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Alexander Yan Pan 4/17/2012

Transforming cochlear supporting cells into auditory hair cells for inner ear sensory organ regeneration

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2012

Abstract

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The cochlear sensory domain, known as the organ of Corti, within the mammalian inner ear is composed of sensory hair cells and interdigitating non-sensory supporting cells. The hair cells are the primary receptor cells responsible for detecting and transmitting sound signal. Loss of these cells in humans leads to permanent hearing loss, since mammalian hair cells cannot be regenerated. In contrast, lower vertebrate classes exhibit spontaneous regeneration of hair cells following damage by proliferation and conversion of the supporting cells. Atoh1 is a hair cell differentiation factor, and previous studies have shown that overexpressing Atoh1 can induce the expression of hair cell markers in cochlear non-sensory cells. However, these cells are usually located outside the sensory region domain. Furthermore, our previous study suggests that Atoh1 has a very limited ability to induce hair cell differentiation in non-sensory supporting cells within the sensory domain in the postnatal mammalian inner ear. In many regenerative species, hair cell regeneration in the mature cochlea occurs only after the loss of existing hair cells. Therefore, I hypothesized that the presence of existing hair cells inhibits Atoh1-mediated conversion of supporting cells in mammals. To test this hypothesis, I first developed an efficient hair cell ablation protocol involving two different ototoxic aminoglycosidic drugs. I then induced Atoh1 expression first throughout the cochlear epithelium, then specifically within the supporting cells. I show that ablating the endogenous hair cells modestly increases the number of hair cells converted from non-sensory supporting cells. Together, this data indicates that the *in vitro* cochlear explant model can be used to efficiently test

various conditions for hair cell regeneration and that the competency of supporting cells conversion into hair cells may be modulated to achieve hair cell regeneration.

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Acknowledgements

I would first and foremost like to thank Dr. Ping Chen for guiding me through this process and providing me with this great research opportunity. I would also like to thank Dr. Michael Kelly for his incredible mentoring, guidance, and support. Finally, I would like to thank all of my committee members, Dr. Andreas Fritz, Dr. Nicole Gerardo, who provided a great deal of help with the statistical analyses, and Dr. Amanda Starnes for their patience and constant encouragement.

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ABSTRACT

The cochlear sensory domain, known as the organ of Corti, within the mammalian inner ear is composed of sensory hair cells and interdigitating non-sensory supporting cells. The hair cells are the primary receptor cells responsible for detecting and transmitting sound signal. Loss of these cells in humans leads to permanent hearing loss, since mammalian hair cells cannot be regenerated. In contrast, lower vertebrate classes exhibit spontaneous regeneration of hair cells following damage by proliferation and conversion of the supporting cells. Atoh1 is a hair cell differentiation factor, and previous studies have shown that overexpressing Atoh1 can induce the expression of hair cell markers in cochlear non-sensory cells. However, these cells are usually located outside the sensory region domain. Furthermore, our previous study suggests that Atoh1 has a very limited ability to induce hair cell differentiation in non-sensory supporting cells within the sensory domain in the postnatal mammalian inner ear. In many regenerative species, hair cell regeneration in the mature cochlea occurs only after the loss of existing hair cells. Therefore, I hypothesized that the presence of existing hair cells inhibits Atoh1-mediated conversion of supporting cells in mammals. To test this hypothesis, I first developed an efficient hair cell ablation protocol involving two different ototoxic aminoglycosidic drugs. I then induced Atoh1 expression first throughout the cochlear epithelium, then specifically within the supporting cells. I show that ablating the endogenous hair cells modestly increases the number of hair cells converted from non-sensory supporting cells. Together, this data indicates that the *in vitro* cochlear explant model can be used to efficiently test various conditions for hair cell regeneration and that the competency of supporting cells conversion into hair cells may be modulated to achieve hair cell regeneration.

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INTRODUCTION

Our sensation of hearing is a complex process and involves the passage of air vibrations from the external ear to the middle ear and finally into the internal ear. Sound travels as vibrations in the air, and these sound vibrations first pass through the structures of the outer ear: the pinna and the external auditory meatus. The shape and structure of the external ear amplifies the sound waves and focuses them upon the tympanic membrane, which is more commonly referred to as the eardrum. The amplified sound waves vibrate the eardrum, which then vibrates the malleus, incus, and stapes, the three bones of the middle ear. The vibrations in the stapes vibrates a small membrane at the base of the cochlea, known as the oval window, and this transmits vibrations to the fluids within the cochlea. The cochlea is a coiled structure within the inner ear, and it is within this structure that sounds are converted into neural activity. The portion of the cochlea closest to the oval window is considered the base of the cochlea, while the portion at the top end of the coil is considered the apex.

The sensory region of the cochlea that is primarily responsible for converting sound into nerve impulses lies along the basilar membrane. This region, known as the organ of Corti, consists of two types of cells: sensory hair cells and non-sensory supporting cells. Auditory nerve fibers connect sensory hair cells with more central brain structures. There are stiff, hair-like structures at the tips of each of these hair cells known as stereocilia. The heights of these stereocilia increase over the surface of the hair cell, forming an inclined plane. The tectorial membrane lies atop the sensory hair cells, and the tips of the stereocilia actually protrude into grooves on the bottom of this membrane. Therefore, when sound vibrations move the incompressible fluids within the cochlea, this shifts the basilar

membrane, and the positioning of the stereocilia are shifted in relation to the tectorial membrane. Depending on the direction the stereocilia bend, ion channels within the hair cells will either open or close. This change in ion concentration within the hair cell then leads to the increase or decrease in the firing rate of the auditory nerve fibers.

External stimuli such as loud noises, infection, and exposure to ototoxic substances can cause damage to the sensory hair cells within the organ of Corti. Neomycin and gentamicin are two members of the aminoglycosidic class of antibiotic drugs known to have ototoxic effects. Neomycin has been found to primarily kill cochlear hair cells, while gentamicin primarily kills hair cells in the vestibular portion of the inner ear (Selimoglu 2007). Still, these two drugs act to kill hair cells through a common mechanism. It has been suggested that aminoglycosides are taken up by hair cells through endocytosis at the apical surface and/or through the transduction channels. Once inside the hair cells, the drugs generate reactive oxygen species and activate the c-Jun N-terminal kinase pathway. This is followed by the release of cytochrome-c from the mitochondria, and finally, the activation of caspases and nucleases followed closely by the appearance of pyknotic nuclei in the hair cells (Warchol 2010).

Unlike in birds, fish, and amphibians, mammalian hair cells are unable to regenerate (Warchol 2011). As a result, when mammals lose these sensory hair cells, they experience permanent deafness. One method the avian auditory sensory epithelium is able to regenerate their hair cells upon loss of existing hair cells is through “direct transdifferentiation”, where existing supporting cells change cell fate to become hair cells (Stone and Cotanche, 2007). Although it is still unclear why similar conversions of mature

supporting cells to hair cells do not occur in mammals, several avenues of investigation are being pursued to promote mammalian hair cell regeneration (Brigande and Heller 2009).

