(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 June 2008 (26.06.2008)

PCT

(10) International Publication Number
WO 2008/076556 A2

(51) International Patent Classification:
C12N 5/08 (2006.01)

(21) International Application Number:
PCT/US2007/084654

(22) International Filing Date:
14 November 2007 (14.11.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/859,041 15 November 2006 (15.11.2006) US

(71) Applicant *(for all designated States except US)*: **MASSACHUSETTS EYE & EAR INFIRMARY** [US/US]; 243 Charles Street, Boston, MA 02114 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **EDGE, Albert** [US/US]; 50 Fisher Avenue, Brookline, MA 02445 (US).

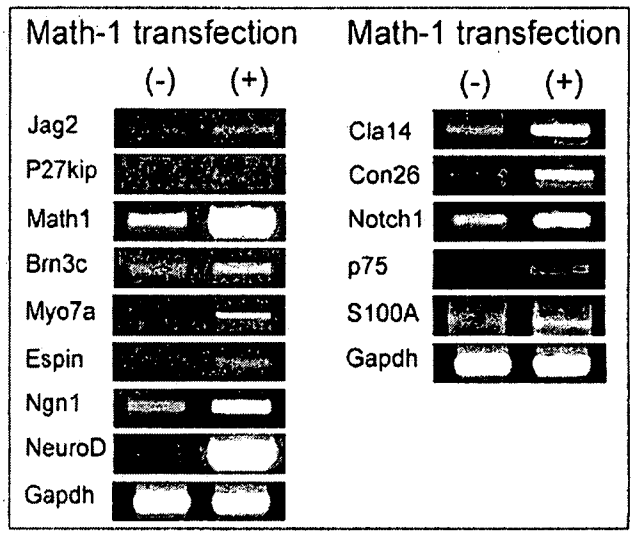
(74) Agents: **FREEMAN, John, W.** et al.; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, MN 55440-1022 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

(54) **Title:** GENERATION OF INNER EAR CELLS



4B

(57) **Abstract:** Methods for generating cells of the inner ear, e.g., hair cells and supporting cells, from stem cells, e.g., mesenchymal stem cells, are provided, as well as compositions including the inner ear cells. Methods for the therapeutic use of the inner ear cells for the treatment of hearing loss are also described.

WO 2008/076556 A2

GENERATION OF INNER EAR CELLS

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/859,041, filed on November 15, 2006, the entire contents of which are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. F33 DC006789, RO1 DC007174, and P30 DC05209 from the National Institute on Deafness and other Communicative Disorders (NIDCD) of the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

This invention relates to methods using bone marrow mesenchymal stem cells to regenerate inner ear cells, e.g., hair cells and supporting cells, to treat inner ear damage.

BACKGROUND

A source of sensory cells and neurons for regeneration of inner ear cells would provide a valuable tool for clinical application because neurons and hair cells could be employed in cell replacement therapy for hearing loss. Recent work has shown that hair cells and neurons can be differentiated from endogenous stem cells of the inner ear (Li et al., Nat Med 9, 1293-1299 (2003); Rask-Andersen et al., Hear Res 203, 180-191 (2005)) and other work has shown that endogenous cells of the sensory epithelium can be converted to hair cells when the proneural transcription factor, Atoh1, is expressed exogenously (Izumikawa et al., Nat Med 11, 271-276 (2005); Zheng and Gao, Nat Neurosci 3, 580-586 (2000)) and yet the endogenous stem cells of the inner ear do not spontaneously generate hair cells. Injection of whole bone marrow to reconstitute a lethally irradiated mouse resulted in engraftment of these cells in areas occupied by inner ear mesenchymal cells and fibrocytes but did not yield hair cells (Lang et al., J Comp Neurol 496, 187-201 (2006)).

SUMMARY

The present invention is based, at least in part, on the discovery of methods that can be used to induce stem cells to differentiate into hair cells and supporting cells. Thus, described herein are methods for providing populations of hair cells and/or supporting cells, compositions comprising said cells, and methods of use thereof, e.g., for the treatment of subjects who have or are at risk of developing a hearing loss.

In one aspect, the invention provides methods for providing populations of hair cells and/or supporting cells. The methods include:

obtaining a population of stem cells with neurogenic potential;

culturing the stem cells under conditions sufficient to induce the differentiation of at least some of the stem cells into inner ear progenitor cells, and doing one (or more) of the following:

(i) inducing the expression of Atoh1 in the inner ear progenitor cells, in an amount and for a time sufficient to induce at least some of the inner ear progenitor cells to differentiate into hair cells;

(ii) contacting the inner ear progenitor cells with an inhibitor of Notch signalling (e.g., a gamma-secretase inhibitor or inhibitory nucleic acid), in an amount and for a time sufficient to induce at least some of the inner ear progenitor cells to differentiate into hair cells; or

(iii) culturing the inner ear progenitor cells in the presence of chick otocyst cells for a time and under conditions sufficient for at least some of the inner ear progenitor cells to differentiate into hair cells,

thereby providing populations of hair cells and/or supporting cells.

In some embodiments, the methods include isolating the inner ear progenitor cells, hair cells, and/or supporting cells, e.g., to provide a purified population thereof.

In some embodiments, the inner ear progenitor cells express nestin, sox2, musashi, Brn3C, Pax2, and Atoh1.

In some embodiments, the hair cells express one or more genes selected from the group consisting of Atoh1, jagged 2, Brn3c, p27Kip, Ngn1, NeuroD, myosin VIIa and espin. In some embodiments, the hair cells express jagged 2, Brn3c, myosin VIIa and espin. In some embodiments, the hair cells express F-actin in a V pattern on the apical surface of the cells.

In some embodiments, the supporting cells express one or more of claudin14, connexin 26, $p75^{Trk}$, Notch 1, and S100A.

In some embodiments, the methods further include transplanting the hair cells or supporting cells into a subject in need thereof, e.g., into or near the sensory epithelium of the subject. In some embodiments, the population of stem cells is obtained from a subject in need of the transplant.

Also described herein are isolated populations of hair cells, supporting cells, and inner ear progenitor cells obtained by a method described herein.

In another aspect, the invention features methods for treating a subject who has or is at risk for developing a disorder, e.g., a hearing disorder or vestibular disorder, wherein the disorder is treatable with a transplant of hair cells and/or supporting cells, the method comprising transplanting cells obtained by a method described herein into the cochlea of the subject, thereby treating the subject. In these embodiments, it is preferable if the population of stem cells was obtained from the subject in need of the transplant.

In some embodiments, inducing the expression of Atoh1 in the cells comprises inducing the expression of exogenous Atoh1 in the cells, e.g., by transducing the cells with a vector encoding a Atoh1 polypeptide, e.g., a plasmid vector or a viral vector, e.g., an adenovirus, lentivirus, or retrovirus.

In some embodiments, inducing the expression of exogenous Atoh1 in the stem cells comprises increasing expression of endogenous Atoh1, e.g., by increasing activity of the Atoh1 promoter or by replacing the endogenous Atoh1 promoter with a more highly active promoter.

In some embodiments, culturing the stem cells in the presence of chick otocyst cells for a time and under conditions sufficient for at least some of the stem cells to differentiate into hair cells comprises culturing the stem cells in medium comprising IGF, EGF, and FGF.

In some embodiments, the stem cells used in the methods described herein are mesenchymal stem cells. In some embodiments, the stem cells used in the methods described herein are human stem cells.

As noted, the invention also features cells isolated by a method described herein, as well as compositions containing them.

Methods for treating subjects (*e.g.*, mammals such as humans) who have, or who are at risk for developing, a hearing loss, are also described herein. These methods include administering a cell or population of cells (as described herein; *e.g.*, a population of hair cells obtained by differentiating a population of stem cells) to the ear of the patient, e.g., to the cochlea. The administered cells may be obtained by the methods described herein, and the starting material may be stem cells obtained from the patient to be treated.

There may be certain advantages to the use of the cells described herein for the treatment of hearing loss. For example, the stem cells can be obtained from humans for clinical applications. Because the stem cells can be harvested from a human, and in particular can be harvested from the human in need of treatment, the immunological hurdles common in xeno- and allotransplantation experiments can be largely avoided.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a row of four photomicrographs of bone marrow MSCs from passage 3 immunostained with antibodies against CD44, CD45, CD34 and Sca-1 followed by secondary antibodies against mouse immunoglobulins labeled with TRITC (medium gray, shown in red in the original). Staining for CD34 and CD45 was negative, but CD44 and Sca-1 were expressed. Nuclei were stained with DAPI (darker gray, blue in the original).

FIG. 1B is a row of four photomicrographs of bone marrow MSCs from passage 3 immunolabeled for CD44 (first panel, medium gray, shown in red in the

original) and nestin (second panel, lighter gray, shown in green). The third panel is a DAPI nuclear stain (blue in original). The merged image in the right-most panel shows co-staining of a population of cells with both markers (lightest gray, yellow/orange in the original)

FIG. 1C is a row of four photomicrographs of bone marrow MSCs from passage 3 stained for co-expression of Sca-1 (first panel, red in the original) and nestin (second panel, green in the original). Merged image in the right-most panel shows co-staining.

FIG 1D is a row of four plots showing the results of analysis of bone marrow MSCs by chip flow cytometry indicating the ratio of immunopositive cells for each of the listed antibodies (CD44, first panel; Sca-1, second panel; CD34, third panel; and CD45, last panel); axes are "Fluorescence" and "No. of events."

FIG. 1E is a pair of photomicrographs showing the potential for lineage differentiation, as demonstrated by formation of chondrocytes and extracellular matrix after treatment of bone marrow MSCs with TGF-β. Cells that grew out from a micro-aggregate (left) were stained for type II collagen (right).

FIG. 1F is a pair of photomicrographs showing the differentiation of bone marrow MSCs to neurons by differentiation in serum-free medium containing neuronal growth supplements and bFGF. Staining for neurofilament (NF-M) is shown in these cells.

FIG. 2A is a gel showing the results of genetic analysis for neural progenitor markers by RT-PCR of MSCs treated with IGF-1, EGF and bFGF for 14 days followed by analysis. MSC (bone marrow MSCs), NP (neural progenitors at 2 wks after induction of progenitor formation). The genes analyzed are shown to the left of the gel.

FIGs. 2B-C are two sets of four photomicrographs showing that the neural progenitor marker, nestin, visualized by immunohistochemistry using a secondary antibody labeled with FITC (top right panel in 2B and 2C, shown in green in the original), was co-expressed with CD44 (2B, top left panel, shown in red in the original) and with Sca-1 (2C, top left panel, shown in red in the original). DAPI is shown in blue (lower left panel in each figure). Scale bars are 50 μm. Merged images in the lower right panel of each figure show coexpression of nestin and CD44 (2B) or Sca1 (2C) (all of the cells appeared green in the original, indicating coexpression).

FIG. 3A is a gel showing the results of genetic analysis by RT-PCR of precursor cells incubated in NT3, FGF and BDNF (which support neuronal and sensory cell progenitors in the inner ear). The gene profiles included expression of *Oct4, nestin, Otx2,* and *Musashi*, as well as proneural transcription factors, *GATA3, NeuroD, Ngn1, Atoh1, Brn3c,* and *Zic2*. These cells did not express hair cells genes, *myosin VIIa* and *espin.*

FIG. 3B is a gel showing the results of genetic analysis by RT-PCR of the cells obtained after induction with NT3, FGF, and BDNF. Genes characteristic of supporting cells (*claudin14, connexin 26, p75^{Trk}, Notch 1,* and *S100A*) were also observed. These progenitor cells thus had expression profiles characteristic of neuronal or sensory progenitors. Genes analyzed are shown to the left of the gels.

FIG. 4A is a photomicrograph showing exogenous expression of *Atoh1* in bone marrow MSCs; expression was observed in cells and nuclei (green in the original) due to the expression of GFP from the vector.

FIG. 4B is a gel showing the results of gene expression in cells transfected with *Atoh1* followed by treatment of the cells with NT3, FGF and BDNF. The results indicate that this protocol gave rise to progenitor cells that subsequently matured into cells expressing hair cell genes, including *espin, myosin VIIa, jagged 2,* and *Brn3c,* and *p27Kip*, in addition to the proneural genes, *Ngn1* and *NeuroD.*

FIG. 4C is a gel showing the results of further genetic analysis of the cells under the differentiating conditions described in 4B; the results showed that the cells also expressed *S100A, p75^{Trk}, claudin 14, connexin 26,* and *Notch1*, consistent with some cells having a supporting cell phenotype.

FIG. 4D is a photomicrograph of an MSC cell line selected in Zeocin; the cells had a high percentage of GFP expression when cultured in serum (green in original).

FIG. 4E is a row of 4 photomicrographs of cells stained for Myo7a (first panel), Math1/Atoh1 (second panel), or DAPI (third panel); the last panel is a merged image. After differentiation, the number of hair cell-like cells per DAPI nucleus rose and these cells stained for myosin VIIa (shown in red in the first panel) and Atoh1 (shown in green in the second panel; arrows in the second and last panels).

FIG. 4F is two rows of 4 photomicrographs of an *Atoh1* expressing cell line differentiated to cells with nuclei that were immunopositive for Brn3c (second column, green in original; indicated by arrowheads) and cytoplasm positive for

myosin VIIa (first column, red in original; indicated by arrows). Nuclei were stained with DAPI (third column, blue in orignal).

FIG. 4G is a row of three photomicrographs showing that the differentiated cells were positive for F-actin which protruded from the apex of the cell in the shape of a stereocilia bundle (arrow).

FIG. 4H is a row of three photomicrographs showing that F-actin staining was arranged in a characteristic V pattern on the apical surface.

FIG. 5A is a gel showing the results of genetic analysis of bone marrow MSC derived progenitors were co-cultured for 21 days with chick otocyst cells that had been treated with mitomycin C (Mito C); the results showed that expression of *jagged 2, p27Kip, Atoh1, Brn3c, myosin VIIa* and *espin* was increased, whereas the expression of these genes in chick cells was undetectable. Chick otocyst cells that had been fixed by incubation with paraformaldehyde were less effective (PFA) than the unfixed cells but did cause differentiation of the progenitors. Conditioned medium from the chick cells (Cnd Med) had no effect (levels of expression of these markers similar to previously shown data for differentiating conditions).

