Methods and products for biasing cellular development

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Kiminobu Sugaya
University of Central Florida

Angel Alvarez
University of Central Florida

Amelia Marutle
University of Central Florida

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* cited by examiner

Primary Examiner — Nancy T Vogel
(74) Attorney, Agent, or Firm — Timothy H. Van Dyke; Beusse, Wolter, Sunks & Maire, PLLC

ABSTRACT

Methods are described that bias cells, such as potent and multipotent stem cells, by transfection with a nucleic acid sequence, to differentiate to a desired end-stage cell or a cell having characteristics of a desired end-stage cell. In particular embodiments, human neural stem cells are transfected with vectors comprising genes in the homeobox family of transcription factor developmental control genes, and this results in a greater percentage of resultant transformed cells, or their progeny, differentiating into a desired end-stage cell or a cell having characteristics of a desired end-stage cell.

5 Claims, 14 Drawing Sheets
FIG. 4

FIG. 5
Cells were double-immunofluorescence stained with
a) βIII-tubulin (red) and b) CHAT (green), markers for cholinergic
neurons. c) co-localization of βIII-tubulin & CHAT.
Blue signal is a counter staining for nuclei by DAPI.

FIG. 12
Cells were double-immunofluorescence stained with a) βIII-tubulin (green) and b) ChAT (red), markers for cholinergic neurons. c) co-localization of βIII-tubulin & ChAT. (20x magnification) DAPI signal is a counter staining for nuclei. d) Insert for non-specific staining for βIII-tubulin & ChAT, respectively.

FIG. 13
a) βIII-tubulin (green) and b) ChAT (red), markers for cholinergic neurons. c) Co-localization of βIII-tubulin & ChAT. Blue signal is a counter staining for nuclei by DAPI. (40 x magnification)
Cells were double-immunofluorescence stained with 

\[ \text{II-tubulin (green)} \]

and 

\[ \text{nuclei counterstaining by DAPI (blue)} \]

No 

\[ \text{ChAT-positive neurons} \]

were observed. (a-b) 10 x and (c) 20 x magnification, respectively.
METHODS AND PRODUCTS FOR BLAISING CELLULAR DEVELOPMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Ser. No. 60/621, 483 filed Oct. 22, 2004; and is a divisional application of U.S. application Ser. No. 13/330,957, filed Dec. 20, 2011, which is a divisional application of U.S. application Ser. No. 11/258, 603, filed Oct. 24, 2005, which hereby incorporated in their entirety.

FIELD OF INVENTION

The present invention is directed to methods and systems directed to altering the differentiation of a cell, more particularly to biasing a multipotent stem cell by transferring the cell with a nucleic acid sequence comprising a desired gene, the gene being expressed so that the cell, or its progeny, differentiate to a desired end-stage cell.

BACKGROUND

Proper cellular function and differentiation depends on intrinsic signals and extracellular environmental cues. These signals and cues vary over time and location in a developing organism (i.e., during embryogenesis), and remain important in developing and differentiating cells during postnatal growth and in a mature adult organism. Thus, in a general sense, the interplay of the dynamically changing set of intracellular dynamics (such as manifest by intrinsic chemical signaling and control of gene expression) and environmental influences (such as signals from adjacent cells) determine cellular activity. The cellular activity so determined is known to include cell migration, cell differentiation, and the manner a cell interacts with surrounding cells.

The use of stem cells and stem-cell-like cells of various types for cell replacement therapies, and for other cell-introduction-based therapies, is being actively pursued by a number of researchers. Embryonic stem cells from a blastocyst stage are frequently touted for their pluripotency—that is, their ability to differentiate into all cell types of the developing organism. Later-stage embryonic stem cells, and certain cells from generative areas of an adult organism, are identified as more specialized, multipotent stem cells. These cells include cells that are able to give rise to a succession of a more limited subset of mature end-stage differentiated cells of particular types or categories, such as hematopoietic, mesenchymal, or neuroectodermal end-stage differentiated cells. For example, a multipotent neural stem cell may give rise to one or more neuron cell types (i.e., cholinergic neuron, dopaminergic neuron, GABAergic neurons), which includes their specific cell classes (i.e., a basket cell or a chandelier cell for GABAergic neurons), and to non-neuron glial cells, such as astrocytes and dendrocytes.

Further along the path of differentiation are cells derived from multipotent stem cells. For example, derivatives of a localized, non-migrating neuroectodermal type stem cell may migrate but, compared to their multipotent parent, have more limited abilities to self-renew and to differentiate (See Stem Cell Biology, Marshak, Gardner & Gottlieb, Cold Spring Harbor Laboratory Press, 2001, particularly Chapter 18, p. 407). Some of these cells are referred to a neuron-restricted precursors ("NRPs"), based on their ability, under appropriate conditions, to differentiate into neurons. There is evidence that these NRPs have different subclasses, although this may reflect different characteristics of localized multipotent stem cells (Stem Cell Biology, Marshak et al., pp. 418-419).

One advantage of use of multipotent and more committed cells further along in differentiation, compared to pluripotent embryonic stem cells, is the reduced possibility that some cells introduced into an organism from such source will form a tumor (Stem Cell Biology, Marshak et al., p. 407). However, a disadvantage of cells such as cell types developed from multipotent stem cells, for instance, embryonic progenitors, is that they are not amenable to ongoing cell culture. For instance, embryonic neural progenitor cells, which are able to differentiate into neurons and astrocytes, are reported to survive only one to two months in a cell culture.

Generally, it is known in the art that the lack of certain factors critical to differentiation will result in no or improper differentiation of a stem cell. Researchers also have demonstrated that certain factors may be added to a culture system comprising stem cells, such as embryonic stem cells, so that differentiation is directed to a desired, stable end-stage differentiated cell proceeds. It also is known in the art to introduce and express a transcription factor gene, Nurr1, into embryonic stem cells, and then process the cells through a five-step differentiation method (Kim, Jong-Hoon et al., Dopamine Neurons Derived from Embryonic Stem Cells Function in an Animal Model of Parkinson’s Disease, Nature, 418:50-56 (2002)), resulting in differentiated cells having features of dopaminergic cells. However, the starting cell for this was an embryonic stem cell, and the differentiation process through to a cell having the features of a dopaminergic neuron, requires substantial effort that includes the addition and control of endogenous factors. In addition, the starting cell is an early-stage embryonic stem cell having pluripotency, there is a relatively higher risk that some cells implanted from this source will become tumorigenic.

Also, without being bound to a particular theory, it is believed that to the extent a particular method of differentiation results in a greater percentage of cells that are dedicated or predetermined to differentiate to a desired functional cell type (i.e., a cholinergic neuron), this reduces the chance of tumor formation after introduction of cells derived from such method. As disclosed herein, embodiments of the present method that utilize multipotent stem cells as the starting material provide an increased percentage of cells predisposed (i.e., biased) to or differentiated to a desired cell type. This is believed to provide for reduced risk of tumor formation equivalent to or superior to the use of more differentiated cells such as NRPs.

There are many possible applications for methods, compositions, and systems that provide for improved differentiation of stem cells to a desired functional, differentiated cell. For example, not to be limiting, millions of people suffer from deafness and balance defects caused by damage to inner ear hair cells (IEHCs), the primary sensory receptor cells for the auditory and vestibular system after exposure to loud noises, antibiotics, or antitumor drugs. Since IEHCs rarely regenerate in mammals, any damage to these organs is almost irreversible, precludes any recovery from hearing loss, and results in potentially devastating consequences. Current therapies utilizing artificial cochlear implants or hearing aids may partially improve but not sufficiently restore hearing. Therefore, cell therapy to replace the damaged IEHC may be the most promising venues today. In the past, IEHC production from progenitor cells from the vestibular sensory epithelium of the bullfrog [Cristofalo, 1998 #28] and possible existence of IEHC progenitors in mammalian cochlear sensory epithelium [Kojima, 2004 #29] has been reported. However limited quantity of IEHC progenitor prevents clinical
application of this type of cell to treat deafness. Thus novel technology to produce IEHCs from other cell sources is needed.

While stem cells are known to be the building blocks responsible for producing all of a body’s cells, the specific differentiation process towards toIEHC lineage is not clear. Embryonic stem cells transplanted into the inner ear of adult mice or embryonic chickens did not differentiate into IEHCs [Sakamoto, 2004 #19]. Neural stem cells (NSCs) grafted into the modulus of cisplatin-treated cochlea of mice only differentiated into glial or neuronal cells within the cochlea [Tamura, 2004 #18]. In order to produce IEHCs from these stem cells, modification or direction of the cell fate decision may be needed.

Another possible application for methods, compositions, and systems of the present invention is biasing Human Neural Stem Cells (“HNSCs”) to differentiate to cholinergic neurons, or to cells having characteristics of cholinergic neurons. Such biasing would provide for an improved percentage of such stem cells in a culture vessel to differentiate to this desired end-stage nerve cell. Improvements to the percentage of cells that are known to be biased to differentiate to this end-stage neuron cell, or to cells having characteristics of a cholinergic neuron, may lead to improvements both in research and treatment technologies for diseases and conditions that involve degeneration or loss of function of cholinergic neurons. Alzheimer’s disease is one example of a malady known to be associated with degeneration of the long-projecting axons of cholinergic neurons.

Thus, there is a need in the art to improve the compositions, methods and systems that provide biased and/or differentiated cells from stem cells or stem-cell-like cells. More particularly, a need exists to obtain a higher percentage of desired cells from a pre-implantation cell culture, such as starting from multipotent stem cells and obtaining a higher percentage of cells committed to differentiate to a specified type of functional nerve cell, such as cholinergic neurons or inner ear hair cells. The present invention addresses these needs.

**BRIEF DESCRIPTION OF THE FIGURES**

FIGS. 1-16 are appended hereto, are part of the specification, and are described herein and/or on the figures themselves.

FIG. 1 A Hath1 gene (SEQ ID NO:4) was amplified from the *homo sapiens* BAC clone RP11-680J17 by PCR and then cloned it into a mammalian expression directional cloning vector, pcDNAHismax TOPO TA. FIG. 2 The hair cell marker calretinin was identified on certain cells via immunocytochemistry.

FIG. 3 Hath 1 full length mRNA was amplified by RT-PCR and digested with ApaI, which cuts position 441 of Hath1ORF. Expecting fragment sizes are 441 bp and 624 bp. M: 100 bp marker; 5, 6, without transfection (control); 7, 8: transfected with mammalian expression vector containing Hath2. FIG. 4 The presence of the actual protein calretinin on the cell surface was determined via Western Blot. Seven days for differentiation was allowed before any analysis of the cells. Protein was isolated from the cells and calretinin was identified in the cell isolate.

