Methods and materials for increasing potency of cells (CON 1)

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METHODS AND MATERIALS FOR INCREASING POTENCY OF CELLS

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(WO 2004/072226) via the application file for complete search history.

Field of Classification

CPC ........................................... 435/377; 435/455
USPC ........................................... 435/377; 435/455

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Attorney, Agent, or Firm — Timothy H. Van Dyke; Beusse, Wolter, Sunks & Maira, P.A.

ABSTRACT

Disclosed herein are methods and materials for producing a more developmentally potent cell from a less developmentally potent cell. Specifically exemplified herein are methods that comprise introducing an expressible dedifferentiating polynucleotide sequence into a less developmentally potent cell, wherein the transfected less developmentally potent cell becomes a more developmentally potent cell capable of differentiating to a less developmentally potent cell of its lineage of origin or a different lineage.

1 Claim, 17 Drawing Sheets
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OTHER PUBLICATIONS


* cited by examiner
PRIOR ART

FIG 1.
FIG. 2

[A] Mesenchymal stem cells pre-transfection
[B] Nanog-transfected mesenchymal stem cells 12 days post-transfection
[C] Nanog-transfected mesenchymal stem cells 4 weeks post-transfection
Green: βIII-tubulin, Red: GFAP, Blue: DAPI
Green: βIII-tubulin, Red: GFAP, Blue: DAPI

FIG. 7
FIG. 9: NanogP8 Sequence

```plaintext
actaacctgtggagtttttagttgactccacaaaccatggattttattctaaactactcatgaacatg
atatgacttggaggctgccttggaagctgctggggaaggccttaatgtaatacagcagacc
sgdgyfvlngemvqqsckmhmstplqeglnvqqtactagtattttagtacctcaacaaacattgattaattattctaatcactctcatgaacatgc
caacctgaagacgtgtga
```
Treatment with 10, 3 or 1 uM of 5azaC for 21 days, 5 days coculture.
Electrophoresis 050115
Troponin I and hANP

3 weeks of treatment (3uM BrdU or 5-azaC) and coculture for 7 days

Lane 2: ladder
Lane 3: Troponin I or GATA-4 low RNA of BrdU treatment
Lane 4: Troponin I or GATA-4 high RNA of BrdU treatment
Lane 5: Troponin I or GATA-4 low RNA of 5azaC treatment
Lane 6: Troponin I or GATA-4 high RNA of 5azaC treatment
Lane 7: ladder
Lane 8: hANP or MLC-2v low RNA of BrdU treatment
Lane 9: hANP or MLC-2v high RNA of BrdU treatment
Lane 10: hANP or MLC-2v low RNA of 5azaC treatment
Lane 11: hANP or MLC-2v high RNA of 5azaC treatment
Electrophoresis 050116
Troponin I and hANP

Lane 1: 100bp ladder
Lane 2: 3uM combined treatment 3 weeks
Lane 3: 1uM combined treatment 3 weeks
Lane 4: 3uM 5azaC 3 weeks
Lane 5: 3uM BrdU 3 weeks
Lane 6: 3uM Control (nt 12/27)
Lane 7: 3uM combined treatment 3 weeks
Lane 8: 1uM combined treatment 3 weeks
Lane 9: 3uM 5azaC 3 weeks
Lane 10: 3uM BrdU 3 weeks
Lane 11: 3uM Control (nt 12/27)
Lane 12: 100bp ladder

FIG. 12

GATA-4 and MLC-2v
January 24, 2005

GATA-4 and MLC-2v

Lane 1: DNA ladder
Lane 2: 10uM 5azaC treatment
Lane 3: 3uM 5azaC treatment
Lane 4: 1uM 5azaC treatment
Lane 5: 3uM 5azaC treatment
Lane 6: 3uM BrdU treatment
Lane 7: 10uM 5azaC treatment
Lane 8: 3uM 5azaC treatment
Lane 9: 1uM 5azaC treatment
Lane 10: 3uM 5azaC treatment
Lane 11: 3uM BrdU treatment
Lane 12: DNA ladder

FIG. 13

Troponin I and hANP

Lane 1: DNA ladder
Lane 2: 10uM 5azaC treatment
Lane 3: 3uM 5azaC treatment
Lane 4: 1uM 5azaC treatment
Lane 5: 3uM 5azaC treatment
Lane 6: 3uM BrdU treatment
Lane 7: 10uM 5azaC treatment
Lane 8: 3uM 5azaC treatment
Lane 9: 1uM 5azaC treatment
Lane 10: 3uM 5azaC treatment
Lane 11: 3uM BrdU treatment
Lane 12: DNA ladder
Nanog vector sequence analysis

FIG. 17
METHODS AND MATERIALS FOR INCREASING POTENCY OF CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 11/258,401; filed Oct. 24, 2005, which issued as U.S. Pat. No. 8,192,988; and which claims priority to U.S. Provisional Application No. 60/621,901 filed Oct. 22, 2004 and 60/650,438 filed Feb. 4, 2005, both of which are incorporated herein in their entirety.

BACKGROUND OF THE INVENTION

The use of stem cells for the treatment of neurodegenerative conditions offers the hope of curing diseases like Alzheimer’s and Parkinson’s by means of transplantation [1]. However, major obstacles regarding cell procurement, directing cell fate and avoiding immune response hinder clinical development [2-4]. Research has focused on both adult and embryonic stem cells and attempted to balance limitations in regulating their development and preventing immune response. Increased potency of stem cells can be achieved by epigenetic modifications through nucleotide derivatives [5] and their lineage can be directed by gene transfection [6,7].

