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61/378,518 31 August 2010 (31.08.2010) US(71) Applicant (for all designated States except US): **KYOTO UNIVERSITY** [JP/JP]; 36-1, Yoshida-honmachi, Sakyo-ku, Kyoto-shi, Kyoto, 6068501 (JP).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **YAMANAKA, Shinya** [JP/JP]; c/o Center for iPS Cell Research and Application, Kyoto University, 53, Kawahara-cho, Shogoin, Sakyo-ku, Kyoto-shi, Kyoto, 6068507 (JP). **MIURA, Kyoko** [JP/JP]; c/o Graduate School of Medicine, Kyoto University, Yoshida-konoecho, Sakyo-ku, Kyoto-shi, Kyoto, 6068501 (JP). **OHNUKI, Mari** [JP/JP]; c/o Graduate

School of Medicine, Kyoto University, Yoshida-konoecho, Sakyo-ku, Kyoto-shi, Kyoto, 6068501 (JP).

(74) Agents: **HIRAKI, Yusuke** et al.; Kamiya-cho MT Bldg. 19F, 3-20, Toranomon 4-chome, Minato-ku, Tokyo, 1050001 (JP).

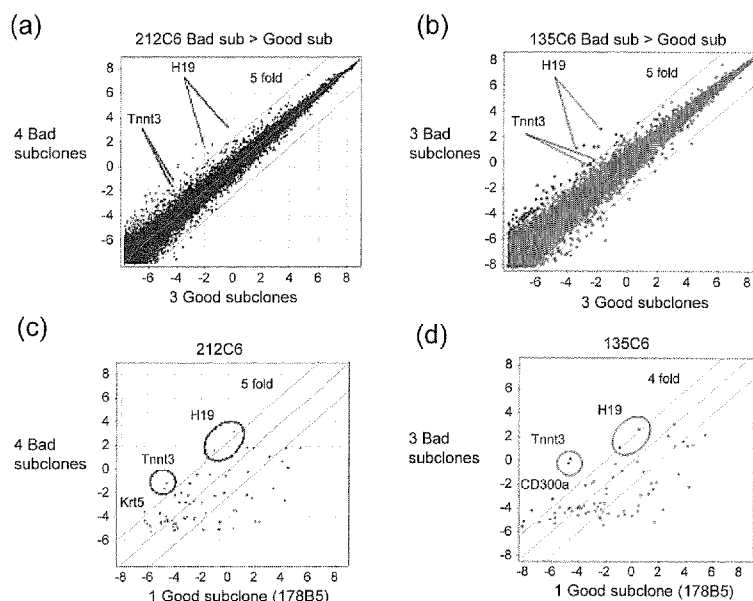
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[Continued on next page]

(54) Title: METHOD FOR SCREENING DIFFERENTIATION-RESPONSIVE INDUCED PLURIPOTENT STEM CELL

[Fig. 2]



(57) Abstract: The present invention provides a method for screening for a differentiation-responsive induced pluripotent stem cell in a pluripotent stem cell sample, comprising measuring at least one of expression levels of H19 and/or Tnnt3 genes in the sample, or DNA methylation of H19 and/or Tnnt3 gene expression regulating regions in the sample, thus screening differentiation-responsive cell based on its property.



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Description

Title of Invention: METHOD FOR SCREENING DIFFERENTIATION-RESPONSIVE INDUCED PLURIPOTENT STEM CELL

Technical Field

[0001] The present invention relates to a method for screening an induced pluripotent stem cell with high safety. More specifically, the present invention relates to a method for selecting an induced pluripotent stem cell having low tumorigenesis, wherein the method comprises measuring expression of H19 and/or Tnnt3 genes in the induced pluripotent stem cell, or DNA methylation of H19 and/or Tnnt3 gene expression regulating regions in the induced pluripotent stem cell, in order to eliminate differentiation-resistant cells without allowing persistent presence of the undifferentiated cells.

Background Art

[0002] In recent years, mouse and human induced pluripotent stem cells (iPS cells) have been successively established. Yamanaka et al. have induced iPS cells by introducing Oct3/4, Sox2, Klf4, and c-Myc genes into mouse-derived fibroblasts so as to enable the forced expression of such genes (Patent Literature 1 and Non-Patent Literature 1). Subsequently, it has been revealed that iPS cells can also be prepared using 3 of the above factors eliminating the c-Myc gene (Non-Patent Literature 2). Furthermore, Yamanaka et al. have succeeded establishing iPS cells by introducing the above 4 genes into human skin-derived fibroblast cells, as in mouse fibroblast cells (Patent Literature 1 and Non-Patent Literature 3). Meanwhile, Thomson et al. have prepared human iPS cells using Nanog and Lin28 instead of Klf4 and c-Myc (Patent Literature 2 and Non-Patent Literature 4). The thus obtained iPS cells are prepared using somatic cells from a patient to be treated, which cells can subsequently be differentiated into cells of different tissues. Thus, it is expected that iPS cells will be used as rejection-free grafting materials in the field of regenerative medicine.

[0003] However, when iPS cells were differentiated into neural progenitor cells which were then transplanted into the brain of a mouse, tumorigenesis was confirmed (Non-Patent Literature 5). Such tumorigenesis has been reported as follows (Non-Patent Literature 5). Upon tumorigenesis, tumors were not always formed in all iPS cell-derived nerve cells. Because iPS cell lines differ in responsiveness to induction of differentiation, they were divided into clones with good responsiveness to differentiation and thus with high safety (i.e., differentiation-responsive clones), and clones with poor induction of differentiation and thus with high tumorigenesis (i.e., differentiation-resistant clones).

Responsiveness differs depending on which types of original somatic cells are used (Non-Patent Literature 5).

- [0004] However, with regard to difference in differentiation responsiveness, the mechanism that causes such difference has not yet been elucidated. A method for establishing iPS cells that will never lead to tumorigenesis has not yet been established.

Citation List

Patent Literature

- [0005] PTL 1: WO 2007/069666 A1
PTL 2: WO 2008/118820 A2

Non Patent Literature

- [0006] NPL 1: Takahashi, K. and Yamanaka, S., Cell, 126: 663-676 (2006)
NPL 2: Nakagawa, M. et al., Nat. Biotechnol., 26: 101-106 (2008)
NPL 3: Takahashi, K. et al., Cell, 131: 861-872 (2007)
NPL 4: Yu, J. et al., Science, 318: 1917-1920 (2007)
NPL 5: Miura K. et al., Nat Biotechnol., 27: 743-745 (2009)

Summary of the Invention

- [0007] An object of the invention is to provide a means for efficiently selecting a safe induced pluripotent stem cell (iPS cell) that is appropriate for clinical application. Specifically, an object of the present invention is to provide a means for selectively eliminating iPS cells exhibiting resistance to differentiation, which cells maintain pluripotency even after induction of differentiation.
- [0008] The present inventors have now studied expression of genes for iPS cells having various genetic backgrounds in order to achieve the above object. As a result, the present inventors have now found that differentiation-resistant iPS cells and differentiation-responsive iPS cells can be distinguished from each other using H19 gene, Tnnt3 gene, or both genes (also, referred to as "H19 and/or Tnnt3 genes"). The present inventors have also now confirmed that the H19 and/or Tnnt3 genes can be used as indicators for eliminating iPS cells that may cause tumorigenesis and for screening a sample of iPS cells for differentiation-responsive iPS cells with high safety.
- [0009] The present inventors have further now found that screening of differentiation-responsive iPS cells can be similarly performed by measuring DNA methylation in the gene expression regulating region for the H19 gene.
- [0010] Based on the above results, the present inventors have now found that differentiation-resistant iPS cells that can cause tumorigenesis can be eliminated or removed through detection of the expression of H19 gene and/or Tnnt3 gene or the DNA methylation in the H19 and/or Tnnt3 gene expression regulating regions. The present inventors have thus completed the present invention.

Specifically, the present invention includes the following characteristics.

{1} A method for screening for a differentiation-responsive induced pluripotent stem cell in a pluripotent stem cell sample, comprising the following steps of:

(1) measuring at least one of expression levels of H19 and/or Tnnt3 genes in the sample; and

(2) selectively eliminating an induced pluripotent stem cell in which the at least one of expression levels of H19 and/or Tnnt3 genes is not less than that in a differentiation-responsive control cell or in which the at least one of expression levels of H19 and/or Tnnt3 genes is not less than that in a differentiation-resistant control cell.

{2} A method for screening for a differentiation-responsive induced pluripotent stem cell in a pluripotent stem cell sample, comprising the following steps of:

(1) measuring DNA methylation of H19 and/or Tnnt3 expression regulating regions in the sample; and

(2) selectively eliminating an induced pluripotent stem cell in which a degree of DNA methylation of each expression regulating region in the chromosome is lower than that for a differentiation-responsive control cell or in which a degree of DNA methylation of each expression regulating region in the chromosome is not more than that for a differentiation-resistant control cell.

{3} The method according to {1} or {2} above, wherein the differentiation-responsive induced pluripotent stem cell is for use in transplantation of a cell.

{4} The method according to {3} above, wherein the differentiation-responsive induced pluripotent stem cell is for use in transplantation of neural cell.

