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(54) **Title**: METHOD OF INDUCING THE DIFFERENTIATION OF GERMLINE STEM CELLS, METHOD OF EXPANDING THE CELLS, AND CULTURE MEDIA THEREFOR

(57) **Abstract**: Provided are a method of inducing the differentiation of germline stem cells possessing the capabilities of self-regeneration and spermiogenesis from pluripotent stem cells such as iPS cells, a method of maintaining and expanding the germline stem cells, and culture media therefor. Specifically provided are a method of producing Oct4-positive Vasa-positive germline stem cells (GR cells), comprising culturing pluripotent stem cells in the presence of (a) bone morphogenetic protein 4 (BMP4) and (b) one or more growth factors selected from among glial cell-derived neurotrophic factor (GDNF), epithelial cell growth factor (EGF) and stem cell factor (SCF), and a method of expanding GR cells, comprising culturing GR cells in the presence of GDNF, EGF, SCF and basic fibroblast growth factor (bFGF).

DESCRIPTION

METHOD OF INDUCING THE DIFFERENTIATION OF GERMLINE STEM CELLS, METHOD OF EXPANDING THE CELLS, AND CULTURE MEDIA THEREFOR

Technical Field

[0001]

The present invention relates to a method of inducing the differentiation of primordial germ cell (PGC)-like cells from a pluripotent stem cell, a method of maintaining and expanding the PGC-like cells, and a culture medium used for the methods.

Background of the Invention

[0002]

In addition to attacking cancer cells, anticancer agents and radiations used for anticancer therapy are known to cause serious adverse reactions on normal highly active cells and tissues. Anticancer agents mostly exhibit their therapeutic effects by selective toxicity based on the higher proliferative activity of cancer cells than that of normal cells. Hence, anticancer agents even attack normal cells in vigorous proliferation, such as gastrointestinal mucosa, hair root cells, myelocytes, and spermatogonia. For example, adriamycin is known to cause orchiopathies characterized by necrosis of spermatogonia.

Pharmaceuticals that lessen the adverse reactions to anticancer agents have been developed by gene recombination technology one after another. For example, granulocyte colony stimulating factor (G-CSF) is a groundbreaking medicine for avoiding bone marrow suppression as an adverse reaction, enabling more aggressive cancer chemotherapy. However, no drugs have been found to date that are effective in preventing/treating the orchiopathies due to the adverse reactions to anticancer agents or radiotherapies.

[0003]

Meanwhile, 30% of cases of infertility reportedly have its causal factor on the male side (i.e., azoospermia, the absence of living sperms in the semen, or oligozoospermia, a

subnormal concentration of sperms in the semen). The Japan Society of Obstetrics and Gynecology permits artificial insemination by donor, a procedure in which a sperm sample from a donor other than the woman's mate is deposited to directly into the uterus, although its application is limited to infertility treatment for a married couple. However, many people view the provision of sperms from a donor other than husband for infertility treatment as being ethically problematic, not only for an unmarried woman, but also for a married couple.

[0004]

As a solution to the male infertility due to adverse reactions to anticancer therapy, a method is available wherein sperms are collected before treatment and preserved under freezing until use; however, this is ineffective in childhood cancer patients, who do not permit sperm preservation. In 1994, Brinster et al. succeeded in transplanting spermatogonial stem cells in vivo [Brinster, R.L. et al., Proc. Natl. Acad. Sci. USA, 91: 11298-11302 (1994)]. Specifically, transplanting testicular cells, including stem cells, from a donor mouse into the seminiferous tubule of an infertile male mouse led to spermiogenesis from the donor cells. The infertile male mouse was mated with a female mouse, and offspring derived from the donor was obtained. Furthermore, Shinohara et al. established a method of culturing spermatogonial stem cells in vitro for a long time using a culture medium containing glial cell-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), epithelial cell growth factor (EGF), and basic fibroblast growth factor (bFGF), and named the cell line obtained Germline Stem (GS) cells [Pamphlet for International Patent Publication WO 2004/092357; Kanatsu-Shinohara, M. et al., Biol. Reprod., 69: 612-616 (2003)]. Transplanting this line of GS cells to the testis resulted in spermiogenesis and the generation of offspring. Hence, provided that a line of GS cells is established from testicular tissue collected by

biopsy before treatment and preserved, autologous transplantation of the GS cells to the testis after treatment makes it possible to avoid infertility due to adverse reactions to cancer treatment. This method is also effective in childhood cancer patients, who do not permit sperm preservation.

For patients who have already contracted an orchiopathy making them unable to form sperms, however, it can be impossible to collect spermatogonial stem cells at one time in sufficient numbers to induce GS cells, so that multiple biopsies must be taken; collecting a large amount of testicular tissue specimens poses the risk of causing testicular atrophy.

[0005]

In recent years, mouse and human induced pluripotent stem cells (iPS cells) have been established one after another [Pamphlets for International Patent Publications WO 2007/069666 and WO 2008/118820; Takahashi, K. and Yamanaka, S., Cell, 126: 663-676 (2006); Nakagawa, M. et al., Nat. Biotechnol., 26: 101-106 (2008); Takahashi, K. et al., Cell, 131: 861-872 (2007); Yu, J. et al., Science, 318: 1917-1920 (2007)]. Human iPS cells can be differentiated into cells of various tissues after being generated using cells derived from a patient to be treated, and are therefore expected to serve as transplantation materials free of graft rejections in the field of regenerative medicine. Successful generation of iPS cells using three factors excluding the c-Myc gene [Takahashi, K. and Yamanaka, S., Cell, 126: 663-676 (2006)] and the ability to induce iPS cells using a plasmid or episomal vector without integration of reprogramming factors in the genome [Okita, K. et al., Science, 322: 949-953 (2008); Yu, J. et al., Science, 324: 797-801 (2009)] are increasing the expectations for clinical application of human iPS cells. Because iPS cells can easily be established from, for example, dermal fibroblasts and the like, it would be possible to avoid the

risky procedure of collecting a source of cells, such as by multiple biopsies of the testis, provided that sperms or progenitor cells thereof are efficiently differentiation-induced from human iPS cells and expanded. This is expected to lead to more safe spermiogenesis in male infertility patients.

Summary of the Invention

Problems to Be Solved by the Invention

[0006]

It is an object of the present invention to provide a method of inducing the differentiation of germline stem cells possessing the capabilities of self-regeneration and spermiogenesis from pluripotent stem cells such as iPS cells, a method of maintaining and expanding the germline stem cells, and culture media used therefor.

Means of Solving the Problems

[0007]

The present inventors inserted reporter genes (GFP and RFP) downstream of the respective expression control regions of the undifferentiated cell-specific Oct3/4 (Oct4) gene and the mouse homolog of the germ cell lineage-specific Vasa gene (Mouse vasa homolog; hereinafter referred to as Mvh) to create a transgenic (Tg) mouse wherein undifferentiated cells and germ cells are visualized. The inventors conducted extensive investigations using iPS cells induced from the mouse and ES cells as an experimental system, in an attempt to establish culturing conditions that allow Oct4-GFP-positive Mvh-RFP-positive ($Oct4^+/Mvh^+$) cells to be induced efficiently, and culturing conditions that allow the cells to be maintained and expanded in the $Oct4^+/Mvh^+$ state. As a result, the present inventors found that the differentiation of $Oct4^+/Mvh^+$ cells can be induced efficiently by culturing pluripotent stem cells in the presence of BMP4, GDNF, EGF, and stem cell factor (SCF), and that $Oct4^+/Mvh^+$ cells can be expanded, while maintaining their double-positive state, by culturing the cells in the

presence of GDNF, EGF, bFGF, and SCF, preferably in the further presence of one or more factors selected from the group consisting of hepatocyte growth factor (HGF), interleukin 2 (IL-2), and fibroblast growth factor 9 (FGF9). Characterization of the $Oct4^+/Mvh^+$ cells thus obtained (named GR cells) revealed the identity of the cells as a cell line reflecting the characteristics of primordial germ cells (PGC) in the later stage of migration. Furthermore, the present inventors found that these GR cells, when transplanted into the seminal duct of an infertile mouse, survived for a long time and did not show tumor formation, confirming their identity as unipotent stem cells destined to become germline cells, and have developed the present invention.

[0008]

Accordingly, the present invention relates to the following:

(1) A method of producing an Oct4-positive Vasa-positive germline stem cell, comprising culturing a pluripotent stem cell in the presence of (a) bone morphogenetic protein 4 (BMP4) and (b) one or more growth factors selected from among glial cell-derived neurotrophic factor (GDNF), epithelial cell growth factor (EGF), and stem cell factor (SCF).

(2) The method according to (1) above, wherein the method comprises culturing a pluripotent stem cell in the presence of the BMP4, GDNF, EGF, and SCF.

(3) The method according to (1) or (2) above, wherein the pluripotent stem cell is cultured in the presence of feeder cells.

(4) The method according to (3) above, wherein the growth factors are supplied by the feeder cells.

(5) The method according to any one of (1) to (4) above, wherein the pluripotent stem cell is an iPS cell or ES cell.

(6) The method according to any one of (1) to (5) above, wherein the pluripotent stem cell is derived from human or mouse.

(7) An iPS cell-derived Oct4-positive Vasa-positive germline stem cell obtained by the method according to any one of (1) to (6) above.

(8) An inducer of differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, comprising (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF, and SCF, in combination.

(9) An inducer of differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, comprising BMP4, GDNF, EGF, and SCF in combination.

(10) The inducer according to (8) or (9) above, wherein the inducer comprises a cell that produces the growth factors.

(11) A culture medium for inducing differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, supplemented with the inducer according to (8) or (9) above.

(11b) A kit for inducing differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, comprising a medium containing a basal medium and one or more components selected from Table 1 and the inducer according to any one of (8) to (10) above.

(12) A method of expanding an Oct4-positive Vasa-positive germline stem cell, comprising culturing the germline stem cell in the presence of GDNF, EGF, SCF, and basic fibroblast growth factor (bFGF).

(13) The method according to (12) above, wherein the method comprises culturing the germline stem cell in the presence of one or more factors selected from among hepatocyte growth factor (HGF), interleukin 2 (IL-2), and fibroblast growth factor 9 (FGF9).

(14) The method according to (12) or (13) above, wherein the germline stem cell is cultured in the presence of feeder cells.

(15) The method according to any one of (12) to (14) above, wherein the germline stem cell has been differentiation-induced from a pluripotent stem cell.

(16) An expansion support agent for an Oct4-positive Vasa-positive germline stem cell, comprising GDNF, EGF, SCF, and bFGF in combination.

(17) The agent according to (16) above, further comprising one or more factors selected from among HGF, IL-2, and FGF9.

(18) A culture medium for expanding an Oct4-positive Vasa-positive germline stem cell, supplemented with the agent according to (16) or (17) above.

(18b) A culture medium for expanding an Oct4-positive Vasa-positive germline stem cell, consisting of a medium containing a basal medium and one or more optionally chosen components selected from Table 1, and one or more components selected from Table 2 added thereto.

(19) A method of allowing an infertile animal to form sperms, comprising transplanting an Oct4-positive Vasa-positive germline stem cell obtained by the method according to any one of (1) to (6) above, the Oct4-positive Vasa-positive germline stem cell according to (7) above, or an Oct4-positive Vasa-positive germline stem cell expanded by the method according to any one of (12) to (15) above, to the testis of the recipient animal of the same species as the cell.

(20) The method according to (19) above, wherein the germline stem cell is derived from an iPS cell generated from a somatic cell of an infertile animal.

(21) The method according to (19) or (20) above, wherein the recipient animal is a human or mouse.

(22) A therapeutic agent for male infertility comprising an Oct4-positive Vasa-positive germline stem cell obtained by the method according to any one of (1) to (6) above, the Oct4-positive Vasa-positive germline stem cell according to (7) above, or an Oct4-positive Vasa-positive germline stem cell expanded by the method according to any one of (12) to (15) above.

(23) The agent according to (22) above, wherein the agent is to be administered to an individual from which spermatogonial

stem cells are difficult to collect.

Effect of the Invention

[0009]

According to the present invention, it is possible to induce germline stem cells capable of spermiogenesis from iPS cells that can easily be generated from somatic cells such as skin cells, and to maintain and expand the same induced. Therefore, even patients with non-obstructive azoospermia, a disease that poses the problem of difficulty and/or risk in collecting spermatogonial stem cells, can be allowed to form sperms conveying their own genetic information.

Brief Description of the Drawings

[0010]

Fig. 1 is a photographic representation of iPS cells established from MEFs derived from an Oct4-GFP/Mvh-RFP Tg fetal mouse. The upper panel shows images of the iPS clone 522A3, established by transfection with the three genes Oct3/4, Sox2 and Klf4. The lower panel shows images of the iPS clone 522B2, established by transfection with the four genes Oct3/4, Sox2, Klf4 and Nanog.

Fig. 2 is a photographic representation of electrophoresis showing the results of genomic PCR analysis of the established iPS clones 522A1 to A4 and 522B1 to 522B4.

Fig. 3 is a photographic representation of electrophoresis showing the results of an examination of the expression of undifferentiation markers by RT-PCR analysis in the established iPS clones 522A1 to A4 and 522B1 to 522B4.

Fig. 4 is a photographic representation showing the results of a histological analysis of teratomas formed by subcutaneously injecting an established iPS clone into immunodeficient mice.

Fig. 5 is a photographic representation of embryoid bodies (EB) formed from ES cells and iPS cells (clone 522B2) by a conventional method of differentiation induction.

Fig. 6 shows a photograph showing the morphology of M15-

4GF cells obtained by transferring the GDNF, mSCF and EGF genes to M15-BMP4 cells (left panel), and an electrophoregram showing the results of RT-PCR analysis of M15-4GF (right panel).

Fig. 7 is a photographic representation of cell masses obtained by suspension-culturing established iPS cells and M15-4GF cells in the medium of Fig. 27 (without Supplement) (upper panel), and Oct4-GFP-positive / Mvh-RFP-positive colonies obtained by dissociating the cell masses with trypsin and collagenase, and culturing them in the medium of Fig. 27 (with Supplement) on feeder cells (lower panel).

Fig. 8 is a photographic representation of Oct4-GFP-positive / Mvh-RFP-positive colonies that are stably proliferating under the culturing conditions of Fig. 27.

Fig. 9 is a photographic representation showing that an Oct4-GFP-positive / Mvh-RFP-positive colony exhibited an alkaline phosphatase activity, which serves as a cell surface marker both in undifferentiated cells and in germ cells.

Fig. 10 is a photographic representation of cells obtained by culturing an Oct4-GFP-positive / Mvh-RFP-positive colony under iPS cell culturing conditions (upper panel), and cells obtained by culturing an Oct4-GFP-positive / Mvh-RFP-positive colony under germ stem (GS) cell culturing conditions (lower panel).

Fig. 11 is a photographic representation of cells obtained by culturing undifferentiated iPS cells under iPS cell culturing conditions (upper panel), and cells obtained by culturing undifferentiated iPS cells directly under the maintenance culture conditions of Fig. 27 without the process of differentiation induction (lower panel).

Fig. 12 is a photographic representation of Oct4-GFP-positive / Mvh-RFP-positive germ cell-like cells (GR cells) (upper panel), and Oct4-GFP-positive / Mvh-RFP-negative apparently undifferentiated cells (Gsp cells) (lower panel).

Fig. 13 is a photographic representation of

electrophoresis showing the results of genomic PCR analysis of GR cells and Gsp cells. For control, iPS cells and EK cells were also analyzed.

Fig. 14 is a photographic representation of electrophoresis showing the results of Southern blot analysis of GR cells and Gsp cells. For control, iPS cells and EK cells were also analyzed.

Fig. 15 is a graphic representation of the results of total cell counting of iPS cells and GR cells in culture as counted every two days.

Fig. 16 is a photographic representation of the results of cultivation of GR cells on feeder cells (MEF) or in the absence of feeders on gelatin-, laminin- or fibronectin-coated plates.

Fig. 17 is a photographic representation of the results of an examination of immunodeficient nu/nu mice receiving subcutaneously transplanted GR cells or Gsp cells to determine whether tumors would be formed at transplantation sites. For control, iPS cells and EK cells were also transplanted in the same way.

Fig. 18 is a photographic representation of electrophoresis showing the results of RT-PCR analysis of the expression of exogenous transgenes and corresponding endogenous genes in GR cells and Gsp cells. For control, iPS cells, EK cells and Fbx-iPS cells [Cell, 126, 663-676 (2006)] were also analyzed.

Fig. 19 is a graphic representation of the results of quantitation by realtime PCR of the expression of exogenous transgenes and corresponding endogenous genes in GR cells and Gsp cells. For control, iPS cells and EK cells were also analyzed.

Fig. 20 is a photographic representation of electrophoresis showing the results of RT-PCR of the expression of germ cell marker genes in GR cells and Gsp cells. For control, iPS cells, EK cells and the testis were also

analyzed.

Fig. 21, like Fig. 20, is a photographic representation of electrophoresis showing the results of RT-PCR of the expression of germ cell marker genes.

Fig. 22 shows the results of a comparison of the comprehensive gene expression in iPS cells and GR cells using DNA microarrays. "Up" indicates genes whose expression increased in GR cells compared with iPS cells; "Down" indicates genes whose expression decreased in GR cells compared with iPS cells.

Fig. 23 is a photographic representation of electrophoresis showing the results of Western blot analysis of the expression of the proteins of germ cell marker genes in GR cells and Gsp cells. For control, iPS cells and EK cells were also analyzed.

Fig. 24 shows the results of an analysis of DNA methylation statuses in GR cells and Gsp cells by bisulphite genomic sequencing. The cells were examined for the female imprint genes Igf2r, Lit1 and SNRPN and the male imprint gene H19. For control, iPS cells and EK cells were also analyzed.

Fig. 25 is a photographic representation of the results of a histological analysis of the testis from a W/Wv mouse receiving a GR cell graft transplanted into the testis. "Empty" indicates a histological image of a site where the transplanted cells did not engraft; "Engraftment" indicates a histological image of the site where the transplanted cells engrafted.

Fig. 26 is a photographic representation of Oct4-GFP-negative / Mvh-RFP-positive EK cells cultured under the culturing conditions of Fig. 27 (upper panel) and ES cell-derived Oct4-GFP-positive / Mvh-RFP-positive GR cells cultured under the culturing conditions of Fig. 27 (lower panel).

Fig. 27 shows a table of components of the medium used to induce the differentiation of Oct4-GFP-positive / Mvh-RFP-positive cells from iPS cells or ES cells (without Supplement)

and to maintain-culture the same (with Supplement).

[Modes for Embodying the Invention]

[0011]

The present invention provides a method of inducing germline stem cells from a pluripotent stem cell, and a method of maintaining and expanding the germline stem cells. The germline stem cells differentiation-induced from a pluripotent stem cell by the present invention are characterized by the co-expression of both the Oct3/4(Oct4) gene, which is a marker of undifferentiated cells, and the Vasa gene, which is specific for germ cell lineage (Oct4-positive Vasa-positive, sometimes abbreviated $Oct4^+/Vasa^+$). The Vasa gene is a gene that encodes ATP-dependent RNA helicase, which has a DEAD box, identified by an analysis of the germ cell aplasia mutation in the drosophila. Its homologs have been cloned in various mammals, including mice (Proc. Natl. Acad. Sci. USA, 91: 12258-62, 1994), rats (Rvh; Biochem. Biophys. Res. Commun., 207: 405-10, 1995), pigs (Mol. Reprod. Dev., 72: 320-8, 2005), and humans (Proc. Natl. Acad. Sci. USA, 97: 9585-90, 2000), [annotation analysis predicts the presence of homologs in chimpanzees (XM_517757) and dogs (XM_544339)]; all these homologs are known to be expressed specifically in germ cells. Mammalian vasa homologs are also known as DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4) genes. Herein, homologs of the drosophila vasa gene are generically referred to as Vasa genes, with a particular homolog, in the case of mice, for example, denoted as the mouse vasa homolog (Mvh).

As shown by the results of various characterization analyses in Examples below, the germline stem cells (also referred to as GR cells) of the present invention are PGC-like cells that reflect the characteristics of primordial germ cells (PGC) in the later stage of migration (just before or just after penetration into the fetal genital primordium). The germline stem cells are stem cells that can be maintained and expanded under the maintain-culturing conditions of the

present invention (possessing the capability of self-regeneration), and are destined to become germline cells because they are capable of survive for a long time without forming tumors and without being eliminated when transplanted to the testis.

[0012]

I. Pluripotent stem cells

The pluripotent stem cells that serve as a starting material in the present invention are not particularly limited, as far as they are undifferentiated cells having "a potential for self-regeneration" to proliferate while maintaining the undifferentiated state, and "pluripotency", the capability of differentiating into all the three primary germ layers of the embryo; examples include iPS cells and ES cells, as well as embryonic germ (EG) cells derived from primordial germ cells, multipotent germline stem (mGS) cells isolated in the process of establishment and cultivation of GS cells from testicular tissue, multipotent adult progenitor cells (MAPC) isolated from the bone marrow and the like. The ES cells may be ones resulting from nuclear reprogramming of somatic cells. Although iPS cells or ES cells are preferred, mGS cells and MAPC are also preferred because they can be acquired from live-born individuals. The methods of the present invention are applicable to optionally chosen mammals for which any pluripotent stem cells have been established or are establishable; examples include humans, mice, monkeys, pigs, rats, dogs and the like, with preference given to humans or mice.

[0013]

II. How to produce pluripotent stem cells

An example production of iPS cells suitable as pluripotent stem cells in the present invention is described below, but is not to be construed as limiting the scope of the present invention.

(A) Source of somatic cells

Any cells other than germ cells of mammalian origin (e.g., humans, mice, monkeys, pigs, rats and the like) can be used as starting material for producing iPS cells in the present invention. Examples include keratinizing epithelial cells (e.g., keratinized epidermal cells), mucosal epithelial cells (e.g., epithelial cells of the superficial layer of tongue), exocrine gland epithelial cells (e.g., mammary gland cells), hormone-secreting cells (e.g., adrenomedullary cells), cells for metabolism or storage (e.g., liver cells), intimal epithelial cells constituting interfaces (e.g., type I alveolar cells), intimal epithelial cells of the obturator canal (e.g., vascular endothelial cells), cells having cilia with transporting capability (e.g., airway epithelial cells), cells for extracellular matrix secretion (e.g., fibroblasts), constrictive cells (e.g., smooth muscle cells), cells of the blood and the immune system (e.g., T lymphocytes), sense-related cells (e.g., rod cells), autonomic nervous system neurons (e.g., cholinergic neurons), sustentacular cells of sensory organs and peripheral neurons (e.g., satellite cells), nerve cells and glia cells of the central nervous system (e.g., astroglia cells), pigment cells (e.g., retinal pigment epithelial cells), progenitor cells (tissue progenitor cells) thereof and the like. There is no limitation on the degree of cell differentiation, the age of an animal from which cells are collected and the like; even undifferentiated progenitor cells (including somatic stem cells) and finally differentiated mature cells can be used alike as sources of somatic cells in the present invention. Examples of undifferentiated progenitor cells include tissue stem cells (somatic stem cells) such as nerve stem cells, hematopoietic stem cells, mesenchymal stem cells, and dental pulp stem cells.

[0014]

The choice of mammalian individual as a source of somatic cells is not particularly limited; however, when the desired germline stem cells (GR cells) are to be used for the

treatment of infertility, it is preferable to collect somatic cells from the patient. Meanwhile, when the GR cells are to be used for gene therapy for germ cells and the like, it is preferable, from the viewpoint of preventing graft rejections, to collect somatic cells from the patient or another person with the same or substantially the same HLA type as that of the patient. "Substantially the same" as used here means that the HLA type of the donor matches with that of the patient to the extent that the transplanted cells, which have been obtained by inducing the differentiation of iPS cells derived from the donor's somatic cells, can engraft when transplanted to the patient with the use of an immunosuppressant and the like. For example, it includes an HLA type wherein major HLAs (e.g., the three loci of HLA-A, HLA-B and HLA-DR, four loci further including HLA-Cw) are identical and the like (the same applies below). When the GR cells are not to be administered (transplanted) to a human, but used as, for example, a source of cells for screening for evaluating drug susceptibility or adverse reactions in a patient's testis, it is likewise desirable to collect the somatic cells from the patient or another person with the same genetic polymorphism correlating with the drug susceptibility or adverse reactions.

[0015]

Somatic cells isolated from a mammal can be pre-cultured using a medium known per se suitable for their cultivation according to the choice of cells before being subjected to the step of nuclear reprogramming. Examples of such media include, but are not limited to, minimal essential medium (MEM) containing about 5% to 20% fetal calf serum (FCS), Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium, and the like. When a transfer reagent such as cationic liposome, for example, is used in bringing the somatic cell into contact with a nuclear reprogramming substance (and an iPS cell establishment efficiency improver if required), it is sometimes preferable that the medium have been replaced with a

serum-free medium so as to prevent the transfer efficiency from decreasing.

[0016]

(B) Nuclear reprogramming substances

In the present invention, "a nuclear reprogramming substance" can be a proteinous factor(s) capable of inducing iPS cells from a somatic cell or a nucleic acid that encodes the same (including forms incorporated in a vector). A nuclear reprogramming substance used in the present invention may be a gene described in WO 2007/069666. More specifically, examples include Oct3/4, Klf4, Klf1, Klf2, Klf5, Sox2, Sox1, Sox3, Sox15, Sox17, Sox18, c-Myc, L-Myc, N-Myc, TERT, SV40 Large T antigen, HPV16 E6, HPV16 E7, Bmi1, Lin28, Lin28b, Nanog, Esrrb and Esrrg. These reprogramming substances may be used in combination when establishing iPS cells; the combination comprises at least one, two, or three of the aforementioned reprogramming substances, with preference given to a combination comprising four. Specifically, examples include the following combinations (hereinafter, only the names for proteinous factors are shown).

(1) Oct3/4, Klf4, Sox2, c-Myc (here, Sox2 is replaceable with Sox1, Sox3, Sox15, Sox17 or Sox18; Klf4 is replaceable with Klf1, Klf2 or Klf5; c-Myc is replaceable with L-Myc or N-Myc)

(2) Oct3/4, Klf4, Sox2, c-Myc, TERT, SV40 Large T antigen (hereinafter, SV40LT)

(3) Oct3/4, Klf4, Sox2, c-Myc, TERT, HPV16 E6

(4) Oct3/4, Klf4, Sox2, c-Myc, TERT, HPV16 E7

(5) Oct3/4, Klf4, Sox2, c-Myc, TERT, HPV16 E6, HPV16 E7

(6) Oct3/4, Klf4, Sox2, c-Myc, TERT, Bmi1

(7) Oct3/4, Klf4, Sox2, c-Myc, Lin28

(8) Oct3/4, Klf4, Sox2, c-Myc, Lin28, SV40LT

(9) Oct3/4, Klf4, Sox2, c-Myc, Lin28, TERT, SV40LT

(10) Oct3/4, Klf4, Sox2, c-Myc, SV40LT

(11) Oct3/4, Esrrb, Sox2, c-Myc (Esrrb is replaceable with Esrrg)

(12) Oct3/4, Klf4, Sox2

(13) Oct3/4, Klf4, Sox2, TERT, SV40LT

(14) Oct3/4, Klf4, Sox2, TERT, HPV16 E6

(15) Oct3/4, Klf4, Sox2, TERT, HPV16 E7

(16) Oct3/4, Klf4, Sox2, TERT, HPV16 E6, HPV16 E7

(17) Oct3/4, Klf4, Sox2, TERT, Bmi1

(18) Oct3/4, Klf4, Sox2, Lin28

(19) Oct3/4, Klf4, Sox2, Lin28, SV40LT

(20) Oct3/4, Klf4, Sox2, Lin28, TERT, SV40LT

(21) Oct3/4, Klf4, Sox2, SV40LT

(22) Oct3/4, Esrrb, Sox2 (Esrrb is replaceable with Esrrg)

In (1)-(22) above, L-Myc can be used in place of c-Myc, and Lin28b can be used in place of Lin28.

