of two anti-bodies to supporting cell markers, supporting cell antigen (SCA; Kruger et al., 1999) and  $\beta$ -tectorin precursor (Goodyear et al., 1996) (**Fig. 4H–J**).

In basilar papillae cultured for 7 days after transfection with the *Atoh1* reporter plasmid, 56 ( $\pm 9$ )% of GFP+ cells were myosin 6+. In contrast, only 4 ( $\pm 5$ )% of GFP+ cells were myosin 6+ after transfection of the negative control plasmid (pShuttle-IRES-GFP) (**Fig. 4B**). These numbers were significantly different ( $p \leq 0.05$ ). Our observations demonstrate that supporting cells with *Atoh1* transcriptional activity were more likely to differentiate as hair cells than more randomly sampled cells, but activation of *Atoh1* is not sufficient to drive all supporting cells to the hair cell fate. We were surprised to find that the percentage of cells with *Atoh1* enhancer activity that were hair cell-like decreased significantly in cultures between 7 and 11 days post-electroporation ( $p \leq 0.05$ ) (**Fig. 4B**). At 7 days, 56 ( $\pm 9$ )% of cells with *Atoh1* activity were myosin 6+, while at 11 days, only 18 ( $\pm 6$ )% were myosin 6+. Since most electroporated cells were located in the neural half of the epithelium, we were unable to systematically assess if this decrease in young hair cells occurred in a regional manner. We examined whether cell death may be involved by labeling basilar papillae for TUNEL at 9, 10, and 11 days post-electroporation ( $n = 7, 4,$  and 6 BPs at each time-point, respectively). TUNEL+ cells were present in small numbers at all times (data not shown). However, we noted no clear evidence that GFP+ cells were dying. Further, numbers of TUNEL+ cells seemed to be similar across all time-points. Additional study will be required to elucidate the mechanisms underlying the decrease in differentiating hair cells over time.

### 3.3. Inactivation of notch enables more *Atoh1*-activated supporting cells to transdifferentiate

Activation of the notch receptor antagonizes *Atoh1* transcription during development of the mouse organ of Corti (Lanford et al., 2000). Notch activity also appears to modulate *Atoh1* expression and supporting cell transdifferentiation into hair cells during regeneration in the mature basilar papilla (Daudet et al., 2009). To address if notch signaling also blocks *Atoh1* enhancer activity, we included the gamma secretase inhibitor DAPT (or DAPT vehicle, DMSO) in cultures for 7 days after delivery of the *Atoh1* reporter construct (pShuttle-J2Xn-GFP). Gamma secretase is a multi-unit protease required for all canonical notch activation and signal transduction (reviewed in Selkoe and Kopan, 2003), and 10  $\mu$ M DAPT blocks notch activity in cultured basilar papillae (Daudet et al., 2009). DAPT treatment caused a substantial increase in GFP+ cells across the papilla (**Fig. 5A,B**), suggesting that notch activity may normally block the *Atoh1* enhancer.

We also examined if notch prevents *Atoh1*-activated supporting cells from transdifferentiating into hair cells. The proportion of GFP+ cells (cells with *Atoh1* activity) that became myosin 6+ (i.e., developed hair cell features) was 73 ( $\pm$ 9) % in DAPT-treated cultures versus 45 ( $\pm$ 11) % in DMSO-treated cultures (**Fig. 5C-E**). This difference was statistically significant ( $p \leq 0.05$ ). Thus, inactivation of notch pushed more cells with *Atoh1* activity to transdifferentiate into hair cells than would otherwise do so. However, a substantial number of supporting cells with *Atoh1* activity still failed to transdifferentiate, which suggests a notch-independent mechanism may prevent some supporting cells from transdifferentiating into hair cells.

To determine if more *Atoh1*-activated cells may acquire hair cell features if more time were allotted while inhibiting notch, basilar papillae were cultured with DAPT or DMSO for 11 days after delivery of the *Atoh1* reporter. We found that 30 ( $\pm$ 7) % developed myosin 6 immunoreactivity when DAPT was present, which is significantly more than was seen when DMSO only was present (18 ( $\pm$ 7) %), as anticipated. However, in both groups, numbers were significantly reduced compared to those seen after a shorter period of DAPT treatment (73 ( $\pm$ 9) % with DAPT versus 45 ( $\pm$ 11) % with DMSO, at 7 days) (**Fig. 5E**). These data collectively show that, over time, the proportion of *Atoh1*-activated cells that maintain hair cell phenotypes is reduced. This is similar to what we saw with true controls (no DMSO added) in **Fig. 4**. These data suggest that, although supporting cells demonstrate transcriptional activity

of *Atoh1*, these cells may not sustain their differentiation as a hair cell or survive, even under conditions when notch activity is reduced.

### **3.4. *Atoh1* overexpression pushes many, but not all supporting cells to transdifferentiate**

In the next set of experiments, we examined the degree to which forced overexpression of *Atoh1* drives supporting cells to transdifferentiate into hair cells. In initial experiments, we electroporated basilar papillae at 2 days post-streptomycin with the pShuttle-Math1-IRES-GFP plasmid, which encodes full-length mouse *Atoh1* homologue (*Math1*) and GFP under control of the CMV promoter (see *Materials and Methods* for details). This plasmid should drive ATOH1 expression up to or above normal physiological levels. To verify this, we examined basilar papillae at 2 days after electroporation of pShuttle-Math1-IRES-GFP plasmid. Supporting cells were indeed highly immunoreactive for ATOH1 using two different anti-ATOH1 antibodies (**Fig. 6A-B''**).

To examine the fate of supporting cells with forced mouse *Atoh1* overexpression, we allowed basilar papillae to remain in culture for 7 days after electroporation with pShuttle-Math1-IRES-GFP or control plasmid (pShuttle-IRES-GFP). GFP+ cells were scored as myosin 6+ or myosin 6-. In basilar papillae transfected with pShuttle-Math1-IRES-GFP, 66 ( $\pm 7$ ) % of GFP+ cells were myosin 6+, compared to 4 ( $\pm 5$ ) % in basilar papillae transfected with control plasmid (**Fig. 6C-E**). Thus, overexpression of mouse *Atoh1* significantly increased the number of chicken supporting cells that normally transdifferentiated into hair cells, but it was not sufficient to drive all supporting cells to do so.

### **3.5. *Atoh1* overexpression increases a supporting cell's likelihood to enter the cell cycle**

In the regenerating chicken basilar papilla, approximately 20% of supporting cells with ATOH1 immunoreactivity were mitotically active (Cafaro et al., 2007). We investigated whether ATOH1 levels influence supporting cell entry into the cell cycle. Basilar papillae were cultured with streptomycin for 2 days and electroporated with pShuttle-Math1-IRES-GFP. Then, basilar papillae were cultured an additional 3 days without streptomycin, with BrdU present in media for the last 2 days in culture. After this treatment, 17 ( $\pm 6$ ) % of GFP+ cells were BrdU+ (**Fig. 7B**). In contrast, after transfection with negative control plasmid (pShuttle-IRES-GFP), only 6 ( $\pm 4$ ) % of GFP+ cells were BrdU+ (**Fig. 7A**). These numbers

were significantly different ( $p \leq 0.05$ ). These findings demonstrate that driving high ATOH1 levels substantially increases a supporting cell's likelihood of entering the cell cycle after hair cell damage.

#### **4. Discussion**

This study used *in vitro* organ culture techniques to examine the role of *Atoh1* in hair cell regeneration in the avian basilar papilla. First, we examined endogenous *Atoh1* transcriptional activity and its correlation with the acquisition of the hair cell phenotype. Second, we examined the effect of increasing ATOH1 levels on hair cell differentiation, using two complementary strategies, notch pathway inhibition and *Atoh1* overexpression. Third, we examined the effect of *Atoh1* overexpression on supporting cell division.

##### **4.1. Only half of supporting cells with elevated *Atoh1* transcriptional activity successfully transdifferentiate into hair cells**

ATOH1 is a powerful regulator of hair cell differentiation that is both necessary and sufficient for embryonic hair cell production in the inner ear (Bermingham et al., 1999; Zheng and Gao, 2000; Gubbels et al., 2008). We found that *Atoh1* transcripts are very low in the undamaged mature basilar papilla. However, *Atoh1* is transcriptionally upregulated in supporting cells one day after gentamicin treatment, and *Atoh1* transcripts are elevated in many supporting cells by 2 days post-gentamicin. Similar results were found with ATOH1 protein (Cafaro et al., 2007). Yet, only some supporting cells will transdifferentiate into new hair cells, by either mitotic or non-mitotic processes (Roberson et al., 1996). Using cell tracing analysis, we found that only 56% of transfected supporting cells with elevated *Atoh1* enhancer activity converted into hair cell-like cells after 7 days in culture, while many cells with elevated *Atoh1* enhancer activity maintained supporting cell features. Prolongation of the culture period did not result in differentiation of more hair cells. Rather, the percent of cells with *Atoh1* activity that were hair cell-like at 11 days (18%) was significantly lower than at 7 days (56%). The reason for this shift is unclear, but it suggests that some cells with hair cell features may have reverted to a supporting cell phenotype or died. A high degree of plasticity in the mature chicken basilar papilla has been demonstrated (reviewed in Morest and Cotanche, 2004), so the idea that some young hair cells may dedifferentiate, redifferentiate, or die during the process of regeneration is not new. Using the current methods, we were unable to

examine dedifferentiation or redifferentiation of new hair cells. Although we detected dying cells in regenerating basilar papillae during the period of interest, we obtained no definitive evidence that hair cell precursors were dying. Therefore, each of these possible mechanisms remains untested and should be addressed in future studies.

The exact role of the early, broad upregulation of *Atoh1* transcription in the process of determination of hair cell fate remains unclear. One previous study suggested *Atoh1* defines a prosensory equivalence group in the developing mouse organ of Corti (Woods et al., 2004). The early reactivation of *Atoh1* in supporting cells after hair cell damage in mature animals may represent a re-establishment of this equivalence group, to render cells competent for new sensory cell production. Regardless of the role of early *Atoh1* reactivation in regeneration, it is clear that many supporting cells with *Atoh1* activation are subsequently diverted away from the hair cell fate.

#### **4.2. High *Atoh1* expression is not sufficient to assure supporting cell transdifferentiation**

We hypothesized that some supporting cells with *Atoh1* activation do not fully transdifferentiate into hair cells because they fail to accumulate sufficient ATOH1 to complete the conversion. To investigate this, we used two methods to drive up *Atoh1* levels in supporting cells: inhibition of notch activity or direct overexpression of *Atoh1*. As anticipated, an increased number of supporting cells acquired hair cell properties when *Atoh1* levels were amplified via either method, compared to controls. However, many supporting cells with elevated *Atoh1* maintained their phenotype. This observation indicated that, in some supporting cells, high *Atoh1* expression was insufficient to drive transdifferentiation. Additionally, we found that some supporting cells that upregulated *Atoh1* and acquired hair cell features did not maintain their hair cell identity over time. Related observations in mature inner ear epithelia have been reported by other investigators. In adult rat utricles, only 71% of supporting cells transfected with full-length *Atoh1* developed immunoreactivity for the hair cell marker, myosin 7a (Shou et al., 2003). In adult mouse utricles, transduction of supporting cells with *Atoh1* significantly increased, but did not fully restore, hair cell numbers (Staecker et al., 2007). Inactivation of notch with DAPT was sufficient to promote hair cell regeneration in only limited regions of the post-hatch chicken basilar papilla (Daudet et al., 2009) and the

adult mouse utricle (Lin et al., 2011). Perhaps most significant with respect to developing treatments for sensorineural deafness and balance deficits, *Atoh1* overexpression in the highly damaged, flat organ of Corti stimulated no new hair cell differentiation (Izumikawa et al., 2008). These results point to a potential shortcoming of *Atoh1* overexpression or notch inhibition as therapeutic tools for inducing hair cell regeneration in mammals. Therefore, it is important to elucidate the factors besides notch that limit supporting cell responsiveness to *Atoh1* misexpression.

Several factors may antagonize the full conversion of supporting cells into lasting hair cells following *Atoh1* misexpression. Some supporting cells may be terminally differentiated. Or, signals from the microenvironment may prevent ATOH1 accumulation in some cells. For example, bone morphogenetic proteins (BMPs) block *Atoh1* expression in the developing chicken inner ear (Pujades et al., 2006), and they promote ATOH1 degradation in other cells (Zhao et al., 2008). Transcriptional repressors of *Atoh1* may be differentially active among supporting cells in the injured basilar papilla. Doetzlhofer et al. (2009) showed that transdifferentiation in supporting cell subtypes of the developing mouse organ of Corti is blocked by distinct transcriptional repressors. The differentiated state of Deiters' cells and inner phalangeal cells is maintained by the bHLH transcription factors HES1 and HES5, which repress sensory differentiation in response to notch activity (reviewed in Kageyama et al., 2008). By contrast, pillar cells are maintained collectively by HES1/5 and HEY2. Distinct extracellular signals (notch and fibroblast growth factor) work to regulate expression of these transcription factors in each cell type. Additional bHLH transcription factors, such as neurogenin1 and neuroD, appear to antagonize *Atoh1* transcription during development of the mouse inner ear (Raft et al., 2007; Jahan et al., 2010; Fritsch et al., 2011). An interplay between these transcription factors is likely to also be active while cells transdifferentiate and attain stable fates following hair cell damage in the mature chicken basilar papilla. Future studies should seek to characterize both intrinsic and extrinsic signals controlling *Atoh1* expression and other signals influencing transdifferentiation. It is likely that the most effective strategy for stimulating hair cell regeneration in the mammal will require additional manipulations aside from *Atoh1* misexpression alone.

#### **4.3. High *Atoh1* expression increases the likelihood that supporting cells will enter the cell cycle**

We were surprised to find that *Atoh1* overexpression appears to increase a supporting cell's likelihood of entering the cell cycle. ATOH1 is a proneural transcription factor that controls the state of cellular differentiation. This function for ATOH1 is well documented in the developing inner ear (e.g., Bermingham et al., 1999; Zheng and Gao, 2000; Woods et al., 2004). Its role in regulating cell division remains largely unexplored. In the developing cochlea, ATOH1 protein becomes detected after hair cell progenitors have exited the cell cycle (Chen et al., 2002), which argues against a role for ATOH1 in controlling cell cycle in that organ. By contrast, ATOH1 is expressed in some dividing supporting cells in the regenerating chicken basilar papilla and utricle (Cafaro et al., 2007). Recently, a proneural bHLH transcription factor similar to ATOH1, called ASCL1, was shown to drive proliferation of neural progenitors by positively regulating E2f1 and FoxM1, which control cell cycle entry and cell cycle progression, respectively (Castro and Guillemot, 2011). In addition, factors downstream from ASCL1 can rapidly induce cell cycle exit. This finding suggests that proneural transcription factors such as ATOH1 could play direct roles in controlling the supporting cell's proliferative status or capacity. This possibility should be more carefully explored in future studies.

#### **4.4. Additional Considerations**

It is important to acknowledge that this study utilized primarily *in vitro* organ culture methods to examine the role of ATOH1 in hair cell regeneration. While both mitotic and non-mitotic hair cell regeneration proceed in organ cultures of the basilar papilla (Shang et al., 2010), culture conditions may significantly alter normal processes associated with cellular division and/or differentiation. Further, although current studies point to the contrary, the use of mouse-derived plasmids to examine chicken cellular behavior may have unseen limitations and therefore may have important implications for the observations we made in this study. Future studies should seek to further elucidate the role of ATOH1 in hair cell regeneration using *in vivo* models.

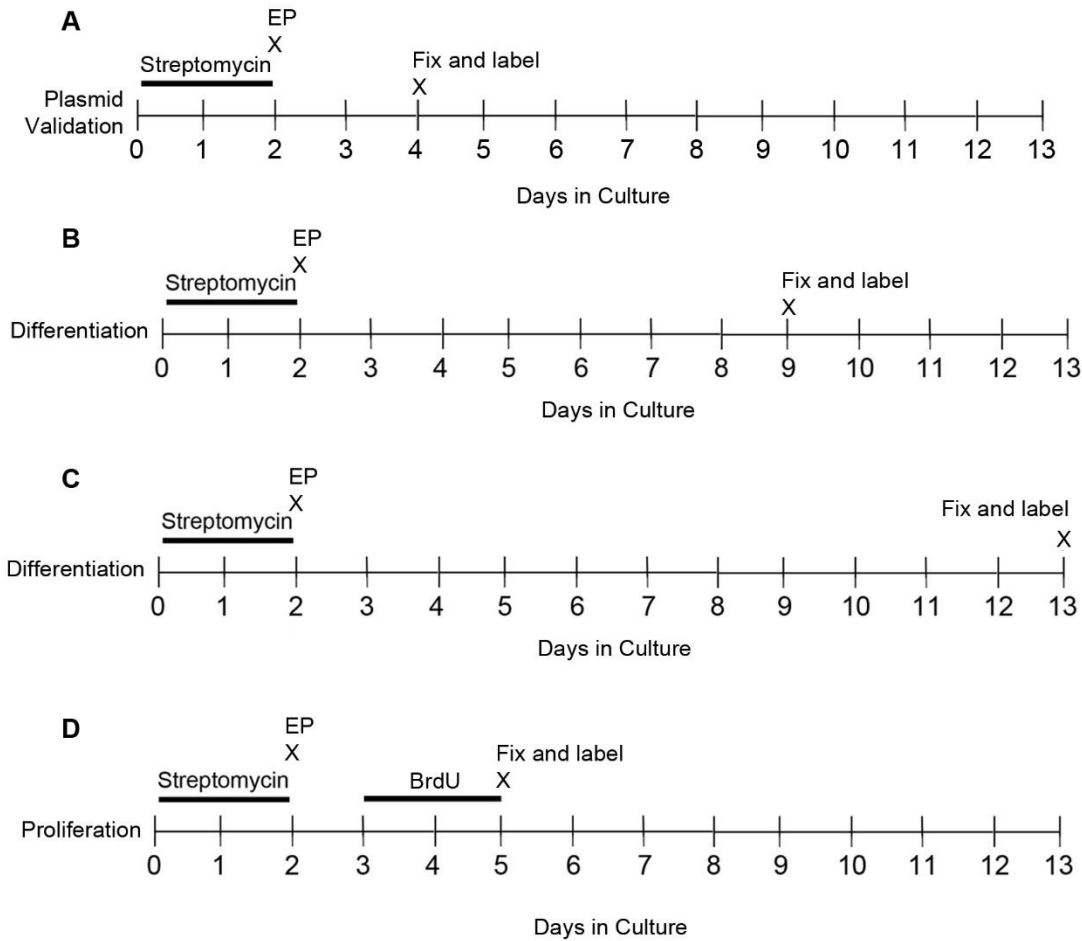
## 5. Conclusions

In the post-hatch chicken basilar papilla, *Atoh1* transcription is broadly upregulated in supporting cells after hair cell damage, but only a little more than half of these cells appear to acquire the hair cell fate. Forced upregulation of *Atoh1* by gene transfection or notch pathway inhibition is not sufficient to push all supporting cells to transdifferentiate into hair cells. Further, *Atoh1* overexpression increases the likelihood of supporting cell re-entry into the cell cycle. Overall, it appears that elevated *Atoh1* or inhibition of notch signaling is insufficient to drive and maintain transdifferentiation of supporting cells into hair cells. It will be important to identify additional factors besides notch activity that antagonize supporting cell transdifferentiation into hair cells and to define the mechanisms by which ATOH1 influences supporting cell division.

Days in culture (ICC Label)	pShuttle-IRES-GFP		pShuttle-Math1-IRES-GFP		pShuttle-J2Xn-GFP		pShuttle-J2Xn-GFP, with DMSO		pShuttle-J2Xn-GFP, with DAPT	
	BPs	GFP+ cells	BPs	GFP+ cells	BPs	GFP+ cells	BPs	GFP+ cells	BPs	GFP+ cells
7 days (Myo6/GFP)	6	152 total 9-75/BP	15	271 total 6-72/BP	8	221 total 4-82/BP	5	325 total 25-98/BP	10	641 total 3-228/BP
11 days (Myo6/GFP)	-	-	-	-	12	528 total 5-89/BP	9	1262 total 8-292/BP	12	1896 total 17-668/BP
3 days (BrdU/GFP)	15	233 total 3-53/BP	15	599 total 3-127/BP	-	-	-	-	-	-

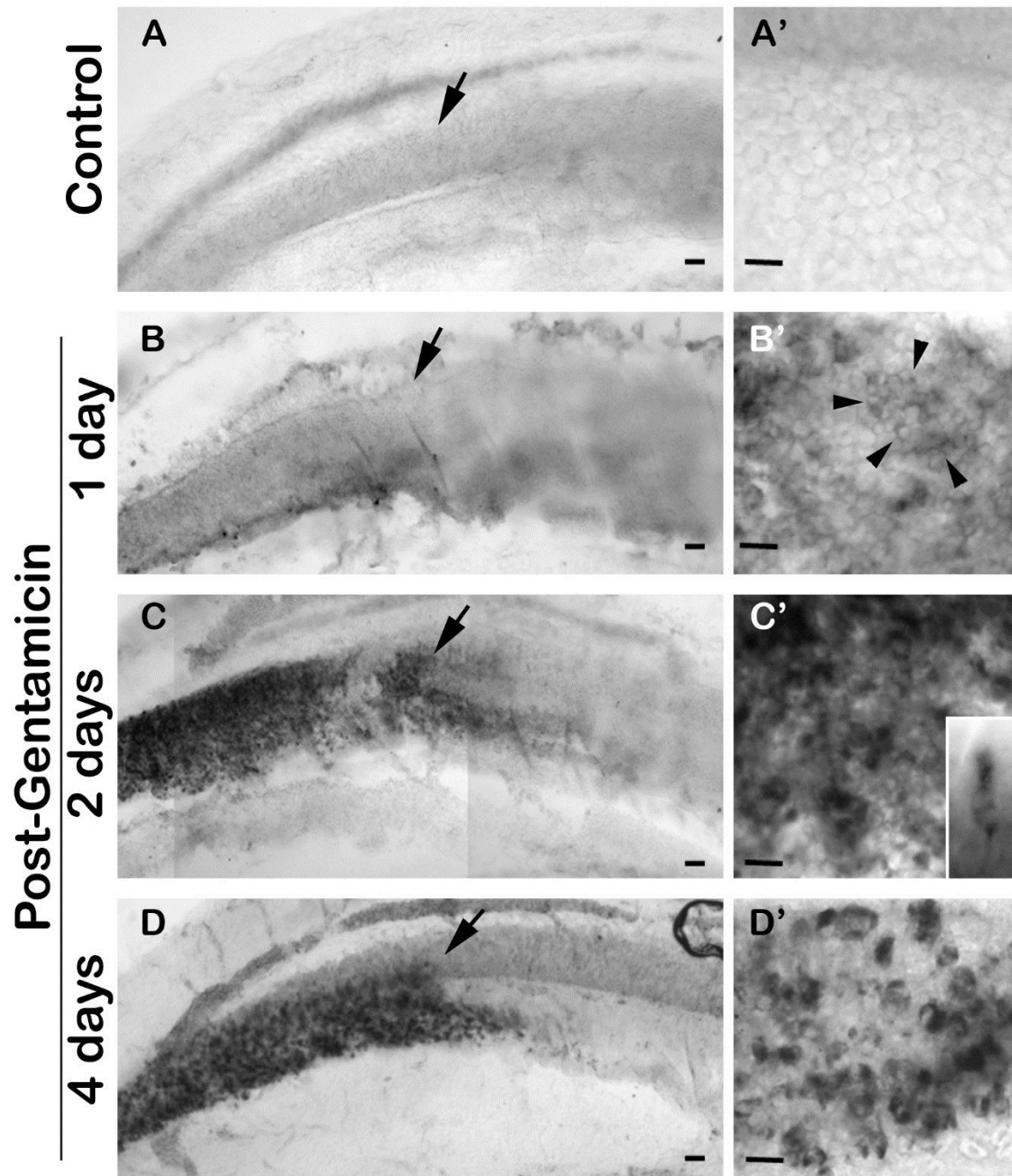
**Table 1**

Numbers of basilar papillae (BPs) and GFP+ cells used for quantitative analyses for each experimental group. Experimental groups (including plasmid used and additional treatments) are provided in the top row. For each experimental group, we also indicate the number of BPs and the number of GFP+ cells that were analyzed. GFP+ cells are expressed as the total number analyzed for the group (i.e., pooled across all of the BPs in the group) or as the range of GFP+ cells analyzed per BP. The time of fixation and the immunolabeling scheme (in parentheses) for each group are shown in the left column.



