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(54) **MULTIPOTENT STEM CELLS DERIVED FROM HUMAN ADIPOSE TISSUE AND CELLULAR THERAPEUTIC AGENTS COMPRISING THE SAME**

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(30) **Foreign Application Priority Data**

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(52) **U.S. Cl.** **435/325**

(57) **ABSTRACT**

Human adipose tissue-derived multipotent adult stem cells are provided, which are characterized by the ability to be maintained in an undifferentiated state for a long period of time by forming spheres and which have high proliferation rates. Also provided are methods for isolating and maintaining the adult stem cells, and methods for differentiating the multipotent adult stem cells into nerve cells, fat cells, cartilage cells, osteogenic cells, muscle cells, endothelial cells, hepatic cells and insulin-releasing pancreatic β-cells. Also provided are cellular therapeutic agents for treating osteoarthritis, osteoporosis, nerve disease, diabetes and for forming breast tissue, which contain differentiated cells or the adult stem cells.

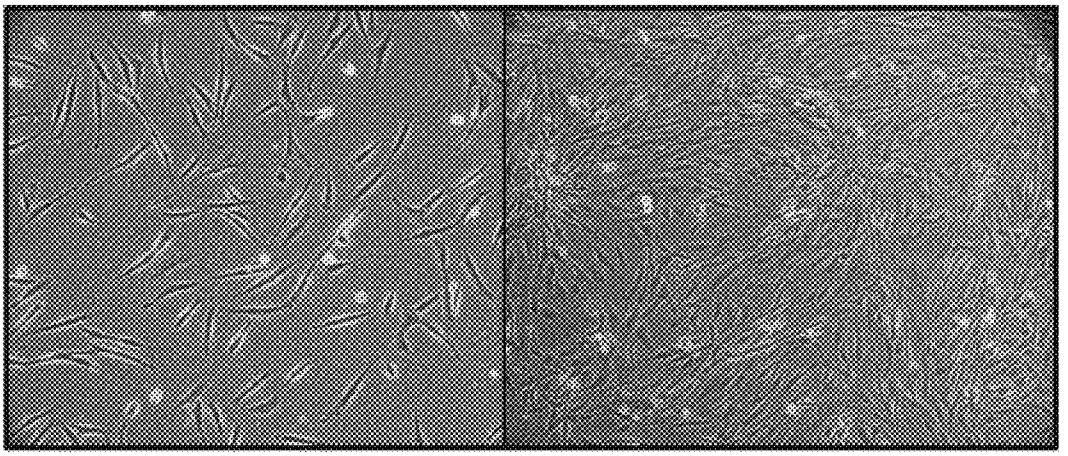


FIG. 1

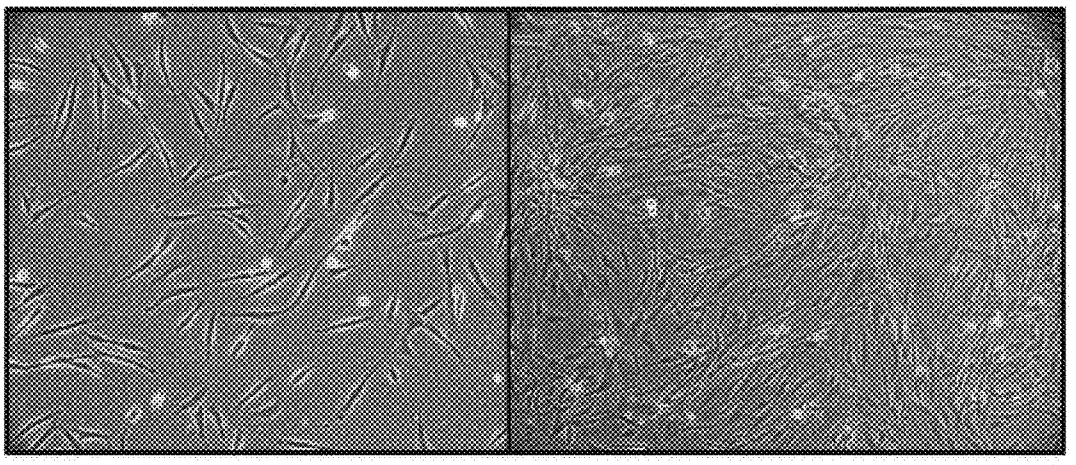
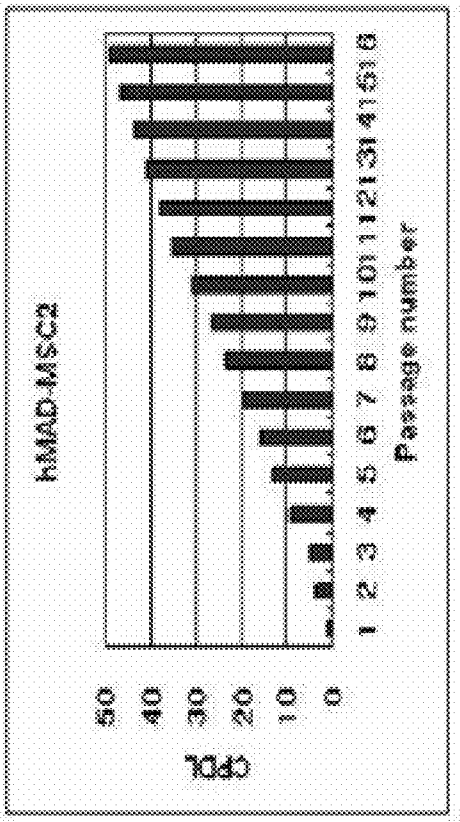


FIG. 2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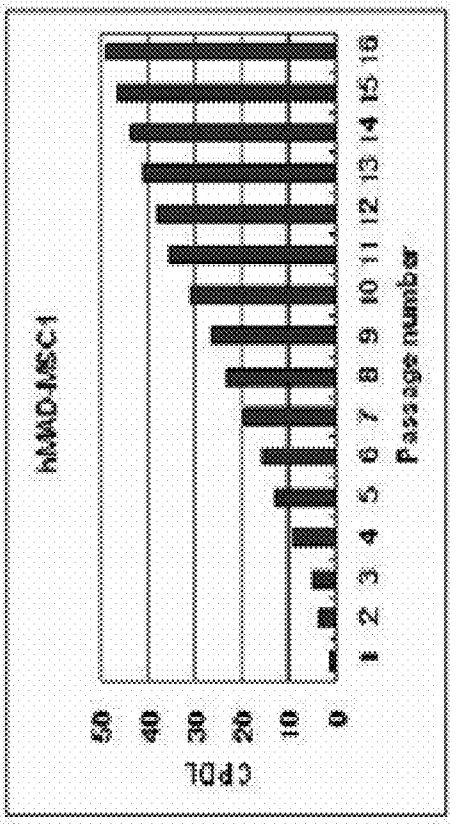
CPDL
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Passage number

A-2


hMAD-MSC1
CPDL
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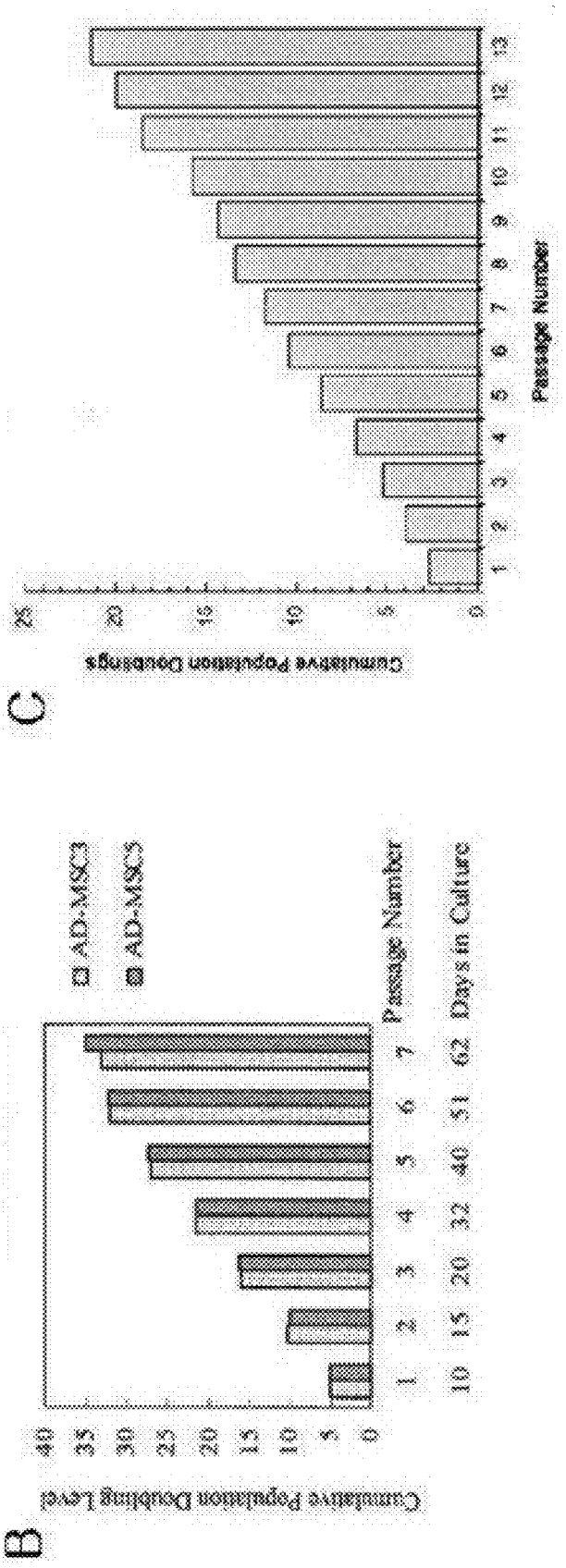
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Passage number

A-1

FIG. 2 (cont.)


B
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AD-MSC3
AD-MSC5
Passage Number
Days in Culture
C
Cumulative Population Doublings
Passage Number

FIG. 3

Negative

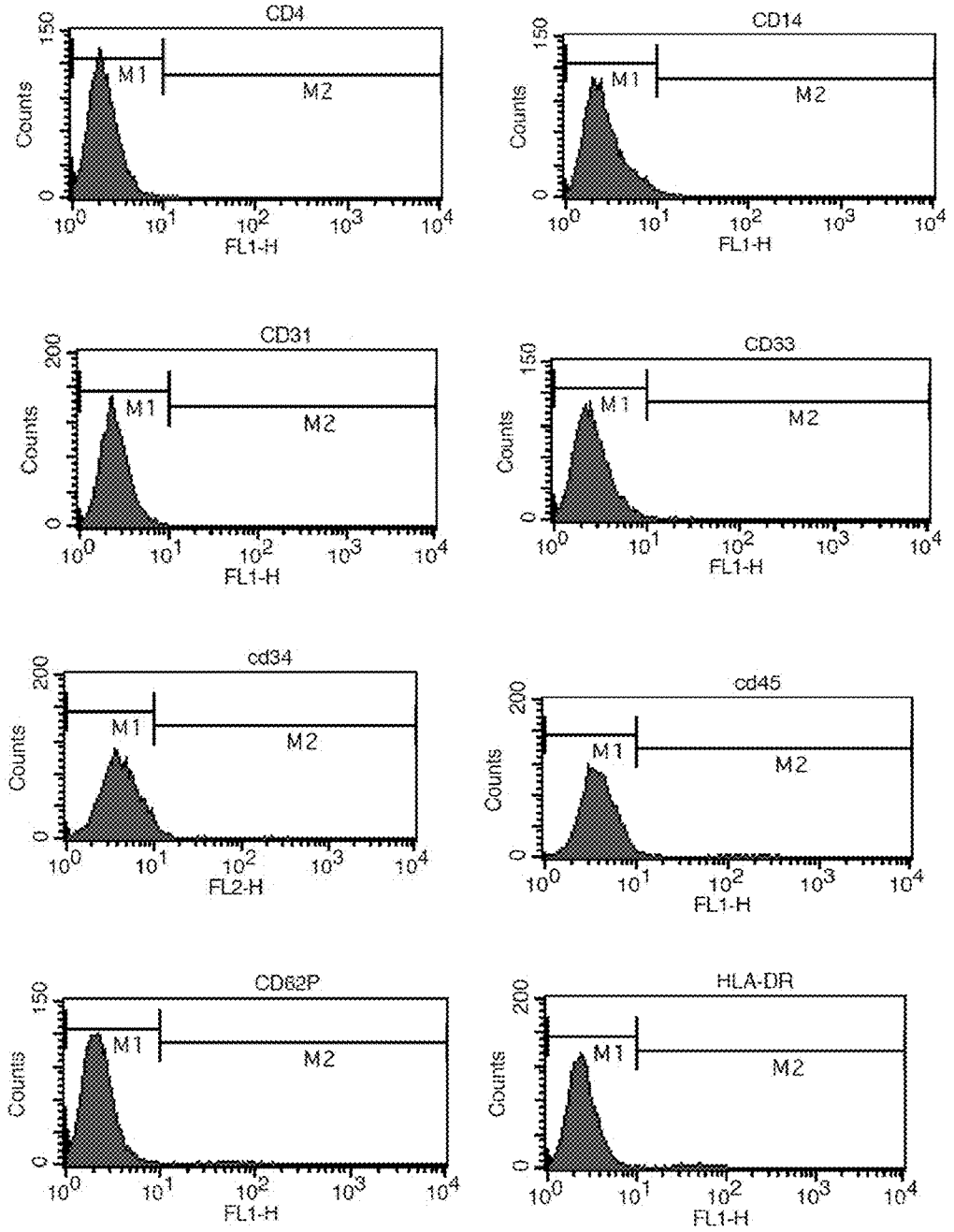


FIG. 3 (cont.)