Patients currently suffering from neurodegenerative conditions have limited treatment options. Conventional drug therapy helps delay or reduce the symptoms of disease but is unable to restore complete functionality of the brain or repair damaged tissue. Through stem cell-based therapies, scientists aim to transplant cells in order to regenerate damaged tissue and restore proper function. However, the best source of stem cells for transplantation remains an unresolved issue; with debate focusing around embryonic or adult derived stem cells. Embryonic stem cells can be readily differentiated to multiple neuronal fates but pose the risk of tumor formation or immune response; whereas adult stem cell technology is easily accessible, but provides limited capacity for transdifferentiation. An optimal approach may be to increase cellular plasticity of adult stem cells for use in autologous transplantation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the vector system for cloning nanog according to the teachings in Example 1.

FIG. 2 shows images of cells before and after transfection with nanog: A: shows mesenchymal stem cells pre-transfection; B: shows nanog-transfected mesenchymal stem cells 12 days post-cells 12 days post-transfection; C: shows nanog-transfected mesenchymal stem cells 4 weeks post-cells 4 weeks post-transfection. FIG. 3 shows images of transfected mesenchymal stem cells 9 days (A and B) and 2 months (C and D) post-transfection.

FIG. 4 shows images of cells in culture in accord with one embodiment of the subject invention. FIG. 5 shows images of Co-culturing experiments which demonstrated that embryoid body-like clusters began differentiation within 48 hours (A). Control cells with our empty vector treatments failed to show any signs of neural differentiation (B). Embryoid-like bodies adhered to membrane and differentiation occurred as neural cells migrated radially outward (C).

FIG. 6-7 show images of the clustering of nanog transfected cells.

FIG. 8 shows images of MeSC-derived neurons and astrocytes. FIG. 9 shows sequence of Nanog encoding polynucleotide and corresponding polypeptide sequence.

FIGS. 10-13 show gel images of gene expression in cells subjected to various treatments demonstrating an ability to increase potency of mesenchymal stem cells and differentiation into cardiac cells.

FIGS. 14-16 show photograph images of cells subjected to various treatments.

FIG. 17 shows a schematic representation of the nanog sequence cloned inside a CMV mammalian promoter vector.

DETAILED DESCRIPTION

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 publications, scientific publications, and other references referenced herein are hereby incorporated by reference in this application, in their entirety to the extent not inconsistent with the teachings herein.

Reference to particular buffers, media, reagents, cells, culture conditions and the like, or to some subclass of same, is not intended to be limiting, but should be read to include all such related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture medium for another, such that a different but known way is used to achieve the same goals as those to which the use of a suggested method, material or composition is directed.

It is important to an understanding of the present invention to note that all technical and scientific terms used herein, unless defined herein, are intended to have the same meaning as commonly understood by one of ordinary skill in the art. The techniques employed herein are also those that are known to one of ordinary skill in the art, unless stated otherwise. For purposes of more clearly facilitating an understanding of the invention as disclosed and claimed herein, the following definitions are provided.

The differentiation of stem cells along multiple lineages has been intensely studied given their great therapeutic potential. However, the mechanisms that underlie proliferation, self-renewal and differentiation in cells with the capacity for further development remains poorly understood. A recently discovered gene, nanog, is required to sustain pluripotency in embryonic stem cells and acts concomitantly with embryonic transcription factor Oct-4, yet utilizes a Stat3-3 independent mechanism. The subject invention is based on the inventor’s discovery that gene transfection of adult stem cells with nanog, an embryonic stem cell gene maintaining pluripotency [8,9], can allow for the production of neurons and astrocytes from bone marrow cells via a two-step process. First, mesenchymal stem cells are modified by nanog transfection, and the cells form embryoid-like bodies. Then cells are committed to neuronal lineage in a co-culture system with differentiated neural stem cells separated by a semi-permeable membrane.

This technology may be a means of generating effective autologous stem cell transplants to improve neuroreplacement strategies. The inventors have discovered that adult stem cells can be dedifferentiated through introduction and expression of the nanog gene or other dedifferentiating genes.

Thus, in one embodiment, the invention provides methods for making a more developmentally potent cell from a less developmentally potent cell. In a typical embodiment, the method comprises the step of introducing an expressible
dedifferentiating polynucleotide sequence into a less developmentally potent cell, wherein the transfected less developmentally potent cell becomes a more developmentally potent cell capable of differentiating to a less developmentally potent cell of its lineage of origin or a different lineage. In certain embodiments, the inventive methods further comprise the step of co-culturing the transfected less developmentally potent cell with neural-lineage cells or media conditioned with neural-lineage cells, wherein the transfected cells become a more developmentally potent cells capable of differentiating to a less developmentally potent cells of its lineage of origin or a different lineage.

In the practice of one embodiment of the invention, the phenotype of the less developmentally potent cell is changed when it becomes a more developmentally potent cell. Thus, the invention provides methods for changing a first phenotype of a less developmentally potent cell into a second phenotype of more developmentally potent cell. The change from a certain potency to a higher level of potency is considered “dedifferentiation” in accord with the teachings herein. In preferred embodiments, the less developmentally potent cell is a stem cell, more preferably a hematopoietic stem cell, a neural stem cell, an epithelial stem cell, an epidermal stem cell, a retinal stem cell, an adipose stem cell and a mesenchymal stem cell.