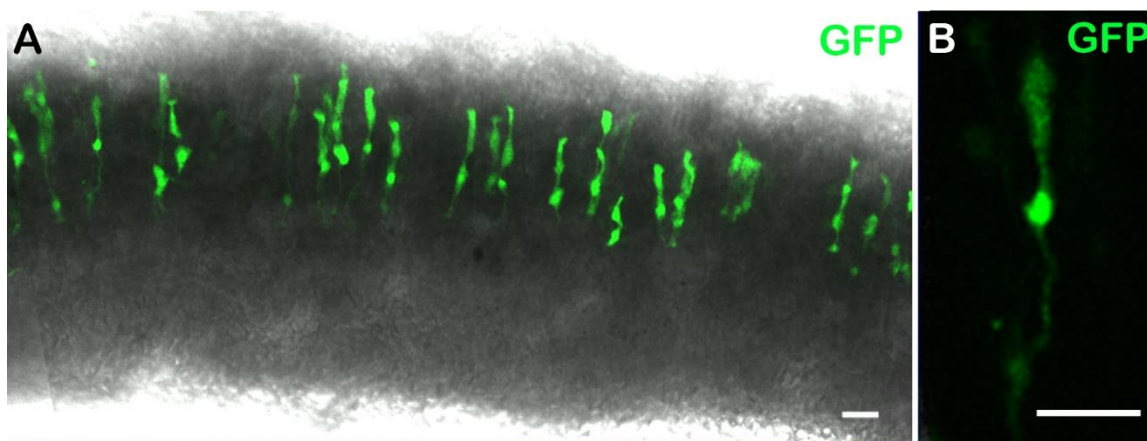
**Fig. 1. Experimental design.** The four experimental designs (A-D) used in this study are described. For all four designs, basilar papillae were explanted at day 0 and cultured with streptomycin for 2 days, at which point electroporation (EP) was performed. After EP, different steps were followed, as indicated for each experiment. A. Basilar papillae were fixed 2 days after EP (equivalent to 4 days in culture). This design was used for: 1) examination of general transfection patterns using pMES-IRES-GFP; 2) co-localization of Atoh1 enhancer reporter and ATOH1 protein using pShuttle-J2Xn-GFP; or 3) examination of effects of Atoh1 overexpression on ATOH1 protein using pShuttle-Math1-IRES-GFP or control vector (pShuttle-IRES-GFP). B. Basilar papillae were fixed 7 days post-EP (equivalent to 9 days in culture). This design was used to: 1) follow the fate of supporting cells with Atoh1 transcriptional activity using pShuttle-J2Xn-GFP (in control media, or media containing DMSO or DAPT) or control vector, pShuttle-IRES-GFP; or 2) examine effects of Atoh1 overexpression on supporting cell transdifferentiation using pShuttle-

Math1-IRES-GFP or control vector pShuttle-IRES-GFP. C. Basilar papillae were fixed 11 days post-EP (equivalent to 13 days in culture). This design was used to follow the fate of supporting cells with Atoh1 transcriptional activity using pShuttle-J2Xn-GFP in: 1) control media, 2) media containing DMSO, or 3) media containing DAPT. D. After EP, basilar papillae were maintained for 1 day with no additives, followed by 2 days with BrdU, and then fixed on the third day after EP (equivalent to 5 days in culture). This design was used to examine effects of Atoh1 overexpression on supporting cell proliferation using pShuttle-Math1-IRES-GFP or control vector pShuttle-IRES-GFP.

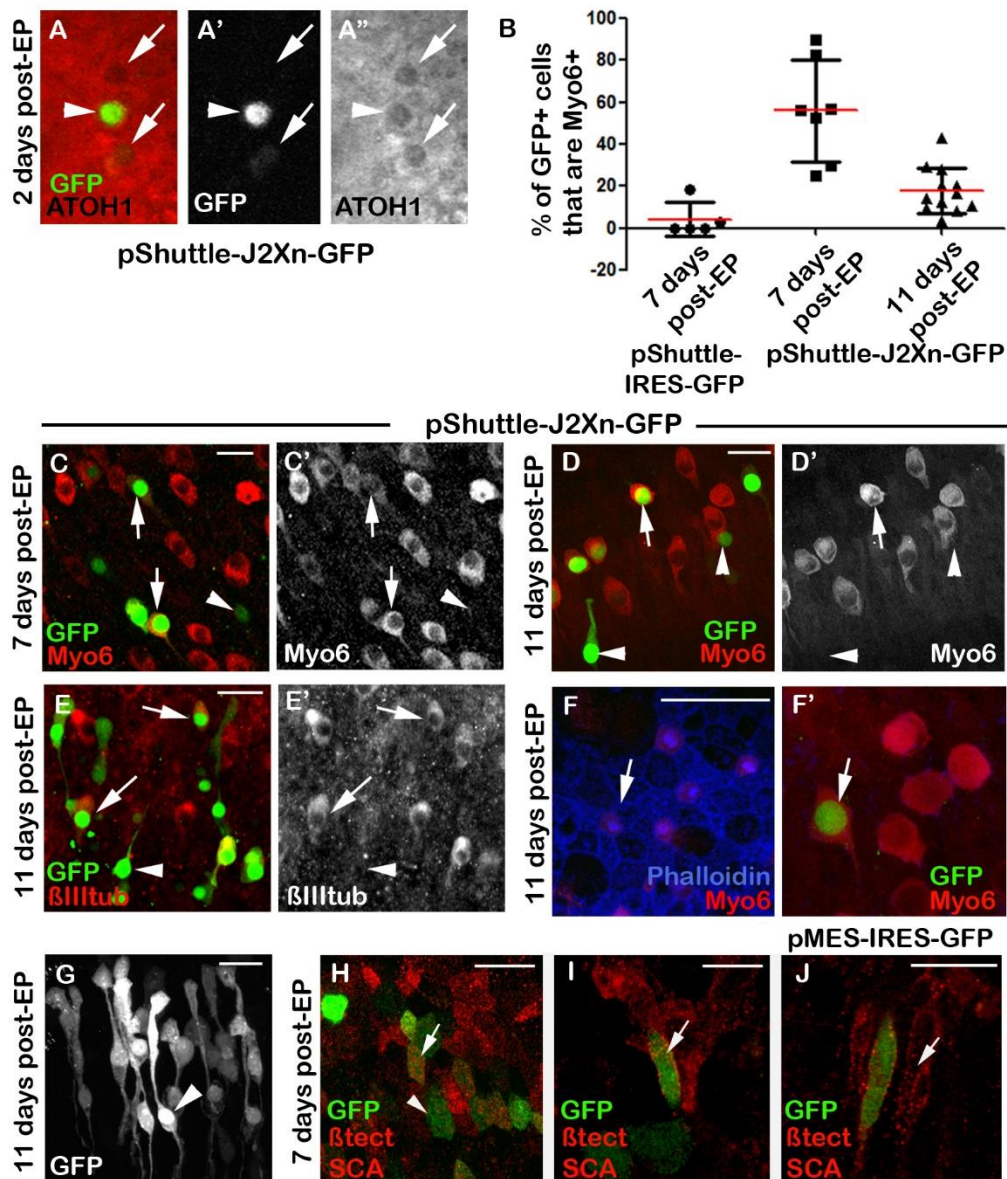


**Fig. 2.** *Atoh1* expression in control and damaged basilar papillae. A-D show low-magnification views of the basilar papilla from control chickens (A) and from chickens at 1 day (B), 2 days (C), and 4 days (D) post-gentamicin that had *Atoh1* mRNA labeling via *in situ* hybridization. Arrows indicate transitional zone between hair cell damage and loss (on left side, which is proximal) and little or no hair cell damage or loss (on the right side, which is apical). A'-D' show higher magnification images of areas within the damaged

region (to the left of the arrow in A-D). Arrowheads in B' indicate areas of hybridization. The inset in C' shows a pair of presumed immature hair cells with elevated *Atoh1* hybridization. Scale bars = 15  $\mu$ m.

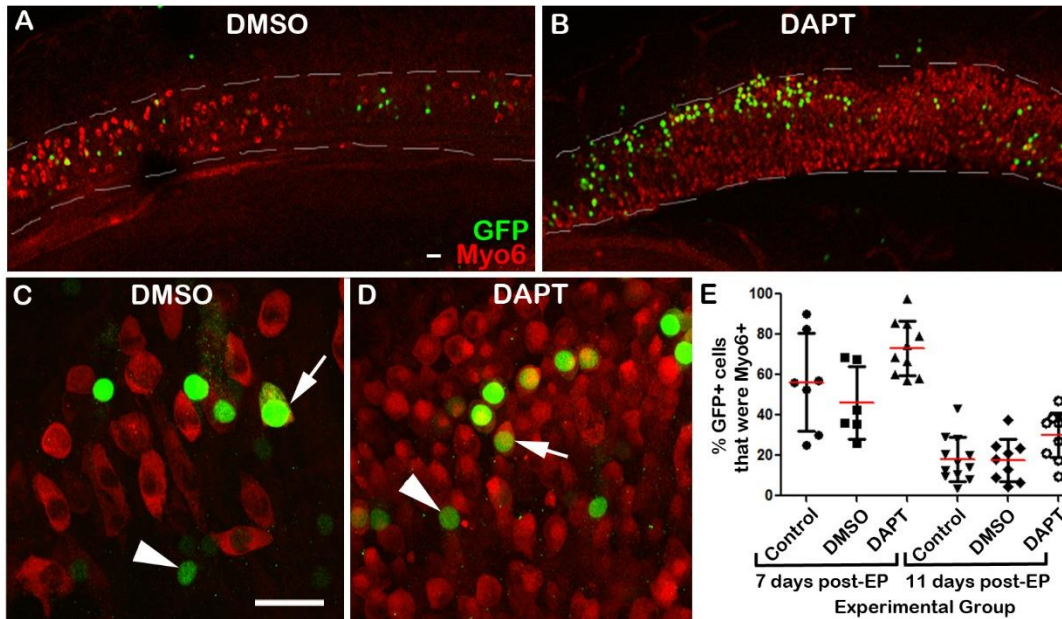


**Fig. 3.** Electroporation of supporting cells in the drug-damaged basilar papilla. A. Low-magnification image of a basilar papilla with many supporting cells transfected with the pMES-IRES-GFP construct. Gray is transmitted light signal, and the dark gray shape defines the basilar papilla. The neural side is toward the top, and the apical end is toward the right. B. An exemplary GFP+ cell, with an elongated shape characteristic of a supporting cell. Scale bars = 15 mm.

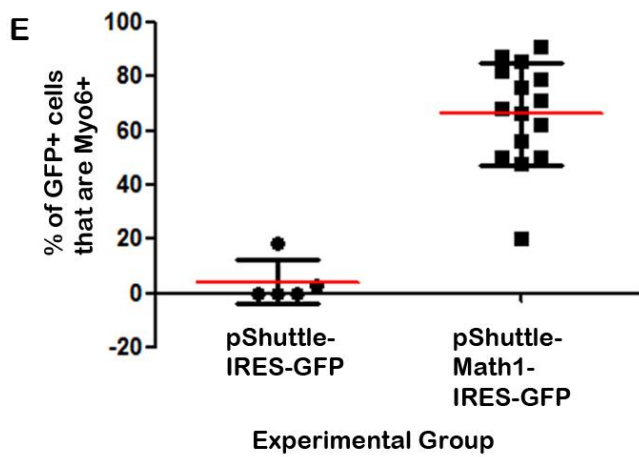
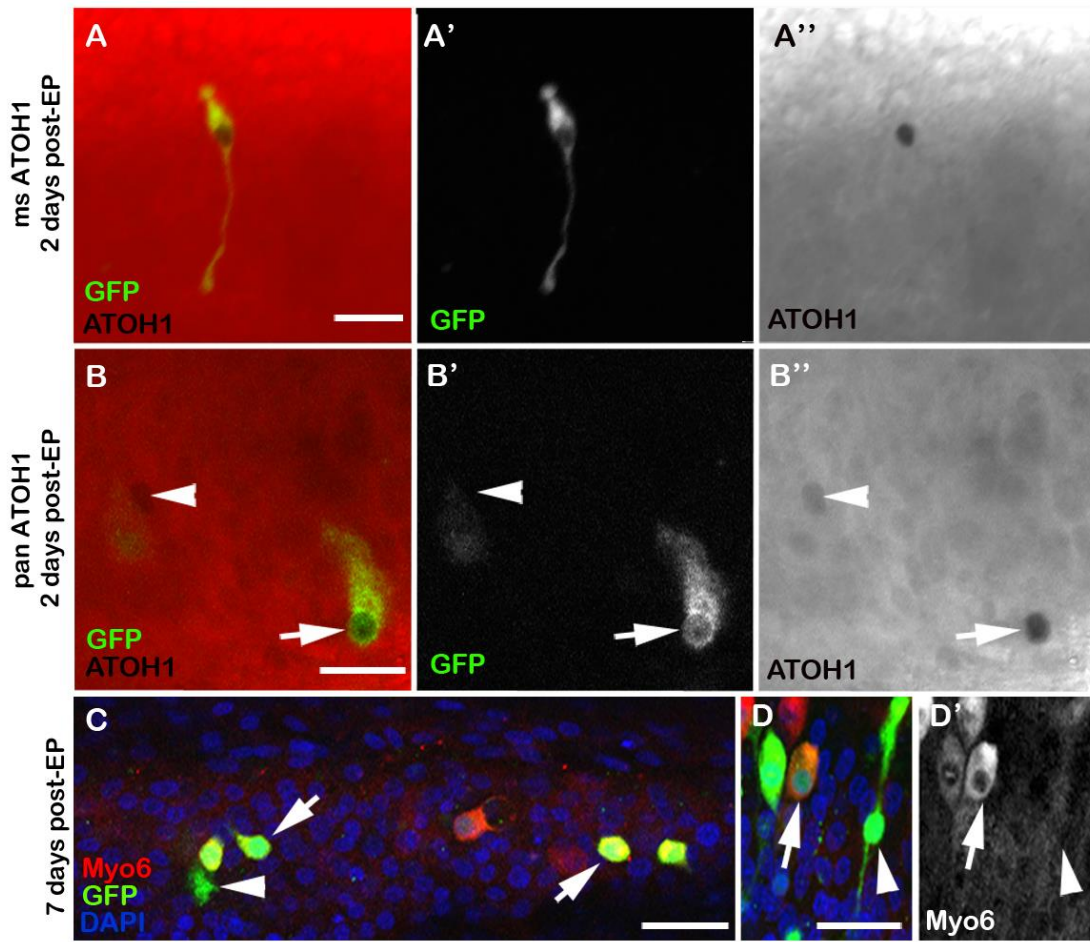


**Fig. 4.** Fate of supporting cells with *Atoh1* transcriptional activity. Panels A, C-I show basilar papillae transfected with pShuttle-J2Xn-GFP and maintained in culture for different periods. J shows tissue electroporated with pMES-IRES-GFP and cultured for 7 days afterward. A-A'' show supporting cells transfected with pShuttle-J2Xn-GFP and fixed 2 days after electroporation, then labeled with polyclonal antibodies to ATOH1. The arrowhead points to a GFP+/ATOH1+ cell, and arrows point to GFP-/ATOH1+

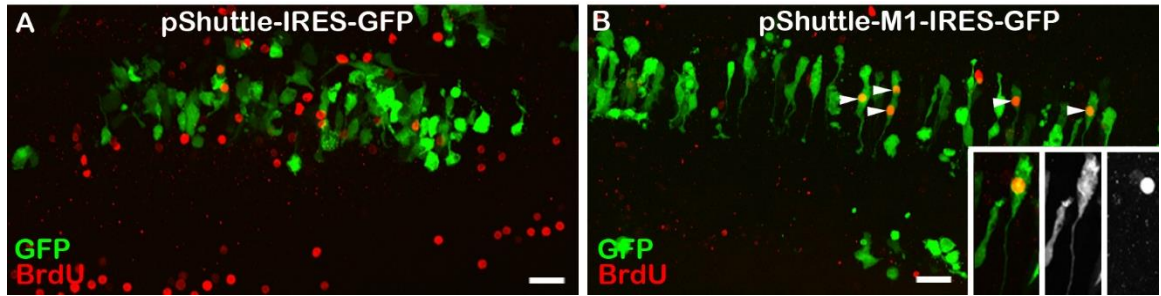
cells. Note the similar level of ATOH1 immunolabeling between GFP+ and GFP- cells. B is a graph of the % of GFP+ cells that were Myo6+ (averaged across basilar papillae) for each experimental group. Each point on the graph represents the average % for a given basilar papilla. Horizontal red bars indicate the weighted average for the group. Error bars indicate one standard deviation from the mean. The numbers of basilar papillae and GFP+ cells used for this analysis are detailed in **Table 1**. C, C' show myosin 6 (Myo6) and GFP labeling in the same optical slice from a basilar papilla fixed at 7 days post-electroporation (EP). Arrows point to examples of Myo6+/GFP+ cells, and arrowheads indicate examples of Myo6-/GFP+ cells. D, D' follow the format set in A-A', except tissue was fixed at 11 days post-EP. E, E' show  $\beta$ III tubulin ( $\beta$ III tub) and GFP labeling in the same optical slice from a culture fixed at 11 days post-EP. Arrows point to examples of  $\beta$ III tub+/GFP+ cells, and arrowheads point to examples of  $\beta$ III tub-/GFP+ cells. F, F0 show phalloidin, Myo6, and/or GFP labeling in the same visual field from a culture fixed at 11 days post-EP. F is focused at the level of the hair cell stereocilia, and F0 is focused at the hair cell nucleus. Arrow points to a Myo6+/GFP+ cell with a distinct bundle of phalloidin+ immature stereocilia. G. GFP labeling in a basilar papilla at 11 days post-EP. Arrow points to a cell with the classic supporting cell shape. H-J show labeling with a cocktail of anti-SCA and anti- $\beta$ -tectorin ( $\beta$ tect) precursor antibodies at 7 days after EP with pShuttle-J2Xn-GFP (H, I) or pMES-IRES-GFP (J). H is an en face view of the luminal surface of the basilar papilla, and I, J are vertical slices through the epithelium. Arrows point to SCA- $\beta$ tect+/GFP+ cells, and arrowhead points to SCA- $\beta$ tect-/GFP+ cells. Scale bars = 15  $\mu$ m.



**Fig. 5.** Effect of notch inhibition on the fate of cells with *Atoh1* transcriptional activation. A-D. Images from basilar papillae cultured with either DMSO (A, C) or 10  $\mu$ M DAPT (B, D) that were labeled with antibodies to GFP and myosin 6 (Myo6) at 7 days post-EP. In A, B, note the higher numbers of GFP+ cells and Myo6+ cells in the DAPT-treated epithelium. Arrows in C,D point to examples of GFP+/Myo6+ cells, and arrowheads point to examples of GFP+/Myo6- cells. E shows a graph of the % of GFP+ cells that were Myo6+ (averaged across basilar papillae) for each experimental group. Each point on the graph represents the average % for a given basilar papilla. Horizontal red bars indicate the weighted average for the group. Error bars indicate one standard deviation from the mean. The numbers of basilar papillae and GFP+ cells used for this analysis are detailed in **Table 1**. Scale bars = 15  $\mu$ m.



**Fig. 6.** Fate of supporting cells with *Atoh1* overexpression. All basilar papillae shown in this figure were electroporated with pShuttle-Math1-IRES-GFP. A-A". Immunolabeling for GFP and mouse ATOH1 in the same optical slice of a basilar papilla at 2 days post-EP. B-B". Immunolabeling for GFP and pan-ATOH1 (including chicken and mouse ATOH1) in the same optical slice of a basilar papilla at 2 days post-EP. Arrows show a GFP+ cell and arrowheads show a GFP- cell. C demonstrates a typical field in the basilar papilla imaged for this experiment, with the arrows pointing to examples of GFP+/Myo6+ cells and the arrowhead pointing to an example of a GFP+/Myo6- cell. Panels D-D' show the same optical field, with a GFP+ supporting cell that maintained its phenotype 7 days post-EP (arrowheads) and a GFP+ cell that converted into a hair cell (arrows). E is a graph of the % of GFP+ cells that were Myo6+ (averaged across basilar papillae) for each experimental group. Each point on the graph represents the average % for a given basilar papilla. Horizontal red bars indicate the weighted average for the group. Error bars indicate one standard deviation from the mean. The numbers of basilar papillae and GFP+ cells used for this analysis are detailed in **Table 1**. Scale bars = 15  $\mu$ m.



**Fig. 7.** Effect of *Atoh1* overexpression on supporting cell proliferation. Images show GFP and BrdU labeling in the middle region of basilar papillae that were electroporated with either pShuttle-IRES-GFP (A) or pShuttle-Math1 (M1)-IRES-GFP (B). The neural side of the basilar papilla is toward the top, and apical end of the basilar papilla is toward the right. Arrowheads in B point to cells that are GFP+/BrdU+. Insets in B show an example of a cell that is GFP+/BrdU+. Scale bars = 15  $\mu$ m.

## Chapter 3

### Bone Morphogenetic Protein 4 Antagonizes Hair Cell Regeneration After Damage in the Avian Auditory Epithelium

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#### ABSTRACT

Permanent hearing loss is often a result of damage to cochlear hair cells, which mammals are unable to regenerate. Non-mammalian vertebrates such as birds regenerate hair cells and restore hearing function. To examine mechanisms that allow for hair cell regeneration in the avian inner ear, we examined expression and function of bone morphogenetic protein 4 (BMP4) in the chicken auditory epithelium (basilar papilla) after hair cell destruction with aminoglycoside antibiotics. BMP4 regulates inner ear morphogenesis and antagonizes hair cell development in sensory organs. In mature basilar papillae, *Bmp4* mRNA is highly expressed in hair cells and is lost upon hair cell destruction. Receptors for BMP4, and effectors of BMP4 signaling, are also transcriptionally altered after hair cell loss. Experiments using organotypic cultures demonstrate BMP4 antagonizes damage-induced upregulation of *Atoh1* – a transcription factor that drives hair cell differentiation and blocks hair cell regeneration – without affecting cell survival. By contrast, noggin, a BMP4 inhibitor, increased numbers of regenerated hair cells. These results demonstrate BMP4 is a potent antagonist of hair cell regeneration in the chicken basilar papilla, perhaps through inhibition of *Atoh1* transcription.

## **SIGNIFICANCE STATEMENT**

Sensorineural hearing loss (SNHL) is caused by loss of auditory hair cells, the sensory receptors for hearing. Hair cells are not regenerated in any mammals, but regeneration would likely improve hearing in humans with SNHL. Since birds spontaneously regenerate hair cells, they provide opportunities to study how hair cell regeneration is regulated. ATOH1 is a transcription factor that promotes mammalian hair cell regeneration when misexpressed in hair cell progenitors in some contexts. This study sought to identify factors restricting ATOH1 efficacy in driving supporting cells to form new hair cells in chickens. We show that bone morphogenetic factor 4 (BMP4) blocks upregulation of *Atoh1* in progenitor cells after damage and prevents hair cell regeneration without impacting cell survival.

## **INTRODUCTION**

Humans sustain permanent sensorineural hearing loss through loss or dysfunction of cochlear hair cells, neurons of the cochlear nerve, and/or nuclei of the central auditory nervous system. Hair cells are sensory mechanoreceptors for hearing that are located in the inner ear. They are easily damaged by ototoxic drugs, noise exposure, and aging. Excessive damage to hair cells leads to their extrusion from the auditory epithelium (Winther, 1970; Ryan et al., 1980; Bird et al., 2010). Mature mammals cannot replace cochlear hair cells (e.g., Roberson and Rubel, 1994; Chardin and Romand, 1995; Sobkowicz et al., 1997), and therefore hearing deficits caused by hair cell loss are permanent. By contrast, birds regenerate auditory hair cells and restore hearing within a few weeks (Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; reviewed in Bermingham-McDonogh and Rubel, 2003; Stone and Cotanche, 2007). Avian auditory hair cells reside in the basilar papilla (BP), a sensory epithelium located in the cochlear duct. Replacement hair cells are derived from adjacent supporting cells by either mitotic division or direct transdifferentiation, during which supporting cells phenotypically convert into hair cells without dividing.

The mechanisms that regulate hair cell regeneration in mature animals are largely unknown. During vertebrate embryogenesis, the transcriptional activator ATOH1 drives expression of many hair cell-specific genes (Cai et al., 2015) and is necessary for both hair cell differentiation and survival (Bermingham et al., 1999; Itoh and Chitnis, 2001; Chen et al., 2002; Millimaki et al., 2007; Pan et al.,

2012; Cai et al., 2013; Chonko et al., 2013). *Atoh1* is transcribed at a low level in developing hair cell progenitors (Bermingham et al., 1999; Woods et al., 2004). *Atoh1* transcripts and protein become elevated in nascent hair cells and diminish once hair cells mature (e.g., Chen et al., 2002; Woods et al., 2004). ATOH1 is re-activated during hair cell regeneration. Shortly after hair cell damage is initiated, almost all supporting cells (hair cell progenitors) in the area of damage upregulate *Atoh1* transcription (Lewis et al., 2012). Only a subpopulation of supporting cells accumulate ATOH1 protein (Cafaro et al., 2007; Cotanche and Kaiser, 2010; Lewis et al., 2012) and go on to transdifferentiate into hair cells. ATOH1 overexpression drives higher rates of supporting cell division and direct transdifferentiation in the chicken BP (Lewis et al., 2012) and promotes regeneration of hair cell-like cells in mammalian hair-cell epithelia (e.g., Kawamoto et al., 2003; Shou et al., 2003) after damage at mature stages.

