Tympanic border cells are Wnt-responsive and can act as progenitors for postnatal mouse cochlear cells

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SUMMARY
Permanent hearing loss is caused by the irreversible damage of cochlear sensory hair cells and nonsensory supporting cells. In the postnatal cochlea, the sensory epithelium is terminally differentiated, whereas tympanic border cells (TBCs) beneath the sensory epithelium are proliferative. The functions of TBCs are poorly characterized. Using an Axin2lacZ Wnt reporter mouse, we found transient but robust Wnt signaling and proliferation in TBCs during the first 3 postnatal weeks, when the number of TBCs decreases. In vivo lineage tracing shows that a subset of hair cells and supporting cells is derived postnatally from Axin2-expressing TBCs. In cochlear explants, Wnt agonists stimulated the proliferation of TBCs, whereas Wnt inhibitors suppressed it. In addition, purified Axin2lacZ cells were clonogenic and self-renewing in culture in a Wnt-dependent manner, and were able to differentiate into hair-cell-like and supporting cell-like cells. Taken together, our data indicate that Axin2-positive TBCs are Wnt responsive and can act as precursors to sensory epithelial cells in the postnatal cochlea.

KEY WORDS: Stem cells, Regeneration, Development, Inner ear, β-Catenin, Hair cells, Mouse

INTRODUCTION
The integrity of hair cells and supporting cells in the mammalian organ of Corti is required for hearing function. Because the postnatal sensory organ lacks the capacity to repair or regenerate, disorders causing cell loss in the cochlear sensory epithelium (SE) result in permanent hearing loss. In mice, cell proliferation in the developing organ of Corti terminates on embryonic day 14 (Ruben, 1967). Although various sensory and non-sensory cell types continue to mature until hearing function begins in the second postnatal week, these cells remain mitotically inactive. Recent evidence suggests, however, that the postnatal mammalian cochlea contains a relatively uncharacterized population of cells that retain a transient proliferative potential, as measured in vitro by cell-derived floating spheres (Malgrange et al., 2002; Oshima et al., 2007; Savary et al., 2008; Shi et al., 2012; Zhang et al., 2007) or by culturing the cells on specific feeder cells (Chai et al., 2012; Sinkkonen et al., 2011; White et al., 2006). Cultured cochlear cell-derived spheres are thought to be derived from stem/progenitor cells, whereas the sphere-derived cells can differentiate in vitro into cells exhibiting hair cell and supporting cell phenotypes (Oshima et al., 2007). However, the proliferative potential of this endogenous pool of cochlear progenitor cells rapidly declines during the first 3 postnatal weeks (Oshima et al., 2007; White et al., 2006). At present, there is limited understanding of both the origin of these cochlear progenitor cells and the mechanisms that regulate their proliferative capacity, although experimental evidence suggests that a subpopulation of cochlear supporting cells behave as progenitor cells in vitro (Sinkkonen et al., 2011; White et al., 2006).

The Wnt/β-catenin signaling pathway is essential in maintaining homeostasis of many tissues (Logan and Nusse, 2004). Active Wnt signaling marks endogenous stem cells in the gastrointestinal system (Barker et al., 2007; Ootani et al., 2009), integumentary system (Jaks et al., 2008) and the mammary gland (Zeng and Nusse, 2010). Expression of Axin2, a downstream target and feedback inhibitor of the Wnt pathway, reliably reports active canonical Wnt signaling in many tissues (Jho et al., 2002; Lustig et al., 2002). By manipulating the Wnt pathway with exogenous agonists, several investigators have reported proliferation and expansion of resident stem/progenitor cell populations (Kalani et al., 2008; Ootani et al., 2009; Zeng and Nusse, 2010). Although components of the Wnt pathway are expressed in the mammalian postnatal cochlea (Daudet et al., 2002; Shah et al., 2009), their roles in mediating Wnt signaling in inner ear progenitor cells are incompletely understood. Here, we tested whether active canonical Wnt signaling marks and promotes proliferation of cochlear progenitor cells. We found that the Wnt target gene Axin2 marks a previously poorly characterized cochlear cell population: tympanic border cells (TBCs). Both in vivo and in vitro, TBCs retain proliferative capacity, which is promoted by Wnt activation and suppressed by Wnt inhibition. By lineage tracing and culturing sorted cells, we found that Axin2lacZ-positive TBCs are able to generate new hair cells and supporting cells in vivo and in vitro.

MATERIALS AND METHODS
Mice
Wild-type and Axin2lacZ mice (Jho et al., 2002; Lustig et al., 2002) in CD1 background, Pax2-Cre mice (provided by A. Groves, Baylor College of Medicine, Houston, TX, USA) (Ohyama and Groves, 2004), Actin-GFP mice (stock #007075) (Okabe et al., 1997), Actin-DsRed mice (stock #006051) (Vintersten et al., 2004), R26Rep-Cre mice (stock #007576) (Muzumdar et al., 2007) and R26tdTomato mice (stock #007914) (Madisen et al., 2010) (all from the Jackson Laboratory); and Axin2CreERTmice (van
Amorongen et al., 2012) were used. For lineage tracing, pups were injected intraperitoneally with tamoxifen (0.05-1.00 mg/25 g dissolved in corn oil) (Sigma). Intraperitoneal injection of EdU (50 mg/kg; Invitrogen) was performed once per day for 2 days or twice per day for 3 days. The latter regimen, when combined with tamoxifen, caused ~33% lethality. Cochlea were processed for cryosectioning and immunostaining as described below, and EdU detection was performed per product protocol using an Alexa Fluor 555 Imaging Kit. All protocols were approved by the Animal Care and Use Committee of Stanford University School of Medicine.