Atoh1 is a basic helix-loop-helix (bHLH) transcription factor known to be required for the differentiation of inner ear sensory hair cells from sensory precursor cells within the cochlear epithelium (Bermingham et al. 1999, Chen et al. 2002). Recent studies have shown that overexpression of Atoh1 has been successful in generating new hair cells when delivered to cochlear epithelial cells (Zheng and Gao 2000, Woods et al. 2004, Gubbels et al. 2009, Kawamoto et al. 2003, Shou et al. 2003, and Kelly et al. 2012). Within these studies, it appears that columnar epithelial cells from the greater epithelial ridge, which is one of two ridges that compose the cochlea and that lies closer to the medial side of the cochlea, are relatively easily converted into sensory hair cells following overexpression of Atoh1. It is important, however, to determine the conversion competency of supporting cells in the sensory region. Importantly, these non-sensory supporting cells are the only ones that remain within the organ of Corti following loss of hair cells. While there has been some evidence that shows that Atoh1 overexpression within these postnatal supporting cells can lead to very limited conversion to hair cells (Izumikawa et al. 2005, Kelly et al. 2012), they also may not have the same competency as the developmentally transient cells of the greater epithelial ridge. Since most non-mammalian vertebrates appear to be capable of regenerating hair cells from supporting cells following injury (Warchol 2011), it is possible that the ablation of the existing hair cells increases the competency of supporting cells to undergo Atoh1-mediated hair cell generation.

For this honors thesis project, I have used an *in vitro* model of hair cell ablation and Atoh1 delivery to compare the competency of supporting cells to Atoh1-mediated hair cell

conversion with and without the prior ablation of endogenous hair cells. I used a transgenic mouse line, Math1-GFP, which labels hair cell nuclei (Chen et al. 2002), to visualize the ablation efficiency of drug treatments using time-lapse imaging. I then used an inducible Atoh1 transgenic mouse line to drive Atoh1 expression in combination with various tissue-specific Cre mouse lines to direct the Atoh1 expression domain. After inducing Atoh1 overexpression throughout the cochlear epithelium, I observed a large number of ectopic hair cells, but it was difficult to visualize the location of the sensory region. I then used a different Cre recombinase mouse line to direct Atoh1 overexpression specifically to the supporting cells and saw that there were new ectopic hair cells within the sensory region that appeared to originate from the supporting cells following hair cell ablation. These results could provide insight into the mechanisms necessary for supporting cell conversion to hair cells and the role Atoh1 plays in the process. It can also reveal potential future therapeutic uses for Atoh1 following deafness.

EXPERIMENTS/METHODS

Organotypic culture of postnatal mouse cochleae

Cochleae were harvested from postnatal day 0 (P0) or postnatal day 1 (P1) mice, and the lateral wall, Reissner's membrane, and tectorial membrane were removed. The cochleae were then split into three pieces of approximately equal lengths, corresponding with the basal, middle, and apical turns of the cochlea. These three segments were placed on poly-D-lysine coated glass bottom dishes with the lumenal surface pointing upwards. Cultures were incubated in a supplemented DMEM-F12 media (10% FBS, 2% B27 supplement, 1% penicillin) overnight before adding aminoglycosidic drug and doxycycline when appropriate.

Transgenic mouse models

Math1-GFP (Chen et al. 2002) mice were used to allow visualization of hair cells. These animals carry a transgene that allows the expression of green fluorescent protein (GFP) in hair cells.

TgAtoh1 (Kelly et al. 2012) mice were used to induce the expression of Atoh1 temporally and spatially. Foxg1^{cre} (Hebert et al. 2000) mice were used to induce the expression of Atoh1 throughout the cochlear epithelium, while GFAP^{cre} (Casper and McCarthy 2006) mice were used to induce the expression of Atoh1 specifically in supporting cells.

Characterization of ototoxic drug conditions

Cochleae from P0/P1-stage Math1-GFP mice were harvested and set up as organ cultures. Following the overnight incubation in supplemented DMEM-F12 media, the

cultures were then incubated within complete media containing an aminoglycosidic drug (either gentamicin or neomycin). The drug media was left on for 1 or 2 days, and then it was replaced with the supplemented DMEM-F12 media for 2 days (if the drug media was left on for 1 day) or for only 1 day (if the drug media was left on for 2 days). Stock 50 mg/mL gentamicin solution was added to the supplemented culture media for final concentrations of 3 mg/mL and 2 mg/mL. Neomycin was added to supplemented culture media for final concentrations of 1.5 mM, 1 mM, 0.8 mM, 0.6 mM, and 0.4 mM.

Induction of transgenic Atoh1 in transgenic cochlear explant cultures

Cochleae from transgenic P0 or P1 mice containing the rtTa/pTM1/Cre transgenes were harvested and set up as organ cultures. Following treatment with the aminoglycosidic drug-containing media for 1 day, cultures were then incubated in supplemented DMEM-F12 media for 4 days with 1 mg/mL doxycycline.

Immunohistochemistry and imaging

For the drug condition experiments, the primary antibodies used were: MyoVI (Proteus Biosciences Cat: 25-6791) to mark hair cells, Sox2 (Santa Cruz Biotech, Cat: sc-17320) to mark supporting cells, and Hoechst (Molecular Probes), which localizes to cell nuclei. For hair cell regeneration experiments, the same primary antibodies were used with the addition of AF488-Phal (Molecular Probes, Cat: A12379) to visualize the stereociliary bundles. The secondary antibodies used were Cy5-conjugated Donkey-anti-Rabbit (Jackson IR, Cat: 711-175-152) and Rhodamine-conjugated Donkey-anti-Goat.

Once the samples were mounted, imaging was done with either an Olympus IX71 inverted microscope with a Zeiss high-resolution monochrome AxioCam HRm camera or scanned using a Zeiss LSM510 confocal.

Cell counts and statistical analyses

Cell counts were taken by analyzing 200 pixel by 200 pixel portions of the base, middle, and apical regions of the cochlea. All images were taken at the same objective, zoom, and scan resolution. Images from Math1-GFP 1DIV and 4DIV live cultures were examined, and each instance of GFP expression was assumed to be a single hair cell.

To assess the impact of aminoglycosidic drug treatment on hair cell ablation, I used general linear models with quasibinomial distribution to compare the number of hair cells before drug treatment and after drug treatment. Minimal model comparison was used to remove non-significant terms.

RESULTS

Neomycin and gentamicin efficiently ablate hair cells in cochlear explants

To test whether the ablation of endogenous hair cells increases the ability of supporting cells to transdifferentiate into new hair cells following Atoh1 overexpression, I first tested two different aminoglycosidic drugs, neomycin and gentamicin, at various concentrations to determine the most effective method for the efficient ablation of endogenous hair cells. In order to develop an efficient hair cell ablation protocol, I tested five different concentrations of neomycin: 1.5 mM, 1.0 mM, 0.8 mM, 0.6 mM, and 0.4 mM. Cochleae from P0 neonatal mice were split into three separate parts to resemble the basal, middle, and apical regions, and they were set up as organ cultures. The Math1-GFP mouse line was used during these experiments because it provided the opportunity to track hair cell death in live culture. For both time periods, both 1.5 mM and 1.0 mM neomycin appear to be too concentrated, as they appear to kill not only the endogenous hair cells, but also the neighboring supporting cells as well, in the basal and middle regions. The apical region appears unaffected. Diminished GFP expression from the live cultures suggests that the hair cells are dying within twenty-four hours of adding aminoglycoside. The drastically reduced expressions of MyoVI and Sox2 in the experimental cultures compared to the control cultures provide evidence that cells of both types are dying. 0.8 mM and 0.6 mM neomycin, however, appeared to be suitable choices for efficient hair cell ablation. Images from the live cultures reveal a decrease in GFP expression following aminoglycoside treatment (Fig. 1). In the basal and middle regions, there is a significant decrease in MyoVI expression, but Sox2 expression remains comparable to control cultures (Figs. 2H,L,P,T and 3H,L,P,T). Interestingly, the GFP expression and MyoVI expression are not completely

absent. Instead, small patches remain (Fig. 2G arrowhead). Again, hair cell death is only evident within the basal and middle regions; the apical region appears unaffected by the addition of neomycin (Fig. 4). Finally, 0.4 mM neomycin does not appear to be a high enough concentration to kill hair cells. Both MyoVI and Sox2 expression remained robust, and there was not a significant decrease in the number of hair cells (Figs. 2U-b, 3U-b, 4U-b).

