Intrinsic regenerative potential of murine cochlear supporting cells

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The lack of cochlear regenerative potential is the main cause for the permanence of hearing loss. Albeit quiescent in vivo, dissociated non-sensory cells from the neonatal cochlea proliferate and show ability to generate hair cell-like cells in vitro. Only a few non-sensory cell-derived colonies, however, give rise to hair cell-like cells, suggesting that sensory progenitor cells are a subpopulation of proliferating non-sensory cells. Here we purify from the neonatal mouse cochlea four different non-sensory cell populations by fluorescence-activated cell sorting (FACS). All four populations displayed proliferative potential, but only lesser epithelial ridge and supporting cells robustly gave rise to hair cell marker-positive cells. These results suggest that cochlear supporting cells and cells of the lesser epithelial ridge show robust potential to de-differentiate into prosensory cells that proliferate and undergo differentiation in similar fashion to native prosensory cells of the developing inner ear.

The mammalian cochlea is a beautiful and complex organ containing sensory hair cells and a great variety of non-sensory cells. Sensory hair cells are vulnerable to ototoxic insults such as traumatic noise, certain drugs, and the effects of aging. This vulnerability combined with lack of hair cell regeneration is the leading cause of incurable hearing loss affecting hundreds of millions of patients worldwide. Cochlear non-sensory cells are highly specialized, for example to provide mechanical support or essential physiological accessory functions such as regulation of ion homeostasis. In non-mammalian vertebrates, such as birds, the non-sensory supporting cells that are closely associated with hair cells serve as somatic stem cells with ability to regenerate lost hair cells and hearing. Mammalian supporting cells, however, are mitotically quiescent and do not replace lost hair cells. Nevertheless, several reports have shown that cochlear non-sensory cells including supporting cells are able to proliferate and to serve as progenitor cells with the ability to grow into epithelial patches containing hair cell- and supporting cell-like cells. This regenerative potential is rare. Typically less than 1% of the dissociated cells are able to grow into colonies, and only a small fraction of these colonies ultimately give rise to hair cell marker-expressing cells. Furthermore, the proliferative ability of dissociated cochlear cells is transient and it ceases after the second postnatal week in mice.

Here we investigated the proliferative capacity as well as the potential to re-differentiate into sensory epithelia of different cell populations isolated from the neonatal cochlea. We used fluorescence-activated cell sorting (FACS) to purify 4 non-sensory cell populations as well as sensory hair cells. All four non-sensory cell populations showed strong proliferative potential. Notably, the cells representing the lesser epithelial ridge (LER, or outer sulcus) including the organ of Corti supporting cells were not only proliferative but also robustly gave rise to epithelial cell patches harboring hair cell- and supporting cell-like cells. These results suggest that the cells that are closely associated with the sensory hair cells appear to have the strongest intrinsic regenerative capacity, whereas other cochlear non-sensory cells are proliferative, but less capable of serving as hair cell and supporting cell-progenitors.

Results

Purification of different neonatal organ of Corti cell types. To mark different types of supporting cells, we utilized the surface markers CD271, CD326, and CD146, which are differentially expressed in the postnatal day (P) 3 cochlea (Fig. 1a). CD271 (p75 neurotrophin receptor) labels pillar and Hensen’s cells, CD326 (epithelial cell adhesion molecule, EpCAM) was detectable in all supporting cells and sensory hair cells and has previously been shown to be highly expressed in the newborn mouse inner ear, and CD146 (melanoma cell adhesion molecule, MCAM), which labeled the cells of the greater epithelial ridge and afferent neurite-associated cells below the
organ of Corti basilar membrane. Because the expression pattern of all three CD markers was dynamic during neonatal maturation of the organ of Corti, we focused our analyses on organ of Corti preparations from P3, which is within the neonatal two-week period wherein some cochlear cells display in vitro capacity for de-differentiation, proliferation, and multipotency to differentiate into supporting cell- and hair cell-like cells\(^6\).

