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(54) **Title**: METHOD FOR SELECTING CLONE OF INDUCED PLURIPOTENT STEM CELLS

(57) **Abstract**: To efficiently identify and select a clone from clones of induced pluripotent stem cells (iPS cell) having low tumor formation rate in vivo when allowed to differentiate and transplanted in a living body, iPS cells of the clones are induced to differentiate, undifferentiated cells among the cells after the induction of differentiation are detected, and a clone having the content of the undifferentiated cell below a control is selected.

Description

Title of Invention: METHOD FOR SELECTING CLONE OF INDUCED PLURIPOTENT STEM CELLS

Technical Filed

[0001] The present invention relates to methods for selecting a clone of induced pluripotent stem cells, and the clones selected by the selection methods.

Background Art

[0002] The induced pluripotent stem cells (iPS cells) can be produced by introducing reprogramming factors into somatic cells (K. Takahashi and S. Yamanaka, Cell 126 (4), 663, 2006; WO2007/069666). The iPS cells thus produced have been confirmed to possess pluripotency, for example, by the fact that iPS cells contribute to the germline of chimeric mice (K. Okita, et al., Nature 448 (7151), 313, 2007; M. Wernig, et al., Nature 448 (7151), 318, 2007). Since the iPS cells can be produced by using cells derived from a patient to be treated and then induced to differentiate into cells of intended tissues, they are expected in the field of regenerative medicine to serve as a transplantation material free from rejection.

[0003] However, in chimeric mice derived from the iPS cells thus produced and their offsprings, possibility that a tumor is formed due to reactivation of the introduced c-myc gene cannot be denied. Thus, in order to reduce their tumor formation ability, methods without using a retrovirus carrying c-Myc gene have been developed to produce iPS cells, although their efficiency of induction is relatively low (M. Nakagawa, et al., Nat Biotechnol 26 (1), 101, 2008; M. Wernig, et al., Cell Stem Cell 2 (1), 10, 2008; WO2008/118820; WO2009/057831). Nonetheless, in consideration of the high efficiency with using c-Myc, it is more desirable to develop a method for selecting iPS cells with low tumor formation ability while using a retrovirus carrying c-Myc.

[0004] Similarly, although various methods have been developed for practical application of producing iPS cells, such as a method of preparing iPS cells adaptable to production of chimeric mice without employing selection by a drug (A. Meissner, et al., Nat Biotechnol 25(10), 1177 2007) and a method of produce iPS cells using cells from various tissues (Aoi, T. et al., Science 321, 699-702, 2008), the iPS cells thus produced are not always suitable for transplantation.

[0005] Accordingly, it is expected to establish methods not only for producing iPS cells but also for selecting iPS cells with high safety.

Summary of Invention

Technical Problem

[0006] The present invention is intended to provide methods for efficiently identifying and selecting clones of iPS cells having low tumor formation rate in vivo when allowed to differentiate and transplanted in a living body, as well as the clones selected by the methods and kits for the selection.

Solution to Problem

[0007] In one embodiment of the present invention, a method for selecting a clone of induced pluripotent stem cells (iPS cells) includes the steps of detecting an undifferentiated cell in the clones after induction of differentiation of the iPS cells, and selecting a clone based on the result of the detection. In the step of detecting an undifferentiated cell, formation of teratoma may be examined, or promoter activity of an undifferentiated cell-specific gene may be detected, and more preferably a content of cells in which the promoter activity is detected in the clone may be measured. The induction of differentiation may include allowing the iPS cells of the clones to form a primary neurosphere or a secondary neurosphere. In the step of detecting the promoter activity, the iPS cells may have a marker gene whose expression is regulated by the promoter of the undifferentiated cell-specific gene, wherein expression of the marker gene may be detected. The marker gene preferably encodes a fluorescent protein, a luminescent protein or an enzyme. In the step of selecting the clone, expression of an endogenous undifferentiated cell-specific gene may be detected. The undifferentiated cell-specific gene may be, but not limited to, Nanog gene.

[0008] In the step of selecting iPS cells, a content of cells where transcription of an undifferentiated cell-specific gene is activated in the clones may be measured in multiple times, and a clone of iPS cells in which an average of the measurements is less than 0.042% or the measurements are less than 0.066% in all generations of the iPS cells examined may be selected.

[0009] Another embodiment of the present invention provides a method for producing a clone of iPS cells, comprising the step of selecting a clone from clones of iPS cells by any one of the abovementioned methods.

[0010] Another embodiment of the present invention provides a clone of iPS cells selected by any one of the abovementioned methods.

[0011] In a further embodiment of the present invention, a kit for selecting a clone of iPS cells contains a reagent to detect promoter activity of an undifferentiated cell-specific gene. The reagent may detect a transcription product transcribed from the undifferentiated cell-specific gene or a gene encoding a fluorescent protein, a luminescent protein or an enzyme, or a reagent to detect a peptide translated from the transcription product. The undifferentiated cell-specific gene may be, but not limited to, Nanog gene.

[0012] The iPS cells from which the clone is selected may have been obtained by introducing at least one gene in a gene family selected from the group consisting of Oct gene family, Sox gene family, Klf gene family, Myc gene family, Nanog gene, Sall gene family and Lin28 gene. Preferably, the member of the Oct gene family is Oct3/4; the member of the Sox gene family is Sox2; the member of the Klf gene family is Klf4; the member of the Myc gene family is c-Myc or L-Myc; and the member of the Sall gene family is Sall4 or Sall1. More preferably, the iPS cells have been obtained by introducing Oct3/4 gene, Sox2 gene, Klf4 gene and c-myc gene.

[0013] ==CROSS REFERENCE TO RELATED APPLICATIONS==

This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/217,362, filed on May 29, 2009, which is incorporated herein by reference. All of the documents cited in the specification is also incorporated herein by reference.

Advantageous Effects of Invention

[0014] The present invention can provide methods for efficiently identifying and selecting clones of iPS cells with high safety, as well as clones selected by the methods.

Brief Description of Drawings

[0015] [fig.1]Fig. 1 shows formation of SNSs from mouse iPS cells. (a) Photographs of secondary neurospheres derived from ES cells (1A2), MEF-iPS cells (178B2), TTF-iPS cells (212C5) or Hep-iPS cells (238C2). Scale bar = 200 um. (b) Immunohistochemical analyses of Tuj1 (a marker for neurons), GFAP (a marker for astrocytes) and CNPase (a marker for oligodendrocytes) in the cells induced to differentiate from SNSs. Scale bar = 100 um. (c) Immunohistochemical analyses of neurons (using NeuN), astrocytes (using GFAP) and oligodendrocytes (using APC) in a NOD/SCID mouse at 4 weeks after transplantation of SNSs derived from MEF-iPS cells (38C2). Scale bar = 50 um (10 um in the inset).

[fig.2]Fig. 2 shows the contents of undifferentiated cells in SNSs derived from ES cells and iPS cells. (a) Comparison of the contents of Nanog-GFP positive cells in SNSs derived from ES cells, MEF-iPS cells, TTF-iPS cells, and Hep-iPS cells. (b) More detailed comparison of the contents of Nanog-GFP positive cells in the SNSs derived from ES cells, MEF-iPS cells, TTF-iPS cells, and Hep-iPS cells. (c) Comparison of the contents of Nanog-GFP positive cells in the SNSs derived from the iPS cells established with or without using Myc. No significant difference was observed in a Mann-Whitney U-test between the respective groups. (d) Comparison of the contents of Nanog-GFP positive cells in the SNSs derived from the iPS cells established with or without selection in terms of expression of Nanog. No significant difference was observed in a Mann-Whitney U-test between respective groups.

[fig.3]Fig. 3 shows formation of teratomas by the SNSs derived from ES cells or iPS

cells. (a) Observation period of transplanted mice divided into four groups according to teratoma formation. A diamond- indicates a mouse without forming teratoma (n=140); a diamond+ indicates a mouse that formed teratoma with a diameter of 0.1 to 5.7 mm (least tertile, n=29); a diamond++ indicates a mouse that formed teratoma with a diameter of 5.8-8.2 mm (second tertile, n=29); and a diamond+++ indicates a mouse that formed teratoma with a diameter of 8.3 mm or more (most tertile, n=29). Filled diamonds indicate dead or weakened mice, whereas open diamonds indicate healthy mice. (b) Diameters of teratomas in the SNSs derived from ES cell clones or iPS cell clones.
[fig.4]Fig. 4 shows histological images of teratomas generated by SNSs derived from TTF-iPS cells (256D4). Photographs obtained by hematoxylin-eosin staining following formalin fixation, paraffin embedding and sectioning.
[fig.5]Fig. 5 shows a correlation between the origin of iPS cells and teratoma formation of SNSs. (a) Comparison of diameters of teratomas generated by SNSs derived from MEF-iPS cells, TTF-iPS cells, Hep-iPS cells, Stm-iPS cells or ES cells. Kruskal-Wallis tests and Scheffe tests were employed for the comparison between the respective five groups. *** indicates $p<0.0001$. (b) Comparison of ratios of each state of being dead or weakened (in black) and healthy (in white) among the SNSs derived from MEF-iPS cells, TTF-iPS cells, Hep-iPS cells, Stm-iPS cells and ES cells. Chi-square tests were employed for the comparison between the respective two groups. ** indicates $p<0.01$ and *** indicates $p<0.0001$. (c) Comparison of diameters of teratomas generated by SNSs derived from iPS cells established with or without using c-Myc. A dead or weakened mouse is indicated by a black diamond, whereas a healthy mouse is indicated by a white diamond. No significant difference was observed in a Mann-Whitney U-test between the respective groups. (d) Comparison of diameters of teratomas generated by SNSs derived from iPS cells established with or without selection. A dead or weakened mouse is indicated by a black diamond, whereas a healthy mouse is indicated by a while diamond. No significant difference was observed in a Mann-Whitney U-test between the respective groups. (e) Correlation between the contents of Nanog positive cells in SNSs and the diameters of teratoma. The mice transplanted with SNSs were divided according to the contents of Nanog-GFP positive cells: (0 to 0.018%, least tertile, 46 mice), (0.019 to 0.556%, second tertile, 48 mice) and (0.556% or more, most tertile, 45 mice).
[fig.6]Fig. 6 shows expression of introduced genes in SNSs and teratomas. RT-PCR analyses of RNAs isolated from undifferentiated cells (Un.), SNSs derived from cell clones or teratomas (178B5-MEF-iPS, 256H13-TTF-iPS, 256H17-TTF-iPS) by using primer pairs for amplifying either of coding regions of four introduced genes ("total"), a transcript from an endogenous gene only (endo), or a transcript from an introduced

gene only (tg). Clone 4-3, a TTF-Fbxo15-iPS cell, was used as a positive control for expression of an introduced gene.

