Defined populations of inner ear progenitor cells show limited and distinct capacities for differentiation into hair cells, neurons, and glia

By
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Abstract

Despite the fact that mammalian hair cells and neurons do not naturally regenerate *in vivo*, progenitor cells exist within the postnatal inner ear that can be manipulated to generate hair cells and neurons. This work reveals the differentiation capabilities of distinct inner ear progenitor populations and pinpoints cell types that can become cochlear hair cells, vestibular hair cells, neurons, and CNS glia.

We expanded and differentiated cochlear and vestibular progenitors from mice (postnatal days 1-3) and analyzed the cells for expression of mature properties by RT-PCR, immunostaining, and patch clamping. Whereas previous reports suggested that inner ear stem cells may be pluripotent and/or revert to a more neural stem cell fate, we find that cells from each organ type differentiated into cells with characteristics of the respective organ. Only cochlear-derived cells expressed the outer-hair-cell protein, prestin, while only vestibular-derived cells expressed the vestibular extracellular matrix marker, otopetrin. Since Atoh1 expression is consistently found in new hair cells, we used an Atoh1-nGFP mouse line to identify hair cell candidates. We find that cells expressing Atoh1 also expressed key transduction, hair bundle, and synaptic genes needed for proper function. Whole-cell patch clamp recordings showed that Atoh1-nGFP+ cells derived from both cochlear and vestibular tissue had voltage-gated ion channels that were typical of postnatal hair cells. Only vestibular-derived Atoh1-nGFP+ cells, however, had I_h, a hyperpolarization-activated current typical of native vestibular hair cells but not native cochlear hair cells.

Lineage tracing studies with known supporting cell and glial cell markers showed that progenitor capacity of cochlear supporting cells positive for Lgr5 (Lgr5+ cells) was limited to differentiation into hair cell-like cells but not neuron-like cells. In contrast, glial cells positive for PLP (PLP1+ cells) from the auditory nerve differentiated into multiple cell types, with properties of neurons, astrocytes, or mature oligodendrocytes but not hair cells. Thus, PLP+ progenitor cells within the auditory nerve are limited to neuronal or glial fates but have greater potency than Lgr5+ progenitors, which only formed hair cell-like cells.

In summary, this work identifies distinct populations of post-natal inner ear progenitors and delineates their capacity for differentiation and maturation.

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Introduction

In America alone, over 35 million people suffer from hearing loss (15% of the population) (Kochkin 2009). At some point in their lives, 35% of Americans suffer from vestibular/balance issues such as vertigo, dizziness, and disorientation, problems that are correlated with a risk of falling and severely reduced quality of life (Agrawal 2009). Over 90% of all hearing loss cases reflect hair cell or auditory nerve damage, which is typically caused by factors such as noise exposure, ototoxic drugs, viral/bacterial infections, and aging (NIDCD). Hair cell loss and noise exposure can also lead to auditory nerve degeneration (Spoendlin 1975, Kujawa et al. 2009). Cases of vestibular compromise are often treated with steroid injections or labyrinthectomies (inner ear removal), with the latter treatment always leading to hearing loss.

Although fish and birds regenerate hair cells lost to damage, mammals do not; thus, hair cell and neuronal death from disease or trauma leads to permanent loss of hearing or balance cues. Present remedies for hearing loss include hearing aids and cochlear implants, which have limitations. Both devices can only stimulate the residual sensory cells and/or neurons of the damaged inner ear, and because of electrical current spread, cochlear implants provide limited frequency resolution. Both remedies leave the patient with poor sound localization and musical perception, and improvements in speech perception vary widely (Eddington 2011). Thus, understanding biological mechanisms that could lead to hearing and balance restoration would significantly advance therapy options. In recent years efforts have focused on developing ways to regenerate the sensory cells and neurons of the inner ear. Despite the lack of hair cell regeneration, populations of progenitors have been demonstrated within the mammalian inner ear (Fig. 1). Prior work has suggested that inner ear stem cells are

pluripotent and can be isolated, cultured, and differentiated into hair cells and neural cells (Li et al. 2003, Oshima et al. 2007, Martinez-Monedero et al. 2008). Later work demonstrated that Lgr5+ cells are the hair cell precursors within the cochlea (Chai et al. 2011, Shi et al. 2012). We hypothesize that, rather than a single pluripotent or multipotent stem cell type, there are separate pools of inner ear progenitors within the inner ear that give rise to vestibular hair cell-like cells, cochlear hair-cell like cells, and neuron-like cells.

Aim 1: Examine cochlear and vestibular stem cell's differentiation capabilities *in vitro*.

Hair cells and supporting cells from each organ differ in their gene expression. For instance, outer hair cells of the cochlea express prestin, while supporting cells of the vestibular organs express otopetrin. Through the use of PCR and immunostaining, we can use these differences to determine which cells types are created from each organ's differentiated progenitors, and also determine if they express key genes that are needed for proper physiological function.

Hair cells commonly express large numbers of voltage-gated ion channels in particular families (e.g., inward and outwardly rectifying potassium (K) channels, sodium (Na) channels, and calcium (Ca) channels); the exact composition is specific to hair cell subtype and can be used to recognize the cells' identity. With the patch clamp method, we recorded voltage-activated currents from differentiating progenitor cells and examined the data for similarities to whole-cell currents from native subpopulations of hair cells. With an Atoh1-nGFP mouse line (nuclear GFP), we record from Atoh1-expressing cells to see if progenitors from each tissue give rise to particular hair cell subtypes based on accepted electrophysiological criteria.

Aim 2: Examine potency and differentiation capabilities of inner ear progenitors.

Shi et al. (2012) determined that Lgr5+ cells in the mouse cochlea gave rise to hair cells, whereas Lgr5-negative (Lgr5–) cells did not. Since prior work suggested that inner ear cells when cultured as neurospheres (described in Methods) may revert back to a neural stem cell fate (Li et al. 2003, Oshima et al. 2007) or perhaps even a pluripotent state, we set out to determine by lineage tracing if Lgr5+ cells were capable of becoming other cell types, such as neurons.

Previous work had also shown that a neural stem cell population arises from inner ear neurosphere culture (described in Methods) (Li et al. 2003, Oshima et al. 2007, Martinez-Monedero et al. 2008). We set out to identify which progenitor cells were responsible for forming neurons and glia. Work by Gomez-Casati et al. (2010) revealed that inner ear Schwann cells express PLP1. Using their PLP-Cre mouse line, we found that the Schwann cells of the inner ear generated both neurons and two types of CNS glia.

Background and Significance

Stem cells and the inner ear

In the beginning of embryogenesis the fertilized egg goes through cell divisions to form the morula. These cells are totipotent because they can form all tissues of the organism and the placenta, which allow it to generate an intact animal. These cells then progress to the blastocyst stage, which contains the inner cell mass. The inner cell mass is the source of pluripotent embryonic stem cells, which can differentiate to form the specialized tissues of all three germ layers, but cannot generate a fertile adult mouse. A grown organism has adult stem cells which repair, replenish, and maintain normal turnover of regenerative organs, such as the complete regeneration that occurs every 2-5 days within the intestinal epithelium (Barker et al. 2007, Barker et al. 2008). These adult stem cells are called multipotent and are limited to generating the cell types of a particular tissue.

The mammalian inner ear contains a population of stem cells that generate the highly specialized sensory and supporting cells of the vestibular and auditory sensory epithelia. The cochlea, however, lacks adult stem cells to allow regeneration and repair; adult mammalian vestibular epithelia may have some low-level regenerative capability (Burns et al. 2012) but it is inadequate to prevent significant hair cell loss with aging (Rauch et al 2001). We are interested in what gives various tissues different regenerative capabilities, and to what extent a specific progenitor cell can give rise to and repair diverse tissue types. The theory on stem cell potency has often been described by Conrad Waddington's "epigenetic landscape" theory (Fig. B1) (Waddington 1957). The theory illustrates the successive restriction of initially totipotent cells to pluripotency, with further commitment to a certain lineage as the stem cell moves down the

path of differentiation. The inner ear provides a valuable tool to study this progression because isolated stem cells from the cochlea, vestibule, and ganglia are thought to be able to generate both hair cells and neurons (Li et al. 2003, Oshima et al. 2007, Martinez-Monedero et al. 2008) and to form all three germ layers (Li et al. 2003). In this research project we use a combination of molecular signaling manipulations, patch clamping, and detection of gene expression to analyze the ability of inner ear progenitor cells to differentiate into the specialized cellular fates of the various inner ear organs.

The inner ear has two parts, the cochlea (hearing) and vestibule (balance), both of which have hair cells, so named for their apical microvillar bundles ("hair bundles"). The cochlea and vestibular organs each have two different hair cell subtypes. In the cochlea, the signals we perceive as sound are transmitted by inner hair cells (IHCs), and the signal is amplified and tuned by outer hair cells (OHCs). The vestibular organs have type I and type II hair cells, which have different shapes, synaptic contacts, and microvillar bundle characteristics. Electrophysiology experiments with the patch clamp method enable us to distinguish between these four hair cell subtypes based on their ion channels and transduction properties, and also how these properties are acquired in normal development. However, the developmental signals determining if an immature hair cell acquires a vestibular or cochlear fate remain elusive, and even less is known about how these subtypes emerge.

Hair cell differentiation and development depends on Atoh1, a basic helix-loop-helix (bHLH) transcription factor that is regulated through the Notch signaling pathway (Bermingham et al. 1999; Zheng & Gao 2000; Woods et al. 2004, Izumikawa et al. 2005). Previous work has used the nuclear expression of the Atoh1 transcription factor to identify hair cells that have

differentiated from stem cells. In an analogous fashion, Atoh1 is necessary for intestinal progenitors to develop into epithelial secretory cells such as goblet, Paneth, and enteroendocrine cells (Yang et al. 2001). In either case, when a cell expresses Atoh1, it also expresses the Dll1/Jagged2 ligand that binds to the Notch receptors of its neighboring cells, suppressing their Atoh1 expression. The resulting "lateral inhibition" mechanism specifies cells that express Atoh1 as hair cells (ear) or secretory cells (intestine), while the neighboring cells remain supporting cells (ear) or enterocytes (intestine) (Yang et al. 2001). This phenomenon is necessary to establish normal morphology in both of these organs.

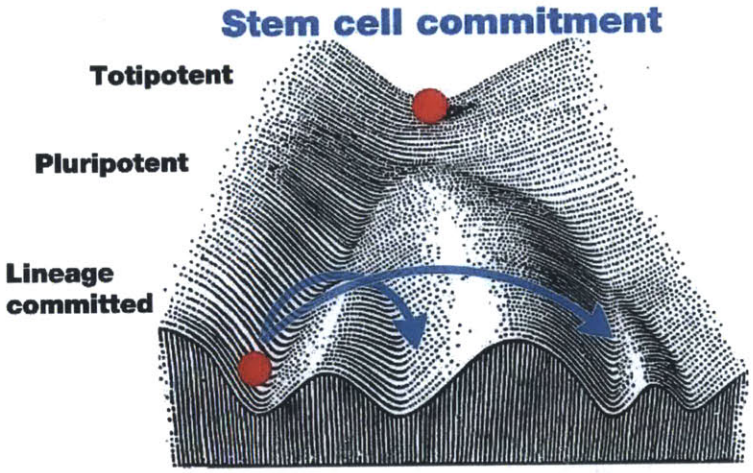

Figure B1: Waddington's "epigenetic landscape" theory suggests that stem cells behave like a marble rolling down a hill. As the marble proceeds down the hill it must commit to various troughs, with each trough representing another degree of commitment (i.e. decreased potency). The further down the hill the marble rolls, the more difficult it is to change paths (represented by the blue arrows). This corresponds to the increased difficulty for a cell to change fates as it becomes more committed (Image modified by McLean; original from Waddington 1957).

Potential for therapy

Efforts to advance therapy for hearing loss and poor vestibular function have focused on developing ways to regenerate the hair cells of the inner ear so that the natural biological mechanisms of hearing can be restored. The hair bundles are moved by sound, in the case of the auditory organ, and head motions, in the case of vestibular organs. Motion of the bundle evokes an electrical signal in the hair cell, which is relayed by eighth-nerve fibers to the brain. Although hair cells regenerate after damage in fish and birds, hair cells do not regenerate in mammals, and hair cell death from disease or trauma leads to permanent loss of hearing or balance cues.

Despite the fact that mammalian hair cells do not naturally regenerate *in vivo*, populations of stem cells exist within the postnatal inner ear of mice (Fig. 1). Previous work has suggested that these inner ear stem cells may be pluripotent (Li et al. 2003), based on several lines of experimentation. Progenitors were isolated from the utricle and cultured in the presence of the growth factors IGF and EGF to form neurospheres. The neurospheres were then differentiated, at which point cells with the properties of hair cells, neurons and glia were obtained. In addition, co-culturing neurospheres with a mesodermal cell line allowed them to differentiate into cells that stained positively for known muscle proteins. Furthermore, co-culturing the neurospheres with a totipotent cell line allowed the colonies to form cells that stained positively for myogenic markers. When lineage-tagged neurospheres were injected into stage 4 chicken embryos, which allows the cells to be distributed during gastrulation and exposes the cells to various inductive environments, the neurosphere cells were incorporated into all three germ layers.

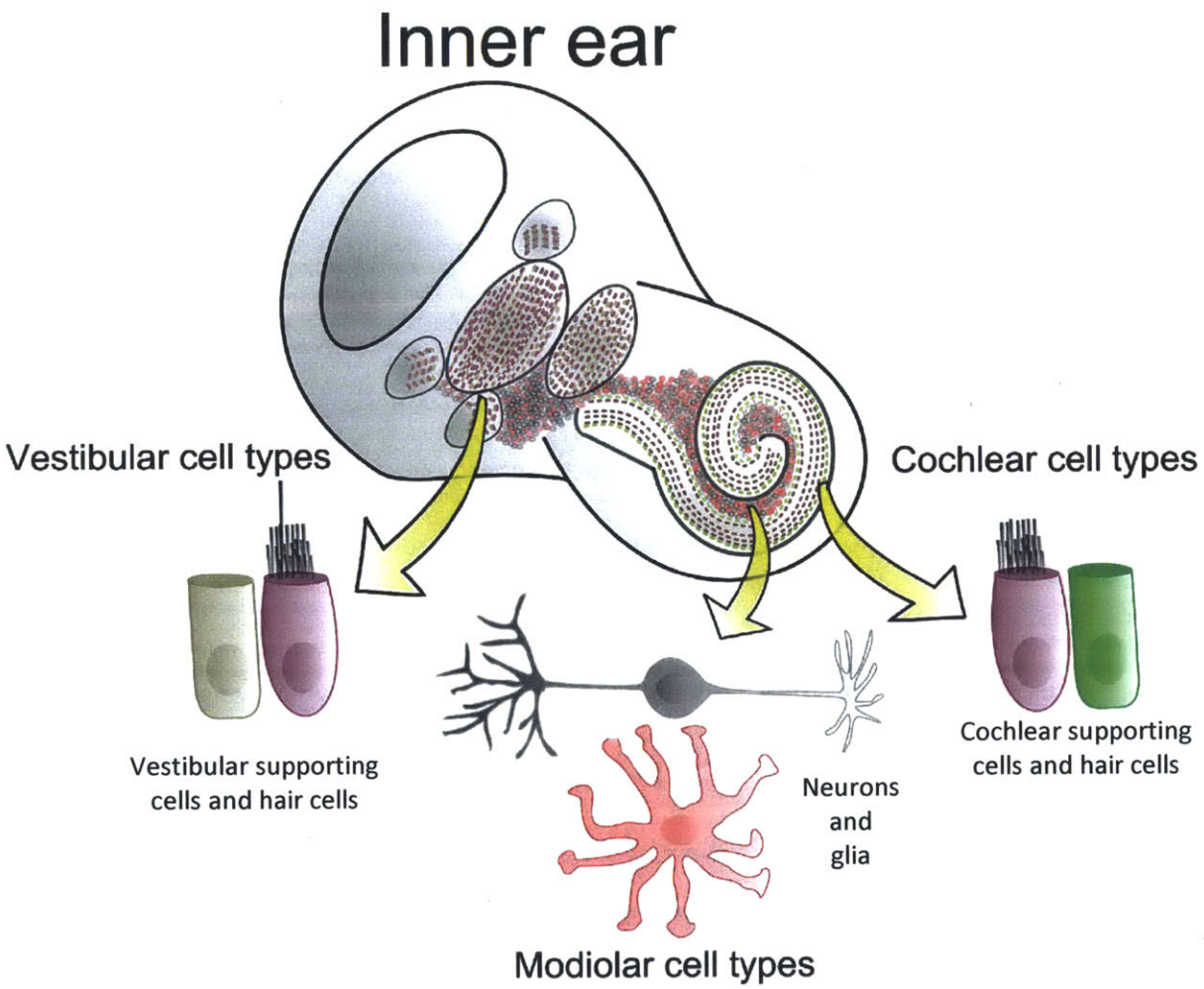

Figure 1: Analysis of inner ear progenitor cells
The potency and differentiation characteristics of 3 inner ear tissues are analyzed. Hair cell-like cells that are produced from cochlear and vestibular tissue are analyzed and compared. This work also analyzes the potency of Lgr5+ cells within the cochlea (green) and PLP+ glial cells within the auditory nerve (red).