FIG. 5B is a set of three photomicrographs showing that expression of Atoh1 (Math-1, middle panel, green in original) and myosin VIIa (top panel, red in original) in cells from a *Atoh1-GFP* mouse showed green fluorescence corresponding to the induction of this marker in the nucleus and had expression of myosin VIIa in the cytoplasm.

FIG. 6A is a set of four photomicrographs showing an increase in fluorescence (green in original) indicating the conversion of bone marrow cells to cells expressing Atoh1. The cells stained for Atoh1 (Math1, bottom left, green in original), myosin VIIa (top left, red in original) and DAPI (top right, blue in original). A merged image is shown in on the bottom right panel.

FIG. 6B is a photomicrograph showing that Atoh1-expressing cells were found incorporated into the tissue of the chick otic epithelium. The hair cells of the chick were stained with the chick-specific marker, HCA (white in original) and myosin VIIa (red in original), whereas the Atoh-1 expressing mouse cells were green due to expression of GFP (arrows).

FIG. 6C is a set of four photomicrographs showing a lack of cell fusion, demonstrated by the presence of HCA (arrowhead, lower panels) in cells that did not

have green fluorescence and of Atoh1-GFP (arrow, right column) exclusively in cells that did not stain for HCA, a marker for chicken cells. No cells with both GFP and HCA were observed in these experiments. Scale bars are 100 μm.

FIG. 7 is a gel showing the results of genetic analysis of cells after inhibition of Notch signaling with an inhibitor of γ-secretase increases expression of hair cell markers. Gene expression in MSCs treated with a γ-secretase inhibitor showed that loss of Notch signaling increased Atoh1 expression. The timing of inhibition was critical: γ-secretase inhibitor added at d1 of differentiation in vitro for a total of 10 days led to an increase in hair cell markers, myosin VIIa and espin, whereas inhibitor added at d3 did not induce hair cell markers.

DETAILED DESCRIPTION

Although stem cells are present in the inner ear (Li et al., Trends Mol Med 10, 309-315 (2004); Li et al., Nat Med 9, 1293-1299 (2003); Rask-Andersen et al., Hear Res 203, 180-191 (2005)), hair cells do not regenerate after damage, and, therefore, a source of cells that could potentially be used for cell transplantation in a therapeutic replacement of these sensory cells has important implications for treatment of sensorineural hearing loss. Bone marrow has been harvested and used extensively in clinical applications and is a highly desirable source, because cells from a patient's bone marrow could potentially be transplanted without the problem of immune rejection. The present methods include a treatment regimen for hearing loss including transplantation of hair cells obtained by methods described herein.

By a combination of growth factor stimulation and expression of the transcription factor, Atoh1, that is required for hair cell formation in the inner ear, the present inventors demonstrate herein that stem cells, e.g., mesenchymal stem cells derived from bone marrow, can be induced to differentiate into hair cells. In addition, the neurosensory progenitors obtained from bone marrow can be converted to sensory cells by co-culture with cells of the developing sensory epithelium, even in the absence of Atoh1 expression.

Stem cells in bone marrow are known to be the precursors for all lymphoid and erythroid cells, but mesenchymal stem cells in bone marrow also act as precursors to bone, cartilage, and fat cells (Colter et al., Proc Natl Acad Sci U S A 97, 3213-3218 (2000); Pittenger et al., Science 284, 143-147 (1999)). In addition to mesenchymal

tissues, these stem cells have been shown to give rise to cells of other lineages including pancreatic cells (Hess et al., Nat Biotechnol 21, 763-770 (2003)), muscle cells (Doyonnas et al., Proc Natl Acad Sci U S A 101, 13507-13512 (2004)) and neurons (Dezawa et al., J Clin Invest 113, 1701-1710 (2004); Hermann et al., J Cell Sci 117, 4411-4422 (2004); Jiang et al., Proc Natl Acad Sci U S A 100 Suppl 1, 11854-11860 (2003)). The evidence provided herein demonstrates an extended range of cell fates available for these bone marrow-derived cells that includes cells of the neurosensory lineage, even including differentiation to inner ear hair cells.

Methods for Generating Cells of the Inner Ear

Methods of generating cells of the inner ear are provided, including progenitor cells and differentiated inner ear cells including hair cells and supporting cells. Stem cells are unspecialized cells capable of extensive proliferation. Stem cells are pluripotent and are believed to have the capacity to differentiate into most cell types in the body (Pedersen, Scientif. Am. 280:68 (1999)), including neural cells, muscle cells, blood cells, epithelial cells, skin cells, and cells of the inner ear (e.g., hair cells and cells of the spiral ganglion). Stem cells are capable of ongoing proliferation in vitro without differentiating. As they divide, they retain a normal karyotype, and they retain the capacity to differentiate to produce adult cell types.

Hematopoietic stem cells resident in bone marrow are the source of blood cells, but in addition to these hematopoietic stem cells, the bone marrow contains mesenchymal stem cells (MSCs) that can differentiate into cell types of all three embryonic germ layers (Colter et al., Proc Natl Acad Sci U S A 97, 3213-3218 (2000); Doyonnas et al., Proc Natl Acad Sci U S A 101, 13507-13512 (2004); Herzog et al., Blood 102, 3483-3493 (2003); Hess et al., Nat Biotechnol 21, 763-770 (2003); Jiang et al., Nature 418, 41-49 (2002); Pittenger et al., Science 284, 143-147 (1999)). This has been demonstrated in vivo in studies that track transplanted bone marrow cells to specific tissues where they differentiate into the resident tissue type (Mezey et al., Proc Natl Acad Sci U S A 100, 1364-1369 (2003); Weimann et al., Proc Natl Acad Sci U S A 100, 2088-2093 (2003)).

Many of these cells have been used for transplantation and are a preferred source of new cells for therapies because the transplanted cells are immunologically matched when harvested from a patient to be treated and because they have been extensively used in clinical applications so that their safety is known.

Stem cells can differentiate to varying degrees. For example, stem cells can form cell aggregates called embryoid bodies in hanging drop cultures. The embryoid bodies contain neural progenitor cells that can be selected by their expression of an early marker gene such as Sox1 and the nestin gene, which encodes an intermediate filament protein (Lee et al., Nat. Biotech. 18:675-9, 2000).

Neurogenic Stem Cells

Inner ear cells or inner ear cell progenitors can be generated from mammalian stem cells. As described herein, stem cells suitable for use in the present methods can be any stem cell that has neurogenic potential, i.e., any stem cell that has the potential to differentiate into a neural cell, e.g., neurons, glia, astrocytes, retinal photoreceptors, oligodendrocytes, olfactory cells, hair cells, supporting cells, and the like. Neurogenic stem cells, including human adult stem cells such as bone marrow mesenchymal stem cells, can be induced to differentiate into inner ear progenitor cells that are capable of giving rise to mature inner ear cells including hair cells and supporting cells. Neurogenic stem cells useful in the methods described herein can be identified by the expression of certain neurogenic stem cell markers, such as nestin, sox1, sox2, and musashi. Alternatively or in addition, these cells express high levels of helix-loop-helix transcription factors NeuroD, Atoh1, and neurogenin1.

Examples of neurogenic stem cells include embryonic stem cells or stem cells derived from mature (e.g., adult) tissue, such as the ear (e.g., inner ear), central nervous system, blood, skin, eye or bone marrow. In some embodiments, the stem cells are mesenchymal stem cells. Any of the methods described herein for culturing stem cells and inducing differentiation into inner ear cells (e.g., hair cells or supporting cells) can be used.

Stem cells useful for generating cells of the inner ear can be derived from a mammal, such as a human, mouse, rat, pig, sheep, goat, or non-human primate. For example, stem cells have been identified and isolated from the mouse utricular macula (Li et al., Nature Medicine 9:1293-1299, 2003).

Generation of Neural Progenitor Cells

There are a number of induction protocols known in the art for inducing differentiation of stem cells with neurogenic potential into neural progenitor cells, including growth factor treatment (e.g., treatment with EGF, FGF, and IGF, as

described herein) and neurotrophin treatment (e.g., treatment with NT3 and BDNF, as described herein). Other differentiation protocols are known in the art; see, e.g., Corrales et al., J. Neurobiol. 66(13):1489-500 (2006); Kim et al., Nature 418, 50-6 (2002); Lee et al., Nat Biotechnol 18, 675-9 (2000); and Li et al., Nat Biotechnol 23, 215-21 (2005).

As one example of an induction protocol, the stem cells are grown in the presence of supplemental growth factors that induce differentiation into progenitor cells. These supplemental growth factors are added to the culture medium. The type and concentration of the supplemental growth factors is be adjusted to modulate the growth characteristics of the cells (e.g., to stimulate or sensitize the cells to differentiate) and to permit the survival of the differentiated cells such as neurons, glial cells, supporting cells or hair cells.

Exemplary supplementary growth factors are discussed in detail below, and include, but are not limited to basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF). Alternatively, the supplemental growth factors can include the neurotrophic factors neurotrophin-3 (NT3) and brain derived neurotrophic factor (BDNF). Concentrations of growth factors can range from about 100 ng/mL to about 0.5 ng/mL (e.g., from about 80 ng/mL to about 3 ng/mL, such as about 60 ng/mL, about 50 ng/mL, about 40 ng/mL, about 30 ng/mL, about 20 ng/mL, about 10 ng/mL, or about 5 ng/mL).

Neural progenitor cells produced by these methods include inner ear progenitor cells, i.e., cells that can give rise to inner ear cells such as hair cells and supporting cells. Inner ear progenitor cells can be identified by the expression of marker genes such as nestin, sox2, and musashi, in addition to certain inner-ear specific marker genes Brn3C, Pax2, and Atoh1. The invention includes purified populations of inner ear progenitor cells expressing nestin, sox2, musashi, Brn3C, Pax2, and Atoh1. These inner ear progenitor cells are lineage committed, and can be induced to further differentiate into hair cells and supporting cells by a method described herein.

Progenitor cells prepared by a method described herein can optionally be frozen for future use.

Cell Culture Methods

In general, standard culture methods are used in the methods described herein. Appropriate culture medium is described in the art, such as in Li et al. (supra). For example, stem cells can be cultured in serum free DMEM/high-glucose and F12 media (mixed 1:1), and supplemented with N2 and B27 solutions and growth factors. Growth factors such as EGF, IGF-1, and bFGF have been demonstrated to augment sphere formation in culture. In vitro, stem cells often show a distinct potential for forming spheres by proliferation of single cells. Thus, the identification and isolation of spheres can aid in the process of isolating stem cells from mature tissue for use in making differentiated cells of the inner ear. The growth medium for cultured stem cells can contain one or more or any combination of growth factors. This includes leukemia inhibitory factor (LIF) which prevents the stem cells from differentiating. To induce the cells (and the cells of the spheres) to differentiate, the medium can be exchanged for medium lacking growth factors. For example, the medium can be serum-free DMEM/high glucose and F12 media (mixed 1:1) supplemented with N2 and B27 solutions. Equivalent alternative media and nutrients can also be used. Culture conditions can be optimized using methods known in the art.

Differentiation by Expression of Atoh1

As described herein, expression of Atoh1 in stem-cell derived progenitor cells was sufficient to drive them into adopting hair cell markers. Studies of Atoh1 expression in the ear have indicated that this helix-loop-helix transcription factor occupies a key place in the hierarchy of inner ear transcription factors for differentiation of hair cells.

Atoh1 nucleic acids and polypeptides are known in the art, and described in, for example, U.S. Pat. Nos. 6,838,444 and 7,053,200, and P.G. PUB. Nos. 2004/0237127 and 2004/0231009, all to Zoghbi et al., all incorporated by reference in their entirety. In some embodiments, the Atoh1 is, or is at least 80%, 85%, 90%, 93%, or 95% identical to, human atonal homolog 1 (ATOH1); ATH1; and HATH1 (for additional information see Ben-Arie et al., Molec. Genet. 5: 1207-1216 (1996); Bermingham et al., Science 284: 1837-1841 (1999); OMIM *601461; UniGene Hs.532680; GenBank Accession Nos. NM_005172.1 (nucleic acid) and NP_005163.1 (polypeptide)). Other species can also be used, e.g., Mouse Atoh1 (also known as

Math1, GenBank Acc. No. NM_007500.2), chicken Atoh1 (also known as Cath1; GenBank Acc. No. AF467292.1).

The human Atoh1 mRNA (CDS = - 1065) and polypeptide sequences are as follows:

```
     1 atgtcccgcc tgctgcatgc agaagagtgg gctgaagtga aggagttggg agaccaccat
    61 cgccagcccc agccgcatca tctcccgcaa ccgccgccgc cgccgcagcc acctgcaact
   121 ttgcaggcga gagagcatcc cgtctacccg cctgagctgt ccctcctgga cagcaccgac
   181 ccacgcgcct ggctggctcc cactttgcag ggcatctgca cggcacgcgc cgcccagtat
   241 ttgctacatt ccccggagct gggtgcctca gaggccgctg cgccccggga cgaggtggac
   301 ggccgggggg agctggtaag gaggagcagc ggcggtgcca gcagcagcaa gagccccggg
   361 ccggtgaaag tgcgggaaca gctgtgcaag ctgaaaggcg gggtggtggt agacgagctg
   421 ggctgcagcc gccaacgggc cccttccagc aaacaggtga atggggtgca gaagcagaga
   481 cggctagcag ccaacgccag ggagcggcgc aggatgcatg ggctgaacca cgccttcgac
   541 cagctgcgca atgttatccc gtcgttcaac aacgacaaga agctgtccaa atatgagacc
   601 ctgcagatgg cccaaatcta catcaacgcc ttgtccgagc tgctacaaac gcccagcgga
   661 ggggaacagc caccgccgcc tccagcctcc tgcaaaagcg accaccacca ccttcgcacc
   721 gcggcctcct atgaaggggg cgcgggcaac gcgaccgcag ctggggctca gcaggcttcc
   781 ggagggagcc agcggccgac cccgcccggg agttgccgga ctcgcttctc agccccagct
   841 tctgcgggag ggtactcggt gcagctggac gctctgcact tctcgacttt cgaggacagc
   901 gccctgacag cgatgatggc gcaaaagaat ttgtctcctt ctctccccgg gagcatcttg
   961 cagccagtgc aggaggaaaa cagcaaaact tcgcctcggt cccacagaag cgacggggaa
  1021 ttttcccccc attcccatta cagtgactcg gatgaggcaa gttag (SEQ ID NO:1)

MSRLLHAEEWAEVKELGDHHRQPQPHHLPQPPPPPQPPATLQAREHPVYP
PELSLLDSTDPRAWLAPTLQGICTARAAQYLLHSPELGASEAAAPRDEVD
GRGELVRRSSGGASSSKSPGPVKVREQLCKLKGGVVVDELGCSRQRAPSS
KQVNGVQKQRRLAANARERRRMHGLNHAFDQLRNVIPSFNNDKKLSKYET
LQMAQIYINALSELLQTPSGGEQPPPPPASCKSDHHHLRTAASYEGGAGN
ATAAGAQQASGGSQRPTPPGSCRTRFSAPASAGGYSVQLDALHFSTFEDS
ALTAMMAQKNLSPSLPGSILQPVQEENSKTSPRSHRSDGEFSPHSHYSDS
DEAS (SEQ ID NO:2)
```

