DESCRIPTION

METHOD OF EFFICIENTLY ESTABLISHING INDUCED PLURIPOTENT STEM CELLS

Technical Field

The present invention relates to a method of improving the efficiency of establishment of induced pluripotent stem cells (hereinafter referred to as iPS cells) and reagents therefor, more specifically to a method of improving the efficiency of establishment of iPS cells using members of the GLIS family and members of the Klf family, and reagents therefor and the like.

Background of the Invention

In recent years, mouse and human iPS cells have been established one after another. Takahashi and Yamanaka induced iPS cells by transferring the Oct3/4, Sox2, Klf4 and c-Myc genes into fibroblasts from a reporter mouse wherein the neomycin resistance gene is knocked-in into the Fbx15 locus, and forcing the cells to express the genes [Takahashi, K. and Yamanaka, S., Cell, 126: 663-676 (2006)]. Okita et al. succeeded in establishing iPS cells (Nanog iPS cells) that show almost the same gene expression and epigenetic modification profiles as those of embryonic stem (ES) cells, by creating a transgenic mouse having the green fluorescent protein (GFP) and puromycin resistance genes integrated into the locus of Nanog, whose expression is more localized in pluripotent cells than the expression of Fbx15, forcing fibroblasts from the mouse to express the above-mentioned four genes, and selecting cells that are puromycin-resistant and GFP-positive cells [Okita, K. et al., Nature, 448: 313-317 (2007)]. Similar results were obtained by other groups [Wernig, M. et al., Nature, 448: 318-324 (2007); Maherali, N. et al., Cell Stem Cell, 1: 55-70 (2007)]. Thereafter, it was revealed that iPS cells could also be produced with 3 factors other than the c-Myc gene [Nakagawa, M. et al., *Nat. Biotethnol.*,

26: 101-106 (2008)].

Furthermore, Takahashi et al. [Takahashi, K. et al., *Cell, 131*: 861-872 (2007)] succeeded in establishing iPS cells by introducing the same 4 genes as those used in the mouse into human skin fibroblasts. On the other hand, Yu et al. produced human iPS cells using Nanog and Lin28 in place of Klf4 and c-Myc [Yu, J. et al., Science, 318: 1917-1920 (2007)]. Hence, it has been demonstrated that iPS cells comparable to ES cells in terms of pluripotency can be produced in both humans and mice, by transferring defined factors into somatic cells.

Since then, a wide variety of attempts have been made to increase the efficiency of iPS cell establishment, including iPS cells established by transferring TERT and SV40 large T antigen (known as a human cell immortalization genes), along with the four factors Oct3/4, Sox2, Klf4 and c-Myc [Park, I.H. et al., *Nature, 451*: 141-146 (2008)], iPS cells established with the addition of Nanog and Lin28 to the foregoing four factors [Liao, J. et al., *Cell Research, 18*: 600-603 (2008)], and iPS cells established with the addition of UTF1 to the foregoing four or three factors other than c-Myc [Zhao, Y. et al., *Cell Stem Cell, 3*: 475-479 (2008)]. However, the situation stands wherein no satisfactory improvement has been achieved.

Summary of the Invention

The present inventors conducted a comprehensive investigation in search of genes that can be used to establish iPS cells, as substitutes for Klf4, not only out of genes expressed specifically in pluripotent cells such as ES cells, but also from a broader range of gene libraries of transcription factors. The inventors thus succeeded in efficiently establishing iPS cells by transferring a gene belonging to the GLIS family (e.g., GLIS1), a gene belonging to the PTX family (e.g., PITX2), or the DMRT-like family B with proline-rich C-terminal 1 gene (DMRTB1), along with the

three genes Oct3/4, Sox2 and c-Myc, to mouse and human dermal fibroblasts, and identified these transcription factors as novel nuclear reprogramming substances capable of functionally substituting for Klf4 (US Provisional Application No. 61/208,853, filed on February 27, 2009 and US Provisional Application No. 61/276,123, filed on September 8, 2009).

Next, the present inventors investigated the effects of these Klf4 substitute factors GLIS1, PITX2 and DMRTB1 used in combination with Klf4 on the establishment of iPS cells. As an unexpected result, PITX2 and DMRTB1 exhibited absolutely no additional effect when combined with Klf4, whereas combined use of GLIS1 and Klf4 produced a dramatic synergistic effect on the establishment of iPS cells in both mouse and human cells. The present inventors conducted further investigations based on these findings, and have developed the present invention.

Accordingly, the present invention provides the following:

[1] A method of improving iPS cell establishment efficiency, comprising contacting the following (1) and (2):

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same,

with a somatic cell.

[2] The method according to [1] above, wherein the substances (1) above include GLIS family zinc finger 1 (GLIS1) or a nucleic acid that encodes the GLIS1.

[3] The method according to [1] or [2] above, wherein the substances (2) above include Klf4 or a nucleic acid that encodes the Klf4.

[4] An iPS cell establishment efficiency improver comprising the following (1) and (2):

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same.

[5] The improver according to [4] above, wherein the substances (1) above include GLIS1 or a nucleic acid that encodes the GLIS1.

[6] The improver according to [4] or [5] above, wherein the substances (2) above include Klf4 or a nucleic acid that encodes the Klf4.

[7] A method of producing an iPS cell, comprising contacting the following (1), (2) and (3):

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same,

(3) a nuclear reprogramming substance capable of inducing an iPS cell from a somatic cell by being combined with the substances (1) and (2) above,

with a somatic cell.

[8] The method according to [7] above, wherein the substances (1) above include GLIS1 or a nucleic acid that encodes the GLIS1.

[9] The method according to [7] or [8] above, wherein the substances (2) above include Klf4 or a nucleic acid that encodes the Klf4.

[10] The method according to any one of [7] to [9] above, wherein the nuclear reprogramming substance (3) above is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Myc family, members of the Lin28 family, Nanog, and nucleic acids that

encode the same.

[11] The method according to any one of [7] to [9] above, wherein the nuclear reprogramming substance (3) above includes Oct3/4 or a nucleic acid that encodes the same.

[12] The method according to [11] above, wherein the nuclear reprogramming substance (3) above includes Oct3/4 and Sox2 or nucleic acids that encode the same.

[13] The method according to [11] above, wherein the nuclear reprogramming substance (3) above includes Oct3/4, Sox2 and c-Myc or nucleic acids that encode the same.

[14] An agent for iPS cell induction from a somatic cell, comprising the following (1), (2) and (3):

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same,

(3) a nuclear reprogramming substance capable of inducing an iPS cell from a somatic cell by being combined with the substances (1) and (2) above.

[15] The agent according to [14] above, wherein the substances (1) above include GLIS1 or a nucleic acid that encodes the GLIS1.

[16] The agent according to [14] or [15] above, wherein the substances (2) above include Klf4 or a nucleic acid that encodes the Klf4.

[17] The agent according to any one of [14] to [16] above, wherein the nuclear reprogramming substance (3) above is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Myc family, members of the Lin28 family, Nanog, and nucleic acids that encode the same.

[18] The agent according to any one of [14] to [16] above, wherein the nuclear reprogramming substance (3) above includes

Oct3/4 or a nucleic acid that encodes the same.

[19] The agent according to [18] above, wherein the nuclear reprogramming substance (3) above includes Oct3/4 and Sox2 or nucleic acids that encode the same.

[20] The agent according to [18] above, wherein the nuclear reprogramming substance (3) above includes Oct3/4, Sox2 and c-Myc or nucleic acids that encode the same.

[21] An iPS cell comprising the following (1) and (2):

(1) one or more nucleic acids selected from the group consisting of exogenous nucleic acids that encode members of the GLIS family,

(2) one or more nucleic acids selected from the group consisting of exogenous nucleic acids that encode members of the Klf family.

[22] The iPS cell according to [21] above, wherein the exogenous nucleic acids are integrated in a genome.

[23] A method of producing a somatic cell, comprising treating the iPS cell according to [21] or [22] above to induce it to differentiate into a somatic cell.

[24] A method of producing a somatic cell, comprising the following (1) and (2):

(1) the step of producing an iPS cell by the method according to any one of [7] to [13] above, and

(2) the step of treating the iPS cell obtained through the step (1) above to induce it to differentiate into a somatic cell.

[25] A use of the following (1) and (2) to improve the efficiency of establishment of iPS cells:

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same.

[26] A use of one or more substances selected from the group

consisting of members of the GLIS family and nucleic acids that encode the same to improve the efficiency of establishment of iPS cells, wherein the substances, along with one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same, are contacted with a somatic cell.

[27] A use of the following (1), (2) and (3) to produce an iPS cell:

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same,

(3) a nuclear reprogramming substance capable of inducing an iPS cell from a somatic cell by being combined with the substances (1) and (2) above.

[28] A use of the following (1) and (2) to produce an iPS cell:

(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same,

(2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same, wherein the factors, along with a nuclear reprogramming substance capable of inducing an iPS cell from a somatic cell by being combined with the substances (1) and (2) above, are contacted with a somatic cell.

[29] A use of (1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same to produce an iPS cell, wherein the substances, along with (2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same, and a nuclear reprogramming substance capable of inducing an iPS cell from a somatic cell

by being combined with the substances (1) and (2) above, are contacted with a somatic cell.

[30] A use of the iPS cell according to [21] or [22] above in producing a somatic cell.

[31] The iPS cell according to [21] or [22] above, wherein the iPS cell serves as a source of cell in producing a somatic cell.

The iPS cell establishment efficiency improver of the present invention is capable of remarkably improving the efficiency of establishment of an iPS cell from a somatic cell, as stated above, and is therefore useful in, for example, applications to human transplantation medicine by autologous transplantation.

Brief Description of the Drawings

Fig. 1 is a schematic diagram showing the steps for narrowing down entry clones by function from human Gateway® entry clones (N. Goshima et al., Nature methods, 2008).

Fig. 2 outlines the procedures used to prepare a transcription factor library for screening for somatic cell reprogramming factor from an entry clone of transcription factor.