[0017]

Any combination that does not fall in (1) to (22) above but comprises all the constituents of any one of (1) to (22) above and further comprises an optionally chosen other substance can also be included in the scope of "nuclear reprogramming substances" in the present invention. Provided that the somatic cell to undergo nuclear reprogramming is endogenously expressing one or more of the constituents of any one of (1) to (22) above at a level sufficient to cause nuclear reprogramming, a combination of only the remaining constituents excluding the one or more constituents can also be included in the scope of "nuclear reprogramming substances" in the present invention.

[0018]

Of these combinations, the four factors Oct3/4, Sox2, Klf4 and c-Myc (or L-Myc) and the three factors Oct3/4, Sox2, and Klf4 exemplify preferable nuclear reprogramming substances. The five or four factors consisting of these combinations plus Lin28 (or Lin28b), and the six or five factors consisting of these combinations plus SV40 Large T antigen are also preferred.

[0019]

Information on mouse and human cDNA sequences of the aforementioned nuclear reprogramming substances is available with reference to the NCBI accession numbers mentioned in WO 2007/069666 (in the publication, Nanog is described as ECAT4; mouse and human cDNA sequence information on Lin28, Lin28b, Esrrb, Esrrg, and L-Myc can be acquired by referring to the following NCBI accession numbers, respectively); those skilled in the art are easily able to isolate these cDNAs.

Name of gene	Mouse	Human
Lin28	NM_145833	NM_024674
Lin28b	NM_001031772	NM_001004317
Esrrb	NM_011934	NM_004452
Esrrg	NM_011935	NM_001438
L-Myc	NM_008506	NM_001033081

When a proteinous factor is used as it is as a nuclear reprogramming substance, it can be prepared by inserting the cDNA obtained into an appropriate expression vector, transferring the vector into a host cell, culturing the cell, and recovering the recombinant proteinous factor from the culture. Meanwhile, when a nucleic acid that encodes a proteinous factor is used as a nuclear reprogramming substance, the cDNA obtained is inserted into a viral vector, plasmid vector, episomal vector or the like to construct an expression vector, which is subjected to the step of nuclear reprogramming.

[0020]

(C) How to transfer nuclear reprogramming substances to somatic cells

To transfer a nuclear reprogramming substance in the form of a proteinous factor to a somatic cell, transfer of a nuclear reprogramming substance to a somatic cell can be achieved using a method known per se for protein transfer into a cell. Such methods include, for example, the method using a protein transfer reagent, the method using a protein transfer domain (PTD) or cell penetrating peptide (CPP) fusion protein,

the microinjection method and the like. Protein transfer reagents are commercially available, including those based on a cationic lipid, such as BioPOTER Protein Delivery Reagent (Gene Therapy Systems), Pro-Ject™ Protein Transfection Reagent (PIERCE) and ProVectin (IMGENEX); those based on a lipid, such as Profect-1 (Targeting Systems); those based on a membrane-permeable peptide, such as Penetrain Peptide (Q biogene) and Chariot Kit (Active Motif), GenomONE (ISHIHARA SANGYO KAISHA, LTD.) utilizing HVJ envelope (inactivated hemagglutinating virus of Japan) and the like. The transfer can be achieved per the protocols attached to these reagents, a common procedure being as described below. A nuclear reprogramming substance is diluted in an appropriate solvent (e.g., a buffer solution such as PBS or HEPES), a transfer reagent is added, the mixture is incubated at room temperature for about 5 to 15 minutes to form a complex, this complex is added to cells after exchanging the medium with a serum-free medium, and the cells are incubated at 37°C for one to several hours. Thereafter, the medium is removed and replaced with a serum-containing medium.

Developed PTDs include those using transcellular domains of proteins such as drosophila-derived AntP, HIV-derived TAT [Frankel, A. et al., Cell 55, 1189-93 (1988); Green, M. and Loewenstein, P.M., Cell 55, 1179-88 (1988)], Penetratin [Derossi, D. et al., J. Biol. Chem. 269, 10444-50 (1994)], Buforin II [Park, C.B. et al., Proc. Natl Acad. Sci. USA 97, 8245-50 (2000)], Transportan [Pooga, M. et al., FASEB J. 12, 67-77 (1998)], MAP (model amphipathic peptide) [Oehlke, J. et al., Biochim. Biophys. Acta. 1414, 127-39 (1998)], K-FGF [Lin, Y.Z. et al., J. Biol. Chem. 270, 14255-14258 (1995)], Ku70 [Sawada, M. et al., Nature Cell Biol. 5, 352-7 (2003)], Prion [Lundberg, P. et al., Biochem. Biophys. Res. Commun. 299, 85-90 (2002)], pVEC [Elmquist, A. et al., Exp. Cell Res. 269, 237-44 (2001)], Pep-1 [Morris, M.C. et al., Nature Biotechnol. 19, 1173-6 (2001)], Pep-7 [Gao, C. et al., Bioorg. Med. Chem.

10, 4057-65 (2002)], SynBl [Rousselle, C. et al., Mol. Pharmacol. 57, 679-86 (2000)], HN-I [Hong, F.D. & Clayman, G L. Cancer Res. 60, 6551-6 (2000)], and HSV-derived VP22. CPPs derived from the PTDs include polyarginines such as 11R [Cell Stem Cell, 4:381-384 (2009)] and 9R [Cell Stem Cell, 4:472-476 (2009)].

[0021]

A fusion protein expression vector incorporating a cDNA of the nuclear reprogramming substance and the PTD or CPP sequence is prepared to allow recombinant expression, and the fusion protein is recovered and used for transfection. The transfection can be performed in the same manner as the above except that no protein transfer reagent is added.

[0022]

Microinjection, a method of placing a protein solution in a glass needle having a tip diameter of about 1 μm, and injecting the solution into a cell, ensures the transfer of the protein into the cell.

[0023]

The protein transferring operation can be performed one or more optionally chosen times (e.g., once or more to 10 times or less, or once or more to 5 times or less and the like). Preferably, the transferring operation can be performed twice or more (e.g., 3 times or 4 times) repeatedly. The time interval for repeated transferring operation is, for example, 6 to 48 hours, preferably 12 to 24 hours.

[0024]

When iPS cell establishment efficiency is emphasized, it is preferable that the nuclear reprogramming substance be used not as a proteinous factor, but in the form of a nucleic acid that encodes the same. The nucleic acid may be a DNA or RNA, and may be a DNA/RNA chimera. The nucleic acid may be double-stranded or single-stranded. Preferably, the nucleic acid is a double-stranded DNA, particularly a cDNA.

A cDNA of the nuclear reprogramming substance is inserted

into an appropriate expression vector comprising a promoter capable of functioning in the host somatic cell. Useful expression vectors include, for example, viral vectors such as retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, herpesvirus and Sendai virus, plasmids for the expression in animal cells (e.g., pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo) and the like.

[0025]

The type of a vector to be used can be chosen as appropriate according to the intended use of the GR cell to be differentiation-induced from the iPS cell obtained. For example, useful vectors include adenoviral vectors, plasmid vectors, adeno-associated viral vectors, retroviral vectors, lentiviral vectors, Sendai viral vectors, episomal vectors and the like.

[0026]

Examples of promoters used in expression vectors include the EF1α promoter, the CAG promoter, the SRα promoter, the SV40 promoter, the LTR promoter, the CMV (cytomegalovirus) promoter, the RSV (Rous sarcoma virus) promoter, the MoMuLV (Moloney mouse leukemia virus) LTR, the HSV-TK (herpes simplex virus thymidine kinase) promoter and the like, with preference given to the EF1α promoter, the CAG promoter, the MoMuLV LTR, the CMV promoter, the SRα promoter and the like.

[0027]

The expression vector may contain as desired, in addition to a promoter, an enhancer, a polyA addition signal, a selection marker gene, a SV40 replication origin and the like. Examples of selection marker genes include the dihydrofolate reductase gene, the neomycin resistance gene, the puromycin resistance gene and the like.

[0028]

Nucleic acids that are nuclear reprogramming substances (reprogramming genes) may be separately integrated onto respective expression vectors, and two or more, preferably two

or three, different genes may be integrated onto a single expression vector. It is preferable to choose the former mode when using a retrovirus or lentiviral vector, which offers high gene transfer efficiency, and to choose the latter mode when using a plasmid, adenovirus or episomal vector or the like. Furthermore, an expression vector incorporating two or more different genes and an expression vector incorporating one gene alone may be used in combination.

[0029]

In the above-described procedure, when a plurality of reprogramming genes (e.g., two or more, preferably two or three, genes selected from among Oct3/4, Sox2, Klf4, and c-Myc) are integrated into one expression vector, these genes can preferably be integrated into the expression vector via a sequence enabling polycistronic expression. Using a sequence enabling polycistronic expression makes it possible to more efficiently express a plurality of genes integrated in one expression vector. Useful sequences enabling polycistronic expression include, for example, the 2A sequence of foot-and-mouth disease virus (PLoS ONE 3, e2532, 2008, Stem Cells 25, 1707, 2007), the IRES sequence (U.S. Patent No. 4,937,190) and the like, with preference given to the 2A sequence.

[0030]

An expression vector comprising a reprogramming gene can be introduced into a cell by a technique known per se according to the choice of vector. In the case of a viral vector, for example, a plasmid containing the nucleic acid is introduced into an appropriate packaging cell (e.g., Plat-E cell) or a complementary cell line (e.g., 293-cells), the viral vector produced in the culture supernatant is recovered, and the vector is infected to the cell by a method suitable for the viral vector. For example, specific means using a retroviral vector are disclosed in WO2007/69666, Cell, 126, 663-676 (2006) and Cell, 131, 861-872 (2007). Specific means using a lentiviral vector is disclosed in Science,

318, 1917-1920 (2007). When GR cells differentiation-induced from iPS cells are utilized for medical purposes such as infertility treatment and germ cell gene therapy, the expression (reactivation) of the reprogramming gene potentially increases the risk of carcinogenesis in sperm and testicular tissue regenerated from GR cells derived from iPS cell. Therefore, the reprogramming gene is preferably expressed transiently, without being integrated into the chromosome of the cells. From this viewpoint, the use of an adenoviral vector, whose integration into chromosome is rare, is preferred. Specific means using an adenoviral vector is described in Science, 322, 945-949 (2008). Because an adeno-associated viral vector is also low in the frequency of integration into chromosome, and is lower than adenoviral vectors in terms of cytotoxicity and inflammation-inducibility, it can be mentioned as another preferred vector. Because a Sendai viral vector is capable of being stably present outside the chromosome, and can be degraded and removed using an siRNA as required, it is preferably utilized as well. Regarding a Sendai viral vector, one described in J. Biol. Chem., 282, 27383-27391 (2007) or Japanese Patent 3602058 can be used.

[0031]

When a retroviral vector or a lentiviral vector is used, even if silencing of the transgene occurs, it would possibly become reactivated; therefore, for example, a method can be used preferably wherein the nucleic acid that encodes the nuclear reprogramming substance is cut out using the Cre-loxP system, when the vector becomes no longer necessary. That is, with loxP sequences arranged on both ends of the nucleic acid in advance, iPS cells are induced, thereafter Cre recombinase is allowed to act on the cells using a plasmid vector or adenoviral vector, and the region sandwiched by the loxP sequences can be cut out. Because the enhancer-promoter sequence of the LTR U3 region possibly upregulates the host gene in the vicinity thereof by insertion mutation, it is more

preferable to avoid the expression control of the endogenous genes by the LTR outside of the loxP sequence remaining in the genome without being cut out, using a 3'-self-inactivated (SIN) LTR prepared by deleting the sequence, or substituting the sequence with a polyadenylation sequence such as of SV40. Specific means using the Cre-loxP system and SIN LTR is disclosed by Chang et al. in Stem Cells, 27: 1042-1049 (2009).

[0032]

Meanwhile, being a non-viral vector, a plasmid vector can be transferred into a cell using the lipofection method, liposome method, electroporation method, calcium phosphate co-precipitation method, DEAE dextran method, microinjection method, gene gun method and the like. Specific means using a plasmid as a vector are described in, for example, Science, 322, 949-953 (2008) and the like.

[0033]

When a plasmid vector, an adenoviral vector or the like is used, the transfection can be performed one or more optionally chosen times (e.g., once to 10 times, once to 5 times, and the like). When two or more different expression vectors are introduced into a somatic cell, it is preferable that all these expression vectors be introduced into the somatic cell at one time. Even in this case, however, the transfection can be performed one or more optionally chosen times (e.g., once to 10 times, once to 5 times, and the like); preferably, the transfection can be repeatedly performed twice or more (e.g., 3 times or 4 times).

[0034]

When an adenovirus or a plasmid is used, the transgene can get integrated into chromosome; therefore, it is eventually necessary to confirm the absence of insertion of the gene into chromosome by Southern blotting or PCR. For this reason, as with the aforementioned Cre-loxP system, it can be advantageous to use a means wherein the transgene is integrated into chromosome and then removed. In another

preferred mode of embodiment, a method can be used wherein the transgene is integrated into chromosome using a transposon, thereafter transposase is allowed to act on the cell using a plasmid vector or adenoviral vector so as to completely eliminate the transgene from the chromosome. Examples of preferable transposons include piggyBac, a transposon derived from a lepidopterous insect, and the like. Specific means using the piggyBac transposon is disclosed by Kaji, K. et al. in Nature, 458: 771-775 (2009), and by Woltjen et al. in Nature, 458: 766-770 (2009).

Another preferable non-integration type vector is an episomal vector, which is capable of self-replication outside of the chromosome. Specific means using an episomal vector is disclosed by Yu et al. in Science, 324, 797-801 (2009).

[0035]

Examples of episomal vectors used in the present invention include a vector comprising sequences required for autonomous replication from EBV, SV40 and the like as vector components. Specifically, vector components required for autonomous replication are a replication origin and a gene that encodes a protein that binds to the replication origin to control the replication, exemplified by the replication origin oriP and the EBNA-1 gene for EBV, and by the replication origin ori and the SV40 large T antigen gene for SV40.

[0036]

An episomal expression vector comprises a promoter that controls the transcription of reprogramming genes. As the promoter, the same promoters as those mentioned above can be used. The episomal expression vector may further comprise an enhancer, a polyA addition signal, a selection marker gene and the like if desired, as described above. Examples of selection marker genes include the dihydrofolate reductase gene, the neomycin resistance gene and the like.

[0037]

An episomal vector can be transferred into a cell using,

for example, the lipofection method, liposome method, electroporation method, calcium phosphate co-precipitation method, DEAE dextran method, microinjection method, gene gun method and the like. Specifically, methods described in Science, 324: 797-801 (2009) and the like can be used.

[0038]

Whether the episomal vector has been removed from the iPS cell can be confirmed by performing Southern blot analysis or PCR analysis using a portion of the vector as a probe or primer, with the episome fraction isolated from the iPS cell as a template, and determining the presence or absence of a band or the length of the band detected. The episome fraction can be prepared by a method obvious in the art; for example, methods described in Science, 324: 797-801 (2009) and the like, can be used.

[0039]

(D) Functional inhibitors of p53

In the present invention, it is more preferable to contact, in addition to the above-described nuclear reprogramming substances, a functional inhibitor of p53, with a somatic cell. As mentioned herein, "a functional inhibitor of p53" may be any substance, as far as it is capable of inhibiting either (a) the function of the p53 protein or (b) the expression of the p53 gene. That is, not only substances that act directly on the p53 protein to inhibit the function thereof and substances that act directly on the p53 gene to inhibit the expression thereof, but also substances that act on a factor involved in p53 signal transduction to result in the inhibition of the function of the p53 protein or the expression of the p53 gene, are also included in the scope of "a functional inhibitor of p53" as mentioned herein. Preferably, the functional inhibitor of p53 is a substance that inhibits the expression of the p53 gene, more preferably an expression vector that encodes an siRNA or shRNA against p53.

[0040]

Examples of substances that inhibit the function of the p53 protein include, but are not limited to, a chemical inhibitor of p53, a dominant negative mutant of p53 or a nucleic acid that encodes the same, an anti-p53 antagonist antibody or a nucleic acid that encodes the same, a decoy nucleic acid comprising a consensus sequence of a p53-responsive element, a substance that inhibits the p53 pathway, and the like. Preferably, a chemical inhibitor of p53, a dominant negative mutant of p53 or a nucleic acid that encodes the same, and a p53 pathway inhibitor can be mentioned.

[0041]

(D1) Chemical inhibitors of p53

Examples of chemical inhibitors of p53 include, but are not limited to, p53 inhibitors typified by pifithrin (PFT)-α and -β, which are disclosed in WO 00/44364, PFT-μ disclosed by Storm et al. in Nat. Chem. Biol. 2, 474 (2006), analogues thereof and salts thereof (e.g., acid addition salts such as hydrochlorides and hydrobromides, and the like) and the like. Thereof, PFT-α and analogues thereof [2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-p-tolylethanone, HBr (trade name: Pifithrin-α) and 1-(4-nitrophenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2H)-benzothiazolyl)ethanone, HBr (trade name: Pifithrin-α, p-Nitro)] and PFT-β and analogues thereof [2-(4-methylphenyl)imidazo[2,1-b]-5,6,7,8-tetrahydrobenzothiazole, HBr (trade name: Pifithrin-α, Cyclic) and 2-(4-nitrophenyl)imidazo[2,1-b]-5,6,7,8-tetrahydrobenzothiazole (trade name: Pifithrin-α, p-Nitro, Cyclic)], PFT-μ [phenylacetylenylsulfonamide (trade name: Pifithrin-μ)] are commercially available from Merck Company.

[0042]

Contact of a chemical inhibitor of p53 with a somatic cell can be performed by dissolving the inhibitor at an appropriate concentration in an aqueous or non-aqueous solvent, adding the solution of the inhibitor to a medium suitable for

cultivation of somatic cells isolated from a human or mouse [for example, minimal essential medium (MEM) comprising about 5% to 20% fetal bovine serum, Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium and the like] so that the inhibitor concentration will fall in a range that fully inhibits the function of p53 and does not cause cytotoxicity, and culturing the cells for a given period. The inhibitor concentration varies depending on the kind of inhibitor used, and is chosen as appropriate over the range of about 0.1 nM to about 100 nM. Duration of contact is not particularly limited, as far as it is sufficient to achieve cell nuclear reprogramming; usually, the inhibitor may be allowed to be co-present in the medium until a positive colony emerges.

[0043]

The p53 gene is known as a cancer suppressor gene; permanent functional inhibition of p53 potentially possibly increases the risk of carcinogenesis. Chemical inhibitors of p53 are useful, not only because of the advantage of permitting introduction into cells simply by the addition to the medium, but also because of the ability to cancel the functional inhibition of p53, easily and quickly, by removing the medium containing the inhibitor after induction of iPS cells.

[0044]

(D2) Dominant negative mutants of p53

The choice of dominant negative mutant of p53 is not particularly limited, as far as the mutant is capable of competitively acting against the wild-type p53 protein being endogenously present in somatic cells to inhibit the function thereof; examples include p53P275S, resulting from point mutation of the proline at the 275-position (in the case of humans, 278-position) located in the DNA-binding region of mouse p53 to serine [de Vries, A., Proc. Natl. Acad. Sci. USA, 99, 2948-2953 (2002)]; p53DD, resulting from deletion of the

amino acids at the 14-301-positions of mouse p53 (in human p53, corresponds to the 11-304-positions) [Bowman, T., Genes Develop., 10, 826-835 (1996)], and the like. Other known mutants include, for example, p53S58A, resulting from point mutation of the serine at the 58-position of mouse p53 (in the case of humans, 61-position) to alanine; p53C135Y, resulting from point mutation of the cysteine at the 135-position of human p53 (in the case of mice, 132-position) to tyrosine; p53A135V, resulting from point mutation of the alanine at the 135-position of mouse p53 (in the case of humans, 138-position) to valine; p53R172H, resulting from point mutation of the arginine at the 172-position (in the case of humans, 175-position) to histidine; p53R270H, resulting from point mutation of the arginine at the 270-position (in the case of humans, 273-position) to histidine; p53D278N, resulting from point mutation of the aspartic acid at the 278-position of mouse p53 (in the case of humans, 281-position) to asparagine, and the like; these can be used in the same way.

[0045]

A dominant negative mutant of p53 can be obtained by, for example, the technique described below. First, an appropriate oligonucleotide is synthesized as a probe or primer on the basis of mouse or human p53 cDNA sequence information, and a mouse or human p53 cDNA is cloned from a mRNA, cDNA or cDNA library derived from a mouse or human cell or tissue, using the hybridization method or the (RT-)PCR method, and is subcloned into an appropriate plasmid. In a form wherein a codon of the site into which a mutation is to be introduced (for example, in the case of p53P275S, cct, the codon that encodes Pro at the 275-position) is replaced with a codon that encodes another desired amino acid (for example, in the case of p53P275S, tct, the codon that encodes Ser), a primer comprising the site is synthesized, and inverse PCR is performed using this primer with the plasmid incorporating the p53 cDNA as a template, whereby a nucleic acid that encodes

the desired dominant negative mutant is acquired. In the case of a deletion mutant like p53DD, a primer may be designed outside the site to be deleted, and inverse PCR may be performed as described above. By introducing the thus-obtained nucleic acid that encodes the dominant negative mutant into a host cell, culturing the cell, and recovering the recombinant protein from the resulting culture, the desired dominant negative mutant can be acquired.

[0046]

Contact of a dominant negative mutant with a somatic cell can be achieved in the same way as with the above-described case of a proteinous nuclear reprogramming substance. As described above, permanent functional inhibition of p53 possibly increases the risk of carcinogenesis; however, because a dominant negative mutant of p53 undergoes degradation by protease in the transfected cell and disappears gradually, and correspondingly the function of p53 endogenously occurring in the cell is restored, the use of the mutant protein can be suitable in cases where high safety is required as in the case where the iPS cells obtained are utilized for therapeutic purposes.

[0047]

(D3) Nucleic acids that encode dominant negative mutants of p53

In another preferred mode of embodiment of the present invention, the functional inhibitor of p53 is a nucleic acid that encodes a dominant negative mutant of p53. The nucleic acid may be a DNA or RNA or a DNA/RNA chimera, and is preferably a DNA. The nucleic acid may be double-stranded or single-stranded. A cDNA that encodes a dominant negative mutant of p53 can be cloned by the technique described above with respect to preparation of the mutant protein.

The cDNA isolated can be inserted into an appropriate expression vector and transferred to a somatic cell in the same way as the above-described case of nucleic acids that are

nuclear reprogramming substances (reprogramming genes).

[0048]

(D4) p53 pathway inhibitors

Here, the term p53 pathway is used with a meaning including all upstream signal cascades that can activate p53 and all downstream signal cascades mediated by activated p53. Therefore, p53 pathway inhibitors include all substances that inhibit any one of the aforementioned signal transduction pathways. In a preferred mode of embodiment, however, the p53 pathway inhibitor is a substance that inhibits the expression or function (Myc inhibitory activity) of p21, whose transcription is activated by p53; examples include siRNA, shRNA, antisense nucleic acids, ribozymes and the like against p21. These nucleic acids that inhibit the expression of p21 can be designed and synthesized and introduced into a somatic cell in the same manner as the method for siRNA, shRNA, antisense nucleic acids, and ribozymes against p53 described below. The nucleic acids may be provided in the form of a vector that expresses them; the vector can be constructed and introduced into a somatic cell in the same manner as the method for a vector that expresses an siRNA, shRNA, antisense nucleic acid, or ribozyme against p53 described below.

[0049]

In another preferred mode of embodiment, the p53 pathway inhibitor is a substance that inhibits the ARF-MDM2-p53 pathway. Examples of ARF-MDM2-p53 pathway inhibitors include MDM2, which binds directly to p53 to promote the nuclear export or ubiquitination thereof, or a nucleic acid that encodes the same, $p19^{ARF}$, which inhibits the action of MDM2 on p53, a substance that inhibits the expression or function of ATM (ataxia-telangiectasia mutated) (for example, siRNAs and shRNAs against these factors) and the like.

[0050]

(D5) Other substances

Examples of other substances that inhibit the function of

the p53 protein include an anti-p53 antagonist antibody and a nucleic acid that encodes the same. The anti-p53 antagonist antibody may be a polyclonal antibody or a monoclonal antibody. The isotype of the antibody is not particularly limited, and is preferably IgG, IgM or IgA, particularly preferably IgG. The antibody may be, in addition to a complete antibody molecule, for example, a fragment such as Fab, Fab', or $F(ab')_2$, a conjugate molecule prepared by a gene engineering technique, such as scFv, scFv-Fc, minibody, or diabody, or a derivative thereof modified with a molecule having protein-stabilizing action, such as polyethylene glycol (PEG). An anti-p53 antagonist antibody can be produced using p53 or a partial peptide thereof as an antigen, by a method of antibody or anti-serum production known per se. Examples of publicly known anti-p53 antagonist antibodies include PAb1801 (Oncogene Science Ab-2), DO-1 (Oncogene Science Ab-6) [Gire and Wynford-Thomas, Mol. Cell. Biol., 18, 1611-1621 (1998)] and the like. A nucleic acid that encodes an anti-p53 antagonist antibody can be isolated from a hybridoma that produces an anti-p53 monoclonal antibody by a conventional method. The H-chain and L-chain genes obtained may be joined together to prepare a nucleic acid that encodes a single-chain antibody.

[0051]

As another substance that inhibits the function of the p53 protein, an anti-p21 antagonist antibody or a nucleic acid that encodes the same can be mentioned. An anti-p21 antagonist antibody and a nucleic acid that encodes the same can also be prepared as with the aforementioned anti-p53 antagonist antibody and nucleic acid that encodes the same.

Still another substance that inhibits the function of the p53 protein is a decoy nucleic acid comprising a consensus sequence of p53-responsive element [e.g., Pu-Pu-Pu-G-A/T-T/A-C-Py-Py-Py (Pu: purine base, Py: pyrimidine base)]. Such a nucleic acid can be synthesized on the basis of the aforementioned base sequence information using an automated

DNA/RNA synthesizer. Alternatively, such a decoy nucleic acid is commercially available [e.g., p53 transcription factor decoy (GeneDetect.com)].

An anti-p53 antagonist antibody or an anti-p21 antagonist antibody, as with a dominant negative mutant of p53, and a nucleic acid that encodes the antibody, as with a nucleic acid that encodes the mutant, can be introduced into a cell. The aforementioned decoy nucleic acid can be introduced into a cell by the lipofection method and the like.

[0052]

Meanwhile, examples of substances that inhibit the expression of the p53 gene include siRNAs or shRNAs against p53, vectors that express an siRNA or shRNA against p53, antisense nucleic acids against p53 and ribozymes against p53, and the like, with preference given to siRNAs and shRNAs against p53 and vectors that express an siRNA or shRNA.

[0053]

(D6) siRNAs and shRNAs against p53

An siRNA against p53 can be designed on the basis of mouse or human p53 cDNA sequence information, in accordance with, for example, the rules proposed by Elbashir et al. [Genes Dev., 15, 188-200 (2001)]. The target sequence for the siRNA is, as a general rule, AA+(N)19, but may be AA+(N)21 or NA+(N)21. The 5′ end of the sense strand need not to be AA. Although the position of the target sequence is not particularly limited, it is desirable that the target sequence be selected between 5′-UTR and about 50 bases from the start codon, as well as from a region other than 3′-UTR. The GC content of the target sequence is also not particularly limited, but the content is preferably about 30% to about 50%; a sequence with no irregularity in GC distribution and with only a few repeats is desirable. When a polIII system promoter is used as the promoter in designing a vector that expresses an siRNA or shRNA of (b2) below, a sequence of 4 or more bases of T or A in succession should not be chosen, so as to prevent

polymerase transcription from ceasing.