The mouse Atoh1 mRNA (CDS = 196 – 1251) and polypeptide sequences are as follows:

```
     1 tcgacccacg cgtccgccca cgcgtccgga tctccgagtg agaggggcag ggtcagagga
    61 ggaaggaaaa aaaaatcaga ccttgcagaa gagactagga aggtttttgt tgttgttgtt
   121 cggggcttat ccccttcgtt gaactgggtt gccagcacct cctctaacac ggcacctccg
   181 agccattgca gtgcgatgtc ccgcctgctg catgcagaag agtgggctga ggtaaaagag
   241 ttgggggacc accatcgcca tccccagccg caccacgtcc cgccgctgac gccacagcca
   301 cctgctaccc tgcaggcgag agaccttccc gtctacccgg cagaactgtc cctcctggat
   361 agcaccgacc cacgcgcctg gctgactccc actttgcagg gcctctgcac ggcacgcgcc
   421 gcccagtatc tgctgcattc tcccgagctg ggtgcctccg aggccgcggc gccccgggac
   481 gaggctgaca gccagggtga gctggtaagg agaagcggct gtggcggcct cagcaagagc
   541 cccgggcccg tcaaagtacg ggaacagctg tgcaagctga agggtggggt tgtagtggac
   601 gagcttggct gcagccgcca gcgagcccct tccagcaaac aggtgaatgg ggtacagaag
   661 caaaggaggc tggcagcaaa cgcaagggaa cggcgcagga tgcacgggct gaaccacgcc
   721 ttcgaccagc tgcgcaacgt tatcccgtcc ttcaacaacg acaagaagct gtccaaatat
   781 gagaccctac agatggccca gatctacatc aacgctctgt cggagttgct gcagactccc
   841 aatgtcggag agcaaccgcc gccgcccaca gcttcctgca aaaatgacca ccatcacctt
   901 cgcaccgcct cctcctatga aggaggtgcg ggcgcctctg cggtagctgg ggctcagcca
   961 gccccgggag ggggcccgag acctaccccg ccccgggcctt gccggactcg cttctcaggc
  1021 ccagcttcct ctgggggtta ctcggtgcag ctggacgctt tgcacttccc agccttcgag
  1081 gacagggccc taacagcgat gatggcacag aaggacctgt cgccttcgct gcccgggggc
  1141 atcctgcagc ctgtacagga ggacaacagc aaaacatctc ccagatccca cagaagtgac
  1201 ggagagtttt ccccccactc tcattacagt gactctgatg aggccagtta ggaaggcaac
  1261 agctccctga aaactgagac aaccaaatgc ccttcctagc gcgcgggaag ccccgtgaca
  1321 aatatccctg cacccttaa tttttggtct gtggtgatcg ttgttagcaa cgacttgact
  1381 tcggacggct gcagctcttc caatcccctt cctcctacct tctccttcct ctgtatgtag
  1441 atactgtatc attatatgta cctttacgtg gcatcgtttc atggtccatg ctgccaatat
```

```
1501 gctgctaaaa tgtcgtatct ctgcctctgg tctgggtttc acttatttta taccttggga
1561 gttcatcctt gcgtgttgcg ctcactcaca aataagggag ttagtcaatg aagttgtttc
1621 cccaactgct tgagacccgc attgggtact ttactgaaca cggactattg tgttgttaaa
1681 atgcaggggc agataagagt atctgtagag cttagacacc aagtgtgtcc agcagtgtgt
1741 ctagcggacc cagaatacac gcacttcatc actggccgct gcgccgcctt gaagaaactc
1801 aactgccaat gcagagcaac ttttgatttt aaaaacagcc actcataatc attaaactct
1861 ttgcaaatgt ttgtttttgc aaatgaaaat taaaaaaaaa catgtagtgt caaaggcatt
1921 tggtcaattt tattttgctt tgttaacatt agaaaagtta tttattattg cgtatttgga
1981 cccatttcta cttaattgcc tttttttac attttctact cgagatcgtt ttattttgat
2041 ttagcaaatc cagttgccat tgctttatgt atgtatgctc ttttacaaat gataaaataa
2101 actcggaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaa (SEQ ID NO:3)

MSRLLHAEEWAEVKELGDHHRHPQPHHVPPLTPQPPATLQARDLLVRRSG
CGGLSKSPGPVKVREQLCKLKGGVVVDELGCSRQRAPSSKQVNGVQKQRR
LAANARERRRMHGLNHAFDQLRNVIPSFNNDKKLSKYETLQMAQIYINAL
SELLQTPNVGASSGGYSVQLDALHFPAFEDRALTAMMAQKDLSPSLPGGI
LQPVQEDNSKTSPRSHRSDGEFSPHSHYSDSDEAS (SEQ ID NO:4)
```

The chicken Cath1 mRNA (CDS = 1 – 717) and polypeptide sequences are as follows:

```
   1 atggccccag gaggtagcga gtgttgttgc agtgatgccg cgcacatcac ttggaggcag
  61 tgggagtaca cgcacgagaa ccaactgtgc gtggcaggaa ctgtcagcag gatgaggccc
 121 aggacgtggg tctgcaccgg atctttgtgg gaccaggaag cgggaattac tttgatgggc
 181 ccccaaatac ccaaagtgga tgaggcagga gtgatgaccc acccggcaag gtcgctttgc
 241 agcactgggg cacatccgtg tcccggggtg gtcgtgctgc ccacgggtgg gatagggcag
 301 ccttcaaaga agctctccaa gtacgagacg ctgcagatgg cgcaaatcta catcagcgcc
 361 ctcgccgagc ttctgcacgg gccgcccgcg ccccccgagc cgcccgccaa ggccgagctc
 421 cgcggggccc ccttcgagcc tcccccgccg ccccctcctc cgccgccccg cgcctcgccc
 481 cccgcgcccg ccaggactcg cttccccccg gcggcggccg cgggcggttt cgcggcgctt
 541 ctcgagccgc tgcgcttccc ttctttcccg gcgcagaaag cgccttctcc cgcgctgctc
 601 ctggggccgc ccgcgccgca gcagcccgag aggagcaaag cgtcgccgcg ctctcaccgc
 661 agcgacgggg agttctcgcc gcgctcccac tacagtgact cggacgaggc cagctag
(SEQ ID NO:5)

MAPGGSECCCSDAAHITWRQWEYTHENQLCVAGTVSRMRPRTWVCTGSLWDQEAGI
TLMGPQIPKVDEAGVMTHPARSLCSTGAHPCPGVVVLPTGGIGQPSKKLSKYETLQ
MAQIYISALAELLHGPPAPPEPPAKAELRGAPFEPPPPPPPPPPRASPPAPARTRF
PPAAAAGGFAALLEPLRFPSFPAQKAPSPALLLGPPAPQQPERSKASPRSHRSDGE
FSPRSHYSDSDEAS (SEQ ID NO:6)
```

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes is at least 80% of the length of the reference sequence, and in some embodiments is at least 90% or 100%. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is

equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

For purposes of the present invention, the comparison of sequences and determination of percent identity between two sequences can be accomplished using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

In some embodiments, the methods include expressing in the cells a Atoh1 polypeptide encoded by a nucleic acid that hybridizes to the human Atoh1 mRNA under stringent conditions. As used herein, the term "stringent conditions" describes conditions for hybridization and washing. Stringent conditions as used herein are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C. See, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (2006).

In some embodiments, the methods include expressing exogenous Atoh1 in a stem cell. This can be achieved, for example, by introducing an expression vector in the cell. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked and can include a plasmid, cosmid or viral vector. The vector can be capable of autonomous replication or it can integrate into a host DNA. Viral vectors include, e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses.

A vector can include a Atoh1 nucleic acid in a form suitable for expression of the nucleic acid in a host cell. Generally, the expression vector includes one or more regulatory sequences operatively linked to the nucleic acid sequence to be expressed. The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences. The design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors can be introduced into host cells using methods known in the art, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection,

lipofection, or electroporation. See, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (2006).

In the present methods, the Atoh1 polypeptide expressed in the stem cells will have the ability to induce differentiation of mesenchymal stem cells to hair cells and/or supporting cells, as described herein.

Differentiation by Culturing with Chick Otocysts

Also as described herein, the stem cell-derived progenitor cells also responded to physical contact with developing otocyst cells from the chicken embryo by differentiating into sensory epithelial cells, without the requirement for exogenous *Atoh1*. This was evidenced by nGFP expression from a *Atoh1* enhancer-GFP reporter construct and co-expression of myosin VIIa after co-culture and differentiation, as described herein. Neurons that express markers of sensory cells have been induced from bone marrow MSCs in previous work by incubation with otocyst and hindbrain-conditioned medium (Kondo et al., Proc Natl Acad Sci U S A 102, 4789-4794 (2005)) from embryonic mice.

Thus, the methods described herein can include contacting progenitor cells with otocyst cells, e.g., cells isolated from E3 embryonic chicks, as described herein.

In some embodiments, the methods include culturing the progenitor cells with the otocyst cells in a ratio of about 50,000 cells per confluent layer of otocyst cells, or by injection of 100,000 cells into an intact otocyst (see Examples, below). Alternatively, the stem cells can be cultured in the presence of chick otocyst-conditioned media, which can be produced using methods known in the art, e.g., using media that has been in contact with a culture of chick otocysts for at about four days.

Differentiation by Inhibition of Notch Signalling

Notch is a plasma membrane receptor, and the Notch pathway consists of Notch and its ligands, as well as intracellular proteins that transmit the Notch signal to the nucleus. Included in the Notch pathway are the transcription factors that bear the effector function of the pathway.

Notch signaling plays a role in lateral inhibition, in which one cell is singled out from a cell cluster for a given fate (e.g., differentiation into a hair cell, for example). Differentiation is inhibited in those cells not selected to differentiate, resulting in the prevention of a specified fate commitment on the part of most of the

cells of a cluster. Lateral inhibition occurs repeatedly during development. Central to this process is binding to the Notch receptor of one of several ligands, including Delta, Scabrous and Serrate. Ligand binding to Notch ligand triggers a chain of intracellular events resulting in lateral inhibition. A review of the Notch pathway can be found at Artavanis-Tsakonas et al., Science 268: 225-232 (1995). As described herein, inhibition of Notch in the inner ear progenitor cells described herein results in differentiation of the cells into hair cells and supporting cells.

Thus, in some embodiments of the methods described herein, progenitor cells are grown in the presence of a Notch signalling pathway inhibitor. Exemplary Notch pathway inhibitors include γ-secretase inhibitors, of which a number are known in the art (e.g., arylsulfonamides (AS), dibenzazepines (DBZ), benzodiazepines (BZ), N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT), L-685,458 (Sigma-Aldrich), and MK0752 (Merck). A useful concentration will depend on the inhibitor chosen.

Other Notch inhibitors include inhibitory nucleic acids (e.g., small interfering RNAs, antisense oligonucleotides, and morpholino oligos; methods for designing, making, and using them are known in the art, e.g., gene walk methods for selecting and optimizing inhibitory sequences, see, e.g., Engelke, RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology, (DNA Press, 2004); Mol, Antisense Nucleic Acids and Proteins, (CRC, 1994); Sioud, Ribozymes and Sirna Protocols (Methods in Molecular Biology), (Humana Press; 2nd edition 2004); and Philips, Antisense Therapeutics (Methods in Molecular Medicine), (Humana Press 2004)) targeting Notch (see, e.g., Presente et al., Proc. Nat. Acad. Sci. 101(6):1764-1768 (2004); Ivanov et al., Proc. Nat. Acad. Sci. 101(46):16216-16221 (2004)) or its ligands, i.e., Delta or Jagged (see, e.g., Patzel et al., Nature Biotechnology 23, 1440 - 1444 (2005); Purow et al., Cancer Research 65:2353-2363 (2005); or Stallwood et al., J. Immunol. 177:885-895 (2006)). Alternatively, the cells can be modified to express m-Numb (GenBank Acc. No. NP_001005743.1) or disheveled (Dvl; the human homologs are at GenBank Acc. No. NM_004421.2 (variant 1); NM_004422.2 (variant 2); and NM_004423.3 (variant 3), both endogenous inhibitors of Notch signalling.

Assaying Differentiation

A variety of methods can be utilized to determine that a stem cell has differentiated into a progenitor cell, or into a cell of the inner ear, e.g., a hair cell or

supporting cell. For example, the cell can be examined for the expression of a cell marker gene. Hair cell marker genes include myosin VIIa (myoVIIa), Atoh1, α9 acetylcholine receptor, espin, parvalbumin 3, and Brn3c. Supporting cell markers include claudin14, connexin 26, p75Trk, Notch 1, and S100A. Pluripotent stem cells generally do not express these genes. A stem cell that propagates and produces a cell expressing one or more of these genes, has produced a hair cell, i.e., the stem cell has differentiated at least partially into a hair cell. A stem cell that has differentiated into an inner ear progenitor cell (a precursor of hair cells) expresses early ear marker genes such as nestin, sox2, musashi, Brn3C, Pax2, and Atoh1. A progenitor cell can express one or more of these genes. The progenitor cells can be propagated in serum-free medium in the presence of growth factors. Removal of growth factors and expression of Atoh1, or co-culture with chick otocysts, will induce the cells to differentiate further, such as into hair cells and supporting cells.