Fig. 3 is a photographic representation of the morphology of GFP-positive colonies obtained by transferring a total of 4 different genes, i.e., 3 genes (Oct3/4, Sox2, c-Myc) and G06 (gene code name: GLIS1), H08 (gene code name: DMRTB1) or H10 (gene code name: PITX2), into Nanog-GFP mouse dermal fibroblasts by means of retrovirus. "Klf-G6-1" indicates an iPS cell clone obtained by transferring G06 (gene code name: GLIS1) along with the 3 genes; "Klf-H8-2" indicates an iPS cell clone obtained by transferring H08 (gene code name: DMRTB1) along with the 3 genes; "Klf-H10-1" and "Klf-H10" indicate iPS cell clones obtained by transferring H10 (gene code name: PITX2) along with the 3 genes. P0 shows photographs

taken at the time of colony establishment; P1 shows photographs for the 1st generation (24 wells); P2 shows photographs for the 2nd generation (6 wells). For each set of three photographs, the left panel shows an image of GFP-positive colonies, the central panel shows a phase-contrast image, and the right panel shows a superposed photograph of the GFP-positive colony image and the phase-contrast image. Only Klf-H10-1 was established by the Reseed method, whereas the others were established by the MSTO method.

Fig. 4 is a photographic representation of the morphology of GFP-positive colonies obtained by transferring a total of 4 different genes, i.e., 3 genes (Oct3/4, Sox2, c-Myc) and F09 (gene code name: IRX6), G06 (gene code name: GLIS1), H08 (gene code name: DMRTB1) or H10 (gene code name: PITX2), into Nanog-GFP mouse dermal fibroblasts by means of retrovirus, as of the time of establishment of the colonies. "Klf-F9" indicates an iPS cell clone obtained by transferring F09 (gene code name: IRX6) along with the 3 genes; "Klf-G6-1" and "Klf-G6-2" indicate iPS cell clones obtained by transferring G06 (gene code name: GLIS1) along with the 3 genes; "Klf-H8-1" and "Klf-H8-2" indicate iPS cell clones obtained by transferring H08 (gene code name: DMRTB1) along with the 3 genes; "Klf-H10" indicates an iPS cell clone obtained by transferring H10 (gene code name: PITX2) along with the 3 genes. "Reseed" shows the results obtained by the Reseed method; "MSTO" shows the results obtained by the MSTO method.

Fig. 5 is a photographic representation of the results of genomic-PCR on the G6-1 (Klf-G6-1), H8-2 (Klf-H8-2) and H10 (Klf-H10) iPS cell clones, wherein "skin" indicates the fibroblast used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene integrated into pMXs.

Fig. 6 is a photographic representation of the results of genomic-PCR on an H10 (Klf-H10) iPS cell clone other than that shown in Figure 5. In Figure 6, "skin" indicates the

fibroblast used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene integrated into pMXs.

Fig. 7 is a photographic representation of the results of RT-PCR on the G6-1 (Klf-G6-1), H8-2 (Klf-H8-2) and H10 (Klf-H10) iPS cell clones, wherein "skin" indicates the fibroblast used as a source of somatic cells; "ES" and "iPS" indicate mouse ES cells and iPS cells; "Sox2 RT-" is a negative control.

Fig. 8 is a photographic representation of the results of RT-PCR on an H10 (Klf-H10) iPS cell clone other than that in Figure 7. In this figure, "skin" indicates the fibroblast used as a source of somatic cells; "ES" and "iPS" indicate mouse ES cells and iPS cells; "Sox2 RT-" is a negative control.

Fig. 9 is a graphic representation of the results of counting colonies of iPS cells (GFP-positive cells) established by transferring a combination of 2 factors (Oct3/4, Sox2) or 3 factors (Oct3/4, Sox2, Klf4) with G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2), into Nanog-GFP mouse dermal fibroblasts. The results of three (four for the control only) independent experiments are summarized.

Fig. 10 shows the number of Nanog-GFP-positive colonies from indicated factor-transduced skin fibroblasts 22 days after infection.

Fig. 11 shows the ratio of Nanog-GFP-positive colonies from indicated factor-transduced skin fibroblasts 22 days after infection. The graph shows the mean of three independent experiments with standard deviation (error bar). **: $p<0.01$

Fig. 12 shows Nanog-GFP-positive colonies from skin fibroblasts (P0; passage 0). Fluorescent images (left); Phase-contrast images (middle); Marged images (right)

Fig. 13 shows the number of Nanog-GFP-positive colonies from the indicated factor-transduced MEFs 20 days after infection. After 3 days of infection, fibroblasts were reseeded on feeder cells.

Fig. 14 shows the ratio of Nanog-GFP-positive colonies

from indicated factor-transduced MEFs 20 days after infection. The graph represents the mean of three independent experiments with standard deviation (error bar). **: $p<0.01$

Fig. 15 shows Nanog-GFP-positive colonies from MEFs (P0; passage 0). Fluorescent images (left); Phase-contrast images (middle); Marged images (right)

Fig. 16 is a graphic representation of the results of counting colonies of iPS cells (ES-like cells) established by transferring a combination of 3 factors (Oct3/4, Sox2, c-Myc) with Klf4 and/or G6 (GLIS1) into adult human dermal fibroblasts (HDF), wherein "104" and "105" indicate the results for $5x10^4$ cells/100 mm dish reseeded onto feeder cells, and for $5x10^5$ cells/100 mm dish, respectively. The results of three independent experiments are summarized.

Fig. 17 is a graphic representation of the results of counting colonies of non-iPS cells (non-ES-like cells) established by transferring a combination of 3 factors (Oct3/4, Sox2, c-Myc) with Klf4 and/or G6 (GLIS1) into adult human dermal fibroblasts (HDF), wherein "104" and "105" indicate the results for $5x10^4$ cells/100 mm dish reseeded onto feeder cells, and for $5x10^5$ cells/100 mm dish, respectively. The results of three independent experiments are summarized.

Fig. 18 is a photographic representation of phase-contrast images of iPS colonies (ES-like colonies) established with Oct3/4, Sox2, c-Myc, Klf4 and G6.

Fig. 19 shows the number of ESC-like colonies from indicated factor-transduced human dermal fibroblasts (upper: 5×10^4 cells, lower: 5×10^5 cells) approximately 30 days after infection.

Fig. 20 shows the ratio of ESC-like colonies from indicated factor-transduced human dermal fibroblasts (upper: 5×10^4 cells, lower: 5×10^5 cells) approximately 30 days after infection. The graphs show the mean of three independent experiments with standard deviation (error bar). *: $p<0.05$; **: $p<0.01$

Fig. 21 shows human ESC-like colonies generated by OSK + GLIS1.

Fig. 22 shows the genomic-PCR analyses of transduced genes in established human iPS clones. AHDF: adult human dermal fibroblast

Fig. 23 shows the RT-PCR analyses of ESC-marker genes in human iPSCs generated by OSK + GLIS1. AHDF: adult human dermal fibroblast; 201B7: human iPS clone generated by OSKM

Fig. 24 shows scatter plots comparing global gene expression between iPSCs generated with OSK + GLIS1 and adult HDFs (upper), and between OSK + GLIS1-transduced iPSCs and OSKM-transduced iPSCs (lower), as determined by DNA microarray. The correlation coefficient (R^2) was calculated.

Fig. 25 shows teratoma formation of human iPSCs generated with OSK + GLIS1.

Fig. 26 shows the expression of GLIS1 in various mouse tissues. The total RNA isolated from each mouse tissue was examined by quantitative RT-PCR. The graph shows the mean of four independent experiments with standard deviation (error bar).

Fig. 27 shows the quantitative RT-PCR analyses of endogenous GLIS1 in skin fibroblasts exposed to GLIS1 shRNAs. The graph represents the mean of two independent experiments with average error (error bar).

Fig. 28 shows the effect of GLIS1 shRNAs on iPSC establishment efficiency by 3 reprogramming factors (OSK). Four weeks after transduction of OSK into skin fibroblasts with or without GLIS1 shRNA, the numbers of Nanog-GFP-positive colonies were counted.

Detailed Description of the Invention

The present invention provides a method of improving the efficiency of establishment of iPS cells, comprising contacting (1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode

the same, and (2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same (hereinafter also referred to as establishment efficiency improving factors of the present invention), with the somatic cell, in the step of nuclear reprogramming of a somatic cell. Because the somatic cell nuclear reprogramming is achieved by contacting a nuclear reprogramming substance with a somatic cell, the present invention also provides a method of producing an iPS cell, comprising contacting (3) a nuclear reprogramming substance capable of inducing an iPS cell from a somatic cell by being combined with the substances (1) and (2) above (hereinafter also simply referred to as a nuclear reprogramming substance), along with the substances (1) and (2) above, with a somatic cell. Herein, a case wherein iPS cells cannot be established with the substance (3) above (nuclear reprogramming substance) alone, but can be established when the nuclear reprogramming substance, along with iPS cell establishment efficiency improving factor of the present invention, is contacted with a somatic cell, is also deemed "an improvement of the efficiency of establishment".

(a) Sources of somatic cells

Any cells other than germ cells of mammalian origin (e.g., humans, mice, monkeys, bovines, pigs, rats, dogs etc.) can be used as starting material for the production of iPS cells in the present invention. Examples include keratinizing epithelial cells (e.g., keratinized epidermal cells), mucosal epithelial cells (e.g., epithelial cells of the superficial layer of tongue), exocrine gland epithelial cells (e.g., mammary gland cells), hormone-secreting cells (e.g., adrenomedullary cells), cells for metabolism or storage (e.g., liver cells), intimal epithelial cells constituting interfaces (e.g., type I alveolar cells), intimal epithelial cells of the obturator canal (e.g., vascular endothelial cells), cells having cilia with transporting capability (e.g., airway

epithelial cells), cells for extracellular matrix secretion (e.g., fibroblasts), constrictive cells (e.g., smooth muscle cells), cells of the blood and the immune system (e.g., T lymphocytes), sense-related cells (e.g., bacillary cells), autonomic nervous system neurons (e.g., cholinergic neurons), sustentacular cells of sensory organs and peripheral neurons (e.g., satellite cells), nerve cells and glia cells of the central nervous system (e.g., astroglia cells), pigment cells (e.g., retinal pigment epithelial cells), progenitor cells (e.g., tissue progenitor cells) thereof and the like. There is no limitation on the degree of cell differentiation, the age of an animal from which cells are collected and the like; even undifferentiated progenitor cells (including somatic stem cells) and finally differentiated mature cells can be used alike as sources of somatic cells in the present invention. Examples of undifferentiated progenitor cells include tissue stem cells (somatic stem cells) such as nerve stem cells, hematopoietic stem cells, mesenchymal stem cells, and dental pulp stem cells.

