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(54) Title: METHOD OF EFFICIENTLY ESTABLISHING INDUCED PLURIPOTENT STEM CELLS

(57) Abstract: Provided is a method of improving the efficiency of iPS cell establishment, comprising bringing one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR into contact with a somatic cell, particularly a somatic cell of adult derivation, in the step of nuclear reprogramming of the somatic cell. Also provided are a method of producing an iPS cell comprising the step of bringing the factor(s) and a nuclear programming substance into contact with a somatic cell, an iPS cell comprising a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc, that can be obtained by the method of producing an iPS cell, and a method of somatic cell production by forcing the iPS cell to differentiate.

DESCRIPTION

METHOD OF EFFICIENTLY ESTABLISHING INDUCED PLURIPOTENT STEM CELLS

Technical Field of the Invention

The present invention relates to a method of improving the efficiency of establishment of induced pluripotent stem cells (hereinafter referred to as iPS cells) and reagents therefor, more specifically to factors [genes (proteins), low-molecular compounds, medium ingredients] that improve the efficiency of establishment of iPS cells from a somatic cell of adult derivation and a method of improving the efficiency of establishment of iPS cells using these factors. The present invention also relates to a method of producing iPS cells that do not exhibit differentiation resistance using these factors.

Background of the Invention

In recent years, mouse and human iPS cells have been established one after another. Takahashi and Yamanaka induced iPS cells by transferring the Oct3/4, Sox2, Klf4 and c-Myc genes into fibroblasts from a reporter mouse wherein the neomycin resistance gene is knocked-in into the Fbx15 locus, and forcing the cells to express the genes [1]. Okita et al. succeeded in establishing iPS cells (Nanog iPS cells) that show almost the same gene expression and epigenetic modification profiles as those of embryonic stem (ES) cells by creating a transgenic mouse having the green fluorescent protein (GFP) and puromycin-resistance genes integrated into the locus of Nanog, whose expression is more localized in pluripotent cells than the expression of Fbx15, forcing fibroblasts from the mouse to express the above-mentioned four genes, and selecting puromycin-resistant and GFP-positive cells [2]. Similar results were obtained by other groups [3, 4]. Thereafter, it was revealed that iPS cells could also be produced with three of the factors other than the c-Myc gene

[5].

Furthermore, Takahashi et al. succeeded in establishing iPS cells by transferring into human skin fibroblasts the same four genes as those used in the mouse [6]. On the other hand, Yu et al. produced human iPS cells using Nanog and Lin28 in place of Klf4 and c-Myc [7]. Park et al. produced human iPS cells using TERT, which is known as the human cell immortalizing gene, and the SV40 large T antigen, in addition to the four factors Oct3/4, Sox2, Klf4 and c-Myc [8]. Hence, it has been demonstrated that iPS cells comparable to ES cells in terms of pluripotency can be produced in both humans and mice, by transferring defined factors into somatic cells.

While a wide variety of attempts have been made to increase the efficiency of iPS cell establishment, the efficiency of establishment of iPS cells from a somatic cell of adult derivation, in particular, is much lower than that from a fetal somatic cell, and there is a demand for an improvement of the former efficiency.

Furthermore, while many different iPS cell clones have been established using a wide variety of combinations of the above-described reprogramming factors and various origins of somatic cells and methods of selection to date, the origin of somatic cell was recently shown to be a key to the differentiation potential of iPS cells [9]. Specifically, it was shown that iPS cells derived from TTF (adult tail tissue derived fibroblast), unlike iPS cells derived from MEF (mouse embryonic fibroblast), include some differentiation-resistant iPS cells even when differentiated into neurospheres, forming a teratoma after transplantation. Although the mechanism of this differentiation resistance is unclear, it is thought to be of paramount importance to establish iPS cells of high differentiation potential (not exhibiting differentiation resistance) from a somatic cell of adult derivation, when bearing in mind transplantation therapy using iPS cells.

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Summary of the Invention

It is an object of the present invention to provide a means of improving the efficiency of establishment of iPS cells, especially from a somatic cell of adult derivation, and a method of efficiently producing iPS cells using the means.

The present inventors transfected TTFs with various candidate genes, along with the 4 genes Oct3/4, Sox2, Klf4, and c-Myc, or cultured TTFs transfected with the 4 genes only in the presence of a candidate low-molecular compound or medium ingredient, and examined the cells to determine whether the establishment efficiency was improved. As a result, the present inventors found that several genes (Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc), a low-molecular compound (VPA), and a medium ingredient (KSR) have higher iPS cell establishment efficiency improving effects on TTFs than on MEFs.

Because TTFs are lower than MEFs in both iPS cell establishment efficiency (reprogramming efficiency) and differentiation potential, a gene and the like that even improve the differentiation potential of iPS cells are possibly present among the aforementioned genes and the like that improve the efficiency of establishment of iPS cells from a TTF.

The present inventors conducted further investigations based on these findings, and have developed the present invention.

Accordingly, the present invention provides:

[1] a method of improving iPS cell establishment efficiency, comprising bringing one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR into contact with a somatic cell in a nuclear reprogramming step;

[2] the method according to [1] above, wherein the somatic cell is a somatic cell of adult derivation;

[3] an iPS cell establishment efficiency improver comprising a factor selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR;

[4] the agent according to [3] above, wherein the improver is to be used to produce iPS cells from a somatic cell of adult derivation;

[5] a method of producing iPS cells, comprising the step of bringing one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR, and a nuclear reprogramming substance into contact with a somatic cell;

[6] the method according to [5] above, wherein the nuclear reprogramming substance is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Klf4 family, members of the Myc family, members of the Lin28 family, and Nanog, as well as nucleic acids that encode the same;

[7] the method according to [5] above, wherein the nuclear reprogramming substance includes Oct3/4 or a nucleic acid that encodes the same;

[8] the method according to [7] above, wherein the nuclear reprogramming substance consists of Oct3/4, Sox2, Klf4, and L-myc or c-Myc, or nucleic acids that encode the same;

[9] the method according to any one of [5] to [8] above, wherein the somatic cell is a somatic cell of adult derivation;

[10] an iPS cell inducer from a somatic cell, comprising a factor selected from the group consisting of Dppa2, Sall4, Utf1,