X-gal staining and cryosectioning
Tissues were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in phosphate-buffered solution (PBS, pH 7.4) for 30 minutes on ice and subsequently washed with 2 mM MgCl₂ (in PBS) before incubation with X-gal reagents at 37°C for 35 minutes. For cryosectioning, cochlea were similarly fixed and stained, then treated in a sucrose gradient (10-30% in PBS). Tissues were serially treated with sucrose/CTC mixture (1:1, 1:3, then 0:1) in a vacuum chamber for 1 hour at room temperature. Tissues were then sectioned at 10 µm and processed for immunohistochemistry. Decalcification with EDTA (0.5 M in PBS) was performed for cochlea from mice aged P7 or older.

Cell sorting
As previously described (Jan et al., 2011), cochlea from P0-P2 Axin2CreERT mice were isolated, with the stria vasculare and spiral ganglia removed before incubation in 0.125% trypsin (Invitrogen; in PBS for 8 minutes) and then in trypsin inhibitor/DNase I cocktail (1:1; 10 mg/ml; Worthington Biochehm). Following trituration, cells were passed through a 40 µm filter and labeled with 3-carboxyumbelliferyl β-D-galactopyranoside (CUG, 1:50 for 25 minutes; Marker Gene Technologies) and propidium iodide (1 µg/ml; Sigma). Wild-type cochlea were used to determine background labeling levels in each sort. Using a BD Aria FACs cytometer (BD Biosciences), we consistently achieved over 90% cell viability and over 93% purity for sorted cells as measured via re-sort analysis. For staining, sorted cells were plated on fibronectin (Sigma)-coated slides (2 hours at room temperature) and removing the SE via thermolysin treatment (0.5 mg/ml in HBSS; Sigma) for 25 minutes; Marker Gene Technologies) and propidium iodide (1 µg/ml; Abcam Bioscience). Following trituration, cells were passed through a 40 µm filter before incubation in 0.125% trypsin (Invitrogen; in PBS for 3 minutes at 37°C). Quantification of propidium iodide-labeled cells found ~70% cell death with this passaging protocol.

Enzyme-linked immunosorbent assay (ELISA)
Cochlea cultured in defined conditions were lysed and homogenized. A BCA Protein Assay kit was used to determine protein levels and a FluorReporter lacZ/Galactosidase Quantification kit (both Molecular Probes) was used to determine β-galactosidase activity. Manufacturer’s protocols were followed.

Immunohistochemistry
Tissues were fixed for 30-60 minutes in 4% paraformaldehyde (in PBS, pH 7.4) prior to incubation with primary antibodies overnight at 4°C. We used antibodies against the following markers: myosin 7a (1:400; Proteus Bioscience), Ki67 (1:400; ThermoScientific), phosphohistone 3 (1:400; Millipore), pancytokeratin (1:200; Sigma), parvalbumin (1:100; Sigma), calretinin (1:1000; Millipore), fibronectin (1:400; BD Biosciences), β-galactosidase (1:100,000; Promega), Jagged 1 (1:800; Santa Cruz Biotechnology), Sox2 (1:200; Santa Cruz Biotechnology), espin (1:1000; a gift from A. Hudspeth, Rockefeller University, New York, NY, USA), Prox1 (1:300; Millipore), E-cadherin, vimentin (both 1:400; Sigma), p27Kip1 (1:400; Fisher Scientific), GFP (1:1000; Abcam) and Brn4 (1:500; a gift from B. Cresslaw, University of Pennsylvania, Philadelphia, PA, USA). The secondary antibodies were conjugated with FITC, TRITC, or Cy5 (1:500; Invitrogen). Images were acquired using epifluorescent or confocal microscopy (Axioplan 2, Zeiss, Germany) and analyzed with Photoshop CS4 (Adobe Systems). Three-dimensional reconstruction was performed using Velocity software (v5.3.0; Improvis).

Whole-organ cultures
Cochlea were cultured as previously described (Chai et al., 2011). Briefly, organs were cultured in growth factor-enriched, serum-free media (GM) consisting of DMEM/F12, N2 (1:100), B27 (1:50; all from Invitrogen), bFGF (1 ng/ml), IGF-1 (50 ng/ml), EGF (20 ng/ml), heparan sulfate (50 ng/ml) and ampicillin (50 µg/ml; all from Sigma) (Oshima et al., 2007) in four-well Petri dishes (Greiner Bio-one) with media replenished every 1-2 days. R-spondin 1 (1 µg/ml; R&D Systems), purified Wnt3a (200 ng/ml), Fz8CRD (25 µg/ml) and EdU (25 ng/ml; Invitrogen) were added. The synthesis and purification of Wnt3a and Fz8CRD have been previously described (DeAlmeida et al., 2007; Willert et al., 2003).

