DESCRIPTION

NOVEL NUCLEAR REPROGRAMMING SUBSTANCE

Technical Field of the Invention

The present invention relates to a novel nuclear reprogramming substance and use thereof, more specifically to a novel nuclear reprogramming substance that can substitute for Klf4, and a method of establishing induced pluripotent stem (hereinafter referred to as iPS) cells using the same.

Background of the Invention

In recent years, mouse and human iPS cells have been established one after another. Yamanaka et al. identified genes expressed specifically in pluripotent cells such as ES cells and germ cells by analyzing the EST database, and conducted functional analyses using the knockout mouse technique and the like. Taking into consideration some reports by other research groups, they selected 24 genes as candidate substances that induce pluripotency (reprogram the nucleus) in somatic cells [WO 2007/069666 A1; Takahashi, K. and Yamanaka, S., Cell, 126: 663-676 (2006)]. They induced iPS cells by introducing these 24 genes into fibroblasts (MEF) from a reporter mouse wherein the neomycin resistance gene is knocked-in into the Fbx15 locus and forcing the cells to express these genes, by means of retrovirus. They proceeded to narrow the coverage of the genes that are most important to nuclear reprogramming by transferring 23 out of the 24 genes, and eventually identified the four genes Oct3/4, Sox2, Klf4 and c-Myc as essential factors for nuclear reprogramming in somatic cells [WO 2007/069666 A1; Takahashi, K. and Yamanaka, S., Cell, 126: 663-676 (2006)].

In addition, Yamanaka et al. succeeded in establishing iPS cells (Nanog iPS cells) that show almost the same gene expression and epigenetic modification profiles as those in embryonic stem (ES) cells and preparing a chimeric mouse, by producing a transgenic mouse wherein the green fluorescent

protein (GFP) and puromycin-resistance genes are integrated into the locus of Nanog, whose expression is more localized in pluripotent cells than Fbx15 expression, forcing MEF derived from the mouse to express the above-mentioned four genes, and selecting puromycin-resistant and GFP-positive cells [Okita, K. et al., Nature, 448: 313-317 (2007)]. Thereafter, it was revealed that iPS cells could also be produced with three factors other than the c-Myc gene, which also contributes to the germline of chimeric mouse [Nakagawa, M. et al., Nat. Biotethnol., 26: 101-106 (2008)].

Furthermore, Yamanaka et al. succeeded in establishing iPS cells by introducing the same four genes or three genes as those used in the mouse into human skin fibroblasts [WO 2007/069666 A1; Takahashi, K. et al., Cell, 131: 861-872 (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 introducing defined factors into somatic cells.

Of the four genes Oct3/4, Sox2, Klf4 and c-Myc, Oct3/4 and Sox2 are reportedly essential for maintaining self-renewal and pluripotency of ES cells, and c-Myc has also been reported to be involved in maintaining self-renewal and pluripotency of ES cells. Meanwhile, Klf4 belongs to the family of Krüppel-like factor (Klf), a transcriptional factor that controls various biological processes, including proliferation, differentiation, development, and apoptosis [McConnell, B.B. et al., Bioassays, 29: 549-557 (2007)], but details of its functions remain unclear. Epiblast stem cells (EpiSC) established from the epiblast of post-implantation embryo, unlike ES cells, are incapable of forming a chimeric embryo even when injected into a host blastocyst. In EpiSC, however, Oct3/4 and Sox2 are expressed at levels similar to those in ES cells, whereas the Klf4 gene is expressed at remarkably lower levels. Recently, it was reported that by transferring the Klf4 gene alone into EpiSC, a nature similar to that of ES cells can be acquired [Guo, G. et al., Development, 136: 1063-1069 (2009)].

Since ES cells exhibit no morphological changes even when Klf4 is knocked down by RNAi [Nakatake, Y. et al., Mol. Cell. Biol., 26: 7772-7782 (2006)], however, Klf4 may be unessential to the maintenance of the undifferentiated state of ES cells. Yamanaka et al. hypothesized that the same four genes 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)]. A group of Thomson et al. reported that human iPS cells could be generated using Nanog and Lin28 in place of Klf4 and c-Myc [WO 2008/118820 A2; Yu, J. et al., Science, 318: 1917-1920 (2007)]; the function of Klf4 can be thought to have many common aspects compared with Nanog.

When ES cells are treated with retinoic acid to induce their differentiation, not only expression of Klf4, but also expression of Klf2 and Klf5 decrease. Taking note of this fact, Jiang et al. 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 transcriptional factors and epigenetic regulatory factor, along with the three genes Oct3/4, Sox2 and c-Myc, into MEF; they confirmed that Klf2 and Klf5 can substitute for Klf4, and found that Esrrb, an orphan nuclear receptor similar to estrogen receptors, is also capable of substituting for Klf4 [Feng, B. et al., Nat. Cell Biol., 11: 197-203 (2009)].

Summary of the Invention

In finding clinical applications for iPS cells, it is of paramount importance to elucidate all details of the nuclear reprogramming mechanism. To identify unknown nuclear reprogramming substances that can substitute for existing

nuclear reprogramming substances of public knowledge is of great significance not only in helping elucidate the nuclear reprogramming mechanism, but also in developing a process for establishing iPS cells best suited for clinical applications. Accordingly, it is an object of the present invention to identify a novel nuclear reprogramming substance, particularly a novel nuclear reprogramming substance that can substitute for Klf4, and provide a novel method of establishing iPS cells using the same.

To accomplish the object, the present inventors performed comprehensive gene analysis that can be used to establish iPS cells as substitutes for Klf4, out of gene libraries not only of genes expressed specifically in pluripotent cells such as ES cells, but also of a broader range of transcriptional factors. As a result, the inventors found that iPS cells can be established efficiently when a gene belonging to the IRX family, a gene belonging to the GLIS family, a gene belonging to the PTX family or the DMRT-like family B with proline-rich C-terminal, 1 (DMRTB1) gene, along with the three genes Oct3/4, Sox2 and c-Myc, is transferred into adult mouse skin fibroblasts or MEF, identified these transcriptional factors as novel nuclear reprogramming substances that can functionally substitute for Klf4, and developed the present invention.

Accordingly, the present invention provides the following:

[1] A method of producing iPS cells, comprising the step of transferring into a somatic cell the following (1) and (2):

(1) one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same,

(2) a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

[2] The method according to [1] above, wherein the substances mentioned in (1) above include at least one kind of substance selected from the group consisting of iroquois homeobox protein

6 (IRX6), GLIS family zinc finger 1 (GLIS1), paired-like homeodomain transcription factor 2 isoform b (PITX2), DMRTB1 and nucleic acids that encode the same.

[3] The method according to [1] above, wherein the substance mentioned in (2) above is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Myc family, the Nanog and Lin families, and nucleic acids that encode the same.

[4] The method according to [1] above, wherein the substance mentioned in (2) above is Oct3/4.

[5] The method according to [1] above, wherein the substance mentioned in (2) above is Oct3/4 and Sox2.

[6] The method according to [1] above, wherein the substance mentioned in (2) above is Oct3/4 and c-Myc.

[7] The method according to [1] above, wherein the substance mentioned in (2) above is Oct3/4, Sox2 and c-Myc.

[8] An inducer of iPS cells from a somatic cell, comprising the following (1) and (2):

(1) one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same,

(2) a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

[9] The inducer according to [8] above, wherein the substances mentioned in (1) above include at least one kind of substance selected from the group consisting of IRX6, GLIS1, PITX2, DMRTB1 and nucleic acids that encode the same.

[10] An iPS cell containing an extraneous nucleic acid that encodes one or more kinds of factors selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family and DMRTB1.

[11] The iPS cell according to [10] above, containing an extraneous nucleic acid that encodes one or more kinds of factors selected from the group consisting of IRX6, GLIS1, PITX2

and DMRTB1.

[12] The iPS cell according to [10] above, wherein the at least one kind of extraneous nucleic acid is integrated in the genome.

[13] A method of producing somatic cells, comprising treating the iPS cell according to [10] above to induce the differentiation thereof into somatic cells.

[14] An inducer of iPS cells from a somatic cell, comprising one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same, wherein the inducer is transferred into a somatic cell, along with a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

[15] A use of one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same, for producing iPS cells, wherein the substance(s) is transferred into a somatic cell, along with a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

[16] A substance selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same, as an inducer of iPS cells from a somatic cell, wherein the substance is transferred into a somatic cell, along with a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

[17] A use of the iPS cell according to [10] above in producing somatic cells.

[18] The iPS cell according to [10] above as a source of cells in producing somatic cells.

According to the present invention, it has been shown that members of the IRX family, members of the GLIS family, members of the PTX family and DMRTB1 are capable of functionally substituting for Klf4 in nuclear reprogramming. Investigating

the mechanisms of actions of these nuclear reprogramming substances is expected to promote the elucidation of the nuclear reprogramming mechanism. The fact that genes other than those expressed specifically in ES cells have been newly identified as nuclear reprogramming substances by the present invention suggests that other nuclear reprogramming substances that are now unknown will be found in the future.