{5} A kit for measuring expression levels of H19 and/or Tnnt3 genes to select a differentiation-responsive induced pluripotent stem cell, comprising at least one nucleic acid or artificial nucleic acid, which is complementary to a sequence of each gene.

{6} A microarray for measuring expression levels of H19 and/or Tnnt3 genes to select a differentiation-responsive induced pluripotent stem cell, comprising at least one nucleic acid or artificial nucleic acid, which is complementary to a sequence of each gene.

{7} A kit for measuring DNA methylation of H19 and/or Tnnt3 expression regulating regions to select a differentiation-responsive induced pluripotent stem cell, comprising:

(1) probes or primers specific for H19 and/or Tnnt3 expression regulating regions; and

(2) methylated DNA of the H19 and/or Tnnt3 expression regulating regions of a differentiation-responsive cell as a negative control, or unmethylated DNA of the H19 and/or Tnnt3 expression regulating regions of a differentiation-resistant cell as a positive control.

{8} Use of at least one of nucleic acids comprising H19 and/or Tnnt3 expression regulating regions, for screening for a differentiation-responsive artificial pluripotent

cell in a pluripotent stem cell sample.

[0011] Through the use of the present invention, differentiation-resistant iPS cells are selectively eliminated, while differentiation-responsive cells can be screened for without leaving undifferentiated cells to remain, which thus makes it possible to induce the differentiation into cells and tissues with high safety for transplantation. Accordingly, the present invention would be very useful for the application of iPS cells to regenerative medicine.

Brief Description of Drawings

[0012] [fig.1a]Fig. 1a shows photographs (left, phase-contrast images; and right, EGFP fluorescence images) showing the state of the remaining undifferentiated cells of SNS (secondary neurosphere) when differentiation was induced from the Tail-Tip Fibroblast (TTF)-derived tumoral iPS cell line (212C6), as detected for Nanog-EGFP positive cells. In Fig. 1(a), the term "Good subclone" indicates a differentiation-responsive subclone and the term "Bad subclone" indicates a differentiation-resistant subclone.

[fig.1b]Fig. 1b shows photographs (left, phase-contrast images; and right, EGFP fluorescence images) showing the state of remaining undifferentiated cells of SNS (secondary neurosphere) when differentiation was induced from a hepatocyte (Hep)-derived tumoral iPS cell line (135C6), as detected for Nanog-EGFP positive cells. In Fig. 1(b), the term "Good subclone" indicates a differentiation-responsive subclone and the term "Bad subclone" indicates a differentiation-resistant subclone.

[fig.2]Fig. 2 shows the results of a comparative microarray analysis of gene expression levels affected by a difference in resistance to differentiation. Upper panels (a) and (b) show scatter plots of gene expression in subclones of iPS cell clones 212C6 (panel a) and 135C6 (panel b) divided into a differentiation-resistant group and a differentiation-responsive group, and in these panels, gene expression patterns were compared between the two groups. Black lines indicate that the gene expression level changed by 5 times or greater. Lower panels (c) and (d) show scatter plots of gene expression in differentiation-resistant subclones of iPS cell clones 212C6 (panel c) and 135C6 (panel d), as compared with the gene expression pattern in iPS cell differentiation-responsive 178B5 clone. Green lines indicate that the gene expression level changed by 5 times (c) or 4 times (d) or greater. In Fig. 2, the term "Good subclone" indicates a differentiation-responsive subclone, the term "Bad subclone" indicates a differentiation-resistant subclone, and the term "Good clone" indicates a differentiation-responsive clone.

[fig.3]Fig. 3 is a schematic diagram showing the arrangement of H19 and Tnnt3 genes in an imprinting domain of the F5 region on mouse chromosome 7.

[fig.4]Fig. 4 shows the results of analyzing by a bisulfite method the state of CpG methylation in H19-DMR of ES cells (1A2), MEF, TTF and iPS cells (212-C6, 135-C6). Black circles indicate methylated CpG and open circles indicate unmethylated CpG. Good subclones are differentiation-responsive subclones, from upper left column, iPS-TTF-FB/Ng-212C6 subclone#3, iPS-Hep-FB/Ng-135C6 subclone#28, iPS-Hep-FB/Ng-135C6 subclone#13, and iPS-Hep-FB/Ng-135C6 subclone#26, and, from upper right column, iPS-TTF-FB/Ng-212C6 subclone#11, iPS-Hep-FB/Ng-135C6 subclone#29, iPS-Hep-FB/Ng-135C6 subclone#48, and iPS-TTF-FB/Ng-212C6 subclone#13. Bad subclones are differentiation-resistant subclones, from upper left column, iPS-TTF-FB/Ng-212C6 subclone#5, iPS-TTF-FB/Ng-212C6 subclone#14, iPS-Hep-FB/Ng-135C6 subclone#37, and iPS-Hep-FB/Ng-135C6 subclone#44, and, from upper right column, iPS-TTF-FB/Ng-212C6 subclone#6, iPS-TTF-FB/Ng-212C6 subclone#12, iPS-TTF-FB/Ng-212C6 subclone#17, and iPS-Hep-FB/Ng-135C6 subclone#40.

Description of Embodiments

[0013] The present invention provides a method and a kit for screening for differentiation-responsive induced pluripotent stem cells.

[0014] Stem cells are defined as undifferentiated cells having two properties, "self replicating ability" and "differentiation potency." The term "differentiation-resistant induced pluripotent stem cells" refers to induced pluripotent stem cells (iPS cells) exhibiting abnormally strong "self replicating ability" from among the above two properties, "differentiation potency" and "self replicating ability." Specifically, this term refers to induced pluripotent stem cells that have a high risk of giving rise to tumors when cells obtained via differentiation induction are transplanted, since some cells retain pluripotency because of their self replications although most cells differentiate into cells of interest, upon differentiation into cells of interest. In other words, this term refers to induced pluripotent stem cells that are likely to give rise to tumors to a degree higher than that generally observed for normal embryonic stem cells (ES cells) when they are induced to differentiate into cells of interest, which are then transplanted into a host. In contrast, the term "differentiation-responsive induced pluripotent stem cell" refers to an induced pluripotent stem cell that has good responsiveness to differentiation induction, can be differentiated into cells of interest without allowing undifferentiated cells to remain, and present a low risk of tumorigenesis after transplantation. Specifically, in situations in which such cells are differentiated into cells of interest, which are transplanted into a host, this term refers to induced pluripotent stem cells that are unlikely to give rise to tumors in a manner similar to normal embryonic stem cells.

I. Method for producing iPS cells

Induced (artificial) pluripotent stem (iPS) cells can be prepared by introducing specific nuclear reprogramming substances in the form of DNAs or proteins into somatic cells. iPS cells are somatic cell-derived artificial stem cells having properties almost equivalent to those of ES cells, such as pluripotency and proliferation potency via autonomous replication (K. Takahashi and S. Yamanaka (2006) *Cell*, 126: 663-676; K. Takahashi et al. (2007) *Cell*, 131: 861-872; J. Yu et al. (2007) *Science*, 318: 1917-1920; M. Nakagawa et al. (2008) *Nat. Biotechnol.*, 26: 101-106; international publication WO 2007/069666). A nuclear reprogramming substance may be a gene specifically expressed in ES cells, a gene playing an important role in maintenance of undifferentiation of ES cells, or a gene product thereof. Examples of such nuclear reprogramming substances include, but are not limited to, 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 upon establishment of iPS cells. Such combination may contain at least one, two, or three reprogramming substances above and preferably contains 4 reprogramming substances above.

- [0015] The nucleotide sequence information of the mouse or human cDNA of each of the above nuclear reprogramming substances and the amino acid sequence information of a protein encoded by the cDNA can be obtained by referring to NCBI accession numbers described in WO 2007/069666. Also, the mouse and human cDNA and amino acid sequence information of L-Myc, Lin28, Lin28b, Esrrb, and Esrrg can be readily obtained by accessing to the NCBI accession numbers as described below. Persons skilled in the art can prepare desired nuclear reprogramming substances by conventional techniques based on the cDNA or amino acid sequence information.