Positive

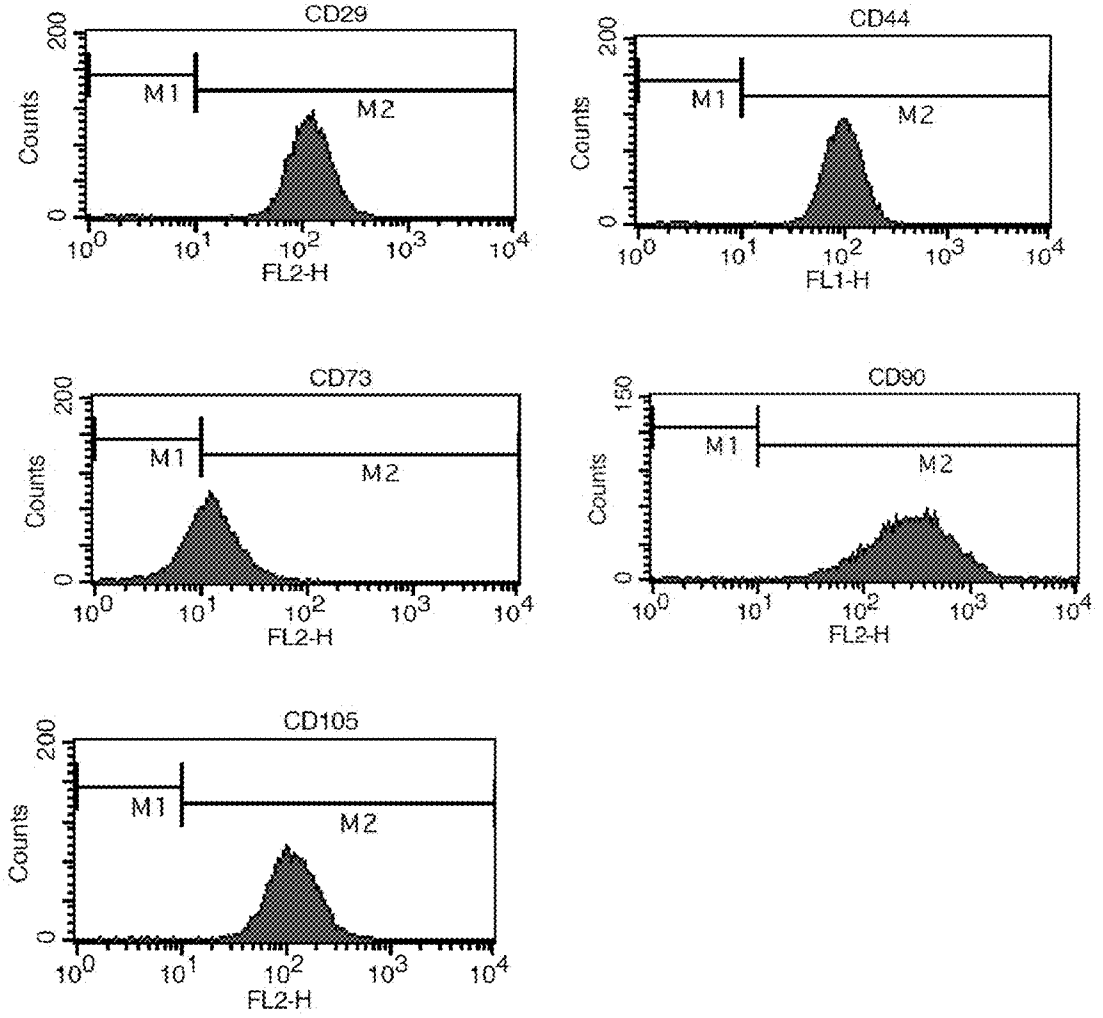
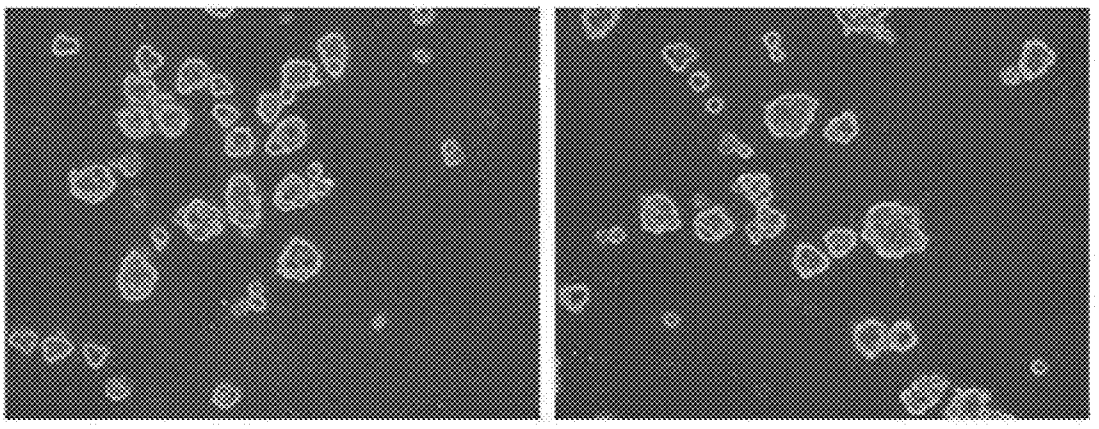
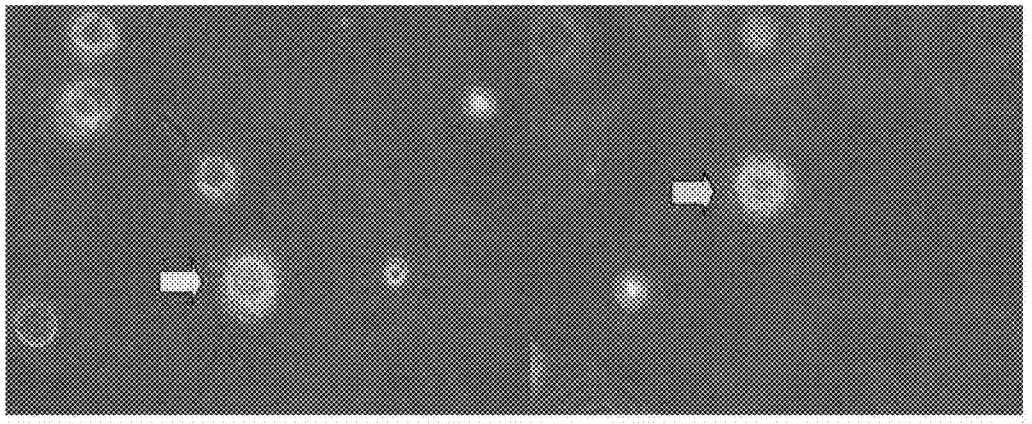


FIG. 4



7 days

FIG. 5



Day 12

FIG. 6

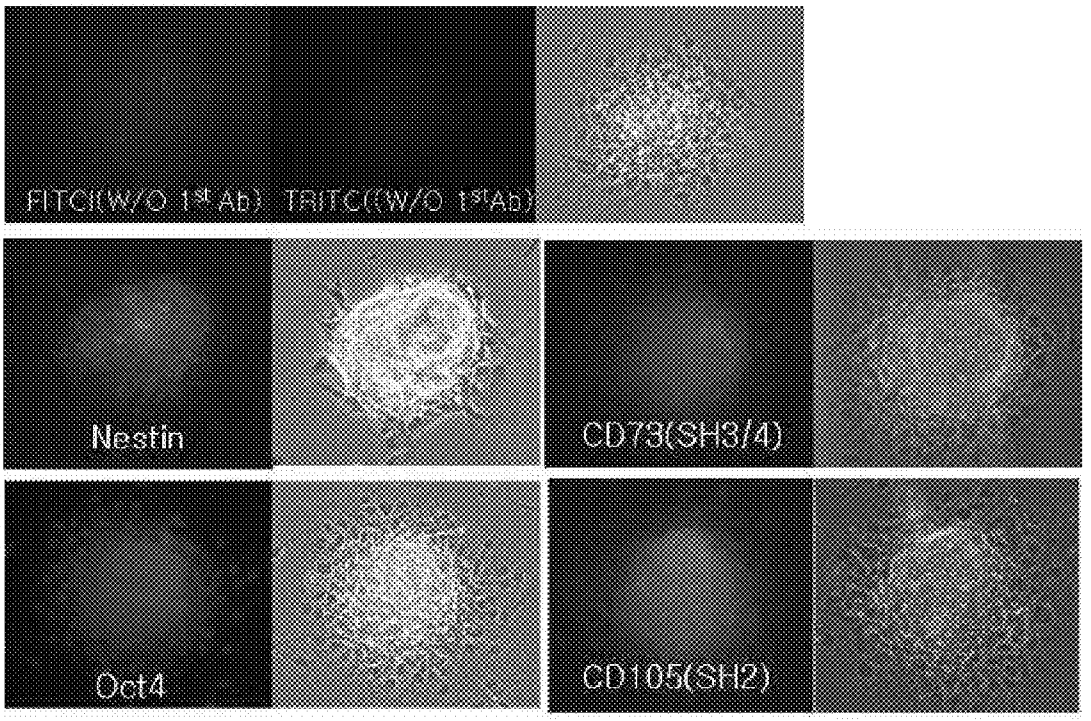


FIG. 7

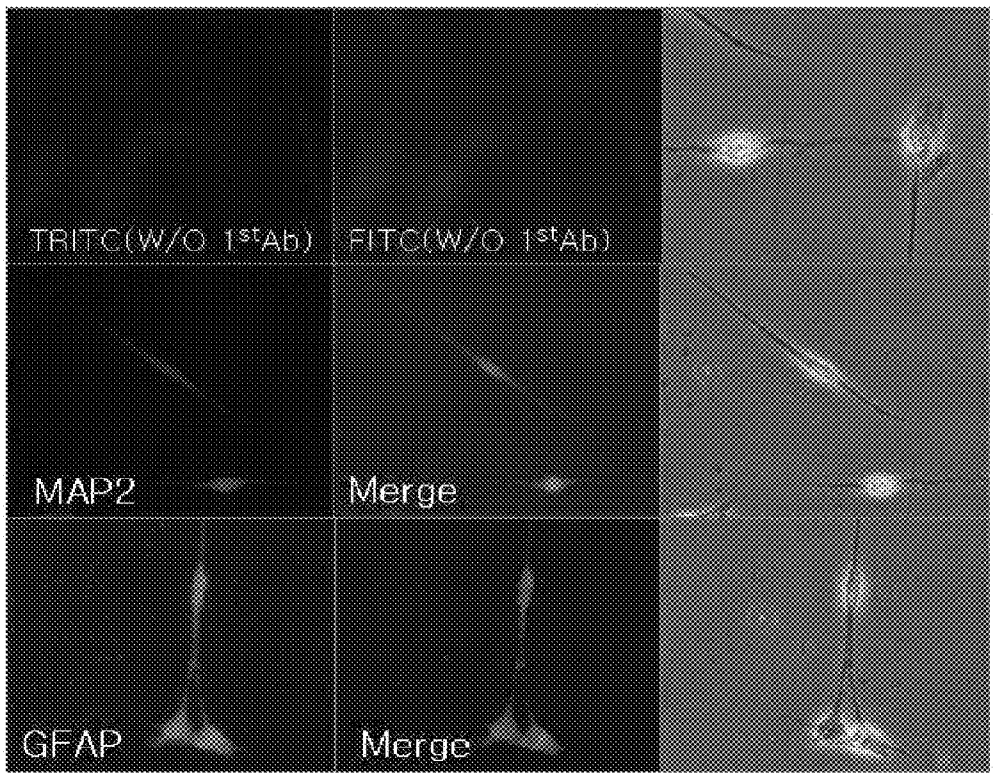
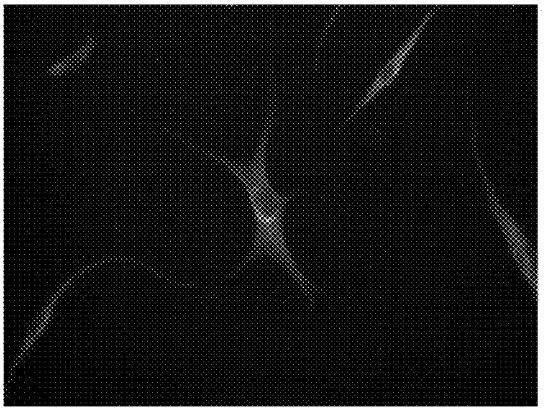
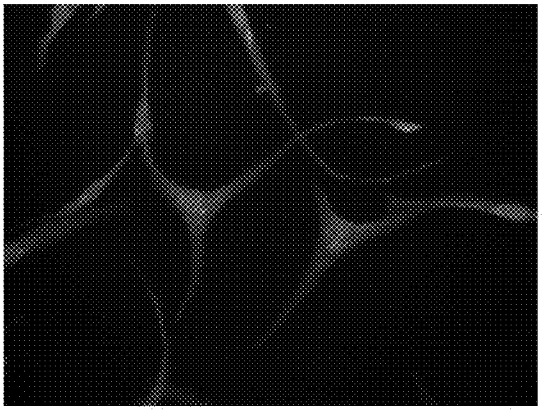