The choice of mammal individual as a source of somatic cells is not particularly limited; however, when the iPS cells obtained are to be used for regenerative medicine in humans, it is particularly preferable, from the viewpoint of prevention of graft rejection, to collect the somatic cells from a patient or another person with the same or substantially the same HLA type as that of the patient. "Substantially the same HLA type" as used herein means that the HLA type of donor matches with that of patient to the extent that the transplanted cells, which have been obtained by inducing differentiation of iPS cells derived from the donor's somatic cells, can be engrafted when they are transplanted to the patient with use of immunosuppressant and the like. For example, it includes an HLA type wherein major HLAs (e.g., the three major loci of HLA-A, HLA-B and HLA-DR) are identical (hereinafter the same meaning shall apply) and

the like. When the iPS cells obtained are not to be administered (transplanted) to a human, but used as, for example, a source of cells for screening for evaluating a patient's drug susceptibility or adverse reactions, it is likewise desired to collect the somatic cells from the patient or another person with the same genetic polymorphism correlating with the drug susceptibility or adverse reactions.

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

(b) iPS cell establishment efficiency improving factors of the present invention

In the present invention, the GLIS family is a Kruppel-like zinc finger family having five C2H2 (Cys_2-His_2-type) Zinc finger regions, which was named after its similarity to Gli transcription factors [Glis= Gli similar, Kim, Y.S. et al., *J. Biol. Chem.*, *277*(34), 30901-30913 (2002)]. The GLIS family is membered by transcription factors that positively or negatively control the expression of various genes in the process of embryogenesis. Examples of members of this gene family include, but are not limited to, GLIS family zinc

finger 1 (GLIS1), GLIS2, GLIS3 and the like, with preference given to GLIS1. Note that GLIS1 is a gene not expressed in mouse ES cells.

Although the members of the GLIS family used in the present invention may be proteins derived from cells or tissues [e.g., cells or tissues of thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas or prostate, corresponding precursor cells, stem cells or cancer cells thereof, and the like] of optionally chosen mammals (e.g., humans, mice, rats, monkeys, bovines, horses, pigs, dogs and the like) or nucleic acids that encode the same, preference is given to those derived from a human or mouse cell or tissue.

Information on the amino acid sequences and cDNA sequences of members of the GLIS family of human and mouse origin can be acquired with reference to the NCBI accession numbers shown in Table 1. Those skilled in the art are easily able to isolate nucleic acids that encode the respective proteins on the basis of the cDNA sequence information, and to produce recombinant proteins as required.

Table 1

Gene code name	Humans		Mice	
	cDNA	Protein	cDNA	Protein
GLIS1	NM_147193 (SEQ ID NO:1)	NP_671726 (SEQ ID NO:2)	NM_147221 (SEQ ID NO:3)	NP_671754 (SEQ ID NO:4)
GLIS2	NM_032575	NP_115964	NM_031184	NP_112461
GLIS3	NM_001042413	NP_001035878	NM_175459	NP_780668

A natural or artificial mutant protein having an identity of 90% or more, preferably 95% or more, more preferably 98% or more, particularly preferably 99% or more, to each amino acid sequence shown above, and possessing an iPS cell establishment efficiency improving effect equivalent to that of the wild-type protein, and a nucleic acid that encodes the same, can also be utilized as an iPS cell establishment efficiency improving factor of the present invention. Here, the effect in

improving the efficiency of establishment of iPS cells can be verified by comparing the number of emerging iPS cell colonies between a case wherein only specified reprogramming factors (e.g., the 2 factors Oct3/4 and Sox, the 3 factors consisting of the 2 factors and c-Myc, and the like) are transferred to the somatic cell, and a case wherein in addition to transferring the reprogramming factors, an iPS cell establishment efficiency improving factor of the present invention is contacted with the somatic cell.

Regarding the members of the GLIS family of the present invention and nucleic acids that encode the same, any one of the factors belonging to the family may be used alone, and two or more may be used in combination.

The Klf (Krüppel-like factor) family is membered by transcription factors that control various biological processes such as proliferation, differentiation, genesis, and apoptosis [McConnell, B.B. et al., *Bioassays, 29*: 549-557 (2007)], but their functions remain to be clarified in detail. Examples of members of this gene family include, but are not limited to, Klf1, Klf2, Klf4, Klf5 and the like, with preference given to Klf4. As stated above, the GLIS family has five C2H2 type Zinc finger regions, whereas the Klf family has three C2H2 type Zinc finger regions.

Yamanaka et al. hypothesized that the same four genes (Oct3/4, Sox2, Klf4 and c-Myc) could be substituted by other genes belonging to the same respective families, and showed that iPS cells could be established even when Klf4 was replaced with Klf1, Klf2 or Klf5 [WO 2007/069666 A1; Nakagawa, M. et al., Nat. Biotethnol., 26: 101-106 (2008)]. When ES cells are treated with retinoic acid to induce their differentiation, not only Klf4, but also Klf2 and Klf5 decrease their expression. Taking note of this fact, a group of Jiang et al. recently knocked down Klf2, Klf4 and Klf5 simultaneously, and found that differentiation was induced in the ES cells, showing that at least some of the members of the

Klf family, such as Klf2 and Klf5, can functionally substitute for Klf4 in ES cells [Jiang, J. et al., Nat. Cell Biol., 10: 353-360 (2008)]. They proceeded to transfer the Klf2 or Klf5 gene, or other transcription factors or epigenetic regulatory factors, along with the three genes Oct3/4, Sox2 and c-Myc, into MEF, confirming that Klf2 and Klf5 can substitute for Klf4, and finding that Esrrb, an orphan nuclear receptor resembling to estrogen receptors, is also capable of substituting for Klf4 [Feng, B. et al., Nat. Cell Biol., 11: 197-203 (2009)]. These findings lead to the notion that Klf1, Klf2, Klf5, and even Esrrb, also possess the effect of Klf4 confirmed in Examples given herein (an improvement of the efficiency of establishment of iPS cells with the use in combination with the GLIS family).

Although the members of the Klf family used in the present invention may be proteins derived from cells or tissues [e.g., cells or tissues of thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas or prostate, corresponding precursor cells, stem cells or cancer cells thereof, and the like] of optionally chosen mammals (e.g., humans, mice, rats, monkeys, bovines, horses, pigs, dogs and the like) or nucleic acids that encode the same, preference is given to those of human or mouse origin.

Information on the amino acid sequences and cDNA sequences of members of the Klf family of human and mouse origin can be acquired with reference to the NCBI accession numbers shown in Table 2. Those skilled in the art are easily able to isolate nucleic acids that encode the respective proteins on the basis of the cDNA sequence information, and to produce recombinant proteins as required.

Table 2

Gene code name	Humans		Mice	
	cDNA	Protein	cDNA	Protein
Klf1	NM_006563	NP_006554	NM_010635	NP_034765
Klf2	NM_016270	NP_057354	NM_008452	NP_032478
Klf4	NM_004235 (SEQ ID NO:5)	NP_004226 (SEQ ID NO:6)	NM_010637 (SEQ ID NO:7)	NP_034767 (SEQ ID NO:8)
Klf5	NM_001730	NP_001721	NM_009769	NP_033899

A natural or artificial mutant protein having an identity of 90% or more, preferably 95% or more, more preferably 98% or more, particularly preferably 99% or more, to each amino acid sequence shown above, and possessing an iPS cell establishment efficiency improving effect equivalent to that of the wild-type protein, and a nucleic acid that encodes the same, can also be utilized as an iPS cell establishment efficiency improving factor of the present invention.

Regarding the members of the Klf family of the present invention and nucleic acids that encode the same, any one of the factors belonging to the family may be used alone, and two or more may be used in combination.

Provided that the somatic cell to undergo nuclear reprogramming is endogenously expressing one or more of the constituents of any one of members of the GLIS family, or members of the Klf family, which are the above-described iPS cell establishment efficiency improving factors of the present invention, at a level sufficient to improve the establishment efficiency, a combination of only the remaining constituents excluding the endogenously expressed constituents can also be included in the scope of "iPS cell establishment efficiency improving factor" in the present invention.

Transfer of an iPS cell establishment efficiency improving factor of the present invention in the form of a protein to a somatic cell can be achieved using a method known per se for protein transfer into a cell. Such methods include,

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

Developed PTDs include those using transcellular domains of proteins such as drosophila-derived AntP, HIV-derived TAT (Frankel, A. et al, *Cell 55*, 1189-93 (1988) or Green, M. & Loewenstein, P. M. *Cell 55*, 1179-88 (1988)), Penetratin (Derossi, D. et al, *J. Biol. Chem. 269*, 10444-50 (1994)), Buforin II (Park, C. B. et al. *Proc. Natl Acad. Sci. USA 97*, 8245-50 (2000)), Transportan (Pooga, M. et al. *FASEB J. 12*, 67-77 (1998)), MAP (model amphipathic peptide) (Oehlke, J. et al. *Biochim. Biophys. Acta. 1414*, 127-39 (1998)), K-FGF (Lin, Y. Z. et al. *J. Biol. Chem. 270*, 14255-14258 (1995)), Ku70 (Sawada, M. et al. *Nature Cell Biol. 5*, 352-7 (2003)), Prion

(Lundberg, P. et al. *Biochem. Biophys. Res. Commun. 299*, 85-90 (2002)), pVEC (Elmquist, A. et al. *Exp. Cell Res. 269*, 237-44 (2001)), Pep-1 (Morris, M. C. et al. *Nature Biotechnol. 19*, 1173-6 (2001)), Pep-7 (Gao, C. et al. *Bioorg. Med. Chem. 10*, 4057-65 (2002)), SynB1 (Rousselle, C. et al. *Mol. Pharmacol. 57*, 679-86 (2000)), HN-I (Hong, F. D. & Clayman, G L. *Cancer Res. 60*, 6551-6 (2000)), and HSV-derived VP22. CPPs derived from the PTDs include polyarginines such as 11R (*Cell Stem Cell, 4*,381-384 (2009)) and 9R (*Cell Stem Cell, 4*, 472-476 (2009)).

A fused protein expression vector incorporating cDNA of an iPS cell establishment efficiency improving factor of the present invention and PTD sequence or CPP sequence is prepared, and recombination expression is performed using the vector. The fused protein is recovered and used for transfer. Transfer can be performed in the same manner as above except that a protein transfer reagent is not added.

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

Other useful methods of protein transfer include electroporation, the semi-intact cell method [Kano, F. et al. *Methods in Molecular Biology*, Vol. 322, 357-365 (2006)], transfer using the Wr-t peptide [Kondo, E. et al., *Mol. Cancer Ther.* 3(12), 1623-1630 (2004)] and the like.

