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

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\textbf{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 are restricted in their differentiation capacity, we assessed the phenotypes of differentiated progenitor cells isolated from three compartments of the mouse inner ear – the vestibular and cochlear sensory epithelia and the spiral ganglion – by measuring electrophysiologial properties and gene expression. Lgr5\textsuperscript{+} 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. PLP1\textsuperscript{+} 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.

\textbf{KEY WORDS:} Hair cell, Neuron, Neural stem cell, Cochlea, Vestibular, Spiral ganglion, Mouse

\textbf{INTRODUCTION}

Impaired hearing (15\% of the population) and balance (35\%) associated 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 to be 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 electrophysiologial 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\textsuperscript{+} cells in the neonatal OC caused proliferation of Lgr5\textsuperscript{+} supporting cells as well as their differentiation to HCs (Shi et al., 2013). HC regeneration in the neonate also occurred from Lgr5\textsuperscript{+} 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 investigated 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 PLP1⁺ 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 by cell division (Materials and 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 PLP1, 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

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 third passage. HCs were recognized by their expression of the HC markers Atoh1-nGFP (visualized by GFP fluorescence) and myosin VIIA (visualized by immunostaining). Third-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 h. 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 by confocal microscopy over the entire seeding area. Cochlear spheres that were differentiated for 3 h gave rise to no Atoh1-nGFP-expressing cells; 14 days of differentiation produced an average of 32±12 Atoh1-nGFP⁺ cells (n=3 cultures). For vestibular spheres, 3 h of differentiation produced one Atoh1-nGFP⁺ cell (n=2 cultures), substantially fewer than the average after 14 days (326±294) of myosin VIIA-positive cells derived from cochlear progenitors and 73% (58/79) from vestibular progenitors had protruding structures that were labeled by phalloidin (Fig. 2A–F), which stains filamentous actin, the most abundant protein in stereocilia. Of phalloidin-positive structures, 50% (27/54) of myosin VIIA-positive cells derived from cochlear progenitors and 73% (58/79) from vestibular progenitors were immunoreactive for plasma membrane Ca²⁺-ATPase 2 (PMCA2; also known as Atp2b2; Fig. 2C, red) and primary afferent neurons (gray). HC-like cells produced from vestibular and cochlear compartments were analyzed, and the potencies of Lgr5⁺ cells within the OC and PLP1⁺ glial cells within the spiral ganglion examined.

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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 (Fig. 2I, asterisks) 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.
Fig. 2. Progenitor cells derived from vestibular organs and OC can differentiate into cells that express HC proteins and other typical HC characteristics. On the left are cartoons of the major HC types and some of their key proteins found in vestibular (top) and cochlear (bottom) intact, mature inner ear epithelia. (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 the Ca²⁺-binding proteins parvalbumin (B), oncomodulin (Ocm; D, arrowhead) which is specific to type I vestibular HCs in vivo, and calretiln (Calret; E); phalloidin-positive apical structures that are reminiscent of cuticular plates and stereocilia (A-C); and elongated structures that, like the kinocilium 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; arrowhead shows bundles) cell membrane and arrow points to white structure where three labels colocalize); and elongated structures that, like the kinocilium 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; arrowhead shows diffuse staining in the membrane and arrow points to colocalization of all three labels) 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; outlined), and also form an epithelial-like web of actin around other cells (asterisk), consistent with non-sensory epithelium. Phalloidin stain alone shown in insets in A and F. Scale bars in E,F apply to all micrographs.

To investigate whether any differentiated HC-like cells assume a specialized fate, we labeled them with antibody against prestin (Slc26a5), 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 postnatal day (P) 0 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%). By 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). Ten percent (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 Ca²⁺-binding protein calretiln (calbindin 2) is strongly expressed in immature HCs as well as in mature type II vestibular HCs and cochlear inner HCs (IHCs) (Duchi et al., 1994; Desai et al., 2005; Li et al., 2008). The calretiln antibody labeled one-third of Atoh1⁺nGFP⁺ cells (5/15 cells, one experiment) derived from vestibular tissue (Fig. 2E; Table 1). By contrast, no cochlear Atoh1⁺nGFP⁺ cells (0/26) were calretiln 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 might indicate differentiation limitations in each organ’s progenitor cell population.