Gentamicin drug tests revealed similar results. The concentrations tested were 3 mg/mL and 2 mg/mL gentamicin. Both concentrations appear to be effective for hair cell ablation, while also keeping the supporting cells alive. As a result of these drug tests, I determined that 0.8 mM neomycin and 2 mg/mL gentamicin would be the most appropriate drug conditions to be used for the subsequent hair cell regeneration experiments (Fig. 5).

In addition to testing different concentrations of aminoglycosidic drug, I also tested different time durations of drug incubation; organ cultures were incubated in each of the different concentrations for either 1 day or 2 days. The extra day of incubation did not appear to make a significant difference between the number of hair cells ablated in the 0.8 mM and 0.6 mM concentrations, however (Figs. 2,3,4). In cultures treated with 0.4 mM neomycin, incubating the cultures in aminoglycoside for an extra day appeared to begin to cause changes in the sensory region (Fig. 2Y-b, Fig. 3Y-b).

After selecting 1-day treatments of 0.8 mM neomycin and 2 mg/mL gentamicin as appropriate hair cell ablation parameters, statistical analyses were performed to ensure the statistical significance of the decrease in the number of hair cells. Due to variation and the limited number of samples, significant decreases in the number of hair cells were evident only within the basal region ($df=6$, $F=23$, $p=0.0014$) and in the apical region when

treated with 0.8 mM neomycin. Within the basal region, both gentamicin and neomycin had a significant effect (t=5.026, p=0.00239 for 2 mg/mL gentamicin; t=4.701, p=0.00332 for 0.8 mM neomycin) (Fig. 6A). Although large decreases in GFP expression were seen in the middle region, these observed changes were not enough to imply a statistically significant decrease in the number of hair cells (df=6, F=3, p=0.123) (Fig. 6B). Still, the immunohistochemistry results provide adequate evidence to suggest that the middle region can be used for our purposes. Within the apical region, only treatment with neomycin was significantly different from control (df=7, t=2.194, p=0.064 for gentamicin; df= 7, t=2.590, p=0.036 for neomycin) (Fig. 6C).

Inducing overexpression of Atoh1 throughout the cochlear epithelium induces regeneration of new hair cells

To test if ablating the existing hair cells affects the competency of the supporting cells to Atoh1-mediated hair cell conversion, I used a transgenic mouse model that allows inducible expression of Atoh1. With this model, a tetracycline-response element, or TRE, regulates the expression of Atoh1. This TRE is only active when doxycycline binds to a reverse-tetracycline transactivator (rtTa). In addition, this rtTa, as well as an eGFP reporter, is controlled by a tissue-specific Cre recombinase, which excises a stop cassette and activates expression. In order to test if hair cell ablation affects the competency of supporting cell transdifferentiation into new hair cells following Atoh1 overexpression, I used our transgenic mouse model with the Foxg1^{cre} line to induce Atoh1 expression throughout the cochlear epithelium. It has previously been shown that inducing Atoh1 overexpression is sufficient to generate ectopic hair cells in postnatal mice cochleae (Kelly et al. 2012). Since all cells throughout the cochlear epithelium express Foxg1^{cre}, Atoh1

should be overexpressed by all cells in the cochlea. When compared with controls in which Atoh1 is not overexpressed, transgenic cultures exhibited a greatly increased amount of MyoVI-positive cells, regardless of whether or not the endogenous hair cells were ablated (Fig. 9). However, in cultures where the endogenous hair cells were not ablated, the new ectopic hair cells do not appear in the original sensory region; instead, they seem to appear more medially to the sensory region, in what is known as Kölliker's organ region (Fig. 9A). However, these results expose a critical limitation of the Foxg1^{cre} line. Since the original hair cells have been eliminated, it is difficult to trace where the original sensory region was (Fig. 9E-H). Therefore, a new model was needed in order to keep track of the location of the sensory region.

Inducing overexpression of Atoh1 specifically in supporting cells leads to new ectopic hair cells within the sensory region

In order to be able to locate the sensory region following hair cell ablation, the Foxg1^{cre} line was replaced with a GFAP^{cre} line. GFAP, or glial fibrillary acidic protein, is an intermediate filament and is expressed in specific populations of glial cells, including astrocytes and non-myelinating Schwann cells. GFAP expression has been seen in supporting cells in both the inner and outer hair cell areas, but not within the hair cells themselves (Rio et al. 2002), so with this specific transgenic model, the eGFP reporter should be able to mark the location of the sensory region. Also, since Atoh1 should only be overexpressed strictly within the supporting cells, rather than throughout the cochlear epithelium as with the Foxg1^{cre} line, it allows one to lineage trace: we are now able to see where the ectopic hair cells originate from depending on their eGFP expression.

Overexpressing Atoh1 with this GFAP^{cre} model also managed to generate ectopic hair cells; however, the number of new hair cells was much less compared to those generated from the Foxg1^{cre} line (Fig. 10F). Regrettably, the GFAP GFP expression was not complete and was highly variable. However, in most cultures, there was enough GFP expression to provide an estimate of where the sensory region was located. Unlike transgenic Foxg1^{cre} cultures where a large number of ectopic hair cells were observed following doxycycline treatment, cultures from transgenic GFAP^{cre} samples did not generate any new hair cells (Fig. 10A); only those cultures that were treated with aminoglycosidic drug prior to doxycycline treatment exhibited signs of ectopic hair cells (Fig. 10F,K). Interestingly, these new ectopic hair cells were also GFP-positive, suggesting they are of supporting cell origin (Fig. 10J,O). Another noteworthy result is that samples with physical damage also appear to regenerate hair cells following doxycycline treatment. In the middle region of a control transgenic GFAP^{cre} sample, the morphology of the organ of Corti is disrupted, perhaps due to a mistake during dissection. Interestingly, there were a number of MyoVI-positive cells, but only approximately half of them were GFP-positive, suggesting these are the new ectopic hair cells, while the MyoVI-positive/GFP-negative cells were endogenous hair cells (Fig. 10). In conclusion, the GFAP^{cre} line allowed us to see that ectopic hair cells originated from supporting cells and that when the endogenous hair cells were ablated, the ectopic hair cells were capable of being regenerated within the original sensory region.

DISCUSSION

The purpose of this study was to explore the effect that hair cell ablation had on the competency of supporting cells to Atoh1-mediated hair cell conversion. Inducing overexpression of Atoh1 had previously been successful in generating ectopic hair cells (Zheng and Gao 2000, Woods et al. 2004, Gubbels et al. 2009, Kawamoto et al. 2003, Shou et al. 2003, and Kelly et al. 2012); however, these new hair cells were most widespread within the non-sensory Kölliker's organ. In the non-mammalian vertebrate inner ear sensory systems, natural hair cell regeneration is observed following injury to the inner ear. I therefore aimed to test whether ablating the endogenous hair cells within the postnatal mouse cochleae *in vitro* would lead to regenerated hair cells with Atoh1 delivery. I first developed an efficient hair cell ablation protocol utilizing two different aminoglycosidic drugs: neomycin and gentamicin. I then utilized two specific Cre recombinases to direct Atoh1 expression: first throughout the entire cochlear epithelium and then specifically within the supporting cells. My results suggested that ablating the endogenous hair cells prior to induction of Atoh1 overexpression increases the competency of supporting cells to transdifferentiate into hair cells within the sensory region.