Description of Embodiments

[0016] Embodiments of the present invention accomplished based on the above-described findings are hereinafter described in detail by giving examples. Where there is no particular explanations in embodiments or examples, methods described in standard sets of protocols such as J. Sambrook, E. F. Fritsch & T. Maniatis (Ed.), Molecular cloning, a laboratory manual (3rd edition), Cold Spring Harbor Press, Cold Spring Harbor, New York (2001); F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J.G. Seidman, J. A. Smith, K. Struhl (Ed.), Current Protocols in Molecular Biology, John Wiley & Sons Ltd., or their modified/changed methods are used. When using a commercial reagent kit or a measuring apparatus, unless otherwise explained, protocols attached to them are used.

[0017] The object, characteristics, advantages of the present invention as well as the idea thereof are apparent to those skilled in the art from the descriptions given herein, and the present invention can be easily worked by those skilled in the art based on the descriptions given herein. It is to be understood that the embodiments and specific examples of the invention described hereinbelow are to be taken as preferred examples of the present invention. These descriptions are only for illustrative and explanatory purposes and are not intended to limit the invention to these embodiments or examples. It is further apparent to those skilled in the art that various changes and modifications may be made based on the descriptions given herein within the intent and scope of the present invention disclosed herein.

[0018] == Induced Pluripotent Stem Cells ==

An induced pluripotent stem cell (iPS cell) refers to a cell having pluripotency and self-reproducing ability, which is artificially induced by reprogramming a differentiated cell other than germline cells (such as egg cells, sperm cells and their precursor cells such as oogonia and spermatogonia) or undifferentiated cells derived from embryos at early stages of development (such as embryonic stem cells). The differentiated cell may be derived from an embryo, a fetus, or an adult, and may originate from any animal species, such as mice or humans. The characteristics of the differentiated cell are not particularly limited as long as the cell has at least partly lost endogenous totipotency of a fertilized cell. Examples of the differentiated cell include fibroblasts, epithelial cells, hepatocytes, etc.

[0019] The method for the reprogramming is not particularly limited, but in a preferred method cells are induced to obtain pluripotency and self-reproduction ability by introducing a nuclear reprogramming factor. For example, any of the reprogramming

methods described in WO2005/080598, WO2007/069666, WO2008/118820 and WO2009/057831 may be used. The disclosures of these publications are incorporated herein by reference.

[0020] The nuclear reprogramming factor is not particularly limited, but preferred is at least one product of a gene in a family selected from each of the Oct gene family, Klf gene family, Sox gene family, Myc gene family, Sall gene family, Nanog gene family (mouse NM_028016, human NM_024865) and Lin gene family. The genes belonging to the Oct gene family include Oct3/4 (mouse NM_013633, human NM_002701), Oct1A (mouse NM_198934, human NM_002697) and Oct6 (mouse NM_011141, human NM_002699); those belonging to the Klf gene family include Klf1 (mouse NM_010635, human NM_006563), Klf2 (mouse NM_008452, human NM_016270), Klf4 (mouse NM_010637, human NM_004235) and Klf5 (mouse NM_009769, human NM_001730); those belonging to the Sox gene family include Sox1 (mouse NM_009233, human NM_005986), Sox2 (mouse NM_011443, human NM_003106), Sox3 (mouse NM_009237, human NM_005634), Sox7 (mouse NM_011446, human NM_031439), Sox15 (mouse NM_009235, human NM_006942), Sox17 (mouse NM_011441, human NM_022454) and Sox18 (mouse NM_009236, human NM_018419); those belonging to the Myc gene family include c-Myc(mouse NM_010849, human NM_002467), N-Myc (mouse NM_008709, human NM_005378) and L-Myc (mouse NM_008506, human NM_001033081); those belonging to the Sall gene family include Sall1 (mouse NM_021390, human NM_002968) and Sal4 (mouse NM_175303, human NM_020436); and those belonging to the Lin gene family include Lin28 (mouse NM_145833, human NM_024674) and Lin28b (mouse NM_001031772, human NM_001004317). As the nuclear reprogramming factor, other kinds of gene products may also be used, and examples include an immortalization-inducing factor.

[0021] More preferably, the nuclear reprogramming factor may include at least a product of one or more genes selected from Oct3/4 gene, Klf4 gene, Sox2 gene, c-Myc gene, L-Myc gene, Sall4 gene, Sall1 gene, Nanog gene and Lin28 gene.

[0022] While these genes are herein represented by mouse and human sequences with reference to their Accession Numbers registered at the National Center for Biotechnology Information (NCBI), they are all highly conserved among the vertebrates, and therefore a gene represented herein includes its homologues unless a name of a particular animal is indicated. Moreover, mutated genes including those with polymorphism are also encompassed as long as they have a function comparable to that of the wild-type gene product.

[0023] == Methods for Producing iPS Cells ==

In order to produce iPS cells by using nuclear reprogramming factors, they are

preferably introduced into a somatic cell. The number of nuclear reprogramming factors to be included is two, three, preferably four, or more than four. A preferable combination of the factors is either the combination of Oct3/4 gene, Sox2 gene and Klf4 gene, or the combination of Oct3/4 gene, Sox2 gene, Klf4 gene and c-myc gene.

[0024] In order to introduce a nuclear reprogramming factor, in the case where it is a protein functioning in a cell, a gene encoding the protein is preferably incorporated into an expression vector, which is introduced into a target differentiated cell such as a somatic cell, so that the protein is intracellularly expressed (the gene transduction method). The expression vector to be used is not particularly limited, but preferred examples include plasmid vectors, viral vectors and artificial chromosome vectors (Suzuki N. et al., J. Biol. Chem. 281(36):26615, 2006), and preferred examples of the viral vectors include adenovirus vectors, Sendai virus vectors, retrovirus vectors and lentivirus vectors. Alternatively, the protein may be introduced into cells by binding a peptide called protein transduction domain (PTD) to the protein, which is added to a culture medium (the protein transduction method). Also, the protein can be introduced by, for example, utilizing any of various protein introducing agents (such as Chariot™ and Bioporter™) for introducing a purified protein. In the case where the factor is a protein secreted extracellularly, the factor may be added to a medium for culturing differentiated cells during the production of iPS cells. If any of the nuclear reprogramming factors is expressed in the differentiated cells to be reprogrammed, such factors do not need to be introduced exogenously.

[0025] In some cases, a cytokine or a chemical compound may be added for the purpose of substituting a nuclear reprogramming factor or for the purpose of improving the efficiency of induction. Examples of the cytokine include SCF (stem cell factor), bFGF, Wnt family and LIF (leukemia inhibitory factor), and examples of the chemical compound include histone deacetylase inhibitor, DNA methylation inhibitor, MEK inhibitor, GSK3beta inhibitor TGF receptor inhibitor and ROCK inhibitor (WO 2009/117439).

[0026] After the reprogramming factors are introduced into somatic cells, the cells may be transferred onto feeder cells and cultured. The feeder cells are not particularly limited, but examples include mouse embryonic fibroblasts (MEFs). Media preferably used for the culture include those suitable for culturing cells of the animal species from which the somatic cells are derived, and in the case of human cells for example, a preferred medium is the DMEM/F12 medium supplemented with 20% serum substitute, 2mM L-glutamine, 1 x 10^{-4} M non-essential amino acids, 1 x 10^{-4} M 2-mercaptoethanol, 0.5% penicillin and streptomycin, and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF).

[0027] The iPS cells are then isolated from the differentiated cells to which the nuclear re-

programming factors have been introduced by, foe example, selecting cells expressing an undifferentiated cell-specific gene or utilizing morphology of the cells as a marker. The method for selecting cells expressing the undifferentiated cell-specific gene is not particularly limited. In the case where the undifferentiated cell-specific gene encodes an intracellular protein, one of marker genes, such as GFP gene, galactosidase gene and drug resistance genes such as neomycin resistance gene, hygromycin resistance gene and puromycin resistance gene, may be knocked-in to the downstream of a promoter of the undifferentiated cell-specific gene to be expressed as a fusion protein, and cells expressing the marker gene may be selected. In the case where the marker is a drug resistance gene, the desired cells can be easily isolated by a selection using the drug. In the case where the undifferentiated cell-specific gene encodes a membrane protein, cells expressing the protein can be selected by using a specific antibody to the protein or by utilizing an enzymatic activity of the protein. It should be noted that an undifferentiated cell-specific gene as used herein indicates a gene specifically expressed in an embryonic stem cell (ES cell) and known to those skilled in the art, and examples include those disclosed in, for example, WO2005/080598, WO2007/069666, WO2008/118820, WO2009/057831 and Nat Biotechnol. 25, 803, 2007. The undifferentiated cell-specific gene is selected preferably from a group consisting of Oct3/4, Sox2, Nanog, Lin28, Rex1, UTF1, Eras, Fgf4, TDGF, Cripto, Dax1, ESG1, GDF3, Sall4, Fbx15, SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 and alkaline phosphatases (such as TRA-2-54 and TRA-2-49) with consideration of animal species (for example, SSEA-1 is specific for mouse, whereas SSEA-4, TRA-1-60 and TRA-1-81 are specific for human). A preferable undifferentiated cell-specific gene is Fbx15 gene or Nanog gene. In the case where cellular morphology is utilized as a marker, the selection may be conducted by, for example, utilizing formation of colonies as a marker.

[0028] Cell populations or cell lines or clones thus isolated from the reprogrammed cells can be used as the iPS cells.

[0029] In the present description, the words of cell populations, cell lines and clones are indistinguishable, unless otherwise specified.

[0030] == Methods for Identifying/Selecting iPS Cells ==

In one embodiment of the present invention, methods are provided for inducing differentiation of cells into a certain cell type and then selecting iPS cells suitable for cell supplement therapy. Specifically, the iPS cells obtained by any of the abovementioned methods are appropriately treated for induction of differentiation, presence of an undifferentiated cell is detected, and the iPS cells are selected based on the result of the detection. The method for detecting an undifferentiated cell may be, but not limited to, the method to examine formation of teratoma from the cells after the induction of dif-

ferentiation, or the method to detect promoter activity of the undifferentiated cell-specific gene. The iPS cells to be selected are preferably cloned as a population of cells having homogeneous genetic information. The undifferentiated cell-specific gene in this method may be the same as the abovementioned undifferentiated cell-specific gene, and is preferably Nanog gene.

[0031] Formation of teratoma may be examined by, for example, transplanting the cells induced to differentiate into an immunodeficient animal, dissecting a tissue at the site of the transplantation at 4 to 45 weeks after the transplantation, and observing a structure of any of fetal or mature tissues of endodermal, mesodermal or ectodermal origin, such as a tumor, cartilage, smooth muscle, mucous gland, respiratory organ, digestive organ and nervous tissue.

[0032] The immunodeficient animal may be, but not limited to, nude mice or NOD/SCID mice. The site to which the cells induced to differentiate are transplanted is preferably hypodermis, an inside of testis or corpus striatum.