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

When iPS cell establishment efficiency is emphasized, it is preferable that an iPS cell establishment efficiency

improving factor of the present invention be used not as a protein, but in the form of a nucleic acid that encodes the same. The nucleic acid may be a DNA or RNA, and may be a DNA/RNA chimera, with preference given to a DNA. The nucleic acid may be double-stranded or single-stranded. In the case of a double strand, the same may be a double-stranded DNA, a double-stranded RNA, or a DNA/RNA hybrid. Preferably, the nucleic acid is a double-stranded DNA, particularly a cDNA.

A nucleic acid-based iPS cell establishment efficiency improving factor of the present invention can be cloned from, for example, a cDNA derived from cells or tissues [e.g., cells or tissues of thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas or prostate, corresponding precursor cells, stem cells or cancer cells thereof, and the like] of humans or other mammals (e.g., mouse, rats, monkeys, pigs, dogs and the like), according to a conventional method.

Transfer of an iPS cell establishment efficiency improving factor of the present invention to a somatic cell can be achieved using a method known per se for gene transfer to cells. A nucleic acid that encodes an iPS cell establishment efficiency improving factor of the present invention is inserted into an appropriate expression vector comprising a promoter capable of functioning in a host somatic cell. Useful expression vectors include, for example, viral vectors such as retrovirus, lentivirus, adenovirus, adeno-associated virus, herpesvirus and Sendai virus, plasmids for the expression in animal cells (e.g., pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo) and the like.

The type of a vector to be used can be chosen as appropriate according to the intended use of the iPS cell to be obtained. Useful vectors include adenovirus vector, plasmid vector, adeno-associated virus vector, retrovirus vector, lentivirus vector, Sendai virus vector and the like.

Examples of promoters used in expression vectors include

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

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

Regarding the nucleic acids that encode iPS cell establishment efficiency improving factors of the present invention, any one may be integrated onto an expression vector alone, and some in combination may be integrated onto one expression vector. Furthermore, the nucleic acid(s) may be integrated onto one expression vector along with one or more reprogramming genes.

In the above-described procedure, when genes of iPS cell establishment efficiency improving factors and reprogramming factors of the present invention are integrated in combination into one expression vector, these genes can preferably be integrated into the expression vector via a sequence enabling polycistronic expression. Using a sequence enabling polycistronic expression makes it possible to more efficiently express a plurality of genes integrated in one expression vector. Useful sequences enabling polycistronic expression include, for example, the 2A sequence of foot-and-mouth disease virus (SEQ ID NO:9; PLoS ONE 3, e2532, 2008, Stem Cells 25, 1707, 2007), the IRES sequence (U.S. Patent No. 4,937,190) and the like, with preference given to the 2A sequence.

An expression vector comprising a nucleic acid that

encodes an iPS cell establishment efficiency improving factor of the present invention can be introduced into a cell by a technique known per se according to the choice of vector. In the case of a viral vector, for example, a plasmid containing the nucleic acid is introduced into an appropriate packaging cell (e.g., Plat-E cell) or a complementary cell line (e.g., 293-cells), the viral vector produced in the culture supernatant is recovered, and the vector is infected to the cell by a method suitable for the viral vector. For example, specific means using a retroviral vector are disclosed in WO2007/69666, *Cell, 126*, 663-676 (2006) and *Cell, 131*, 861-872 (2007). Specific means using a lentivirus vector is disclosed in *Science, 318*, 1917-1920 (2007). When iPS cells are utilized as a source of cells for regenerative medicine, the expression (reactivation) of an iPS cell establishment efficiency improving factor of the present invention or the activation of an endogenous gene present in the vicinity of the site where the exogenous gene is integrated potentially increases the risk of carcinogenesis in tissues regenerated from differentiated cells of iPS cell derivation. Therefore, the nucleic acid that encodes an iPS cell establishment efficiency improving factor of the present invention is preferably expressed transiently, without being integrated into the chromosome of the cells. From this viewpoint, use of an adenoviral vector, whose integration into chromosome is rare, is preferred. Specific means using an adenoviral vector is described in *Science, 322*, 945-949 (2008). Because an adeno-associated viral vector is also low in the frequency of integration into chromosome, and is lower than adenoviral vectors in terms of cytotoxicity and inflammation-inducibility, it can be mentioned as another preferred vector. Because Sendai viral vector is capable of being stably present outside the chromosome, and can be degraded and removed using an siRNA as required, it is preferably utilized as well. Regarding a Sendai viral vector, one described in *J. Biol. Chem., 282*,

27383-27391 (2007), *Proc. Jpn. Acad., Ser. B 85*, 348-362 (2009) or JP-B-3602058 can be used.

When a retroviral vector or a lentiviral vector is used, even if silencing of the transgene has occurred, it possibly becomes reactivated. Therefore, for example, a method can be used preferably wherein a nucleic acid encoding an iPS cell establishment efficiency improving factor of the present invention is cut out using the Cre-loxP system, when becoming unnecessary. That is, with loxP sequences arranged on both ends of the nucleic acid in advance, iPS cells are induced, thereafter the Cre recombinase is allowed to act on the cells using a plasmid vector or adenoviral vector, and the region sandwiched by the loxP sequences can be cut out. Because the enhancer-promoter sequence of the LTR U3 region possibly upregulates a host gene in the vicinity thereof by insertion mutation, it is more preferable to avoid the expression regulation of the endogenous gene by the LTR outside of the loxP sequence remaining in the genome without being cut out, using a 3′-self-inactivated (SIN) LTR prepared by deleting the sequence, or substituting the sequence with a polyadenylation sequence such as of SV40. Specific means using the Cre-loxP system and SIN LTR is disclosed in Soldner et al., *Cell, 136*: 964-977 (2009), Chang et al., *Stem Cells, 27*: 1042-1049 (2009) and the like.

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

When a plasmid vector, an adenovirus vector and the like are used, the transfection can be performed once or more optionally chosen times (e.g., once to 10 times, once to 5 times or the like). When two or more kinds of expression

vectors are introduced into a somatic cell, it is preferable that these all kinds of expression vectors be concurrently introduced into a somatic cell; however, even in this case, the transfection can be performed once or more optionally chosen times (e.g., once to 10 times, once to 5 times or the like), preferably the transfection can be repeatedly performed twice or more (e.g., 3 times or 4 times).

Also when an adenovirus or a plasmid is used, the transgene can get integrated into chromosome; therefore, it is eventually necessary to confirm the absence of insertion of the gene into chromosome by Southern blotting or PCR. For this reason, like the aforementioned Cre-loxP system, it can be advantageous to use a means wherein the transgene is integrated into chromosome, thereafter the gene is removed. In another preferred mode of embodiment, a method can be used wherein the transgene is integrated into chromosome using a transposon, thereafter a transposase is allowed to act on the cell using a plasmid vector or adenoviral vector so as to completely eliminate the transgene from the chromosome. As examples of preferable transposons, piggyBac, a transposon derived from a lepidopterous insect, and the like can be mentioned. Specific means using the piggyBac transposon is disclosed in Kaji, K. et al., *Nature, 458*: 771-775 (2009), Woltjen et al., *Nature, 458*: 766-770 (2009).

Another preferable non-integration type vector is an episomal vector, which is capable of self-replication outside the chromosome. Specific means using an episomal vector is disclosed in Yu et al., *Science, 324*, 797-801 (2009). As required, an expression vector may be constructed by inserting a nucleic acid that encodes an iPS cell establishment efficiency improving factor of the present invention into an episomal vector having loxP sequences placed in the same orientation on the 5′ and 3′ sides of the vector constituent essential for the replication of the episomal vector, and this can be transferred to a somatic cell.

Examples of the episomal vector include a vector comprising as a vector component a sequence derived from EBV, SV40 and the like necessary for self-replication. The vector component necessary for self-replication is specifically exemplified by a replication origin and a gene that encodes a protein that binds to the replication origin to control the replication; examples include the replication origin oriP and the EBNA-1 gene for EBV, and the replication origin ori and the SV40 large T antigen gene for SV40.

The episomal expression vector contains a promoter that controls the transcription of a nucleic acid that encodes an iPS cell establishment efficiency improving factor of the present invention. The promoter used may be as described above. The episomal expression vector may further contain as desired an enhancer, a polyadenylation signal, a selection marker gene and the like, as described above. Examples of the selection marker gene include the dihydrofolate reductase gene, the neomycin resistance gene and the like.

The loxP sequences useful in the present invention include, in addition to the bacteriophage P1-derived wild type loxP sequence (SEQ ID NO:10), optionally chosen mutant loxP sequences capable of deleting the sequence flanked by the loxP sequence by recombination when placed in the same orientation at positions flanking a vector component necessary for the replication of the transgene. Examples of such mutant loxP sequences include lox71 (SEQ ID NO:11), mutated in 5′ repeat, lox66 (SEQ ID NO:12), mutated in 3′ repeat, and lox2272 and lox511, mutated in spacer portion. Although the two loxP sequences placed on the 5′ and 3′ sides of the vector component may be identical or not, the two mutant loxP sequences mutated in spacer portion must be identical (e.g., a pair of lox2272 sequences, a pair of lox511 sequences). Preference is given to a combination of a mutant loxP sequence mutated in 5′ repeat (e.g., lox71) and a mutant loxP sequence mutated in 3′ repeat (e.g., lox66). In this case, the loxP

sequences remaining on the chromosome as a result of recombination have double mutations in the repeats on the 5′ side and 3′ side, and are therefore unlikely to be recognized by Cre recombinase, thus reducing the risk of causing a deletion mutation in the chromosome due to unwanted recombination. When the mutant loxP sequences lox71 and lox66 are used in combination, each may be placed on any of the 5′ and 3′ sides of the aforementioned vector component, but it is necessary that the mutant loxP sequences be inserted in an orientation such that the mutated sites would be located at the outer ends of the respective loxP sequences.

Each of the two loxP sequences is placed in the same orientation on the 5′ and 3′ sides of a vector constituent essential for the replication of the transgene (i.e., a replication origin, or a gene sequence that encodes a protein that binds to the replication origin to control the replication). The vector constituent flanked by the loxP sequences may be either the replication origin or a gene sequence that encodes a protein that binds to a replication origin to control the replication, or both.

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

Whether or not the vector component necessary for the replication of the transgene has been removed from the iPS cell can be confirmed by performing a Southern blot analysis or PCR analysis using a nucleic acid comprising a nucleotide sequence in the vector component and/or in the vicinity of loxP sequence as a probe or primer, with the episome fraction isolated from the iPS cell as a template, and determining the presence or absence of a band or the length of the band

detected. The episome fraction can be prepared by a method obvious in the art; for example, methods described in *Science*, *324*: 797-801 (2009) and elsewhere can be used.