Previous studies have shown that isolated cochlear, vestibular, and modiolar progenitors can be cultured long term and survive through seven passages. Cochlear and vestibular spheres gave rise to hair cell-like cells, supporting cells, and neurons *in vitro*, as

indicated by immunostaining for typical hair cell proteins such as Atoh1 and Myosin VIIA and neuronal genes such as Tuj and GFAP (Li et al. 2003, Oshima et al., 2007, Martinez-Monedero et al., 2008, Martinez-Monedero 2007). From the voltage dependence of whole-cell currents recorded from the generated hair cell-like cells, it was suggested that the cells most closely resembled embryonic hair cells (Oshima et al. 2007). Similarly, generated neurons were found to have similar, yet smaller, currents than neonatal neurons (Martinez-Monedero et al., 2008). Modiolar spheres were shown to produce a much higher yield of neural cell types when compared to the sensory organs, and although hair cell-like cells were reported, they occurred at an extremely low rate (<.1% of all cells)(Oshima et al 2007).

Thus, substantial progress has been made in discovering the underlying mechanisms of hair cell and neuronal development and in establishing reliable methods for their generation (though typically at low yields). These studies attempted to determine whether a latent capacity for regeneration was present in the inner ear and much of the work was done with unknown and possibly mixed types of progenitors. To identify regenerated hair cells, previous work used markers that are specific to hair cells within the native inner ear; however, many of these markers are expressed elsewhere in the body and are therefore not necessarily indicative of hair cells if newly created cells are in fact derived from pluripotent stem cells. Oshima et al. (2007) described prestin expression via RT-PCR in differentiated cochlear spheres, but otherwise previous work did not address whether specific subtypes of hair cells could be developed, which can be detected by looking for specific gene expression (e.g. prestin for OHCs, VGLUT3 for IHCs, A-current for type II hair cells). In summary, previous studies found that stem

cells may exist within the mouse inner ear but did not address whether they comprise different populations or whether they differ in their potency and differentiation capabilities.

More specifically, previous studies used immunostaining against the proteins Atoh1, espin, parvalbumin3, and myosin VIIA to identify hair cells; against Tuj to identify neurons; and against GFAP to identify glia. However, Atoh1 and Myosin VIIA are expressed in all hair cell subtypes and GFAP is expressed in some peripheral and CNS glia, as well as supporting cells (Rio et al. 2002). Furthermore, Atoh1 is known to be expressed in the Merkel cells of the skin, secretory cells of the gut, granular neurons, spinal cord interneurons, and, transiently, in supporting cells of the ear (Lewis et al. 2012). Similarly, Myosin VIIA is known to be expressed in the secretory cells of the gut, testis, lung, kidneys, and retina. Thus, reliance on these proteins to identify progenitor-derived hair cells can be questioned because other cell types would be expected to express one or both of these proteins. Similarly, GFAP expression alone doesn't determine if cells are glia or supporting cells. This issue is further complicated if, as suggested by Li et al. (2003), inner ear stem cells are pluripotent. If this is the case, it is possible that the cells differentiated from inner ear stem cells could be any of the previously mentioned cell types. Therefore, using the current protein expression standards could lead to misidentifications.

Thus, there is a need for a more extensive analysis of progenitor-derived hair cell-like cells and neural like cells. First, proper identification and understanding of what drives stem cells to a particular hair cell fate would move the field one step closer to being able to target specific hearing or balance ailments that are rooted in loss or damage of a certain hair cell subtype. In addition, a more thorough analysis is needed to determine if the newly created cells

possess all the machinery to function as legitimate hair cells, a step that would be necessary for proper hearing or balance restoration in future therapies.

Much is known about specific hair cell subtype characteristics, in terms of how function and gene expression progress through aging. Much of what is known about this particular area of hair cell function is owed to patch clamping experiments, in which a fine glass electrode is used to measure current through one or many ion channels of a cell. More specifically, one can measure a single transduction channel's response while deflecting the hair bundle with a probe (Beurg et al. 2006), or the current through thousands of channels activated by voltage. Furthermore, previous work had identified specific channel types that contribute to behavior of hair cell subtypes. For example, type I vestibular cells express the $Na_V1.5$ sodium channel, and this expression changes with development (Wooltorton et al. 2007). Similarly, previous work has shown how each hair cell subtype follows a specific program of ion channel acquisition and expression, with the program and end product being unique to each subtype (Rüsch et al. 1998, Marcotti et al 2003, Géléoc et al. 2004, Hurley et al. 2006). Electrophysiology can provide information that is not available from immunohistochemistry. In all, the patch clamp method provides a tool that can greatly aid in the characterization of hair cell-like cells. Figure B2 summarizes the dynamic physiological changes that cochlear hair cells undergo as they develop.

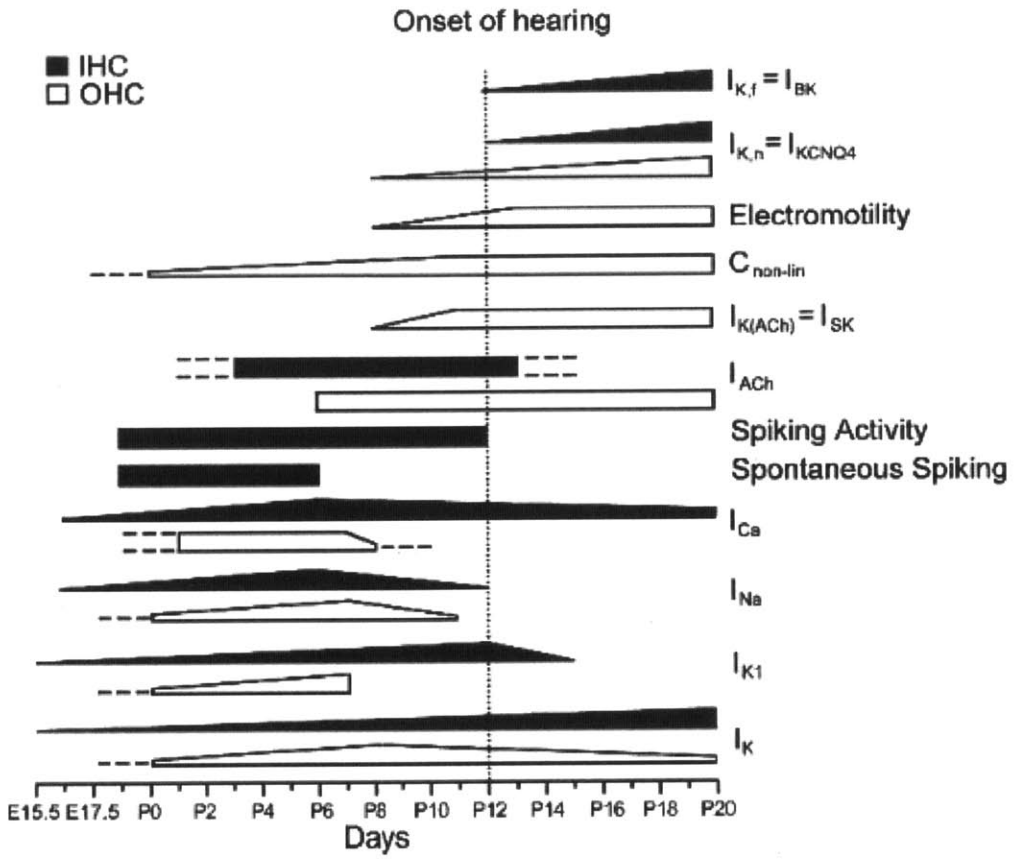

Figure B2: Extensive research into native hair cell development has revealed the time course for how hair cells acquire physiological characteristics.
Inner hair cells are indicated by white fill, while outer hair cells are indicated by black fill. Ramps show the increase or decrease of a current or other functional characteristic over time. Dashed lines indicate uncertainty or projections of behavior based on in vitro results. (Figure from: Goodyear et al. 2006)

Recent experiments in hair cell and glial gene expression have created new ways to identify specific cell types. For instance, the motor protein prestin, which is only found in OHCs, has a large rise in expression as the animal nears hearing (Belyantseva et al. 2000). Some other examples of protein markers that help distinguish hair cell subtypes include calretinin (expressed in immature hair cells, mature inner hair cells (IHCs) and type II hair cells), VGLUT3

(expressed in IHCs and vestibular hair cells), and alpha3 tubulin (found in kinocilia, which are present on immature hair cells and mature vestibular hair cells). In addition, the proteins O4 and O1 are selective markers for immature non-myelinating oligodendrocytes and for mature myelinating oligodendrocytes, respectively (Sommer and Schachner 1980, Sommer and Schachner 1982), and Aldhl1l is a specific astrocyte marker (Cahoy et al. 2008). These glial markers could help determine whether inner ear progenitors are capable of forming glia from outside their lineage. Furthermore, immunostaining and other molecular methods such as RT-PCR can help identify functional hair cells. Looking for expression of functional proteins such as VGLUT3 in synaptic vesicles, $Ca_v1.3$ channels in the presynaptic membrane, Ctbp2 (in the ribbon synapse), AChr9 (part of the acetylcholine receptor found on OHCs), and many others, can indicate whether the new hair cells have the potential to respond to stimuli in a similar manner to native hair cells. As a further example, we can use the PCR method to determine whether stem cell-derived hair cells express components of the transduction channel by taking advantage of the recent identification of TMC1 and TMC2 as channel proteins (Kawashima et al. 2011, Pan et al. 2013). In order for newly created hair cells to function properly in stem cell therapies, these critical proteins must be generated.

By combining the techniques of molecular biology, biophysics, and lineage tracing, we are able to examine the differentiation of inner ear progenitors. Building on previous work that showed how to generate hair cell-like cells and neural-like cells *in vitro*, our studies analyze the process of acquiring a certain cellular fate and whether these cells possess the cellular machinery needed to be fully functional. The potency and differentiation characteristics of three inner ear tissues are analyzed: cochlea, vestibule, and the modiolus (auditory ganglion).

Hair cell-like cells from cochlear and vestibular tissue are compared, and the potencies of Lgr5+ cells within the cochlea and PLP+ glial cells within the spiral ganglion are investigated.

Research Plan

To test whether different populations of progenitors are responsible for creating vestibular hair cell-like cells, cochlear hair cell-like cells, and neural cell types, we separated and cultured inner ear tissues in parallel and analyzed their gene expression and function (Fig. P1).

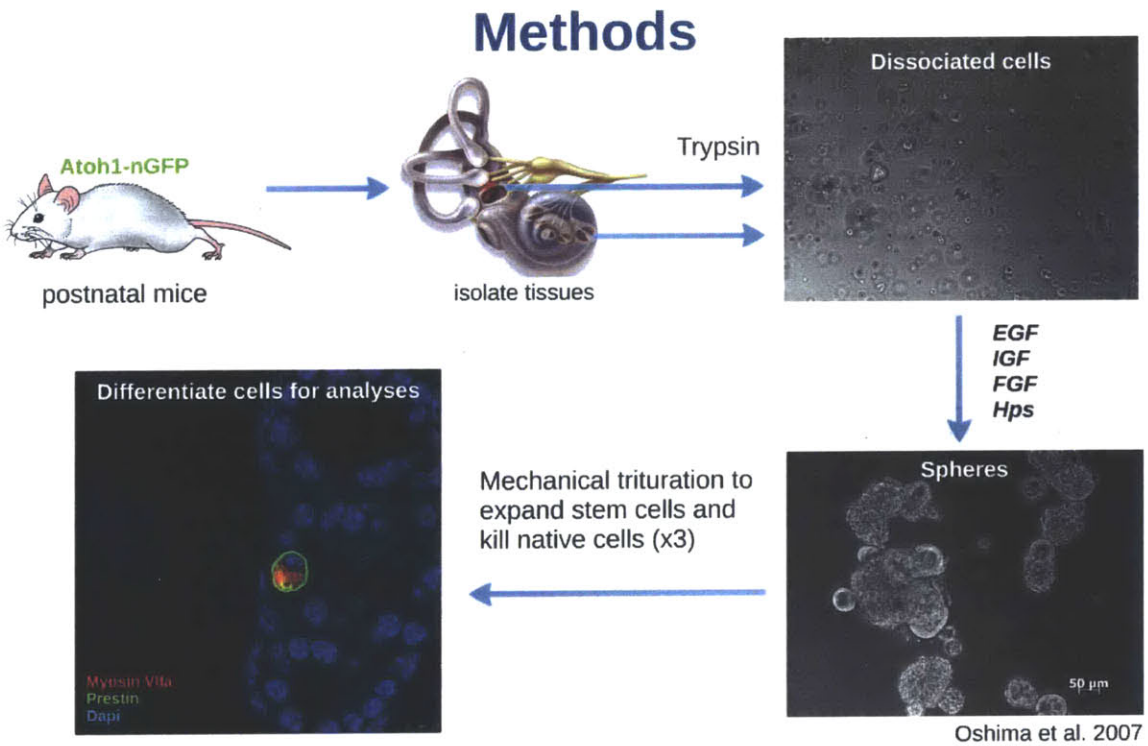

Figure P1: **Our stem cell isolation protocol.**
The inner ear organs are dissected from postnatal mice and cultured in parallel for the remainder of the experiments. The cells are cultured with growth factors that are known to expand stem cells. Spheres are triturated every few days to expand stem cells and kill native cells. Growth factors are then removed, which allows the stem cells to differentiate. After various time points we can analyze the cells that are present. Methods derived from Oshima et al. (2007).

To test the differentiation capabilities of specific cellular populations, we took tissues from mouse lines that enable lineage tracing. Lgr5+ supporting cells were tested by crossing Lgr5-EGFP-IRES-creERT2 mice with td-tomato reporter mice and looking for tomato expression (red cells, Fig. 1) in hair cell-like or neuron-like cells following our usual neurosphere preparation and differentiation. We also crossed Plp-cre/ERT2 and td-tomato reporter mice to determine if glial cells of the spiral ganglia are capable of producing hair cells or if they are limited to producing neurons or glia. The various strain crosses and the experiments we did with them are shown in Table P1.

Table P1: Mouse strains

Strain	Analysis
Atoh1-nGFP (nuclear GFP)	Immunostaining to look for hair cell markers. Targeting candidates for patch clamping.
Lgr5-EGFP-IRES-creERT2+ Tomato reporter	Lineage tracing (immunostaining) to determine the cell types that arise from Lgr5+ cells.
Plp-cre-ERT + Tomato reporter	Lineage tracing (immunostaining) to determine the cell types that arise from Plp+ cells.

We analyzed the gene expression in the differentiated cells by RT-PCR and immunostaining. In Table P2 we list the genes we examined, the method of detection, and the cell type and location of the expression.