Identification of a hair cell or hair cell progenitor (e.g., a hair cell, supporting cell, or progenitor cell that differentiated from a stem cell) can be facilitated by the detection of expression of marker genes as described herein. Detection of the products of gene expression can be by immunocytochemistry. Immunocytochemistry techniques involve the staining of cells or tissues using antibodies against the appropriate antigen. In this case, the appropriate antigen is the protein product of the tissue-specific gene expression. Although, in principle, a first antibody (i.e., the antibody that binds the antigen) can be labeled, it is more common (and improves the visualization) to use a second antibody directed against the first (e.g., an anti-IgG). This second antibody is conjugated either with fluorochromes, or appropriate enzymes for colorimetric reactions, or gold beads (for electron microscopy), or with the biotin-avidin system, so that the location of the primary antibody, and thus the antigen, can be recognized. The protein marker can also be detected by flow cytometry using antibodies against these antigens, or by Western blot analysis of cell extracts.

Alternatively or in addition, gene expression can be analyzed directly, e.g., using PCR methods known in the art, including quantitative PCR, e.g., quantitative RT-PCR, which can be used to detect and compare levels of expression.

Methods of Treatment

The methods described herein can be used to generate cells for therapeutic use. Treatment methods include generating cells of the inner ear (*e.g.*, hair cells or supporting cells) from stem cells, using a method described herein, for transplantation into an ear of a human in need thereof. Transplantation of the cells into the inner ear of a subject can be useful for restoring or improving the ability of the subject to hear, or for decreasing the symptoms of vestibular dysfunction. Inner ear cells derived from stem cells according to the methods described herein need not be fully differentiated to be therapeutically useful. A partially differentiated cell that improves any symptom of a hearing disorder in a subject is useful for the therapeutic compositions and methods described herein.

A human having a disorder of the inner ear, or at risk for developing such a disorder, can be treated with inner ear cells (hair cells or supporting cells) generated from stem cells using a method described herein. In a successful engraftment, at least some transplanted hair cells, for example, will form synaptic contacts with spiral ganglion cells, and integrate into the sensory epithelium of the inner ear. To improve the ability of the cells to engraft, the stem cells can be modified prior to differentiation. For example, the cells can be engineered to overexpress one or more anti-apoptotic genes in the progenitor or differentiated cells. The Fak tyrosine kinase or Akt genes are candidate anti-apoptotic genes that can be useful for this purpose; overexpression of FAK or Akt can prevent cell death in spiral ganglion cells and encourage engraftment when transplanted into another tissue, such as an explanted organ of Corti (see for example, Mangi et al., Nat. Med. 9:1195-201 (2003)). Neural progenitor cells overexpressing $\alpha_v\beta_3$ integrin may have an enhanced ability to extend neurites into a tissue explant, as the integrin has been shown to mediate neurite extension from spiral ganglion neurons on laminin substrates (Aletsee et al., Audiol. Neurootol. 6:57-65 (2001)). In another example, ephrinB2 and ephrinB3 expression can be altered, such as by silencing with RNAi or overexpression with an exogenously expressed cDNA, to modify EphA4 signaling events. Spiral ganglion neurons have been shown to be guided by signals from EphA4 that are mediated by cell surface expression of ephrin-B2 and -B3 (Brors et al., J. Comp. Neurol. 462:90-100 (2003)). Inactivation of this guidance signal may enhance the number of neurons that reach their target in an adult inner ear. Exogenous factors such as the

neurotrophins BDNF and NT3, and LIF can be added to tissue transplants to enhance the extension of neurites and their growth towards a target tissue in vivo and in ex vivo tissue cultures. Neurite extension of sensory neurons can be enhanced by the addition of neurotrophins (BDNF, NT3) and LIF (Gillespie et al., NeuroReport 12:275-279 (2001)). A Sonic hedgehog (Shh) polypeptide or polypeptide fragment (e.g., SHH-N), can also be useful as an endogenous factor to enhance neurite extension. Shh is a developmental modulator for the inner ear and a chemoattractant for axons (Charron et al., Cell 113:11 23 (2003)).

Any human experiencing or at risk for developing a hearing loss is a candidate for the treatment methods described herein. For example, the human can receive a transplant of inner ear hair cells or supporting cells generated by a method described herein. A human having or at risk for developing a hearing loss can hear less well than the average human being, or less well than a human before experiencing the hearing loss. For example, hearing can be diminished by at least 5, 10, 30, 50% or more. The human can have sensorineural hearing loss, which results from damage or malfunction of the sensory part (the cochlea) or the neural part (the auditory nerve) of the ear, or conductive hearing loss, which is caused by blockage or damage in the outer and/or middle ear, or the human can have mixed hearing loss, which is caused by a problem in both the conductive pathway (in the outer or middle ear) and in the nerve pathway (the inner ear). An example of a mixed hearing loss is a conductive loss due to a middle-ear infection combined with a sensorineural loss due to damage associated with aging.

The subject can be deaf or have a hearing loss for any reason or as a result of any type of event. For example, a human can be deaf because of a genetic or congenital defect; for example, a human can have been deaf since birth, or can be deaf or hard-of-hearing as a result of a gradual loss of hearing due to a genetic or congenital defect. In another example, a human can be deaf or hard-of-hearing as a result of a traumatic event, such as a physical trauma to a structure of the ear, or a sudden loud noise, or a prolonged exposure to loud noises. For example, prolonged exposures to concert venues, airport runways, and construction areas can cause inner ear damage and subsequent hearing loss. A human can experience chemical-induced ototoxicity, wherein ototoxins include therapeutic drugs including antineoplastic agents, salicylates, quinines, and aminoglycoside antibiotics, contaminants in foods or

medicinals, and environmental or industrial pollutants. A human can have a hearing disorder that results from aging, or the human can have tinnitus (characterized by ringing in the ears).

The cells can be administered by any suitable method. For example, to restore hearing, inner ear cells generated by a method described herein can be transplanted, such as in the form of a cell suspension, into the ear by injection, such as into the luminae of the cochlea. See, e.g., the methods described in Corrales et al., J. Neurobiol. 66(13):1489-500 (2006) and Hu et al., Experimental Cell Research 302:40– 47 (2005). Injection can be, for example, through the round window of the ear or through the bony capsule surrounding the cochlea. The cells can be injected through the round window into the auditory nerve trunk in the internal auditory meatus or into the scala tympani. In a preferred embodiment, the cells are administered into or near the sensory epithelium of the subject, e.g., into a fluid (perilymph)-filled space above or below the sensory epithelium, i.e., the scala media, scala tympani, or scala vestibuli.

Alternatively, a human suitable for the therapeutic compositions and methods described herein can include a human having a vestibular dysfunction, including bilateral and unilateral vestibular dysfunction. Vestibular dysfunction is an inner ear dysfunction characterized by symptoms that include dizziness, imbalance, vertigo, nausea, and fuzzy vision and may be accompanied by hearing problems, fatigue and changes in cognitive functioning. Vestibular dysfunction can be the result of a genetic or congenital defect; an infection, such as a viral or bacterial infection; or an injury, such as a traumatic or nontraumatic injury. Vestibular dysfunction is most commonly tested by measuring individual symptoms of the disorder (e.g., vertigo, nausea, and fuzzy vision). In these embodiments, the inner ear cells generated by a method described herein can be transplanted, such as in the form of a cell suspension, e.g., by injection, into an organ of the vestibular system, e.g., the utricle, ampulla and sacculus. The cells would generally be injected into the perilymph of these organs or into the vestibule (which connects the 3 organs).

Following treatment with an inner ear cell or inner ear cell progenitor as described herein, the human can be tested for an improvement in hearing or in other symptoms related to inner ear disorders. Methods for measuring hearing are well-known and include pure tone audiometry, air conduction, and bone conduction tests.

These exams measure the limits of loudness (intensity) and pitch (frequency) that a human can hear. Hearing tests in humans include behavioral observation audiometry (for infants to seven months), visual reinforcement orientation audiometry (for children 7 months to 3 years) and play audiometry for children older than 3 years. Oto-acoustic emission testing can be used to test the functioning of the cochlear hair cells, and electro-cochleography provides information about the functioning of the cochlea and the first part of the nerve pathway to the brain.

The therapeutic compositions and methods described herein can be used prophylactically, such as to prevent hearing loss, deafness, or other auditory disorder associated with loss of inner ear function. For example, a composition containing a differentiation agent can be administered with a second therapeutic, such as a therapeutic that may effect a hearing disorder. Such ototoxic drugs include the antibiotics neomycin, kanamycin, amikacin, viomycin, gentamycin, tobramycin, erythromycin, vancomycin, and streptomycin; chemotherapeutics such as cisplatin; nonsteroidal anti-inflammatory drugs (NSAIDs) such as choline magnesium trisalicylate, diclofenac, diflunisal, fenoprofen, flurbiprofen, ibuprofen, indomethacin, ketoprofen, meclofenamate, nabumetone, naproxen, oxaprozin, phenylbutazone, piroxicam, salsalate, sulindac, and tolmetin; diuretics; salicylates such as aspirin; and certain malaria treatments such as quinine and chloroquine.

For example, a human undergoing chemotherapy can also be administered an inner ear cell or inner ear cell progenitor as described herein, by a method described herein. The chemotherapeutic agent cisplatin, for example, is known to cause hearing loss. Therefore, a composition containing a differentiation agent can be administered with cisplatin therapy to prevent or lessen the severity of the cisplatin side effect. An inner ear cell or inner ear cell progenitor as described herein can be administered before, after and/or simultaneously with the second therapeutic agent. The two treatments generally will be administered by different routes of administration.

The compositions and methods featured in the invention are appropriate for the treatment of hearing disorders resulting from sensorineural hair cell loss or auditory neuropathy. For example, patients with sensorineural hair cell loss experience the degeneration of cochlear hair cells, which frequently results in the loss of spiral ganglion neurons in regions of hair cell loss, and may also experience loss of supporting cells in the organ of Corti, and degeneration of the limbus, spiral ligament,

and stria vascularis in the temporal bone material. Such patients may benefit particularly from administration of supporting cells and/or hair cells into the inner ear.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Sensory Progenitors from Mesenchymal Stem Cells

Mesenchymal stem cells were obtained from mouse bone marrow by culturing adherent cells from the marrow under high serum conditions.

Briefly, cells were obtained from bilateral femurs and tibias of 4 week old C57BL/6 or *Atoh1-nGFP* mice (Helms et al., Development 127, 1185-1196 (2000)) by flushing out the bone marrow with MEM-α (Gibco/BRL) containing 10% fetal bovine serum (FBS; BioWhittaker, Cambrex, NY) and1 mM glutamine (Gibco/BRL). Pelleted cells were resuspended and mixed with RBC lysis buffer (Gibco/BRL). Approximately 5×10^6 cells were cultured on a 10 cm dish overnight in MEM-α with 9% horse serum, 9% FBS, 1% Gluta-Max (Invitrogen) and 100 units/ml penicillin and streptomycin (100 µg/ml, Sigma) at 37°C in a 5% CO_2 atmosphere. Nonadherent hematopoietic stem cells were removed, leaving adherent bone marrow stromal cells. When the cells became confluent, trypsinization was performed and the cells were cultured and passaged three to five times, with media changes every 3-4 days. These cells are referred to as mesenchymal stem cells (MSC).

Immunohistochemistry was performed as follows. Cells were fixed for 10 min with 4% paraformaldehyde in PBS. Immunostaining was initiated by rehydrating and blocking the sections for 1 h with 0.1% Triton X-100 in PBS supplemented with 1% BSA and 5% goat serum (PBT1). Fixed and permeabilized cells or rehydrated sections were incubated overnight in PBT1. CD34, CD44, CD45, Sca-1 antibodies (BD Biosciences) diluted 1: 40 were used for the characterization of extracted bone marrow cells. Hair cells and bone marrow progenitors were characterized using monoclonal antibody to chick hair cell specific antigen diluted 1:500 (gift from Guy Richardson (Bartolami et al., J Comp Neurol 314, 777-788 (1991)); polyclonal antibody to myosin VIIa, 1:500 (Oshima et al., J Assoc Res Otolaryngol. 8(1):18-31 (2007)); monoclonal antibody to nestin, 1,000 (Developmental Studies Hybridoma Bank, Iowa City, IA); polyclonal antibody to parvalbumin 3, 1:2,000 (Heller et al., J

Assoc Res Otolaryngol 3, 488-498 (2002)); monoclonal antibody to Atoh1, 1:100 (Developmental Studies Hybridoma Bank); monoclonal antibody to neurofilament M, 1:200 (Chemicon); Polyclonal antibody to collagen type II, 1:40 (Chemicon); polyclonal antibody to Brn3c (Covance, Princeton); Cy-5 conjugated F-actin 1:1000 (Molecular probe). Samples were washed three times for 20 min each with PBS. Anti-rabbit, anti-guinea pig and anti-mouse secondary antibodies conjugated with FITC-, TRITC-, and Cy-5- (Jackson ImmunoResearch) were used to detect primary antibodies. The samples were counterstained with DAPI for 10 min (Vector Laboratories) and viewed by epifluorescence microscopy (Axioskop 2 Mot Axiocam, Zeiss) or confocal microscopy (TCS, Leica). The counting of immunopositive cells was performed by counting 300 cells in 20 randomly selected microscopic fields and significance was calculated by Student's t-test.

Flow cytometric analysis was also performed. MSC were incubated with antibodies to CD34, CD44, CD45 or Sca-1 (BD Biosciences) and further incubated with secondary anti-mouse antibody conjugated to TRITC. Data were acquired and analyzed using an Agilent 2100 Bioanalyzer system and flow cytometry chips (Agilent Technology Inc., Palo Alto, CA). The reference window was set so that fluorescence from the secondary antibody alone was less than 2%.

The MSCs were negative for CD34 and CD45, markers for hematopoietic stem cells in bone marrow (Jiang et al., Nature 418, 41-49 (2002); Pittenger et al., Science 284, 143-147 (1999)) and positive for CD44 and Sca-1, markers for MSCs (Dezawa et al., J Clin Invest 113, 1701-1710 (2004)). Sca-1 was present on 5.2% of the cells and CD44 was present on 11.5% of the cells based on immunohistochemistry and the percentages determined by flow cytometry were similar (Fig. 1A and 1D and Table 1). We detected co-expression of CD44 and nestin as well as Sca-1 and nestin on a small percentage of the cells (Fig. 1B and 1C).