Gene name Mouse Human

L-Myc NM_008506 NM_001033081

Lin28 NM_145833 NM_024674

Lin28b NM_001031772 NM_001004317

Esrrb NM_011934 NM_004452

Esrrg NM_011935 NM_001438

These nuclear reprogramming substances may be introduced in the form of protein or mature mRNA into somatic cells by a technique such as lipofection, binding with a cell membrane-permeable peptide, or microinjection. Alternatively, they may also be introduced in the form of DNA into somatic cells by techniques such as techniques using a vector such as virus, plasmid, or artificial chromosome, lipofection, techniques using a liposome, or microinjection. Examples of viral vectors include a retrovirus vector, a lentivirus vector (see *Cell*, 126, pp.663-676, 2006; *Cell*, 131, pp.861-872, 2007; and

Science, 318, pp. 1917-1920, 2007), an adenovirus vector (see Science, 322, 945-949, 2008), an adeno-associated virus vector, and a Sendai virus vector (see Proc Jpn Acad Ser B Phys Biol Sci. 85, 348-62, 2009). Also, examples of an artificial chromosome vector include a human artificial chromosome (HAC), a yeast artificial chromosome (YAC), and a bacterial or phage artificial chromosome (BAC or PAC). As the plasmid, plasmids for mammalian cells can be used (Science, 322: 949-953, 2008). A vector can contain regulatory sequences such as a promoter, an enhancer, a ribosome binding sequence, a terminator, and a polyadenylation site, so that a nuclear reprogramming substance can be expressed. A vector may further comprise, if necessary, a selection marker sequence such as a drug resistance gene (e.g., a neomycin resistance gene, an ampicillin resistance gene, and a puromycin resistance gene), a thymidine kinase gene, and a diphtheria toxin gene, and a reporter gene sequence such as a green fluorescent protein (GFP), beta-glucuronidase (GUS), and FLAG. Also, in order to cleave a gene encoding a nuclear reprogramming substance or a gene encoding a nuclear reprogramming substance linked to a promoter, after introduction into somatic cells, the above vector may have LoxP sequences located before and after said gene. Furthermore, the above vector may also comprise EBNA-1 and oriP, or Large T and SV40ori sequences so that they can be episomally present as a result of replication even if they are not incorporated into the chromosome of a cell.

- [0016] Upon nuclear reprogramming, to improve the efficiency for inducing iPS cells, in addition to the above factors, histone deacetylase (HDAC) inhibitors {e.g., low-molecular weight inhibitors such as valproic acid (VPA) (see Nat. Biotechnol., 26(7): 795-797 (2008)), trichostatin A, sodium butyrate, MC 1293, and M344, and nucleic acid expression inhibitors such as siRNA and shRNA against HDAC (e.g., HDAC1 siRNA Smartpool™ (Millipore) and HuSH 29mer shRNA Constructs against HDAC1 (OriGene))}, DNA methyltransferase inhibitors (e.g., 5'-azacytidine) (see Nat. Biotechnol., 26(7): 795-797 (2008)), G9a histone methyltransferase inhibitors {e.g., low-molecular-weight inhibitors such as BIX-01294 (see Cell Stem Cell, 2: 525-528 (2008)) and nucleic acid expression inhibitors such as siRNA and shRNA against G9a (e.g., G9a siRNA (human) (Santa Cruz Biotechnology))}, L-channel calcium agonists (e.g., Bayk8644) (see Cell Stem Cell, 3, 568-574 (2008)), p53 inhibitors (e.g., siRNA and shRNA against p53) (see Cell Stem Cell, 3, 475-479 (2008)), Wnt Signaling (e.g., soluble Wnt3a) (see Cell Stem Cell, 3, 132-135 (2008)), cytokines such as LIF or bFGF, ALK5 inhibitors (e.g., SB431542) (see Nat Methods, 6: 805-8 (2009)), mitogen-activated protein kinase signaling inhibitors, glycogen synthase kinase-3 inhibitors (see PloS Biology, 6(10), 2237-2247 (2008)), miRNA such as miR-291-3p, miR-294, and miR-295 (see R. L. Judson et al., Nat. Biotech., 27: 459-461 (2009)), for example, can be used.

- [0017] Examples of a culture medium for inducing iPS cells include, but are not limited to, (1) a DMEM, DMEM/F12, or DME medium containing 10-15% FBS (these media may further appropriately contain LIF, penicillin/streptomycin, puromycin, L-glutamine, nonessential amino acids, beta-mercaptoethanol, and the like), (2) a medium for ES cell culture containing bFGF or SCF, such as a medium for mouse ES cell culture (e.g., TX-WES medium (Thromb-X)), and a medium for primate ES cell culture (e.g., a medium for primate (human & monkey) ES cells, ReproCELL, Kyoto, Japan).
- [0018] An example of culture methods is as follows. Somatic cells are brought into contact with nuclear reprogramming substances (DNAs or proteins) on a DMEM or DMEM/F12 medium containing 10% FBS at 37 degrees C in the presence of 5% CO₂ and are cultured for about 4 to 7 days. Subsequently, the cells are reseeded on feeder cells (e.g., mitomycin C-treated STO cells or SNL cells). About 10 days after contact between the somatic cells and the nuclear reprogramming factors, cells are cultured in a bFGF-containing medium for primate ES cell culture. About 30-45 days or more after the contact, iPS cell-like colonies can be formed. Cells may also be cultured under conditions in which the oxygen concentration is as low as 5%-10% in order to increase the efficiency for inducing iPS cells.
- [0019] Alternatively, cells may be cultured using a DMEM medium containing 10% FBS (which may further appropriately contain LIF, penicillin/streptomycin, L-glutamine, nonessential amino acids, beta-mercaptoethanol, and the like) on feeder cells (e.g., mitomycin C-treated STO cells or SNL cells). After about 25-30 days or more, ES cell-like colonies can be formed.
- [0020] During the above culture, medium exchange with fresh medium is preferably performed once a day from day 2 after the start of culture. In addition, the number of somatic cells to be used for nuclear reprogramming is not limited, but ranges from approximately 5×10^3 to approximately 5×10^6 cells per culture dish (100 cm²).
- [0021] When a gene such as drug resistance gene is used as a marker gene, cells expressing the marker gene can be selected by culturing the cells in a medium (selection medium) containing the relevant drug. Also, cells expressing the marker gene can be detected when the marker gene is a fluorescent protein gene, through observation with a fluorescence microscope, by adding a luminescent substrate in the case of a luminescent enzyme gene, or adding a chromogenic substrate in the case of a chromogenic enzyme gene.
- [0022] The term "somatic cells" as used herein may refer to any cells other than germ cells from mammals (e.g., humans, mice, monkeys, pigs, and rats). Examples of such somatic cells include keratinizing epithelial cells (e.g., keratinizing epidermal cells), mucosal epithelial cells (e.g., epithelial cells of the surface layer of tongue), exocrine

epithelial cells (e.g., mammary glandular cells), hormone-secreting cells (e.g., adrenal medullary cells), cells for metabolism and storage (e.g., hepatocytes), boundary-forming luminal epithelial cells (e.g., type I alveolar cells), luminal epithelial cells of internal tubules (e.g., vascular endothelial cells), ciliated cells having carrying capacity (e.g., airway epithelial cells), cells for secretion to extracellular matrix (e.g., fibroblasts), contractile cells (e.g., smooth muscle cells), cells of blood and immune system (e.g., T lymphocytes), cells involved in sensation (e.g., rod cells), autonomic nervous system neurons (e.g., cholinergic neurons), sensory organ and peripheral neuron supporting cells (e.g., satellite cells), nerve cells and glial cells of the central nervous system (e.g., astroglial cells), chromocytes (e.g., retinal pigment epithelial cells), and precursor cells thereof (tissue precursor cells). Without particular limitation concerning the degree of cell differentiation, the age of an animal from which cells are collected, or the like, both undifferentiated precursor cells (also including somatic stem cells) and terminally-differentiated mature cells can be similarly used as the source of somatic cells in the invention. Examples of undifferentiated precursor cells include tissue stem cells (somatic stem cells) such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells, and dental pulp stem cells.

[0023] In the present invention, individual mammals from which somatic cells are collected are not particularly limited but are preferably humans.

II. Method for screening iPS cells

According to the present invention, differentiation-resistant iPS cells are selected and eliminated using at least one high-level expression of H19 and/or Tnnt3 genes, or using low DNA methylation of expression regulating regions containing these genes (of the above-established iPS cells), as an indicator, in an induced pluripotent stem cell sample, so that only differentiation-responsive cells can be screened for.

[0024] The H19 gene is non-coding RNA and is known as an imprinting gene. The Tnnt3 gene is known as a troponin T gene, the above human- and mouse-derived gene sequence information can be obtained by accessing to the NCBI (U.S.) accession numbers listed in Table 1.

[0025]

[Table 1]

Genes as indicators

Gene name	NCBI Accession No.	
	Mouse	Human
H19	NR_001592 (SEQ ID NO:7)	NR_002196 (SEQ ID NO:8)
Tnnt3	NM_011620 (SEQ ID NO:9)	NM_006757 (SEQ ID NO:10)

The human and mouse H19 and Tnnt3 genes may have not only the nucleotide sequences as indicated in Table 1 but also nucleotide sequences having at least 95%, preferably at least 98%, more preferably at least 99% identity to the nucleotide sequence of SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, or SEQ ID NO. 10. The "% identity" can be determined by using the BLAST algorithm (as provided by the NCBI, USA). Thus, the H19 and Tnnt3 genes may comprise naturally occurring mutations such as substitutions, deletions, insertions, or additions.

[0026] Examples of a method for detecting the above genes include, but are not limited to, Northern blotting, hybridization such as in situ hybridization, an RNase protection assay, a PCR method, a real-time PCR method, and a microarray method.

[0027] Detection can be preferably performed by extracting total RNA containing mRNA from a biological sample, obtaining mRNA using a poly T column, synthesizing cDNA by reverse transcription reaction, amplifying using phage or PCR cloning, and then performing hybridization with a probe of about 20-70 mer or larger in length complementary to the target DNA or quantitative PCR using about 20-30 mer primers, for example. As a label for hybridization or PCR, a fluorescent label can be used. As the fluorescent label, cyanines, fluorescamine, rhodamine, or derivatives thereof such as Cy3, Cy5, FITC, and TRITC can be used.

