Distinct capacity for differentiation to inner ear cell types by progenitor cells of the cochlea and vestibular organs

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Summary statement

Progenitor cells isolated from the cochlear and vestibular sensory epithelia and the spiral ganglion, showed distinct capacity for differentiation, acquiring phenotypic properties of their respective compartments of origin.
Abstract
Disorders of hearing and balance are most commonly associated with damage to cochlear and vestibular hair cells or neurons. Although these cells are not capable of spontaneous regeneration, progenitor cells in the hearing and balance organs of the neonatal mammalian inner ear have the capacity to generate new hair cells after damage. To investigate whether these cells were restricted in their differentiation capacity, we assessed the phenotypes of differentiated progenitor cells isolated from three compartments of the inner ear - the vestibular and cochlear sensory epithelia and the spiral ganglion - by measuring electrophysiological properties and gene expression. Lgr5+ progenitor cells from the sensory epithelia gave rise to hair cell-like cells, but not neurons or glial cells. Newly created hair cell-like cells had hair bundle proteins, synaptic proteins, and membrane proteins characteristic of the compartment of origin. PLP+ glial cells from the spiral ganglion were identified as neural progenitors, which gave rise to neurons, astrocytes, and oligodendrocytes, but not hair cells. Thus, distinct progenitor populations from the neonatal inner ear differentiate to cell types associated with their organ of origin.

Introduction
Impaired hearing (15% of the population) and balance (35%) correlating with a risk of falling and severely reduced quality of life (Agrawal et al., 2009) most commonly reflect sensory hair cell (HC) loss (Wong and Ryan, 2015) or auditory synapse degeneration (Kujawa and Liberman, 2009; Spoendlin, 1975). The permanence of hearing loss is likely related to the lack of regenerative capacity in the adult cochlea, where HC damage is not followed by differentiation of new cells. However, recent demonstrations of spontaneous HC regeneration in neonatal cochlea suggest the continued presence of progenitor cells (Bramhall et al., 2014; Cox et al., 2014). Replacement of HCs in the neonate is achieved primarily by transdifferentiation of supporting cells and not by proliferation of progenitor cells. A low level of regeneration of vestibular HCs has also been observed but decreases with age (Burns et al., 2012) and is not sufficient to compensate for loss of HCs (Burns et al., 2012; Rauch et al., 2001).

Neural progenitors that give rise to different brain regions during development are initially broadly specified and then become increasingly restricted to specific fates corresponding to location, as has been shown by the maintenance of regional phenotypes by cells cultured as neurospheres (Hitoshi et al., 2002). In a similar process, early embryonic progenitors in the otic placode are specified to make the hearing and balance organs of the inner ear - the cochlea and vestibular organs - with specialized neurons, sensory HCs, and supporting cells. This occurs through a well-choreographed series of steps involving both innate
genetic programs and inductive cues (Barald and Kelley, 2004; Groves et al., 2013; Raft and Groves, 2015). In previous studies, cells isolated from cochlear, vestibular, and neural inner ear compartments in the early postnatal mouse formed spheres that were thought to be multipotent, i.e. capable of generating neurons, HCs, and glia (Li et al., 2003; Malgrange et al., 2002; Martinez-Monedero et al., 2008; Oshima et al., 2007) or were even reported to be pluripotent, i.e., capable of forming all cell types in the organism (Li et al., 2003). The putative HCs expressed electrophysiological properties of embryonic HCs (Oshima et al., 2007) and the putative neurons were glutamatergic and had properties of cochlear afferent neurons (Martinez-Monedero et al., 2008). The cells within the spheres, however, whether generated from cochlear, vestibular, or spiral ganglion compartments of the inner ear, were heterogeneous, and it was not possible to determine whether single progenitor cells gave rise to multiple differentiated types or whether several progenitor populations existed within a given inner ear compartment.

Evidence from the intact cochlear epithelium (organ of Corti, OC) suggests that postnatal supporting cells are not uniform. A subset of supporting cells in the early postnatal mouse OC expresses Lgr5 (Chai et al., 2012; Shi et al., 2012), a marker of stem cells of the intestinal epithelium (Barker et al., 2007). Lgr5 increases a cell’s responsiveness to Wnt by potentiating the signal transmitted through Frizzled (de Lau et al., 2011). Forced activation of the Wnt pathway in Lgr5+ cells in the neonatal OC caused proliferation of Lgr5+ supporting cells as well as their differentiation to HCs (Shi et al., 2013). HC regeneration in the neonate also occurred from Lgr5+ cells and required Wnt pathway activation, whether spontaneous or induced by Notch inhibition (Bramhall et al., 2014). We used Lgr5 as a marker for the HC progenitors in the cochlea and vestibular systems in these experiments to determine the cell types produced from spheres during differentiation.

We also asked whether specific inner ear cell types could be generated from any compartment or only from the compartment of origin, i.e., spiral ganglion, vestibular epithelium or OC. Potency of neural stem cells has been difficult to determine without adequate markers to use for tracing the lineage of progenitor cells and for the differentiated progeny, and, as a result, similar questions have remained unresolved in several neural and non-neural stem cell compartments (Fuentealba et al., 2015). Here, by using gene expression analysis and physiological properties, as well as lineage tracing of putative progenitors in the spiral ganglion, OC, and vestibular epithelia, we could identify nine distinct cell types, which allowed us to determine whether the progenitor cells were uni-, multi-, or pluripotent. Our results indicate that postnatal inner ear progenitors are restricted to the lineages of their respective organs of origin, although newly identified PLP+ progenitor cells within the auditory nerve also form glial cell types of the central nervous system.
Results

Progenitor cells from three compartments in the inner ear

We performed experiments on the differentiation of progenitors from three compartments of the inner ear (Fig. 1) from the early postnatal mouse: the OC, the vestibular epithelia (the utricular macula, saccular macula, and semicircular canal cristae combined), and the spiral ganglion (containing Schwann cells and cell bodies of cochlear neurons). We cultured cells in conditions that produced spheres via cell division (Methods). We then differentiated the spheres for 14-70 days to allow significant maturation and used distinct markers and electrophysiological features to achieve cell-specific identification as shown in Figs. 2-4. We used lineage tracing with Lgr5 and PLP, respectively, to follow cell fates of epithelial and spiral ganglion progenitors (described in Figs. 5-7). The combination of progenitor-specific lineage tags and cell-specific markers allowed us to identify the cell types produced by progenitor cells in each compartment of these closely related cell types, which had not been possible in previous experiments (Li et al., 2003; Oshima et al., 2007).