My attempts to develop an efficient drug ablation protocol yielded interesting results beyond the basic goal of killing hair cells whilst keeping the supporting cells alive. First, hair cells were more readily ablated within the basal region. Significant decreases in hair cells were not seen within the middle region, and only 0.8 mM neomycin treatment yielded significant results in the apical region. The reason for this may be attributed to the mechanism that aminoglycosidic drugs induce hair cell death. As mentioned before, aminoglycosides enter hair cells through their mechanotransduction cells and/or through endocytosis at the apical surface. Therefore, since these cochleae are from neonatal P0

mice, the hair cells in the middle and apical regions may not yet be fully developed, so aminoglycosides cannot enter and induce cell death (Lelli et al. 2009). Also, in a number of cultures, the outer hair cells appeared more easily ablated than the inner hair cells. This observation has been seen before in other aminoglycoside studies, and it has been suggested that there were different modes of hair cell death in the inner hair cells as compared to the outer hair cells (Taylor et al. 2007).

Another noteworthy observation is the GFP and MyoVI expression following immunostaining. In cultures treated with the optimal drug dosages (0.8 mM neomycin and 2 mg/mL gentamicin), the GFP and MyoVI expression was not completely absent, which would suggest the complete removal of hair cells, but small GFP-positive and MyoVI-positive puncta that were smaller than comparable healthy hair cells remain (Fig. 5E,I and Fig. 5G arrowheads). Although the presence of GFP and MyoVI expression may suggest that the hair cells remain, the morphology of these pieces suggests that they are simply dying hair cells. 0.8 mM neomycin and 2 mg/mL gentamicin are still appropriate drug conditions to efficiently ablate hair cells.

After developing an appropriate aminoglycosidic drug scheme, hair cell regeneration experiments were done with the Foxg1^{cre} and GFAP^{cre} lines. While the ability to clearly define the sensory region following hair cell ablation was an important difference between the GFAP^{cre} and Foxg1^{cre} lines, another noteworthy difference was the number and location of ectopic hair cells, both with and without hair cell ablation. Even without hair cell ablation, inducing overexpression of Atoh1 throughout all cochlear epithelial cells with the Foxg1^{cre} system resulted in a large number of new hair cells within Kölliker's organ. Without ablating hair cells, overexpressing Atoh1 with the GFAP^{cre} line, however, did not

result in any new hair cells, not even in the more competent Kölliker's organ. A possible explanation for this is that Atoh1 is simply overexpressed in a greater number of convertible cell types in the Foxg1^{cre} system, while in the GFAP^{cre} system, Atoh1 is only overexpressed in the supporting cell population. In cultures where the endogenous hair cells are ablated, however, MyoVI-positive cells appeared within the organ of Corti, suggesting the generation of new ectopic hair cells. These results would suggest that ablating the existing hair cells does in fact affect the competency of supporting cells to be converted to hair cells. This may be due to the elimination of lateral inhibition, which consists of the inhibitory interactions between adjacent progenitor cells. As the mammalian cochlea develops, the organ of Corti must become organized, so hair cells will prevent their neighbors from differentiating into hair cells (Lanford et al. 1999). This lateral inhibition is due primarily to Notch signaling. Notch signaling is important during inner ear development for regulating supporting cell fate. It has also been shown that inhibiting Notch leads to an increase in hair cell number at the expense of supporting cells. (Takebayashi et al. 2007).

A noteworthy observation with the GFAP^{cre} samples is the variable GFP expression within the sensory region. Rio et al. (2002) notes that the GFP expression of GFAP demonstrated high levels of fluorescence within the organ of Corti at P3. However, since our tissue samples were taken at P0, this may explain the incomplete distribution of GFP expression in the GFAP^{cre} samples. Still, it is interesting to note that in cultures where the endogenous hair cells were ablated, the cells that did express GFP also expressed MyoVI. Again, the existence of these GFP-positive/MyoVI-positive cells suggests two things: (1) the

ectopic hair cells are now being regenerated within the sensory region and (2) these ectopic hair cells are of supporting cell origin.

Finally, although these results are promising and provide evidence that ablating the endogenous hair cells can increase the competency of supporting cells for conversion to hair cells, it is still important to note that there was not a very large amount of new ectopic hair cells. Therefore, one can conclude that simply eliminating the endogenous hair cell population is only a part of the mechanism that could lead to the regeneration of hair cells within the sensory region following Atoh1 overexpression, but it is not the sole factor responsible. Also, judging from the lack of distinct Phalloidin staining, stereociliary bundles appeared to be absent from the ectopic hair cells, and MyoVI expression appeared to be decreased in ectopic hair cells when compared to endogenous hair cells (Fig. 11A,C). To explore the possibility of regenerating more new hair cells to recreate a functional sensory region, additional experiments need to be done in the future. These experiments would include a longer Doxycycline induction in order to prolong the period of Atoh1 overexpression. Also, extra time in culture would allow extra time for the $GFAP^{cre}$ to be expressed in more of the supporting cells in the organ of Corti.

Overall with this project, I was able to develop an efficient hair cell ablation protocol, one that not only kills hair cells, but also preserves the supporting cell population. Initial hair cell regeneration experiments featuring the $Foxg1^{cre}$ line reproduced results from previous successful hair cell regeneration experiments. However, they exposed the crucial limitation of the $Foxg1^{cre}$ system, i.e. the difficulty of locating the sensory region following hair cell ablation. To overcome these limitations, I induced Atoh1 overexpression specifically within the supporting cells by utilizing the $GFAP^{cre}$ line. Results from these

experiments demonstrated the possibility of regenerating hair cells within the sensory region and that these cells are of supporting cell origin. The results of this work showed that ablating the endogenous hair cells was important for regenerating hair cells. This provides evidence that when utilizing Atoh1 overexpression in potential future therapeutic uses to restore hearing within mammals, the presence of existing hair cells may hinder the Atoh1-mediated conversion of non-sensory supporting cells into hair cells.

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ACKNOWLEDGEMENTS

I would first and foremost like to thank Dr. Ping Chen for guiding me through this process and providing me with this great research opportunity. I would also like to thank Dr. Michael Kelly for his incredible mentoring, guidance, and support. Finally, I would like to thank all of my committee members, Dr. Andreas Fritz, Dr. Nicole Gerardo, who provided a great deal of help with the statistical analyses, and Dr. Amanda Starnes for their patience and constant encouragement.