RESULTS

Wnt signaling in the postnatal cochlea
We used an Axin2CreERT reporter mouse (Jho et al., 2002; Lustig et al., 2002) to detect active canonical Wnt signaling in the postnatal cochlea. As a feedback inhibitor, Axin2 is expressed in cells with active Wnt signaling (Jho et al., 2002). We established that Axin2CreERT mice had no detectable phenotypes in the auditory system, as the mice had normal cochlear morphology and function (Fig. 1A; supplementary material Fig. S1A). Analysis of cryosections and whole mounts of neonatal cochlea revealed that TBCs underneath the SE on the basilar membrane express LacZ (Fig. 1A,B; supplementary material Fig. S1E). In situ hybridization demonstrated robust Axin2 expression in the TBCs (supplementary material Fig. S1B) (Chai et al., 2011), corroborating the Axin2CreERT reporter gene activity data. TBCs extend from the lateral cochlear wall to the habenula perforata (Fig. 1B; supplementary material Fig. S1C,D). To further characterize the TBCs, we examined expression of markers of proliferation, hair cells, supporting cells, epithelial cells and mesenchymal cells. In contrast to the P1 SE cells, TBCs were proliferative (Ki67 positive) and did not express markers of
shown to contribute to the cochlear ductal epithelium (Basch et al., 2007; Ohyama and Groves, 2004). However, their relationship with TBCs is unclear. Crossing the Pax2-Cre mouse strain with the R26RmTmG double reporter strain allowed us to examine the identity of cells derived from Pax2 expressing cells. The presence of Cre recombinase initiates expression of mGFP reporter while switching off the mTomato signal. In the P1 cochlea, mGFP-positive cells occupied the majority of the SE, including hair cells and supporting cells, whereas TBCs were not labeled (Fig. 1J; supplementary material Fig. S1F; Table 1). In contradistinction, Brn4 expression is undetectable from the sensory epithelium and robust in the periotic mesenchymal cells in the embryonic period, and was transiently present in TBCs in the early postnatal period (supplementary material Fig. S2A-C) (Ahn et al., 2009; Phippard et al., 1999). We performed lineage tracing of Brn4-expressing cells using the B4OE-Cre;R26RmTmG mouse strain, and found a subset of TBCs traced in the P1 cochlea (not shown). These results show that TBCs originate from Brn4-expressing cells outside the cochlear ductal epithelium.

**Ongoing proliferation in the early postnatal cochlea**

Because the SE continues to mature during the first 3 weeks of postnatal development, we further investigated TBCs during this period. When the thymidine analog 5-ethyl-2’-deoxyxuridine (EdU) was administered for 2 days, robust label uptake was seen in TBCs in the P3 wild-type cochlea (6.8±1.2 EdU-positive TBCs per turn), less frequent uptake in the P9 cochlea (1.8±0.9) and there was rare uptake at P23 (0.7±1.2); at all three ages, there was no uptake into the SE cells (Fig. 2A-D). These data show that TBCs proliferate until the second week of postnatal development, in contrast to the quiescent SE cells. Interestingly, the number of TBCs declined during the first 3 postnatal weeks, and they cease to proliferate between the first and second postnatal week, while the anatomy of this region gradually transitions from a three- to four-cell layered structure at P3 to a one- to two-cell layer by the second postnatal week (Fig. 2A-C,E).

Concomitantly with the decline in proliferation and quantity of TBCs, mRNA expression levels of the Wnt target genes Axin2 and Sp5 in the cochlea decreased, whereas expression of the outer hair cell gene Prestin (Slic26a5) was elevated. Decreased expression of Sox2, a supporting cell marker, correlated with the natural maturation of the organ of Corti (Fig. 2F). The hair cell-specific gene Brn3.1 is important for hair cell specification (Erkman et al., 1996) and its expression levels did not change significantly. In Axin2mTomato/ mouse cochlea, LacZ was expressed at P3 and declined over the subsequent 2-3 weeks (Fig. 2G-J; supplementary material Fig. S1C,D).

These experiments show that TBCs beneath the organ of Corti display a robust but transient proliferative potential during the first postnatal week in vivo, after which proliferation rapidly decreases. This decline in proliferation is accompanied by decreased expression levels of Wnt target genes as well as increased expression of mature cell markers. These observations led to the hypothesis that high levels of Wnt signaling might be indicative of progenitor cell features, and we therefore investigated whether Axin2-positive cells can behave as progenitor cells for the SE.

**A subset of sensory epithelial cells are derived from Axin2-positive tympanic border cells**

The architecture of the organ of Corti is dynamic during the first 3 postnatal weeks, and SE cell types are distinguished by their anatomic locations and specific markers (Fig. 3A-C). Using lineage-tracing

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**Fig. 1. Axin2 expression in tympanic border cells and their lineage origin.** (A) Cryosection of X-gal stained P3 Axin2flox/flox cochlea demonstrating intense LacZ (Axin2) expression among tympanic border cells (TBCs) beneath the basilar membrane (dotted line). Myosin 7a marks outer and inner hair cells (OHCs and IHCs). (B) The location of TBCs in the cochlea in relation to the sensory epithelium (SE). (C) TBCs are proliferative and express Ki67. (DE) Expression of jagged 1 (Jag1) and Sox2 was restricted to SE-supporting cells and absent in TBCs. (F-I) Anti-β-galactosidase antibody was used to detect Sox2 was restricted to SE-supporting cells and absent in TBCs. ( Asterisks) in the P1 galactosidase antibody was used to detect Sox2 was restricted to SE-supporting cells and absent in TBCs. ( Fig. 1G) Anti-β-galactosidase antibody was used to detect Sox2 was restricted to SE-supporting cells and absent in TBCs. (C) The location of TBCs in the cochlea in relation to the sensory epithelium (SE). (D) Whole-mount cochlea from P1 Pax2-Cre;R26RmTmG demonstrating traced mGFP-positive cells in the SE but not in TBCs. (J) Reconstructed cross-section of z-stack images. Asterisk indicates TBCs. BC, Boettcher cells; CC, Claudius cells; HEC, Hensen’s cells; DC, Deiters’ cells; HP, habenula perforata; LER, lesser epithelial ridge; GER, greater epithelial ridge. Scale bars: 25 µm in A,J,J; 50 µm in C-I.

