Directed differentiation of mouse cochlear neural progenitors in vitro

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Lin J, Feng L, Hamajima Y, Komori M, Burns TC, Fukudome S, Anderson J, Wang D, Verfaillie CM, Low WC. Directed differentiation of mouse cochlear neural progenitors in vitro. Am J Physiol Cell Physiol 296: C441–C452, 2009. First published December 17, 2008; doi:10.1152/ajpcell.00324.2008.—Multipotent cochlear neural progenitors (CNPs) in the organ of Corti hold the promise for cell replacement in degenerative hearing disorders. However, not much is known about the CNPs and the specific conditions for their differentiation. Here we isolate the CNPs from the postnatal day 1 organ of Corti in mice and demonstrate their capability to self-renew and to differentiate into hair cell-like and neuronal cell-like phenotypes under the guidance of sonic hedgehog (SHH), epidermal growth factor (EGF), retinoic acid (RA), and brain-derived neurotrophic factor (BDNF), herein termed SERB (abbreviation of SHH, EGF, RA, and BDNF) in an asymmetric or symmetric manner from clonal isolates. Differentiation of CNPs into hair cells by SERB was dependent on the ERK signaling pathway, whereas the differentiation of CNPs into neurons by SERB was not. This work develops a new in vitro methodology for the maintenance and self-regeneration of CNPs for future design of regenerative strategies for hearing disorders.

most nonmammalian vertebrates (amphibians and birds) retain the capability to generate new hair cells throughout life. In chicken and birds, the loss of hair cells triggers the production of new hair cells through mitosis of supporting cells that survive at the lesion site and hearing is restored (7, 27). This is attributed to factors released from damaged cells that trigger hair cell regeneration (8). These inductive factors, however, remain to be elucidated.

In contrast, similar loss of sensory epithelial cells in the mammalian inner ear leads to very limited or no production of new hair cells, which raises the question of whether cochlear neural progenitors (CNPs) or cochlear stem cells exist in adult mammals. It is generally accepted that the production of hair cells normally ceases before birth (7). In recent years, however, hair cell progenitors were isolated from the mammalian organ of Corti shortly after birth (10, 14, 23, 25). Sphere-forming cells, capable of differentiating into hair cells, were successfully isolated from the rat postnatal day 0 (P0) organ of Corti (23), the mouse P1 to P21 organ of Corti (20, 24), and the adult mouse utricle (19, 24), suggesting the existence of cochlear stem cells or CNPs in the organ of Corti. The pluripotency and self-renewal of vestibular stem cells have been shown (19), but the multipotent and renewal capability of cochlear stem cells remains to be determined. Due to this reason, it is plausible to call these sphere-forming cells or otospheres isolated from the postnatal day organ of Corti in mammalians as multipotent neural progenitors or CNPs, instead of cochlear stem cells. In this study, we used clonal analysis of CNPs to demonstrate their multipotency whereby CNPs may contain subpopulations in which one subpopulation differentiates into a distinct phenotype and the other, another distinct phenotype. Stem cells or progenitor cells appear to be quiescent in the normal mammalian organ of Corti and do not respond to damage or lesions. The reason for this is not clear, but it may involve a variety of inhibitory genes (or cell cycle inhibitors) such as p19ink(46) (6, 38), retinoblastoma (Rb1; Ref. 28), and Hes1 (36) that create an adverse situation for stem cell or CNP proliferation and differentiation. However, stem cells proliferate, differentiate, and self-renew in vitro, when isolated from the vestibular tissue of mammals (19), which adds support to the notion that proliferation and differentiation of stem cells or CNPs are inhibited for proliferation and differentiation in the organ of Corti. Therefore, exogenous stimuli of growth factors and cytokines may be needed to remove inhibition and activate the proliferation and differentiation of existing stem cells or CNPs in the mammalian organ of Corti.

What are likely candidate factors for promoting the proliferation and differentiation of stem cells or CNPs? Sonic hedgehog [SHH (S)] is involved in the development of the inner ear (21), and inhibition of SHH bioactivity with specific antibodies results in the loss of the ventral inner ear structure (4), which gives rise to cochlea. Retinoic acid [RA (R)] stimulates the regeneration of mammalian auditory hair cells (17). Epidermal growth factor [EGF (E)] has been shown to stimulate the replacement of hair cells after aminoglycoside ototoxic damage in rat cochlear organotypic cultures (39). In addition, brain-derived neurotrophic factor [BDNF (B)] is an important neurotrophin in the central and peripheral nervous systems (22, 31) that contributes to cell differentiation, neurogenesis, and survival of auditory neurons (31).

In this study, we hypothesized that a combination of the growth factors mentioned above (SERB) may be capable of inducing the proliferation and specification of clonal CNPs into hair cell-like and neuron-like phenotypes. To test this hypothesis, we isolated CNPs from the P1 organ of Corti and used SERB for directing the proliferation and differentiation of CNPs in a two-step protocol in vitro with SERB for 14 days (step 1) and without SERB for 7 days (step 2). We found that expression of hair cell markers in CNPs increased during proliferation in the presence of SERB at step 1 but that profound differentiation did not occur until after withdrawal of SERB at step 2. Importantly, resulting hair cell-like phenotype...
demonstrated some mature morphological features such as hair bundle-like structures. To explore which pathways participate in the generation of hair cell-like phenotype from CNPs, several specific signaling pathway inhibitors (LY294002, PD98059, and SB225002) were used. Results indicated that the ERK pathway is required for the generation of hair cell phenotype.