(c) Nuclear reprogramming substances

In the present invention, "a nuclear reprogramming substance" refers to any substance(s) capable of inducing an iPS cell from a somatic cell, which may be composed of any substance such as a proteinous factor or a nucleic acid that encodes the same (including forms integrated in a vector), or a low-molecular compound, when transferred to the somatic cell, or when contacted with the somatic cell along with establishment efficiency improving factors of the present invention [(1) one or more substances selected from the group consisting of members of the GLIS family and nucleic acids that encode the same, and (2) one or more substances selected from the group consisting of members of the Klf family and nucleic acids that encode the same]. As a known nuclear reprogramming substance that is a proteinous factor or a nucleic acid that encodes the same, the following combinations, for example, are preferable (hereinafter, only the names for proteinous factors are shown).

(1) Oct3/4, Klf4, c-Myc

(2) Oct3/4, Klf4, c-Myc, Sox2 (Sox2 is replaceable with Sox1, Sox3, Sox15, Sox17 or Sox18; Klf4 is replaceable with Klf1, Klf2 or Klf5; c-Myc is replaceable with T58A (active mutant), or L-Myc)

(3) Oct3/4, Klf4, c-Myc, Sox2, Fbx15, Nanog, ERas, TclI

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

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

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

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

(8) Oct3/4, Klf4, c-Myc, Sox2, TERT, Bmil

[For more information on the factors shown above, see WO 2007/069666 (for information on replacement of Sox2 with Sox18

2007/069666 (for information on replacement of Sox2 with Sox18 and replacement of Klf4 with Klf1 or Klf5 in the combination (2) above, see *Nature Biotechnology, 26*, 101-106 (2008)); for the combination "Oct3/4, Klf4, c-Myc, Sox2", see also *Cell, 126*, 663-676 (2006), *Cell, 131*, 861-872 (2007) and the like; for the combination "Oct3/4, Klf2 (or Klf5), c-Myc, Sox2", see also *Nat. Cell Biol., 11*, 197-203 (2009); for the combination "Oct3/4, Klf4, c-Myc, Sox2, hTERT, SV40 LT", see also *Nature, 451*, 141-146 (2008).]

(9) Oct3/4, Klf4, Sox2 (see *Nature Biotechnology, 26*, 101-106 (2008))

(10) Oct3/4, Sox2, Nanog, Lin28 (see *Science, 318*, 1917-1920 (2007))

(11) Oct3/4, Sox2, Nanog, Lin28, hTERT, SV40LT (see *Stem Cells, 26*, 1998-2005 (2008))

(12) Oct3/4, Klf4, c-Myc, Sox2, Nanog, Lin28 (see *Cell Research 18* (2008) 600-603)

(13) Oct3/4, Klf4, c-Myc, Sox2, SV40LT (see *Stem Cells, 26*, 1998-2005 (2008))

(14) Oct3/4, Klf4 (see *Nature 454*:646-650 (2008)、*Cell Stem Cell, 2*, 525-528 (2008)))

(15) Oct3/4, c-Myc (see *Nature 454*:646-650 (2008))

(16) Oct3/4, Sox2 (see *Nature, 451*, 141-146 (2008), WO2008/118820)

(17) Oct3/4, Sox2, Nanog (see WO2008/118820)

(18) Oct3/4, Sox2, Lin28 (see WO2008/118820)

(19) Oct3/4, Sox2, c-Myc, Esrrb (Here, Esrrb can be substituted by Esrrg, see *Nat. Cell Biol., 11*, 197-203 (2009))

(20) Oct3/4, Sox2, Esrrb (see *Nat. Cell Biol., 11*, 197-203 (2009))

(21) Oct3/4, Klf4, L-Myc (see *Proc. Natl. Acad. Sci. U S A., 107(32)*, 14152-14157 (2010))

(22) Oct3/4, Klf4, Sox2, L-Myc, Lin28 (see WO2011/016588)

(23) Oct3/4, Nanog

(24) Oct3/4 (*Cell 136*: 411-419 (2009)、*Nature*, 08436,

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(25) Oct3/4, Klf4, c-Myc, Sox2, Nanog, Lin28, SV40LT (see *Science, 324*: 797-801 (2009))

In (1)-(25) above, Oct3/4 may be replaced with another member of the Oct family, for example, Oct1A, Oct6 or the like. Sox2 (or Sox1, Sox3, Sox15, Sox17, Sox18) may be replaced with another member of the Sox family, for example, Sox7 or the like. Furthermore, provided that c-Myc or Lin28 is included as a nuclear reprogramming substance in the combinations (1)-(25) above, L-Myc or Lin28B can be used in place of c-Myc or Lin28, respectively.

When the combinations of factors (1)-(25) above include members of the Klf family, the "nuclear reprogramming substance" used in combination with an iPS cell establishment efficiency improving factor of the present invention is suitably one containing a factor other than these members of the Klf family. When the combinations (1)-(25) above do not include a member of the Klf family, nuclear reprogramming substances used in combination with iPS cell establishment efficiency improving factors of the present invention may be a combination of the factors.

Combinations further comprising another optionally chosen substance, in addition to the aforementioned nuclear reprogramming substances, are also suitably used as a "nuclear reprogramming substance" in the present invention. Provided that the somatic cell to undergo nuclear reprogramming is endogenously expressing one or more of the constituents of any one of (1) to (25) above at a level sufficient to cause nuclear reprogramming, a combination of only the remaining constituents excluding the one or more constituents can also be included in the scope of "nuclear reprogramming substances" in the present invention.

Of these combinations, one or more substances selected from among members of the Oct family, members of the Sox family, members of the Myc family, members of the Lin28 family

and Nanog, for example, are preferable nuclear reprogramming substances, with greater preference given to the combination of Oct3/4 and Sox2, the combination of Oct3/4, Sox2 and c-Myc, the combination of Oct3/4, Sox2 and L-Myc, or the combination of Oct3/4, Sox2, L-Myc and Lin28.

While promoting the establishment of iPS cells, c-Myc also promotes the generation of non-iPS transformed cells (partially reprogrammed cells, nullipotent transformed cells). The present inventors not only demonstrated that co-expressing GLIS1 with Oct3/4, Sox2 and Klf4 dramatically promotes the establishment of iPS cells from mouse and human adult skin fibroblasts, but also revealed that GLIS1, unlike c-Myc, does not promote the aforementioned genesis of non-iPS transformed cells. Therefore, it is particularly preferable to use GLIS1 without using c-Myc.

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

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

A proteinous factor for use as a nuclear reprogramming substance can be prepared by inserting the cDNA obtained into an appropriate expression vector, introducing the vector into a host cell, and recovering the recombinant proteinous factor from the cultured cell or its conditioned medium. Meanwhile, when a nucleic acid that encodes a proteinous factor is used

as a nuclear reprogramming substance, the cDNA obtained is inserted into a viral vector, episomal vector or plasmid vector in the same manner as with the above-described case of the nucleic acid-based iPS cell establishment efficiency improving factor of the present invention to construct an expression vector, which is subjected to the nuclear reprogramming step. The aforementioned Cre-loxP system or piggyBac transposon system can also be utilized as required. When two or more nucleic acids that encode two or more proteinous factors are transferred to a cell as nuclear reprogramming substances, the different nucleic acids may be carried by separate vectors, or the plurality of nucleic acids may be joined in tandem to obtain a polycistronic vector. In the latter case, to allow efficient polycistronic expression, it is desirable that the 2A self-cleaving peptide of foot-and-mouth disease virus be inserted between the nucleic acids (see, for example, *Science, 322*, 949-953, 2008).

Contact of a nuclear reprogramming substance with a somatic cell can be achieved (a) in the same manner as with the above-described proteinous iPS cell establishment efficiency improving factor of the present invention when the substance is a proteinous factor, or (b) in the same manner as with the above-described nucleic acid-based iPS cell establishment efficiency improving factor of the present invention when the substance is a nucleic acid that encodes a proteinous factor. (c) When the nuclear reprogramming substance is a low-molecular compound, contacting with somatic cells can be achieved by dissolving the low-molecular compound at an appropriate concentration in an aqueous or non-aqueous solvent, adding the solution to a medium suitable for cultivation of somatic cells isolated from human or the other mammals [e.g., minimal essential medium (MEM) comprising about 5 to 20% fetal bovine serum, Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium, and the like] so that the nuclear reprogramming substance concentration will

fall in a range that is sufficient to cause nuclear reprogramming in somatic cells and does not cause cytotoxicity, and culturing the cells for a given period. The nuclear reprogramming substance concentration varies depending on the kind of nuclear reprogramming substance used, and is chosen as appropriate over the range of about 0.1 nM to about 100 nM. Duration of contact is not particularly limited, as far as it is sufficient to cause nuclear reprogramming of the cells; usually, the nuclear reprogramming substance may be allowed to be co-present in the medium until a positive colony emerges.

(d) Other iPS cell establishment efficiency improvers

In recent years, various substances that improve the efficiency of establishment of iPS cells, which has traditionally been low, have been proposed one after another. When contacted with a somatic cell along with the aforementioned iPS cell establishment efficiency improving factor of the present invention, other iPS cell establishment efficiency improvers are expected to further raise the efficiency of establishment of iPS cells.

Examples of the other iPS cell establishment efficiency improvers include, but are not limited to, histone deacetylase (HDAC) inhibitors except for VPA [e.g., low-molecular inhibitors such as trichostatin A (TSA), sodium butyrate, MC 1293, and M344, nucleic acid-based expression inhibitors such as siRNAs and shRNAs against HDAC (e.g., HDAC1 siRNA Smartpool® (Millipore), HuSH 29mer shRNA Constructs against HDAC1 (OriGene) and the like), and the like], DNA methyltransferase inhibitors [e.g., 5'-azacytidine (5'-azaC) [*Nat. Biotechnol.*, *26*(7): 795-797 (2008)], G9a histone methyltransferase inhibitors [e.g., low-molecular inhibitors such as BIX-01294 (*Cell Stem Cell*, *2*: 525-528 (2008)), nucleic acid-based expression inhibitors such as siRNAs and shRNAs against G9a [e.g., G9a siRNA (human) (Santa Cruz Biotechnology) and the like) and the like], L-channel calcium agonists (e.g., Bayk8644) [*Cell Stem Cell*, *3*, 568-574 (2008)], p53 inhibitors

[e.g., siRNA, shRNA, dominant negative mutants etc. against p53 (*Cell Stem Cell, 3,* 475-479 (2008)); Nature 460, 1132-1135 (2009))], Wnt signaling activator (e.g., soluble Wnt3a) [*Cell Stem Cell, 3,* 132-135 (2008)], 2i/LIF [2i is an inhibitor of mitogen-activated protein kinase signaling and glycogen synthase kinase-3, *PloS Biology,* 6(10), 2237-2247 (2008)], ES cell-specific miRNA [e.g., miR-302-367 cluster (*Mol. Cell. Biol.* doi:10.1128/MCB.00398-08); miR-302 (*RNA* (2008) 14: 1-10); miR-291-3p, miR-294 and miR-295 (*Nat. Biotechnol.* 27: 459-461 (2009)] and the like. As mentioned above, the nucleic acid-based expression inhibitors may be in the form of expression vectors harboring a DNA that encodes an siRNA or shRNA.