P3 Math1-nGFP mouse cochlear sensory epithelia\(^6\), consisting of greater and lesser epithelial ridges (GER & LER) including the organ of Corti with nGFP-positive hair cells, were dissociated (Fig. 1b), dissociated into single cells, and labeled with propidium iodide and the three CD marker antibodies. The cell suspension was subjected to FACS purification. 83.6 ± 2.8% of the total input of cells were viable, determined by exclusion of propidium iodide. Only viable cells were collected into 5 distinct populations: GFP\(^+\) cells (Fig. 1c), GFP\(^+\)/CD271\(^{High}\) (Fig. 1c\'), as well as GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{High}\) and GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\), and GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\) (Fig. 1c\''). The sorted populations were re-analyzed via flow cytometry, revealing >95% purity. The different populations were subjected to gene array-based mRNA expression analysis, which confirmed a high degree of homogeneity among the three independent biological samples for each group (Fig. 1d). The gene array data analysis also revealed that each population was distinctly different from the others, showing enrichment of sensory hair cell markers in GFP\(^+\) cells, monocyte/macrophage markers in GFP\(^+\)/CD271\(^{High}\) cells\(^11,12\), as well as LER and supporting cell markers such as BMP4, Prox1, p75, and FGFR3\(^13,14\) in GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{High}\) and GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\) (Fig. 2). GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{High}\) cells expressed genes that are either specific for the GER such as Crabp1/2\(^\dagger\), or strongly enriched in the GER region such as Foxg1\(^\dagger\), or expressed in homologous regions such as Cdhl, which is found in homologous cells in the avian basilar papilla\(^15\). In contrast, the GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\) cell population consisted of cells expressing sub-basilar membrane cell markers including Mpz, Mial, and Agpl\(^16\). Based on this analysis, we hypothesize that flow cytometric cell separation reliably enriched for organ of Corti sensory hair cells, monocytes/macrophages, LER and supporting cells, GER cells, as well as sub-basilar membrane cells (Fig. 3).

Dissociated cells above the basilar membrane display greater proliferative potential than sub-basilar membrane cells. We tested the proliferative potential of the different cochlear cell subtypes in clonal floating colony (sphere) formation assays, in which the cells are cultured non-adherently at low density\(^16\). After 5 days in culture, the GFP\(^+\)/CD271\(^{High}\) and GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{High}\)/CD146\(^{Low}\) cells gave rise to 5.1 ± 0.8 and 5.1 ± 1.4 spheres, respectively, per 300 cells plated (Fig. 4a), which is a 2–4-fold enrichment of sphere formation capacity when compared with our previous reports using cochlear sensory epithelium cells in identical conditions without enrichment of supporting cell subtypes\(^2\). 1.5 ± 0.6 of 300 GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\)/CD146\(^{Low}\) cells (n=6) grew into spheres, whereas only 0.07 ± 0.4 spheres per 300 GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\) cells formed spheres. All spheres, except for irregular shaped ones that formed from GFP\(^+\)/CD271\(^{Low}\)/CD326\(^{Low}\) cells, were initially solid and transitioned into hollow spheres when cultured for longer periods, similar to previous reports of cochlear sphere types\(^9\). When we included 5-ethyl-2'-deoxyuridine (EdU) in the culture media during the first 36h of the sphere formation period, we found that approximately 90% of the sphere cells had incorporated the thymidine analogue during S-phase, which shows that the spheres formed by mitotic cell proliferation (Fig. 4b). At low plating concentrations of 3 cells/μl that are regarded as clonal analysis\(^17,25\), we assume that the majority of spheres that formed were clonal.

Sphere formation assays have been mainly used for isolation of progenitor and stem cells from neural tissue\(^24,26,27\), but the growth potential of epithelial cells might not be adequately revealed with non-adherent assays. Consequently, we employed an attached cell colony formation assay as alternative method to evaluate the proliferative and differentiation potential FACS-isolated cochlear cell populations. The cells were cultured for 3–5 days on mitotically inactivated feeder cells derived from a primary culture of chicken