**MATERIALS AND METHODS**

**Media Used**

For isolation of CNPs from the P1 organ of Corti, DMEM/F-12 + 1% N₂ (GIBCO) was used as the basal media for CNPs in this study. N₂ was added to basal media for selective growth of neural and neuronal cell growth. N₂ was chosen because it has been successfully used for culture of neural stem cells (NSCs) from the brain in our earlier study (30). E + b were added to the above basal media for stimulating CNP proliferation in primary cultures of cells isolated from the P1 organ of Corti (growth media). For maintenance of CNPs after isolation, MEM (Sigma) was used, which is supplemented with serum, etc. (see Table 1: MEM media). MEM media were chosen because of their capability to maintain the rat cochlear progenitor hair cells (25). For “priming” of CNPs toward hair cells or neuronal cells, SERB was added to CNPs for induction of hair cell and neuronal cell markers (induction media). Induction of the CNP differentiation process consisted of a two-step procedure: cells were cultured in the above induction media for 2 wk followed by withdrawal of SERB from the induction media for 1 wk. All the media used in this study are detailed in Table 1. Specific signaling pathway inhibitors for phosphoinositide 3-kinase (PI3K, LY294002), p38 mitogen-activated protein kinase (p38; SB225002), and ERK (PD98059) were purchased from Calbiochem and used for inhibition of the PI3K, p38, and ERK pathways, which are important for cell proliferation and differentiation in the central and peripheral nervous systems. Non-pharmaceutical concentrations used in this study were from our pilot studies and/or referenced studies. NSCs from embryonic mouse brain were cultured as previously described (30).

**CNP Isolation and Maintenance**

The animal use protocol was approved by the Institutional Animal Care and Use Committee at the University of Minnesota. C57BL/6 mice were dissected out from three mice and placed into growth media were used for CNP isolation in this study. Procedures for isolation of cells from the postnatal organ of Corti were performed as previously described (25), with some modifications. Briefly, two organs of Corti from a P1 mouse were dissected out from three mice and placed into growth media (not counting pilot studies). Cells in the specimens were physically dissociated by pipetting, placed into two 35-mm cell culture dishes, and cultured in an incubator with 5% CO₂ at 37°C. Cell culture medium change was made on a 2-day interval basis starting at day 1 and changing at day 3. After 3–5 days of culture, cells in floating or loosely adherent were physically removed and transferred to new culture dishes. Within 7–10 days, single cells gradually grew into multiple cellular spheres. Cells in cellular spheres were dissociated by pipetting and divided into new plates every 7–10 days at a cell density of <1–2 × 10⁴ cells/mm² or 7–10 spheres per 35-mm dish with media changes as above. Simultaneously, cells were grown on eight-well chamber slides or in T-25 flasks on days 1, 5, and 7 for morphology observation or were harvested for evaluation of their cellular identities by RT-PCR and immunohistochemistry. Isolation of CNPs from mice was performed in triplicate, and representative data are presented.

**Clonal Analysis of CNPs**

From the fifth passage culture of CNPs, ~30 single cells were diluted in 18 ml of MEM media, divided into 90 wells (~200 ul per well) of a 96-well plate, and cultured in MEM media until appearance of cell clones, as previously described (25). The experiment was performed in duplicate. Growth of single clones was examined under a contrast microscope on a daily basis. Single clones were counted and documented. After establishment of single-cell clones, CNPs from individual clones were cultured on eight-well chamber slides with 5 μM bromodeoxyuridine (Brdu) added to growth media at the beginning of experiment. CNPs derived from single clones were cultured in growth media for 1, 3, and 6 days (in triplicate) with media changed as above. Cells were fixed with 100% alcohol at room temperature for 6 min on days 1, 3, and 6, respectively. Cellular spheres on the chamber slides were stained with anti-Brdu antibody (sheep IgG; cat. no. ab1895) and secondary antibody (donkey ant SHEEP, Santa Cruz) conjugated to tetramethyl rhodamine isothiocyanate (TRITC) for evaluating proliferating cells and then were stained with DAPI for demonstrating total cell nuclei on slides. For cellular division experiment, 10–100 CNPs per well were plated onto eight-well chamber slides in growth media containing Brdu at 5 μM. Individual adherent CNPs were identified and marked for analysis over time. Cell proliferation was documented under a contrast microscope on a regular basis, three times per day at 8 AM, 12 PM, and 6 PM, respectively, for identification of cell colonies developed from an individual CNP. Cells were fixed with 100% ethanol as above and stained with espin antibody for evaluation of CNPs, myosin VIIa antibody for evaluation of newly proliferated hair cell progenitors, β3-tubulin for evaluation of newly proliferated neuron progenitors, and then they were stained with DAPI for demonstration of cellular nuclei.

**CNP Proliferation and Gene Expression**

Cellular proliferation was determined with 1) viable cell counting by Trypan blue exclusion on day 14; 2) RT-PCR for determination of whether cellular proliferation of CNPs by SERB is associated with upregulation of hair cell markers (myosin VIIa, Math1, Brn3.1, espin, and p19Ink4d) on day 4; 3) measurements of Brdu incorporation on day 14; and 4) progression of cell cycles by flow cytometry on day 14.