HC proteins in cells differentiated from inner ear progenitors

Differentiated spheres contained cells that stained for HC markers, such as the transcription factor Atoh1 (Bermingham et al., 1999), detected in an Atoh1-nGFP mouse (Lumpkin et al., 2003), myosin VIIA (Grati and Kachar, 2011; Hasson et al., 1995) and the Ca^{2+} binding protein parvalbumin (Yang et al., 2004). We refer to these cells as "hair-cell like cells." Atoh1-nGFP+ cells were a small fraction (~1%, 876/92443) of all cells in the differentiated spheres. 51% of Atoh1-nGFP+ cells double-stained for another HC marker such as myosin VIIA, which is similar to the 44% yield of <1% of total cells in culture found in a previous study (Oshima et al., 2007). 67% (285/423) of myosin VIIA-positive cells derived from vestibular progenitors and 81% (690/850) of myosin VIIA-positive cells derived from cochlear progenitors had protruding structures that labelled with phalloidin (Fig. 2A-C,F,H), which stains filamentous actin, the most abundant protein in stereocilia. Of phalloidin-positive structures, 50% (27/54) from cochlear progenitors and 73% (58/79) from vestibular progenitors were immunoreactive for plasma membrane Ca^{2+}-ATPase 2 (PMCA2; Fig. 2C,H), which is abundant in postnatal HC bundles (Chen et al., 2012; Dumont et al., 2001; Hill et al., 2006). PMCA2-like immunoreactivity was strong at the base of the actin-positive structures (Fig. 2C,H, arrows), as reported for HCs (Chen et al., 2012), with more diffuse immunoreactivity present in the membrane of the cell body (Fig. 2C,H). Some of the phalloidin-positive structures resemble actin-containing “cytocauds”, a malformation of stereocilia that has been described in animals with inner ear mutations that affect hair bundles (Kanzaki et al., 2002).
In addition to the actin-based stereocilia, vestibular hair bundles and immature cochlear hair bundles have a single kinocilium that is microtubule-based and contains acetylated α-tubulin (Li et al., 2008; Ogata and Slepecky, 1995). In our differentiated cells, immunoreactivity to acetylated α3 tubulin was rare: only 4% (9/289) of vestibular hair-cell like cells (Fig. 2B; Table 1) and no cochlear hair-cell like cells (0/312) were stained. Phalloidin-positive rings near Atoh1-nGFP+ cells (asterisk, Fig. 2I) are reminiscent of actin rings that typically surround supporting cells in HC epithelia (Burns and Corwin, 2014), suggesting that spheres form partially organized epithelia during differentiation.

<table>
<thead>
<tr>
<th>Staining target</th>
<th>Cochlear-derived</th>
<th>Vestibular-derived</th>
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<tbody>
<tr>
<td></td>
<td>Cell count</td>
<td>%</td>
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<tr>
<td>Phalloidin (actin; hair bundles)</td>
<td>690/850 MyoVIIa+ cells</td>
<td>81</td>
</tr>
<tr>
<td>α3 tubulin (kinocilia)</td>
<td>0/312 MyoVIIa+ cells</td>
<td>0</td>
</tr>
<tr>
<td>PMCA2 (bundles)</td>
<td>27/54 actin bundles</td>
<td>50</td>
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<tr>
<td>Oncomodulin (type I vestibular HC)</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Calretinin (type II vestibular HC, IHC)</td>
<td>0/26 Atoh1+ cells</td>
<td>0</td>
</tr>
<tr>
<td>Prestin (OHC)</td>
<td>58/95 Atoh1+ cells</td>
<td>61</td>
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To investigate whether any differentiated HC-like cells assume a specialized fate, we labeled them with antibody against prestin, a membrane protein that is specifically expressed in cochlear outer HCs (OHCs) and not in vestibular organs (Zheng et al., 2000). In the OC, expression increases between P0 and the onset of hearing (~P12 in mice) (Belyantseva et al., 2000). OC-derived Atoh1-nGFP+ cells showed robust prestin-like immunoreactivity in the cell membrane, always in conjunction with myosin VIIA-immunoreactivity (Fig. 2G). The percentage of cells that were prestin-positive (61%) is roughly consistent with the percentage of OHCs in the cochlea (~75%). In contrast, none of 1302 Atoh1-nGFP+ cells generated from vestibular organs expressed prestin (Table 1). These data suggest that each inner-ear stem cell population is strongly biased to differentiate into a specific HC 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 HCs in the central zones of vestibular epithelia (Simmons et al., 2010) (Fig. 2D). 10% (8/83) of myosin VIIA-positive cells derived from vestibular progenitors showed oncomodulin-like immunoreactivity, the same as the percentage of HCs in the mature mouse utricular epithelium that are striolar type I HCs (366/3613; Li et al., 2008). The Ca\textsuperscript{2+}-binding protein, calretinin, is strongly expressed in
immature HCs as well as mature type II vestibular HCs and cochlear inner HCs (IHCs) (Dechesne et al., 1994; Desai et al., 2005; Li et al., 2008). Calretinin antibody labeled one-third of Atoh1-nGFP+ cells (5/15 cells, one experiment) derived from vestibular tissue (Fig. 2E; Table 1). In contrast, no cochlear Atoh1-nGFP+ cells (0/26) were calretinin-positive; thus, if any cells were differentiating along the IHC path, they did not acquire all IHC properties.

In summary, immunostaining for the HC markers Atoh1 and myosin VIIA showed that some differentiating cells in spheres acquired proteins expected of HCs. Of these cells, many had protrusions containing f-actin and PMCA2, typical of stereocilia, but very few expressed the kinocilium marker, α3 tubulin. The expression of prestin by a large fraction of HC-like cells derived from the OC but not by any cells derived from vestibular tissue suggests that the inner ear progenitor cells of the neonatal mouse are not fully competent to form HCs of all types, but rather have fates restricted to their inner ear compartment of origin. This may indicate differentiation limitations in each organ’s progenitor cell population.

Expression of hair-cell specific genes

We next used RT-PCR to detect mRNA that is essential for proper HC functions and would further specify HC type. We probed differentiated spheres of OC and vestibular origin for mRNA specific to: the mechanosensory apparatus (Tmc proteins), hair bundle (espins), electromotility in OHCs (prestin), the presynaptic apparatus (proteins of synaptic ribbons and vesicles), and the postsynaptic apparatus (receptors for cholinergic efferent input) (Fig. 3A). We also tested for expression of genes related to supporting cell function, in particular secretion of the extracellular matrices (otogelin and otopetrin). The spheres were differentiated for 14 days and expression is compared to control data from native tissues prepared acutely at P5. Representative gels for these probes are shown in Figure 3B and the results are summarized here.

In HCs, Tmc proteins are essential for mechanoelectrical transduction (Pan et al., 2013). In both vestibular and cochlear native tissues at P5, we detected expression of mRNA for both isoforms, Tmc1 and Tmc2, as expected. Vestibular-derived differentiated cells expressed Tmc1 and Tmc2, and OC-derived cells expressed Tmc1. The lack of Tmc2 in OC-derived cells may indicate differentiation of the transduction complex, consistent with the in vivo time course: Tmc2 disappears from the OC at about the onset of hearing (Pan et al., 2013), and the spheres were differentiated for 14 days at a minimum.