Table P2: Genes analyzed

Gene	Immunostain or PCR	Localization
Myosin VIIA	Both	All hair cells
VGLUT3	PCR	Inner hair cells, vestibular hair cells
Prestin	Both	Outer hair cells
Phalloidin	Immunostain	Actin; Hair bundles, cuticular plate, actin rings
Calretinin	Immunostain	Immature hair cells, mature inner hair cells, type II hair cells
Alpha3 tubulin	Immunostain	Kinocilia; Immature hair cells & vestibular hair cells
PMCA2	Immunostain	Hair bundles of all hair cells
Oncomodulin	Both	Type I hair cells
Pchd15	PCR	Hair bundle tip links
Cdh23	PCR	Hair bundle tip links
$Ca_V1.3$	PCR	Basal portion of hair cells, associated with ribbon synapse
Chrna9	PCR	Hair cells, associated with efferent synapse
Ribeye (ctbp2)	Both	Hair cell synaptic ribbon
Otogelin	PCR	Supporting cells
Otopetrin	PCR	Vestibular supporting cells only
TMC1	PCR	Transduction channel
TMC2	PCR	Transduction channel
Espin1	PCR	Hair bundles
Espin4	PCR	Hair bundles
Myosin 1c	PCR	Hair bundles
Lgr5	PCR	Subset of supporting cells

In addition, we used the patch clamp technique to identify currents that determine if the hair cells are functional and what subtype they may be. Examples of native hair cell currents from different hair cell-subtypes are shown in Figure P2. We selected hair cell candidates to patch by their Atoh1-nGFP expression. Examples of electrophysiological characteristics looked for are listed in Table P3.

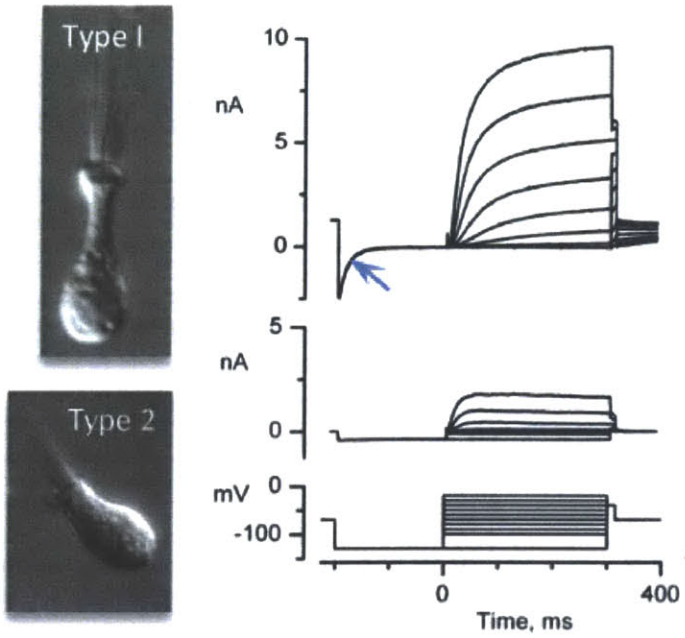

Figure P2: Examples of currents recorded from type I and type II vestibular hair cells.
A distinguishing characteristic is that type I hair cells have larger currents than type II hair cells. Type I hair cells also have the negatively-activating current $G_{K,L}$ (blue arrow). Image is courtesy of K.M. Hurley & R.A. Eatock, unpublished.

Table P3: Patch clamp experiments

Experiment	Characteristic Identification
Differences in K^+ currents	Size and kinetics can help determine maturation state and subtype – e.g., large A-current in extrastriolar type II hair cells
K^+ current blockers (ex. TEA, IBTX)	Can determine K^+ current types present, which can indicate cell type and maturity
Use large hyperpolarizing steps (from ?60mV to ?120mV) to look for slowly activating I_h	Presence of I_h may indicate vestibular hair cell type.
Current clamp recordings (to look at voltage behavior)	Resting potential, which is well characterized in developing hair cells and shifts with age. Voltage responses to current steps can help identify cell type and maturation – e.g., young hair cells spike, mature hair cells do not.

By combining these genetic, molecular, and electrophysiology techniques, this body of work should help determine the differentiation capabilities of cochlear and vestibular stem cells, and their functional state.

Methods

Isolation of stem cells from the inner ear

To gather progenitors for proliferation and hair cell-like cell generation, we extracted progenitor cells from the neonatal mouse inner ear. All animal studies were conducted under an approved institutional protocol according to National Institutes of Health guidelines. For each experiment, the cochleae and the vestibular organs of 6-8 CD1 or Atoh1-nGFP pups (age p1-p4) were dissected in HBSS and kept separate from each other for the remainder of the protocol. This separation allows us to analyze each tissue's developmental properties separately. In the case of the cochleae, the organ of Corti (OC) was separated from the stria vascularis and the modiolus. The OC and vestibular tissues were transferred to the dissociating medium TrypLE (Life Technologies) for 11-13 min at 37°C and then manually dissociated with a pipette. The triturated cells were then passed through a 70-μm cell strainer to remove tissue and bone debris. Single cells were cultured in a 1:1 mixture of DMEM/high-glucose medium and F12, supplemented with N2, B27 (Invitrogen), EGF (20 ng/mL; Chemicon), bFGF (10 ng/mL; Chemicon), IGF-1 (50 ng/mL; Chemicon), and heparin sulfate (50 ng/mL; Sigma). Single cells were maintained in ultralow-cluster plates (Costar) for several days in culture to obtain floating spheres. The spheres were then passaged every 3-4 days; a 100-μl pipette was used to triturate the cell suspension 20-25 times.

After passage, the cells were placed in fresh culture medium supplemented as described above. For each experiment, the cells were passaged three times to eliminate any hair cells or neurons that may have been carried over during the trituration process.

Mouse strains for lineage tracing

In addition to isolating each organ, cell type potency was analyzed for Lgr5+ cells of the cochlea and PLP+ cells of the ganglia. Male Lgr5-EGFP-IRES-creERT2 (http://jaxmice.jax.org/strain/008875.html) and male PLP-cre-ERT mice (http://jaxmice.jax.org/strain/005975.html) were crossed with female td-tomato reporter mice (http://jaxmice.jax.org/strain/007909.html) in order to lineage trace the cells that resulted from each cell type. Mother mice were injected with 600 µl tamoxifen (50mg/ml) on the day of birth and 1st day post birth (d0 and d1). Pups were dissected at p3 and were identified as either genetically positive or negative based on their fluorescence. Spheres were generated from the OC of Lgr5+ mice and the modiolar tissue of PLP+ mice.

Differentiation and treatment of spheres

To generate differentiated cells for future assays, 3rd generation spheres were plated in 4-well plates (Greiner) on round 10 mm glass coverslips coated with poly-L-lysine (Cultrex). Attachment took place overnight in DMEM-high glucose/F12 (mixed 1:1, GIBCO) with N2 and B27 (Invitrogen). Spheres were differentiated in these conditions for 7-70 days, with fresh culture medium being applied every 2-3 days to maintain optimal culture conditions. Cells were then harvested for PCR analyses, immunostained for further gene expression analyses, or used for patch

clamping experiments.

Control spheres

To ensure that the results obtained were not significantly affected by hair cells that were carried over during the trituration process that survived throughout culture, I counted the number of hair cells that were still present after the 3rd passage (see appendix). For immunostaining analyses, 3rd generation spheres were seeded in the same experimental culturing conditions as were used for experiments, but instead were only allowed to differentiate for 3 hours. This seeding time allows the spheres to adhere to the culturing surface, but should be insufficient to allow the cells to differentiate. All controls were analyzed for expression of Atoh1-nGFP and myosin VIIA. Controls were viewed via confocal microscopy (Leica) over the entire seeding area. For PCR analyses, 3rd generation spheres were collected from floating cultures and Myosin VIIA expression was analyzed (n=3 cultures).

Electrophysiology

We performed patch-clamping experiments to test for functionality and to phenotypically assess between the cellular subtypes that are developed from the cochlea and vestibular tissue. Prior to recording, the cell culture solution was replaced with L-15 (Leibowitz 15) medium (supplemented with 10 mM HEPES, pH 7.3, ~320 mmol/kg osmolarity). In all experiments, we recorded from single cells using the whole-cell patch method at room temperature (22–25°C). The pipette solution contained (in mM): 135 KCl, 3.5 $MgCl_2$, 5 Na_2ATP, 10 HEPES, 10 EGTA, 0.1 Na-cAMP, 0.1 Li-GTP. The solution's pH was adjusted to 7.4 by adding 15 mM KOH. Osmolality of the solution was 280 ± 5 mmol/kg. Recording pipettes were pulled from borosilicate glass and heat

polished to a resistance of 3-9 MΩ.

Currents were recorded with a patch-clamp amplifier (Axopatch 200B or the Multiclamp 700; Molecular Devices, Sunnyvale, CA). Series resistance (R_S) was estimated and compensated 20–90% with the intrinsic circuitry of the amplifier. Currents were filtered with an eight-pole low-pass Bessel filter with a corner frequency of 2 kHz and sampled at more than twice the filter frequency with a Digidata 1440 board (Molecular Devices), controlled by Clampex software (version 10.1; Molecular Devices).

Analyses and fits were done with Origin software (version 9; OriginLab Software, Northampton, MA), which uses a Levenberg–Marquardt least-squares fitting algorithm. All cells considered for analysis had a <100MΩ patch resistance. To obtain the activation curve for a current, we stepped to a series of test potentials and measured the "tail current" at –40 mV, that is, the current immediately upon stepping to –40 mV after each iterated test step. Plotting the tail currents against the test-step voltage produced sigmoidal "activation curves", which show how the conductance producing the current depends on voltage. Such curves could be fit with a Boltzmann function (Equation 1), where I(V) is current at voltage V, I_{min} and I_{max} are minimum and maximum currents, $V_{1/2}$ is voltage corresponding to half-maximal activation, and S is the voltage corresponding to an e-fold increase in I(V).

Eq. 1

$$I(V_m) = \frac{I_{max} - I_{min}}{1 + \exp\left(\frac{V_{1/2} - V_m}{S}\right)} + I_{min}$$

The time course of current activation was approximated by the fit of a monoexponential decay function (Origin 9.0).

Immunohistochemistry

Immunohistochemistry was performed In order to visualize and quantify the differentiated cells that express mature/functional genes. To accomplish this, differentiated spheres were fixed at room temperature in 4% paraformaldehyde/PBS for 15-20 min and then washed in PBS. Permeabilization of the cellular membrane and blocking was performed with blocking solution (0.3% Triton X-100, 15% heat inactivated goat or donkey serum in PBS) for 1 h. Diluted primary antibody (0.1% Triton X-100, and 10% heat inactivated goat or donkey serum in PBS) was applied overnight at 4° C. Primary antibody dilutions are listed in Table 1. Secondary antibodies (Alexafluor 488, 568, and 647-conjugated; Invitrogen) were used at 1:500 dilution for detection of primary antibodies. Nuclei were visualized with 4,6-diamidino-2-phenylindole (Vector Laboratories). Staining was visualized with confocal microscopy (TCD, Leica). All cellular counts of gene expression were performed manually.

Table 1. Antibodies

Primary antibody	Dilution	Source	Vendor
Myosin VIIA	1:500	Rabbit Polyclonal	Proteus Biosciences
Sox2	1:300	Goat polyclonal	Santa Cruz
VGLUT3	1:1000	Guinea Pig polyclonal	Chemicon
Prestin	1:400	Goat Polyclonal	Santa Cruz
Phalloidin	1:500	Fluorescent labeled toxin	Invitrogen
Calretinin	1:300	Rabbit polyclonal	Chemicon
Ocm (N-19)	1:100	Goat polyclonal	Santa Cruz
Alpha3 tubulin	1:1000	Mouse monclonal	Sigma
PMCA2	1:200	Rabbit Polyclonal	Thermo Scientific
O1	1:50	Mouse monoclonal	eBioscience
O4	1:50	Mouse monoclonal	R&D systems
Aldhl1l	1:80	Rabbit Polyclonal	abcam

RT-PCR

We used RT-PCR to quickly and sensitively analyze gene expression in the various tissues. First, we extracted RNA from the inner ears of CD1 mice aged E13.5, P3, P16, or from differentiated spheres, using the RNeasy Maxi Kit (Qiagen) according to the manufacturer's instructions. RNA was denatured at 65˚C for 5 min. For reverse transcription, ImProm II (Promega) was used with random hexamers. The reverse transcription conditions were 25°C for 5 min followed by 42° C for 60 min. The reaction was terminated at 70°C for 15 min. To the resulting cDNA we added primers for various inner ear proteins, which are listed below. The amplified products were then separated on a 2% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator.

Primers

Cdh15
CTGGGGCCAGTATGACGATG
TGTACCGTTTCGACTCTCTTCA

Cdh23
ACAGTCAATGCCACGGATCAA
GGAGAGTGCTCGTAGATGTTAGT

VGLUT3
CTCTTGTCAGCTGTCCCACA
CAGTCCTGTTTTTCCCCAGA

Prestin
ACAGTGTGGATGTCGTTGGA
CAGGTTGACGATCACAATGG

Espin1
CCACAGGCTACCTCTCTTGC
AGCAGCCACTTCACCACATC

Espin4
ATGGGCAATAGCTTGGAACAC
GCGAGGACATACCCGAGTC

Oncomodulin
CTGACCACTGTTGGGGAGTT
GCTCTGGAACCTCTGTAGG

Otopetrin
CTGCTCTGGATGCTGTGGTA
CAAAACGATGGTGATGTTGC

Gapdh
AACACAGTCCATGCC
TCCACCACCCTGTTGCTG

Otogelin
CCATGCAGGGTGTGTATGAG
GGCATTGCAGTCTTCTGTCA

CTBP2 A domain (Ribeye)
TGCTGGACCTTTGCAGACAC
CACCGTGTACGAGGTGGAGT

Lgr5
CCTACTCGAAGACTTACCCAGT
GCATTGGGGTGAATGATAGCA

CaV1.3
AAGGGCTACCTGGACTGGAT
CCACACACCACAAAGCAATC

Myo1c
TCTTCATCCGATTTCCCAAG
CCACACCATGTTCTTCATGC

Alpha9(Chrna9)
GGAACCAGGTGGACATATTCAAT
GCAGCCGTAGGAGATGACG

Tmc1
CTGTCCCACCCTGTTTGACT
TCACGAAACATGCTCTGAGG

Tmc2
TGGCTACAGCTTGATGATCG
GTTTCAAACAGAGGGGGACA

Results

In order to identify gene expression that may indicate the level of maturity, function, and which hair cell subtypes are being created from a given tissue source's progenitors, we performed immunostaining experiments, RT-PCR, and patch clamping assays. We first isolated progenitors from the cochlea or vestibule, allowed them to proliferate and form spheres, then differentiated the spheres for 14-70 days in order to allow significant maturation. We found that differentiated spheres contained cells that stained positively for hair cell markers, such as Atoh1 (Bermingham et al. 1999; Zheng & Gao 2000; Woods et al. 2004, Izumikawa et al. 2005), myosin VIIA (Hasson et al. 1995), and parvalbumin, a Ca^{2+}-binding protein found in vestibular type I hair cells (Demêmes et al. 1993) and cochlear inner hair cells (Pack et al. 1995) (Table 2). In the results below, we focus on the other markers expressed by cells expressing either Atoh1, myosin VIIA, or parvalbumin, which we refer to as "hair-cell like cells". We found that 51% of Atoh1-nGFP+ cells (<1% of total cells in culture) double-stained for another hair cell marker such as myosin VIIA, which is similar to the 44% yield (<1% of total cells in culture) found by Oshima et al. (2007).

Hair Bundle proteins: 67% (285/423) of myosin VIIA-positive cells derived from vestibular progenitors had phalloidin-positive stereocilia protrusions; phalloidin stains filamentous actin, which is the major constituent protein in hair bundles (Fig. 2A; Table 2). Similarly, 81% (690/850) of myosin VIIA positive cells derived from cochlear progenitors had phalloidin-positive bundle-like protrusions (Fig. 2F; Table 2). Staining for α3 tubulin, which is expressed in the single kinocilium found on vestibular hair cells and immature cochlear hair

cells (Ogata et al. 1995), was much more rare than staining for actin: α3 tubulin antibody labeled no cochlear bundle-like structures (0/312), and 4% (9/289) of vestibular bundle-like structures showed evidence of a3 tubulin expression (Fig. 2B; Table 2). This suggests that bundle formation is imperfect, as is also suggested by the structures themselves.