Table 1. Cell culture media used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Component</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>Basal media</td>
<td>DMEM/F-12 + 1% N₂*</td>
<td>Isolation of CNPs from the postnatal day 1 ear</td>
</tr>
<tr>
<td>Growth media</td>
<td>DMEM/F-12 + 1% N₂ + EGF + bFGF</td>
<td>Growth of neurospheres</td>
</tr>
<tr>
<td>MEM media</td>
<td>MEM + supplements†</td>
<td>Subcloning and maintenance of CNPs</td>
</tr>
<tr>
<td>Induction media</td>
<td>DMEM/F-12 + 1% N₂ + SERB§</td>
<td>Induction of hair cell and neuronal makers</td>
</tr>
<tr>
<td>Differentiation media</td>
<td>DMEM/F-12 + 1% N₂ + SERB + (+/-)§</td>
<td>Induction of CNP growth and differentiation</td>
</tr>
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* N₂ contained 25 μg/ml bovine insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putrescine, and 30 nM sodium selenite (cat. no. 17502-048; GIBCO) and DMEM/F-12 (cat. no. 11320; Invitrogen). Growth media contained 10 ng/ml EGF and 10 ng/ml basic FGF (cat. no. 236-EG and 3139-FB; R&D Systems). MEM (cat. no. M2279) + supplements contained 20 nM HEPES, 2 mM L-glutamine, 10 mM nonessential amino acid, 0.4 μM hydrocortisone, 5 μg/ml insulin, 2.5 μg/ml transferrin, 10 μg/ml EGF, and 10% FBS (cat. no. 30-2030 and lot no. 3000950; ATCC). SERB contained 5 nM sonic hedgehog (SHH), 10 μg/ml EGF, 10⁻⁶ Trans-retinoic acid (RA), and 10 ng/ml brain-derived neurotrophic factor (BDNF; cat. nos. 461-SH, 236-EG, and 248-BD; R&D Systems; and cat. no. R2625; Sigma). § Differentiation process consisted of a 2-step procedure: cells were cultured in induction media for 2 wk followed by withdrawal of SERB for 1 wk, which allows cochlear neural progenitors (CNPs) to be “primed,” expanded, and then differentiated.
Trypan blue exclusion. Cells were cultured in growth media starting at $5 \times 10^5$ per T-25 flask; treated with and without EGF (E) + basic fibroblast growth factor (b), S, E, R, B, or SERB for 14 days; and harvested for cell counts after staining with Trypan blue as previously described (26).

**Cell cycle progression.** Cells in flasks were treated with and without E + b, S, E, R, B, or SERB for 14 days and harvested for cellular DNA quantitation by flow cytometry and cell counts by Trypan blue exclusion as above. For cell cycle analysis, $-3 \times 5 \times 10^5$ cells were suspended in 100 µl of 40 µg/ml DNase-free RNase A with 100 µl of 200 µg/ml propidium iodide (DNA dye) added, incubated for 30 min at room temperature, and analyzed on an FACS Calibur (Becton Dickinson). Singlets with DNA amount at $2 \times$ were defined as G0/G1 phase cells, $>2 \times$ and $<4 \times$ as S phase cells, $4 \times$ as G2/M phase cells, and $<2 \times$ as sub-G1 cells (e.g., apoptotic cells). Approximately 20,000 cells were measured per specimens, and the experiment was performed in triplicate. Data were analyzed with Flowjo (Tree Star) and are means ± SD.

**BrdU incorporation.** Cells were cultured on chamber slides at $5 \times 10^5$ cells per well in basal media containing 5 µM of BrdU; treated with and without S, E, R, B, or SERB for 4 days; fixed with 100% ethanol for 6 min; and stained sequentially with sheep anti-BrdU antibody (abcam; cat no. ab1893, 1:2,000 dilution) for 60 min, donkey anti-sheep secondary antibody conjugated to FITC (Santa Cruz) for 90 min, and then DAPI, with each step washed by PBS three times. Representative BrdU incorporation data are presented.

**RT-PCR.** Cells were cultured in flasks at $5 \times 10^5$ cells per flask, treated with and without E + b, S, E, R, B, or SERB for 4 days; and harvested for RT-PCR. Briefly, total RNA was isolated from the above harvested cells using RNA MiniPrep kit (Stratagene). Residual genomic DNA in total RNA samples was digested with DNases according to the manufacturer's manual followed by DAPI stain for demonstration of cellular nuclei, and then the number of apoptotic cells (moderate green) per high power field against total cells (DAPI stained) was counted while cells were viewed under a fluorescence microscope. Briefly, cells were cultured on chamber slides as above and stained with YO-PRO-1 dye as instructed by the manufacturer’s manual. The results were expressed as positive cells per 10,000 cells. Nonspecific IgG was used as an antibody control. To study the signaling pathways involved in BrdU differentiation, specific pathway inhibitors were used. Briefly, cells were cultured in flasks; treated with SERB for 14 days in the presence of PD98059, LY294002, and SB225002, respectively, and incubated with basal media (omitting SERB) for 7 days in the presence of PD98059, LY294002, and SB225002, respectively, and followed by incubation with FITC-conjugated secondary antibody on ice for 20 min, washed with 0.5% saponin in PBS, incubated with FITC-conjugated secondary antibody on ice for 20 min, washed with 0.5% saponin in PBS, incubated with and stained with YO-PRO-1 dye as instructed by the manufacturer’s manual. The results were expressed as positive cells per 10,000 cells. Nonspecific IgG was used as an antibody control. To study the signaling pathways involved in BrdU differentiation, specific pathway inhibitors were used. 

**CNP Differentiation.** Cellular differentiation is usually associated with a reduction of BrdU incorporation, cell growth arrest, apoptosis, and terminal cell marker expression and was therefore evaluated by 1) BrdU incorporation, 2) apoptosis assay by YO-PRO-1 stain, 3) cell cycle by flow cytometry, and 4) hair cell marker expression by immunohistochemistry and fluorescence-associated cell sorting (FACS) as well as RT-PCR as described above.

**BrdU incorporation.** Cells were cultured on eight-well chamber slides in T25-flasks; treated with and without S, E, R, B, or SERB for 14 days; then incubated with basal media (without SERB) for 7 days with addition of 5 µM BrdU for the last 2 days; and harvested for evaluation of BrdU incorporation as above.