Brief Description of the Drawings

Figure 1 is a schematic diagram showing the steps to the selection of entry clones by function from the human Gateway® entry clone (N. Goshima et al., Nature methods, 2008).

Figure 2 shows a flow chart of generating a transcriptional factor expression library for somatic cell reprogramming factor screening from an entry clone of transcriptional factor.

Figure 3 is a photographic representation of the morphology of GFP-positive colonies obtained by transferring a total of 4 kinds of genes, i.e., 3 genes (Oct3/4, Sox2, c-Myc) and G06 (gene name: GLIS1), H08 (gene name: DMRTB1) or H10 (gene name: PITX2), into Nanog-GFP mouse skin fibroblasts by means of retrovirus. "Klf-G6-1" indicates an iPS cell clone obtained by transferring G06 (gene name: GLIS1) along with the 3 genes; "Klf-H8-2" indicates an iPS cell clone obtained by transferring H08 (gene name: DMRTB1) along with the 3 genes; "Klf-H10-1" and "Klf-H10" indicate iPS cell clones obtained by transferring H10 (gene name: PITX2) along with the 3 genes. P0 shows images taken at the time of colony establishment; P1 shows images for the 1st generation (24 wells); P2 shows images for the 2nd generation (6 wells). For each set of three photographs, the left panel shows an image of a GFP-positive colony, the central panel shows a phase-contrast image, and the right panel shows a superposed photograph of the GFP-positive colony image and phase-contrast image. Only Klf-H10-1 was established by the Reseed method, whereas the others were established by the MSTO method.

Figure 4 is a photographic representation of the morphology of GFP-positive colonies obtained by transferring a total of 4 kinds of genes, i.e., 3 genes (Oct3/4, Sox2, c-Myc) and F09 (gene name: IRX6), G06 (gene name: GLIS1), H08 (gene name: DMRTB1) or H10 (gene name: PITX2), into Nanog-GFP mouse skin 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 name: IRX6) along with the 3 genes; "Klf-G6-1" and "Klf-G6-2" indicate iPS cell clones obtained by transferring G06 (gene name: GLIS1) along with the 3 genes; "Klf-H8-1 and "Klf-H8-2" indicate iPS cell clones obtained by transferring H08 (gene name: DMRTB1) along with the 3 genes; "Klf-H10" indicates an iPS cell clone obtained by transferring H10 (gene 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.

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

Figure 6 is a photographic representation of the results of genomic-PCR on another H10 (Klf-H10) iPS cell clone other than that in Figure 5. In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 7 is a photographic representation of the results of RT-PCR on G6-1 (Klf-G6-1), H8-2 (Klf-H8-2) and H10 (Klf-H10) iPS cell clones. 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.

Figure 8 is a photographic representation of the results of RT-PCR on another 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.

Figure 9 is a graphic representation of the results of counting the number of colonies of iPS cells (GFP-positive cells) established by transferring a combination of 3 factors (Oct3/4, Sox2, c-Myc) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse skin fibroblasts. The results of six independent experiments are summarized.

Figure 10 is a graphic representation of the results of counting the number of colonies of iPS cells (GFP-positive cells) established by transferring a combination of 2 factors (Oct3/4, Sox2) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse skin fibroblasts. The results of two independent experiments are summarized.

Figure 11 is a photographic representation of colonies of iPS cells established by transferring a combination of 3 factors (Oct3/4, Sox2, c-Myc) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse skin fibroblasts, and colonies of iPS cells established by transferring a combination of 2 factors (Oct3/4, Sox2) and G6 (GLIS1) or H8 (DMRTB1) into Nanog-GFP mouse skin fibroblasts. P0 shows images taken at the time of colony establishment; P1 shows images for the 1st generation; P2 shows images for the 2nd generation. For each set of three photographs, the left panel shows an image of a GFP-positive colony, the central panel shows a phase-contrast image, and the right panel shows a superposed photograph of the GFP-positive colony image and phase-contrast image.

Figure 12 is a photographic representation of the results of genomic-PCR on the iPS cell clones shown in Figure 11. In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 13 is a photographic representation of the results

of RT-PCR on the iPS cell clones shown in Figure 11. In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "ES" and "iPS" indicate mouse ES cells and iPS cells established using 4 factors (Oct3/4, Sox2, c-Myc, Klf4).

Figure 14 is a photographic representation of colonies of iPS cells established by transferring a combination of 2 factors (Oct3/4,c-Myc) and H10 (PITX2) into Nanog-GFP mouse skin fibroblasts. P0 shows images taken at the time of colony establishment; P1 shows images for the 1st generation; P2 shows images for the 2nd generation. For each set of three photographs, the left panel shows a GFP-positive colony image, the central panel shows a phase-contrast image, and the right panel shows a superposed photograph of the GFP-positive colony image and phase-contrast image.

Figure 15 is a photographic representation of the results of genomic-PCR on the iPS cell clones shown in Figure 14. In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 16 is a photographic representation of the results of RT-PCR on the iPS cell clones shown in Figure 14. In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "ES" and "iPS" indicate mouse ES cells and iPS cells established using 4 factors (Oct3/4, Sox2, c-Myc, Klf4).

Figure 17 is a graphic representation of the results of counting the number of colonies of iPS cells (GFP-positive cells) established by transferring a combination of 3 factors (Oct3/4, Sox2, c-Myc) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse MEF. The results of four independent experiments are summarized.

Figure 18 is a graphic representation of the results of counting the number of colonies of iPS cells (GFP-positive cells) established by transferring a combination of 2 factors

(Oct3/4, Sox2) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse MEF. The number of colonies established in 2(OS)+H10 was 1 for the MSTO method and 1 for the Reseed method.

Figure 19 is a photographic representation of colonies of iPS cells established by transferring a combination of 3 factors (Oct3/4, Sox2, c-Myc) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse MEF, and colonies of iPS cells established by transferring a combination of 2 factors (Oct3/4, Sox2) and G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2) into Nanog-GFP mouse MEF. P0 shows images taken at the time of colony establishment; P1 shows images for the 1st generation; P2 shows images for the 2nd generation. The photographs of OS+G6, OS+H8, and OS+H10 are as of P0. For each set of three photographs, the left panel shows a GFP-positive colony image, the central panel shows a phase-contrast image, and the right panel shows a superposed photograph of the GFP-positive colony image and phase-contrast image.

Figure 20 is a photographic representation of the results of genomic-PCR on the iPS cell clones shown in Figure 19 (3 factors + G6, H8 or H10). In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 21 is a photographic representation of the results of RT-PCR on the iPS cell clones shown in Figure 19 (3 factors + G6, H8 or H10). In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "ES" and "iPS" indicate mouse ES cells and iPS cells established using 4 factors (Oct3/4, Sox2, c-Myc, Klf4).

Figure 22 is a photographic representation of the results of genomic-PCR on teratomas prepared by subcutaneous injection of iPS clones established from adult mouse (Nanog-GFP mouse) skin fibroblasts (G6-1 clone, G6-6 clone, H8-2 clone, H10 clone) into immunodeficient mice. In this figure, "skin" indicates the fibroblast used as a source of somatic cells, and "plasmid"

indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 23 shows histologically stained images (hematoxylin-eosin stain) of teratomas prepared by subcutaneous injection of G6-1 clone or G6-6 clone into immunodeficient mice.

Figure 24 shows histologically stained images (hematoxylin-eosin stain) of teratomas prepared by subcutaneous injection of H8-2 clone or H10 clone into immunodeficient mice.

Figure 25 shows a scatter plot showing the results of DNA microarray analyses performed to determine whether or not a difference in gene expression pattern is present between K-G6 and MEF, or between K-G6 and 4F gene (fold change line: 2-fold).

Figure 26 shows a scatter plot showing the results of DNA microarray analyses performed to determine whether or not a difference in gene expression pattern is present between K-H8 and MEF, or between K-H8 and 4F (fold change line: 2-fold).

Figure 27 shows a scatter plot showing the results of DNA microarray analyses performed to determine whether or not a difference in gene expression pattern is present between K-H10 and MEF, or between K-H10 and 4F gene (fold change line: 2-fold).

Figure 28 shows the results of clustering performed on the basis of the correlation coefficient between respective cells.

Figure 29 is a photographic representation of a colony image and alkaline phosphatase stain image of iPS cells established by transferring 3 genes (Oct3/4, Sox2, c-Myc) and G6 (GLIS1) into HDF. For control, a colony image of iPS cells established with 4 genes (Oct3/4, Sox2, c-Myc, Klf4) is also shown.

Figure 30 is a photographic representation of a 2nd-generation colony of iPS cells established by transferring 3 genes (Oct3/4, Sox2, c-Myc) and G6 (GLIS1) into HDF. For control, a colony image of iPS cells established with 4 genes (Oct3/4, Sox2, c-Myc, Klf4) is also shown.

Figure 31 is a photographic representation of the results of genomic-PCR on an iPS cell clone established by transferring

3 genes (Oct3/4, Sox2, c-Myc) and G6 (GLIS1) into HDF. In this figure, "4 factors" indicates an iPS cell established with 4 genes (Oct3/4, Sox2, c-Myc, Klf4), "AHDF" indicates the adult skin fibroblasts used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated in pMXs.