FIGURES

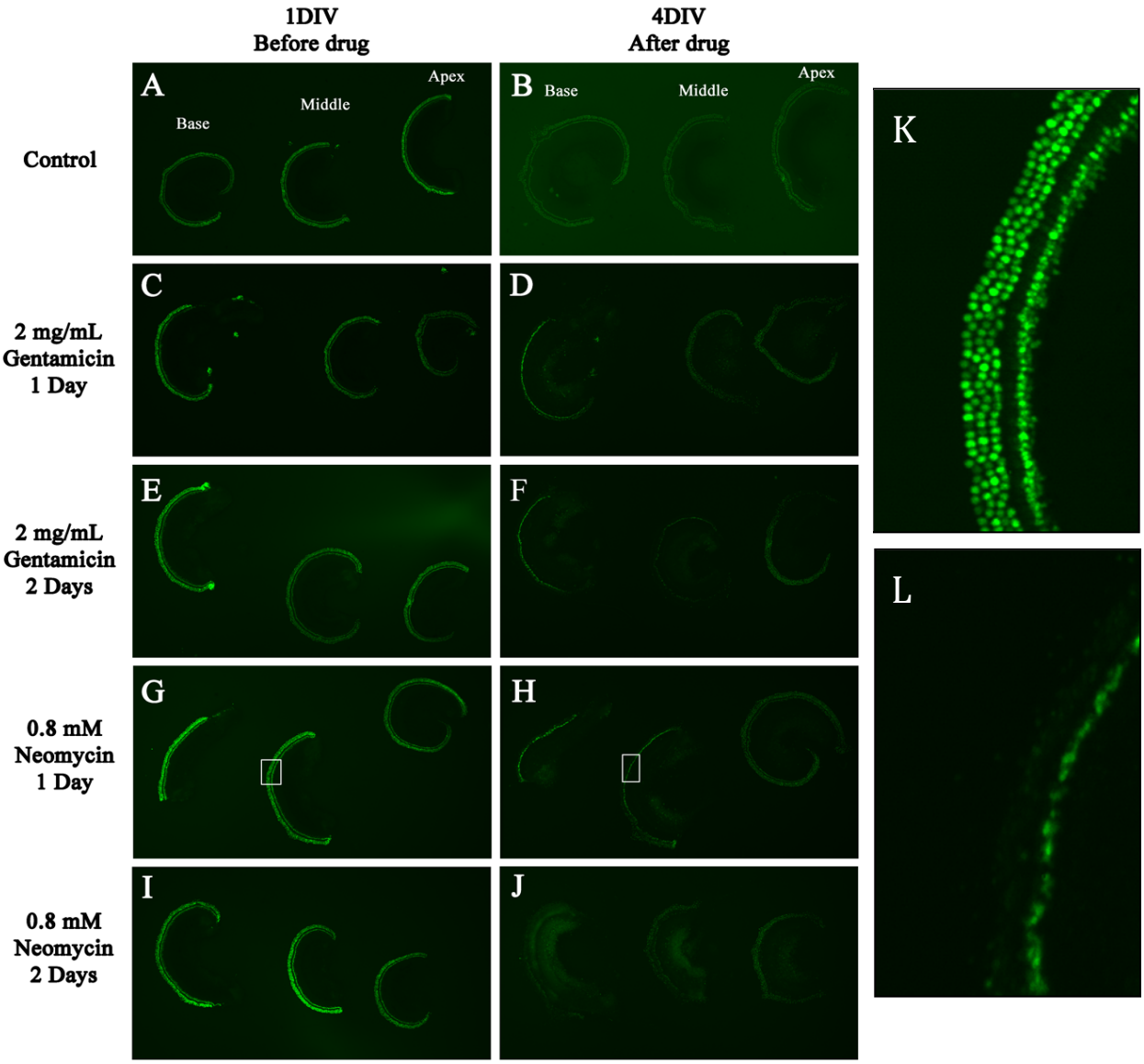

Fig. 1.

Aminoglycosidic drugs ablate hair cells in Math1-GFP mice.

(**A-B**) Control cultures were incubated in supplemented DMEM-F12 media for the duration of the experiment. GFP expression, which marks the hair cells, remains evident at the end of the incubation period. (**C-J**) Cultures treated with aminoglycosides exhibited a decrease in GFP expression following the addition of drug; the apex, however appeared unaffected. (**K**) Close-up image of the area marked by the white box from (G) demonstrating complete GFP expression in four rows of hair cells before the addition of aminoglycoside. (**L**) Close-up image of the area marked by the white box from (H) demonstrating the extent of GFP expression following the addition of aminoglycoside suggesting a diminished number of hair cells.

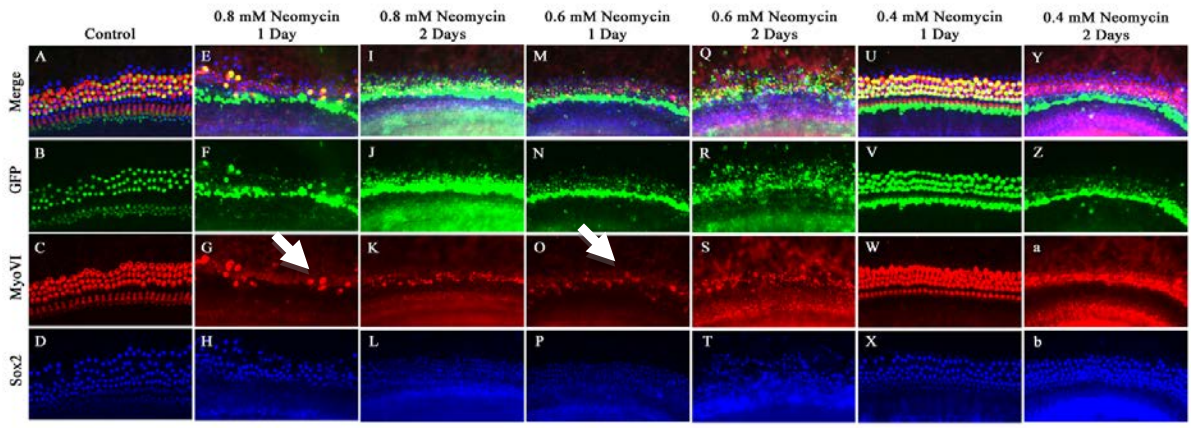

Fig. 2. Hair cell ablation within the basal region following neomycin treatment at different concentrations and for different time periods.

(**A-D**) Immunohistochemistry results in control cultures that were not treated with neomycin. GFP (green) is localized to hair cell nuclei, while MyoVI (red) shows the morphology of hair cells. Sox2 (blue) marks the surrounding nonsensory supporting cells. (**E-H**) Immunohistochemistry results in cultures treated with 0.8 mM neomycin for 1 day. (**I-L**) Immunohistochemistry results in cultures treated with 0.8 mM neomycin for 2 days. (**M-P**) Immunohistochemistry results in cultures treated with 0.6 mM neomycin for 1 day. (**Q-T**) Immunohistochemistry results in cultures treated with 0.6 mM neomycin for 2 days. (**U-X**) Immunohistochemistry results in cultures treated with 0.4 mM neomycin for 1 day. (**Y-b**) Immunohistochemistry results in cultures treated with 0.4 mM for 2 days. Arrowheads in (G) and (O) show the small puncta of MyoVI expression following addition of aminoglycoside.