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**Figure 1** | Dissection of the neonatal organ of Corti into 5 distinct cell populations. a, Immunohistochemical staining with CD271, CD326, and CD146 of middle-turn cryosections of fixed P3 Math1-nGFP cochlea. Primary antibodies were visualized with Cy5-conjugated secondary antibodies (shown in red), the hair cells are nGFP-positive (shown in green). b, Fragment of a typical P3 Math1-nGFP cochlear duct used for cell dissociation and FACS analysis. The lesser (LER) and greater epithelial ridges (GER) are indicated; hair cells are nGFP-positive. c, FACS regimen. 2.3 ± 0.2% of the total viable cells were GFP\(^+\) (green box). c\', 2.6 ± 0.3% of the GFP\(^+\) cells were CD271\(^{High}\) (red box), and c\'', 7.2 ± 0.7% of the GFP\(^+\)/CD271\(^{Low}\) cells were CD326\(^{Low}\)/CD146\(^{Low}\) (light blue box), 21.2 ± 2.0% were CD326\(^{Low}\)/CD146\(^{High}\) (dark blue box), and 63.2 ± 2.9% were CD326\(^{High}\) (yellow box) (n=9). d, Shown is a heat map representing similarity and divergence in the gene expression levels of the 1,000 most divergent genes among 13 array data sets obtained with independent sorted cochlear cell populations.
utricles and the number of colonies identified with antibodies to E-cadherin was counted and assessed for EdU incorporation. GFP+/CD271+/CD326- cells had low capacity for colony formation, whereas cells from the three other populations robustly grew into colonies of 10–50 cells (Fig. 4c–e). GFP+/CD271+/CD146low cells, which were less potent to give rise to spheres (Fig. 4a), were as potent as the GFP+ cells (shown in green) represent the inner and outer hair cells (IHC, OHC), GFP+/CD271low (shown in red) represent monocytes/macrophages, GFP+/CD271low/CD326+/CD146low (light blue) represent LER and organ of Corti supporting cells, GFP+/CD271low/CD326+/CD146high (dark blue) represent GER cells, and GFP+/CD271low/CD326- (yellow) represent cells located below the basilar membrane.

Figure 2 | Selected genes and their maximum differential expression within the 5 groups of FACS-sorted cells. Markers known to be specific for hair cells are indicated with light green, markers known to be specific for other cell types or cochlear regions are indicated as follows: monocytes/macrophages in light red, sub-basilar membrane in light yellow, IER and supporting cells in light blue, and GER in purple. In all cases, the maximum expression indicated by x-fold enrichment over the average of all other populations matched the distinct population, which is indicated with saturated colors.
protrusions displayed the typical staircase architecture normally associated with sensory hair cells (Fig. 5h).

Previous reports showed that hair cell-like cells, derived from cochlear cell populations, differentiate either from proliferating progenitors or they develop by a phenotypical conversion from a differentiated supporting cell. Because the colonies that grew from GFP<sup>+</sup>/CD271<sup>−</sup>/CD326<sup>−</sup>/CD146<sup>−</sup> cells were mostly EdU-positive, we hypothesized that most hair cell-like cells were derived from proliferating progenitor cells. To label hair cell-like cells generated from proliferating progenitor cells, we added EdU throughout the colony formation phase. We found that presence of EdU, even at very low concentration (1 μM), strongly inhibited the occurrence of nGFP-positive cells in GFP<sup>+</sup>/CD271<sup>−</sup>/CD326<sup>−</sup>/CD146<sup>−</sup> cell-derived colonies. The thymidine analog BrdU had a similar detrimental effect, which we had reported previously. We consequently restricted the time period in which EdU was present in the cultures to a 36h period at the beginning of the culture, and we found EdU-positive hair cell-like cells when we analyzed the maturing colonies at day 10 (Fig. 6a). This finding, combined with the evidence that many colonies are derived from single or just a few proliferating cells (Fig. 4c), supports our conclusion that hair cell-like cells differentiate from progenitors that are derived from individual proliferating GFP<sup>+</sup>/CD271<sup>−</sup>/CD326<sup>−</sup>/CD146<sup>−</sup> cells.