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hair cells (myosin 7a) or supporting cells (jagged 1 and Sox2) (Fig. 1C-E). Furthermore, unlike the SE cells, which expressed E-cadherin and cytokeratin, TBCs were devoid of these epithelial cell markers (Fig. 1F,G). The mesenchymal markers vimentin and fibronectin were also absent in TBCs (Fig. 1H,I). Anti-β-galactosidase antibody was used to detect Sox2 was restricted to SE-supporting cells and absent in TBCs. ( Asterisks) in the P1 galactosidase antibody was used to detect Sox2 was restricted to SE-supporting cells and absent in TBCs. (C) The location of TBCs in the cochlea in relation to the sensory epithelium (SE). (D) Whole-mount cochlea from P1 Pax2-Cre;R26RmTmG demonstrating traced mGFP-positive cells in the SE but not in TBCs. (J) Reconstructed cross-section of z-stack images. Asterisk indicates TBCs. BC, Boettcher cells; CC, Claudius cells; HEC, Hensen’s cells; DC, Deiters’ cells; HP, habenula perforata; LER, lesser epithelial ridge; GER, greater epithelial ridge. Scale bars: 25 µm in A,J,J; 50 µm in C-I.
experiments, we tested the hypothesis that TBCs can act as progenitor cells for the cochlear SE in vivo during this period. We generated an Axin2<sup>CreERT<sub>2</sub></sup> mouse strain (van Amerongen et al., 2012) and crossed it with the R26R<sup>mTmG</sup> reporter strain to examine cells produced by Axin2-positive TBCs. A single injection of tamoxifen on P1 or P3 activated Axin2-driven Cre recombinase to initiate mGFP reporter activity while switching off the mTomato signal 2 days post injection (DPI) (Fig. 3D; supplementary material Fig. S3A,B; Movies 1, 2).

mGFP expression was first observed in the TBCs at 2 DPI and was not detected in cochleae of mice that received drug vehicle alone (Fig. 3D,G). At 5 and 7 DPI, mGFP-positive cells occupied the SE and constituted distinct supporting cell types, which are specified by their anatomical locations (Fig. 3E,H,I; supplementary material Fig. S3C-F). No apical-basal gradient of traced cells was observed (not shown). Quantitative analyses showed that the number of mGFP-positive cells within the population of sensory hair cells, supporting cells, and greater and lesser epithelial ridges (GER and LER) significantly increased from P3 to P8 (*P* < 0.05, Table 1). These cells remained integrated in the P15 cochleae (at 12 DPI) and included Deiters’ and pillar cells (DC/PC, *P* < 0.05), greater epithelial ridge cells (GER, including inner phalangeal cells; *P* < 0.01), and lesser epithelial ridge cells (LER, including Hensen’s, Claudius and Boettcher cells, *P* < 0.01).

Table 1. Quantitative analysis of traced cells*

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Age</th>
<th>OHC</th>
<th>IHC</th>
<th>TBC</th>
<th>DC/PC</th>
<th>GER</th>
<th>LER</th>
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<tbody>
<tr>
<td>Pax2-Cre;R26R&lt;sup&gt;mTmG&lt;/sup&gt;</td>
<td>P1 (n=4)</td>
<td>1254±94</td>
<td>403±39</td>
<td>0</td>
<td>2208±104</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Axin2&lt;sup&gt;CreERT&lt;sub&gt;2&lt;/sub&gt;&lt;/sup&gt;;R26R&lt;sup&gt;mTmG&lt;/sup&gt;</td>
<td>P3 (n=7)</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>2.7±10.5</td>
<td>290±77</td>
<td>53.2±39.9</td>
</tr>
<tr>
<td></td>
<td>P8 (n=5)</td>
<td>1.2±0.8</td>
<td>2.0±0.7</td>
<td>++</td>
<td>84.0±42.0</td>
<td>1868±99</td>
<td>845±67</td>
</tr>
<tr>
<td></td>
<td>P8 (n=2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

*Cell counts per cochlea. Data are means±s.d. In comparison with those of P3, cochleae from P8 Axin2<sup>CreERT<sub>2</sub></sup>;R26R<sup>mTmG</sup> mice show significantly more traced outer hair cells (OHC, *P* < 0.05), inner hair cells (IHC, *P* < 0.01), Deiters’ and pillar cells (DC/PC, *P* < 0.05), greater epithelial ridge cells (GER, including inner phalangeal cells; *P* < 0.01), and lesser epithelial ridge cells (LER, including Hensen’s, Claudius and Boettcher cells, *P* < 0.01).†Tamoxifen (0.05 mg/25 g) administered at P1. §Corn oil only. ++, robust labeling; TBC, tympanic border cells.

![Fig. 2. Proliferation and Wnt signaling decreased with age in the postnatal cochlea.](image) (A-C) Cryosections of cochleae from mice administered EdU once daily for 2 days. EdU-labeled TBCs decreased from P3 to P9, and further decreased from P9 to P23. (D) Quantification of EdU-positive TBCs (n=3-10). (E) The number of TBCs (between the lateral cochlear wall and habenula perforata) decreases with age, n=6 for each age. (F) Using RT-PCR and qPCR, cochleae demonstrated a significant decrease in the Wnt target genes (Axin2 and Sp5) (P<0.005 for both) between P2 and P15; experiments were carried out in triplicate. Levels of the hair cell marker (Bm3.1) remained constant (P=0.24). Levels of the supporting cell marker (Sox2) decreased (P<0.05) and those of the outer hair cell gene Prestin increased (P<0.001). (G-J) Axin2<lacZ> expression in the postnatal cochlea decreased with age. Scale bars: 25 µm in A-C, 200 µm in G-J. Data are means±s.d. Asterisk indicates statistical significance.
and stereociliary hair bundles on their apical surfaces (Fig. 3J-L; supplementary material Fig. S3E,F; Movies 1, 2). Outer hair cell stereocilia are organized in a V-shaped pattern, which is observed in mGFP-positive outer hair cells (Fig. 3L; supplementary material Movies 1, 2). The finding that both inner and outer hair cells were mGFP-positive further supports the idea that TBCs have the potential to generate multiple cell types in the SE.