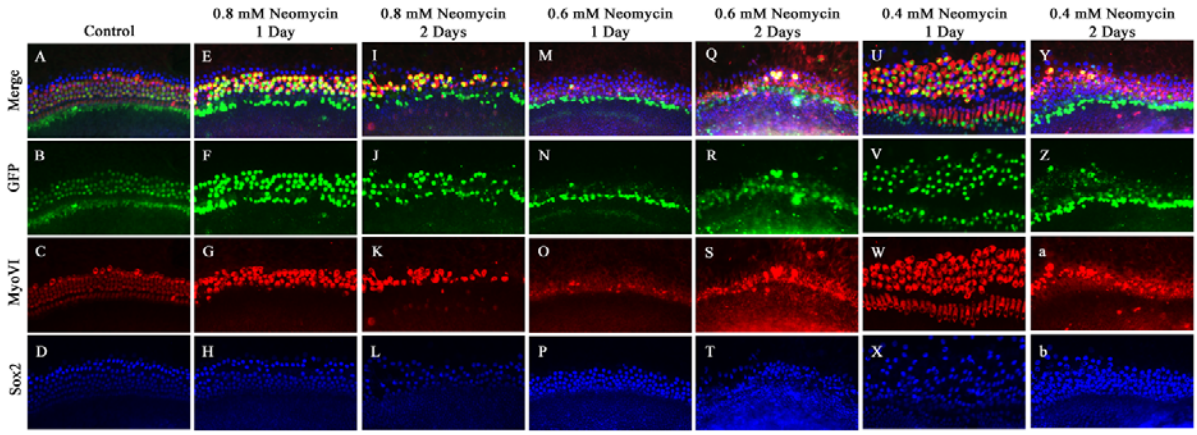

Fig. 3. Hair cell ablation within the middle region of cultures treated with neomycin at different concentrations and for different time periods.

(**A-D**) Immunohistochemistry results in control cultures that were not treated with neomycin. GFP (green) is localized to hair cell nuclei, while MyoVI (red) shows the morphology of hair cells. Sox2 (blue) marks the surrounding nonsensory supporting cells. (**E-H**) Immunohistochemistry results in cultures treated with 0.8 mM neomycin for 1 day. (**I-L**) Immunohistochemistry results in cultures treated with 0.8 mM neomycin for 2 days. (**M-P**) Immunohistochemistry results in cultures treated with 0.6 mM neomycin for 1 day. (**Q-T**) Immunohistochemistry results in cultures treated with 0.6 mM neomycin for 2 days. (**U-X**) Immunohistochemistry results in cultures treated with 0.4 mM neomycin for 1 day. (**Y-b**) Immunohistochemistry results in cultures treated with 0.4 mM for 2 days.

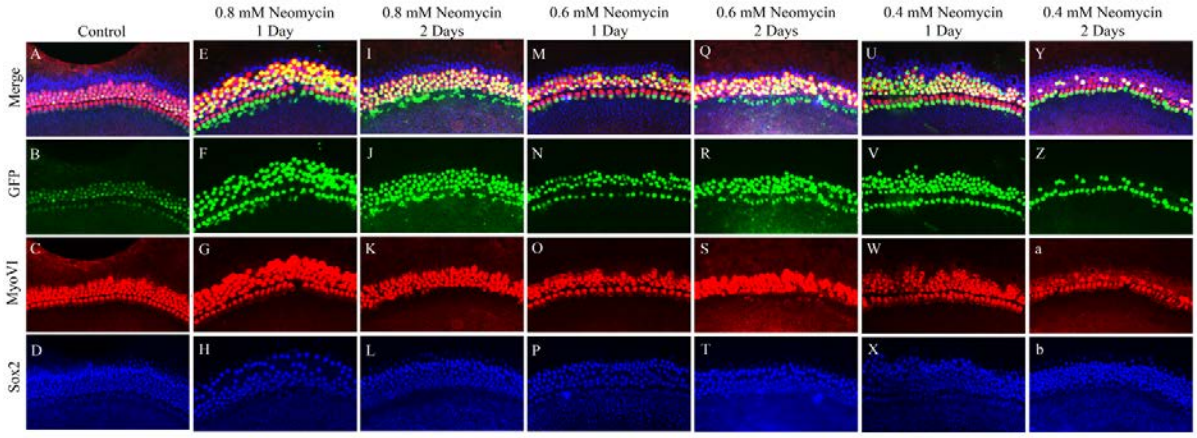

Fig. 4. Hair cell ablation within the apical region of cultures treated with neomycin at different concentrations and for different time periods.
(**A-D**) Immunohistochemistry results in control cultures that were not treated with neomycin. GFP (green) is localized to hair cell nuclei, while MyoVI (red) shows the morphology of hair cells. Sox2 (blue) marks the surrounding nonsensory supporting cells. (**E-H**) Immunohistochemistry results in cultures treated with 0.8 mM neomycin for 1 day. (**I-L**) Immunohistochemistry results in cultures treated with 0.8 mM neomycin for 2 days. (**M-P**) Immunohistochemistry results in cultures treated with 0.6 mM neomycin for 1 day. (**Q-T**) Immunohistochemistry results in cultures treated with 0.6 mM neomycin for 2 days. (**U-X**) Immunohistochemistry results in cultures treated with 0.4 mM neomycin for 1 day. (**Y-b**) Immunohistochemistry results in cultures treated with 0.4 mM for 2 days.

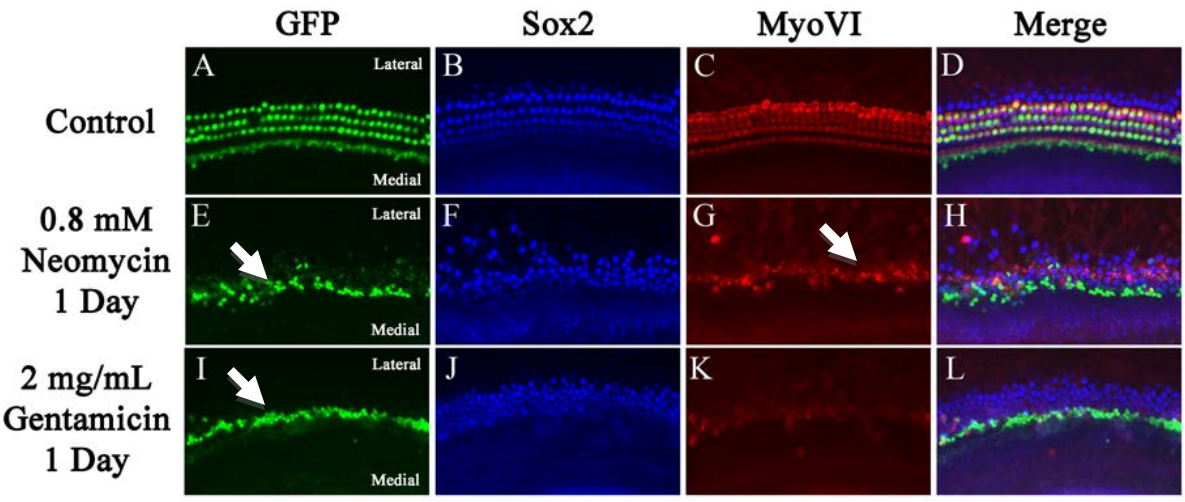

Fig. 5. 0.8 mM neomycin or 2 mg/mL gentamicin conditions are optimum for efficient hair cell ablation in the culture.

(**A-D**) Immunohistochemistry results from control cultures reveal the normal morphology of the sensory region. GFP (green) and MyoVI (red) mark hair cells, while Sox2 (blue) marks the supporting cells. (**E-H**) Immunohistochemistry results from cultures treated with 0.8 mM neomycin for 1 day demonstrated diminished GFP and MyoVI expression while Sox2 expression remains suggesting specific hair cell loss. (**I-L**) Immunohistochemistry results from cultures treated with 2 mg/mL gentamicin for 1 day show similar results to cultures treated with 0.8 mM neomycin for 1 day. Decreased GFP and MyoVI expression suggest loss of hair cells, while Sox2 expression suggests remaining supporting cell population. The arrowheads in (E,I) show the small puncta of GFP expression present following the addition of aminoglycosidic drug, and the arrowhead in (G) shows the small puncta of MyoVI expression after aminoglycoside addition.