Table 1. Co-Expression of CD44 and Sca-1 with Nestin in Mesenchymal Stem Cells

	pre-induction (%)	post-induction (%)
Nestin (+) cells	4.7 ± 0.8	14.2 ± 2.0
CD44 (+) cells	11.5 ± 1.6	11.9 ± 1.8
Sca-1 (+) cells	5.2 ± 1.5	5.0 ± 0.4
CD 44 & nestin (+) cells	3.4 ± 0.9	9.9 ± 0.9
Sca-1 & nestin (+) cells	2.8 ± 1.2	4.3 ± 0.5

Positive cells were counted in relation to total nuclei stained by DAPI. Data are mean ± SE for 10 separate experiments. The increase in cells staining with nestin was significant

($p < 0.001$) as was the increase in the cells staining for both nestin and CD44 ($p < 0.001$) and nestin and Sca-1 ($p < 0.05$).

We confirmed the previously reported capacity of MSCs to be converted to chondrocytes (Pittenger et al., Science 284, 143-147 (1999)) and neurons (Dezawa et al., J Clin Invest 113, 1701-1710 (2004)). For chondrogenic differentiation, MSC were formed into a micropellet and cultured in DMEM with 10 ng/ml TGFbeta1, 6.25 ug/ml transferrin and 6.25 ug/ml insulin for 2 weeks. Their potential to differentiate into chondrocytes is demonstrated in Fig. 1E. For neuronal differentiation, MSC were cultured in DMEM/F12 1:1 containing N2/B27 supplement with bFGF (10 ng/ml) for 14 days and for 7 days without FGF. This resulted in differentiation to neurons (Dezawa et al., J Clin Invest 113, 1701-1710 (2004)) as shown by neuronal markers (Fig. 1F).

To determine whether otic vesicle growth factors that are important in the early development of inner ear progenitor cells could have a similar effect on MSCs, we removed the serum from the MSCs after 3-5 passages and cultured the cells in serum-free medium containing IGF-1, EGF and bFGF.

For the induction of progenitor cells, passage 3-5 MSC were trypsinized and transferred to 6-well plates or 4 well plates (BD Bioscience) coated with poly-L-ornithine and gelatin or fibronectin (Sigma) at 5×10^4 cells/ml. Cells were cultured for 5-7 days, and then cultured in serum-free medium composed of DMEM/F12 1:1 containing N2/B27 supplements (Invitrogen). For progenitor cell induction, we used a combination of EGF (20 ng/ml) and IGF (50 ng/ml; R&D Systems, Minneapolis, MN) for 2 weeks followed by the addition of bFGF (10 ng/ml) plus the other growth factors for an additional 2 weeks, or a combination of NT3 (30 ng/ml) and bFGF (10 ng/ml) for 4-5 days followed by NT3 (30 ng/ml) and BDNF (10 ng/ml) for 7 days.

Semiquantitative RT-PCR was performed as follows. Total RNA was extracted with the RNAeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For reverse transcription, 6 μg of total RNA was used with SuperScript III transcriptase (Invitrogen) and oligo-dT primers. The PCR cycling conditions were optimized in pilot experiments. Specific cycling parameters were: initial denaturation step at 94°C for 2 minutes, denaturation 94 °C for 30 seconds, annealing temperature optimized between 56-60°C for 30 seconds, extension 72°C for 60 seconds, extension 72°C for 60 seconds, and followed by 7 minutes of terminal extension at 72°C after the last cycle. The number of cycles was optimized

between 30 and 35, and conditions were kept constant for each primer. The presented data are from experiments repeated at least 5 times. Control PCR without reverse transcriptase did not produce specific bands. The primer pairs and cDNA product lengths were as follows:

Table 2: RT-PCR - Primer Pairs and cDNA Product Length

cDNA target	Forward primer	SEQ ID NO:	Reverse primer	SEQ ID NO:	Expected product length
Oct4	ATG GCT GGA CAC CTG GCT TCA G	7.	TTA ACC CCA AAG CTC CAG GTT C	8.	1033 bp
Otx2	CCA TGA CCT ATA CTC AGG CTT CAG G	9.	GAA GCT CCA TAT CCC TGG GTG GAA AG	10.	211 bp
Sox2	CAC CCG GGC CTC AAC GCT CAC G	11.	TCC CCT TCT CCA GTT CGC AGT CCA	12.	414 bp
Pax2	CCA AAG TGG TGG ACA AGA TTG CC	13.	GGA TAG GAA GGA CGC TCA AAG AC	14.	544 bp
Pax6	AGA CTT TAA CCA AGG GCG GT	15.	TAG CCA GGT TGC GAA GAA CT	16.	589 bp
Nestin	AAC AGA GAT TGG AAG GCC GCT GGC	17.	CTT CAG AAA GGC TGT CAC AGG AG	18.	392 bp
Musashi	ATG GAG ACT GAC GCG CCC CAG	19.	ATC TTC TTC GTC CGA GTG AC	20.	332 bp
GATA3	CCT CCG ACG GCA GGA GTC	21.	ACC GTA GCC CTG ACG GAG TTT	22.	319 bp
Math1	AGA TCT ACA TCA ACG CTC TGT C	23.	ACT GGC CTC ATC AGA GTC ACT G	24.	449 bp
Neurogenin-1	TGG TGT CGT CGG GGA AC	25.	AAG GCC GAC CTC CAA ACC TC	26.	400 bp
NeuroD	ACG GGC TGA ACG CGG CGC TGG AC	27.	TGA AAG AGA AGT TGC CAT TGA TG	28.	513 bp
Brn3c	GCC ATG CGC CGA GTT TGT C	29.	ATG GCG CCT AGA TGA TGC	30.	714 bp
Espin	CAG CCT	31.	TGA CCT	32.	475 bp

	GAG TCA CCG CAG CCT C		GTC GCT GCC AGG GCG CG		
Myo7a	CTC CCT CTA CAT CGC TCT GTT CG	33.	AAG CAC CTG CTC CTG CTC GTC CAC G	34.	628 bp
Zic1	GGC CAA CCC CAA AAA GTC GTG	35.	GAG AGC TGG GGT GCG TGT AGG A	36.	425 bp
Zic2	GGC GGC GCA GCT CCA CAA CCA GTA	37.	TTG CCA CAG CCC GGG AAA GGA CAG	38.	405 bp
TrkB	TTG CCC CTT CCC CTT TTA T	39.	CGC TTG CTC GCT CTC GT	40.	46 bp
TrkC	ACC CGC ATC CCA GTC AT	41.	TCC CGG TGT ACA AAG TGC	42.	521 bp
P27Kip	CTG GAG CGG ATG GAC GCC AGA C	43.	CGT CTG CTC CAC AGT GCC AGC	44.	525 BP
Jag2	GTC CTT CCC ACA TGG GAG TT	45.	GTT TCC ACC TTG ACC TCG GT	46.	
Notch1	AGA GAT GTG GGA TGC AGG AC	47.	CAC ACA GGG AAC TTC ACC CT	48.	306 BP
P75	GTC GTG GGC CTT GTG GCC	49.	CTG TGA GTT CAC ACT GGG G	50.	
S100	GCC AAC CGT GTG CTG CTG	51.	ACG TCG AGA CTG GGC AAG G	52.	423 bp
Cla14	CCA GCA CAG CGG TCC AG	53.	GGG GCA CGG TTG TCC TTG TAG	54.	664 bp
Con26	CGG AAC CAG AGA TAG GAC CTA C	55.	CTA AGC ACG GGT TGC CTC ATC C	56.	824 bp
Gapdh	AAC GGG AAG CCC ATC ACC	57.	CAG CCT TGG CAG CAC CAG	58.	442 bp

When the expression of neural progenitor cell markers in the resulting cultures was assessed, *Otx2*, *nestin*, *Sox2*, and *Musashi* were expressed in increased amounts in these cells, which are subsequently referred to herein as progenitor cells, relative to MSCs based on RT-PCR (Fig. 2A). *Pax6* was found in the progenitor cells but not in the MSCs (Fig. 2A). *Pax2* was not expressed. A low level of *Pax5* was detected but

Pax8 was not expressed (data not shown). A similar pattern of expression was seen for the stem cell marker, *Oct4*, which was expressed in the progenitor cells but interestingly, given its role in maintaining the pluripotency of stem cells, was not found in the MSCs. The increase in expression of *nestin* in the progenitor cells relative to the MSCs (Fig. 2A) was confirmed by immunohistochemistry (Figs. 2B and 2C and Table 1) and was significant ($p < 0.001$). Additional markers of the hair cell and neural lineages (*Atoh1, Brn3c, GATA3)* and neuronal markers *(TrkB* and *TrkC*) were also expressed in the progenitors (Fig. 2A).

Because of the expression of *TrkB* and *TrkC* in the progenitor cell populations, we tested whether incubation with NT-3 and BDNF, the neurotrophins that bind to these receptors, would increase the yield of progenitor cells or alter the expression of genes for hair cell or neuronal fate. We found an increase in expression of *Otx2*, *Sox2*, *nestin,* and *Musashi* under these conditions as well as an increase in *Oct4* expression (Fig. 3A), indicating that the cells may have adopted a neural progenitor cell fate. The neurotrophin-mediated conversion to progenitor cells had a more rapid time course that we found for EGF, IGF-1 and bFGF alone. The expression of proneural transcription factors, *NeuroD* and *Ngn1*, as well as neural and hair cell lineage markers, *GATA3*, *Atoh1*, and *Brn3c*, were also increased and the expression of *Ngn1* and *NeuroD*, which select for a neural over a hair cell fate in the inner ear (Kim et al., Development 128, 417-426 (2001); Matei et al., Dev Dyn. 234(3):633-50 (2005)) were higher when NT-3 and BDNF were included in the differentiation medium. Other transcription factors expressed in the otic precursors during development, *Zic2* and *Pax6*, were elevated in the progenitor cells relative to the MSCs, and *Zic1* expression was not observed. This suggests that NT-3 and BDNF induced the formation of cells of a neural lineage that were potentially destined to become both neurons and hair cells. However, the cells were not converted to hair cells or neurons because markers for these cells were not found (Fig. 3A, hair cell markers myosin VIIa and espin). We also tested for the expression of genes characteristic of other epithelial cells in the cochlea such as supporting cells, because the progenitors for hair cells can include or give rise to these cells and found that the progenitors expressed *S100A*, $p75^{trk}$, *claudin 14, connexin 26,* and *Notch1*.

The observation of supporting cell markers from the MSC-derived progenitor cells after growth factor induction may be correlated to their origin from a common

progenitor during *in vivo* development (Matei et al., Dev Dyn. 234(3):633-50 (2005); Satoh and Fekete, Development 132, 1687-1697 (2005)). Since hair cells can be induced to develop from supporting cells after introduction of the *Atoh1* gene (Izumikawa et al., Nat Med 11, 271-276 (2005); Zheng and Gao, Nat Neurosci 3, 580-586 (2000)), the role of supporting cells as potential progenitors for hair cells via transdifferentiation has been discussed (Izumikawa et al., Nat Med 11, 271-276 (2005)). The expression of supporting cell genes may reflect an intermediate or accompanying stage on the way to becoming hair cells; in *Atoh1* knockout mice undifferentiated cells with markers of supporting cells have been observed to activate the *Atoh1* gene (Fritzsch et al., Dev Dyn 233, 570-583 (2005); Woods et al., Nat Neurosci 7, 1310-1318 (2004)). Alternatively, supporting cells could be induced by the developing hair cells: ectopic hair cells in the greater epithelial ridge induced supporting cell markers in surrounding cells (Woods et al., Nat Neurosci 7, 1310-1318 (2004)). The MSCs could be induced to become hair cell progenitors by bFGF, EGF and IGF-1, factors that potentially stimulate the *in vivo* formation of these progenitors (Leon et al.,. Endocrinology 136, 3494-3503 (1995); Pauley et al., Dev Dyn 227, 203-215 (2003); Zheng et al., J Neurosci 17, 216-226 (1997)), and these progenitors were able to give rise to hair cells after overexpression of *Atoh1*. An increase in expression of neural progenitor markers could be caused by expansion of the cells that express these markers or by differentiation of MSCs to the neural progenitor phenotype.

As described herein, MSC-derived progenitor cells expressed neurotrophin receptors. BDNF and NT-3 play important roles in maturation of inner ear neurons (Fritzsch et al., J Neurosci 17, 6213-6225 (1997); Pirvola and Ylikoski, Curr Top Dev Biol 57, 207-223 (2003)), and in differentiation of neural stem cells to neurons (Ito et al., J Neurosci Res 71, 648-658 (2003)), and we therefore tested whether the fate of the progenitors could be modulated by neurotrophins. Incubation with these factors resulted in enrichment of progenitors that could be converted to hair cells by subsequent *Atoh1* overexpression (Izumikawa et al., Nat Med 11, 271-276 (2005); Zheng and Gao, Nat Neurosci 3, 580-586 (2000)) or co-culture with chick otocyst cells. Since NT-3 and BDNF were found to increase both *Atoh1* expression and differentiation in neural stem cells (Ito et al., J Neurosci Res 71, 648-658 (2003)), neurotrophins could directly increase differentiation of MSCs or could increase their competence to respond to overexpressed *Atoh1*.

Analysis of the progenitor cells obtained from the MSCs revealed parallels with natural development of the inner ear sensory epithelia. The MSC-derived progenitors expressed *Sox2*, which must be present for subsequent hair cell differentiation in the developing otocyst (Kiernan et al., Nature 434, 1031-1035 (2005)). The expression of *Atoh1* in cells that did not have myosin VIIa and the appearance of myosin VIIa at later time points is consistent with the order of their expression during development based on immunohistochemistry (Chen et al., Development 129, 2495-2505 (2002)). The lack of *Pax2* expression was surprising since the paired box transcription factor is ubiquitously expressed in the otocyst (Burton et al., Dev Biol 272, 161-175 (2004); Li et al., J Neurobiol 60, 61-70 (2004)). This may suggest that *Pax2* is not required or that it can be replaced by another factor for the conversion of MSCs to hair cells. *Pax5* was detected and may substitute for *Pax2* based on their functional equivalence (Bouchard et al., Development. 127(5):1017-28 (2000)). This is consistent with the analysis of the *Pax2* null mouse (Burton et al., Dev Biol 272, 161-175 (2004)), which appears to develop hair cells despite severe disruption of the normal morphology of the cochlea. The lack of *Zic1* expression relative to *Zic2* is also found during development of a hair cell phenotype as compared to sensory neurons in the otocyst (Warner et al., Dev Dyn 226, 702-712 (2003)) and is thus consistent with the development of a hair cell phenotype. The identification of inductive molecules on chick otocyst cells that are not present in conditioned media will provide further insights into hair cell differentiation.