FIG. 5 Cells transfected with Hath1 and grown to allow for differentiation were visualized using Transmission Electron Microscopy. A subset of the cells exhibited distinct hair-like projections. These were the actual hairs from the transfected HNSCs that differentiated into cells having this characteristic feature of IEHCs.

**FIG. 6** is another view of hair-like projections as shown in FIG. 5.

FIG. 7 LA-N-2 cells treated with RA expressed Lhx8, βIII-tubulin, and ChAT. FIG. 7A shows LA-N-2 cells stained red indicating the presence of βIII-tubulin. FIG. 7B shows LA-N-2 cells stained green indicating the presence of ChAT. FIG. 7C shows LA-N-2 cells stained green indicating the presence of NGF (blue stain indicating counter-staining for nuclei by DAPI). FIG. 7D (insert) shows non-specific staining for ChAT.

FIG. 8. The Gbx1 cDNA sequence (SEQ ID NO:9) is inserted into the enhanced green fluorescent protein (EGFP) vector pEGFP-C1 ((BD Biosciences Clontech) at the EcoRI site within the vector’s multiple cloning site, which is 3’ of a CMV promoter and the EGFP gene.

FIG. 9: Phase contrast micrographs of LA-N2 cells. FIG. 9a: LA-N2 cells grow in clusters as adherent fibroblasts-like cells, occasionally cells extend short processes and form neuronal-like networks. FIG. 9b: LA-N2 cells treated with 10-6 µM retinoic acid.

FIG. 10: Lhx8 expression in the LA-N-2 and HNSCs cells. FIG. 10a: Shows gene expression with RT-PCR analysis of LA-N-2 cells treated with ten µM RA showed an increased expression of Lhx8 (394 bp) and ChAT (splice variants –600 and 400 bp, respectively, compared with non-treated cells. FIG. 10b: represents RT-PCR analysis of Lhx8 expression in HNSCs 48 hours post-transfection.

FIG. 11: In vitro differentiation of Lhx8-transfected HNSCs. HNXCs transfected with Lhx8 (a) in co-culture with LA-N-2 cells and serum-free conditions differentiated mostly neurons with long extended processes after 10-14 days (b-f).

FIG. 12: Differentiated HNSCs/Lhx8 in co-culture with LA-N-2 cells. Cells were double-immunofluorescence stained with (a) βIII-tubulin (red) and (b) ChAT (green), markers for colinergic neurons (c) two localization of βIII-tubulin and ChAT. Blue signal is a counter staining for nuclei by DAPI.

FIG. 13: Differentiated HNSCs/Lhx8 in co-culture with LA-N-2 cells. A) βIII-tubulin (green) and b) ChAT (red), markers for cholinergic neurons, c) co-localization of βIII-tubulin and ChAT. Blue signal is a counter staining for nuclei by DAPI.

FIG. 15: Differentiated HNSCs in co-culture with LA-N-2 cells. Cells were double-immunofluorescence stained with (a) βIII-tubulin (green) and (b) ChAT (red), markers for colinergic neurons (c) co-localization of βIII-tubulin and ChAT. (20x magnification) blue signal is a counter staining for nuclei by DAPI (40x magnification).

FIG. 16: Differentiated non-transfected HNSCs in co-culture with LA-N-2 cells. Cells were double-immunofluorescence stained with (a-b) βIII-tubulin (green) and (c) GFAP (red) markers for neurons and astrocytes, respectively. Blue signal is a counter staining for nuclei by DAPI.

**DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION**

In reviewing the detailed disclosure which follows, and the specification more generally, it should be borne in mind that all patents, patent applications, patent publications, technical
by "neural stem cell" (NSC) is meant a cell that (i) has the potential of differentiating into at least two cell types selected from a neuron, an astrocyte, and an oligodendrocyte, and (ii) exhibits self-renewal, meaning that at a cell division, at least one of the two daughter cells will also be a stem cell. Generally, the non-stem cell progeny of a single NSC are capable of differentiating into neurons, astrocytes, Schwann cells, and oligodendrocytes. Hence, a stem cell such as a neural stem cell is considered "multipotent" because its progeny have multiple differentiation pathways. Under certain conditions an NSC also may have the potential to differentiate as another non-neuronal cell type (e.g., a skin cell, a hematopoietic cell, a smooth muscle cell, a cardiac muscle cell, a skeletal muscle cell, a bone cell, a cartilage cell, a pancreatic cell or an adipocyte).

By "Human Neural Stem Cell" (HNNSC) is meant a neural stem cell of human origin. A HNSSC may be of fetal origin, or adult origin from a neural source, or may be derived from other cell sources, such as by de-differentiating a cell of mesenchymal origin. As to the latter, for example see U.S. application serial number 2003/019989, which is incorporated by reference, inter alia, specifically for this teaching. HNSSCs of the invention are distinguished from natural HNSSCs by their adaptation for proliferation, migration and differentiation in mammalian host tissue when introduced thereto.

By a "population of cells" is meant a collection of at least ten cells. A population may consist of at least twenty cells, or of at least one hundred cells, or of at least one thousand or even one million cells. Because the NSCs of the present invention exhibit a capacity for self-renewal, they can be expanded in culture to produce a collection of large numbers of cells.

By "potent cell" is meant a stem cell that has the capability to differentiate into a number of different types of end-stage cell types, and to self-renew, and may include stem cells classified as pluripotent, multipotent, or cells more differentiating than multipotent (i.e., a dedicated progenitor) under different stem cell classification schemes.

By "a presumptive end-stage cell" is meant a cell that has acquired characteristics of a desired end-stage cell type, but which has not been conclusively identified as being the desired end-stage cell. A presumptive end-stage cell possesses at least two, and often more, morphological and/or molecular phenotypic properties of the desired end-stage cell.

that biases the same cells to differentiate to a desired cell type or to a particular tissue in a living organism. In such embodiments, this improves a differentiation ratio so that a higher percentage of cells introduced into a particular cell medium, a tissue culture, or a living organism in a particular location differentiate into the end-stage differentiated cell type that is desired. Without being bound to a particular theory, this is believed to increase the probability of success and overall effectiveness, and to decrease the risks associated with implantation of cells obtained from embryonic stem cells or embryonic-cell-like cells.

While not meant to be limiting as to the type of nucleic acid sequence introduced, examples herein utilize introduction to a cell of a nucleic acid sequence comprising a homebox gene. This is a gene group that includes a number of known developmental control genes. A homebox gene is a gene containing an approximately 180-base-pair segment (the “homebox”) that encodes a protein domain involved in binding to (and thus regulating the expression of) DNA. The homebox segment is remarkably similar in many genes with different functions. However, specific homebox genes are known to operate at different stages, and in different tissue environments, to yield very different specific results. For example, in relatively early embryological development in the vertebrate embryo, expression of genes of the Hox family of homeobox genes appears to affect development of the brain based on position along the anterior/posterior axis. This is believed to control identity and phenotypic specializations of individual rhombomeres. (Fundamental Neuroscience, Zigmond et al., p. 435). Later in development, LIM homebox gene expression is associated with the projection pattern of developing primary motor neurons, and more generally, expression of a particular combination of LIM homebox genes appears to be related to motor neuron subtype identity and to targeting specificity (Fundamental Neuroscience, Zigmond et al., p. 507). Also, some LIM homeobox genes appear to affect developmental progression, rather than fate, of motor neurons, which suggests a role of cell-to-cell signaling in the embryo to fully effectuate the differentiation in vivo (Fundamental Neuroscience, Zigmond et al., pp. 443-444). These highly specialized and variable roles for homebox genes in general, and for LIM family homeobox genes more particularly, demonstrate the subtle, specific, and highly variable effects that these genes may have on cell and tissue development and differentiation.

Further with regard to function of homebox genes, these genes encode transcriptional regulators that play critical roles in a variety of developmental processes. Although the genetic and developmental mechanisms that control the formation of forebrain cholinergic neurons are just beginning to be elucidated, it is known that the vast majority of forebrain cholinergic neurons derive from a region of the subcortical telencephalon that expresses the Nlhx2-1 homebox gene.

It has recently been reported that Nlhx2-1 appears to specify the development of the basal telencephalon by positively regulating transcription factors such as the LIM-homeobox genes Lhx8 (also known as I.3 or Lhx7) and Gbx1, which are associated with the development of cholinergic neurons in the basal forebrain (Zhao et al., 2003; Asbreuk et al., 2002, Waters et al., 2003).

In the spinal cord, Isl1, Lhx1, Lhx3 and Lhx4 have been shown to be important for the development of spinal cord cholinergic neurons (Pfafl et al., 1996; Sharma et al., 1998; Kania et al., 2000). Given that the spinal cord cholinergic neurons are reported to require multiple LIM-homeobox genes for their development, it is expected that Lhx8 is not the only LIM-homeobox gene that is required in generating telencephalic cholinergic neurons. Other candidates are Lhx6 and Isl1, which are also expressed in the basal telencephalon (Marin et al., 2000). Also, it is suggested that Dlx1/2 and
Mash, though not directly regulating Lhx8, participate in controlling the number of cholinergic neurons that are formed in the telencephalon (Marin et al., 2000).

Thus, at a minimum, developmental control genes that may be used in the present invention to transfect cells to bias those cells (or their progeny) to differentiate to a desired end-stage cell type, here that cell type being cholinergic neurons, include, but are not limited to Lhx8, Gbx1, Lhx6, Isl1, Dlx1/2 and Mash.

The Human Neural Stem Cells (HNSCs), such as discussed in the examples below, are obtained from cultures that were started from clones obtained from human fetal brain tissue. One lineage was obtained by isolating individual cells from neurospheres of a fetal brain tissue sample obtained from Cambrex, and ultimately identifying one multipotent stem cell for clonal propagation. A second lineage was obtained by isolating a desired multipotent cell from a 9-week old fetal brain (Christopher L. Brannen and Kiminobu Sugaya, Neurureport 11, 1123-8 (2000)). The HNSCs so obtained were maintained in serum-free medium, and have been demonstrated to have the capability to differentiate into neurons and glial cells such as astrocytes and dendrocytes.

The following examples are provided to further disclose the genesis, operation, scope and uses of embodiments of the present invention. These examples are meant to be instructive, and illustrative, and not to be limiting as to the scope of invention as claimed herein. These examples are to be considered with the referred to drawings.