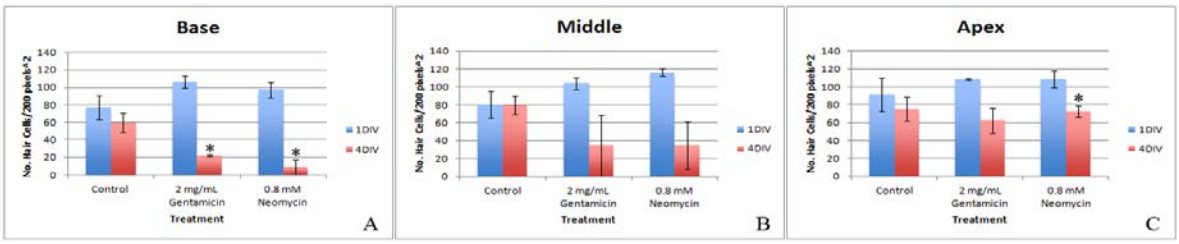

Fig. 6. Aminoglycosidic drug treatment leads to a gradient decrease of hair cells along the length of the cochlear duct.

(**A**) Statistical analyses of cell counts taken from 200 pixel by 200 pixel regions within the base of the cochlea reveals that treatment of cultures with 2 mg/mL gentamicin or 0.8 mM neomycin for 1 day are sufficient to cause a significant decrease in the number of hair cells. (**B**) 2 mg/mL gentamicin and 0.8 mM neomycin also cause hair cell loss, but to a lesser extent within the middle region. (**C**) Hair cell loss within the apical region is only significant when cultures are treated with 0.8 mM neomycin.

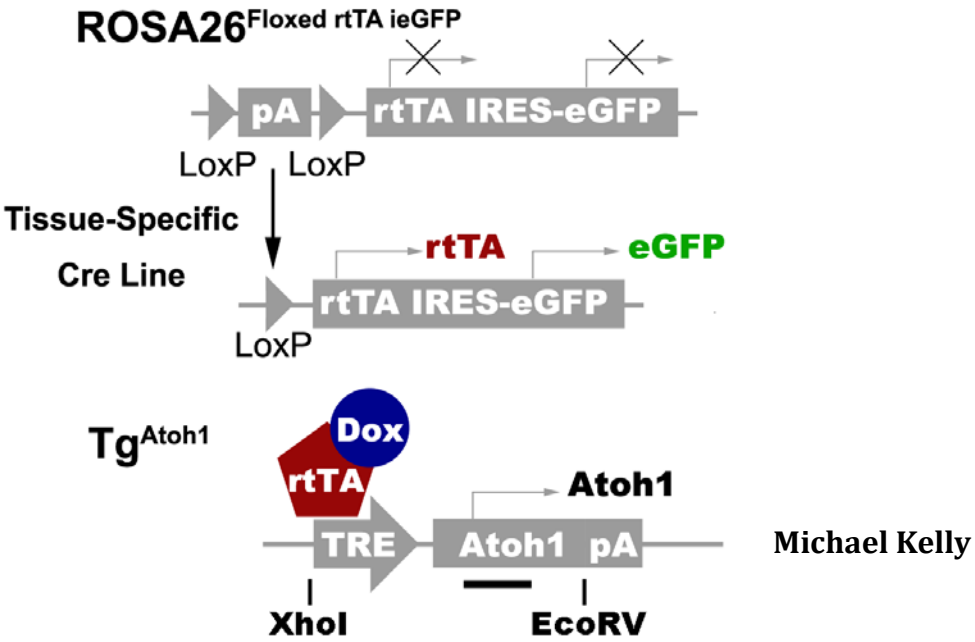

Fig. 7. Diagram illustrating the transgenic Atoh1 model*.
Atoh1 expression is regulated by a tetracycline-response element, which is only active when doxycycline binds to a reverse-tetracycline transactivator (rtTa). Additionally, this rtTa, along with an eGFP reporter, is regulated by a tissue-specific Cre recombinase, which allows induction of Atoh1 expression within specific tissue. With the combination of tissue- or cell-specific Cre and the timing of doxycycline addition, cell-type specific and temporal control of Atoh1 induction can be achieved.
*Adapted from Kelly et al., 2012 J. Neuroscience, in press. (Alexander Pan is a contributor of the paper.)

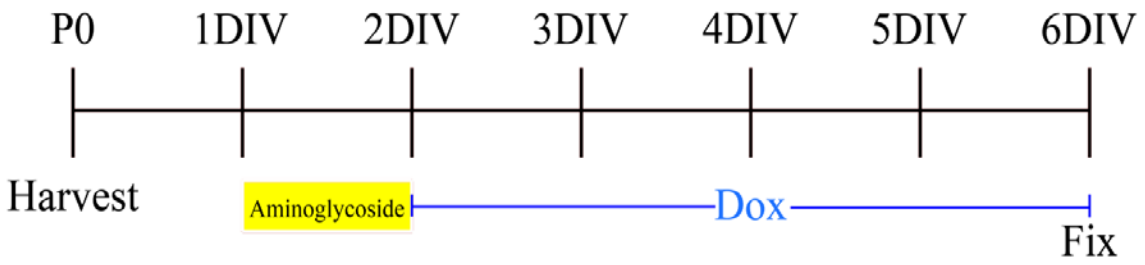

Fig. 8. Timeline diagram for hair cell regeneration experiments.
Neonatal mice were harvested at the P0 stage, and their cochleae were set up as organ cultures. After the cultures incubated in supplemented DMEM-F12 media for 1 day, an aminoglycosidic drug was added for 1 day. 1 mg/mL doxycycline-containing supplemented DMEM-F12 media was then added at 2DIV, and this doxycycline induction lasted for 4 days. After a total of 6 days *in vitro*, the cultures were fixed, stained, mounted, and imaged.