Figure 32 is a photographic representation of the results of RT-PCR on an iPS cell clone established by transferring 3 genes (Oct3/4, Sox2, c-Myc) and G6 (GLIS1) into HDF. In this figure, "4 factors" and "AHDF" are identical to those in Figure 31, "KhES1" indicates human ES cells, and "201B7" indicates iPS cells established in the past [Cell, 131:861-872 (2007)].

Figure 33 is a photographic representation of colony images and alkaline phosphatase stain images of iPS cells established by transferring 2 genes (Oct3/4, Sox2) and H8 (DMRTB1), or 3 genes (Oct3/4, Sox2, c-Myc) and H10 (PITX2), into DP31. For control, a colony image of iPS cells established with 4 genes (Oct3/4, Sox2, c-Myc, Klf4) is also shown.

Figure 34 is a photographic representation of a 1st-generation colony of iPS cells established by transferring 2 genes (Oct3/4, Sox2) and H8 (DMRTB1) into DP31.

Figure 35 is a photographic representation of the results of genomic-PCR on an iPS cell clone established by transferring 2 genes (Oct3/4, Sox2) and H8 (DMRTB1) into DP31. In this figure, "DP31" indicates the dental pulp stem cell clone used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 36 is a photographic representation of the results of RT-PCR on an iPS cell clone established by transferring 2 genes (Oct3/4, Sox2) and H8 (DMRTB1) into DP31. In this figure, "DP31" indicates the dental pulp stem cell clone used as a source of somatic cells, "hES" indicates human ES cells, and "201B7" indicates iPS cells established in the past [Cell, 131:861-872 (2007)].

Figure 37 is a photographic representation of a 1st-

generation colony of iPS cells established by transferring 3 genes (Oct3/4, Sox2, c-Myc) and H10 (PITX2) into DP31.

Figure 38 is a photographic representation of the results of genomic-PCR on an iPS cell clone established by transferring 3 genes (Oct3/4, Sox2, c-Myc) and H10 (PITX2) into DP31. In this figure, "DP31" indicates the dental pulp stem cell clone used as a source of somatic cells, and "plasmid" indicates positive controls prepared by amplifying each gene incorporated into pMXs.

Figure 39 is a photographic representation of the results of RT-PCR on an iPS cell clone established by transferring 3 genes (Oct3/4, Sox2, c-Myc) and H10 (PITX2) into DP31. In this figure, "DP31" indicates the dental pulp stem cell clone used as a source of somatic cells, "hES" indicates human ES cells, and "201B7" indicates iPS cells established in the past [Cell, 131:861-872 (2007)].

Figure 40 shows a histologically stained image (hematoxylin-eosin stain) of a teratoma prepared by injecting an iPS cell clone established with 3 genes (Oct3/4, Sox2, c-Myc) and G6 (GLIS1) into an Scid mouse testis.

Detailed Description of the Invention

The present invention provides a novel nuclear reprogramming substance that can substitute for Klf4 and a method of producing iPS cells by transferring the substance and a nuclear reprogramming substance capable of inducing iPS cells from a somatic cell when combined with Klf4, into a somatic cell.

(a) Novel nuclear reprogramming substance (substitute for Klf4)

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 incorporated in a vector), or a low-molecular compound. A nuclear reprogramming substance that can substitute for Klf4, identified by the present invention, is a protein out of members of the IRX family, members of the GLIS

family, members of the PTX family and DMRT-like family B with proline-rich C-terminal, 1 (DMRTB1), or a nucleic acid that encodes the same.

The IRX (iroquois homeobox) family has a homeobox domain, and is thought to play a multiple role during the pattern formation process in the vertebral embryo. Examples of members of this gene family include, but are not limited to, iroquois homeobox protein 1 (IRX1), IRX2, IRX3, IRX4, IRX5, IRX6 and the like, with preference given to IRX6. IRX6 is a gene not expressed in human and mouse ES cells.

The GLIS family comprises transcriptional factors having five C2H2 type zinc finger regions, and controlling the expression of various genes in the process of embryogenesis positively or negatively. 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. GLIS1 is a gene not expressed in mouse ES cells.

The PTX family has a homeobox domain, and is involved in organogenesis and determination of lateral asymmetry. Examples of members of this gene family include, but are not limited to, paired-like homeodomain transcription factor 1 (PITX1), PITX2, PITX3 and the like, with preference given to PITX2. PITX2 is known to occur in three isoforms (isoforms a, b and c). Although any isoform can be used, isoform b, for example, is preferably used.

DMRT-like family B with proline-rich C-terminal, 1 (DMRTB1) is a transcriptional factor of unknown function having a doublesex DNA binding motif. DMRTB1 is a gene not expressed in human and mouse ES cells.

Although the members of the IRX family, members of the GLIS family, members of the PTX family and DMRTB1 used in the present invention may be proteins derived from optionally chosen mammals (e.g., humans, mice, rats, monkeys, bovines, horses, pigs, dogs and the like) or nucleic acids that encode the same, proteins or nucleic acids of human or mouse origin are preferred.

Information on the amino acid sequences and cDNA sequences of the above-described nuclear reprogramming substances of human or mouse origin can be acquired by referring 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 name	Human		Mouse	
	cDNA	Protein	cDNA	Protein
IRX1	NM_024337	NP_077313	NM_010573	NP_034703
IRX2	NM_033267	NP_150366	NM_010574	NP_034704
IRX3	NM_024336	NP_077312	NM_008393	NP_032419
IRX4	NM_016358	NP_057442	NM_018885	NP_061373
IRX5	NM_005853	NP_005844	NM_018826	NP_061296
IRX6	NM_024335 (SEQ ID NO:1)	NP_077311 (SEQ ID NO:2)	NM_022428 (SEQ ID NO:3)	NP_071873 (SEQ ID NO:4)
GLIS1	NM_147193 (SEQ ID NO:5)	NP_671726 (SEQ ID NO:6)	NM_147221 (SEQ ID NO:7)	NP_671754 (SEQ ID NO:8)
GLIS2	NM_032575	NP_115964	NM_031184	NP_112461
GLIS3	NM_001042413	NP_001035878	NM_175459	NP_780668
PITX1	NM_002653	NP_002644	NM_011097	NP_035227
PITX2 (isoform a)	NM_153427	NP_700476	NM_001042504	NP_001035969
PITX2 (isoform b)	NM_153426 (SEQ ID NO:9)	NP_700475 (SEQ ID NO:10)	NM_011098 (SEQ ID NO:11)	NP_035228 (SEQ ID NO:12)
PITX2 (isoform c)	NM_000325	NP_000316	NM_001042502	NP_001035967
PITX3	NM_005029	NP_005020	NM_008852	NP_032878
DMRTB1	NM_033067 (SEQ ID NO:13)	NP_149056 (SEQ ID NO:14)	XM_205469 (SEQ ID NO:15)	XP_205469 (SEQ ID NO:16)

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 a potential for nuclear reprogramming as a substitute for Klf4 equivalent to that of the wild-type protein and a nucleic acid that encodes the same, can

also be utilized as the nuclear reprogramming substance of the present invention that can substitute for Klf4.

Out of members of the IRX family, members of the GLIS family, members of the PTX family and DMRTB1 (including nucleic acids that encode the same), any one kind alone may be used, and two kinds or more may be used in combination.

(b) Nuclear reprogramming substance capable of inducing iPS cells when combined with Klf4

Currently, the following combinations of nuclear reprogramming substances comprising Klf4 are known to be capable of inducing iPS cells from a somatic cell (hereinafter, only the names of protein factors are given).

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

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

(3) Oct3/4, Klf4, c-Myc, Sox2, Fbx15, Nanog, Eras, ECAT15-2, Tcl1, β-catenin (active mutant S33Y)

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

(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 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, Klf4, c-Myc, Sox2, hTERT, SV40 Large T", see also *Nature, 451,* 141-146 (2008).]

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

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

(11) Oct3/4, Klf4, c-Myc, Sox2, SV40 Large T (see also *Stem*

Cells, 26, 1998-2005 (2008))

(12) Oct3/4, Klf4 [see also *Nature, 454,* 646-650 (2008); *Cell Stem Cell, 2*: 525-528 (2008)]

(13) Oct3/4, Klf4, L-Myc

Therefore, preferable combinations of nuclear reprogramming substances capable of inducing iPS cells when combined with Klf4 are combinations of the same substances as (1)-(13) above, but excluding Klf4, i.e.,

(i) Oct3/4, c-Myc

(ii) Oct3/4, c-Myc, Sox2 (Sox2 is replaceable with Sox1, Sox3, Sox15, Sox17 or Sox18; c-Myc is replaceable with T58A (active mutant), N-Myc, or L-Myc)

(iii) Oct3/4, c-Myc, Sox2, Fbx15, Nanog, Eras, ECAT15-2, TclI, β-catenin (active mutant S33Y)

(iv) Oct3/4, c-Myc, Sox2, TERT, SV40 Large T

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

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

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

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

(ix) Oct3/4, Sox2

(x) Oct3/4, c-Myc, Sox2, Nanog, Lin28

(xi) Oct3/4, c-Myc, Sox2, SV40 Large T

(xii) Oct3/4, and

(xiii) Oct3/4, L-Myc. With regard to (i)-(xiii) above, Oct3/4 can be replaced with other members of the Oct family, e.g., Oct1A, Oct6 and the like. Furthermore, Sox2 (or Sox1, Sox3, Sox15, Sox17, Sox18) can be replaced with other members of the Sox family, e.g., Sox7 and the like.