Of the aforementioned constituents of nuclear reprogramming substances, SV40 large T, for example, can also be included in the scope of iPS cell establishment efficiency improvers because they are auxiliary factors unessential for the nuclear reprogramming of somatic cells. While the mechanism of nuclear reprogramming remains unclear, it does not matter whether auxiliary factors, other than the factors essential for nuclear reprogramming, are deemed nuclear reprogramming substances or iPS cell establishment efficiency improvers. Hence, because the somatic cell nuclear reprogramming process is taken as an overall event resulting from contact of nuclear reprogramming substances and an iPS cell establishment efficiency improver with somatic cells, it does not seem always essential for those skilled in the art to distinguish between the two.

Contact of an iPS cell establishment efficiency improver with a somatic cell can be achieved in the same manner as with the above-described iPS cell establishment efficiency improving factor of the present invention and nuclear reprogramming substance when the improver is (a) a proteinous factor, (b) a nucleic acid that encodes the proteinous factor, or (c) a low-molecular compound, respectively.

iPS cell establishment efficiency improvers, including iPS cell establishment efficiency improving factors of the present invention, may be contacted with the somatic cell simultaneously with the nuclear reprogramming substance, and either one may be contacted in advance, as far as the efficiency of iPS cell establishment from a somatic cell improves significantly compared with the efficiency obtained in the absence of the substance. In an embodiment, for example, when the nuclear reprogramming substance is a nucleic acid that encodes a proteinous factor and the iPS cell establishment efficiency improver is a chemical inhibitor, the iPS cell establishment efficiency improver can be added to the medium after the cell is cultured for a given length of time following the gene transfer treatment, because the nuclear reprogramming substance involves a given length of time lag from the gene transfer treatment to the mass-expression of the proteinous factor, whereas the iPS cell establishment efficiency improver is capable of rapidly acting on the cell. In another embodiment, when a nuclear reprogramming substance and an iPS cell establishment efficiency improver are both used in the form of a viral or plasmid vector, for example, both may be simultaneously introduced into the cell.

(e) Improving the establishment efficiency by culture conditions

iPS cell establishment efficiency can further be improved by culturing the cells under hypoxic conditions in the nuclear reprogramming process for somatic cells (see *Cell Stem Cell*, 5, p237-241(2009)). As mentioned herein, the term "hypoxic conditions" means that the ambient oxygen concentration as of the time of cell culture is significantly lower than that in the atmosphere. Specifically, conditions involving lower oxygen concentrations than the ambient oxygen concentrations in the 5-10% CO_2/95-90% air atmosphere, which is commonly used for ordinary cell culture, can be mentioned; examples include conditions involving an ambient oxygen concentration of 18% or

less. Preferably, the ambient oxygen concentration is 15% or less (e.g., 14% or less, 13% or less, 12% or less, 11% or less and the like), 10% or less (e.g., 9% or less, 8% or less, 7% or less, 6% or less and the like), or 5% or less (e.g., 4% or less, 3% or less, 2% or less and the like). The ambient oxygen concentration is preferably 0.1% or more (e.g., 0.2% or more, 0.3% or more, 0.4% or more and the like), 0.5% or more (e.g., 0.6% or more, 0.7% or more, 0.8% or more, 0.95% or more and the like), or 1% or more (e.g., 1.1% or more, 1.2% or more, 1.3% or more, 1.4% or more and the like).

Although any method of creating a hypoxic state in a cellular environment can be used, the easiest way is to culture cells in a CO_2 incubator permitting adjustments of oxygen concentration, and this represents a suitable case. CO_2 incubators permitting adjustment of oxygen concentration are commercially available from various manufacturers (e.g., CO_2 incubators for hypoxic culture manufactured by Thermo scientific, Ikemoto Scientific Technology, Juji Field, Wakenyaku etc.).

The time of starting cell culture under hypoxic conditions is not particularly limited, as far as iPS cell establishment efficiency is not prevented from being improved compared with the normal oxygen concentration (20%). Although the culture may be started before the somatic cell is contacted with an iPS cell establishment efficiency improving factor of the present invention and a nuclear reprogramming substance, or at the same time as the contact, or after the contact, it is preferable, for example, that the culture under hypoxic conditions be started just after the somatic cell is contacted with the iPS cell establishment efficiency improving factor of the present invention and a nuclear reprogramming substance, or after a given time interval after the contact [e.g., 1 to 10 (e.g., 2, 3, 4, 5, 6, 7, 8 or 9) days].

The duration of cultivation of cells under hypoxic conditions is not particularly limited, as far as iPS cell

establishment efficiency is not prevented from being improved compared with the normal oxygen concentration (20%); examples include, but are not limited to, periods of 3 days or more, 5 days or more, 7 days or more or 10 days or more, and 50 days or less, 40 days or less, 35 days or less or 30 days or less and the like. Preferred duration of cultivation under hypoxic conditions varies depending on ambient oxygen concentration; those skilled in the art can adjust as appropriate the duration of cultivation according to the oxygen concentration used. In an embodiment of the present invention, if iPS cell candidate colonies are selected with drug resistance as an index, it is preferable that a normal oxygen concentration be restored from hypoxic conditions before starting drug selection.

Furthermore, preferred starting time and preferred duration of cultivation for cell culture under hypoxic conditions also vary depending on the choice of nuclear reprogramming substance used, iPS cell establishment efficiency at normal oxygen concentrations and the like.

(f) Selection and identification of iPS cells

After being contacted with an iPS cell establishment efficiency improving factor of the present invention and a nuclear reprogramming substance (and another iPS cell establishment efficiency improver), the cell can, for example, be cultured under conditions suitable for culturing ES cells. In the case of mouse cells, the culture is carried out with the addition of leukemia inhibitory factor (LIF) as a differentiation suppression factor to an ordinary medium. Meanwhile, in the case of human cells, it is desirable that basic fibroblast growth factor (bFGF) and/or stem cell factor (SCF) be added in place of LIF. Usually, the cell is cultured in the co-presence of mouse embryonic fibroblasts (MEF) treated with radiation or an antibiotic to terminate the cell division, as feeder cells. Usually, STO cells and the like are commonly used as MEFs, but for inducing iPS cells, SNL cells

[McMahon, A.P. & Bradley, A. *Cell 62*, 1073-1085 (1990)] and the like are commonly used. Co-culture with the feeder cells may be started before contact with the iPS cell establishment efficiency improving factor and nuclear reprogramming substance of the present invention, at the time of the contact, or after the contact (for example, 1-10 days later).

A candidate colony of iPS cells can be selected by a method with drug resistance and reporter activity as indicators, and also by a method based on macroscopic examination of morphology. As an example of the former, a colony positive for drug resistance and/or reporter activity is selected using a recombinant cell wherein a drug resistance gene and/or a reporter gene is targeted to the locus of a gene highly expressed specifically in pluripotent cells (for example, Fbx15, Nanog, Oct3/4 and the like, preferably Nanog or Oct3/4). As examples of such recombinant cells, a mouse-derived MEF and TTF wherein the βgeo (which encodes a fusion protein of β-galactosidase and neomycin phosphotransferase) gene is knocked-in to the Fbx15 gene locus (Takahashi & Yamanaka, *Cell, 126*, 663-676 (2006)), or a transgenic mouse-derived MEF and TTF wherein green fluorescent protein (GFP) gene and the puromycin resistance gene are integrated in the Nanog gene locus (Okita et al., *Nature, 448*, 313-317 (2007)) and the like can be mentioned. Meanwhile, methods for selecting a candidate colony by macroscopic examination of morphology include, for example, the method described by Takahashi et al. in *Cell, 131*, 861-872 (2007). Although methods using reporter cells are convenient and efficient, colony selection by macroscopic examination is desirable from the viewpoint of safety when iPS cells are prepared for the purpose of human treatment.

The identity of the cells of the selected colony as iPS cells can be confirmed by positive responses to Nanog (or Oct3/4) reporters (puromycin resistance, GFP positivity and the like), as well as by the formation of a visible ES cell-

like colony, as described above; however, to increase the accuracy, it is possible to perform tests such as alkaline phosphatase staining, analyzing the expression of various ES-cell-specific genes, and transplanting the cells selected to a mouse and confirming teratoma formation.

When a nucleic acid that encodes an iPS cell establishment efficiency improving factor of the present invention is transferred to a somatic cell, the iPS cell obtained is a novel cell that is distinct from conventionally known iPS cells in that the exogenous nucleic acid is contained therein. In particular, if when the exogenous nucleic acid is transferred to the somatic cell using a retrovirus, lentivirus or the like, the exogenous nucleic acid is usually integrated in the genome of the iPS cell obtained, so that the character of containing the exogenous nucleic acid is stably retained.

(g) Use applications for iPS cells

The iPS cells thus established can be used for various purposes. For example, by utilizing a method of differentiation induction reported with respect to pluripotent stem cells such as ES cells (e.g., methods of differentiation induction include a method described in JP-A-2002-291469 for nerve stem cells, a method described in JP-A-2004-121165 for pancreatic stem-like cells, and a method described in JP-T-2003-505006 for hematopoietic cells; methods of differentiation induction by formation of embryoid body include a method described in JP-T-2003-523766), differentiation into various cells (e.g., myocardial cells, blood cells, nerve cells, vascular endothelial cells, insulin-secreting cells and the like) from iPS cells can be induced. Therefore, inducing iPS cells using a somatic cell collected from a patient or another person of the same or substantially the same HLA type would enable stem cell therapy by autologous transplantation, wherein the iPS cells are differentiated into desired cells (that is, cells of an affected organ of the

patient, cells that have a therapeutic effect on the disease, and the like), which are transplanted to the patient. Furthermore, because functional cells (e.g., hepatocytes) differentiated from iPS cells are thought to better reflect the actual state of the functional cells *in vivo* than do corresponding existing cell lines, they can also be suitably used for *in vitro* screening for the effectiveness and toxicity of pharmaceutical candidate compounds and the like.