FIG. 8



TUJ1



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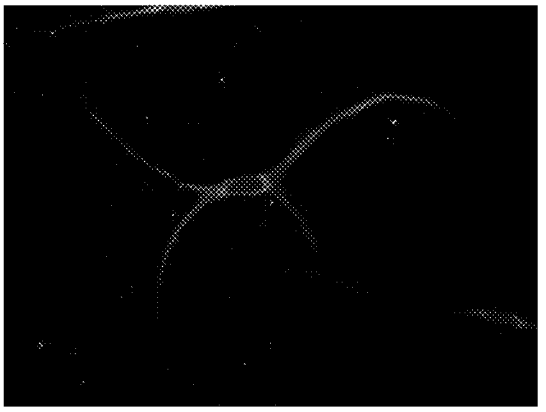
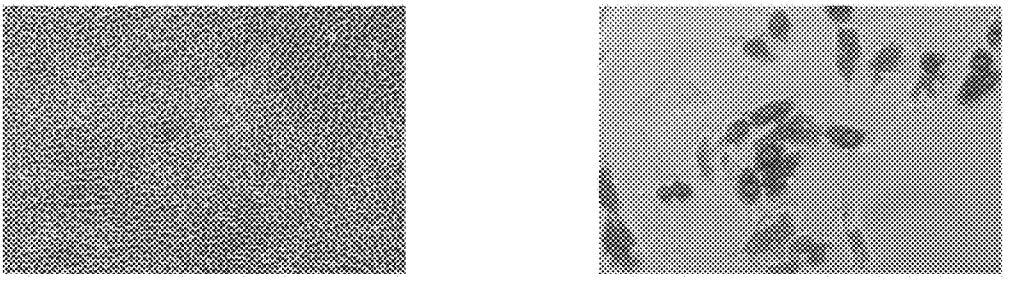


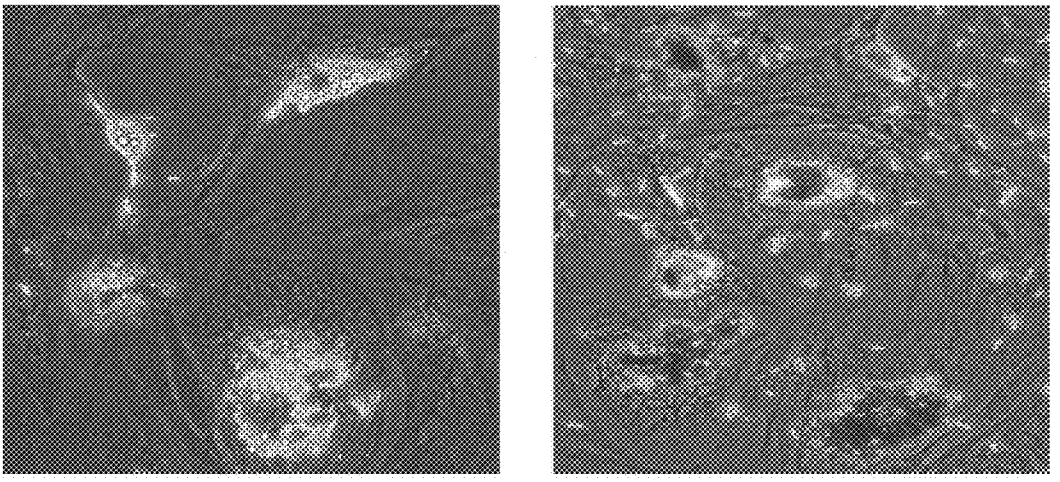
SOX2

FIG. 9



Control Differentiation

FIG. 10



(A) Phase contrast (B) Oil Red O stain

FIG. 11

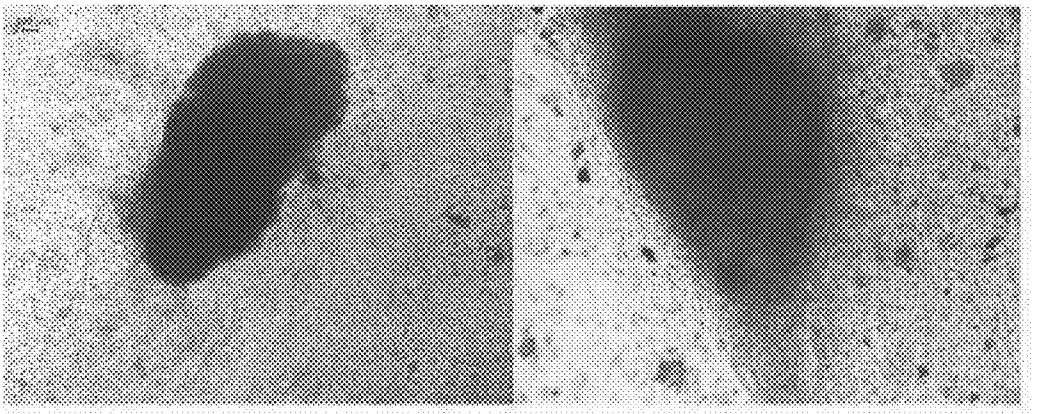


FIG. 12

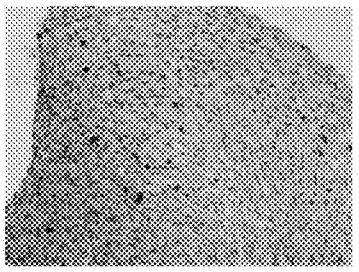
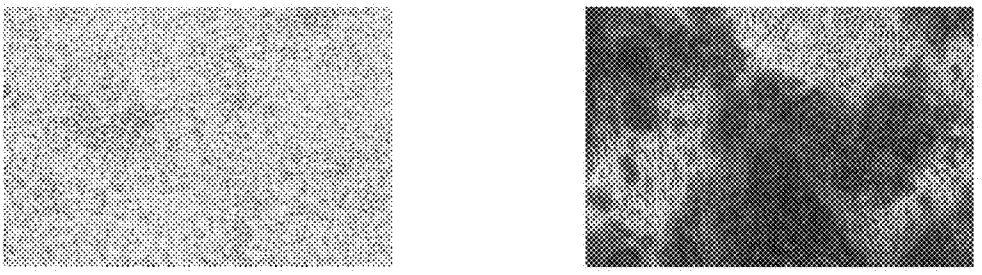