β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR, and a nuclear programming substance;
[11] the inducer according to [10] above, wherein the nuclear reprogramming substance is selected from the group consisting
5 of members of the Oct family, members of the Sox family, members of the Klf4 family, members of the Myc family, members of the Lin28 family, and Nanog, as well as nucleic acids that encode the same;
[12] the inducer according to [10] above, wherein the nuclear
10 reprogramming substance includes Oct3/4 or a nucleic acid that encodes the same;
[13] the inducer according to [12] above, wherein the nuclear reprogramming substance consists of Oct3/4, Sox2, Klf4, and L-Myc or c-Myc, or nucleic acids that encode the same;
15 [14] the inducer according to any one of [10] to [13] above, wherein the somatic cell is a somatic cell of adult derivation;
[15] an iPS cell containing an exogenous nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc;
[16] the iPS cell according to [15] above, wherein the
20 exogenous nucleic acid is integrated in the genome;
[17] a method of producing a somatic cell, comprising performing a differentiation induction treatment on the iPS cell according to [15] or [16] above to cause the iPS cell to differentiate into a somatic cell;
25 [18] a method of producing a somatic cell, comprising the steps of:
(1) producing an iPS cell by the method according to any one of [5] to [9] above, and
(2) performing a differentiation induction treatment on the iPS
30 cell obtained through the step (1) to cause the iPS cell to differentiate into a somatic cell;
[19] a use of one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR for improving
35 the efficiency of establishment of iPS cells;

[20] the use according to [19] above, wherein the use is for producing an iPS cell from a somatic cell of adult derivation;
[21] a use of one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR for producing an iPS cell, wherein the factor(s), along with a nuclear programming substance, is(are) brought into contact with a somatic cell;
[22] the use according to [21] above, wherein the nuclear reprogramming substance is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Klf4 family, members of the Myc family, members of the Lin28 family, and Nanog, as well as nucleic acids that encode the same;
[23] the use according to [21] above, wherein the nuclear reprogramming substance includes Oct3/4 or a nucleic acid that encodes the same;
[24] the use according to [23] above, wherein the nuclear reprogramming substance consists of Oct3/4, Sox2, Klf4, and L-Myc or c-Myc, or nucleic acids that encode the same;
[25] the use according to any one of [21] to [24] above, wherein the somatic cell is a somatic cell of adult derivation;
[26] a use of the iPS cell according to [15] or [16] above in producing a somatic cell; and
[27] the iPS cell according to [15] or [16] above as a source of cells for producing a somatic cell.

Because the iPS cell establishment efficiency improving factors of the present invention are capable of remarkably improving the efficiency of establishment of iPS cells from a somatic cell of adult derivation, as stated above, they are useful in, for example, applications to human transplantation medicine by autotransplantation. Furthermore, these factors can include those that even improve the differentiation potential of iPS cells; utilizing such a factor makes it possible to provide an iPS cell as a source of cells for producing safe graft cells

with reduced risks for tumorigenesis.

Brief Description of the Drawings

Fig. 1 is a graph showing the number of GFP-positive colonies (iPS cell colonies) that emerged after a total of five different genes consisting of the 4 genes (Oct3/4, Sox2, Klf4, c-Myc) and each candidate gene were transferred to mouse TTFs, indicated as values relative to the number of colonies obtained with transfer of the 4 genes only. The data shown are means for values obtained in four independent experiments.

Fig. 2 is a graph showing the number of GFP-positive colonies (iPS cell colonies) that emerged after a total of five different genes consisting of the 4 genes (Oct3/4, Sox2, Klf4, c-Myc) and each candidate gene were transferred to MEFs, indicated as values relative to the number of colonies obtained with transfer of the 4 genes only. The data shown are means for values obtained in four independent experiments.

Fig. 3 is a graph showing the number of GFP-positive colonies (iPS cell colonies) that emerged when TTFs transfected with the 4 genes (Oct3/4, Sox2, Klf4, c-Myc) only were cultured in the presence of VPA, 5′azaC or TSA, or cultured using the KSR medium.

Detailed Description of the Invention

The present invention provides a method of improving the efficiency of iPS cell establishment by bringing one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR (hereinafter also referred to as the establishment efficiency improving factors of the present invention) into contact with a somatic cell in the step of nuclear reprogramming of the somatic cell. Here, the nuclear reprogramming of the somatic cell is achieved by transferring a nuclear programming substance to the somatic cell; therefore, the present invention also provides a method of producing an iPS cell by bringing the

factor(s) and a nuclear programming substance into contact with a somatic cell. Herein, cases where iPS cells cannot be established by merely transferring a nuclear reprogramming substance alone to a somatic cell, but can be established by bringing a nuclear reprogramming substance along with the establishment efficiency improving factors of the present invention into contact with a somatic cell, are also deemed as corresponding to "an improvement of establishment efficiency."

(a) Source of somatic cells

In the present invention, any cells other than germ cells of mammalian origin (e.g., humans, mice, monkeys, pigs, rats etc.) can be used as starting material for the production of iPS cells. 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), contractile cells (e.g., smooth muscle cells), cells of the blood and the immune system (e.g., T lymphocytes), sense-related cells (e.g., rod cells), autonomic nervous system neurons (e.g., cholinergic neurons), sustentacular cells of sensory organs and peripheral neurons (e.g., satellite cells), nerve cells and glia cells of the central nervous system (e.g., astroglia cells), pigment cells (e.g., retinal pigment epithelial cells), progenitor cells thereof (tissue progenitor cells) and the like. There is no limitation on the degree of cell differentiation, the age of the 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 neural stem cells, hematopoietic stem cells, mesenchymal stem cells, and dental pulp stem cells.

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

Because the establishment efficiency improving factors of the present invention are capable of remarkably improving the efficiency of establishment of iPS cells from somatic cells of adult derivation, which is reportedly generally lower than somatic cells of fetal derivation in both iPS cell establishment efficiency (reprogramming efficiency) and differentiation potential, the method of the present invention is particularly

useful when a somatic cell of adult derivation is used as a source of somatic cells.

Before being subjected to the step of nuclear reprogramming, somatic cells separated from a mammal 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) containing about 5 to 20% fetal calf serum, Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium and the like. When using, for example, a transfection reagent such as a cationic liposome in contacting the cell with the establishment efficiency improving factors of the present invention and a nuclear reprogramming substance (and another iPS cell establishment efficiency improver as required), it is sometimes preferable that the medium be previously replaced with a serum-free medium to prevent a reduction in the transfer efficiency.

(b) The establishment efficiency improving factors of the present invention

The Dppa2, Sall4, Utf1, β-catenin, Stat3 and N-Myc are proteins comprising the same or substantially the same amino acid sequences as the amino acid sequences shown by SEQ ID NO:2 or 4, SEQ ID NO:6 or 8, SEQ ID NO:10 or 12, SEQ ID NO:14 or 16, SEQ ID NO:18 or 20 and SEQ ID NO:22 or 24, respectively. These proteins may be a protein isolated and purified from a cell or tissue [for example, cell or tissue of thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver or prostate, its precursor cell, stem cell or cancer cell or the like] by known protein separation techniques. They may also be proteins chemically synthesized on the basis of the amino acid sequences shown by SEQ ID NO:2 or 4, SEQ ID NO:6 or 8, SEQ ID NO:10 or 12, SEQ ID NO:14 or 16, SEQ ID NO:18 or 20 and SEQ ID NO:22 or 24, respectively, or recombinant proteins produced by a transformant incorporating a nucleic acid having the nucleotide sequence encoding the

above-described amino acid sequence. Alternatively, they may also be proteins biochemically synthesized using a cell-free translation system with the nucleic acid as a template.