Differentiated progenitors and native tissues all expressed myosin 1c, which plays a key role in transducer adaptation in vestibular HCs (Gillespie, 2004; Holt et al., 2002), and the tip-link proteins, cadherin 23 and protocadherin 15, which are critical to transduction (Kazmierczak et al., 2007). Differentiated progenitor cells from both vestibular and OC tissues
expressed two isoforms of espin, espin1 and espin4, which are associated with stereociliary elongation. Given that espin4 arises postnatally (Sekerkova et al., 2006), its presence further suggests that some hair-cell-like cells had differentiated significantly.

We also found that differentiated progenitors from both tissues expressed genes that are specific to afferent and efferent synaptic functions. At afferent synapses from HCs onto neurons, Ca\(^{2+}\) enters through Ca\(V\)1.3 calcium channels (Dou et al., 2004; Platzer et al., 2000) in the pre-synaptic HC membrane to promote release of glutamate from vesicles clustered around presynaptic ribbons. As shown in Figure 3B, differentiated spheres from both tissues expressed the genes for vesicular glutamate transporter gene Vglut3, which packages glutamate into hair-cell synaptic vesicles (Peng et al., 2013; Seal et al., 2008; Wang et al., 2007); for C-terminal binding protein 2, Ctbp2, which encodes the major component of pre-synaptic ribbons, ribeye (Schmitz et al., 2000); and for Cacna1d, the gene coding for Ca\(V\)1.3 channels. HCs are innervated by efferent nerve fibers of brainstem origin, which release acetylcholine onto hair-cell specific cholinergic receptors containing \(\alpha9\) subunits encoded by Chrna9 (Elgoyhen and Franchini, 2011; Luo et al., 1998). Differentiated spheres from both tissues expressed Chrna9 (Fig. 3B). Expression of molecules specialized for inner ear synaptic transmission indicates that HC differentiation took place.

Consistent with our antibody staining, and confirming previous RT-PCR results on differentiated spheres (Oshima et al., 2007), the cells from both cochlea and vestibular organs expressed the hair-cell marker myosin VIIA, while cochlear spheres alone expressed the OHC marker prestin (Fig. 3B). Expression of the calcium-binding protein, oncomodulin (Ocm gene), was detected in both cochlear and vestibular tissues, as expected from \textit{in vivo} reports, where it is selectively expressed by vestibular type I cells and cochlear IHCs (Fig. 3B).

**Expression of supporting cell genes**

Both otogelin (El-Amraoui et al., 2001) and Lgr5 are expressed by supporting cells in both the OC and vestibular epithelia, and otopetrin is specific to peripheral supporting cells of vestibular epithelia (Kim et al., 2010). Again the differentiated spheres showed expression appropriate to their origins: spheres derived from both cochlear and vestibular tissue expressed mRNA for otogelin and Lgr5, and only vestibular-derived spheres expressed otopetrin (Fig. 3B). Thus, sphere differentiation yields markers of inner-ear supporting cells in addition to HC markers.

In summary, both cochlear and vestibular progenitor cells can differentiate into cells that express components of the transduction apparatus, the hair bundle, and pre- and post-synaptic machinery, consistent with substantially differentiated HCs. The selective expression of prestin by OC-derived spheres and of Tmc2 and otopetrin by vestibular-derived spheres suggests that the progenitors from each tissue are constrained to differentiate into their native
Voltage-gated currents in differentiated hair-cell like cells

We conducted electrophysiological experiments on newly differentiated hair-cell like cells to further test the hypothesis that they form distinct cellular subtypes that are limited to those of their native organ, and to determine if their electrical properties resemble those reported for native HCs. With the patch clamp method, we recorded voltage-dependent whole-cell currents from HC-like cells, which we recognized in the recording dish by their expression of Atoh1-nGFP. Most Atoh1+ cells produced large outwardly rectifying currents in response to depolarization, with amplitudes and time courses that are qualitatively within the range of the outward delayed rectifying K⁺ current (I_{KD}) of HCs (Fig. 4A). We observed such currents in all (34/34) Atoh1+ cells derived from vestibular organs (Fig. 4A.1, A.2) and in almost all (29/32, 91%) of Atoh1+ cells derived from the cochlea (e.g., Fig. 4A.3). In 7 Atoh1-negative cells tested (6 cochlear, 1 vestibular), none had HC-like currents (e.g., Fig. 4A.4).

To quantify the voltage dependence of steady-state outwardly rectifying currents, we made and fit tail-current activation curves (Fig. 4A.5) from records like those in Figure 4A (Methods). For both tissues of origin, I_{KD} activated around −60 mV and had activation midpoints (V_{1/2} values) typical of mouse HCs of the first postnatal week (Marcotti et al., 2003a; Rüsch et al., 1998; reviewed in Eatock and Hurley, 2003), with means of −21 ± 3 mV (SE) and −33 ± 2 mV for 3 cochlear and 5 vestibular cells, respectively. Negatively activating K⁺ currents (I_{Kn} and I_{K,L}) that normally emerge in specific hair cells during the pre-hearing period in mice (Geleoc et al., 2004; Marcotti et al., 1999; Rusch et al., 1998) were not detected here.

Outward currents in cochlear cells had activation time courses in the range recorded from neonatal cochlear HCs in ex vivo OC preparations (times to half-maximum current 10-20 ms) (Marcotti et al., 1999; Marcotti et al., 2003a). The activation time courses of outward currents of seven vestibular-derived cells (Fig. 4B.1) fell into two groups (Fig. 4B.2) with values close to those of type I and type II hair cells in the postnatal mouse utricle (Rüsch et al., 1998), consistent with differentiation of the vestibular progenitor cells into vestibular hair cell types. The outward currents of vestibular-derived cells showed a range of inactivation time courses, also consistent with reports from early postnatal mouse utricles (Holt et al., 1999; Rusch et al., 1998): 4 of 32 cells had strongly inactivating currents (A currents, I_{A}, Fig. 4D) while others did not show fast inactivation (Fig.4A, top).

Vestibular-derived Atoh1+ cells also resembled native vestibular HCs in expressing sizeable h currents, I_{h}, carried by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Fig. 4C). In vivo, vestibular HCs acquire I_{h} postnatally. Its size increases dramatically at P3–P4 in the mouse utricle (Horwitz et al., 2010; Horwitz et al., 2011; Rusch et
We observed $I_h$ in 22/34 (65%) of Atoh1-nGFP+ cells derived from vestibular tissue. 0 of 32 cochlear-derived cells had detectable $I_h$ (Fig. 4C.1), consistent with native cochlear HCs (Horwitz et al., 2010; Horwitz et al., 2011). This result suggests that only the vestibular progenitors were capable of becoming vestibular HCs.