Labeling for PMCA2 (Fig. 2C; Table 2), a Ca^{2+}-ATPase that is enriched in postnatal hair cell bundles (Dumont et al. 2001, Hill et al. 2006, Chen et al. 2012), was more frequent: 50% (27/54) of cochlear bundle-like structures and 73% (58/79) of vestibular bundle-like structures were PMCA2+ (Fig. 2H; Table 2). The PMCA2 immunoreactivity was strongest in the basal portion of the stereocilia (Fig. 2C, 2H), PMCA2 antibody also labeled the basolateral cell membrane, a result that aligns with findings of Chen et al. (2012) (Fig. 2C, 2H).

Other markers: Actin rings near the Atoh1-nGFP+ cells were reminiscent of the rings that typically surround supporting cells (Fig. 2I). This observation suggests that the spheres form a partially organized epithelium during differentiation.

In addition, we stained for markers such as prestin, which is specifically expressed in the outer hair cells (OHCs) of the organ of Corti (OC), and shows increased expression at the onset of hearing (~P12 in mice). Organ of Corti-derived cells showed robust expression of prestin, while the vestibular-derived Atoh1-nGFP+ cells did not (Table 2). 61% (58/95) of Atoh1-nGFP+ cells from the OC were also prestin-positive. Of these prestin cells, 100% were also myosin VIIA-positive (Fig. 2G). In contrast, 0% of the 1302 Atoh1-nGFP+ cells generated from the vestibular organs expressed prestin (Table 2). These data suggest that each inner-ear stem cell population

is strongly biased to differentiate into a specific hair cell type – i.e., that the potency of each tissue is limited to cells specific to that tissue.

To test whether the vestibular spheres had a similar capacity for generating specific cell types, we stained for oncomodulin, a marker of type I hair cells in the vestibular organ (Fig. 2D; Simmons et al. 2010). 10% (8/83) of myosin VIIA+ cells derived from vestibular progenitors stained positive for oncomodulin; this is less than the expected fraction of type I hair cells in the total hair cell population of rodent inner ear organs (~60%, Rüsch et al. 1998). (We did not test cochlear-derived cells for oncomodulin, which is selectively expressed by outer hair cells in cochlear tissue, because we used prestin instead.) We found that 33% (5/15) of Atoh1-nGFP+ cells derived from vestibular tissue stained positively for calretinin, which is expressed in immature hair cells as well as mature type II hair cells (Figure 2E; Table 2; Desai et al. 2005, Li et al. 2008). No cochlear Atoh1-nGFP+ cells stained positively for calretinin (0/26) (Table 2), suggesting that if IHCs were present, as indicated by parvalbumin staining, they were not expressing calretinin at a high enough level for detection (Dechesne et al. 1994).

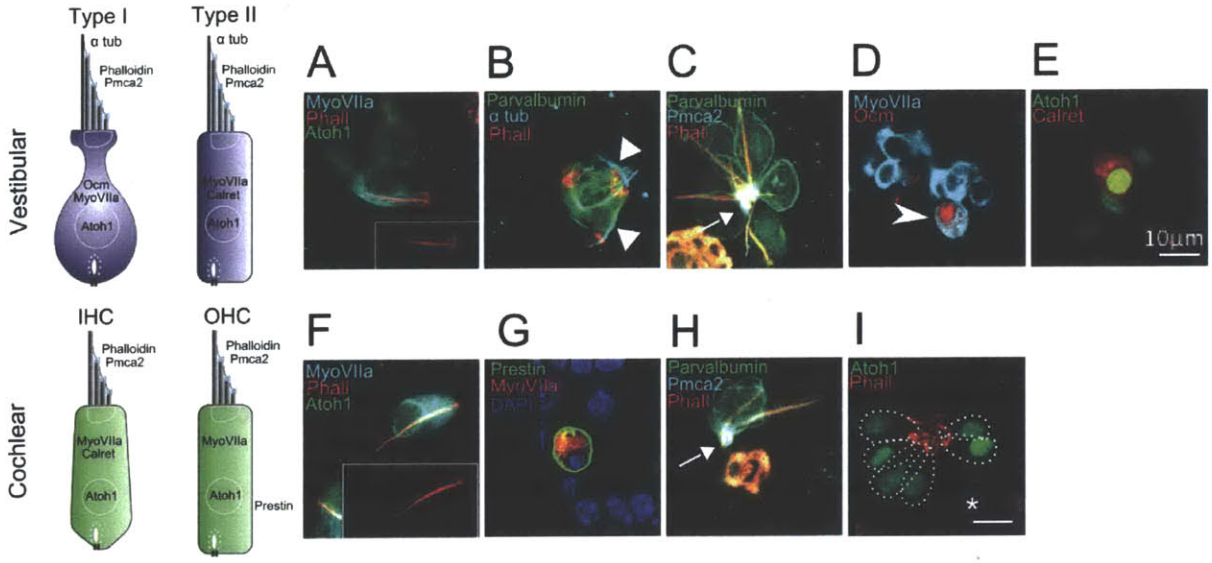

Figure 2: Organ of Corti and vestibular derived stem cells are capable of differentiating to form cells that express hair cell proteins and possess typical hair cell characteristics

A) Vestibular-derived hair cell-like cells possessed actin rich cuticular plates with bundle-like structures emitting from the apical surface. Here, hair cells were recognized by their expression of parvalbumin, a Ca^{2+}-binding protein found in type I hair cells within the vestibular organs.

B) Vestibular cells also possessed kinocilia-like structures, as indicated by α3 acetylated tubulin staining (white triangle).

C) PMCA2 labeled the cell membrane of vestibular cells, but was most robust at the bases portion of the bundle-like structures (arrow).

D) Vestibular-derived cells also expressed oncomodulin (arrowhead), which is found in type I hair cells.

E) Some vestibular-derived hair cell-like cells (recognized by Atoh1 expression, green) also expressed calretinin, a Ca^{2+}-binding protein which is found in immature hair cells and type II hair cells.

F) Cochlear-derived hair cell-like cells (recognized by myosin VIIA expression and Atoh1 expression) had bundle-like structures emanating from the apical surface.

G) Many organ of Corti-derived cells that expressed myosin VIIA (a hair cell marker) also expressed prestin, the electromotility motor specific to outer hair cells. Note the strong expression in the membrane, consistent with outer hair cell expression patterns.

H) PMCA2 antibody labeled the cell membrane of cochlear-derived cells and was robustly expressed at the basal portion of the bundle-like structure (arrow). The hair cells shown here also express parvalbumin, a Ca^{2+}-binding protein found in IHCs within the cochlea.

I) Actin rings were also seen in cochlear-derived spheres. Here they are seen at the apices of a group of Atoh1+ hair cell-like cells (green) and also on other cells (asterisk), indicating an epithelial like fate that is not hair cell.

Table 2: Summary of counts for hair cell protein immunolocalization

	Source			
Gene	**Cochlear cell count**	**%**	**Vestibular cell count**	**%**
Phalloidin (filamentous actin; hair bundles)	690/850 myosin VIIA+ cells	81%	285/423 myosin VIIA+ cells	67%
α3 tubulin (kinocilia on immature and vestibular hair cells)	0/312 myosin VIIA+ cells	0%	9/289 myosin VIIA+ cells	4%
PMCA2 (bundles)	50% 27/54 actin bundles	50%	58/79 actin bundles	73%
Oncomodulin (type I hair cells)	N/A	N/A	8/83 myosin VIIA+ cells	10%
Calretinin (vestibular type II, immature hair cells, IHCs)	0/26 Atoh1-nGFP+ cells	0%	5/15 Atoh1-nGFP+	33%
Prestin (OHC marker)	58/95 Atoh1-nGFP+ cells	61%	0/1302 Atoh1-nGFP+ cells	0%

In summary, immunolocalization showed that many cells in spheres differentiated into possible hair cell-like cells, as indicated by expression of Atoh1, myosin VIIA, or parvalbumin. Of these cells, many had protrusions that express proteins that are prominent in hair bundles: f-actin and PMCA2, but few expressed the kinocilia marker, β3 tubulin. The expression of prestin by a large fraction of hair cell-like cells derived from the organ of Corti but not by any cells derived from vestibular tissue suggests that the progenitor cells in particular organs of the neonatal mouse are not fully competent to form hair cells of all types, but rather have fates

restricted to their inner ear compartment. This may indicate differentiation limitations in each tissue's progenitor cell population.

Expression of hair-cell specific genes

Because the newly generated cells expressed several key hair cell genes and possessed hair cell features, we next used RT-PCR to determine the presence or absence of genes that would provide key machinery for proper hair cell function. The genes we looked for are indicated in Figure 3, where a band indicates that a given gene was being expressed in a particular tissue, and a lack of a band indicates that the gene was not expressed. We chose genes specific for mechanoelectrical transduction, for electromotility (prestin), the presynaptic apparatus (e.g. proteins of synaptic ribbons and vesicles), and the postsynaptic apparatus (e.g. postsynaptic to cholinergic efferent input). We also tested for expression of genes related to supporting cell function, in particular secretion of the extracellular matrices (e.g. otogelin, otopetrin).

Transduction apparatus. We found that differentiated organ of Corti-derived progenitors expressed the vital transduction channel component, Tmc1 but not Tmc2, whereas the vestibular-derived cells expressed both Tmc1 and Tmc2. Expression of these two proteins is different in vivo, as TMC2 is initially expressed in both organs, but disappears in the cochlea near the onset of hearing (Kawashima et al. 2013). Differentiated progenitor cells from both organs also expressed Espn1 and Espn4, the two ESPIN isoforms that are associated with bundle elongation; Espn4 arises postnatally (Fig. 3B)(Sekerková et al. 2004, Sekerková et al.

2006, Sekerková et al. 2006), consistent with the newly generating cells acquiring a certain level of differentiation. Differentiated progenitors from both tissues also expressed myosin 1c, a gene that contributes to setting the resting current in hair cells and is critical to the adaptation response associated with hair bundle deflection (Fig. 2B) (Holt et al. 2002, Gillespie and Cyr 2004, Stauffer et al 2005). The differentiated progenitors from both tissues also expressed Cdh23 and Pchd15, the two components of the hair cell tip-links that bind the stereocilia and are critical to transduction (Fig. 3B)(Siemens et al. 2004, Kazmierczak et al. 2007).

Synaptic transmission. We also found that differentiated progenitors from both tissues expressed genes associated with both afferent and efferent synaptic function. The differentiated cultures produced the cholinergic receptor, Chrna9, which is found in all hair cells and mediates the response to cholinergic terminals from efferent neurons in the brainstem (Elgoyhen et al. 1994, 2001, Luo et al. 1998, Zuo et al. 1999, Simmons and Morley 1998, Morley and Simmons 2002, Simmons 2002). They also produced the glutamate transporter VGLUT3, which fills synaptic vesicles with the excitatory neurotransmitter glutamate for afferent signaling (Fig. 3B) (Wang et al. 2007, Zhang et al. 2007, Seal et al. 2008, Peng et al. 2013). In addition, the differentiated cultures expressed $Ca_V1.3$ calcium channels, which admit calcium ions to initiate glutamate release from hair cells (Fig. 3B) (Platzer et al. 2000, Schnee and Ricci 2003, Brandt et al. 2003, Brandt et al. 2005), which ultimately leads to neurotransmitter release. Similarly, the differentiated cultures expressed ribeye, the portion of the Ctbp2 protein that is highly specific to ribbon synapses (Schmitz et al. 2000). Both differentiated tissues also expressed oncomodulin, a calcium binding protein that is found in type I hair cells within vestibular tissue and outer hair cells of the cochlea (Fig. 3B) (Simmons et al. 2010).

Other. Both differentiated cultures expressed myosin VIIA, a hair cell marker protein. Similar to our staining results, we found that only differentiated cochlear cultures expressed the outer hair cell protein prestin (Fig. 3B). Whereas both differentiated tissues expressed the supporting cell genes otogelin and Lgr5, only vestibular-derived cultures expressed the supporting cell gene otopetrin, which is specific to the vestibular epithelia (Fig. 3B).

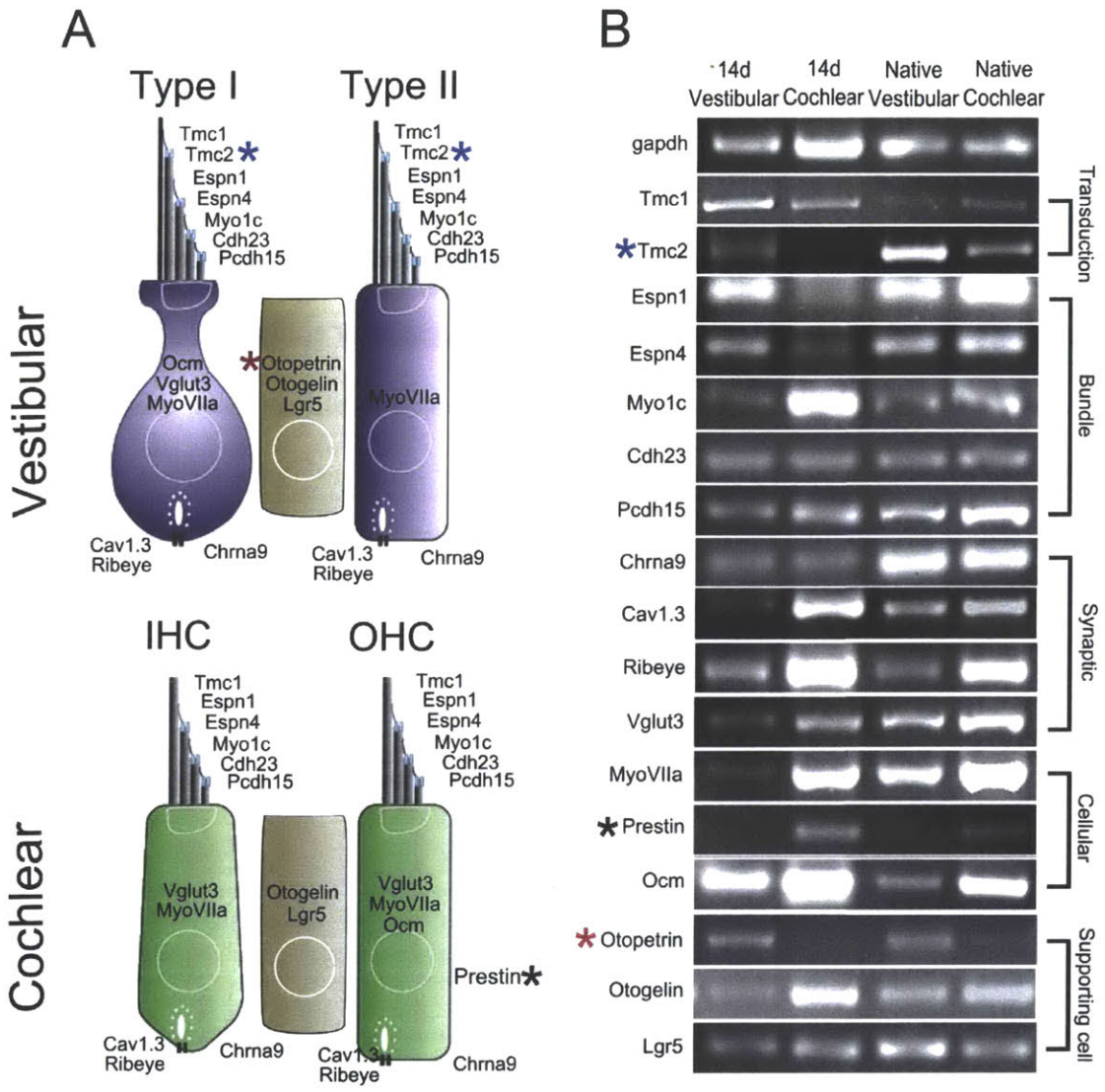

Figure 3: Differentiated organ of Corti and vestibular stem cells express genes that are specific to their derived tissue and are required for proper physiological function

A) A schematic showing the genes that were analyzed and their location of expression within each tissue and cell type.

B) Differentiated cells from both tissues expressed the hair cell gene myosin VIIA, hair bundle associated genes (Espn1, Espn4, Myo1c, Cdh23, Pchd15), genes associated with synaptic function (Chrna9, Cav1.3, ribeye, VGLUT3, Ocm), and the supporting cell genes Otogelin and Lgr5. The differentiated cells from each tissue differed in that only cochlear derived cells expressed Prestin (*) and Tmc1 alone, whereas only vestibular derived tissue expressed otopetrin (*) and the combination of Tmc1 and Tmc2 (*). All results were confirmed with a minimum of n=5 cultures.