FIG. 13



Control Differentation

FIG. 14

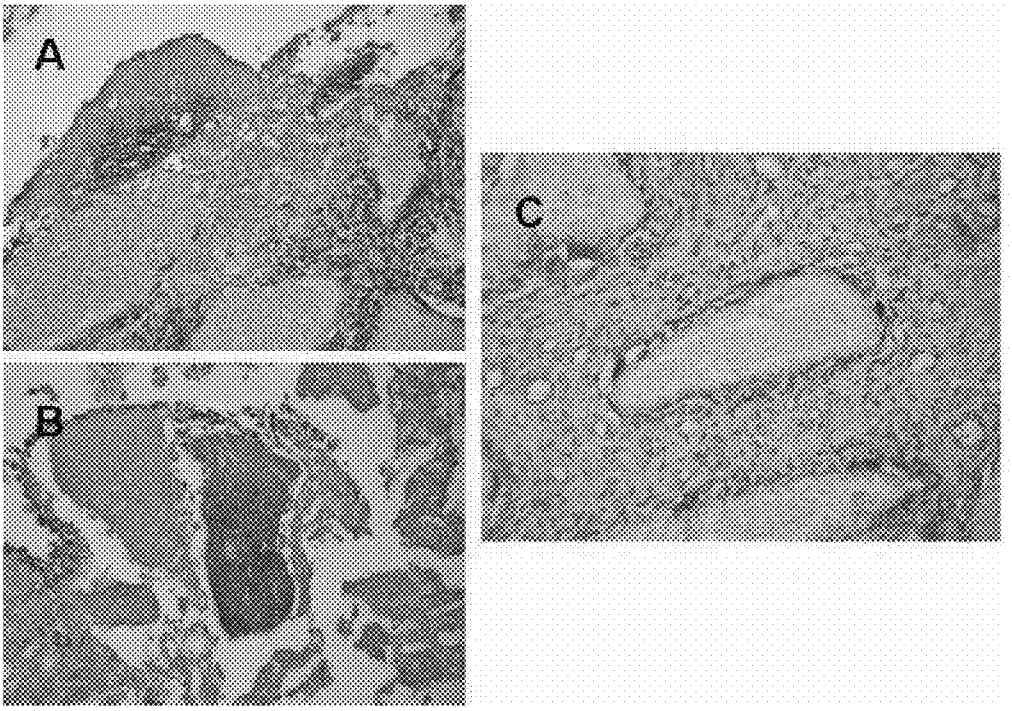


FIG. 15

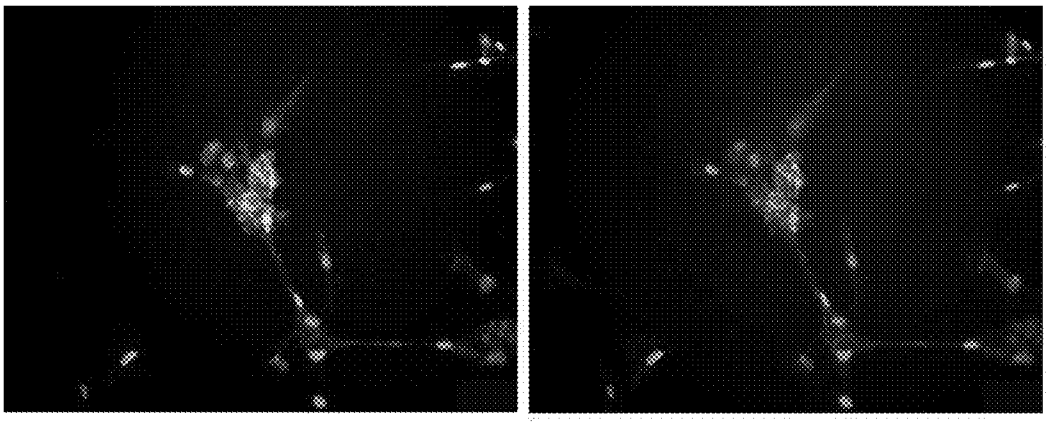


FIG. 16

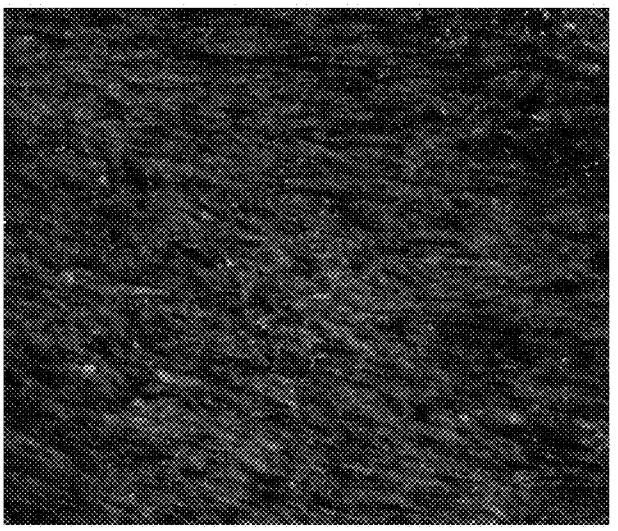


FIG. 17

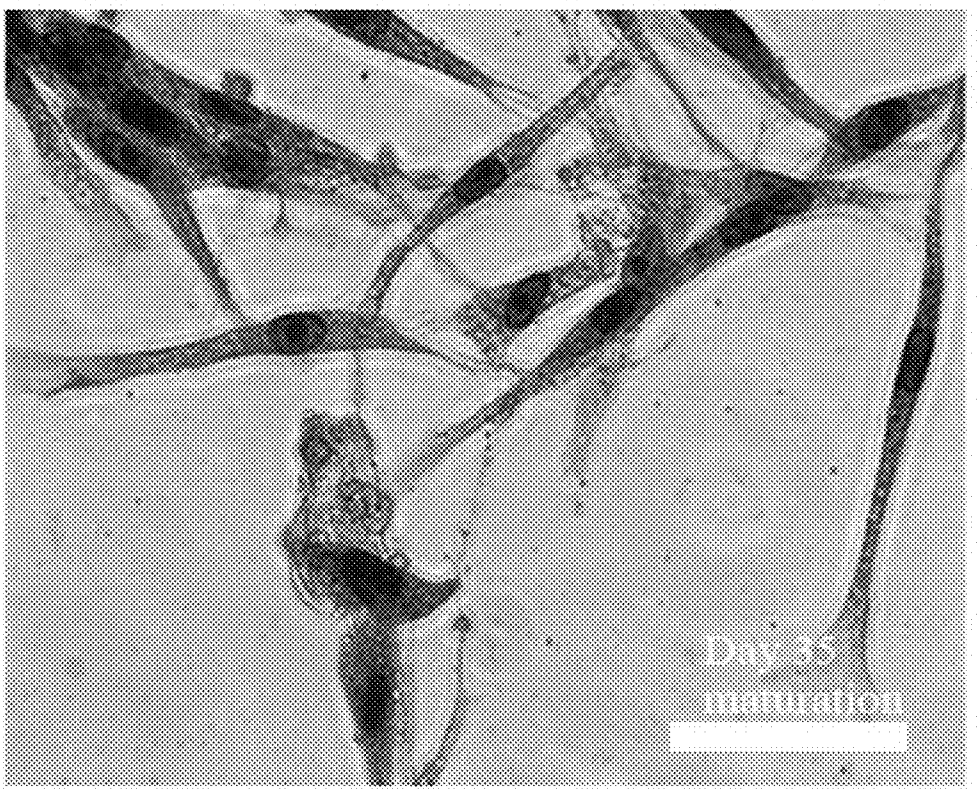


FIG. 18

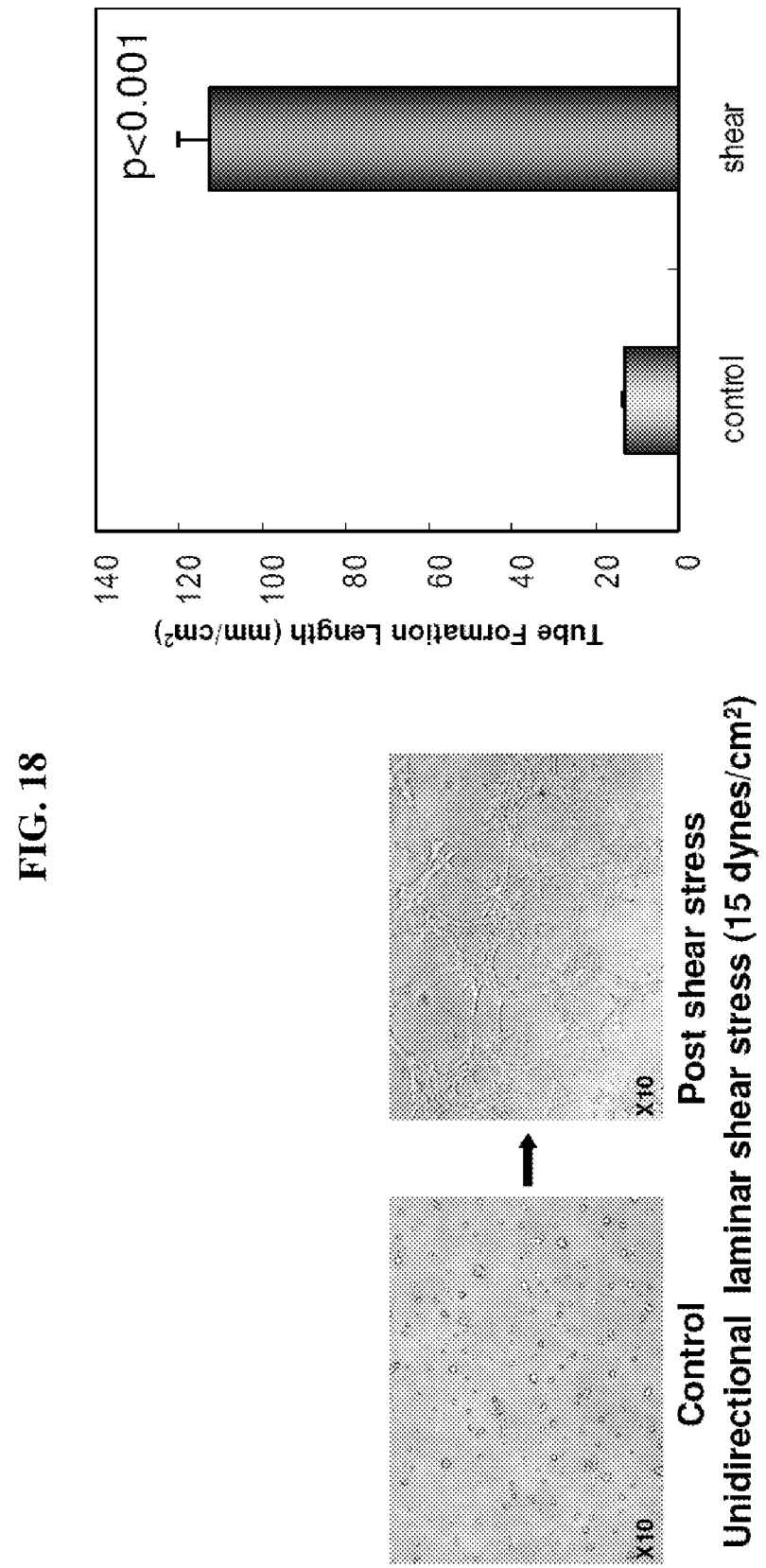

Tube Formation Length (mm/cm²)
0
20
40
60
80
100
120
140
p<0.001
control
shear
Control
Unidirectional laminar shear stress (15 dynes/cm²)
Post shear stress
X10
X10

FIG. 19

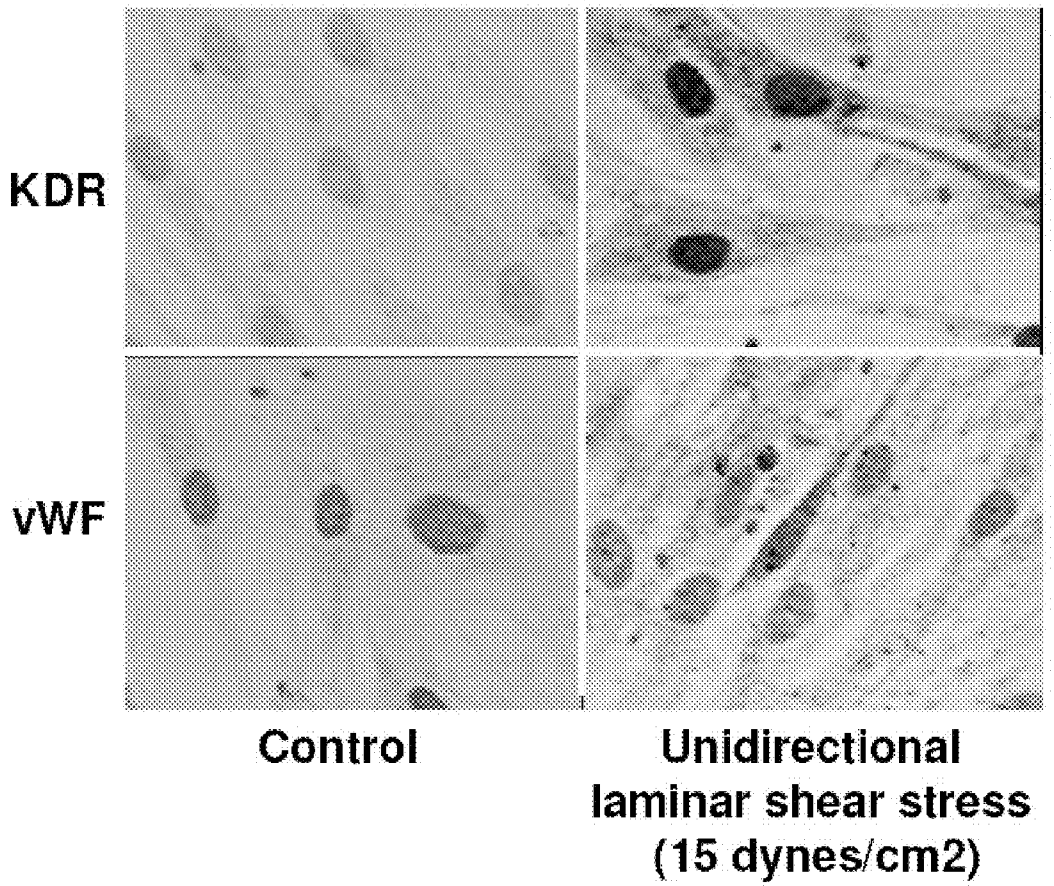
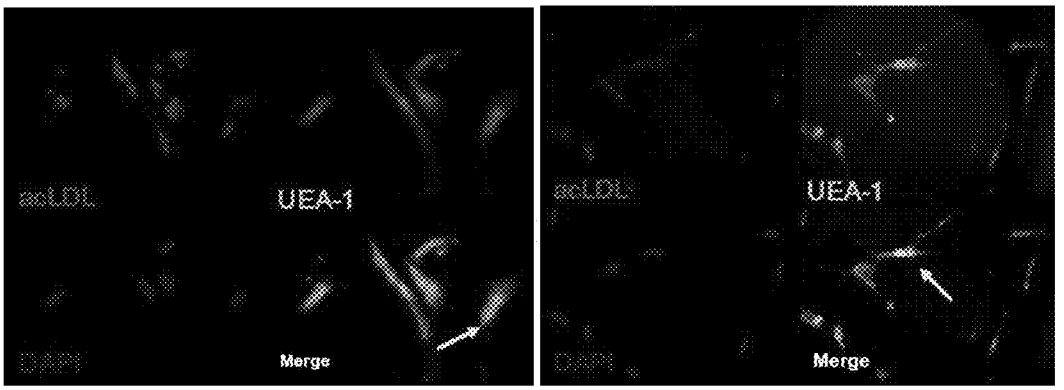

KDR
vWF
Control
Unidirectional laminar shear stress (15 dynes/cm2)

FIG. 20


acLDL
UEA-1
DAPI
Merge
acLDL
UEA-1
DAPI
Merge

MULTIPOTENT STEM CELLS DERIVED FROM HUMAN ADIPOSE TISSUE AND CELLULAR THERAPEUTIC AGENTS COMPRISING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. patent application Ser. No. 11/313,083, filed on Dec. 20, 2005 and issued as U.S. Pat. No. 7,807,461, which claims the benefit of Korean Patent Application No. 10-2005-0109502 filed on Nov. 16, 2005, in the Korean Intellectual Property Office. The disclosures of all of the foregoing applications are hereby incorporated herein by reference in their respective entireties, for all purposes, and the priority of all such applications is hereby claimed under the provisions of 35 U.S.C. §120.

TECHNICAL FIELD

[0002] The present invention relates to multipotent adult stem cells derived from human adipose tissue, and more particularly, to human breast adipose tissue-derived multipotent adult mesenchymal stem cells, which can be maintained in a non-differentiated state for a long period of time by forming spheres and have high proliferation rates. Also, the present invention relates to a method for isolating and maintaining the adult stem cells, a method for differentiating the adult stem cells into nerve cells, fat cells, cartilage cells, osteogenic cells, insulin-releasing pancreatic beta-cells, muscle cells, endothelial cells and hepatic cells and a cellular therapeutic agent for treating osteoarthritis, osteoporosis, diabetes, muscle disease, vascular disease and hepatic disease and a cellular therapeutic agent for forming breast tissue.

BACKGROUND ART

[0003] 21st biotechnology presents the possibility of new solutions to the food, environment and health problems, with the ultimate object of promoting human prosperity. In recent years, the technology of using stem cells has been considered as a new way to treat incurable diseases. Formerly, organ transplantation, gene therapy, etc., were presented for the treatment of incurable human diseases, but their efficient use has not been made due to immune rejection, a short supply of organs, an insufficient development of vectors, and an insufficient knowledge of disease genes.

[0004] For this reason, with increasing interests in stem cell studies, it has been recognized that totipotent stem cells having the ability to form all the organs by proliferation and differentiation can not only treat most of diseases but also fundamentally heal organ injuries. Also, many scientists have suggested the applicability of stem cells for the regeneration of all the organs and the treatment of incurable diseases, including Parkinson's disease, various cancers, diabetes and spinal damages.

[0005] Stem cells refers to cells having not only self-replicaiton ability but also the ability to differentiate into at least two cells, and can be divided into totipotent stem cells, pluripotent stem cells, and multipotent stem cells.

[0006] Totipotent stem cells are cells having totipotent properties capable of developing into one perfect individual, and these properties are possessed by cells up to the 8-cell stage after the fertilization of an oocyte and a sperm. When these cells are isolated and transplanted into the uterus, they can develop into one perfect individual.

[0007] Pluripotent stem cells, which are cells capable of developing into various cells and tissues derived from the ectodermal, mesodermal and endodermal layers, are derived from an inner cell mass located inside of blastocysts generated 4-5 days after fertilization. These cells are called "embryonic stem cells" and can differentiate into various other tissue cells but not form new living organisms.

[0008] Multipotent stem cells, which are stem cells capable of differentiating into only cells specific to tissues and organs containing these cells, are involved not only in the growth and development of various tissues and organs in the fetal, neonatal and adult periods but also in the maintenance of homeostasis of adult tissue and the function of inducing regeneration upon tissue damage. Tissue-specific multipotent cells are collectively called "adult stem cells".