Judging from these facts combined together, a nuclear reprogramming substance capable of inducing iPS cells when combined with Klf4 is preferably selected from among members of the Oct family (e.g., Oct3/4, Oct1A, Oct6), members of the Sox family (e.g., Sox2, Sox1, Sox3, Sox7, Sox15, Sox17, Sox18), members of the Myc family (e.g., c-Myc, n-Myc, L-Myc), and the Nanog and members of Lin families (e.g., Lin28, Lin28b). More

preferably, a combination that comprises at least Oct3/4 and may optionally further comprise Sox2 and/or c-Myc [i.e., any one of (a) Oct3/4, (b) Oct3/4+Sox2, (c) Oct3/4+c-Myc, and (d) Oct3/4+Sox2+c-Myc] can be used, and this may be used in further combination with Nanog and/or Lin28. Here, a combination comprising L-Myc in place of c-Myc also represents a preferred embodiment.

Any combination other than (i) to (xiii) above but comprising all the constituents of any one thereof and further comprising any other optionally chosen substance can also be included in the scope of "nuclear reprogramming substances" in the present invention. For example, members of the Klf family (e.g., Klf1, Klf2, Klf5) or other known substitutional factors (e.g., member of the Esrr family such as Esrrb, Esrrg and the same) may be used in combination as other substances. Provided that the somatic cells to undergo nuclear reprogramming express one or more of the constituents of any one of (i) to (xiii) above endogenously at a level sufficient to cause nuclear reprogramming, the combination of the remaining constituents only, excluding the one or more constituents expressed can also be included in the scope of "nuclear reprogramming substances capable of inducing iPS cells when combined with Klf4" in the present invention.

Out of these combinations, a combination of the 2 factors Oct3/4 and Sox2 [i.e., (ix) above] or a combination of the 3 factors Oct3/4, Sox2 and L-Myc [i.e., (ii) above] is preferred with the use of the resulting iPS cells for therapeutic purposes in mind. Meanwhile, when the use of the iPS cells for therapeutic purposes is not in mind (e.g., use as a research tool such as for drug discovery screening, and the like), a combination of the four factors Oct3/4, c-Myc (or L-Myc), Sox2 and Lin28, or a combination of 5 factors consisting of the same 4 factors and Nanog [i.e., (x) above] is preferred.

Information on the mouse and human cDNA sequences of the aforementioned individual proteinous factors is available with

reference to the NCBI accession numbers mentioned in WO 2007/069666 (in the publication, Nanog is described as ECAT4). Information on mouse and human cDNA sequences of Lin28 can be acquired with reference to NCBI accession number NM_145833 and NM_024674, respectively. Information on mouse and human cDNA sequences of Lin28b can be acquired with reference to NCBI accession number NM_001031772 and NM_001004317, respectively; information on mouse and human cDNA sequences of L-Myc can be acquired with reference to NCBI accession number NM_008506 and NM_001033081, respectively; those skilled in the art are able to easily isolate these cDNAs. When used as the nuclear reprogramming substance, the protein factor itself can be prepared by inserting the cDNA obtained into an appropriate expression vector, transferring the vector into a host cell, culturing the cell, and recovering a recombinant proteinous factor from the resulting culture. Meanwhile, when the nuclear reprogramming substance used is a nucleic acid that encodes a proteinous factor, the cDNA obtained is inserted into a viral or plasmid vector to construct an expression vector, and the vector is subjected to the step of nuclear reprogramming.

Nuclear reprogramming substances capable of inducing iPS cells when combined with Klf4 include not only combinations of the above-described conventionally known proteinous factors or nucleic acids that encode the same, but also combinations of proteinous factors that will be newly discovered or nucleic acids that encode the same, and can further include combinations comprising a non-proteinous factor such as a low-molecular compound, as far as it is capable of converting a somatic cell to an iPS cell when introduced into the somatic cell along with Klf4.

(c) Sources of somatic cells

Any cells, but other than germ cells, of mammalian origin (e.g., humans, mice, monkey, swine, rat 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 thereof (tissue progenitor cells) and the like. There is no limitation on the degree of cell differentiation, age of 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 individual mammal 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, that somatic cells are patient's own cells or collected from another person (donor) having the same or substantially the same HLA type as that of the patient. Here, the statement that the HLA type is "substantially the same" means that there is an agreement of the HLA types to the extent

that allows a cell graft to survive in a patient receiving cells obtained by inducing differentiation from the somatic cell-derived iPS cell, transplanted with the use of an immunosuppressant and the like. Examples include cases where the primary HLA types (e.g., 3 loci HLA-A, HLA-B and HLA-DR) are the same and the like (the same applies below). 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 desirable to collect the somatic cells from the patient or another person with the same genetic polymorphism correlating with the drug susceptibility or adverse reactions.

Somatic cells separated from a mammal such as mouse or human can be pre-cultured using a medium known per se suitable for the cultivation thereof, depending on the kind of the cells. Examples of such media include, but are not limited to, a minimal essential medium (MEM) comprising about 5 to 20% fetal calf serum, 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 cell into contact with a nuclear reprogramming substance (and, as required, also with the iPS cell establishment efficiency improver described below), it is sometimes preferable to exchange the medium with a serum-free medium in order to prevent transfer efficiency reductions.

(d) How to transfer nuclear reprogramming substance into somatic cell

Transfer of the "nuclear reprogramming substance that can substitute for Klf4" described in (a) above and the "nuclear reprogramming substance capable of inducing iPS cells when combined with Klf4" described in (b) above into a somatic cell can be performed using a method of protein transfer into cells which is known per se, provided that the substance is a proteinous factor. 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 (Genlantis), Pro-Ject™ Protein Transfection Reagent (PIERCE), PULSin™ delivery reagent (Polyplus-transfection) 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), Chariot Kit (Active Motif), and GenomONE (Ishihara Sangyo), which employs HVJ envelop (inactivated Sendai virus), and the like. The transfer can be achieved per the protocols attached to these reagents, a common procedure being as described below. Nuclear reprogramming substance(s) is(are) diluted in an appropriate solvent (e.g., a buffer solution such as PBS or HEPES), a transfer reagent is added, the mixture is incubated at room temperature for about 5 to 15 minutes to form a complex, this complex is added to cells after exchanging the medium with a serum-free medium, and the cells are incubated at 37°C for one to several hours. Thereafter, the medium is removed and replaced with a serum-containing medium.

Developed PTDs include those using transcellular domains of proteins such as drosophila-derived AntP, HIV-derived TAT (Frankel, A. et al, Cell 55, 1189-93 (1988); Green, M. & 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. PTD-derived CPPs include polyarginines such as 11R [Cell Stem Cell, 4:381-384(2009)] and 9R [Cell Stem Cell, 4:472-476(2009)].

A fusion protein expression vector incorporating a cDNA of a nuclear reprogramming substance and a PTD or CPP sequence is prepared to allow the recombinant expression of the fusion protein, and the fusion protein is recovered for use in for transfer. This transfer can be achieved as described above, except that no protein transfer reagent is 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 transfer 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 transfer operation can be performed twice or more (e.g., 3 times or 4 times) repeatedly. The time interval for repeated transfer is, for example, 6 to 48 hours, preferably 12 to 24 hours.

If emphasis is placed on iPS cell establishment efficiency, it is preferable to use the nuclear reprogramming substance in the form of a nucleic acid that encodes the same, rather than as the proteinous factor itself. The nucleic acid may be a DNA, an RNA, or a DNA/RNA chimera, and may be double-stranded or single-stranded. Preferably, the nucleic acid is a double-stranded DNA, particularly cDNA.

A cDNA of a nuclear reprogramming substance 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 kind of vector used can be chosen as appropriate according to the intended use of the iPS cells obtained. Useful vectors include, for example, adenovirus vectors, plasmid vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, Sendai virus vectors and the like.

Examples of promoters used in expression vectors include the EF1alpha 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 EF1alpha 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 useful selectable marker genes include the dihydrofolate reductase gene, the neomycin resistant gene, the puromycin resistant gene and the like.

The nucleic acids as nuclear reprogramming substances (reprogramming genes) may be separately integrated into different expression vectors, or 2 kinds or more, preferably 2 to 3 kinds, of genes may be incorporated into a single expression vector. Preference is given to the former case with the use of a retrovirus or lentivirus vector, which offer high gene transfer efficiency, and to the latter case with the use of a plasmid, adenovirus, or episomal vector and the like. Furthermore, an expression vector incorporating two kinds or

more of genes and another expression vector incorporating one gene alone can be used in combination.