Many HCs have an inwardly rectifying potassium current, $I_{K1}$, which contributes to setting a HC's resting membrane potential during development. Type I vestibular HCs express $I_{K1}$ up to P4 and likely afterwards, while type II vestibular HCs express $I_{K1}$ throughout the maturation process (Rusch et al., 1998). 60% (19/32) of the Atoh1-nGFP+ vestibular-derived cells expressed $I_{K1}$: a fast inward current at the start of a hyperpolarizing voltage step, which rapidly deactivated after a depolarizing step (Fig. 4C.2). Within the cochlea, $I_{K1}$ increases in size in IHCs from E15 to P12, the onset of hearing, then declines rapidly, disappearing by P15 (Marcotti et al., 2003a). It disappears from OHCs about a week earlier (Marcotti et al., 1999). In our experiments, $I_{K1}$ was not detected in cochlear HC-like cells (Fig. 4A.3); they either never expressed $I_{K1}$ or had passed through that stage of differentiation.

In vestibular-derived Atoh1+ cells, large hyperpolarizing voltage steps evoked inward currents that followed a double-exponential time course reflecting the two components, $I_{K1}$ and $I_h$ (Fig. 4C.2). The faster time constant (0.99 ± 0.32 ms, n=5) is comparable to values reported for $I_{K1}$, the fast inward rectifier, in early postnatal mouse utricular HCs (Rusch et al., 1998). The slower time constant (92 ± 21 ms, n=6) is comparable to the faster of two time constants for $I_h$ activation in mouse utricular HCs (Horwitz et al., 2011); our 200-ms steps were too brief to substantially activate the slower $I_h$ component.

In some 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 HC literature (Fig 4D). The resting potentials for Atoh1+ cells were $-53 \pm 8$ mV (4 cochlear-derived cells) and $-53 \pm 6$ mV (6 vestibular-derived cells). These values are set in large measure by K+-selective inwardly-rectifying and outwardly-rectifying channels and are within the physiological range for postnatal HCs (Eatock and Hurley, 2003). Both cochlear-derived and vestibular-derived Atoh1+- cells responded to current steps with voltage waveforms within the normal range reported from postnatal HCs. We did not see the mixed Ca$^{2+}$-Na$^+$ spikes that are typical of mouse IHCs in the first postnatal week (Marcotti et al., 2003b). All Atoh1+ cells lacked the voltage-gated Na$^+$ currents of immature HC subtypes (Chabbert et al., 2003; Eckrich et al., 2012; Geleoc et al., 2004; Li et al., 2010; Marcotti et al., 2003b; Oliver et al., 1997; Witt et al., 1994; Wooltorton et al., 2007). Again, these channels either were never expressed or had stopped being expressed by the earliest time point we examined, 12 days of in vitro differentiation.
In all, our results support the hypothesis that the early postnatal inner ear harbors different populations of progenitors with limited differentiation capabilities. Organ-specific progenitors differentiated to Atoh1+ cells with many properties necessary for HC function, some of which are consistent with a relatively mature state: the presence and voltage dependence of delayed rectifiers in all cells; the lack of I_{Na} in all cells; the lack of Tmc2 and I_{K1} and the presence of prestin in cochlear cells; and the presence of I_{K1}, I_{h} and different kinds of outward rectifier in vestibular cells. Other mature properties – notably the distinctive negatively activating outward rectifiers of mature type I HCs and OHCs - failed to develop over the time frame examined.

**Progenitor sub-populations**

We next analyzed the potency of known HC progenitors within the cochlea. Lgr5 is expressed in inner border cells, inner pillar cells, and 3rd row Deiter’s cells (Chai et al., 2012; Shi et al., 2012) (Fig. 5A). Lgr5 was chosen as a marker to use for determining cell fates of neonatal progenitors. By crossing Lgr5-EGFP-IRES-CreER mice with floxed-tdTomato mice (Methods), we were able to trace the lineage of Lgr5+ cells in inner ear spheres (Fig. 5B) that we differentiated. After culture and differentiation, we observed that Lgr5+ lineage-traced cells could form HC-like cells, as indicated by co-localization of myosin VIIA and tdTomato expression (n=20 cells; Fig. 5C). Myosin VIIA+/tdTomato-negative cells were thought to result from incomplete recombination by this Cre (see Shi et al, 2013). To determine if Lgr5+ cells could also form neurons, we stained the differentiated cultures for Tuj1, a tubulin that is highly specific to neurons (Fig. 5D). tdTomato was not co-localized with Tuj1 in any cells (n = 696 Tuj1+ cells), consistent with in situ data that localize Lgr5 to non-neuronal cells in the OC (Chai et al., 2012; Shi et al., 2012).

Since we had previously shown (Martinez-Monedero et al., 2008; Oshima et al., 2007) that neurons could differentiate from inner ear spheres, we set out to identify an inner ear progenitor that gives rise to neurons. We hypothesized that Schwann cells within the inner ear could be the source of the spheres from the spiral ganglion. Because these Schwann cells express PLP (Gomez-Casati et al., 2010), we crossed PLP-Cre-ER mice with tdTomato mice and generated spheres from the spiral ganglion (Fig. 6A). The spiral ganglion comprises the cell bodies of cochlear afferents and Schwann cells, and only the Schwann cells are PLP+. In mature rodents, Schwann cells myelinate the peripheral processes of the bipolar spiral ganglion neurons, their cell bodies, and the initial portions of their central axons (Hurley et al., 2007; Spoendlin, 1975; Toesca, 1996) up to the glia limitans, a transition zone near the cochlear nucleus where CNS glia (oligodendrocytes and astrocytes) take over the myelination (Hurley et al., 2007; Jalenques et al., 1995; Toesca, 1996; Valderrama-Canales et al., 1993).
The PLP-tdTomato-positive progenitor cells gave rise to many labeled cells in 3rd-generation spheres (Fig. 6B). We used PLP to mark spheres in an attempt to determine the potency of the progenitors in the newborn spiral ganglion. After differentiation of the 3rd generation spheres, we stained the cultures for specific markers of neurons and central nervous system (CNS) glia to determine if the PLP+ cells, which in situ would give rise to Schwann cells, also had the capacity to give rise to neurons and CNS glia. Co-staining of tdTomato and antibody to the neuron-specific class III β-tubulin Tuj 1 (n=727 cells; Fig. 6C) indicated that PLP+ glial cells formed neurons. We also observed co-staining of tdTomato and antibody to the early-stage oligodendrocyte marker O4, a sulfated galactosylcerebroside on non-myelinating oligodendrocytes (Sommer and Schachner, 1981; Sommer and Schachner, 1982) (n = 1299 cells, Fig. 6D). The PLP+ cells could also form more mature oligodendrocytes, as indicated by co-staining of tdTomato cells with antibody to O1, a galactocerebroside on terminally differentiated oligodendrocytes that are capable of myelination (Sommer and Schachner, 1981; Sommer and Schachner, 1982) (n=1286 cells, Fig. 6E). Lastly, co-staining of tdTomato and Aldh1L1, an aldehyde dehydrogenase that within the nervous system is highly specific to astrocytes (Cahoy et al., 2008) (n = 531 cells, Fig. 6F), showed that the PLP+ cells could also form astrocytes. PLP+ cells in the differentiated cultures that lacked the markers Tuj1, O1 and O4 had probably differentiated as Schwann cells.