To examine the possibility that delayed Cre activity could lead to reporter activity within the SE, we administered tamoxifen at P8 and found mGFP expression limited to TBCs 2 DPI (supplementary material Fig. S3J), confirming tight regulation of the Axin2 CreERT2 activity. Administration of EdU (50 mg/kg twice daily, P0-2) revealed EdU-positive cells among the TBCs (15.5±3.8%), but none in the organ of Corti at P3 (Fig. 3M; Table 2). Among cells in the lesser (LER) and greater epithelial ridges (GER), 5.0±0.5% and 0.02±0.02% were EdU positive, respectively (n=3). P8 and P15 cochleae revealed that the EdU-labeled cells occasionally contributed to organ of Corti cells (Fig. 3N,O). At P8, EdU labeled significantly more cells: 29.1±6.3% TBCs, 8.6±1.5% GER cells and 2.0±0.5% LER cells (P<0.001 for all) (n=4). At P8, we detected three EdU-positive cells in the organ of Corti. Concurrent EdU and tracing experiments identified occasional EdU, mGFP double-positive SE cells at P8, but also untraced EdU-positive cells within the SE (supplementary material Fig. S3M-O).

### Table 2. Quantitative analysis of EdU-labeled cells*

<table>
<thead>
<tr>
<th>Regions</th>
<th>P3 (n=3)</th>
<th>P8 (n=4)</th>
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<tbody>
<tr>
<td>TBC</td>
<td>15.5±3.8%</td>
<td>29.1±6.3%</td>
</tr>
<tr>
<td>Organ of Corti</td>
<td>0%</td>
<td>0.05±0.7%</td>
</tr>
<tr>
<td>GER</td>
<td>0.02±0.02%</td>
<td>2.0±0.5%</td>
</tr>
<tr>
<td>LER</td>
<td>5.0±0.5%</td>
<td>8.6±1.5%</td>
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*Values represent average percentages of EdU-labeled cells (±s.d.) among all DAPI-positive cells within each region: organ of Corti (hair cells, Deiters' cells and pillar cells), tympanic border cells (TBCs), greater epithelial ridge (GER, including inner phalangeal cells) and lesser epithelial ridge (LER, including Hensen's cells, Claudius cells and Boettcher cells). P0-2 wild-type mice were injected with EdU (total of six injections) and cochleae harvested at P3 or P8. Cell counting was performed on each cochlear turn in sections. No apical-basal gradient was detected. The number of EdU-labeled cells significantly increased from P3 to P8 in the TBCs, GER and LER (P<0.001 for all).
Together, these data suggest that Axin2-positive TBCs are multipotent progenitor cells in the postnatal mouse cochlea.

**Isolated Axin2hi cells from the cochlea form clonal colonies**

To test the in vitro potential of Axin2-positive TBCs, we used flow cytometry to isolate cochlear Axin2-expressing cells for further analyses (Jan et al., 2011). Using cochleae from P0-2 Axin2lacZ/+ mice, we labeled and isolated the most fluorescent cells (top 16.1±0.6%), expressing high levels of the Wnt target genes Axin2 and Sp5, and the fewest fluorescent cells (bottom 23.2±2.3%), which expressed hair cell (Brn3.1) and supporting cell (p27Kip1 and Sox2) genes (Fig. 4A,B,H; supplementary material Fig. S4A). We designed parameters to isolate the least fluorescent cells to minimize contamination and allow comparisons between the two groups based on the levels of Axin2 expression. These two groups are henceforth referred to as Axin2hi and Axin2lo cells, respectively. To confirm cell purity, we immunostained cells immediately post-sort and found that the Axin2hi cells contained over 93% β-galactosidase-positive cells, 0% myosin 7a-positive hair cells, 0% Prox1-positive supporting cells, 75% Brn4-positive cells and <0.1% other supporting cell types (Sox2-, Jag1- or p27Kip1-positive; n=3 sorts with 2500-5000 DAPI-positive cells analyzed) (Fig. 4C-G; supplementary material Fig. S4B-F).

We first compared the proliferative capacity of Axin2hi and Axin2lo cells using a colony formation assay: cells were seeded in serum-free, N2/B27-supplemented media onto feeder cells harvested from embryonic chicken utricles (supplementary material Fig. S4G). The use of embryonic otic mesenchymal tissues and deprivation of growth factors has been shown to promote differentiation of inner ear progenitor cells (Chai et al., 2012; Doetzlhofer et al., 2004; Oshima et al., 2010; Sinkkonen et al., 2011; White et al., 2006). After 10 days in vitro, Axin2hi cells formed cytokeratin-positive colonies more frequently than Axin2lo cells (88.2±19.4 versus 22.0±6.2, P<0.0001, n=5). (K-M) Axin2hi cells isolated from both Actin-GFP-positive Axin2lacZ/+ cochleae and Actin-GFP negative Axin2lacZ/+ cochleae were mixed 1:1 and cultured. After 10 days, 94% of cytokeratin-positive colonies were monochromatic, suggesting Axin2hi colonies were highly clonal (n=3). Scale bars: 25 µm. Data are mean±s.d. Asterisks indicate statistical significance.