In the context above, when a plurality of genes are incorporated in one expression vector, these genes can preferably be inserted into the expression vector via an intervening sequence enabling polycistronic expression. By using an intervening sequence enabling polycistronic expression, it is possible to more efficiently express a plurality of genes incorporated in one kind of expression vector. Useful sequences enabling polycistronic expression include, for example, the 2A sequence of foot-and-mouth disease virus (SEQ ID NO:2; PLoS ONE 3, e2532, 2008, Stem Cells 25, 1707, 2007), IRES sequence (U.S. Patent No. 4,937,190) and the like, with preference given to the 2A sequence.

An expression vector harboring a nucleic acid as a nuclear reprogramming substance can be introduced into a cell by a technique known per se according to the choice of the 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 cells) 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, it is preferable that the reprogramming gene be expressed transiently, without being integrated into the chromosome of the cells because the expression (reactivation) of the reprogramming gene possibly increases the risk of carcinogenesis in the tissues regenerated from a differentiated cell from an iPS cell. From this viewpoint, use of an adenoviral vector, whose integration into chromosome is rare, is preferred. Specific means using an adenoviral vector

is disclosed in *Science, 322*, 945-949 (2008). Because adeno-associated virus 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 Sendai viral vector, one described in *J. Biol. Chem., 282*, 27383-27391 (2007), *Proc. Jpn. Acad., Ser. B 85*, 348-362(2009) or JP Patent No. 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 that encodes a nuclear reprogramming substance is cut out using the Cre/loxP system, when it has become unnecessary. That is, with a loxP sequence 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 Chang et al., *Stem Cells, 27*: 1042-1049 (2009).

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 or adenovirus vector or the like is used, gene transfer can be performed once or more optionally chosen times (e.g., once to 10 times, or once to 5 times). 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 preferred non-recombination type vector is an episomal vector autonomously replicable outside the chromosome. A specific procedure for using an episomal vector is disclosed by Yu et al. in *Science, 324*, 797-801 (2009). As required, an

expression vector may be constructed by inserting a reprogramming gene into an episomal vector having loxP sequences placed in the same orientation at both the 5′ and 3′ sides of the vector element essential for the replication of the episomal vector, and this may be transferred into a somatic cell.

Examples of the episomal vector include vectors comprising a sequence required for its autonomous replication, derived from EBV, SV40 and the like, as a vector element. Specifically, the vector element required for its autonomous replication is a replication origin or a gene that encodes a protein that binds to the replication origin to regulate its replication; examples include the replication origin oriP and EBNA-1 gene for EBV, and the replication origin ori and SV40 large T antigen gene for SV40.

The episomal expression vector contains a promoter that controls the transcription of the reprogramming gene. The promoter used can be the same promoter as the above. The episomal expression vector may further comprise an enhancer, poly-A addition signal, selection marker gene and the like as desired, as described above. Examples of selection marker gene include the dihydrofolate reductase gene, neomycin resistance gene and the like.

Examples of loxP sequences that can be used in the present invention include a bacteriophage P1-derived wild-type loxP sequence (SEQ ID NO:17), and an optionally chosen mutant loxP sequence capable of deleting the sequence between the loxP sequences by recombination when placed in the same orientation at positions sandwiching the vector element essential for the replication of the reprogramming gene. Examples of mutant loxP sequences include lox71 (SEQ ID NO:18), which has a mutation in the 5′ side repeat sequence, lox66 (SEQ ID NO:19), which has a mutation in the 3′ side repeat sequence, lox2272, lox511 and the like which have a mutation in the spacer moiety thereof. Although the two loxP sequences placed on the 5′ and 3′ sides of the vector element may be identical or not, an identical one

(e.g., a pair of lox2272 sequences, a pair of lox511 sequences) is used in the case of mutant loxP sequences having a mutation in the spacer region thereof. Preferably, a combination of a mutant loxP sequence having a mutation in the 5′ side repeat sequence (e.g., lox71) and a mutant loxP sequence having a mutation in the 3′ side repeat sequence (e.g., lox66) is used. In this case, the loxP sequence remaining on the chromosome after the recombination has double mutations in the repeat sequences on the 5′ and 3′ sides, and is therefore unlikely to be recognized by Cre recombinase; therefore, the risk of causing a deletion mutation in the chromosome due to unwanted recombination is reduced. When both lox71 and lox66 are used, either mutant loxP sequence may be placed on the 5′ and 3′ sides of the aforementioned vector element, but it is necessary that the mutant loxP sequence be inserted in the orientation such that the mutated site is located at the outer end of the loxP sequence.

The two loxP sequences are placed in the same orientation on the 5′ and 3′ sides of a vector element essential for the replication of the reprogramming gene (i.e., replication origin, or a gene sequence that binds to the replication origin to regulate its replication). The vector element between the loxP sequences may be either a replication origin, or a gene sequence that encodes a protein that binds to the replication origin to regulate its replication, or both.

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

Whether or not the vector element required for replication of reprogramming gene has been removed from the iPS cell can be determined by performing Southern blot analysis or PCR analysis using a nucleic acid comprising a base sequence inside the

vector element and/or in the vicinity of the loxP sequence as a probe or primer, with an episome fraction isolated from the iPS cell as the template, to examine for the presence or absence of a band or the length of the band detected. An episome fraction can be prepared using a method well known in the art, for example, the method described in *Science, 324*: 797-801 (2009).

When the nuclear reprogramming substance capable of inducing iPS cell by combination with Klf4 is a low-molecular compound, introducing thereof into a somatic cell can be achieved by dissolving the substance 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 mouse [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 combinations thereof, 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.

(e) iPS cell establishment efficiency improvers

In recent years, a wide variety of substances that improve the efficiency of establishment of iPS cells, which has traditionally been low, have been proposed one after another. Therefore, in addition to the above-described nuclear reprogramming substance, the efficiency of establishment of iPS cell can be expected to be increased by bringing these iPS cell establishment efficiency improvers into contact with a somatic

cell.

Examples of iPS cell establishment efficiency improvers include, but are not limited to, histone deacetylase (HDAC) inhibitors [e.g., valproic acid (VPA) (*Nat. Biotechnol., 26*(7): 795-797 (2008)], low-molecular inhibitors such as trichostatin A, sodium butyrate, MC 1293, and M344, nucleic acid-based expression inhibitors such as siRNAs and shRNAs against HDAC (e.g., HDAC1 siRNA Smartpool® (Millipore), HuSH 29mer shRNA Constructs against HDAC1 (OriGene) and the like), and the like], DNA methyltransferase inhibitors (e.g., 5'-azacytidine) [*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 and shRNA against p53 (*Cell Stem Cell, 3*, 475-479 (2008)), UTF1 [*Cell Stem Cell, 3*, 475-479 (2008)], Wnt Signaling inducers (e.g., soluble Wnt3a) [*Cell Stem Cell, 3*, 132-135 (2008)], 2i/LIF [2i is an inhibitor of mitogen-activated protein kinase signaling and glycogen synthase kinase-3, *PloS Biology, 6*(10), 2237-2247 (2008)], ES cell-specific miRNAs [e.g., miR-302-367 cluster (Mol. Cell. Biol. doi:10.1128/MCB.00398-08), miR-302 (RNA (2008) 14: 1-10), miR-291-3p, miR-294 and miR-295 (these three described in 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.

Among the constituents of the aforementioned nuclear reprogramming substances, SV40 large T and the like, for example, can also be included in the scope of iPS cell establishment efficiency improvers because they are deemed not essential, but auxiliary, factors for somatic cell nuclear reprogramming. In the situation of the mechanisms for nuclear programming

remaining unclear, the auxiliary factors, which are not essential for nuclear reprogramming, may be conveniently considered as nuclear reprogramming substances or iPS cell establishment efficiency improvers. Hence, because the somatic cell nuclear reprogramming process is understood as an overall event resulting from contact of nuclear reprogramming substance(s) and iPS cell establishment efficiency improver(s) with a somatic cell, it seems unnecessary for those skilled in the art to always distinguish between the nuclear reprogramming substance and the iPS cell establishment efficiency improver.

Contact of an iPS cell establishment efficiency improver with a somatic cell can be achieved as described above for each of cases: (a) the improver is a proteinous factor, (b) the improver is a nucleic acid that encodes the proteinous factor, and (c) the improver is a low-molecular compound.

An iPS cell establishment efficiency improver may be brought into contact with a somatic cell simultaneously with a nuclear reprogramming substance, or either one may be contacted in advance, as far as the efficiency of establishment of iPS cells from the somatic cell is significantly improved, compared with the absence of the improver. 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 after 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 non-viral vector, for example, both may be simultaneously introduced into the cell.

(f) Improving the establishment efficiency by culture conditions

The efficiency of establishment of iPS cells can be further improved by culturing the somatic cells therefor under hypoxic conditions in the step of nuclear reprogramming of the cells. The term hypoxic conditions as used herein means that the oxygen concentration in the ambient atmosphere during cell culture is significantly lower than that in the air. Specifically, such conditions include lower oxygen concentrations than the oxygen concentrations in the ambient atmosphere of 5-10% CO_2/95-90% air, which is commonly used for ordinary cell culture; for example, oxygen concentrations of 18% or less in the ambient atmosphere are applicable. Preferably, the oxygen concentration in the ambient atmosphere is 15% or less (e.g., 14% or less, 13% or less, 12% or less, 11% or less and the like), 10% or less (e.g., 9% or less, 8% or less, 7% or less, 6% or less and the like), or 5% or less (e.g., 4% or less, 3% or less, 2% or less and the like). The oxygen concentration in the ambient atmosphere is preferably 0.1% or more (e.g., 0.2% or more, 0.3% or more, 0.4% or more and the like), 0.5% or more (e.g., 0.6% or more, 0.7% or more, 0.8% or more, 0.9% or more and the like), or 1% or more (e.g., 1.1% or more, 1.2% or more, 1.3% or more, 1.4% or more and the like).