The isolation of progenitor cells that can give rise to the tissue of origin, as observed in the inner ear (Li et al., Trends Mol Med 10, 309-315 (2004); Li et al., Nat Med 9, 1293-1299 (2003a)), might be predicted and yet the cells do not regenerate after damage, possibly because of the decrease in number of inner ear stem cells after birth (Oshima et al., J Assoc Res Otolaryngol. 8(1):18-31 (2007)). Therefore, a source of cells to provide replacements for these sensory cells is highly desirable. The *in vivo* role of MSCs in regeneration generally remains uncertain although bone marrow could act as a source of new cells in organs with few progenitors. Despite the demonstration that cells from bone marrow migrate into the brain and heart in adults (Mezey et al., Proc Natl Acad Sci U S A 100, 1364-1369 (2003); Weimann et al., Proc Natl Acad Sci U S A 100, 2088-2093 (2003)) and differentiate into neurons in the brain, hematopoietic stem cells from bone marrow were not converted to

cardiomyocytes after injection (Murry et al., Nature 428, 664-668 (2004)) and conversion to neurons was extremely rare (Wagers et al., Science 297, 2256-2259 (2002); Weimann et al., Proc Natl Acad Sci U S A 100, 2088-2093 (2003)). The most successful attempts at regeneration by adult stem cells from other tissues have been obtained after a lesion Doyonnas et al., Proc Natl Acad Sci U S A 101, 13507-13512 (2004); Edge, Transplant Proc 32, 1169-1171 (2000); Hess et al., Nat Biotechnol 21, 763-770 (2003); Pagani et al., J Am Coll Cardiol 41, 879-888 (2003)) and tissue damage may be required to see cell replacement by bone marrow-derived cells. Whether bone marrow-derived cells play any regenerative role in the sensory or peripheral nervous system in a spontaneous response to damage *in vivo* is an unanswered question, but, although low-level replacement of hair cells by bone marrow cells *in vivo* cannot be ruled out, spontaneous replacement of sensory cells is unlikely to be significant given the lack of hair cell regeneration seen in the adult cochlear and vestibular systems (Hawkins and Lovett, Hum Mol Genet 13(Spec No 2):R289-296 (2004); White et al., Nature 441, 984-987 (2006)).

Example 2: Transfection with an *Atoh1* Expression Plasmid Converts Progenitors to Hair Cells

To test whether the progenitor cells could act as inner ear precursor cells, it was evaluated whether overexpression of *Atoh1*, a transcription factor that is known to push competent progenitors to a hair cell fate (Izumikawa et al., Nat Med 11, 271-276 (2005); Zheng and Gao, Nat Neurosci 3, 580-586 (2000)), would increase the expression of hair cell markers.

The efficiency of *Atoh1* transfection was tested by counting green fluorescent cells after transfection with a vector coding for GFP expression in addition to *Atoh1*. We constructed a vector containing the Atoh1 coding sequence under EF1alpha-promotor control in the pTracer-EF vector (Invitrogen) that has a GFP-Zeocin fusion sequence under the CMV promoter. Gene transfection was done in the progenitor cell state or as MSC using LIPOFECTAMINE™ transfection reagent (Sigma). Cells were cultured in Zeocin (Invitrogen) to obtain stable transfectants. Transfected MSC were cultured in the serum-free conditions with combinations of growth factors.

When MSCs were transfected, as many as 2% of the cells were GFP positive at 24 hours (Fig. 4A). RT-PCR at day 14 showed that the transfected cell population expressed markers of developing sensory epithelia, such as *p27Kip*, *Brn3c* and

jagged2, and mature hair cells markers, *myosin VIIa* and *espin* (Fig. 4B) as well as increased expression of *Ngn1* and *NeuroD*. We also detected expression of supporting cell markers, *S100A*, *p75^{Trk}*, *claudin 14*, *connexin 26*, and *Notch1*, indicating that the progenitor cells could give rise to hair cells and supporting cells (Fig. 4C). Selection of MSC transfectants with stable *Atoh1* expression increased the percentage of GFP-positive cells (Fig. 4D). Incubation of these cells in the growth factors described above followed by immunohistochemistry yielded cells with expression of Atoh1 and myosin VIIa respectively in 7.7% and 7.1% of the total cells (Fig. 4E). Differentiation under growth factor stimulation gave rise to cells with Brn3c in the nucleus and myosin VIIa in the cytoplasm (Fig. 4F). These cells were positive for both markers in the same cells, with 92% of the Atoh1-positive cells showing staining for myosin VIIa, and 77% of the Brn3c-positive cells showing staining for myosin VIIa. Examination of the myosin VIIa positive cells for F-actin (Fig. 4G and H) indicated that some of the cells (4.9% of the myosin VIIa–positive cells) had developed protrusions at their apical poles. These protrusion had the polarized appearance of stereociliary bundles and were positive for espin (Fig. 4G).

Atoh1 expression led to strong expression of helix-loop-helix transcription factors, *Ngn1* and *NeuroD*. Several previous studies have indicated that *Atoh1* expression can increase these transcription factors. In mouse cerebellum *Atoh1* expression leads to overexpression of *NeuroD* (Helms et al., Mol Cell Neurosci 17, 671-682 (2001)). In zebrafish *NeuroD* is not expressed in the absence of *Atoh1* (Sarrazin et al., Dev Biol 295, 534-545 (2006)) and is required for hair cell formation. The related mouse achaete-scute (*Mash1*) upregulates *Ngn1* (Cau et al., Development 124, 1611-1621 (1997)). However, *Ngn1* was downregulated by overexpression of *Atoh1* in chick neural tube (Gowan et al., Neuron 31, 219-232 (2001)).

These data demonstrate that overexpression of Atoh1 in growth-factor induced progenitor cells induces the differentiation of a percentage of those cells to hair cells.

Example 3: Conversion of Progenitors to Hair Cells is Stimulated by Developing Otocyst Cells

To test whether the developing otocyst produced factors that would increase the differentiation of MSCs to hair cells, co-culture experiments of E3 chick otocyst cells with MSCs were performed.

Embryos of the white leghorn strain (Charles River) were harvested 72 hours after placing fertilized eggs onto rocking platforms in a humidified incubator maintained at 38°C. The dissection of otocysts from the extracted embryos was done in cooled PBS, pH 7.2, after removal of periotic mesenchymal tissues. The otocysts were trypsinized and dissociated to single cells for plating and 2×10^4 cells were cultured overnight in four-well plates in 10% FBS. One day after plating, the otocyst cells were fixed with 4% paraformaldehyde for 20 minutes, or inactivated with mitomicin C (10 μg/ml) for 3 hours, then washed 4 times with PBS. Conditioned medium from the cultured cells was collected and frozen prior to use on progenitors cells. Progenitor cells (5×10^4 cells/ml) induced in serum-free medium with growth factors, were overlaid on the chick otocyst cells and cultured for 5-7 days with EGF/IGF, followed by 10 days with EGF/IGF/FGF and withdrawal of growth factors for 5-10 more days. The cells were analyzed by RT-PCR or immunohistochemistry as described herein.

After culture in the presence of the chick otocyst cells for 21 days, increased expression of *myosin VIIa, jagged2, p27Kip, Brn3c* and *Atoh1* by RT-PCR was found (Fig. 5A). The factor(s) was unlikely to be a secreted molecule because fixation of the cells did not diminish their ability to promote differentiation after exposure for 14 days, while conditioned medium was ineffective in 14 days (Fig. 5A). Conversion of the stem cells to hair cells could be followed by appearance of green fluorescence in the cultures using MSCs derived from transgenic *Atoh1-nGFP* mice that express a nuclear version of enhanced GFP when *Atoh1* enhancer elements are activated (Chen et al., Development 129, 2495-2505 (2002); Lumpkin et al., Gene Expr Patterns 3, 389-395 (2003)). These green cells were observed in the co-cultures with chick otocyst cells (Fig. 5B) and the cells were co-labeled with antibody to myosin VIIa.

The otocyst from E3 chick embryos were used for injection of progenitor cells. The dissected otocysts were transferred into 7 ml of serum-free DMEM/F12 1:1 containing N2 and B27 on a gelatin-coated tissue culture dish. After attachment of intact otocysts, progenitor cells from MSC (5×10^7 cells/ml) were injected into the otocyst with a micropipette in 2 μl of medium. The left otic vesicles did not receive cell grafts and served as controls. The otocysts were harvested after 10-14 days, fixed 30 min in paraformaldehyde (4% in PBS), cryoprotected overnight in sucrose (30% in

PBS), embedded in TissueTek (EMS) and serially sectioned (16 μm) with a cryostat (CM3050, Leica, Nussloch, Germany).

When the progenitor cells were injected into chick otocysts obtained at E3, conversion of progenitors to cells with hair cell properties (5% of the myosin VIIa-positive cells were positive for nGFP) was observed (Fig. 6A). The murine hair cells were seen to incorporate into the hair cell bearing epithelia of the developing chicken otocyst as detected by expression of GFP (Fig. 6B). One possible explanation for the expression of hair cell genes by the MSC-derived cells in co-culture is fusion with chick cells. To rule this out we labeled the cells with an antibody to chick hair cell antigen (Bartolami et al., J Comp Neurol 314, 777-788 (1991)). Native chick hair cells could be detected lining the internal cavity of the otocyst (51% of 1,352 cells from 15 otocyst injections that stained for myosin VIIa were positive for chick hair cell antigen), and the cells that expressed nGFP and hair cell markers did not co-express chick hair cell antigen (Fig. 6C) and were therefore of mouse origin and not the product of cell fusion.

These experiments, performed in an attempt to understand how contact of the MSCs with developing otocyst cells provided a signal that induced their differentiation to hair cells, demonstrated that the inductive effect was through a cell surface molecule as opposed to a secreted factor. Injection of the MSC into the developing otocyst *in vitro* indicated that hair cells that differentiated from the stem cells were integrated into the chick otocyst epithelium, demonstrating that the environment provided by developing chicken otocyst cells could guide differentiation and integration of suitable progenitor cells. The instructive influence has also been seen previously with inner ear-derived stem cells and murine ES cell-derived progenitor cells (Li et al., Trends Mol Med 10, 309-315 (2004); Li et al., Nat Med 9, 1293-1299 (2003); Li et al., Proc Natl Acad Sci U S A 100, 13495-13500 (2003).

The effect of the co-incubation with otocyst cells may be simply to activate *Atoh1* expression and a sufficient amount of *Atoh1* may be required to allow hair cell differentiation since the MSCs had low levels of *Atoh1* but did not have detectable sensory epithelial cell markers. This type of high level expression could be needed for Atoh1 to overcome the level of preexisting endogenous inhibitors that interact with Atoh1 protein. The murine cells could be clearly distinguished from the chick hair cells that differentiated at the same time by their expression of nGFP and by

immunolabeling of the chick hair cells with a species-specific antibody. The cells were never co-stained (based on examination of 1,352 cells), indicating that the mouse hair cells had differentiated from stem cells and did not arise from cell fusion.

Example 4: Inhibition of Notch Signaling Induces Differentiation of Hair Cells

The Notch pathway maintains the alternating pattern of hair cells and supporting cells in vivo by suppressing the differentiation of hair cells from supporting cells and activation of Notch in the embryo appears to block development of hair cells from progenitors.

To examine the effect of the Notch pathway on the differentiation of hair cells, the NT3/BDNF treated progenitors were incubated with a γ-secretase inhibitor. Analysis of gene expression in the progenitors made by incubation with NT3, BDNF, FGF and subsequently treated with the γ-secretase inhibitor demonstrated that loss of the notch signaling increased Atoh1 expression. Atoh1 levels rose compared to the treatment with growth factors alone based on RT-PCR when the inhibitor was used at 1 μM (Fig. 7). The timing of the addition of the inhibitor was essential with inhibition at a later stage (after 3 days of differentiation in vitro) causing less induction of hair cell markers than inhibition starting at day 0 and continuing for 10 days. At the low concentration, γ-secretase inhibitor activates ngn1 and NeuroD and causes no increase in Atoh1 or hair cell markers. At higher concentration, the γ-secretase inhibitor increases Atoh1 and Brn3c expression. The increased Atoh1 appeared to be able to produce hair cells as the cells expressed markers for the hair cells such as myosin7a, p27Kip. As HLH transcription factors mediate the effects of the Notch pathway, this result is consistent with the role of Notch and suggests a mechanism for preventing hair cell differentiation under normal conditions.

Example 5: Inhibition of Hair Cell Differentiation in Human Stem Cells

To determine if human mesenchymal stem cells (hMSCs) can be differentiated into inner ear cell types including hair cells or sensory neurons, human bone marrow cells from healthy adults were evaluated.

The human bone marrow cells were harvested and cultured as plated on tissue culture plastic for 16 hours, and nonadherent hematopoietic stem cells were aspirated.

First, the adherent cells were cultured in αMEM containing 9% horse serum and 9% fetal bovine serum and were negative for blood-forming cell markers, CD34 and CD45. These cells gave rise to chondrocytes expressing type II and IV collagen after culture in the presence of TGFβ, transferrin and insulin.

Culture of hMSCs in DMEM/F12 medium containing N2 and B27 without serum in the presence of NT-3, BDNF, Sonic hedgehog and retinoic acid for 10 days gave rise to cells that expressed neurosensory progenitor markers detected by RT-PCR, Musashi, nestin, Pax6, Brn3a, NeuroD, Ngn1, and GATA3, and sensory neuron markers, peripherin and TrkC. These differentiated hMSCs were positive for β-III tubulin (2.1% of the total cells were positive based on immunohistochemistry) and, of these cells, 28% co-stained for peripherin and 31% co-stained for Brn3a.

For the differentiation to hair cells, hMSCs were transfected with human Atoh1 in an expression vector with a selectable marker for eukaryotic cells. The selected progenitor cells expressed Atoh1 and, after differentiation in DMEM/F12 medium containing N2 and B27 with NT-3 and BDNF for 10 days, expressed hair cell markers, Atoh1, myosin VIIa, p27Kip, Jag2 and espin based on RT-PCR.

The ability of these cells to engraft in an organ of Corti, the Atoh1-transfected cells were co-cultured with an *ex vivo* organ of Corti from mouse. This gave rise to cells expressing myosin VIIa and espin that were detected by immunostaining, i.e., differentiated hair cells. When the *ex vivo* mouse organ of Corti was treated with toxins to induce hair cell degeneration, co-cultured bone marrow-derived cells were observed to engraft in the mouse sensory epithelium, thus demonstrating the ability of cells obtained.