[0033] The method for examining promoter activity of an undifferentiated cell-specific gene is not particularly limited. In the case where the iPS cells have a reporter gene whose expression is regulated by a promoter of an undifferentiated cell-specific gene, expression of the reporter gene may be examined. In an alternative embodiment, the endogenous expression of an undifferentiated cell-specific gene may be examined for promoter activity of the undifferentiated cell-specific gene. Examples of the reporter gene include those encoding fluorescent proteins such as green fluorescent protein (GFP), yellow fluorescent protein (YFP) and blue fluorescent protein (BFP), luminescent proteins such as aequorin, and enzymes such as luciferase, beta-galactosidase, alkaline phosphatase and horse radish peroxidase (HRP).

[0034] The iPS cells having a reporter gene whose expression is regulated by a promoter of an undifferentiated cell-specific gene may be produced from differentiated cells by any of methods known to those skilled in the art such as a homologous recombination method, in which the cells are genetically modified by replacing a coding region of the undifferentiated cell-specific gene with a sequence of the reporter gene. Alternatively, the iPS cells may be produced from differentiated cells that have been genetically modified by inserting a sequence of the reporter gene into the locus of the undifferentiated cell-specific gene so that a fusion protein is formed from the protein or a fragment thereof encoded by the undifferentiated cell-specific gene and the protein encoded by the reporter gene. Alternatively, the iPS cells having the reporter gene may be produced by a homologous recombination which involves genetic modification to directly replace the coding region of the undifferentiated cell gene with the reporter gene or to insert the reporter gene into the locus of the undifferentiated cell gene. In an alternative embodiment, the iPS cells having the reporter gene may be produced by in-

troducing, into the iPS cells, a construct containing a nucleotide sequence in which the promoter region of the undifferentiated cell-specific gene is linked with the reporter gene. A DNA fragment containing the promoter region can be isolated from a genomic DNA or a genome library by any of methods known to those skilled in the art based on the result of a promoter analysis on the undifferentiated cell-specific gene to be used. The construct may be prepared using a plasmid vector, a virus vector or an artificial chromosome vector (Suzuki N. et al., J. Biol. Chem. 281(36):26615, 2006).

[0035] In the case where expression of the reporter gene or the endogenous undifferentiated cell-specific gene is to be detected, a transcription product (such as hnRNA, mRNA etc.) may be detected by, for example, PCR method, LAMP method or Northern hybridization method. Alternatively, a translation product (such as a peptide, a modified peptide etc.) may be detect by, for example, RIA method, IRMA method, EIA method, ELISA method, LPIA method, CLIA method or immunoblotting method. The transcription product or the translation product is preferably detected in a quantitative manner.

[0036] In the method to detect the promoter activity of the undifferentiated cell-specific gene, expression of the reporter gene or the expression of the endogenous undifferentiated cell-specific gene is preferably measured by flow cytometer etc. to quantitate a content of cells having activated promoter of the undifferentiated cell-specific gene in the clone of the cells. In this case, the content of the cells having the activated promoter is calculated as the ratio of such cells in the clone of the cells having been induced to differentiate.

[0037] In the case where the content of the cells, which have been induced to differentiate and in which the promoter of the undifferentiated cell-specific gene has been activated, is measured for the selection of a clone of the iPS cells, it is preferable to select clones of the iPS cells in which the content of the cells having the activated promoter is below a control content of cells having the activated promoter in a clone of the cells that would not substantially cause tumor formation when transplanted into a living body. As for the control content, it is preferable to examine tumor formation and examine the content as shown in Table 1 after any of available cell lines is transplanted into a living body, and adopt a content predetermined so that both values of sensitivity and specificity as shown in Table 1 are equal to or more than 0.5, 0.6, 0.7, 0.8, 0.9, 0.95 or 0.99. Preferably, the value of sensitivity is more than 0.9, 0.95, 0.99 or 1. More preferably the values of the sensitivity and specificity are both 1. It should be noted that the both values of the sensitivity and specificity being 1 indicate that tumor formation would not occur if the content is below the determined control content. The cell line to be transplanted is preferably a cell into which an iPS cell or an ES cell is induced to differentiate.

[Table 1]

	Number of clones positive for tumor formation	Number of clones negative for tumor formation
Number of clones in which the content of the cells having the activated promoter is above a control content	A	C
Number of clones in which the content of the cells having the activated promoter is below a control content	B	D
	Sensitivity = A/(A+B)	Specificity = D/C+D

[0038] In another embodiment, the detection of the promoter activity may be conducted more than once for each of iPS cells to be examined, in which case an average or a maximum value of the measured contents may be compared with the control content. In the case where the average of the contents is to be compared with the control content, 0.042% is preferably adopted as the control content. In the case where the maximum value of the contents is to be compared with the control content, 0.082%, 0.066%, 0.051% or 0.019% is preferably adopted as the control content. More preferably, 0.066% is adopted as the control content.

[0039] In another embodiment where a transcription product or translation product of the undifferentiated cell-specific gene is quantified after the induction of differentiation, amounts of the transcription product or amounts of the translation product in a clone of the iPS cells may be compared with a respective control and the clones having the amount below a control may be selected. As for the control amount of transcription product or translation product, it is preferable to measure tumor formation and the amount of transcription product or translation product as shown in Table 2 after any of available cell line is transplanted into a living body, and adopt a predetermined content with which both values of sensitivity and specificity as shown in Table 2 are equal to or more than 0.5, 0.6, 0.7, 0.8, 0.9, 0.95 or 0.99. Preferably, the value of sensitivity is more than 0.9, 0.95, 0.99 or 1. More preferably the values of the sensitivity and specificity are both 1. It should be noted that both values of the sensitivity and specificity being 1 indicate that tumor formation would not occur if the content is below the predetermined control amount of transcription product or translation product. The cell line to be transplanted is preferably a cell into which an iPS cell or an ES cell is induced to differentiate.

[Table 2]

	Number of clones positive for tumor formation	Number of clones negative for tumor formation
Number of clones with trascription (or translation) products more than control transciption (or tranlsation) products	A	C
Number of clones with trascription (or translation) products less than control transciption (or tranlsation) products	B	D
	Sensitivity = A/(A+B)	Specificity = D/C+D

[0040] While the method for inducing differentiation of iPS cells or ES cells is not particularly limited, a preferable method is to induce them to differentiate into neural stem cells or neural progenitor cells by forming neurospheres in which cells are spherically clustered. More preferred is the method to induce the cells to form secondary neurospheres. The induction method for iPS cells into neural stem cells may be the same as the methods used for ES cells and known to those skilled in the art (Okada Y, et al., Stem cells 26 (12), 3086, 2008; Japanese Patent Application Laid-open Publication No.2002-291469). The iPS cells to be treated may be cultured in suspension in the presence of a low level (10^{-9} M to 10^{-6} M) of retinoic acid to thereby induce formation of an embryoid body (EB). Alternatively, the medium for culturing the iPS cells may be supplemented with Noggin protein. Specifically, a culture supernatant from mammalian cultured cells to which Xenopus Noggin has been introduced and induced to transiently express the Noggin protein may be used as is (1 to 50% (v/v)), or a recombinant Noggin protein (about 1 ug/ml) may be added to the medium. Then, the EBs thus obtained are dissociated and cultured in a serum-free medium supplemented with FGF-2 (10 to 100 ng/ml) and B27 to allow them to form primary neurospheres. Further, the primary neurospheres may be dissociated to allow them to form secondary neurospheres under the same conditions. The neurosphere dissociation - neurosphere formation step may be repeated to further form higher-order neurospheres.

[0041] The clones of the iPS cells thus selected can be used as a material for the cell supplement therapy.

[0042] == Kits ==

The kit for selecting clones of iPS cells according to the present invention includes a reagent to detect promoter activity of an undifferentiated cell-specific gene.

[0043] As described earlier, the examples of the methods for detecting promoter activity of an undifferentiated cell-specific gene include PCR method, LAMP method, Northern hybridization method, RIA method, IRMA method, EIA method, ELISA method, LPIA method, CLIA method and immunoblotting method. Since all of these detection methods have been publicly known, a person skilled in the art can appropriately

include a primer, a probe, an antibody, an enzyme substrate, a reagent and/or the like in the kit in accordance with the detection method to be employed. Also, a vector or recombination construct that contains a promoter of an undifferentiated cell-specific gene and a marker gene whose expression is regulated by the promoter may be included. The recombination construct may be produced by any of the methods known to those skilled in the art. In the case where the undifferentiated cell-specific gene is Nanog, the construct may be the targeting vector described in Mitsui K, et al., Cell, 113, 631, 2003, and in the case where the gene is Fbx15, the construct may be the targeting vector described in Tokuzawa Y, et al, Mol Cell Biol., 23(8), 2699, 2003.

Examples

[0044] <Materials and Methods>

<Culture>

Establishment and culture of ES cells and iPS cells were conducted in the conventional methods as described (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; and Aoi, T. et al., Science 321, 699-702, 2008). Following 5 cell lines of established ES cells or iPS cells; 1A2, 212C6, 256D4, 135C6 and 178B5, were subcloned by picking up colonies after weak cell concentration culture. The induction of differentiation into neural cells was conducted in the method by Okada et al. (Okada Y, et al., Stem cells 26 (12), 3086, 2008) with minor modification. In brief, iPS cells were cultured in the presence of 1 x 10^{-8} M retinoic acid to form embryoid bodies (EBs). Subsequently, the EBs were dissociated at 6 days after the addition of retinoic acid, and suspension cultured in the Media hormone mix (MHM) supplemented with B27 and 20ng/ml FGF-2 (Waco) in a culturing flask (Nunc). On day 4 from suspension culture, the suspension cells were transferred from the culturing flask to a Ultra-Low Attachment dish (Corning), and cultured in the same medium for 3 or 4 days. Then primary neurospheres (PNSs) were formed. Subsequently, the prepared PNSs were dissociated by using TrypLESelect and suspension-cultured in the same medium. After 4 days from the suspension culture, the suspension cells were placed on a coverslip coated with poly-L ornithine/fibronectin and cultured in the absence of FGF2 for 5 to 6 days. In this way secondary neurospheres (SNSs) were formed.

<Flow Cytometry>

Undifferentiated iPS cells and iPS-derived SNSs containing Nanog-EGFP reporter (WO2007/069666) were dissociated and subjected to flow cytometry analyses with using FACS Calibur or FACS Aria (both Becton-Dickinson). Dead cells were detected by propidium iodide staining and removed, and the number of EGFP-positive cells was

measured as a ratio in viable cells.