[0028] Upon screening for differentiation-responsive iPS cells, the expression level of either of the H19 and/or Tnnt3 genes detected by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation resistance is designated as a positive reference value. The iPS cells in which the expression level is not less than the positive reference value are selectively eliminated as differentiation-resistant iPS cells.

[0029] Similarly, the expression level of either of the H19 and/or Tnnt3 genes detected by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation responsiveness is designated as a negative reference value. The iPS cells in

which the expression level is higher than the negative reference value may be selectively eliminated as differentiation-resistant iPS cells.

[0030] Also, the expression level of either of the H19 and/or Tnnt3 genes detected by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation resistance is designated as a positive reference value. The iPS cells in which the expression level is less than the positive reference value may be selected as differentiation-responsive iPS cells.

[0031] Also, the expression level of either of the H19 and/or Tnnt3 genes detected by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation responsiveness is designated as a negative reference value. The iPS cells in which the expression level is not more than the negative reference value may be selected as differentiation-responsive iPS cells.

[0032] Furthermore, in the present invention, differentiation-resistant iPS cells may also be selectively eliminated by measuring a degree of the methylation of DNA involved in regulation of the gene expression in the H19 and/or Tnnt3 regions. H19 is known as an imprinting gene. The term "expression regulating region" as used herein comprises such an imprinting control region. An example of the imprinting regulating region is a region (DMR) that is referred to as a CpG island with high cytosine and guanine (i.e., CG) content, wherein its DNA methylation state or pattern in a maternally derived chromosome and that in a paternally derived chromosome are asymmetrical. A preferable example of such a region is the H19-differentially methylated region (H19-DMR). Examples of the H19-DMR include, but are not limited to, in the case of mice, the CG sequence (SEQ ID NO: 1) in the 317-bp sequence between the nucleotide position 837676 and the nucleotide position 837360 in the NCBI NT_039437 sequence on chromosome 7, and in the case of humans, the CG sequence (SEQ ID NO: 2) in the 320-bp sequence between the nucleotide position 84099 and the nucleotide position 84418 on chromosome 11 (NCBI Accession No: AC123789). The nucleotide sequences of SEQ ID NO. 1 and SEQ ID NO. 2 may comprise naturally occurring mutations such as substitutions, deletions, insertions, or additions, so that the nucleotide sequences having mutations have at least 95%, preferably at least 98%, more preferably at least 99% identity to the nucleotide sequence of SEQ ID NO. 1 or SEQ ID NO. 2. The "% identity" can be determined by using the BLAST algorithm (as provided by the NCBI, USA). The above mentioned sequences are subjected to measurement of DNA methylation.

[0033] As the method for measuring DNA methylation, known methods for analyzing methylation can be employed. For example, a method comprising cleaving an unmethylation recognizing sequence with a restriction enzyme is known. When DNA is treated with a methylation-sensitive restriction enzyme, unmethylated recognition sites

are cleaved, the resulting DNA is subjected to electrophoresis, Southern blotting or the like, and then the presence or absence of the methylation of a subject region can be determined depending on differences in the thus detected band lengths.

[0034] Examples of methylation-sensitive restriction enzymes are Sma I and Hpa II, preferably Sma I. An unmethylation-sensitive enzyme that recognizes the same recognition sequence as that of a methylation-sensitive enzyme can be easily found by persons skilled in the art. Such an unmethylation-sensitive enzyme can be used in combination with a methylation-sensitive enzyme.

[0035] Also, in a method for measuring DNA methylation state, bisulfite can be used. When DNA is treated with bisulfite, only unmethylated cytosine is converted to uracil, and methylated cytosine remains unconverted (remains as cytosine). With the use of a bisulfite sequencing method, a genomic region treated with bisulfite is amplified by PCR and then cloned, and then sequencing is performed, so that the presence or absence of DNA methylation can be found. Also, with the use of a COBRA (Combined Bisulfite Restriction Analysis) method, DNA treated with bisulfite is cleaved with a restriction enzyme and then the presence or absence of remaining restriction enzyme recognition sites is examined. Thus, the presence or the absence of DNA methylation can be detected. Moreover, a methylation-specific PCR can also be employed, wherein PCR primers recognize a difference between a sequence before bisulfite treatment and a sequence after bisulfite treatment, and then the presence or absence of methylated DNA or unmethylated DNA is determined depending on the presence or the absence of PCR products. In addition to these examples, a chromosome immunoprecipitation method (ChIP: Chromatin Immuno-Precipitation) may also be employed, by which DNA methylation in a specific region is detected by a method comprising finding or extracting a DNA sequence in a region in which DNA has been methylated, using a methylation-specific oligonucleotide (MSO) microarray or a DNA methylation-specific antibody, and performing PCR, followed by sequencing.

[0036] Upon screening for differentiation-responsive iPS cells, degrees of DNA methylation in both of the maternal and paternal chromosomes measured by the above-described method for iPS cells or embryonic stem cells (ES cells) known to have differentiation resistance are designated as positive reference values. The iPS cells in which the degree of methylation is not more than the positive reference value are selectively eliminated as differentiation-resistant iPS cells.

[0037] Similarly, degrees of DNA methylation of both chromosomes measured by the above-described method for iPS cells or embryonic stem cells (ES cells) known to have differentiation responsiveness are designated as negative reference values. The iPS cells in which the degree of methylation is lower than the negative reference value are selectively eliminated as differentiation-resistant iPS cells.

- [0038] Also, degrees of DNA methylation in both chromosomes measured by the above-described method for iPS cells or embryonic stem cells (ES cells) known to have differentiation resistance are designated as positive reference values. The iPS cells for which the degree of methylation is higher than the positive reference value are screened as differentiation-responsive iPS cells.
- [0039] Also, degrees of DNA methylation in both chromosomes measured by the above-described method for iPS cells or embryonic stem cells (ES cells) known to have differentiation responsiveness are designated as negative reference values. The iPS cells for which the degree of methylation is not less than the negative reference value are screened as differentiation-responsive iPS cells.
- [0040] Also, it is known that in the case of paternal imprinting genes, paternally derived chromosomal DNAs generally come into a methylated state and maternally derived genes alone are expressed. In the present invention, when differentiation-responsive iPS cells are screened for using the expression regulating region of an H19 gene that is a paternal imprinting gene, a degree of the methylation of paternally derived chromosomal DNA measured by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation resistance is designated as a positive reference value. The iPS cells in which the degree of methylation is not more than the positive reference value are selectively eliminated as differentiation-resistant iPS cells.
- [0041] Similarly, a degree of the methylation of paternally derived chromosomal DNA measured by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation responsiveness is designated as a negative reference value. The iPS cells in which the degree of methylation is lower than the negative reference value are selectively eliminated as differentiation-resistant iPS cells.
- [0042] Also, a degree of the methylation of paternally derived chromosomal DNA measured by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation resistance is designated as a positive reference value. The iPS cells for which the degree of methylation is higher than the positive reference value are screened as differentiation-responsive iPS cells.
- [0043] Also, a degree of the methylation of paternally derived chromosomal DNA measured by the above method for iPS cells or embryonic stem cells (ES cells) known to have differentiation responsiveness is designated as a negative reference value. The iPS cells for which the degree of methylation is not less than that indicated by the negative reference value are screened as differentiation-responsive iPS cells.
- [0044] An example of a method for determining a rate of methylated DNAs is as follows. Where the method comprises cleaving an unmethylation recognizing sequence with a restriction enzyme, DNA is fragmented and then quantified by Southern blotting, and the amount of unfragmented DNA is compared with that of fragmented DNA in order

to calculate a rate of methylated DNA. Meanwhile, in the case of the bisulfite sequencing method, an arbitrarily selected chromosome is sequenced. Hence, said rate can be calculated by repeatedly sequencing a template, which is a cloned PCR product, a plurality of times such as 2 or more times, preferably 5 or more times, and more preferably 10 or more times, and then comparing the number of sequenced clones with the number of clones for which DNA methylation has been detected. Where a pyrosequencing method is employed, said rate can also be directly determined based on the rate of cytosine and thymine. Also, in the case of a chromosome immunoprecipitation method (ChIP: Chromatin Immuno-Precipitation) using a DNA methylation-specific antibody, the amount of precipitated target DNA and the amount of DNA before precipitation can be detected by PCR and then compared, so that the rate of methylated DNA can be determined.

III. Application to regenerative medicine

In the present invention, examples of diseases to be treated include, but are not limited to, heart diseases, bone diseases, nervous system diseases, retinal diseases, corneal diseases, hematopoietic disorders, vascular system disorders, immune disorders, muscular diseases, hepatic diseases, and pancreatic diseases. In a preferred embodiment, nervous system diseases are the subject of the present invention, for example.

[0045] Differentiation-responsive induced pluripotent stem cells that are screened for by the method of the present invention are almost not likely to cause tumorigenesis, and thus they can be effectively used in the field of regenerative medicine in order to normalize damaged nervous tissues. Thus, the cells can serve as therapeutic cells for diseases associated with any disorders of neural cells.