A few factors that control the expression of *Atoh1* and hair cell fate acquisition have been defined. For example, Notch signaling limits *Atoh1* transcription and restricts the numbers and positions of hair cells that are formed during development (reviewed in Jahan et al., 2013) and regeneration (Ma et al., 2008; Daudet et al., 2009; Wang et al., 2010; Lin et al., 2011; Atkinson et al., 2014; Staecker et al., 2014). During embryogenesis, development of hair cells is antagonized by bone morphogenetic proteins (BMPs), which are diffusible factors with a wide range of roles in developing and mature tissues (reviewed in Brazil et al., 2015). Inhibition of BMP4 in the chick otocyst increases *Atoh1* transcripts and hair cell numbers, while addition of soluble BMP4 produces the opposite results (Pujades et al., 2006). Inhibition of *Atoh1* transcription by BMPs may be mediated by the transcriptional cofactors *inhibitors of DNA binding (Ids)* (Jones et al., 2006; Kamaid et al., 2010). However, another study had opposing findings; BMP4 increased hair cell numbers in chick otocyst, while inhibition of BMP4 reduced hair cell formation (Li et al., 2005). BMP4 treatment also reduced sensory epithelial cell proliferation and induced apoptosis among developing ganglion cells (Li et al., 2005). Further examination of BMP4's proliferative effects at this early developmental stage reveals that BMP4 reduces the number of progenitor cells through apoptotic cell death (Pujades et al., 2006). The role of BMP4 during hair cell regeneration has not been examined.

We tested the role of BMP4 during auditory hair cell regeneration in post-hatch chickens following treatment with ototoxic antibiotics. We evaluated expression of *Bmp4*, BMP4 receptors, and *Ids 1-4*. We

also tested whether direct application of exogenous BMP4 antagonizes *Atoh1* expression and hair cell regeneration, and assessed if BMP4 treatment *in vitro* promotes hair cell loss. Our results indicate that loss of BMP4 signaling may be an important step required for avian supporting cells to upregulate *Atoh1* and to differentiate into replacement hair cells following hair cell damage.

## **MATERIALS AND METHODS**

### *Animal care and treatment*

Chickens were obtained in two manners. Fertile eggs of chickens (*Gallus gallus*, White Leghorn) were purchased from Charles River Labs (Wilmington, MA) or Featherland Farms (East Pearl Coburg, OR) and stored in a refrigerator for up to one week. Eggs were placed in a humidified incubator until hatching. Alternatively, hatchlings were purchased from Belt Hatchery (Fresno, CA) or Featherland Farms (East Pearl Coburg, OR). Hatchlings were housed in heated brooders with water and food at the Virginia Merrill Bloedel Hearing Research Vivarium. All procedures were approved by the University of Washington Animal Care Committee and conform to federal standards.

### *In vivo experiments: Gentamicin injections*

Post-hatch chicks of 7-10 days old received gentamicin (subcutaneous, 1×300 mg/Kg on 2 consecutive days, Sigma-Aldrich, St. Louis, MI) and were returned to brooders for recovery until 4 or 8 days following the first gentamicin injection. Chickens were euthanized by decapitation. The middle ear was opened, and the columella was removed. For tissue being prepared for *in situ* hybridization (ISH), heads were immersion-fixed in a solution of 0.2mM EGTA and 3.7% formaldehyde in 1X phosphate-buffered saline (PBS) overnight at 4°C. After fixation, cochlear ducts (containing the BP) were dissected and placed in diethylpyrocarbonate (DEPC)-treated PBS for removal of the tegmentum vasculosum and the tectorial membrane. Cochlear ducts were rapidly dehydrated in sequential methanol rinses and stored at -80°C until ISH was performed. For tissue being prepared for immunocytochemistry (ICC), cochlear ducts were removed immediately after decapitation and fixed in buffered 4% paraformaldehyde (Stone and Rubel, 1999) for 30 minutes at room temperature and stored in PBS at 4°C. For all BPs, the

tectorial membrane was mechanically removed by dissection prior to dehydration (for ISH) or prior to storage (for ICC).

#### *In vitro experiments: Organ cultures*

Post-hatch chicks aged between days 7-10 were euthanized by decapitation, and heads rapidly immersed in 70% ethanol for 1 minute. Cochlear ducts were dissected, and the tegmentum vasculosum was removed. Each cochlear duct was placed in an individual well containing 450  $\mu$ L of Dulbecco's Minimal Essential Medium (Sigma-Aldrich, St. Louis, MI) at 37°C in 95% environmental room air/5% CO<sub>2</sub>. The following drugs were included in culture media for each experiment: 1% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% (78  $\mu$ M) penicillin-streptomycin (Sigma-Aldrich, St. Louis, MI), 10 ng/mL BMP4 (R&D Systems, Minneapolis, MN), and 0.5-1  $\mu$ g/mL Noggin (R&D Systems, Minneapolis, MN). Culture media were replaced daily at half-volume for maintenance or full-volume when changing to another stage of the experiment (e.g. transitioning from with aminoglycoside to without aminoglycoside). Cochlear ducts were fixed as described above for ISH or ICC. For each experiment, at least 3 runs were performed. For each run, at least 4 experimental and 4 control organs were included.

#### *Whole-mount in situ hybridization*

Using non-radioactive *in situ* hybridization (ISH), mRNA was detected in whole-mount cochlear ducts from which the tegmentum vasculosum and tectorial membrane had been removed (Stone and Rubel, 1999; Henrique et al., 1995). Digoxigenin (DIG)-conjugated riboprobes were synthesized from plasmids containing fragments or complete cDNA of the following chicken genes: *Bmp4* (obtained from Dr. Doris Wu, NIDCD), *Bmpr1a* and *Bmpr1b* (obtained from Dr. Jeanette Hyer, Department of Neurosurgery, University of California San Francisco), *Bmpr2* (obtained from Dr. Tsutomu Nohno, Department of Molecular and Developmental Biology, Kawasaki Medical School), *Id1*, *Id2*, *Id3*, *Id4* (gift from Dr. Marianne Fraser, Caltech), and *Atoh1* (obtained from Dr. Fernando Giraldez from Pompeu Fabra University, Barcelona, Spain; Pujades et al., 2006). Riboprobes were detected using alkaline phosphatase conjugated anti-DIG antibody and NBT/BCIP substrate (Roche, Indianapolis, IN). To examine gene expression after *in vivo* gentamicin exposure, approximately 4 BPs were examined per

time-point for each gene. To compare gene expression in organ cultures, at least 8 specimens (4 experimental, 4 control) from the same culture batch were processed in parallel, with 2–3 culture runs performed for each experiment. Some whole-mounts were embedded in plastic and sectioned at 2-3  $\mu\text{m}$  to better visualize cellular localization of the hybridization reaction.

#### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

The proximal halves of 10-15 BPs from control (undamaged) chicks and chicks at 4 days post-Gentamicin were isolated and placed in lysis buffer. RNA were extracted, and mRNA was purified and reverse-transcribed to generate cDNA. Quantitative polymerase chain reaction was performed to amplify cDNAs encoding following genes using their primer sets (Invitrogen, Carlsbad, CA):  $\beta$ -actin (for normalization), *BmpR1a* (5' cgattgcttgagcctatctg, 3' tggcaggattgtcttcattcc), *BmpR1b* (5' tgcaggcagaagtcatggag, 3' gcagataagcctctccaacc), and *BmpR2* (5' cggctgtgtgcatttaagga, 3' ctctcgctgattccgtggt). We used the same methods as in a prior study (Daudet et al., 2009). We estimated gene expression level in each sample using the  $2^{-\Delta\Delta\text{Ct}}$  method (Pfaffl, 2001; Colebrooke et al., 2007). The mRNA level of each target gene relative to  $\beta$ -actin was estimated by calculating the  $\Delta\text{Ct}$  ( $\text{Ct}_{\text{Target Gene}} - \text{Ct}_{\beta\text{-actin}}$ ) and then converting to  $2^{-\Delta\text{Ct}}$ . These values were averaged across samples in a group ( $n=3$ ), and statistical differences were examined using ANOVA. To compare mRNA levels between experimental groups, the ratio of the average  $2^{-\Delta\text{Ct}}$  for each treatment group relative to the control group ( $2^{-\Delta\Delta\text{Ct}}$ ) was determined for each gene. This ratio represents a fold change for each gene after damage.

#### *Immunocytochemistry*

Proteins were detected in whole-mount cochlear ducts using indirect immunolabeling. Rabbit anti-myosin VI and anti-myosin VIIa antibodies were purchased from Proteus (Ramona, CA).  $\beta$ III-tubulin antibody was purchased from Covance (Redmond, WA). Secondary antibodies conjugated to fluorophores (Alexa 488, Alexa 594) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Molecular Probes (Eugene, OR). Some cultures included bromodeoxyuridine (BrdU; from Sigma-Aldrich, St. Louis) to evaluate proliferation effects.

### *Dead-cell labeling*

TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling) with Alexa Fluor 488 from ThermoFisher Scientific (Waltham, MA) was used to label cells that were undergoing apoptotic cell death. These cultures were also labeled with the nuclear dye 4',6-diamidino-2-phenylindole, or DAPI to confirm TUNEL was co-localized with nuclear material, as predicted, and to gauge nuclear condensation.

### *Imaging and Quantitative Analyses*

Fluorescent imaging of whole-mount BPs was performed using an Olympus FV-1000 confocal microscope. Brightfield microscopy was performed using a Zeiss Axioplan microscope. For qualitative analyses, at least 4 BPs were examined for each experimental group. For quantitative analyses, the number of BPs analyzed ranged between 4 – 13 per group, which differs based on the treatment group and analysis. The number of BPs per group is noted in Figure Legends.

In each treatment group (no additive, BMP4 or Noggin), we estimated the density of hair cells (myosin VI-positive cells) in each BP. To do this, we counted hair cells within each BP in a distal region of sensory epithelium only. Distal regions were used consistently due to reliable lesion without other overt structural damage due to dissection or removal of tectorial membrane, which is more common in the proximal half of the basilar papilla. We then measured the area of the sensory epithelium for each image as noted by appropriate nuclear DAPI labeling. The number of cells in each image was then divided by the area of the sensory epithelium and multiplied by 10,000  $\mu\text{m}^2$  to demonstrate the number of differentiated or dying cells per unit area for a single BP. On average the sampled area was 10.0% (65,273  $\mu\text{m}^2$ ) of the total area of the BP (650,000  $\mu\text{m}^2$ ). Each BP was included as an individual data point for their respective treatment group and analyzed with one-way ANOVA and a *post-hoc* Bonferroni multiple comparison test. Effects were considered statistically significant if  $p \leq 0.05$ .

To evaluate cell death, TUNEL-positive cells in each BP were imaged in distal regions within each BP and subjected to the same density measure. There were 4-5 BPs analyzed from 2 runs for each

group. We only counted TUNEL particles that were co-localized with DAPI labeling. TUNEL-labeled BPs used one image per BP that was averaged within treatment group and analyzed with one-way ANOVA with a Bonferroni *post-hoc* multiple comparison test. Effects were considered statistically significant if  $p \leq 0.05$ .

## **RESULTS**

### **Hair cells were lost and regenerated in the proximal region of the basilar papilla after ototoxin treatment**

Prior studies demonstrated that injection of post-hatch chickens with the ototoxic antibiotic gentamicin results in complete loss of hair cells in the proximal third-to-half of the basilar papilla (BP) within 3-4 days (Bhave et al., 1995; Janas et al., 1995). At 3-5 days post-gentamicin, hair cell progenitors (supporting cells) are dividing and transdifferentiating into hair cells (Stone and Rubel, 2000; Roberson et al., 2004; Cafaro et al., 2007). Regenerated hair cells begin to emerge in the damaged region by 4 days post-gentamicin (Stone and Rubel, 2000; Roberson et al., 2004).

In this study, chickens received one injection of 200-250 mg/kg gentamicin on two consecutive days and were euthanized at 4 days or 8 days after the first gentamicin injection (post-gentamicin)(Fig. 1A). Control birds received no gentamicin. Hair cells were labeled with antibodies to  $\beta$ III tubulin. Our findings are schematized in Fig. 1B-D; images of immunolabeling for  $\beta$ III tubulin are shown in Fig. 1E-G.

In control birds, hair cells were distributed throughout the BP (Fig. 1B,E). At 4 days post-gentamicin, tubulin-positive hair cells were lost from the proximal region; only tubulin-labeled nerve fibers remain (Fig. 1C,F). By 8 days post-gentamicin, immature regenerated hair cells had emerged in the proximal BP (Fig. 1D,G). Hair cells in the distal region remained undamaged throughout the course of the experiment (Fig 1B-D).

### ***Atoh1* transcripts were upregulated after damage in the region of hair cell loss and enriched in early regenerated hair cells**

One of the earliest markers of regenerating hair cells is *Atoh1* mRNA and protein, which accumulates in supporting cells as they transdifferentiate into hair cells (Cafaro et al., 2007; Lewis et al.,

2012). Using *in situ* hybridization (ISH), we confirmed that *Atoh1* transcripts were expressed at very low levels in control BPs (Fig. 1H). At 4 days post-gentamicin, after hair cell elimination, *Atoh1* transcripts were highly upregulated in many supporting cells (Fig. 1I). *Atoh1* transcripts were expressed in regenerated hair cells at 8 days post-gentamicin, although some hair cells expressed low levels of *Atoh1*, suggesting they were late in differentiation (Fig. 1J).

### ***Bmp4* transcripts were detected in mature and regenerated hair cells but were lost from the basilar papilla upon drug-induced hair cell loss**

The morphogen bone morphogenetic protein 4 (BMP4) is expressed in the sensory epithelia of the developing chick otocyst, where it antagonizes *Atoh1* transcript accumulation (Pujades et al., 2006). To begin to assess if BMP4 has a similar role during hair cell regeneration, we performed whole-mount ISH to detect *Bmp4* mRNA in whole-mount cochlear ducts from chicks at post-hatch day 7-10, before and after hair cell damage by the ototoxin Gentamicin (Fig. 2A). In control (undamaged) conditions, *Bmp4* mRNA was highly expressed in hair cells throughout the BP (Fig. 2B-D), but it was not detected in supporting cells (Fig. 2B). *Bmp4* transcripts were also detected in cells lining the basilar membrane (see Fig. 5). Four days post-gentamicin, *Bmp4* mRNA labeling was eliminated from the proximal (damaged) half of the BP (Fig. 2F), concurrent with hair cell loss. At 8 days post-gentamicin, *Bmp4* mRNA was detected in regenerated hair cells in the proximal region (Fig. 2H). By contrast, *Bmp4* mRNA labeling did not appear to change significantly in the distal region after damage (compare Figs. 2E,G with Fig. 2C).

### **Transcripts for BMP4 receptors were detected in the normal basilar papilla and upregulated after hair cell damage**

To define cells in the mature avian auditory epithelium receiving BMP signaling expression, we performed whole-mount ISH to detect transcripts for three receptors for which BMP4 has a high affinity: *Bmpr1a*, *Bmpr1b*, and *Bmpr2* (Feng et al., 2014; Gorący et al., 2012; Miyazono et al., 2010; van Wijk et al., 2007). We analyzed BPs from untreated control chicks and from chicks at 4 days post-gentamicin. Some cochlear ducts were embedded in plastic and sectioned to better localize transcripts. For

comparison, a matching set of BPs were also immunolabeled for  $\beta$ III tubulin, a hair cell marker (Fig. 3A-C).

In undamaged BPs, transcripts for all three BMP4 receptors were localized to hair cells and supporting cells (Fig. 3D,G,J). Transcripts for *Bmpr1b* and *Bmpr2* appeared to be more present at higher levels in hair cells than supporting cells (Fig. 3G,J). At 4 days post-gentamicin, labeling for transcripts for all three receptors - *Bmpr1a*, *Bmpr1b*, and *Bmpr2* - increased in the proximal, damaged region (Fig. 3F,I,L) relative to the distal, undamaged region (Fig. 3E,H,K).

We quantitatively analyzed transcripts for *Bmpr1a*, *Bmpr1b*, and *Bmpr2* using quantitative reverse transcription polymerase chain reaction (qRT-PCR), comparing the proximal half of control BPs with the proximal half of BPs at 4 days post-Gentamicin. In response to hair cell damage, *Bmpr1a* transcripts were increased 3.4-fold ( $\pm 0.8$  standard error), *Bmpr1b* transcripts were increased 1.4-fold ( $\pm 0.4$  standard error), and *Bmpr2* transcripts were 2.1-fold ( $\pm 0.5$  standard error). These results were consistent with ISH findings and demonstrate that receptors for BMP4 are expressed in BP cells and are significantly upregulated after hair cell loss.

### **Transcripts for *Ids* were detected in the basilar papilla under normal conditions and altered after damage**

We used ISH to examine transcripts for *inhibitor of DNA binding (Id)* transcription factors, which are activated by BMPs in the developing sensory epithelia in the inner ear (Kamaid et al., 2010). In undamaged BPs, supporting cells express transcripts for *Id1* (Fig. 4A,B,C), *Id2* (Fig. E,F,G), and *Id3* (Fig. 4I,J,K). *Id4* transcripts were expressed in hair cells and supporting cells (Fig. 4M,N,O). At 4 days post-gentamicin, *Id1* transcripts were increased in supporting cells in the proximal region after hair cell loss (Fig. 4D). By contrast, transcripts for *Id2* and *Id3* were reduced in the proximal region after hair cell loss (Fig. 4H and Fig. 4L, respectively). *Id4* transcripts were retained and possibly upregulated in supporting cells after hair cell loss (Fig. 4P). Together, these data demonstrate that *Id1-4* transcripts are expressed in the mature BP and are dynamic during hair cell regeneration. In particular, transcripts for *Id2* and *Id3* decrease after hair cell damage, concurrent with reduced *Bmp4* expression.

### **Spatial distribution of transcripts for *Atoh1* and BMP4 pathway genes after hair cell loss in the BP**

Fig. 5 provides a partial summary of the spatial patterns of gene expression described so far for the BP at 4 days post-Gentamicin. Labeling for the hair cell marker myosin VIIa demonstrates hair cells were completely lost from the proximal, damaged half of the BP but remained intact in the distal, undamaged half (Fig. 5A). *Bmp4* transcripts showed a similar spatial pattern (Fig. 5B), although there was retention of *Bmp4* mRNA labeling in the damaged half among cells lining the basilar membrane. Transcripts for the BMP4 target gene, *Id3*, were also reduced in the damaged half (Fig. 5C). By contrast, *Atoh1* and *Bmpr1a* transcripts were increased in supporting cells in the damaged half but were not changed relative to controls in the undamaged end (Fig. 5D,E).

### **BMP4 reduced *Atoh1* transcripts in the damaged BP**

Results presented so far indicate that, under normal conditions when no new hair cells are being formed, *Bmp4* is expressed in hair cells and very little *Atoh1* is expressed in supporting cells. Upon hair cell loss, *Bmp4* expression is lost from the proximal BP, while *Atoh1* mRNA expression is highly upregulated there. These observations suggest BMP4 antagonizes *Atoh1* expression and prevents supporting cell transdifferentiation when hair cells are intact, but upon hair cell damage, the loss of BMP4 signaling enables *Atoh1* to be upregulated in supporting cells, which initiates hair cell regeneration. We reasoned that add-back of BMP4 during hair cell loss would prevent *Atoh1* mRNA accumulation. To test this hypothesis, cochlear ducts were explanted and treated in culture for two days with the ototoxic antibiotic streptomycin, which eliminates hair cells throughout the entire BP, triggering hair cell regeneration (Shang et al., 2010). After rinsing off streptomycin, cultures were treated for the next two days with (1) no additive, (2) recombinant BMP4 protein, or (3) recombinant noggin, an antagonist of BMP4 (Gerlach et al., 2000)(Fig. 6A). BPs were then processed for ISH to assess changes in *Atoh1* mRNA (Figure 6A).

In cultures without additive, *Atoh1* mRNA expression was detected in regenerated hair cells throughout the BP (Figure 6B). BMP4 treatment strongly reduced *Atoh1* mRNA in the BP (Fig. 6C), while

noggin treatment appeared to increase *Atoh1* mRNA (Fig. 6D). These results show BMP4 antagonizes the increase of *Atoh1* transcripts that are triggered by hair cell loss, while inhibition of BMPs may promote an increase in *Atoh1* expression.

### **Treatment with BMP4 did not increase apoptotic cell death**

One possible interpretation for the reduction of *Atoh1* transcripts in response to early BMP4 treatment is damage or death of supporting cells or early hair cell precursors. To address this possibility, cochlear ducts were treated with streptomycin for either 1 or 2 days in culture with or without BMP4 present. Cochlear ducts were fixed and processed for TUNEL, which marks apoptotic cells, and DAPI, a dye that labels all cell nuclei. We calculated the average density of TUNEL-positive/DAPI-positive particles in the mid-distal region of BPs from each experimental group. We chose to analyze two time-points because we were not certain how rapidly cell death would be detected, were it to happen.

We found no significant effects of BMP4 treatment on cell death in either 1-day or 2-day treatment groups (Fig. 7). ANOVA revealed differences in variance across the groups ( $p < 0.05$ ), and *post-hoc* analysis demonstrated this difference was not attributable to treatment but rather to an increase in the number of TUNEL-positive cells at 1 day *in vitro* compared 2 days *in vitro*. A larger degree of cell death seen at 1-day was likely attributable to hair cell death in response to aminoglycosides. Our results indicate BMP4 did not increase the rate of either hair cell or supporting cell apoptosis.

### **BMP4 antagonized hair cell regeneration**

Next, we tested if BMP4 treatment decreases the number of hair cells that are regenerated in the chick BP. Cochlear ducts were explanted and cultured for two days with streptomycin. Subsequently, organs were cultured for 6 days with BMP4, noggin, or nothing (Fig. 6E). Cochlear ducts were processed for immunohistochemistry to evaluate changes in hair cell numbers using antibodies to myosin VI, which selectively label mature (Hasson et al., 1997) and regenerating (Shang et al., 2010) hair cells.

In control cultures with no additive, regenerated hair cells were present in the mid-distal region of the BP (Fig. 6F,I). Treatment with BMP4 caused a reduction in the average density of hair cells in the mid-distal region of the BP (Fig. 6G,I), while treatment with noggin increased hair cell density (Fig. 6H,I).

One-way ANOVA demonstrated a significant effect of treatment ( $p < 0.05$ ), while *post-hoc* analysis revealed controls were different from BMP-treated organs ( $p < 0.05$ ) and noggin-treated organs ( $p < 0.05$ ).

## DISCUSSION

Our results demonstrate that, after destruction of auditory hair cells in post-hatch chickens, BMP4 antagonizes *Atoh1* transcription or transcript accumulation, and this effect is accompanied by reduced hair cell regeneration. We present the following model to account for our findings and those of other laboratories (Fig. 8).

In the undamaged BP (Fig. 8A,A'), hair cells (HC) secrete BMP4 (blue) that binds BMP receptors on nearby supporting cells (SC, purple). BMP4 signal transduction in supporting cells triggers transcription of *Ids* and other effectors (not shown), which maintain low transcripts for *Atoh1*. Upon hair cell death, BMP4-mediated inhibition of *Atoh1* in supporting cells is eliminated (Fig. 8B,B'), and *Atoh1* transcription is increased in supporting cells (Fig. 8C,C'). Supporting cells that accumulate *Atoh1* transcripts transdifferentiate into hair cells (Fig. 8D,D'). When regenerated hair cells mature to the point of secreting BMP4, (Fig. 8E,E') *Atoh1* transcription in supporting cells is reduced once more. Eventually, BMP4's steady-state inhibition of *Atoh1* is re-established when regenerated hair cells reach maturity (Fig. 8F,F').

The role of BMP4 in controlling hair cell differentiation has been studied in the context of development. Sensory patches in the chicken inner ear express *Bmp4* (Wu and Oh, 1996). Once hair cells differentiate, BMP4 expression is limited to hair cells (Wu and Oh, 1996). Treatment of chicken otocysts with BMP4 leads to downregulation of *Atoh1* (Pujades et al., 2006) and increased *Id1-3* expression (Kamaid et al., 2010), with inhibition of BMPs through noggin application producing inverse results (Pujades et al., 2006; Kamaid et al., 2010). Further, their forced overexpression of *Id3* resulted in reduction of *Atoh1*, suggesting that BMP4 acts through *inhibitors of DNA binding* to antagonize *Atoh1* (Kamaid et al., 2010). Both the avian and mammalian otocyst express BMP4 broadly at early stages of development, which becomes localized to focal points to induce patterning of the auditory epithelium: the chicken otocyst limits BMP4 expression to anterior and posterior ends of the otocyst (Wu and Oh, 1996), and the mammalian otocyst limits BMP4 expression to the abneural edge (Morsli et al., 1998; Ohyama et

al., 2010). Despite these similarities, there are notable differences in developmental expression between the avian and mammalian auditory epithelia.

BMP4 expression continues to outline the shape of the cochlea through development (Morsli et al., 1998), possibly to further assist with patterning of the cochlea. Although BMP4 expression of mature cochlear hair cells has not yet been examined in mammals, developmental BMP4 expression becomes localized to outer sulcus cells and is not observed in developing hair cells (Morsli et al., 1998; Ohyama et al., 2010). Addition of BMP4 to developing mammalian otocysts produced variable results, with some reports of increased hair cell numbers and reduced proliferation (Li et al., 2005) which opposes results that indicate BMP4 induces *Id2* expression along with other markers for outer sulcus cell fate in a dose-dependent manner (Ohyama et al., 2010). The current study did not evaluate proliferative effects of BMP4, and this remains open for further investigation. When BMP signaling was reduced through genetic manipulation of BMP receptors in the developing mammalian cochlea, the cochlear duct is shortened and mild vestibular defects occur (Ohyama et al., 2010). With the demonstrable concentration-dependent effect of BMP4 in both developing mammalian and avian systems (Pujades et al., 2006; Ohyama et al., 2010) and varying results of BMP4 addition to the developing mammalian cochlea (Li et al., 2005, Ohyama et al., 2010), the function of BMP4 in the mature regenerative basilar papilla was previously unknown. Many studies are currently investigating the effects of *Atoh1* introduction to mammalian inner ear tissue as a method for hair cell differentiation after damage. With identified factors, such as BMP4, known to inhibit *Atoh1* (Pujades et al., 2006) or hair cell differentiation (Ohyama et al., 2010) in developing auditory epithelia and regenerative basilar papilla, further investigation of these factors in the mature mammalian organ of Corti is warranted.