The "protein comprising substantially the same amino acid sequence as the amino acid sequence shown by a SEQ ID NO", refers to a protein comprising an amino acid sequence having a identity of about 80% or more, preferably about 90% or more, more preferably about 95% or more, to the amino acid sequence shown by the SEQ ID NO, and having substantially the same quality of activity as a protein comprising the amino acid sequence shown by the SEQ ID NO. As used herein, "activity" refers to an effect of improving iPS cell establishment efficiency and includes an effect of improving differentiation potential of iPS cell when a protein comprising the amino acid sequence shown by the SEQ ID NO has such improving effect. "Substantially the same quality" means that the effects are equivalent to or greater than those which a protein comprising the amino acid sequence shown by the SEQ ID NO exerts. The iPS cell establishment efficiency-improving effect can be confirmed by comparing the numbers of iPS cell colonies emerged between when given reprogramming factors (e.g., 3 factors consisting of Oct3/4, Sox2 and Klf4, 4 factors consisting of said 3 factors and additional c-Myc, etc.) alone are introduced into a somatic cell and when the iPS cell establishment efficiency-improving factors of the present invention in addition to the reprogramming factors are introduced into a somatic cell.

Examples of the Dppa2, Sall4, Utf1, β-catenin, Stat3 and N-Myc of the present invention also include proteins comprising (i) an amino acid sequence having one or more amino acids (for example, about 1 to 10, preferably 1 to several (5, 4, 3 or 2) amino acids) deleted from the amino acid sequence shown by each SEQ ID NO (in the case of Dppa2, SEQ ID NO:2 or 4, in the case of Sall4, SEQ ID NO:6 or 8, in the case of Utf1, SEQ ID NO:10 or 12, in the case of β-catenin, SEQ ID NO:14 or

16, in the case of Stat3, SEQ ID NO:18 or 20, in the case of N-Myc, SEQ ID NO:22 or 24), (2) an amino acid sequence having one or more amino acids (for example, about 1 to 10, preferably 1 to several (5, 4, 3 or 2) amino acids) added to the amino acid sequence shown by each SEQ ID NO (same as above), (3) an amino acid sequence having one or more amino acids (for example, about 1 to 10, preferably 1 to several (5, 4, 3 or 2) amino acids) inserted in the amino acid sequence shown by each SEQ ID NO (same as above), (4) an amino acid sequence having one or more amino acids (for example, about 1 to 10, preferably 1 to several (5, 4, 3 or 2) amino acids) substituted by other amino acids in the amino acid sequence shown by each SEQ ID NO (same as above), or (5) an amino acid sequence comprising a combination thereof, and having substantially the same quality of activity as a protein comprising the amino acid sequence shown by the SEQ ID NO.

When an amino acid sequence is inserted, deleted or substituted as described above, the position of the insertion, deletion or substitution is not subject to limitation, as long as a protein comprising the resulting amino acid sequence retains an effect of improving iPS cell establishment efficiency.

Preferable examples of Dppa2 protein include, for example, mouse Dppa2 consisting of the amino acid sequence shown by SEQ ID NO:2 (RefSeq Accession No. NP_082891.1), human Dppa2 consisting of the amino acid sequence shown by SEQ ID NO:4 (RefSeq Accession No. NP_620170.3) and orthologs thereof in other mammals, as well as naturally occurring allelic mutants, polymorphic variants and artificially activated mutants thereof. It is preferable to use Dppa2 homologous to the animal species of the target somatic cell.

Preferable examples of Sall4 protein include, for example, mouse Sall4 consisting of the amino acid sequence shown by SEQ ID NO:6 (RefSeq Accession No. NP_780512.2), human Sall4 consisting of the amino acid sequence shown by SEQ ID NO:8

(RefSeq Accession No. NP_065169.1) and orthologs thereof in other mammals, as well as naturally occurring allelic mutants, polymorphic variants and artificially activated mutants thereof. It is preferable to use Sall4 homologous to the animal species of the target somatic cell.

Preferable examples of Utf1 protein include, for example, mouse Utf1 consisting of the amino acid sequence shown by SEQ ID NO:10 (RefSeq Accession No. NP_033508.1), human Utf1 consisting of the amino acid sequence shown by SEQ ID NO:12 (RefSeq Accession No. NP_003568.2) and orthologs thereof in other mammals, as well as naturally occurring allelic mutants, polymorphic variants and artificially activated mutants thereof. It is preferable to use Utf1 homologous to the animal species of the target somatic cell.

Preferable examples of β-catenin protein include, for example, mouse β-catenin consisting of the amino acid sequence shown by SEQ ID NO:14 (RefSeq Accession No. NP_031640.1), human β-catenin consisting of the amino acid sequence shown by SEQ ID NO:16 (RefSeq Accession No. NP_001895.1) and orthologs thereof in other mammals, as well as naturally occurring allelic mutants, polymorphic variants and artificially activated mutants thereof. It is preferable to use β-catenin homologous to the animal species of the target somatic cell.

Preferable examples of Stat3 protein include, for example, mouse Stat3 consisting of the amino acid sequence shown by SEQ ID NO:18 (RefSeq Accession No. NP_998824.1), human Stat3 consisting of the amino acid sequence shown by SEQ ID NO:20 (RefSeq Accession No. NP_644805.1) and orthologs thereof in other mammals, as well as naturally occurring allelic mutants, polymorphic variants and artificially activated mutants thereof. It is preferable to use Stat3 homologous to the animal species of the target somatic cell.

Preferable examples of N-Myc protein include, for example, mouse N-Myc consisting of the amino acid sequence shown by SEQ ID NO:22 (RefSeq Accession No. NP_032735.3), human N-Myc

consisting of the amino acid sequence shown by SEQ ID NO:24 (RefSeq Accession No. NP_005369.2) and orthologs thereof in other mammals, as well as naturally occurring allelic mutants, polymorphic variants and artificially activated mutants thereof. It is preferable to use N-Myc homologous to the animal species of the target somatic cell.