The target sequence candidates selected on the basis of the above-described rules are examined for homology to sequences of 16-17 bases in succession in mRNAs other than the target, using a homology search software program such as BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), so as to confirm the specificity of the target sequences selected. For the target sequences for which the specificity has been confirmed, a double-stranded RNA consisting of a sense strand having a 3'-terminal overhang of TT or UU in 19-21 bases after AA (or NA), and an antisense strand having a sequence complementary to the 19-21 bases and a 3'-terminal overhang of TT or UU, is designed as an siRNA. Also, an shRNA can be designed by optionally choosing as appropriate a linker sequence capable of forming a loop structure (for example, about 8-25 bases), and ligating the aforementioned sense and antisense strands via the linker sequence.

[0054]

Sequences of siRNAs and/or shRNAs can be searched for using search software programs available at no cost on various websites. Examples of such sites include, but are not limited to, the siRNA Target Finder (http://www.ambion.com/jp/techlib/misc/siRNA_finder.html) and insert design tool for pSilencer™ Expression Vector (http://www.ambion.com/jp/techlib/misc/psilencer_converter.html), both provided by Ambion, and the GeneSeer (http://codex.cshl.edu/scripts/newsearchhairpin.cgi), provided by RNAi Codex; and similar search is possible on the websites of QIAGEN, Takara Bio, SiSearch, Dharmacon, Whitehead Institute, Invitrogen, Promega and the like.

[0055]

An siRNA against p53 can be prepared by synthesizing a sense strand oligonucleotide and antisense strand oligonucleotide designed as described above using an automated DNA/RNA synthesizer and, for example, denaturing the

oligonucleotides in an appropriate annealing buffer solution at about 90°C to about 95°C for about 1 minute, thereafter annealing the same at about 30°C to about 70°C for about 1 hour to about 8 hours. An shRNA against p53 can be prepared by synthesizing oligonucleotides having an shRNA sequence, designed as described above, using an automated DNA/RNA synthesizer, and allowing the same to self-anneal as described above.

[0056]

Although the nucleotide molecules that constitute the siRNA or shRNA may be natural-type RNAs, the molecules can comprise various chemical modifications in order to increase the stability (chemical and/or to-enzyme) or specific activity (affinity for mRNA). For example, to prevent degradation by hydrolases, such as nuclease, the phosphoric acid residue (phosphate) of each nucleotide that constitutes the antisense nucleic acid can be substituted with, for example, a chemically modified phosphoric acid residue such as phosphorothioate (PS), methylphosphonate, or phosphorodithionate. The hydroxyl group at the 2'-position of the sugar (ribose) of each nucleotide may be replaced with -OR [R represents, for example, CH_3(2'-O-Me), $CH_2CH_2OCH_3$(2'-O-MOE), $CH_2CH_2NHC(NH)NH_2$, $CH_2CONHCH_3$, CH_2CH_2CN or the like]. Furthermore, a base moiety (pyrimidine, purine) may be chemically modified; examples of such modifications include introduction of a methyl group or a cationic functional group into the 5-position of the pyrimidine base, substitution of the 2-position carbonyl group with thiocarbonyl and the like.

[0057]

Regarding the conformation of the sugar moiety of RNA, two types are dominant: C2'-endo (S type) and C3'-endo (N type); in a single-stranded RNA, the sugar moiety occurs in an equilibrium of both, but when a double strand is formed, the conformation is fixed at the N type. Therefore, BNA (LNA) (Imanishi, T. et al., Chem. Commun., 1653-9, 2002; Jepsen, J.S.

et al., Oligonucleotides, 14, 130-46, 2004) and ENA (Morita, K. et al., Nucleosides Nucleotides Nucleic Acids, 22, 1619-21, 2003), which are RNA derivatives wherein the conformation of the sugar moiety is fixed at the N type by bridging the 2' oxygen and 4' carbon so as to confer strong bindability to the target RNA, can also be used preferably.

However, because replacing all ribonucleoside molecules in natural-type RNA with modified type molecules can lead to the loss of RNAi activity, it is necessary to introduce a nucleoside modified to the minimum possible extent that allows the RISC complex to function.

[0058]

An siRNA against p53 can also be purchased from, for example, Ambion (e.g., Ambion Cat# AM16708, siRNA ID# 69659, 69753, 69843, 187424, 187425, 187426), Santa Cruz (e.g., Santa Cruz Cat# sc-29436, 44219) and the like.

An siRNA and shRNA against human p53 can also be designed and synthesized using one of the aforementioned search software programs, by inputting human p53 cDNA sequence information (e.g., Refseq. No. NM_000546) and the like as a query, or can also be purchased from Ambion and the like. Specifically, the shRNA against human p53 described in Science, 296, 550-553 (2002) and the like can be mentioned.

[0059]

Contact of an siRNA or shRNA against p53 with a somatic cell can be achieved by, as in the case of plasmid DNA, introducing the nucleic acid into the cell using the liposome method, polyamine method, electroporation method, beads method and the like. The method using a cationic liposome is the most common and offers high transfer efficiency. In addition to common transfection reagents such as Lipofectamine2000 and Oligofectamine (Invitrogen), for example, transfer reagents suitable for introduction of an siRNA, such as the GeneEraser™ siRNA transfection reagent (Stratagene), are also commercially available.

[0060]

(D7) Vectors that express an siRNA or shRNA against p53

Vectors that express an siRNA are available in the tandem type and the stem loop (hairpin) type. The former is the type in which an expression cassette for a sense strand of an siRNA and an expression cassette for an antisense strand are ligated tandem, each strand being expressed in the cell and undergoing annealing to form a double-stranded siRNA (dsRNA). Meanwhile, the latter is the type in which an expression cassette for an shRNA is inserted into a vector, the shRNA being expressed in the cell and undergoing processing by a dicer to form a dsRNA. Although a polII system promoter (for example, immediate-early promoter of CMV) may be used as the promoter, it is common practice to use a polIII system promoter in order to allow the accurate transcription of short RNA. As the polIII system promoter, mouse and human U6-snRNA promoters, human H1-RNase P RNA promoter, human valine-tRNA promoter and the like can be mentioned. As a transcription termination signal, a sequence of four or more T residues in succession is used.

[0061]

The siRNA or shRNA expression cassette thus constructed is then inserted into a plasmid vector, episomal vector, viral vector or the like. As such vectors, the same as those described with respect to nucleic acids that are nuclear reprogramming substances (reprogramming genes) can be utilized preferably (viral vectors such as retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, herpesviruses, and Sendai virus; animal cell expression plasmids, episomal vectors and the like). The vector used can be chosen as appropriate according to the intended use of the GR cells differentiation-induced from the iPS cell obtained, as in the case of reprogramming genes. Because permanent functional inhibition of p53 potentially increases the risk of carcinogenesis, as described above, it is preferable to use a vector capable of transiently expressing p53 and leaving the

cell soon after establishment of iPS cells (e.g., plasmid vector and the like), when the GR cells are used for medical purposes in humans. Alternatively, as an expression vector that encodes an shRNA against p53, a viral vector such as a retrovirus, a plasmid vector, an episomal vector and the like prepared on the basis of a commercially available plasmid (for example, pMKO.1-puro p53 shRNA2: #10672, commercially available from Addgene, and the like) or the like can also be used. The aforementioned Cre-loxP system or piggyBac transposon system can also be utilized as required.

[0062]

Contact of a vector that expresses an siRNA or shRNA against p53 with a somatic cell is achieved by introducing a plasmid vector, episomal vector or viral vector prepared as described above into the cell. Transfer of these genes can be achieved by the same technique as that described with respect to reprogramming genes.

[0063]

(D8) Other substances

Other substances that inhibit the expression of the p53 gene include antisense nucleic acids and ribozymes against p53.

The antisense nucleic acid may be a DNA or RNA or a DNA/RNA chimera. When the antisense nucleic acid is a DNA, an RNA:DNA hybrid formed by a target RNA and the antisense DNA is capable of being recognized by endogenous RNase H to cause selective degradation of the target RNA. Therefore, in the case of an antisense DNA to direct degradation by RNase H, the target sequence may be not only a sequence in p53 mRNA, but also a sequence in the intron region of the initial transcription product of the p53 gene. The length of the target region for the antisense nucleic acid is not particularly limited, as far as hybridization of the antisense nucleic acid results in the inhibition of the translation into the p53 protein; the target region may be the entire sequence or a partial sequence of p53 mRNA, and may be a sequence of

about 15 bases for the shortest, or of the entire sequence of the mRNA or initial transcription product for the longest. Considering the ease of synthesis, antigenicity, transferability in cells and other issues, an oligonucleotide consisting of about 15-40 bases, particularly about 18 to 30 bases, is preferable. Positions of the target sequence include, but are not limited to, 5′- and 3′-UTR, vicinities of the start codon and the like.

A ribozyme refers to an RNA possessing an enzyme activity to cleave a nucleic acid in the narrow sense, and is herein understood to be used as a concept encompassing DNA, as far as the ribozyme possesses sequence-specific nucleic acid cleavage activity. One of the most versatile ribozymes is a self-splicing RNA found in infectious RNAs such as viroid and virusoid, and the hammerhead type, the hairpin type and the like are known. The hammerhead type exhibits enzyme activity with about 40 bases in length, and it is possible to specifically cleave the target mRNA by making several bases at both ends adjoining to the hammerhead structure portion (about 10 bases in total) be a sequence complementary to the desired cleavage site of the mRNA.

[0064]

An antisense nucleic acid or a ribozyme can be synthesized using an automated DNA/RNA synthesizer. The nucleotide molecules that constitute them may also have the same modifications as those for siRNA, so as to increase the stability, specific activity and the like.

Alternatively, the antisense nucleic acid or ribozyme can also be used in the form of a nucleic acid that encodes the same, as in the case of siRNA.

[0065]

The aforementioned functional inhibitor of p53 need to be brought into contact with a somatic cell in a way sufficient to inhibit the function of p53 in the step of somatic cell nuclear reprogramming. As far as this requirement is met, the

nuclear reprogramming substance and the functional inhibitor of p53 may be brought into contact with the somatic cell simultaneously, or either one may be contacted in advance. In a mode of embodiment of the present invention, for example, when the nuclear reprogramming substance is a nucleic acid that encodes a proteinous factor, and the functional inhibitor of p53 is a chemical inhibitor, the former involves a given length of time lag from the transfection treatment to the mass-expression of the proteinous factor, whereas the latter is capable of rapidly inhibiting the function of p53. Therefore, after the cell is cultured for a given length of time after the transfection treatment, the chemical inhibitor of p53 can be added to the medium. In another mode of embodiment, for example, when the nuclear reprogramming substance and the functional inhibitor of p53 are both used in the form of a viral vector, plasmid vector, episomal vector or the like, both may be introduced into the cell simultaneously.

[0066]

(E) Other iPS cell establishment efficiency improvers

By bringing, in addition to the above-described reprogramming factors and functional inhibitors of p53, another publicly known iPS cell establishment efficiency improver into contact with a somatic cell, the efficiency of establishment of iPS cells is expected to be increased more. Examples of such iPS cell establishment efficiency improvers include, but are not limited to, histone deacetylase (HDAC) inhibitors [e.g., low-molecular inhibitors such as valproic acid (VPA) (Nat. Biotechnol., 26(7): 795-797 (2008)), trichostatin A, sodium butyrate, MC 1293, and M344; nucleic acid-based expression inhibitors such as siRNAs and shRNAs against HDAC (e.g., HDAC1 siRNA Smartpool (registered trademark) (Millipore), HuSH 29mer shRNA Constructs against HDAC1 (OriGene) and the like); and the like], G9a histone methyltransferase inhibitors [e.g., low-molecular inhibitors such as BIX-01294 (Cell Stem Cell, 2: 525-528 (2008)); nucleic

acid-based expression inhibitors such as siRNAs and shRNAs against G9a (e.g., G9a siRNA (human) (Santa Cruz Biotechnology) and the like; and the like], L-calcium channel agonists (e.g., Bayk8644) [Cell Stem Cell, 3, 568-574 (2008)], UTF1 [Cell Stem Cell, 3, 475-479 (2008)], Wnt Signaling (e.g., soluble Wnt3a) [Cell Stem Cell, 3, 132-135 (2008)], 2i/LIF [2i is an inhibitor of mitogen-activated protein kinase signaling and glycogen synthase kinase-3, PloS Biology, 6(10), 2237-2247 (2008)], ES cell-specific miRNAs [e.g., miR-302-367 cluster (Mol. Cell. Biol. doi:10.1128/MCB.00398-08, WO2009/075119), miR-302 (RNA (2008) 14: 1-10], miR-291-3p, miR-294 and miR-295 [Nat. Biotechnol. 27: 459-461 (2009)] and the like. As mentioned above, the nucleic acid-based expression inhibitors may be in the form of expression vectors harboring a DNA that encodes an siRNA or shRNA.

[0067]

Of the aforementioned constituents of nuclear reprogramming substances, SV40 Large T antigen and the like, for example, can also be encompassed in the scope of iPS cell establishment efficiency improvers because they are factors not essential but auxiliary for somatic cell nuclear reprogramming. While the situation stands in which the mechanism behind nuclear reprograming is unknown, it does not matter whether auxiliary factors other than the factors essential for nuclear reprogramming are positioned as nuclear reprogramming substances or iPS cell establishment efficiency improvers. Hence, the process of somatic cell nuclear reprogramming is dealt with as an overall event resulting from contact of a nuclear reprogramming substance and an iPS cell establishment efficiency improver with a somatic cell; therefore, it seems unnecessary for those skilled in the art to ensure that both are clearly distinguished from each other.

[0068]

Contact of these other iPS cell establishment efficiency improvers with somatic cells can be achieved as described

above with respect to functional inhibitors of p53, according to the case where the improver is (a) a proteinous factor, (b) a nucleic acid that encodes the proteinous factor, or (c) a low-molecular compound, respectively.

The other iPS cell establishment efficiency improvers may be brought into contact with the somatic cells simultaneously with the nuclear reprogramming substance, and either one may be contacted in advance, as far as the efficiency of iPS cell establishment from a somatic cell improves significantly compared with the efficiency obtained in the absence of the substance. The iPS cell establishment efficiency improver can be contacted with a somatic cell at the same timing as that described above with respect to functional inhibitors of p53, according to the properties of the improver.

[0069]

(F) Improving iPS cell establishment efficiency by choosing culturing conditions

The efficiency of establishment of iPS cells can be further improved by culturing somatic cells under hypoxic conditions in the step of nuclear reprogramming thereof. The term hypoxic conditions as used herein means that the oxygen concentration in the ambient atmosphere during cell culture is significantly lower than that in the air. Specifically, such conditions include lower oxygen concentrations than the oxygen concentrations in the ambient atmosphere of 5-10% CO_2/95-90% air, which is commonly used for ordinary cell culture; for example, oxygen concentrations of 18% or less in the ambient atmosphere are applicable. Preferably, the oxygen concentration in the ambient atmosphere is 15% or less (e.g., 14% or less, 13% or less, 12% or less, 11% or less and the like), 10% or less (e.g., 9% or less, 8% or less, 7% or less, 6% or less and the like), or 5% or less (e.g., 4% or less, 3% or less, 2% or less and the like). The oxygen concentration in the ambient atmosphere is preferably 0.1% or more (e.g., 0.2% or more, 0.3% or more, 0.4% or more and the like), 0.5% or

more (e.g., 0.6% or more, 0.7% or more, 0.8% or more, 0.9% or more and the like), or 1% or more (e.g., 1.1% or more, 1.2% or more, 1.3% or more, 1.4% or more and the like).

[0070]

There is no limitation on how to create hypoxic conditions in a cellular environment; the easiest of suitable methods is to culture cells in a CO_2 incubator that allows control of oxygen concentrations. Such CO_2 incubators are commercially available from a number of manufacturers of equipment (e.g., CO_2 incubators for hypoxic culture manufactured by Thermo Scientific, Ikemoto Scientific Technology, Juji Field Inc., and Wakenyaku Co., Ltd. can be used).

[0071]

The timing of beginning cell culture under hypoxic conditions is not particularly limited, as far as it does not interfere with improving the efficiency of establishment of iPS cells compared with that obtained at a normal oxygen concentration (20%). The starting time may be before or after contact of nuclear reprogramming substances with a somatic cell, and may be at the same time as the contact. For example, it is preferable that cell culture under hypoxic conditions be begun just after contacting a nuclear reprogramming substance with a somatic cell, or after a given time (e.g., 1 to 10 (e.g., 2, 3, 4, 5, 6, 7, 8 or 9) days) following the contact.

[0072]

The duration of cell culture under hypoxic conditions is not particularly limited, as far as it does not interfere with improving the efficiency of establishment of iPS cells compared with that obtained at a normal oxygen concentration (20%); examples include, but are not limited to, between 3 days or more, 5 days or more, 7 days or more or 10 days or more, and 50 days or less, 40 days or less, 35 days or less or 30 days or less. The preferred duration of cell culture under hypoxic conditions also varies depending on the oxygen

concentration in the ambient atmosphere; those skilled in the art can adjust as appropriate the duration of cell culture according to the oxygen concentration used. In an embodiment of the present invention, when iPS cell candidate colonies are selected with drug resistance as an indicator, it is preferable that a normal oxygen concentration be restored from hypoxic conditions by the start of drug selection.

Furthermore, the preferred starting time and duration of cell culture under hypoxic conditions also vary depending on the choice of nuclear reprogramming substances used, the efficiency of establishment of iPS cells under conditions involving a normal oxygen concentration, and other factors.

[0073]

After the nuclear reprogramming substance and functional inhibitor of p53 (and another iPS cell establishment efficiency improver as necessary) are brought into contact with the cell, the cell can be cultured under conditions suitable for the cultivation of, for example, ES cells. In the case of mouse cells, the cultivation is carried out with the addition of Leukemia Inhibitory Factor (LIF) as a differentiation suppressor to an ordinary medium. Meanwhile, in the case of human cells, it is desirable that basic fibroblast growth factor (bFGF) and/or stem cell factor (SCF) be added in place of LIF.

Usually, the cells are cultured in the co-presence of mouse embryonic fibroblasts treated with radiation or an antibiotic to terminate their cell division, as feeder cells. Usually, the STO cell line (ATCC CRL-1503) and other lines of mouse embryonic fibroblasts are commonly used as feeders. For inducing iPS cells, however, SNL cells (SNL76/7 STO cells; ECACC 07032801) [McMahon, A.P. & Bradley, A., Cell 62, 1073-1085 (1990)], which are prepared by stably incorporating the neomycin resistance gene and the LIF gene into STO cells, and the like are commonly used. However, because human iPS cell establishment efficiency sometimes improves more when using

mouse embryonic fibroblasts in primary culture (MEF), the use of MEF is also preferable. Mitomycin C-treated MEFs are commercially available from Millipore Company and ReproCELL Company. Co-culture with these feeder cells may be started before contact of the nuclear reprogramming substance, at the time of the contact, or after the contact (e.g., 1-10 days later).

[0074]

(G) Selection and identification of iPS cells

A candidate colony of iPS cells can be selected by a method with drug resistance and reporter activity as indicators, and also by a method based on visual examination of morphology. In the former method, a colony positive for drug resistance and/or reporter activity is selected using a recombinant somatic cell wherein a drug resistance gene and/or a reporter gene is targeted to the locus of a gene highly expressed specifically in pluripotent cells (e.g., Fbx15, Nanog, Oct3/4 and the like, preferably Nanog or Oct3/4). Examples of such recombinant somatic cells include MEFs from a mouse having the β-geo gene (which encodes a fusion protein of β-galactosidase and neomycin phosphotransferase) knocked-in to the Fbx15 locus [Takahashi & Yamanaka, Cell, 126, 663-676 (2006)], MEFs from a transgenic mouse having the green fluorescent protein (GFP) gene and the puromycin resistance gene integrated in the Nanog locus [Okita et al., Nature, 448, 313-317 (2007)] and the like. Because the GR cells of the present invention highly express the Oct3/4 gene (Oct4-positive), it is more preferable to use cells wherein a reporter gene that encodes a visualizing protein such as GFP or RFP is knocked in to the Oct3/4 locus, in the method with reporter activity as an index. Meanwhile, examples of the latter method based on visual examination of morphology include the method described by Takahashi et al. in Cell, 131, 861-872 (2007). Although the method using reporter cells is convenient and efficient, it is desirable from the viewpoint

of safety that colonies be selected by visual examination when the GR cells differentiation-induced from iPS cells are prepared for the purpose of human treatment.

[0075]

The identity of the cells of the selected colony as iPS cells can be confirmed by positive responses to an Oct 4 (or Nanog) reporter (puromycin resistance, GFP positivity and the like), as well as by the formation of a visible ES cell-like colony, as described above. However, to ensure higher accuracy, it is possible to perform tests such as alkaline phosphatase staining, analyzing the expression of various ES-cell-specific genes, and transplanting the selected cells to a mouse and confirming the formation of teratomas.

[0076]

(H) Other methods of producing pluripotent stem cells

Examples of available methods of generating ES cells include, but are not limited to, methods in which a mammalian inner cell mass in the blastocyst stage is cultured [see, for example, Manipulating the Mouse Embryo A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1994)], methods in which an early embryo prepared by somatic cell nuclear transfer is cultured [Wilmut et al., Nature, 385, 810 (1997); Cibelli et al., Science, 280, 1256 (1998); Akira Iritani et al., Protein, Nucleic Acid and Enzyme, 44, 892 (1999); Baguisi et al., Nature Biotechnology, 17, 456 (1999); Wakayama et al., Nature, 394, 369 (1998); Wakayama et al., Nature Genetics, 22, 127 (1999); Wakayama et al., Proc. Natl. Acad. Sci. USA, 96, 14984 (1999); Rideout III et al., Nature Genetics, 24, 109 (2000)] and the like. ES cells are available from specified organizations, and commercial products may be purchased. For example, the human ES cells KhES-1, KhES-2 and KhES-3 are available from the Institute for Frontier Medical Sciences, Kyoto University.

When using somatic cell nuclear transplantation, the choice of somatic cell and the source of somatic cells to be

collected are the same as those in the above-described case of iPS cells.

EG cells can be induced by isolating primordial germ cells by a conventional method, and culturing the cells in the presence of LIF, bFGF and SCF. mGS cells can be prepared from testicular cells according to a method described in WO 2005/100548. Multipotent adult progenitor cells (MAPC) can be isolated from the bone marrow according to a method described in J. Clin. Invest. 109:337-346 (2002).

[0077]

III. Induction of differentiation from pluripotent stem cells to GR cells

Any medium useful for animal cell culture can be used as the basal medium for differentiation induction of GR cells. Examples include the Neurobasal medium, Neural Progenitor Basal medium, NS-A medium, BME medium, BGJb medium, CMRL 1066 medium, Glasgow MEM medium, Improved MEM Zinc Option medium, IMDM medium, Medium 199 medium, Eagle MEM medium, α-MEM medium, DMEM medium, DMEM/F12 medium, Ham medium, RPMI 1640 medium, Fischer's medium, and mixed media thereof. More preferably, the basal medium is the Neurobasal medium. These media may contain a serum [e.g., fetal calf serum (FCS), human serum and the like] or not. Alternatively, a serum substitute additive [e.g., Knockout Serum Replacement (KSR) (produced by Invitrogen Company) and the like] may be used. A serum addition concentration can be chosen as appropriate over the range of 0 to 20%; preferably, a serum-free or low-serum (e.g., 0 to 5%, preferably 0 to 2%) medium can be used.

[0078]

One of the media described above may be used as supplemented with medium additives, for example, serum proteins [e.g., albumins such as bovine serum albumin (BSA) and human serum albumin (HSA) and the like], reducing agents (e.g., 2-mercaptoethanol and the like), growth factors (e.g., insulin, bFGF, HGF, FGF9 and the like), stem cell

differentiation suppressants (e.g., LIF, Wnt, TGF-β and the like), iron sources (e.g., transferrin and the like), minerals (e.g., sodium selenite), amino acids (e.g., non-essential amino acids such as glutamine, alanine, asparagine, serine, aspartic acid, cysteine, glutamic acid, glycine, proline, and tyrosine), vitamins (e.g., choline chloride, pantothenic acid, folic acid, nicotinamide, pyridoxal hydrochloride, riboflavin, thiamine hydrochloride, ascorbic acid, biotin, inositol and the like), sugars (e.g., glucose and the like), organic amines (e.g., putrescine and the like), steroids (e.g., progesterone, β-estradiol and the like), antibiotics (e.g., penicillin, streptomycin and the like), interleukins (e.g., IL-1, IL-2, IL-3, IL-6 and the like), adhesive factors (e.g., heparin, heparan sulfate, collagen, fibronectin and the like), organic acids (e.g., pyruvic acid, succinic acid, lactic acid and the like) or salts thereof, buffering agents (e.g., HEPES and the like), nutritive additives (e.g., B27 supplement, N2 supplement, StemPro supplement and the like) and the like.

In a preferred embodiment, one or more factors selected from among the medium additives listed in Table 1 are used as added to any one of the basal media described above. Those skilled in the art are able to add these factors at appropriate concentrations to the basal medium; for example, the concentrations (final concentrations) shown in the "Medium component" panel in Fig. 27 may be chosen.

[0079]

[Table 1]

B27 supplement (Invitrogen)
L-glutamine
Penicillin/streptomycin
Bovine serum albumin
Non-Essential Amino Acids (Invitrogen)
Sodium pyruvate
Vitamin Solution (Invitrogen)
Insulin-Transferrin-Selenium Supplement (Invitrogen)
D-(+)-glucose
Progesterone
β-Estradiol
2-Mercaptoethanol
Sodium DL-lactate
Putrescine dihydrochloride
Fetal bovine serum

[0080]

The method of the present invention for inducing differentiation from pluripotent stem cells to GR cells comprises culturing pluripotent stem cells in the presence of (a) bone morphogenetic protein 4 (BMP4) and (b) one or more growth factors selected from among glial cell-derived neurotrophic factor (GDNF), epithelial cell growth factor (EGF) and stem cell factor (SCF). Preferably, the pluripotent stem cells are cultured in the presence of three factors, i.e., BMP4 and two growth factors selected from among GDNF, EGF and SCF, more preferably in the presence of the four factors BMP4, GDNF, EGF and SCF. These growth factors may be used as added to one of the above-described media to obtain a differentiation induction medium, or cells that produce the factors may be used as feeder cells and co-cultured with the pluripotent stem cells.

[0081]

Although the BMP4, GDNF, EGF and SCF used may be derived from an optionally chosen mammal (e.g., humans, mice, monkeys, pigs, rats, dogs and the like, preferably humans or mice), it is preferable to use one derived from the same species as the pluripotent stem cells. In particular, when human GR cells are used for therapeutic purposes in humans, it is desirable that the human GR cells be induced under conditions wherein no heterologous components are present (xeno-free); therefore, human-derived proteins are preferably used as these growth factors.

[0082]

When BMP4, GDNF, EGF or SCF is used as added to the medium, these growth factors may be ones isolated/purified from cells [e.g., nerve cells, glial cells, hepatocytes, splenocytes, pancreatic β cells, myelocytes, mesangial cells, Langerhans' cells, epidermal cells, epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, fibrocytes, myocytes, adipocytes, immune cells (e.g., macrophages, T cells, B cells, natural killer cells, mast cells, neutrophils, basophils, eosinophils, monocytes), megakaryocytes, synovial cells, chondrocytes, bone cells, osteoblasts, osteoclasts, mammary gland cells or interstitial cells, or corresponding precursor cells, stem cells or cancer cells thereof, and the like] of a mammal that produces the same (e.g., humans, mice, monkeys, pigs, rats, dogs and the like) or any tissues where such cells are present [e.g., brain, parts of brain (e.g., olfactory bulb, amygdaloid nucleus, basal ganglia, hippocampus, thalamus, hypothalamus, cerebral cortex, medulla oblongata, cerebellum), spinal cord, hypophysis, stomach, pancreas, kidney, liver, gonad, thyroid, gall-bladder, bone marrow, adrenal gland, skin, muscles (e.g., smooth muscle, skeletal muscle), lung, gastrointestinal tract (e.g., large intestine, small intestine), blood vessel, heart, thymus, spleen, submandibular gland, peripheral blood, prostate, testis, ovary, placenta, uterus, bone, joint,

adipose tissues (e.g., white adipose tissue, brown adipose tissue) and the like] by a method of protein separation and purification known per se (e.g., methods based on differences in solubility, such as salting-out and solvent precipitation; methods based mainly on differences in molecular weight, such as dialysis, ultrafiltration, gel filtration, and SDS-polyacrylamide gel electrophoresis; methods based on differences in electric charge, such as ion exchange chromatography; methods based on specific affinity, such as affinity chromatography; methods based on differences in hydrophobicity, such as reverse phase high performance liquid chromatography; methods based on differences in isoelectric point, such as isoelectric focusing; and the like; these methods may be combined as appropriate). Although the growth factors may also be proteins synthesized chemically or synthesized biochemically in a cell-free translation system, they are preferably recombinant proteins produced by a transformant incorporating a nucleic acid that encodes one of these proteins.