[0046] Examples of the diseases include, but are not limited to, ischemic cerebrovascular diseases (e.g., stroke), brain trauma, spinal injury, motor neurologic diseases, neurodegenerative diseases, retinitis pigmentosa, age-related macular degeneration, inner ear hearing loss, multiple sclerosis, amyotrophic lateral sclerosis, spinocerebellar degeneration, Huntington's disease, Alzheimer's disease, Parkinson's disease, epilepsy, and schizophrenia.

[0047] Also, when the cells are used as remedies or therapeutics, cell purity should desirably be increased. Examples of a method for increasing cell purity include: a method for sorting a target cell, such as a flow cytometry method; and treatment with an anticancer agent-containing medium. The flow cytometry method comprises flowing cell particles through a very thin liquid stream at a high rate, irradiating the cell particles with a laser beam, and then measuring light such as fluorescence emitted from the particles (when the cells are fluorescence-labeled in advance) or scattered light. The flow cytometry system has a cell sorter to make it possible to screen for and separate a

target cell. Fluorescence labeling of cells can be conducted using a (fluorescence-labeled) antibody specific for neural progenitor cells, such as an anti-Nestin antibody and an anti-Musashi-1 antibody. Also, undifferentiated cells can be eliminated by treatment with an anticancer agent-containing medium. Examples of the anticancer agent include, but are not limited to, Mitomycin-C, 5-Fluorouracil, Adriamycin, and Methotrexate.

[0048] Cells are preferably transplanted to a desired site, but a site for transplantation is not limited thereto. A composition containing cells screened by the method of the present invention may be administered or transplanted to any site, as long as treatment or prevention for the desired site is finally possible. Transplantation to a site of disorder can be performed by techniques as described in *Nature Neuroscience*, 2: 1137 (1999) or *N Engl J Med.*, 344: 710-9 (2001), for example.

IV. Kit for screening of iPS cells

The present invention provides a kit useful for screening iPS cells with high safety. The kit for screening iPS cells according to the present invention comprises a kit for measuring genes, a microarray, or a kit for measuring DNA methylation, for the above-described measurement methods.

[0049] The kit for measuring genes can comprise nucleic acid probes or artificial nucleic acid probes of about 20-70 mer or more in length that are complementary to target DNA or mRNA of a gene, or primers of about 20-30 mer in length.

[0050] An artificial nucleic acid probe may comprise a modified nucleotide (e.g., conversion of adenine, cytosine, guanine or thymine to a modified nucleotide such as inosine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, or 2,6-diaminopurine), conversion of phosphodiester linkage to phosphorothioate linkage, conversion of the 2'-hydroxyl group of ribose to 2'-O-methyl group or 2'-O-methoxyethyl group, LNA (Locked Nucleic Acid) or BNA (Bridged Nucleic Acid) having a structure with crosslinking at positions 2' and 4' of ribose via methylene groups, PNA (Peptide Nucleic Acid) wherein the main chain comprises peptide linkage of N-(2-aminoethyl)glycine, and the like (e.g., A.A. Koshikin et al., *Tetrahedron* 54: 3607 (1998), S. Obika et al., *Tetrahedron Lett.* 39: 5401 (1998), P.E. Nielson et al., *Science* 254: 1497 (1991), JP Patent Publication (Kokai) No. 2010-150280 A, and JP Patent Publication (Kokai) No. 2010-090159 A).

[0051] The tool for screening iPS cells according to the present invention may include a microarray prepared by attaching these probes to a carrier.

[0052] The kit for measuring DNA methylation can contain a methylation-sensitive restriction enzyme and/or a methylation-insensitive restriction enzyme. When DNA is treated with a methylation-sensitive restriction enzyme, an unmethylated recognition site is cleaved. The thus generated DNA can be subjected to electrophoresis then

Southern blotting or the like, and the presence or absence of methylation in a subject region is determined depending on differences in detected band lengths.

[0053] Also, the kit may also comprise a bisulfite reagent for detection of methylation of cytosine nucleotides utilizing the bisulfite reaction. The kit may further comprise reagents and a microarray for use in the MSO (methylation-specific oligonucleotide) microarray method utilizing the bisulfite reaction (Izuho Hatada, *Experimental Medicine*, Vol. 24, No. 8 (Extra Number), pp. 212-219 (2006)).

[0054] Also, the kit can comprise probes or primers specific to a subject region (e.g., either of H19 and/or Tnnt3 gene expression regulating regions) regardless of the presence or the absence of bisulfite treatment. In the methylation specific oligonucleotide (MSO) microarray method, PCR is performed for DNA treated with bisulfite by selecting sequences (containing no CpG sequences), as primers, that remain unaltered regardless of methylation. As a result, unmethylated cytosine is amplified as thymine and methylated cytosine is amplified as cytosine.

[0055] Also, the kit for measuring DNA methylation can comprise reagents for amplifying a gene expression regulating region. Specifically, the kit can appropriately comprise components required for PCR, such as DNA polymerase, an appropriate buffer, a magnesium salt, and dNTPs.

[0056] Furthermore, the kit for screening iPS cells can comprise methylated DNA occurring in H19 and/or Tnnt3 expression regulating regions of a differentiation-responsive cell as a negative control. Also, the kit can comprise the unmethylated DNA of differentiation-resistant H19 and/or Tnnt3 expression regulating regions in a differentiation-resistant cell as a positive control.

[0057] The kit can also comprise reagents for H19 and/or Tnnt3 extraction, reagents for gene extraction, or reagents for chromosome extraction, for example. Also, a kit of the present invention may comprise means for discrimination analysis such as documents or instructions containing procedures for discrimination analysis, a program for implementing the procedures for discrimination analysis by a computer, the program list, a recording medium containing the program recorded therein, which is readable by the computer (e.g., flexible disk, optical disk, CD-ROM, CD-R, and CD-RW), and an apparatus or a system (e.g., computer) for implementation of discrimination analysis.

[0058] The present invention will be further described in detail by examples as follows, but the scope of the present invention is not limited by these examples.

Examples

[0059] Cells:

ES cells (1A2) were cultured and iPS cells shown in Table 2 were established and cultured by conventional methods (see Takahashi K and Yamanaka S, *Cell* 126 (4),

663, 2006; Okita K, et al., Nature 448 (7151), 313, 2007; Nakagawa M, et al., Nat Biotechnol 26 (1), 101, 2008; Aoi, T. et al., Science 321, 699-702, 2008; and Okita K, et al., Science 322, 949, 2008).

[0060] In Table 2, "origin" indicates somatic cells serving as origins, "MEF" indicates Mouse Embryonic Fibroblast, "TTF" indicates Tail-Tip Fibroblast, and "Hep" indicates hepatocytes. All iPS cells used herein contained Nanog-EGFP reporter incorporated therein, so that in the case of the iPS cells in undifferentiated state, EGFP was found to become positive due to Nanog expression.

[0061] [Table 2]

iPS cell name	Origin	Factor
178B5	MEF	Oct3/4, Sox2, Klf4
212C6	TTF	Oct3/4, Sox2, Klf4, c-Myc
135C6	Hep	Oct3/4, Sox2, Klf4, c-Myc

The thus established iPS cells were induced to differentiate into SNS, and then whether or not Nanog (that is, an undifferentiated-state-specific gene) was expressed was examined. As a result, it was revealed that in the case of clones (212C6 and 135C6 cell lines) expressing Nanog-EGFP in SNS, undifferentiated cells remained after induction of differentiation. Such cells were differentiation-resistant cell lines that may cause tumorigenesis in the future.

Discrimination of differentiation-responsive cells and differentiation-resistant cells

The above differentiation-resistant clones, the 212C6 cell line (Unsafe clone TTF-4f-Nanog selection-212C6) and the 135C6 cell line (Unsafe clone Hep-4f-Nanog selection-135C6), which may cause tumorigenesis, were seeded on feeder cells at low density, and then colonies formed after the cell growth were established and subclones were obtained therefrom. Next, as described in Miura K. et al., Nat Biotechnol., 27: 743-745 (2009), these subclones were induced to differentiate into SNS (secondary neurosphere) and then whether or not Nanog (that is, an undifferentiated-state-specific gene) was expressed was examined. The presence of subclones that had not expressed Nanog and the presence of subclones that had expressed Nanog were revealed (Fig. 1). Hence, subclones that had not expressed Nanog in SNS were identified as differentiation-responsive cells and subclones that had expressed Nanog in SNS were identified as differentiation-resistant cells (which had remained as undifferentiated cells).

Expression analysis for subclones:

Microarray analysis was conducted to examine if there were differences in gene expression pattern arising from differences in differentiation resistance of iPS cells.

[0062] Analysis was conducted according to a conventional method by extracting RNA and then performing gene expression profiling for genes that had been expressed in cells using a mouse GE array (Agilent).

[0063] Genes expressed by subclones (Table 3) from iPS cell clones (212C6 and 135C6) grouped into differentiation-responsive cells and differentiation-resistant cells as described above were detected, and then comparison between the groups was conducted.