**GFP<sup>+</sup>/CD271<sup>−</sup>/CD326<sup>−</sup>/CD146<sup>−</sup>-derived hair cell-bearing patches mature similar to inner ear sensory epithelium.** Inner ear sensory hair cells and supporting cells arise from common progenitor cells. Hair cells are always associated with supporting cells, which express the transcription factor Sox2 that is required for sensory epithelial development and expressed in the vast majority of mouse inner ear supporting cells. During inner ear development, Sox2 is expressed in prosensory cells that give rise to hair- and supporting cells, and it becomes downregulated in nascent and maturing hair cells but remains expressed in supporting cells. After 3 days in culture, Sox2-expressing cells were mainly detectable in colonies derived from GFP<sup>+</sup>/CD271<sup>−</sup>/CD326<sup>−</sup>/CD146<sup>−</sup> cells and increased steadily when assessed after 7, 10, and 14 days in vitro (Fig. 6b). The other inner ear-derived FACS-purified cell populations harbored only few Sox2-expressing cells, which did not increase significantly during the 14 days culture period. In “young” GFP<sup>+</sup>/CD271<sup>−</sup>/CD326<sup>−</sup>/CD146<sup>−</sup>-derived colonies after 3 and 7 days in vitro, we found that many nGFP-positive nascent hair cell-like cells co-expressed Sox2, but usually with lower intensity than surrounding nGFP-negative cells (Fig. 6c). After 14 days in vitro, less than 10% of nGFP-expressing cells were Sox2-positive (Fig. 6c,d), suggesting that Sox2 is initially expressed in hair- and supporting cell progenitors, and becomes downregulated in more mature hair cell-like cells as seen in native developing sensory epithelia.

**Discussion**

In this study, we used antibodies to cell surface proteins to label dissociated cells of the neonatal organ of Corti and closely associated tissues. Cells that were differentially marked with these antibodies were sorted into four populations of non-sensory cells and one sensory hair cell population, which was identified by nGFP expression. Because our main interest was in ascertaining the regenerative potential of different cochlear non-sensory cell populations, we focused on
the four non-sensory populations. Nevertheless, we also tested the survival and proliferation potential of sorted cochlear hair cells, represented by the GFP cell population, and we found that disassociated hair cells were neither able to form colonies nor did they survive culturing on mitotically inactivated chicken utricle stromal feeder cells. Gene array analyses revealed that the four non-sensory populations were distinctively different from each other.

Surprisingly, the cells that labeled with CD271 antibody to p75

**Figure 5** | Hair cell-like cells. a, Upregulation of nGFP in FACS-sorted non-sensory cell populations (n=3–9). Color codes are indicated in Figs. 1 and 2. *** indicates p<0.0001. b, Expression of multiple hair cell markers in a subset of nGFP-positive hair cell-like cells. c, Merge of a light microscopic image with a fluorescent image showing 4 nGFP-positive cells in a colony derived from GFP / CD271 / CD326 / CD146™ cells. d, The same colony as in (c), visualized with SEM. e,f,g, Apical protrusions emanating from the cells labeled in (d) with e, f, and g. The arrow points to a hair bundle-like protrusion in f. In g, the arrow points to a thicker protrusion, a presumptive kinocilium-like structure. h, Higher magnification image of a hair bundle-like protrusion.