[0083]

For example, information on human and mouse cDNA sequences of BMP4, GDNF, EGF and SCF is available with reference to the NCBI accession numbers mentioned shown below; those skilled in the art are easily able to isolate these cDNAs by a conventional method on the basis of the information.

Name of gene	Human	Mouse
BMP4	NM_001202	NM_007554
GDNF	NM_000514	NM_010275
EGF	NM_001963	NM_010113
SCF	NM_000899	NM_013598

It is possible to insert the cDNA thus obtained into an appropriate expression vector, introduce the vector into a host cell (e.g., animal cells, insect cells, Bacillus subtilis, yeast, Escherichia coli and the like), culture the resulting transformant, and isolate/purify the desired recombinant

protein from the resulting culture supernatant using the above-described techniques of protein separation and purification combined as appropriate. Recombinant proteins of BMP4, GDNF, EGF and SCF are commercially available.

[0084]

The BMP4, GDNF, EGF or SCF used to induce the differentiation of the GR cells of the present invention may be a protein having an amino acid sequence different from the amino acid sequence encoded by one of the above-described cDNA sequences, as far as it possesses the capability of inducing the differentiation of GR cells from pluripotent stem cells by being combined with other factors. Specifically, the BMP4, GDNF, EGF or SCF used to induce the differentiation of GR cells means a protein that comprises:

(a) an amino acid sequence having a homology of about 90% or more to the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers;

(b) an amino acid sequence resulting from substitution and/or deletion and/or insertion and/or addition of 1 to 20 amino acids in the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers;

(c) an amino acid sequence of an ortholog in another mammal of a human or mouse protein consisting of the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers;

(d) an amino acid sequence in a human or mouse protein consisting of the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers or a splice variant, allelic mutant or polymorph (e.g., SNP and the like) of the ortholog of (c) above; or

(e) a portion (fragment) of one of the amino acid sequences (a) to (d) above,

and that possesses the capability of inducing the differentiation of GR cells from pluripotent stem cells by being combined with other factors.

Here, "homology" means a ratio (%) of identical amino acid residues and similar amino acid residues to all overlapping amino acid residues in the optimal alignment where two amino acid sequences are aligned using a mathematical algorithm known in the technical field (preferably, the algorithm considers introduction of gaps on one or both of the sequence for the best alignment). "A similar amino acid" means an amino acid having similar physicochemical properties; examples thereof include amino acids classified under the same group, such as aromatic amino acids (Phe, Trp, Tyr), aliphatic amino acids (Ala, Leu, Ile, Val), polar amino acids (Gln, Asn), basic amino acids (Lys, Arg, His), acidic amino acids (Glu, Asp), amino acids having a hydroxyl group (Ser, Thr) and amino acids having a small side-chain (Gly, Ala, Ser, Thr, Met). Substitution by such similar amino acids is expected not to change the phenotype of the protein (i.e., conservative amino acid substitution). Specific examples of the conservative amino acid substitution are known in the technical field and described in various documents [see, for example, Bowie et al., Science, 247:1306-1310 (1990)]. Amino acid sequence homology as mentioned herein can be calculated using the homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy=10; gap allowed; matrix=BLOSUM62; filtering=OFF).

[0085]

The BMP4, GDNF, EGF or SCF used to induce the differentiation of GR cells in (a) above is more preferably a protein comprising an amino acid sequence having an identity of about 95% or more, more preferably about 97% or more, particularly preferably about 98% or more, to the amino acid sequence encoded by the cDNA sequences shown by one of the aforementioned NCBI accession numbers.

[0086]

The BMP4, GDNF, EGF or SCF used to induce the

differentiation of GR cells in (b) above can preferably a protein comprising (i) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids deleted from the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, (ii) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids added to the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, (iii) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids inserted into the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, (iv) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids substituted by other amino acids in the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, or (v) an amino acid sequence comprising a combination thereof, and that possesses the capability of inducing the differentiation of GR cells from pluripotent stem cells by being combined with other factors.

When an amino acid sequence is inserted, deleted or substituted as described above, the position of the insertion, deletion or substitution is not particularly limited, as far as the differentiation induction activity for the GR cells is retained.

[0087]

In an embodiment of the present invention, another protein of the BMP family, for example, BMP8b, BMP2, BMP7 and the like, may be used in place of, or in addition to, BMP4 when inducing differentiation from pluripotent stem cells to GR cells. Preferably, BMP4 and BMP8b are used in combination. In another embodiment, it is also possible to use the GDNF equivalents described in WO 2004/092357 in place of, or in addition to, GDNF when inducing differentiation from

pluripotent stem cells to GR cells.

[0088]

When BMP4 is used as added to the medium, the BMP4 concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the BMP4 concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less. When another protein of the BMP family is used in combination with BMP4, it is preferable that the BMPs be added to obtain a total concentration in the aforementioned range.

[0089]

When GDNF is used as added to the medium, the GDNF concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the GDNF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less. When a GDNF equivalent is used in combination with GDNF, it is preferable that the GDNF and the equivalent be added to obtain a total concentration in the aforementioned range.

[0090]

When EGF is used as added to the medium, the EGF concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the EGF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less.

[0091]

When SCF is used as added to the medium, the SCF

concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the SCF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less.

[0092]

When (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF are used as added to the medium, the components (a) and (b) may be provided separately from the above-described basal medium or the medium comprising the basal medium and the above-described medium additives added thereto, and used as added to the medium just before use. Alternatively, these components may be provided in the form of a differentiation induction medium already containing the components, as far as they do not adversely affect the stability and the like of the growth factors and other components of the medium.

[0093]

In a preferred embodiment of the present invention, at least one factor, preferably all the factors used, out of the factors used in inducing the differentiation of the GR cells of the present invention, i.e., (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF, are supplied from feeder cells, rather than added to the medium. Although the feeder cells may be mammalian cells that produce these growth factors by nature, it is more preferable to use recombinant cells that are introduced with genes encoding the growth factors and overexpress the growth factors. Although a plurality of kinds of cells that express different growth factors can be used in combination, it is preferable to use one kind of cells that express all the growth factors to be supplied from the feeders. Cells that can be used as the host include, but are not limited to, cells that have traditionally

been suitably used as feeder cells. For example, mouse embryonic fibroblasts (MEF) treated with radiations or antibiotics to terminate their cell division [e.g., STO cell line (ATCC CRL-1503) and the like] and the like are commonly used as feeders, but SNL cells, which are prepared by stably incorporating the neomycin resistance gene and the LIF gene into STO cells (SNL76/7 STO cells; ECACC 07032801) and the like are also preferable. It is also preferable to use cells of the same species as the pluripotent stem cells as feeders. For example, when GR cells are to be induced from human pluripotent stem cells, human dermal fibroblasts (HDF), human dental pulp stem cells and the like can be used as feeders.

[0094]

For example, when BMP4 is to be supplied from feeder cells, M15-BMP4 cells [Proc. Natl. Acad. Sci. USA, 100: 11457-11462 (2003)], for example, can be used as the feeder cells. Furthermore, when one or more growth factors selected from among GDNF, EGF and SCF are also to be supplied from feeder cells, it is also possible to use transformant cells obtained by introducing an expression vector harboring nucleic acids that encode the one or more growth factors selected from among GDNF, EGF and SCF, for example, into M15-BMP4 cells. For example, in an Example below, a viral vector harboring the nucleic acids that encode GDNF, EGF and SCF is introduced into M15-BMP4 cells to create cells that overexpress the four growth factors BMP4, GDNF, EGF and SCF (M15-4GF).

[0095]

Cultivation for inducing differentiation from pluripotent stem cells to GR cells can, for example, be performed as described below.

Any culture vessel for cell culture can be used in this step of differentiation induction. Such culture vessels include, for example, flasks, tissue culture flasks, dishes, Petri dishes, tissue culture dishes, multi-dishes, microplates, micro-well plates, multi-plates, multi-well plates, chamber

slides, tubes, trays, culturing bags, and roller bottles. The culture vessel can be non-cell-adhesive (weakly cell-adhesive) or cell-adhesive, depending on the method of cultivation (suspension culture or adhesion culture). A cell-adhesive culture vessel is a culture vessel whose surface is coated with a cell support substrate to improve adhesion of cells (pluripotent stem cells or feeder cells). Such cell support substrates include, for example, collagen, gelatin, Matrigel, poly-L-lysine, poly-D-lysine, laminin, fibronectin and the like.

[0096]

Pluripotent stem cells are seeded to the above-described culture vessel to obtain a cell density of, for example, about 0.5 to 50×10^4 cells/cm^2, preferably about 1 to 10×10^4 cells/cm^2, and cultured, for example, in a CO_2 incubator under an atmosphere of about 1% to about 10%, preferably about 2% to about 5%, CO_2 concentration, at about 30°C to about 40°C, preferably about 37°C, for about 1 to 8 weeks, preferably about 2 to 6 weeks. The cultivation may be performed by suspension culture or adhesion culture, and preferably by suspension culture. When at least one factor of (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF (differentiation induction growth factors of the present invention) are supplied from feeder cells, the pluripotent stem cells and feeder cells are mixed in a ratio of 1:10 to 10:1, preferably 1:5 to 5:1, more preferably 1:2 to 2:1, seeded to the culture vessel, and subjected to suspension culture. Alternatively, the pluripotent stem cells may be seeded to the culture vessel to which feeder cells have been seeded in advance, and co-cultured. Even when all of the differentiation induction growth factors of the present invention are used as added to the medium, the pluripotent stem cells may be cultured in the presence of feeder cells. In this case, useful feeder cells include ordinary MEF [e.g., STO cell line (ATCC CRL-1503) and the like], SNL cells (SNL76/7

STO cells; ECACC 07032801), HDF, dental pulp stem cells and the like treated with radiations or an antibiotic to terminate their cell division.

[0097]

Differentiation from pluripotent stem cells to GR cells can be confirmed with an Oct4-positive and Vasa-positive response as an index. For example, when using pluripotent stem cells incorporating a reporter gene for visualization downstream of the expression control regions of the two genes (for example, pluripotent stem cells derived from the Oct4-GFP/Vasa-RFP knock-in mouse described in an Example below), color development, fluorescence or the like due to the expression of the reporter gene may be detected to confirm the induction of Oct4-positive Vasa-positive germline stem cells, i.e., GR cells. Oct4-positive Vasa-positive ($Oct4^+/Vasa^+$) cells can be sorted and isolated from other undifferentiated cells ($Oct4^+/Vasa^-$) and cells that have differentiated into somatic cells ($Oct4^-$) by flow cytometry (FACS). When suspension culture is performed using feeder cells, suspended cell masses containing the feeder cells are formed; therefore, for example, trypsin/EDTA, collagenase and the like are added to dissociate the cell masses, and the resulting cell suspension is seeded to, and incubated on, a culture vessel coated with a cell support substrate, after which suspended cells are recovered, whereby the feeder cells can be separated and removed.

Meanwhile, bearing in mind therapeutic use in humans, it is undesirable to use pluripotent stem cells wherein a reporter gene is knocked in at the Oct4 and Vasa gene loci; therefore, the expression of the Oct4 and Vasa genes themselves must be detected. In this case, the detection can be achieved by analyzing the phenotype of the surface antigen of the cells using a cell sorter and antibodies against an undifferentiated cell surface marker corresponding to the expression of Oct4 and against a germ line cell surface marker

corresponding to the expression of the Vasa protein or Vasa. For example, undifferentiated cell surface markers include SSEA-1, Forssman antigen, β1- and α6-integrin and the like, and germline cell surface markers include EpCAM, CD9, EE2, c-kit and the like. The expression of Oct4 and other transcription factors may also be examined as required.

[0098]

The present invention also provides iPS cell-derived Oct4-positive Vasa-positive germline stem cells, i.e., GR cells, obtained by the method described above. To date, there have been some reported cases where spermiogenesis in an infertile mouse was realized by transplanting Vasa-positive primordial germ cell (PGC)-like cells induced from ES cells [Proc. Natl. Acad. Sci. USA, 100: 11457-11462 (2003)] or a cell line (GS cells) permitting long-time cultivation induced from spermatogonial stem cells (e.g., WO 2004/092357 and the like) to the testis of the mouse. As shown in an Example below, however, germline stem cells capable of causing spermiogenesis could not be established from iPS cells when using these conventional culturing conditions; iPS cell-derived germline stem cells were obtained for the first time by using the method of differentiation induction of the present invention.

[0099]

As stated above, in the method of GR cell differentiation induction of the present invention, (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF are added to the medium, or supplied from feeder cells. Accordingly, the present invention also provides an inducer of differentiation from pluripotent stem cells to Oct4-positive Vasa-positive germline stem cells (GR cells), comprising (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF in combination. Preferably, the GR cell differentiation inducer of the present invention comprises two factors or more of GDNF, EGF and SCF, more preferably all of the three factors. These growth factors may be provided in

solution in water or an appropriate buffer solution, and may be provided as a lyophilized powder and dissolved in an appropriate solvent just before use. These components may stand as separate reagents to form a kit, and may be provided as a single reagent comprising a blend of two or more, as far as they do not adversely affect each other.

The GR cell differentiation inducer of the present invention can further comprise physiologically acceptable carriers, excipients, antiseptics, stabilizers, binders, solubilizers, nonionic surfactants, buffering agents, preservatives, antioxidants and the like.

[0100]

In another preferred embodiment, the GR cell differentiation inducer of the present invention comprises cells that produce (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF. Examples of the cells include recombinant cells that overexpress these growth factors, obtained by introducing an expression vector harboring nucleic acids that encode the growth factors into host cells. Because the cells, as feeder cells, are co-cultured with pluripotent stem cells, it is preferable to use cells that have traditionally been used as feeder cells in the relevant technical field, as the host cells. Specifically, the cells described above can be mentioned. Cells that produce (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF and SCF can be provided in a state suspended at an appropriate cell density in an appropriate medium (e.g., the above-described basal media and the like), or in a state preserved under freezing by a conventional method.

[0101]

The present invention also provides a GR cell differentiation induction medium comprising any one of the above-described basal media or a medium prepared by supplementing the basal medium with any one of the above-described medium additives, and the above-described GR cell

differentiation inducer of the present invention added to the medium. The differentiation induction medium may be provided as supplemented with the differentiation inducer, and the differentiation inducer may stand as a separate reagent to form a kit to be added to the medium just before use.

[0102]

IV. How to maintain and expand GR cells

The thus-obtained Oct4-positive Vasa-positive germline stem cells (GR cells) can be cultured in the presence of GDNF, EGF, SCF and basic fibroblast growth factor (bFGF) for a long time, while maintaining the Oct4-positive Vasa-positive property. Accordingly, the present invention also provides a method of expanding GR cells, comprising culturing the GR cells in the presence of GDNF, EGF, SCF and bFGF.

[0103]

Although the GR cells used for the method of cell maintenance and expansion of the present invention are not subject to limitations with regard to derivation, as far as they are Oct4-positive Vasa-positive cells destined to become germline cells that are capable of survive for a long time without forming tumors and without being eliminated when transplanted to the testis, the GR cells used are preferably those derived from pluripotent stem cells, more preferably those induced by the above-described method of differentiation induction of the present invention. The GR cells may be provided as an isolated and purified homogenous population of Oct4-positive Vasa-positive cells, and may be provided as a heterogenous population of cells wherein Oct4-negative or Vasa-negative cells are also present (hereinafter, heterogenous cell populations containing GR cells are included in the scope of GR cells unless otherwise stated).

[0104]

The basal medium and optionally chosen medium additives used in the method of the present invention for maintaining and expanding GR cells are the same as those used in the

above-described method of inducing differentiation from pluripotent stem cells to GR cells. As stated below, however, the method of the present invention for maintaining and expanding GR cells is performed in the presence of SCF and bFGF, so that the combined use of LIF can induce the dedifferentiation of GR cells, which are PGC-like cells, into EG cell-like cells; therefore, it is sometimes preferable not to use LIF.

In a preferred embodiment, one or more factors selected from among the medium additives listed in Table 1 are used as added to the basal medium. Those skilled in the art are able to add these factors at appropriate concentrations to the basal medium; for example, the concentrations (final concentrations) shown in the "Medium component" panel in Fig. 27 may be chosen.

[0105]

In the method of the present invention for maintaining and expanding GR cells, GR cells are cultured in the presence of GDNF, EGF, SCF and bFGF. Although these growth factors may be used as added to one of the above-described media to obtain a differentiation induction medium, or cells that produce the factors may be used as feeder cells and co-cultured with the pluripotent stem cells, the factors are preferably used as added to the medium.

[0106]

Although the GDNF, EGF, SCF and bFGF used may be derived from an optionally chosen mammal (e.g., humans, mice, monkeys, pigs, rats, dogs and the like, preferably humans or mice), it is preferable to use one derived from the same species as the GR cells. In particular, when human GR cells are used for therapeutic purposes in humans, it is desirable that the human GR cells be induced under conditions wherein no heterologous components are present (xeno-free); therefore, human-derived proteins are preferably used as these growth factors.

[0107]

When GDNF, EGF, SCF or bFGF is used as added to the medium, these growth factors may be ones isolated/purified from cells of a mammal that produces the same (for example, humans, mice, monkeys, pigs, rats, dogs and the like) or any tissues where such cells are present and the like by a method of protein separation and purification known per se. Although the growth factors may also be proteins synthesized chemically or synthesized biochemically in a cell-free translation system, they are preferably recombinant proteins produced by a transformant incorporating a nucleic acid that encodes one of these proteins. Recombinant proteins of GDNF, EGF and SCF can be prepared by the above-described method. Information on the human and mouse cDNA sequences of bFGF is available with reference to the NCBI accession numbers NM_002006 and NM_008006; those skilled in the art are able to isolate a cDNA of bFGF by a conventional method on the basis of the information, and to easily prepare a recombinant protein in the same way as the above. Recombinant proteins of GDNF, EGF, SCF and bFGF are commercially available.

[0108]

The GDNF, EGF, SCF or bFGF used to maintain and expand the GR cells of the present invention may be a protein having an amino acid sequence different from the amino acid sequence encoded by one of the aforementioned cDNA sequences, as far as it supports the capability of the GR cells of self-replication while maintaining their differentiated state by being combined with other factors. Specifically, the GDNF, EGF, SCF or bFGF used to maintain and expand the GR cells means a protein that comprises:

(a) an amino acid sequence having a homology of about 90% or more to the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers;

(b) an amino acid sequence resulting from substitution and/or deletion and/or insertion and/or addition of 1 to 20 amino acids in the amino acid sequence encoded by the cDNA sequence

shown by one of the aforementioned NCBI accession numbers;

(c) an amino acid sequence of an ortholog in another mammal of a human or mouse protein consisting of the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers;

(d) an amino acid sequence in a human or mouse protein consisting of the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers or a splice variant, allelic mutant or polymorph (e.g., SNP and the like) of the ortholog of (c) above; or

(e) a portion (fragment) of one of the amino acid sequences (a) to (d) above,

and that supports the capability of the GR cells of self-replication while maintaining their differentiated state by being combined with other factors.

Here, "homology" has the same definition as the above described with respect to the BMP4 and the like used to induce the differentiation of GR cells. The GDNF, EGF, SCF or bFGF used to maintain and expand the GR cells in (a) above is more preferably a protein comprising an amino acid sequence having an identity of about 95% or more, more preferably about 97% or more, particularly preferably about 98% or more, with the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers.

[0109]

The GDNF, EGF, SCF or bFGF used to maintain and expand the GR cells in (b) above can preferably a protein comprising (i) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids deleted from the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, (ii) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids added to the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, (iii) an amino acid sequence having 1 to 10,

more preferably one to several (5, 4, 3, or 2) amino acids inserted into the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, (iv) an amino acid sequence having 1 to 10, more preferably one to several (5, 4, 3, or 2) amino acids substituted by other amino acids in the amino acid sequence encoded by the cDNA sequence shown by one of the aforementioned NCBI accession numbers, or (v) an amino acid sequence comprising a combination thereof, and that supports the capability of the GR cells of self-replication while maintaining their differentiated state by being combined with other factors, and the like.

When an amino acid sequence is inserted, deleted or substituted as described above, the position of the insertion, deletion or substitution is not particularly limited, as far as the maintenance and expansion activity for the GR cells is retained.

[0110]

In a mode of embodiment of the present invention, a GDNF equivalent described in WO 2004/092357 may be used in place of, or in addition to, GDNF, when maintaining and expanding GR cells.

[0111]

When GDNF is used as added to the medium, the GDNF concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the GDNF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less. When a GDNF equivalent is used in combination, it is preferable that the GDNF and the equivalent be added to obtain a total concentration in the aforementioned range.

[0112]

When EGF is used as added to the medium, the EGF

concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the EGF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less.

[0113]

When SCF is used as added to the medium, the SCF concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the SCF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less.

[0114]

When bFGF is used as added to the medium, the bFGF concentration is, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more. Also, the bFGF concentration is, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less.

[0115]

When GDNF, EGF, SCF and bFGF are used as added to the medium, these components may be provided separately from the above-described basal medium or the medium comprising the basal medium and the above-described medium additives which are optionally chosen components added thereto, and used as added to the medium just before use, or may be provided as a GR cell maintenance and expansion medium in a form already contained in the medium, as far as they do not adversely affect the stability and the like of the growth factors and

other components of the medium.

[0116]

In another embodiment, at least one factor out of the factors used in the maintenance and expansion of GR cells of the present invention, i.e., GDNF, EGF, SCF and bFGF, can be supplied from feeder cells, rather than added to the medium. Although the feeder cells may be mammalian cells that produce these growth factors by nature, it is more preferable to use recombinant cells that are introduced with genes encoding the growth factors and overexpress the growth factors. Although a plurality of kinds of cells that express different growth factors can be used in combination, it is preferable to use one kind of cells that express all the growth factors to be supplied from the feeders. Cells that can be used as the host are the same cells as those described above with respect to the differentiation induction of GR cells. The recombinant cells can be produced by the same techniques as those described above with respect to the differentiation induction of GR cells.

[0117]

In the method of the present invention for maintaining and expanding GR cells, it is preferable that the GR cells be cultured in the presence of, in addition to the aforementioned four factors (GDNF, EGF, SCF and bFGF), one or more medium additives selected from among other growth factors such as HGF and FGF9 and interleukins such as IL-2.

Each of growth factors such as HGF and FGF9 can be added to the medium at concentrations of, for example, about 0.1 ng/ml or more, preferably about 0.5 ng/ml or more, more preferably about 1 ng/ml or more, particularly preferably about 5 ng/ml or more, and at, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less. Interleukins such as IL-2 can be added to the medium at concentrations of, for example, about 0.01 ng/ml or more,

preferably about 0.1 ng/ml or more, more preferably about 0.5 ng/ml or more, particularly preferably about 1 ng/ml or more, and at, for example, about 100 ng/ml or less, preferably about 50 ng/ml or less, more preferably about 30 ng/ml or less, particularly preferably about 20 ng/ml or less.

[0118]

In the method of the present invention for maintaining and expanding GR cells, it is also preferable to culture the GR cells in the further presence of an androgen (e.g., testosterone and the like) and/or a derivative thereof (e.g., forskolin). These components can be added to the medium, as far as they support the maintenance and expansion of the GR cells and do not adversely affect the survival of the cells; the components can be added to the medium at concentrations of, for example, about 0.001 μM or more, preferably 0.01 μM or more, more preferably 0.05 μM or more, and at about 1000 μM or less, preferably about 500 μM or less, more preferably about 100 μM or less.

[0119]

In another preferred embodiment, one or more factors selected from among the medium additives listed in Table 1 and one or more factors selected from among the medium additives listed in Table 2 are used as added to any one of the above-described basal media. Those skilled in the art are able to add these factors at appropriate concentrations to the basal medium; for example, the concentrations (final concentrations) shown in Fig. 27 may be chosen.

[0120]

[Table 2]

GDNF
EGF
bFGF
SCF
HGF
IL-2
FGF9
Forskolin
Testosterone
StemPro supplement (Invitrogen)

[0121]

Maintenance and expansion culture of GR cells can, for example, be performed as described below.

Any culture vessel for cell culture can be used in this step of cultivation. Such culture vessels include, for example, the same culture vessels as those used in the step of GR cell differentiation induction. The culture vessel can be non-cell-adhesive (weakly cell-adhesive) or cell-adhesive, depending on the method of cultivation (suspension culture or adhesion culture). A cell-adhesive culture vessel is a culture vessel whose surface is coated with a cell support substrate to improve adhesion of cells (pluripotent stem cells or feeder cells). Such cell support substrata include, for example, collagen, gelatin, Matrigel, poly-L-lysine, poly-D-lysine, laminin, fibronectin and the like.

[0122]

The GR cells are seeded to the above-described culture vessel to obtain a cell density of, for example, about 0.5 to 50×10^4 cells/cm^2, preferably about 1 to 10×10^4 cells/cm^2, and cultured, for example, in a CO_2 incubator under an atmosphere of about 1% to about 10%, preferably about 2% to about 5%, CO_2 concentration at about 30°C to about 40°C, preferably about 37°C.

When at least one factor of GDNF, EGF, SCF and bFGF is supplied from feeder cells, the feeder cells are seeded to the culture vessel to obtain a cell density of, for example, about 0.5 to 50×10^4 cells/cm^2, preferably about 1 to 10×10^4 cells/cm^2, and the GR cells are seeded thereonto. Even when all of GDNF, EGF, SCF and bFGF are used as added to the medium, it is preferable that the pluripotent stem cells be cultured in the presence of feeder cells. In this case, feeder cells include ordinary MEFs [e.g., STO cell line (ATCC CRL-1503) and the like], SNL cells (SNL76/7 STO cells; ECACC 07032801), HDF, dental pulp stem cells and the like treated with radiations or an antibiotic to terminate their cell division. By subculturing the cells by a conventional method when the cells become confluent or subconfluent, it is possible to maintain and expand the GR cells for a long time while maintaining their differentiated state.

[0123]

When GR cells are provided as a heterogenous cell population containing Oct4-negative or Vasa-negative cells, homogenous GR cells can be obtained by separating and recovering GR cells from the expanded cell population with an Oct4-positive Vasa-positive response as an index. The GR cells can be separated by the same method as that used to separate the GR cells induced from pluripotent stem cells.

[0124]

The cell population induced from pluripotent stem cells by the method of GR cell differentiation induction of the present invention contains not only GR cells, but also Oct4-positive Vasa-negative undifferentiated cells. When the separated undifferentiated cells were cultured under the above-described GR cell maintenance and expansion conditions, they were stably expanded while maintaining the Oct4-positive Vasa-negative differentiated state; therefore, this undifferentiated cell line was named Gsp cells. Transplantation of Gsp cells to an immunodeficient mouse

resulted in teratoma formation. This finding, combined with the results of various characterization analyses, demonstrated the identity of the Gsp cells as newly established pluripotent stem cells distinct from the starting pluripotent stem cells. Accordingly, the present invention also provides Gsp cells, which are novel pluripotent stem cells induced in the process of GR cell differentiation induction, and are stably maintained and expanded under GR cell maintenance and expansion conditions, and a method of producing and maintaining the same. The Gsp cells, like the mGS cells, which have been discovered in the course of establishing the GS cells, are expected to find a broad range of applications as a substitute for known pluripotent stem cells such as ES cells and iPS cells.