Table 1. Incidence of label for HC proteins

<table>
<thead>
<tr>
<th>Staining target</th>
<th>Cochlear derived</th>
<th>Vestibular derived</th>
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<tr>
<td>Phalloidin (actin; hair bundles)</td>
<td>0/1302 Atoh1⁺ cells (0%)</td>
<td>0/1302 Atoh1⁺ cells (0%)</td>
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<tr>
<td>α3 tubulin (kinocilia)</td>
<td>0/285 MyoVIIA⁺ cells (0%)</td>
<td>0/285 MyoVIIA⁺ cells (0%)</td>
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<tr>
<td>PMCA2 (bundls)</td>
<td>0/285 MyoVIIA⁺ cells (0%)</td>
<td>0/285 MyoVIIA⁺ cells (0%)</td>
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<tr>
<td>Oncomodulin (type I vestibular HC)</td>
<td>0/83 MyoVIIA⁺ cells (10%)</td>
<td>0/83 MyoVIIA⁺ cells (10%)</td>
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<tr>
<td>Calretiln (type II vestibular HC, IHC)</td>
<td>0/1302 Atoh1⁺ cells (0%)</td>
<td>0/1302 Atoh1⁺ cells (0%)</td>
</tr>
<tr>
<td>Prestin (OHC)</td>
<td>0/1302 Atoh1⁺ cells (0%)</td>
<td>0/1302 Atoh1⁺ cells (0%)</td>
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N/A, not applicable.
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 bundles (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 with control data from native tissues prepared acutely at P5. Representative gels for these probes are shown in Fig. 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 might indicate differentiation of vestibular type I cells and cochlear IHCs (Fig. 3B).

Differentiated progenitors and native tissues all expressed myosin VIIA, whereas cochlear spheres alone expressed the OHC marker prestin (Fig. 3B). Expression of the calcium-binding protein oncomodulin (Ocm) was detected in both cochlear and vestibular tissues, as expected from 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 otogelin and Lgr5 mRNA, 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 cell types.

Voltage-gated currents in differentiated hair cell-like cells

We conducted electrophysiological experiments on newly differentiated hair cell-like cells to test further 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<sup>+</sup> cells produced large outwardly rectifying currents (e.g. Fig. 4Ac). In the seven Atoh1-negative cells tested (six cochlear, one vestibular), none had HC-like currents (e.g. Fig. 4Ad).

To quantify the voltage dependence of steady-state outwardly rectifying currents, we made and fit tail-current activation curves...
Methods). For both tissues of origin, IKD 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 by Eatock and Hurley, 2003), with means of -21±3 mV (±s.e.m.) and -33±2 mV for three cochlear and five vestibular cells, respectively. Negatively activating K^+ currents (I_{Ko} and I_{Ko,}), which normally emerge in specific hair cells during the pre-hearing period in mice (Géléoc et al., 2004; Marcotti et al., 1999; Rüsch 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, 2003a). The activation time courses of outward currents of seven vestibular-derived cells (Fig. 4Ba) fell into two groups (Fig. 4Bb) 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; Rüsch et al., 1998): four out of 32 cells had strongly inactivating currents (A currents, I_A; Fig. 4Ab) whereas others did not show fast inactivation (Fig. 4Aa).

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, 2011; Rüsch et al., 1998). We observed I_h in 22/34 (65%) Atoh1-nGFP^+ cells derived from vestibular tissue. None of the 32 cochlear-derived cells had detectable I_h (Fig. 4Ca), consistent with native cochlear HCs (Horwitz et al., 2010, 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 probably afterwards, whereas type II vestibular HCs express I_{K1} during the maturation process (Rüsch et al., 1998). Sixty percent (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. 4Cb). Within the cochlea, I_{K1} increases in size in IHCs from embryonic day (E) 15 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. 4Ac); 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. 4Cb). The faster time constant (0.99±0.32 ms, n=5) is comparable to the faster of two time constants expressed I_{K1}: a fast inward current at the start of a hyperpolarizing voltage step, which rapidly deactivated after a depolarizing step (Fig. 4Cb). Within the cochlea, I_{K1} increases in size in IHCs from embryonic day (E) 15 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. 4Ac); they either never expressed I_{K1} or had passed through that stage of differentiation.

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±8 mV (four cochlear-derived cells) and -53±6 mV (six 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; Gélèoc 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 third row Deiters’ 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 (Materials and 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...
colocalization 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 whether Lgr5+ cells could also form neurons, we stained the differentiated cultures for TuJ1 (Tubb3), a tubulin that is highly specific to neurons (Fig. 5D). tdTomato was not colocalized 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).

Because 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 PLP1 (Gomez-Casati et al., 2010), we crossed PLP1-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 PLP1+. 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; Jalencques et al., 1995; Toesca, 1996; Valderrama-Canales et al., 1993).