In yet further aspects of the invention are provided pharmaceutical compositions comprising said more developmentally potent cells prepared according to the methods of the invention and a pharmaceutically-acceptable carrier or excipient. The invention provides such pharmaceutical compositions comprising said more developmentally potent cells that are tissue stem cells for use in cell or tissue regeneration or for correcting a disease or disorder in a tissue or animal in need thereof.

Thus, the invention also provides methods for using the pharmaceutical compositions provided herein to treat an animal in need thereof by administering the more developmentally potent cells thereto. In certain preferred embodiments, the more developmentally potent cells comprise a cluster of two or more of the more developmentally potent cells. Preferably, the animal has a cortical or neurological deficit that can be treated or ameliorated by administration of said more developmentally potent cells, such as a deficit caused by a neurodegenerative disease, a traumatic injury, ischemia, a developmental disorder, a disorder affecting vision, an injury or disease of the spinal cord, a demyelinating disease, an autoimmune disease, an infection, an inflammatory disease, or a disorder affecting development of the spinal cord, a demyelinating disease, an autoimmune disease, an infection, or a disease of the spinal cord, a demyelinating disease, an autoimmune disease, an infection, or a disease of the spinal cord, a demyelinating disease, an autoimmune disease, an infection, or a disease of the spinal cord, a demyelinating disease.

In yet another embodiment, the invention relates to treating a stem cell, excluding those of neural origin, such that it is converted into a more developmentally potent cell, which enables it to differentiate into the various cell types found in eye tissue, inter alia, choroid, retinal pigment epithelium cells, rod and cone photoreceptor cells, horizontal cells, bipolar neurons, amacrine, ganglion and optic nerve cells. These non-limiting, exemplary cell types found in eye tissue are collectively referred to as retinal cells. The methods comprising the step of contacting more developmentally potent cells of the invention with an effective amount of one or a combination of growth factor selected from the group consisting of TGF-b3, IGF-1 and CNTF for an effective period such that the growth factor-contacted cells can differentiate into retinal cells.

As used herein, the terms “multipotent neural stem cells (MNSCs),” “neural stem cells (NSCs)” and “neural progenitor cells (NPCs)” refer to undifferentiated, multipotent cells of the CNS. Such terms are commonly used in the scientific literature. MNSCs can differentiate into tissue-specific cell types, for example astrocytes, oligodendrocytes, and neurons
when transplanted in the brain. MNSCs of the invention are distinguished from natural MNSCs by their adaptation for proliferation, migration and differentiation in mammalian host tissue when introduced thereto.

As used herein, a “less developmentally potent cell” is a cell that is capable of limited multi-lineage differentiation or capable of single-lineage, tissue-specific differentiation, for example, an untreated mesenchymal stem cell can differentiate into, inter alia, osteocytes and chondrocytes, i.e., cells of mesenchymal lineage, but has only limited ability to differentiate into cells of other lineages (e.g., neural lineage.).

As used herein, a “more developmentally potent cell” is a cell that is readily capable of differentiating into a greater variety of cell types than its corresponding less developmentally potent cell. For example, a mesenchymal stem cell can readily differentiate into osteocytes and chondrocytes but has only limited ability to differentiate into neural or retinal lineage cells (i.e., it is a less developmentally potent cell in this context). Mesenchymal stem cells treated according to the methods of the invention become more developmentally potent because they can readily differentiate into, for example, mesenchymal-lineage and neural-lineage cell types; the plasticity of the cells is increased when treated according to the methods of the invention.

The invention provides methods of delivery and transplantation of the more developmentally potent cells of the invention to ameliorate the effects of age, physical and biological trauma and degenerative disease on the brain or central nervous system of an animal, as well as other tissues such as, for example, retinal tissue. It is well recognized in the art that transplantation of tissue into the CNS offers the potential for treatment of neurodegenerative disorders and CNS damage due to injury. Transplantation of new cells into the damaged CNS has the potential to repair damaged circuitry and provide neurotransmitters thereby restoring neurological function. It is also recognized in the art that transplantation into other tissue, such as eye tissue, offers the potential for treatment of degenerative disorders and tissue damage due to injury. As disclosed herein, the invention provides methods for generating more developmentally potent cells adapted for proliferation, migration and differentiation in mammalian tissue when introduced thereto. The use of more developmentally potent cells in the treatment of neurological disorders and CNS damage, as well as the use of more developmentally potent cells in the treatment of other tissue damage or degeneration, can be demonstrated by the use of established animal models known in the art.

In one embodiment dedifferentiated cells or more developmentally potent cells of the invention can be administered to an animal with abnormal or degenerative symptoms obtained in any manner, including those obtained as a result of age, physical or biological trauma, or neurodegenerative disease and the like, or animal models created by man using recombinant genetic techniques, such as transgenic and “gene knockout” animals.

Recipients of the more developmentally potent cells of the invention can be immunosuppressed, either through the use of immunosuppressive drugs such as cyclosporin, or through local immunosuppression strategies employing locally applied immunosuppressants, but such immunosuppression need not necessarily be a prerequisite in certain immunoprivileged tissues such as, for example, brain and eye tissues.