[0125]

As stated above, in the method of the present invention for maintaining and expanding GR cells, GDNF, EGF, SCF and bFGF are added to the medium. Accordingly, the present invention also provides an expansion support agent for Oct4-positive Vasa-positive germline stem cells (GR cells), comprising GDNF, EGF, SCF and bFGF in combination. Under the GR cell maintenance and expansion conditions of the present invention, Oct4-positive Vasa-negative pluripotent stem cells (Gsp cells) are also stably maintained; therefore, the expansion support agent also serves as an expansion support agent for Gsp cells. Each of the factor GDNF, EGF, SCF, and bFGF may be provided in solution in water or an appropriate buffer solution, and may be provided as a lyophilized powder and dissolved in an appropriate solvent just before use. These components may stand as separate reagents to form a kit, and may be provided as a single reagent comprising a blend of two or more, as far as they do not adversely affect each other.

The GR cell expansion support agent of the present invention can further comprise physiologically acceptable carriers, excipients, antiseptics, stabilizers, binders,

solubilizers, nonionic surfactants, buffering agents, preservatives, antioxidants and the like.

[0126]

In another preferred embodiment, the GR cell expansion support agent of the present invention is combined with one or more, preferably two or more, factors, more preferably all the three factors, selected from among HGF, IL-2 and FGF9, in addition to the four factors GDNF, EGF, SCF and bFGF. Each of these growth factors may be provided in solution in water or an appropriate buffer solution, and may be provided as a lyophilized powder and dissolved in an appropriate solvent just before use. These components may stand as separate reagents to form a kit, and may be provided as a single reagent comprising a blend of two or more, as far as they do not adversely affect each other.

[0127]

The present invention also provides a GR cell maintenance and expansion medium comprising any one of the above-described basal media or a medium prepared by supplementing the basal medium with the above-described medium additives which are optionally chosen components, and the above-described GR cell expansion support agent of the present invention added thereto. The maintenance and expansion medium may be provided as supplemented with the expansion support agent, and the expansion support agent may be provided as a separate reagent as a component of a kit to be added to the medium just before use.

[0128]

The present invention still also provides a method of allowing an infertile animal to form sperms by transplanting GR cells obtained by the above-described method of differentiation induction, or GR cells maintained by the above-described method of maintenance and expansion, to the testis of the infertile animal, wherein the infertile animal is of the same species as the cells. The method is applicable

to an optionally chosen mammal that allows GR cells to be established by the method of differentiation induction and/or method of maintenance and expansion of the present invention. However, when an application to infertility treatment or gene therapy is born in mind, the subject animal is preferably a human. For research purposes, subject animals include mice, rats, guinea pigs, hamsters, gerbils, rabbits, dogs, monkeys and the like that have traditionally been in common use as laboratory animals. For breeding purposes, various farm animals (e.g., bovines, horses, pigs, sheep, goat and the like), companion animals (e.g., dogs, cats and the like) and the like can be mentioned.

[0129]

Transplantation of GR cells to the testis can be achieved by, for example, using the GR cells of the present invention in place of GS cells, by the methods described in WO 2004/092357 and Biol. Reprod., 69: 612-616 (2003).

[0130]

When the method of spermiogenesis of the present invention is used for infertility treatment, it is particularly desirable that the GR cells to be transplanted be induced from the recipient animal individual. To produce sperms derived from a patient, the germline stem cells transplanted for spermiogenesis must be inducible from cells that can be collected after birth. However, iPS cells are faulty in that the establishment efficiency is low, when they are intended to be generated by protein transfer or virus-free gene transfer in view of safety concerns. For this reason, provided that the patient's own spermatogonial stem cells are collectable, it is sometimes advantageous to use GS cells induced from spermatogonial stem cells. However, if it is impossible to collect spermatogonial stem cells, or if collecting spermatogonial stem cells involves a considerable risk because of the necessity for multiple testicular biopsies or for other reasons, and in other cases, it is of paramount

importance to use GR cells prepared by a method of the present invention from iPS cells that are readily inducible from skin cells and the like. Therefore, when the method of spermiogenesis of the present invention is used for infertility treatment, preferred recipients of the GR cells include infertile animals from which spermatogonial stem cells are difficult to collect.

[0131]

When the method of spermiogenesis of the present invention is used for gene therapy, GR cells differentiation-induced from iPS cells or mGS cells induced from a hereditary disease patient cannot be used as they are for transplantation. For this reason, it is necessary to genetically manipulate pluripotent stem cells induced from the patient or GR cells differentiation-induced from the pluripotent stem cells by a method of the present invention to replace the mutant gene with a normal gene, or by other treatment, and to transplant the resulting GR cells to the patient. By allowing sperms derived from the patient, but having the normal gene function, to be formed as described above, it is possible to prevent the patient's mutant gene from descending to his or her posterity to avoid the development of the hereditary disease in the offspring.

[0132]

In the field of breeding, provided that pluripotent stem cells have been established from an individual carrying favorable genetic information, it is possible to induce and expand GR cells from the pluripotent stem cells by a method of the present invention and transplant the GR cells to the testis of another individual to allow the individual to form sperms derived from the pluripotent stem cells, in the event that the excellent ancestry ceases. The excellent ancestry could be maintained by reproduction using the individual.

[0133]

The present invention also provides a therapeutic agent

for male infertility comprising GR cells obtained by the above-described method of differentiation induction or GR cells maintained by the above-described method of maintenance and expansion.

The GR cells of the present invention are produced as a parenteral preparation such as an injection, suspension, drip infusion and the like by being blended with a pharmaceutically acceptable carrier by a conventional means or otherwise. Pharmaceutically acceptable carriers that can be contained in the parenteral preparation include, for example, aqueous solutions for injection, such as physiological saline and isotonic solutions containing glucose or another auxiliary drug (e.g., D-sorbitol, D-mannitol, sodium chloride and the like). The agent of the present invention may be formulated with, for example, a buffering agent (e.g., phosphate buffer solution, sodium acetate buffer solution), a soothing agent (e.g., benzalkonium chloride, procaine hydrochloride and the like), a stabilizer (e.g., human serum albumin, polyethylene glycol and the like), a preservative, an antioxidant and the like. Of course, the GR cells may be used as they are in the form of a suspension in the above-described GR cell maintenance and expansion medium. When the therapeutic agent for infertility of the present invention is prepared as an aqueous suspension, GR cells are suspended in the above-described aqueous liquid to obtain a cell density of about 1.0×10^6 to 1.0×10^8 cells/ml.

The therapeutic agent for male infertility of the present invention may be provided in a state preserved under freezing conditions in common use for freezing preservation of stem cells, and thawed just before use. In this case, serum or a substitute therefor, an organic solvent (e.g., DMSO) and the like may further be contained. In this case, the concentration of serum or substitute therefor is not particularly limited, and can be about 1% to about 30% (v/v), preferably about 5% to about 20% (v/v). The concentration of the organic solvent is

not particularly limited, and can be 0% to about 50% (v/v), preferably about 5% to about 20% (v/v).

[0134]

Because the preparation thus obtained is stable and less toxic, it can be safely administered to mammals such as humans. Although the method of administration is not particularly limited, the preparation is preferably administered by injection or drip infusion, and can be administered into the seminiferous tubule. The therapeutic agent for infertility of the present invention can be administered, for example, at about 1.0×10^5 to about 1×10^7 cells per dose based on GR cells, once or multiple times (e.g., 2 to 10 times) at intervals of about 1 to 2 weeks, for a human infertility patient.

[0135]

The present invention is hereinafter described in more detail by means of the following Examples, which, however, are not to be construed as limiting the scope of the invention.

Examples

[0136]

Example 1: Establishment of iPS cells

A transgenic mouse with undifferentiated cells and germ cells visualized by introduction of the reporter genes Oct4-GFP and Mvh (mouse vasa homolog)-RFP, respectively (Oct4-GFP/Mvh-RFP Tg mouse) was used as an experimental system. The Oct3/4 (Oct4) and mouse vasa genes are known to be expressed specifically in undifferentiated cells and germ cell lineages, respectively. This mouse line was created by spontaneous mating of a Tg mouse generated by micro-injecting a reporter plasmid DNA comprising the expression control region of the mouse Oct4 gene and the GFP gene ligated thereto into a fertilized egg (Oct4-GFP), and a Tg mouse generated by micro-injecting a BAC clone DNA comprising the mouse Mvh genomic gene and the RFP gene inserted thereinto (Mvh-RFP).

The retroviruses used for the reprogramming were prepared by separately introducing retroviral expression vectors (pMXs-

Oct3/4, pMXs-Sox2, pMXs-Klf4, pMXs-Nanog) to Plat-E cells (Morita, S. et al., Gene Ther. 7, 1063-1066 (2000)) that had been seeded to 6-well culture plates (Falcon) at 0.6 x 10^6 cells per well on the previous day. The culture broth used was DMEM/10% FCS [DMEM (Nacalai Tesque) supplemented with 10% fetal bovine serum], and the cells were cultured at 37°C, 5% CO_2. To introduce each vector, 4.5 μL of the FuGene6 transfection reagent (Roche) was added to 100 μL of Opti-MEM I Reduced-Serum Medium (Invitrogen), and the medium was allowed to stand at room temperature for 5 minutes. Subsequently, 1.5 μg of each expression vector was added, and the medium was allowed to stand at room temperature for 15 minutes, after which they were added to a Plat-E cell culture broth. On day 2, the Plat-E cell culture supernatant was replaced with a fresh supply of the medium. On day 3, the culture supernatant was recovered and filtered through a 0.45 μm sterile filter (Whatman), polybrene (Nacalai Tesque) was added to obtain a final concentration of 4 μg/mL, and this was used as the virus liquid.

Fibroblasts (MEFs) were isolated from a fetus (13.5 days after fertilization) of the above-described Oct4-GFP/Mvh-RFP Tg mouse. These MEFs were seeded to 6-well culture plates (Falcon) coated with 0.1% gelatin (Sigma) at 1 x 10^5 cells per well. The culture broth used was DMEM/10% FCS, and the fibroblasts were cultured at 37°C, 5% CO_2. The following day, each retrovirus liquid was added to cause overnight infection for gene transfer of the four genes Oct3/4, Sox2, Klf4 and Nanog, or the three genes Oct3/4, Sox2 and Klf4.

Starting 3 days after the viral infection, the cells were cultured using an ES cell culture medium supplemented with LIF [DMEM (Nacalai Tesque) supplemented with 15% fetal bovine serum, 2 mM L-glutamine (Invitrogen), 100 μM non-essential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Invitrogen), 50 U/mL penicillin (Invitrogen) and 50 μg/mL streptomycin (Invitrogen)]. On day 5 after the infection, the MEF medium was removed, and

the cells were washed by the addition of 1 mL of PBS. After the PBS was removed, 0.25% trypsin/1 mM EDTA (Invitrogen) was added, and a reaction was allowed to proceed at 37°C for about 5 minutes. After the cells floated up, cells were suspended by the addition of an ES cell culture medium and 5 x 10^3 cells were seeded to a 100 mm dish having feeder cells seeded thereto previously. The feeder cells used were SNL cells treated with mitomycin C to terminate their cell division [McMahon, A.P. & Bradley, A. *Cell*, 62, 1073-1085 (1990)]. Cultivation was continued while replacing the ES cell culture medium with a fresh supply of the same medium every two days until a visible colony emerged. Photographs of iPS cells colonies taken on day 17 after the infection are given in Fig. 1. In both cases of 4-gene transfer and 3-gene transfer, Oct3/4-GFP-positive Mvh-RFP-negative iPS cell colonies were established.

Genomic PCR analysis of these iPS clones by a conventional method confirmed the integration of the transferred exogenous genes in all the clones (Fig. 2).

Each iPS clone was examined for the expression of undifferentiation markers by RT-PCR analysis using the Rever Tra Ace kit (Takara). The results are shown in Fig. 3. Every iPS clone exhibited the expression of undifferentiation markers at levels comparable to those in ES cells and Nanog-iPS [Nature, 448, 313-317 (2007)].

Next, a teratoma was formed according to the method described in Cell, 126, 663-676 (2006). Specifically, 1 x 10^6 iPS cells were subcutaneously injected to an immunodeficient mouse; 4 weeks later, a teratoma was isolated. The teratoma was shredded, and fixed in PBS(-) containing 4% formaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin-eosin. The results are shown in Fig. 4. Histologically, the tumor was composed of a plurality of kinds of cells, with neural tissue, epidermal tissue, muscular tissue, cartilage tissue, adipose tissue, and gut-like epithelial tissue noted, demonstrating the multipotency of the

established iPS cells.

[0137]

Example 2: Establishment of differentiation maintenance medium for germline stem cells

To induce the differentiation of the established iPS cells, an attempt was made to form embryoids (EBs) using DMEM/10% FCS. Specifically, 5 x 10^5 iPS cells per mL of culture broth were seeded to 6-well weakly cell-adhesive culture plates (Nunc), and the medium was replaced with a fresh supply of the same medium every two days. Photographs taken on day 14 of the cultivation are given in Fig. 5. Differentiation of some Mvh-RFP-positive germ cells was observed in the EBs derived from the same Oct4-GFP/Mvh-RFP Tg ES cells, whereas no Mvh-RFP-positive cells were found in the EBs derived from the iPS cells.

With this in mind, an attempt was made to improve M15-BMP4 cells, which were used as differentiation support cells in PNAS, 100, 11457-11462 (2003), in order to establish culturing conditions for inducing the differentiation of iPS cells into germ cells. Each of retroviral expression vectors of glial cell-derived neurotrophic factor (GDNF), membrane-binding stem cell factor (mSCF), and epithelial cell growth factor (EGF) (pMXs-GDNF-IP, pMXs-mSCF-IP, pMXs-EGF-IP) was transferred to Plat-E cells by the above-described method, and the M15-BMP4 cells were infected with the virus liquid obtained. To select virus-infected cells, the cells were cultured in the presence of 0.2 mg/mL neomycin and 2.5 μg/mL puromycin, and proliferating cells were newly designated M15-4GF. The morphology of M15-4GF and the results of RT-PCR are shown in Fig. 6. RT-PCR confirmed that the expression of the transferred GDNF, mSCF, and EGF, in addition to BMP4, increased in M15-4GF.

Furthermore, a culture medium suitable for induction of the differentiation of germ cells was prepared. The composition is shown in Fig. 27.

With the Neurobasal medium (Invitrogen) as the basal medium, 1x B-27 Supplement (Invitrogen), 1 x Penicillin-Streptomycin-Glutamine (Invitrogen), 5 mg/mL Bovine Albumin (MP Biomedicals), 0.1 mM Non-Essential Amino Acids (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen), 1x Vitamin Solution (Invitrogen), 1x Insulin-Transferrin-Selenium Supplement (Invitrogen), 6 mg/mL D-(+)-Glucose (Sigma), 60 ng/mL Progesterone (Sigma), 30 ng/mL β-Estradiol (Sigma), 55 μM 2-Mercaptoethanol (Invitrogen), 0.34 μL/mL Sodium DL-lactate (Sigma), 60 μg/mL Putrescine dihydrochloride (Sigma), and 0.1% FCS (Invitrogen) were added (final concentrations are shown; see the "Medium component" panel in Fig. 27). For maintenance culture of the cells after differentiation induction, 15 ng/mL GDNF (R&D systems), 20 ng/mL EGF (AUSTRAL Biologicals), 12.5 ng/mL basic fibroblast growth factor (bFGF) (Wako), 10 ng/mL SCF (R&D systems), 10 ng/mL hepatocyte growth factor (HGF) (PEPROTECH), 5 ng/mL interleukin-2 (IL2) (Roche), 12.5 ng/mL fibroblast growth factor 9 (R&D systems), 10 μM Forskolin (Sigma), 0.1 μM Testosterone (Sigma), and 0.4x StemPro Supplement (Invitrogen) were added to the above-described culture broth (final concentrations are shown; see the "Supplement" panel in Fig. 27).

To induce the differentiation of germ cells, iPS cells and M15-4GF cells were mixed in the same culture broth as the above, but not supplemented with growth factors (Supplement), to obtain a cell density of 5×10^5 cells per mL for each cell type, and seeded to 6-well weakly cell-adhesive culture plates. With medium exchanges every two days, suspension culture was performed; differentiation of Mvh-RFP-positive cells in cell masses was confirmed. On day 28 of the cultivation, suspended cell masses were recovered in a 15 mL tube and washed with PBS, after which 0.25% Trypsin/1 mM EDTA and 0.2 mg/mL collagenase IV (Invitrogen) were added, and a reaction was allowed to proceed at 37°C for about 15 minutes. After addition of DMEM/10% FCS, the resulting suspension was centrifuged (1000

rpm), and the supernatant was replaced with a culture broth supplemented with growth factors (Supplement) for maintenance culture. To remove the M15-4GF cells, the cell suspension was seeded onto gelatin-coated culture plates and allowed to stand for about 30 minutes to 1 hour, after which suspended cells were recovered, seeded onto mitomycin C-treated MEF feeder cells (Millipore), and cultured. As a result, Oct4-GFP-positive / Mvh-RFP-positive colonies emerged (Fig. 7).

The cells of the Oct4-GFP-positive / Mvh-RFP-positive colonies proliferated stably under the above-described culturing conditions, exhibiting a morphology different from that of undifferentiated iPS cells (Fig. 8). Also noted was an activity of alkaline phosphatase, a cell surface marker serving in both undifferentiated cells and germ cells (Fig. 9).

When these Oct4-GFP-positive / Mvh-RFP-positive colonies were cultured under iPS cell culturing conditions (DMEM/10% FCS) or under the GS cell culturing conditions described in Biol. Reprod, 69, 612-616 (2003) (GDNF, LIF, EGF and bFGF added), the proliferation rate decreased compared with the findings obtained under the above-described culturing conditions, and partial cell death and decreased Mvh-RFP fluorescence intensity were noted (Fig. 10). When undifferentiated iPS cells were directly subjected to cultivation under the above-described maintenance culturing conditions without following the differentiation induction process, Mvh-RFP was not induced, although Oct4-GFP regression accompanying somatic cell differentiation and cell death were observed (Fig. 11).

[0138]

Example 3: Characterization of Oct4-GFP-positive / Mvh-RFP-positive cells

When suspended cell masses were dissociated and cultured under the above-described culturing conditions, not only Oct4-GFP-positive / Mvh-RFP-positive germ cell-like cells, but also Oct4-GFP-positive / Mvh-RFP-negative apparently

undifferentiated cells were successfully separated and stably expanded (Fig. 12). The Oct4-GFP-positive / Mvh-RFP-positive cells were designated "GR cells", and the Oct4-GFP-positive / Mvh-RFP-negative cells "Gsp cells".

Genomic PCR analysis of the GR cells and the Gsp cells confirmed the integration of the exogenous genes introduced at the time of generating the iPS cells. In EK cells, which represent an Oct4-GFP-negative / Mvh-RFP-positive germ cell-like cell line that had been differentiation-induced and established from ES cells in the past, no exogenous genes were noted. By contrast, the GR cells and Gsp cells were shown to be derived from iPS cells (Fig. 13). Examining the band pattern of the exogenous genes in Southern blot analysis also confirmed that the GR cells and the Gsp cells were derived from the same clone as the iPS cells (Fig. 14). It was also found, however, that one exogenous gene, Sox2, was lacked in the GR cells.

Undifferentiated iPS cells or GR cells were seeded onto mitomycin C-treated MEF feeder cells that had been seeded to 24-well culture plates at 2.5×10^3 cells per well. Total cell counting taken every two days showed that the GR cells proliferated at a rate about half that of the iPS cells (Fig. 15). To determine the feeder cell dependency of GR cell proliferation, 6-well culture plates were coated with gelatin, laminin, or fibronectin, and GR cells were seeded at 1×10^4 cells per well. As a result, the GR cells proliferated while colonizing when seeded onto MEF feeder cells, whereas under feeder-free conditions, almost no cells adhered to the plates whichever extracellular matrix was used, nor was there any evidence of colonization (Fig. 16).

When tumor cells or undifferentiated iPS cells are subcutaneously transplanted to an immunodeficient nu/nu mouse, a tumor is formed at the transplantation site. Meanwhile, it is known that tumorigenesis is not noted in the transplantation of GS cells, which represent a line of

spermatogonial stem cells. With this in mind, GR cells and Gsp cells were transplanted to immunodeficient mice, which were then examined for tumorigenesis. As a result, it was found that when Gsp cells were transplanted, they cancerated, although the resulting tumors are smaller than those when transplanting iPS cells. Meanwhile, when GR cells were transplanted, no tumorigenesis was noted as with the EK cells derived from ES cells, showing that the GR cells are not cells that have cancerated like embryonic carcinoma (EC) cells (Fig. 17).

Because the iPS cells used for the differentiation induction had the exogenous genes transferred thereto by means of a retrovirus, some of the cells obtained might have been immortalized due to reactivation of the retrovirus. With this in mind, the expression of the exogenous transgenes and corresponding endogenous genes was analyzed by RT-PCR. As a result, there was no evidence of increased abnormal expression of the exogenous transgenes (Fig. 18). Quantitation of the gene expression by realtime PCR revealed that the expression of the exogenous genes in the GR cells decreased compared with the undifferentiated iPS cells (Fig. 19).

Subsequently, the expression of germ cell marker genes in GR cells was examined by RT-PCR. As reported in BMC Dev. Biol., 6, 34 (2006), the expression of ECAT1 and Fgf4 is suppressed in GS cells. However, the expression of these genes was not suppressed in the GR cells. Additionally, ERas, which is normally not expressed in the cells of living organisms, was expressed, demonstrating the identity of the GR cells as established cells in culture. In the GR cells, the expression of Prdm14, Mvh, Plzf, c-Ret and the like increased, whereas the expression of Stra8, Ngn3 and the like decreased. The results are shown in Figs. 20 and 21.

A comparison of the comprehensive gene expression of iPS cells and GR cells using DNA microarrays demonstrated increased expression of genes related to matrix

metalloprotease and chemokines and decreased expression of the extracellular matrix keratin, the cell adhesion factor claudin and the like. This result suggests that in the GR cells, cell motility may have been increased, as in primordial germ cells in the migration stage (Fig. 22).

Also analyzed by Western blotting was the expression of the proteins of germ cell marker genes. The results are shown in Fig. 23. The GR cells were found to be Oct4-GFP-positive, and to undergo suppression at the protein level, despite confirmation by RT-PCR of the occurrence of the transcription of the endogenous Oct4 gene. Also observed in the GR cells was increased expression of the proteins of Nanog, ECAT1, Mvh, and Dnmt3L.

Subsequently, the DNA methylation status was analyzed by bisulphite genomic sequencing. The results are shown in Fig. 24. In EK cells, the methylation control regions of the female imprinted genes Igf2r, SNRPN, and Lit1 are in the demethylated state, whereas the male imprinted gene H19 is in a highly methylated state; this demonstrates the acquisition of an imprint status of the male type. Meanwhile, in GR cells, the methylation control regions of the female imprinted genes exhibited a somatic cell pattern, demonstrating that the elimination of genomic imprinting, which is to be induced in the fetal genital primordium, has not occurred. Interpreted comprehensively, these findings of gene expression and the DNA methylation status led to the speculation that the GR cells represent a cell line that reflects the characteristics of primordial germ cells in the later stage of migration (just before or after penetration into the fetal genital primordium).

To determine whether GR cells possess the capability of spermiogenesis, GR cells were transplanted to the testis of a W/Wv mouse, which is a model of infertility developed by deleting germ cells by a mutation of the c-kit gene (courtesy of Professor Takashi Shinohara at the Faculty of Medicine, Kyoto University), by the method described in Biol. Reprod, 69,

612-616 (2003). Specifically, GR cells were suspended in DMEM/10% FCS at 3×10^7 cells/mL, and injected into the seminiferous tubule of a neonatal W/Wv mouse. Four months later, the testis was extirpated from the recipient, and fixed in PBS(-) containing 4% formaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin-eosin. The results are shown in Fig. 25. While the testis of a W/Wv mouse not undergoing transplantation of the cells showed a histological finding of the absence of reproductive cells, known as "Sertoli cell-only", the transplanted cells engrafted when GR cells were transplanted. The surviving graft cells did not exhibit teratoma formation, nor was there any evidence of clear canceration.

When transplanted to the testis, non-germ cells are unable to adapt to the environment in the testis and hence die or are eliminated. Meanwhile, transplantation of undifferentiated iPS cells or tumor cells leads to tumorigenesis. As stated above, when GR cells were transplanted, the cells were maintained in the testis for a long time of 4 months, with no evidence of tumorigenesis, demonstrating the identity of the GR cells as cells destined to become germline cells. However, the finding differed from the normal spermiogenesis profile. TUNEL staining demonstrated the induction of apoptosis-based cell death in the lumens where mature spermatids after meiosis are normally present.

[0139]

Example 4: Differentiation induction from ES cells to Oct4-GFP-positive / Mvh-RFP-positive cells

The culturing conditions described above were successfully applied to establish a total of five lines of GR cells from iPS cells in independent experiments. The conditions were applicable not only to iPS cells, but also to ES cells. Even when differentiation induction was attempted in the same way using ES cells, Oct4-GFP-positive / Mvh-RFP-positive cells were successfully established (Fig. 26).

Industrial Applicability

[0140]

The methods of GR cell differentiation induction and maintenance and expansion of the present invention make it possible to induce germline stem cells capable of spermiogenesis from iPS cells that can easily be generated from somatic cells such as skin cells, and to maintain and expand the same induced, and are therefore particularly useful in infertility treatment and gene therapy for patients with non-obstructive azoospermia, a condition that poses the problem of difficulty and/or risk in collecting spermatogonial stem cells. The GR cells generated according to the present invention are of high utility for the purpose of conserving animal varieties with favorable characters in the field of breeding.

This application is based on Japanese patent application No. 2010-052384 filed on March 9, 2010, the contents of which are hereby incorporated by reference.

CLAIMS

1. A method of producing an Oct4-positive Vasa-positive germline stem cell, comprising culturing a pluripotent stem cell in the presence of (a) bone morphogenetic protein 4 (BMP4) and (b) one or more growth factors selected from among glial cell-derived neurotrophic factor (GDNF), epithelial cell growth factor (EGF), and stem cell factor (SCF).

2. The method according to claim 1, wherein the method comprises culturing a pluripotent stem cell in the presence of the BMP4, GDNF, EGF, and SCF.

3. The method according to claim 1 or 2, wherein the pluripotent stem cell is cultured in the presence of feeder cells.

4. The method according to claim 3, wherein the growth factors are supplied by the feeder cells.

5. The method according to any one of claims 1 to 4, wherein the pluripotent stem cell is an iPS cell or ES cell.

6. The method according to any one of claims 1 to 5, wherein the pluripotent stem cell is derived from human or mouse.

7. An iPS cell-derived Oct4-positive Vasa-positive germline stem cell obtained by the method according to any one of claims 1 to 6.

8. An inducer of differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, comprising (a) BMP4 and (b) one or more growth factors selected from among GDNF, EGF, and SCF, in combination.

9. An inducer of differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, comprising BMP4, GDNF, EGF, and SCF in combination.

10. The inducer according to claim 8 or 9, wherein the inducer comprises a cell that produces the growth factors.

11. A culture medium for inducing differentiation from a pluripotent stem cell to an Oct4-positive Vasa-positive germline stem cell, supplemented with the inducer according to claim 8 or 9.

12. A method of expanding an Oct4-positive Vasa-positive germline stem cell, comprising culturing the germline stem cell in the presence of GDNF, EGF, SCF, and basic fibroblast growth factor (bFGF).

13. The method according to claim 12, wherein the method comprises culturing the germline stem cell in the presence of one or more factors selected from among hepatocyte growth factor (HGF), interleukin 2 (IL-2), and fibroblast growth factor 9 (FGF9).