Thus, lineage-tracing experiments revealed that neonatal Schwann cells within the ganglion can form multiple cell types, including neuronal and glial cell types (oligodendrocyte-like, and astrocyte-like). No myosin VIIA-expressing cells were observed after sphere differentiation (n = 5 cultures), indicating that progenitor cells of the neonatal spiral ganglion did not differentiate into HC-like cells. Together these methods, using combinations of markers and electrophysiological characteristics to distinguish cell type phenotype, could identify nine distinct cell types arising from the three compartments of the inner ear (Fig. 7).

Discussion

The neonatal inner ear contains progenitor cells with the capacity to regenerate HCs after damage (Bramhall et al., 2014; Cox et al., 2014). The HCs regenerated in the cochlea, both spontaneously after damage or after Notch inhibition or Wnt pathway stimulation, arise from Lgr5+ cells (Bramhall et al., 2014). Progenitor cells from the different inner ear compartments can be differentiated in vitro to cells that resemble HCs, neurons, and glia (Martinez-Monedero et al., 2008; Oshima et al., 2007). HC-like cells derived from utricular progenitors resembled immature vestibular HCs in whole-cell recordings (Oshima et al., 2007), and progenitor-derived neurons had characteristics of auditory neurons (Martinez-Monedero et al., 2006). It was not determined whether these cell types arose from a common pluripotent stem cell as reported
previously (Li et al., 2003), or from multiple progenitor populations with more limited
differentiation capacities. Because the sensory epithelia of the cochlear and vestibular organs
are difficult to separate completely from neural elements, and because the cell types that arise
from these different compartments show much overlap in gene expression, previous work had
not been able to reach agreement about the potency of potential inner ear progenitor cells (Liu
et al., 2014). One paper concluded that cells in the mouse utricle were pluripotent (Li et al.,
2003) and another that neurons and epithelial cells could be obtained from epithelial
compartments (Oshima et al., 2007). Knowing which progenitor cells give rise to which cell
types is of fundamental interest and also may be necessary to regenerate inner ear cells
therapeutically.

Numerous studies of inner ear development show that the otic placode is the source of
both neural and sensory elements of the adult ear (Barald and Kelley, 2004; Kelley, 2006; Raft
and Groves, 2015), and the placode likely contains progenitor cells that divide and become
specified to neural and sensory fates. The otic placode gives rise to the otocyst in the early
stages of inner ear morphogenesis, and the otocyst becomes regionalized into areas that
eventually form the cochlea and vestibular organs (Barald and Kelley, 2004; Fekete and Wu,
2002; Groves and Fekete, 2012; Raft and Groves, 2015), both of which arise from the
prosensory epithelium. In the mouse inner ear, the neural lineage separates from the sensory
lineage at E9.5 (Ma et al., 1998), when the neuroblasts delaminate from the prosensory region.
These proneural cells are infiltrated by cells of the neural crest that give rise to the glia of the
inner ear (Breuskin et al., 2010; D’Amico-Martel and Noden, 1983). Thus, the compartments
that we study here contain progenitors from both neural crest and otic placode and generate
specialized cell types including neurons, glia, and sensory cells.

Progenitor cell markers are widely expressed in the embryo and become restricted to
populations of progenitors as development proceeds. Thus, the time at which progenitors in the
developing cochlea or utricle are labeled for lineage determination is important for the pattern
of labeling. Here we show that activation of Cre for labeling of mouse inner ear progenitor cells
at neonatal time points reveals three distinct populations: Lgr5+ cochlear progenitors, Lgr5+
vestibular progenitors, and PLP+ Schwann cell derivatives.

Knowledge of the differentiation capacity of closely related and physically overlapping
progenitor cell populations is important for an understanding of their functional role in
generating the multiple cell types that comprise a complex tissue. The plasticity of neural
progenitors has been followed during regionalization of the fore-, mid-, and hindbrain (Hitoshi
et al., 2002; Shen et al., 2006). These studies show an increasing level of regionalization, as less
specified stem cells mature into more highly determined progenitors and migrate into their final
positions. Cortical neurons are derived from distinct progenitor cells during development (Tyler
Fate of the progenitors is partially pre-programmed for broad regional identity and partly determined by inductive cues as the progenitors assume their identity (Gage, 2002; Hitoshi et al., 2002; Shen et al., 2006; Zhao et al., 2008). Some of the neural progenitors in the embryo are restricted to playing a developmental role only. Stem cells remain, however, in the dentate gyrus and the subventricular zone, where they give rise to the rostral migratory stream and hippocampal neurons (Kempermann et al., 2015; Kriegstein and Alvarez-Buylla, 2009; Zhao et al., 2008). The capacity of neural stem cells to differentiate to single or multiple cell types has been difficult to assess without sufficient markers for both the progenitors and their cellular progeny.

The neural crest bears considerable resemblance to the placodes containing the stem cells that generate the sensory ganglia of the inner ear. Both give rise to multiple cell types including glutamatergic sensory neurons, as well as peripheral and central glia. Adult DRG, like adult spiral ganglion, gives rise to neurospheres (Li et al., 2007). However, neural crest-derived progenitor cells in DRG maintain their regenerative capacity into adulthood, unlike what has been observed to date in spiral ganglion. DRG cells divide in response to damage and differentiate into neurons at the site of injury (Gallaher et al., 2014; Li et al., 2007).

Understanding the postnatal capacity for regeneration is particularly relevant to poorly regenerating tissues. In the inner ear, well-defined compartments and considerable existing data on cell-specific proteins and electrophysiological phenotypes make this goal achievable. We used a previously identified marker (Lgr5) to trace the lineage of specific OC progenitors. While the mature cochlear epithelium has little regenerative capacity, early postnatal Lgr5+ cells show an ability to both divide and differentiate to HCs. We identified a new marker (PLP) for spiral ganglion progenitors, which we also lineage traced. The PLP+ Schwann cells also show some capacity for cell division in the adult (Lang et al., 2011). We observed the development of distinct HC, neural, and glial subtypes from neonatal progenitors from the different inner ear compartments, suggesting that the differentiation capacity of postnatal inner ear progenitors is limited to cells of the originating tissue (Fig. 7).

The newly created HC-like cells expressed many genes necessary for proper function. In addition to demonstrating immunoreactivity for proteins key to HC-specific functions and morphological characteristics (prestin, espin, oncomodulin, and PMCA2), we showed that the newly created cells expressed species of mRNA that are necessary for mechanotransduction, bundle structure, synaptic function, and Ca\(^{2+}\) buffering. Interestingly, differentiated cochlear cells showed robust prestin expression, a characteristic of OHCs near the onset of hearing (Belyantseva et al., 2000). Similarly, HC-like cells differentiated from the cochlea only expressed the transduction channel-associated gene TMC1, while vestibular cells expressed both TMC1 and TMC2. This resembles the expression pattern in mature HCs within native tissue.
We also observed that newly created HC-like cells acquired the physiological behavior of bona fide HCs, such as resting potentials around \(-55\) mV, large outwardly rectifying currents likely carried by \(K^+\), and, in cells from vestibular tissue, additional currents (\(I_{KA}\), \(I_h\), and \(I_{K1}\)) that occur frequently in mature native vestibular cells. These gene-expression and physiological results are consistent with results from native postnatal HCs beyond the earliest stages of differentiation. It seems likely that immature HC characteristics would have been observed at earlier stages (<12 days) following the start of differentiating culture conditions, but we did not study that period because at early stages the levels of GFP, our marker for \(Atoh1\) expression, were low.

