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Differentiation of Adult Mouse Olfactory Precursor Cells into Hair Cells In Vitro

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ABSTRACT

Many forms of deafness result from degeneration of the sensory cells for hearing, the hair cells in the cochlea. Stem cells offer a potential cell-based therapy for the treatment of deafness. Here, we investigate whether adult olfactory precursor cells can differentiate into hair cells in culture. Precursor cells were isolated from mouse olfactory neuroepithelium, were sphere-forming, showed proliferative capacity, and contained cells expressing neuronal and nonneuronal proteins. To induce differentiation, precursor cells were cocultured with cochlear cells and/or cochlear supernatant. Differentiated precursor cells were immunopositive for specific hair cell markers, including myosin VIIa, FM1-43, calretinin, phalloidin, and espin, and resembled hair cells anatomically and immunocytochemically in culture. The results demonstrate for the first time that adult olfactory precursor cells can differentiate into hair cell-like cells, thus providing a potential autotransplantation therapy for hearing loss. STEM CELLS *2007;25:621–627*

INTRODUCTION

Sensorineural deafness induced by ageing, excessive noise and certain antibiotics accounts for the majority of permanent hearing loss in humans. Hearing loss is caused by the dysfunction of the sensory epithelium (the organ of Corti) within the inner ear (cochlea). It is associated with the irreversible loss of sensory hair cells [1] and spiral ganglion neurons [1]. There is evidence of regeneration of sensory cells in nonmammalian species [2, 3]; however, studies in mammals have failed to find evidence of cochlear regeneration [4].

Several areas of research have addressed the treatment of sensorineural hearing loss. Gene therapy has been applied to protect or generate cochlear hair cells. The insertion and overexpression of neurotrophic factors or oxidative stress-reducing enzymes by viral transfection protects cochlear hair cells from ototoxic trauma in guinea pigs [5, 6]. The overexpression of a transcription factor essential for hair cell development in nonsensory cochlear cells, Math1/Atoh1, via viral transfection, generates new hair cells and substantially improves hearing thresholds in adult deaf guinea pigs [7, 8]. Other treatments for hearing loss include the exogenous delivery of neurotrophic factors in concert with electrical stimulation to increase the survival of spiral ganglion neurons [9, 10]. White et al. [11] have recently shown that postmitotic mammalian supporting cells ($p27^{Kip1}$-positive) have the ability to divide and transdifferentiate into hair cells.

Stem cell therapy has recently been investigated as a potential treatment for hair cell loss. Mouse embryonic stem cells can differentiate into hair cells in the developing inner ear of chick embryos [12], and/or into neuronal cells in the cochlea of deafened guinea pig [13]. Adult stem cells, isolated from the mouse vestibular system, can differentiate into hair cells in the developing inner ear of chick embryos [14], whereas adult neural stem cells survive better and differentiate to a greater extent in deafened versus normal guinea pig cochlea [15]. Some reports show the presence of limited numbers of nestin-positive stem cells in intact mouse organ of Corti [16], and dissociated neonatal rat organ of Corti [17], suggesting that there may be some intrinsic potential for repair.

The olfactory neuroepithelium offers an abundant and easily accessible source of adult stem cells, a significant advantage for future autotransplantation cell therapy. Olfactory receptor neurons are exposed to the external environment and are susceptible to toxic airborne chemicals, infectious pathogens, and physical damage after frontal head trauma. Hence, olfactory receptor neurons are replaced periodically throughout adult life, and also have the capacity to proliferate in response to acute injury. The persistence and ability of the olfactory system to regenerate its neuroepithelium by replacing damaged or dead neurons is unique in the mammalian nervous system. Replacement and proliferation result from the presence of multipotent stem cells in the olfactory neuroepithelium [18–21]. Here, we investigate whether olfactory precursor cells can be isolated from adult mouse and characterized according to current stem cell criteria. Next, we investigate whether olfactory precursor cells can be differentiated into hair cells in vitro.

MATERIALS AND METHODS

Experimental Animals

Adult CBA/CaH mice were bred in-house within the Biological Testing Facility at the Garvan Institute of Medical Research. All experiments were performed with the approval of the Garvan Institute and St. Vincent's Hospital Animal Experimentation and Ethics Committee, in accordance with National Health & Medical Re-

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Table 1. Cell type specific markers, their optimal concentrations and source

Marker	Cell type and distribution	Dilution	Antibody type	Source
Myosin VIIa	Hair cell soma and stereocilia	1:100–200	Rabbit polyclonal	Novus Biologicals
Calretinin	Inner hair cell soma and stereocilia	1:1,400	Rabbit polyclonal	Swant
Prestin	Outer hair cell soma	1:80	Goat polyclonal	Santa Cruz
Math1	Hair cell nuclei	1:50–100	Rabbit polyclonal	Abcam
Espin	Stereocilia	1:50	Mouse monoclonal	BD Transduction Laboratories
Phalloidin	Hair cell soma and stereocilia	1:50	Labelled secondary	Molecular Probes
FM1-43	Hair cell soma and stereocilia	5 μg/ml	Styryl dye	Molecular Probes
β-III-Tubulin	Neuronal soma and neurites	1:100	Mouse monoclonal	Sigma
Keratin	Epithelial cells	1:400	Rabbit polyclonal	Dako
Pax-6	Sustentacular cells, olfactory glandular cells	1:200	Rabbit polyclonal	Chemicon
Glial fibrillary associated protein	Astrocytes and other glial cells	1:800	Rabbit polyclonal	Dako

search Council animal experimentation guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