In certain embodiments, the delivery method of the invention can cause less localized tissue damage to the site of cell damage or malfunction than existing methods of delivery. More developmentally potent cells of the invention can be prepared from the recipient’s own tissue. In such instances, the progeny of the more developmentally potent cells can be generated from dissociated or isolated tissue and proliferated in vitro using the methods described herein. In the case of mesenchymal stem cells (MeSCs), progeny can be generated from MeSCs isolated from, for example, bone marrow. Upon suitable expansion of cell numbers, the stem cells of the invention can be harvested and readied for administration into the recipient’s affected tissue.

There are significant differences in the method of delivery to the brain of the more developmentally potent cells compared to the prior art. One exemplary difference is as follows: the more developmentally potent cells of the invention are transplanted intraventricularly. Further, while the transplantation of one or more separate more developmentally potent cells is efficacious, the more developmentally potent cells of the invention are preferably transplanted in the form of clusters of two or more cells via a surgical procedure or injection using a syringe large enough to leave the clusters substantially intact. The results disclosed in the Examples below indicate that ventricular delivery of more developmentally potent cells of the invention in the form of a cluster of two or more cells can result in migration to the area of damage at the brain and proper neuronal differentiation. Another benefit of intraventricular injection is less tissue destruction, resulting in less localized recruitment of immune cells by the host. This is evidenced by the lack of ventricular distortion, tumor formation, and increased host astrocyte staining without any immunosuppression.

The method of delivery of the more developmentally potent cells of the invention to the brain can be essentially duplicated for other immunoprivileged tissue such as, for example, the eye. Delivery of one or more separate or two or more of the more developmentally potent cells in the form of a cluster via injection using a syringe large enough to leave the any cluster of two or more cells that is present substantially intact can result in migration to the area of damage in the eye and proper tissue-specific differentiation.

In the context of the present application, a polynucleotide 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 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 shown in FIG. 9, 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” designates those which have corresponding properties, particularly with regard to their charge, hydrophobic character, steric properties, etc. Thus, in one embodiment the protein may be from 70% up to less than 100% homologous to nanog.
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 blosum45
or blosum80, may be selected to optimize identity, similarity
or homology scores.

The term “isolated” means separated from its natural envi-
ronment.

The term “polynucleotide” refers in general to polyribo-
nucleotides and polynucleotides, and can denote an
unmodified RNA or DNA or a modified RNA or DNA.

The term “polypeptide” is to be understood to mean pep-
tides or proteins which contain two or more amino acids
which are bound via peptide bonds.

The polypeptides for use in accord with the teachings
herein include polypeptides corresponding to nanog, and also
include those, at least 70% of which, preferably at least 80%
of which, are homologous with the polypeptide corre-
sponding to nanog, and most preferably those which exhibit a
homology of at least 90% to 95% with the polypeptide corre-
sponding to nanog and which have dedifferentiating influ-
ence. See polypeptide sequence provided in FIG. 9. Thus, the
polypeptides may have a homology of from 70% up to
100% with respect to nanog.

As used herein, a “polypeptide sequence exhibiting dedif-
ferentiating influence” is a polypeptide whose presence in the
cell causes an increase in potency, or transformation from a
less developmentally potent cell to a more developmentally
potent cell. Examples of such polypeptide sequences include
the expression products of the nanog gene, and polynucle-
otide sequences that hybridize to the complement of the
sequence in FIG. 9, as well as expression products of the
polynucleotide sequences listed in Table 1 below in Example
3.

The terms “stringent conditions” or “stringent hybridiza-
tion conditions” includes reference to conditions under which
a polynucleotide will hybridize to its target sequence, to a
detectably greater degree than other sequences (e.g., at least
2-fold over background). Stringent conditions are sequence-
dependent and will be different in different circumstances. By
controlling the stringency of the hybridization and/or wash-
ing conditions, target sequences can be identified which are
100% complementary to the probe (homologous probing).
Alternatively, stringency conditions can be adjusted to allow
some mismatching in sequences so that lower degrees of
similarity are detected (heterologous probing).

Typically, stringent conditions will be those in which the
salt concentration is less than about 1.5 M Na ion, typically
about 0.1 to 1.0 M Na ion concentration (or other salts) at pH
7.0 to 8.3 and the temperature is at least about 30° C. for short
probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for
long probes (e.g., greater than 50 nucleotides). Stringent
conditions may also be achieved with the addition of destabiliz-
ing agents such as formamide. Exemplary low stringency
conditions include hybridization with a buffer solution of 30
to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl
sulfate) at 37° C., and a wash in 1× to 2×SSC (20× = SSC = 3.0
M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary
moderate stringency conditions include hybridization in 40 to
45% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in
0.5× to 1×SSC at 55 to 60° C. Exemplary high stringency
conditions include hybridization in 50% formamide, 1 M
NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65°
C.

Specificity is typically the function of post-hybridization
washes, the critical factors being the ionic strength and tem-
perature of the final wash solution. For DNA-DNA hybrids,
the Tm can be approximated from the equation of Meinkoth
and Wahl, Anal. Biochem., 138:267-284 (1984): Tm = 81.5 + 16.6 (log M) - 0.41 (% GC) - 0.61 (% form) -
500/L; where M is the molarity of monovalent cations, % GC
is the percentage of guanosine and cytosine nucleotides in the
DNA, % form is the percentage of formamide in the hybrid-
ization solution, and L is the length of the hybrid in base pairs.
The Tm is the temperature (under defined ionic strength and
pH) at which 50% of a complementary target sequence
hybridizes to a perfectly matched probe. Tm is reduced by
about 1° C. for each 1% of mismatching; thus, Tm, hybrid-
ization and/or wash conditions can be adjusted to hydridize to
sequences of the desired identity. For example, if sequences
with approximately 90% identity are sought, the Tm can be
decreased 10° C. Generally, stringent conditions are selected
to be about 5° C. lower than the thermal melting point (Tm)
for the specific sequence and its complement at a defined
ionic strength and pH. However, severely stringent conditions
may be used, or an appropriate scoring matrix, such as blosum45
or blosum80, may be selected to optimize identity, similarity
or homology scores.