\(Atoh1\) over-expression \textit{in situ} causes trans-differentiation of supporting cells to ectopic HCs that have been called “primordial” because their properties do not depend on the organ in which they reside (Yang et al., 2012). In our method, the expression of \(Atoh1\) was influenced by passive signaling stimulated by removal of growth factors, and a given inner ear organ generated its own HC subtypes. Endogenous signaling within the progenitor cell population of each organ may give rise to cell types more representative of the differentiation capacity of each tissue. Production of HCs can be induced by Wnt activation (Shi et al., 2013; Shi et al., 2014) or Notch inhibition (Doetzloher et al., 2009; Mizutari et al., 2013; Pan et al., 2010; Yamamoto et al., 2006). Notch-mediated lateral inhibition (Daudet and Lewis, 2005; Lanford et al., 1999) and innate Wnt signaling are both critical for the development of HCs (Shi et al., 2013; Shi et al., 2014). The Wnt pathway interacts with the Notch pathway in development (Collu et al., 2014; Shi et al., 2010) and this interaction could regulate the level of expression of \(Atoh1\) and other genes. Bypassing key components of developmental pathways via viral \(Atoh1\) transduction may disrupt mechanisms that control levels of Wnt and Notch factors and thus create “primordial” HCs.

Previous work established that neonatal inner ear tissues can generate both HCs and neurons, but the potency of individual progenitor cells was not established. Our work shows that in the neonatal mouse, HC progenitors and neural progenitors are different populations with different potencies. The cochlea-derived Lgr5-expressing cells produced HC-like cells, consistent with previous work (Chai et al., 2012; Shi et al., 2013; Shi et al., 2012), but did not produce neural cell types. We demonstrated that PLP-expressing glial cells from the neonatal spiral ganglion were capable of differentiating both to neurons and to multiple forms of glia that are outside the otic lineage. CNS cell types (oligodendrocytes) were also seen after differentiation of DRG neurospheres (Binder et al., 2011). Although bipotent, the PLP+ cells in our study did not give rise to HCs, just as Lgr5+ cells did not give rise to neurons. This is an important observation for efforts to regenerate both HCs and neurons. Identification of these distinct progenitor compartments is an important step in fate mapping of the inner ear,
although we cannot rule out the existence of other lineages. Thus, the cochlea, vestibular organs and spiral ganglion progenitor cells in mice are partly committed to specific cell fates even at birth. Neural progenitor cells in the inner ear of neonatal mice give rise to a greater range of cell types than HC progenitors but both have limited potency. This work advances our understanding of the commitment of tissue stem cells to cell fates of the mammalian inner ear.

**Materials and Methods**

**Isolation of progenitor cells from the inner ear.** We extracted progenitor cells from the neonatal mouse inner ear of several mouse lines (Table S1). All animal studies were conducted under an approved institutional protocol according to National Institutes of Health guidelines.

For each experiment, the otic capsules of 6-8 mice (age range postnatal day, P1-4) were carefully extracted from the skull and any brain tissue was discarded. The cochleas and vestibular organs (utricle, saccule, and ampullas combined) were dissected out (Fig. 1) in HBSS and kept separate from each other for the remainder of the protocol, allowing us to analyze each tissue’s developmental properties separately. For cochleas, the OC (sensory epithelium) was separated from the stria vascularis (ion transport epithelium) and the bony modiolus, which houses the spiral ganglion, comprising the cell bodies of auditory nerve fibers and associated Schwann cells (Fig. 1).

The OC and vestibular organs were transferred to the dissociating medium TrypLE (Life Technologies, CA, USA) for 11-13 min at 37°C and then dissociated by trituration with a pipette. The triturated cells were passed through a 70-μm cell strainer to remove tissue and bone debris. Single cells were deposited in ultralow-cluster plates (Corning, NY, USA) and proliferated to produce floating spheres by culturing for several days in a 1:1 mixture of DMEM/high-glucose medium and F12, supplemented with N2, B27 (Life Technologies), EGF (20 ng/mL; Chemicon, MA, USA), bFGF (10 ng/mL; Chemicon), IGF-1 (50 ng/mL; Chemicon), and heparan sulfate (50 ng/mL; Sigma-Aldrich, MO, USA). The spheres were passaged by trituration with a 100-μl pipette, then placed in fresh culture medium. For each experiment, spheres were passaged 3 times at intervals of 3-4 days to eliminate HCs or neurons from the original tissue.

**Mouse strains for lineage tracing.** We analyzed the potency of Lgr5+ cells from the OC and PLP+ cells from the spiral ganglion. Male Lgr5-EGFP-IRES-Cre-ER (jaxmice.jax.org/strain/008875.html) and male PLP-Cre-ER mice (jaxmice.jax.org/strain/005975.html) were crossed with female tdTomato reporter mice (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 (50 mg/ml) on the day of birth (P0) and P1. Pups were dissected at P3 and were identified as positive or negative for the marker of
interest based on cellular fluorescence. Spheres were generated from the OC of Lgr5+ mice and the modiolar tissue (containing the spiral ganglion, including cochlear neuronal cell bodies and glial cells) of PLP+ mice.

**Differentiation and treatment of spheres.** To generate differentiated cells, 3rd generation spheres were plated in 4-well plates (Greiner, Austria) on round 10-mm glass coverslips coated with poly-L-lysine (Trevigen, MD, USA). Attachment took place overnight in DMEM-high glucose/F12 (mixed 1:1, Life Technologies) containing N2 and B27 (Invitrogen); the elimination of growth factors halted cell division and promoted differentiation. Spheres were differentiated in these conditions for 7-70 days, with fresh culture medium applied every 2-3 days to maintain optimal culture conditions. Cells were then harvested for RT-PCR (to analyze gene expression), immunostaining (to identify and count differentiated cells expressing proteins characteristic of specific cell types), or whole-cell patch clamping (to test for functional maturation and expression of voltage-dependent properties that differentiate cochlear and vestibular sensory cells).