In summary, both cochlear and vestibular progenitor cells are capable of differentiating into populations of cells that express transduction channel components (TMC1 and TMC2), hair bundle components (Espn1, Espn4, Myo1c, Cdh23, Pchd15), and genes associated with synaptic function (Chrna9, Cav1.3, ribeye, VGLUT3, Ocm), which may suggest that bonafide hair cells are being formed. Since only cochlear-derived progenitors differentiate to form prestin-expressing cell populations, and only vestibular-derived progenitors differentiate to form otopetrin-expressing cell populations, these results further suggest that progenitors from each tissue are limited to forming their own native cell types.

Voltage-gated currents

We conducted electrophysiological experiments to (1) further test the hypothesis that the newly generated cells form distinct cellular subtypes that are limited to those of their native organ; and (2) determine if these cells could respond to electrical stimuli in a manner similar to functional hair cells. With the patch clamp method, we recorded voltage-dependent whole-cell currents from hair cell-like cells, which we recognized in the recording dish by their expression of Atoh1-nGFP. Most Atoh1-nGFP+ cells produced large outward currents in response to depolarizations, with amplitudes and time courses that are qualitatively within the range of the outward delayed rectifying K^+ current (I_{KD}) of hair cells (Fig. 4A). We observed these currents in 91% (29/32) of Atoh1-nGFP+ cells derived from the cochlea, and in 100% (34/34) of Atoh1-nGFP+ cells derived from vestibular organs (Fig. 5). In 7 Atoh1-nGFP-negative cells tested (6 cochlear, 1 vestibular), none had hair cell-like currents (Figure 4B). In small samples of hair-cell like cells from each tissue, we obtained tail-current activation curves (see Eq. 1 in Methods),

which showed that for both tissues of origin, the outward currents activated around –60 mV and had activation midpoints ($V_{1/2}$ values) that were typical of hair cells within their first postnatal week (Eatock and Hurley 2003). Cochlear $V_{1/2}$ values ranged from –14 mV to –30 mV (mean –21 ± 3 mV, SE, n=3 cells) and vestibular $V_{1/2}$ values ranged from –25 mV to –34 mV (mean= –33 ± 2 mV, n=5 cells). Figure 4C shows a cochlear-derived cell with a $V_{1/2}$ value of –20 mV and a vestibular-derived cell with a $V_{1/2}$ value of –25 mV.

The vestibular-derived cells had currents similar to those found in previous electrophysiological analyses of vestibular type II hair cells (Rüsch & Eatock 1998, Holt et al. 1999) (Fig. 4E). Some cells resembled type II cells from the striolar and central zones of vestibular epithelia in that the outward current did not show fast inactivation and they had a large I_h current (28/32 cells =87%; Fig. 4A, top; Fig. 5). Other cells more closely resembled type II hair cells from the extrastriolar/peripheral epithelial zones in that they had inactivating outward current that resembled A-current (I_A)(4/32 =12%) (Fig. 4D, bottom).

Outward currents in two cochlear cells had "times to half-maximum current" in the same range as previously recorded for neonatal cochlear hair cells (10-20 ms) by Marcotti and Kros (1999, 2003). The outward currents of seven vestibular-derived cells were well fit with monoexponential decay functions with values that fell into two groups. At –45 mV, four cells had relatively slow time constants (49 ± 15 ms; see Figure 4E top, Figure 4F), similar to the delayed rectifier of type I hair cells ($I_{DR,I}$) after the first postnatal week (Rüsch et al. 1998). The other group of cells had a mean time constant of 14.2 ± 1.9 ms (n=3)(Figure 4E bottom, Figure 4F), similar to the delayed rectifier in type II hair cells ($I_{DR,II}$) in the utricular epithelium of postnatal mice (Rüsch et al. 1998).

Vestibular hair cells acquire I_h current postnatally, and the size of the current dramatically increases around P3 –P4 in mouse utricle (Rüsch & Eatock 1998; Horwitz et al. 2010, Horwitz et al. 2011). In our voltage protocol, an I_h -like current was recognized as a slowly increasing inward current during the 150-ms step to –125 mV at the start of each voltage protocol. We observed this I_h -like current in 65% (22/34) of Atoh1-nGFP+ cells derived from vestibular tissue but in 0 of 32 cochlear-derived cells (Fig. 4G; Fig. 5). This result suggests that only vestibular progenitors are capable of becoming vestibular hair cells.

Hair cells also possess an inward-rectifying potassium current known as I_{K1}, which plays a large part in setting a hair cell's resting membrane potential during development (Rüsch et al. 1998, Behrend 1997, Marcotti et al. 1999, 2003). Within the cochlea IHCs express this current from E15-P14, while OHCs express this current from P0-P6 (Marcotti et al. 1999, 2003). Type I vestibular hair cells express I_{K1} up to P4 and likely afterwards, while type II vestibular hair cells express I_{K1} throughout the maturation process (Rüsch et al. 1998). We found that 60% (19/32) Atoh1-nGFP+ vestibular derived cells showed evidence of an I_{K1}-like current, as indicated by the fast inward-rectifying current at the onset of the voltage step and its subsequent deactivation when voltage is stepped to more positive potentials (Fig. 4H; Fig. 5). All hair cell-like cells derived from cochlear tissue lacked evidence of I_{K1} in voltage clamp tests.

The time course of the inward currents evoked by large hyperpolarizing voltage steps could be fit with a double-exponential decay function. For hair-cell like cells from vestibular tissue, 150-ms steps from –65 mV to –125 mV typically elicited inward currents with fast and slow time constants (τ) on the order of 1 ms and 100 ms, respectively: The mean slow τ was 92 ± 21 ms (n=6 cells) (Figure 4H). The mean fast τ was 0.99 ± 0.32 ms (n=5 cells) (Figure

4H). These numbers agree reasonably well with data from vestibular hair cells: The faster time constant we measured is comparable to values reported for I_{K1}, the fast inward rectifier, in early postnatal mouse utricular hair cells by Rüsch et al. (1998). The slower time constant we recorded is comparable to the faster of two time constants for I_h activation in mouse utricular hair cells recorded with much longer voltage steps (Horwitz et al. 2011) - our brief voltage steps did not substantially activate the slower component of I_h activation.

In small samples of the hair-cell like cells, we recorded membrane voltage in current-clamp mode to assess the resting potential and voltage responses to injected current steps for comparison with the literature on hair cell responses (Figure 4I). The resting potentials were similar for Atoh1-nGFP+ cochlear and vestibular cells: –53 ± 8 mV (n=4 cochlear-derived cells) and –53 ± 6 mV (n=6 vestibular-derived cells). These values are set in large measure by the developmental acquisition of K^+-selective inwardly-rectifying and outwardly-rectifying channels (e.g., Eatock and Hurley 2003; Goodyear et al. 2006) and are within the physiological range for postnatal hair cells.

Voltage clamp and current clamp recordings from the vestibular-derived cells lacked clear evidence of the type-I-specific current, $I_{K,L}$ (Rüsch et al. 1998, Holt et al. 1999). Nevertheless, the activation time courses of the delayed rectifiers are consistent with differentiation into type I and type II hair cells (Figure 4E). We also did not see the mixed Ca^{2+}-Na^+ spikes that are typical of mouse inner hair cells in the first postnatal week (Marcotti et al. 2003). All Atoh1-nGFP+ cells lacked the voltage-gated Na^+ currents that are reported in immature hair cell subtypes from both the cochlea and vestibular system (Witt et al. 2004, Oliver et al. 1997, Chabbert et al. 2003, Marcotti et al. 2003, Géléoc et al. 2004, Wooltorton et

al. 2007, Li et al. 2010, Eckrich et al. 2012). A possible explanation is that by 12 days of *in vitro* differentiation, the earliest time that we examined, the hair cell-like cells had advanced beyond the stage of significant Na channel expression.

In all, our results support our hypothesis that the early postnatal inner ear harbors different populations of progenitors with limited differentiation capabilities. We also show that organ-specific progenitors can differentiate to form Atoh1-nGFP+ cells that have many of the electrical characteristics that are necessary for hair cell function.

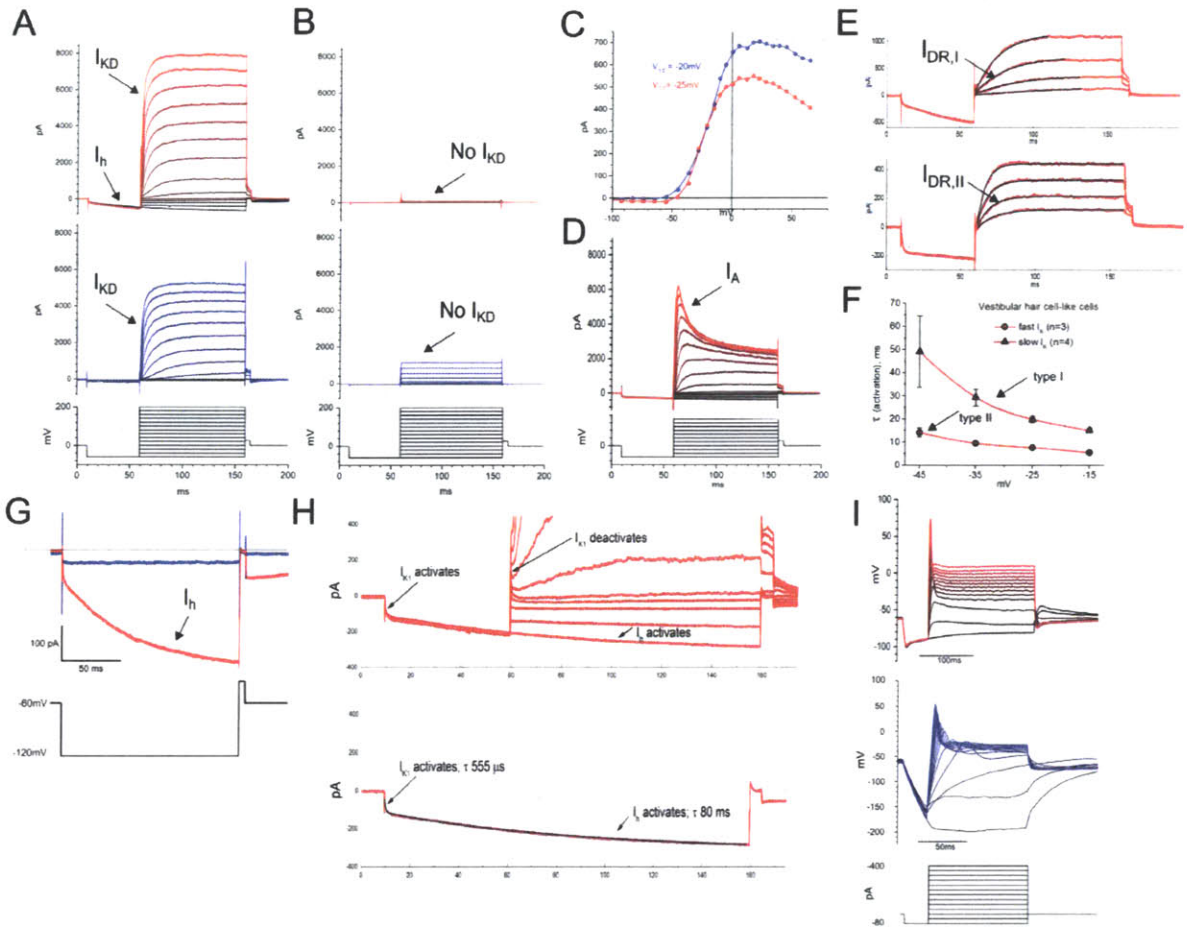

Figure 4: Differentiated organ of Corti and vestibular progenitor cells resemble the hair cells from their native tissues in certain electrophysiological properties.

A) Atoh1-nGFP+ cells derived from both tissues had large voltage-dependent currents. Vestibular-derived cells are in red (top panel) and cochlear-derived cells are in blue (bottom panel). The largest currents were outward currents evoked by depolarizing voltage steps and were reminiscent of the delayed rectifier potassium currents (I_{KD}) found in native hair cells. No sodium currents were detected.

B) Atoh1-nGFP-negative cells did not have currents that resembled those found in native hair cells based on the size and time course of the currents.

C) Tail current activation curves taken at –40 mV. Smooth curves are single-Boltzmann fits (Methods) with midpoints ($V_{1/2}$ values) as given. These are similar to those found in native hair cells within the first postnatal week.

D) Some but not all Atoh1-nGFP+ cells derived from vestibular tissue had inactivating A-current (I_A). This heterogeneity is seen *in vivo* in type II vestibular hair cells.

E) Outward currents from vestibular derived Atoh1-nGFP+ had time courses that resembled the slow type I delayed rectifier ($I_{DR,I}$) or the faster type II delayed rectifier ($I_{DR,II}$).

F) Activation time courses in response to a series of voltage steps revealed that vestibular-derived Atoh1-nGFP+ cells separated into two distinct groups that resembled the time courses seen in native type I and type II hair cells.
G) The vestibular-specific postnatal current I_h was found in 65% of vestibular-derived Atoh1-nGFP+ cells and no cochlear-derived Atoh1-nGFP+ cells.
H) Vestibular hair cells showed evidence of the fast inward rectifier, I_{K1}, in addition to I_h, which activated in response to voltage steps from –65 mV to –125 mV with time constants similar to those reported for native hair cells.
I) Both cochlear-derived and vestibular-derived Atoh1-nGFP+ cells responded to current steps with voltage changes similar to those reported from postnatal hair cells.

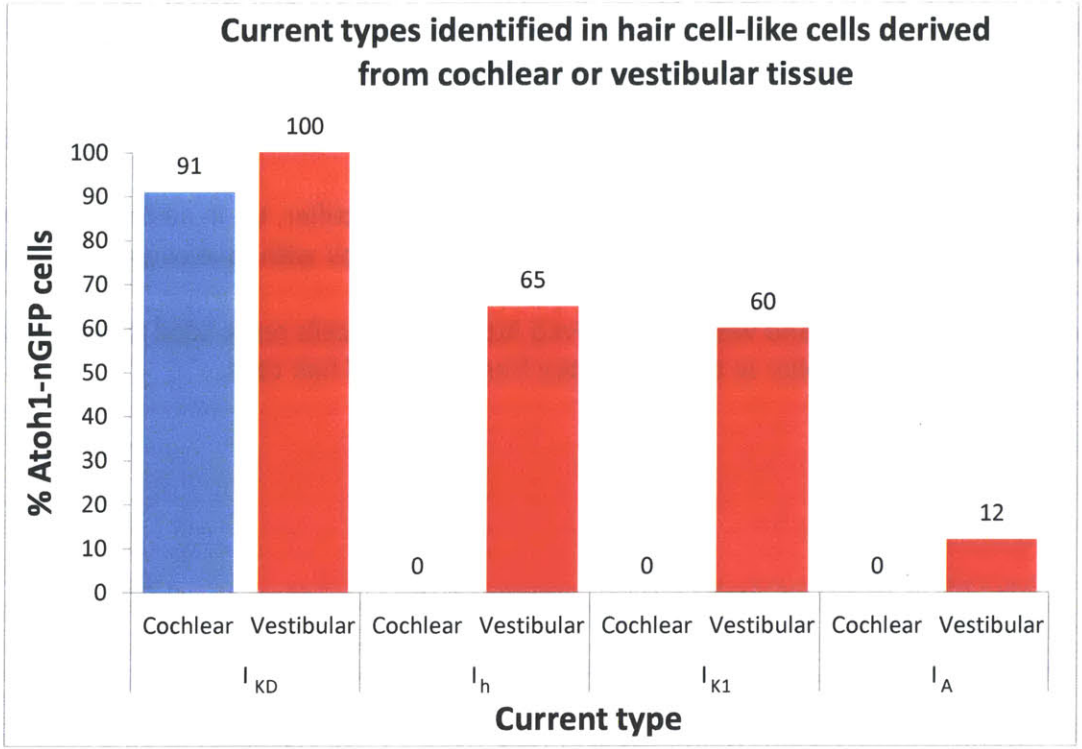

Figure 5: Atoh1-nGFP cells derived from cochlear and vestibular progenitors have different current expression profiles.
I_{KD}) 91% (29/32 Atoh1-nGFP cells) from differentiated cochlear spheres showed evidence of I_{KD}, while 100% (34/34 Atoh1-nGFP cells) from differentiated vestibular cells showed evidence of I_{KD}.
I_h) 0% (0/32 Atoh1-nGFP cells) from differentiated cochlear spheres showed evidence of I_{KD}, while 65% (22/34 Atoh1-nGFP cells) from differentiated vestibular cells showed evidence of I_{KD}.
I_{K1}) 0% (29/32 Atoh1-nGFP cells) from differentiated cochlear spheres showed evidence of I_{KD}, while 60% (19/34 Atoh1-nGFP cells) from differentiated vestibular cells showed evidence of I_{KD}.
I_{KA}) 0% (0/32 Atoh1-nGFP cells) from differentiated cochlear spheres showed evidence of I_{KD}, while 13% (4/34 Atoh1-nGFP cells) from differentiated vestibular cells showed evidence of I_{KD}.

