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***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.

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

7–10-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 is sufficient to drive upregulation of *Atoh1* in chicken basilar papilla (Daudet et al., 2009), or its vehicle DMSO. *In vitro*, hair cell regeneration follows 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, β -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 β -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* homolog (*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 min, 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 min 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- β 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- β -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 1/300; 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 (NiCl₂)

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-min incubation in 1.5% H₂O₂. 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 counterlabeled for DAPI (0.1 mg/ml 4,6-diamidino-2-phenylindole) to detect nuclei. Tissue samples labeled for ATOH1 with NiCl₂ 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 20–60× at 512 pixel × 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 1.5 h. Anti-digoxigenin conjugate label solution was diluted 1:1 with TUNEL blocking solution (Millipore), and incubation time was extended to 1 h.

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* < 0.05. All data are expressed as mean ± 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 β -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 β 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 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 β -tectorin precursor (Goodyear et al., 1996) (Fig. 4H–J).

In basilar papillae cultured for 7 days after transfection with the *Atoh1* reporter plasmid, 56 (± 9)% of GFP+ cells were myosin 6+. In contrast, only 4 (± 5)% of GFP+ cells were myosin 6+ after transfection of the negative control plasmid (pShuttle-IRES-GFP) + (Fig. 4B). These numbers were significantly different ($p = 0.05$). 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 ($p = 0.05$) (Fig. 4B). At 7 days, 56 (± 9)% of cells with *Atoh1* activity were myosin 6+, while at 11 days, only 18 (± 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 $10 \mu\text{M}$ 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 ($p = 0.05$). 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* homolog (*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 (± 7)% of GFP+ cells were myosin 6+, compared to 4 (± 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 are 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 (± 6)% of GFP+ cells were BrdU+ (Fig. 7B). In contrast, after transfection with negative control plasmid (pShuttle-IRES-GFP), only 6 (± 4)% of GFP+ cells were BrdU+ (Fig. 7A). These numbers were significantly different ($p = 0.05$). 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; Fritzsche 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.

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.

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Abbreviations

Atoh1	atonal homolog 1
Myo6	myosin 6
βIII tub	β III tubulin
SCA	supporting cell antigen
βtect	β -tectorin

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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 μ 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 μm .

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 β III tubulin (β III tub) and GFP labeling in the same optical slice from a culture fixed at 11 days post-EP. Arrows point to examples of β III tub+/GFP+ cells, and arrowheads point to examples of β III tub–/GFP+ cells. F, F' 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 F' 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- β -tectorin (β 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- β tect+/GFP+ cells, and arrowhead points to SCA- β tect–/GFP+ cells. Scale bars = 15 μ 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 μ 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 μ 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 μ 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 μ m.

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.

Table 1

Days in culture (ICC Label)	pShuttle-IRES-GFP		pShuttle-Math1-IRES-GFP		pShuttle-J2Xn-GFP		pShuttle-J2Xn-GFP, with DMSO		pShuttle-J2Xn-GFP, with DAPI	
	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	-	-	-	-	-	-