Example 1

This example demonstrates that transfection of a human neural stem cell with Hath1 results in the transfected cell (or its progeny) differentiating into a cell having markers of an inner ear hair cell (IEHC). Hath1 (in humans) and Math1 (in mice) are basic helix-loop-helix transcription factors (and homologs of the Drosophila gene atonal) that are expressed in inner ear sensory epithelia. Since embryonic Math1-null mice failed to generate cochlear and vestibular hair cells, it appears to be required for the generation of inner ear hair cells (Bermingham N A, Hasson B A, Price S D, Vollrath M A, Ben-Arie N, Eatock R A, Bellen H J, Lysakowski A, Zoghbi H Y. 1999. Math1: An essential gene for the generation of inner ear hair cells. Science 284 (June 1): 1837-1841). Fate determination of mammalian IEHC is generally completed by birth. However, overexpression of Math1 in postnatal rat cochlear explant cultures resulted in production of extra hair cells from columnar epithelial cells located outside the sensory epithelium, which normally give rise to inner sulcus cells. Math1 expression also facilitated conversion of postnatal utricular supporting cells into hair cells (Zheng, G L, Gao Wq. 2000. Overexpression of Hath1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neuroscie June; 3(6):580-6). In vivo, Math1 overexpression leads to the appearance of immature hair cells in the organ of Corti and new hair cells adjacent to the organ of Corti in the interdental cell, inner sulcus, and Hensen cell regions, indicating nonsensory cells in the mature cochlea retain the competence to generate new hair cells after over expression of Math1 (Kawamoto K, Ishimoto S, Minoda R, Brough D E, Raphael Y. 2003. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. J Neurosci June 1; 23(11):4395-400). Based on the above-summarized work, it was hypothesized that Hath1 may be necessary, and sufficient as a single introduced gene for expression in a multipotent neural stem cell, to positively affect differentiation to an IEHC, or to a cell having characteristics of an IEHC.

A Hath1 gene (SEQ ID NO:4) was amplified from the homo sapiens BAC clone RP11-680171 by PCR and then cloned it into a mammalian expression directional cloning vector, pcDNAHismax TOPO TA (See FIG. 1; 6xHis tag disclosed as SEQ ID NO: 16). Upon the insertion of the Hath1 gene expression sequence into the directional cloning vector, the expressible sequence was operatively linked to the CMV promoter, and was also positioned upstream (with regard to reading) of a polyadenylation transcription termination site. The clone was confirmed by sequencing of the insert.

An established a non-serum HNSC culture system was utilized to investigate the differentiation of human neural stem cells (HNSCs) within a defined condition. (Christopher L. Brannen and Kiminobu Sugaya, Regeneration and Transplantation, 11:5, 1123-1128 (2000)). The serum-free supplemented growth medium consisted of HAMS-F12 (Gibco, BRL, Burlington, ON), antibiotic/antimycotic mixture (1:100, Gibco), B27 (1:50, Gibco), human recombinant FGF-2 and EGF (20 ng/ml each, R and D Systems, Minneapolis, Minn.), and heparin (5 ug/ml, Sigma, St. Louis, Mo.). Cells were maintained in 20 ml of this medium at 37°C in a 5% CO2, humidified incubation chamber.

The mammalian expression vector containing Hath1 gene was transfected into HNSCs by using the Neuroporter Kit (Gene Therapy Systems, Inc. San Diego, Calif.) and Hath1 gene expression was confirmed by RT-PCR. These Hath1-transfected HNSCs were differentiated for 7 days by the depletion of mitotic factors (FGF-2, EGF) from the culture media. After the differentiation the cells were fixed for immunocytochemistry and Electron Microscopy.

The immunocytochemistry revealed the existence of cells expressing calretinin, a hair cell marker, which were immunoreactive in this culture. These calretinin immunopositive cells resembled morphology of IEHC. The calretinin expression in the culture was also confirmed by Western blot, which showed single band specific to calretinin molecular weight (29 kD). Further electron microscopy analysis of the cells also showed a typical IEHC morphology. These results indicate that HNSCs transfected with a vector comprising a Hath1 gene differentiate into IEHCs or into cells having characteristics of IEHCs. Comparisons with non-transfected controls using Western blot and room temperature PCR showed the presence of Hath1 protein and Hath1 mRNA in cells transfected with Hath1, but not in the controls.

Thus, embodiments of the present method provide for improved approaches to obtain IEHCs, or cell having characteristics of IEHCs, that are derived from HNSCs. Embodiments of the present invention provide a higher percentage of a population of cells biased, or disposed, to differentiate to IEHCs, or to cells having characteristics of IEHCs. The HNSCs utilized in this example are readily and continuously cultured in serum-free culture medium. Without being limited, in vitro and in vivo studies and trials using cells so obtained from HNSCs may include electrophysiological assessment of the cells and investigation of functional recovery after transplantation of the cells into the animal model of deafness. Positive findings in such pre-clinical studies may advance the art farther toward treatment of deafness via cell transplantation therapy using IEHCs produced from HNSCs.

Material and Methods

Hath 1 Transfection

The human Hath1 gene (SEQ ID NO:1) is amplified from the Homo sapiens BAC clone RP11-680371 by PCR, using a forward primer (5'-TCCGAATTCCTGAGCTGCGAGGCTT-3', SEQ ID NO: 14) and reverse primer (5'-GCTCTGTACCTTCCTAACTTGGC-3', SEQ ID NO: 15). The PCR amplification is conducted in 20 µl volumes containing the BAC
clone (100 ng), 1x amplification buffer, 1 µM of each primer, dNTP Mix (250 µM), and Taq DNA Polymerase (2.5 U). The PCR condition is 95° C. (30s), 56° C. (30s), 72° C. (60s) for 35 cycles, with an initial denaturation of 95° C. (5s) and final elongation of 72° C. (15s). The PCR amplified fragment is cloned into a directional pcDNA-Hismax TOPO TA vector and the clone is confirmed by sequencing of the insert. The gene expression of Hath1 is assessed by RT-PCR with the following condition: 95° C. (60s), 56° C. (60s), 72° C. (60s) for 35 cycles, with an initial denaturation of 95° C. (5s) and final elongation of 72° C. (15s). The Hath1 gene is transfected into Human Neural Stem Cells (HNSCs) using the Neuroporter Kit. The Neuroporter kit utilizes a lipid-based transfection system for the use with cultured primary neurons, neuronal cell lines, and glial cells. DNA and Neuroporter are used in a ratio of 10 µg DNA/75 µl Neuroporter. 37.5 µl per well in a 6-well plate and with total volumes of 1.5 mL growth media per well. 10 µg of DNA is added to DNA Diluent to make a total volume of 125 µl; this is incubated for 5' at room temperature. 75 µl of the Neuroporter Reagent is added to serum-free media to make a final volume of 125 µl. These solutions are incubated for 10 minutes to allow Neuroporter/DNA complexes to form, and then added directly to the HNSCs in a 6-well plate. One day later, the media is replaced with fresh growth media; one day later, this is replaced with differentiation media (Basal Medium Eagle) to induce spontaneous differentiation. The cells are cultured for 1-2 weeks in a basal differentiation medium containing Eagle's salts and L-glutamine, which is not supplemented with FGF-2 or EGF, and is serum-free.

RT-PCR

TRizol reagent is used to extract RNA for RT-PCR and protein for a Western Blot. 6 µl of the template RNA is added to 1x Reaction Mix, 1 µM of each Hath1-specific primer, and 1 µl of the RT-Platinum® Taq Mix. The total volume of the solution is 20 µl. The RT-PCR condition is 94° C. (15s), 59° C. (30s), 72° C. (60s) for 40 cycles, with an initial denaturation of 95° C. (30s) and 94° C. (5s).

Immunocytochemistry

The cells are fixed with 4% paraformaldehyde for 30' at room temperature, washed in phosphate-buffered saline (PBS, pH 7.2), then blocked with 3% normal goat serum in PBS containing 0.05% Triton-X100 for 1 hour. The cells are incubated with primary antibody calretinin overnight at 4°C, with a dilution factor of (1:2000) in PBS containing 0.05% Triton-X100.

Following PBST washing, the cells are incubated with secondary antibody biotinylated anti-rabbit made in goat in PBS containing 0.05% Triton-X100 (PBST), with a dilution factor of 1:200. This incubation takes 1 hour. The cells are washed with PBST, and incubated with ABC reagent for 1 hour. Following a PBS wash and staining with DAB for 5-8', the cells are washed with PBS and distilled water, then stained with methyl green (5'). The cells are washed with water, ethanol, and xylene, coverslipped with permount, and ready for viewing with microscopy.

Western Blot

A Western Blot is performed to assay protein expression. The protein is extracted with TRizol reagent. 15 µl of the protein is loaded with the size marker on a PVDF membrane and run at 200 V and 110 mA/gel for 50. The transfer is run overnight at 15V, 170 0 mA at 4°C. The membrane is then washed with PBST 2x10' while rotating, and blocked with 3% milk for 60. This is washed 2x10' with PBST and blocked with the primary antibody calretinin (1:500) overnight at 4°C. After washing 3x5 with PBST the membrane is incubated with the secondary antibody (1:2000) and shaken for 1 hour.

For detection, 7.5 mL of ECL solution is warmed to room temperature and 187.5 µl of solution B is added to solution A. 7.5 mL is added to the membrane at RT for 5'. The membrane is then placed in an x-ray film cassette and exposed as needed for chemiluminescent detection.

Electron Microscopy

Cells were fixed with 3% glutaraldehyde with cacodylate buffer 0.1M, and dehydrated with a series of alcohols beginning with 50% up to 100% absolute ethanol followed by hexamethyldisilazane (HMDS). The cultured cells were allowed to air-dry at room temperature. The specimens were attached to aluminum stubs using double sided carbon coated tape, sputter-coated with Platinum and palladium using the Cressington 208 HR High Resolution Coater. Samples were viewed with a Jeol 6320F Field Emission Microscope (high resolution images) and recorded with a digital camera. Samples were also viewed with the Hitachi Variable pressure microscope in V-P mode (variable pressure mode) and digital images were captured.

Results

A non-serum HNSC culture system was utilized (Christopher L. Brannen and Kiminobu Sugaya, Regeneration and Transplantation, 11:5, 1123-1128 (2000)). This culture system provides for the differentiation and expansion of HNSCs in vitro in the absence of serum. This system provides for the observation of differentiation of HNSCs within a defined condition. These HNSCs have been cultured in a medium consisting of DMEM/F12, antibiotic-antimycotic mixture (1:100), B-27 supplement (1:50), human recombinant FGF-2 and EGF (20 ng/ml each), and heparin (5 µg/ml). These cells have been maintained at 37° C. in a 5% CO2 humidified incubation chamber for more than 3 years in the lab. These cells are CD133—(a stem cell marker, which is known to be expressed in stem cells) positive, and GFAP- and βIII-tubulin-negative before differentiation. Upon differentiation, various differentiated cells typically express glial fibrillary acidic protein (GFAP), or βIII-tubulin, which are glial and neuronal markers, respectively.