Hair cell and supporting cell progenitors

Since it appeared that hair cell progenitors from each organ were limited in their differentiation capabilities, we next decided to analyze the potency of known hair cell progenitors within the cochlea. Shi et al. (2012) showed that Lgr5-expressing cells within the

cochlea formed hair cells, whereas Lgr5-negative cells did not. Since prior work had shown that progenitor cells within the inner ear could generate neurons and glia (Li et al. 2003, Oshima et al. 2007), we set out to determine if the cells responsible for forming hair cells (Lgr5-positive cells) were also capable of forming neurons.

Lgr5 is known to be expressed in inner border cells, inner pillar cells, and 3rd row Deiter's cells (Fig.5A). By crossing Lgr5-EGFP-IRES-creERT2 mice with floxed-tomato mice we were able to trace the lineage of Lgr5+ cells in neurosphere culture (Fig. 6B). After culture and differentiation, we observed that Lgr5 lineage-traced cells could form hair cell-like cells, as indicated by colocalization of myosin VIIA and td-tomato expression (n=20 cells)(Fig. 6C). To determine if Lgr5+ cells could also form neurons, we stained the differentiated cultures for Tuj, a tubulin that is highly specific to neurons. The lack of Tuj and td-tomato colocalization in any cells (n=696 Tuj+ cells) (Fig. 6D) shows that no Lgr5+ cells differentiated to form neurons.

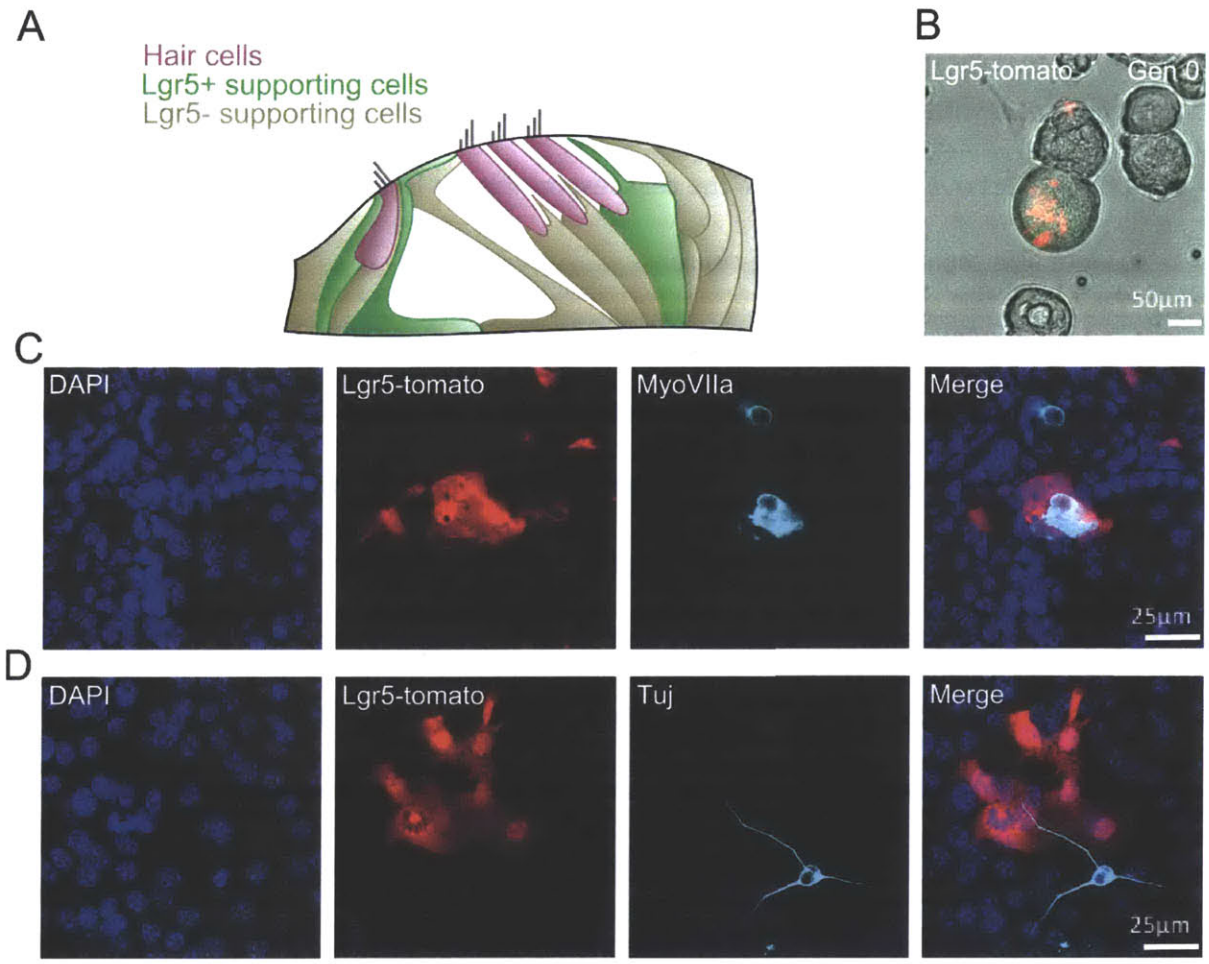

Figure 6: Lgr5+ cells from the postnatal cochlea form hair cells, but not neurons
A) Lgr5 is expressed within the GER, inner border cells, inner pillar cells, and 3rd row Deiter's cells.

B) During initial sphere formation Lgr5-GFP is lost and remains absent throughout the culture process (scale bar, 50 µm).

C) Lineage-traced Lgr5-tomato cells give rise to hair cell-like cells as revealed by MyoVIIA expression.

D) Lgr5+ cells do not form neurons in neurosphere culture. Here Tuj stains a neuron-like cell with processes, and Lgr5+ stains other cells.

Since our results suggested that Lgr5+ cells did not form neurons but previous work had shown that neurons could form from neurosphere culture, we set out to identify an inner ear progenitor that could form neurons. We hypothesized that Schwann cells within the inner ear could be capable of forming neural cell types. Previous work had used lineage tracing studies to show that Schwann cells of the inner ear expressed PLP (Gomez-Casati et al. 2010). Therefore, we conducted lineage tracing of these cells using PLP-Cre-ERT mice crossed with td-tomato mice. We then isolated the modiolar tissue for neurosphere culture to identify td-tomato-positive glial cells among the cells derived from PLP+ progenitor cells (Fig. 7A). The neurospheres contained td-tomato-positive cells showing their derivation from PLP-expressing cells (Fig. 7B).

After differentiation, we stained the cultures for highly specific markers of neurons and central nervous system (CNS) glia to determine if PLP+ cells could form neurons and also glia from outside their native lineage. Our results indicated that PLP+ glial cells formed neurons after going through neurosphere culture, as indicated by co-staining with Tuj and td-tomato (n=727 cells) (Fig. 7C). We also found that PLP+ Schwann cells could form oligodendrocyte-like cells, as indicated by positive staining of td-tomato cells for the early-stage oligodendrocyte marker O4, which labels sulfated galactosylcerebroside on non-myelinating oligodendrocytes (n=1299 cells)(Fig. 7D) (Sommer et al. 1980, Sommer et al. 1982). PLP-expressing cells derived from modiolar tissue could also form more mature oligodendrocytes, as indicated by co-staining of the td-tomato cells with O1, a galactocerebroside antigen found on terminally differentiated oligodendrocytes that are capable of myelination (n=1286 cells)(Fig. 7E) (Sommer et al. 1980, Sommer et al. 1982). Lastly, we found that PLP-expressing cells could also form

astrocytes as indicated by co-staining of td-tomato cells with Aldh1L1, an aldehyde dehydrogenase that is highly specific to astrocytes within the nervous system (n=531 cells)(Fig. 7F) (Cahoy et al. 2008). These lineage tracing assays reveal that Schwann cells within the modiolar tissue can form CNS cell types. In addition, no myosin VIIA-expressing cells were seen after differentiation of these cultures (n=5 cultures), suggesting that the PLP+ cells do not differentiate into hair cell-like cells.

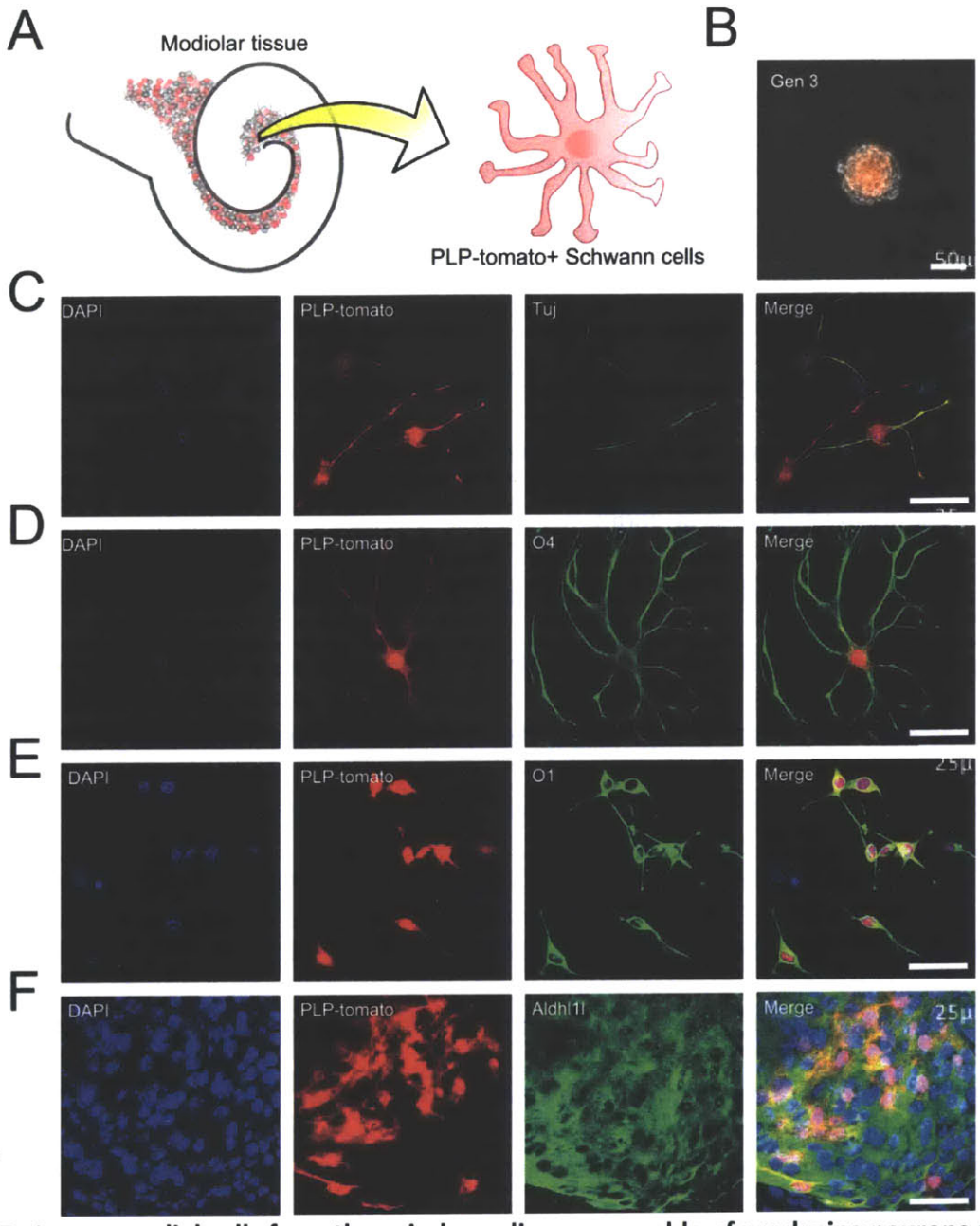

Figure 7: Inner ear glial cells from the spiral ganglion are capable of producing neurons and CNS glia.

A) Glial cells modiolar tissue were lineage traced using the PLP-Cre-ERT mouse, allowing us to trace the fate of these cells through culture.

B) Glial cells (revealed by PLP-td-Tomato staining) were maintained throughout passaging.

C-F) Inner ear glial cells, labeled with td-tomato, were capable of giving rise to:

C) neuron-like cells, as indicated by co-staining for neuron-specific tubulin, tuj;

D) immature non-myelinating oligodendrocytes, as indicated by co-staining for O4;

E) mature myelinating oligodendrocytes, as indicated by co-staining for O1;

F) astrocytes, as indicated by co-staining for Aldhl1l.

Discussion

A previous study suggested that inner ear progenitors are pluripotent (Li et al. 2003). It has also been shown that isolated inner ear progenitor cells can give rise to hair cell-like cells *in vitro*, as indicated by immunostaining for typical hair cell marker proteins such as Atoh1, myosin VIIA, and espin (Oshima et al., 2007; Martinez-Monedero et al., 2008). When whole-cell recordings were made from hair cell-like cells derived from utricular progenitors, it was determined that they most closely resembled immature vestibular hair cells at the late embryonic stage (Oshima et al. 2006).

Although hair cells and neurons had previously been created from postnatal inner ear progenitors that had gone through neurosphere culture (Li et al. 2003, Oshima et al., 2007, Martinez-Monedero et al., 2008, Martinez-Monedero 2007), these studies did not follow the fate of particular progenitor populations and could not determine whether hair cells and neural cells arose from a common progenitor cell. Thus, previous work was not able to define the potency of the three different progenitor sources within the inner ear. Furthermore, it was not known whether the hair cell-like cells that were produced were similar to native cells from the tissue source, if the cells tended to become a recognizable hair cell type (e.g. outer hair cells), or if progenitors from a given organ could form both their native and non-native hair cell types.

Our results suggest that postnatal inner ear progenitors may have a differentiation capacity limited to the hair cells and supporting cells of their native organ after passive differentiation. Based on highly specific characteristics such as prestin expression, otopetrin expression, and I_h current to identify the resulting inner ear epithelial cell types (Figures 2-5), our results suggest that different hair cell and supporting cell subtypes develop from neonatal

progenitors generated from different organs. Furthermore, individual tissues could give rise to multiple sub-types found in the native tissue, such as outer hair cells (Fig. 2,3) striolar and extrastriolar type II hair cells (Fig. 4), or neurons and glia (Fig. 7), although we did not determine if these different subtypes arise from specific subsets of progenitors, e.g. different Lgr5 populations (3rd Deiter, inner boarder, inner pillar) and different zones in the vestibular epithelia (striola vs. extrastriola).

We further expanded on previous work by showing the expression of many genes that are necessary for proper function in differentiated cell types. In addition to immunostaining for functional genes and morphological characteristics, we showed that the newly created cells expressed several transduction-associated genes, bundle-associated genes, synaptic genes, and other hair cell and supporting cell genes. Interestingly, differentiated cochlear cells showed robust prestin expression, a characteristic that is found in outer hair cells near the onset of hearing (~p12)(Belyantseva et al 2000). Similarly, differentiated cochlear spheres only expressed the transduction channel-associated gene TMC1, while vestibular spheres expressed both TMC1 and TMC2 (Fig. 3). This resembles the expression pattern that is typically seen in more mature hair cells within native tissue (Kawashima et al. 2011, Pan et al 2013), and may further support the hypothesis that progenitors from each tissue are limited to their native cell types. In addition to expressing genes that are implicated in physiological function, we also observed that the newly created hair cell-like cells were capable of acquiring the physiological behavior of hair cells, such as resting potentials around –60mV, large outward currents likely carried by K^+, and other currents such as I_{KA}, I_h, and I_{K1} in vestibular cells (Fig. 4). Although cells that were differentiated for less than 12 days were not analyzed, these gene-expression and

physiological results were consistent with native hair cells in the later stage of development (i.e. postnatal or later). It is possible that immature hair cell characteristics (e.g. Na^+ and Ca^{2+} spiking in all hair cell candidates and TMC2 expression in differentiated cochlear cells) would have been observed during earlier stages of differentiation; we were unable to test this hypothesis because at early stages very few cells were Atoh1-nGFP+. We looked at whether increasing differentiation time (12-70 days in vitro) advanced the hair cell-like cells' electrophysiological characteristics, but the small number of cells tested at each stage precluded a definitive answer. A summary of our findings is provided in Table 3.