The term “two different amino acid sequences, the default
settings 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
blosum45 or blosum80, may be selected to optimize identity,
similarity or homology scores.

The term “isolated” means separated from its natural envi-
ronment.

The term “polynucleotide” refers in general to polyribo-
nucleotides and polynucleotides, and can denote an
unmodified RNA or DNA or a modified RNA or DNA.

The term “polypeptide” is to be understood to mean pep-
tides or proteins which contain two or more amino acids
which are bound via peptide bonds.

The polypeptides for use in accord with the teachings
herein include polypeptides corresponding to nanog, and also
include those, at least 70% of which, preferably at least 80%
of which, are homologous with the polypeptide corre-
sponding to nanog, and most preferably those which exhibit a
homology of at least 90% to 95% with the polypeptide corre-
sponding to nanog and which have dedifferentiating influ-
ence. See polypeptide sequence provided in FIG. 9. Thus, the
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detectably greater degree than other sequences (e.g., at least
2-fold over background). Stringent conditions are sequence-
dependent and will be different in different circumstances. By
controlling the stringency of the hybridization and/or wash-
ing conditions, target sequences can be identified which are
100% complementary to the probe (homologous probing).
Alternatively, stringency conditions can be adjusted to allow
some mismatching in sequences so that lower degrees of
similarity are detected (heterologous probing).

Typically, stringent conditions will be those in which the
salt concentration is less than about 1.5 M Na ion, typically
about 0.1 to 1.0 M Na ion concentration (or other salts) at pH
7.0 to 8.3 and the temperature is at least about 30° C. for short
probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for
long probes (e.g., greater than 50 nucleotides). Stringent
conditions may also be achieved with the addition of destabiliz-
ing agents such as formamide. Exemplary low stringency
conditions include hybridization with a buffer solution of 30
to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl
sulfate) at 37° C., and a wash in 1× to 2×SSC (20× = SSC = 3.0
M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary
moderate stringency conditions include hybridization in 40 to
45% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in
0.5× to 1×SSC at 55 to 60° C. Exemplary high stringency
conditions include hybridization in 50% formamide, 1 M
NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65°
C.
nanog-like protein may be one expressed by a polynucleotide sequence introduced in the cell or cellular component, or protein delivered into the cell or cellular component, or protein expressed by an endogenous polynucleotide sequence that has been activated. Nanog expression may be activated by the provision of Oct 4 and/or Sox2, which typically form a dimer. In a specific embodiment, the cellular component is a nucleus, liposome, or mitochondria. Such endogenous polynucleotide sequence or cellular component contacted by nanog or nanog may be removed from a cell or cellular component and introduced into another cell or cellular component.

In another specific embodiment, the invention pertains to increasing the efficacy of nuclear transfer comprising fusing a nucleus with a polynucleotide encoding nanog or nanog-like protein to obtain a treated nucleus and introducing the treated nucleus into a cell. The cell may be any suitable cell but would typically be an ovum with its nucleus removed.

Example 1

Dedifferentiation of Mesenchymal Stem Cells

Introduction

Embryonic stem cells are derived during the blastocyst stage from the inner cell mass of prenatal mammalia; and possess the intrinsic properties of rapid self-renewal and pluripotency. Under the influence of endogenous and extracellular signals, these cells migrate and differentiate during the developmental process. Extracellular signals regulating self-renewal or differentiation have been demonstrated in vitro by differentiating embryonic stem cells into cell types comprising all three germ layers. These varieties include neuronal, pancreatic, cardiac and hematopoietic tissue using well-established culturing protocols. Embryonic stem cells form embryoid bodies, non-adherent proliferating clusters, in the presence of leukemia-inhibitory factor (LIF) and a feeder layer of typically fibroblast cells. Upon removal of LIF or transfer to non-feeder cell cultures, embryoid bodies undergo spontaneous differentiation. Early differentiation is characterized by loss of stem cell-specific surface antigens (SSEA-1) and alkaline phosphatase activity. Additionally, endogenous signals, including regulatory intracellular proteins, continually change throughout development. Numerous gene expression studies show distinct variations among different embryonic and adult stem cells, pointing toward underlying mechanisms responsible for the continual loss of potency corresponding with differentiation. Several key genes, namely Oct-3/4, LIF, DNMT3B and Nanog, are repeatedly shown to be almost exclusively expressed in embryonic stem cells that regulate pluripotency [10-14]. The immediate down-regulation of these genes may explain irreversible loss of potency, making embryonic stem cells an attractive source for clinical therapies. However, serious questions remain concerning the production of these cells in sufficient quantities for therapies, bioethical potential immune response and tumor formation [15].