Preferential differentiation of HNSCs into IEHCs can be induced in vitro by the transfection of Hath1. The human Hath1 gene was amplified from the Homo sapiens BAC clone RP11-680117 by PCR and cloned into a directional pcDNA-Hismax TOPO TA vector. This was confirmed by sequencing of the insert. After confirming expression of the gene by RT-PCR, the Neuroporter kit was utilized to transfect HNSCs. These HNSCs were known to be viable and capable of differentiation, aggregating in neurospheres when multipotent. Once they began the process of differentiation, they left their neurospheres. After allowing 7 days for differentiation, these cells were either stained for hair cell specific markers or assayed for protein expression. Via immunocytochemistry, the hair cell marker calretinin was identified on certain cells (FIG. 2). Via RT-PCR, the expression of this protein was also verified (FIG. 3).

The presence of the actual protein calretinin on the cell surface was determined via Western Blot. Seven days for differentiation was allowed before any analysis of the cells. Protein was isolated from the cells and calretinin was identified in the cell isolate (FIG. 4).

Using Transmission Electron Microscopy, cells transfected with Hath1 and grown to allow for differentiation were visualized. A subset of the cells exhibited distinct hair-like projections. These were the actual hairs from the transfected HNSCs that differentiated into cells having this characteristic feature of IEHCs (FIGS. 5 and 6).
Discussion

In order to replace damaged IEHCs, a renewable source must be created. The HNSCs cultured in serum-free medium were shown to have the ability to become transfected by Hath1 and then differentiate in vitro into IEHCs, or cells having characteristics of IEHCs. In the present example, transfection with and expression of Hath1 appears to be an essential step in the genesis from HNSCs to IEHCs, or cells having characteristics of IEHCs.

Before transfection, HNSCs do not express Hath1. Following transfection with the Neuroporter Kit, they express this gene in their DNA as verified by RT-PCR. They also produce the hair cell specific marker calretinin as verified by immunocytochemistry and Western Blot. Furthermore, actual hairs from the transfected cells can be visualized through electron microscopy. Thus, characteristics of IEHCs are shown by these data, and it appears that these cells either are end-stage IEHCs or are presumptive IEHC cells in that they have at least two characteristics of IEHCs.

Cells expressing IEHC markers and differentiating into cells with hairlike extremities have been generated in this example. These methods, and the cells produced by the methods of the present invention, as shown in this example, advance the art of differentiating multipotent stem cells toward obtaining end-stage neuron-type cells.

Example 2

Introduction

A cholinergic deficit is one of the primary features of Alzheimer’s disease (AD), where there is a marked degeneration of long-projecting axons of cholinergic neurons in the basal forebrain and target areas in the hippocampus and cerebral cortex. Recent progress in stem cell technologies suggest the probability of using neuroreplacement strategies in AD therapy, although several hurdles are implicated: i) is it possible to generate large numbers of cholinergic neurons from stem cells; and ii) can long-projecting cholinergic neurons be replaced? Toward improving the ability to conduct research in the area of cell implantation and replacement therapies, and toward achieving desired results in later-developed therapies, embodiments of the present invention are directed to bias human neural stem cells (HNSCs) to differentiate to cells having characteristics of cholinergic neurons through genetic manipulation of endogenous neural precursors in situ.

The LIM-homeobox gene Lhx8 has been reported to be crucial for the proper development of basal forebrain cholinergic neurons in mouse (Zhao et al., 2003; Mori et al., 2004). Lhx8 is expressed in progenitor and postmitotic cells, suggesting that it may have an important role in specification of neural precursor cells and maintenance of phenotype in differentiating and mature neurons. Furthermore, previous studies using the human neuroblastoma cell line, LA-N-2, have demonstrated that treatment with retinoic acid (RA) further enhances cholinergic characteristics of these cells, thus providing a good in vitro model of cholinergic neurons (Croslan, 1996).

The present example utilizes an in vitro assay cell coculture model with plated RA-differentiated LA-N-2 cells and membrane inserts containing Lhx8-transfected HNSCs, to assess whether the Lhx8-transfected HNSCs adopt a cholinergic neuronal fate. The rationale behind this co-culture model is that HNSCs are influenced by intrinsic as well as extracellular factors in the microenvironment and therefore, able to respond by differentiating into specific cell types according to the environmental cues to which they are exposed. Culture of RA-differentiated LA-N-2 in basal medium under a serum-free condition, results in the release of factors to the Lhx8-transfected HNSCs in co-culture. It should be noted that there is no cell-to-cell contact in this co-culture system. Thus it is reasonable to assume that any modification of the cell fate of the genetically modified HNSCs by the cholinergic-differentiated LA-N-2 cells would come from membrane permeable endogenous factor(s) released from the cholinergic-differentiated LA-N-2 cells.

Materials & Method

HNSCs culture: Human NSCs were originally purchased from BioWhittaker, Walkersville, Md. These cells have been expanded and passaged in a serum-free culture medium containing bFGF and EGF in our laboratory for over three years (Braunen & Sugaya, 2000). The HNSCs were cultured at a density of 50 spheres in 75 cm² culture flasks (Corning, Cambridge, Mass.) in 20 ml of a serum-free supplemented growth medium consisting of HAMS-F12 (Gibco, BRL, Burlington, ON), antibiotic-antimycotic mixture (1:100, Gibco), b27 (1:50, Gibco), human recombinant FGF-2 and EGF (20 µg/ml each, R&D Systems, Minneapolis, Minn.) and heparin (5 µg/ml, Sigma, St. Louis, Mo.) incubated at 37°C in a 5% CO2 humidified incubation chamber (Fisher, Pittsburgh, Pa.). To facilitate optimal growth conditions, HNSCs were sectioned into quarters every 2 weeks and fed by replacing 50% of the medium every 4-5 days.

LA-N-2 Human Neuroblastoma Culture:

LA-N-2 cells were obtained from Dr. Jan Blusztein (Boston University, MA). The cells were cultured in Leibovitz L-15 medium (Gibco, BRL, Burlington, ON) containing 10% fetal calf serum, and antibiotic-antimycotic mixture (Gibco) in a humidified incubator at 37°C without CO2. The medium was replaced every 3 days. For treatment with retinoic acid (RA), the cells were sub-plated at a density of 0.5 x 10⁶ cells/plate using 0.25% trypsin/1 mM EDTA (Gibco, BRL) and allowed to attach overnight. A fresh stock of 4 µM all-trans retinoic acid (RA) (Sigma, St. Louis, Mo.) was prepared in 100% ethanol under amber lighting. RA solution was diluted into culture media (final concentration, 10⁻⁸M) and we replaced the media in the cells with the RA-containing media. The media was changed every 48 h during the differentiation of the cells, which was complete after 7-14 days.

Lhx8 Subcloning:

The mouse cDNA clone for Lhx8 (SEQ ID NO: 7) was kindly provided by Dr Westphal, NIH, Bethesda, Md.) was inserted into the EcoRI site of the pcDNA 3.1/Neo mammalian expression vector (Invitrogen). Insertion was subsequently confirmed by restriction digestion and sequence analysis. This mouse Lhx8 (SEQ ID NO: 7) has high homology to the human sequence (70-80%).

Transfection:

HNSCs were placed in 6-well poly-lysine coated plates and transfected with 4 µg pcDNA 3.1/Lhx8 plasmid using the Neuroporter transfection system (Gene Therapy Systems, see description in Example 1). Upon the insertion of the Lhx8 gene into the plasmid, the transfected HNSCs were placed in serum-free medium, and allowed to differentiate for 10-15 days in culture.
Co-Cultures of Transfected HNSCs and RA-Treated LA-N-2 Cells:

HNSC's (~5×10^4) transfected with pcDNA 3.1/Lhx8 and non-transfected HNSC's (controls) were transfected into cell culture inserts with an appropriate pore size and suspended in basal media (in the absence of FGF-2 and EGF) and without the addition of other extrinsic differentiation factors) over differentiated LA-N-2 cells plated in 6-well plates. For immunocytochemical analyses of HNSCs, the culture insert was removed after 10-20 days of co-culture and the HNSCs were fixed with 4% paraformaldehyde overnight at 4°C. Also, transfected HNSCs were cultured without the presence of differentiated LA-N-2 cells to assess the need for and effectiveness of the co-culturing.

Immunocytochemistry:

Following fixation, HNSCs were briefly washed 3×5 min in Phosphate buffered saline (PBS), then blocked with 3% normal donkey serum in PBS containing 0.05% Tween 20 (PBS-T) and incubated with goat IgG polyclonal anti-human ChAT (1:500, Chemicon), mouse IgG2b monoclonal anti-human βIII-tubulin (1:1000, Sigma) or rabbit anti-human glial filament protein (GFAP) (1:1000, Sigma) overnight at 4°C. The corresponding secondary antibodies (donkey anti-goat, donkey anti-mouse, and donkey anti-rabbit, respectively) conjugated to rhodamine or FITC (Jackson IR Laboratories, CA) were added for a 2 hr incubation at RT in the dark. Cells were then washed with PBS (3×5 min) and mounted with Vectashield with DAPI (Vector Laboratories, CA) for fluorescent microscopic observation. LA-N-2 cells were similarly treated to prepare for microscopic observations. Results showed that the LA-N-2 cells treated with RA expressed Lhx8, βIII-tubulin, and ChAT. This is demonstrated in FIGS. 7A-D. FIG. 7A shows LA-N-2 cells stained red indicating the presence of βIII-tubulin. FIG. 7B shows LA-N-2 cells stained green indicating the presence of ChAT. FIG. 7C shows LA-N-2 cells stained green indicating the presence of NGF (blue stain indicating counter-staining for nuclei by DAPI). FIG. 7D (insert) shows non-specific staining for ChAT.

In vitro, HNSCs expressing the LIM homeobox gene, Lhx8, differentiated into mainly βIII-tubulin and ChAT-positive cells, in co-culture with LA-N-2 cholinergic cells. For the transfected HNSCs cultured without the presence of differentiated LA-N-2 cells, there was no significant difference from the non-transfected HNSCs with regard to the number of cells differentiating to cells having characteristics of cholinergic cells. This demonstrated the need under these experimental conditions for the differentiated LA-N-2 cells (and the factors released by them).