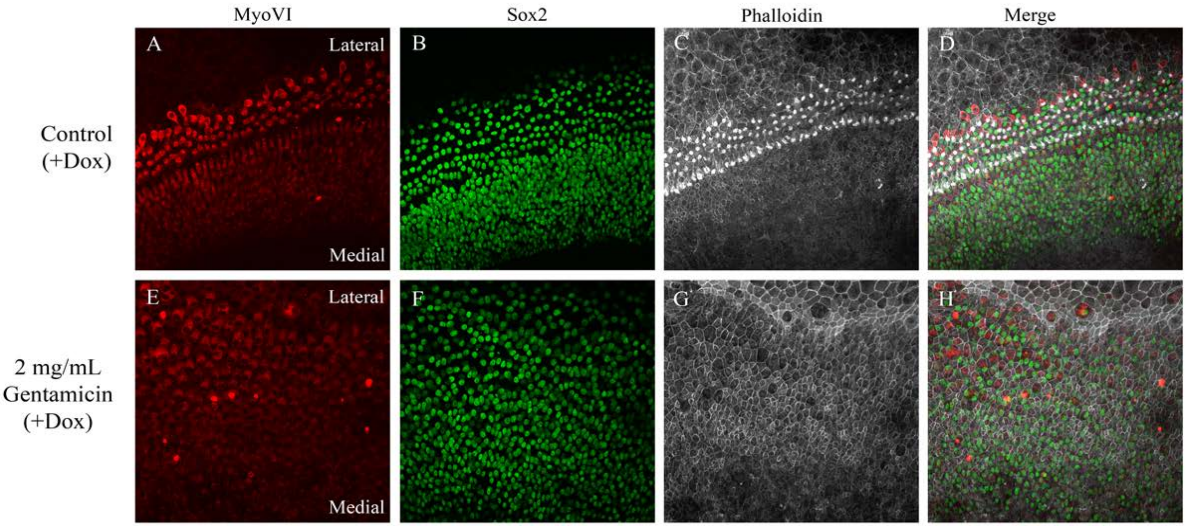

Fig. 9. Induction of Atoh1 throughout the cochlear epithelium leads to an extensive number of new ectopic hair cells.
(**A-D**) Control cultures with induced Atoh1 expression without prior hair cell ablation. Increased MyoVI (red) expression within Kölliker's organ suggests the presence of new ectopic hair cells within this region. (**E-H**) Experimental cultures where Atoh1 expression is induced throughout the cochlear epithelium following prior hair cell ablation by 2 mg/mL gentamicin. Extensive MyoVI (red) expression suggests the presence of ectopic hair cells, but the sensory region is difficult to locate. Phalloidin (white) expression suggests the absence of stereociliary bundles on these ectopic hair cells.

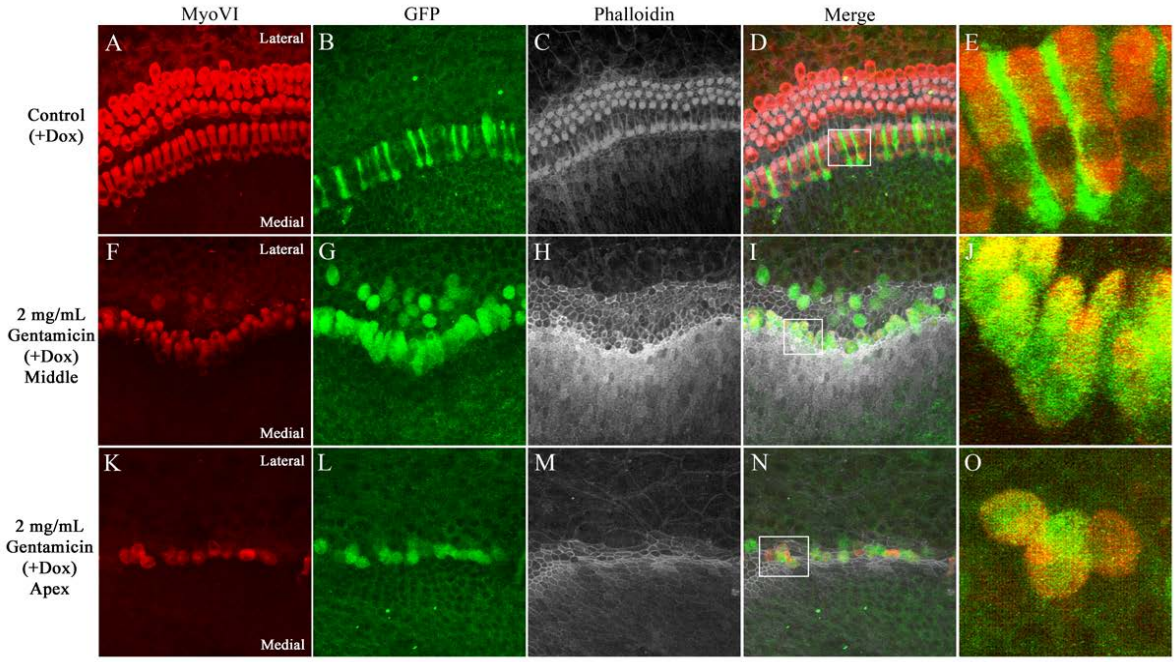

Fig. 10. Induction of Atoh1 expression within the supporting cells leads to regenerated hair cells of supporting cell origin within the sensory region after ablation of endogenous hair cells.

(**A-E**) Cultures from tri-allelic transgenic samples where hair cells were not ablated prior to Atoh1 induction in the supporting cells that are marked by GFP. Judging from the MyoVI (red) expression and GFP expression, there is no evidence of ectopic hair cells generated from supporting cells that have been induced to express Atoh1. The GFP (green) expression appears limited to the supporting cells within the inner hair cell region. Phalloidin (white) expression shows the presence of stereociliary bundles on the endogenous hair cells. (E) A close-up image of the region contained within the white box in (D). There are no cells expressing both MyoVI and GFP, indicating no generation of hair cells from supporting cells without ablation of endogenous hair cells (E). (**F-L**) Images from the middle region of a tri-allelic transgenic sample where Atoh1 is induced and the endogenous hair cells were ablated. The MyoVI-positive cells appear to have GFP expression, suggesting that these MyoVI-positive cells are new ectopic hair cells derived from supporting cells. The close-up image in (L) demonstrates the co-localization of MyoVI and GFP within the hair cells. (**K-O**) A small region of ectopic hair cells within the apical region of the cochlea from tri-allelic animals with Atoh1 induction and ablation of endogenous hair cells. Co-localization of MyoVI and GFP was observed in hair cells in the apical region, similar to the middle region of the cochlea shown in (F-J).

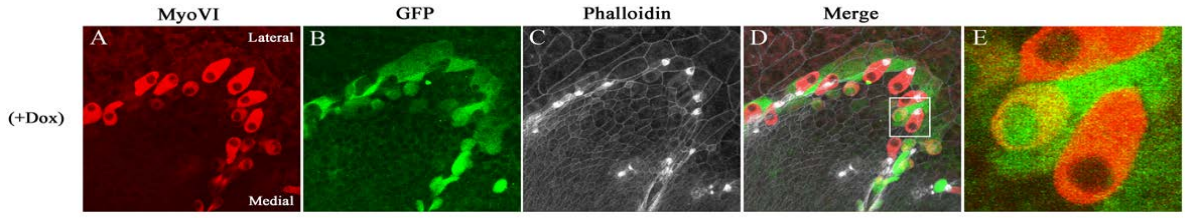

Fig. 11. Physical damage prior to Atoh1 induction also leads to ectopic hair cells within the sensory region.

(**A-E**) Culture from a tri-allelic transgenic sample where the hair cells were not ablated with aminoglycosidic drug, but by physical damage during dissection. Physical injuries during the dissection process damaged the morphology of the organ of Corti, and only a few endogenous hair cells survived in the culture. MyoVI (A), GFP (B), and Phalloidin (C) are shown to mark hair cells, GFP+ cells of supporting cell lineage, and the stereocilia hair bundles, respectively. The overlay of the three is shown in (D). Following Atoh1 induction by $GFAP^{cre}$ specifically in the non-sensory supporting cells, a small number of ectopic hair cells were generated from the supporting cells as demonstrated by the co-localized MyoVI and GFP cells. (C-D) Phalloidin (white) expression (C) reveals stereociliary bundles at the apical surface of endogenous hair cells that are positive for MyoVI (red, D) but negative for GFP (green, D). (E) Close-up image of two endogenous hair cells along with a single new ectopic hair cell that is generated from supporting cell as evidenced by their positive expression of MyoVI but negative expression of GFP or positive expression for both MyoVI and GFP, respectively.