The inventors believe that adult stem cells offer a practicable alternative to the use of embryonic tissue as they are easily harvested and potentially taken from autologous sources to preclude immune response. Stem cell populations have been found in several adult tissues including adipose [16], muscle [17], pancreas [18] and liver [19] and primarily bone marrow [20-23]; all potential sources for cellular transplants [24]. Previous in vitro studies with adult bone marrow-derived stem cells have demonstrated the ability to differentiate into brain [21], liver [23] and cardiac cells [22]. In vivo studies have shown evidence that adult stem cells can migrate and differentiate into various tissues, albeit at extremely low frequencies [20]. However, challenges have been raised over the plasticity of these cells given both the low frequencies of detected cells and new found evidence of cell fusion, in conjunction with false positives [25-27]. An ideal therapeutic alternative may exist if adult cells can be dedifferentiated to an embryonic-like state and reprogrammed to differentiate to a desired cell fate.

Nanog, also referred to as early embryo specific NK (ENK) [28], is a recently discovered gene responsible for maintaining pluripotency in embryonic stem cells [8, 9, 28, 29]. This unique gene and its cousin, Nanog2, are genetically distinct members of the ANTP class of homeodomain proteins [30] and have at least twelve identified pseudogenes [31]. Structurally, Nanog contains three alpha helices encoded within the homeodomain portion and can be divided into three regions with respect to the central homeodomain sequence [30]. The N-terminal region is rich in serine and threonine residues indicating phosphate-regulated transactivation, possibly through SMAD4 interactions, while the C-terminal domain is seven times as active with an unusual motif of equally spaced tryptophans separated by four amino acids, each flanked with serine or threonine residues. Gene expression studies have shown nanog to be active in embryonic stem cells, tumors and some adult tissue. Nanog expression precipitously decreases with differentiation and maintains self-renewal in embryonic stem cells by gene transfection. In culture, nanog guards against differentiation and acts concomitantly with Oct-4, Wnt and BMP-4, yet utilizes a STAT-3 independent mechanism to maintain an undifferentiated state. Inventors believe that the role of nanog in regulating pluripotency makes this gene a potential candidate for increasing the potency of adult stem cells.

Previous studies regulating gene expression in stem cell lines have provided valuable insight into underlying mechanisms of proliferation, self-renewal and differentiation. Gene manipulation experiments can either prevent or enhance differentiation. In particular, differentiation can be prevented in embryonic stem cell lines by over expression of Pem or nanog, genes that regulate pluripotency. Conversely, over-expression of lineage specific gene Nurrl promotes the differentiation of neural 1 stem cell lines to produce dopamine-secreting cells. Taken together, gene vectors can maintain cells in a specific state or allow for lineage committed cells to develop into a specific subpopulation. In one embodiment, the subject invention pertains to a method of dedifferentiating adult stem cells by expressing genes regulating pluripotency to enhance transdifferentiation. This technology allows for adult cells to be used for autologous transplantation and thereby provide a greater understanding of stem cell biology.

FIG. 17 shows a schematic representation of the nanog sequence cloned inside a CMV mammalian promoter vector. The 5'UTR contains an Oct-4 and Sox2 binding region. The nanog protein coding sequence can be divided into an N-terminal, homeodomain and C-terminal region. The C-terminal region can be further subdivided into a C1, Cw and C2 domains. The 3' UTR contains an Alu sequence element.

Methods and Results

Human mesenchymal stem cells (hMeSC) are initially plated in 6-well plates, adhere to the surface and allowed to divide to varying degrees of confluence. They are cultured in serum-DMEM (Dulbecco’s modified Eagle’s medium) containing 10% FCS, 5% H5, 292 mg/ml glutamine, 50 U/ml streptomycin and penicillin (all from Invitrogen).

Cloning of nanog was achieved by first performing polymerase chain reaction with primers corresponding to the
The inventors developed a two-step process of dedifferentiation. Transferring non-adherent cells to wells without a feeder layer resulted in apoptosis, related to either absence of feeder cell proteins of decreased cell density. Cellular transformation occurred in a pattern of transfection, non-adherence, survival and proliferation. To determine if nanog could restore pluripotency in adult cells, nanog transfection of mesenchymal stem cells resulted in cellular transformation occurred in a pattern of transfection, non-adherence, survival and proliferation. Non-adherent clusters proliferated in vitro beyond two months in the presence of remaining adherent mesenchymal stem cells. To determine if nanog could restore pluripotency in adult cells rather than simply maintain the state in embryonic cells, the inventors developed a two-step process of dedifferentiation and development along an alternative lineage, discussed in Example 2 below. Human mesenchymal stem cells were cultured in a six-well culture plates and were allowed to adhere and grow for at least 48 hours to achieve approximately 75% confluence. Cells were subsequently transfected with a mammalian cell vector or control vehicle, cultured and examined. Cells transfected with NANOGP8 became non-adherent and proliferated in the presence of the remaining adherent mesenchymal stem cells.

Example 2

Neuronal Differentiation of Dedifferentiated Mesenchymal Cells

To test whether cells could be dedifferentiated using nanog and committed to an alternate lineage we utilized a co-culture system of differentiated human neural stem cells and transformed mesenchymal cells. Neural stem cells were placed in 12 well plates and differentiated using serum-free basal media as previously described. Neuronal stem cells began to differentiate by becoming adherent and migrating radially outwards from the original neural sphere. Following neural stem cell differentiation, these cells were utilized as feeder cells in our co-culture system by placing modified cells inside co-culture chamber that separated modified stem cells from the feeder layer with a 0.2 µm semipermeable membrane. See FIG. 5. Within 48 hours, transferred cells began to display characteristics of differentiation marked by morphological alterations, membrane adherence and outward migration. Immunohistochemical analysis revealed positive markers for βIII-tubulin and GFAP, showing neuron and glial differentiation. Positive staining for mixed neuronal populations was observed in non-adherent clusters cultured for more than 48 hours, adherent cell masses and individual membrane bound cells.