Non-transfected HNSCs differentiated into mainly βIII-tubulin and GFAP positive cells in co-culture with LA-N-2 cholinergic cells.

With regard to percentage differences between non-transfected cells and transfected cells, in one trial less than two percent of non-transfected cells, and over 40 percent of transfected cells, were observed at the end of the trial to have characteristics of cholinergic neurons.

Conclusions and Comments

Expression of the LIM-homeobox gene Lhx8 triggers HNSCs to adopt a cholinergic neural lineage. Cells having the noted characteristics of cholinergic neurons either are cholinergic neurons or presumptive cholinergic neurons in that they have at least two characteristics of cholinergic neurons.

LA-N-2 cells in co-culture with HNSCs expressing Lhx8, suggest that the microenvironment is also important for the differentiation and survival of cholinergic neurons.

The present invention may provide utility by biasing human neural stem cells through genetically manipulation so that the cells so manipulated may be used in research, including as cells transplantable, such as in experiments, and therapies, including regarding replacing damaged cholinergic neurons.

As to the efficiency of biasing to a desired cell type, and to observing cells having characteristics of a desired end-stage cell type, without being bound to a particular theory, it is believed that the factors that increase the efficiency of biasing by transfection include: 1) inherent properties of the cell to be transfected; 2) inherent efficiency of the selected vector or method of transfection; 3) relative percentage of cells in which the introduced nucleic acid sequence enters the nucleus compared to remains in the cytoplasm; and 4) number of copies of the nucleic acid sequence that are available for expression in the cell. Methods of transfection are well-known in the art, and the use and modification of known approaches to transfection of a cell with a nucleic acid sequence to be expressed therein to improve the percentage of biasing are within the scope of the present invention.

Thus, it is appreciated that in some embodiments of the present invention, a multipotent stem cell is transfected with a desired developmental control gene, and the expression of the gene during in vitro culture biases the differentiation of that cell to a desired end-stage differentiated cell. In other embodiments, the multipotent stem cell may be transfected in vivo with a developmental control gene whose expression biases transfected cells to differentiate into a desired end-stage cell. In any of such embodiments, accessory cells may provide factors that are needed for, or that assist with, the differentiation of the transfected cell. These accessory cells, such as the co-cultured LA-N-2 cells in the above example, need not be in contact with the transfected cells, demonstrating here that the factors are membrane permeable. These factors may include the same factor that is expressed by the transfected gene, or may be other factors known in the art or later determined to be useful in achieving a desired differentiation.

Also, it is appreciated that multipotent stem cells may be cultured without an accessory cell, and may receive factors by direct addition of factors to the culture medium, or such factors may be released by cells at a site of implantation, or may be added to a site of implantation.

Example 3

Using the same vector formation and transfection methods as in Example 2, the Human Lhx8 gene (SEQ ID NO: 6) is transfected into HNSCs. Transfected cells (HNSCs cells) number of copies of the nucleic acid sequence that are available for expression in the cell. Methods of transfection are well-known in the art, and the use and modification of known approaches to transfection of a cell with a nucleic acid sequence to be expressed therein to improve the percentage of biasing are within the scope of the present invention.

Thus, it is appreciated that in some embodiments of the present invention, a multipotent stem cell is transfected with a desired developmental control gene, and the expression of the gene during in vitro culture biases the differentiation of that cell to a desired end-stage differentiated cell. In other embodiments, the multipotent stem cell may be transfected in vivo with a developmental control gene whose expression biases transfected cells to differentiate into a desired end-stage cell. In any of such embodiments, accessory cells may provide factors that are needed for, or that assist with, the differentiation of the transfected cell. These accessory cells, such as the co-cultured LA-N-2 cells in the above example, need not be in contact with the transfected cells, demonstrating here that the factors are membrane permeable. These factors may include the same factor that is expressed by the transfected gene, or may be other factors known in the art or later determined to be useful in achieving a desired differentiation.

Also, it is appreciated that multipotent stem cells may be cultured without an accessory cell, and may receive factors by direct addition of factors to the culture medium, or such factors may be released by cells at a site of implantation, or may be added to a site of implantation.

Example 4

An additional development control gene, Gbx1 sequence (SEQ ID NO: 9), is transfected into HNSCs and is evaluated.
as to its capacity to bias HNSCs to differentiate to cholinergic cells, or to cells having characteristics of cholinergic cells. The Gbx1 cDNA sequence (SEQ ID NO: 9) is inserted into the enhanced green fluorescent protein (EGFP) vector pEGFP-C1 (BD Biosciences Clontech) at the EcoRI site within the vector’s multiple cloning site, which is 3′ of a CMV promoter and the PGFP gene (See FIG. 8; sequence disclosed as SEQ ID NO: 17). Further, in that a question remains as to whether the percentage of biasing is related directly to the percentage of transfection of cells in population of cells exposed to a transfecting vector, the human Lhx8 cDNA (SEQ ID NO:6) independently also is inserted into a second pEGFP-C1 vector. This allows for visualization of both vectors, each bearing an expressible sequence for a different developmental control gene, in cells in respective cell populations into which these vectors are transfected.

Culture methods of the HNSCs into which the Gbx1 and the Lhx8 genes are transfected are as described above in Example 2.

This experiment provides an estimate of the ratio of HNSCs that become cholinergic neurons based on percent transfection of the population. Compared to non-transfected control HNSCs, the transfected cells have characteristics of the desired end-stage differentiated cell type, that is, a cholinergic neuron.

This demonstrates that a number of development control genes, particularly transcription factor genes, may be introduced into a HNSC to bias that cell (or its progeny) to differentiate to a cell having the characteristics of a desired end-stage differentiated neural cell type.

Example 5

Cell sorting technology is combined with the above-described embodiments of the present invention, particularly the vectors of Example 4, to improve the yield and selection of desired cells having the bias to differentiate to a desired end-stage cell (or having already so differentiated). For example, not to be limiting, the introduction of genetic marking such as described above, using EGFP, and the use of Fluorescent Activated Cell Sorter (FACS) techniques is utilized to sort and select cells that have been transfected with the desired developmental control gene (which is linked to a marker on the vector). The FACS technology is well known in the art (See, for example, U.S. patent application number 2002/0127715 A1.)

Using FACS, HNSCs that are transfected with a vector bearing both EGFP and either Gbx1 or Lhx8 are sorted and thereby concentrated. This adds to the utility and effectiveness of the biasing by reducing the number and percentage of cells that are not transfected.

The above examples utilize specific sequences of genes incorporated into respective vectors and introduced into HNSCs. However, the present invention is not meant to be limited to the specifics of these examples. First, in addition to Math1, Hath1, Lhx8 and Gbx1), other developmental control genes of interest include Lhx6, Isl1, Dlx1/2 and Mash. Examples of cDNA sequences, and corresponding translated polypeptide and protein sequences, of these and other developmental control genes are readily obtainable from the GenBank online database (See ncbi.nlm.nih.gov/entrez/query.fcgi), and these are hereby incorporated by reference for that purpose.

Also, as to the nucleic acid sequences comprising the genes of interest, specific sequences of which are provided in the above examples and in the above paragraph, it is appreciated that substantial variation may exist in a nucleic acid sequence for a gene, yet a polypeptide or protein may nonetheless be produced in a cell from one of a number of such variant nucleic acid sequences, wherein such polypeptide or protein has a desired effect on the cell comparable to a polypeptide or protein produced from one of the nucleic acid sequences specified in the above examples. That is, variations may exist in a nucleic acid sequence for a gene yet the variations nonetheless function effectively when substituted for a nucleic acid sequence of a specified gene.

Accordingly, embodiments of the present invention also include and/or employ nucleic acid sequences that hybridize under stringent hybridization conditions (as defined herein) to all or a portion of a nucleic acid sequence represented by any of the SEQ ID Nos. 1-13, or their complements, or to sequences for Isl1, Dlx1/2, Mash, or their complements. The hybridizing portion of the hybridizing nucleic acid sequences is typically at least 15 (e.g., 20, 25, 30, or 50) nucleic acids in length. The hybridizing portion of the hybridizing nucleic acid sequence is at least 80%, e.g., at least 95%, or at least 98%, identical to the sequence of a portion or all of a nucleic acid sequence encoding one of genes identified by the noted Sequence ID numbers, or one of their complements. Hybridizing nucleic acids of the type described herein can be used, for example, as a cloning probe, a primer (e.g., a PCR primer), or a diagnostic probe, as well as for a gene transfected into a cell as described in the examples above.

Hybridization of the oligonucleic acid probe to a nucleic acid sample typically is performed under stringent conditions. Nucleic acid duplex or hybrid stability is expressed as the melting temperature or Tm, which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions. If sequences are to be identified that are related and substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (e.g., SSC or SSPE).

Then, assuming that 1% mismatching results in a 1 °C. decrease in the Tm, the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having >95% identity with the probe are sought, the final wash temperature is decreased by 5 °C.). In practice, the change in Tm can be between 0.5 °C. and 1.5 °C. per 1% mismatch. Stringent conditions involve hybridizing at 68 °C. in 5xSSC/5xDenhardt’s solution/1.0% SDS, and washing in 0.2xSSC/0.1% SDS at room temperature. Moderately stringent conditions include washing in 3xSSC at 42 °C. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Additional guidance regarding such conditions is readily available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, N.Y.; and Ausubel et al. (eds), 1995, Current Protocols in Molecular Biology. (John Wiley & Sons, N.Y.) at Unit 2.10.

The above-specified sequences are not meant to be limiting. For example, provided herein are additional identified sequences for Math1 (SEQ ID Nos:2 and 3), and Hath1 (SEQ ID NO:5). Numerous other similar sequences are known and searchable at GenBank. Also, the methods and compositions disclosed and claimed herein for other sequences may be practiced with Gbx1 (SEQ ID NO:9) and sequences similar to it.

Further, the sequences for introduced genes and polypeptides or proteins expressed by them may also be defined in terms of homology to one of the sequences provided in the above examples and discussion. In the context of the present
application, a nucleic acid sequence is "homologous" with the sequence according to the invention if at least 70%, preferably at least 80%, most preferably at least 90% of its base composition and base sequence corresponds to the sequence specified according to the invention. According to the invention, a "homologous protein" is to be understood to comprise proteins which contain an amino acid sequence at least 70% of which, preferably at least 80% of which, most preferably at least 90% of which, corresponds to the amino acid sequence disclosed in (Gish and States, 1993); wherein corresponds is to be understood to mean that the corresponding amino acids are either identical or are mutually homologous amino acids. The expression "homologous amino acids" denotes those which have corresponding properties, particularly with regard to their charge, hydrophobic character, steric properties, etc. Thus, a protein may be from 70% up to less than 100% homologous to any one of the proteins expressed by one of the disclosed introduced genes.