**Fig. 4. Purified Axin2hi cells display robust proliferative capacity.** (A) P0-P2 Axin2lacZ/+ cochleae were dissociated and labeled with propidium iodide (PI) and CUG to allow for isolation of PI-negative (viable), CUG-positive cells. The flow cytometry plot depicts gates used for isolation of the Axin2hi (red) and Axin2lo (green) cells. (B-H) Isolated CUG-positive cells robustly expressed the Wnt target genes Axin2 and Sp5, and not the hair cell marker Brn3.1 or the supporting cell markers p27Kip1 and Sox2 (P<0.001 for all). Experiments were carried out in triplicate. Immunostaining of CUG-positive cells shows 93.4±0.6% β-gal-positive, 74.9±2.6% Brn4-positive, 0.0% myosin 7a-positive hair cells (0.0%) and rare (<0.1%) (Sox2-, jagged 1-, p27Kip1-, E-cadherin or Prox1-positive) supporting cells. (I,J) After 10 days in vitro, Axin2hi cells formed cytokeratin-positive colonies more frequently than Axin2lo cells (88.2±19.4 versus 22.0±6.2, P<0.0001, n=5). (K-M) Axin2hi cells isolated from both Actin-GFP-positive Axin2lacZ/+ cochleae and Actin-GFP negative Axin2lacZ/+ cochleae were mixed 1:1 and cultured. After 10 days, 94% of cytokeratin-positive colonies were monochromatic, suggesting Axin2hi colonies were highly clonal (n=3). Scale bars: 25 µm. Data are mean±s.d. Asterisks indicate statistical significance.
demonstrate that isolated TBCs from the postnatal cochlea have colony-forming capacity.

**Axin2hi cells differentiate into hair cell-like and supporting cell-like cells in vitro**

The cochlear SE comprises two main cell types: supporting cells and hair cells. After 10 days, colonies derived from the Axin2hi cells uniformly expressed E-cadherin and cytokeratin, both epithelial markers expressed in the SE (Fig. 5A-D). This observation suggests that the isolated TBCs are able to adopt epithelial characteristics in vitro, similar to their behavior in vivo. In the postnatal organ of Corti, Sox2 is a marker of supporting cells and Prox1 is expressed in two subtypes of supporting cells (Deiters’ cells and pillar cells) (Fig. 1E; supplementary material Fig. S6F) (Bermingham-McDonogh et al., 2006; Oesterle et al., 2008). Colonies derived from Axin2hi cells expressed Sox2 and Prox1 (77.5±3.2% and 18.2±0.6%, respectively) with Prox1-positive cells always co-expressing Sox2 (Fig. 5A-B). In addition, jagged 1 was also highly expressed (74.3±3.5%) in TBC-derived colonies (Fig. 5D). Control experiments ascertain that chicken mesenchymal cells do not express used markers: cytokeratin, Prox1, jagged 1, myosin 7a, Sox2 or E-cadherin (supplementary material Fig. S4H-K). As TBCs do not express Sox2, Prox1, jagged 1, E-cadherin or cytokeratin in vivo (Fig. 1D-G), the finding that their progeny can express these markers in vitro implies that isolated TBCs have an enhanced potential to acquire an epithelial phenotype and display markers that are specific for SE supporting cells.

Within the cochlear SE, hair cells are marked by antibodies to myosin 7a (Hasson et al., 1997) and parvalbumin (Hackney et al., 2005). The apical surfaces of hair cells contain highly ordered stereocilia bundles and cuticular plates, which are enriched in filamentous actin and espin, an actin-bundling protein that is essential for hearing function (Zheng et al., 2000). Myosin 7a-positive hair cell-like cells were infrequently noted among Axin2hi colonies (6.9±1.4%); when present, they were always juxtaposed to Sox2-positive cells (Fig. 5A) and detected in 9.4±0.9% of Sox2-positive colonies. Although purified Axin2lo cells initially expressed high levels of the hair cell marker Brn3.1 (Fig. 4B,H), myosin 7a-positive hair cell-like cells were more frequently observed in the Axin2lo colonies after 10 days in vitro (P<0.005) (Fig. 5F). When EdU was present during the first 3 days of the culture period, some of the hair cell-like cells were EdU labeled, suggesting that Axin2hi cells generated new sensory cells via mitotic divisions (Fig. 5E; supplementary material Fig. SSA), although most myosin 7a-positive cells were EdU negative (supplementary material Fig. S5B). The hair cell-like cells also co-expressed parvalbumin and exhibited stereocilia-like structures, as indicated by a polarized expression pattern of filamentous actin and espin (Fig. 5G-I). Interestingly, we observed that a subset of cells within each type of colony remained proliferative (9.2 Ki67-positive cells per Axin2hi colony versus 3.9 per Axin2lo colony; Fig. 5M). Overall, significantly more Ki67-positive colonies were derived from the Axin2lo than from the Axin2hi cell populations (P<0.001) (Fig. 5J-K). Labeling for the M-phase marker phosphohistone 3 further demonstrated that a subset of cells within Axin2hi colonies was...

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**Fig. 5. Purified Axin2hi cells acquire hair cell and supporting cell phenotypes.** (A-D) Axin2hi cells formed colonies uniformly expressed cytokeratin and E-cadherin, whereas 77.5±3.2%, 74.3±3.5% and 15.1±3.2% of all cytokeratin-positive colonies expressed Sox2, jagged 1 and Prox1, respectively (n=3 for each, median=22 Sox2-positive cells, 22 jagged 1-positive cells and 4 Prox1-positive cells per colony). (E) In the presence of EdU, myosin 7a-positive hair cells were observed on cytokeratin-positive colonies (arrow) (n=4). (F) Myosin 7a-positive hair cells were observed more frequently among Axin2lo (median indicates one myosin 7a-positive cell per colony) than Axin2hi colonies (P<0.005, n=4). (G) All myosin 7a-positive hair cell-like cells also expressed parvalbumin. (H) A polarized pattern of filamentous actin on the hair cell-like cells was observed. (I) Two parvalbumin-positive hair cell-like cells with espin expression localized to one pole of the cell. (J,K) Cytokeratin-positive colonies containing actively proliferating Ki67-labeled cells (arrowheads) were noted more commonly in Axin2lo than in the Axin2hi colonies (P<0.001, n=3). (L) Within each Axin2hi colony, a subset of cells expressed the M-phase marker phosphohistone 3 (PH3). (M) There were more Ki67-positive cells in Axin2hi than Axin2lo colonies (P<0.001, n=3). Scale bars: 25 μm in A-E; 10 μm in Fig. 5H; 5 μm in H-I. Data are mean±s.d. Asterisks indicate statistical significance.
proliferating (Fig. 5L). In summary, these findings demonstrate the heterogeneity of colonies originating from the Axin2-positive TBCs; some progeny differentiated into cell types resembling supporting cells and hair cells of the cochlear SE, whereas others remained proliferative. We next investigated whether proliferation of these colony-forming cells is regulated by Wnt signaling.