Transfer of these proteins to a somatic cell can be achieved using a method known per se for protein transfer into a cell, 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 (Gene Therapy Systems), Pro-Ject™ Protein Transfection Reagent (PIERCE) and ProVectin (IMGENEX); those based on a lipid, such as Profect-1 (Targeting Systems); those based on a membrane-permeable peptide, such as Penetrain Peptide (Q biogene) and Chariot Kit (Active Motif), GenomONE (ISHIHARA SANGYO KAISHA, LTD.) utilizing HVJ envelope (inactivated hemagglutinating virus of Japan) and the like. The transfer can be achieved per the protocols attached to these reagents, a common procedure being as described below. The proteinous establishment efficiency improving factor(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) or Green, M. &

Loewenstein, P. M. *Cell 55*, 1179-88 (1988)), Penetratin (Derossi, D. et al, *J. Biol. Chem. 269*, 10444-50 (1994)), Buforin II (Park, C. B. et al. *Proc. Natl Acad. Sci. USA 97*, 8245-50 (2000)), Transportan (Pooga, M. et al. *FASEB J. 12*, 67-77 (1998)), MAP (model amphipathic peptide) (Oehlke, J. et al. *Biochim. Biophys. Acta. 1414*, 127-39 (1998)), K-FGF (Lin, Y. Z. et al. *J. Biol. Chem. 270*, 14255-14258 (1995)), Ku70 (Sawada, M. et al. *Nature Cell Biol. 5*, 352-7 (2003)), Prion (Lundberg, P. et al. *Biochem. Biophys. Res. Commun. 299*, 85-90 (2002)), pVEC (Elmquist, A. et al. *Exp. Cell Res. 269*, 237-44 (2001)), Pep-1 (Morris, M. C. et al. *Nature Biotechnol. 19*, 1173-6 (2001)), Pep-7 (Gao, C. et al. *Bioorg. Med. Chem. 10*, 4057-65 (2002)), SynBl (Rousselle, C. et al. *Mol. Pharmacol. 57*, 679-86 (2000)), HN-I (Hong, F. D. & Clayman, G L. *Cancer Res. 60*, 6551-6 (2000)), and HSV-derived VP22. CPPs derived from the PTDs include polyarginines such as 11R (*Cell Stem Cell, 4*,381-384 (2009)) and 9R (*Cell Stem Cell, 4*, 472-476 (2009)).

A fusion protein expression vector incorporating a cDNA of Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc 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 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 transferring operation can be performed one or more optionally chosen times (e.g., once or more to 10

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

The choice of nucleic acids that encode proteinous establishment efficiency improving factors of the present invention (Dppa2, Sall4, Utf1, β-catenin, Stat3 and N-Myc) (the nucleic acid-based establishment efficiency improving factors of the present invention) is not particularly limited, as far as they encode the above-described Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc protein in the present invention, respectively. The nucleic acid may be a DNA or an RNA, or a DNA/RNA chimera, and is preferably a DNA. The nucleic acid may be double-stranded or single-stranded. In the case of double strands, the nucleic acid may be a double-stranded DNA, a double-stranded RNA or a DNA:RNA hybrid.

A nucleic acid-based establishment efficiency improving factor of the present invention can, for example, be cloned from a cell or tissue [e.g., cells and tissues of thymus, bone marrow, spleen, brain, spinal cord, heart, skeletal muscle, kidney, lung, liver, pancreas or prostate, progenitor cells, stem cells or cancer cells of these cells, and the like] of a human or another mammal (e.g., mouse, rat, monkey, pig, dog and the like) by a conventional method.

Nucleic acids that encode Dppa2 include, for example, nucleic acids comprising the base sequence shown by SEQ ID NO:1 or 3, and nucleic acids that comprise a base sequence capable of hybridizing with a sequence complementary to the base sequence shown by SEQ ID NO:1 or 3 under stringent conditions, and that encode a protein possessing substantially the same quality of activity as the aforementioned Dppa2.

Nucleic acids that encode Sall4 include, for example, nucleic acids comprising the base sequence shown by SEQ ID NO:5 or 7, and nucleic acids that comprise a base sequence capable of

hybridizing with a sequence complementary to the base sequence shown by SEQ ID NO:5 or 7 under stringent conditions, and that encode a protein possessing substantially the same quality of activity as the aforementioned Dppa2.

Nucleic acids that encode Utf1 include, for example, nucleic acids comprising the base sequence shown by SEQ ID NO:9 or 11, and nucleic acids that comprise a base sequence capable of hybridizing with a sequence complementary to the base sequence shown by SEQ ID NO:9 or 11 under stringent conditions, and that encode a protein possessing substantially the same quality of activity as the aforementioned Utf1.

Nucleic acids that encode β-catenin include, for example, nucleic acids comprising the base sequence shown by SEQ ID NO:13 or 15, and nucleic acids that comprise a base sequence capable of hybridizing with a sequence complementary to the base sequence shown by SEQ ID NO:13 or 15 under stringent conditions, and that encode a protein possessing substantially the same quality of activity as the aforementioned β-catenin.

Nucleic acids that encode Stat3 include, for example, nucleic acids comprising the base sequence shown by SEQ ID NO:17 or 19, and nucleic acids that comprise a base sequence capable of hybridizing with a sequence complementary to the base sequence shown by SEQ ID NO:17 or 19 under stringent conditions, and that encode a protein possessing substantially the same quality of activity as the aforementioned Stat3.

Nucleic acids that encode N-Myc include, for example, nucleic acids comprising the base sequence shown by SEQ ID NO:21 or 23, and nucleic acids that comprise a base sequence capable of hybridizing with a sequence complementary to the base sequence shown by SEQ ID NO:21 or 23 under stringent conditions, and that encode a protein possessing substantially the same quality of activity as the aforementioned N-Myc.

Here, "substantially the same quality of activity" has the same definition as in the above-described case of proteinous establishment efficiency improving factors.

A useful nucleic acid capable of hybridizing with a sequence complementary to the base sequence shown by each sequence identification number under stringent conditions is a nucleic acid comprising a base sequence having an identity of about 80% or more, preferably about 90% or more, more preferably about 95% or more, to the base sequence shown by each sequence identification number. Examples of stringent conditions include conditions described in Current Protocols in Molecular Biology, John Wiley & Sons, 6.3.1-6.3.6, 1999, e.g., hybridization with 6×SSC (sodium chloride/sodium citrate)/45°C followed by not less than one time of washing with 0.2×SSC/0.1% SDS/50 to 65°C; those skilled in the art can choose as appropriate hybridization conditions that give equivalent stringency.