Table 3: Summary of findings

	Source		
	Cochlear progenitors	**Vestibular progenitors**	**Auditory nerve progenitors**
Differentiation Capabilities	Limited to cochlear cell types	Limited to vestibular cell types	Capable of forming neurons and glia but no indication of hair-cell like cells
Methods used	Immunostaining, RT-PCR, patch clamp recordings, Lgr5+ lineage tracing	Immunostaining, RT-PCR, patch clamp recordings	Immunostaining, PLP+ lineage tracing
Key findings	*Prestin expression, no otopetrin expression. *Expression of key transduction, hair bundle, and synaptic genes. *Hair cell-like currents that are typical of cochlear hair cells ($I_{K,DR}$, No I_h, No I_{Na}, No spontaneous or evoked spikes). *Resting potentials and current clamp responses typical of postnatal hair cells. *Lgr5+ cells form hair cell-like cells, but not neurons.	*No prestin expression, otopetrin expression. *Expression of key transduction, hair bundle, and synaptic genes. *Hair cell-like currents that are typical of mature or semi-mature vestibular hair cells (multiple $I_{K,DR}$ types; I_h; No I_{Na}, No spikes). *Resting potentials and current clamp responses typical of postnatal hair cells.	PLP+ glial cells can form: *neurons, as indicated by Tuj staining. *immature oligodendrocytes (O4 staining). *mature oligodendrocytes (O1 staining). *astrocytes (Aldhl1l staining).

Previous work using viral transduction or forced expression of Atoh1 differs from the results we obtained via passive differentiation. Previous publications have shown that transducing supporting cells with an Atoh1 adenovirus can make them form hair cell-like cells. Physiological analysis has shown that they acquire vestibular phenotypes, such as an I_h current and kinocilia (Kawamoto et al 2003, Izumikawa et al 2005, Gubbels et al. 2008, Huang et al. 2009, Han et al. 2010, Yang J et al. 2012, Yang SM et al. 2012, Yang J et al. 2013). Although there is no indication that cochlear hair cells express I_h at any point during development (Horwitz et al. 2010, 2011), Atoh1-transduced I_h-expressing hair cell-like cells are sometimes referred to as primitive or primordial, possibly because they do not resemble mature cochlear hair cells. A potential reason that our results do not also show a primordial behavior is that our methods rely on passive signaling, such as Notch lateral-inhibition and innate Wnt signaling, to influence the expression of Atoh1, rather than artificial up-regulation of the gene.

In our experiments, Atoh1-nGFP fluorescence was more robust in vestibular hair cell-like cells than cochlear hair cell-like cells, possibly indicating a higher level of Atoh1 expression (data not shown) and raising the question of whether Atoh1 level helps determine the type of hair cell that forms. Furthermore, the fact that in our conditions a given inner ear organ gives rise to its own hair cell subtypes may suggest that endogenous signaling within the progenitor cell population of each organ affects differentiation capabilities. It is likely that hair cell fate is not determined by one factor alone, but perhaps the interaction of several factors. For instance, previous work has shown that the relative Lgr5 expression differs between supporting cell types (Chai et al. 2011, Shi et al. 2012). Lgr5 is a Wnt-associated gene (Van der Flier et al. 2007), and the Wnt/β-catenin pathway is critical for hair cell development (Shi et al. 2013, 2014).

Furthermore, the Wnt pathway interacts with the Notch pathway in development (Collu et al. 2014). Whereas previous work focused only on the consequences of Wnt activation (Shi et al. 2013, Shi et al. 2013) or Notch inhibition (Bermingham et al. 1999; Zheng & Gao 2000; Woods et al. 2004, Izumikawa et al. 2005, Mizutari et al. 2013) for hair cell development, perhaps the interplay of these pathways regulates the level of Atoh1 expression (in addition to other genes) to help determine hair cell fate. Therefore, bypassing key components of these developmental pathways via viral transduction may disrupt any innate level-control mechanisms, and may be one explanation for the generation of "primordial hair cells" from inner ear supporting cells in that condition.

The up-regulation of β-catenin can lead to the formation of additional hair cells, possibly via its interaction with Atoh1 (Shi et al. 2013, Shi et al. 2014). My results with Lgr5+ cells agree with Cheng et al. (2011) and Shi et al. (2012) in showing that Lgr5-expressing cells of the cochlea are indeed hair cell progenitors. In addition, we determined that the progenitors that form hair cells and the progenitors that form neurons must in fact be different cell types, as no Lgr5 lineage-traced cells went on to form neural cell types (Fig. 6). This result suggests that while tissues can be pluripotent, individual cells may not be.

While previous reports described that inner ear progenitors could revert back to a neural stem cell fate while in neurosphere culture, it was not known whether all progenitors held this ability or if it was limited to a particular population of cells (Malgrange et al. 2002, Oshima et al. 2007). Although this prior work also showed that both neurons and glia could be created from inner ear progenitors, it did not show whether the differentiated cells included bona fide CNS cell types and whether they progressed in maturation. We build on their

observations to show that PLP-expressing glial cells from the inner ear are capable of forming neurons, oligodendrocytes, and astrocytes, all of which are outside the otic lineage. Furthermore, we showed that PLP+ Schwann cells did not give rise to hair cells (Fig. 6). In creating CNS cell types from peripheral glia, our results are similar to previous work showing that neural crest stem cell-like cells isolated from embryonic and neonatal dorsal root ganglia could give rise to CNS cell types after neurosphere culture (Binder et al. 2011).

Why the PLP+ Schwann cells that differentiated from modiolar tissue adopted a CNS fate is unclear. Early studies on chick-quail chimeras showed that glia of the inner ear originate from neural crest cells (Britsch et al. 2001, Breuskin et al. 2010, D'Amico-Martel and Noden 1983). In addition, Freyer et al. (2011) showed that neural crest cells can contribute to forming the otic vesicle during development. These neural crest cells were then shown to form neurons, sensory cells, and supporting cells within the inner ear of mice. In older rodents, it is well documented that Schwann cells myelinate the peripheral axons of the spiral ganglion neurons, cell bodies, and initial portion of the central axons (Spoendlin 1985, Toesca 1996, Hurley et al. 2007). CNS glia, such as oligodendrocytes and astrocytes, myelinate the central axons starting at the peripheral-central glia transition zone (the *glia limitans)*, and terminate at the central synapses within the cochlear nucleus (Spoendlin 1985, Valderrama-Canales et al. 1993, Jalenques et al. 1995, Toesca 1996, Moore and Linthicum 2001, Hurley et al. 2007). It would be interesting to know if there is a relationship between neural crest origin and the adoption of a CNS fate by some progenitor cells that we derived from modiolar tissue. However, if the neural crest's developmental contribution to modiolar cells, supporting cells, and hair cells (Freyer et al. 2011) was the only cause for the adoption of neural characteristics

upon differentiation, one would expect some of the Lgr5+ supporting cells (inner boarder cells, inner pillar calls, and 3rd Deiter cells) and perhaps even hair cells to also have the ability to form CNS glia and neurons, which our data indicates is not the case.

Since our work suggests that progenitors from modiolar tissue give rise to multiple cell types while hair cell progenitors are limited to fates found within their native organs, and that hair cell progenitors and neural progenitors are two distinctively different populations of cells, it is likely that the cells from each population differ in their fate plasticity in response to neurosphere culture. Similar to Shi et al. (2012), we found that Lgr5+ progenitors within the cochlea produce hair cells and supporting cells. However, we found that the Lgr5+ cells did not give rise to neural cells, vestibular hair cell-like cells, or vestibular supporting cells (Fig. 8A). Similarly, vestibular progenitors only gave rise to vestibular hair cell-like cells and supporting cells, and not cochlear cell types (Fig. 8B). Lastly, we found that Schwann cells of the ganglia could give rise to neurons and CNS glia after neurosphere culture (Fig. 8C). In all, this work sheds light on the potency and differentiation capabilities of different progenitor populations within the inner ear, which may aid in targeted differentiation of hair cell or neural cell types for therapeutic purposes. Our results highlight the need for further investigation into the potency and differentiation capabilities of specific subsets of Lgr5+ and PLP+ progenitors.

Future Directions. Although this work clarifies the abilities of various progenitor cells to form hair cells and neural cell types, many questions remain. Our work confirms the findings of Chai et al. (2011) and Shi et al. (2012) that Lgr5+ cells are hair cell progenitors within the cochlea. It is not known whether Lgr5+ cells are hair cell precursors within the vestibular organs, where Lgr5 is expressed at very low level, or whether another Wnt-responsive gene or

BMI1, a gene implicated generally in self-renewal in oncogenesis and development and reported to compensate for loss of Lgr5+ cells in gut regeneration (Yan et al. 2012), is responsible for this role. Whereas this work reveals that PLP-expressing Schwann cells can form neurons and CNS glial cell types, it does not rule out that other inner ear glia may also hold this potential. Further work should be performed to determine if the satellite cells that surround neuronal cell bodies (as opposed to the Schwann cells on axons) also possess the capability to form cell types from outside their lineage.

As in previous studies, in our experiments hair cell-like cells were difficult to generate *in vitro*; yields were approximately 32 Atoh1-nGFP+ cells per experiment from cochlear tissue and 325 Atoh1-nGFP+ cells per experiment from vestibular tissue, with each experiment using tissue from, on average, 7 pups. Future work could explore the possibilities of manipulating pathways that may be involved in Lgr5+ progenitor cell expansion in order to enrich for hair cell candidates. Similarly, experiments can now be conducted to isolate pure populations of PLP+ Schwann cells to explore signaling manipulations that might promote large scale expansion of these cells, and enrich for either neurons, oligodendrocytes, astrocytes, or CNS glia upon differentiation.

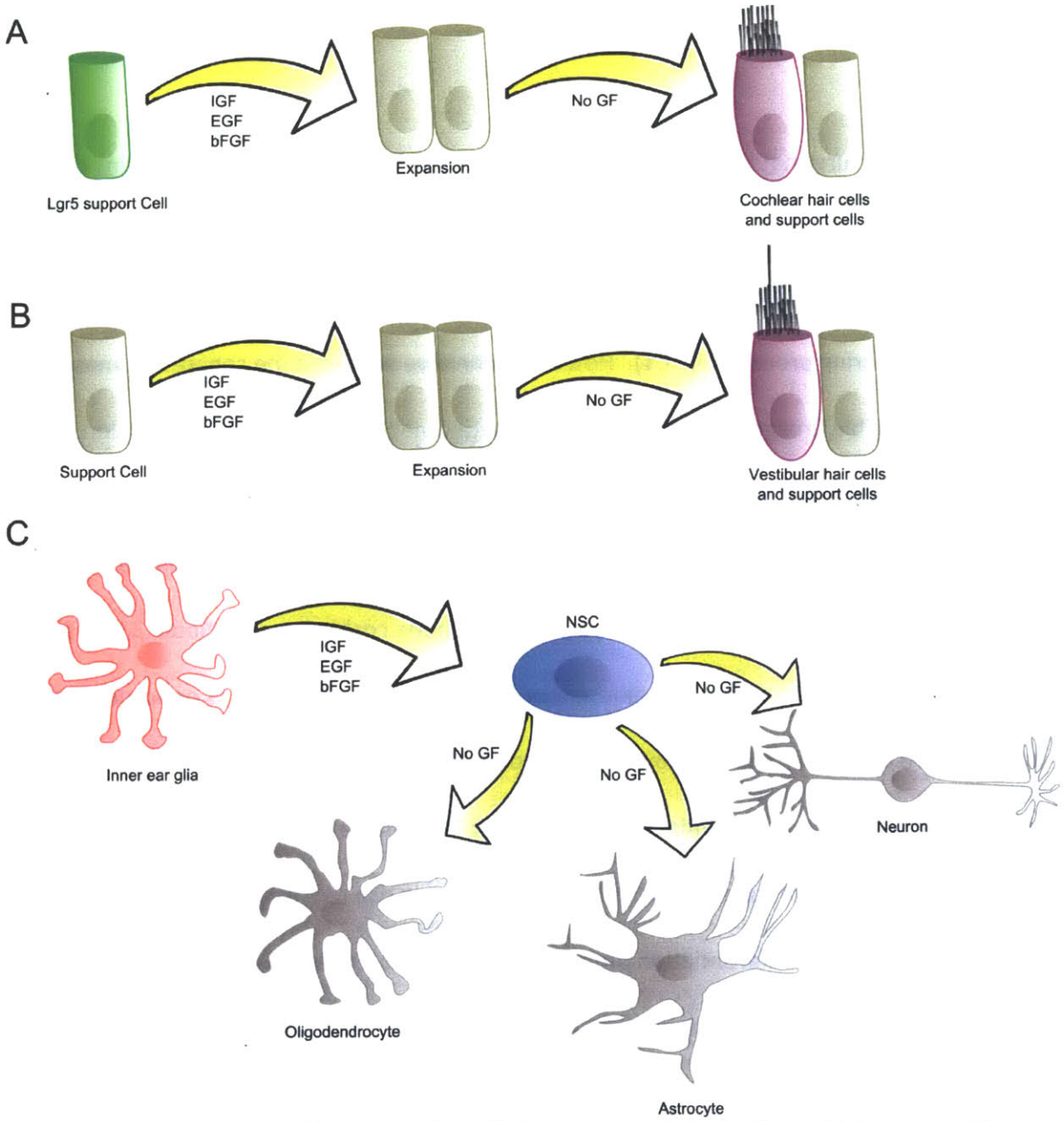

Figure 8: Inner ear stem cell sources show different potency and differentiation capabilities
A) Cochlear supporting cells can give rise to cochlear hair cells and supporting cells but not neurons.
B) Postnatal vestibular supporting cells can give rise to vestibular hair cells and supporting cells.
C) Postnatal glial cells of the inner ear can reset to a more potent state, where they can become neurons and CNS glia.

Appendix

Controls

To make sure that hair cells were not carried over from native inner ear organs during the trituration/culturing process, we performed a series of control experiments. After one passage, we found that a substantial number of hair cells were carried over from the trituration/culturing process (Fig. C1). However, when we passaged the spheres 3 times, carry-over was negligible (images not shown). RT-PCR experiments compared the expression of the generic hair cell marker myosin VIIA in native inner ear tissue and floating spheres that were passaged 3 times. The spheres tested negative for myosin VIIA (Fig C2). To quantify cell numbers carried over, we passaged the cells from both the cochlea and vestibule 3 times, and only allowed them to adhere in the differentiation conditions for 3 hours, as opposed to the typical 14-70 days. This should allow the cells to solidly attach, but should not be sufficient for differentiation to take place. Indeed, when we quantified these results we found that the cochlear spheres that were differentiated for 3 hours gave rise to 0 Atoh1-nGFP expressing cells, while 14 days of differentiation produced an average of 32 Atoh1-nGFP+ cells per experiment (Fig. C3). The 3 hour differentiation of vestibular spheres produced 1 Atoh1-nGFP+ cell in a total of two experiments, which is substantially different than the average of 325.5 ± 107 Atoh1-nGFP+ cells (n=4 cultures) that we obtained following 14 days of differentiation (Fig. C3). In all, these results indicate that our results are due to new hair cell-like cells and not native cells that survived the culturing process.