The present invention is hereinafter described in further detail by means of the following examples, to which, however, the invention is never limited.

Examples

Reference Example 1: Screening for novel reprogramming factors

Approximately 20000 clones of comprehensive human genes were ordered on the basis of human Gateway® entry clones generated by Goshima et al. (the library described by N. Goshima et al. in *Nature methods*, 2008 was used; database published by Y. Maruyama et al. in *Nucleic Acid Res.*, 2009), by the method shown in Fig. 1. Specifically, about 50000 clones containing a full-length ORF, out of the human Gateway® entry clones, were subjected to BLASTP search against 37900 sequences (24200 genes) registered with the NCBI RefSeq, with the criteria of a coverage of 80% or more and an amino acid identity of 95% or more. A sublibrary consisting of about 20000 entry clones involving no sequence overlap in each of the N-type, which has a stop codon at the 3' end thereof, and the F-type, which lacks the stop codon, was thus constructed. These about 20000 ordered entry clones were classified by a bioinformatics technique into protein kinases, protein phosphatases, transcription factors, GPCRs, and other clones; a sublibrary consisting of entry clones of transcription factors (over 50% of all human transcription factors are covered) was constructed (Fig. 1). An expression clone DNA was prepared for each entry clone from this sublibrary of transcription factors by an LR reaction with the pMXs-GW

destination vector, as shown in Fig. 2. This reaction liquor was transferred to Escherichia coli DH5α, which was then cloned to construct a transcription factor expression library (transcription factor expression library for reprogramming factor screening). Each of the human Oct3/4, Sox2, Klf4, c-Myc genes was also integrated into the same pMXs-GW to construct respective expression clones. A recombinant retrovirus was generated from this DNA and used in the following experiment.

An experiment to induce iPS cells was performed using dermal fibroblasts from a Nanog-GFP mouse [Okita et al., *Nature, 448,* 313-317 (2007)]. The experiment was conducted using two systems: a system involving retrovirus infection on MSTO (SNL cells treated with mitomycin C to terminate the cell division thereof) used as feeder cells [hereinafter the MSTO method, *Cell, 126,* 663-676 (2006)] and a system involving infection without using feeder cells, followed by cell reseeding and subsequent cultivation on MSTO [hereinafter the Reseed method, *Nature Biotech., 26,* pp.101-106 (2008)].

For 1st screening, iPS cells were induced using 24-well plates. Nanog-GFP mouse skin fibroblasts were seeded onto gelatin (Reseed method) or MSTO (MSTO method). The following day, the fibroblasts were infected with retroviruses prepared from various plasmids (Day 0). Specifically, the fibroblasts were infected with the three genes Oct3/4, Sox2 and c-Myc and one gene selected from the above-described transcription factor library in the 1:1:1:1 ratio. For negative control, the fibroblasts were infected with the three genes Oct3/4, Sox2 and c-Myc in the 1:1:1 ratio. For positive control, the fibroblasts were infected with the four genes Oct3/4, Sox2, Klf4 and c-Myc in the 1:1:1:1 ratio.

The fibroblasts were cultured with 10% FBS/DMEM until day 2 after the infection, and with the ES medium [Cell, 126, 663-676 (2006)] on day 3 and after. When the fibroblasts were initially seeded onto gelatin (Reseed method), they were reseeded onto MSTO on day 3. Thereafter, while replacing the

medium with a fresh supply of the same medium every two days, puromycin selection was started on day 21, and the cells were examined on day 28. As a result, GFP-positive colonies emerged in the wells incorporating each gene [sample F09 (gene code name: IRX6), sample G06 (gene code name: GLIS1), sample H08 (gene code name: DMRTB1), and sample H10 (gene code name: PITX2)] transferred along with the three genes, confirming the establishment of mouse iPS cells. When iPS induction was again attempted using 6-well plates, GFP-positive colonies emerged likewise; reproducibility was obtained. Photographic images and phase-contrast images of GFP-positive iPS cell colonies taken at the time of colony formation and 1st generation and 2nd generation are shown in Figs. 3 and 4.

These results demonstrate the identify of these four factors as novel reprogramming factors capable of substituting for Klf4. When the same experiment was performed using MEF (mouse embryonic fibroblasts) or HDF (human dermal fibroblasts) in place of adult mouse skin fibroblasts, iPS cells (GFP-positive colonies) were likewise established.

Reference Example 2: Analysis of established mouse iPS cells

The genome was extracted using the Gentra Puregene Cell Kit (QIAGEN), and Genomic-PCR was performed using a PCR enzyme (Takara Ex Taq) and the iPS cells established in Reference Example 1. The results are shown in Figs. 5 and 6. In all the iPS cells established, the presence of only the transgenes on the genome and the absence of other genes on the genome were confirmed. For the G6-1 clone (gene code name: GLIS1), the c-Myc used for the transfer was not inserted onto the genome (Fig. 5). Because retrovirus vectors are not stably expressed unless inserted onto the genome, this clone G6-1 was thought to have been established with the expression of only the three factors Oct3/4, Sox2 and GLIS1.

Next, RT-PCR analysis was performed using the Rever Tra Ace kit (Takara). The results are shown in Figs. 7 and 8. All

the iPS cells established in Reference Example 1 expressed the ES cell-specific marker genes Nanog, Oct3/4, Sox2, Rex1 and ECAT1. These results confirmed the identity of the cells established using the novel reprogramming factors as iPS cells.

Example 1: Establishment of mouse iPS cells with G6 and Klf4 used in combination

(a) Effects of G6 and Klf4 used in combination on the efficiency of establishment of mouse iPS cells

An investigation was conducted to determine whether iPS cells could be established when using G6 (gene code name: GLIS1), H8 (gene code name: DMRTB1) and H10 (gene code name: PITX2), which are novel reprogramming factors capable of substituting for Klf4, identified in Reference Example 1, in combination with Klf4. The experiments were conducted by the Reseed method using Nanog-GFP mouse skin fibroblasts as in Reference Example 1. The combinations of genes used for the gene transfer are shown below.

(1) Oct3/4, Sox2
(2) Oct3/4, Sox2, G6 (gene code name: GLIS1)
(3) Oct3/4, Sox2, H8 (gene code name: DMRTB1)
(4) Oct3/4, Sox2, H10 (gene code name: PITX2)
(5) Oct3/4, Sox2, Klf4
(6) Oct3/4, Sox2, Klf4, G6
(7) Oct3/4, Sox2, Klf4, H8
(8) Oct3/4, Sox2, Klf4, H10

The retroviruses used for the reprogramming were prepared by separately transferring retrovirus expression vectors (pMXs-Oct3/4, pMXs-Sox2, pMXs-Klf4, pMXs-G6, pMXs-H8, pMXs-H10) to Plat-E cells (Morita, S. et al., *Gene Ther.* 7, 1063-1066) that had been seeded at 2.5 x 10^6 cells per 100 mm culture dish (Falcon) on the previous day. The culture broth used was DMEM/10% FCS [DMEM (Nacalai tesque) supplemented with 10% fetal bovine serum], and the cells were cultured at 37°C, 5% CO_2.

To facilitate vector transfer, 27 μL of FuGene6 transfection reagent (Roche) was placed in 300 μL of Opti-MEM I Reduced-Serum Medium (Invitrogen), and the mixture was allowed to stand at room temperature for 5 minutes. Subsequently, 9 μg of each expression vector was added, and the mixture was allowed to further stand at room temperature for 15 minutes, after which it was added to the Plat-E culture broth. On day 2, the Plat-E supernatant was replaced with a fresh supply of the medium. On day 3, the culture supernatant was recovered and filtered through a 0.45 μm sterile filter (Whatman), and polybrene (Nacalai) was added at 4 μg/mL to yield a viral liquid.

The Nanog-GFP mouse skin fibroblasts used were obtained by removing the dermis from a mouse back/abdomen skin, and culturing it on a gelatin-coated dish.

The culture broth used was DMEM/10% FCS, and the fibroblasts were seeded to 100 mm dishes (Falcon) at 8.0×10^5 cells per dish, and cultured at 37°C, 5% CO_2. The following day, each retrovirus liquid [any of the combinations (1) to (8) above] was added to transfer the genes by overnight infection.

On the day after the viral infection, the retrovirus liquid was removed and replaced with DMEM/10% FCS, and the cells were cultured using DMEM/10% FCS until day 3 after the infection. On day 3 after the infection, the medium was removed, and the cells were washed by the addition of 10 mL of PBS. After the PBS was removed, 0.25% trypsin/1 mM EDTA (Invitrogen) was added, and a reaction was allowed to proceed at 37°C for about 5 minutes. After the cells floated up, they were suspended by the addition of an ES cell culture medium [DMEM (Nacalai Tesque) supplemented with 15% fetal bovine serum, 2 mM L-glutamine (Invitrogen), 100 μM non-essential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Invitrogen), 50 U/mL penicillin (Invitrogen) and 50 μg/mL streptomycin (Invitrogen)], and seeded to a 100 mm dish having feeder cells seeded thereto previously.

The feeder cells used were SNL cells treated with mitomycin C to terminate the cell division thereof [McMahon, A.P. & Bradley, A. *Cell*, 62, 1073-1085 (1990)]. Cultivation was continued while replacing the ES cell culture medium with a fresh supply of the same medium every two days until a visible colony emerged; 26 to 28 days after infection, GFP-positive colonies were counted. The results of three independent experiments are shown in Table 3 and Fig. 9 (Fig. 9 is a graphic representation of the results shown in Table 3; the results of four independent experiments are shown for the control only).

Table 3

	DsRed	OS+Mock+Mock	OS+Mock+Q6	OS+Mock+H8	OS+Mock+H10	OS+Klf+Mock	OS+Klf+Q6	OS+Klf+H8	OS+Klf+H10
1st time	0	0	0			4	997		
2nd time	0	0	0	0	0	49	1680	21	48
3rd time	0	0	0	0	0	3	1590	6	2
4th time	0	0		6	0	295		223	102

Even with the addition of G6, H8 or H10 to Oct3/4 and Sox2, iPS cells could not be established, or only a very few iPS cells could be established, under these conditions. When H8 or H10 was added to Oct3/4, Sox2 and Klf4, the iPS colony count did not rise, compared with the absence of the addition (Oct3/4, Sox2 and Klf4). By contrast, when G6 was added to Oct3/4, Sox2 and Klf4, the iPS colony count rose dramatically, at a level much higher than the sum of the colony count obtained with the addition of G6 to Oct3/4 and Sox2 and the colony count obtained with the addition of Klf4 to Oct3/4 and Sox2. Using Klf4 and G6 in combination was shown to be synergistically effective on the efficiency of establishment of iPS cells.