[0009] Adult stem cells are obtained by taking cells from various human organs and developing the cells into stem cells and are characterized in that they differentiate into only specific tissues. However, recently, experiments for differentiating adult stem cells into various tissues, including liver cells, were dramatically successful.

[0010] The multipotent stem cells were first isolated from adult marrow (Jiang et al., *Nature,* 418:41, 2002), and then also found in other various adult tissues (Verfaillie, *Trends Cell Biol.,* 12:502, 2002). In other words, although the marrow is the most widely known source of stem cells, the multipotent stem cells were also found in the skin, blood vessels, muscles and brains (Tomas et al., *Nat. Cell Biol.,* 3:778, 2001; Sampaolesi et al., *Science,* 301:487, 2003; Jiang et al., *Exp. Hematol.,* 30:896, 2002). However, stem cells in adult tissues, such as the marrow, are very rarely present, and such cells are difficult to culture without inducing differentiation, and so difficult to culture in the absence of specifically screened media. Namely, it is very difficult to maintain the isolated stem cells in vitro.

[0011] Recently, adipose tissue was found to be a new source of multipotent stem cells (Cousin et al., *BBRC.,* 301: 1016, 2003; Miranville et al., *Circulation,* 110:349, 2004; Gronthos et al., *J. Cell Physiol.,* 189:54, 2001; Seo et al., *BBRC.,* 328:258, 2005). Namely, it was reported that a group of undifferentiated cells is included in human adipose tissue obtained by liposuction and has the ability to differentiate into fat cells, osteogenic cells, myoblasts and chondroblasts (Zuk et al., *Tissue Eng.,* 7:211, 2001; Rodriguez et al., *BBRC.,* 315:255, 2004). This adipose tissue has an advantage in that it can be extracted in large amounts, and thus, it receives attention as a new source of stem cells, which overcomes the existing shortcomings.

[0012] Also, recent studies using animal model experiments indicate that adipose tissue-derived cells have the abilities to regenerate muscles and to stimulate the differentiation of nerve blood vessels. Thus, these adipose tissue-derived cells have attention as a new source of stem cells.

[0013] Adipose tissue-derived stem cells known till now include human adipose-derived adult stem cells that can differentiate into epithelial cells (Brzoska et al., *BBRC,* 330:142, 2005), human adipose-derived adult stem cells that can differentiate into osteogenic and fat cells (Cao et al., *BBRC,* 332:370, 2005), human adipose-derived adult stem cells that can differentiate into nerve cells (Safford et al., *BBRC,* 294: 371, 2002), rat adipose-derived stem cells that can differentiate into fat cells (Ogawa et al., *BBRC,* 319:511, 2004), rat

adipose-derived stem cells that can differentiate into osteogenic and chondrogenic cells (Ogawa et al., *BBRC,* 313:871, 2004), human adipose-derived stem cells that can differentiate into cartilage cells (*Biomaterials,* 25:3211, 2004), rat adipose-derived stem cells that can differentiate into nerve cells (Fujimura et al., *BBRC,* 333:116, 2005), and adipose-derived stem cells that can differentiate into bone cells, cartilage cells, nerve cells or muscle cells (U.S. Pat. No. 6,777, 231).

[0014] However, most of adipose-derived stem cells known till now are stem cells derived from the adipose tissue of animals other than human beings. Even if they are stem cells derived from human adipose tissue, they have been limited to those derived from tissues obtained by the liposuction of abdominal fat, and the kind of cells differentiated from the stem cells has also been limited. Particularly, isolated stem cells have low proliferation rates and are difficult to maintain in an undifferentiated state for a long period of time, and thus, have been limited in application.

[0015] Accordingly, the present inventors have made extensive efforts to develop multipotent adult stem cells, which have high proliferation rates, can be maintained in a undifferentiated state for a long period of time by forming spheres and can differentiate into more various cells, as a result, found that multipotent stem cells isolated from human adipose tissue can differentiate into various cells, including osteogenic cells, chondrogenic cells, nerve cells, astrocytes, fat cells, insulin-releasing pancreatic beta-cells, muscle cells, endothelial cells and hepatic cells have a very high proliferation rate and can be maintained in an undifferentiated state for a long period of time by forming spheres, thereby completing the present invention.

SUMMARY OF THE INVENTION

[0016] Therefore, it is an object of the present invention to provide human adipose tissue-derived multipotent adult stem cells, which have high proliferation rates and can be maintained in an undifferentiated state for a long period of time by forming spheres, as well as a production method thereof.

[0017] Another object of the present invention is to provide a method for differentiating said multipotent stem cells into nerve cells, astrocytes, cartilage cells, osteogenic cells, insulin-releasing pancreatic beta-cells, muscle cells, endothelial cells and hepatic cells, as well as cellular therapeutic agents containing said differentiated cells or adult stem cells.

[0018] To achieve the above objects, in one aspect, the present invention provides a method for producing adult mesenchymal stem cells, comprising culturing human adipose tissue-derived pellets in a medium containing N-acetyl-L-cysteine (NAC) and then collecting the cultured cells, the adult stem cells being characterized by: (a) showing positive immunological responses to all of CD73, CD90, CD29, CD44 and CD105, and negative immunological responses to all of CD33, CD34, CD45, CD4, CD31, CD62p, CD14 and HLA-DR; (b) growing attached to a plastic material, showing spindle-shaped morphological features, and forming spheres in a medium containing CORM-2 so as to be able to be maintained in an undifferentiated state for a long period of time; and (c) having the ability to differentiate into ectoderm, mesoderm, and/or endoderm-derived cells.

[0019] In the present invention, the NAC-containing medium additionally contains ascorbic acid, calcium, rEGF, BPE, insulin and hydrocortisone.

[0020] In another aspect, the present invention provides a method for maintaining adult stem cells in an undifferentiated state, the method comprising culturing adult stem cells prepared by said method in a medium containing CORM-2 so as to form spheres.

[0021] In the present invention, the CORM-2-containing medium is preferably a serum-free medium, which additionally contains antibiotic antimycotic solution, hydrocortisone, insulin, rEGF, FGF, B27 and β-mercaptoethanol.

[0022] In still another aspect, the present invention provides adult mesenchymal stem cells produced by said method and characterized by: (a) showing positive immunological responses to all of CD73, CD90, CD29, CD44 and CD105, and negative immunological responses to all of CD33, CD34, CD45, CD4, CD31, CD62p, CD14 and HLA-DR; (b) growing attached to a plastic material, showing spindle-shaped morphological features, and forming spheres in a medium containing CORM-2 so as to be able to be maintained in an undifferentiated state for a long period of time; and (c) having the ability to differentiate into ectoderm, mesoderm and/or endoderm-derived cells.

[0023] In the present invention, the adult mesenchymal stem cells are preferably cultured in an undifferentiated state for at least 16 passages, and the mesoderm-derived cells are preferably cartilage cells, osteogenic cells, nerve cells, astrocytes, fat cells, insulin-releasing pancreatic beta-cells, muscle cells, endothelial cells and hepatic cells.

[0024] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into nerve cells, the method comprising the steps of: (a) preincubating the adult mesenchymal stem cells in a DMEM medium containing BME and FBS; and (b) treating the preincubated broth with DMSO and BHA so as to induce differentiation into nerve cells. Also, the present invention provides a cellular therapeutic agent for treating nerve disease, which contains said differentiated nerve cells as active ingredients.

[0025] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into cartilage cells, the method comprising culturing the adult mesenchymal stem cells in an α-MEM medium containing TFG-β1, L-ascorbate-2-phosphate and insulin. Also, the present invention provides a cellular therapeutic agent for treating osteoarthritis, which contains said differentiated cartilage cells as active ingredients.

[0026] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into osteogenic cells, the method comprising culturing the adult mesenchymal stem cells in a medium containing FBS, dexamethasone, L-ascorbae-2-phosphate, and glycerol-2-phosphate. Also, the present invention provides a cellular therapeutic agent for treating bone deficiency, which contains said differentiated osteogenic cells as active ingredients.

[0027] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into osteogenic cells, the method comprising mixing the adult stem cells with tricalcium phosphate (TCP) and isotransplanting the mixture. Also, the present invention provides a cellular therapeutic agent for treating bone deficiency, which contains said differentiated osteogenic cells as active ingredients.

[0028] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into fat cells, the method comprising culturing the adult stem cells in an α-MEM medium containing dexamethasone,

indomethacin, insulin and IBMX. Also, the present invention provides a cellular therapeutic agent for forming breast tissue, which contains said differentiated fat cells as active ingredients.

[0029] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into insulin-releasing pancreatic beta-cells, the method comprising the steps of: (a) culturing the adult stem cells in low-glucose DMEM medium containing nicotinamide, β-mercaptoethanol and FBS for 12-72 hours; and (b) culturing the cultured cells in high-glucose DMEM medium containing nicotinamide, β-mercaptoethanol and FBS for 4-7 days. Also, the present invention provides a cellular therapeutic agent for treating diabetes, which contains said differentiated insulin-releasing pancreatic beta-cells as active ingredients.

[0030] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into muscle cells, the method comprising culturing the adult mesenchymal stem cells in a medium containing hEGF, dexamethasone, and glutamine. Also, the present invention provides a cellular therapeutic agent for treating muscle diseases such as Cardic infarct or urinary incontinence, which contains said differentiated muscle cells as active ingredients.

[0031] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into endothelial cells, the method comprising starving the adult mesenchymal stem cells in a M199 medium, and giving physical shear stress. Also, the present invention provides a cellular therapeutic agent for treating vascular diseases such as Critical limb ischemia or Buerger's disease, which contains said differentiated endothelial cells as active ingredients.

[0032] In still another aspect, the present invention provides a method for differentiating adult mesenchymal stem cells into hepatic cells, the method comprising starving the adult mesenchymal stem cells in a Keratinocyte-SFM medium containing FBS, Fungizone, collagen, heparin and HGM. Also, the present invention provides a cellular therapeutic agent for treating hepatic disease such as liver cirrhosis or acute hepatic disease, which contains said differentiated hepatic cells as active ingredients.

[0033] In still another aspect, the present invention provides a cellular therapeutic agent for treating nerve disease containing the adult mesenchymal stem cells having the ability of differentiation into nerve cells, as active ingredients.

[0034] In still another aspect, the present invention provides a cellular therapeutic agent for treating diabetes containing the adult mesenchymal stem cells having the ability of differentiation into insulin-releasing pancreatic beta-cells, as active ingredients.

[0035] In still another aspect, the present invention provides a cellular therapeutic agent for treating osteoarthritis containing the adult mesenchymal stem cells having the ability of differentiation into cartilage cells, as active ingredients.

[0036] In still another aspect, the present invention provides a cellular therapeutic agent for treating bone deficiency containing the adult mesenchymal stem cells having the ability of differentiation into osteogenic cells, as active ingredients.

[0037] In still another aspect, the present invention provides a cellular therapeutic agent for forming breast tissue containing the adult mesenchymal stem cells having the ability of differentiation into fat cells, as active ingredients.

[0038] In still another aspect, the present invention provides a cellular therapeutic agent for treating muscle diseases such as Cardic infarct or urinary incontinence containing the adult mesenchymal stem cells having the ability of differentiation into muscle cells, as active ingredients.

[0039] In still another aspect, the present invention provides a cellular therapeutic agent for treating vascular diseases such as Critical limb ischemia or Buerger's disease containing the adult mesenchymal stem cells having the ability of differentiation into endothelial cells, as active ingredients.

[0040] In still another aspect, the present invention provides a cellular therapeutic agent for treating hepatic disease such as liver cirrhosis or acute hepatic disease containing the adult mesenchymal stem cells having the ability of differentiation into hepatic cells, as active ingredients.

[0041] The above and other objects, features and embodiments of the present invention will be more clearly understood from the following detailed description and the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] FIG. **1** shows photographs taken at 100× magnification for human adipose tissue-derived multipotent stem cells according to the present invention.

[0043] FIG. **2** shows the cumulative population doubling level (CPDL) of human adipose tissue-derived multipotent stem cells according to the present invention. A-1 and A-2: human adipose tissue-derived multipotent stem cells according to the present invention; and B and C: adipose-derived stem cells according to the prior art.

[0044] FIG. **3** shows a histogram of FACS analysis of adipose-derived mesenchymal stem cells of the present invention.

[0045] FIG. **4** shows photographs taken at 200× magnification for spheres formed at 7 days after culturing human breast adipose tissue-derived multipotent stem cells according to the present invention.

[0046] FIG. **5** is a photograph taken at 200× magnification for the shape of a sphere formed by the proliferation of a stem cell in agar.

[0047] FIG. **6** illustrates photographs taken at 100× magnification, which show the expression of Nestin, Oct4, SH2, SH3/4 in the inventive human adipose tissue-derived multipotent stem cells, which were sphere-cultured in a CORM-2-containing MEBM medium and then immunostained.

[0048] FIG. **7** shows that human adipose tissue-derived multipotent stem cells according to the present invention were differentiated into nerve cells and astrocytes.

[0049] FIG. **8** shows that human adipose tissue-derived multipotent stem cells according to the present invention were differentiated into nerve cells by expression of TUJ1, NSE and SOX2.