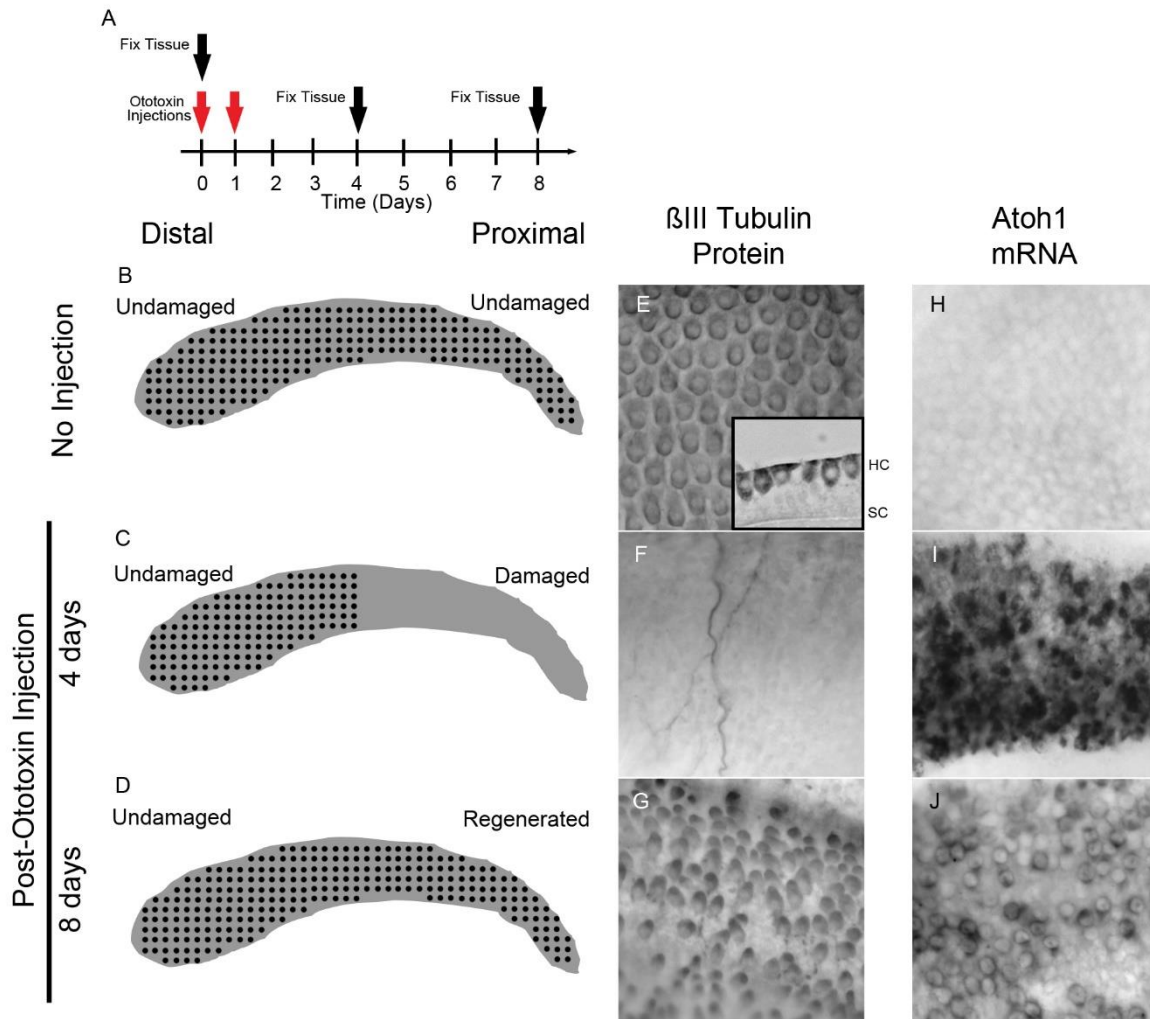
Other members of the bone morphogenetic protein family are present in developing basilar papilla that may contribute to these expression patterns and cellular behavior. BMP2 is present in developing cochlear hair cells, but removal of BMP2 resulted in normal hair cell differentiation with normal hearing (Hwang et al., 2010). BMP2 and BMP4 share the same receptors of *Bmpr1a*, *Bmpr1b*, and *Bmpr2*, and these should be investigated further to evaluate redundancies and relationships of BMPs in developing and regenerating auditory epithelia. Evaluation of additional growth factors among both auditory and vestibular epithelia is encouraged, as BMP7 is also present in developing chicken otocyst

and localizes to vestibular epithelia (Oh et al., 1996). Further evaluation of BMPs and other signaling molecules in mature auditory and vestibular epithelia is warranted given their developmental prevalence and function.

The antagonistic effect of BMP4 on *Atoh1* expression is not limited to auditory epithelia. In the cerebellum, BMP2 and BMP4 inhibit proliferation and increase differentiation of granule neuron precursor cells and medulloblastoma cells through post-transcriptional degradation of *Atoh1* (Zhao et al., 2008). Not only is there high importance for the evaluation of other BMPs such as BMP2, but also evaluation of signaling molecules such as Sonic Hedgehog (SHH). SHH is expressed in developing auditory epithelia and is crucial for cell patterning (Son et al., 2015). The granule neuron progenitor cells were able to overcome BMP-mediated degradation of *Atoh1* through ectopic expression of *Atoh1*, but introduction of downstream factors to SHH such as Gli1 did not restore normal proliferative and differentiating functions to these cells (Zhao et al., 2008). Mechanisms that allow for *Atoh1* degradation and expression should be evaluated alongside BMP and other signaling molecules to determine their proliferative and differentiation effects, not only in auditory epithelia but other systems that demonstrate these capacities such as the cerebellum. Mammalian supporting cells in the cochlea do not upregulate *Atoh1* expression after hair cell damage. Our finding that BMP4 is a potent antagonist of hair cell regeneration in birds suggests it might play a similar role in mammals. To date, no studies have examined BMP4 expression in mature mammalian cochlea either before or after hair cell damage. Future studies should seek to investigate BMP4 expression in the mature healthy and damaged mammalian organ of Corti. Next, treatment of mammalian cochlear tissue should be attempted to determine if the function of BMP4 as a potent inhibitor to *Atoh1* and hair cell differentiation is conserved across mature mammals and non-mammalian vertebrates. Varying concentrations should be evaluated to characterize the dose-dependent effects of BMP4 in auditory epithelium. As previously mentioned, important validation of these findings is needed *in vivo*, as most findings discussed here are limited to *in vitro* manipulations only. To attempt *in vivo* manipulation, investigators are currently searching for effective methods to introduce genetic material or stem cells to the mature organ of Corti, but no studies have attempted *in vivo* manipulation of BMP4 exogenous protein at this time. Previous studies indicate an autoregulatory loop of BMP4 in developing basilar papilla (Pujades et al., 2006) and of *Atoh1* expression in other tissues (Ebert et al., 2003), but

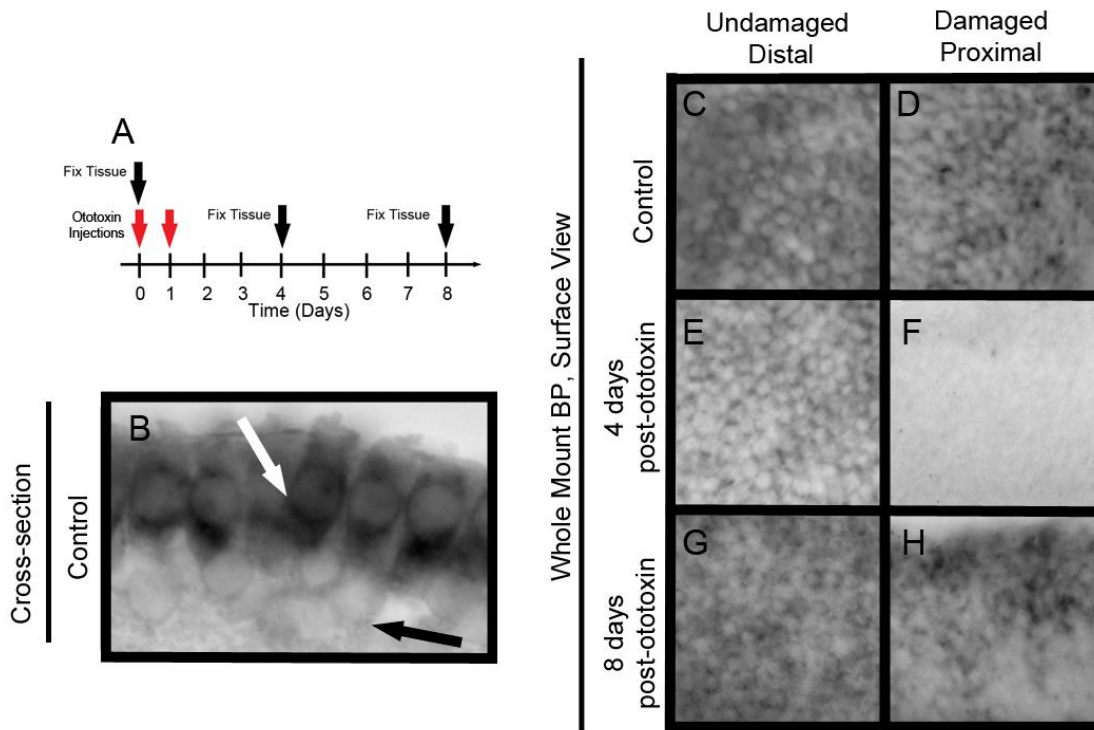
effects of BMP4 with *Atoh1* expression remain unknown in quiescent basilar papilla. Combinatorial treatments of *Atoh1* overexpression with BMP4 inhibition and/or other factors may be considered to further increase efficiency of *Atoh1* expression to overcome these autoregulatory features and produce new hair cells. Other signaling pathways such as previously mentioned Sonic Hedgehog, as well as transforming growth factors, tumor necrosis factors, and many other molecules, should be evaluated to determine effects of differentiation, proliferation, and cell death in both regenerative and non-regenerating auditory epithelia. Given several shared signaling molecules that are activated through these pathways, investigation of these molecules needs to occur both in isolation and in co-treatment paradigms. Research performed regarding interacting pathways and isolated pathways can be evaluated in conjunction to gain a better understanding of functions of these signaling molecules in regenerative auditory epithelia. Future studies and other considerations will be necessary to determine if BMP4 is a reasonable candidate to assist in restoration of hair cells in the mature organ of Corti after hair cell damage.

Although much is left to be characterized in this regenerative system, these data indicate BMP4 serves to antagonize *Atoh1* expression and subsequent hair cell differentiation in the mature avian basilar papilla after hair cell damage.



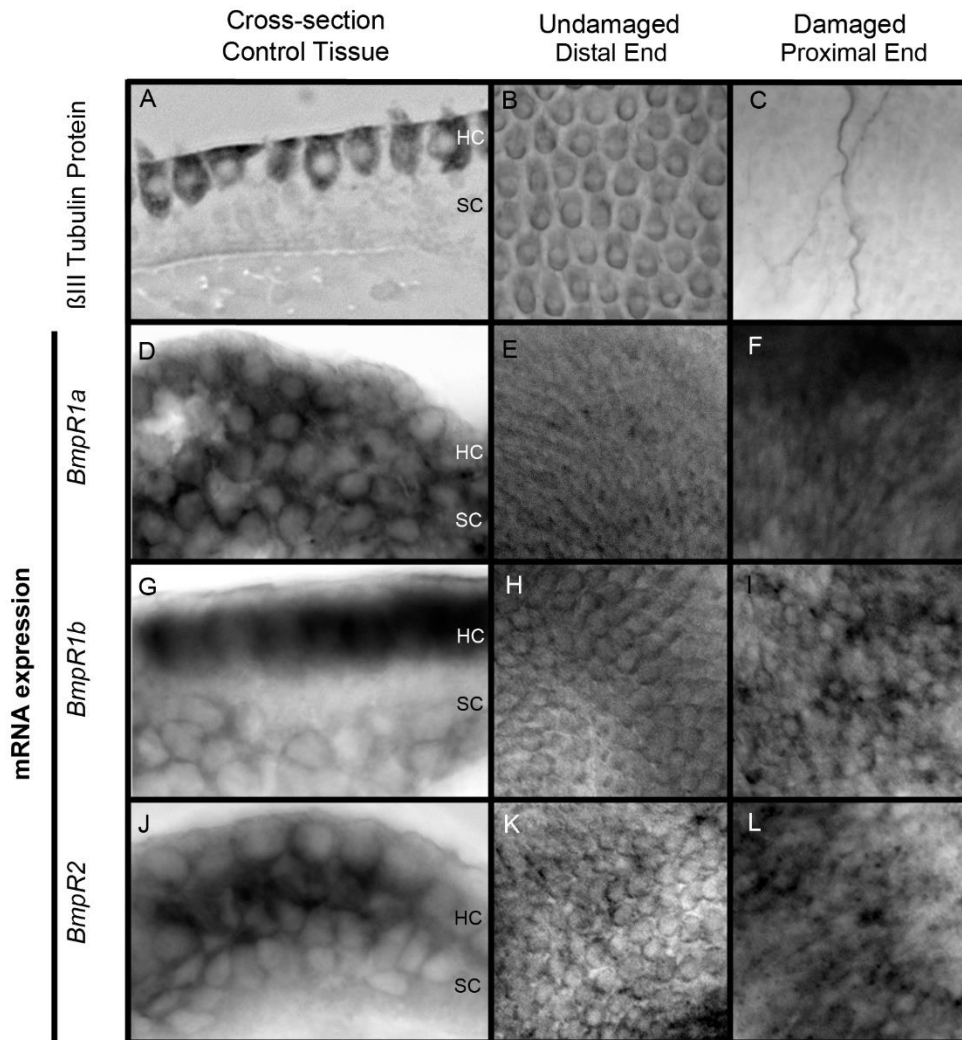
**Figure 1:** Method for *in vivo* hair cell damage and *Atoh1* mRNA detection. (A) Timeline demonstrates the methods used for hair cell damage paradigms. Group 1 (control) was never exposed to ototoxic drug injections (undamaged proximal and distal ends of the BP), and these BPs were removed after fixation. Group 2 was exposed to ototoxic drug injections for 2 consecutive days, then BPs were fixed at 4 days after the first injection. Group 3 was exposed to ototoxic drug injections for 2 consecutive days, then BPs were fixed at 8 days after the first injection. (B) Schematic of undamaged BPs demonstrates hair cells (black dots) along the length of the epithelium. (C) Systemic injection of ototoxic drug results in hair cell damage and extrusion from proximal end of the BPs. (D) Eight days after systemic ototoxic drug injection, hair cells are regenerated at the proximal end of the BP. (E) Image of baseline levels of  $\beta$ III tubulin

labeling among whole mount epithelium, with inset demonstrating a cross-section of the tissue with hair cells labeled with  $\beta$ III tubulin and supporting cells unlabeled. (F)  $\beta$ III tubulin labeling is eliminated from the epithelium in the proximal area of damage. (G)  $\beta$ III tubulin is restored to the epithelium in the proximal area of damage, with immature hair cell morphology noted in this image. (H) *Atoh1* labeling for mRNA is absent or extremely low in undamaged auditory epithelia. (I) *Atoh1* mRNA is upregulated 4 days after ototoxic drug injection. (J) *Atoh1* mRNA is downregulated once hair cells are regenerated 8 days after ototoxic drug injection.



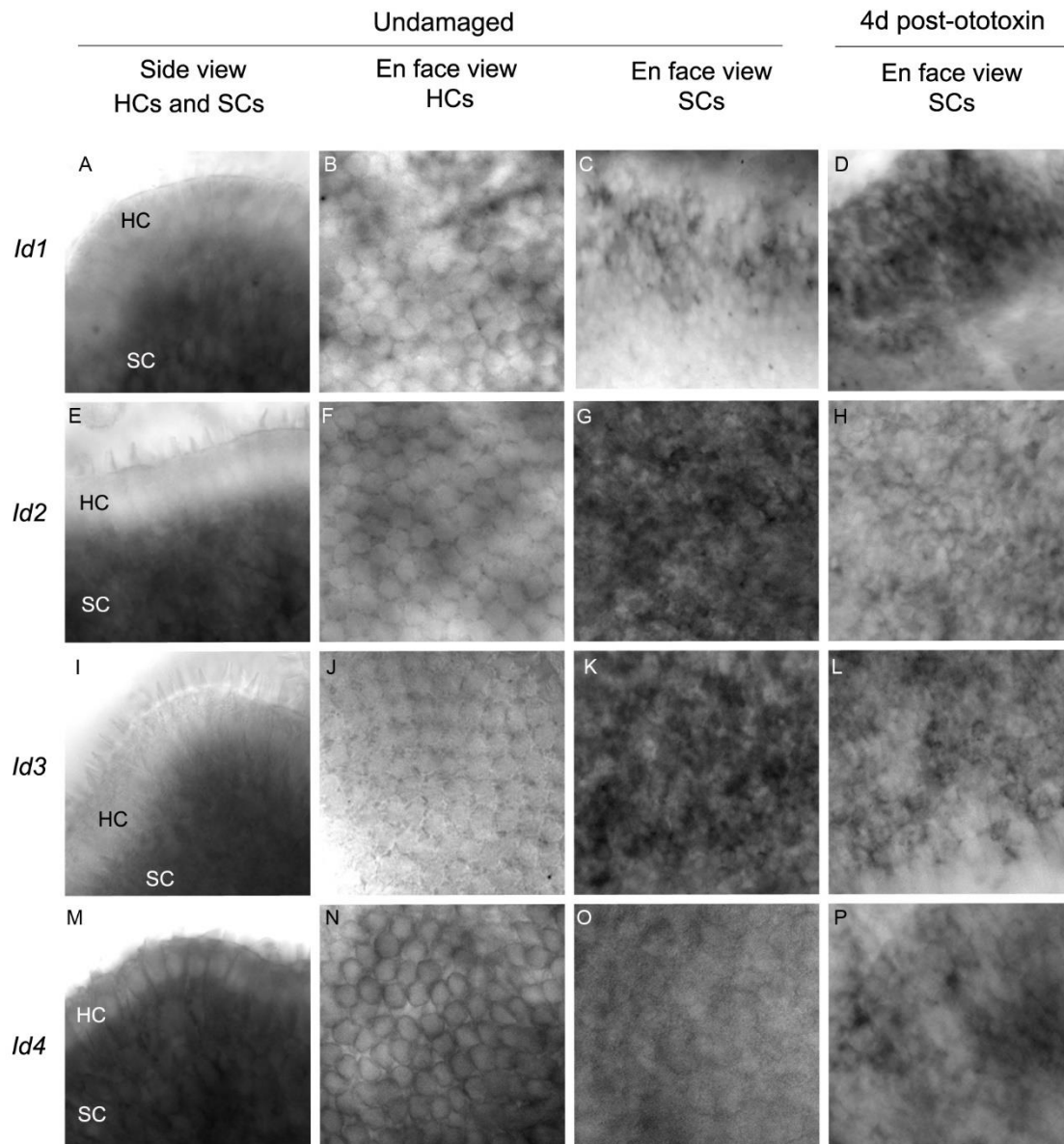
**Figure 2:** *Bmp4* mRNA expression patterns before and after ototoxic damage. (A) Timeline is similar to Figure 1A, with *Bmp4* mRNA analyzed before and after hair cell damage. As demonstrated in Figure 1, Group 1 (control) was never exposed to ototoxic drug injections (undamaged proximal and distal ends of the BP), and these BPs were removed after fixation. Group 2 was exposed to ototoxic drug injections for 2 consecutive days, then BPs were fixed at 4 days after the first injection. Group 3 was exposed to ototoxic drug injections for 2 consecutive days, then BPs were fixed at 8 days after the first injection. (B) Group 1: Baseline levels of *Bmp4* mRNA from a cross-section of undamaged, control BPs. *In situ* hybridization labels *Bmp4* mRNA in hair cell bodies and does not label supporting cells. (C) Group 1: Baseline levels of *Bmp4* mRNA from a whole mount of undamaged, control BPs in the distal region that remains undamaged with the systemic ototoxin drug damage paradigm presented in this paper. (D) Group 1: Baseline levels of *Bmp4* mRNA from a whole mount of undamaged, control BPs in the proximal region that is subject to damage with a systemic ototoxin drug damage paradigm. (E) Group 2: Four days after ototoxic drug damage, *Bmp4* mRNA labeling is maintained in the proximal region where hair cells

remain healthy. (F) Group 2: Four days after ototoxic drug damage, *Bmp4* mRNA labeling is eliminated in the region where hair cells are damaged and extruded from the BP. (G) Group 3: Eight days after ototoxic drug damage, *Bmp4* mRNA labeling remains maintained in the proximal region where hair cells are maintained. (H) Group 3: Eight days after ototoxic drug damage, *Bmp4* mRNA is restored in the distal region where hair cells are regenerating.



**Figure 3:** Expression patterns to receptors for BMP4 before and after systemic ototoxic damage. (A) For reference, a cross-section of undamaged, control tissue is labeled for  $\beta$ III Tubulin protein, which labels hair cells and neurons. Hair cell (HC) bodies are labeled while supporting cells (SC) are unlabeled. (B) For reference, whole mount of the distal end of the BP exposed to systemic ototoxic damage presents a field of undamaged hair cells labeled for  $\beta$ III Tubulin protein. (C) For reference, whole mount of the proximal end of a BP exposed to systemic ototoxic damage presents a field where hair cells are extruded after damage. Two neurons that remain after ototoxic drug damage are shown. (D) Cross-section of undamaged, control tissue is labeled for *Bmpr1a* mRNA. Both hair cells and supporting cells are labeled

through *Bmpr1a in situ* hybridization. (E) Whole mount of the distal end of a BP exposed to systemic ototoxic drug damage presents a field of hair cells and supporting cells that are labeled for *Bmpr1a* mRNA. (F) Whole mount of the proximal end of a BP exposed to systemic ototoxic drug damage presents a field of supporting cells that have highly upregulated *Bmpr1a* in the absence of hair cells. (G) Cross-section of undamaged, control tissue is labeled for *Bmpr1b* mRNA. Both hair cells and supporting cells are labeled for *Bmpr1b*, but hair cells demonstrate a relatively higher labeling when compared to supporting cells. (H) Whole mount of the distal end of a BP exposed to systemic ototoxic drug damage presents a field of hair cells and supporting cells that are labeled for *Bmpr1b* mRNA. (I) Whole mount of the proximal end of a BP exposed to systemic ototoxic drug damage presents a field of supporting cells that have moderately increased their labeling for *Bmpr1b* in the absence of hair cells. (J) Cross-section of undamaged, control tissue is labeled for *Bmpr2* mRNA. Both hair cells and supporting cells are labeled for *Bmpr2* mRNA with relatively higher labeling among hair cells. (K) Whole mount of the distal end of a BP exposed to systemic ototoxic drug damage presents a field of hair cells and supporting cells labeled with *Bmpr2*. (L) Whole mount of the proximal end of a BP exposed to systemic ototoxic drug damage presents a field of supporting cells that have moderately increased their labeling for *Bmpr2* in the absence of hair cells.