[0064] [Table 3]

List of used cells

Cell name	Cell type	Differentiation-resistance
1A2	ES	
iPS-MEF-Ng-212C6	iPS	
iPS-TTF-FB/Ng-212C6		
iPS-TTF-FB/Ng-212C6 subclone#3		Differentiation-responsive
iPS-TTF-FB/Ng-212C6 subclone#6		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#11		Differentiation-responsive
iPS-TTF-FB/Ng-212C6 subclone#12		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#13		Differentiation-responsive
iPS-TTF-FB/Ng-212C6 subclone#14		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#17		Differentiation-resistant
iPS-Hep-FB/Ng-135C6		
iPS-Hep-FB/Ng-135C6 subclone#13		Differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#26		Differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#28		Differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#29		Differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#37		Differentiation-resistant
iPS-Hep-FB/Ng-135C6 subclone#40		Differentiation-resistant
iPS-Hep-FB/Ng-135C6 subclone#44		Differentiation-resistant

The results are shown in Fig. 2.

[0065] For detection of genes specifically expressed in the differentiation-resistant subclone group, first, gene groups found to show changes in gene expression to a degree 5 times or greater than that of the differentiation-responsive subclone group were extracted (upper panels in Fig. 2). From among subsequently extracted gene groups, genes the expression levels of which were low in the differentiation-responsive clone (178B5) and ES cells (1A2) were selected.

[0066] As a result, increased H19 and Tnnt3 gene expression levels were observed in differentiation-resistant subclones. The result was commonly observed in the two clones. Thus, it was suggested that the genes were differentiation-resistance-specific genes. Therefore, it was also suggested that H19 and Tnnt3 would be useful as marker genes for detection of remaining undifferentiated cells that cause tumors.

Confirmation of DNA methylation of H19-DMR:

The 2 above genes are located in the vicinity of the mouse chromosome 7 (Fig. 3). Hence, methylation of the H19-differentially methylated region (DMR) as an expression regulating region for H19 was studied. In the sequence of NCBI NT_039437, DNA methylation in the 317-bp sequence ranging from nucleotide position 837676 to nucleotide position 837360 was determined for CG sequences.

[0067] Genomic DNA was extracted from cells shown in Table 4 by the conventional method, the DNA was subjected to bisulfite treatment using an EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH), and then unmethylated cytosine was substituted with thymine. Subsequently, H19-DMR was amplified by nested-PCR using primers shown in Table 5, fragments were collected, and then TA cloning (Invitrogen) was performed. Plasmids were collected from the thus amplified colonies and then the sequences of the fragments were determined using a sequencer.

[0068] The results are shown in Fig. 4. A tendency of lower proportions of methylated CpG was observed in the differentiation-resistant group, compared with the differentiation responsive-group. Also, such tendency was similar when compared with MEF and TTF, which were differentiated cells.

[0069] It was suggested accordingly that H19 expression in differentiation-resistant subclones was controlled by DNA methylation and abnormal imprinting could occur. It was thus suggested that differentiation-resistant iPS cells that may cause tumorigenesis can be screened for by measuring H19-DMR methylation and then determining whether or not imprinting within the region is normal.

[0070]

[Table 4]

List of used cells

Cell name	Cell type	Differentiation resistance
1A2	ES	
iPS-TTF-FB/Ng-212C6 subclone#3	iPS	Differentiation-responsive
iPS-TTF-FB/Ng-212C6 subclone#5		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#6		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#11		Differentiation-responsive
iPS-TTF-FB/Ng-212C6 subclone#12		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#13		Differentiation-responsive
iPS-TTF-FB/Ng-212C6 subclone#14		Differentiation-resistant
iPS-TTF-FB/Ng-212C6 subclone#17		Differentiation-resistant
iPS-Hep-FB/Ng-135C6 subclone#13		differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#26		differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#28		differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#29		differentiation-responsive
iPS-Hep-FB/Ng-135C6 subclone#37		Differentiation-resistant
iPS-Hep-FB/Ng-135C6 subclone#40		Differentiation-resistant
iPS-Hep-FB/Ng-135C6 subclone#44		Differentiation-resistant
iPS-Hep-FB/Ng-135C6 subclone#48		Differentiation-resistant
MEF		
TTF		

[0071]

[Table 5]

Primers used:

	Primer sequence
1 st PCR	H19-A outer-s: 5'-gat tag ata gta ttg agt ttg ttt gga gt-3' (SEQ ID NO: 3)
	H19-A outer-as: 5'-cct aaa ata cta aac tta aat aac cca ca-3' (SEQ ID NO: 4)
2 nd PCR	H19-A inner-s: 5'-gag aaa ata gtt att gtt tat agt ttt-3' (SEQ ID NO: 5)
	H19-A inner-as: 5'-acc att tat aaa ttc caa tac caa aaa taa-3' (SEQ ID NO: 6)

Claims

- [Claim 1] A method for screening for a differentiation-responsive induced pluripotent stem cell in a pluripotent stem cell sample, comprising the following steps of:
- (1) measuring at least one of expression levels of H19 and/or Tnnt3 genes in the sample; and
 - (2) selectively eliminating an induced pluripotent stem cell in which the at least one of expression levels of H19 and/or Tnnt3 genes is not less than that in a differentiation-responsive control cell or in which the at least one of expression levels of H19 and/or Tnnt3 genes is not less than that in a differentiation-resistant control cell.
- [Claim 2] A method for screening for a differentiation-responsive induced pluripotent stem cell in a pluripotent stem cell sample, comprising the following steps of:
- (1) measuring DNA methylation of H19 and/or Tnnt3 expression regulating regions in the sample; and
 - (2) selectively eliminating an induced pluripotent stem cell in which a degree of DNA methylation of each expression regulating region in the chromosome is lower than that for a differentiation-responsive control cell or in which a degree of DNA methylation of each expression regulating region in the chromosome is not more than that for a differentiation-resistant control cell.
- [Claim 3] The method according to claim 1 or 2, wherein the differentiation-responsive induced pluripotent stem cell is for use in transplantation of a cell.
- [Claim 4] The method according to claim 3, wherein the differentiation-responsive induced pluripotent stem cell is for use in transplantation of a neural cell.
- [Claim 5] A kit for measuring expression levels of H19 and/or Tnnt3 genes to select a differentiation-responsive induced pluripotent stem cell, comprising at least one nucleic acid or artificial nucleic acid, which is complementary to a sequence of each gene.
- [Claim 6] A microarray for measuring expression levels of H19 and/or Tnnt3 genes to select a differentiation-responsive induced pluripotent stem cell, comprising at least one nucleic acid or artificial nucleic acid, which is complementary to a sequence of each gene.
- [Claim 7] A kit for measuring DNA methylation of H19 and/or Tnnt3 expression

regulating regions to select a differentiation-responsive induced pluripotent stem cell, comprising:

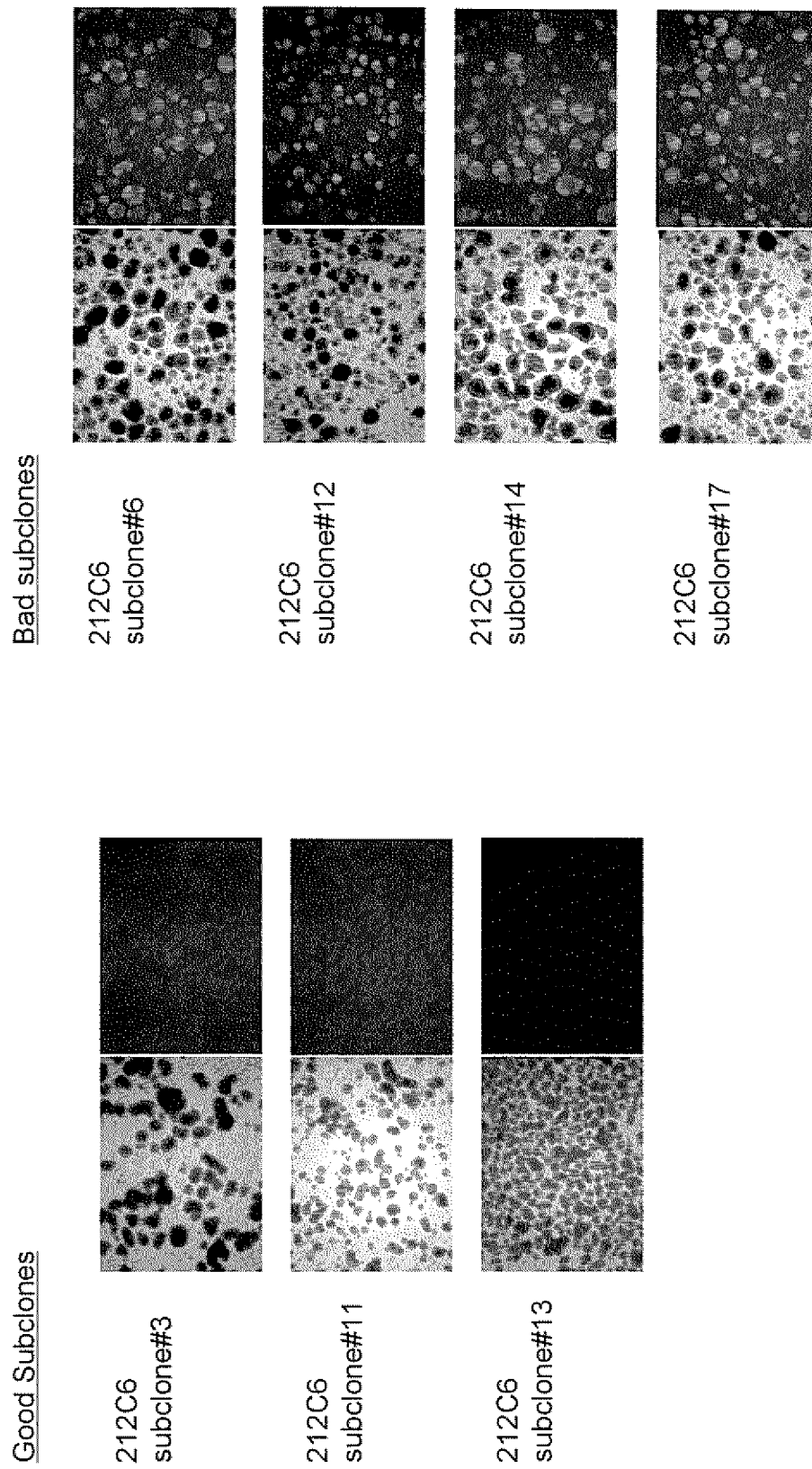
(1) probes or primers specific for H19 and/or Tnnt3 expression regulating regions; and