Primary Adult Olfactory Precursor Cells

Olfactory turbinates were dissected from 10–15 adult CBA/CaH mice aged 6 weeks to isolate olfactory precursor cells. Mice were anesthetized with CO_2 and decapitated, and olfactory turbinates were removed and placed in Dulbecco's modified Eagle medium (DMEM) containing 9.6 mg/ml HEPES buffer. Tissue was centrifuged, and supernatant was removed before placing minced tissue into DMEM containing 1% (wt/vol) bovine serum albumin (BSA; Sigma Chemicals, St. Louis, http://www.sigmaaldrich.com/), 50 μg/ml DNase (Sigma Chemicals), 1 mg/ml hyaluronidase (Sigma Chemicals), 1 mg/ml collagenase (Roche, Australia, http://www.roche-australia.com/), and 5 mg/ml dispase (Roche) for 1 hour at 37°C. Tissue suspension was triturated, filtered (150-μm wire mesh; Small Parts Inc., Miami Lakes, FL, http://www.smallparts.com/), centrifuged, and resuspended in neurobasal medium (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com), containing 10% (wt/vol) dialysed fetal calf serum (Gibco-BRL), 10,000 units/ml penicillin G (Sigma Chemicals), 50 μg/ml gentamycin sulfate (Sigma Chemicals), 20 mM glutamine (CSL, Melbourne, Australia, http://www.csl.com.au), and 2.8 μg/ml cytosine arabinoside (Sigma Chemicals). Cells were filtered twice more through a 40-μm nylon mesh filter (BD Falcon, Franklin Lakes, NJ, http://www.bd.com) for size exclusion and subsequently the olfactory precursor cells were collected on a 10-μm nylon mesh filter (Small Parts Inc.). Cultures were grown at 1×10^5 cells per milliliter at 37°C in 5% CO_2 in neurobasal medium containing B27 supplement (instead of fetal calf serum), 20 ng/ml fibroblast growth factor-2 (Promega, Madison, WI, http://www.promega.com/), and 20 ng/ml epidermal growth factor (Promega) for 7 days. The olfactory neurosphere cultures prepared for coculturing with cochlear cells or cochlear supernatant did not contain growth factors or gentamycin. Under normal growth conditions (without the need for addition of cochlear supernatant or withdrawal of growth factors), the medium was not changed for the duration of the experiments.

For the bromodeoxyuridine (BrdU) incorporation assay, the olfactory neurospheres were incubated with BrdU labeling reagent (Sigma Chemicals) that was added to the growth medium at a final concentration of 50 mM upon plating. Cells were grown in Labtek four-well chamber slides (Nunc, Rochester, NY, http://www.nuncbrand.com) for 5 days at 37°C in 5% CO_2. The cells were fixed in 70% ethanol (in 50 mM glycine buffer) for 20 minutes at −20°C, washed in PBS, and permeabilized with 0.3% Triton X-100 for 15 minutes at room temperature. Cells were incubated with monoclonal anti-BrdU (1:10; Chemicon, Temecula, CA, http://www.chemicon.com/) for 1 hour at room temperature and detected using goat antimouse Alexa Fluor 488 (1:50; Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for 30 minutes at room temperature. Cells were washed in PBS and mounted in Vectashield (Vector, Laboratories, Burlingame, CA, http://www.vectorlabs.com) mounting medium. Fluorescence was visualized using a laser scanning confocal microscope (Leica, Heerbrugg, Switzerland, http://www.leica.com).

Immunochemistry

Hair cells in the intact organ of Corti were characterized by immunohistochemistry using several hair cell specific markers (Table 1). Adult CBA/CaH mice aged 6 weeks ($n = 8$) were given an overdose of intraperitoneal euthal (80 mg/kg) and transcardially perfused with ice-cold normal saline followed by 4% paraformaldehyde (PFA). Cochlear tissue was dissected and processed for paraffin embedding. Cross-sections (6 μm) were collected onto electrostatic glass slides (Menzel-Glaser, Braunschweig, Germany, http://www.menzel.de/). Sections were dewaxed in Histoclear (National Diagnostics, Atlanta, GA, http://www.nationaldiagnostics.com/) and rehydrated through a graded series of alcohols. The cochlear tissue used for anti-myosin VIIa histochemistry was immersion fixed overnight in formalin, cryoprotected, and subsequently sectioned (14 μm) on a cryostat (Leica).

Nonspecific staining was blocked in 10% serum in 1% BSA in PBS for 1 hour. This was followed by incubation with the primary antibodies (Table 1) for 1 hour. Control sections were incubated with 1% normal serum of the same species as the primary antibody and processed in parallel. For immunoperoxidase studies, sections were washed in PBS submerged in 0.3% H_2O_2 for 15 minutes. After washing, sections were incubated with biotinylated goat anti-rabbit, rabbit anti-goat, or horse anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com/) at a concentration of 1:300, washed in PBS, and coverslipped using aquamount (BDH, Merck, Darmstadt, Germany, http://www.merck.com). Immunofluorescent histochemistry was used to detect antimyosin VIIa staining in tissue sections using goat antirabbit (Alexa Fluor 488; 1:50). Double-labeling immunocytochemistry was performed using two primary antibody combinations: anti-calretinin (rabbit) and anti-prestin (goat) and anti-calretinin (rabbit) and anti-β III tubulin (mouse). Sections were blocked in 1% BSA in PBS for 1 hour, followed by incubation with primary antibodies for 1 hour. For the primary antibody incubation of calretinin and prestin, the primary antibodies were incubated sequentially. This was followed by secondary antibody incubation for 30 minutes with goat anti-rabbit (1:50; Alexa Fluor 594; Molecular Probes) and donkey anti-goat (1:50; Alexa Fluor 488), or goat anti-rabbit (1:50; Alexa Fluor 594) and goat anti-mouse (Alexa Fluor 488). Slides were mounted in Vectashield, CA), and images were captured using a Leica DC480 (Leica) digital camera attached to a Zeiss Axiophot microscope (Carl Zeiss, Germany, http://www.zeiss.de/en).