[0050] FIG. **9** shows that human adipose tissue-derived multipotent stem cells according to the present invention were differentiated into fat cells.

[0051] FIG. **10** shows photographs taken at 200× magnification for fat cells differentiated from human adipose tissue-derived multipotent stem cells according to the present invention. A: differentiated phase contrast; and B: stained by oil red O staining.

[0052] FIG. **11** shows photographs taken at 100× magnification for cartilage cells differentiated from human adipose tissue-derived multipotent stem cells according to the present

invention. A: differentiated phase contrast; and B: Alcian blue staining results showing differentiation into cartilage cells.

[0053] FIG. **12** shows that human adipose tissue-derived multipotent stem cells according to the present invention were differentiated into cartilage cells.

[0054] FIG. **13** is immunostaining result showing that human adipose tissue-derived multipotent stem cells according to the present invention were differentiated into osteogenic cells.

[0055] FIG. **14** shows osteogenic cells differentiated from human adipose tissue-derived multipotent stem cells according to the present invention. A: a group treated with TCP alone; B: a group treated with a mixture of TCP and marrow stem cells; and C: a group treated with a mixture of TCP and adipose-derived stem cells.

[0056] FIG. **15** shows immunostaining results for insulin-releasing pancreatic beta-cells differentiated from human adipose tissue-derived multipotent stem cells according to the present invention.

[0057] FIG. **16** shows immunostaining results for muscle cells differentiated from human adipose tissue-derived multipotent stem cells according to the present invention.

[0058] FIG. **17** shows immunostaining results for hepatic cells differentiated from human adipose tissue-derived multipotent stem cells according to the present invention.

[0059] FIG. **18** shows results for endothelial cells differentiated from human adipose tissue-derived multipotent stem cells after shear stress, and results for tube formation length.

[0060] FIG. **19** shows results for endothelial cells differentiated from human adipose tissue-derived multipotent stem cells using expressions of VEGFR-2 and Vwf.

[0061] FIG. **20** shows a pattern for endothelial cells differentiation from human adipose tissue-derived multipotent stem cells.

DETAILED DESCRIPTION OF THE INVENTION, AND PREFERRED EMBODIMENTS

[0062] The present invention relates to multipotent stem cells isolated from human adipose tissue.

Definitions

[0063] As used herein, "stem cell" defines an adult undifferentiated cell that can produce itself and a further differentiated progeny cell.

[0064] As used herein, "mesenchymal stem cell (or MSC)" defines multipotent stem cells that can be differentiated into a variety of cell types including osteoblast, chondrocytes (cartilage cells), adipocyte (fat cells), etc.

[0065] As used herein, the "lineage" of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation.

[0066] As used herein, the term "differentiates or differentiated" defines a cell that takes on a more committed ("differentiated") position within the lineage of a cell.

[0067] As used herein, "a cell that differentiates into a mesodermal, or ectodermal or endodermal lineage" defines a cell that becomes committed to a specific mesodermal, ectodermal or endodermal lineage, respectively.

[0068] Examples of cells that differentiate into ectodermal lineage include, but are not limited to epidermal cells, neurogenic cells, and neurogliagenic cells, astrocyte, etc.

[0069] Examples of cells that differentiate into a mesodermal lineage or give rise to specific mesodermal cells include, but are not limited to, cells that are adipogenic, chondrogenic, cardiogenic, dermatogenic, hematopoetic, hemangiogenic, myogenic, nephrogenic, urogenitogenic, osteogenic, pericardiogenic, or stromal, etc.

[0070] Examples of cells that differentiate into endodermal lineage include, but are not limited to insulin-releasing pancreatic beta cells, pleurigenic cells, and hepatogenic cells, cell that give rise to the lining of the intestine, and cells that give rise to pancreogenic and splanchogenic cells.

[0071] As used herein, a "pluripotent cell" defines a less differentiated cell that can give rise to at least two distinct (genotypically and/or phenotypically) further differentiated progeny cells.

[0072] A "multi-lineage stem cell" or "multipotent stem cell" refers to a stem cell that reproduces itself and at least two further differentiated progeny cells from distinct developmental lineages. The lineages can be from the same germ layer (i.e., mesoderm, ectoderm or endoderm), or from different germ layers. An example of two progeny cells with distinct developmental lineages from differentiation of a multi-lineage stem cell is a myogenic cell and an adipogenic cell (both are of mesodermal origin, yet give rise to different tissues). Another example is a neurogenic cell (of ectodermal origin) and adipogenic cell (of mesodermal origin).

[0073] As used here, "adipose tissue" defines a diffuse organ of primary metabolic importance made-up of white fat, yellow fat or brown fat. The adipose tissue has adipocytes and stroma. Adipose tissue is found throughout the body of an animal. For example, in mammals, adipose tissue is present in the omentum, bone marrow, subcutaneous space and surrounding most organs.

[0074] As used herein "conditioned media" defines a medium in which a specific cell or population of cells have been cultured in, and then removed. While the cells were cultured in said medium, they secrete cellular factors that include, but are not limited to hormones, cytokines, extracellular matrix (ECM), proteins, vesicles, antibodies, and granules. The medium plus the cellular factors is the conditioned medium.

[0075] As used herein "isolated" defines a substance, for example an adipose-derived stem cell, that is separated from contaminants (i.e., substances that differ from the stem cell).

[0076] The present invention provides adipose-derived mesenchymal stem cells and methods for obtaining them from a mesodermal origin (e.g., adipose tissue) and using them. Surprisingly, the inventive ADSCs can differentiate into cells that give rise to more than one type of germ layer, e.g. mesoderm, endoderm or ectoderm, and combinations thereof, and are thus "multilineage" or "multipotent" cells.

[0077] One aspect of the invention pertains to an "adipose-derived mesenchymal stem cell-enriched fraction" that contains adipose-derived stem cells of the invention. Preferably, the adipose-derived mesenchymal stem cell-enriched fraction is substantially free of other cell types (e.g., adipocytes, red blood cells, and other stromal cells, etc.) and extracellular matrix material. More preferably, the adipose-derived mesenchymal stem cell-enriched fraction is completely free of such other cell types and matrix material. The adipose-derived mesenchymal stem cell-enriched fraction is obtained from adipose tissue of a mammal. The preferred embodiment includes an adipose-derived mesenchymal stem cell-enriched fraction obtained from adipose tissue of a higher primate

(e.g., a baboon or ape). The most preferred adipose-derived mesenchymal stem cell-enriched fraction is obtained from human adipose tissue, using the methods described herein.

[0078] In other words, the adipose-derived mesenchymal stem cell-enriched fraction is "a homogenous cell population of the mesenchymal stem cells" that is substantially free of other cell types.

Method of Obtaining the Adipose-Derived Adult Mesenchymal Stem Cells

[0079] The adipose-derived adult mesenchymal stem cells according to the present invention can be isolated from human breast adipose tissue, or from the extracted adipose tissue mass. Or, the stem cells can be isolated from adipose tissue of supernatant by centrifuging an adipose containing-suspension floated in saline solution obtained from liposuction of human adipose tissue.

[0080] In the present invention, multipotent stem cells were first isolated and purified from human breast adipose tissue in the following manner. The isolated human adipose tissue was washed with PBS, and finely cut and then digested in a DMEM medium supplemented with collagenase type 1 (1 mg/ml), at 37° C. for 2 hours. After washing with PBS, the tissue was centrifuged at 1000 rpm for 5 minutes. The supernatant was suctioned off, and the pellets remaining on the bottom were washed with PBS and then centrifuged at 1000 rpm for 5 minutes. The resulting pellets were filtered through a 100 μm mesh to remove debris, followed by washing with PBS. Then, the pellets were incubated in a DMEM medium (10% FBS, 2 mM NAC, 0.2 mM ascorbic acid). After one overnight period, unattached cells were washed off with PBS, and the remaining cells were cultured in a K-NAC media (Keratinocyte-SFM media+2 mM NAC+0.2 mM ascorbic acid+0.09 mM calcium+5 ng/ml rEGF+50 μg/ml BPE+5 μg/ml insulin+74 ng/ml hydrocortisone) while the media were replaced at two-day intervals, thereby obtaining human breast adipose tissue-derived multipotent stem cell solution.

Sphere Formation of Adipose-Derived Adult Mesenchymal Stem Cells

[0081] Meanwhile, for the sphere culture of stem cells, 5×10^4-1×10^5 cells/ml of the isolated adipose tissue-derived multipotent stem cells were seeded into each well of a 6-well plate, which contains MEBM medium (10 μM CORM-2 (tricarbonyldichlororuthenium(II) dimer), B27, 5 ml antibiotic antimycotic solution (100×), 1 μg/ml hydrocortisone, 5 μg/ml insulin, 20 ng/ml EGF, 40 ng/ml FGF and β-mercaptoethanol), as a result, they started to form spheres from 3 days after the seeding. This suggests that the stem cells have high proliferation rates while being maintained in an undifferentiated state.

[0082] Methods of obtaining multipotent stem cells expressing the desired surface antigens from the human adipose tissue-derived stem cell broth obtained above include a FACS method using a flow cytometer with sorting function (*Int. Immunol.*, 10(3):275, 1998), a method using magnetic beads, and a panning method using an antibody specifically recognizing multipotent stem cells (*J. Immunol.*, 141(8):2797, 1998). Also, methods for obtaining multipotent stem cells from a large amount of culture broth include a method where antibodies specifically recognizing molecules expressed on the surface of cells (hereinafter, referred to as "surface antigens") are used alone or in combination as columns.

[0083] Flow cytometry sorting methods may include a water drop charge method and a cell capture method. In any of these methods, an antibody specifically recognizing an antigen on the cell surface is fluorescently labeled, the intensity of fluorescence emitted from an antibody bonded with the molecule expressed on the surface of the cell is converted to an electric signal whereby the expressed amount of the antigen can be quantified. It is also possible to separate cells expressing a plurality of surface antigens by combination of fluorescence types used therefor. Examples of fluorescences which can be used in this case include FITC (fluorescein isothiocyanate), PE (phycoerythrin), APC (allo-phycocyanin), TR (Texas Red), Cy 3, CyChrome, Red 613, Red 670, TRI-Color, Quantum Red, etc.

[0084] FACS methods using a flow cytometer include: a method where the above stem cell broth is collected, from which cells are isolated by, for example, centrifugation, and stained directly with antibodies; and a method where the cells are cultured and grown in a suitable medium and then stained with antibodies. The staining of cells is performed by mixing a primary antibody recognizing a surface antigen with a target cell sample and incubating the mixture on ice for 30 minutes to 1 hour. When the primary antibody is fluorescently labeled, the cells are isolated with a flow cytometer after washing. When the primary antibody is not fluorescently labeled, cells reacted with the primary antibody and a fluorescent labeled secondary antibody having binding activity to the primary antibody are mixed after washing, and incubated on ice water for 30 minutes to 1 hour. After washing, the cells stained with the primary and secondary antibodies are isolated with a flow cytometer.

[0085] Various surface antigens may include hematopoietic-associated antigens, the surface antigens of mesenchymal cells, and antigens specific to nervous system neurons. The hematopoietic-associated antigens include CD34, CD45, etc., the surface antigens of mesenchymal cells include SH-2, SH-3, etc., and the antigens specific to nervous system neurons include NSE, GFAP, etc. The single or combined use of antibodies recognizing the above-described surface antigens allows the desired cells to be obtained.

Adipose-Derived Adult Mesenchymal Stem Cells of the Invention

[0086] The proliferation rate of the isolated human breast adipose tissue-derived multipotent stem cells was examined, as a result, it was found that CPDL was gradually increased up to a passage number of 16, indicating that the stem cells have high proliferation rates.

[0087] The isolated multipotent adult mesenchymal stem cells, (or a homogenous cell population of the mesenchymal stem cells) according to the present invention were analyzed using a flow cytometer, as a result, showed positive responses to CD73, CD90, CD29, CD44, and CD105. Also, the multipotent stem cells showed negative immunological responses to all of CD33, CD34, CD45, CD4, CD31, CD62p, CD14 and HLA-DR.

[0088] The CD73, CD90, CD29, CD44, and CD105 are known for early mesenchymal stem cell markers, especially present on bone marrow mesenchymal stem cells, etc.

[0089] The CD4 is a 55 kD single-chain type I transmembrane glycoprotein expressed on most thymocytes, a subset of

T cells, and monocytes/macrophages. CD4, a member of the Ig superfamily, recognizes antigens associated with MHC class II molecules and participates in cell-cell interactions, thymic differentiation, and signal transduction.

[0090] The CD14 is expressed at high levels on monocytes and macrophages, and at lower levels on granulocytes. Some dendritic cell populations such as interfollicular dendritic cells, reticular dendritic cells, and Langerhans cells have also been reported to express CD14.

[0091] The CD31 is expressed on monocytes, platelets, granulocytes, endothelial cells and lymphocyte subsets.

[0092] The CD33 is a sialoadhesion immunoglobulin superfamily member expressed on myeloid progenitors, monocytes, granulocytes, dendritic cells and mast cells. CD33 is absent on normal platelets, lymphocytes, erythrocytes and hematopoietic stem cells.