(2) methylated DNA of the H19 and/or Tnnt3 expression regulating regions of a differentiation-responsive cell as a negative control, or unmethylated DNA of the H19 and/or Tnnt3 expression regulating regions of a differentiation-resistant cell as a positive control.

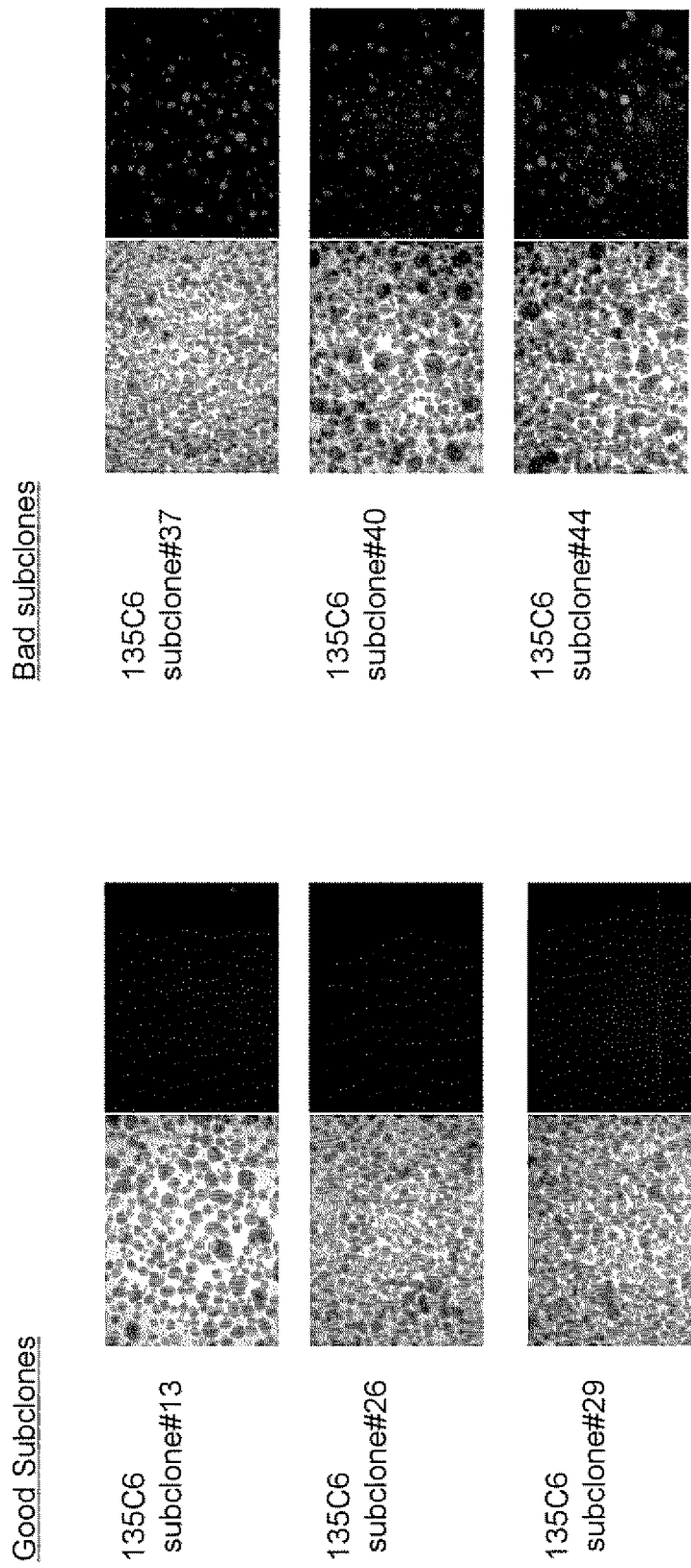
[Claim 8]

Use of at least one of nucleic acids comprising H19 and/or Tnnt3 expression regulating regions, for screening for a differentiation-responsive artificial pluripotent cell in a pluripotent stem cell sample .

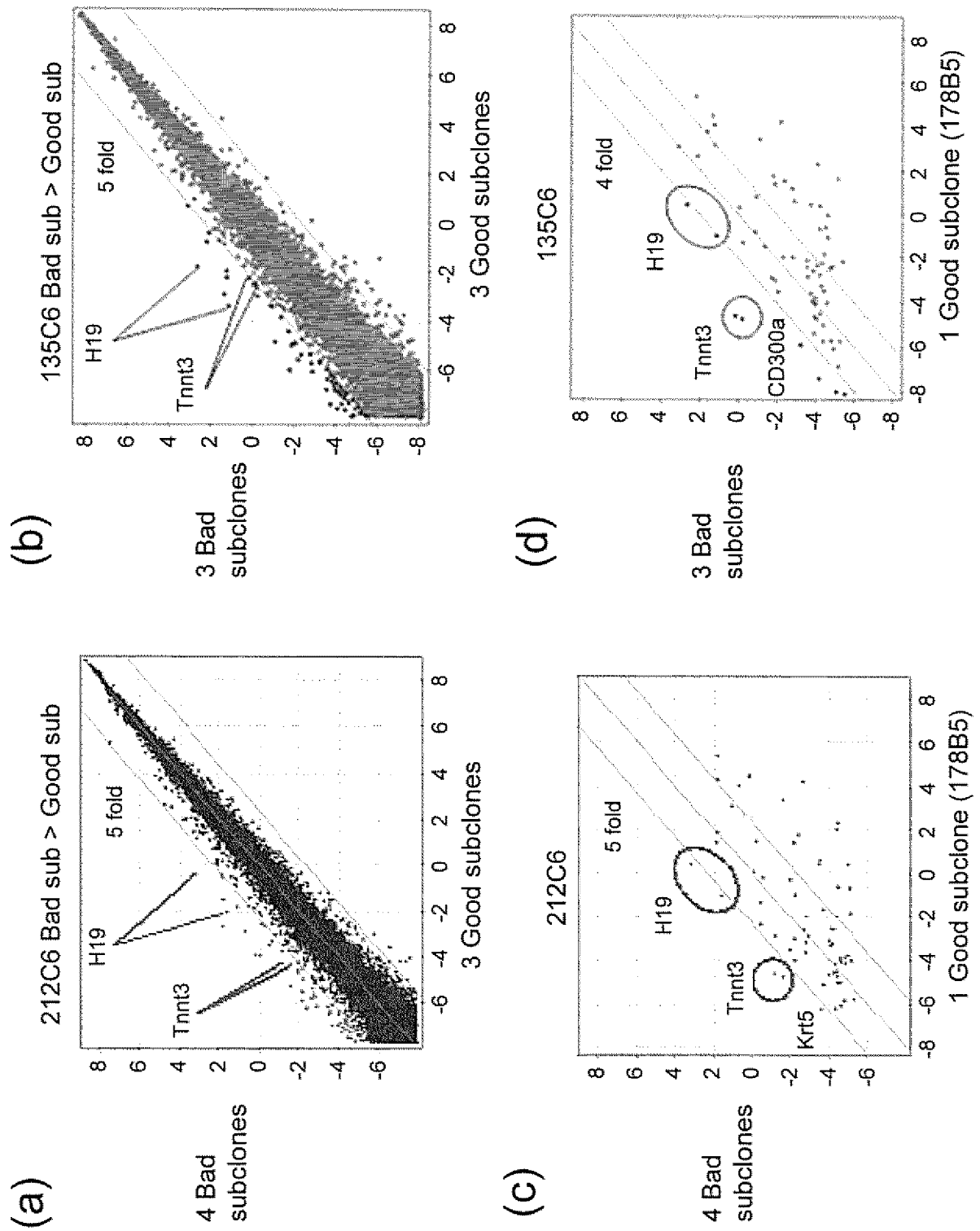
[Fig. 1a]