14. The method according to claim 12 or 13, wherein the germline stem cell is cultured in the presence of feeder cells.

15. The method according to any one of claims 12 to 14, wherein the germline stem cell has been differentiation-induced from a pluripotent stem cell.

16. An expansion support agent for an Oct4-positive Vasa-positive germline stem cell, comprising GDNF, EGF, SCF, and bFGF in combination.

17. The agent according to claim 16, further comprising one or

more factors selected from among HGF, IL-2, and FGF9.

18. A culture medium for expanding an Oct4-positive Vasa-positive germline stem cell, supplemented with the agent according to claim 16 or 17.

19. A method of allowing an infertile animal to form sperms, comprising transplanting an Oct4-positive Vasa-positive germline stem cell obtained by the method according to any one of claims 1 to 6, the Oct4-positive Vasa-positive germline stem cell according to claim 7, or an Oct4-positive Vasa-positive germline stem cell expanded by the method according to any one of claims 12 to 15, to the testis of the recipient animal of the same species as the cell.

20. The method according to claim 19, wherein the germline stem cell is derived from an iPS cell generated from a somatic cell of an infertile animal.

21. The method according to claim 19 or 20, wherein the recipient animal is a human or mouse.

22. A therapeutic agent for male infertility comprising an Oct4-positive Vasa-positive germline stem cell obtained by the method according to any one of claims 1 to 6, the Oct4-positive Vasa-positive germline stem cell according to claim 7, or an Oct4-positive Vasa-positive germline stem cell expanded by the method according to any one of claims 12 to 15.

23. The agent according to claim 22, wherein the agent is to be administered to an individual from which spermatogonial stem cells are difficult to collect.

Fig. 1

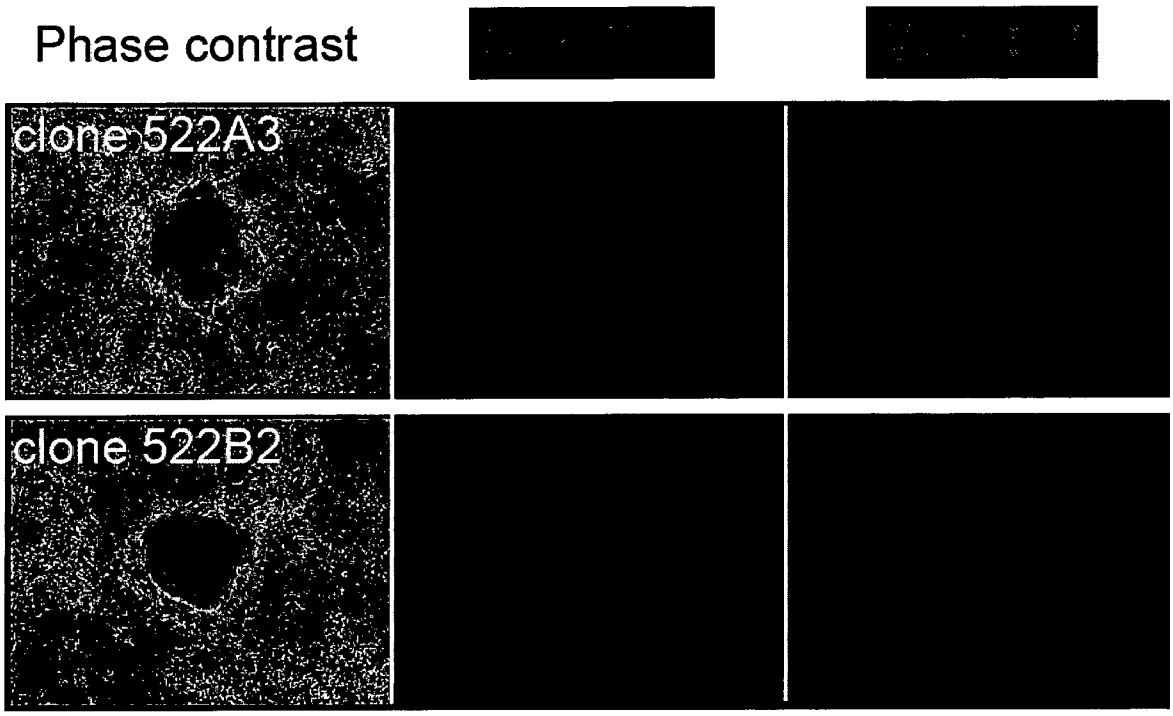
Phase contrast
clone 522A3
clone 522B2

Fig. 2

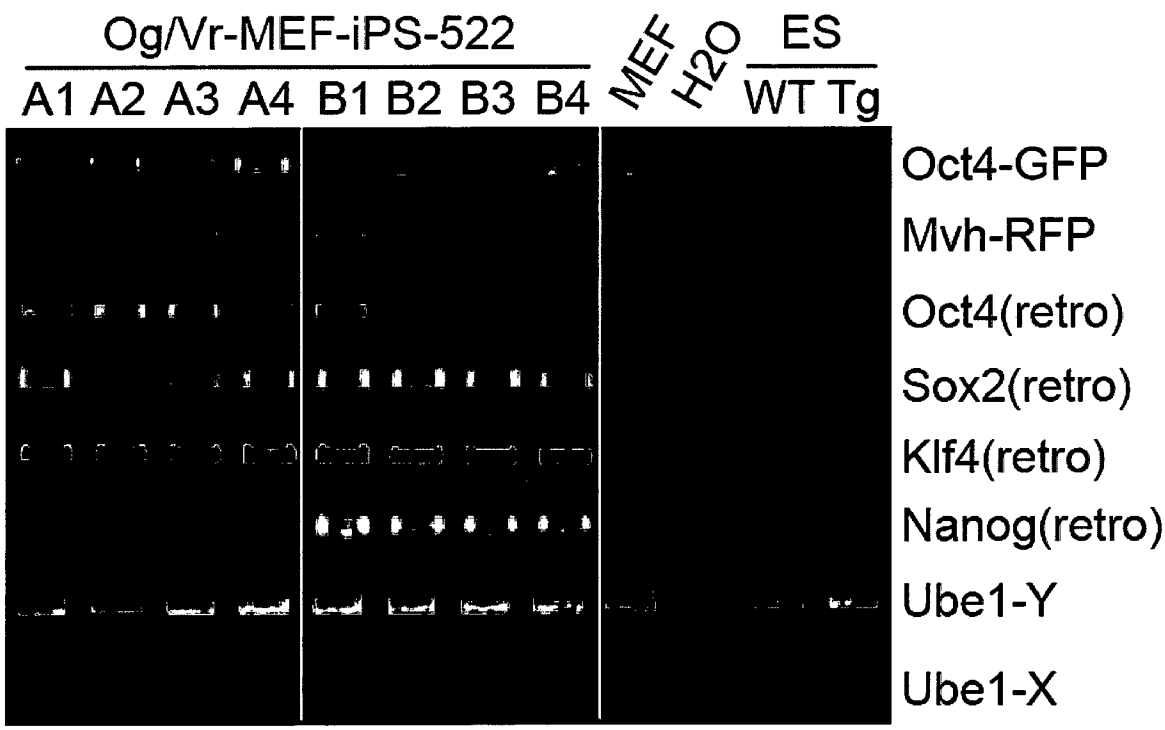
Og/Vr-MEF-iPS-522
A1 A2 A3 A4 B1 B2 B3 B4
MEF
H2O
ES
WT Tg
Oct4-GFP
Mvh-RFP
Oct4(retro)
Sox2(retro)
Klf4(retro)
Nanog(retro)
Ube1-Y
Ube1-X

Fig. 3

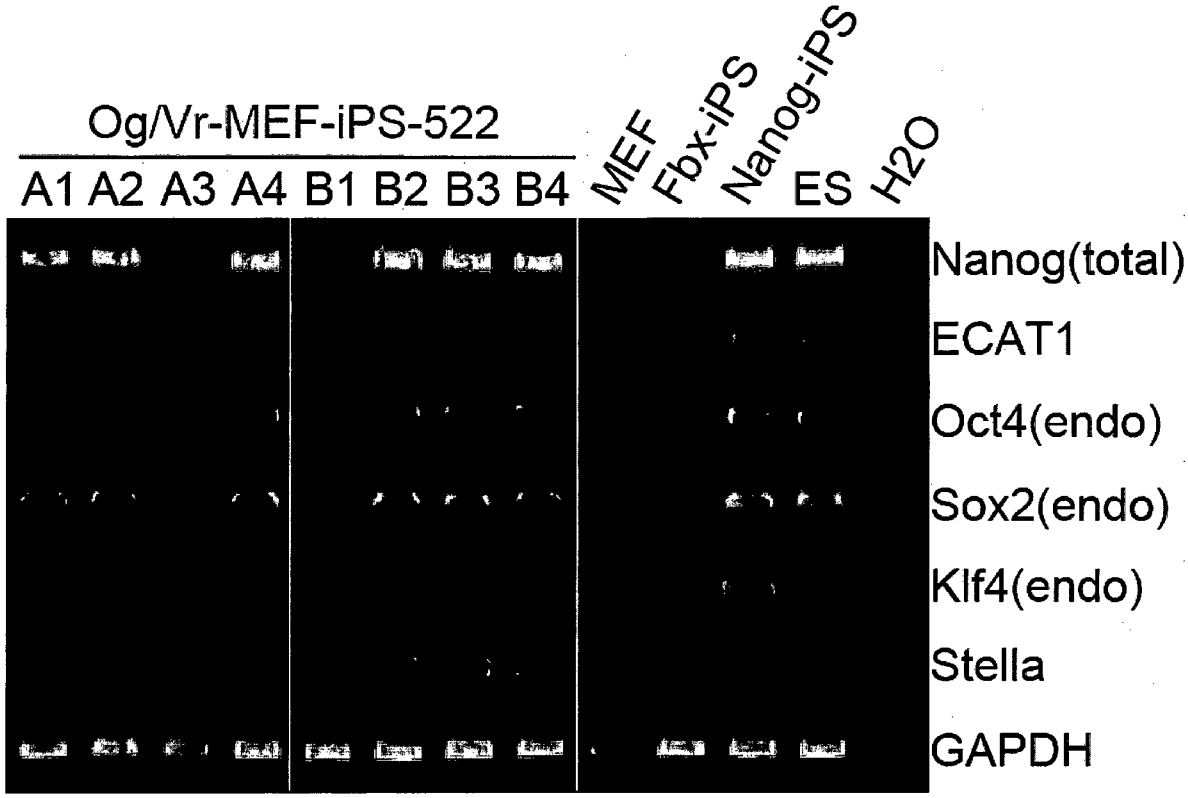
Og/Vr-MEF-iPS-522
A1 A2 A3 A4 B1 B2 B3 B4
MEF
Fbx-iPS
Nanog-iPS
ES
H2O
Nanog(total)
ECAT1
Oct4(endo)
Sox2(endo)
Klf4(endo)
Stella
GAPDH

Fig. 4

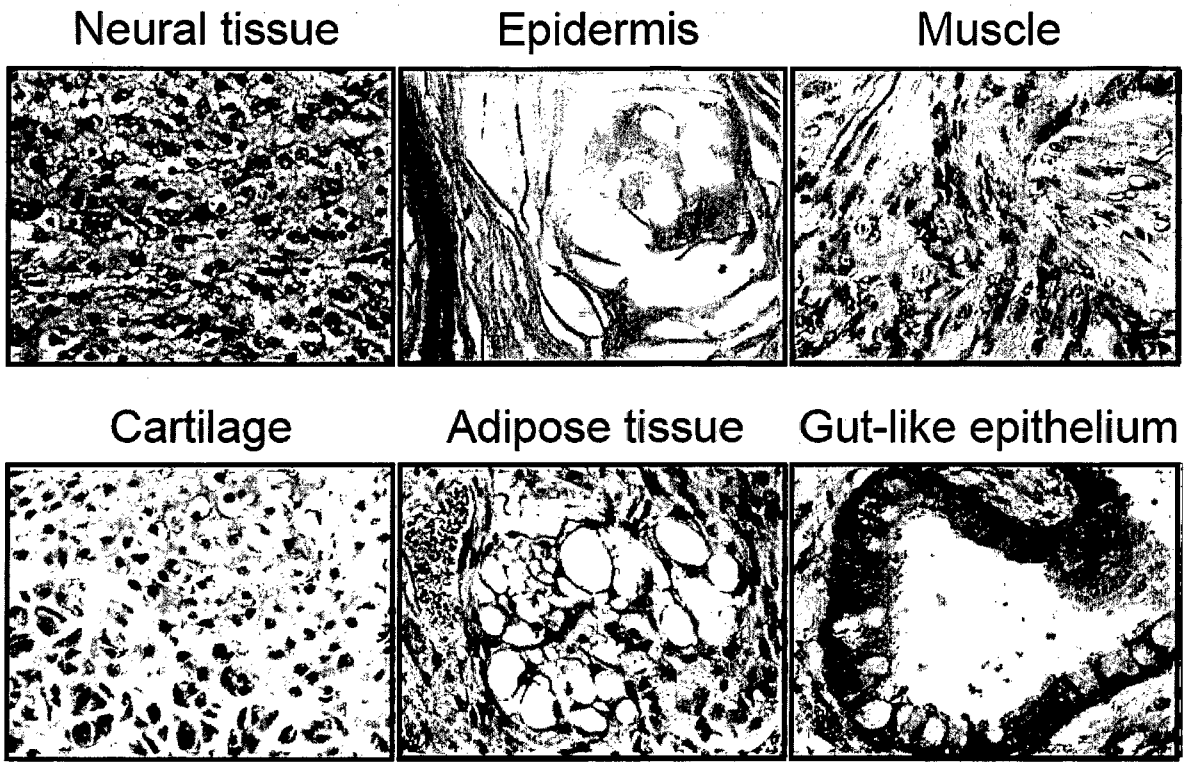
Neural tissue
Epidermis
Muscle
Cartilage
Adipose tissue
Gut-like epithelium

Fig. 5

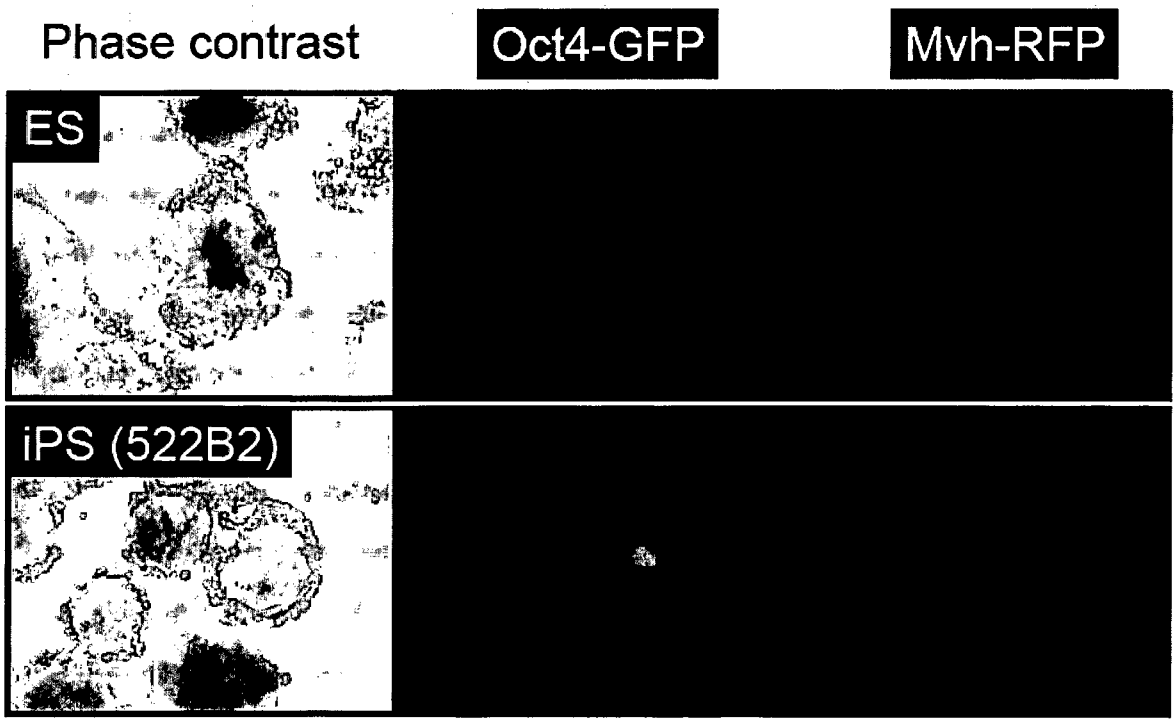
Phase contrast
Oct4-GFP
Mvh-RFP
ES
iPS (522B2)

Fig. 6

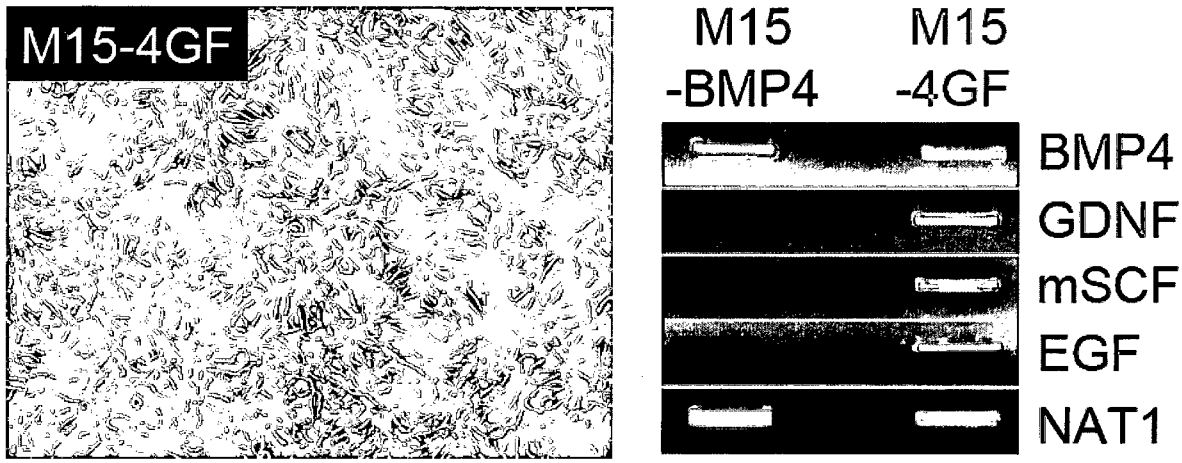
M15-4GF
M15
-BMP4
M15
-4GF
BMP4
GDNF
mSCF
EGF
NAT1

Fig. 7

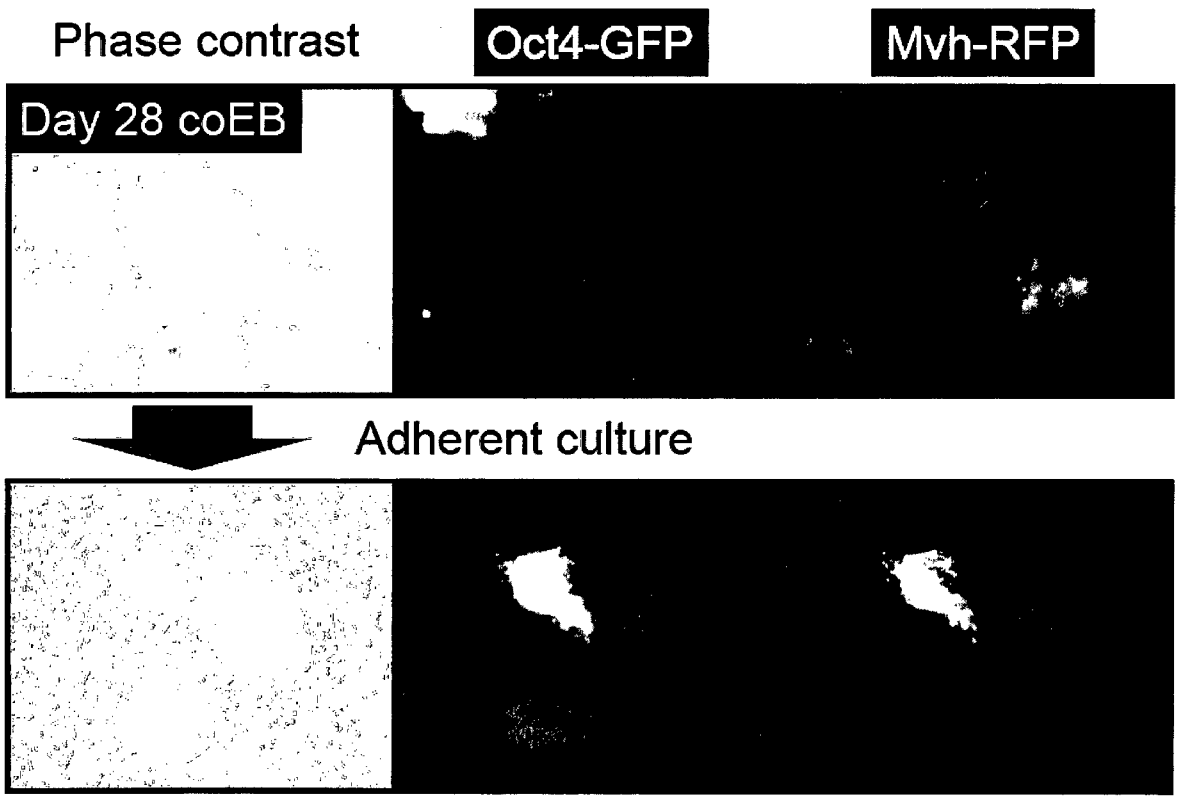
Phase contrast
Oct4-GFP
Mvh-RFP
Day 28 coEB
Adherent culture

Fig. 8

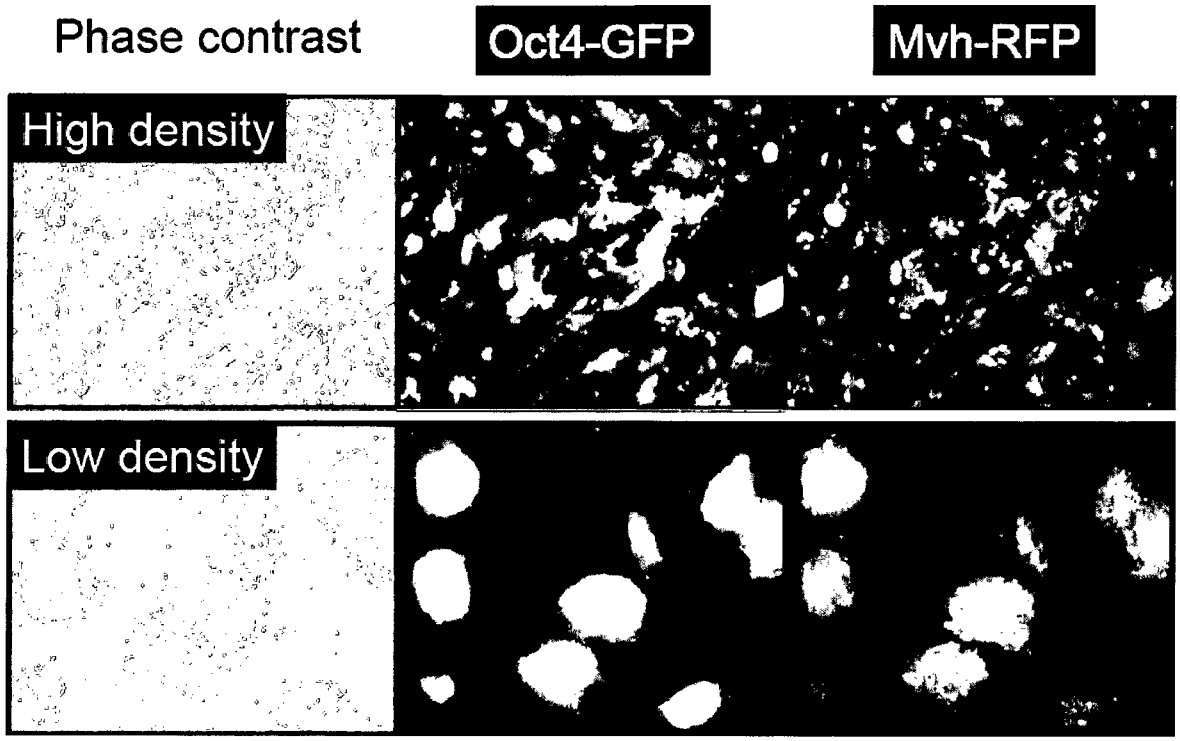
Phase contrast
Oct4-GFP
Mvh-RFP
High density
Low density

Fig. 9

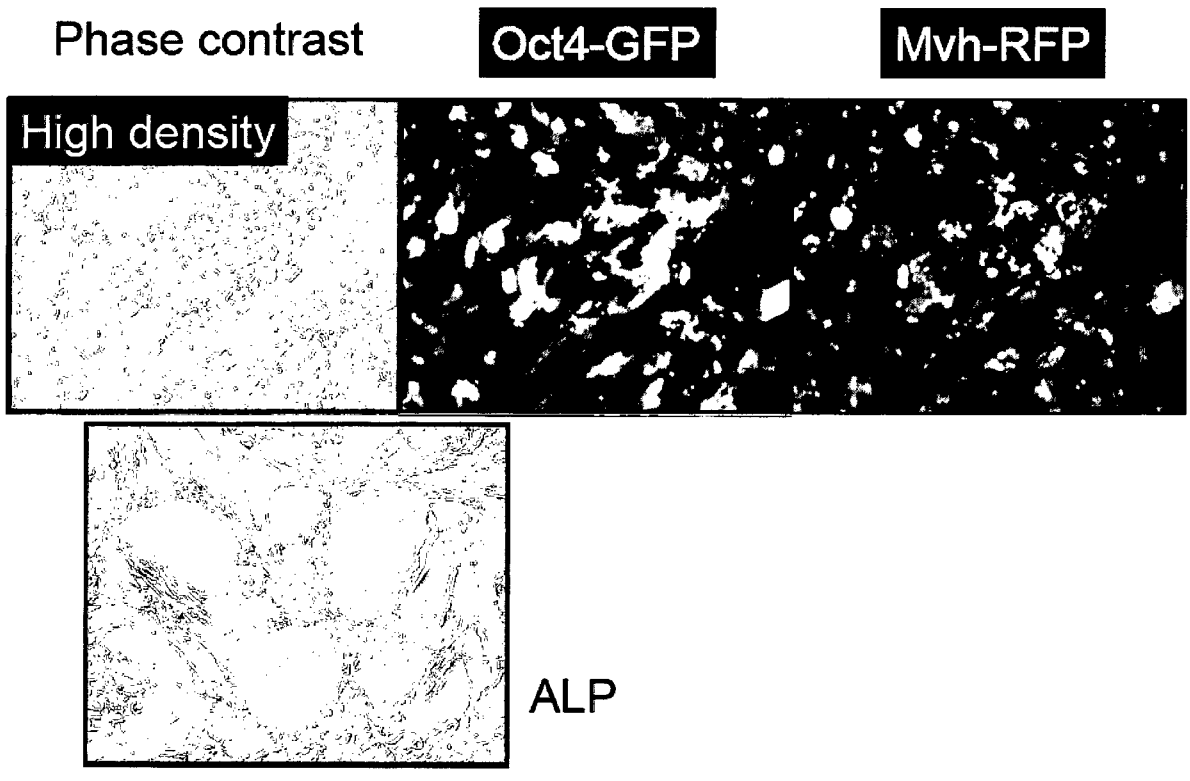
Phase contrast
Oct4-GFP
Mvh-RFP
High density
ALP