**Apoptosis studies.** Cells were cultured on chamber slides; treated with and without S, E, R, B, and SERB for 14 days; and then incubated in basal media without S, E, R, B, and SERB for additional 7 days. At the end of step 2, cells were harvested for evaluation of apoptotic cells by YO-PRO-1 stain. YO-PRO-1 (cat. no. Y3603; Molecular Probes/Invitrogen), 4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethylene]-1-(3'-trimethyl ammoniumpropyl)-quinolinium diiodide, is known to selectively pass through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence. Briefly, cells were cultured on chamber slides as above and stained with YO-PRO-1 dye as instructed by the manufacturer’s manual. The results were expressed as positive cells per 10,000 cells. Nonspecific IgG was used as an antibody control. To study the signaling pathways involved in BrdU differentiation, specific pathway inhibitors were used. Briefly, cells were cultured in flasks; treated with SERB for 14 days in the presence of PD98059, LY294002, and SB225002, respectively, and incubated with basal media (omitting SERB) for 7 days in the presence of PD98059, LY294002, and SB225002, respectively, and followed by incubation with FITC-conjugated secondary antibody on ice for 20 min, washed with 0.5% saponin in PBS, incubated with and stained with YO-PRO-1 dye as instructed by the manufacturer’s manual. The results were expressed as positive cells per 10,000 cells. Nonspecific IgG was used as an antibody control. To study the signaling pathways involved in BrdU differentiation, specific pathway inhibitors were used. 

**Evaluation of hair cell marker regulation.** Cells were cultured in flasks; treated with and without S, E, R, B, and SERB for 14 days; then incubated in basal media without S, E, R, B, and SERB for 7 days; and harvested for evaluation of cell cycle (sub-G1, G0/G1, S, and G2/M) as previously described (26).

**Cell cycle evaluation by flow cytometry.** Cells were cultured in flasks, treated with and without S, E, R, B, or SERB for 14 days; then incubated with basal media (without S, E, R, B, and SERB) for 7 days; and harvested for evaluation of cell cycle (sub-G1, G0/G1, S, and G2/M) as previously described (26).

**Evidence for primary cilium formation in cultured cochlear neural progenitors.** Cells in flasks were treated with and without S, E, R, B, or SERB for 14 days; then incubated with basal media (without SERB) for 7 days; and harvested for evaluation of cell cycle (sub-G1, G0/G1, S, and G2/M) as previously described (26).
stained nuclei. Positive cells against total cells were calculated in cell cultures. In a similar manner, dual-labeled immunohistochemistry was performed.

Primary antibodies used in this study were Sox2 (1:400, S9072; Sigma), Math1 (1:100, ab13483; Abcam); espin (1:400, 616156; BD Biosciences); KCNQ1 (1:100, sc-20816; Santa Cruz) myosin VIIa (1:100, ab3481; Abcam); GFAP (1:500, Z0334; Dako); β3-tubulin (1:400, T8660; Sigma); MAP2 (Sigma, 1:500, M4403); nestin (1:100, 556309; BD Pharmingen); and Id1 (1:400, sc-488; Santa Cruz), and secondary antibodies were used FITC or TRITC-conjugated mouse anti-rabbit, goat anti-mouse IgG (conjugated, Sigma). MAP2 and β3-tubulin double-positive cells were identified as immature neurons (β3-tubulin is considered an immature neuronal marker, although it is also present at lower levels in mature neurons; MAP2 is a more mature neuronal marker). Math1 and myosin VIIa double-positive cells were identified as immature hair cells, whereas espin and rhodamine phallolidin (conjugated to TRITC) myosin VIIa double-positive cells were identified as immature hair cell progenitors. To determine the markers expressed by these cells in the P1 organ of Corti were physically dissociated and plated onto two 35-mm culture dishes with 1 ml of E−b containing media per dish (i.e., DMEM/F-12 supplemented with 1% N2, 20 ng/ml of EGF + 20 ng/ml of basic FGF, hereafter termed growth media). In our earlier study, this serum-free growth media favored growth of NSCs from the neonatal brain (30). Compared with previously used media for culturing otospheres (18, 24, 26), this formula is relatively simple, containing no B27 (a similar formula to N2 for neural cell growth and maintenance), leukemia inhibitory factor, or IGF-I. It is believed that this formula favors growth of immature neuroepithelial cells but not for differentiated cells or nonprogenitor cells. CNP cultures reached ~40% confluence within 1 wk in growth media. In these primary cultures, some cells (4.6 ± 2.4%; n = 3 culture plates) were suspended in the culture media or loosely adherent to culture dishes, while the remaining were firmly adherent. Organ of Corti-derived cells are self-renewing and capable of growing into colonies in growth media. Small, round, and bright cells were found in the primary cultures from the P1 organ of Corti and expanded in growth media after depletion of adherent cells (Fig. 2a). Some of them attached to culture dishes (Fig. 2b). Roughly a third of the enriched floating cells (30.3 ± 8.5%; n = 3 culture plates) remained nonadherent and developed into cellular spheres within 7–10 days (Fig. 2c) when plated at a low cell density (1–2 × 10^3 single cells/mm^2) in growth media, while remaining cells adhered to the plate during the culture (Fig. 2d). Organ of Corti-derived cells were