Homology, sequence similarity or sequence identity of nucleic acid or amino acid sequences may be determined conventionally by using known software or computer programs such as the BestFit or Gap pairwise comparison programs (GGC Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wis. 53711). BestFit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. Gap performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as Bestfit, to determine the degree of sequence homology, similarity or identity, the default setting may be used, or an appropriate scoring matrix may be selected to optimize identity, similarity or homology scores. Similarly, when using a program such as Bestfit to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as blossum45 or blossum80, may be selected to optimize identity, similarity or homology scores.

Alternatively, as used herein, "percent homology" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleic acid searches are performed with the NBLAST program, score=100, wordlength=12, to obtain nucleic acid sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches are performed with the XBLAST program, score=50, wordlength=3, to obtain amino acid sequences homologous to a reference polypeptide. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See ncbi.nlm.nih.gov.

Further, in addition to the homology, as indicated in certain claims (i.e., for some embodiments), is a requirement that the homologous or hybridizable nucleic acid sequence or polypeptide or protein functions analogously to the specified sequence of which it is homologous or with which it is hybridizable. That is, the homologous or hybridizable variant functions to achieve the same result, i.e., to increase the probability of a transfected cell, or the percentage of a number of cells, that are biased to differentiate to a cell, or cells, respectively, having characteristics of a desired end-stage differentiated cell.

While the transfection into HNCS in the above examples uses the Neuroporter approach (Gene Therapy Systems, Inc., San Diego, Calif.), it is appreciated that any known or later-developed method of introduction of a nucleic acid sequence may be employed in the methods and systems, and to produce the compositions, of the present invention. For example, and not to be limiting, introduction of a nucleic acid sequence may be effectuated by stable or transient transfection, lipofection by methods other than Neuroporter, calcium phosphate treatment, electroporation, infection with a recombinant viral vector, and the use of vectors comprising a plasmid construct. Generally and collectively, these methods are considered to be included in the term "means to transfect," in the term "step for transfecting." Also, the use of the particular promoter and polyadenylation transcription termination site are not meant to be limiting, as many promoter and transcription termination sites are known and used routinely in the art.

As to the use of different means to transfect, and in view of the above discussion of the relative percentage of cells biased to cells having characteristics of a desired end-stage cell type, it is appreciated that types of transfection, cells that are transfected, and other factors, including post transfection conditions, affect the percentage of cells ultimately biased. In view of these factors, and considering the importance of the specific developmental control genes that are introduced to a cell in certain embodiments of the present invention, in some embodiments the percentage of transfected cells biased exceeds 40 percent, in other embodiments the percentage of transfected cells biased exceeds 50 percent, in other embodiments the percentage of transfected cells biased exceeds 65 percent, and in other embodiments the percentage of transfected cells biased exceeds 70 percent. However, it also is appreciated that determination of the percentage of cells that are in fact transfected in a given container of cells may be difficult to assess, the performance of the present invention in certain embodiments may be expressed in an alternative manner. That is, in some embodiments of the present invention in which a number of cells has been exposed to a selected method or means of transfection for the purpose of introducing a desired developmental control gene (such as Lhx8), the percentage of total cells that are biased to a desired end-stage cell type, or to a cell having characteristics of a desired end-stage cell type, is at least 35 percent, in other embodiments such percentage of total cells exceeds 50 percent, and in other embodiments such percentage of total cells exceeds 70 percent.

Further, it is appreciated that embodiments of the present invention are described as follows:

1. A neural stem cell, including a human neural stem cell, comprising an introduced nucleic acid sequence having an expressible developmental control gene, the expression of said gene being effective to increase the probability of differentiation of said cell to a desired neural cell type.

2. A neural stem cell, including a human neural stem cell, comprising an introduced nucleic acid sequence having an expressible developmental control gene, the expression of said gene being effective to increase the probability of differentiation of said cell to a cell having characteristics of a cholinergic neuron.

3. A neural stem cell, including a human neural stem cell, comprising an introduced nucleic acid sequence having an expressible developmental control gene, the expres-
transfecting homeobox genes may be practiced with progeni-
tive stem cell; alternatively invention embodiments
multipotent, stem cell may be a pluripotent or a

tion of embodiments of the present invention may be selected
the group consisting of Math! and Hath!. Finally, the devel­
homeobox gene, so that a homeobox gene is transfected to a
the development of cardiac cells.

The developmental control gene in the above first description
of embodiments of the present invention may be selected from
the group consisting of Math!, Hath!, Lhx8, Gbx1, Lhx6, Isl.1, Dix1/2, Mash and Nurrl. The
developmental control gene in the above second description of embodiments of
the present invention may be selected from the group consisting of Lhx8, Gbx1, Lhx6, Isl.1, Dix1/2, and Mash. The
developmental control gene in the above third description of
embodiments of the present invention may be selected from the
group consisting of Math1 and Hath1. Finally, the developed
central control gene in the above fourth description of
embodiments of the present invention may be selected from the
following the methods of the present invention also are con-
sidered to fall within the definitions of pluripo-
tential, of multipotent, and of progenitor cells (i.e., more
differentiated than multipotent yet capable of limited self-
renewal).

Based on the above examples and disclosure, in view of the
knowledge and skill in the art, it also is appreciated that
embodiments of the present invention also are used for any
homeobox gene, so that a homeobox gene is transfected to a
stem cell to effect a biasing of the stem cell to differentiate to
a desired end-stage cell, or to a cell having characteristics of
the end-stage cell. The stem cell may be a pluripotent or a
multipotent stem cell; alternatively invention embodiments
transflecting homeobox genes may be practiced with progeni-
tor cells as described herein. Cells so biased by these genes
following the methods of the present invention also are con-
sidered to fall within the scope of embodiments of the present
invention.

Example 6

Nkx2-5 Biases the Differentiation Toward the
Development of Cardiac Cells

According to another embodiment, transfection with
Nkx2-5 (SEQ ID NO: 12) biases the differentiation toward
the development of cardiac cells. See FIG. 17. Red=human
specific Troponin I, Green=Human cells. Following transfection,
multipotent stem cells were cocultured with rat cardiac
myocytes that provide environmental signals to allow the
transfected cells to develop properly.

Further, and more generally, embodiments of the present
invention may be practiced by transfecting a stem or a pro-
genitor cell with a nucleic acid sequence comprising a develop-
mental control gene, so that the transfecting is effective to
bias the cell to differentiate to a desired end-stage cell, or to a
cell having characteristics of the end-stage cell.

Also, it is appreciated that the methods of the present
invention may be applied to the daughter cells of multipotent
cells, which may have begun some stages of differentiation
but are still capable of being biased by transfection of appro-
perate developmental control genes as described herein, but
by virtue of initiating differentiation (or being less self-renewing)
may be some opinions therefore not be considered to
be multipotent cells. For the purposes of this invention, such
daughter cells, which may be found in culture with the mul-

tipotent stem cells from which they arose, are termed “biassible
progeny cells.”

It is appreciated that embodiments of the present invention
also may be defined and claimed with regard to the polypep-
tide or protein sequences expressed as a result of the transfe-
disclosed and discussed above. For example, not to be
being limiting, the peptide sequences, disclosed as the transla-
tion sequences in the attached Sequence Listing pages,
and their expression in a transfected cell, are used to identify
and/or characterize a characteristic and/or result of embodi-
ments of the present invention. Translation sequences are
obtainable from the respective GenBank database data entries
for cDNAs as described herein, and those database entries are
incorporated by reference for such information.

While a number of embodiments of the present invention
have been shown and described herein in the present context,
such embodiments are provided by way of example only, and
not of limitation. Numerous variations, changes and substi-
tutions will occur to those of skill in the art without materi-
ally departing from the invention herein. For example, the
present invention need not be limited to best mode disclosed
herein, since other applications can equally benefit from the
teachings of the present invention. Also, in the claims, means-
plus-function and step-plus-function clauses are intended to
cover the structures and acts, respectively, described herein
as performing the recited function and not only structural
equivalents or act equivalents, but also equivalent structures
or equivalent acts, respectively. Accordingly, all such modi-
fications are intended to be included within the scope of this
invention as defined in the following claims, in accordance
with relevant law as to their interpretation.

SEQUENCE LISTING

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tctctaaa tgggtctgac tggactgatcc gaggcagag cctagcagcg cggcagcagc 600
tcgcttgag ctcgcccagc acaccccagc ccagctgaca cggcagcagc cgtggcagtc 660	taacactca tggtaaccc acoaatgac ccagctgcca ataagtcata ctaacttccc 720
aaacgtctc cccactccct ttctgtgtgt a 751

<210> SEQ ID NO 9
<211> LENGTH: 1452
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 9
ggagccagtg aagcgaatatt tgggagaagat ggtgcttgcg ggcaggtcg 60
cgcggccgcg ccagcacttt cgctattgag cggagagcgc ggcgcgggcg gcacactcg 120
cagagccgac ccacccgcggcc ccagccgagc ccacccgac ggcgcgctgc cggcgccggc 180
cgcggccgcg ccagcacttt ccacccgac ggcgcgctgc cggcgccggcc ccacccgac 240
cgcggccgcg ccagcacttt ccacccgac ggcgcgctgc cggcgccggc ccacccgac 300
tcgcttgag ctcgcccagc acaccccagc ccagctgaca cggcagcagc cgtggcagtc 360
taatcaca tggtaaccc acoaatgac ccagctgcca ataagtcata ctaacttccc 420
cgcgcgcttc cgcgcgcttc cgcgcgcttc cgcgcgcttc cgcgcgcttc cgcgcgcttc 480
TGGGCAGCG GGTGCCTCG ATGGTGCGCG TGACCCTGCG GCTGGCAGCG