Preferred nucleic acids that encode Dppa2 include a nucleic acid comprising the base sequence that encodes mouse Dppa2, shown by SEQ ID NO:1 (RefSeq Accession No. NM_028615.1), a nucleic acid comprising the base sequence that encodes human Dppa2, shown by SEQ ID NO:3 (RefSeq Accession No. NM_138815.3), orthologs thereof in other mammals, natural allelic mutants and polymorphs thereof, artificial active mutants and the like. It is desirable to use a nucleic acid that encodes the Dppa2 of the same animal species as the donor of the somatic cell to be transfected.

Preferred nucleic acids that encode Sall4 include a nucleic acid comprising the base sequence that encodes mouse Sall4, shown by SEQ ID NO:5 (RefSeq Accession No. NM_175303.3), a nucleic acid comprising the base sequence that encodes human sall4, shown by SEQ ID NO:7 (RefSeq Accession No. NM_020436.3), orthologs thereof in other mammals, natural allelic mutants and polymorphs thereof, artificial active mutants and the like. It is desirable to use a nucleic acid that encodes the Sall4 of the same animal species as the donor of the somatic cell to be transfected.

Preferred nucleic acids that encode Utf1 include a nucleic

acid comprising the base sequence that encodes mouse Utf1, shown by SEQ ID NO:9 (RefSeq Accession No. NM_009482.2), a nucleic acid comprising the base sequence that encodes human Utf1, shown by SEQ ID NO:11 (RefSeq Accession No. NM_003577.2), orthologs thereof in other mammals, natural allelic mutants and polymorphs thereof, artificial active mutants and the like. It is desirable to use a nucleic acid that encodes the Utf1 of the same animal species as the donor of the somatic cell to be transfected.

Preferred nucleic acids that encode β-catenin include a nucleic acid comprising the base sequence that encodes mouse β-catenin, shown by SEQ ID NO:13 (RefSeq Accession No. NM_007614.3), a nucleic acid comprising the base sequence that encodes human β-catenin, shown by SEQ ID NO:15 (RefSeq Accession No. NM_001904.3) or nucleic acids that encode β-catenin S33Y, which is an active mutant thereof, orthologs thereof in other mammals, natural allelic mutants and polymorphs thereof, artificial active mutants and the like. It is desirable to use a nucleic acid that encodes the β-catenin of the same animal species as the donor of the somatic cell to be transfected.

Preferred nucleic acids that encode Stat3 include a nucleic acid comprising the base sequence that encodes mouse Stat3, shown by SEQ ID NO:17 (RefSeq Accession No. NM_213659.2), a nucleic acid comprising the base sequence that encodes human Stat3, shown by SEQ ID NO:19 (RefSeq Accession No. NM_139276.2) or nucleic acids that encode Stat3-C, which is an active mutant thereof, orthologs thereof in other mammals, natural allelic mutants and polymorphs thereof, artificial active mutants and the like. It is desirable to use a nucleic acid that encodes the Stat3 of the same animal species as the donor of the somatic cell to be transfected.

Preferred nucleic acids that encode N-Myc include a nucleic acid comprising the base sequence that encodes mouse N-Myc, shown by SEQ ID NO:21 (RefSeq Accession No. NM_008709.3), a nucleic acid comprising the base sequence that encodes human N-Myc, shown by SEQ ID NO:23 (RefSeq Accession No. NM_005378.4),

orthologs thereof in other mammals, natural allelic mutants and polymorphs thereof, artificial active mutants and the like. It is desirable to use a nucleic acid that encodes the N-Myc of the same animal species as the donor of the somatic cell to be transfected.

Transfer of a nucleic acid-based establishment efficiency improving factor of the present invention to a somatic cell can be achieved using a method of gene transfer to cells known per se. A nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc is inserted into an appropriate expression vector containing a promoter capable of functioning in the 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.

A vector for this purpose can be chosen as appropriate according to the intended use of the iPS cell to be obtained. Useful vectors include adenovirus vector, plasmid vector, adeno-associated virus vector, retrovirus vector, lentivirus vector, Sendai virus vector, episomal vector and the like.

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

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

A nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc may be integrated alone into an expression vector, or along with one or more reprogramming genes into an expression vector. Preference is sometimes given to the former case when using a retrovirus or lentivirus vector, which offer high gene transfer efficiency, and to the latter case when using a plasmid, adenovirus, or episomal vector and the like, but there are no particular limitations.

In the context above, when a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc and one or more reprogramming genes are integrated in one expression vector, these genes can preferably be integrated into the expression vector via a sequence enabling polycistronic expression. By using a sequence enabling polycistronic expression, it is possible to more efficiently express a plurality of genes integrated in one expression vector. Useful sequences enabling polycistronic expression include, for example, the 2A sequence of foot-and-mouth disease virus (SEQ ID NO:25; PLoS ONE3, e2532, 2008, Stem Cells 25, 1707, 2007), the IRES sequence (U.S. Patent No. 4,937,190) and the like, with preference given to the 2A sequence.

An expression vector harboring a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc 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 each 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); when a lentivirus vector is used, a disclosure is available in *Science, 318*, 1917-1920 (2007). When iPS cells are utilized as a source of

cells for regenerative medicine, the expression (reactivation) of Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc or the activation of an endogenous gene present in the vicinity of the site where the exogenous gene is integrated potentially increases the risk of carcinogenesis in tissues regenerated from differentiated cells of iPS cell derivation; therefore, a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc is preferably expressed transiently, without being integrated into the chromosome of the cells. From this viewpoint, use of an adenoviral vector, whose integration into chromosome is rare, is preferred. Specific means using an adenoviral vector is described in *Science, 322*, 945-949 (2008). Because an adeno-associated viral vector is also low in the frequency of integration into chromosome, and is lower than adenoviral vectors in terms of cytotoxicity and inflammation-inducibility, it can be mentioned as another preferred vector. Because Sendai viral vector is capable of being stably present outside the chromosome, and can be degraded and removed using an siRNA as required, it is preferably utilized as well. Regarding a Sendai viral vector, one described in *J. Biol. Chem., 282*, 27383-27391 (2007) and JP-3602058 B 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 later; therefore, for example, a method can be used preferably wherein a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc is cut out using the Cre/loxP system, when becoming unnecessary. That is, with loxP sequences arranged on both ends of the nucleic acid in advance, after iPS cells are induced, 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-inactivating (SIN) LTR prepared by deleting the sequence, or substituting the sequence with a polyadenylation sequence such as of SV40. Specific means using the Cre-loxP system and SIN LTR is disclosed in Soldner et al., *Cell, 136*: 964-977 (2009), Chang et al., *Stem Cells, 27*: 1042-1049 (2009) and the like.