Hair cells in culture were characterized by immunocytochemistry with a similar range of hair cell-specific markers used in the intact organ of Corti. After 7–14 days in culture, cells (cochlear or differentiated precursor cells) were rinsed in phosphate-buffered saline and fixed in 2% paraformaldehyde (ProSciTech, Thuringowa, Australia, http://www.proscitech.com.au). After fixation, cells were permeabilized in 0.1% Triton X-100, rinsed in PBS, blocked in 10% normal serum from the species in which the secondary antibody was made, and then incubated with primary antibodies. Unless otherwise stated, all incubations were at room temperature in a humidified chamber. After three washes in PBS, the cells were incubated with the secondary fluorescent antibodies for 30 minutes in the dark. Slides were mounted in Vectashield and images were captured

using a Leica DC480 digital camera (Leica) attached to a Zeiss Axiophot microscope.

Primary Adult Cochlear Cultures

To characterize hair cells in vitro, primary cultures were prepared from cochlea dissected from three to four adult CBA/CaH mice aged 6 weeks. Although every effort was made to limit the dissection to cochlear tissue, the inclusion of some vestibular tissue cannot be ruled out. Mice were anesthetized with CO_2, and cochleas were removed and placed in Dulbecco's modified Eagle's medium containing 9.6 mg/ml HEPES buffer. The protocol of enzymatic digestion and cell filtration is the same as for primary olfactory neurosphere cultures. Cells were filtered through a 40-μm nylon mesh filter before plating into Labtek tissue culture chamber slides (Nunc) or transwell 12-well plates (Costar Corning, MA). Cultures were plated at 1.25×10^6 cells per milliliter and grown at 37°C in 5% CO_2 in neurobasal medium containing B27 supplement instead of fetal calf serum.

Differentiation of Adult Olfactory Precursor Cells in Culture

After the removal of growth factors, two techniques were used to differentiate olfactory neurospheres into hair cells: coculturing with primary cochlear cultures, or exposure to cochlear supernatant.

Coculturing of primary olfactory neurosphere cultures with primary cochlear cultures was performed in two-well chamber slides and in the 12-well transwell culture system, where a porous 0.4-μm membrane prevents any direct contact between olfactory and cochlear cells, but allows diffusion of soluble factors. Tissue culture supernatant was collected from cochlear cultures (1–21 days in vitro), frozen, and then centrifuged and filtered through a 40-μm mesh before being added, undiluted, to olfactory neurosphere cultures. Control olfactory neurosphere cultures were allowed to differentiate in the absence of supernatant and growth factors.

Quantification of Differentiation of Olfactory Neurospheres into Hair Cell-Like Cells

To quantify the extent to which the olfactory neurospheres differentiated into hair cell-like cells, FM1-43-positive cells (hair cell marker) were counted in the presence or absence of cochlear supernatant. Neurospheres were treated with and without supernatant, allowed to differentiate for 11 days, then exposed to FM1-43 for 1 minute, washed thoroughly in PBS, fixed in 2% paraformaldehyde, permeabilized (0.1% Triton X-100), and labeled with propidium iodide (10 μg/ml; Sigma Chemicals). Cells were examined using a Zeiss Axiophot microscope. Fields were chosen at random ($n = 27$ with supernatant treatment; $n = 22$ without supernatant treatment), and the number of FM1-43-positive cells was counted and calculated as a percentage of the total number of propidium iodide-positive differentiated cells.

RESULTS

Characterization of Primary Olfactory Precursor Cells

Olfactory precursor cells were prepared from dissociated primary cell cultures ($n = 16$) of adult mouse olfactory turbinates. Precursor cells proliferated to form tight clusters of cells (neurospheres) after 24 hours in culture. Neurospheres were typically 100–200 μm in diameter, but could reach 500 μm in diameter (Fig. 1A). Single round cells radiating from the neurosphere were commonly seen (Fig. 1A). Some cells within the neurospheres were immunopositive for the BrdU antibody, a marker of the S-phase of the cell cycle (Fig. 1B). Trituration of neurospheres and single cell disposition experiments using cell sorting (FACS Calibur, BD) gave rise to secondary neurospheres, which provides additional evidence that neurospheres were self-replicating (data not shown). Upon removal of growth factors, olfactory neurospheres spontaneously differentiated into cells showing positive immunoreactivity for the neuronal antibody marker (β III tubulin), the epithelial cell marker (keratin), the sustentacular cell/glandular cell marker (Pax-6), and the glial cell marker (glial fibrillary associated protein; Fig. 1C–1F). Some cells remained unlabeled and are as yet unidentified. In summary, olfactory precursor cells were able to form spheres, showed proliferative capacity, and were able to differentiate into neuronal and non-neuronal cell types.