The PLP1-tdTomato-positive progenitor cells gave rise to many labeled cells in third-generation spheres (Fig. 6B). We used PLP1 to mark spheres in an attempt to determine the potency of the progenitors in the newborn spiral ganglion. After differentiation of the third-generation spheres, we stained the cultures for specific markers of neurons and CNS glia to determine whether the PLP1+ 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 neuron-specific class III β-tubulin (n=727 cells; Fig. 6C) indicated that PLP1+ glial cells formed neurons. We also observed co-staining of tdTomato and an antibody for the early-stage oligodendrocyte marker O4, a sulfated galactosylcerebrosides on non-myelinating oligodendrocytes (Sommer and Schachner, 1981, 1982) (n=1299 cells; Fig. 6D). The PLP1+ cells could also form more mature oligodendrocytes, as indicated by co-staining of tdTomato with antibody to O1, a galactocerebrosides on terminally differentiated oligodendrocytes that are capable of myelination (Sommer and Schachner, 1981, 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 PLP1+ cells could also form astrocytes. PLP1+ 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 (Bramlhall 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 allowed consensus to be met regarding 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 for the regeneration of inner ear cells for therapeutic use.

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 probably 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 PLP1+ 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 fewer 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 et al., 2015). The 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 dorsal root ganglion (DRG), like adult spiral ganglion, gives rise to neurospheres (Li et al., 2007). However, neural crest-derived progenitor cells in DRGs 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 (Gallagher 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. Although 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 (PLP1) for spiral ganglion progenitors, which we also lineage traced. The PLP1+ 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 Ca2+ 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 family member TMC1, whereas vestibular cells expressed both TMC1 and TMC2. This resembles the expression pattern in mature HCs within native tissue (Kawashima et al., 2011; Pan et al., 2013). 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 probably carried by K+, and, in cells from vestibular tissue, additional currents (IKA, I0 and IK1) 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 overexpression in situ causes trans-differentiation of supporting cells to ectopic HCs, which 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, 2014) or Notch inhibition (Doetzlhofer et al., 2009; Mizutari et al., 2013; Pan et al., 2010; Yamamoto et al., 2006). Notch-mediated lateral inhibition (Daudet and Lewis, 2005; Lamford et al., 1999) and innate Wnt signaling are both crucial for the development of HCs (Shi et al., 2013, 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 might 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, 2012), but did not produce neural cell types. We demonstrated that PLP1-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 PLP1+ 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 do 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 six to eight mice (age range P1-4) were carefully extracted from the skull and any brain tissue was discarded. The cochleas and vestibular organs (utricule, 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 the developmental properties of each tissue 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) 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) 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), bFGF (10 ng/ml; Chemicon), IGFl (50 ng/ml; Chemicon) and heparan sulfate (50 ng/ml; Sigma-Aldrich). The spheres were passaged by trituration with a 100-μl pipette, then placed in fresh culture medium. For each experiment, spheres were passaged three 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 PLP1+ cells from the spiral ganglion. Male Lgr5-EGFP-IRES-Cre-ER (jaxmice.jax.org/strain/008875.html) and male PLP1-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 PLP1+ mice.

**Differentiation and treatment of spheres**

To generate differentiated cells, third-generation spheres were plated in 4-well plates (Greiner, Austria) on round 10-mm glass coverslips coated with poly-L-lysine (Trevigen). 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).

**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) according to the manufacturer’s instructions, denatured at 65°C for 5 min, and reverse-transcribed using ImProm II (Promega) 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 third generation were assessed for expression of myosin Vla 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 block 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 (DAPI; Vector Laboratories). Staining was visualized by 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 proteins 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, 0.1 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). Series resistance (Rs) was compensated 20-90% with the intrinsic circuitry of the amplifier. CURRENTS WERE RECORDED WITH A PATCH-CAPLAMP AMPLIFIER (AXOPATCH 200B OR MULTICLAMP 700; MOLECULAR DEVICES). SERIES RESISTANCE (RS) WAS COMPENSATED 20-90% WITH THE INTRINSIC CIRCUITRY OF THE AMPLIFIER.
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 performed with Origin software (version 9; OriginLab Software). 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, i.e. 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 (Eqn 1), where $I(V)$ is current at voltage $V$. $I_{\text{max}}$ and $I_{\text{min}}$ 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 $I$:

$$I(V) = \frac{I_{\text{max}} - I_{\text{min}}}{1 + \exp[(V_{1/2} - V)/S]} + I_{\text{min}}.$$  \(1\)

The time course of current activation was fit with a mono-exponential decay function. Averaged values are presented as mean±s.e.m.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.139840.supplemental

References


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**

<table>
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<tr>
<th>Mouse line</th>
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<th>Background</th>
<th>Source</th>
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<td>N/A</td>
<td>Charles River</td>
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Table S2: Primers for RT-PCR experiments
**Table S3. Primary antibodies**

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