<Production of Lentivirus and Infection to Secondary Neurospheres>

For transplantation into a brain of a NOD/SCID mouse, cells were labeled with pCSII-EF-MCS-IRES2-Venus which was constructed by using a third-generation lentivirus vector derived from HIV-1 with inactivated self-propagation (Miyoshi H, et al., J Virol 72 (10), 8150, 1998). For production of lentivirus, HEK-293T cells were transfected with either pCSII-EF-MCS-IRES2-Venus, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev (all in Miyoshi H, et al., J Virol 72 (10), 8150, 1998), and culture supernatant containing respective viral particles was recovered. The viral particles were concentrated by centrifugation at 25000rpm, 4°C for 1.5 hour. The concentrated viral particles were added to the culture medium during the formation of SNS from PNS.

<Transplantation into Brain of NOD/SCID Mouse>

The neurospheres introduced with Venus by using the lentivirus (pCSII-EF-MCS-IRES2-Venus) were transplanted by using a glass micropipette on a stereotactic introducer in a conventional method (Ogawa D, et al., J Neurosci Res, 2008). In this method, the tip of the pipette was inserted into a right corpus striatum (1 mm rostral and 2 mm lateral from bregma and 3 mm deep from dura mater) of a 6 week-old female NOD/SCID mouse and 3ml of the suspension of SNS cells (2×10^5 cells) was injected.

[0045] Example 1: Differentiation of Respective iPS Cells into Neurons

36 clones of iPS cells were divided according to (1) the origins of iPS cells; i.e. MEF (Mouse Embryonic Fibroblast), TTF (Tail Tip Fibroblast), Hep (Hepatocyte) or Stm (Stomach epithelial cell); (2) with or without introduction of c-Myc-containing retrovirus; and (3) with or without selection in terms of expression of Nanog or Fbxo15. Profiles and results of analyses of each clone are shown in Tables 3 and 4. As control, the following three ES cell clones were used (RF8 (Meiner, V.L. et al., Proc Natl Acad Sci U S A., 93(24):14041, 1996); 1A2, a subclone thereof having Nanog-EGFP reporter (Okita K, et al., Nature 448 (7151), 313, 2007); and EB3 having an Oct3/4 blasticidin resistant reporter gene (Mol Cell Biol., 22(5):1526, 2002 and Okada Y, et al., Stem cells 26 (12), 3086, 2008)). When these iPS cells and ES cells were allowed to form neurospheres containing neural stem cells and/or precursor cells (NS/PC), each of the iPS clones and most of the ES cells formed primary neurospheres (PNS) in the presence of FGF2 after formation of embryoid bodies. When the PNS were dissociated and secondary neurospheres were formed (Fig. 1a), three of Hep-iPS clones and one of Stm-iPS clones did not form a SNS (Table 3). The SNSs derived from iPS cells and ES cells were differentiated in vitro into three types of neural cells, i.e. neurons, astrocytes and oligodendrocytes (Fig. 1b). These SNSs survived also in

vivo, and differentiated into the three types of neural cells (Fig. 1c). The iPS cells thus possess the potency to differentiate into neural cells as ES cells do, regardless of the cell type of their origin or presence/absence of the c-Myc-containing retrovirus or selection.

[Table 3]

iPS line				SNS formation			Transplantation of SNSs					
							Diameter of teratoma (mm)					
Origin	Selection	cMyc	clone's name	Trial	EGFP+ cells in SNSs (%)	Average of EGFP+ cells in SNSs (%)	≧8.3	5.8-8.2	0.1-5.7	0	Analyzed numberof mice	Teratoma+ / total
MEF	Nanog	+	20D17	1st	ND	0.0454075	0	0	3	0	3	15/21
				2nd	ND		0	3	2	0	5	
				3rd	0.00363		0	0	0	4	4	
				4th	0.049		0	0	0	2	2	
				5th	0.11		0	0	3	0	3	
				6th	0.019		0	0	4	0	4	
			38D2	1st	ND	0.103805	0	0	3	0	3	13/17
				2nd	ND		0	1	2	0	3	
				3rd	0.026		0	0	3	0	3	
				4th	0.00143		0	0	0	2	2	
				5th	0.00779		0	0	0	2	2	
				6th	0.38		0	0	4	0	4	
			38C2	1st	ND	0	0	0	0	4	4	0/17
				2nd	ND		0	0	0	7	7	
				3rd	0		0	0	0	4	4	
				4th	0		0	0	0	2	2	
		−	178B5	1st	ND	0	0	0	0	3	3	0/7
				2nd	0		0	0	0	4	4	
			178B1	1st	0.0012	0.0006	0	0	0	3	3	0/6
				2nd	0		0	0	0	3	3	
			178B2	1st	0.0012	0.0006	0	0	0	3	3	0/6
				2nd	0		0	0	0	3	3	
	w/o		506GN1	1st	0.0052	0.0026	0	0	0	2	2	0/4
				2nd	0		0	0	0	2	2	
			506GN2	1st	0.0719	0.03695	0	0	0	2	2	0/4
				2nd	0.002		0	0	0	2	2	
			506GN3	1st	0.0383	0.01915	0	0	0	2	2	0/4
				2nd	0		0	0	0	2	2	
			506GN4	1st	0.066	0.042	0	2	0	1	3	2/5
				2nd	0.018		0	0	0	2	2	
			506GN5	1st	0.339	0.175	2	1	0	0	3	3/5
				2nd	0.011		0	0	0	2	2	
			506GN6	1st	0.063	0.032	0	0	0	2	2	0/4
				2nd	0.001		0	0	0	2	2	

TTF	Nanog	+	212B2	1st	0.6613	0.52015	2	1	0	0	3	6/6
				2nd	0.379		2	1	0	0	3	
			212C5	1st	0.5581	0.39905	3	0	0	0	3	4/4
				2nd	0.24		0	1	0	0	1	
			212C6	1st	4.182	4.6225	3	1	0	0	4	6/6
				2nd	5.063		2	0	0	0	2	
		−	335D1	1st	0.1593	0.09215	0	0	0	3	3	0/5
				2nd	0.025		0	0	0	2	2	
			335D3	1st	0.01	4.4153333	0	0	0	2	2	2/5
				2nd	12.829		0	1	0	0	1	
				3rd	0.407		1	0	0	1	2	
			212D1	1st	2.4327	2.79885	1	2	0	0	3	5/5
				2nd	3.165		1	0	1	0	2	
			212D2	1st	1.6079	10.61855	3	0	0	0	3	4/4
				2nd	19.6292		0	1	0	0	1	
	w/o	−	256H13	1st	−	−	1	0	1	0	2	2/2
			256H18	1st	−	−	0	3	0	0	3	4/4
				2nd	−		1	0	0	0	1	
			256D4	1st	20.1087	10.33805	1	3	0	0	4	7/7
				2nd	0.5674		2	1	0	0	3	
			256D7	1st	1.6644	0.87435	2	0	2	0	4	6/7
				2nd	0.0843		1	1	0	1	3	
Hep	Nanog	+	135C4	1st	0.12	0.389	0	0	0	1	1	1/5
				2nd	1.013		0	1	0	1	2	
				3rd	0.034		0	0	0	2	2	
			135C6	1st	0.975	4.6476667	0	3	0	0	3	7/7
				2nd	12		1	1	0	0	2	
				3rd	0.968		2	0	0	0	2	
			103C1	1st	ND (carrying CAG-EGFP)	−	0	0	0	2	2	1/5
				2nd			1	0	0	0	1	
				3rd			0	0	0	2	2	
			103C2	1st	ND (carrying CAG-EGFP)	−	0	0	0	2	2	0/2
		−	390B1	1st	Failed to form neurospheres							
				2nd								
				3rd								

			390B3	1st	Failed to form neurospheres						
				2nd							
	Fbx	+	92A3	1st	ND (carrying CAG-EGFP)	-	0	0	0	2	2	0/6
				2nd			0	0	0	3	3	
				3rd			0	0	0	1	1	
			98A1	1st	ND (carrying CAG-EGFP)	-	0	0	0	2	2	0/5
				2nd			0	0	0	2	2	
				3rd			0	0	0	1	1	
	w/o	-	238C1	1st	ND	-						
				2nd								
				3rd								
			238C2	1st	1.625	1.1517333	0	0	0	2	2	1/6
				2nd	1.2232		0	0	0	2	2	
				3rd	0.607		1	0	0	1	2	
Stm	Fbx	+	99-1	1st	ND (carrying CAG-EGFP)	-	0	0	0	2	2	0/3
				2nd			0	0	0	1	1	
			99-3	1st	ND (carrying CAG-EGFP)	-	0	0	0	2	2	0/5
				2nd			0	0	0	2	2	
				3rd			0	0	0	1	1	
			116-5	1st	ND (carrying CAG-EGFP)	-	formed few neurospheres					
				2nd								
				3rd								
1A2 Nanog-EGFP-RF8-ES			1A2	1st	ND	0.0146667	0	0	0	3	3	0/19
				2nd	0		0	0	0	4	4	
				3rd	0		0	0	0	4	4	
				4th	0.081		0	0	0	2	2	
				5th	0		0	0	0	2	2	
				6th	0		0	0	0	2	2	
				7th	0.007		0	0	0	2	2	
RF8				1st	-	-	0	0	0	2	2	0/7
				2nd	-		0	0	0	2	2	
				3rd	-		0	0	0	3	3	
EB3				1st	-	-	0	1	1	2	4	4/8
				2nd	-		0	0	0	2	2	
				3rd	-		0	2	0	0	2	