Co-culturing experiments showed embryoid body-like clusters began differentiation within 48 hours. Control cells with our vector treatments failed to show any signs of neural differentiation. Immunohistochemical staining revealed nanog transfected samples intensely stained positive for βIII-tubulin and GFAP, indicating neuron and astrocyte differentiation. See FIGS. 6-7. Originally, nanog-transformed cells remain in a cluster of differentiating neural cells that continually radiate outward (See FIGS. 6-7). FIG. 8 shows MeSC derived neurons and astrocytes.

Example 3

Dedifferentiation of Cells Utilizing Genes Affecting Pluripotency

Following the transfection and evaluation protocol provided above in Example 1, PCR products of the genes in Table 1 are evaluated for their ability to dedifferentiate cells, particularly mesenchymal stem cells.

### TABLE 1

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<th>Description</th>
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<td>Nanog</td>
<td>15 markers for</td>
<td>nanog pseudogene 8 (NANOG8)</td>
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<td></td>
<td></td>
<td>a segment containing no introns and sharing 99 percent homology with nanog.</td>
</tr>
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<td></td>
<td></td>
<td>The deduced protein product is almost identical with two differing amino acids.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The protein can be divided into three distinct domains at the N-terminal, homeodomain and C-terminal as previously described.</td>
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<tr>
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<td></td>
<td>The gene product encodes for a nanog-like protein with a 305 amino acid serine/threonine-rich sequence. The protein can be divided into three distinct domains at the N-terminal, homeodomain and C-terminal as previously described.</td>
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<td>The deduced protein product is almost identical with two differing amino acids.</td>
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**REFERENCES**


derivatives BrdU and 5-azaC and/or forced expression of embryonic stem cell gene nanog. Following treatment, cells were plated in 6-well culture plates and expanded in serum-DMEM (Delbecco’s Modified Eagle’s Medium) containing 10% non-conditioned FBS and antibiotics/antimycotics. MeSCs were treated with varying concentrations (1-10 uM) of BrdU and/or 5-azaC for 3 weeks or transfected with mammalian expression vector containing a nanog encoding vector. Cell media was changed every three days prior to co-culture with cardiac cells. Cardiomyocytes were expanded and grown to near confluence in serum-DMEM with antibiotics and antimycotics. To differentiate cardiac cells, serum media was transfected with mammalian expression vector containing a nanog encoding vector. Cardiomyocytes were expanded and grown to near confluence in serum-DMEM containing only antibiotics and allowed to differentiate. Co-cultures were created by combining MeSCs with cardiac cells and culturing in cardiac media. Following co-culture, cells were treated with TRizol and gene expression was assessed using RT-PCR and cardiac specific primers. Gel electrophoresis of samples revealed expression of cardiac specific genes following treatment. The first (Electrophoresis 050101, FIG. 10) shows screens for each primer tested. The key shows which sample is in a given lane. Negative control represents untreated mesenchymal stem cells and Positive controls 1x and 2x are nanog transfected cells (2x indicates that well received twice the number of rat cardiac cells, not twice the amount of nanog transfected cells). Each sample was co-cultured with rat cardiomyocytes and primers are human specific and represent markers of cardiomyocyte related gene expression. The second data set (Electrophoresis 050115, FIG. 11) shows gene expression for each primer following 3 uM treatment of either BrdU or 5azaC. The high and low RNA is because we had low cell numbers and tested one well (low RNA) against combining two wells of equal treatment (high RNA). The third attachment (Electrophoresis 050116, FIG. 12) shows the effects of three weeks treatment of combined (3 uM or 1 uM of both 5azaC and BrdU), 3 uM of either 5azaC or BrdU, or nanog transfected cells (marked “control”). The poor quality is the result of low cell numbers and the use of a different RT-PCR kit (BioRad instead of the usual Invitrogen). See also FIG. 13, FIGS. 14-16 pertain to photographs of MeSCs treated with 3 uM of 5azaC (A 050123 3 uM 5azaC 3 weeks MSC.jpg, FIG. 14), of 3 uM of BrdU (A 050123 3 uM BrdU 3 weeks MSC.jpg, FIG. 15) and of nanog transfected cells combined with rat cardiac cells in co-culture (A 41227 of ntMSC 1213 2.jpg, FIG. 16). The cell differentiation is due to environmental signals and cell to cell contacts. The inventors demonstrate that treatment with nucleotide derivatives and/or nanog transfection provides for cardiac differentiation of mesenchymal stem cells. Accordingly, an embodiment of the invention pertains to a method of increasing the potency of a cell comprising introducing a gene comprising nanog activity, optionally in conjunction with treatment of such cell with a compound, such as a nucleotide derivative, known to exert a dedifferentiating influence on cells.
Gln Leu Cys Val Leu Arg Asp Arg Glu Tyr Leu Ser
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ctc cag cag att cca gaa gca ctc aac tcc aac ctc agc tac aac
Leu Glu Glu Met Glu Leu Ser Arg Cys Tyr Leu Glu Ser Tyr Lys
125 130 135