GCCGCCAGCG AGACCGCGAG AGAGGAGAAG GGGCTCAGCG GGTGACAGCG

AGACGAGCTT CCTGCAAGCG TCTGAGACT GTTTCAGCG CACCGGCAGCG

<200> SEQUENCE: 10

GACGGGTGC CGGCGGCGCG GCGGCACCAC GTTTAGGAGA GGGCGGCGCG

GCCGCCAGCG AGACCGCGAG AGAGGAGAAG GGGCTCAGCG GGTGACAGCG

AGACYTGTGC TCTGAGACT GTTTCAGCG CACCGGCAGCG

<400> ORGANISM: Homo sapiens
US 9,127,291 B2

-continued

agggaaggga ccgcgtggc gcgcacctga ccgcacacct cccgactct 1140
cggggggaga aaggggtccc aacatgcacc tggactcccct gatatttgcac tcaactctctg 1200
cggagacctg gaaactttttcttgccccag gcggcttgtg ttcctgcttggtt gagagcgt gg 1260
tgacgccgct gagctgtggc caaggaggtggt gtcctcggcact gtggggtgtccc gatctgcccc 1320
caggggagtc ctggctggag cccgcagcacc ccgctgtatt tagttttttat cctattgcttg 1380
taagaaatga cgctggcctt cccattaaag agatgcgctt gaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1440

<210> SEQ ID NO 11
<211> LENGTH: 1406
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 11

gagcgccccg ggcagagggg gcccggggag caggggggcg gcccccactc cggccgggtg 60
cccggccct gggccctgcg tggctggatgg atcgcggggc cagccggactg tattgtgggt 120
cctgctcgag cagagcgccc tagcgctgcc tctgtggagt tggcggcggct gacgcggctc 180
gagcgccccg gcacgccct ctgctgacgt gacgcgggct ggcctttggc gatgtacctc 240
gagcaggcct gcagggccca gcggccgctc cactcgggtt gggcggcggt ggtgtgtctt 300
gggctctgcc cagagccctg tctgtggaa aagagcagcg gggcggagcg cgcagctcctc 360
accagccgag gcgtacaggc gctaggggcc accttccaga gcgcgcgcct gggccacctg 420
agcgcagccg agagattgcg gcgtgctggcc gcccgctgctg gtcgctgctt gtcgctgctc 480
ttcaagacc ggccgcggcct atggccgacc gcgggcggcg ggcgctgtgt gtcgccgctc 540
aagagcctcg ctggggggcag gctctttgccc gccgcgcgctg gcggctgggct 600
aagctctcg aogccaaagt ggcgcggcccc ggtcgttggcc gtcgctgcgg cgcgctcccc 660
tttctcctcg cttcagcacc gcgtcgcgctg ggcgctgcgg cgggtggtgg 720
cccagcacgc ctgcgctgcg ctcagctggg tctcgcgtgc ggcgctgccc gcgggcgggtc 780
ccgagccgcg gcggcggcgg gcggcggcgg cggccggcgg ggcggtggttg 840
gtcgcgcgct gcgcgccgtg ctcgctgcgt gtcgctggtg gcggcgggct gcggcgggct 900
gctctcttc ctcgctcgc gcgtgctggtc tgggacgtgc gcgtgctgctg 960
aagagcaccg aagccgcttg ctcgctgcgt gcgtgctgctg ggcgctgccc gcgggcgggtc 1020
aacattctgc gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct 1080
ccttcgctcg acgcggcgcg gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct 1140
cgggctgcc gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct 1200
agagcctctg ctcgctgcgt gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct 1260
tcgccctggc ctcgctgcgt gcgcgcgcct gcgcgcgcct gcgcgcgcct gcgcgcgcct 1320
ccgcccttgc aagccgcttg ctcgctgcgt gcgcgcgcct gcgcgcgcct gcgcgcgcct 1380
ggcgctgcct aaaaaaaaaaaaaaaaaaaaa 1406

<210> SEQ ID NO 12
<211> LENGTH: 20
**SEQ ID NO 12**
**LENGTH:** 20
**TYPE:** DNA
**ORGANISM:** Artificial Sequence
**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic primer

**SEQUENCE:**
tgctggcagt tgcgtgtct

**SEQ ID NO 13**
**LENGTH:** 24
**TYPE:** DNA
**ORGANISM:** Artificial Sequence
**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic primer

**SEQUENCE:**
ctggctttgg atgattgacg
tccgatctgg agcgtccgag cctt

gcttctgtca ccttcctaac ttgcc

gcttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
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gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc
gttctgtca cttcctaac ttgcc

**SEQ ID NO 16**
**LENGTH:** 94
**TYPE:** DNA
**ORGANISM:** Artificial Sequence
**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic polynucleotide

**SEQUENCE:**
tacaagtccg gactcagatc tcgagctcaag cgttcaattc gccagtcag cgggtaccgc

**SEQ ID NO 11**
**LENGTH:** 6
**TYPE:** PRT
**ORGANISM:** Artificial Sequence
**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic 6xHis tag

**SEQUENCE:**
His His His His His

**SEQ ID NO 17**
**LENGTH:** 94
**TYPE:** DNA
**ORGANISM:** Artificial Sequence
**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic polynucleotide

**SEQUENCE:**
tacaagtccg gactcagatc tcgagctcaag cgttcaattc gccagtcag cgggtaccgc
ggccgagggat coacgggat tagataactg atca
<210> SEQ ID NO 18
<211> LENGTH: 351
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 18

Met Ser Arg Leu Leu His Ala Glu Glu Trp Ala Glu Val Lys Glu Leu
1 5 10 15
Gly Asp His Arg His Pro Gln Pro His His Val Pro Pro Leu Thr
20 25 30
Pro Gln Pro Pro Ala Thr Leu Gln Ala Arg Asp Leu Pro Val Tyr Pro
35 40 45
Ala Glu Leu Ser Leu Leu Asp Ser Thr Asp Pro Arg Ala Trp Leu Thr
50 55 60
Pro Thr Leu Gln Gly Leu Cys Thr Ala Arg Ala Ala Gly Tyr Leu Leu
65 70 75 80
His Ser Pro Glu Leu Gly Ala Ser Glu Ala Ala Pro Arg Asp Glu
85 90 95
Ala Asp Ser Glu Gly Leu Val Arg Arg Ser Gly Cys Gly Gly Leu
100 105 110
Ser Lys Ser Pro Gly Pro Val Lys Val Arg Glu Gln Leu Cys Lys Leu
115 120 125
Lys Gly Gly Val Val Asp Leu Gln Val Gly Cys Ser Arg Arg Glu Arg Ala
130 135 140
Pro Ser Ser Lys Gln Val Asn Gly Val Gln Gly Arg Arg Leu Ala
145 150 155 160
Ala Asn Ala Arg Glu Arg Arg Met His Gly Leu Asn His Ala Phe
165 170 175
Asp Gln Leu Arg Asn Val Ile Pro Ser Gln Asn Asp Lys Lys Leu
180 185 190
Ser Lys Tyr Glu Thr Leu Gln Met Ala Gln Ile Tyr Ile Asn Ala Leu
195 200 205
Ser Glu Leu Leu Gln Thr Pro Asn Val Gly Glu Gln Pro Pro Pro Pro
210 215 220
Thr Ala Ser Cys Lys Asn Asp His His His Leu Arg Thr Ala Ser Ser
225 230 235 240
Tyr Glu Gly Ala Gly Ala Ser Ala Val Ala Gly Ala Gln Pro Ala
245 250 255
Pro Gly Gly Gly Pro Arg Pro Pro Thr Pro Pro Gly Cys Arg Thr Arg
260 265 270
Phe Ser Gly Pro Ala Ser Ser Gly Gly Tyr Ser Val Gln Leu Asp Ala
275 280 285
Leu His Phe Pro Ala Phe Glu Asp Ala Leu Thr Ala Met Met Ala
290 295 300
Gln Lys Asp Leu Ser Pro Ser Leu Pro Gly Gln Ile Leu Gln Pro Val
305 310 315 320
Gln Glu Asp Asn Ser Lys Thr Pro Arg Ser His Arg Ser Asp Gly
325 330 335
Glu Phe Ser Pro His Ser His Tyr Ser Asp Ser Asp Glu Ala Ser
340 345 350