Thus, human MSCs are a potential alternative for cell-based treatment of hearing loss, as they can be differentiated into inner ear cell types including hair cells or sensory neurons, and can be successfully engrafted into structures of the inner ear.

Example 6: Math1-Estrogen Receptor (ER) Fusion Constructs

One alternative to constitutive expression of Math1 is to use a conditional or inducible system of gene expression, to upregulate Math1 with an inducer that is added to the cell medium or cochlear environment. An inducible model is particularly useful when investigating the temporal effects of gene expression.

This Example describes a system in which administration of tamoxifen, a synthetic estrogen agonist, induces expression of Math1. A Math1-estrogen receptor

(ER) fusion protein, where the ER has been mutated so that it selectively binds to tamoxifen rather than estrogen, is constitutively expressed. In the absence of tamoxifen, the Math1-ER construct remains quiescent within the cytosol where it is inactivated by heat shock proteins. The addition of tamoxifen to the transfected cells results in a dose-dependent localization of the Math1-ER construct to the nucleus where it is transcribed leading to increased expression of Math1. The sequence of Math1 is given above.

The sequence of ER used is as follows (SEQ ID NO:59):

ATGTCCAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTCGATGCAACGAGTGAT
GAGGTTCGCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATG
CTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAA
CCTGAAGATGTTCGCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAA
CATTTGGGCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTT
TCACTGGTTATGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTCTAGCG
TTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATATACGT
AATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTTACGTATAGCCGAAATTGCCAGGATCAGGGTT
AAAGATATCTCACGTACTGACGGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGC
ACCGCAGGTGTAGAGAAGGCACTTAGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCT
GGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGTGTTGCCGCGCCATCT
GCCACCAGCCAGCTATCAACTCGCGCCCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGC
GCTAAGGATGACTCTGGTCAGAGATACCTGGCCTGGTCTGGACACAGTGCCCGTGTCGGAGCCGCGCGA
GATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGGTGGCTGGACCAATGTAAATATT
GTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATGGTGCGCCTGCTGGAAGATGGGGAT
CAGGCTGGTGCCATGGGCGATCCACGAAATGAAATGGGTGCTTCAGGAGACATGAGGGCTGCCAACCTT
TGGCCAAGCCCTCTTGTGATTAAGCACACTAAGAAGAATAGCCCTGCCTTGTCCTTGACAGCTGACCAG
ATGGTCAGTGCCTTGTTGGATGCTGAACCGCCCATGATCTATTCTGAATATGATCCTTCTAGACCCTTC
AGTGAAGCCTCAATGATGGGCTTATTGACCAACCTAGCAGATAGGGAGCTGGTTCATATGATCAACTGG
GCAAAGAGAGTGCCAGGCTTTGGGGACTTGAATCTCCATGATCAGGTCCACCTTCTCGAGTGTGCCTGG
CTGGAGATTCTGATGATTGGTCTCGTCTGGCGCTCCATGGAACACCCGGGGAAGCTCCTGTTTGCTCCT
AACTTGCTCCTGGACAGGAATCAAGGTAAATGTGTGGAAGGCATGGTGGAGATCTTTGACATGTTGCTT
GCTACGTCAAGTCGGTTCCGCATGATGAACCTGCAGGGTGAAGAGTTTGTGTGCCTCAAATCCATCATT
TTGCTTAATTCCGGAGTGTACACGTTTCTGTCCAGCACCTTGAAGTCTCTGGAAGAGAAGGACCACATC
CACCGTGTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGGCCAAAGCTGGCCTGACTCTGCAG
CAGCAGCATCGCCGCCTAGCTCAGCTCCTTCTCATTCTTTCCCATATCCGGCACATGAGTAACAAACGC
ATGGAGCATCTCTACAACATGAAATGCAAGAACGTGGTACCCCTCTATGACCTGCTCCTGGAGATGTTG
GATGCCCACCGCCTTCATGCCCCAGCCAGTCGCATGGGAGTGCCCCCAGAGGAGCCCAGCCAGACCCAG
CTGGCCACCACCAGCTCCACTTCAGCACATTCCTTACAAACCTACTACATACCCCCGGAAGCAGAGGGC
TTCCCCAACACGATCTGA

ADDITIONAL REFERENCES

Kicic et al., J Neurosci 23, 7742-7749 (2003).

Ma et al., J Assoc Res Otolaryngol 1, 129-143 (2000).

Wang et al., Nature 422, 897-901 (2003).

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended

to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of providing a population of hair cells, the method comprising:
   obtaining a population of stem cells with neurogenic potential;
   culturing the stem cells under conditions sufficient to induce the differentiation of at least some of the stem cells into inner ear progenitor cells;
   inducing the expression of Atoh1 in the inner ear progenitor cells, in an amount and for a time sufficient to induce at least some of the inner ear progenitor cells to differentiate into hair cells,
   thereby providing a population of hair cells.
2. A method of providing a population of hair cells, the method comprising:
   obtaining a population of stem cells with neurogenic potential;
   culturing the stem cells under conditions sufficient to induce the differentiation of at least some of the stem cells into inner ear progenitor cells;
   contacting the inner ear progenitor cells with an inhibitor of Notch signalling, in an amount and for a time sufficient to induce at least some of the inner ear progenitor cells to differentiate into hair cells,
   thereby providing a population of hair cells.
3. A method of providing a population of hair cells, the method comprising:
   obtaining a population of stem cells with neurogenic potential;
   culturing the stem cells under conditions sufficient to induce the differentiation of at least some of the stem cells into inner ear progenitor cells;
   culturing the inner ear progenitor cells in the presence of chick otocyst cells for a time and under conditions sufficient for at least some of the inner ear progenitor cells to differentiate into hair cells, thereby providing a population of hair cells.
4. The method of claim 2, wherein the inhibitor of Notch signalling is a gamma-secretase inhibitor.
5. The method of any of claims 1-3, further comprising isolating the progenitor cells.
6. The method of any of claims 1-3, further comprising isolating the hair cells.
7. The method of any of claims 1-3, wherein the inner ear progenitor cells express nestin, sox2, musashi, Brn3C, Pax2, and Atoh1.
8. The method of any of claims 1-3, wherein the hair cells express one or more genes selected from the group consisting of Atoh1, jagged 2, Brn3c, p27Kip, Ngn1, NeuroD, myosin VIIa and espin.

9. The method of any of claims 1-3, wherein the hair cells express jagged 2, Brn3c, myosin VIIa and espin.
10. The method of any of claims 1-3, wherein the hair cells express F-actin in a V pattern on the apical surface of the cells.
11. A method of providing a population of supporting cells, the method comprising:
obtaining a population of stem cells with neurogenic potential;
culturing the stem cells under conditions sufficient to induce the differentiation of at least some of the stem cells into inner ear progenitor cells;
one or more of:
(i) inducing the expression of Atoh1 in the inner ear progenitor cells, in an amount and for a time sufficient to induce at least some of the inner ear progenitor cells to differentiate into supporting cells;
(ii) contacting the inner ear progenitor cells with an inhibitor of Notch signalling, in an amount and for a time sufficient to induce at least some of the progenitor cells to differentiate into supporting cells; or
(iii) culturing the inner ear progenitor cells in the presence of chick otocyst cells for a time and under conditions sufficient for at least some of the inner ear progenitor cells to differentiate into supporting cells,
thereby providing a population of supporting cells.
12. The method of claim 11, further comprising isolating the supporting cells.
13. The method of claim 1, wherein the supporting cells express one or more of claudin14, connexin 26, $p75^{Trk}$, Notch 1, and S100A.
14. The method of claim 11, wherein the inner ear progenitor cells express nestin, sox2, musashi, Brn3C, Pax2, and Atoh1.
15. The method of any of claims 1-14, further comprising transplanting the cells into a subject in need thereof.
16. The method of claim 15, wherein the population of stem cells is obtained from a subject in need of the transplant.
17. An isolated population of hair cells obtained by the method of claim 6.
18. An isolated population of supporting cells obtained by the method of claim 12.
19. A method of treating a subject who has or is at risk for developing a disorder that is treatable with a transplant of hair cells and/or supporting cells, the method

comprising transplanting the cells of claims 17 or 18 into the cochlea of the subject, thereby treating the subject.

20. The method of claim 19, wherein the population of stem cells was obtained from the subject in need of the transplant.
21. The method of any of claims 1 or 11, wherein inducing the expression of Atoh1 in the cells comprises inducing the expression of exogenous Atoh1 in the cells.
22. The method of claim 21, wherein inducing the expression of exogenous Atoh1 in the cells comprises transducing the cells with a vector encoding a Atoh1 polypeptide.
23. The method of claim 22, wherein the vector is a viral vector.
24. The method of claim 22, wherein the vector is a plasmid vector.
25. The method of claim 21, wherein inducing the expression of exogenous Atoh1 in the stem cells comprises increasing expression of endogenous Atoh1.
26. The method of claim 3 or 11, wherein culturing the stem cells in the presence of chick otocyst cells for a time and under conditions sufficient for at least some of the stem cells to differentiate into hair cells comprises culturing the stem cells in medium comprising IGF, EGF, and FGF.
27. The method of any of the preceding claims, wherein the stem cells are mesenchymal stem cells.
28. The method of any of the preceding claims, wherein the stem cells are human stem cells.

Figures 1A-1C

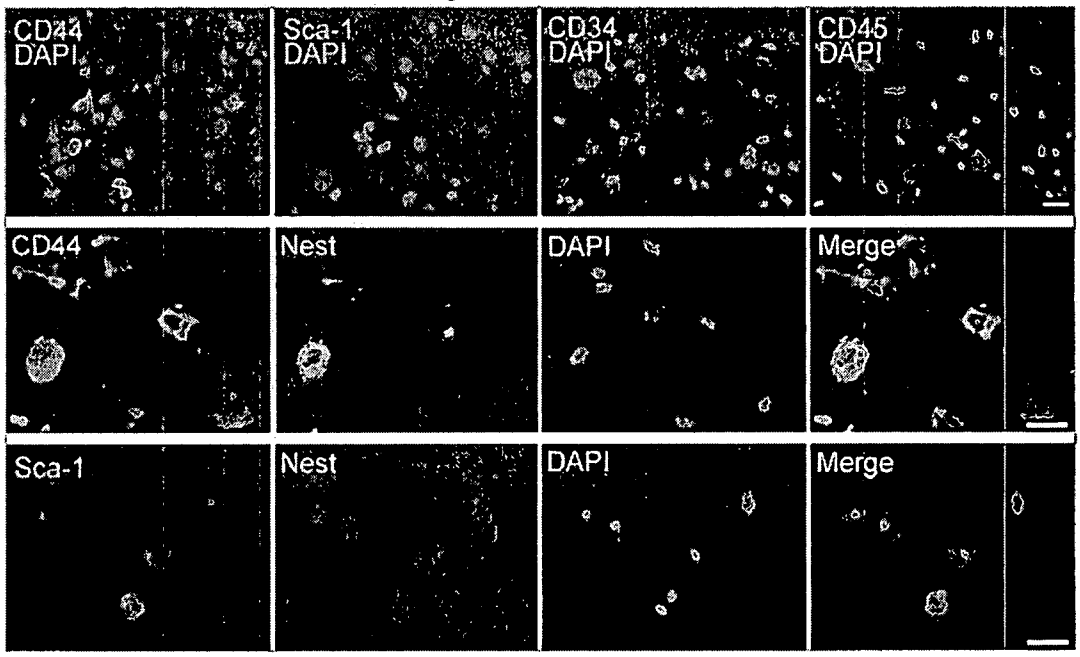


Figure 1E

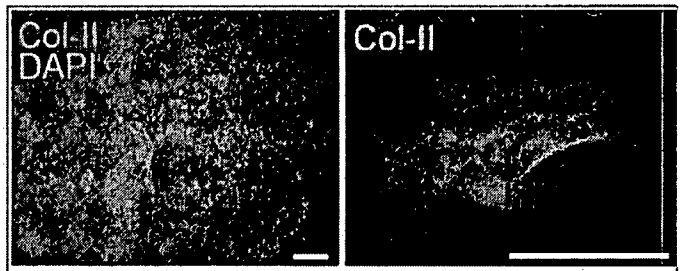


Figure 1F

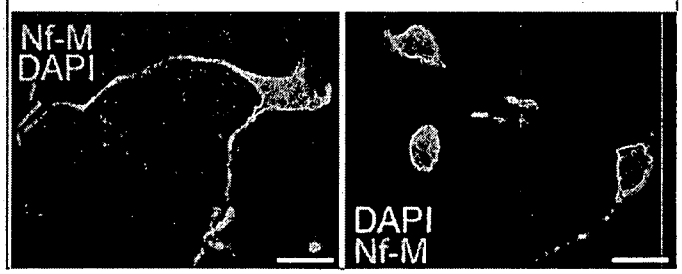
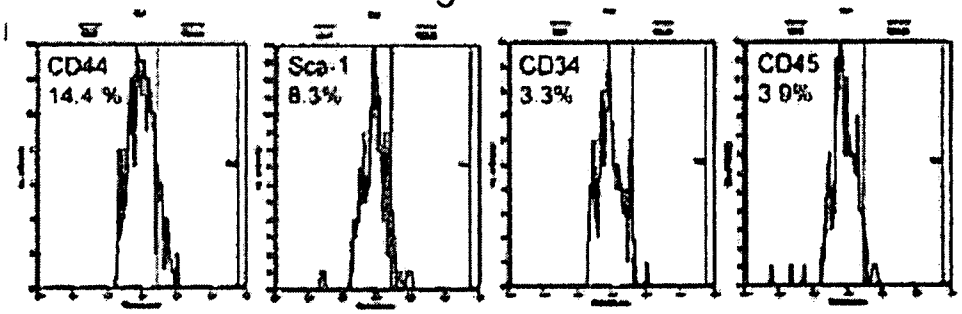