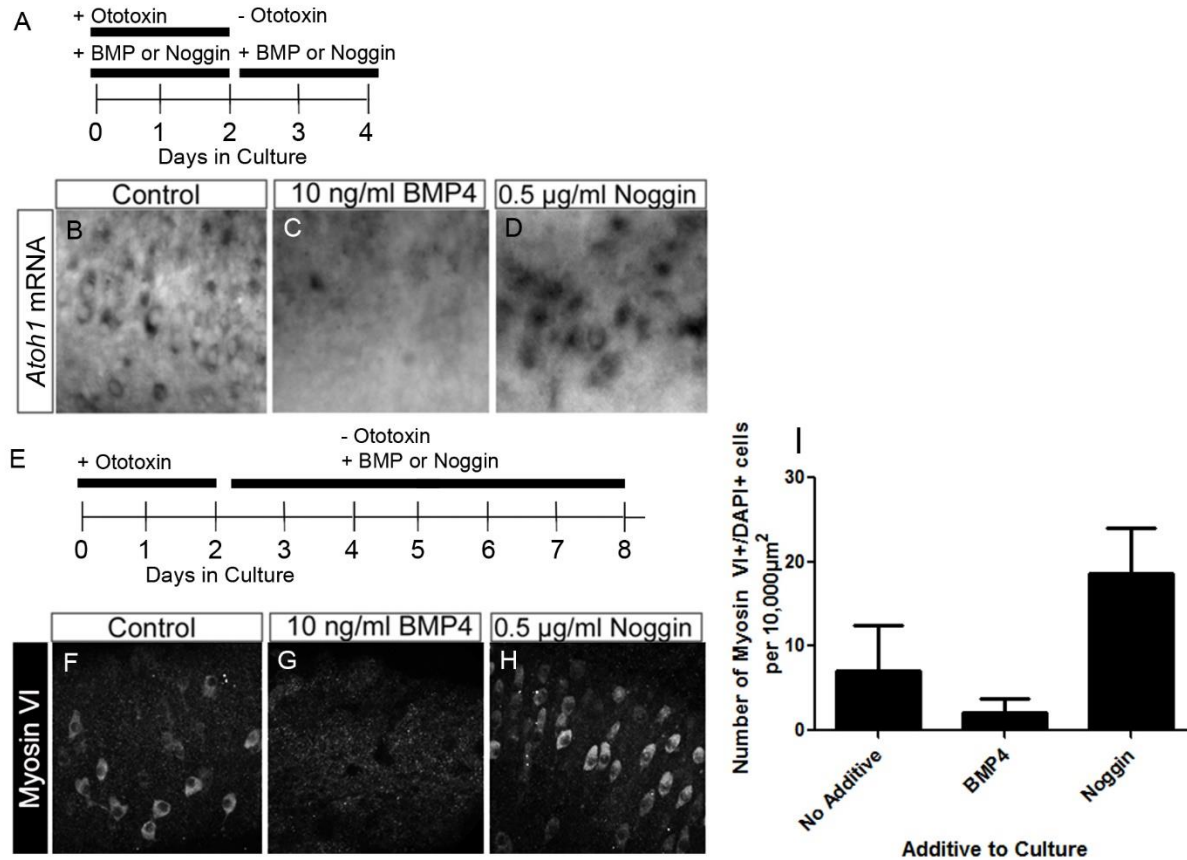
**Figure 4:** Expression patterns for *inhibitors of DNA binding (Id)* before and after systemic ototoxic drug damage among whole mount BPs. (A) A side view of undamaged BPs demonstrates mRNA labeling of *Id1* among supporting cells with relatively light labeling among hair cells. (B) En face imaging of the hair cell layer of undamaged BPs demonstrates light labeling of *Id1* mRNA. (C) En face imaging of the supporting cell layer of undamaged BPs demonstrates supporting cells moderately labeled with *Id1* mRNA. (D) Four days after systemic ototoxic drug exposure, en face imaging of the supporting cell layer demonstrates relatively higher *Id1* mRNA labeling in the proximal region where hair cells were extruded

from the BP. (E) A side view of undamaged BPs demonstrates mRNA labeling of *Id2* among supporting cells with essentially no labeling among hair cells. (F) En face imaging of the hair cell layer of undamaged BPs demonstrates light labeling of *Id2* mRNA. (G) En face imaging of the supporting cell layer of undamaged BPs demonstrates supporting cells with intense labeling of *Id2* mRNA. (H) Four days after systemic ototoxic drug exposure, en face imaging of the supporting cell layer demonstrates relatively lower *Id2* mRNA labeling in the proximal region where hair cells were extruded from the BPs. (I) A side view of undamaged BPs demonstrates mRNA labeling of *Id3* among supporting cells with essentially no labeling among hair cells. (J) En face imaging of the hair cell layer of undamaged BPs demonstrates light labeling of *Id3* mRNA. (K) En face imaging of the supporting cell layer of undamaged BPs demonstrates supporting cells with intense labeling of *Id3* mRNA. (L) Four days after systemic ototoxic drug exposure, en face imaging of the supporting cell layer demonstrates relatively lower *Id3* mRNA labeling in the proximal region where hair cells were extruded from the BP. (M) A side view of undamaged BPs demonstrates mRNA labeling of *Id4* among supporting cells with relatively light labeling among hair cells. (N) En face imaging of the hair cell layer of undamaged BPs demonstrates relatively light labeling of *Id4* mRNA. (O) En face imaging of the supporting cell layer of undamaged BPs demonstrates sparse labeling of *Id4* mRNA. (P) Four days after systemic ototoxic drug exposure, en face imaging of the supporting cell layer demonstrates relatively higher *Id4* mRNA expression in the proximal region where hair cells were extruded from the BP when compared to control tissue.



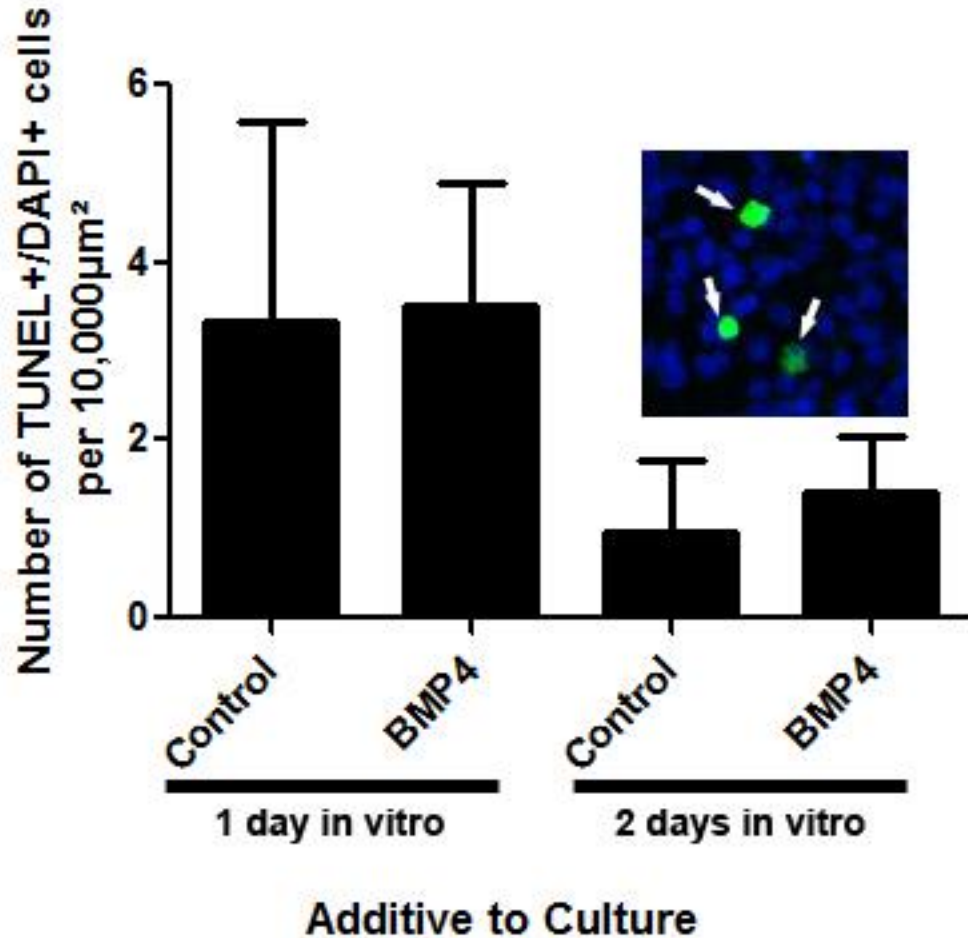
**Figure 5:** Imaging the whole BP four days after systemic ototoxic drug exposure. (A) Myosin VIIa protein labeling is provided as a reference to demonstrate the robust presence of hair cells at the distal end (left side of image) of the BP. The proximal end of the BP (right side of image) demonstrates the absence of hair cells after systemic ototoxic drug exposure. (B) *Bmp4* mRNA labels hair cells at the distal,

undamaged end of the BP while *Bmp4* mRNA labeling is absent in the proximal region where hair cells are eliminated with exception to labeling of cells that line the basilar membrane. (C) *Id3* mRNA labels supporting cells at the distal, undamaged end of the BP while *Id3* mRNA labeling is lessened at the proximal end of the BP after hair cells are eliminated. (D) *Atoh1* mRNA is absent in the distal, undamaged end of the BP while *Atoh1* mRNA labeling is highly upregulated among supporting cells after hair cells are eliminated. (E) *Bmpr1a* mRNA label is relatively low at the distal, undamaged end of the BP while *Bmpr1a* mRNA label is greatly increased at the proximal end of the BP after hair cells are eliminated.

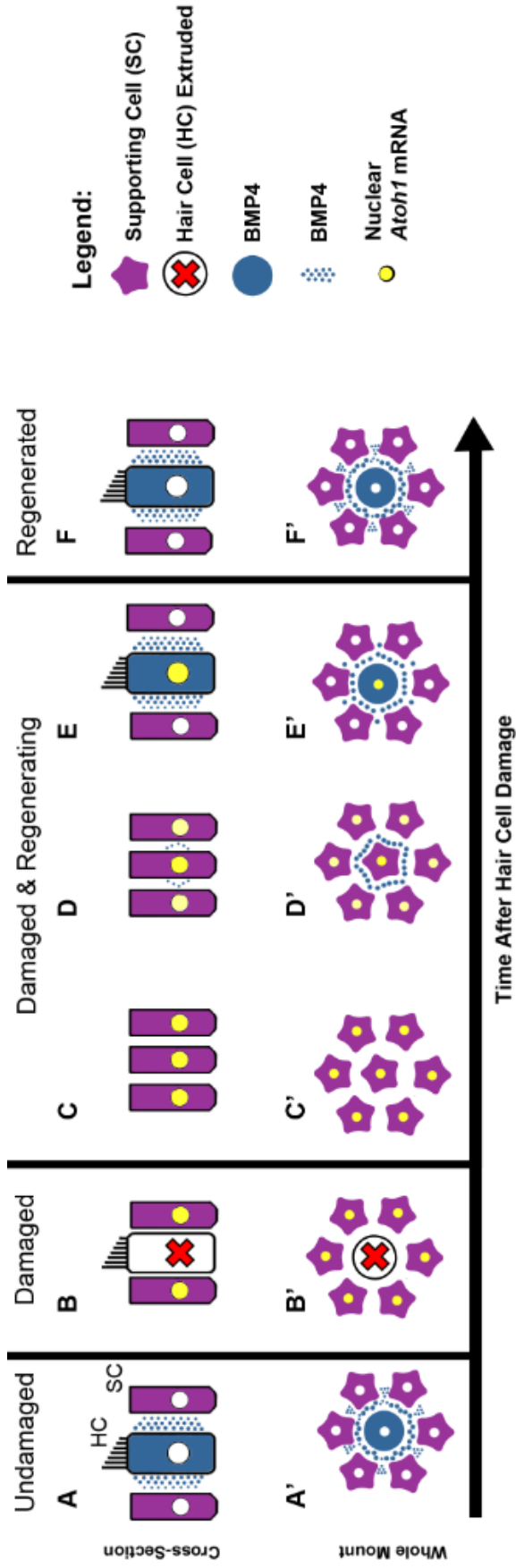


**Figure 6:** BMP4 antagonizes *Atoh1* mRNA expression and hair cell regeneration. (A) To evaluate BMP4 effects on *Atoh1* expression, exogenous BMP4 protein or its inhibitor Noggin were added to cultured BPs along with aminoglycoside for 2 days, then allowed to recover in the presence of BMP4, noggin, or no additive (control) for 2 additional days. (B) Baseline levels of *Atoh1* mRNA expression were evaluated through *in situ* hybridization after hair cells are damaged throughout the epithelium as a result of *in vitro* ototoxic treatment. (C) In the presence of BMP4 protein, *Atoh1* mRNA expression after hair cell death is greatly reduced. (D) In the presence of noggin, inhibitor to BMP4, *Atoh1* expression after hair cell death is moderately increased. (E) To evaluate BMP4 effects on hair cell regeneration, ototoxin was first applied to *in vitro* BPs to eliminate hair cells throughout the epithelium. Basilar papillae recovered for 6 days in the presence of no additive, exogenous BMP4 protein or its inhibitor noggin. Cultures were then fixed and evaluated for hair cell number through immunofluorescent labeling of myosin VI. (F) Baseline hair cell

numbers were evaluated in the absence of BMP4 or noggin additive after hair cell elimination ( $n = 7$ ). (G) In the presence of BMP4 protein ( $n=13$ ), BPs greatly reduce their capacity for regenerating hair cell numbers as demonstrated through sparse myosin VI labeling. (H) In the presence of noggin protein ( $n=5$ ), BPs greatly increase their regenerated hair cell numbers as indicated through high numbers of myosin VI labeling. (I) Comparison of the density of regenerated hair cells per treatment group reveals BMP4 treatment significantly reduces the number of hair cells that regenerate after damage to the BP ( $p<0.05$ ) while noggin treatment significantly increases the number of hair cells that regenerate ( $p<0.05$ ).



**Figure 7:** BMP4 treatment leads to no significant difference in supporting cell apoptosis. ANOVA analysis of the number of supporting cells co-labeled with both TUNEL, a marker for apoptosis, and DAPI, a nuclear marker, reveals significant differences in variance among treatment groups. However, *post-hoc* analysis reveals no significant differences in the number of supporting cells subjected to apoptotic cell death within treatment groups (BMP4 vs. aminoglycoside only) and within culture period (1 day vs. 2 day). For reference, total number of BPs included in analysis per group are as follows: 1 day with BMP4 treatment, n=4; 1 day with aminoglycoside only, n=5; 2 days with BMP4 treatment, n=5; 2 days with aminoglycoside only, n=4.



## Chapter 4

### Discussion and Application of Current Findings

Because the prevalence of hearing loss due to cochlear hair cell damage is very high, understanding mechanisms for cochlear hair cell regeneration is crucial to developing therapeutic strategies for these sensorineural hearing disorders. This dissertation examined the basilar papilla of the chicken, which is structurally and functionally similar to the organ of Corti in mammals except that it naturally regenerates hair cells after ototoxic drug damage *in vivo* and *in vitro*. I studied the expression and function of two molecules during avian hair cell regeneration – the transcription factor ATOH1 and the cell-to-cell signaling molecule BMP4. Roles of both molecules have been well-studied in development but not in avian hair cell regeneration. During embryogenesis, ATOH1 is necessary and sufficient for hair cell differentiation, while BMP4 antagonizes *Atoh1* expression. My goal was to increase understanding of ATOH1's role in promoting hair cell regeneration and to test BMP4's ability to antagonize *Atoh1* expression (and therefore hair cell regeneration) after damage in the mature basilar papilla.

My results confirm *Atoh1* is broadly upregulated among supporting cells after hair cell damage in the mature basilar papilla, as previous studies have shown (Cafaro et al., 2007). While *Atoh1* transcriptional activity is upregulated in nearly all supporting cells in the area of hair cell damage, only a subset of supporting cells transdifferentiate as hair cells. Forced expression of *Atoh1* causes most supporting cells to become hair cells, suggesting accumulation of ATOH1 is a necessary step in supporting cell transdifferentiation that is naturally blocked to ensure appropriate numbers of and patterning of hair cells and supporting cells. My studies show that two signaling pathways, notch and BMP, antagonize ATOH1 expression. Inhibition of Notch or BMP4 activity causes more supporting cells to transdifferentiate, while BMP4 blocks this process. *Atoh1* transcript is restricted by notch signaling during regeneration. Together, the results presented here indicate that, while ATOH1 contributes to the ability for supporting cells to transdifferentiate into hair cells in the regenerating basilar papilla, notch and BMP4 antagonize this process.

Prior to the beginning of these studies, *Atoh1*-related effects upon proliferation in the mature auditory system were largely unknown. Several supporting cells were noted to express *Atoh1* during cell

division in the regenerative basilar papilla (Cafaro et al., 2007) and proliferation due to *Atoh1* expression is noted in several other contexts (Mulvaney and Dabdoub, 2012). During development of the organ of Corti, cell proliferation is completed prior to the onset of *Atoh1* expression (Chen et al., 2002), suggesting *Atoh1* is not required for proliferation of sensory progenitors. Although *Atoh1* is not required for proliferation in that context, the second chapter of this dissertation demonstrates that forced *Atoh1* overexpression among supporting cells in the damaged basilar papilla leads to increased proliferation without cell death. Supporting these data, *Atoh1* was found to increase proliferation in the normally postmitotic mammalian cochlear epithelium (Kelly et al., 2012) and utricle (Gao et al., 2016). BMP4 treatments decrease proliferation of sensory progenitor cells and trigger cell death (Pujades et al., 2006). In my study in Chapter 3, I did not test the function of BMP4 upon supporting cell division, but I did determine that BMP4 treatments do not cause supporting cell death.

Several studies have shown *Atoh1* misexpression in the developing organ of Corti forces supporting cells to transdifferentiate into hair cells (Zheng and Gao, 2000; Woods et al., 2004; Gubbels et al., 2008). There is considerable controversy with respect to whether *Atoh1* has this capacity in adult mammals. Misexpression of *Atoh1* in the organ of Corti of adult rodents using viral-mediated transfer resulted in extremely limited numbers of new hair cells with stereociliary bundles consistent with immature inner hair cells that result in variable restoration of auditory brainstem response thresholds (Kawamoto et al., 2003; Izumikawa et al., 2005; Atkinson et al., 2014). By contrast, genetic activation of *Atoh1* among supporting cells in the adult organ of Corti results in increased numbers of new hair cells throughout the length of the cochlea (Kelly et al., 2012) that are mechanotransductive as indicated by FM4-64FX uptake (Liu et al., 2012). While these new hair cells survived for 2 months in vivo, they lacked the appropriate morphology and polarity of native hair cells (Liu et al., 2012). It is not clear why such different results have been reported. One possibility is that unknown side-effects of each method negatively impact hair cell differentiation and/or survival. Another possibility is that cellular context of the cells receiving *Atoh1* misexpression affect the outcome. For instance, supporting cells in the vestibular system of mammals appear to transdifferentiate readily into hair cells in response to *Atoh1* misexpression (Shou et al., 2003; Staecker et al., 2014; Gao et al., 2016), suggesting fewer antagonists to ATOH1 accumulation may be present. It is possible that inhibitory factors such as notch or BMP4 reduce the impact of *Atoh1*

misexpression in the organ of Corti. Clearly, further studies in mammals are needed to determine how to increase the efficacy of *Atoh1* as a potential treatment for hearing loss.

These studies demonstrate the potency of *Atoh1* in its increase of hair cell differentiation and proliferation while being subjected to other signaling factors such as notch and BMP4. However, limitations of these studies include solely *in vitro* manipulation of BMP4 or notch that was not verified *in vivo*. It is currently unknown whether BMP4 expression is present in mature mammalian cochlea, as the tissue has not yet been evaluated in either undamaged or damaged epithelia. This study focused on mRNA transcript, but further studies that examine protein expression may provide additional information regarding the mechanisms for BMP4-mediated degradation of *Atoh1* or other factors that may restrict *Atoh1* expression. Given the complex regulation of hair cell differentiation and proliferation, and the current data that demonstrate *Atoh1* is limited by other growth factors or signaling molecules, additional study to identify regulators of *Atoh1* is necessary to understand the avian regenerative system and the mammalian auditory epithelia as hair cell regeneration is approached in cochlear tissue.

Although *Atoh1* transfection alone leads to limited regeneration of auditory hair cells, a combination of therapies may result in better outcomes. Consideration of notch, BMP4, and other heretofore unknown factors that limit ATOH1 accumulation and subsequent hair cell differentiation is necessary for investigators developing *Atoh1*-based therapies in the mammalian inner ear to restore hearing and balance.

## References

- Adler, H.J., Kenealy, J.F., Dedio, R.M., and Saunders, J.C. (1992). Threshold shift, hair cell loss, and hair bundle stiffness following exposure to 120 and 125 dB pure tones in the neonatal chick. *Acta Otolaryngol.* 112, 444–454.
- Adler H.J., Komeda M., Raphael Y. (1997). Further evidence for supporting cell conversion in the damaged avian basilar papilla. *Int J Dev Neurosci* 15, 375-385.
- Adler H.J., Raphael Y. (1996). New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. *Neurosci Lett* 205, 17-20.
- Atkinson, P.J., Wise, A.K., Flynn, B.O., Nayagam, B.A., and Richardson, R.T. (2014). Hair cell regeneration after ATOH1 gene therapy in the cochlea of profoundly deaf adult guinea pigs. *PLoS ONE* 9, e102077.
- Baird R.A., Steyger P.S., Schuff N.R. (1996). Mitotic and nonmitotic hair cell regeneration in the bullfrog vestibular otolith organs. *Ann N Y Acad Sci* 781, 59-70.
- Batts, S.A., Shoemaker, C.R., and Raphael, Y. (2009). Notch signaling and Hes labeling in the normal and drug-damaged organ of Corti. *Hear. Res.* 249, 15–22.
- Ben-Arie N., McCall A.E., Berkman S., Eichele G., Bellen H.J., Zoghbi H.Y. (1996). Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. *Hum Mol Genet* 5, 1207-16.
- Ben-Arie N., Hassan B.A., Bermingham N.A., Malicki D.M., Armstrong D., Matzuk M., Bellen H.J., Zoghbi H.Y. (2000). Functional conservation of atonal and Math1 in the CNS and PNS. *Development* 127,1039-1048.
- Bermingham N.A., Hassan B.A., Price S.D., Vollrath M.A., Ben-Arie N., Eatock R.A., Bellen H.J., Lysakowski A., Zoghbi H.Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* 284, 1837-1841.
- Bermingham, N.A., Hassan, B.A., Wang, V.Y., Fernandez, M., Banfi, S., Bellen, H.J., Fritsch, B., and Zoghbi, H.Y. (2001). Proprioceptor pathway development is dependent on Math1. *Neuron* 30, 411–422.
- Bermingham-McDonogh, O., and Rubel, E.W. (2003). Hair cell regeneration: winging our way towards a sound future. *Curr. Opin. Neurobiol.* 13, 119–126.
- Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* 3, 517–530.
- Bhave, S.A., Stone, J.S., Rubel, E.W., and Coltrera, M.D. (1995). Cell cycle progression in gentamicin-damaged avian cochleas. *J. Neurosci. Off. J. Soc. Neurosci.* 15, 4618–4628.
- Bird, J.E., Daudet, N., Warchol, M.E., and Gale, J.E. (2010). Supporting cells eliminate dying sensory hair cells to maintain epithelial integrity in the avian inner ear. *J. Neurosci. Off. J. Soc. Neurosci.* 30, 12545–12556.
- Brazil, D.P., Church, R.H., Surae, S., Godson, C., and Martin, F. (2015). BMP signalling: agony and antagonism in the family. *Trends Cell Biol.* 25, 249–264.
- Brignull, H.R., Raible, D.W., and Stone, J.S. (2009). Feathers and fins: non-mammalian models for hair cell regeneration. *Brain Res.* 1277, 12–23.
- Cafaro, J., Lee, G.S., and Stone, J.S. (2007). Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Dev. Dyn.* 236, 156–170.
- Cai, T., Jen, H.-I., Kang, H., Klisch, T.J., Zoghbi, H.Y., and Groves, A.K. (2015). Characterization of the transcriptome of nascent hair cells and identification of direct targets of the Atoh1 transcription factor. *J. Neurosci. Off. J. Soc. Neurosci.* 35, 5870–5883.
- Cai, T., Seymour, M.L., Zhang, H., Pereira, F.A., and Groves, A.K. (2013). Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. *J. Neurosci.* 33, 10110–10122.
- Castro D.S., Guillemot F. (2011). Old and new functions of proneural factors revealed by the genome-wide characterization of their transcriptional targets. *Cell Cycle* 10, 4026-31.
- Chardin, S., and Romand, R. (1995). Regeneration and mammalian auditory hair cells. *Science* 267, 707–711.
- Chen P., Johnson J.E., Zoghbi H.Y., Segil N. (2002). The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* 129, 2495-2505.