[Table 4]

iPS line				Differentiationin into neural tri-lineage			Transplantation of SNSs				Chimera		
							Histology of teratoma						
Origin	Selection	cMyc	clone's name	Neuron (Tuj1^{+})	Astrocyte (GFAP^{+})	Oligo-dendrocyte (CNPase^{+} or O4^{+})	N	Ectderm	Mesoderm	Endoderm	Embryo	Adult	Germ line transmission
MEF	Nanog	+	20D17	+	+	+	1	+ neural cell	+ muscle	+ duct	+	+	+
			38D2	+	+	+		ND	ND	ND	+	+	−
			38C2	+	+	+	−				+	+	−
		−	178B5	+	+	+	−				+	+	+
			178B1	+	+	+	−				+	+	−
			178B2	+	+	+	−				+	+	−
	w/o		506GN1	ND	ND	ND	−				ND	ND	ND
			506GN2	ND	ND	ND	−				ND	ND	ND
			506GN3	ND	ND	ND	−				ND	ND	ND
			506GN4	ND	ND	ND	1	+ neural cell	−	+ duct	ND	ND	ND
			506GN5	ND	ND	ND	1	+ neural cell	−	+ duct	ND	ND	ND
			506GN6	ND	ND	ND	−				ND	ND	ND
TTF	Nanog	+	212B2	+	+	+	1	+ neural tube	+ cartilage	+ duct	+	+	−
			212C5	+	+	+	1	+ neural tube	+ cartilage muscle	+ duct	+	+	−
			212C6	+	+	+	1	+ neural cell	+ muscle	+ duct	+	+	−
		−	335D1	+	+	+	−				+	+	−
			335D3	+	+	+	1	+ neural cell	+ cartilage muscle	+ duct	+	+	−
			212D1	+	+	+	1	+ neural tube keratinized epithelium	+/− adipocyte−	+ duct	+	+	−
			212D2	+	+	+	1	+ neural cell	+ cartilage bone	+ duct	+	+	−

	w/o	−	256H13	+	+	+	1	+ neural tube keratinized epithelium	+/− cartilage-muscle-like	+ duct	+	+	−
			256H18	+	+	+	1	+ neural tube	+/− cartilage-like	+ duct	+	+	−
			256D4	+	+	+	1	+ neural cell keratinized epithelium	+ muscle cartilage	+ duct	ND	ND	ND
			256D7	+	+	+	1	+ neural tube keratinized epithelium	+ muscle	+ duct	ND	ND	ND
Hep	Nanog	+	135C4	+	+	+	1	+ neural cell	−	+ duct	+	+	−
			135C6	+	+	+	1	+ neural cell	+ cartilage	+ duct	+	+	−
			103C1	+	+	+	1	+ neural cell	+ cartilage	+ duct	+	+	+
			103C2	+	+	+	−				+	+	−
		−	390B1	−	−	−	Failed to form neurospheres				ND	ND	ND
			390B3	−	−	−	Failed to form neurospheres				ND	ND	ND
	Fbx	+	92A3	+	+	+	−				+	+	−
			98A1	+	+	+	−				+	+	−
	w/o	−	238C1	−	−	−					ND	ND	ND
			238C2	+	+	+	1	+ neural cell keratinized epithelium	+ bone	+ duct	ND	ND	ND
Stm	Fbx	+	99-1	+	+	+	−				+	+	+
			99-3	+	+	+	−				+	+	+
			116-5	+	−	−	Failed to form neurospheres				+	+	−
1A2 Nanog-EGFP-RF8-ES			1A2	+	+	+	−						
RF8				+	+	+	−						
EB3				+	+	+	1	+ neural cell	−	−			

[0046] Example 2: Activity of Nanog Promoter

It was examined how many undifferentiated cells are present in the SNSs.

In order to evaluate undifferentiated cells by flow cytometer, MEF-iPS cells, TTF-iPS cells, Hep-iPS cells and ES cells, all of them having Nanog-EGFP reporter, were

used. These iPS cells have been established in various ways in the presence/absence of c-Myc-containing retrovirus, with/without selection in terms of the expression of Nanog, and the like. The SNSs derived from the MEF-iPS cells contained little or no Nanog-EGFP-positive cell irrespective of the presence of the c-Myc-containing retrovirus or the selection method (0 to 0.38%). This result is similar to that of the ES cell. On the other hand, the SNSs derived from the TTF-iPS cells contained a significantly larger number of Nanog-EGFP-positive undifferentiated cells (0.025 to 20.1%) in comparison to the cells derived from the MEF-iPS cells. The SNSs derived from the Hep-iPS cells also contained a larger number of Nanog-EGFP-positive undifferentiated cells (0.034% to 12.0%) in comparison to the cells derived from the MEF-iPS cells (Fig. 2a and Fig. 2b). The contents of the Nanog-EGFP-positive undifferentiated cells in the SNSs did not show a significant difference irrespective of the presence/absence of the c-Myc-containing retrovirus (Fig. 2c) or the Nanog expression selection (Fig. 2d).

[0047] Example 3: Tumor Formation after Transplantation of SNS from Respective iPS Cells

In order to evaluate the SNSs derived from ES cells and iPS cells in vivo, the SNSs derived from each of the clones were transplanted into a corpus striatum of a NOD/SCID mouse in which tumor formation was examined (Fig. 3a and Fig. 3b). Mice which died or weakened after the transplantation were then dissected and healthy mice were dissected in 4 to 45 weeks after the transplantation.

Among 34 mice transplanted with SNSs derived from three ES cell clones, three died or weakened by tumor. The remaining 31 mice were also dissected, among which 30 mice did not have a tumor and only one was found to have a small tumor.

On the other hand, among 100 mice transplanted with SNSs derived from 12 MEF-iPS cell clones, nine died or weakened within 19 weeks after the transplantation. Tumor was found in eight of these nine mice. The remaining mice were also dissected, among which 66 mice did not have a tumor, whereas 25 mice showed tumors of various sizes.

Among 55 mice transplanted with SNSs derived from 11 TTF-iPS cell clones, 46 mice died or weakened within 9 weeks after the transplantation. In the remaining nine mice, no tumor was found.

Among 36 mice transplanted with SNSs derived from 7 Hep-iPS cell clones, 13 mice died or weakened within 17 weeks after the transplantation. Tumor was found in 10 of these 13 mice. The remaining 23 mice were also dissected, none of which had a tumor.

Further, 8 mice were transplanted with SNSs derived from two Stm-iPS cell clones, in none of which a tumor was observed in 16 weeks after the transplantation.

Histological analyses showed that these tumors contained various types of cells of

endodermal, mesodermal or ectodermal origin, such as epithelial cells of striated muscle or tubular structure, cornified epithelial cells, cartilages and neuronal cells (Fig. 4). Further, the tumors contained large number of undifferentiated cells. Therefore, they are considered to be either teratoma or teratocarcinoma (hereinafter collectively referred to as teratoma). Survival of the transplanted cells was also recognized in normal tissues other than the teratomas in the brain.
Statistical analyses of these results indicated that the SNSs derived from the TTF-iPS cells formed significantly larger teratoma than the SNSs derived from the other iPS cells or ES cells (Fig. 5a). Further, the SNSs derived from the TTF-iPS cells and the Hep-iPS cells showed significantly larger ratio of died or weakened mice after transplantation (shown in black in the graph) in comparison to the SNSs derived from the other cells (MEF-iPS cell, Stm-iPS cell or ES cells) (Fig. 5b). The use of c-Myc-containing retrovirus (Fig. 5c) or the Nanog expression selection (Fig. 5d) was not significantly relevant with the size of the tumor. In contrast, a significant correlation was found between the diameter of the teratoma and the content of Nanog-EGFP-positive cells in the SNS (Fig. 5e). Although possible contribution of reactivation of the introduced c-Myc-containing retrovirus to tumor formation had been suspected, no reactivation of c-Myc or other introduced gene in the SNS or the teratoma was observed (Fig. 6; the primers used are shown in Table 5).
[Table 5]

gene name	type	sequence	Seq No
Oct3/4 (endogenous)	sense	TCTTTCCACCAGGCCCCCGGCTC	1
	anti-sense	TGCGGGCGGACATGGGGAGATCC	2
Oct3/4 (exogenous)	sense	TTGGGCTAGAGAAGGATGTGGTTC	3
	anti-sense	TTATCGTCGACCACTGTGCTGCTG	4
c-Myc (endogenous)	sense	TGACCTAACTCGAGGAGGAGCTGGAATC	5
	anti-sense	TTATGCACCAGAGTTTCGAAGCTGTTCG	6
c-Myc (exogenous)	sense	CAGAGGAGGAACGAGCTGAAGCGC	7
	anti-sense	TTATCGTCGACCACTGTGCTGCTG	8
Sox2 (endogenous)	sense	TAGAGCTAGACTCCGGGCGATGA	9
	anti-sense	TTGCCTTAAACAAGACCACGAAA	10
Sox2 (exogenous)	sense	GGTTACCTCTTCCTCCCACTCCAG	11
	anti-sense	TTATCGTCGACCACTGTGCTGCTG	12
Klf4 (endogenous)	sense	CCAACTTGAACATGCCCGGACTT	13
	anti-sense	TCTGCTTAAAGGCATACTTGGGA	14
Klf4 (exogenous)	sense	GCGAACTCACACAGGCGAGAAACC	15
	anti-sense	TTATCGTCGACCACTGTGCTGCTG	16
β-actin	sense	CGTGGGCCGCCCTAGGCACCA	17
	anti-sense	TTGGCCTTAGGGTTCAGGGGG	18

[0048] Example 4: Activity of Nanog Promoter in subclone.
To understand the reason of occurring partial differentiation in one iPS cell clone, subclones were established from 4 iPS cell clones, each of which had different background, and an ES cell line as control (Table 6 to 10).

[Table 6]

iPS line					SNS formation	
Origin	Selection	cMyc	clone's name	sub clone's	GFP+ cells in SNSs (%)	
					1st	2nd
TTF	Nanog	+	212C6	parent	1.592	1.472
				#3	0.015	0.254
				#5	4.551	6.27
				#6	36.754	21.121
				#10	18.899	0.79
				#11	0.038	0.223
				#12	–	16.977
				#13	0.002	0.042
				#14	44.962	38.719
				#17	33.005	20.59
1A2					0	0.001

[Table 7]

iPS line					SNS formation		
Origin	Selection	cMyc	clone's name	sub clone's	GFP+ cells in SNSs (%)		
					1st	2nd	3rd
TTF	w/o	–	256D4	parent	2.64	10.238	35.47
				#1	6.485	–	–
				#2	4.025	–	–
				#3	3.509	–	–
				#4	5.026	–	–
				#5	4.999	–	–
				#6	16.544	–	–
				#7	5.245	–	–
				#8	22.13	–	–
				#9	24.755	–	–
				#10	3.598	–	–
				#32	–	0.004	0.049
				#33	–	0.045	0.079
				#34	–	6.424	8.354
				#36	–	38.339	41.689
				#39	–	12.032	18.794
				#41	–	0.639	3.114
				#45	–	17.782	17.289
				#47	–	0.034	0.013
				#48	–	22.438	1.303
1A2					0.001	0	0.034

[Table 8]

iPS line					SNS formation	
Origin	Selection	cMyc	clone's name	sub clone's	GFP+ cells in SNSs (%)	
					1st	2nd
Hep	Nanog	+	135C6	parent	2.45	0.156
				#13	0.483	0.007
				#26	0.053	0
				#28	0.427	0.038
				#29	0.268	0.005
				#30	8.051	0.351
				#37	11.684	0.658
				#40	10.242	3.519
				#44	8.445	0.79
				#48	1.307	0.024
1A2					0.017	0

[Table 9]

iPS line					SNS formation	
Origin	Selection	cMyc	clone's name	sub clone's	GFP+ cells in SNSs (%)	
					1st	2nd
MEF	Nanog	−	178B5	parent	0	0.005
				#1	0	0.001
				#2	0.002	0
				#3	0	0.003
				#4	0	0.01
				#5	0.009	0
				#6	0	0.001
				#7	0.003	0.001
				#8	0	0.047
				#9	0	0.022
				#10	0.001	0.002
1A2					0	0.034

[Table 10]

ES line		SNS formation	
	sub clone's	GFP+ cells in SNSs (%)	
		1st	2nd
1A2	parent	0	0.015
	#1	0	0.036
	#2	0	0.014
	#3	0	0
	#4	0	0.002
	#5	0	0.001
	#6	0	0.007
	#7	0	0.051
	#8	0.001	0.017
	#9	0.001	0.029
	#10	0	0

Undifferentiated cells in SNSs derived from the subclones of iPS cell lines were evaluated with GFP expression substituted for Nanog expression by using flow cytometer. Then some subclones contained lower content of GFP positive cells but very few subclones contained higher content than the parent cell lines (212C6, 256D4

and 135C6) which were contained a substantial number of undifferentiated cells after formation of SNSs. On the other hand, there were little GFP positive cells in subclones from 178B5 which hardly contained undifferentiated cells after formation of SNSs and here also very few subclones contained higher content than the parent cell line. It has been thus confirmed that the clones have a tendency of reducing the content of GFP-positive cells during passages.