**Control spheres.** To ensure that results were not significantly affected by HCs carried over from the original dissociated tissue, we counted the number of HCs that were still present after the 3rd passage. HCs were recognized by their expression of the HC markers Atoh1-nGFP (visualized by GFP fluorescence) and myosin VIIA (visualized by immunostaining). 3rd-generation spheres were seeded in the same experimental culturing conditions as were used for differentiation analysis experiments, but instead were only allowed to differentiate for 3 hours. This seeding time allowed the spheres to adhere to the culturing surface, but was too short for the cells to differentiate. The cells were viewed via confocal microscopy (Leica, SP5, Germany) over the entire seeding area. Cochlear spheres that were differentiated for 3 hours gave rise to 0 Atoh1-nGFP expressing cells; 14 days of differentiation produced an average of 32 ± 12 Atoh1-nGFP+ cells (n = 3 cultures). For vestibular spheres, 3 hours of differentiation produced 1 Atoh1-nGFP+ cell (n = 2 cultures), substantially fewer than the average after 14 days: 326 ± 107 Atoh1-nGFP+ cells (n = 4 cultures). We also did RT-PCR analysis on 3rd generation spheres collected from floating cultures (n = 3 cultures) to look for expression of the hair-cell marker, myosin VIIA. These spheres were positive for GAPDH but negative for the hair-cell marker, myosin VIIA, unlike spheres that were differentiated longer (see Results). In all, these results indicate that our results on differentiated inner ear spheres reflect production of new HC-like cells and are not caused by native cells that survived the culturing process, consistent with previous work using this method (Martinez-Monedero et al., 2008).
**RT-PCR.** We used RT-PCR to analyze gene expression. RNA was extracted from the inner ears of P5 CD1 mice or from differentiated spheres, using the RNeasy Maxi Kit (Qiagen, CA, USA) according to the manufacturer’s instructions, denatured at 65°C for 5 min, and reverse-transcribed using lmProm II (Promega, WI, USA) and random hexamer primers. The reverse transcription conditions were 25°C for 5 min followed by 42°C for 60 min, and the reaction was terminated at 70°C for 15 min. To the resulting cDNA, we added primers (Table S2) for various inner ear proteins. The primers were taken from PrimerBank (pga.mgh.harvard.edu/primerbank) or designed with Primer3 (bioinfo.ut.ee/primer3) and where possible, were intron-spanning to preclude amplification of genomic DNA. The amplified products were then separated on a 2% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator. Spheres at the 3rd generation were assessed for expression of myosin VIIa in the absence of differentiation (Fig. S1). Lanes with GAPDH primers and water were run as positive controls for the tissue and negative controls for contamination, respectively.

**Immunohistochemistry.** We used immunohistochemistry to identify and count differentiated cells that expressed proteins characteristic of specific cell types. Differentiated spheres were fixed at room temperature in 4% paraformaldehyde/PBS for 15-20 min; washed in PBS; permeabilized and blocked for 1 h in blocking solution (0.3% Triton X-100 and 15% heat inactivated goat or donkey serum in PBS); and exposed to diluted primary antibody (in 0.1% Triton X-100 and 10% heat inactivated goat or donkey serum in PBS) overnight at 4°C. Primary antibodies, their target proteins, and antibody dilutions are listed in Table S3. Secondary antibodies (Alexafluor 488, 568, and 647-conjugated; Invitrogen) were diluted 1:500 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.

**Electrophysiology.** We used the patch clamp method to record whole-cell currents and voltages from differentiating cells to test for functional maturation and expression of voltage-dependent properties that differentiate cochlear and vestibular sensory cells. 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). Recordings were made at room temperature (22–25°C). The pipette solution contained (in mM): 135 KCl, 3.5 MgCl₂, 5 Na₂ATP, 10 HEPES, 10 EGTA, 0.1 Na-cAMP, 0.1 Li-GTP; pH was adjusted to 7.4 by adding 15 mM KOH; 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 Multiclamp 700; Molecular Devices, Sunnyvale, CA). Series resistance ($R_s$) was 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). All cells considered for analysis had >100 MΩ input resistance. To obtain the voltage dependence of activation (activation curve) for a current, we stepped through an iterated series of test potentials and measured the tail current at $-40$ mV, that is, the current immediately upon stepping to $-40$ mV after each test step. Plotting tail current against test-step voltage produced sigmoidal activation curves, which show how the conductance depends on voltage. Activation curves were fit with a Boltzmann function (Eq. 1), where $I(V)$ is current at voltage $V$, $I_{\text{min}}$ and $I_{\text{max}}$ are minimum and maximum currents, $V_{1/2}$ is voltage at half-maximal activation, and $S$ is the voltage change corresponding to an e-fold increase in $I$.

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

The time course of current activation was fit with a mono-exponential decay function. Averaged values are presented as means ± SE.
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Competing interests. No competing interests declared

Author contributions. WM, RAE AE: Designed research, WM, DM: Performed research, WM, RAE, AE: Analyzed data, WM, RAE, AE: Wrote the paper. All authors read and revised the paper.