- Chonko, K.T., Jahan, I., Stone, J., Wright, M.C., Fujiyama, T., Hoshino, M., Fritsch, B., and Maricich, S.M. (2013). Atoh1 directs hair cell differentiation and survival in the late embryonic mouse inner ear. *Dev. Biol.* 381, 401–410.
- Colebrooke, R.E., Chan, P.M., Lynch, P.J., Mooslehner, K., and Emson, P.C. (2007). Differential gene expression in the striatum of mice with very low expression of the vesicular monoamine transporter type 2 gene. *Brain Res.* 1152, 10–16.
- Corwin J.T., and Cotanche D.A. (1988). Regeneration of sensory hair cells after acoustic trauma. *Science* 240, 1772-1774.
- Contrera, K.J., Betz, J., Li, L., Blake, C.R., Sung, Y.K., Choi, J.S., and Lin, F.R. (2016). Quality of life after intervention with a cochlear implant or hearing aid. *Laryngoscope*.
- Cotanche, D.A. (1987). Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. *Hear. Res.* 30, 181–195.
- Cotanche, D.A., and Kaiser, C.L. (2010). Hair cell fate decisions in cochlear development and regeneration. *Hear. Res.* 266, 18–25.
- Cotanche, D.A., Petrell, A., and Picard, D.A. (1991). Structural reorganization of hair cells and supporting cells during noise damage, recovery and regeneration in the chick cochlea. *Ciba Found. Symp.* 160, 131–142; discussion 142–150.
- Cousillas, H., and Rebillard, G. (1985). Age-dependent effects of a pure tone trauma in the chick basilar papilla: evidence for a development of the tonotopic organization. *Hear. Res.* 19, 217–226.
- Cox, R.M., Alexander, G.C., Johnson, J., and Rivera, I. (2011). Cochlear dead regions in typical hearing aid candidates: prevalence and implications for use of high-frequency speech cues. *Ear Hear* 32, 339–348.
- Cruikshanks, K.J., Wiley, T.L., Tweed, T.S., Klein, B.E., Klein, R., Mares-Perlman, J.A., and Nondahl, D.M. (1998). Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The Epidemiology of Hearing Loss Study. *Am. J. Epidemiol.* 148, 879–886.
- Cruz, R.M., Lambert, P.R., and Rubel, E.W. (1987). Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. *Arch. Otolaryngol. Head Neck Surg.* 113, 1058–1062.
- Dallos, P., and Evans, B.N. (1995). High-frequency motility of outer hair cells and the cochlear amplifier. *Science* 267, 2006–2009.
- Daudet, N., Gibson, R., Shang, J., Bernard, A., Lewis, J., and Stone, J. (2009). Notch regulation of progenitor cell behavior in quiescent and regenerating auditory epithelium of mature birds. *Dev. Biol.* 326, 86–100.
- Davis, H. (1983). An active process in cochlear mechanics. *Hear. Res.* 9, 79–90.
- Dickman, J.D., and Lim, I. (2004). Posture, head stability, and orientation recovery during vestibular regeneration in pigeons. *J. Assoc. Res. Otolaryngol.* 5, 323–336.
- Doetzlhofer, A., Basch, M.L., Ohyama, T., Gessler, M., Groves, A.K., and Segil, N. (2009). Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. *Dev. Cell* 16, 58–69.
- Du, X., Li, W., Gao, X., West, M.B., Saltzman, W.M., Cheng, C.J., Stewart, C., Zheng, J., Cheng, W., and Kopke, R.D. (2013). Regeneration of mammalian cochlear and vestibular hair cells through Hes1/Hes5 modulation with siRNA. *Hear. Res.* 304, 91–110.
- Duckert, L.G., and Rubel, E.W. (1990). Ultrastructural observations on regenerating hair cells in the chick basilar papilla. *Hear. Res.* 48, 161–182.
- Ebert, P.J., Timmer, J.R., Nakada, Y., Helms, A.W., Parab, P.B., Liu, Y., Hunsaker, T.L., and Johnson, J.E. (2003). Zic1 represses Math1 expression via interactions with the Math1 enhancer and modulation of Math1 autoregulation. *Development* 130, 1949–1959.
- Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., and Turner, D.L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* 127, 693–702.
- Feng, J., Gao, J., Li, Y., Yang, Y., Dang, L., Ye, Y., Deng, J., and Li, A. (2014). BMP4 enhances foam cell formation by BMPR-2/Smad1/5/8 signaling. *Int. J. Mol. Sci.* 15, 5536–5552.
- Forge, A., Li, L., Corwin, J.T., and Nevill, G. (1993). Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science* 259, 1616–1619.
- Forge, A., Li, L., and Nevill, G. (1998). Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. *J. Comp. Neurol.* 397, 69–88.

- Fritzsch, B., Jahan, I., Pan, N., Kersigo, J., Duncan, J., and Kopecky, B. (2011). Dissecting the molecular basis of organ of Corti development: Where are we now? *Hear. Res.* 276, 16–26.
- Froymovich, O., Rebala, V., Salvi, R.J., and Rassael, H. (1995). Long-term effect of acoustic trauma on distortion product otoacoustic emissions in chickens. *J. Acoust. Soc. Am.* 97, 3021–3029.
- Gao, Z., Kelly, M.C., Yu, D., Wu, H., Lin, X., Chi, F.-L., and Chen, P. (2016). Spatial and Age-Dependent Hair Cell Generation in the Postnatal Mammalian Utricle. *Mol. Neurobiol.* 53, 1601–1612.
- Gerlach, L.M., Hutson, M.R., Germiller, J.A., Nguyen-Luu, D., Victor, J.C., and Barald, K.F. (2000). Addition of the BMP4 antagonist, noggin, disrupts avian inner ear development. *Dev. Camb. Engl.* 127, 45–54.
- Gifford, R.H., Dorman, M.F., Shallop, J.K., and Sydlowski, S.A. (2010). Evidence for the expansion of adult cochlear implant candidacy. *Ear Hear* 31, 186–194.
- Gifford, R.H., Dorman, M.F., Brown, C., and Spahr, A.J. (2012). Hearing, Psychophysics, And Cochlear Implantation: Experiences Of Older Individuals With Mild Sloping To Profound Sensory Hearing Loss. *J Hear Sci* 2, 9–17.
- Girod, D.A., Duckert, L.G., and Rubel, E.W. (1989). Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. *Hear. Res.* 42, 175–194.
- Girod, D.A., Park, R.H., Park, D.L., and Durham, D. (2000). Changes in the avian cochlea after single high-dose gentamicin. *Am J Otolaryngol* 21, 379–388.
- Goodyear, R., Killick, R., Legan, P.K., and Richardson, G.P. (1996). Distribution of beta-tectorin mRNA in the early posthatch and developing avian inner ear. *Hear. Res.* 96, 167–178.
- Gorący, I., Safranow, K., Dawid, G., Skonieczna-Żydecka, K., Kaczmarczyk, M., Gorący, J., Loniewska, B., and Ciechanowicz, A. (2012). Common genetic variants of the BMP4, BMPR1A, BMPR1B, and ACVR1 genes, left ventricular mass, and other parameters of the heart in newborns. *Genet. Test. Mol. Biomark.* 16, 1309–1316.
- Gubbels, S.P., Woessner, D.W., Mitchell, J.C., Ricci, A.J., and Brigande, J.V. (2008). Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. *Nature* 455, 537–541.
- Hashino, E., and Salvi, R.J. (1993). Changing spatial patterns of DNA replication in the noise-damaged chick cochlea. *J. Cell Sci.* 105 (Pt 1), 23–31.
- Hasson, T., Gillespie, P.G., Garcia, J.A., MacDonald, R.B., Zhao, Y., Yee, A.G., Mooseker, M.S., and Corey, D.P. (1997). Unconventional myosins in inner-ear sensory epithelia. *J. Cell Biol.* 137, 1287–1307.
- Hawkins, J.E. (1973). Comparative otopathology: aging, noise, and ototoxic drugs. *Adv. Otorhinolaryngol.* 20, 125–141.
- Hawkins, J.E., Johnsson, L.G., Stebbins, W.C., Moody, D.B., and Coombs, S.L. (1976). Hearing loss and cochlear pathology in monkeys after noise exposure. *Acta Otolaryngol.* 81, 337–343.
- Helms, A.W., and Johnson, J.E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* 125, 919–928.
- Helms, A.W., Abney, A.L., Ben-Arie, N., Zoghbi, H.Y., and Johnson, J.E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* 127, 1185–1196.
- Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* 375, 787–790.
- Hwang, C.H., Guo, D., Harris, M.A., Howard, O., Mishina, Y., Gan, L., Harris, S.E., and Wu, D.K. (2010). Role of bone morphogenetic proteins on cochlear hair cell formation: analyses of Noggin and Bmp2 mutant mice. *Dev. Dyn.* 239, 505–513.
- Irvine, D.R.F., Brown, M., Kamke, M.R., and Rubel, E.W. (2009). Effects of restricted basilar papillar lesions and hair cell regeneration on auditory forebrain frequency organization in adult European starlings. *J. Neurosci.* 29, 6871–6882.
- Itoh, M., and Chitnis, A.B. (2001). Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. *Mech. Dev.* 102, 263–266.
- Izumikawa, M., Minoda, R., Kawamoto, K., Abrashkin, K.A., Swiderski, D.L., Dolan, D.F., Brough, D.E., and Raphael, Y. (2005). Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nat. Med.* 11, 271–276.
- Izumikawa, M., Batts, S.A., Miyazawa, T., Swiderski, D.L., and Raphael, Y. (2008). Response of the flat cochlear epithelium to forced expression of Atoh1. *Hear. Res.* 240, 52–56.

- Jahan, I., Pan, N., Kersigo, J., and Fritzsche, B. (2010). Neurod1 suppresses hair cell differentiation in ear ganglia and regulates hair cell subtype development in the cochlea. *PLoS ONE* 5, e11661.
- Jahan, I., Pan, N., Kersigo, J., and Fritzsche, B. (2013). Beyond generalized hair cells: molecular cues for hair cell types. *Hear. Res.* 297, 30–41.
- Janas, J.D., Cotanche, D.A., and Rubel, E.W. (1995). Avian cochlear hair cell regeneration: stereological analyses of damage and recovery from a single high dose of gentamicin. *Hear. Res.* 92, 17–29.
- Jarman, A.P., Brand, M., Jan, L.Y., and Jan, Y.N. (1993a). The regulation and function of the helix-loop-helix gene, *asense*, in *Drosophila* neural precursors. *Development* 119, 19–29.
- Jarman, A.P., Grau, Y., Jan, L.Y., and Jan, Y.N. (1993b). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell* 73, 1307–1321.
- Johnsson, L.G., and Hawkins, J.E. (1976). Degeneration patterns in human ears exposed to noise. *Ann. Otol. Rhinol. Laryngol.* 85, 725–739.
- Jones, J.M., Montcouquiol, M., Dabdoub, A., Woods, C., and Kelley, M.W. (2006). Inhibitors of differentiation and DNA binding (*Ids*) regulate *Math1* and hair cell formation during the development of the organ of Corti. *J. Neurosci. Off. J. Soc. Neurosci.* 26, 550–558.
- Jørgensen, J.M., and Mathiesen, C. (1988). The avian inner ear. Continuous production of hair cells in vestibular sensory organs, but not in the auditory papilla. *Naturwissenschaften* 75, 319–320.
- Kageyama, R., Ohtsuka, T., and Kobayashi, T. (2008). Roles of *Hes* genes in neural development. *Dev. Growth Differ.* 50 Suppl 1, S97–103.
- Kamaid, A., Neves, J., and Giráldez, F. (2010). *Id* gene regulation and function in the prosensory domains of the chicken inner ear: a link between *Bmp* signaling and *Atoh1*. *J. Neurosci.* 30, 11426–11434.
- Kawamoto, K., Ishimoto, S.-I., Minoda, R., Brough, D.E., and Raphael, Y. (2003). *Math1* gene transfer generates new cochlear hair cells in mature guinea pigs *in vivo*. *J. Neurosci.* 23, 4395–4400.
- Kelly, M.C., Chang, Q., Pan, A., Lin, X., and Chen, P. (2012). *Atoh1* directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea *in vivo*. *J. Neurosci.* 32, 6699–6710.
- Kruger, R.P., Goodyear, R.J., Legan, P.K., Warchol, M.E., Raphael, Y., Cotanche, D.A., and Richardson, G.P. (1999). The supporting-cell antigen: a receptor-like protein tyrosine phosphatase expressed in the sensory epithelia of the avian inner ear. *J. Neurosci.* 19, 4815–4827.
- Lanford, P.J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T., and Kelley, M.W. (1999). Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat. Genet.* 21, 289–292.
- Lanford, P.J., Shailam, R., Norton, C.R., Gridley, T., and Kelley, M.W. (2000). Expression of *Math1* and *HES5* in the cochleae of wildtype and *Jag2* mutant mice. *J. Assoc. Res. Otolaryngol.* 1, 161–171.
- Leake, P.A., Hradek, G.T., Rebscher, S.J., and Snyder, R.L. (1991). Chronic intracochlear electrical stimulation induces selective survival of spiral ganglion neurons in neonatally deafened cats. *Hear. Res.* 54, 251–271.
- Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* 6, 3–10.
- Lewis, R.M., Hume, C.R., and Stone, J.S. (2012). *Atoh1* expression and function during auditory hair cell regeneration in post-hatch chickens. *Hear. Res.* 289, 74–85.
- Li, H., Corrales, C.E., Wang, Z., Zhao, Y., Wang, Y., Liu, H., and Heller, S. (2005). *BMP4* signaling is involved in the generation of inner ear sensory epithelia. *BMC Dev. Biol.* 5, 16.
- Lin, V., Golub, J.S., Nguyen, T.B., Hume, C.R., Oesterle, E.C., and Stone, J.S. (2011). Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J. Neurosci.* 31, 15329–15339.
- Lippe, W., and Rubel, E.W. (1983). Development of the place principle: tonotopic organization. *Science* 219, 514–516.
- Liu, Z., Dearman, J.A., Cox, B.C., Walters, B.J., Zhang, L., Ayrault, O., Zindy, F., Gan, L., Roussel, M.F., and Zuo, J. (2012). Age-dependent *in vivo* conversion of mouse cochlear pillar and deiters' cells to immature hair cells by *atoh1* ectopic expression. *J. Neurosci.* 32, 6600–6610.
- Lumpkin, E.A., Collisson, T., Parab, P., Omer-Abdalla, A., Haeberle, H., Chen, P., Doetzlhofer, A., White, P., Groves, A., Segil, N., et al. (2003). *Math1*-driven GFP expression in the developing nervous system of transgenic mice. *Gene Expr. Patterns* 3, 389–395.
- Ma, E.Y., Rubel, E.W., and Raible, D.W. (2008). Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. *J. Neurosci. Off. J. Soc. Neurosci.* 28, 2261–2273.

- Maass, J.C., Gu, R., Basch, M.L., Waldhaus, J., Lopez, E.M., Xia, A., Oghalai, J.S., Heller, S., and Groves, A.K. (2015). Changes in the regulation of the Notch signaling pathway are temporally correlated with regenerative failure in the mouse cochlea. *Front Cell Neurosci* 9, 110.
- Marean, G.C., Burt, J.M., Beecher, M.D., and Rubel, E.W. (1998). Auditory perception following hair cell regeneration in European starling (*Sturnus vulgaris*): frequency and temporal resolution. *J. Acoust. Soc. Am.* 103, 3567–3580.
- Marsh, R.R., Xu, L.R., Moy, J.P., and Saunders, J.C. (1990). Recovery of the basilar papilla following intense sound exposure in the chick. *Hear. Res.* 46, 229–237.
- Masterson, E.A., Tak, S., Themann, C.L., Wall, D.K., Groenewold, M.R., Deddens, J.A., and Calvert, G.M. (2013). Prevalence of hearing loss in the United States by industry. *Am. J. Ind. Med.* 56, 670–681.
- Matei, V., Pauley, S., Kaing, S., Rowitch, D., Beisel, K.W., Morris, K., Feng, F., Jones, K., Lee, J., and Fritzsche, B. (2005). Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. *Dev. Dyn.* 234, 633–650.
- Millimaki, B.B., Sweet, E.M., Dhasan, M.S., and Riley, B.B. (2007). Zebrafish *atoh1* genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. *Dev. Camb. Engl.* 134, 295–305.
- Miyazono, K., Kamiya, Y., and Morikawa, M. (2010). Bone morphogenetic protein receptors and signal transduction. *J. Biochem. (Tokyo)* 147, 35–51.
- Morest, D.K., and Cotanche, D.A. (2004). Regeneration of the inner ear as a model of neural plasticity. *J. Neurosci. Res.* 78, 455–460.
- Morrison, A., Hodgetts, C., Gossler, A., Hrabé de Angelis, M., and Lewis, J. (1999). Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. *Mech. Dev.* 84, 169–172.
- Morsli, H., Choo, D., Ryan, A., Johnson, R., and Wu, D.K. (1998). Development of the mouse inner ear and origin of its sensory organs. *J. Neurosci.* 18, 3327–3335.
- Müller, M., and Smolders, J.W. (1998). Hair cell regeneration after local application of gentamicin at the round window of the cochlea in the pigeon. *Hear. Res.* 120, 25–36.
- Müller, M., and Smolders, J.W. (1999). Responses of auditory nerve fibers innervating regenerated hair cells after local application of gentamicin at the round window of the cochlea in the pigeon. *Hear. Res.* 131, 153–169.
- Mulvaney, J., and Dabdoub, A. (2012). *Atoh1*, an essential transcription factor in neurogenesis and intestinal and inner ear development: function, regulation, and context dependency. *J. Assoc. Res. Otolaryngol.* 13, 281–293.
- Murray, S.S., Brochmann Murray, E.J., Wang, J.C., and Duarte, M.E.L. (2016). The history and histology of bone morphogenetic protein. *Histol. Histopathol.* 11774.
- Niemiec, A.J., Raphael, Y., and Moody, D.B. (1994). Return of auditory function following structural regeneration after acoustic trauma: behavioral measures from quail. *Hear. Res.* 79, 1–16.
- Oesterle, E.C., Tsue, T.T., Reh, T.A., and Rubel, E.W. (1993). Hair-cell regeneration in organ cultures of the postnatal chicken inner ear. *Hear. Res.* 70, 85–108.
- Oh, S.H., Johnson, R., and Wu, D.K. (1996). Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. *J. Neurosci.* 16, 6463–6475.
- Ohyama, T., Basch, M.L., Mishina, Y., Lyons, K.M., Segil, N., and Groves, A.K. (2010). BMP signaling is necessary for patterning the sensory and nonsensory regions of the developing mammalian cochlea. *J. Neurosci.* 30, 15044–15051.
- Pan, N., Jahan, I., Kersigo, J., Duncan, J.S., Kopecky, B., and Fritzsche, B. (2012). A novel *Atoh1* “self-terminating” mouse model reveals the necessity of proper *Atoh1* level and duration for hair cell differentiation and viability. *PLoS One* 7, e30358.
- Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45.
- Pujades, C., Kamaid, A., Alsina, B., and Giraldez, F. (2006). BMP-signaling regulates the generation of hair-cells. *Dev. Biol.* 292, 55–67.
- Raft, S., Koundakjian, E.J., Quinones, H., Jayasena, C.S., Goodrich, L.V., Johnson, J.E., Segil, N., and Groves, A.K. (2007). Cross-regulation of *Ngn1* and *Math1* coordinates the production of neurons and sensory hair cells during inner ear development. *Development* 134, 4405–4415.
- Raphael, Y. (1992). Evidence for supporting cell mitosis in response to acoustic trauma in the avian inner ear. *J. Neurocytol.* 21, 663–671.

- Rinkwitz, S., Bober, E., and Baker, R. (2001). Development of the vertebrate inner ear. *Ann. N. Y. Acad. Sci.* 942, 1–14.
- Roberson D.W. (1996). Light microscopic evidence that direct transdifferentiation gives rise to new hair cells in regenerating avian auditory epithelium. *Aud Neurosci* 2, 195-205.
- Roberson, D.W., Alosi, J.A., and Cotanche, D.A. (2004). Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. *J. Neurosci. Res.* 78, 461–471.
- Roberson, D.W., and Rubel, E.W. (1994). Cell division in the gerbil cochlea after acoustic trauma. *Am. J. Otol.* 15, 28–34.
- Roberson, D.F., Weisleder, P., Bohrer, P.S., and Rubel, E.W. (1992). Ongoing production of sensory cells in the vestibular epithelium of the chick. *Hear. Res.* 57, 166–174.
- Ruben, R.J. (1967). Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Otolaryngol. Suppl* 220, 1–44.
- Ryals, B.M., and Rubel, E.W. (1988). Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* 240, 1774–1776.
- Ryals, B.M., and Westbrook, E.W. (1990). Hair cell regeneration in senescent quail. *Hear. Res.* 50, 87–96.
- Ryals, B.M., and Westbrook, E.W. (1994). TEM analysis of neural terminals on autoradiographically identified regenerated hair cells. *Hear. Res.* 72, 81–88.
- Ryan, A., and McGee, T.J. (1977). Development of hearing loss in kanamycin treated chinchillas. *Ann. Otol. Rhinol. Laryngol.* 86, 176–182.
- Ryan, A.F., Woolf, N.K., and Bone, R.C. (1980). Ultrastructural correlates of selective outer hair cell destruction following kanamycin intoxication in the chinchilla. *Hear. Res.* 3, 335–351.
- Salvi, R.J., Chen, L., Trautwein, P., Powers, N., and Shero, M. (1998). Hair cell regeneration and recovery of function in the avian auditory system. *Scand Audiol Suppl* 48, 7–14.
- Schacht, J., Talaska, A.E., and Rybak, L.P. (2012). Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention. *Anat Rec (Hoboken)* 295, 1837–1850.
- Selkoe, D., and Kopan, R. (2003). Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. *Annu. Rev. Neurosci.* 26, 565–597.
- Shailam, R., Lanford, P.J., Dolinsky, C.M., Norton, C.R., Gridley, T., and Kelley, M.W. (1999). Expression of proneural and neurogenic genes in the embryonic mammalian vestibular system. *J. Neurocytol.* 28, 809–819.
- Shang, J., Cafaro, J., Nehmer, R., and Stone, J. (2010). Supporting cell division is not required for regeneration of auditory hair cells after ototoxic injury in vitro. *J. Assoc. Res. Otolaryngol.* 11, 203–222.
- Shou, J., Zheng, J.L., and Gao, W.Q. (2003). Robust generation of new hair cells in the mature mammalian inner ear by adenoviral expression of Hath1. *Mol. Cell. Neurosci.* 23, 169–179.
- Sobkowicz, H.M., August, B.K., and Slapnick, S.M. (1997). Cellular interactions as a response to injury in the organ of Corti in culture. *Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci.* 15, 463–485.
- Son, E.J., Ma, J.-H., Ankamreddy, H., Shin, J.-O., Choi, J.Y., Wu, D.K., and Bok, J. (2015). Conserved role of Sonic Hedgehog in tonotopic organization of the avian basilar papilla and mammalian cochlea. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3746–3751.
- Sprinzi, G.M., and Riechelmann, H. (2010). Current trends in treating hearing loss in elderly people: a review of the technology and treatment options - a mini-review. *Gerontology* 56, 351–358.
- Staecker, H., Schlecker, C., Kraft, S., Praetorius, M., Hsu, C., and Brough, D.E. (2014). Optimizing atoh1-induced vestibular hair cell regeneration. *Laryngoscope* 124 Suppl 5, S1–S12.
- Staecker H., Praetorius M., Baker K., Brough D.E. (2007) Vestibular hair cell regeneration and restoration of balance function induced by math1 gene transfer. *Otol Neurotol* 28, 223-231.
- Stone, J.S., Leaño, S.G., Baker, L.P., and Rubel, E.W. (1996). Hair cell differentiation in chick cochlear epithelium after aminoglycoside toxicity: in vivo and in vitro observations. *J. Neurosci.* 16, 6157–6174.
- Stone J.S., Cotanche D.A. (1994). Identification of the timing of S phase and the patterns of cell proliferation during hair cell regeneration in the chick cochlea. *J Comp Neurol* 341, 50-67.
- Stone J.S., Cotanche D.A. (2007). Hair cell regeneration in the avian auditory epithelium. *Int J Dev Biol* 51, 633-647.
- Stone J.S., Rubel E.W. (1999). Delta1 expression during avian hair cell regeneration. *Development* 126, 961-73.

- Stone J.S., Rubel E.W. (2000). Temporal, spatial, and morphologic features of hair cell regeneration in the avian basilar papilla. *J Comp Neurol* 417, 1-16.
- Strelioff, D., Flock, A., and Minser, K.E. (1985). Role of inner and outer hair cells in mechanical frequency selectivity of the cochlea. *Hear. Res.* 18, 169–175.
- Sweeney, A.D., Hunter, J.B., Carlson, M.L., Rivas, A., Bennett, M.L., Gifford, R.H., Noble, J.H., Haynes, D.S., Labadie, R.F., and Wanna, G.B. (2016). Durability of Hearing Preservation after Cochlear Implantation with Conventional-Length Electrodes and Scala Tympani Insertion. *Otolaryngol Head Neck Surg* 154, 907–913.
- Tateya, T., Imayoshi, I., Tateya, I., Ito, J., and Kageyama, R. (2011). Cooperative functions of Hes/Hey genes in auditory hair cell and supporting cell development. *Dev. Biol.* 352, 329–340.
- Tong, K.K., Ma, T.C., and Kwan, K.M. (2015). BMP/Smad signaling and embryonic cerebellum development: stem cell specification and heterogeneity of anterior rhombic lip. *Dev. Growth Differ.* 57, 121–134.
- Trautwein, P., Salvi, R.J., Miller, K., Shero, M., and Hashino, E. (1996). Incomplete recovery of chicken distortion product otoacoustic emissions following acoustic overstimulation. *Audiol. Neurootol.* 1, 86–103.
- Tucci, D.L., and Rubel, E.W. (1990). Physiologic status of regenerated hair cells in the avian inner ear following aminoglycoside ototoxicity. *Otolaryngol Head Neck Surg* 103, 443–450.
- Turner, C.W., and Cummings, K.J. (1999). Speech audibility for listeners with high-frequency hearing loss. *Am J Audiol* 8, 47–56.
- Van Wijk, B., Moorman, A.F.M., and van den Hoff, M.J.B. (2007). Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovasc. Res.* 74, 244–255.
- Wang, G.-P., Chatterjee, I., Batts, S.A., Wong, H.T., Gong, T.-W., Gong, S.-S., and Raphael, Y. (2010). Notch signaling and Atoh1 expression during hair cell regeneration in the mouse utricle. *Hear. Res.* 267, 61–70.
- Warchol, M.E., and Corwin, J.T. (1996). Regenerative proliferation in organ cultures of the avian cochlea: identification of the initial progenitors and determination of the latency of the proliferative response. *J. Neurosci.* 16, 5466–5477.
- Winther, F.O. (1970). X-ray irradiation of the inner ear of the guinea pig. An electron microscopic study of the degenerating outer hair cells of the organ of Corti. *Acta Otolaryngol. (Stockh.)* 69, 61–76.
- World Health Organization. (2015). Deafness and hearing loss. Fact sheet N°300. Updated March 2015. Retrieved from <http://www.who.int/mediacentre/factsheets/fs300/en/>
- Woods, C., Montcouquiol, M., and Kelley, M.W. (2004). Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nat. Neurosci.* 7, 1310–1318.
- Wu, D.K., and Oh, S.H. (1996). Sensory organ generation in the chick inner ear. *J. Neurosci.* 16, 6454–6462.
- Xiang, M.L., Mu, M.Y., Pao, X., and Chi, F.L. (2000). The reinnervation of regenerated hair cells in the basilar papilla of chicks after kanamycin ototoxicity. *Acta Otolaryngol.* 120, 912–921.
- Zhang, T., Dorman, M.F., Gifford, R., and Moore, B.C.J. (2014). Cochlear dead regions constrain the benefit of combining acoustic stimulation with electric stimulation. *Ear Hear* 35, 410–417.
- Zhao H., Ayrault O., Zindy F., Kim J.H., Roussel M.F. (2008) Post-transcriptional down-regulation of Atoh1/Math1 by bone morphogenic proteins suppresses medulloblastoma development. *Genes Dev* 22, 722-727.
- Zhao, L.-D., Guo, W.-W., Lin, C., Li, L.-X., Sun, J.-H., Wu, N., Ren, L.-L., Li, X.-X., Liu, H.-Z., Young, W.-Y., et al. (2011). Effects of DAPT and Atoh1 overexpression on hair cell production and hair bundle orientation in cultured Organ of Corti from neonatal rats. *PLoS ONE* 6, e23729.
- Zheng, J.L., and Gao, W.Q. (2000). Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* 3, 580–586.
- Zine, A., Aubert, A., Qiu, J., Therianos, S., Guillemot, F., Kageyama, R., and de Ribaupierre, F. (2001). Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* 21, 4712–4720.