Accordingly since the clones do not gain new potential of resistant against induction of differentiation, the content of the undifferentiated cells in a clone of iPS cells, which is measured at a certain time point, is useful for judging the tumor formation rate that an offspring of the clone inherently has.

[0049] Example 5: Standard value for evaluation of iPS cell lines.

There was a significant correlation between the occurrence of tumorigenesis and the content of Nanog-positive cells in the SNSs; hence the content of the Nanog-positive cells might be useful for prospective evaluation of tumorigenecity by transplantation of the SNSs derived from iPS cells. When a certain control value of percentage of the content is set, the sensitivity and specificity for judgement as catching the tumorigenesis are shown in Table 11.

[Table 11]

iPS line	SNS formation		Transplantation of SNSs	Evaluation by tumor formation	Evaluation by maximum value (control maximum value)				
clone's name	EGFP+ cells in SNSs (%)	Average of EGFP+ cells in SNSs (%)	Teratoma+ / total		(0.082%)	(0.066%)	(0.051%)	(0.019%)	(0.042%)
20D17	0.00363 0.049 0.11 0.019	0.0454075	15/21	+	+	+	+	+	+
38D2	0.026 0.00143 0.00779 0.38	0.103805	13/17	+	+	+	+	+	+
38C2	0 0	0	0/17	-	-	-	-	-	-
178B5	0	0	0/7	-	-	-	-	-	-
178B1	0.0012 0	0.0006	0/6	-	-	-	-	-	-
178B2	0.0012 0	0.0006	0/6	-	-	-	-	-	-
506GN1	0.0052 0	0.0026	0/4	-	-	-	-	-	-
506GN2	0.0719 0.002	0.03695	0/4	-	-	+	+	+	-
506GN3	0.0383 0	0.01915	0/4	-	-	-	-	+	-
506GN4	0.066 0.018	0.042	2/5	+	-	+	+	+	+
506GN5	0.339 0.011	0.175	3/5	+	+	+	+	+	+
506GN6	0.063 0.001	0.032	0/4	-	-	-	+	+	-
212B2	0.6613 0.379	0.52015	6/6	+	+	+	+	+	+
212C5	0.5581 0.24	0.39905	4/4	+	+	+	+	+	+
212C6	4.182 5.063	4.6225	6/6	+	+	+	+	+	+
335D1	0.1593 0.025	0.09215	0/5	-	+	+	+	+	+
335D3	0.01 12.829 0.407	4.41533333	2/5	+	+	+	+	+	+
212D1	2.4327 3.165	2.79885	5/5	+	+	+	+	+	+
212D2	1.6079 19.6292	10.61855	4/4	+	+	+	+	+	+
256D4	20.1087 0.5674	10.33805	7/7	+	+	+	+	+	+
256D7	1.6644 0.0843	0.87435	6/7	+	+	+	+	+	+
135C4	0.12 1.013 0.034	0.389	1/5	+	+	+	+	+	+
135C6	0.975 12 0.968	4.64766667	7/7	+	+	+	+	+	+
238C2	1.625 1.2232 0.607	1.15173333	1/6	+	+	+	+	+	+
1A2	0 0 0.081 0 0 0.007	0.0176	0/19	-	-	+	+	+	-
sensitivity					0.93	1	1	1	1
specificity					0.9	0.7	0.6	0.5	0.9

The high sensitivity shows low degree of false negative of the judgement, and the high specificity shows low degree of false positive of the judgement. The control maximum value was used in the case of comparing the maximum value among results of a

plurality of trials. Similarly, the control average value was used in the case of comparing the average of results of a plurality of trials. The control maximum value of 0.082% was determined by using value of ES cells as a control known not to substantially cause tumor formation. The control maximum value of 0.066% was determined by using value of 506GN4, because the value is the minimum highest value in the case of tumorigenesis. The control maximum value of 0.051% was determined by using value of 1A2 subclone case (shown in Table 10) as a control known not to substantially cause tumor formation. The control maximum value of 0.019% was useful, because a significant correlation was found between the diameter of the teratoma and the SNS with the content of Nanog-EGFP-positive cells more than 0.019% (Fig. 5e). The control average value of 0.042% was determined by using value of 506GN4, because the value is the lowest maximum value in the case of tumorigenesis. It should be noted that, in Table 11, "+" shows tumorigenesis for evaluation by tumor formation and over the control value for evaluation by maximum value, and "-" shows no tumorigenesis and under the control value. There were some false judgments for evaluating tumorigenesis by using the control maximum value, but the reliability of the judgment is very high.
To summarize, an induction of differentiation into neural cells in vitro has been confirmed to be a sensitive method for evaluating iPS cell clones and subclones.

Claims

[Claim 1] A method for selecting a clone from clones of induced pluripotent stem cells (iPS cells), comprising the steps of:
detecting an undifferentiated cell in the clones after induction of differentiation of the iPS cells in the clones, and
selecting the clone based on the result of the detection.

[Claim 2] The method according to Claim 1, wherein the detection step comprises examining whether the cells after the induction of differentiation form teratoma when transplanted into a living body.

[Claim 3] The method according to Claim 1, wherein the detection step comprises detecting promoter activity of an undifferentiated cell-specific gene in the cells after the induction of differentiation.

[Claim 4] The method according to Claim 3, wherein the detection step comprises measuring a content of the cells detected as having the promoter activity in the clone of the cells.

[Claim 5] The method according to any one of Claims 1 to 4, wherein the induction of differentiation comprises allowing the cells to form a primary neurosphere or a secondary neurosphere.

[Claim 6] The method according to Claim 4, wherein a clone is selected if the content of the detected cells in the clone is below a content of cells with the promoter activity in a control clone, the control clone being a clone of iPS cells or embryonic stem cells known not to substantially cause tumor formation when transplanted into a living body.

[Claim 7] The method according to Claim 4, wherein a clone is selected if the content of the detected cells in the clone of the cells is less than 0.042% in average.

[Claim 8] The method according to Claim 4, wherein a clone is selected if the content of the detected cells in the clone is less than 0.066% in all generation examined.

[Claim 9] The method according to any one of Claims 3, 4 and 6 to 8, wherein the detection of the promoter activity comprises detecting expression of a marker gene, the expression being regulated by the promoter of the undifferentiated cell-specific gene.

[Claim 10] The method according to Claim 9, wherein the marker gene encodes a fluorescent protein, a luminescent protein or an enzyme.

[Claim 11] The method according to any one of Claims 3, 4 and 6 to 8, wherein the detection of the promoter activity comprises detecting expression of an

endogenous undifferentiated cell-specific gene.

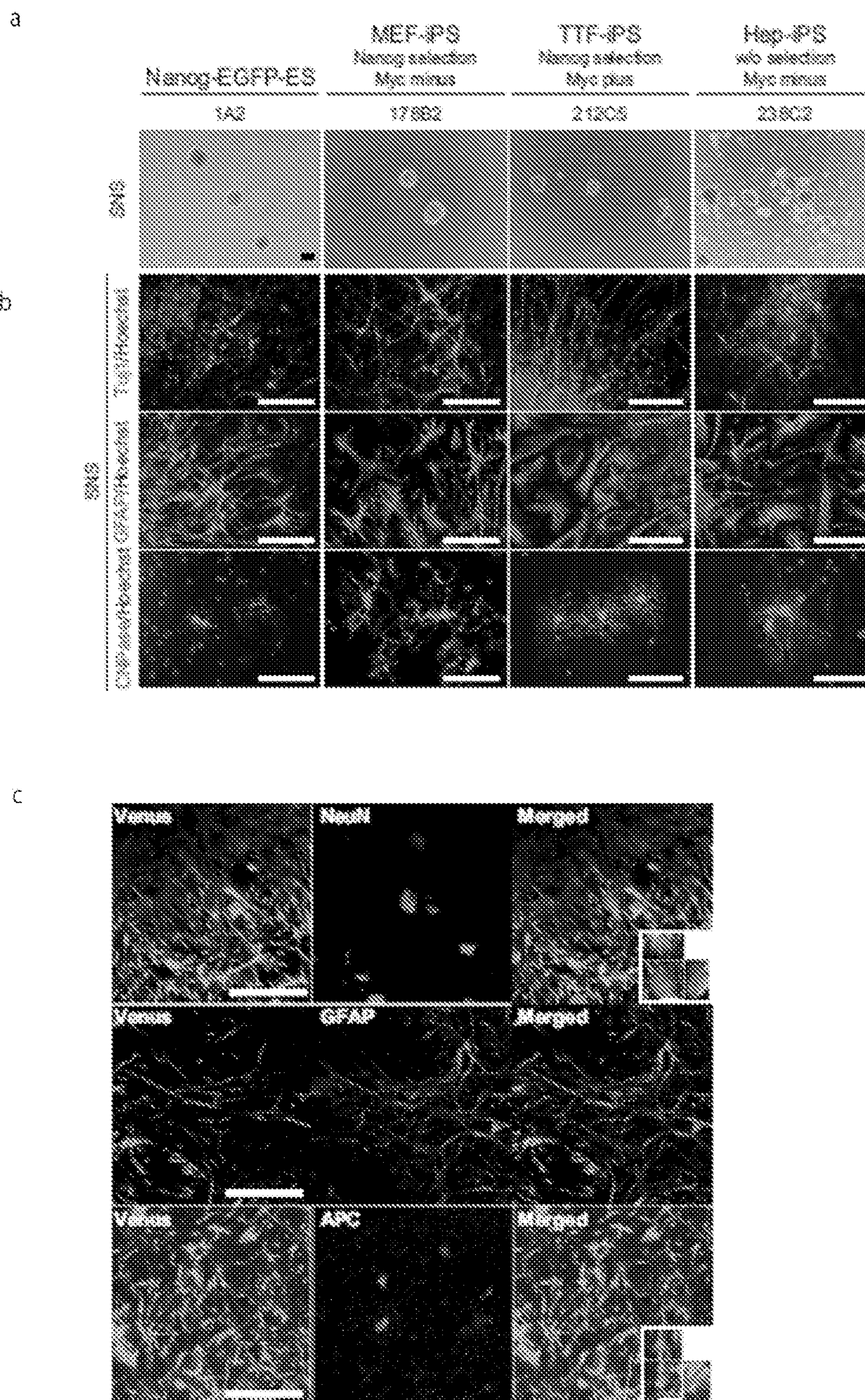
[Claim 12] The method according to any one of Claims 3 to 11, wherein the undifferentiated cell-specific gene is Nanog gene.