Fig. 10

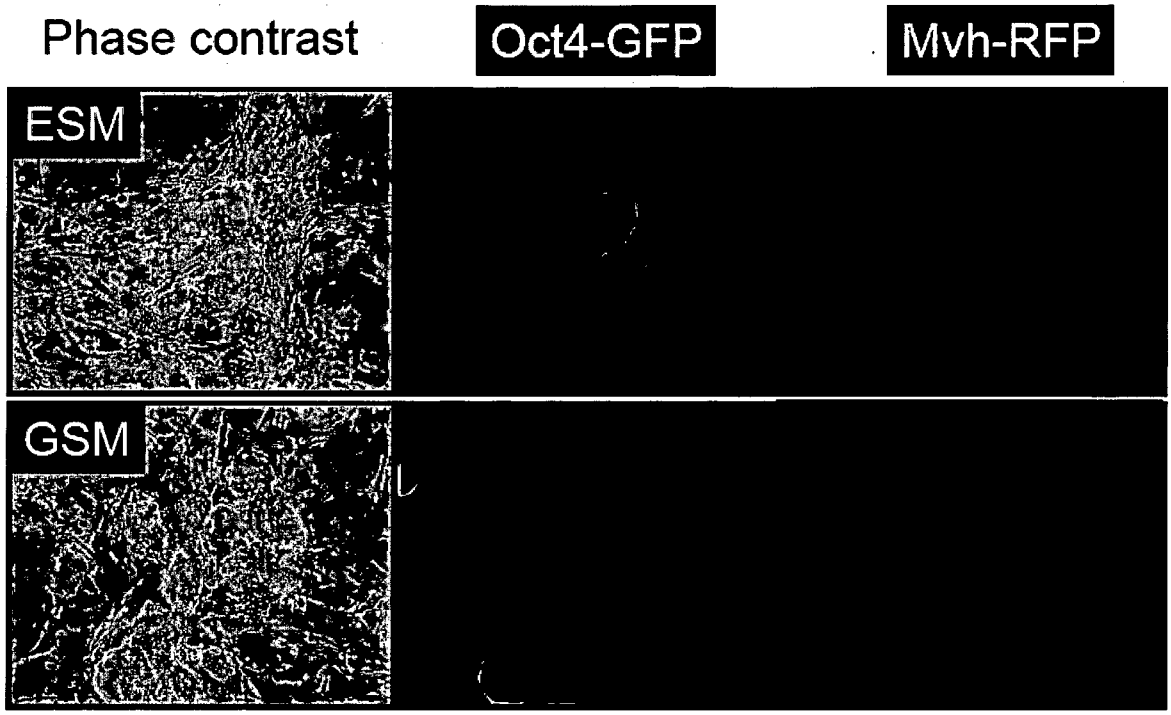

Fig. 11

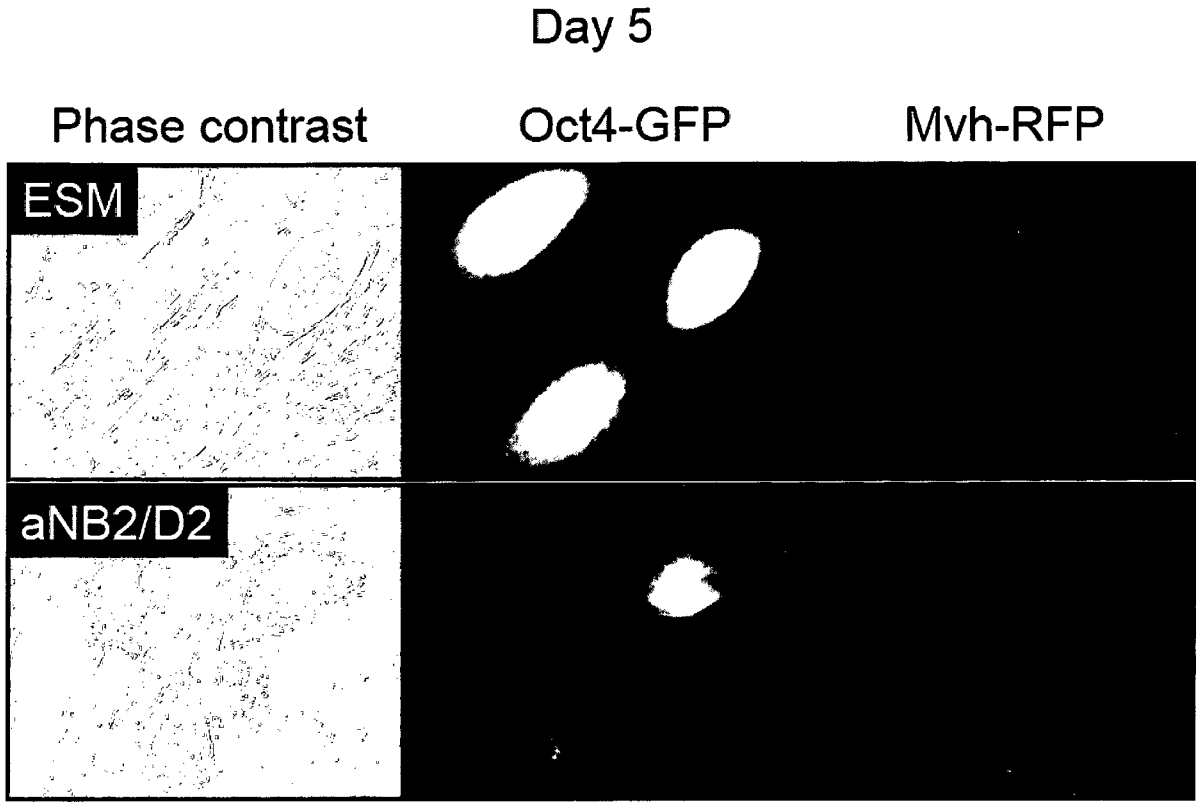
Day 5
Phase contrast
Oct4-GFP
Mvh-RFP
ESM
aNB2/D2

Fig. 12

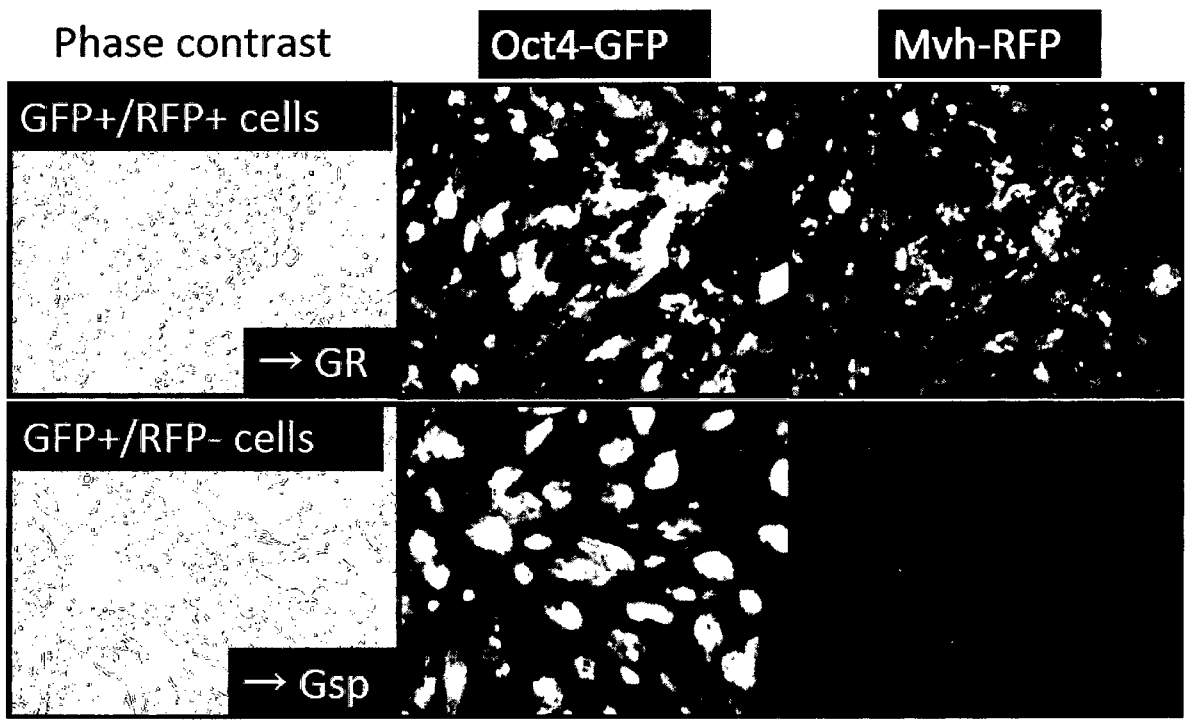
Phase contrast
Oct4-GFP
Mvh-RFP
GFP+/RFP+ cells
→ GR
GFP+/RFP- cells
→ Gsp

Fig. 13

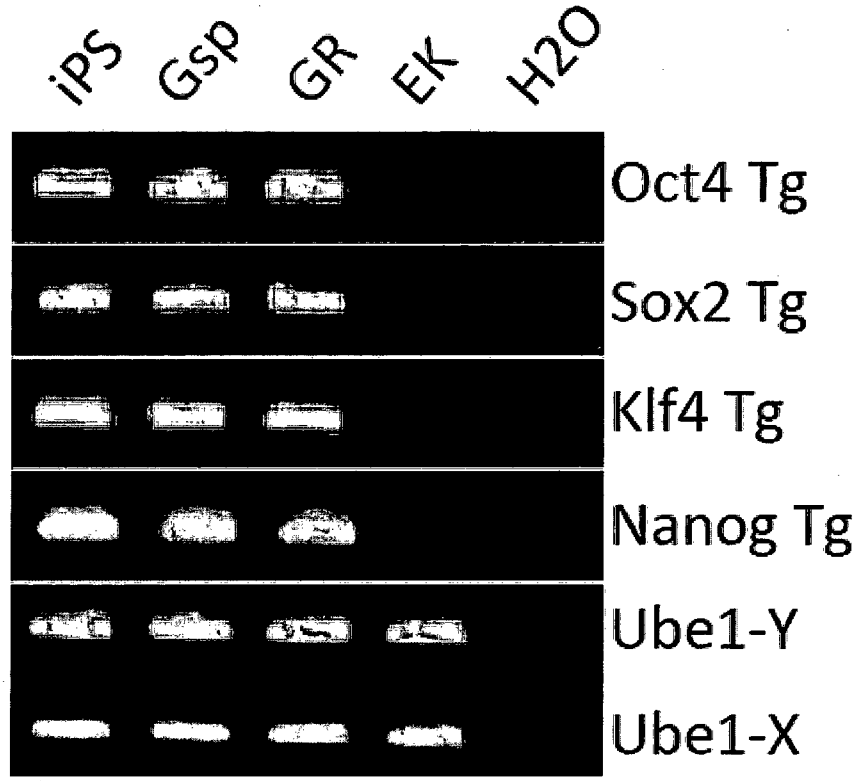

Fig. 14

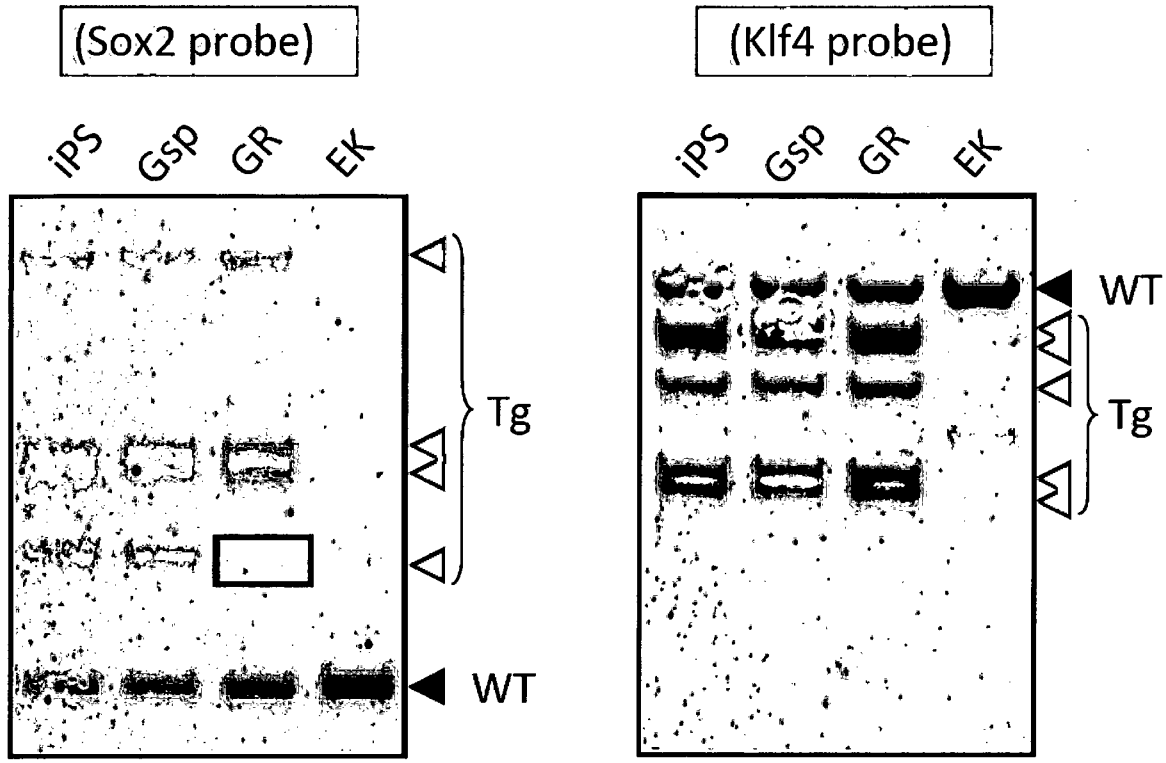
(Sox2 probe)
(Klf4 probe)
iPS
Gsp
GR
EK
Tg
WT

Fig. 15

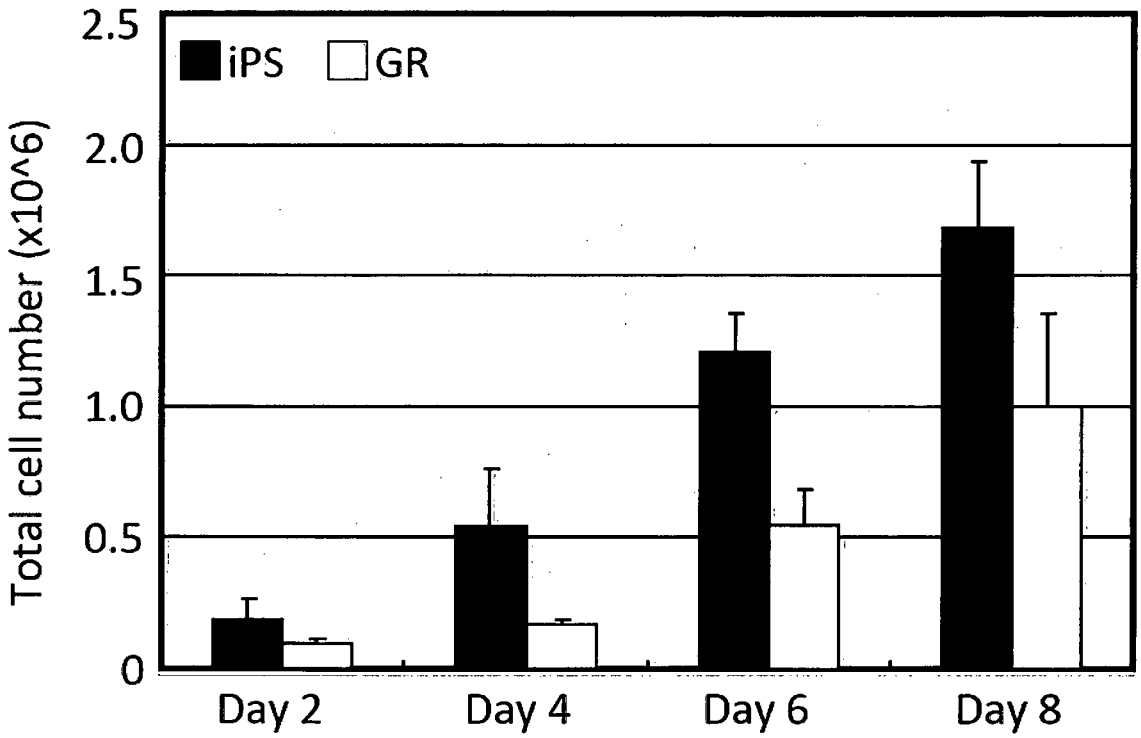
iPS
GR
Total cell number (x10^6)
2.5
2.0
1.5
1.0
0.5
0
Day 2
Day 4
Day 6
Day 8

Fig. 16

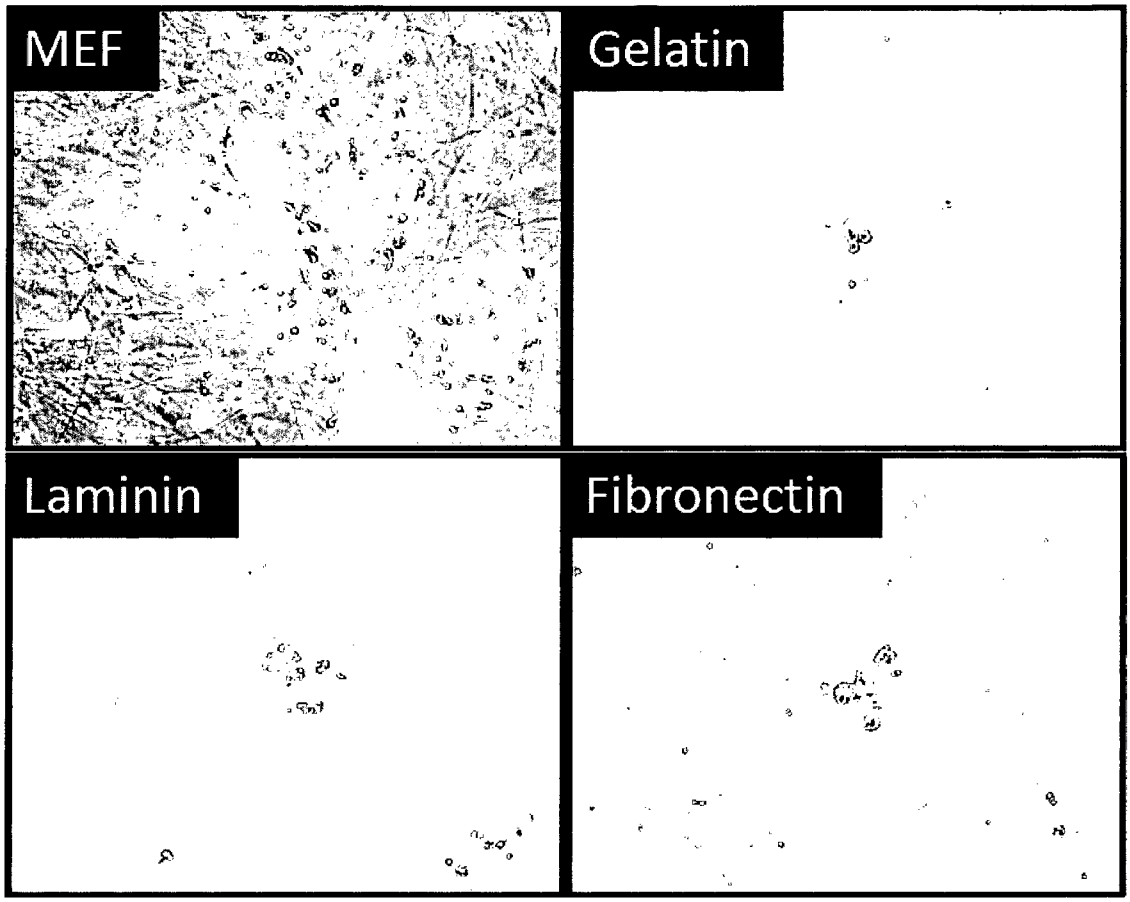

Fig. 17

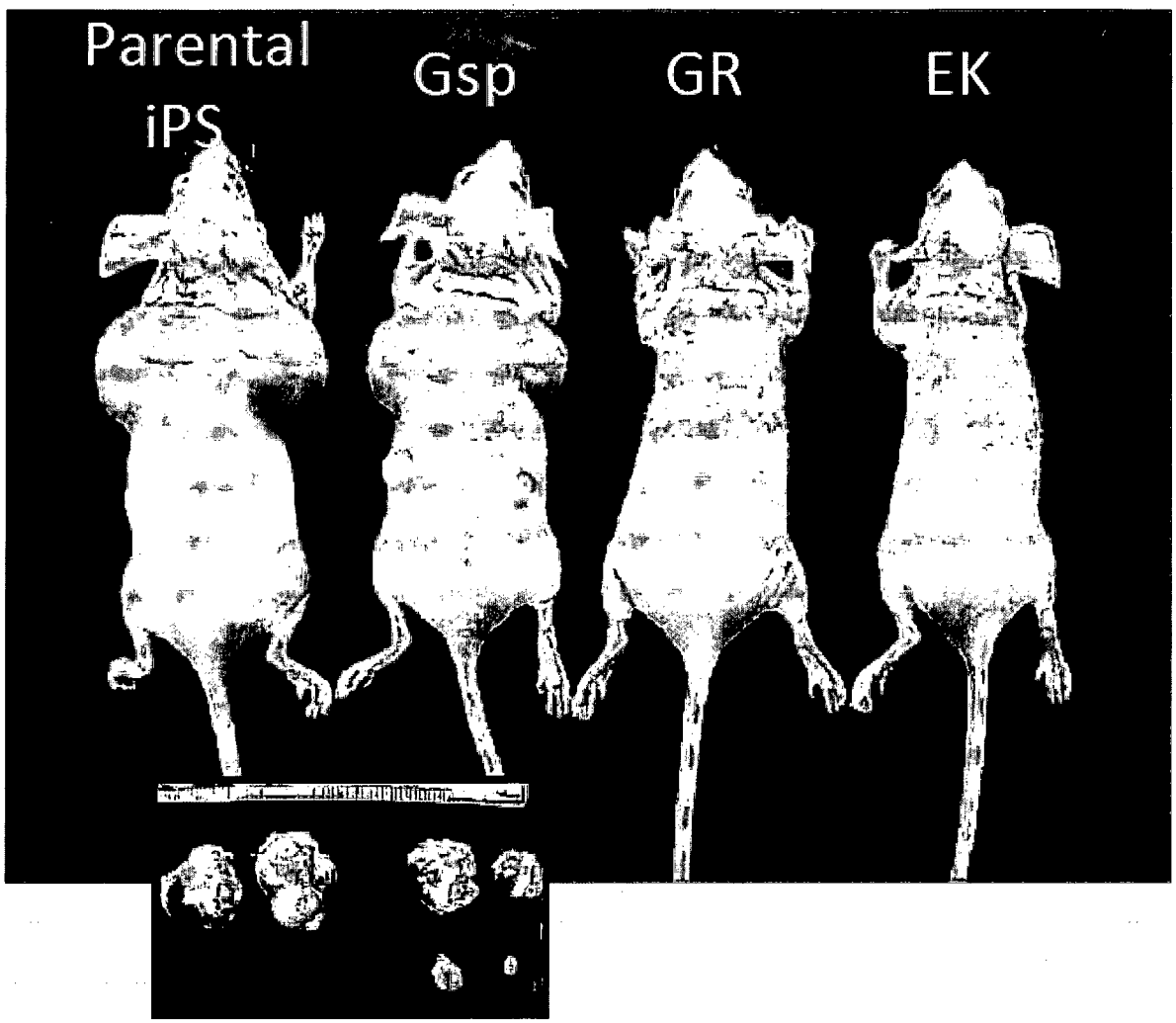

Fig. 18

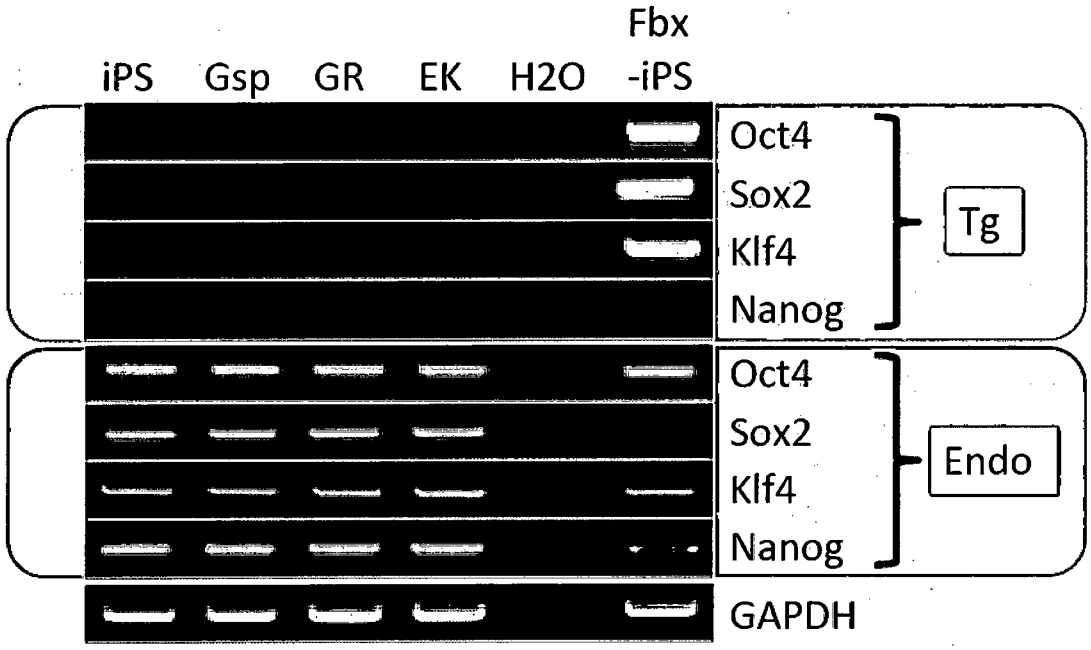
iPS
Gsp
GR
EK
H2O
Fbx
-iPS
Oct4
Sox2
Klf4
Nanog
Tg
Oct4
Sox2
Klf4
Nanog
Endo
GAPDH

Fig. 19

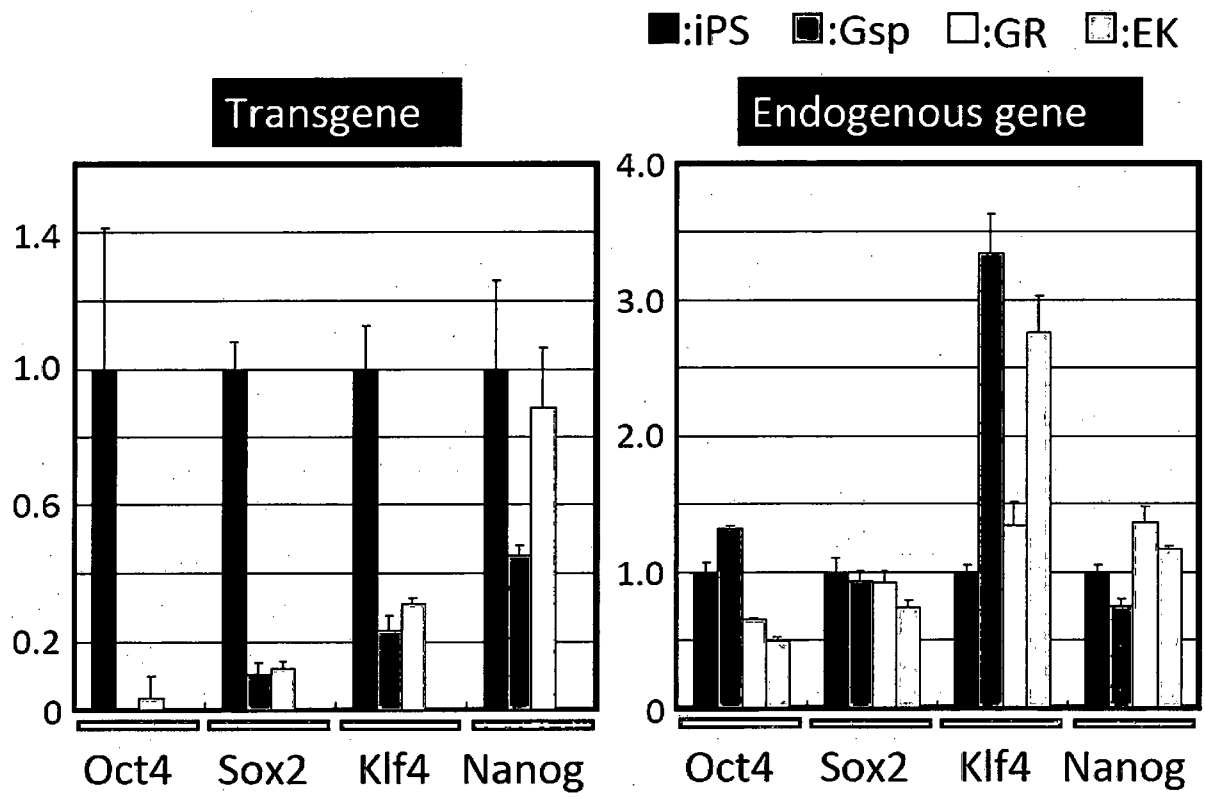
■:iPS ■:Gsp □:GR □:EK
Transgene
Endogenous gene
1.4
1.0
0.6
0.2
0
4.0
3.0
2.0
1.0
0
Oct4
Sox2
Klf4
Nanog
Oct4
Sox2
Klf4
Nanog