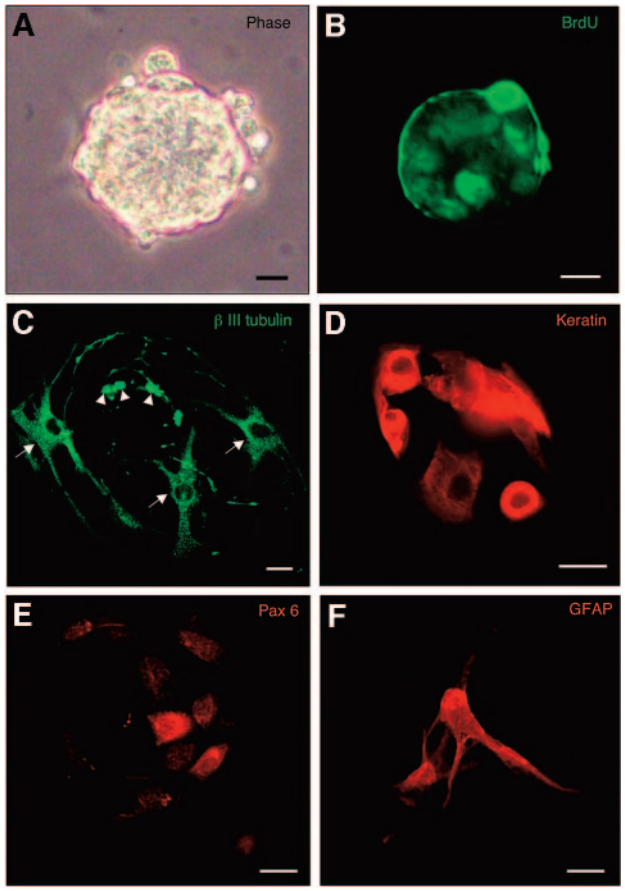

Figure 1. Olfactory neurospheres show hallmarks of precursor cells. **(A):** Olfactory neurospheres consist of three-dimensional clusters of densely packed olfactory precursor cells after 24–48 hours in culture. **(B):** Olfactory neurospheres are self-renewing, as shown by incorporation of BrdU and fluorescent staining of individual nuclei within the olfactory neurosphere. Olfactory neurospheres spontaneously differentiated into **(C)** two morphologically different types of neuron, stellate (arrow) and spherical soma (arrowheads) that were immunopositive for β III tubulin; **(D)** neuroepithelial cells that were immunopositive for keratin; **(E)** olfactory cells of non-neuronal lineage (sustentacular, glandular, or basal cells) that were immunopositive for Pax-6; and **(F)** glial cells that were immunopositive for GFAP. Scale bars = 20 μm in **(A–D)** 10 μm in **(E–F)**. Abbreviations: BrdU, bromodeoxyuridine; GFAP, glial fibrillary-associated protein.

Characterization of Hair Cells in the Organ of Corti

Hair cells in the adult organ of Corti (cochlea, $n = 9$) were analyzed in thin sections of cochlea using markers of inner and outer hair cells. Inner hair cells and spiral ganglion nerve fibers showed positive immunoreactivity to the calcium-binding protein calretinin (Fig. 2A). No staining was observed in outer hair cells with the calretinin antibody. Outer hair cells were positively labeled with prestin, a transmembrane motor protein present in these cells (Fig. 2B). Double labeling immunofluo-

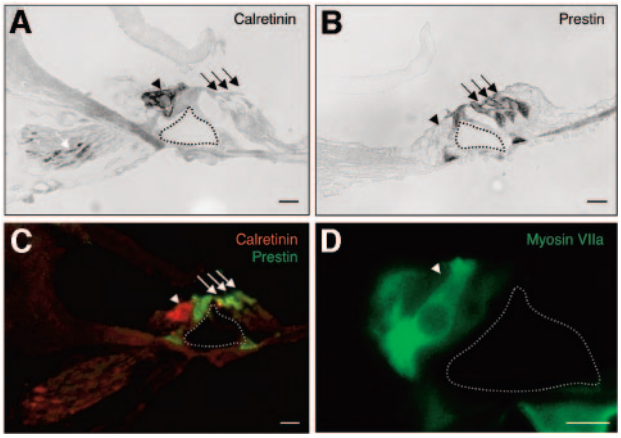

Figure 2. Characterization of hair cells in adult mouse organ of Corti. **(A):** Photomicrograph of cross-section of organ of Corti showing positive immunoreactivity for calretinin in the inner hair cell (arrowhead) and auditory nerve fibers (white arrow), but not outer hair cells (arrows). **(B):** Positive immunoreactivity for prestin in outer hair cells only (arrows). The inner hair cell is negative (arrowhead). **(C):** Double-labeling immunohistochemistry showing distinct labeling of inner hair cells with calretinin (red; white arrowhead), and outer hair cells with prestin (green; white arrows). **(D):** Myosin VIIa immunofluorescence is seen in the cytoplasm of the inner hair cell (arrowhead). Scale bars = 10 μm in **(A–D)**.

rescence shows there was no colocalization of calretinin and prestin in the adult mouse organ of Corti, confirming the specificity of these antibody markers (Fig. 2C). Myosin VIIa, a motor protein essential to sensory epithelia, labeled the cytoplasm of the inner hair cell shown in Figure 2D.

Characterization of Hair Cells in Culture

Dissociated primary cell cultures (n = 11) from adult cochlea were characterized according to morphology and immunocytochemistry. Round immature cells, alone or in clusters, were observed on initial plating of primary mouse cochlear cultures. After 7–14 days in vitro, cells developed into two different types: pear-shaped cells with ciliary tufts and centrally located round nuclei, and bipolar cells with oval soma and distal tufts (Fig. 3A). Several different fluorescent antibody markers were used to characterize cochlear cells in culture. Antibodies were specific for hair cells in the cochlea, and some were additionally specific for inner versus outer hair cells. The Math1 antibody labeled the nuclei of hair cells that appeared in the cochlear cultures 6 days postplating (Fig. 3B). The stereocilia and cytoplasm of pear-shaped cells with bipolar processes labeled positively with the myosin VIIa antibody (Fig. 3C). Phalloidin labeled the extracellular rim and stereocilia of a pear-shaped cell with bipolar ciliary tufts (Fig. 3D). The antibody for prestin showed cytoplasmic immunoreactivity in a cell where no processes or ciliary tufts were detected (Fig. 3E). In addition, hair cells were labeled with FM1-43, a vital fluorescent dye that enters living hair cells either via transduction channels on the stereocilia or through membrane recycling at the apex of inner and outer hair cells or the base of inner hair cells. Fluorescent labeling for FM1-43 was observed in a pear-shaped cell with a unipolar tuft (Fig. 3F).