**Wnt proteins stimulate proliferation of Axin2-positive cells in vitro**

Because Axin2 is a marker of active canonical Wnt signaling, we tested whether addition of Wnt proteins would enhance the proliferative capacity of Axin2-positive TBCs. Wnt3a treatment increased the number of Ki67-positive Axin2<sup>hi</sup> colonies, whereas addition of the Wnt inhibitor Fz8CRD, a soluble analog of Frizzled receptors that can inhibit the activity of Wnt proteins (DeAlmeida et al., 2007), suppressed the formation of Ki67-positive Axin2<sup>lo</sup> colonies (Fig. 6A-E; supplementary material Table S1). Ki67-positive colonies were rare in the Axin2<sup>lo</sup> group and their numbers were not affected by Wnt3a/Fz8CRD treatment, suggesting that the Axin2<sup>hi</sup> and not Axin2<sup>lo</sup> cells were competent to respond to Wnt proteins. Individual Axin2<sup>hi</sup> colonies, but not Axin2<sup>lo</sup> colonies, demonstrated an increase in Ki67-positive cells and in Axin2 expression in response to Wnt3a treatment (Fig. 6A-G; supplementary material Table S1). However, the number of colonies with myosin 7a-positive hair cells did not change significantly with Wnt3a/Fz8CRD treatment (supplementary material Fig. S5C). These data suggest that Wnt proteins promote proliferation and expansion of the Axin2<sup>hi</sup> cells. To further examine this possibility, we cultured purified Axin2<sup>hi</sup> and Axin2<sup>lo</sup> cells from Actin-DsRed-positive Axin2<sup>2loc/z;hi</sup> cochlea in serum-free N2/B27 media containing Wnt3a proteins without other factors. We measured proliferative capacity of the cells by quantifying DsRed-positive colonies and found that the Axin2<sup>hi</sup> cell lineage had a significantly higher colony-forming capacity than Axin2<sup>lo</sup> cell lineage (P<0.01, one-way ANOVA). Wnt3a treatment further increased the number of colonies over multiple passages (P<0.05) (Fig. 6H-J). However, Axin2<sup>lo</sup> colonies failed to respond to Wnt3a treatment. After two cycles of expansion, the number of Axin2<sup>lo</sup> colonies increased 319-fold in comparison with those from the Axin2<sup>hi</sup> cell lineage, and this difference further increased to 924-fold with Wnt3a treatment. Similar to their first generation precursors, these expanded colonies from the Axin2<sup>hi</sup> cell population displayed an epithelial phenotype, expressed cytokeratin, and expressed markers for hair cells (myosin 7a) and supporting cells (Sox2) (Fig. 6K,L). All Sox2-positive and myosin 7a-positive cells formed within colonies were Actin-DsRed positive, indicating that they are derived from mouse tissues and not chicken mesenchymal cells (supplementary material Fig. S5D,E). With expansion via passaging, the percentage of Sox2-positive colonies (~60%) was maintained with Wnt3a supplementation (supplementary material Fig. S5F), whereas that of myosin 7a-positive colonies declined (first generation=8.9±0.4%, second generation=5.0±1.2%, third generation=1.2±0.5%; none detected in subsequent generations). These findings lend credence to the notion that Wnt proteins act as growth factors to purified Axin2<sup>hi</sup> TBCs from the cochlea.

We further investigated the effects of endogenous Wnt signals on the Axin2<sup>-</sup>-positive TBCs in cochlear explants, where architecture of the organ of Corti is preserved. We manipulated the Wnt pathway with Wnt3a, R-spondin 1 and Fz8CRD. R-spondin 1 is an alternative Wnt agonist that acts synergistically with endogenous Wnt proteins (Kim et al., 2006; Ootani et al., 2009). Wnt3a or R-spondin 1 treatment robustly enhanced TBC proliferation (Fig. 7A-C; supplementary material Fig. S6A-C; Movie 3). Quantitative analyses of Ki67- or EdU-positive cells indicated that Wnt activation induced a doubling of the baseline proliferation, and that this mitogenic effect was evident for at least 5 days in vitro (Fig. 7D; supplementary material Table S2). Like isolated Axin2<sup>lo</sup> cells, TBCs were competent to respond to exogenous Wnt agonists by showing a marked increase in proliferation, which was not observed in the SE. In addition, proliferation among TBCs was highly dependent on Wnt signaling as Fz8CRD effectively reduced EdU-positive and Ki67-positive cells and suppressed Axin2 expression (Fig. 7E-I; supplementary material Fig. S6D,E; Table S2). We used ELISA to quantify lacZ (Axin2) expression in cochlear cultures and found it to be significantly enhanced by Wnt3a treatment (P<0.01) and...
inhibited by Fz8CRD treatment \( (P<0.005) \) (Fig. 7J). Based on these observations, we conclude that the proliferative capacity of \( Axin2 \)-positive TBCs is dependent on Wnt signaling.