**Fig. 1.** Location of cochlear neural progenitors (CNPs) in the developing organ of Corti in mice. With the use of Id1, nestin, Sox2, and glial fibrillary acidic protein (GFAP) as CNP markers, positive cells were identified from the otocyst epithelium at embryonic day 12 (E12; a–d) and the organ of Corti at postnatal day 1 (P1; e–h). Red arrowhead points to inner hair cell position and white arrowhead to outer hair cell position in the organ of Corti (e–h). It is noted that there are some cells positive for Id1, nestin, Sox2, and GFAP in the supporting cell position (e–h), underneath the nascent hair cells. GFAP staining was stronger in the spiral ganglion (SG) area than in the organ of Corti at P1 (h). Bar = 10 μm.
capable of self-renewal in maintenance media (45 passages at the time of this report) and maintained the same morphology and gene/protein expression profile (see below). These small, bright round cells expressed the primitive neuroectodermal cell markers Id1, nestin, Sox1, Sox2, Msi, GFAP, and Pax6 (Fig. 2e, lane 5) compared with neural stem cells (NSCs; e, lane 3) and NSC colony (e, lane 4). Negative control fibroblasts (Ctrl; e, lane 2) and PCR control (e, lane 1, omitting reverse-transcription enzyme) remained negative. GAPDH, housekeeping loading control. Immunohistochemistry demonstrated that nestin/Sox2 (g), myosin VIIa/Id1 (h), nestin/Math1 (i), and espin/GFAP (j–l) were detected in CNP spheres. Nonspecific IgG was negative (m, immunohistochemistry control). Bar = 10 μm.

Expression of nestin, Sox2, myosin VIIa, Id1, Math1, espin, and GFAP in the cellular spheres was confirmed by immunohistochemistry (Fig. 2, g–m). Both adherent and floating cells observed after enrichment of primary CNPs maintained expression of CNP markers and could be passaged either together or separately. When passaged separately, subsequent cultures from either cell type consistently contained a mixture of both floating and adherent cells, suggesting that adherent cells can give rise to floating cells and that both are CNPs. A higher percentage of cells tended to be adherent when cultures were maintained at a high density or in media that contained serum. Cultures were passaged on a weekly basis; culture media were changed on a 2-day interval. It was noted that adherent CNPs were different from those adherent cells in primary cultures because adherent CNPs expressed the immature neuroepithelial cell markers nestin, Id1, Msi, GFAP, and Sox2, whereas primary adherent cells did not.

Formation of neurospheres in growth media is a hallmark of NSCs. To study whether CNPs are able to form similar neurospheres in a clonal manner, single CNPs were obtained from the fifth passage (adherent CNPs) using a limiting dilution method as previously described (25). Wells containing a single cell were marked for subsequent analysis. A single cell alone failed to grow in growth media; however, it was able to grow in MEM media that contained serum. Thirteen clones were established via this method from a total of 180 wells (2 plates), all of which had similar morphology.

Serum Exposure Induces Clone Formation in CNPs

Cells were consistently adherent in MEM media; however, after clonal cultures were replated in serum-free growth media, they reformed spheres within 7 days in a time-dependent manner. That sphere formation accompanied by cellular proliferation was demonstrated by BrdU incorporation (Fig. 3A; BrdU+ cells overlapped with DAPI), suggesting that sphere formation occurred via proliferation for the majority of the...
Characterization of clonally derived CNPs

If multiple-passaged adherent CNPs are stem cells, they should be able to both self-renew and generate multiple differentiated cell types. In this experiment, we used clonal cultures that were cloned from the passage 5 of CNPs as described above for examination of their capability to differentiate into distinct hair cell progenitors and neuron progenitors. As noted above, clonal cultures could only be obtained in serum-containing MEM media, as isolated single cells failed to self-renew in serum-free growth media. We used this fact to investigate the generation of differentiated progeny from multiple individual CNPs. CNPs from clone #5 were plated at clonal density (10–100 cells) in eight-well chamber slides, cultured in growth media with addition of 5 μM BrdU, and harvested on days 1, 3, and 6 for immunohistochemistry. Espin was used as a CNP marker (37) because it is expressed in the developing cochlear tissues but not in NSCs. Myosin VIIa was used as hair cell progenitor marker (29), and β3-tubulin was used as a newly generated neuron marker. It was found that division of adherent CNPs in growth media generates daughter cells in either an asymmetric manner (8.2 ± 3.5%; n = 12; Fig. 4, d–f) or in a symmetric division manner (91.3 ± 8.4%; n = 12; Fig. 4, a–c). In cells that underwent asymmetric division, cultures began on day 1 with a single CNP positive for espin (Fig. 4g). On day 3, one daughter cell was positive for espin,
while the other was negative for espin (Fig. 4h). By day 6, one cell was positive for β3-tubulin, while the other was positive for myosin VIIa (Fig. 4i). BrdU confirmed that the two cells were newly born (Fig. 4j). In symmetric divisions, a single CNP on day 1 (Fig. 4k) divided into two identical daughter cells on day 3 (Fig. 4l) that expressed myosin VIIa on day 6 (Fig. 4m). These two identical cells were also both BrdU positive (Fig. 4n). These data suggest that a single CNP can produce two distinct cells via an asymmetric division or two identical cells via a symmetric division, thereby confirming the dual potentiality of CNPs after clonal isolation and repeated passaging. In some cases, further proliferation of myosin VIIa positive cells occurred yielding clones containing several myosin VIIa positive cells (data not shown). In all cases, cells maintained an immature morphology in growth media for CNPs even after cessation of proliferation.

These findings reveal that clonal CNPs can undergo further divisions in a symmetric manner to form myosin VIIa positive cells. Given that myosin VIIa expressing cells were capable of continued proliferation, we speculate that these may correspond to committed hair cell progenitors. In these studies, the majority of CNPs (~91%) underwent symmetric division, producing only myosin VIIa or only β3-tubulin positive progeny. A minority of CNPs (~8%) underwent asymmetric divisions, giving rise to both immature neural cells and immature hair cells (progenitors).