Figure 1D



Figures 2A-C

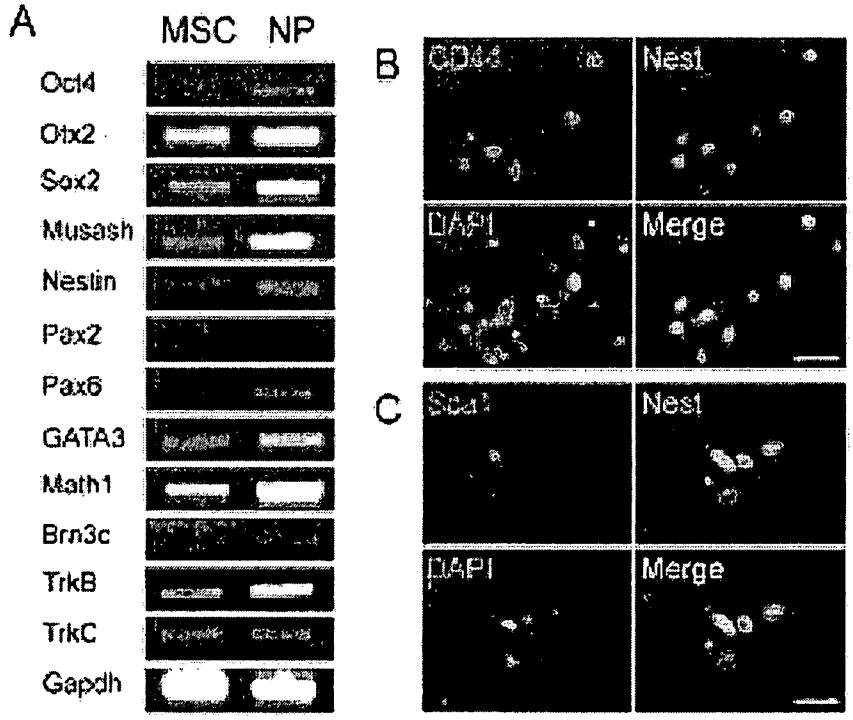

A
MSC
NP
Oct4
Otx2
Sox2
Musash
Nestin
Pax2
Pax6
GATA3
Math1
Brn3c
TrkB
TrkC
Gapdh
B
CD44
Nes1
DAPI
Merge
C
Sca1
Nes1
DAPI
Merge

Figure 3A

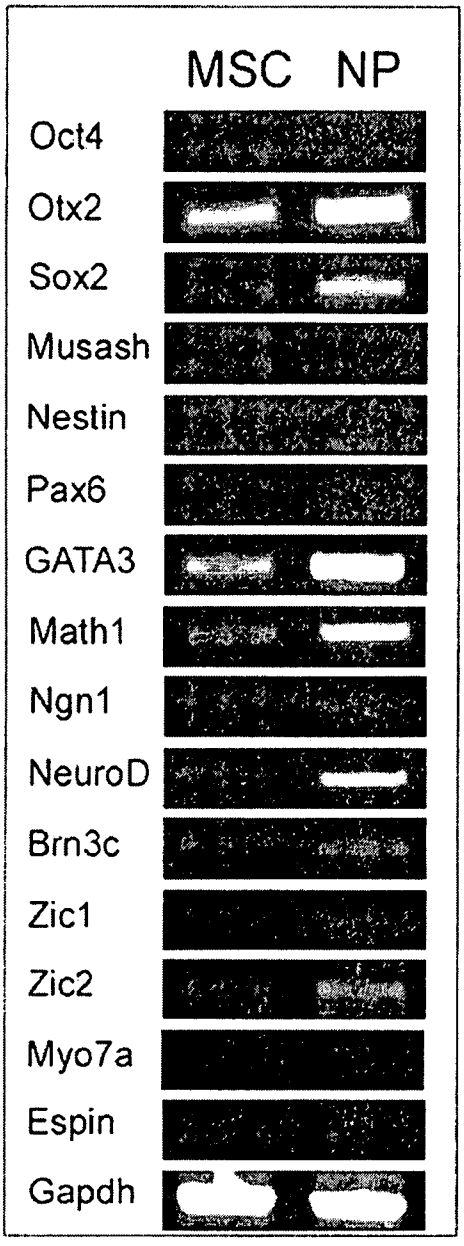


Figure 3B

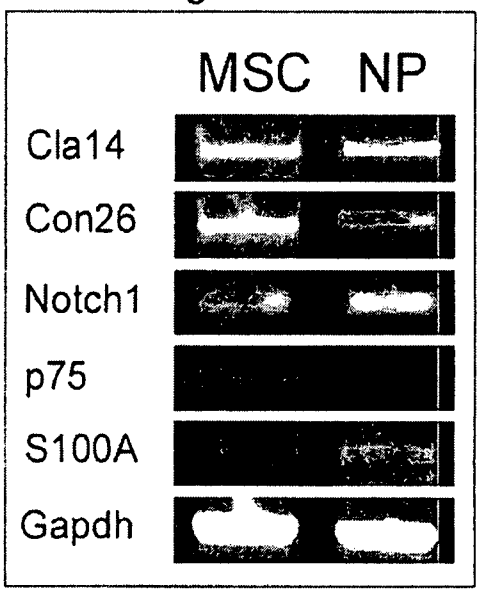


Figure 4A

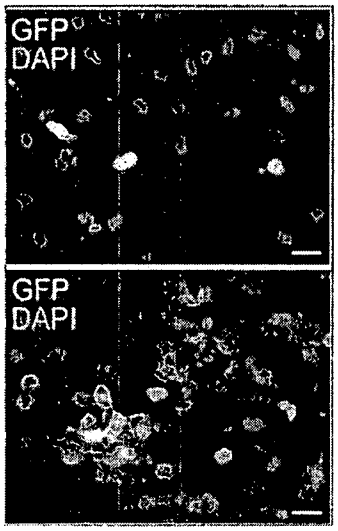


Figure 4D

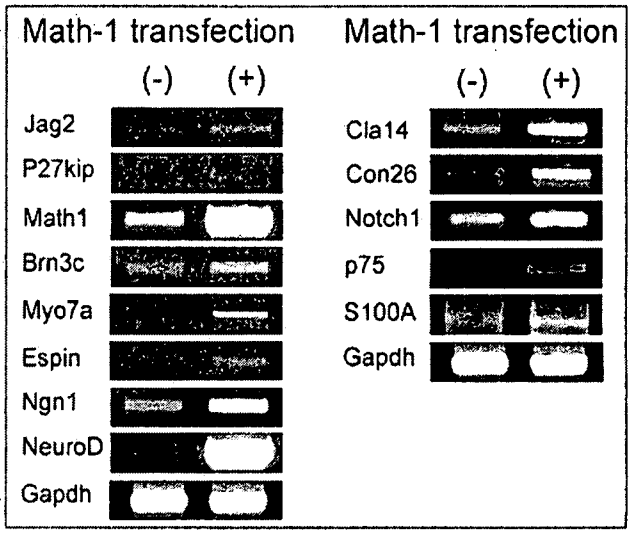


Figure 4B

Figure 4C

Figure 4E (top row)

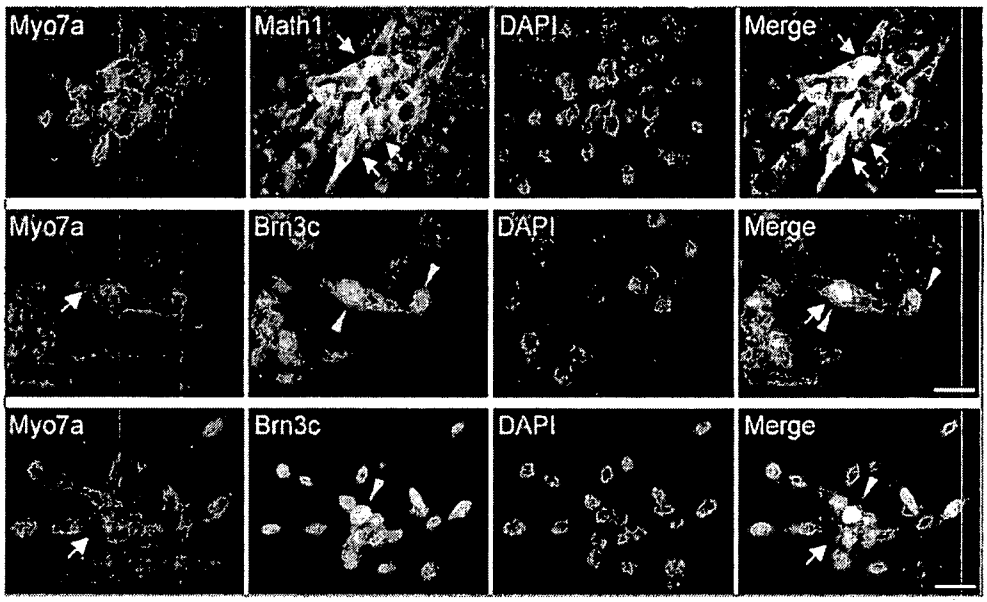


Figure 4F (bottom 2 rows)

Figure 4G (top row)

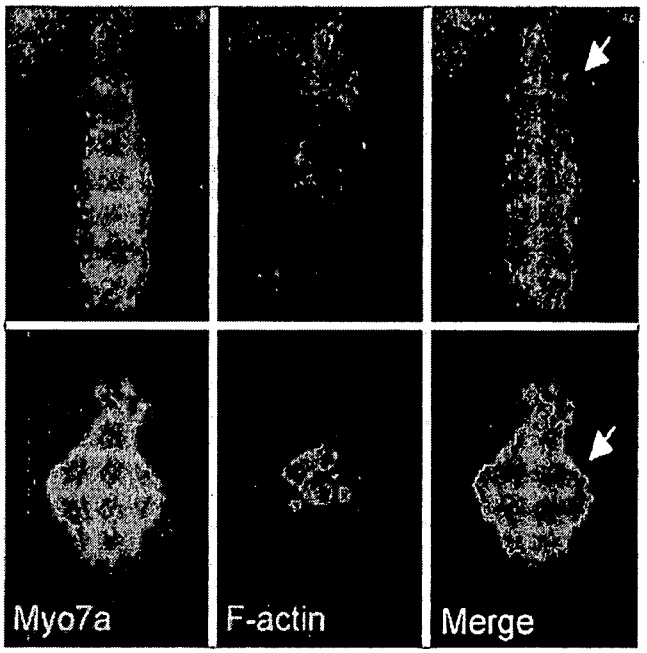


Figure 4H (bottom row)

Figure 5A

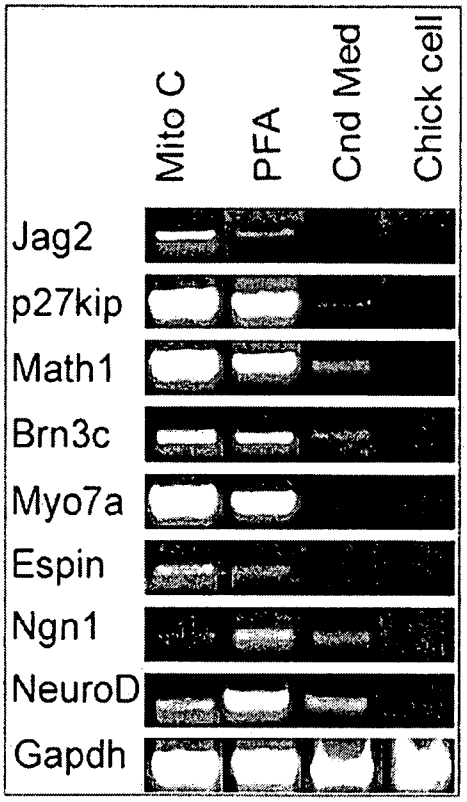


Figure 5B

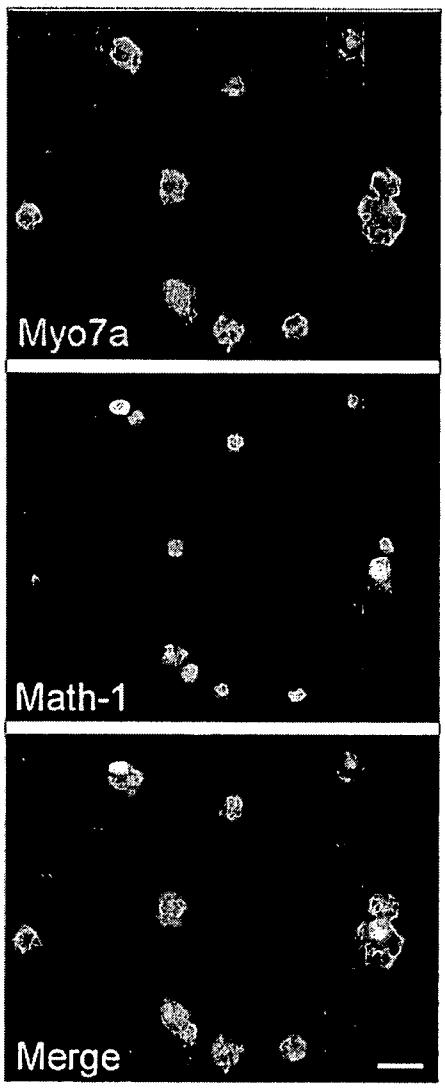


Figure 6A-C

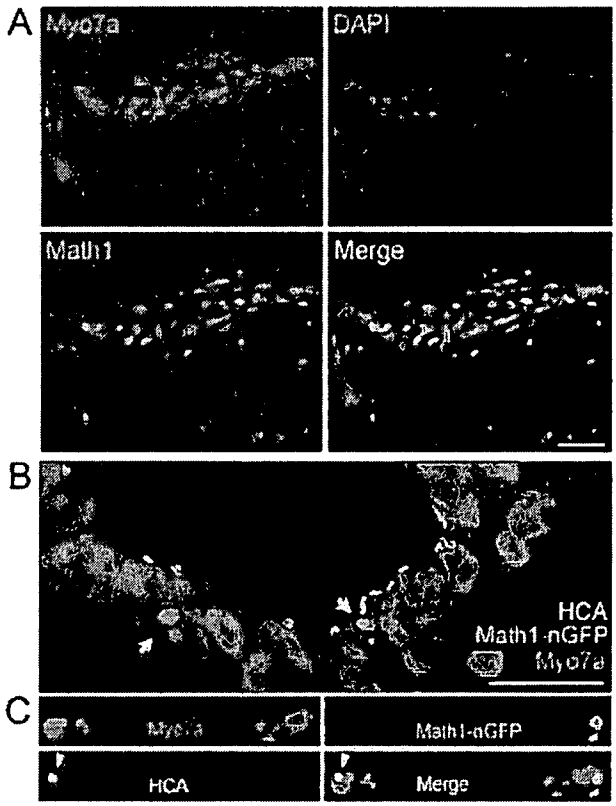


Figure 7