Differentiation of Adult Precursor Cells into Hair Cell-Like Cells

Differentiation of precursor cells was observed when cells were cocultured with primary cochlear cultures (experiments, n = 7) or exposed to cochlear supernatant (experiments, n = 8). Ini-

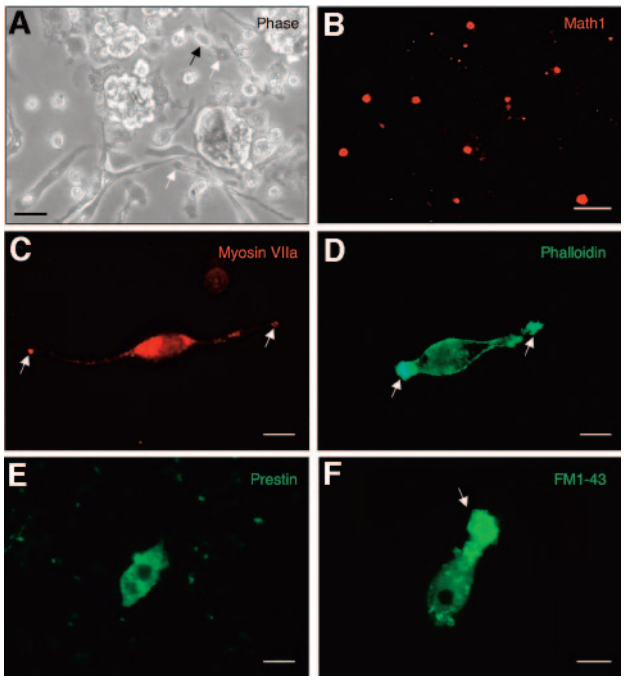

Figure 3. Characterization of hair cells in primary cochlear cultures using specific hair cell markers. **(A):** Phase contrast photomicrograph showing different morphologies of cell types within primary adult cochlear cultures. In particular, there are pear-shaped cells (black arrow) and bipolar cells (white arrow) with stereociliary-like tufts (arrowhead). **(B):** Fluorescence immunolabeling of Math1-positive nuclei in hair cells after 6 days in culture. **(C):** Fluorescence immunocytochemistry showing myosin VIIa-positive cytoplasmic staining of a bipolar cell. The stereocilia tufts are also myosin VIIa positive (arrowhead). **(D):** Fluorescence immunochemistry showing a phalloidin-positive cell with a pear-shaped soma, and ciliary tufts (arrowhead), similar in appearance to inner hair cells. **(E):** Anti-prestin antibody labels the cytoplasm of a cochlear cell in culture. **(F):** FM1-43 labels stereocilia transduction channels, and basal and apical membrane recycling in a pear-shaped cell with a centrally located spherical nucleus. Scale bars = 20 μm in **(A)**, 10 μm in **(B–F)**.

tially, precursor cells and cochlear cultures were cocultured in direct contact (experiments, n = 3). Immunocytochemistry using anti-myosin VIIa showed positively labeled pear-shaped cells and bipolar cells (Fig. 4A). Subsequently, coculturing using a transwell system (experiments, n = 4) was used to prevent direct contact of precursor and cochlear cells while allowing diffusion of soluble factors. As a control, olfactory precursor cells were cocultured with medium alone in the transwell system and did not differentiate. When cocultured with cochlear cells in the transwell, precursor cells were transformed from round, immature cells with no processes to cells with unipolar or bipolar processes after 7–14 days in culture. The differentiated precursor cells possessed a different morphology from that of olfactory neurons in culture (personal observations) [21]. In all transwell experiments, adult precursor cells differentiated into myosin VIIa-positive cells, suggesting that a diffusible factor may be able to induce differentiation of adult precursor cells into hair cell-like cells (Fig. 4B).

The presence of a diffusible differentiation factor was investigated further by supernatant treatment. The supernatant from cochlear cultures was collected, filtered and added to olfactory precursor cell cultures, whereas control cultures were allowed to differentiate in the absence of cochlear supernatant. After 7–14 days of treatment, the precursor cells differentiated into bipolar cells whose soma and stereocilia were positively labeled for phalloidin, myosin VIIa, espin, calretinin, and pres-