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

The timing of beginning cell culture under hypoxic conditions is not particularly limited, as far as it does not interfere with improving the efficiency of establishment of iPS cells compared with that obtained at a normal oxygen concentration (20%). The starting time may be before or after

contact of nuclear reprogramming substances with a somatic cell, and may be at the same time as the contact. For example, it is preferable that cell culture under hypoxic conditions be begun just after contacting a nuclear reprogramming substance with a somatic cell, or after a given time (e.g., 1 to 10 (e.g., 2, 3, 4, 5, 6, 7, 8 or 9) days) following the contact.

The duration of cell culture under hypoxic conditions is not particularly limited, as far as it does not interfere with improving the efficiency of establishment of iPS cells compared with that obtained at a normal oxygen concentration (20%); examples include, but are not limited to, between 3 days or more, 5 days or more, 7 days or more or 10 days or more, and 50 days or less, 40 days or less, 35 days or less or 30 days or less. The preferred duration of cell culture under hypoxic conditions also varies depending on the oxygen concentration in the ambient atmosphere; those skilled in the art can adjust as appropriate the duration of cell culture according to the oxygen concentration used. In an embodiment of the present invention, when iPS cell candidate colonies are selected with drug resistance as an indicator, it is preferable that a normal oxygen concentration be restored from hypoxic conditions by the start of drug selection.

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

After the nuclear reprogramming substance(s) (and iPS cell establishment efficiency improver(s)) is(are) brought into contact with the cell, the cell can be cultured under conditions suitable for the cultivation of, for example, ES cells. In the case of mouse cells, the cultivation is carried out with the addition of Leukemia Inhibitory Factor (LIF) as a differentiation suppressor to an ordinary medium. Meanwhile, in the case of human cells, it is desirable that basic fibroblast

growth factor (bFGF) and/or stem cell factor (SCF) be added in place of LIF. Usually, the cells are cultured in the co-presence of mouse embryo-derived fibroblasts (MEFs) treated with radiation or an antibiotic to terminate the cell division thereof, 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 feeder cells may be started before contact of the nuclear reprogramming substance, at the time of the contact, or after the contact (e.g., 1-10 days later).

A candidate colony of iPS cells can be selected by a method with drug resistance and reporter activity as indicators, and also by a method based on visual examination of morphology. As an example of the former, a colony positive for drug resistance and/or reporter activity is selected using a recombinant somatic cell wherein a drug resistance gene and/or a reporter gene is targeted to the locus of a gene highly expressed specifically in pluripotent cells (e.g., Fbx15, Nanog, Oct3/4 and the like, preferably Nanog or Oct3/4). Examples of such recombinant somatic cells include MEFs from a mouse having the βgeo (which encodes a fusion protein of β-galactosidase and neomycin phosphotransferase) gene knocked-in to the Fbx15 locus [Takahashi & Yamanaka, *Cell, 126*, 663-676 (2006)], MEFs from a transgenic mouse having the green fluorescent protein (GFP) gene and the puromycin resistance gene integrated in the Nanog locus [Okita et al., *Nature, 448*, 313-317 (2007)] and the like. Meanwhile, examples of the latter method based on visual examination of morphology include the method described by Takahashi et al. in *Cell, 131*, 861-872 (2007). Although the method using reporter cells is convenient and efficient, it is desirable from the viewpoint of safety that colonies be selected by visual examination when iPS cells are prepared for the purpose of human treatment. When the two factors Oct3/4 and Sox2 are used as nuclear reprogramming substances capable of inducing

iPS cells by combination with Klf4, the resulting colonies are mostly of iPS cells of high quality comparable to ES cells, although the number of clones established decreases, so that iPS cells can be efficiently established even without using reporter cells.

The identity of the cells of a selected colony as iPS cells can be confirmed by positive responses to the above-described 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; however, to increase the accuracy, it is possible to perform tests such as alkaline phosphatase staining, analysis of the expression of various ES-cell-specific genes, and transplantation of the selected cells to a mouse and confirmation of teratoma formation.

When a nuclear reprogramming substance capable of substituting for Klf4 is transferred into a somatic cell in the form of a nucleic acid that encodes a protein selected from among members of the IRX family, members of the GLIS family, members of the PTX family and DMRTB1, 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 the exogenous nucleic acid is transferred into 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.

The iPS cells thus established can serve various purposes. For example, differentiation of the iPS cells into a wide variety of cells (e.g., myocardial cells, blood cells, nerve cells, vascular endothelial cells, insulin-secreting cells and the like) can be induced by means of a reported method of differentiation induction of ES cells. Therefore, inducing iPS cells using somatic cells collected from a patient or another person of the same or substantially the same HLA type would enable stem cell therapy based on transplantation, wherein the

iPS cells are differentiated into desired cells (cells of an affected organ of the patient, cells having a therapeutic effect on disease, and the like), and the differentiated cells are transplanted to the patient. Furthermore, because functional cells (e.g., liver cells) 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

[Example 1: Screening of a novel reprogramming factor]

Human Gateway® entry clones (The library described in N. Goshima et al., Nature Methods, 2008 was used. Database is disclosed in Y. Maruyama et al., Nucleic Acid Res., 2009.) produced by Goshima et al. were used to consrtuct approximately 20000 clone contigs of human comprehensive genes according to the method described in Figure 1. More specifically, blastp search of approximately 50000 clones containing the full length ORF from the human Gateway® entry clones was performed against the NCBI RefSeq 37900 sequences (24200 genes) using the criteria of 80% or better coverage, and amino acid identity of 95% or more, and a sublibrary was constructed consisting of approximately 20000 entry clones which did not have overlapping sequences within either an N-type having a stop codon at the 3′ end of the ORF, or an F-type not having a stop codon. These approximately 20000 entry clone contigs were classified using bioinformatics techniques into protein kinases, protein phosphatases, transcription factors, GPCRs, or other groups of clones, and a sublibrary consisting of entry clones of transcription factors (covering not less than 50% of all human transcription factors) was constructed (Figure 1). From this

sublibrary of transcription factors, expression clone DNA was produced for every entry clone by LR reaction with a pMXs-GW destination vector as shown in Figure 2, and this reaction solution was transfected into E. coli DH5α, cloned and a transcription factor expression library (transcription factor expression library for screening reprogramming factors) was constructed. Furthermore, human genes, Oct3/4, Sox2, Klf4 and c-Myc, were respectively integrated into the same pMXs-GW, and each expression clone was constructed. Recombinant retroviruses were produced from these DNA, which were used for the following experiments.

Induction experiments of iPS cells were carried out using dermal fibroblasts of Nanog-GFP mouse (Okita et al., Nature, 448, 313-317(2007)). In this regard, experiments were carried out in two systems: one is a system in which infection of retroviruses was performed on MSTO (mytomicin-C treated SNL cells which have stopped cell division) feeder cells (hereinafter called the MSTO method, Cell, 126, 663-676 (2006)), and the other is a system in which feeder cells were not used at the time of infection, and cultivation was performed on MSTO after the cells were reseeded post infection (hereinafter called the Reseed method, Nature Biotech., 26, 101-106 (2008)).

For the first screening, iPS cell induction was carried out in 24-well plates. Nanog-GFP mouse dermal fibroblasts were seeded on Gelatin (Reseed method) or MSTO (MSTO method), and the next day, were infected with retroviruses produced by various plasmids (Day 0). Specifically, the cells were infected with the 3 genes, Oct3/4, Sox2 and c-Myc, and 1 gene from the aforementioned library of transcription factors at a ratio of 1:1:1:1. As a negative control, the cells were infected with the 3 genes, Oct3/4, Sox2 and c-Myc, at a ratio of 1:1:1. As a positive control, the 4 genes, Oct3/4, Sox2, Klf4 and c-Myc, at a ratio of 1:1:1:1.

The cells were cultivated in 10% FBS/DMEM until the second day from infection, and were cultivated in ES culture medium

(Cell, 126, 663-676 (2006)) from day 3. When the cells were first seeded onto Gelatin (Reseed method), they were reseeded onto MSTO on day 3. Thereafter, the culture medium was replaced every 2 days, and these cells were subject to puromycin selection from day 21, and observed on day 28. As a result, in the wells in which the gene of sample F09 (gene name: IRX6), sample G06 (gene name: GLIS1), sample H08 (gene name: DMRTB1), or sample H10 (gene name: PITX2) was respectively transfected with the 3 genes, GFP-positive colonies appeared and the establishment of mouse iPS cells was confirmed. Furthermore, when iPS induction was performed again using 6-well plates, GFP-positive colonies also appeared and reproducibility was confirmed. GFP-positive colony image and the phase contrast image of respective iPS cells at the time of colony formation, passage 1 and passage 2, are shown in Figures 3 and 4.