[Claim 13] The method for producing a clone of iPS cells, comprising the steps of:
(1)introducing at least one gene selected from the group consisting of Oct gene family, Sox gene family, Klf gene family, Myc gene family, Nanog gene, Sall gene family and Lin gene family into a somatic cell and producing iPS cells;
(2)establishing a plurality of clones of the iPS cells produced at step (1); and
(3)selecting a clone by the method of claim 1 to 12.

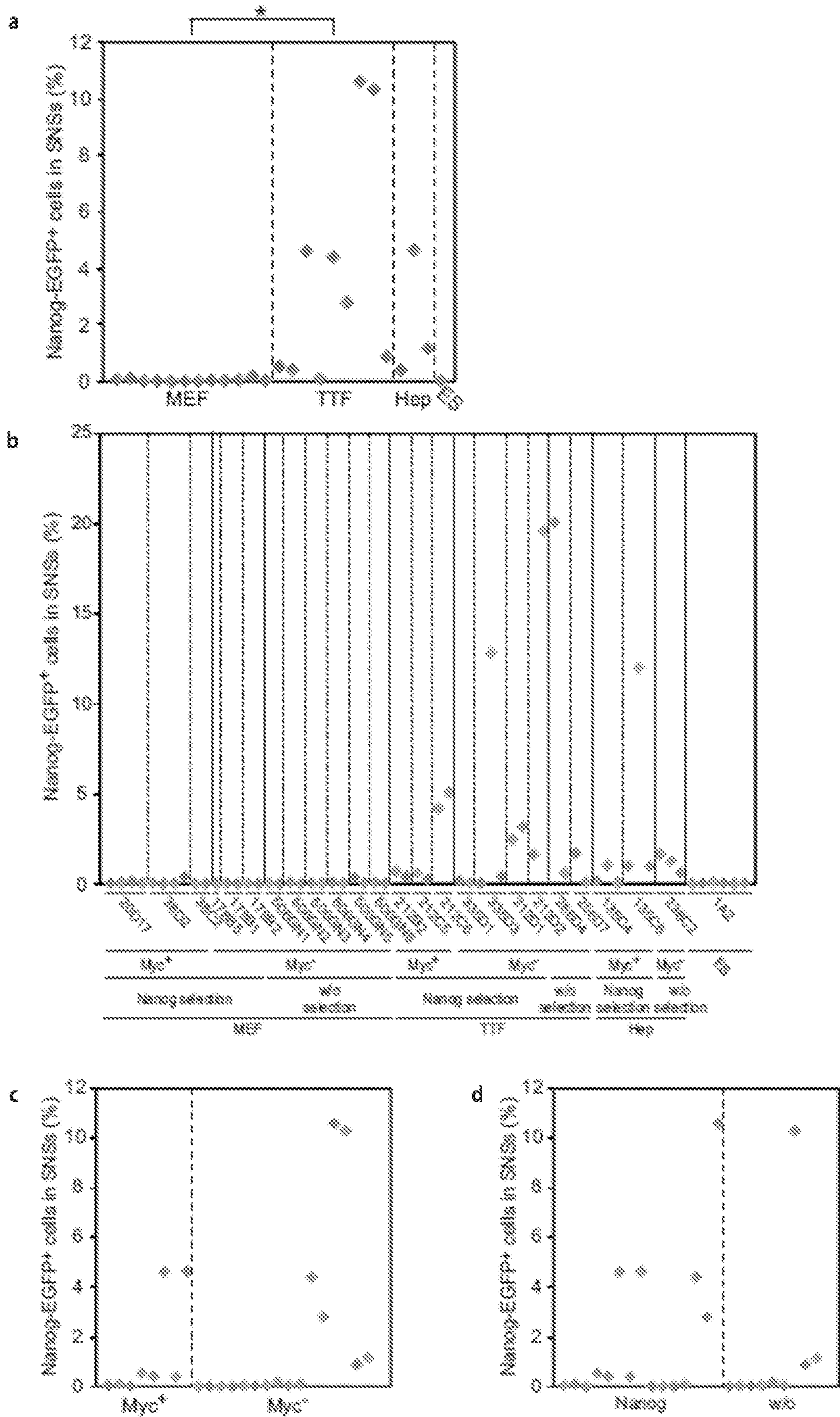
[Claim 14] The method according to Claim 13, wherein the introduced gene at step (1) is selected from the group consisting of Oct3/4 gene, Sox2 gene, Klf4 gene, c-myc gene, L-Myc gene, Nanog gene, Sall4, Sall1 gene and Lin28 gene.

[Claim 15] The method according to Claim 13 or 14, wherein the introduced gene at step (1) is selected from the group consisting of Oct3/4 gene, Sox2 gene, Klf4 gene and c-myc gene.

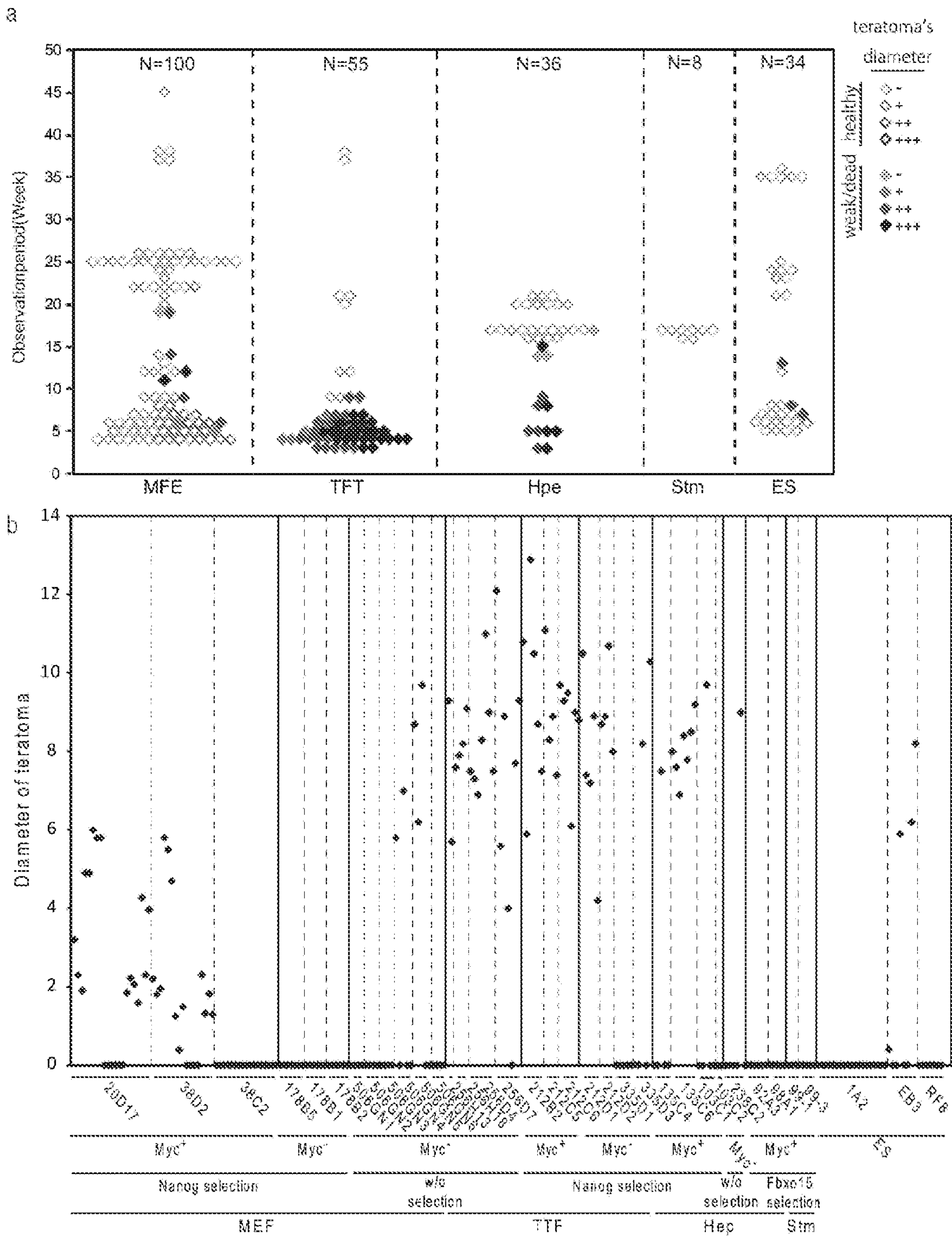
[Fig. 1]