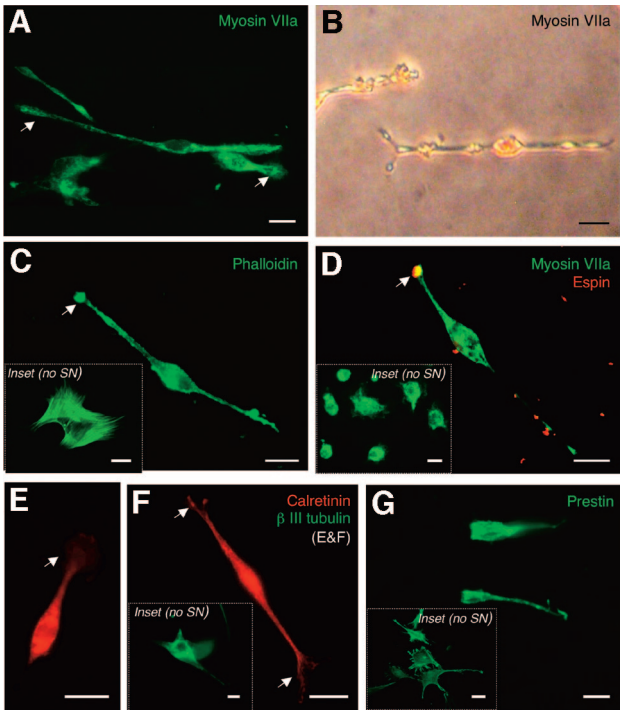

Figure 4. Differentiation of olfactory neurospheres into hair cell-like cells using coculturing and supernatant treatment. **(A):** Coculturing of cochlea and olfactory precursor cells resulted in myosin VIIa positive bipolar and pear-shaped cells with stereociliary tufts (arrowhead) **(B):** Diaminobenzidine immunocytochemistry showing a myosin VIIa-positive cell after coculturing olfactory neurospheres and cochlear cells for 21 days in the transwell system, which allows diffusion of soluble factors. **(C):** Fluorescence immunocytochemistry showing a phalloidin-positive cell with stereocilia tuft (arrowhead) after 14 days treatment of olfactory neurospheres with supernatant from cochlear cultures. The absence of SN results in phalloidin-positive but morphologically dissimilar cells (inset). **(D):** Double immunolabeling of a myosin VIIa- and espin-positive bipolar cell after treatment of olfactory neurospheres with cochlear supernatant. Espin is colocalized with myosin VIIa in the stereocilia (arrowhead). The absence of supernatant produces myosin-positive cells with no processes (inset). **(E–F):** Calretinin-positive pear-shaped and bipolar cells with a stereociliary tufts (arrowhead) observed after treatment of olfactory neurospheres with cochlear supernatant. Calretinin-positive cells are negative for the neuronal marker β-III-tubulin, which labels neurons derived from control cultures (inset). **(G):** Unipolar, rectangular-shaped, prestin-positive cells were derived from supernatant-treated olfactory precursor cells. In the absence of supernatant, only filamentous, prestin-positive cells with multiple processes were observed (inset). Scale bars = 10 μm in **(A–G)** and inset **(G)**; 5 μm in insets **(C)**, **(D)**, and **(F)**. Abbreviation: SN, supernatant.

tin (Fig. 4C–4G). Using the same antibody markers, cells in control cultures without supernatant show different morphologies from the unipolar or bipolar hair cell-like cells (inset 4C–4G). In double-labeling experiments, the bipolar cells labeled positively for calretinin (inner hair cell marker), and negatively for β-III-tubulin (neuronal marker), suggesting that the bipolar cells were not spiral ganglion neurons (Fig. 4E–4F).

Quantification of Differentiated Olfactory Precursors into Hair Cell-Like Cells

The number of hair cell-like cells produced by addition of cochlear supernatant to olfactory precursor cells was quantified using FM1-43 to label hair cell-like cells and propidium iodide to determine the total number of cells. Microscope analysis and quantification in random fields showed that most cells had differentiated, and a small population of cells were FM1-43

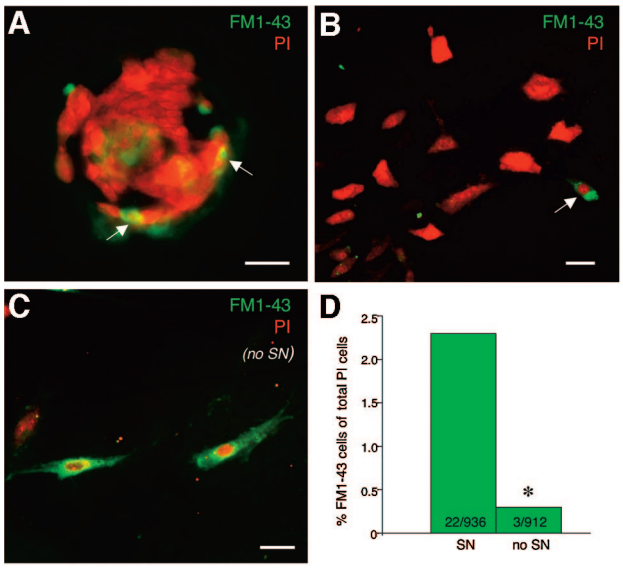

Figure 5. Quantification of hair cell-like cells. **(A):** The percentage of differentiated cells that formed hair cell-like cells after cochlear SN treatment was quantified using FM1-43 (green) to label hair cell-like cells and PI (red) to label total number of cells (only differentiated cells were included in total cell counts). A differentiating olfactory neurosphere contains two FM1-43 positively labeled cells (arrows). **(B):** After supernatant treatment, FM1-43-positive cells (green, arrow) were counted among total differentiated cells (red). **(C):** Without supernatant treatment, olfactory neurospheres differentiated into rare and dissimilar FM1-43-positive cells with different immunostaining (perinuclear) and morphology (multiprocess, no stereociliary tufts). **(D):** Summary data showing that the addition of supernatant caused a significantly greater amount of differentiation, as 2.3% of total differentiated cells were FM1-43-positive cells when treated with supernatant (22 FM1-43-positive cells of 936 PI-positive differentiated cells), whereas, in control experiments without supernatant, 0.3% of differentiated cells were FM1-43-positive cells (three FM1-43-positive cells of 912 PI-positive differentiated cells; * = $p < .001$). Scale bars = 10 μm in **A–C**. Abbreviations: PI, propidium iodide; SN, supernatant.

positive (Fig. 5A, 5B). Some of the FM1-43-positive cells seemed to be radiating directly from the remnants of an olfactory neurosphere (Fig. 5A). When no cochlear supernatant was added, but precursor cells were allowed to differentiate, a significantly reduced number of cells were FM1-43 positive (Fig. 5C). These cells showed perinuclear localization of FM1-43 and had different morphology from that of FM1-43 cells observed in the presence of supernatant. Summary data show that the addition of supernatant caused a significantly greater amount of differentiation ($p < .001$, χ^2 test), as 2.3% (22 of 936 cells) of total differentiated cells were FM1-43-positive cells after cochlear supernatant treatment. In contrast, 0.3% (3 of 912 cells) were FM1-43-positive without supernatant treatment (Fig. 5D).