From the above results, it was revealed that these 4 factors are novel reprogramming factors which substitute Klf4. Meantime, when MEF was used instead of adult mouse dermal fibroblasts, iPS cells (GFP-positive colonies) were also established.

[Example 2: Analysis of established mouse iPS cells]

Genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and using a PCR enzyme (Takara Ex Taq), genomic-PCR was performed using the iPS cells established in Example 1. The results are shown in Figures 5 and 6. In all iPS cells, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome. Meantime, in the G6-1 clone (gene name: GLIS1), c-Myc which was used for transfection, had not been inserted into the genome (Figure 5). Since retrovirus vectors are not stably expressed unless they are inserted into the genome, it is presumed that this G6-1 clone was established by the expression of only the 3 factors of Oct3/4, Sox2 and GLIS1.

Next, RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results are shown in Figures 7 and 8. The iPS cells established in Example 1 all expressed the ES cell specific marker genes Nanog, Oct3/4, Sox2, Rex1 and ECAT1. From these results, the cells that were established using the novel reprogramming factors were confirmed to be iPS cells.

[Example 3: Establishment and analysis of mouse iPS cells (2)]

Using the same method as Example 1, mouse iPS cells were established by introducing the following reprogramming factors. Nanog-GFP mouse dermal fibroblasts which were the same as in Example 1 were used, and both the MSTO method and the Reseed method were carried out.

(1) Oct3/4, Sox2, c-Myc, and G6 (gene name: GLIS1)
(2) Oct3/4, Sox2, c-Myc, and H8 (gene name: DMRTB1)
(3) Oct3/4, Sox2, c-Myc, and H10 (gene name: PITX2)
(4) Oct3/4, Sox2, and G6
(5) Oct3/4, Sox2, and H8
(6) Oct3/4, Sox2, and H10

The numbers of GFP-positive colonies were counted 28 days after gene transfection. The results of the above (1)-(3) are shown in Figure 9 (it is a summarized result of 6 experiments). While no colonies were established using only 3 factors (Oct3/4, Sox2, c-Myc), colonies were established by adding the reprogramming factor of the present invention (GLIS1, DMRTB1 or PITX2). A particularly remarkable effect was observed when DMRTB1 was added. From these results, these factors of the present invention were confirmed to be reprogramming factors.

The results of the above (4)-(6) are shown in Figure 10 (it is a summarized result of 2 experiments). While no colonies were established with only 2 factors (Oct3/4, Sox2), colonies were established by adding the reprogramming factor of the present invention (GLIS1 or DMRTB1). Meantime, colonies were not observed in the 2 experiments in which PITX2 was added.

GFP-positive colony image and the phase contrast image of respective iPS cells at the time of colony formation (P0), passage 1 (P1) and passage 2 (P2), are shown in Figure 11.

Next, genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and using a PCR enzyme (Takara Ex Taq), genomic-PCR was performed using the iPS cells established above. The results are shown in Figure 12. In all iPS cells, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome. Meantime, c-Myc which was used for transfection, had not been inserted into the genome in the experiment with 3 factors + G6 (GLIS1) (the leftmost lane of Figure 12). Since retrovirus vectors are not stably expressed unless they are inserted into the genome, it is presumed that this clone was established by the expression of only the 3 factors of Oct3/4, Sox2 and GLIS1.

Next, RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results are shown in Figure 13. The iPS cells established above, all expressed the ES cell specific marker genes Nanog, Oct3/4, Sox2, Rex1 and ECAT1. From these results, the cells that were established using the novel reprogramming factors were confirmed to be iPS cells.

[Example 4: Establishment and analysis of mouse iPS cells (3)]

Using the same method as Example 3, mouse iPS cells were established by introducing Oct3/4 and c-Myc in combination with the reprogramming factors of the present invention (G6 (GLIS1), H8 (DMRTB1) or H10 (PITX2)). As a result, in the combination of Oct3/4, c-Myc and H10 (PITX2), GFP-positive colonies (iPS colonies) were detected. GFP-positive colony image and the phase contrast image of iPS colonies at the time of colony formation (P0), passage 1 (P1) and passage 2 (P2), are shown in Figure 14.

Next, genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and using a PCR enzyme (Takara Ex Taq), genomic-PCR was performed using the iPS cells established above. The results

are shown in Figure 15. In the established iPS cells, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome.

Next, RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results are shown in Figure 16. The iPS cells established above, expressed the ES cell specific marker genes Nanog, Oct3/4, Sox2, Rex1 and ECAT1. From these results, the cells that were established using the novel reprogramming factors were confirmed to be iPS cells.

[Example 5: Establishment and analysis of mouse iPS cells (4)]

Fibroblasts (MEF) were isolated from fetal Nanog-GFP mouse (13.5 days after fertilization). The following reprogramming factors were introduced into these MEF using the same technique as Examples 3 and 4.

(1) Oct3/4, Sox2, c-Myc, and G6 (gene name: GLIS1)
(2) Oct3/4, Sox2, c-Myc, and H8 (gene name: DMRTB1)
(3) Oct3/4, Sox2, c-Myc, and H10 (gene name: PITX2)
(4) Oct3/4, Sox2, and G6
(5) Oct3/4, Sox2, and H8
(6) Oct3/4, Sox2, and H10

The numbers of GFP-positive colonies were counted 28 days after gene transfection. The results of the above (1)-(3) are shown in Figure 17 (it is a summarized result of 4 experiments). While hardly any colonies were established using only 3 factors (Oct3/4, Sox2, c-Myc), colonies were established by adding the reprogramming factor of the present invention (GLIS1, DMRTB1 or PITX2), with a particularly remarkable effect observed when DMRTB1 was added.

The results of the above (4)-(6) are shown in Figure 18 (result of 1 experiment). While no colonies were established using only 2 factors (Oct3/4, Sox2), colonies were established by adding the reprogramming factor of the present invention

(GLIS1, DMRTB1 or PITX2). A particularly remarkable effect was observed when DMRTB1 was added.

GFP-positive colony image and the phase contrast image of respective iPS cells at the time of colony formation (P0), passage 1 (P1) and passage 2 (P2), are shown in Figure 19.

Next, genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and using a PCR enzyme (Takara Ex Taq), genomic-PCR was performed using the iPS cells established above. The results of the above (1)-(3) are shown in Figure 20. In all iPS cells, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome.

Next, RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results of the above (1)-(3) are shown in Figure 21. The iPS cells established above, all expressed the ES cell specific marker genes Nanog, Oct3/4, Sox2, Rex1 and ECAT1. From these results, the cells that were established using the novel reprogramming factors were confirmed to be iPS cells.

[Example 6: Production of teratomas and generation of chimeric mice]

The following iPS cell clones which were established in the aforementioned Examples from the dermal fibroblasts of adult mice (Nanog-GFP mice), were used to produce teratomas.

- G6-1 clone: established with the 3 factors, Oct3/4, Sox2, and G6 (GLIS1)
- G6-6 clone: established with the 4 factors, Oct3/4, Sox2, c-Myc, and G6 (GLIS1)
- H8-2 clone: established with the 4 factors, Oct3/4, Sox2, c-Myc, and H8 (DMRTB1)
- H10 clone: established with the 4 factors, Oct3/4, Sox2, c-Myc, and H10 (PITX2)

The production of teratomas was carried out according to the method described in Cell, 126, 663-676 (2006). Specifically,

1×10^6 iPS cells were injected subcutaneously into immunodeficient mice, from which teratomas were isolated after 4 weeks. From these teratomas, genome was extracted in the same manner as described above, and genomic-PCR was performed using a PCR enzyme (Takara Ex Taq). The results are shown in Figure 22. In all teratomas, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome.

Next, these teratomas were cut and fixed in PBS(-) containing 4% formaldehyde. Paraffin embedded tissues were sliced and stained with hematoxylin-eosin. The results are shown in Figures 23 and 24. From a histological point of view, the tumors consisted of multiple types of cells, and since adipose tissue, striated muscular tissue, keratosis tissue, ciliated columnar epithelium tissue, nerve tissue, cartilage, collagen fiber tissue, smooth muscle tissue and the like were observed, the pluripotency of iPS cells was demonstrated.

Furthermore, as a result of microinjection of these iPS cells into blastocysts derived from ICR mouse, adult chimeras were generated.

[Example 7: Microarray analysis]

DNA microarray analysis was performed to investigate whether there is a difference in gene expression between the iPS cells established using the novel reprogramming factors of the present invention (G6, H8, H10), and the iPS cells established using the conventional 4 factors (Oct3/4, Sox2, Klf4, c-Myc). Analysis was performed according to the technique described in Cell, 131, 861-872 (2007), using the iPS cells described below and total RNA derived from MEF.