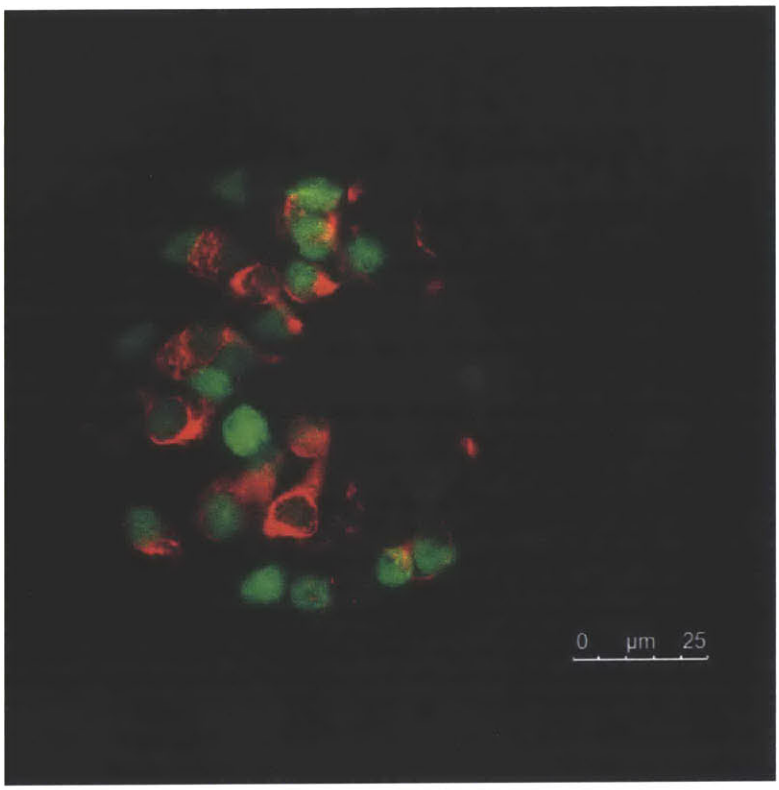

Figure C1: Spheres generated after 1 passage (1st generation) have substantial hair cell carry over, as shown by the Atoh1-nGFP+ nuclei (green) and myosin VIIA expression (red).

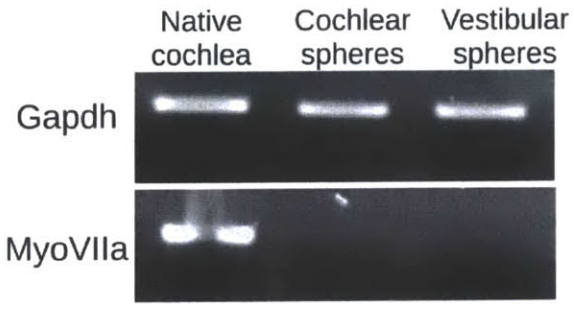

Figure C2: PCR results for myosin VIIA in native inner ear tissue versus progenitor spheres taken directly from culture (repeated in n=3 cultures). The lack of myosin VIIA in the acutely prepared spheres indicates that hair cells were absent immediately after sphere preparation and before differentiation.

3rd Generation Contro			
	Total Atoh1+	Number of Experiments	Average Atoh1+ cells per experiment
Cochlea (14d diff)	95	3	31.6 ±12.2 (SE)
Cochlea (3hr diff)	0	2	0
Vestibule (14d diff)	1302	4	325 ±107 (SE)
Vestibule (3hr diff)	1	2	.5

Figure C3: Gene identification results from differentiated spheres are due to newly created hair cell like cells, and not hair cells being carried over through the stem cell isolation and culture process. The number of cells at 3 hours is the number carried over; at 14 hours, new cells have formed. Stem cells passaged 3 times showed no carry- over for the cochlea, and only one cell was carried over for the vestibular organs in all of the experiments. This is in strong contrast to the average of 32 cells generated per experiment for the cochlea and 325 cells generated per experiment for the vestibular organs.

PLP-lineage tracing specificity

In order to verify our PLP lineage tracing results, we must show that PLP is not expressed in neurons within the modiolus. In order to do this we followed the above lineage tracing protocol, isolated both cochlear and modiolar tissue, then stained the modiolar and cochlear tissue for neuronal and/or hair cell markers. Low magnification confocal microscopy revealed that Tuj+ and PLP-tomato lineage traced cells were located throughout the entire modiolar tissue (Fig. C4). Higher magnification confirmed that PLP+ Schwann cells and Tuj+ neurons were two distinct populations of cells (Fig. C5). Isolated cochlear tissue revealed that Tuj+ neurons extended to the inner and outer hair cell regions, while PLP+ glia extended to the *habenula perforata*, which coincides with the typical myelination patter within the cochlea (Fig. C6).

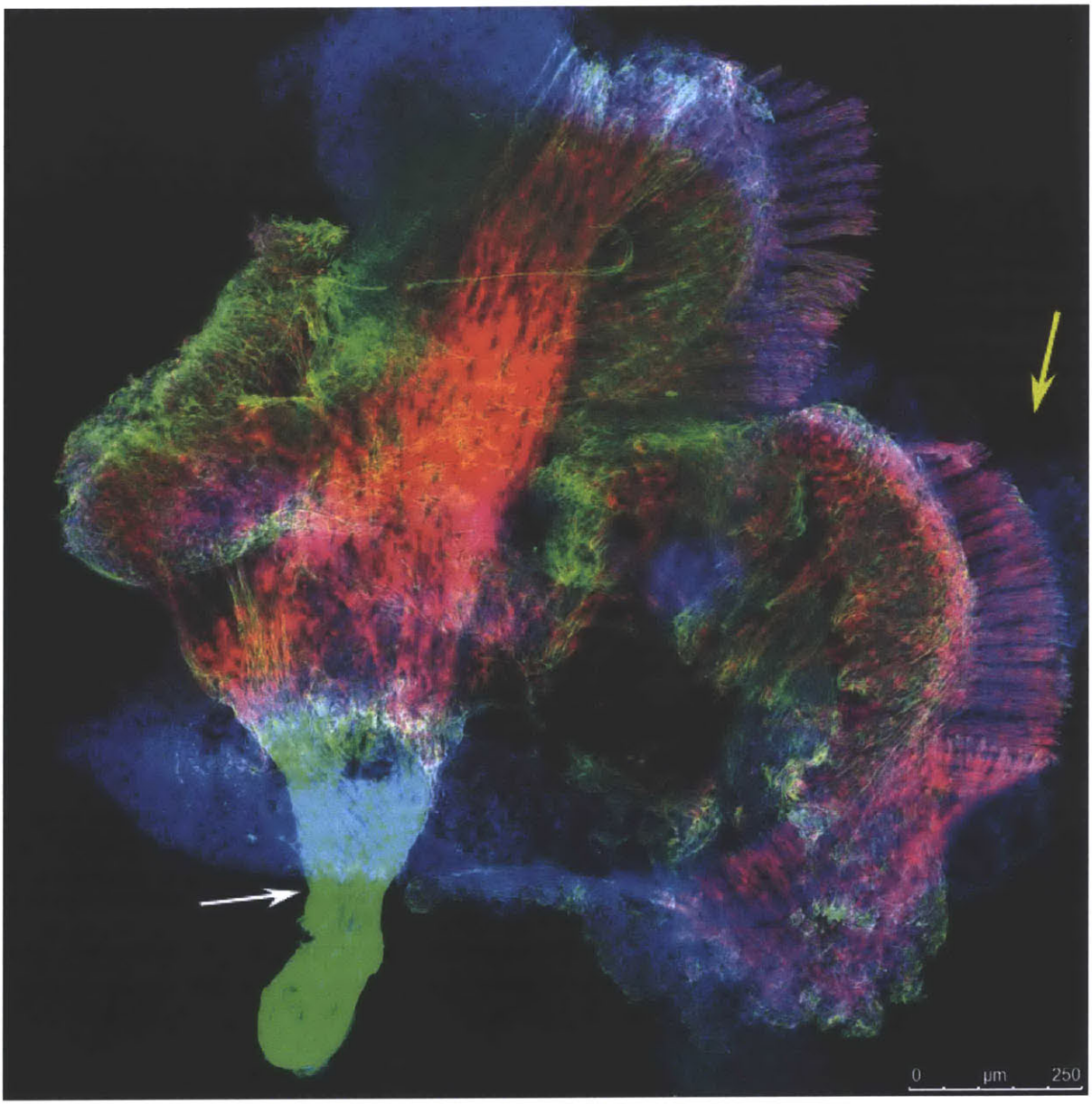

Figure C4: PLP+ Schwann cells and Tuj+ neurons are expressed throughout the cochlear modiolar tissue. Isolated modiolar tissue from PLP-lineage traced mice reveals that PLP+ glia (red) extend throughout the tissue but do not appear to co-localize with neurons stained with Tuj (green). Nuclei are stained with DAPI (blue). The white arrow indicates the trunk of the modiolus, which extends upward. The yellow arrow indicates where the organ of Corti would be, which spirals around this structure.

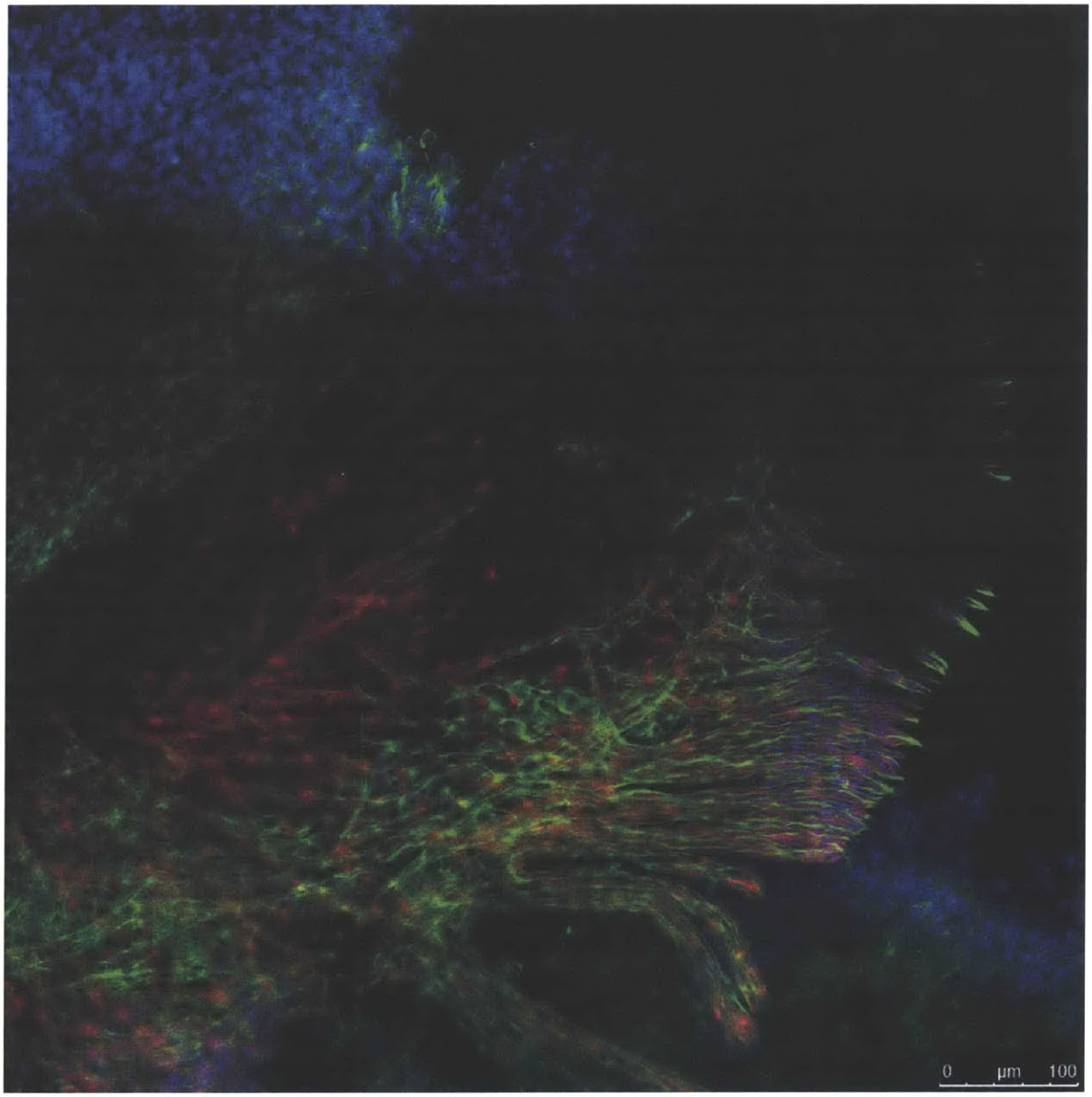

Figure C5: PLP+ cells and Tuj+ cells are two distinct populations of cells within the modiolar tissue. Higher magnification images reveal that Tuj+ neurons (green) and PLP+ Schwann cells (red) are distinct populations of cells within modiolar tissue and do not colocalize. Myelinating PLP+ cells and Tuj+ neurons are intertwined. Scale bar 100µm.

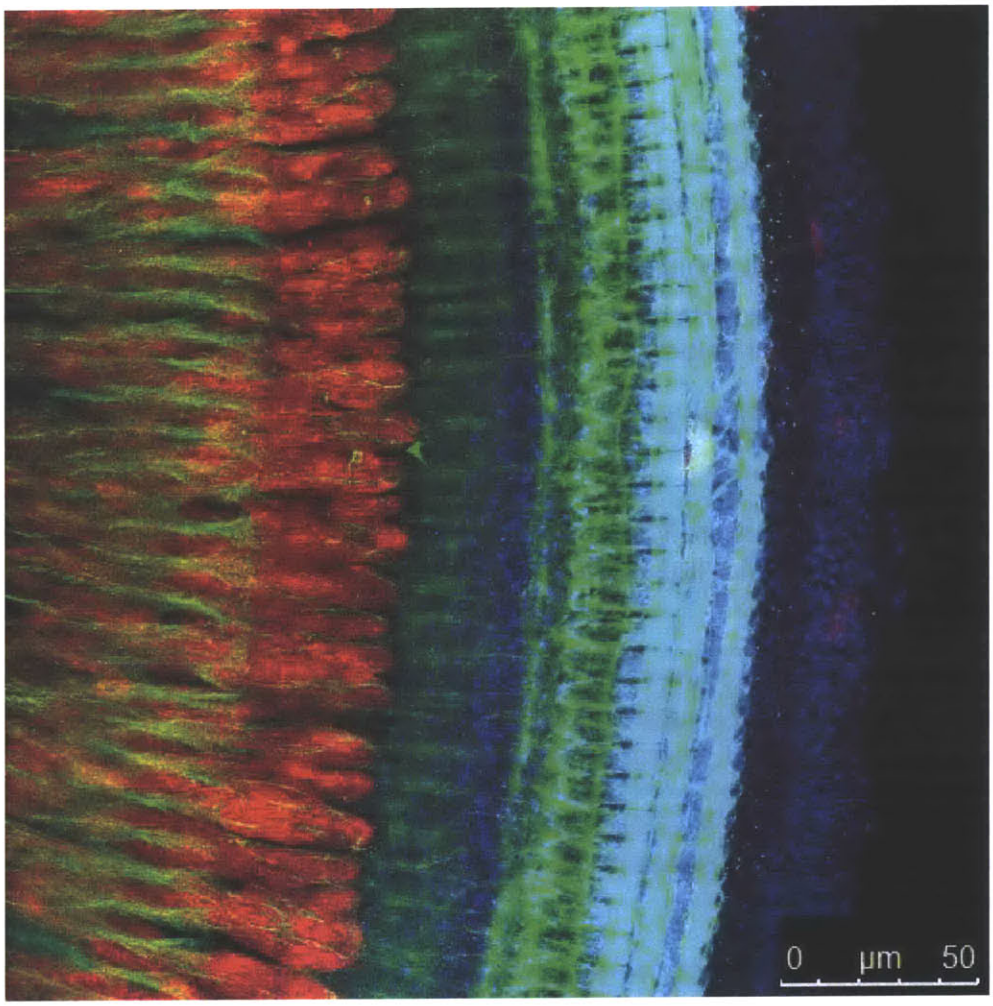

Figure C6: PLP-expression within the cochlea. PLP+ glial cells terminate at the *habenula perforata* within the cochlea, while Tuj+ neurons (green) extend to both the inner and outer hair cell regions (cyan).

Acknowledgments

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