**Figure 6** | Hair cell-like cells originate from proliferating and Sox2-positive cells. a, EdU incorporation into hair cell-like cells after 10 days in vitro. b, Number of Sox2-expressing cells in FACS-sorted non-sensory cell populations (n=3–6). Color codes are indicated in Figs. 1 and 2. * and ** indicate p<0.05 and p<0.001, respectively. c, Co-labeling for nGFP and Sox2 in colonies derived from GFP / CD271 / CD326 / CD146™ cells at 7 and 14 days in vitro. d, Fraction of nGFP expression of Sox2-positive cells in GFP / CD271 / CD326 / CD146™ colonies at 3, 7, 10, and 14 days in vitro, indicated in percent.
neurotrophin receptor (GFP+/CD271high) consisted of a population of cells that were enriched for monocytes/macrophages and not the anticipated population of pillar and Hensen’s cells. This conclusion was based on the results of our gene array analyses, which detected extremely high differential expression of monocyte and macrophage markers in this population, although this does not exclude the possibility that other cell types were also present. Absence of pillar and Hensen’s cells in this population was further supported by differential upregulation of mRNA encoding p75 neurotrophin receptor in the GFP+/CD271high/CD326+/CD146low population when compared with the other populations. Thus, pillar and Hensen’s cells were not present in the CD271high population, but were found in the cell population that presumably consisted of supporting cells and LER. The CD271 non-specificity was unexpected because a previous study has used antibodies to p75 neurotrophin receptor for FACS sorting and has demonstrated by quantitative PCR that pillar and Hensen’s cells are starkly enriched. Differences in the enzymatic cell dissociation procedure or in primary and secondary antibody specificity are presumably the culprit for the unspecific labeling that we observed in our experiments. Nevertheless and despite this non-specificity, CD271-labeled cells consisted of a defined cell population highly enriched for macrophage marker gene expression. Since macrophages can be found in the cochlea, particularly after damage11, it seems unlikely that the putative pillar and Hensen’s cells are truly monocytes/macrophages. We have, therefore, speculated that these cells represent a valid group of cochlear cells and we decided to include them in our analysis. Because we suspect that pillar and Hensen’s cells are present in the GFP+/CD271low/CD326+/CD146low population, one could argue that CD271 selection is not necessary for the present experimental procedure to purify the most potent regenerative cell population. Monocytes and macrophages in this case would likely be segregating to the CD326+ cell population.

The three remaining groups of non-sensory cells were initially divided by differential CD326 labeling. Our immunohistochemical analysis confirmed a previous report that the CD326 antigen EpCAM is expressed in all cochlear epithelial cells12,13. Based on this expression pattern, we expected that CD326+ cells consist of epithelial cells of the organ of Corti and adjacent tissues, but not mesenchymal and nerve-associated cells located below the basilar membrane. Conversely, we hypothesized that the CD326+ cell population would consist of cells located below the basilar membrane. Our gene array analysis supports this hypothesis. Within the CD326+ cell population, we further discriminated between cells that expressed higher levels of CD146 and cells that expressed lower levels of CD146. We excluded ~2% of cells whose CD146 expression levels were mid-range, between the CD146high and the CD146low population. Gene array analysis revealed that the CD146high and CD146low populations were distinctively different. Markers for GER cells were upregulated in CD146high cells, whereas all markers that one would expect in organ of Corti supporting cells and the LER were highly expressed in the CD146low population. Interestingly, both cell populations displayed proliferative potential in sphere and colony formation assays, but only CD146low cells were able to generate sensory patches that robustly gave rise to hair- and supporting cell-like cells. The CD146low population consisting of LER and supporting cells, however, was the only cell population derived from the P3 organ of Corti that was able to proliferate and to generate patches of epithelial cells that robustly gave rise to hair and supporting cell-like cells. With regard to the potential of GER cells to generate hair cell-like cells, it is interesting to note that post-natal ectopic expression of Atoh1 in GER cells leads to the differentiation of new hair cell-like cells14, which shows that these cells are certainly competent to differentiate into sensory epithelial cells. Likewise, coculture of GER-derived cells with mesenchymal cells resulted in upregulation of individual hair cell markers in some cells15, a result consistent with our interpretation that GER and also sub-basilar membrane cell populations harbor a few cells with capacity to give rise to hair and supporting cell-bearing epithelial patches. The most potent cell population with respect to hair cell and supporting cell generation, however, was defined by expression of CD326 and low levels of CD146.