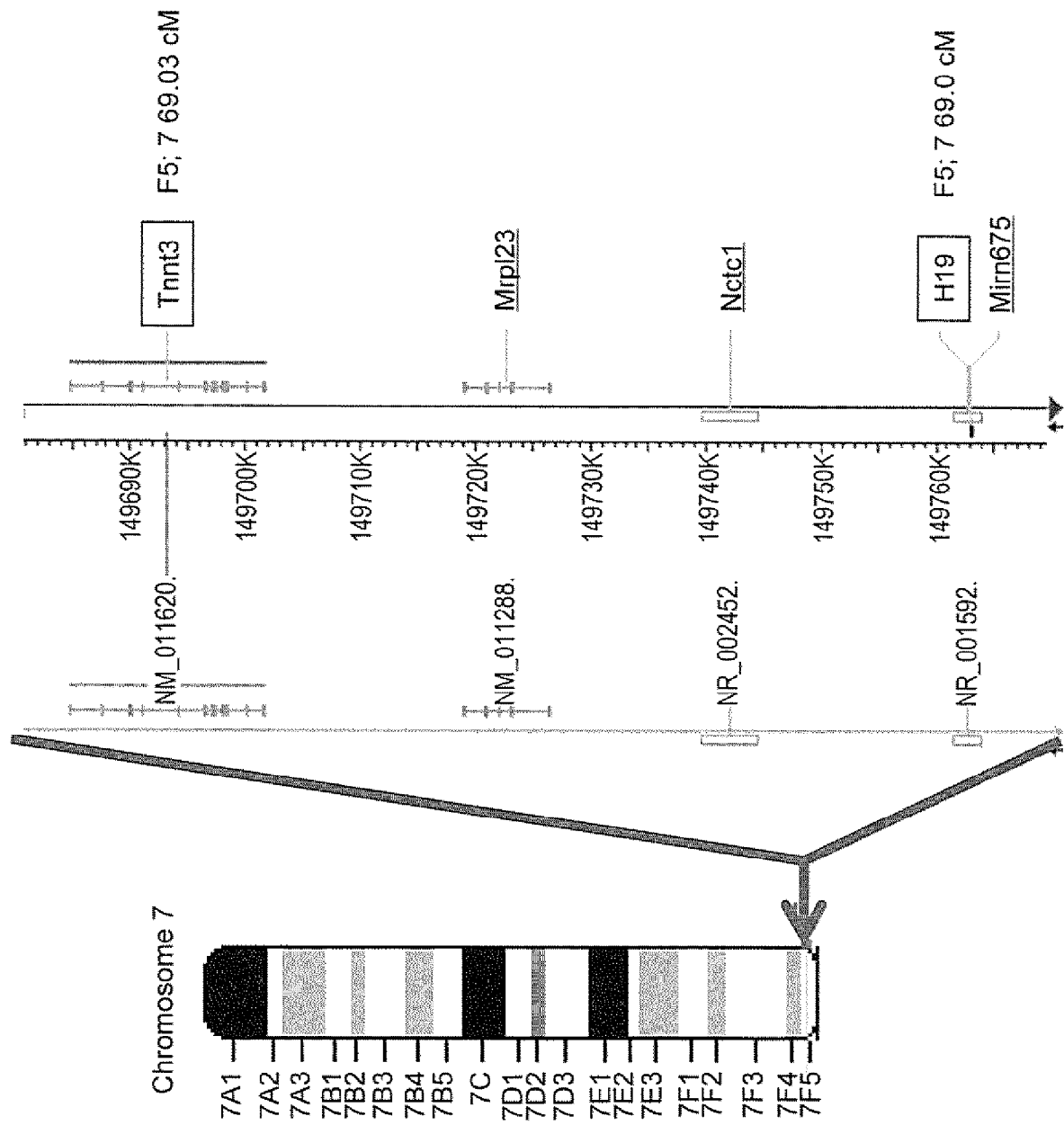
[Fig. 1b]



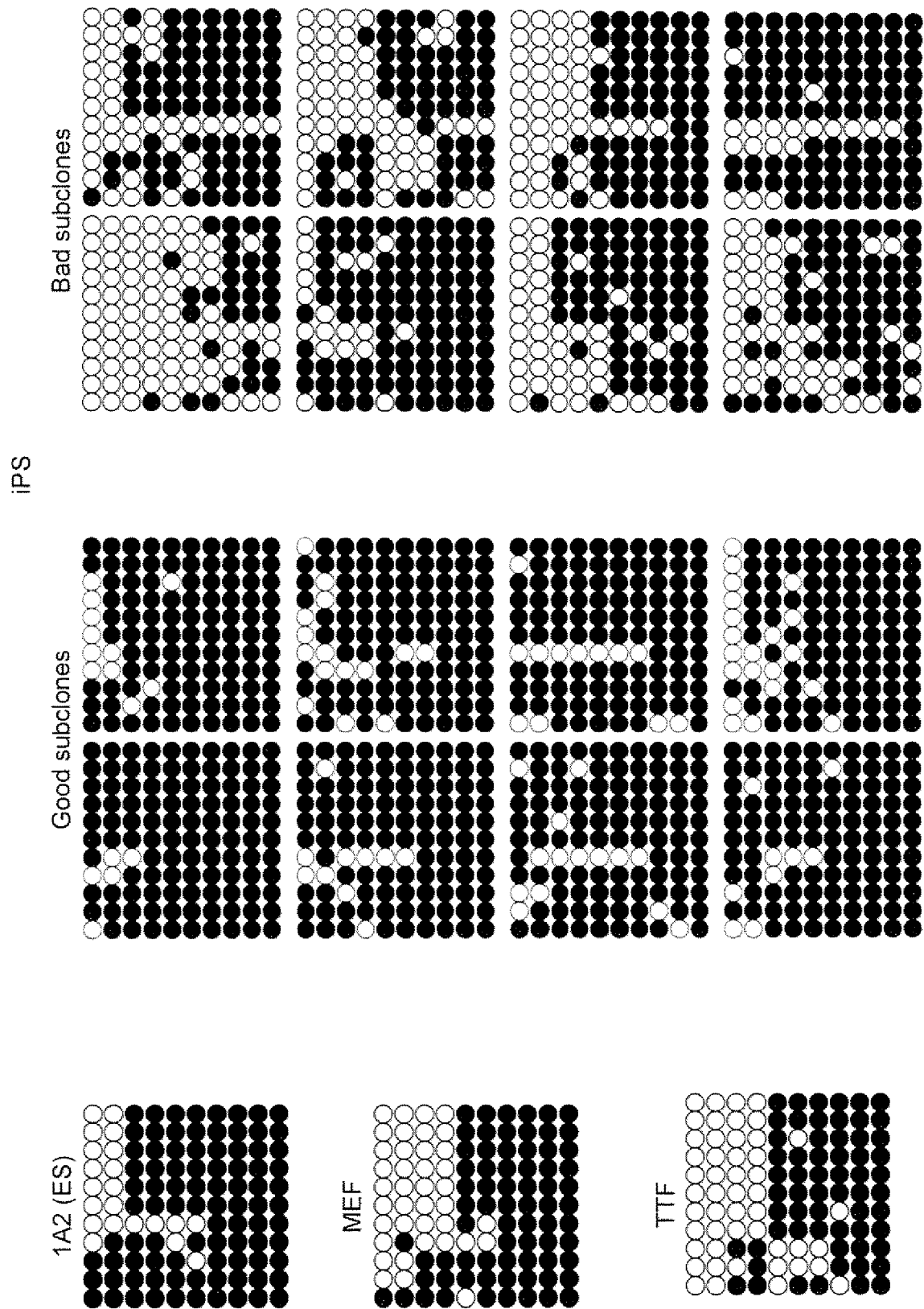
[Fig. 2]



[Fig. 3]



[Fig. 4]



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/004891

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N15/09 (2006.01) i

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. C12N15/09

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), Science Direct, Wiley InterScience, CiNii

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PICK, M. et al, Clone- and gene-specific aberrations of parental imprinting in human induced pluripotent stem cells, Stem cells (Dayton, Ohio), 2009, Vol.27, No.11, p.2686-90	1-8
A	YAMANAKA, S., A fresh look at iPS cells, Cell, 2009.04.03, Vol.137, No.1, pp.13-17	1-8
A	MIURA, K. et al, Variation in the safety of induced pluripotent stem cell lines, Nature biotechnology, 2009, Vol.27, No.8, p.743-5	1-8



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

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"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

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Name and mailing address of the ISA/JP

Japan Patent Office

3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan

Authorized officer

Kyoko YAMAMOTO

Telephone No. +81-3-3581-1101 Ext. 3448

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/004891

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TSUJI-TAKAYAMA, K. et al, Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells , Biochem Biophys Res Commun, 2004, Vol.323, No.1, p.86-90	1-8
A	YUAN, L. et al, An extended region of biallelic gene expression and rodent-human synteny downstream of the imprinted H19 gene on chromosome 11p15.5, Human molecular genetics, 1996, Vol.5, No.12, p.1931-7	1-8