SERB Proliferates Clonally Derived CNPs and Induces Their Expression of Hair Cell Markers

These preliminary experiments suggested that clonally isolated CNPs may have the potential to differentiate toward both hair cell and neuronal lineages. We thus devised a protocol that we hypothesized would optimize subsequent differentiation into a hair cell-like phenotype by use of growth factors involved in cochlear development, namely SERB (DMEM/F-12 + 1% N2 + SERB). To test this hypothesis, clonally isolated CNPs grown for three passages in growth media were incubated with SERB in a two-step protocol [e.g., SERB incubation for 14 days (step 1) and withdrawal of SERB for 7 days (step 2)]. Concentrations of SHH, EGF, RA, and BDNF used in this study are based on the referenced studies (2, 16, 17, 19), with some modifications in our study for the induction of hair cell markers (as discussed below, see Supplemental Fig. S1–S3; supplemental data for this article are available online at the Am J Physiol Cell Physiol website.). The factors present in step 1 should specify CNPs toward a hair cell fate, while withdrawal of these factors in step 2 should force cells to undergo differentiation because of withdrawal of growth factors (leading to cell cycle exit). It was found that CNPs in the presence of growth factors (E + b or SERB) were active in proliferation and stayed in an immature state within the step 1. The comparison between step 1 and step 2 was made...
fluorescence-associated cell sorting (FACS) method of quantification. Approximately 37.4 and E in cultures at the end of control or nondifferentiated CNPs (lane 3 by RT-PCR (SERB (lane 3 of cells underwent apoptosis in control basal media (basal), E + b, SHH, EGF, RA, BDNF-treated cells, whereas ~15% (n = 3) of cells underwent apoptosis in SERB-treated cells after withdrawal of SERB (C). *P < 0.05; arrowheads pointing to apoptotic cells. Bar = 10 μm applying all in C.

immunologically, morphologically, and genetically (see Supplemental Figs. S1–S3).

To study whether SERB promotes proliferation in association with the upregulation of hair cell markers, CNPs were incubated with SERB for 4 days and harvested for evaluation of cell proliferation by BrdU incorporation and hair cell marker expression by RT-PCR. It was found that the expression of hair cell markers was increased by day 4 compared with growth media (E + b; Fig. 5A). Cells actively proliferated in media containing SERB, E + b, or E, as evidenced by incorporation of BrdU (Fig. 5B). It is noted that the cyclin-dependent kinase inhibitor (p19^Ink4d) was not expressed in CNPs during SERB treatment, similar to what we had seen with E + b treatment, but was expressed in cells after SERB treatment at the end of step 2. At the end of step 1, flow cytometry was performed for quantitative evaluation of cell cycle progression. The data indicated that the majority of CNPs were in a state of the S or G2/M phase of the cell cycle (Fig. 5C) and that cell numbers were significantly higher in growth media (E + b), SERB-, and EGF-treated cells than in basal media (DMEM/F-12 + 1% N2) controls (Fig. 5D). These data suggest that SERB not only promotes proliferation but also promotes specification toward a hair cell fate.

**SERB Withdrawal Differentiates Clonally Derived CNPs**

CNPs must exit the cell cycle to differentiate. To promote cell differentiation, CNPs were incubated with basal medium (DMEM/F-12 + 1% N2) for 7 days after the 14 days of SERB induction. It is expected that cells are permitted for differentiation after withdrawal of SERB for 7 days. BrdU incorporation indicated that proliferation was significantly reduced in SERB-treated CNPs after withdrawal of SERB compared with controls (Fig. 6A). The majority of CNPs in the SERB-treated group were in the G0/G1 phase at day 7 after SERB withdrawal, with ~15% cells undergoing apoptotic cell cycle arrest (Fig. 6B). To verify the level of apoptosis, CNPs were stained with the apoptotic marker YO-PRO-1 for evaluation of apoptotic cells or caspase 3 for the apoptosis process activation. The results indicated that 3.4 ± 0.5% of CNPs (n = 3) underwent apoptosis in the basal media control that received no growth factors during step 1 or 2, whereas 15.3 ± 3.5% of CNPs (n =

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**Fig. 6.** Cell proliferation is decreased after withdrawal of SERB. At the end of step 2, the percentage of BrdU-labeled cells was significantly lower in SERB-treated cultures than in E + b, SHH-, EGF-, RA-, BDNF-treated or control (Ctrl, no factor) cultures (A) by immunohistochemistry. The number of S and G2/M phase cells in cultures was significantly lower in SERB-treated cells from which SERB was withdrawn in the last 7 days than in E + b, SHH-, EGF-, RA-, BDNF-treated, or basal media-treated control cells (B) by flow cytometry. YO-PRO-1 stain on cell cultures indicated that ~3% (n = 3) of cells underwent apoptosis in control basal media (basal), E + b, SHH, EGF, RA, BDNF-treated cells, whereas ~15% (n = 3) of cells underwent apoptosis in SERB-treated cells after withdrawal of SERB (C). *P < 0.05; arrowheads pointing to apoptotic cells. Bar = 10 μm applying all in C.