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

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

Also when an adenovirus or a plasmid is used, the transgene can get integrated into chromosome; therefore, it is eventually necessary to confirm the absence of insertion of the gene into chromosome by Southern blotting or PCR. For this reason, like the aforementioned Cre-loxP system, it can be advantageous to use a means wherein the transgene is integrated into chromosome, thereafter the gene is removed. In another preferred mode of embodiment, a method can be used wherein the transgene is integrated into chromosome using a transposon, thereafter a transposase is allowed to act on the

cell using a plasmid vector or adenoviral vector so as to completely eliminate the transgene from the chromosome. As examples of preferable transposons, piggyBac, a transposon derived from a lepidopterous insect, and the like can be mentioned. Specific means using the piggyBac transposon is disclosed in Kaji, K. et al., *Nature, 458*: 771-775 (2009), Woltjen et al., *Nature, 458*: 766-770 (2009).

Another preferable non-integration type vector is an episomal vector, which is autonomously replicable outside the chromosome. Specific means with the use of an episomal vector is described by Yu et al. in *Science, 324*, 797-801 (2009). As appropriate, an expression vector in which a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc is inserted into an episomal vector having loxP sequences placed in the same orientation on the 5′ and 3′ sides of the vector constituent essential for the replication of the episomal vector can be constructed and introduced into a somatic cell.

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

The episomal expression vector harbors a promoter that controls the transcription of a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc. Useful promoters include those mentioned above. The episomal expression vector, like the aforementioned vectors, may further contain as desired an enhancer, a polyA addition signal, a selection marker gene and the like. Examples of useful selection marker genes include the dihydrofolate reductase gene, the neomycin resistance gene and the like.

The loxP sequences useful in the present invention include, in addition to the bacteriophage P1-derived wild type loxP sequence (SEQ ID NO:26), optionally chosen mutant loxP sequences capable of deleting the sequence flanked by the loxP sequence by recombination when placed in the same orientation at positions flanking a vector component necessary for the replication of the introduced gene. Examples of such mutant loxP sequences include lox71 (SEQ ID NO:27), mutated in 5′ repeat, lox66 (SEQ ID NO:28), mutated in 3′ repeat, and lox2272 and lox511, mutated in spacer portion. Although the two loxP sequences placed on the 5′ and 3′ sides of the vector component may be identical or not, the two mutant loxP sequences mutated in spacer portion must be identical (e.g., a pair of lox2272 sequences, a pair of lox511 sequences). Preference is given to a combination of a mutant loxP sequence mutated in 5′ repeat (e.g., lox71) and a mutant loxP sequence mutated in 3′ repeat (e.g., lox66). In this case, the loxP sequences remaining on the chromosome have double mutations in the repeats on the 5′ side and 3′ side as a result of recombination, and are therefore unlikely to be recognized by Cre recombinase, thus reducing the risk of causing a deletion mutation in the chromosome due to unwanted recombination. When the mutant loxP sequences lox71 and lox66 are used in combination, each may be placed on any of the 5′ and 3′ sides of the aforementioned vector component, but it is necessary that the mutant loxP sequences be inserted in an orientation such that the mutated sites would be located at the outer ends of the respective loxP sequences. Although a preferred episomal vector of the present invention is a self-removal vector early shedding from the cell even without being acted on by Cre recombinase, there are possibly exceptional cases where longer time is taken for the episomal vector to be shed from the cell. It is preferable, therefore, that the loxP sequences be designed in preparation for risks such as unwanted recombination due to Cre recombinase treatment.

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

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

Whether or not the vector component necessary for the replication of the introduced gene has been removed from the iPS cell can be confirmed by performing a Southern blot analysis or PCR analysis using a nucleic acid comprising a nucleotide sequence in the vector component as a probe or primer, with the episome fraction isolated from the iPS cell as a template, and determining the presence or absence of a band or the length of the band detected. The episome fraction can be prepared by a method well known in the art; for example, methods described in *Science, 324*: 797-801 (2009) and elsewhere can be used.

The present invention also provides valproic acid (VPA), which is a histone deacetylase (HDAC) inhibitor, and a serum substitute reagent (KSR) used as an ingredient for serum-free media (hereinafter also referred to as the chemical establishment efficiency improving factors of the present invention) as other iPS cell establishment efficiency improving factors.

The VPA of the present invention can include salts and

derivatives thereof, as far as a level of HDAC inhibitory activity equivalent to, or higher than, the activity of the free form, is retained. Salts of VPA include salts with alkali metals such as sodium and potassium, salts with alkaline earth metals such as calcium and magnesium, and the like. Although the VPA can be used in a range of concentrations that are sufficient to improve iPS cell establishment efficiency without causing cytotoxicity, for example, at concentrations of 0.01 to 1.5 mM, preferably 0.05 to 1 mM, more preferably 0.1 to 0.5 mM.

KSR is commercially available from Invitrogen Company. Although KSR can be used in a range of concentrations that are sufficient to improve iPS cell establishment efficiency without causing cytotoxicity, for example, at concentrations of 2 to 30w/w%, preferably 5 to 25w/w%, more preferably 10-20w/w%.

Contact of a chemical establishment efficiency improving factor of the present invention with a somatic cell can be performed by dissolving the factor at an appropriate concentration in an aqueous or non-aqueous solvent, adding the solution of the factor to a medium suitable for cultivation of somatic cells isolated from a human or another mammal [e.g., minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium (if KSR is not used as an improving factor, about 5 to 20% fetal bovine serum may be contained) and the like] so that the factor concentration will fall in the above-described range, and culturing the cells for a given period. Duration of contact is not particularly limited, as far as it is sufficient to achieve nuclear reprogramming of the somatic cell; for example, the factor may be allowed to be co-present in the medium until a positive colony emerges.

(c) Nuclear reprogramming substances

As used herein, "a nuclear reprogramming substance" can include any substance such as a proteinous factor, a nucleic acid that encodes the same (including a form integrated in a vector) or a low molecular weight compound, as long as it can

induce an iPS cell from a somatic cell upon its contact with the somatic cell together with the iPS cell establishment efficiency improving factors of the present invention. When the nuclear reprogramming substance is a proteinous factor or a nucleic acid that encodes the same, the following combinations, for example, are preferable (hereinafter, only the names for proteinous factors are shown).

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

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

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

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

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

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

(7) Oct3/4, Klf4, c-Myc, Sox2, TERT, HPV6 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 and replacement of Klf4 with Klf1 or Klf5 in the combination (2) above, see *Nature Biotechnology, 26*, 101-106 (2008)); for the combination "Oct3/4, Klf4, c-Myc, Sox2", see also *Cell, 126*, 663-676 (2006), *Cell, 131*, 861-872 (2007) and the like; for the combination "Oct3/4, Klf2 (or Klf5), c-Myc, Sox2", see also *Nat. Cell Biol., 11*, 197-203 (2009); for the combination "Oct3/4, Klf4, c-Myc, Sox2, hTERT, SV40 LT", see also *Nature, 451*, 141-146 (2008).]