Discussion

Our results show that adult precursor cells can be isolated from adult mouse olfactory neuroepithelium. The precursor cells formed three-dimensional neurospheres that contained proliferating and multipotent cells (formed neurons, neuroepithelial cells, sustentacular/glandular cells, and glia). It is now recognized that neurospheres are dynamic motile systems, not necessarily of clonal origin [23, 24]. The precursor cells fulfill the criteria of previously isolated adult neural stem cells [25, 26]. Adult neural stem cells isolated from the striatum [26, 27], dentate gyrus [28], and spinal cord [29] show a regenerative

capacity, yet are rare and not easily isolated for cell-based therapies. In contrast, adult olfactory precursor cells are abundant and are readily accessible from the olfactory neuroepithelium.

Precursor cells differentiated into hair cell-like cells by cochlear coculturing or supernatant treatment. Hair cells were first characterized in intact organ of Corti and dissociated cochlea cultures using the specific hair cell markers Math1, myosin VIIa, calretinin, prestin, FM1-43, espin, and phalloidin. Precursor cells differentiated into myosin VIIa-, calretinin-, phalloidin-, espin-, and prestin-positive cells after coculturing of precursor cells with cochlear cells or treatment with supernatant collected from cochlear cultures. It should be noted that mammalian type I and type II vestibular hair cells label positively for myosin VIIa [30], phalloidin [31], and calretinin [32], and contain actin in the superficial area around the stereocilia bundle [33]. As such, we conclude that the olfactory precursor cells gave rise to hair cell-like cells, and we are unable to distinguish between vestibular or cochlear hair cells. Electrophysiological characterization of the hair cell-like cells would be able to identify the different types of hair cells.

The differentiation of precursor cells by cochlear supernatant suggests the presence of soluble factors capable of inducing differentiation. The identity and source of the soluble factors is yet to be determined. There is increasing evidence that neurotrophic factors, namely brain-derived neurotrophic factor [9], glial cell line-derived neurotrophic factor, and transforming growth factor β1 [5] are important for growth and maintenance of hair cells and spiral ganglion neurons. Furthermore, epidermal growth factor, insulin-like growth factor 1, and basic fibroblast growth factor are able to differentiate embryonic stem cells into cells expressing hair cell markers [12]. It has been suggested that the supporting cells in the organ of Corti could be the source of neurotrophic factors [34, 35]. If this is the case, then the organ of Corti may provide the relevant trophic environment for transplanted stem cells. The importance of a trophic environment has been confirmed by the differentiation of mouse embryonic vestibular progenitor cells into hair cells only when situated near developing hair cell patches [14].

Here we have confirmed the multipotency of the precursor cells: differentiated cells were immunopositive for hair cell markers, neuronal markers, an epithelial cell marker, a sustentacular/glandular cell marker, and a glial cell marker. When the precursor cells were allowed to differentiate spontaneously by withdrawal of growth factors, large, stellate-shaped neurons and small, oval-shaped neurons were labeled with the neuronal marker β-III-tubulin (Fig. 1C). Olfactory neurons also label positively for β-III-tubulin, but have spherical soma and complex neuritic processes in culture (personal observations) [22]. Hair cells in intact organ of Corti and hair cell-like cells arising from precursor cells were β-III-tubulin negative (Fig. 4E–4F). Together, these results suggest that cochlear supernatant treatment did not cause precursor cells to differentiate into olfactory or spiral ganglion neurons.

Others have successfully differentiated embryonic stem cells into hair cells in vitro. Li et al. [12] showed that, upon differentiation, embryonic stem cells expressed the hair cell markers Math1, myosin VIIa, espin, and Brn3.1. Others have studied survival and differentiation of embryonic stem cells in vivo after cross-species transplantation. Undifferentiated mouse embryonic and adult stem cells successfully transplanted into mice, rats, guinea pigs, and chicks [14, 36–40] show differentiation into hair cells [13] or into neuronal and ectoderm cells [38, 39]. Undifferentiated adult neural stem cells survived for a limited time in the inner ear of rats and guinea pigs [14, 41], perhaps due to lack of essential growth factors, thus emphasizing the importance of environmental cues for survival and differentiation of stem cells. In our experiments, the dissection and dissociation of the cochlear tissue may cause the release of neurotrophic factors into the supernatant, which are responsible for differentiation of olfactory precursor cells into hair cells.

Our results with adult olfactory precursor cells raise the possibility for autologous transplantation in humans by providing a viable environment for stem cell survival and bypassing issues of immune rejection. Human olfactory precursor cells have been previously shown to differentiate into multiple lineages [42]. Our results suggest for the first time that adult olfactory precursors can differentiate into hair cell-like cells in the presence of extrinsic factors, thus providing a significant potential treatment for hearing loss.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