Colonies derived from the CD326+/CD146low population consisting of LER and supporting cells did not only express markers for hair cells and supporting cells, but also gave rise to hair cell marker-positive cells with hair bundle-like protrusions. SEM revealed that these hair cell-like cells were not well integrated into the epithelial patches and that the hair bundles were disorganized. Our serum-free culture conditions were obviously sufficient for de-differentiation, proliferation and colony formation, as well as differentiation into sensory patches, but we presume that maintenance of nascent hair cells and proper maturation will require different conditions. In fact, we observed a decrease of hair cell marker-expressing cells when we maintained the cultures with nGFP-positive cells beyond the 14 day differentiation period. This suggests that proper hair cell maturation and survival requires a switch to culture conditions optimized for maintaining in vitro-generated hair cell-like cells. Similar observations were made with embryonic and induced pluripotent stem cell-generated sensory patches16. On the other hand, the culture conditions used for colony generation were sufficient to establish prosensory cell clusters defined by expression of Sox2, increase of the number of Sox2-expressing cells, followed by downregulation of Sox2 in nascent hair cell-like cells identified by expression of nGFP/Atoh1. Downregulation of Sox2 in Atoh1-expressing nascent hair cells has been described as a permissive step for hair cell differentiation17. Observing a parallel process in LER/supporting cell-derived colonies suggests that the cells in these clusters undergo a process of prosensory cell differentiation that is similar to nascent development of inner ear sensory epithelia. It is interesting in this regard that Sox2 expression was initially high in the LER/supporting cell population, based on our gene array analysis, but Sox2 became downregulated after the cells were plated, and then was upregulated in the differentiating epithelial patches. This pattern of disappearing and reappearing of developmentally expressed genes, combined with the proliferative capacity, suggest that these cells underwent a form of de-differentiation. The triggers for this de-differentiation, which does not happen in vivo, are likely to be found in cell dissociation, which leads to a proliferative response, for example, of adult mouse utricle supporting cells18. Growth factors are certainly augmenting this response19.

In conclusion, our study revealed that non-sensory cells of the neonatal organ of Corti and surrounding cochlear tissue have robust proliferative capacity when taken out of the context of the coherent epithelium. The different cell types, isolated by distinct surface markers, were able to de-differentiate and proliferate. The population consisting of LER and supporting cells displayed a stronger ability than other non-sensory cells to proliferate into colonies with nascent sensory patches. The identification of molecular markers that can be used to separate LER cells from supporting cells will be an important next step to further analyze the regenerative potential of different cell types of the cochlear epithelium.
4% paraformaldehyde with 50 mM CaCl₂ and 20 mM MgCl₂ in 0.1M HEPES buffer were conducted under protocols approved by the Animal Care and Use Committee of females to gain large litter size. The resultant Math1/nGFP heterozygous mice passed through a 40 monoclonal mouse antibodies CD146 (1:1000, PE, Biologend, 134704) and CD326 (1:1000, PE/Cy7, Bialou, 118201), and with rabbit polyclonal CD271 primary antibody (1:2000, Chemicon, AB1554). Thereafter cells were collected by centrifugation at 3000 rpm for 5 min, washed once in FACS buffer, and incubated for 25 min with secondary goat anti-rabbit antibody (1:250, Jackson Immunoresearch, 111-495-144). Cells were diluted 1:28 with FACS buffer, and propidium iodide (Sigma) was added at a final concentration of 1μg/ml to label nonviable cells. Cell viability of all experiments combined was 83.6±2.8% (n=9). Cocleahae from wild type CD-1 mice were used to determine background levels of labeling for every sort. FACS was performed with a BD FACSaria III flow cytometer using a 100 μm nozzle (BD Biosciences). Forward and side scattering was used to exclude debris and cell aggregates such as doublets.

Sphere formation. The cells were cultured at a density of 3 cells/μl in serum-free media consisting of DMEM/F12 (Gibco), supplemented with N2 and B27, bFGF (10 ng/ml), IGF (50 ng/ml), heparan sulfate (50 ng/ml), and amniotic fluid mouse antibodies CD146 (1:1000, PE, Biologend) or in 96-well suspension culture plates (Greiner Bio-one). For proliferation assays EdU (Invitrogen) was present at 10 μM for a 36h period at the beginning of the culture. EdU was imaged using Click-it labeling (Invitrogen) according to the instructions provided by the manufacturer.

Adherent colony formation assay and cell differentiation. 5,000 FACS-sorted cells were plated in 4-well plates containing mitomycin C (Sigma) at 0.01mg/ml and 100μl of mitomycin C in DMEM/F12 with 5% FBS for 3hr, washed 3× in media and then used for otic cell differentiation.