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References


Figure 1. Progenitor cells were analyzed from three inner ear tissues: vestibular epithelia (1), containing sensory hair cells (magenta) and supporting cells (beige), the cochlear OC (2), comprising sensory HCs (magenta) and Lgr5 supporting cells (green), and the spiral ganglion (3) comprising Schwann cells (red) and primary afferent neurons (gray). HC-like cells produced from vestibular and cochlear compartments are analyzed, and the potencies of Lgr5+ cells within the OC and PLP+ glial cells within the spiral ganglion are examined.
Figure 2. Progenitor cells derived from vestibular organs (A-E) and OC (F-I) can differentiate into cells that express HC proteins and other typical HC characteristics. At the left are cartoons of the major HC types and some of their key proteins found in intact, mature inner ear epithelia: vestibular (top) and cochlear (bottom). A-E, Vestibular-derived cells identified as HC-like by Atoh1 expression, had other HC-like features, including strong intracellular labeling for myosin VIIa (A,D) and Ca^{2+}-binding proteins: parvalbumin (B), oncomodulin (ocm, D, arrowhead) which is specific to type I vestibular HCs in vivo, and calretnin (E); phalloidin-positive apical structures that are reminiscent of cuticular plates and stereocilia (A-C, inset in A, phalloidin stain alone) and, like stereocilia, stain for PMCA2 (C, arrow points to white structure where 3 labels co-localize); and elongate structures that, like the kinocilia of vestibular hair bundles, are immunoreactive for α3 acetylated tubulin (B, multiple cells with multiple tubulin-positive structures, e.g., arrowheads). F-I, Cochlear-derived HC-like cells, recognized by expression of Atoh1 (F,I), myosin VIIA (F,G) or parvalbumin (H), had phalloidin-stained (F,I) or PMCA2-immunoreactive (H) bundle-like structures emanating from the apical surface. Many showed strong immunoreactivity in the lateral membrane to prestin (G), the electromotility motor specific to OHCs. I, Actin rings were also seen in cochlear-derived spheres. Here, they are at the apices of a cluster of Atoh1+ HC-like cells (green), and also form an epithelial-like web of actin around other cells (asterisk), consistent with non-sensory epithelium. Scale bars in E,I apply to all micrographs.
Figure 3. Differentiated OC and vestibular cultures expressed genes that are specific to their tissue of origin and are required for proper physiological function. **A**, Schematic showing the genes that were analyzed and their approximate in vivo localization within each tissue, cell type, and cellular compartment (hair bundle, cytoplasm of the cell body, or synapse). **B**, Differentiated cells from both tissues expressed the HC gene myosin VIIA, hair-bundle associated genes (Espn1, Espn4, Myo1c, Cdh23, Pchd15), genes associated with synaptic function (Chrna9, Vglut3 and genes for Cav1.3 and Ribeye), and Ca²⁺ binding (Ocm), and the supporting cell genes otogelin and Lgr5. Cells of vestibular and cochlear origin differed in prestin, otopetin and Tmc2 expression. All results were confirmed with 5 or more sphere cultures. Native cochlear and vestibular data were from tissues at P5. Marker lanes and lanes for water (negative control) and GAPDH (positive control) are not shown. (n=5 per gene)
Figure 4. Differentiated OC and vestibular progenitor cells resemble the HCs from their tissues of origin in certain electrophysiological properties. A, Whole-cell currents evoked by voltage steps (bottom panels) in Atoh1-nGFP+ cells derived from vestibular tissue (A.1, A.2, 24 days in vitro, DIV) and OC (A.3, 57 DIV), and in an Atoh1-nGFP-negative cell (A.4). In Atoh1-nGFP+ cells, depolarizing voltage steps evoked large outward currents reminiscent of the delayed rectifier potassium currents (I\textsubscript{DR}) found in native HCs (n = 29 vestibular, n = 32 cochlear). Some (A.2) but not all (see A.1) Atoh1-nGFP+ cells from vestibular tissue had inactivating I\textsubscript{A} (n = 4). Currents in Atoh1-nGFP-negative cells (A.4) were small and did not resemble those of native HCs (n = 1 vestibular, n = 6 cochlear). (A.5) Tail current activation curves taken at −40 mV, for the cells in A.1-A.3 and another cochlear-derived Atoh1-nGFP+ cell (62 DIV). Smooth curves, single-Boltzmann fits. B, Outward currents from vestibular-derived Atoh1-nGFP+ cells (B.1) had activation time courses that separated into two groups (B.2) with time courses of native type I HCs (slow) and type II HCs (fast) (n=3 slow, n=4 fast). C, I\textsubscript{h} was found in 65% of vestibular-derived Atoh1-nGFP+ cells and no cochlear-derived Atoh1-nGFP+ cells (C.1). Vestibular HCs also appeared to express the fast inward rectifier, I\textsubscript{K1} (C.2). In C.2, arrowheads point to fast activation of I\textsubscript{K1} and slower activation of I\textsubscript{h} at −125 mV; arrow points to fast deactivation of I\textsubscript{K1} after step from −125 mV to −45 mV (n = 19 I\textsubscript{K1}, n = 22 I\textsubscript{h}). D, Vestibular-derived (D.1) and
cochlear-derived (D.2) Atoh1-nGFP+ cells responded to current steps with heterogeneous voltage changes that are within the range of results from postnatal HCs (n = 6 vestibular, n = 4 cochlear).
Figure 5. Lgr5+ cells from the postnatal cochlea form HCs but not neurons. A, Schematic cross-section of the OC. Lgr5 is expressed in inner border cells (IBC), inner pillar cells (IPC), and Deiters’ cells (DC). B, Lgr5-tomato cells were present in cultured spheres after 3 passages and before differentiation. C, Lineage-traced Lgr5-tdTomato cells gave rise to HC-like cells that were immunoreactive for myosin VIIA (n=20 MyoVIIa+ cells). D, Lgr5+ cells did not form neurons in sphere culture (n=696 Tuj+ cells). Here antibody to Tuj1 stains a neuron-like cell with processes, and other cells are Lgr5-tdTomato-positive.
Figure 6. Glial cells from the spiral ganglion are capable of producing neurons and CNS glia. 

A, Glial cells (Schwann cells) were lineage traced using the PLP-Cre-ERT mouse. 

B, Glial cells (PLP-tdTomato-positive, red) were maintained through 3 passages (generations, “Gen”) before differentiation. 

C-F, Spiral ganglion-derived glial cells, labeled with tdTomato, were capable of giving rise to: 

C, neuron-like cells (immunoreactive for Tuj1, n = 727 cells); 

D, immature non-myelinating oligodendrocytes (immunoreactive for O4, n = 1299 cells); 

E, mature myelinating...
oligodendrocytes (immunoreactive for O1, n = 1286 cells); F, astrocytes, (immunoreactive for Aldh1L1, n = 531 cells).
Figure 7. Progenitor cells from the neonatal mouse inner ear showed different potency and differentiation capabilities. Single cells were expanded to form spheres, then differentiated by removing growth factors. 

A, Lgr5+ cochlear progenitor cells gave rise to inner (IHCs) and outer (OHCs) cochlear HCs and supporting cells but not neurons. 

B, Postnatal vestibular progenitor cells gave rise to vestibular type I and type II HCs and supporting cells. 

C, Postnatal glial cells of the spiral ganglion (SG) became neurons and CNS glia in addition to Schwann cells. Markers and currents are shown according to the following hierarchy: distinguishing between cell types within a tissue (organ of Corti, vestibular epithelia or spiral ganglion) in blue; distinguishing between two tissues in green; and distinguishing both cell type and tissue type in magenta.
Supplementary Information

Figures

Figure S1. The cochlear and vestibular spheres at the 3rd generation prior to differentiation had no detectable myosin VIIa (MyoVIIa). Expression was seen after differentiation (Fig. 3). The native cochlea was the positive control for myosin VIIa expression. (n=3 cultures)

Tables

Table S1. Mouse lines

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<td>Proteus Biosciences (25-6790)</td>
</tr>
<tr>
<td>vGlut3</td>
<td>HC pre-synaptic protein</td>
<td>1:1000</td>
<td>Guinea pig polyclonal</td>
<td>Chemicon (AB5421)</td>
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<tr>
<td>Prestin</td>
<td>OHC electromotility</td>
<td>1:400</td>
<td>Goat polyclonal</td>
<td>Santa Cruz (sc-22692)</td>
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<tr>
<td>Phalloidin</td>
<td>Actin</td>
<td>1:500</td>
<td>Fluorescent labeled toxin</td>
<td>Invitrogen (R415)</td>
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<td>Calretinin</td>
<td>HCs</td>
<td>1:300</td>
<td>Rabbit polyclonal</td>
<td>Chemicon (AB5054)</td>
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<tr>
<td>Oncomodulin, Ocm</td>
<td>OHC, central-zone VHC I</td>
<td>1:100</td>
<td>Goat polyclonal</td>
<td>Santa Cruz (sc-7446)</td>
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<tr>
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<td>IHC</td>
<td>1:1000</td>
<td>Mouse monoclonal</td>
<td>Sigma (P3088)</td>
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<td>α3 tubulin</td>
<td>Kinocilium</td>
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<td>Mouse monoclonal</td>
<td>Sigma (T7451)</td>
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<td>Thermo Scientific (PA1-915)</td>
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<td>eBioscience (14-6506-82)</td>
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<td>R&amp;D Systems (MAB1326)</td>
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<td>Astrocyte</td>
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<td>Abcam (ab87117)</td>
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