(b) Comparison of the improving effects of GLIS1 and c-Myc on the establishment of mouse iPS cells using three reprogramming factors (OSK)

We then compared the ability of GLIS1 and c-Myc to promote iPSC generation with OSK. In adult mouse skin fibroblasts, the effect of GLIS1 is comparable to that of c-Myc, as judged by the number of GFP-positive colonies that were formed (Fig. 10). We also observed a synergistic increase in the number of GFP-positive colonies when both GLIS1 and c-Myc were co-introduced with OSK (Fig. 10).

We next analyzed the ratio of GFP positive colonies to total colonies that emerged after transduction. An one-way repeated-measures ANOVA test and a post-hoc Bonferroni test were used for the analyses. Differences were considered to be statistically significant for P-values of less than 0.05 (*) or 0.01 (**). The results are shown in Fig. 11. Importantly, GLIS1 specifically promoted the generation of GFP-positive colonies, but not GFP-negative colonies, which represent either partially reprogrammed cells or transformed cells (Fig. 11). In contrast, c-Myc increased the number of GFP-negative colonies more prominently than GFP-positive colonies (Fig. 11). This undesirable effect of c-Myc was counteracted when GLIS1

was co-expressed. Similar results were obtained with mouse embryonic fibroblasts (MEF) (Fig. 13 and Fig. 14). GFP-positive colonies are shown in Fig. 12 and Fig. 15.

We also confirmed the iPS cells established with OSK + GLIS1 from MEF are germline-competent.

Example 2: Establishment of human iPS cells with G6 and Klf4 used in combination

(a) Effects of G6 and Klf4 used in combination on the efficiency of establishment of human iPS cells

An investigation was conducted using adult human dermal fibroblasts (HDF) to determine whether the synergistic effect of Klf4 and G6 (GLIS1) used in combination is also noted in human cells. The combinations of genes used for the gene transfer are shown below.

(1) Oct3/4, Sox2, c-Myc

(2) Oct3/4, Sox2, c-Myc, Klf4

(3) Oct3/4, Sox2, c-Myc, G6 (gene code name: GLIS1)

(4) Oct3/4, Sox2, c-Myc, Klf4, G6

HDF was forced to express the mouse ecotropic virus receptor Slc7a1 gene using lentivirus (pLenti6/UbC-Slc7a1) as described by Takahashi, K. et al. in *Cell, 131*: 861-872 (2007). These cells ($2.6x10^5$ cells/60 mm dish) were transfected with genes in the combinations (1) to (4) above using retrovirus as described by Takahashi, K. et al. in *Cell, 131*: 861-872 (2007). Six days after the viral infection, the cells were recovered and re-seeded onto feeder cells ($5x10^4$ cells or $5x10^5$ cells/100 mm dish). The feeder cells used were SNL cells treated with mitomycin C to terminate the cell division thereof [McMahon, A.P. & Bradley, A. *Cell*, 62, 1073-1085 (1990)]. Starting seven days after the infection, the cells were cultured in a primate ES cell culture medium (ReproCELL) supplemented with 4 ng/mL recombinant human bFGF (WAKO). 30 to 35 days after the infection, ES cell-like colonies were counted. The results of three independent experiments are shown in Fig. 16 (ES-like

colonies) and Fig. 17 (non-ES-like colonies). Phase-contrast images of iPS colonies established with Oct3/4, Sox2, c-Myc, Klf4 and G6 are shown in Fig. 18. Compared with adding Klf4 to Oct3/4, Sox2 and c-Myc and adding G6 (GLIS1) to Oct3/4, Sox2 and c-Myc, adding both Klf4 and G6 to Oct3/4, Sox2 and c-Myc resulted in the emergence of a much larger number of ES cell-like colonies (Fig. 16). These colonies exhibited an ES cell-like morphology (Fig. 18). In short, in human cells as well, a synergistic effect on the efficiency of establishment of iPS cells was noted when Klf4 and G6 were used in combination.

(b) Comparison of the improving effects of GLIS1 and c-Myc on the establishment of human iPS cells using three reprogramming factors (OSK)

We then compared the ability of GLIS1 and c-Myc to promote iPSC generation with OSK in the same manner as described in Example 1(b). In human adult fibroblasts, GLIS1 showed a similar effect to a comparable degree to c-Myc and promoted the generation of ESC-like colonies when co-introduced with OSK (Fig. 19). Significantly, GLIS1 specifically promoted the generation of ESC-like colonies, but not non-ESC-like colonies. In contrast, c-Myc increased the number of non-ESC-like colonies more prominently than ESC-like colonies (Fig. 20). Human ESC-like colonies generated by OSK + GLIS1 are shown in Fig. 21.

Then, genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and genomic-PCR was performed using a PCR enzyme (Takara Ex Taq). The results are shown in Fig.22. We confirmed the presence of transgenes in the established human iPSC lines (Fig. 22). RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results are shown in Fig. 23. Cells generated by OSK + GLIS1 expressed undifferentiated ESC marker genes including *Oct3/4*, *Sox2*, *Nanog*, and *Rex1* (Fig. 23). We next performed DNA microarray analyses. Total RNAs were labelled with Cy3 and were hybridized to a Whole Human Genome Microarray (Agilent) according to the manufacturer's protocol.

Arrays were scanned using the G2505C Microarray Scanner System (Agilent). Data were analysed using the GeneSpring GX11.0.1 software program (Agilent). The results are shown in Fig. 24. Cells established with OSK + GLIS1 were similar in global gene expression to iPSCs generated with OSKM (Fig. 24). We then performed teratoma formation as previously described (*Cell*, *131*(5), 861-872 (2007)). Cells generated by OSK + GLIS1 produced teratomas containing various tissues of all three germ layers (Fig. 25). These results demonstrated that GLIS1 strongly and specifically promoted the generation of human iPSCs by OSK.

Example 3: Expression and functional analysis of GLIS1

We then examined the expression pattern of GLIS1. The analyses of mouse expressed sequence tag (EST) databases predicted that GLIS1 representation was biased towards the zygote, especially in the fertilized ovum (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.331757; as of April 24, 2010). In addition, the Gene Expression Data provided by the MGI showed moderate GLIS1 expression in metaphase II oocytes and weak expression in the 2-cell embryo, and no expression was detected in the 8-cell to E4.5 embryos (http://www.informatics.jax.org/searches/expression.cgi?32989; as of April 24, 2010). These web-based analyses strongly indicated the specific expression of GLIS1 in oocytes and one-cell embryos. To experimentally confirm these findings, we isolated total RNAs from unfertilized eggs, 1-cell embryos, 2-cell embryos, and blastocysts, as well as from several adult mouse tissues including the kidney, placenta, brain, lung, liver, spleen, and ovary. In addition, we used total RNAs isolated from mouse ESCs, MEFs, and adult skin fibroblasts. The real-time PCR analyses detected the highest expression of GLIS1 in the one-cell embryos and unfertilized eggs (Fig. 26). Modest expression levels were detected in 2-cell embryos and

placental tissues (Fig. 26). Weak expression was present in several tissues including the kidney, ovary, ESCs, MEFs and skin fibroblasts (Fig. 26). These data confirmed that GLIS1 RNA is enriched in unfertilized eggs and one-cell embryos.

We next examined whether the endogenous GLIS1 in fibroblasts, although expressed at low levels, plays a role during iPSC generation by OSK. To this end, we constructed several retroviral vectors to express GLIS1 shRNA. The shRNA-mediated knockdown was performed as previously described (*Nature, 460*(7259), 1132-1135 (2009)). We found that shRNA2 (target sequence (positions 822-842 of SEQ ID NO:3): ggcctcaccaaccctgcacct; SEQ ID NO:13) and shRNA6 (target sequence (positions 1457-1477 of SEQ ID NO:3): gcccttcaatgcccgctacaa; SEQ ID NO:14) effectively suppressed GLIS1 when transfected into adult mouse skin fibroblasts, whereas shRNA4 (target sequence (positions 857-877 of SEQ ID NO:3): gggcaatgaacccatctcaga; SEQ ID NO:15) was less effective (Fig. 27, A paired *t*-test was used for the statistical analyses). We then introduced each of these shRNAs together with OSK into fibroblasts containing the Nanog-GFP reporter. We found that shRNA2 and shRNA6 significantly decreased the number of GFP-positive colonies (Fig. 28). A weaker effect was observed with shRNA4. This result suggests that the endogenous GLIS1 plays a supportive role during iPSC generation by OSK.

While the present invention has been described with emphasis on preferred embodiments, it is obvious to those skilled in the art that the preferred embodiments can be modified. The present invention intends that the present invention can be embodied by methods other than those described in detail in the present specification. Accordingly, the present invention encompasses all modifications encompassed in the gist and scope of the appended "CLAIMS."

In addition, the contents disclosed in any publication

cited herein, including patents and patent applications, are hereby incorporated in their entireties by reference, to the extent that they have been disclosed herein.

This application is based on U.S. provisional patent applications Nos. 61/305,107 and 61/379,949, the contents of which are hereby incorporated by reference.

CLAIMS

1. A method of producing an iPS cell, comprising culturing a somatic cell transfected with nucleic acids that

encode the following (1), (2), (3), and (4):

(1) GLIS1,

(2) Oct3/4,

(3) a member of the Klf family selected from the group consisting of Klf1, Klf2, Klf4, and Klf5,

(4) a member of the Sox family selected from the group consisting of Sox1, Sox2, Sox3, Sox15, and Sox17,

thereby producing an iPS cell.

2. The method according to claim 1, wherein the member of the Klf family is Klf4.

3. The method according to claim 1, wherein the somatic cell has been transfected with a further nucleic acid that encodes a reprogramming substance selected from the group consisting of a member of the Myc family, a member of the Lin28 family, and Nanog,

wherein the member of the Myc family is selected from the group consisting of c-Myc, L-Myc and N-Myc, and

wherein the member of the Lin28 family is selected from the group consisting of Lin28 and Lin28b.

4. The method according to claim 1, wherein the member of the Sox family is Sox2.

5. The method according to claim 1, wherein the member of the Myc family is c-Myc.

6. A method of producing a somatic cell, comprising the following (1) and (2):

(1) the step of producing an iPS cell by the method according to claim 1, and

(2) the step of treating the iPS cell obtained through the step

(1) above to induce it to differentiate into a somatic cell.