**Appendix: Acoustics Today Article**

**Regeneration of auditory hair cells:**

**A potential treatment for hearing loss on the horizon**

Rebecca M. Lewis, Edwin W Rubel, and Jennifer S. Stone

Short title: Regeneration of auditory hair cells

Teaser: Regeneration of cochlear hair cells is being investigated as a potential therapy for hearing impairments.

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## **Introduction**

The process of hearing involves a complex chain of events, each one is important to ensure proper detection and processing of sounds. In the first step, sound waves traveling through the environment enter the ear canal and vibrate the eardrum. This energy is transmitted through the three bones of the middle ear to the inner ear. Within the inner ear, the energy derived from sound waves is transmitted to the basilar membrane of the cochlea, upon which lies the sensory organ for hearing, the organ of Corti (Figure 1, 2A).

The organ of Corti is composed of sensory hair cells, as well as a group of specialized cell types, collectively called supporting cells, and the peripheral processes of auditory neurons. Hair cells are sensory receptors. Responding to the mechanical signals derived from sound waves, hair cells transduce this energy into electrical signals that are transmitted via the auditory nerve to the brain. In the normal human ear, there are about 3,000 inner hair cells and 12,000 outer hair cells (Bredberg, 1967). Inner hair cells (Figure 2B) are the true sensory receptors. Upon stimulation inner hair cells activate auditory nerve fibers that in turn activate auditory brainstem nuclei. The major function of outer hair cells (Figure 2C) is to modulate the function of the organ of Corti by enhancing signal processing of low intensity auditory signals. These two types of hair cells work together such that the auditory nerve transmits highly selective information about the frequency, timing, and intensity of sounds to the brain. Supporting cells are non-sensory cells that neighbor and isolate hair cells from one another. These non-sensory cells work with the surrounding structures to provide physical and molecular support to this elaborate sensory epithelium.

Hearing loss can result from failure of acoustic signals to reach the inner ear (conductive hearing loss) or from damage to any part of the inner ear or the central auditory pathways in the brain (sensorineural hearing loss, or SNHL). Conductive hearing loss is usually treated by medical or surgical means. The most common form of SNHL results from damage or dysfunction of hair cells of the organ of Corti. When hair cells in the mammalian cochlea die, they do not regenerate; this form of SNHL is permanent (Figure 3). If hearing loss is moderate, patients can be fit with hearing aids, which amplify sounds to enhance hearing. If it is severe, patients can receive cochlear implants to bypass the injured hair cells and directly stimulate the auditory nerve. Neither form of treatment restores normal hearing or addresses the cause of hearing loss – the missing hair cells.

Around thirty years ago, the discovery that hair cells regenerate in birds raised the possibility that we could someday find a way to replace hair cells in mammals, including humans. Since that time, many advances in our understanding of hair cell regeneration in birds, fishes, and mammals have been achieved. This article reviews the current state of research in the field of hair cell regeneration. Due to space limitations, we have removed all but the most essential citations. For further details and relevant

citations, we encourage readers to examine the many review papers related to this field (e.g., Warchol, 2011; Groves et al., 2013; Rubel et al., 2013).

### **Cellular Processes of Hair Cell Damage and Regeneration**

The sensory epithelium of the cochlea is a cytoarchitecturally elegant and delicate structure (Figure 1). The hair cells are commonly damaged by a variety of environmental events, some of which are known, including acoustic overstimulation from loud or prolonged noise, or concussive stimuli. Several different types of medications kill hair cells when administered at high doses or for prolonged periods. These include, but are not limited to, aminoglycoside antibiotics, such as gentamicin, and heavy metal anti-cancer drugs, such as cisplatin. Hair cells also die as we age; in most cases, this is due to unknown causes. Finally, genetic mutations exist that cause hair cells to die during embryonic development or at later stages of life.

Until 1985, it was believed that regeneration of inner ear hair cells was not possible in vertebrates. While studying processes of hair cell damage in the chicken auditory epithelium, however, investigators noted a reappearance of hair cells in the area of damage. The immature morphology of these cells appeared similar to that of embryonic hair cells in the cochlea of chickens (Cotanche, 1987; Cruz et al., 1987). During this same period, it was also discovered that regeneration of hair cells occurs readily in the vestibular portions of the avian inner ear (Jørgensen and Mathiesen, 1988). Soon, researchers learned that the hair cells of the inner ear and lateral line system of fish, frogs, and salamanders also readily regenerate after damage, which led to the conclusion that regeneration occurs in hair cell epithelia of all vertebrates except mammals. Further analysis revealed that the supporting cells that normally surround hair cells are the source of these newly differentiating hair cells. Supporting cells may either mitotically divide to achieve hair cell differentiation or phenotypically convert to a hair cell in a process called direct transdifferentiation (Figure 4; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Roberson et al., 1996). With these two methods of replacing hair cells, non-mammalian vertebrates provide valuable models to study these processes and their ability to restore hearing after sustained SNHL.

In mammals, the situation is quite different. When hair cells die in the mature mammalian organ of Corti, supporting cells fill in the gaps where hair cells were located to form permanent scars, and no new hair cells are formed. Moreover, supporting cells neither divide nor convert into hair cells after hair cell damage (e.g., Roberson and Rubel, 1994; Chardin and Romand, 1995).

In contrast to the organ of Corti, adult mammals can spontaneously replace a small number of hair cells in the vestibular organs of the inner ear. New hair cells are largely formed by non-mitotic regeneration (Forge et al., 1998; Kawamoto et al., 2009; Golub et al., 2012). There appears to be a small degree of supporting cell division triggered in response to hair cell loss (Li and Forge, 1997; Kuntz and Oesterle, 1998), but no newly formed cells become replacement hair cells (Oesterle et al., 2003).

The big challenge facing researchers today is to determine why hair cells are not readily regenerated in mammals. Regeneration could fail in the adult cochlea because the hearing organ loses the population of progenitor cells capable of forming new hair cells during development. Alternatively, cells with the potential to replace hair cells may exist in the cochlea but are unable to respond to damage due to active inhibition or lack of a stimulatory substance.

### **Stimulating Native Progenitors to Form New Hair cells in the Adult Cochlea**

Researchers have examined whether the cells capable of forming new hair cells still exist in the cochlea of mature mammals. Many tissues in our body undergo continual renewal. One common feature of these tissues is they contain stem cells that divide and form new specialized cells throughout life. Several lines of evidence show that the cochlea and vestibular organs possess stem-like progenitors to hair cells during early development but lose them as the organs mature. Consistent with this, new hair cells can be formed by supporting cells from the organ of Corti of neonatal mammals (White et al., 2006; Oshima et al., 2007; Cox et al., 2014), but not in adult mammals (e.g., Roberson and Rubel, 1994; Forge et al., 1998).

Investigators are using three general strategies to identify ways to trick supporting cells in the mature mammalian inner ear to regenerate hair cells. First, we are finding clues in cochlear development. Hair cells in the organ of Corti form during the embryonic period through a complex series

of cellular steps controlled by a cascade of molecular interactions. Some researchers have postulated that, before any cell in the mature cochlea can form a new hair cell, it will need to re-live these same stages of development.

Second, we look to other regenerative tissues. Many tissues in the body are continuously replaced under normal conditions and/or after damage, including cells in the skin, intestine, and some regions of the brain. We reason that many of the molecular cascades leading to regeneration in these other tissues could be co-opted to trigger regeneration in the cochlea.

Third, using the new tools of molecular genetics, we can directly query the molecular cascades that are activated in the sensory epithelia of non-mammalian vertebrates that do regenerate hair cells, such as birds and fishes. The section below, we describe several genes and signaling pathways that met one or more of these criteria and were evaluated for their capacity to stimulate hair cell regeneration in mammals. These analyses revealed signaling molecules that are important for facilitating regeneration.

#### *Forced Atoh1 expression: Pushing mature supporting cells to transdifferentiate into hair cells*

A proneural transcription factor named atonal homolog 1 (Atoh1) is a potential therapeutic agent for promoting hair cell regeneration. Atoh1 acts to help direct the generation of hair cell-specific proteins that give the hair cell its morphological and physiological identity (Cai et al., 2015). When the gene encoding Atoh1 is deleted, hair cells in the organ of Corti do not form (Bermingham et al., 1999). Thus, Atoh1 is a very powerful activator of hair cell features and could trigger cells to transdifferentiate into hair cells.

In tissues that regenerate hair cells, Atoh1 expression is activated in supporting cells shortly after hair cell damage (Cafaro et al., 2007; Wang et al., 2010; Lin et al., 2011). In cultured auditory organs from chickens, forced expression of Atoh1 influences supporting cells to form new hair cells by promoting division and direct transdifferentiation (Lewis et al., 2012). In rodents, forced expression of Atoh1 by viral injection into the organ of Corti or nearby regions of developing mice forces more cells to differentiate as hair cells (Zheng and Gao, 2000; Gubbels et al., 2008). These findings suggested Atoh1 misexpression might be sufficient to trigger supporting cells to transdifferentiate into hair cells after damage in the cochlea of adult mammals. Indeed, some studies suggest Atoh1 may drive production of

new hair cells in auditory (Izumikawa et al., 2005) and vestibular (Schlecker et al., 2011) organs, which might result in small improvements in hearing and balance function.

However, recent studies are less encouraging. Misexpression of Atoh1 in pillar and Deiters' cells - two supporting cell subtypes (Figure 1) - in the mature mouse cochlea stimulates early stages of transdifferentiation into hair cells, but this process is not completed and many "forced" cells die (Liu et al., 2012). Indeed, Atkinson et al. (2015) noted no significant improvement in hearing after virally-induced Atoh1 misexpression in the organs of Corti of guinea pigs. Hence, an important current challenge is to determine what factors limit the ability of Atoh1 to drive hair cell regeneration in the mature cochlea. Currently, a human clinical trial testing the ability of viral infection of Atoh1 to improve hearing is underway. Results are not available at this time.

#### *Suppression of Notch signaling: Can this enhance Atoh1's pro-regenerative effects?*

As discussed above, it is evident that, while Atoh1 misexpression reliably promotes supporting cells and other cells around the organ of Corti to become hair cells in neonatal mammals, unidentified factors appear to hinder Atoh1's effects in the mature organ of Corti. One likely suspect is the Notch receptor (Lewis, 1998). Notch is a receptor on the surface of cells that is activated by molecules on adjacent cells (Figure 5).

Notch has many functions in a variety of cells, but its most pertinent role with respect to hair cell regeneration is inhibition of hair cell formation. During development, Notch ligands are expressed in young hair cells and influence surrounding supporting cells to maintain their identity rather than differentiate into hair cells (reviewed in Kelley, 2006). Notch signaling executes this function, at least in part, by blocking Atoh1 synthesis (Lanford et al., 2000). In the developing cochlea, inhibition of Notch signaling results in a significant increase in the number of hair cells (e.g., Hayashi et al., 2008; Doetzlhofer et al., 2009). Similar effects of Notch inhibition have been documented during hair cell regeneration in fishes (Ma et al., 2008), birds (Daudet et al., 2009), and mouse vestibular organs (Lin et al., 2011). One study suggests infusion of Notch inhibitors into live mice can promote supporting cells to convert into hair cells in the organ of Corti of adult mice after hair cell damage (Mizutani et al., 2013). However, another study clearly describes a precipitous loss of efficacy of Notch inhibitors to stimulate hair

cell regeneration (Maass et al., 2015). Hopefully, these apparently conflicting interpretations of Notch inhibition will be resolved in future studies.

#### *Lifting the blockade on supporting cell division in native progenitors*

As discussed above, supporting cells in the mature organ of Corti are strongly inhibited from dividing, even after hair cells have been killed. Although Atoh1 misexpression and/or Notch inhibition appear to encourage supporting cells to form hair cell-like cells in mature animals, neither treatment has a significant effect on supporting cell division. Therefore, as a therapy alone, either manipulation would likely deplete supporting cells, which would almost certainly reduce function of the organ of Corti. Investigators are attempting to determine how to promote supporting cells to divide mitotically and either replace themselves or form new hair cells. At this point, there are no known manipulations that have these effects in the mature organ of Corti. However, we know some ways in which supporting cell division can be promoted in the young cochlea.

For cochlear supporting cells to divide, they must exit their normal state of mitotic inactivity and enter the cell cycle. p27<sup>Kip1</sup> is a molecule that blocks progenitor cells (or supporting cells) in the organ of Corti of mice from dividing during embryonic and postnatal development. Embryonic deletion of the gene encoding p27<sup>Kip1</sup> causes an excess of cells to be formed in the organ of Corti, including hair cells (Chen and Segil, 1999; Löwenheim et al., 1999). In mature mice, blocking the synthesis of p27<sup>Kip1</sup> causes a small but significant increase in cell division in some types of supporting cells in the organ of Corti (Oesterle et al., 2011). Inhibition of p27<sup>Kip1</sup> and similar molecules is under investigation as a way to promote mammalian hair cell regeneration. It is particularly important at this stage that investigators determine if p27<sup>Kip1</sup> deletion in adult rodents leads to the production of functional, stable hair cells.

Activity of p27<sup>Kip1</sup> and other regulators of cell division is controlled by extracellular signaling molecules. One set of molecules that drives cell division in many tissues are Wnts, which bind receptors on the surface of cells and activate a transcriptional co-activator called  $\beta$ -catenin (reviewed in Jansson et al., 2015). Wnt/ $\beta$ -catenin signaling is required for progenitor cell division during cochlear development; when inhibited, significantly fewer hair cells form (Shi et al., 2014). Forced overexpression

of Wnt promotes supporting cells in the organ of Corti to divide in very young, but not in mature, mice (Chai et al., 2012; Shi et al., 2013). Therefore, activation of Wnt alone cannot overcome other inhibitory signals present in the mature mammalian organ of Corti. By contrast, pharmacological activation of Wnt promotes hair cell regeneration in lateral line functional neuromasts of larval zebrafish (Head et al., 2013; Jacques et al., 2014).

Epidermal growth factor (EGF) is another molecule that drives supporting cell division in supporting cells in the organ of Corti of neonatal mice, as well as in supporting cells in the regenerating auditory epithelium of mature chickens (White et al., 2012). Treatment of cultured organs of Corti with EGF in newborn rats increases the formation of supernumerary hair cells (Lefebvre et al., 2000). Once again, this effect rapidly declines with age.

#### *Could transient or combinatorial treatments improve hair cell regeneration?*

As discussed above, we now know several powerful genes or signaling pathways that, when manipulated in very young rodents, cause supporting cells to divide and form new hair cells. But, these same manipulations have very little effect, or even deleterious effects, in mature rodents. These findings tell us that promotion of hair cell regeneration in mature humans will be more challenging than originally thought. One strategy scientists are testing is whether transient activation or suppression of gene activity has a better outcome than sustained alterations. During development, signals turn on and off in cells, while many of the manipulations discussed above are permanent and therefore unnatural. Modern techniques for transient gene silencing, such as siRNA, might enhance effects of treatment by better recapitulating nature. Another hypothesis being tested is whether combinatorial manipulations of genes and pathways can more effectively promote regeneration than single manipulations. This has proven to be fruitful in the cochlea of neonatal rodents in experiments that activate Atoh1 and inhibit Notch simultaneously (Zhao et al., 2011) or activate Atoh1 and Wnt simultaneously (Kuo et al., 2015). These dual approaches acknowledge the complexity of growth regulation in mature tissues, as well as the critical interactions that occur between pathways.

## **Transplantation of Cells to Replace Hair Cells**

In the prior section, we discussed strategies for promoting native cells in the damaged organ of Corti to divide or directly transdifferentiate in order to replace lost hair cells. It is possible, however, that a responsive population may not persist in the adult cochlea. On the other hand, we may fail to find appropriate treatments to stimulate resident cells to regenerate hair cells. In either case, it will be necessary to adopt an alternative approach – to transplant cells to the inner ear that can replace hair cells. The obvious choice is to transplant stem cells, which have the potential to divide and differentiate into a range of mature cell types. Stem cells can be grown in a dish and guided toward a desired cell fate (in this case, hair cell) by certain chemical agents or culture conditions. Stem cells hold great promise for treating several types of pathology, including heart disease, blindness, and leukemia.

Some of the first studies to test the usefulness of different types of stem cells to replace damaged hair cells were performed with pluripotent stem cells or neural stem cells derived from mouse embryos. Li et al. (2003) conditioned mouse embryonic stem cells with various compounds in culture to drive them to differentiate hair cell-like features. Upon transplantation into the embryonic chicken ear, conditioned cells incorporated into hair-cell epithelia and acquire hair cell-like properties. Fujino et al. (2004) found that neural stem cells introduced into cultured inner ear organs from rats integrate into the sensory epithelia of vestibular organs but not the cochlea. Subsequently, Oshima et al. (2010) identified treatments that drive induced pluripotent stem cells (derived from fibroblasts) to differentiate advanced features of hair cells in culture, including hair bundles and mechanotransduction currents. More recently, stem cells from human embryos were found to be capable of forming hair cell-like cells in culture (Ronaghi et al., 2014).

The true test of a stem cell's therapeutic usefulness is whether it can become integrated into the organ of Corti, become innervated by the auditory nerve, differentiate mature features, and survive. Introduction of stem cells to the organ of Corti is a challenge, since the organ is surrounded by a fluid-filled cavity that is embedded within the temporal bone and is easily disrupted by surgical intervention. It would seem very difficult to place transplanted cells into the organ of Corti, given the tiny nature and delicacy of the tissue, and the fact that fluid barriers would need to be disrupted. Nonetheless, several approaches for cell delivery are under investigation. Scientists have introduced embryonic stem cells into the fluids of the organ of Corti (scala media) and into

the perilymphatic spaces surrounding the scala media (Coleman et al., 2006; Hildebrand et al., 2005). While some stem cells seem to persist in these spaces and integrate into some tissues around them, there is little evidence that stem cells integrate into the organ of Corti. However, Parker et al. (2007) reported that neural stem cells injected into the noise-damaged cochlea became incorporated into the sensory epithelium. Clearly, more studies are needed to identify ways to coax stem cells to integrate into damaged hair cell epithelia, acquire mature features, and restore function.

### **Clinical Considerations**

While progress toward hair cell regeneration has been significant given the limited time elapsed since its discovery, several challenges remain in order to determine how effective hair cell replacement could be for improving hearing in humans. For instance, we do not know how many hair cells of each type must be regenerated in order to adequately restore hearing in impaired individuals. While we know inner hair cells are critical, we can only guess how well they will restore hearing in the absence of outer hair cells. Many forms of hearing loss are caused by selective destruction of outer hair cells; regeneration of outer hair cells alone could be helpful in such patients. Further, we lack the capability to accurately test which type of cells need repair in patients. This assessment requires development of more cell-specific and non-invasive diagnostic procedures. In addition, high-resolution imaging of the inner ear enabling quantitative assessment of each cell type would be very helpful and is currently under investigation. Although there are challenges to restoring hair cells after damage in mammals, many hurdles have already been conquered with promising research on the horizon to introduce a potential treatment for hearing loss.

## References

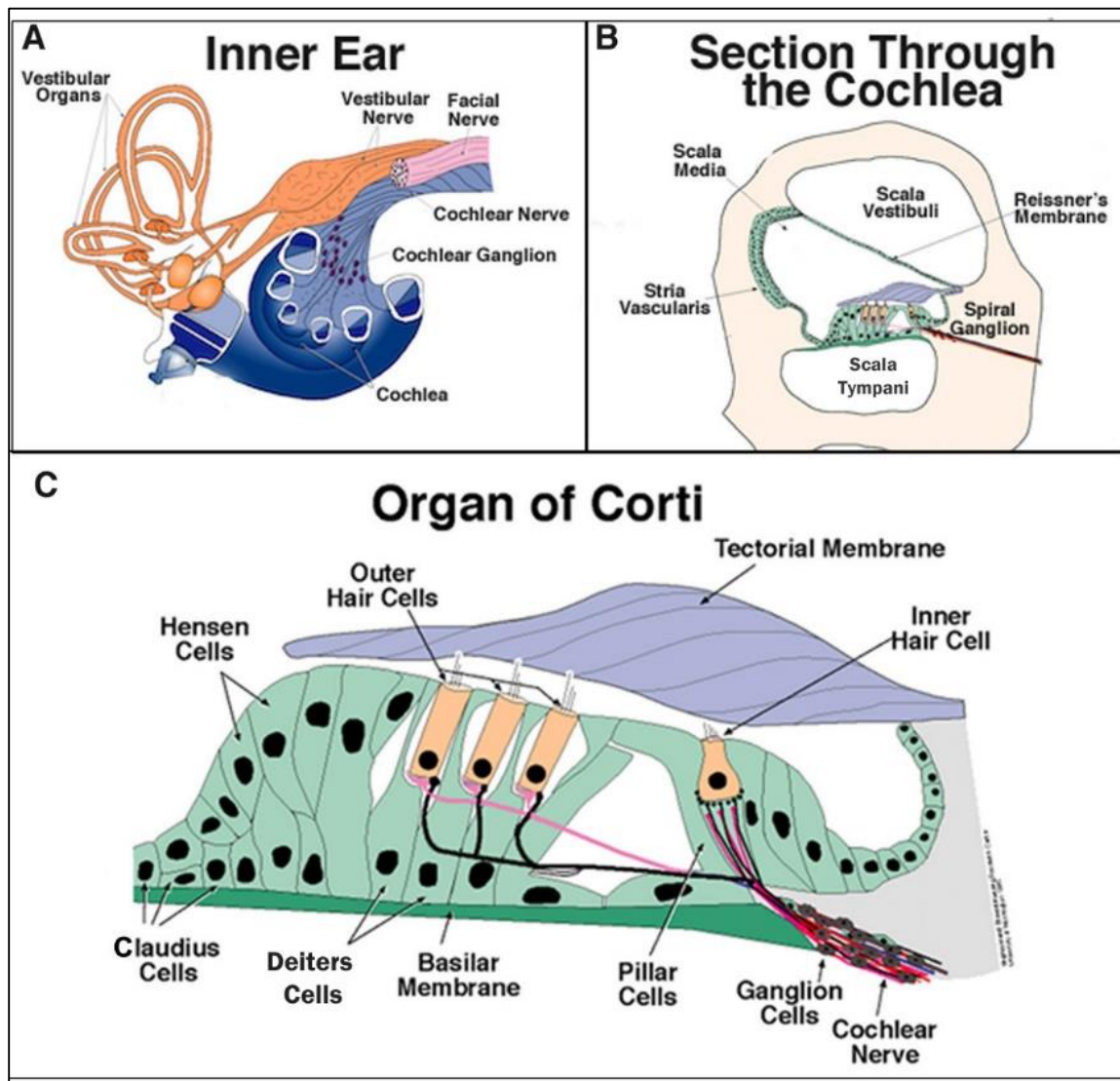
- Atkinson, P.J., Huarcaya Najarro, E., Sayyid, Z.N., and Cheng, A.G. (2015). Sensory hair cell development and regeneration: similarities and differences. *Development* 142, 1561–1571.
- Bermingham, N.A., Hassan, B.A., Price, S.D., Vollrath, M.A., Ben-Arie, N., Eatock, R.A., Bellen, H.J., Lysakowski, A., and Zoghbi, H.Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* 284, 1837–1841.
- Bredberg, G. (1967). The human cochlea during development and ageing. *The Journal of Laryngology and Otology* 81, 739–758.
- Cafaro, J., Lee, G.S., and Stone, J.S. (2007). Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Developmental Dynamics* 236, 156–170.
- Cai, T., Jen, H.-l., Kang, H., Klisch, T.J., Zoghbi, H.Y., and Groves, A.K. (2015). Characterization of the transcriptome of nascent hair cells and identification of direct targets of the Atoh1 transcription factor. *The Journal of Neuroscience* 35, 5870–5883.
- Chai, R., Kuo, B., Wang, T., Liaw, E.J., Xia, A., Jan, T.A., Liu, Z., Takeito, M.M., Oghalai, J.S., Nusse, R., et al. (2012). Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea. *Proceedings of the National Academy of Sciences* 109, 8167–72
- Chardin, S., and Romand, R. (1995). Regeneration and mammalian auditory hair cells. *Science* 267, 707–711.
- Chen, P., and Segil, N. (1999). p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 126, 1581–1590.
- Coleman, B., Hardman, J., Coco, A., Epp, S., de Silva, M., Crook, J., and Shepherd, R. (2006). Fate of embryonic stem cells transplanted into the deafened mammalian cochlea. *Cell Transplantation* 15, 369–380.
- Corwin, J.T., and Cotanche, D.A. (1988). Regeneration of sensory hair cells after acoustic trauma. *Science* 240, 1772–1774.
- Cotanche, D.A. (1987). Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. *Hearing Research* 30, 181–195.
- Cox B.C., Chai R., Lenoir A., Liu Z., Zhang L., Nguyen D.H., Chalasani K., Steigelman K.A., Fang J., Rubel E.W., Cheng A.G., Zuo J. (2014). Spontaneous hair cell regeneration in the neonatal mouse cochlea in vivo. *Development* 141(4), 816–29.
- Cruz, R.M., Lambert, P.R., and Rubel, E.W. (1987). Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. *JAMA Otolaryngology—Head & Neck Surgery* 113, 1058–1062.
- Daudet, N., Gibson, R., Shang, J., Bernard, A., Lewis, J., and Stone, J. (2009). Notch regulation of progenitor cell behavior in quiescent and regenerating auditory epithelium of mature birds. *Developmental Biology* 326, 86–100.
- Doetzlhofer, A., Basch, M.L., Ohyama, T., Gessler, M., Groves, A.K., and Segil, N. (2009). Hey2 regulation by FGF provides a Notch-independent mechanism for maintaining pillar cell fate in the organ of Corti. *Developmental Cell* 16, 58–69.
- Forge, A., Li, L., and Nevill, G. (1998). Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. *The Journal of Comparative Neurology* 397, 69–88.
- Fujino, K., Kim, T.-S., Nishida, A.T., Nakagawa, T., Omori, K., Naito, Y., and Ito, J. (2004). Transplantation of neural stem cells into explants of rat inner ear. *Acta Oto-Laryngologica Supplementum* 31–33.
- Golub, J.S., Tong, L., Ngyuen, T.B., Hume, C.R., Palmiter, R.D., Rubel, E.W., and Stone, J.S. (2012). Hair cell replacement in adult mouse utricles after targeted ablation of hair cells with diphtheria toxin. *The Journal of Neuroscience* 32, 15093–15105.
- Groves A.K., Zhang K.D., Fekete D.M. (2013). The genetics of hair cell development and regeneration. *Annual Review of Neuroscience* 36, 361–81
- Gubbels, S.P., Woessner, D.W., Mitchell, J.C., Ricci, A.J., and Brigande, J.V. (2008). Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. *Nature* 455, 537–541.
- Hayashi T., Kokubo H., Hartman B.H., Ray C.A., Reh T.A., Bermingham-McDonogh O. (2008). Hesr1 and Hesr2 may act as early effectors of Notch signaling in the developing cochlea. *Developmental Biology* 316, 87–99.