<210> SEQ ID NO 19
<211> LENGTH: 351
<212> TYPE: PRT
<213> ORGANISM: Mus musculus
Met Ser Arg Leu Leu His Ala Glu Glu Trp Ala Glu Val Lys Glu Leu 1    5    10    15
Gly Asp His Arg His Pro Gln Pro His His Val Pro Pro Leu Thr  20   25   30
Pro Gln Pro Pro Ala Thr Leu Gln Ala Arg Asp Leu Pro Val Tyr Pro  35   40   45
 Ala Glu Leu Ser Leu Leu Asp Ser Thr Asp Pro Arg Ala Trp Leu Thr  50   55   60
 Pro Thr Leu Gln Gly Leu Cys Thr Ala Arg Ala Ala Gln Tyr Leu Leu  65   70   75   80
 His Ser Pro Glu Leu Gly Ala Ser Glu Ala Ala Pro Arg Asp Glu  85   90   95
 Ala Asp Ser Gln Gly Glu Leu Val Arg Arg Ser Gly Cys Gly Gly Leu 100  105  110
 Ser Lys Ser Pro Gly Pro Val Lys Val Arg Glu Gin Leu Cys Lys Leu 115  120  125
 Lys Gly Gly Val Val Asp Leu Gly Cys Ser Arg Gin Gin Arg Ala  130  135  140
 Pro Ser Ser Lys Gin Val Asn Gin Gin Lys Gin Gin Arg Gin Leu Ala  145  150  155  160
 Ala Asn Ala Arg Glu Arg Arg Met His Gly Leu Asn His Ala Phe  165  170  175
 Asp Gin Leu Arg Asn Val Ile Pro Ser Phe Asn Asp Lys Leu  180  185  190
 Ser Lys Tyr Glu Thr Leu Gin Met Ala Gin Ile Tyr Ile Asn Ala Leu 195  200  205
 Ser Glu Leu Leu Gin Thr Pro Asn Val Gly Gin Gin Pro Pro Pro Pro 210  215  220
 Thr Ala Ser Cys Lys Asn Asp His His Leu Arg Thr Ala Ser Ser  225  230  235  240
 Tyr Glu Gly Gly Ala Gly Ala Ser Ala Val Ala Gly Ala Gin Pro Ala  245  250  255
 Pro Gly Gly Pro Arg Pro Thr Pro Pro Gly Cys Arg Thr Arg  260  265  270
 Phe Ser Gly Pro Ala Ser Ser Gly Gly Tyr Ser Val Gin Leu Asp Ala  275  280  285
 Leu His Phe Pro Ala Phe Glu Asp Ala Leu Thr Ala Met Met Ala  290  295  300
 Gln Lys Gin Leu Ser Pro Ser Leu Pro Gly Gly Ile Leu Gin Pro Val  305  310  315  320
 Gln Glu Gin Asn Ser Lys Thr Ser Pro Gin Gin His Gin Gin Ser Gin  325  330  335
 Glu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gins
Gly Asp His His Arg Gln Pro Gln Pro His His Leu Pro Gln Pro Pro 20 25 30
Pro Pro Pro Gln Pro Pro Ala Thr Leu Gln Ala Arg Glu His Pro Val 35 40 45
Tyr Pro Pro Glu Leu Ser Leu Leu Asp Ser Thr Asp Pro Arg Ala Trp 50 55 60
Leu Ala Pro Thr Leu Gln Ile Cys Thr Ala Arg Ala Ala Gln Tyr 65 70 75 80
Leu Leu His Ser Pro Glu Leu Gly Ala Ser Glu Ala Ala Pro Arg 85 90 95
Asp Glu Val Asp Gly Arg Gly Leu Val Arg Ser Ser Gly Gly 100 105 110
Ala Ser Ser Ser Lys Ser Pro Gly Pro Val Lys Val Arg Glu Gln Leu 115 120 125
Cys Lys Leu Lys Gly Gln Val Val Asp Glu Leu Gly Cys Ser Arg 130 135 140
Gln Arg Ala Pro Ser Ser Lys Gln Val Asn Gly Val Val Lys Gln Arg 145 150 155 160
Arg Leu Ala Ala Asn Ala Arg Arg Arg Met His Gly Leu Asn 165 170 175
His Ala Phe Asp Glu Leu Arg Asn Val Ile Pro Ser Phe Asn Asn Asp 180 185 190
Lys Lys Leu Ser Lys Tyr Glu Thr Leu Gln Met Ala Gln Ile Tyr Ile 195 200 205
Asn Ala Leu Ser Glu Leu Gln Thr Pro Ser Gly Gly Glu Gln Pro 210 215 220
Pro Pro Pro Pro Pro Ala Ser Cys Lys Ser Asp His His His Leu Arg Thr 225 230 235 240
Ala Ala Ser Tyr Glu Gly Ala Gly Asn Ala Thr Ala Ala Gly Ala 245 250 255
Gln Gln Ala Ser Gly Gly Ser Gln Arg Pro Thr Pro Pro Gly Ser Cys 260 265 270
Arg Thr Arg Phe Ser Ala Pro Ala Ser Ala Gly Tyr Ser Val Gln 275 280 285
Leu Asp Ala Leu His Phe Ser Thr Leu Gly Asp Ser Ala Leu Thr Ala 290 295 300
Met Met Ala Glu Asn Leu Ser Pro Ser Leu Pro Gly Ser Ile Leu 305 310 315 320
Gln Pro Val Gln Glu Asn Ser Lys Thr Ser Pro Arg Ser His Arg 325 330 335
Ser Asp Gly Glu Phe Ser Pro His Ser His Tyr Ser Asp Ser Asp Glu 340 345 350
Ala Ser

<210> SEQ ID NO 21
<211> LENGTH: 354
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 21
Met Ser Arg Leu Leu His Ala Glu Glu Trp Ala Glu Val Lys Glu Leu 1 5 10 15
Gly Asp His His Arg Gln Pro Gln Pro His His Leu Pro Gln Pro Pro 20 25 30
Pro Pro Pro Gln Pro Pro Ala Thr Leu Gln Ala Arg Glu His Pro Val
Tyr Pro Pro Glu Leu Ser Leu Leu Asp Ser Thr Asp Pro Arg Ala Trp
Leu Ala Pro Thr Leu Gln Gly Ile Cys Thr Ala Arg Ala Ala Gln Tyr
Leu Leu His Ser Pro Glu Leu Gly Ala Ser Glu Ala Ala Ala Pro Arg
Asp Glu Val Asp Gly Arg Gly Leu Val Arg Arg Ser Ser Gly Gly
Ala Ser Ser Ser Lys Ser Pro Gly Pro Val Lys Val Arg Glu Gln Leu
Cys Lys Leu Lys Gly Val Val Val Asp Glu Leu Gly Cys Ser Arg
Gln Arg Ala Pro Ser Ser Lys Gin Val Asn Gin Val Gin Lye Gin Arg
Arg Leu Ala Ala Asn Ala Arg Glu Arg Arg Met His Gly Leu Asn
His Ala Phe Asp Gin Leu Arg Asn Val Ile Pro Ser Phe Asn Asn Asp
Lys Lys Leu Ser Lys Tyr Glu Thr Leu Gin Met Ala Gin Ile Tyr Ile
Asn Ala Leu Ser Glu Leu Gin Thr Pro Ser Gly Gln Gin Gin Pro
Pro Pro Pro Ala Ser Cys Lys Ser Asp His His His Leu Arg Thr
Ala Ala Ser Tyr Glu Gly Ala Ala Gly Asn Ala Thr Ala Ala Gly Ala
Gln Gln Ala Ser Gly Gin Ser Gin Arg Pro Thr Pro Pro Gly Ser Cys
Arg Thr Arg Phe Ser Ala Pro Ala Ser Ala Gly Tyr Ser Val Gin
Leu Asp Ala Leu His Phe Thr Phe Glu Asp Ser Ala Leu Thr Ala
Met Met Ala Gin Lys Asn Leu Ser Pro Ser Leu Pro Gly Ser Ile Leu
Gln Pro Val Gin Glu Gin Asn Ser Lys Thr Ser Pro Arg Ser His Arg
Ser Asp Gly Gin Phe Ser Pro His Ser His Tyr Ser Asp Ser Aep Glu
Ala Ser

<210> SEQ ID NO 22
<211> LENGTH: 396
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22
Met Gin Ile Leu Ser Arg Cys Gin Gly Leu Met Ser Glu Gln Cys Gly
1      5       10       15
Arg Thr Thr Ala Leu Ala Ala Gly Arg Thr Arg Lys Gly Ala Gly Glu
20      25       30
Glu Gin Leu Val Ser Pro Glu Gly Ala Gly Asp Gin Aep Ser Cys Ser
35      40       45
Ser Ser Ala Pro Leu Ser Pro Ser Ser Ser Pro Arg Ser Met Ala Ser
Gly Ser Gly Cys Pro Pro Gly Lys Cys Val Cys Asn Ser Cys Gly Leu
Glu Ile Val Asp Lys Tyr Leu Leu Lys Val Asn Asp Leu Cys Trp His
Val Arg Cys Leu Ser Cys Ser Val Cys Arg Thr Ser Leu Gly Arg His
Thr Ser Cys Tyr Ile Lys Asp Lys Asp Ile Phe Cys Lys Leu Asp Tyr
Phe Arg Arg Tyr Gly Thr Arg Cys Ser Arg Cys Gly Arg His Ile His
Ser Thr Asp Trp Val Arg Ala Lys Gly Asn Val Tyr His Leu Ala
Cys Phe Ala Cys Phe Ser Cys Lys Arg Gin Leu Ser Thr Gly Glu Glu
Phe Ala Leu Val Glu Glu Val Leu Cys Arg Val His Tyr Asp Cys
Met Leu Asp Asn Leu Lys Arg Gin Val Glu Gin Asn Gly Asn Gly Ile Ser
Val Glu Gly Ala Leu Thr Glu Gin Asp Val Asn His Pro Lys Pro
Ala Lys Arg Ala Arg Thr Ser Phe Thr Ala Asp Gin Leu Gin Val Met
Gln Ala Gin Phe Ala Gin Asp Asn Asn Pro Gin Ala Gin Thr Leu Gin
Lys Leu Ala Glu Arg Thr Gly Leu Ser Arg Arg Val Ile Gin Val Trp
Phe Gin Asn Cys Arg Ala Arg Gin His Lys His Val Ser Pro Asn His
Ser Ser Ser Thr Pro Val Thr Ala Val Pro Arg Leu Ser Pro
Pro Met Leu Glu Ala Val Tyr Ser Ala Tyr Val Pro Gin Asp Gly
Thr Met Leu Thr Ala Leu His Ser Tyr Met Asp Ala His Ser Pro Thr
Thr Leu Gly Leu Gin Pro Leu Leu Pro His Ser Met Thr Gin Leu Pro
Ile Ser His Thr

<210> SEQ ID NO: 23
<211> LENGTH: 365
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 23
Met Tyr Trp Lys Ser Asp Gin Met Phe Val Cys Lys Leu Glu Gly Lys
Glu Met Pro Glu Leu Ala Val Pro Arg Glu Met Cys Pro Gly Leu Met
Ser Glu Cys Gly Arg Pro Ala Gly Ala Gly Arg Thr Arg Lys
Gly Ser Gly Glu Gly Leu Val Asn Pro Glu Gly Ala Gly Asp Glu
What is claimed is:

1. A method of biasing differentiation of a neural stem cell comprising introducing an expression construct comprising a Hath1 gene sequence into the neural stem cell in vitro, wherein expression of the Hath1 gene sequence is effective to bias the neural stem cell to a desired end-stage cell type, in vitro, wherein the desired end-stage cell type comprises characteristics of an inner-ear hair cell, the characteristics comprising inner ear hair cell projections.

2. A method of biasing differentiation of a neural stem cell in vitro comprising:
   a. providing the neural stem cell;
   b. preparing a nucleic acid sequence comprising a promoter operatively linked to an expressible sequence that comprises a Hath1 gene sequence, the nucleic acid sequence comprising a transcription termination site; and
   c. transfecting said neural stem cell with said nucleic acid sequence, in vitro; wherein expression of the expressible sequence results in biasing the neural stem cell to a desired end-stage cell type, in vitro, wherein the desired end-stage cell type comprises characteristics of an inner-ear hair cell, the characteristics comprising inner ear hair cell projections.

3. A method of biasing differentiation of one or more cells in a population of cells comprising neural stem cells, comprising:
   a. providing the population of cells in a vessel;
   b. adding to the vessel a plurality of copies of a nucleic acid sequence comprising Hath1 gene sequence under conditions to express said gene sequence; wherein expression of the gene sequence in one or more cells transfected, in vitro, with a copy of the nucleic acid sequence results in biasing the neural stem cell to a desired end-stage cell type, in vitro, wherein the desired end-stage cell type comprises characteristics of an inner-ear hair cell, the characteristics comprising inner ear hair cell projections.
sequence is effective to bias the one or more cells to a desired end-stage cell type, wherein the desired end-stage cell type comprises characteristics of an inner-ear hair cell, the characteristics comprising inner ear hair cell projections.

4. The method of claim 3, the population of cells additionally comprising one or more biasable progeny cells.

5. The method of claim 3, the neural stem cells comprising human neural stem cells.

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