**Fig. 7.** CNPs differentiate into hair cell- and neuron-like cells upon withdrawal of SERB. Cobblestone-like islands and neuron-like cells were found in cell cultures 7 days after withdrawal of SERB (A). The expression of myosin VIIa, Bm3.1, Math1, espin, p19^Ink4d (Ink4d), TRPA1, and KCNQ mRNA transcripts was upregulated but nestin downregulated at the end of step 2 (lane 4) compared with those in original CNPs (lane 2) and/or 4-day cultures with treatment of SERB (lane 3) by RT-PCR (B). Both espin and F-actin positive structures were occasionally seen in culture (C). The coexpression of both espin and KCNQ1 was also observed (F). Approximately 9.7 ± 1.6% were both myosin VIIa and Math1 positive (D and G) and ~11.8 ± 2.3% both β3-tubulin and MAP2 positive (E and G) at the end of step 2 by immunohistochemistry (IHC). In contrast, more positive cells were found in SERB-induced cell cultures using the more sensitive fluorescence-associated cell sorting (FACS) method of quantification. Approximately 37.4 ± 4.3% of cells were myosin VIIa and Math1 double positive (H and I) in cultures at the end of step 2, whereas ~12.4 ± 2.3% of cells were β3-tubulin and MAP2 double positive (H and J) at the end of step 2. C, nonspecific IgG control or nondifferentiated CNPs (I and J). Ctrl, control (no SERB induction).
3) underwent apoptosis 7 days after SERB withdrawal from cell cultures (Fig. 6C).

Differentiation occurred in step 2, with the appearance of neuron-like and cobblestone-like cells (Fig. 7A). RT-PCR revealed upregulation of myosin VIIa, Math1, Brn3.1, espin, TRPA1, and KCNQ at the end of step 2 relative to cultures 4 days into step 1 or untreated cultures, while nestin expression decreased in step 1 and remained low at the end of step 2.
relative to CNP cultures in growth media (Fig. 7B). Continued expression of nestin by PCR after step 2 may reflect persistence of some undifferentiated CNPs in cultures. Differentiated cells were nestin negative by immunocytochemistry (data not shown). Remarkably, some cells showed highly mature morphology with espin and F-actin in hair bundle-like structures (Fig. 7C).

By immunocytochemistry, cobblestone-like cells expressed myosin VIIa and Math1 (Fig. 7D) and coexpression of ion channel (KCNO1) and espin (Fig. 7F). The β3-tubulin and MAP2 were observed in cells with immature neuron-like morphology (Fig. 7E). Some cells at the end of step 2 (9.7 ± 1.6%, n = 3) clearly expressed both myosin VIIa and Math1, while others (11.8 ± 2.3%, n = 3) expressed both β3-tubulin and MAP2 by immunocytochemistry (Fig. 7G). Nonspecific IgG control antibody staining is shown in Supplemental Fig. S2. We used FACS on 10,000 cells to quantify the fraction of neural and hair cells: 37.4 ± 4.3% of cells (n = 3) were both myosin VIIa and Math1 positive and 12.4 ± 2.3% of cells (n = 3) were both β3-tubulin and MAP2 positive (Fig. 7, H–I). The difference of percent positive cells between immunohistochemistry and FACS is explained by the fact that the two blind observers counted only highly positive cells, while FACS detects cells with lower levels of expression. Hair cell-like differentiation was accompanied by upregulation of p19Ink4d expression (Fig. 7B), which drives cells out of the cell cycle and prevents them from entering the cell cycle again (6).

**DISCUSSION**

In recent years, sphere-forming cells have been isolated from the rat and mouse postnatal organ of Corti and have been shown to differentiate into myosin VIIa positive cells or primitive hair cell-like cells in vitro (23, 24). However, these sphere-forming cells are renewable for approximately 7–10 passages (19, 24). These studies prompt us to examine whether there are self-renewable cells that can extend to indefinite passages and possess the similar potential to differentiate into hair cells and neurons that are lost in degenerative hearing disorders, assuming that the both spiral ganglion neurons and hair cells share the same stem cell/progenitor derived from the otocyst epithelial cells that are positive for nestin, Sox2, GFAP, and Id1. In addition, we would like to know whether conditions are capable of differentiating into distinct phenotypes: neural and hair cell-like phenotypes at the single cell level, which are the most desired for cell replacement in sensorineural hearing loss. We further show that CNPs cultured under these conditions are able to expand more than 45 passages, like a cell line, with their morphology, gene expression profile, and multipotency remaining unchanged after clonal isolation and extensive cultivation. Our data suggest that these CNPs are located in the otocyst epithelium and P1 organ of Corti. Perhaps, this is a potential lineage for cell replacement in degenerative hearing disorders.

CNPs are capable of growing in DMEM/F-12 when mixed with 1% N2, a component for neural and/or neuronal growth in vitro, and supplemented with neuroepithelial growth factors (EGF and/or basic FGF), which have been shown to induce the

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**ERK Pathway Differentiates CNPs Into Hair Cells**

In recognition of the importance of EGF in proliferation of CNPs, we specifically blocked the ERK pathway with PD98059 (Fig. 8A). The ERK pathway is extensively involved in cell proliferation and differentiation, especially in the central nervous system. For experimental controls, two other signaling pathways: p38 and PI3K involved in cellular differentiation and survival were blocked with specific inhibitors (SB22502 and LY294002, respectively). Briefly, cells were incubated with SERB in the presence of the ERK, p38, and Akt pathway inhibitors (PD98059, SB225002, and LY294002, respectively; each at 20 μM) for 14 days followed by basal media for 7 days with pathway inhibitors but without SERB and were harvested for quantitative evaluation of hair cell and neuronal cell marker expression by FACS. It was found that PD98059 significantly reduced the expression of Math1, myosin VIIa, and espin but not MAP2 and β3-tubulin at the end of step 2 (Fig. 8B). SB225002 showed a significant enhancement in MAP2 but not others (Fig. 8B), whereas addition of LY294002 to cell cultures resulted in a significant increase in espin and MAP2 (Fig. 8B). These data suggest that the ERK pathway significantly reduces the number of CNPs adopting a hair cell fate, without reducing the number of cells that adopt a neuronal fate.
generation of neuroepithelial cells from embryonic stem cells in an independent study (19), or of growing cellular spheres from the postnatal day (1–21) organ of Corti, stria vascularis, and spiral ganglion tissues with addition of B27 (24). However, the growth media do not favor the growth of a single CNP nor self-renewal of CNPs. A new method for the maintenance of CNPs has been described in this study, namely, growth media for selecting CNPs from primary cultures followed by MEM media for clonal isolation and self-renewal in vitro. The growth media do not support the growth of CNPs for an extended period of time unless supplemented with additional factors such as SHH, RA, and BDNF. Sphere-forming cells disappear in the mouse P21 day organ of Corti (24) possibly due to the lack of robust growth factor and cytokine support. Our data indicate that sphere-forming cells and multiple-passaged adherent CNPs share the self-renewal capability and the potential to differentiate into distinct phenotypes in vitro. This suggests that these CNPs, irrespective of passages, are renewable and inducible. This is the first demonstration of renewable CNPs in vitro for >45 passages.