REFERENCES

1 Raphael Y. Cochlear pathology, sensory cell death and regeneration. Br Med Bull 2002;63:25–38.
2 Stone JS, Rubel EW. Cellular studies of auditory hair cell regeneration in birds. Proc Natl Acad Sci U S A 2000;97:11714–11721.
3 Morest DK, Cotanche DA. Regeneration of the inner ear as a model of neural plasticity. J Neurosci Res 2004;78:455–460.
4 Forge A, Li L, Nevill G. Hair cell recovery in the vestibular sensory epithelia of mature guinea pigs. J Comp Neurol 1998;397:69–88.
5 Kawamoto K, Yagi M, Stover T et al. Hearing and hair cells are protected by adenoviral gene therapy with TGF-beta1 and GDNF. Mol Ther 2003;7:484–492.
6 Kawamoto K, Sha SH, Minoda R et al. Antioxidant gene therapy can protect hearing and hair cells from ototoxicity. Mol Ther 2004;9:173–181.
7 Izumikawa M, Minoda R, Kawamoto K, et al. Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nat Med 2005;11:271–276.
8 Kawamoto K, Ishimoto S, Minoda R et al. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. J Neurosci 2003;23:4395–4400.
9 Shepherd RK, Coco A, Epp SB et al. Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss. J Comp Neurol 2005;486:145–158.
10 Gillespie LN, Shepherd RK. Clinical application of neurotrophic factors: The potential for primary auditory neuron protection. Eur J Neurosci 2005;22:2123–2133.
11 White PM, Doetzlhofer A, Lee YS et al. Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. Nature 2006;441:984–987.
12 Li H, Roblin G, Liu H et al. Generation of hair cells by stepwise differentiation of embryonic stem cells. Proc Natl Acad Sci U S A 2003;100:13495–13500.
13 Coleman B, Fallon JB, Pettingill LN et al. Auditory hair cell explant co-cultures promote the differentiation of stem cells into bipolar neurons. Exp Cell Res 2007;313:232–243.
14 Li H, Liu H, Heller S. Pluripotent stem cells from the adult mouse inner ear. Nat Med 2003;9:1293–1299.
15 Hu Z, Wei D, Johansson CB et al. Survival and neural differentiation of

adult neural stem cells transplanted into the mature inner ear. Exp Cell Res 2005;302:40–47.
16 Lopez IA, Zhao PM, Yamaguchi M et al. Stem/progenitor cells in the postnatal inner ear of the GFP-nestin transgenic mouse. Int J Dev Neurosci 2004;22:205–213.
17 Malgrange B, Belachew S, Thiry M et al. Proliferative generation of mammalian auditory hair cells in culture. Mech Dev 2002;112:79–88.
18 Othman MM, Klueber KM, Roisen FJ. Identification and culture of olfactory neural progenitors from GFP mice. Biotech Histochem 2003; 78:57–70.
19 Roisen FJ, Klueber KM, Lu CL et al. Adult human olfactory stem cells. Brain Res 2001;890:11–22.
20 Mackay-Sim A, Kittel P. Cell dynamics in the adult mouse olfactory epithelium: A quantitative autoradiographic study. J Neurosci 1991;11: 979–984.
21 Graziadei PP, Graziadei GA. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 1979;8:1–18.
22 Pixley SK. Characterization of olfactory receptor neurons and other cell types in dissociated rat olfactory cell cultures. Int J Dev Neurosci 1996;14:823–839.
23 Wang TY, Sen A, Behie LA et al. Dynamic behavior of cells within neurospheres in expanding populations of neural precursors. Brain Res 2006;1107:82–96.
24 Singec I, Knoth R, Meyer RP et al. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat Methods 2006;3:801–806.
25 Bartlett PF, Brooker GJ, Faux CH et al. Regulation of neural stem cell differentiation in the forebrain. Immunol Cell Biol 1998;76:414–418.
26 Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–1710.
27 Rietze RL, Valcanis H, Brooker GF et al. Purification of a pluripotent neural stem cell from the adult mouse brain. Nature 2001;412:736–739.
28 Gage FH, Ray J, Fisher LJ. Isolation,. characterization, and use of stem cells from the CNS. Annu Rev Neurosci 1995;18:159–192.
29 Shihabuddin LS, Ray J, Gage FH. FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. Exp Neurol 1997;148: 577–586.
30 Bermingham-McDonogh O, Oesterle EC, Stone JS et al. Expression of Prox1 during mouse cochlear development. J Comp Neurol 2006;496: 172–186.
31 Berggren D, Liu W, Frenz D et al. Spontaneous hair-cell renewal following gentamicin exposure in postnatal rat utricular explants. Hear Res 2003;180:114–125.
32 Desai SS, Ali H, Lysakowski A. Comparative morphology of rodent vestibular periphery. II. Cristae ampullares. J Neurophysiol 2005;93: 267–280.
33 Sans A, Atger P, Cavadore C et al. Immunocytochemical localization of myosin, tropomyosin and actin in vestibular hair cells of human fetuses and cats. Hear Res 1989;40:117–125.
34 von Bartheld CS, Patterson SL, Heuer JG et al. Expression of nerve growth factor (NGF) receptors in the developing inner ear of chick and rat. Development 1991;113:455–470.
35 Zhang M, Ding D, Salvi R. Expression of heregulin and ErbB/Her receptors in adult chinchilla cochlear and vestibular sensory epithelium. Hear Res 2002;169:56–68.
36 Hakuba N, Hata R, Morizane I et al. Neural stem cells suppress the hearing threshold shift caused by cochlear ischemia. Neuroreport 2005; 16:1545–1549.
37 Hildebrand MS, Dahl HH, Hardman J et al. Survival of partially differentiated mouse embryonic stem cells in the scala media of the guinea pig cochlea. J Assoc Res Otolaryngol 2005;6:341–354.
38 Sakamoto T, Nakagawa T, Endo T et al. Fates of mouse embryonic stem cells transplanted into the inner ears of adult mice and embryonic chickens. Acta Otolaryngol Suppl 2004:48–52.
39 Regala C, Duan M, Zou J et al. Xenografted fetal dorsal root ganglion, embryonic stem cell and adult neural stem cell survival following implantation into the adult vestibulocochlear nerve. Exp Neurol 2005;193: 326–333.
40 Coleman B, Hardman J, Coco A et al. Fate of embryonic stem cells transplanted into the deafened mammalian cochlea. Cell Transplant 2006;15:369–380.
41 Ito J, Kojima K, Kawaguchi S. Survival of neural stem cells in the cochlea. Acta Otolaryngol 2001;121:140–142.
42 Murrell W, Feron F, Wetzig A et al. Multipotent stem cells from adult olfactory mucosa. Dev Dyn 2005;233:496–515.

Differentiation of Adult Mouse Olfactory Precursor Cells into Hair Cells In Vitro

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