- K-G6: iPS cell established by transfection of the 4 genes, Oct3/4, Sox2, c-Myc and G6, into MEF (passage 5)
- K-H8: iPS cell established by transfection of the 4 genes, Oct3/4, Sox2, c-Myc and H8, into MEF (passage 5)
- K-H10: iPS cell established by the transfection of the 4 genes,

Oct3/4, Sox2, c-Myc and H10, into MEF (passage 5)

• 4F: iPS cell established by the transfection of the 4 genes, Oct3/4, Sox2, c-Myc and Klf4, into MEF (passage 5)

• MEF: MEF cell used for transfection (passage 1)

The results of the scatter plot are shown in Figures 25-27 (fold change line: 2-fold). Additionally, the correlation coefficient between each cell is shown in Table 2, and the result of clustering based on the correlation coefficient is shown in Figure 28.

Table 2

Array Name	K-G6-MEF	K-H8-MEF	K-H10-MEF	4F-MEF	MEF
K-G6-MEF	1	0.997012	0.99035835	0.9905535	0.8930298
K-H8-MEF	0.997012	1	0.9897563	0.9940246	0.89402026
K-H10-MEF	0.99035835	0.9897563	1	0.9888173	0.89811915
4F-MEF	0.9905535	0.9940246	0.9888173	1	0.8940898
MEF	0.8930298	0.89402026	0.89811915	0.8940898	1

From the fact that the iPS cells that were established using the novel reprogramming factors of the present invention all showed gene expression patterns similar to those of the iPS cells that were established by using the conventional 4 factors, it was shown that the iPS cells of the present invention are comparable to the iPS cells that are established with the 4 factors, i.e., that the novel reprogramming factors of the present invention substitute Klf4.

[Example 8: Establishment and analysis of human iPS cells (1)]

According to the method described in Takahashi, K. et al., Cell, 131: 861-872 (2007), mouse ecotropic virus receptor Slc7a1 gene was expressed in adult or neonatal human dermal fibroblasts (HDF) using lentiviruses (pLenti6/UbC-Slc7a1). 4 genes, Oct3/4, Sox2, c-Myc and G6 (GLIS1), were transfected into these cells (1×10^5 cells/well, 6 well plate) using retroviruses, according to the method described in Takahashi, K. et al., Cell, 131: 861-872 (2007). Additionally, transfection of 4 genes, Oct3/4, Sox2, c-Myc and Klf4, was performed as a control.

The cells were collected 6 days after virus infection, and reseeded onto feeder cells (5×10^5 cells/100 mm dish). mitomycin C treated SNL cells which have stopped cell division (McMahon, A. P. & Bradley, A. Cell 62, 1073-1085 (1990)) were used as the feeder cells. From the 7th day after infection, the cells were cultivated in a primate ES cell culture medium (ReproCELL) containing 4 ng/ml of recombinant human bFGF (WAKO). The colony image of the iPS cells around 35 days from infection is shown in Figure 29, and the colony image of passage 2 iPS cells is shown in Figure 30. iPS cells established with Oct3/4, Sox2, c-Myc and G6 (GLIS1) showed ES cell-like morphology that was similar to that of the iPS cells that were established with Oct3/4, Sox2, c-Myc and Klf4. Furthermore, they were positive for alkaline phosphatase activity.

Next, genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and using a PCR enzyme (Takara Ex Taq), genomic-PCR was performed using the iPS cells established above. The results are shown in Figure 31. In the established iPS cells, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome.

Next, RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results are shown in Figure 32. The established iPS cells all expressed the ES cell specific marker genes Oct3/4, Sox2 and Rex1. From these results, the cells that were established using the novel reprogramming factor G6 (GLIS1) were confirmed to be iPS cells.

[Example 9: Establishment and analysis of human iPS cells (2)]

Using the same technique as Example 8, 3 genes, Oct3/4, Sox2 and H8 (DMRTB1), or 4 genes, Oct3/4, Sox2, c-Myc and H10 (PITX2), were transfected into dental pulp stem cells (J. Dent. Res., 87(7): 676-681 (2008)) using a retrovirus. In addition, 4 genes, Oct3/4, Sox2, c-Myc and Klf4, were transfected as a control.

Using the same technique as Example 8, colony image of the cultivated iPS cells around 35 days from infection is shown in Figure 33, and the colony images of passage 1 iPS cells are shown in Figure 34 (Oct3/4, Sox2, H8) and Figure 37 (Oct3/4, Sox2, c-Myc, H10). The established iPS cells showed ES cell-like morphology that was similar to that of the iPS cells that were established with Oct3/4, Sox2, c-Myc and Klf4. Furthermore, they were positive for alkaline phosphatase activity.

Next, genome was extracted using QIAGEN "Gentra Puregene Cell Kit", and using a PCR enzyme (Takara Ex Taq), genomic-PCR was performed using the iPS cells established above. The results are shown in Figure 35 (Oct3/4, Sox2, H8) and Figure 38 (Oct3/4, Sox2, c-Myc, H10). In the established iPS cells, it was confirmed that only the transfected genes were inserted into the genome and the genes that were not used for transfection were not inserted into the genome.

Next, RT-PCR analysis was performed using Rever Tra Ace kit (Takara). The results are shown in Figure 36 (Oct3/4, Sox2, H8) and Figure 39 (Oct3/4, Sox2, c-Myc, H10). The established iPS cells expressed the ES cell specific marker genes Oct3/4, Sox2, Nanog and Rex1. From these results, the cells that were established using the novel reprogramming factors H8 (DMRTB1), and H10 (PITX2) were confirmed to be human iPS cells.

[Example 10: Production of teratomas]

Human iPS cells established by the introduction of the 4 factors Oct3/4, Sox2, c-Myc and G6 (GLIS1) into HDF, were inserted into the testis of Scid mouse and tested for their pluripotent differentiation. Specifically, the aforementioned iPS cells were first cultivated in a primate ES cell culture medium (ReproCELL, Cosmo Bio) containing recombinant human bFGF (4 ng/ml) and Rho kinase inhibitor Y-27632 (10 μM). 1 hour later, the cells were treated with collagen IV and collected, and then recovered by centrifugation, and suspended in DMEM/F12 containing Y-27632 (10 μM). 1/4 amount of the confluent cells

(100 mm dish) was injected into the testis of a Scid mouse. 2-3 months later, the tumor was cut and fixed using PBS(-) containing 4% formaldehyde. Paraffin embedded tissue was sliced and stained with hematoxylin-eosin. The result is shown in Figure 40. From a histological point of view, the tumor consisted of multiple types of cells, and since it had differentiated into 3 germ layers such as the cartilage, epithelia smooth muscle, epithelia, and nerve tissue, the pluripotency of iPS cells was demonstrated.

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."

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 application Nos. 61/208,853 and 61/276,123, the contents of which are hereby incorporated by reference.

CLAIMS

1. A method of producing iPS cells, comprising the step of transferring into a somatic cell the following (1) and (2):
(1) one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same,
(2) a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

2. The method according to claim 1, wherein the substances mentioned in (1) above include at least one kind of substance selected from the group consisting of iroquois homeobox protein 6 (IRX6), GLIS family zinc finger 1 (GLIS1), paired-like homeodomain transcription factor 2 isoform b (PITX2), DMRTB1 and nucleic acids that encode the same.

3. The method according to claim 1, wherein the substance mentioned in (2) above is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Myc family, the Nanog and Lin families, and nucleic acids that encode the same.

4. The method according to claim 1, wherein the substance mentioned in (2) above is Oct3/4.

5. The method according to claim 1, wherein the substance mentioned in (2) above is Oct3/4 and Sox2.

6. The method according to claim 1, wherein the substance mentioned in (2) above is Oct3/4 and c-Myc.

7. The method according to claim 1, wherein the substance mentioned in (2) above is Oct3/4, Sox2 and c-Myc.

8. An inducer of iPS cells from a somatic cell, comprising the following (1) and (2):

(1) one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same,

(2) a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

9. The inducer according to claim 8, wherein the substances mentioned in (1) above include at least one kind of substance selected from the group consisting of IRX6, GLIS1, PITX2, DMRTB1 and nucleic acids that encode the same.

10. An iPS cell containing an extraneous nucleic acid that encodes one or more kinds of factors selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family and DMRTB1.

11. The iPS cell according to claim 10, containing an extraneous nucleic acid that encodes one or more kinds of factors selected from the group consisting of IRX6, GLIS1, PITX2 and DMRTB1.

12. The iPS cell according to claim 10, wherein the at least one kind of extraneous nucleic acid is integrated in the genome.

13. A method of producing somatic cells, comprising treating the iPS cell according to claim 10 to induce the differentiation thereof into somatic cells.

14. An inducer of iPS cells from a somatic cell, comprising one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same, wherein the inducer is transferred into a somatic cell,

along with a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

15. A use of one or more kinds of substances selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same, for producing iPS cells, wherein the substance(s) is transferred into a somatic cell, along with a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

16. A substance selected from the group consisting of members of the IRX family, members of the GLIS family, members of the PTX family, DMRTB1, and nucleic acids that encode the same, as an inducer of iPS cells from a somatic cell, wherein the substance is transferred into a somatic cell, along with a substance capable of inducing iPS cells from a somatic cell when combined with Klf4.

17. A use of the iPS cell according to claim 10 in producing somatic cells.

18. The iPS cell according to claim 10 as a source of cells in producing somatic cells.