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

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

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

(12) Oct3/4, Klf4, c-Myc, Sox2, Nanog, Lin28 (see *Cell Research* (2008) 600-603)
(13) Oct3/4, Klf4, c-Myc, Sox2, SV40LT (see also *Stem Cells, 26*, 1998-2005 (2008))
5 (14) Oct3/4, Klf4 (see *Nature 454*:646-650 (2008), Cell Stem Cell, 2:525-528 (2008)))
(15) Oct3/4, c-Myc (see *Nature 454*:646-650 (2008))
(16) Oct3/4, Sox2 (see Nature, 451, 141-146 (2008), WO2008/118820)
10 (17) Oct3/4, Sox2, Nanog (see WO2008/118820)
(18) Oct3/4, Sox2, Lin28 (see WO2008/118820)
(19) Oct3/4, Sox2, c-Myc, Esrrb (Here, Essrrb can be substituted by Esrrg, see *Nat. Cell Biol., 11*, 197-203 (2009))
(20) Oct3/4, Sox2, Esrrb (see *Nat. Cell Biol., 11*, 197-203
15 (2009))
(21) Oct3/4, Klf4, L-Myc (see *Proc. Natl. Acad. Sci. U S A., 107*, 14152-14157 (2010))
(22) Oct3/4, Nanog
(23) Oct3/4 (*Cell 136*: 411-419 (2009); *Nature*, 08436,
20 doi:10.1038 published online(2009))
(24) Oct3/4, Klf4, c-Myc, Sox2, Nanog, Lin28, SV40LT (see *Science, 324*: 797-801 (2009))

In (1)-(24) above, Oct3/4 may be replaced with another member of the Oct family, for example, Oct1A, Oct6 or the like.
25 Sox2 (or Sox1, Sox3, Sox15, Sox17, Sox18) may be replaced with another member of the Sox family, for example, Sox7 or the like. Furthermore, in (1) to (24) above, when c-Myc or Lin28 is included as a nuclear reprogramming factor, L-Myc or Lin28B can be used in place of c-Myc or Lin28, respectively.

30 Any combination that does not fall in (1) to (24) above but comprises all the constituents of any one of (1) to (24) above and further comprises an optionally chosen other substance can also be included in the scope of "nuclear reprogramming substances" in the present invention. Provided
35 that the somatic cell to undergo nuclear reprogramming is

endogenously expressing one or more of the constituents of any one of (1) to (24) above at a level sufficient to cause nuclear reprogramming, a combination of only the remaining constituents excluding the one or more constituents can also be included in the scope of "nuclear reprogramming substances" in the present invention.

Of these combinations, a combination of at least one, preferably two or more, more preferably three or more, selected from among Oct3/4, Sox2, Klf4, c-Myc or L-Myc, Nanog, Lin28 or Lin28B, and SV40LT, is a preferable nuclear reprogramming substance.

Particularly, when the iPS cells obtained are to be used for therapeutic purposes, a combination of the three factors Oct3/4, Sox2 and Klf4 [combination (9) above] or a combination of the four factors Oct3/4, Sox2, Klf4 and L-Myc [combination (2) above] are preferably used. When the iPS cells obtained are not to be used for therapeutic purposes (e.g., used as an investigational tool for drug discovery screening and the like), in addition to the three factors consisting of Oct3/4, Sox2 and Klf4 and the four factors consisting of Oct3/4, Sox2, Klf4 and L-Myc, four factors consisting of Oct3/4, Sox2, Klf4 and c-Myc, five or six factors consisting of Oct3/4, Sox2, Klf4 and c-Myc/L-Myc as well as Nanog and/or Lin28/Lin28b, or six or seven factors consisting of the above five or six factors and additional SV40 Large T antigen are exemplified.

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

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

A proteinous factor for use as a nuclear reprogramming substance can be prepared by inserting the cDNA obtained into an appropriate expression vector, introducing the vector into a host cell, and recovering the recombinant proteinous factor from the cultured cell or its conditioned medium. Meanwhile, when the nuclear reprogramming substance used is a nucleic acid that encodes a proteinous factor, the cDNA obtained is inserted into a viral vector, episomal vector, or plasmid vector to construct an expression vector, and the vector is subjected to the step of nuclear reprogramming. As appropriate, the above-mentioned Cre-loxP system or piggyBac transposon system can be utilized. When two or more nucleic acids encoding proteinous factors are introduced into a cell, respective nucleic acids can be carried in separate vectors. Alternatively, a polycistronic vector can be constructed by ligating a plurality of nucleic acids in tandem. In latter, it is preferable that 2A self-cleaving peptide from a foot-and-mouth disease virus (*Science, 322*, 949-953, 2008) is ligated between the nucleic acids to allow for an efficient polycistronic expression.

Contact of a nuclear reprogramming substance with a somatic cell can be achieved as with the aforementioned proteinous establishment efficiency improving factor (a) when the substance is a proteinous factor; as with the aforementioned nucleic acid-based establishment efficiency improving factors of the present invention (b) when the substance is a nucleic acid that encodes the proteinous factor of (a); and as with the aforementioned chemical establishment efficiency improving factors of the present invention (c) when

the substance is a low-molecular weight compound.

(d) Other iPS cell establishment efficiency improvers

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

Examples of other iPS cell establishment efficiency improvers include, but are not limited to, histone deacetylase (HDAC) inhibitors other than VPA [e.g., 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, shRNA, dominant negative mutant and the like against p53 (*Cell Stem Cell, 3*, 475-479 (2008); Nature 460, 1132-1135 (2009)), Wnt Signaling (e.g., soluble Wnt3a) [*Cell Stem Cell, 3*, 132-135 (2008)], 2i/LIF [2i is an inhibitor of mitogen-activated protein kinase signaling and glycogen synthase kinase-3, *PloS Biology, 6*(10), 2237-2247 (2008)], (for example, miR-302-367 cluster (Mol. Cell. Biol. doi:10.1128/MCB.00398-08)、miR-302 (RNA (2008) 14: 1-10), miR-291-3p, miR-294 and miR-295 (Nat. Biotechnol. 27: 459-461 (2009)) and the like. As mentioned

above, the nucleic acid-based expression inhibitors may be in the form of expression vectors harboring a DNA that encodes an siRNA or shRNA.

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 regarding the establishment efficiency improving factors of the present invention for each of three 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 weight compound.

An iPS cell establishment efficiency improver, including the establishment efficiency improving factors of the present invention, 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 plasmid vector, for example, both may be simultaneously introduced into the cell.