Fig. 20

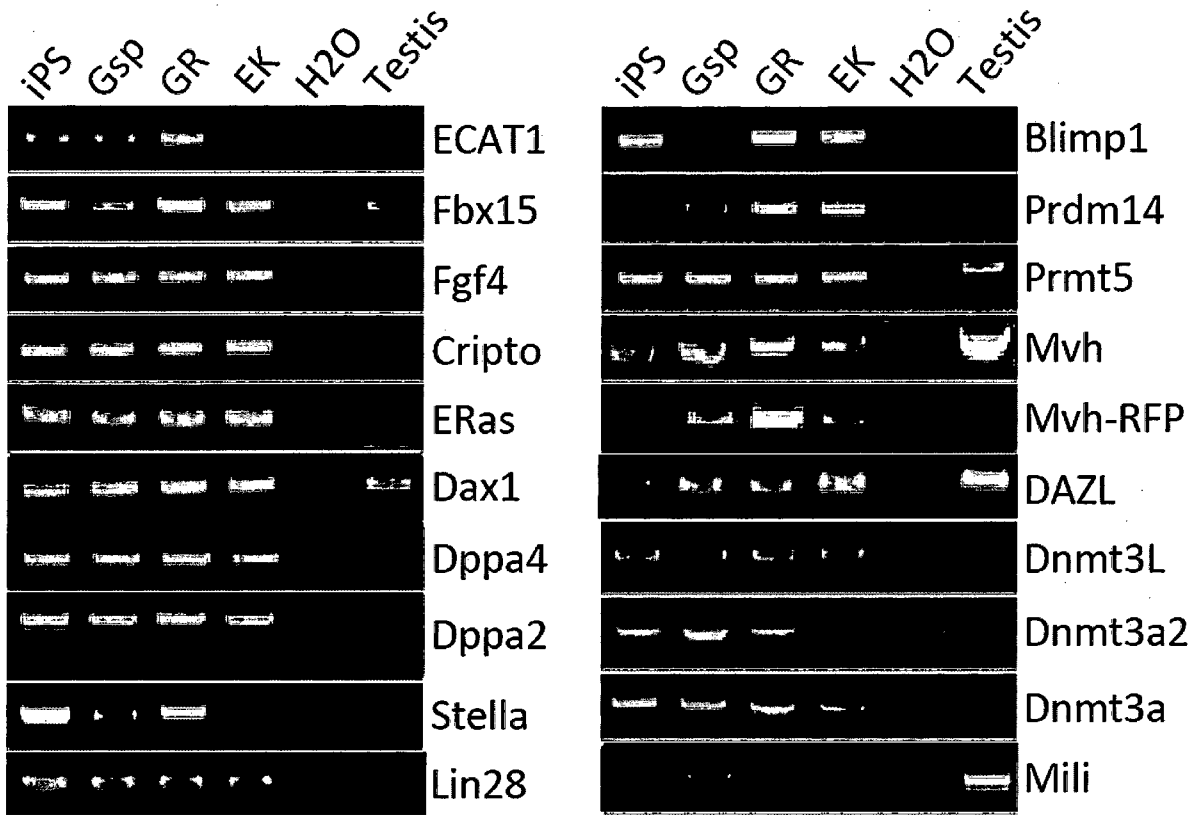
iPS
Gsp
GR
EK
H2O
Testis
ECAT1
Fbx15
Fgf4
Cripto
ERas
Dax1
Dppa4
Dppa2
Stella
Lin28
iPS
Gsp
GR
EK
H2O
Testis
Blimp1
Prdm14
Prmt5
Mvh
Mvh-RFP
DAZL
Dnmt3L
Dnmt3a2
Dnmt3a
Mili

Fig. 21

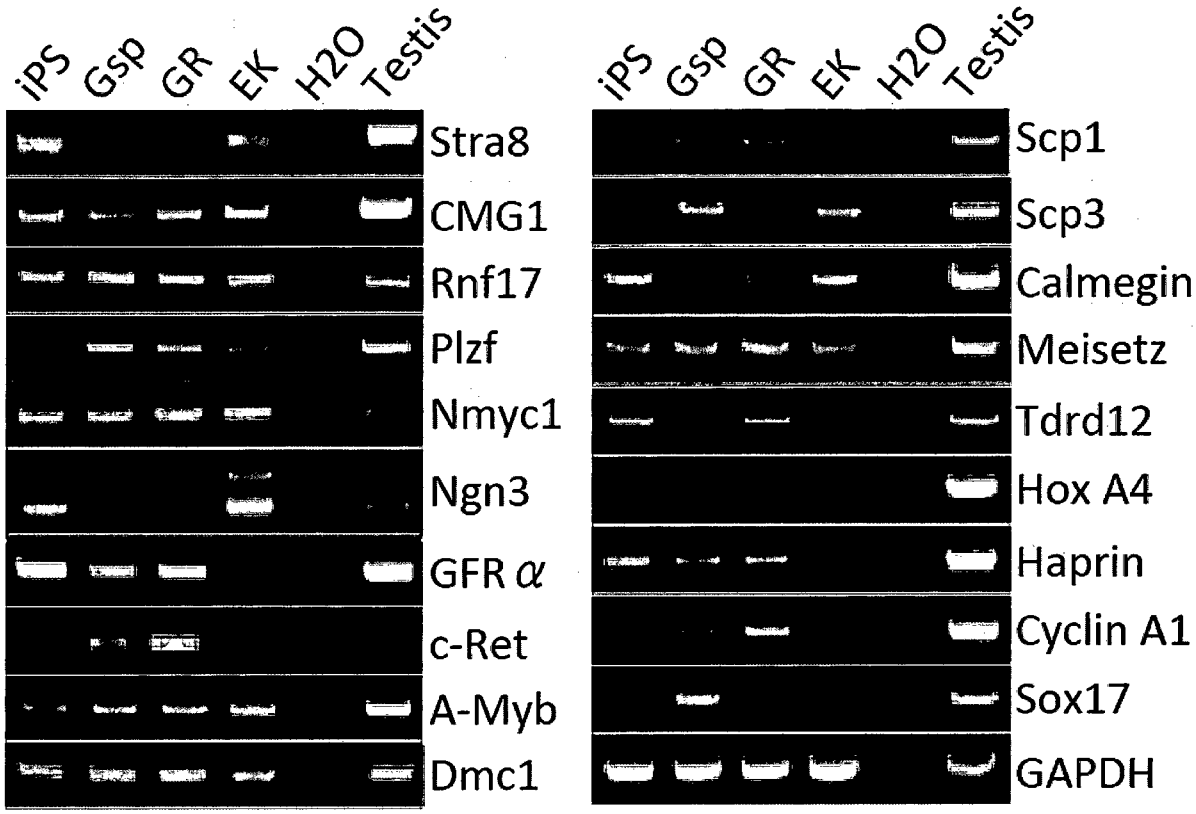
iPS
Gsp
GR
EK
H2O
Testis
Stra8
CMG1
Rnf17
Plzf
Nmyc1
Ngn3
GFR α
c-Ret
A-Myb
Dmc1
iPS
Gsp
GR
EK
H2O
Testis
Scp1
Scp3
Calmegin
Meisetz
Tdrd12
Hox A4
Haprin
Cyclin A1
Sox17
GAPDH

Fig. 22

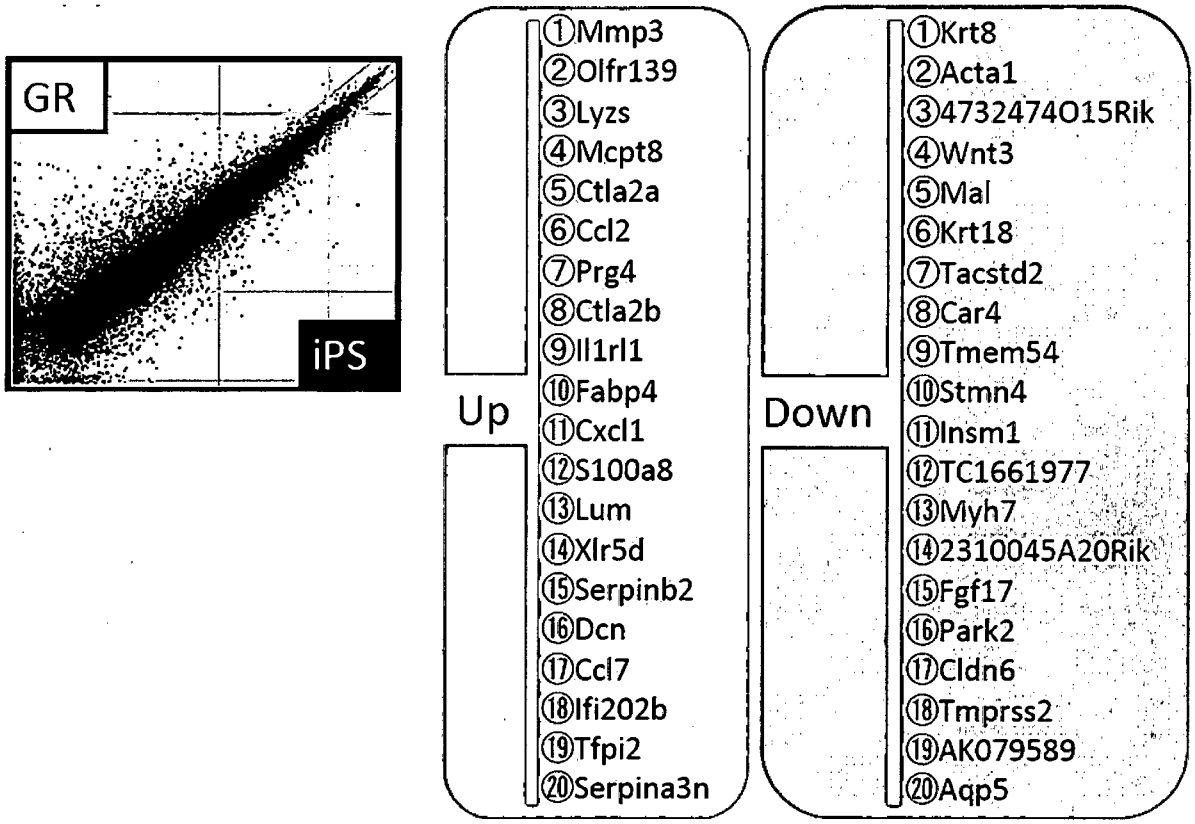
GR
iPS
Up
①Mmp3
②Olfr139
③Lyzs
④Mcpt8
⑤Ctla2a
⑥Ccl2
⑦Prg4
⑧Ctla2b
⑨Il1rl1
⑩Fabp4
⑪Cxcl1
⑫S100a8
⑬Lum
⑭Xlr5d
⑮Serpinb2
⑯Dcn
⑰Ccl7
⑱Ifi202b
⑲Tfpi2
⑳Serpina3n
Down
①Krt8
②Acta1
③4732474O15Rik
④Wnt3
⑤Mal
⑥Krt18
⑦Tacstd2
⑧Car4
⑨Tmem54
⑩Stmn4
⑪Insm1
⑫TC1661977
⑬Myh7
⑭2310045A20Rik
⑮Fgf17
⑯Park2
⑰Cldn6
⑱Tmprss2
⑲AK079589
⑳Aqp5

Fig. 23

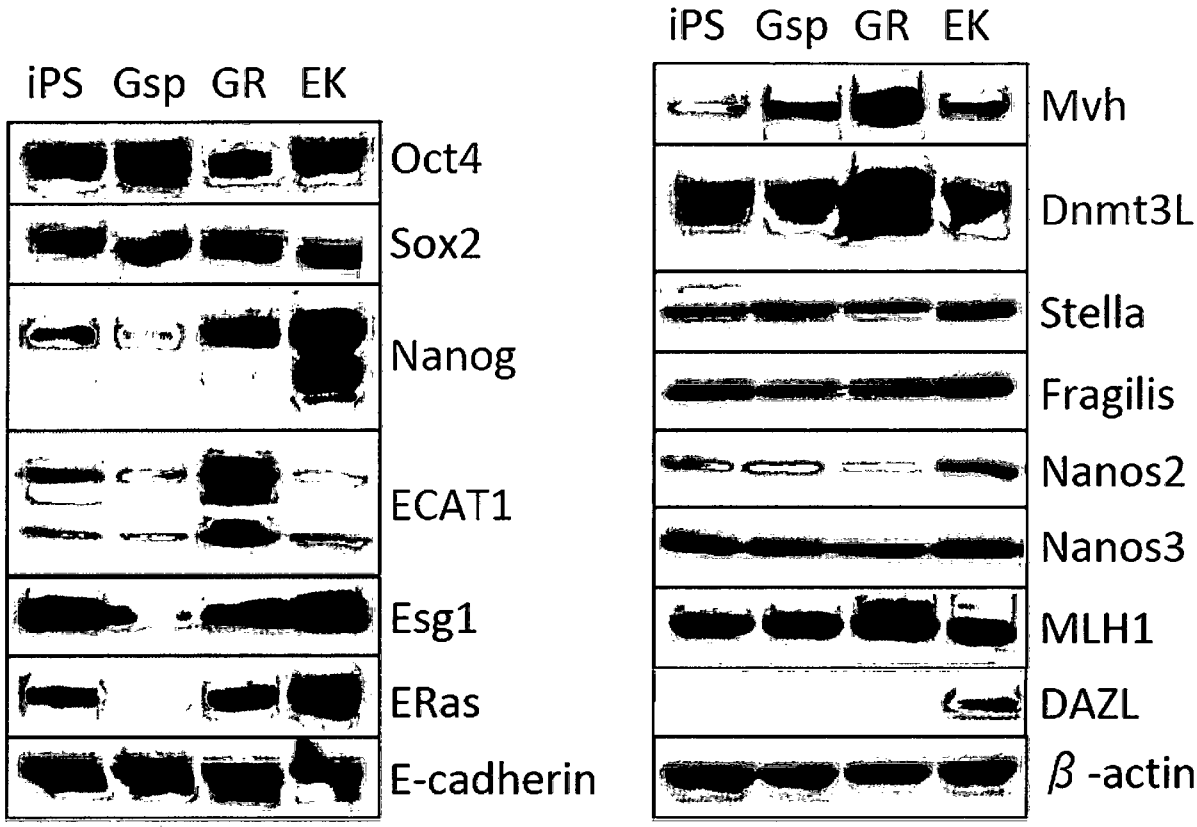
iPS Gsp GR EK
Oct4
Sox2
Nanog
ECAT1
Esg1
ERas
E-cadherin
iPS Gsp GR EK
Mvh
Dnmt3L
Stella
Fragilis
Nanos2
Nanos3
MLH1
DAZL
β -actin

Fig. 24

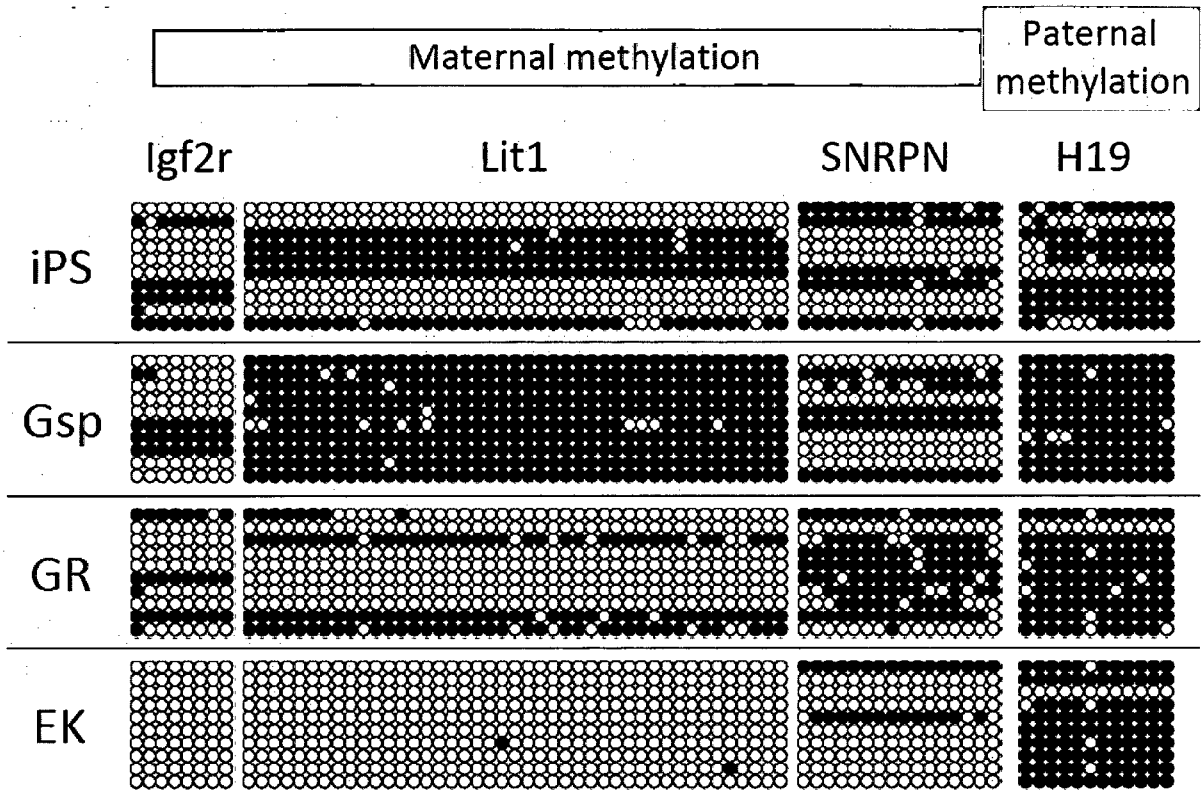
Maternal methylation
Paternal methylation
Igf2r
Lit1
SNRPN
H19
iPS
Gsp
GR
EK

Fig. 25

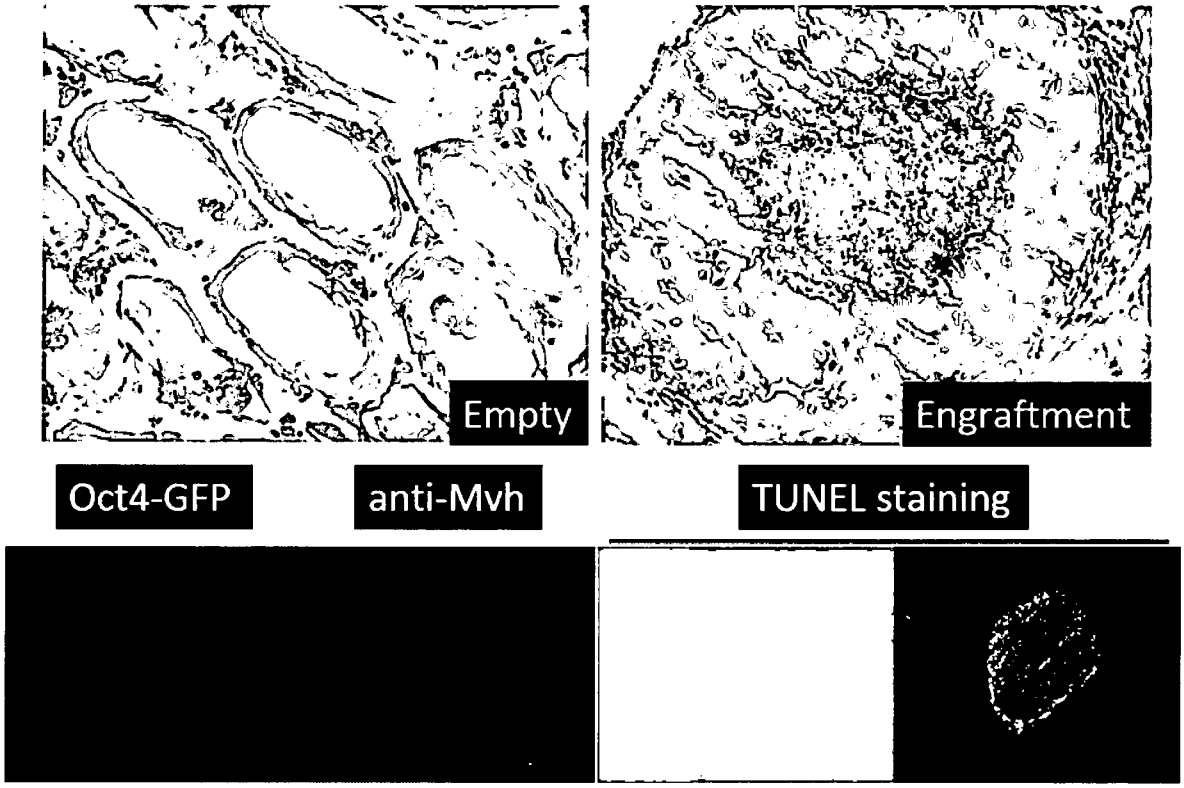
Empty
Engraftment
Oct4-GFP
anti-Mvh
TUNEL staining

Fig. 26

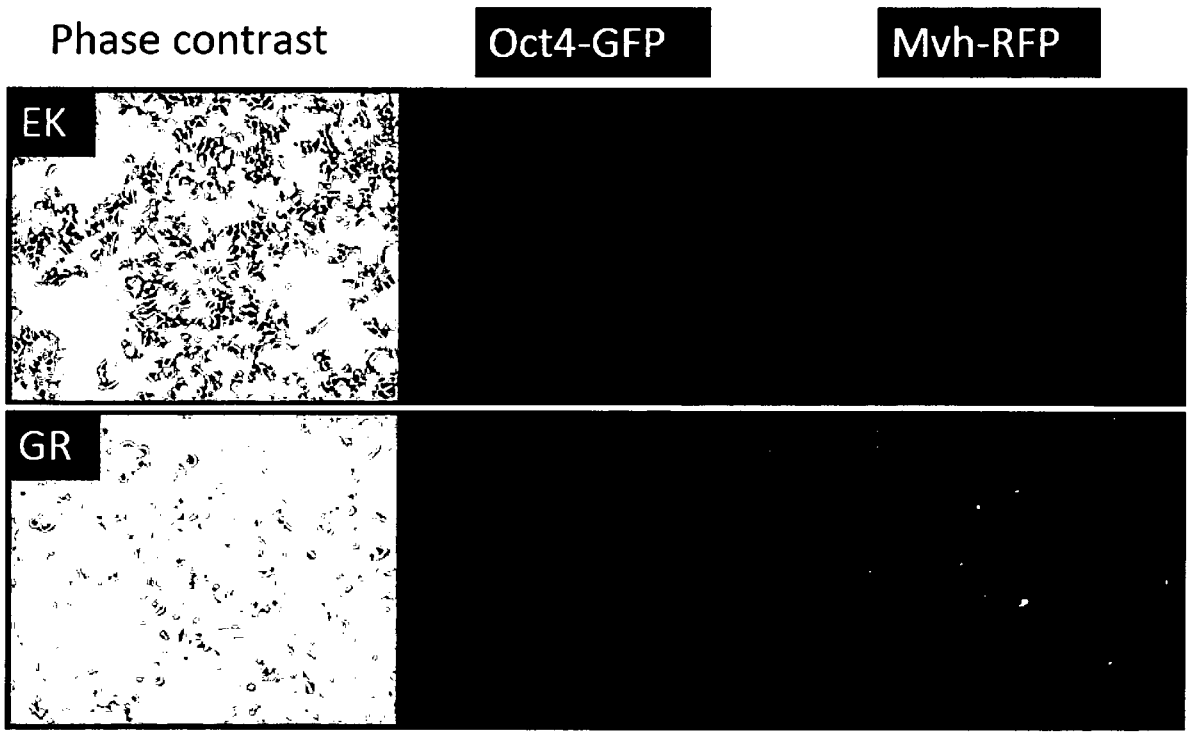
Phase contrast
Oct4-GFP
Mvh-RFP
EK
GR

Fig. 27

Medium component	Supplement
Neurobasal medium	GDNF (15 ng/ml)
B-27 supplement (1x)	EGF (20 ng/ml)
L-Glutamin/Penicillin /Streptomycin (1x)	bFGF (12.5 ng/ml)
BSA (5 mg/ml)	SCF (10 ng/ml)
Non-essential amino acid (0.1 mM)	HGF (10 ng/ml)
Sodium pyruvate (1 mM)	IL-2 (5 ng/ml)
Vitamins (1x)	FGF9 (12.5 ng/ml)
ITS supplement (1x)	Forskolin (10 μM)
Glucose (6 mg/ml)	Testosterone (0.1 μM)
Progesterone (60 ng/ml)	StemPro supplement (0.4x)
β-Estradiol (30 ng/ml)	
2-mercaptoethanol (55 μM)	
FBS (0.1%)	
Lactic acid (0.34 μl/ml)	
Putresine (60 μg/ml)	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/054828

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N5/00, A01K67/027, A61K35/48, A61P15/08, C07K14/47, C07K14/475, C07K14/485, C07K14/51, C12N5/0735

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2011
Registered utility model specifications of Japan 1996-2011
Published registered utility model applications of Japan 1994-2011

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS/MEDLINE/WPIDS(STN), JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	IMAMURA,M et al., Induction of primordial germ cells from mouse induced pluripotent stem cells derived from adult hepatocytes. Mol Reprod Dev. 2010 Sep, vol.77(9), pp.802-811	1-18,22,23
X/Y	YAMAUCHI,K et al., In vitro germ cell differentiation from cynomolgus monkey embryonic stem cells. PLoS One. 2009, vol.4(4), e5338	1-11,22,23/12-18
X/Y	TOYOOKA,Y et al., Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci U S A. 2003 Sep 30, vol.100(20), pp.11457-11462	1-11,22,23/12-18

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26.05.2011	07.06.2011

Name and mailing address of the ISA/JP
Japan Patent Office
3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan

Authorized officer
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4N 9123
Telephone No. +81-3-3581-1101 Ext. 3488

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/054828

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KUBOTA,H et al., Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod. 2004 Sep, vol.71(3), pp.722-731	12-18
A	PARK,TS et al., Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells. Stem Cells. 2009 Apr, vol.27(4), pp.783-795	1-18,22,23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/054828

CLASSIFICATION OF SUBJECT MATTER
C12N5/00(2006.01)i, A01K67/027(2006.01)i, A61K35/48(2006.01)i,
A61P15/08(2006.01)i, C07K14/47(2006.01)i, C07K14/475(2006.01)i,
C07K14/485(2006.01)i, C07K14/51(2006.01)i, C12N5/0735(2010.01)i

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/054828

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: 19-21
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claims 19-21 relates to a method for treatment of the human body by therapy, which does not require an intentional search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.