[0093] The CD34 is a sialomucin molecule that is expressed on primitive hematopoietic stem cells and down-regulated as they differentiate into mature cells. Although its precise function remains unknown, the pattern of expression of CD34 suggests that it plays a significant role in early hematopoiesis.

[0094] The CD45 is a tyrosine phosphatase expressed on the plasma membrane of all hematopoietic cells, except erythrocytes and platelets.

[0095] The CD62P is rapidly transported from its cytoplasmic stores in the Weibel-Palade bodies of endothelial cells or the alpha-granules of platelets to the surface. CD62P initiates the adhesive interaction between endothelial cells and neutrophils and monocytes during the inflammatory reaction and is also involved in the interaction of platelets with monocytes and neutrophils.

[0096] HLA-DR is expressed on the surface of human antigen presenting cells (APC) including B cells, monocytes, macrophages, DCs, and activated T cells. HLA-DR is a heterodimeric transmembrane protein composed of α and β subunits and plays an important role in the presentation of peptides to $CD4^+$ T lymphocytes.

Differentiation into Ectodermal, Mesodermal, and Endodermal lineage

[0097] The adipose-derived mesenchymal stem cells of the invention can be differentiated into two or more distinct lineages from different germ layers such as endodermal and mesodermal, for example hepatocytes and adipocytes.

[0098] The adipose-derived mesenchymal stem cells of the invention can be differentiated into cells of two or more lineages, for example adipogenic, chondrogenic, cardiogenic, dermatogenic, hematopoetic, hemangiogenic, myogenic, nephrogenic, neurogenic, neuralgiagenic, urogenitogenic, osteogenic, pericardiogenic, peritoneogenic, pleurogenic, splanchogenic, and stromal developmental phenotypes. While such cells can retain two or more of these different linages (or developmental phenotypes), preferably, such ADSCs can be differentiated into three or more different lineages. The most preferred ADSCs can be differentiated into four or more lineages.

[0099] The adipose-derived mesenchymal stem cells have the capacity to differentiate into ectoderm-derived cells, ectodermal tissues, such as neurogenic tissue, and neurogliagenic tissue.

[0100] The adipose-derived mesenchymal stem cells of the invention have the capacity to differentiate into mesoderm-derived cells, mesodermal tissues, such as mature adipose tissue, bone, various tissues of the heart (e.g., pericardium, epicardium, epimyocardium, myocardium, pericardium, valve tissue, etc.), dermal connective tissue, hemangial tissues (e.g., corpuscles, endocardium, vascular epithelium, etc.), hematopeotic tissue, muscle tissues (including skeletal muscles, cardiac muscles, smooth muscles, etc.), urogenital tissues (e.g., kidney, pronephros, meta- and meso-nephric ducts, metanephric diverticulum, ureters, renal pelvis, collecting tubules, epithelium of the female reproductive structures (particularly the oviducts, uterus, and vagina), mesodermal glandular tissues (e.g., adrenal cortex tissues), and stromal tissues (e.g., bone marrow). Of course, inasmuch as the adipose-derived mesenchymal stem cells can retain potential to develop into a mature cell, it also can realize its developmental phenotypic potential by differentiating into an appropriate precursor cell (e.g., a preadipocyte, a premyocyte, a preosteocyte, etc.).

[0101] In another embodiment, the adipose-derived mesenchymal stem cells have the capacity to differentiate into endoderm-derived cells, endodermal tissues, such as pleurogenic tissue, and splanchnogenic tissue, and hepatogenic tissue, and pancreogenic tissue.

[0102] In another embodiment, the inventive adipose-derived mesenchymal stem cells can give rise to one or more cell lineages from one or more germ layers such as neurogenic cells (of ectodermal origin) and myogenic cells (of mesodermal origin).

[0103] In preferred embodiment, the inventive adipose-derived mesenchymal stem cells can differentiates into neurogenic cell, astrocyte, cartilage cell, osteogenic cells, fat cells, insulin-releasing pancreatic beta cell, muscle cell, endothelial cells and hepatic cell, etc.

[0104] In addition, the present invention relates to a cellular therapeutic agent for treating various diseases containing the adipose-derived mesenchymal stem cells, as active ingredients.

[0105] For example, the present invention provides a cellular a cellular therapeutic agent for treating nerve disease containing the adult mesenchymal stem cells, which have the ability of differentiation into nerve cells, or, the differentiated nerve cells therefrom as active ingredients.

[0106] The present invention provides a cellular therapeutic agent for treating diabetes containing the adult mesenchymal stem cells, which have the ability of differentiation into insulin-releasing pancreatic beta-cells, or, the differentiated insulin-releasing pancreatic beta-cells therefrom as active ingredients.

[0107] The present invention provides a cellular therapeutic agent for treating osteoarthritis containing the adult mesenchymal stem cells, which have the ability of differentiation into cartilage cells, or, the differentiated cartilage cells therefrom as active ingredients.

[0108] The present invention provides a cellular therapeutic agent for treating bone deficiency containing the adult mesenchymal stem cells, which have the ability of differentiation into osteogenic cells, or, the differentiated osteogenic cells therefrom as active ingredients.

[0109] The present invention provides a cellular therapeutic agent for forming breast tissue containing the adult mesenchymal stem cells, which have the ability of differentiation into fat cells, or, the differentiated fat cells therefrom as active ingredients.

[0110] The present invention provides a cellular therapeutic agent for treating muscle disease (e.g. Cardic infarct or urinary incontinence) containing the adult mesenchymal

stem cells, which have the ability of differentiation into muscle cells, or, the differentiated muscle cells therefrom as active ingredients.

[0111] The present invention provides a cellular therapeutic agent for treating vascular disease (e.g. Critical limb ischemia or Buerger's disease) containing the adult mesenchymal stem cells, which have the ability of differentiation into endothelial cells or the differentiated endothelial cells therefrom as active ingredients.

[0112] The present invention provides a cellular therapeutic agent for treating hepatic disease (e.g. liver cirrhosis or acute hepatic disease) containing the adult mesenchymal stem cells, which have the ability of differentiation into hepatic cells, or, the differentiated hepatic cells therefrom as active ingredients.

[0113] The inventive adipose-derived mesenchymal stem cells are useful for tissue engineering, wound repair, in vivo and ex vivo tissue regeneration, tissue transplantation, treatment of various diseases and other methods that require cells that can differentiate into a variety of phenotypes and genotypes, or can support other cell types in vivo or in vitro.

EXAMPLES

[0114] Hereinafter, the present invention will be described in more detail by examples. It is to be understood, however, that these examples are for illustrative purpose only and are not construed to limit the scope of the present invention.

Example 1

Isolation of Multipotent Stem Cells from Adipose Tissue

[0115] Adipose tissue was isolated from women's breast tissue distributed by Breast Cancer Center, Seoul National University, and washed with PBS and then finely cut. The cut tissue was digested in DMEM media supplemented with collagenase type 1 (1 mg/ml), at 37° C. for 2 hours. The digested tissue was washed with PBS and then centrifuged at 1000 rpm for 5 minutes. The supernatant was suctioned off, and the pellets remaining on the bottom were washed with PBS and then centrifuged at 1000 rpm for 5 minutes. The resulting pellets were filtered through a 100 μm mesh to remove debris, followed by washing with PBS. The resulting cells were incubated in a DMEM medium (10% FBS, 2 mM NAC, 0.2 mM ascorbic acid). After one overnight period, unattached cells were washed with PBS, and cultured in Keratinocyte-SFM media (containing 2 mM NAC, 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, 50 μg/ml BPE, 5 μg/ml insulin and 74 ng/ml hydrocortisone) while the media were replaced at two-day intervals, thus isolating multipotent stem cells. FIG. **1** shows photographs taken at 100× magnification for the human adipose tissue-derived multipotent stem cells isolated as described above.

Example 2

Examination of Proliferation Rate of Adipose Tissue-Derived Stem Cells

[0116] Adipose tissue was obtained from each of different human breast tissue samples according to the isolation method as described in Example 1. In order to examine the proliferation rate of multipotent stem cells derived from the isolated human breast adipose tissue, 2×10^5 of the cells were seeded into a T-75 flask and then measured for CPDL (cumulative population doubling level) and expressed as a function of passage number. CPDL is an index indicative of the proliferation rate of cells and expressed as the following equation.

CPDL=ln(Nf/Ni)/ln2, wherein Ni: the initial number of seeded cells; and Nf: the final number of cells.

[0117] As a result, as shown in "A-1" and "A-2" of FIG. **2**, the adult stem cells (hMAD-MCS1 and hMAD-MCS2) according to the present invention showed a CPDL value of about 50 at a passage number of 16.

[0118] Meanwhile, "B" and "C" of FIG. **2** show the CPDL values of the prior human adipose tissue-derived stem cells (Lin et al., *Stem Cells and Development,* 14:92, 2005; Zuk et al., *Tissue Eng.,* 7:211, 2001) as a function of passage number. As shown in FIG. **2**, the CPDL values of the cells were 30-35 and 21 at passage numbers of 7 and 13, respectively.

[0119] These results suggest that the adult stem cells according to the present invention have very high proliferation rates.

Example 3

Immunological Characteristics of Adipose-Derived Multipotent Stem Cells

[0120] The adipose tissue-derived multipotent stem cells obtained in Example 1 were washed with PBS and treated with trypsin. The treated cells were collected and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and then washed with a mixture of 2% FBS and PBS, followed by centrifugation at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were suspended in PBS, and 1×10^5 cells for each sample were dispensed into a well plate. An antibody (R-phycoerythrin-conjugated mouse anti-human monoclonal antibody) was placed into each well and incubated on ice for 40 minutes. After the incubation, the medium was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cells were washed with PBS and centrifuged at 1000 rpm for 5 minutes. Once again, the supernatant was removed, and the cells were washed with PBS and centrifuged at 1000 rpm for 5 minutes. After removing the supernatant, the cells were fixed with 1% paraformaldehyde and analyzed using a flow cytometer.

TABLE 1

FACS analysis of surface antigens of adipose-derived stem cells

Antigen	AD-MSCs
CD73	+
CD90	+
CD29	+
CD44	+
CD105	+
CD33	−
CD34	−
CD45	−
CD4	−
CD31	−
CD62p	−
CD14	−
HLA-DR	−

[0121] As a result, as shown in Table 1, the adipose tissue-derived adult stem cells according to the present invention showed positive responses of 91% to CD73, 97% to CD90, 96% to CD29, 83% to CD44, and 80% to CD105. Also, the

inventive stem cells showed negative immunological responses to all of CD33, CD34, CD45, CD4, CD31, CD62p, CD14 and HLA-DR.

[0122] The histograms for the FACS results are shown in FIG. **3**. The single peak in the histogram demonstrates that mesenchymal stem cells or mesenchymal stem cell-enriched fractions are a homogenous cell population.

Example 4

Sphere Formation of Adipose Tissue-Derived Multipotent Stem Cells

[0123] 5×10^4-1×10^5/ml of the human breast adipose tissue-derived multipotent stem cells obtained in Example 1 were seeded into each well of a 6-well plate containing a serum-free MEBM medium containing 10 μM CORM-2, 5 ml antibiotic antimycotic solution (100×), 1 μg/ml hydrocortisone, 5 μg/ml insulin, 20 ng/ml EGF, 40 ng/ml FGF, B27 and β-mercaptoethanol. As a result, the cells started to form the shape of spheres from 3-7 days after the seeding, and as shown in FIG. **4** and FIG. **5**, the cells proliferated to form spheres even at 7-10 days after the seeding.

[0124] Also, the stem cells according to the present invention were cultured in agar. As a result, as shown in FIG. **5**, the cells formed spheres.

[0125] Meanwhile, 5×10^4 stem cells obtained in Example 1 were seeded into each well of a 24-well plate and measured for the number of spheres at each passage number (see Table 2). As a result, as shown in Table 2, the cells maintained spheres, indicating that the cells can be proliferated and maintained for a long period of time. Also, as shown in FIG. **6**, Oct4 was positively expressed, indicating that the cells have a high proliferation rate while being maintained in an undifferentiated state.

TABLE 2

Passage number	Number of spheres
1	270
2	260
3	271

Example 5

Immunostaining Analysis of Adipose Tissue-Derived Stem Cells

[0126] The adipose tissue-derived stem cell spheres obtained in Example 4 were washed three times with PBS and fixed with 4% paraformaldehyde-containing PBS for 30 minutes. After washing three times with PBS, the spheres were permeated with PBS containing 0.1% Triton-X100 for 10 minutes. After being washed three times with PBS, the spheres were allowed to react with 10% NGS for 1 hour and then with PBS containing a primary antibody overnight. After washing three times with PBS, the spheres were allowed to react with a secondary antibody in a dark room for 1 hour. After being washed three times with PBS, the spheres were mounted.

[0127] As a result, as shown in FIG. **6**, the multipotent stem cell spheres according to the present invention showed positive responses to all of Nestin, which can be regarded as a marker of nerve progenitor cells, Oct4, which can be regarded as a marker of undifferentiated cells, and SH2(CD105) and SH3/4(CD73), which are markers of mesenchymal stem cells.