- Head, J.R., Gacioch, L., Pennisi, M., and Meyers, J.R. (2013). Activation of canonical Wnt/ $\beta$ -catenin signaling stimulates proliferation in neuromasts in the zebrafish posterior lateral line. *Developmental Dynamics* 242, 832–846.
- Hildebrand, M.S., Dahl, H.-H.M., Hardman, J., Coleman, B., Shepherd, R.K., and de Silva, M.G. (2005). Survival of partially differentiated mouse embryonic stem cells in the scala media of the guinea pig cochlea. *Journal of the Association for Research in Otolaryngology* 6, 341–354.
- Izumikawa, M., Minoda, R., Kawamoto, K., Abrashkin, K.A., Swiderski, D.L., Dolan, D.F., Brough, D.E., and Raphael, Y. (2005). Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nature Medicine* 11, 271–276.
- Jacques, B.E., Montgomery, W.H., Uribe, P.M., Yatteau, A., Asuncion, J.D., Resendiz, G., Matsui, J.I., and Dabdoub, A. (2014). The role of Wnt/ $\beta$ -catenin signaling in proliferation and regeneration of the developing basilar papilla and lateral line. *Developmental Neurobiology* 74, 438–456.
- Jansson, L., Kim, G.S., and Cheng, A.G. (2015). Making sense of Wnt signaling-linking hair cell regeneration to development. *Frontiers in Cellular Neuroscience* 9, 66.
- Jørgensen, J.M., and Mathiesen, C. (1988). The avian inner ear. Continuous production of hair cells in vestibular sensory organs, but not in the auditory papilla. *Naturwissenschaften* 75, 319–320.
- Kawamoto, K., Izumikawa, M., Beyer, L.A., Atkin, G.M., and Raphael, Y. (2009). Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity. *Hearing Research* 247, 17–26.
- Kelley, M.W. (2006). Regulation of cell fate in the sensory epithelia of the inner ear. *Nature Reviews Neuroscience* 7, 837–849.
- Kuntz A.L., Oesterle E.C. (1998). Transforming growth factor alpha with insulin stimulates cell proliferation in vivo in adult rat vestibular sensory epithelium. *The Journal of Comparative Neurology* 399, 413-23.
- Kuo, B.R., Baldwin, E.M., Layman, W.S., Taketo, M.M., and Zuo, J. (2015). In Vivo Cochlear Hair Cell Generation and Survival by Coactivation of  $\beta$ -Catenin and Atoh1. *The Journal of Neuroscience* 35, 10786–10798.
- Lanford, P.J., Shailam, R., Norton, C.R., Gridley, T., and Kelley, M.W. (2000). Expression of Math1 and HES5 in the cochleae of wildtype and Jag2 mutant mice. *Journal of the Association for Research in Otolaryngology* 1, 161–171.
- Lefebvre, P.P., Malgrange, B., Thiry, M., Van De Water, T.R., and Moonen, G. (2000). Epidermal growth factor upregulates production of supernumerary hair cells in neonatal rat organ of corti explants. *Acta Oto-laryngologica* 120, 142–145.
- Lewis, J. (1998) Notch signalling and the control of cell fate choices in vertebrates. *Seminars in Cell & Developmental Biology* 9, 583-9.
- Lewis, R.M., Hume, C.R., and Stone, J.S. (2012). Atoh1 expression and function during auditory hair cell regeneration in post-hatch chickens. *Hearing Research* 289, 74-85.
- Li, L., and Forge, A. (1997). Morphological evidence for supporting cell to hair cell conversion in the mammalian utricular macula. *International Journal of Developmental Neuroscience* 15, 433–446.
- Li, H., Roblin, G., Liu, H., and Heller, S. (2003). Generation of hair cells by stepwise differentiation of embryonic stem cells. *Proceedings of the National Academy of Sciences* 100, 13495–13500.
- Lin, V., Golub, J.S., Nguyen, T.B., Hume, C.R., Oesterle, E.C., and Stone, J.S. (2011). Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *The Journal of Neuroscience* 31, 15329–15339.
- Liu, Z., Dearman, J.A., Cox, B.C., Walters, B.J., Zhang, L., Ayrault, O., Zindy, F., Gan, L., Roussel, M.F., and Zuo, J. (2012). Age-dependent in vivo conversion of mouse cochlear pillar and deiters' cells to immature hair cells by atoh1 ectopic expression. *The Journal of Neuroscience* 32, 6600–6610.
- Löwenheim, H., Furness, D.N., Kil, J., Zinn, C., Gültig, K., Fero, M.L., Frost, D., Gummer, A.W., Roberts, J.M., Rubel, E.W., et al. (1999). Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti. *Proceedings for the National Academy of Sciences* 96, 4084–4088.
- Ma, E.Y., Rubel, E.W., and Raible, D.W. (2008). Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. *The Journal of Neuroscience* 28, 2261–2273.
- Maass, J.C., Gu, R., Basch, M.L., Waldhaus, J., Lopez, E.M., Xia, A., Oghalai, J.S., Heller, S., and Groves, A.K. (2015). Changes in the regulation of the Notch signaling pathway are temporally correlated with regenerative failure in the mouse cochlea. *Frontiers in Cellular Neuroscience* 9, 110.

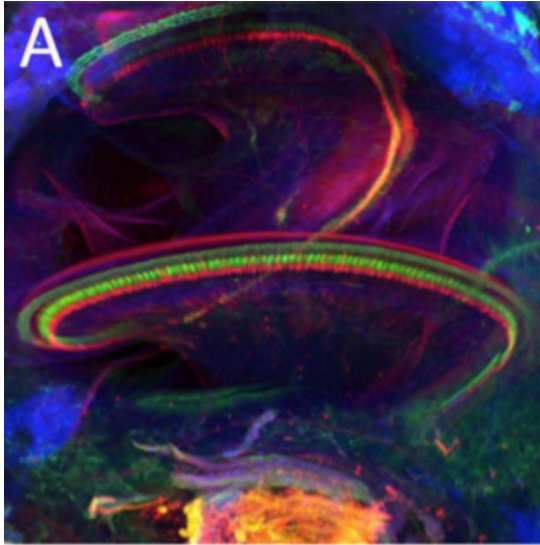
- McLean, W.J., Smith, K.A., Glowatzki, E., and Pyott, S.J. (2009). Distribution of the Na,K-ATPase alpha subunit in the rat spiral ganglion and organ of corti. *Journal of the Association for Research in Otolaryngology* 10, 37–49.
- Mizutani, K., Fujioka, M., Hosoya, M., Bramhall, N., Okano, H.J., Okano, H., and Edge, A.S.B. (2013). Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. *Neuron* 77, 58–69.
- Oesterle, E.C., Cunningham, D.E., Westrum, L.E., and Rubel, E.W. (2003). Ultrastructural analysis of [<sup>3</sup>H]thymidine-labeled cells in the rat utricular macula. *The Journal of Comparative Neurology* 463, 177–195.
- Oesterle, E.C., Chien, W.-M., Campbell, S., Nellimarla, P., and Fero, M.L. (2011). p27(Kip1) is required to maintain proliferative quiescence in the adult cochlea and pituitary. *Cell Cycle* 10, 1237–1248.
- Oshima, K., Grimm, C.M., Corrales, C.E., Senn, P., Martinez Monedero, R., Géléoc, G.S.G., Edge, A., Holt, J.R., and Heller, S. (2007). Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. *Journal of the Association for Research in Otolaryngology* 8, 18–31.
- Oshima, K., Shin, K., Diensthuber, M., Peng, A.W., Ricci, A.J., and Heller, S. (2010). Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. *Cell* 141, 704–716.
- Parker MA, Corliss DA, Gray B, Anderson JK, Bobbin RP, Snyder EY, Cotanche DA (2007) Neural stem cells injected into the sound-damaged cochlea migrate throughout the cochlea and express markers of hair cells, supporting cells, and spiral ganglion cells. *Hearing Research* 232, 29-43.
- Roberson, D.W., and Rubel, E.W. (1994). Cell division in the gerbil cochlea after acoustic trauma. *American Journal of Otolaryngology* 15, 28–34.
- Roberson, D., Kreig, S., and Rubel, E.W. (1996). Light microscopic evidence that direct transdifferentiation gives rise to new hair cells in regenerating avian auditory epithelium. *Auditory Neuroscience* 2, 195–205.
- Ronaghi, M., Nasr, M., Ealy, M., Durruthy-Durruthy, R., Waldhaus, J., Diaz, G.H., Joubert, L.-M., Oshima, K., and Heller, S. (2014). Inner ear hair cell-like cells from human embryonic stem cells. *Stem Cells and Development* 23, 1275–1284.
- Rubel, E.W., Furrer, S.A., and Stone, J.S. (2013). A brief history of hair cell regeneration research and speculations on the future. *Hearing Research* 297, 42–51.
- Ryals, B.M., and Rubel, E.W. (1988). Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* 240, 1774–1776.
- Schlecker, C., Praetorius, M., Brough, D.E., Presler, R.G., Hsu, C., Plinkert, P.K., and Staecker, H. (2011). Selective atonal gene delivery improves balance function in a mouse model of vestibular disease. *Gene Therapy* 18, 884–890.
- Shi, F., Hu, L., and Edge, A.S.B. (2013). Generation of hair cells in neonatal mice by  $\beta$ -catenin overexpression in Lgr5-positive cochlear progenitors. *Proceedings of the National Academy of Sciences* 110, 13851–13856.
- Shi, F., Hu, L., Jacques, B.E., Mulvaney, J.F., Dabdoub, A., and Edge, A.S.B. (2014).  $\beta$ -Catenin is required for hair-cell differentiation in the cochlea. *The Journal of Neuroscience* 34, 6470–6479.
- Wang, G.-P., Chatterjee, I., Batts, S.A., Wong, H.T., Gong, T.-W., Gong, S.-S., and Raphael, Y. (2010). Notch signaling and Atoh1 expression during hair cell regeneration in the mouse utricle. *Hearing Research* 267, 61–70.
- Warchol ME (2011). Sensory regeneration in the vertebrate inner ear: differences at the levels of cells and species. *Hearing Research* 273, 72-9.
- White, P.M., Doetzlhofer, A., Lee, Y.S., Groves, A.K., and Segil, N. (2006). Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature* 441, 984–987.
- White, P.M., Stone, J.S., Groves, A.K., and Segil, N. (2012). EGFR signaling is required for regenerative proliferation in the cochlea: conservation in birds and mammals. *Developmental Biology* 363, 191–200.
- Zhao, L.-D., Guo, W.-W., Lin, C., Li, L.-X., Sun, J.-H., Wu, N., Ren, L.-L., Li, X.-X., Liu, H.-Z., Young, W.-Y., et al. (2011). Effects of DAPT and Atoh1 overexpression on hair cell production and hair bundle orientation in cultured Organ of Corti from neonatal rats. *PLoS One* 6, e23729.
- Zheng, J.L., and Gao, W.Q. (2000). Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nature Neuroscience* 3, 580–586.

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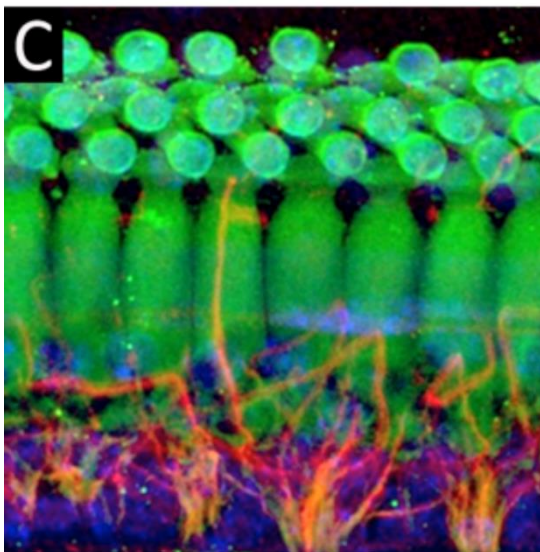
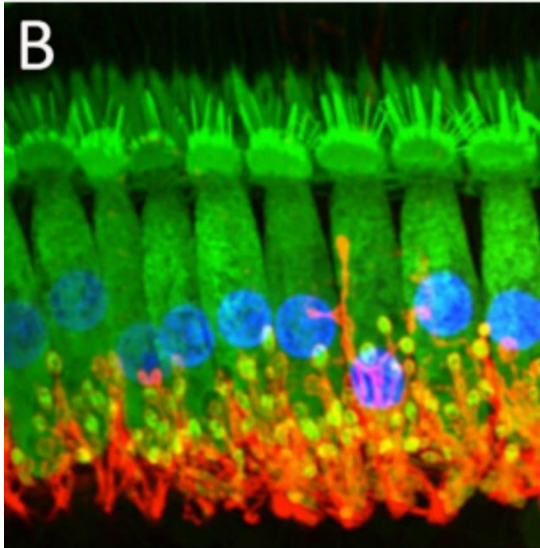
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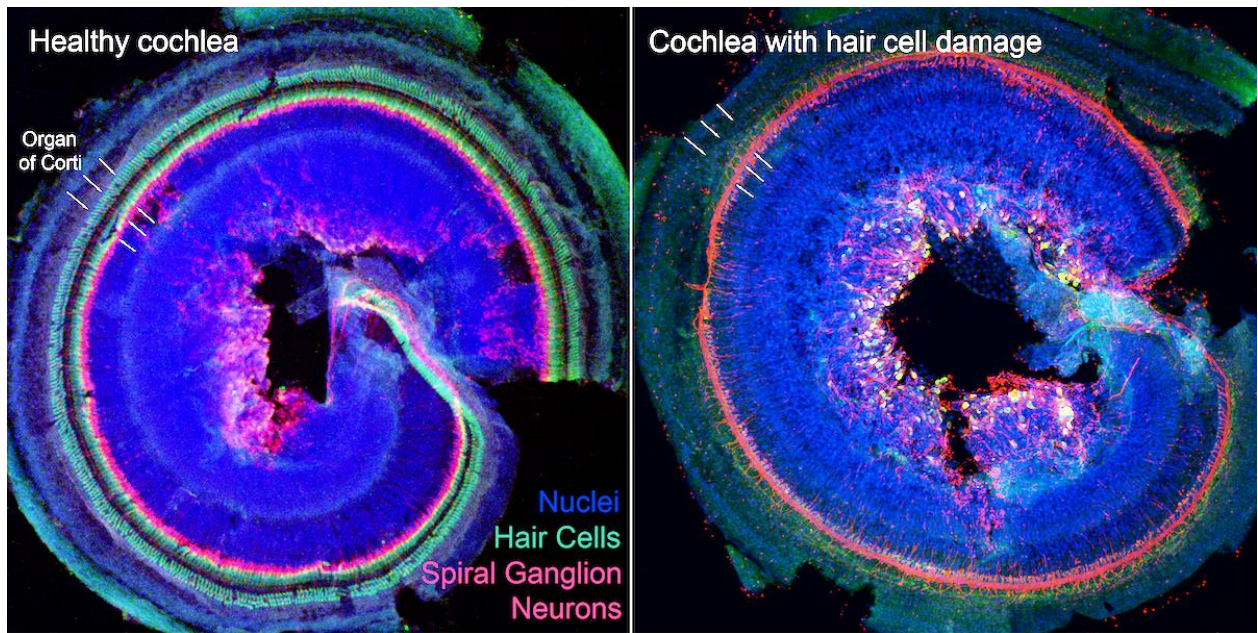


**Figure 1:** Schematic diagrams principal structures of the inner ear tissues (A), a slice through one turn of the cochlea (B) and the Organ of Corti (C). Note in the Organ of Corti a single inner hair cell and three outer hair cells are shown along with the supporting cells. This pattern is repeated about 3000 times along the spiraled cochlea in humans.

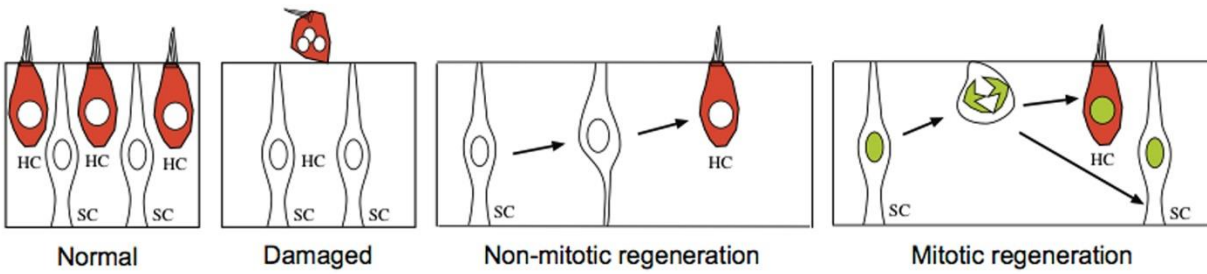


**Figure 2:** Top panel demonstrates mammalian cochlea with hair cells in green and spiral ganglion neurons in red. Middle panel illuminates inner hair cells and stereocilia in green, with nuclei in blue and afferent processes of neurons in red (McLean et al., 2009). Bottom panel shows the tops of the the three rows of outer hair cells in green dots at the top and the tubular single row of inner hair cells innervated by neural process in red/orange.

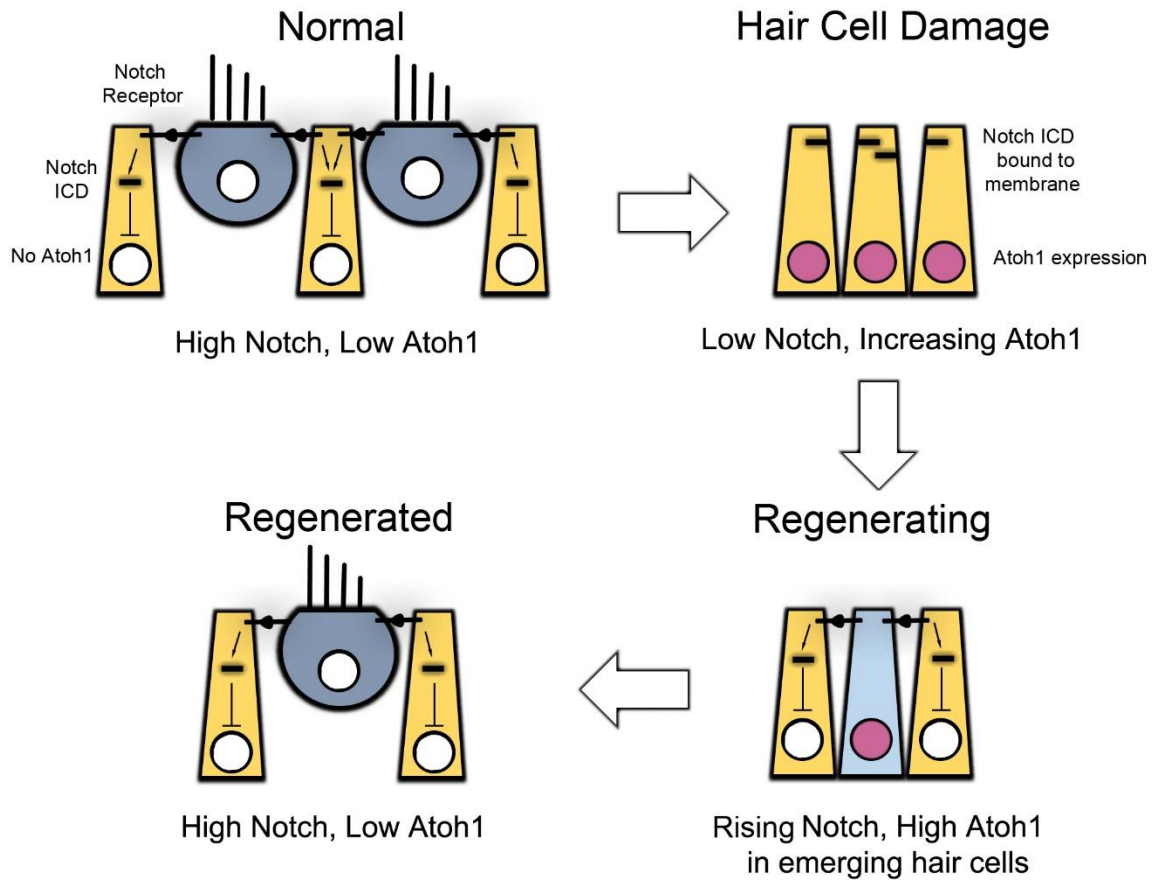




**Figure 3:** The left panel demonstrates a surface view of a healthy cochlea with hair cells in green, neural processes in red, and nuclei in blue. The right panel demonstrates a damaged cochlea, which no longer contains hair cells but preserved neural processes and nuclei. Organ of Corti boundaries are marked with white arrows.



**Figure 4:** The undamaged auditory epithelium of the bird contains hair cells (HC; red) interdigitated with supporting cells (SC; white). Upon damage, hair cells are removed from the epithelium and supporting cells are triggered to regenerate hair cells. Non-mitotic regeneration allows a supporting cell to change its shape and genetic profile to that of a hair cell. Mitotic regeneration requires a supporting cell to divide and differentiate two daughter cells, a hair cell and a supporting cell.



**Figure 5:** Expression patterns of the Notch receptor and Atoh1 transcription factor in supporting cells (yellow) and hair cells (blue) under normal, damaged, and regenerating conditions. In supporting cells in undamaged epithelia, there is high Notch receptor activity and the Notch Intracellular domain (Notch ICD) travels to the nucleus, inhibiting Atoh1 expression. In supporting cells after hair cell damage, Notch receptor activity is reduced, Notch ICD remains at the membrane, and Atoh1 levels increase, driving the supporting cell to transdifferentiate into a hair cell. Once the new hair cell matures, Notch activity is increased again, and Atoh1 transcription is reduced to normal levels.