**DISCUSSION**

Sensory hair cells are mechanoreceptors that are required for auditory function; their irreversible loss leads to permanent hearing loss. Previously thought to lack regenerative capacity, the early postnatal cochlea was recently shown to harbor cells with colony-forming capacity and the potential to generate new hair cell-like cells \textit{in vitro} (Chai et al., 2012; Oshima et al., 2007; Savary et al., 2008; Shi et al., 2012; Sinkkonen et al., 2011; White et al., 2006; Zhang et al., 2007). In defined culture conditions, supporting cells isolated from SE exhibit both of these qualities and therefore are prime candidates as progenitor cells (Sinkkonen et al., 2011; White et al., 2006). \textit{In vivo}, we and others have found no evidence of label incorporation in the organ of Corti immediately after EdU injection, suggesting that proliferation is either rare or non-existent. Conversely, robust proliferation is seen among TBCs, a distinct, yet poorly characterized, heterogeneous population that consists of cells with different potentials for proliferation and differentiation. Lineage tracing and thymidine analog labeling results further suggest that the postnatal organ of Corti remains dynamic, contradicting the previous assumption that hair cell formation is complete by E16.5 in mice (Kelley, 2007) and failed detection of proliferated cells in the postnatal organ of Corti (Ruben, 1967).

As demonstrated for somatic stem cells in other organ systems (Barker et al., 2010; Ootani et al., 2009; Willert et al., 2003; Zeng and Nusse, 2010), and recently in the embryonic and postnatal cochlea (Chai et al., 2012; Jacques et al., 2012; Shi et al., 2012), Wnt agonists act as growth factors for TBCs. However, Wnt inhibition decreases the self-renewal capacity of TBCs, but it does not affect the frequency of hair cell differentiation, which may operate in a Wnt-independent manner. Interestingly, we frequently observed supporting cells among the progeny of TBCs \textit{in vitro} as well as \textit{in vivo}, suggesting that TBCs more readily differentiate into supporting cells than into sensory hair cells in both microenvironments. The mechanisms regulating this differentiation are currently unknown and warrant further investigation.

Our lineage tracing and thymidine analog labeling results further suggest that the postnatal organ of Corti remains dynamic, contradicting the previous assumption that hair cell formation is complete by E16.5 in mice (Kelley, 2007) and failed detection of proliferated cells in the postnatal organ of Corti (Ruben, 1967). Instead, our findings correspond with data from other mammalian species suggesting that postnatal hair cell addition occurs (Kaltenbach and Falzarano, 1994; Mu et al., 1997). Given that our results are surprising, it is important to point out that the incorporation of proliferating cells into the SE is rare (Table 2). Moreover, detecting proliferating cells resulted from six injections of EdU over a 3-day period, in contrast to fewer rounds of potentially more cytotoxic thymidine analogs used previously by others (Lee et al., 2006; Ruben, 1967). One candidate mechanism for such cell addition is migration of TBCs across the basilar membrane. In the embryonic inner ear, gaps in extracellular matrix proteins may mediate the process of delamination when neuroblasts migrate out of the otic epithelium (Davies, 2011). Moreover, neuroepithelial derivatives migrate into the otic epithelium through gaps in the basal lamina (Freyer et al., 2011). These gaps are characterized by the absence of fibronectin, which is also observed in the basilar membrane in the neonatal cochlea (Fig. 11). Together, our data support the notion that TBCs may also serve as a pool of progenitors for SE cells in the postnatal cochlea.

Although the current study examines the \( Axin2 \)-positive TBCs as a single cell population, it is more likely that TBCs are a heterogeneous population that consists of cells with different potentials for proliferation and differentiation. Lineage tracing and thymidine analog labeling experiments showed that the progeny of
Axin2-positive TBCs in the P1-3 cochlea appear in the SE in older cochlea, while others remain in the TBC region. Likewise, purified Axin2-positive cells generated colonies with differentiated SE cells and Ki67-positive proliferative cells in vitro. In vivo, the low number of EdU-positive traced cells may have resulted from the stochastic nature of lineage tracing and the limited efficiency of the EdU labeling assay, which may be better addressed by using mosaic analysis with double markers (MADM) in future experiments (Zong et al., 2005).

Our results raise several important questions, including how is cell fate of individual TBCs determined, what is the mechanism underlying the decrease in TBCs during the first 3 weeks of postnatal development, and what are the roles of the Axin2-positive cells that have been demonstrated to surround the embryonic cochlear duct (Chai et al., 2011)? Do TBCs from older animals retain such progenitor cell potentials? It is plausible to consider applying Wnt agonists to TBCs in the adult cochlea in an attempt to rekindle such progenitor cell potentials. Stimulation of progenitor cell capacity in the mature cochlea is of clinical interest, especially in the context of regenerating the damaged organ of Corti. Although it has been reported that TBCs show increased proliferation post-injury through 3H-thymidine uptake in adult mammals (Roberson and Rubel, 1994; Sobkowicz et al., 1992), no regeneration of sensory cells was noted. The current study raises the possibility that enhancing Wnt signaling may promote regeneration of the damaged inner ear, an approach that has shown promise in the context of bone regeneration (Minecraft et al., 2010).

In summary, we have characterized tympanic border cells as Wnt-dependent progenitor cells and have demonstrated their potential to become sensory epithelial cells both in vitro and in vivo. We postulate that TBCs may potentially serve as a reservoir of cells that function as a fail-safe mechanism for the intricate organization of the organ of Corti during early postnatal development. This may explain the proliferative nature of these cells and their subsequent decrease in proliferation and decline in number as the organ matures. Nonetheless, there exists a remnant quiescent population of TBCs in the adult cochlea that may serve as potential targets of regenerative therapeutics. However, any future therapy will require our full understanding of the mechanisms that help guide TBCs to migrate and differentiate into functional sensory cells.

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Competing interests statement
The authors declare no competing financial interests.

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