Example 6

Differentiation of Adipose-Derived Multipotent Stem Cells into Nerve Cells and Astrocytes

[0128] The adipose tissue-derived multipotent stem cells obtained in Example 1 were preincubated in a DMEM medium supplemented with 1 mM BME and 10% FBS, for 24 hours. After the preincubation, the stem cells were incubated in a medium for inducing nerve cell differentiation, containing 1% DMSO and 100 μM BHA (butylated hydrxyanisole), for 90 minutes, so as to induce differentiation into nerve cells, followed by immunostaining (FIG. **7**). As a result, as shown in FIG. **7**, the human adipose tissue-derived multipotent stem cells according to the present invention showed positive responses to GFAP (glial fibrillary acidic protein), which is an antigen specific to astrocytes in the nervous system, and MAP2 (microtubule-associated protein2), which is a nerve cell-specific substance.

[0129] Photographs on the first line in FIG. **7** show results for a negative control group, which indicate that differentiated cells do not show the fluorescence of FITC and TRITC by themselves. The MAP2 photograph at the left side of the second line shows the red fluorescence of TRITC, indicating that MAP2 was expressed. From the phase contrast photograph and the Merge photograph, it was found that the red fluorescence was a fluorescence emitted from cells in which MAP2 was expressed. Also, the GFAP photograph at the left side of the third line showed the green fluorescence of FITC, and from the phase contrast photograph and the Merge photograph, it was seen that the green fluorescence was a fluorescence emitted from cells in which GFAP was expressed.

[0130] Further, as a result of immunostaining using TUJ1, NSE, and SOX2 known for nerve cell-specific marker, FIG. **8** shows the expression of TUJ1, NSE, and SOX2 by the differentiation of nerve cell from adipose-derived mesenchymal stem cells obtained in the Example 1. These results suggest that the human adipose-derived multipotent stem cells according to the present invention differentiate into nerve cells and astrocytes.

Example 7

Differentiation of Adipose-Derived Multipotent Stem Cells into Fat Cells

[0131] The adipose tissue-derived multipotent stem cells obtained in Example 1 were incubated in an α-MEM medium containing 5% FBS, 1 μM dexamethasone, 200 μM indomethacin, 10 μg/ml insulin and 0.5 mM IBMX (3-isobutyl-1-methylxanthine) for 2 weeks to induce differentiation into fat cells and then analyzed using an oil red O staining method. FIG. **9** shows the differentiation of fat cells from the adipose-derived mesenchymal stem cells obtained in the Example 1. Further, FIG. **10** also shows result of phase contrast and Oil red O staining method.

[0132] As a result, as shown in FIGS. **9** and **10**, it was observed that the human adipose tissue-derived multipotent stem cells were differentiated into fat cells.

Example 8

Differentiation of Adipose-Derived Multipotent Stem Cells into Cartilage Cells

[0133] 10^7 cells/ml of the adipose tissue-derived multipotent stem cells obtained in Example 1 were dispensed into

each center of a 24-well plate in an amount of 10 μl. Then, the cells were incubated in an α-MEM medium containing 5% FBS, 10 ng/ml TFG-β1, 50 μM L-ascorbate-2-phosphate and 6.25 μg/ml insulin for 2 weeks so as to induce differentiation into cartilage cells. Then, whether the multipotent stem cells were differentiated into cartilage cells was analyzed using the Alcian blue staining method. As a result, as shown in FIG. **11**, the human adipose tissue-derived multipotent stem cells were differentiated into cartilage cells.

[0134] As another method, after the induction of the differentiation, toluidine blue O staining method was used. As a result, as shown in FIG. **12**, lacuna (vacant space in cells) as a character for cartilage cells was identified, thus, it demonstrates the differentiation of the cartilage cells.

Example 9

Differentiation of Adipose-Derived Multipotent Stem Cells into Osteogenic Cells

[0135] 10^7 cells/ml of the adipose tissue-derived multipotent stem cells obtained in Example 1 were dispensed into each center of a 24-well plate in an amount of 10 μl. Then, the cells were incubated in a medium containing 10% FBS, 0.1 μM Dexamethasone, 0.2 mM L-ascorbate-2-phosphate, 10 mM Glycerol 2-Phosphate for 2 weeks so as to induce differentiation into osteogenic cells. Then, whether the multipotent stem cells were differentiated into osteogenic cells was analyzed using the Alizatin red S staining method. As a result, as shown in FIG. **13**, red staining was shown, and calcification (as a character for ostogenic cells) was identified. Therefore, the human adipose tissue-derived multipotent stem cells were differentiated into cartilage cells.

[0136] Further, 10^7 cells/ml of the adipose tissue-derived adult stem cells obtained in Example 1 were mixed with TCP (tricalcium phosphate) and isotransplanted subcutaneously into dogs. After 14 days, the tissue was treated and analyzed using the H&E stain method. As a result, as shown in FIG. **14**, a group (A) treated with TCP alone showed the permeation of inflammatory cells into a portion around TCP, and a group (B) treated with a mixture of TCP and marrow stem cells showed inflammatory responses remaining intact around TCP. However, in a group (C) treated with a mixture of TCP and adipose-derived stem cells, most of TCP was absorbed, and typical initial osteogenesis was observed, and osteoblast-like cells, multinuclear osteoclast-like cells and bone matrixes were also observed. These results indicate that the human adipose tissue-derived multipotent stem cells were differentiated into osteogenic cells.

Example 10

Differentiation of Adipose-Derived Multipotent Stem Cells into Insulin-Releasing Pancreatic β-Cells

[0137] The adipose tissue-derived multipotent stem cells obtained in Example 1 were incubated in low-glucose DMEM medium containing 10 mmol/L nicotinamide, 1 mmol/L β-mercaptoethanol and 10% FBS for 24 hours, and then incubated in high-glucose DMEM medium containing 10 mmol/L nicotinamide, 1 mmol/L β-mercaptoethanol and 5% FBS for 5 days, so as to induce differentiation into insulin-releasing pancreatic beta-cells. After inducing the differentiation, the cells were analyzed by immunostaining, and the results are shown in FIG. **15**. As shown in FIG. **10**, C-peptide and insulin were present in the cells. As known in the art, proinsulin, which is divided into insulin and C-peptide, is produced in insulin-releasing pancreatic beta-cells. Thus, the above results indicate that the adipose tissue-derived multipotent stem cells according to the present invention were differentiated into insulin-releasing pancreatic beta-cells.

Example 11

Differentiation of Adipose-Derived Multipotent Stem Cells into Muscle Cells

[0138] The adipose tissue-derived multipotent stem cells obtained in Example 1 were preincubated in DMEM medium containing 10% FBS so that the cells are grown as 50% distribution (confluency). After the preincubation, the cells were incubated in SkGM medium (Lonza, Inc.) containing 0.1% hEGF, 0.1% dexamethasone, 0.1% GA-1000, 2% L-Glutamine for 14 days, so as to induce differentiation into muscle cells. After inducing the differentiation, the cells were analyzed by immunostaining, and the results are shown in FIG. **16**. As shown in FIG. **16**, the cells showed positive response to Myosin which was known for a specific marker for muscle cell. In FIG. **16**, the results show green color, indicating that myosin was expressed. Thus, the above results indicate that the adipose tissue-derived multipotent stem cells according to the present invention were differentiated into muscle cells.

Example 12

Differentiation of Adipose-Derived Multipotent Stem Cells into Hepatic Cells

[0139] The adipose tissue-derived multipotent stem cells obtained in Example 1 were incubated in Keratinocyte-SFM medium containing 2 mM NAC, 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, 50 μg/ml BPE, 5 μg/ml insuline and 74 ng/ml hydrocortisone for 2 days. After 2 days, the differentiation was induced by adding 10% FBS, 1% Fungizone, 1 mg/ml Collagen, 5 μg/ml Heparin, HGM (×200 conc), 0.1% ITS+premix to the Keratinocyte-SFM medium. After the induction to the differentiation for 2 weeks, the cells were cultured in medium containing Keratinocyte-SFM medium, 10% FBS, 1% Fungizone, lmg/ml Collagen, 2% Dexamethasone, 0.1% ITS+premix, 0.5% Oncostatin M, 20 ng/ml HGF for further several days, so as to induce differentiation into hepatic cells. After inducing the differentiation, the whether the multipotent stem cells were differentiated into hepatic cells were analyzed by Periodic acid Schiff staining method. As shown in FIG. **17**, it was identified that the adipose tissue-derived multipotent stem cells according to the present invention were differentiated into hepatic cells.

Example 13

Differentiation of Adipose-Derived Multipotent Stem Cells into Endothelial Cells

[0140] The adipose tissue-derived multipotent stem cells obtained in Example 1 were starved in M199 medium (0.5% FBS-low, phenol red) for 4 hours, and were received physical shear stress at 5 dynes/cm^2 for 18 hours. After 18 hours, the cells were counted and 10^5 cells/Ml and dispensed in matrigel-coated 48-well plate with KSFM medium and, after 6 hours, tube formation was identified (in vitro tube formation analyzing method).

[0141] As shown in FIG. **17**, the differentiation of endothelial cells was induced. FIG. **17** also illustrates the result of tube formation length analysis.

[0142] The differentiation of endothelial cells by the sphere stress to adipose-derived mesenchymal stem cells was induced and such differentiation was identified using cell immunostaining. As shown in FIG. **18**, the differentiation was identified using the dark brown staining of the vascular endothelial growth factor receptor 2 (VEGFR-2, KDR) and von Willebrand factor (Vwf) which were endothelial specific cell surface markers related with vascular formation.

[0143] As shown in FIG. **19**, after treatment of shear stress and growth factor (10 ng VEGF, 1 μg hydrocotisone, 3 μg bovine brain extract and 5% FBS) to identify the pattern for endothelial cell differentiation, the absorption of ulex europaeus agglutinin 1 (UEA-1, green fluorescence stained) and acetylated low density lipoprotein (Ac-LDL, red fluorescence stained) in cells were identified, thus, it was similar to endothelial cells (shown in yellow arrow)

[0144] Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

INDUSTRIAL APPLICABILITY

[0145] As described in detail above, although the multipotent stem cells according to the present invention are adult stem cells, they can differentiate into more various kinds of cells than those differentiated from the prior adipose-derived adult stem cells. Particularly, the inventive adult multipotent stem cells have the ability to differentiate into nerve cells, astrocytes, fat cells, chondrogenic cells, osteogenic cells, insulin-releasing pancreatic beta-cells, muscle cells, endothelial cells or hepatic cells and are effective in treating osteoporosis, osteoarthritis, nerve disease, diabetes, etc., and also useful for the formation of breast tissue. Also, the inventive adult stem cells form spheres in a serum-free medium, so that they can be isolated with high purity, maintained in an undifferentiated state for a long period of time and have a high proliferation rate. Thus, the inventive adult stem cells are useful as cellular therapeutic agents.

What is claimed is:

1. Adult mesenchymal stem cells characterized by:

(a) showing positive immunological responses to all of CD73, CD90, CD29, CD44 and CD105, and negative immunological responses to all of CD33, CD34, CD45, CD4, CD31, CD62p, CD14 and HLA-DR;

(b) growing attached to a plastic material, showing spindle-shaped morphological features, and forming spheres in a medium containing CORM-2 so as to be able to be maintained in an undifferentiated state for a long period of time; and

(c) having the ability to differentiate into ectoderm, mesoderm and/or endoderm-derived cells.

2. The adult mesenchymal stem cells according to claim **1**, wherein the adult mesenchymal stem cells are cultured in an undifferentiated state for at least 16 passages.

3. The adult mesenchymal stem cells according to claim **1**, wherein the ectoderm-derived cells are selected from the group consisting of: nerve cells and astrocytes.

4. The adult mesenchymal stem cells according to claim **1**, wherein the mesoderm-derived cells are selected from the group consisting of: cartilage cells, osteogenic cells, endothelial cells, muscle cells and fat cells.

5. The adult mesenchymal stem cells according to claim **1**, wherein the endoderm-derived cells are selected from the group consisting of: insulin-releasing pancreatic beta-cells and hepatic cells.

6. A cellular therapeutic agent for treating nerve disease containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into nerve cells, or the differentiated nerve cells therefrom, as active ingredients.

7. A cellular therapeutic agent for treating diabetes containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into insulin-releasing pancreatic beta-cells, or the differentiated insulin-releasing pancreatic beta-cells therefrom, as active ingredients.

8. A cellular therapeutic agent for treating osteoarthritis containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into cartilage cells, or the differentiated cartilage cells therefrom, as active ingredients.

9. A cellular therapeutic agent for treating bone deficiency containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into osteogenic cells, or the differentiated osteogenic cells therefrom, as active ingredients.

10. A cellular therapeutic agent for forming breast tissue containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into fat cells, or the differentiated fat cells therefrom, as active ingredients.

11. A cellular therapeutic agent for treating muscle-related disease containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into muscle cells, or the differentiated muscle cells therefrom, as active ingredients.

12. A cellular therapeutic agent for treating hepatic disease containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into hepatic cells, or the differentiated hepatic cells therefrom, as active ingredients.

13. A cellular therapeutic agent for treating vascular disease containing the adult mesenchymal stem cells of claim **1**, which have the ability of differentiation into endothelial cells, or the differentiated endothelial cells therefrom, as active ingredients.

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