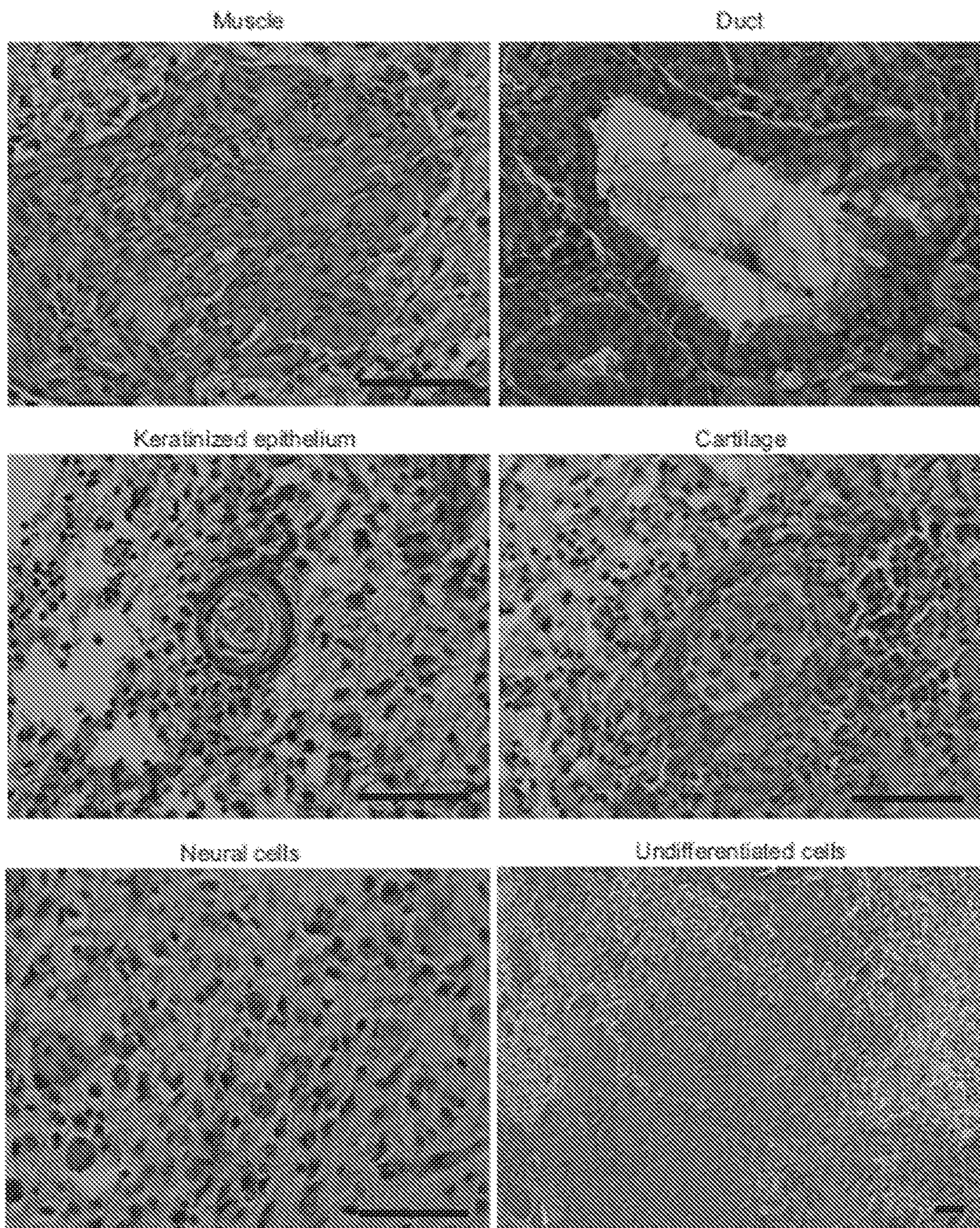
[Fig. 2]



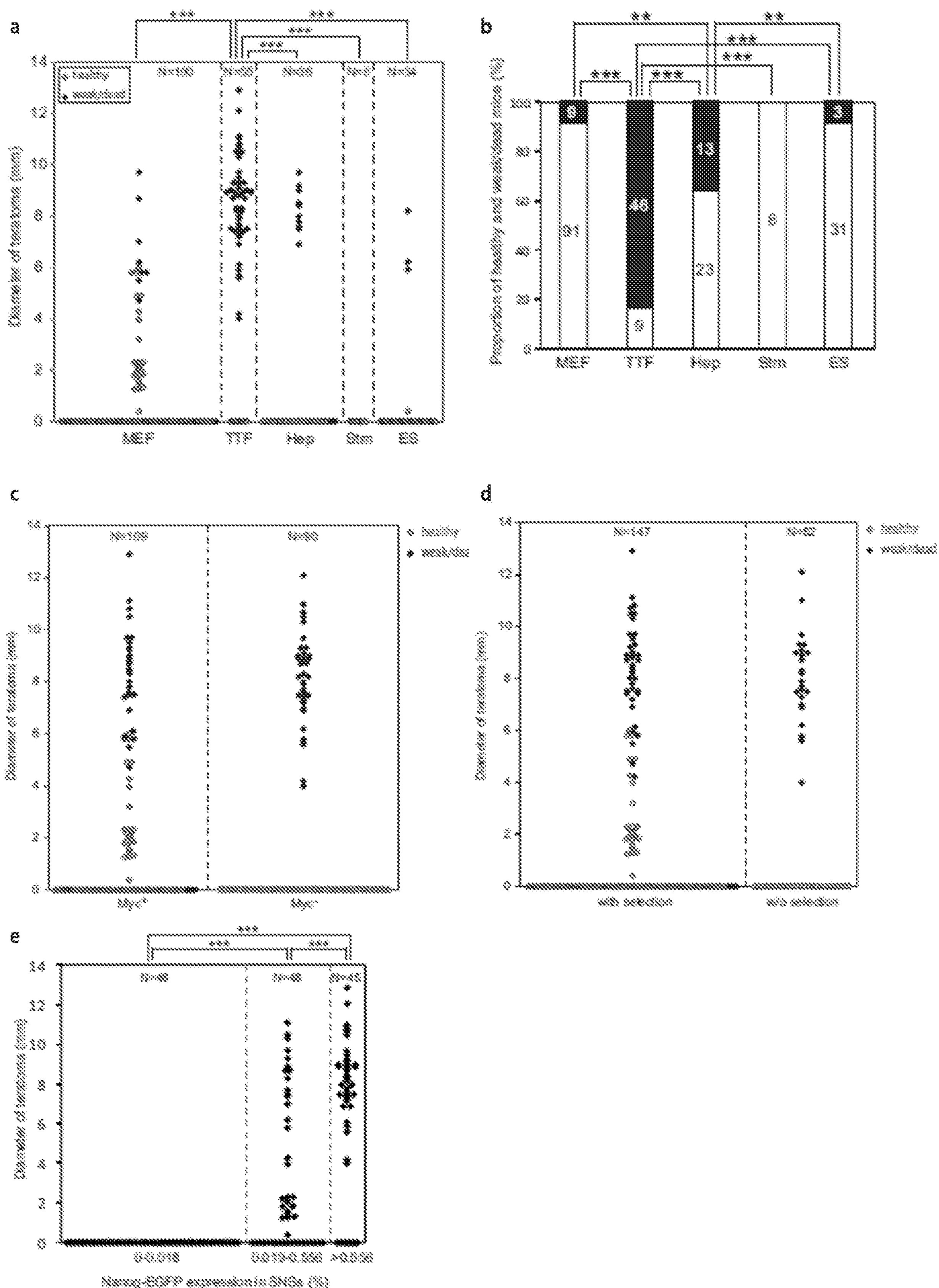
[Fig. 3]



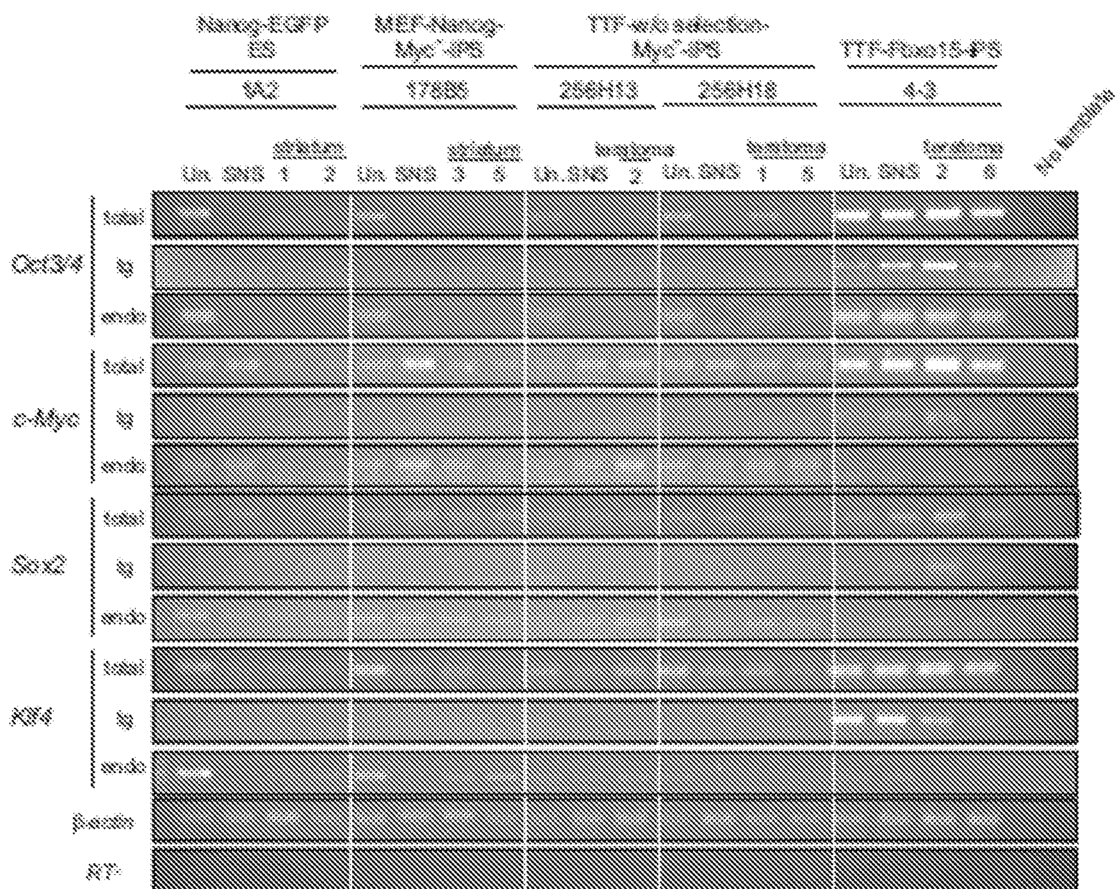
[Fig. 4]



[Fig. 5]



[Fig. 6]



INTERNATIONAL SEARCH REPORT

International application No. PCT/JP2010/003620

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N5/0735(2010.01)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. 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 1971-2010
Registered utility model specifications of Japan 1996-2010
Published registered utility model applications of Japan 1994-2010

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), 医学・薬学予稿集全文データベース

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/ Y	YAMANAKA,S.,A fresh look at iPS cells. Cell. 2009 Apr 3, vol.137(1), pp.13-17	1,2,13-15/ 3-12
Y	EIGES,R et al., Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. Curr Biol. 2001 Apr 3, vol.11(7), pp.514-518	3-15
Y	OKADA,Y et al., Spatiotemporal recapitulation of central nervous system development by murine embryonic stem cell-derived neural stem/progenitor cells. Stem Cells. 2008 Dec, vol.26(12), pp.3086-3098	5,12-15

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

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Date of the actual completion of the international search	Date of mailing of the international search report
17.08.2010	NAGAI KEIKO 24.08.2010

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/003620

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OKITA,K et al., Generation of germline-competent induced pluripotent stem cells. Nature. 2007 Jul 19, vol.448(7151), pp.313-317	12-15
A	HENTZE,H et al., Cell therapy and the safety of embryonic stem cell-derived grafts. Trends Biotechnol. 2007 Jan, vol.25(1), pp.24-32	1-15
A	OSAFUNE,K et al., Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotechnol. 2008 Mar, vol.26(3), pp.313-315	1-15
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P,A	AOI,T. Quality variation among mouse induced pluripotent stem cells. 27th Annual Meeting of the Japan Human Cell Society. 2009 Nov, A4, #S1-4	1-15