Immunocytochemistry. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Non-specific binding sites were blocked for 1hr in 1.0% Triton X-100, 1.0% bovine serum albumin, and 5% heat-inactivated goat serum in PBS. The cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing 3 times with PBS, the cells were incubated for 60 min with fluorochrome-conjugated monoclonal mouse antibodies CD146 (1:1000, PE, Biologend, 134704) and CD326 (1:1000, PE/Cy7, Bialou, 118201), and with rabbit polyclonal CD271 primary antibody (1:2000, Chemicon, AB1554). Thereafter cells were collected by centrifugation at 3000 rpm for 5 min, washed once in FACS buffer, and incubated for 25 min with secondary goat anti-rabbit antibody (1:250, Jackson Immunoresearch, 111-495-144). Cells were diluted 1:28 with FACS buffer, and propidium iodide (Sigma) was added at a final concentration of 1μg/ml to label nonviable cells. Cell viability of all experiments combined was 83.6±2.8% (n=9). Cocleahae from wild type CD-1 mice were used to determine background levels of labeling for every sort. FACS was performed with a BD FACSaria III flow cytometer using a 100 μm nozzle (BD Biosciences). Forward and side scattering was used to exclude debris and cell aggregates such as doublets.

Stereocellular feeder cells. Cells were prepared from 20 embryonic day 15 chicken utricles whose sensory epithelia were removed after 40 min treatment with 0.5 mg/ml trypsin/EDTA (Sigma) in DMEM/F12 at 37°C. The pieces of stereocellular tissue were washed in PBS and transferred into a 150 μl drop of 0.125% trypsin/EDTA in PBS and incubated for 5 min at 37°C. After adding DMEM/F12 media supplemented with 10% FBS and 50 μg/ml ampicillin, the cells were gently trituated and cultured until 80%–90% confluence in a 1.25 mm 96-well tissue culture plate (Greiner Bio-one). For proliferation assays EdU (Invitrogen) was present at 10 μM for a 36h period at the beginning of the culture. EdU was imaged using Click-it labeling (Invitrogen) according to the instructions provided by the manufacturer.

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Immunocytochemistry. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Non-specific binding sites were blocked for 1hr in 1.0% Triton X-100, 1.0% bovine serum albumin, and 5% heat-inactivated goat serum in PBS. The fixed cells were incubated overnight at 4°C with diluted antibodies: 1:1000 for monoclonal rat to Uvomorulin/E-cadherin (Sigma), 1:300 for polyclonal goat antibody to Sox2 (Santa Cruz), 1:1000 for polyclonal guinea pig antibody to myosin VIIa, 1:1000 for polyclonal rabbit antibody to espin1, and 1:100 for monoclonal mouse antibody to Uvomorulin/E-cadherin (Pirrotta, et al., 2001). The cells were then blocked with 5% heat-inactivated goat serum (Jackson Immunoresearch) and subsequently incubated with primary antibodies. Nuclei were visualized with 4’,6-diamidino-2-phenylindole (DAPI). Images were acquired with a Zeiss Axioimager fluorescence microscope.

Scanning electron microscopy. The cells were fixed for 4h with 2% glutaraldehyde / 4% paraformaldehyde with 50 mM CaCl₂ and 20 mM MgCl₂ in 0.1M HEPES buffer (pH = 7.4), post-fixed with 1% aqueous OsO₄, dehydrated in a graded ethanol series, and finally dried by critical point drying with liquid CO₂ (Autosamdri-815 from Toyo-Soda, Makuhari). After specimens were coated with 100 nm of gold using a Denton Desk II Sputter Coater, and viewed with a Hitachi S-3400N variable pressure SEM operated under high vacuum at 5–10 kV at a working distance of 7–10 mm. Chemicals were supplied by Electron Microscopy Sciences (Hatfield, PA).
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Author contributions
STS and SH conceived the study and wrote the manuscript. STS, SH, TAJ, BHH, AGC, and KO participated in study design. STS, RL, and KO conducted the experiments shown in Figs. 1a,b. STS, RC, TAJ, RL, FG, WS, and KO conducted the experiments shown in Figs. 1c, 4, 5 and 6. STS, RL, and BHH conducted the experiments shown in Figs. 1d and 2. AGC prepared Fig. 3. All authors reviewed and edited the manuscript.

Additional information
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