The stem cell identity of the CNPs described in this study is evidenced first by their capability to clonally generate cellular spheres from single CNPs. CNPs express multiple neuroepithelial cell markers: nestin, Sox2, GFAP, and Msi, a gene expression profile similar to that of NSCs neurospheres, although additional genes such as espin and Pax2 are also expressed in CNPs. Second, these CNPs self-renew while maintaining their original neuroepithelial cell marker expression profile, morphology, and behavior. Third, these CNPs are capable of differentiating into distinct phenotypes at the single cell level even after extended cultivation, yielding at least two distinct phenotypes including neural cell- and hair cell-marker positive cohorts from single cell-derived cultures. These cells are en route to terminal differentiation but are not considered as mature because mature cell markers for a neuron (SA100 and Neu) are not detectable and hallmark morphology for a hair cell (hair bundle) is extremely rare. The capability of CNPs to differentiate into supporting and glial cells is an intriguing aspect that needs further studies.

For induction of CNP differentiation, the SERB multiple factors were chosen because of their individual effects on the development of hair cells (17, 21, 31, 39). Another reason is that CNPs grown in growth media for 14 days followed by withdrawal of growth factors (E + b) for 7 days did not appear to induce sufficient differentiation as expected, suggesting that EGF and basic FGF are not sufficient for determination of a hair cell fate. Consistent with this, Malgrange et al. (23) demonstrated that adhesion of cellular spheres (also termed otosphere) onto an extracellular matrix protein (poly-γ-ornithine and fibronectin)-coated surface and withdrawal of mitogenic growth factors did not initiate differentiation of progenitor cells into either hair cells or supporting cells. Directed proliferation and differentiation of CNPs are of importance for the development of stem cell replacement therapies. In organotypic cultures, none of the individual growth factors alone are able to induce hair cell regeneration. This may be due to limited effects of individual factors on removing inhibitory cues for cellular proliferation, for example, Hes1, Rb1, and p15INK4d (6, 28, 36). The combination of multiple factors such as SERB appears to be potent for stimulating proliferation and specification of CNPs in vitro. It is interesting that SERB is capable of extending CNPs from 3–5 passages to more than 10 passages when compared with growth media, contributing to CNP survival and antisenescence. As evidenced in this study, priming of CNPs with appropriate factors (SERB) in step 1 is accompanied by cellular proliferation and hair cell marker expression at the same time, suggesting that SERB not only induces proliferation but also promotes specification toward a hair cell fate or a neuronal fate.

Interestingly, PD98059 inhibits the ERK signaling and subsequently blocks the expression of Math1, myosin VIIa, espin but not β3-tubulin and MAP2. This suggests that the SERB-induced ERK signaling pathway is important for CNP hair cell growth and differentiation but less so for CNP neuron growth and differentiation. An independent study (39) from the literature verified the importance of EGF in the generation of hair cells in the cochlear explants and embryonic otocyst homogenates, adding support to the notion that EGF plays a role in guiding CNP differentiation toward hair cells. Our most recent data suggest that SHH also favors a fate of CNP adapting to hair cells (data not shown).

One of the key actions of SERB is its induction of the Math1 and Brn3.1 mRNA or protein expression in CNPs. Math1 is known to play an important role in the initial cochlear sensory epithelial cell generation (3). Introduction of Math1 cDNA into a damaged organ of Corti has been shown to generate new hair cells in the guinea pig (13, 15, 35). Recently, functional hair cells have been produced in the mouse cochlea by in utero gene transfer of Math1 (11). These studies strongly suggest that upregulation of the Math1 gene by relevant growth factors such as SERB may lead to the generation of new hair cells in damaged cochleas. Brn3.1 (also termed Brn3c or POU4f3) is known as a key transcription factor for hair cell differentiation, maturation, and survival. Early stage mouse embryos lacking the Brn3.1 gene develop hair cell progenitors, but these degenerate before birth (34). Consistent with this, humans with the mutation of the Brn3.1 gene have normal hearing in their early stage of life but progressive hearing loss begins between the age of 18 and 30 (33). Induction of cellular proliferation by EGF via the ERK pathway (5) and SHH perhaps via the cdc2 gene (32), promotion of differentiation by RA via the retinoid X receptor (12), and cellular survival by BDNF via the PI3K-Akt pathway (9) may in part explain the synergistic actions of SERB in the generation of hair cell- and neuron-like phenotypes in vitro. However, the exact role of individual components in the SERB cocktail remains to be elucidated in the future studies.

In summary, we identified CNPs in the postnatal organ of Corti and demonstrated their capability to proliferate and differentiate into hair cell- and neuron-like phenotypes under the influence of SERB, suggesting a potential of using cell replacement strategy as a means to treat sensorineural hearing loss.

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