438

cag gtg aag acc tgt ttc cag aac cag aga att aag tgt gaa agg tgg
Gln Val Lys Thr Trp Phe Glu Arg Met Lys Ser Tyr Arg Trp
140 145 150

486

cag aca aac tgt ccc aag aat agc aat ggt gtc acg cag aag gcc
Gln Lys Arg Thr Pro Thr Leu Ser Arg Tyr Arg Cys Asp
155 160 165

534

tca gca cct acc tac ccc agc ctc tac tct tcc tac cac cag gga tgc
Ser Ala Pro Thr Tyr Pro Ser Tyr Leu Tyr Ser Ser Tyr His Glu Cys
170 175 180 185

582

cct tgt aac cgg act ggg aac ctt cca atg tgt agc aag cag acc tgt
Leu Val Arg Pro Thr Tyr Pro Arg Cys Tyr Ser Arg Asp Arg Cys
190 195 200

630

aac aat tca acc tgt agc aac cag acc cag aac atc cag tcc tgt agc
Aam Arg Ser Thr Trp Ser Arg Cys Tyr Ser Arg Asp Arg Cys
205 210 215

678

aac cac tcc tgt aac act cag acc tgt cgg aac taa ctc aag tgt aag aac
cat cag aag ctc gaa gaa cag cag
Aam His Ser Arg Ser Asp Ser Eas Arg Leu Leu Leu Leu Arg Arg Cys
220 225 230

726

cag gcc tgt aac agt ccc ttc tat aac tgt gga gag gaa tct ctt cag
Gln Ala Arg Asp Pro Tyr Leu Ala Cys Tyr Leu Glu Glu Ser Leu Glu
235 240 245

774

tcc tgc atc aag ctc cag cca aat tct ctt gcc aag tgt gaa gct
Ser Cys Met His Arg Pro Pro Leu Ser Tyr Tyr Leu Glu Leu Ala
250 255 260 265

822

gcc tgt gaa gtt ggt gaa ggc ctt aat gta ata cag cag acc act
Ala Leu Glu Ala Ala Gly Glu Gly Tyr Leu Leu Leu Leu Tyr Leu Cys
270 275 280

870

agg tat ttt agt act cca caa acc atg gat tta ttc cta aac tac tcc
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918

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965

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Glu Asn Ser Val Ala Lys Lys Glu Asp Lys Val Pro Val Lys Lys Gin 85 90 95
Lys Thr Arg Thr Val Phe Ser Ser Thr Gin Leu Cys Val Leu Ann Asp 100 105 110
Arg Phe Gln Arg Gln Lys Tyr Leu Ser Leu Gln Gin Met Gin Glu Leu 115 120 125
Ser Ann Ile Leu Ann Leu Tyr Lys Gin Val Lys Thr Thr Phe Gin 130 135 140
Ann Ser Ann Gly Val Thr Gin Lys Ala Ser Ala Pro Thr Tyr Pro Ser 165 170 175
Leu Tyr Ser Ser Tyr His Gin Gly Cys Leu Val Ann Pro Thr Gly Ann 180 185 190
Leu Pro Met Trp Ser Ann Gin Thr Trp Ann Ann Ser Thr Thr Ann 195 200 205
Gln Thr Gin Asn Ile Gin Ser Trp Ser Asn His Ser Thr Gin 210 215 220
Thr Trp Cys Thr Gin Ser Trp Ann Gin Ala Trp Ann Ser Pro Phe 225 230 235 240
Tyr Asn Cys Gly Glu Ser Gin Gin Ser Gin Ser Cys Gin Thr Gin 245 250 255
Ann Ser Pro Ala Ser Asp Leu Gin Ala Ala Leu Glu Ala Ala Gly Gin 260 265 270
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  65  70  75  80
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Leu Leu Ser Glu Thr Glu Lys Arg Pro Phe Ile Asp Glu Ala Lys Arg
85 90 95
Leu Arg Ala Leu His Met Lys Glu His Pro Lys Tyr Tyr Arg Pro
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Arg Arg Lys Thr Lys Thr Met Lys Lys Tyr Tyr Thr Leu Pro
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Ala His Met Asn Gly Trp Ser Asn Gly Ser Tyr Ser Met Glu Asp
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Ser Ser His Ser Arg Ala Pro Cys Glu Ala Gly Asp Leu Arg Asp
260 265 270
Met Ile Ser Met Tyr Leu Pro Gly Ala Glu Val Pro Gln Pro Ala Ala
275 280 285
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atgcaggttg acacgcggcc taattttata tagcttttgt tcgatcccaa ctttccattt
tgttcagata aaaaaaacca tgaaattact gtgtttgaaa tattttctta tggtttgtaa
tatttctgta aatttattgt gatattttaa ggttttcccc cctttatttt ccgtagttgt
tatttaaaag atttcgtct gcattttttg aacagcctg cegagatct ccgtatatat
ttgacctaat atcccctca taccaggtac attttcaact taagttttta ctccattatg
cacagtttgac gataaataaa ttttggaat atggacactg aaaaaaaaa aaaaaaaaa
```
What is claimed is:

1. A method of transforming an adult mesenchymal stem cell into a pluripotent cell, said method comprising introducing into said adult mesenchymal stem cell an oct4 gene and a sox2 gene and culturing said adult mesenchymal stem cell under conditions to produce expression of said oct4 and sox2 genes, wherein said expression results in said adult mesenchymal stem cell becoming a pluripotent cell.