(e) Improving establishment efficiency by culture conditions

iPS cell establishment efficiency can further be improved by culturing the cells under hypoxic conditions in the nuclear reprogramming process for somatic cells (*Cell Stem Cell, 5*(3): 237-241 (2009); WO 2010/013845). As mentioned herein, the term "hypoxic conditions" means that the ambient oxygen concentration as of the time of cell culture is significantly lower than that in the atmosphere. Specifically, conditions involving lower oxygen concentrations than the ambient oxygen concentrations in the 5-10% CO_2/95-90% air atmosphere, which is commonly used for ordinary cell culture, can be mentioned; examples include conditions involving an ambient oxygen concentration of 18% or less. Preferably, the ambient oxygen concentration is 15% or less (e.g., 14% or less, 13% or less, 12% or less, 11% or less and the like), 10% or less (e.g., 9% or less, 8% or less, 7% or less, 6% or less and the like), or 5% or less (e.g., 4% or less, 3% or less, 2% or less and the like). The ambient oxygen concentration is preferably 0.1% or more (e.g., 0.2% or more, 0.3% or more, 0.4% or more and the like), 0.5% or more (e.g., 0.6% or more, 0.7% or more, 0.8% or more, 0.9% 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).

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

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

The duration of cultivation of cells under hypoxic conditions is not particularly limited, as far as iPS cell establishment efficiency is not prevented from being improved compared with the normal oxygen concentration (20%); examples include, but are not limited to, periods of 3 days or more, 5 days or more, 7 days or more or 10 days or more, and 50 days or less, 40 days or less, 35 days or less or 30 days or less and the like. Preferred duration of cultivation under hypoxic conditions varies depending on ambient oxygen concentration; those skilled in the art can adjust as appropriate the duration of cultivation according to the oxygen concentration

used. In an embodiment of the present invention, if iPS cell candidate colonies are selected with drug resistance as an index, it is preferable that a normal oxygen concentration be restored from hypoxic conditions before starting drug selection.

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

(f) Selection and confirmation of iPS cell

After being contacted with nuclear reprogramming substance(s) and the iPS cell establishment efficiency improving factors of the present invention (and other iPS cell establishment efficiency improving factors), 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 (MEF) treated with radiation or an antibiotic to terminate the cell division thereof, as feeder cells. MEF in common use as feeders include the STO cell and the like; for induction of an iPS cell, the SNL cell [McMahon, A. P. & Bradley, A. Cell 62, 1073-1085 (1990)] and the like are commonly used. Co-culture with these feeder cells may be started before contact of the establishment efficiency improving factors of the present invention and 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 or TTFs 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 or TTFs 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 method of selecting candidate colonies 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.

The identity of the cells of a selected colony as iPS cells can be confirmed by positive responses to a Nanog (or Oct3/4) reporter (puromycin resistance, GFP positivity and the like) as well as by the formation of a visible ES cell-like colony, as described above. However, to ensure higher accuracy, it is possible to perform tests such as alkaline phosphatase staining, analyzing the expression of various ES-cell-specific genes, and transplanting the cells selected to a mouse to confirm the formation of teratomas.

When a nucleic acid that encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc is introduced into a somatic cell, the iPS cell obtained is a novel cell distinct from conventionally

known iPS cells because of the containment of the exogenous nucleic acid. In particular, when the exogenous nucleic acid is introduced into the somatic cell using a retrovirus, lentivirus or the like, the exogenous nucleic acid is usually integrated in 5 the genome of the iPS cell obtained, so that the phenotype of containing the exogenous nucleic acid is stably retained.

(g) Use of iPS cell

The iPS cells thus established can be used for various purposes. For example, by utilizing a method of 10 differentiation induction reported with respect to ES cells (for example, see JP 2002-291469 as a method for inducing differentiation into nerve stem cells, JP 2004-121165 as a method for inducing differentiation into pancreatic stem-like cells, JP 2003-505006 as a method for inducing differentiation 15 into hematopoietic cells, JP 2003-523766 as a differentiation induction method via embryonic body formation), differentiation into various cells (e.g., myocardial cells, blood cells, nerve cells, vascular endothelial cells, insulin-secreting cells and the like) from iPS cells can be induced. Therefore, inducing 20 iPS cells using a somatic cell collected from a patient or another person of the same or substantially the same HLA type would enable stem cell therapy by autogeneic or allogeneic transplantation, wherein the iPS cells are differentiated into desired cells (that is, cells of an affected organ of the 25 patient, cells that have a therapeutic effect on disease, and the like), which are transplanted to the patient. Furthermore, because functional cells (e.g., hepatocytes) differentiated from iPS cells are thought to better reflect the actual state of the functional cells *in vivo* than do corresponding existing 30 cell lines, they can also be suitably used for *in vitro* screening for the effectiveness and toxicity of pharmaceutical candidate compounds and the like.

(h) Improvement of the differentiation potential

As stated above, somatic cells of adult derivation are 35 lower than somatic cells of fetal derivation in both iPS cell

establishment efficiency (reprogramming efficiency) and differentiation potential; therefore, a factor that even improves the differentiation potential of iPS cells is possibly present among factors capable of remarkably improving the efficiency of establishment of iPS cells from a somatic cell of adult derivation. Whether an establishment efficiency improving factor of the present invention improves the differentiation potential of iPS cells [the lower the prevalence of cells that are kept in the undifferentiated state when differentiation-induced (i.e., exhibit differentiation resistance), the higher the differentiation potential] can be determined by, for example, transplanting to a mouse a differentiated cell obtained by differentiation induction of an iPS cell prepared using the establishment efficiency improving factor of the present invention, and examining the mouse for tumorigenesis over time. It is also possible to use a method of determining the differentiation potential improving effect, without transplantation, more quickly and conveniently, wherein an iPS cell is first differentiation-induced under appropriate conditions, thereafter the resulting cells are cultured again under conditions for maintaining the undifferentiated state, and the prevalence of undifferentiated cells is determined and compared with a control value (US Provisional Patent Application No. 61/239,297).

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: Effects of transfer of various candidate genes on establishment of iPS cells

Mice bearing a Nanog reporter were used as an experimental system. The Nanog reporter used was prepared by inserting the green fluorescent protein (EGFP) and puromycin resistance genes into the Nanog locus of a BAC (bacterial

artificial chromosome) purchased from BACPAC Resources [Okita K. et al., Nature 448, 313-317(2007)]. The mouse Nanog gene is expressed specifically in pluripotent cells such as ES cells and early embryos. The mouse iPS cells that have become positive for this reporter are known to be nearly equivalent to ES cells in terms of differentiation potential. Mouse embryonic fibroblasts (MEFs) and tail tissue fibroblasts (TTFs) obtained from a Nanog reporter mouse bearing this Nanog reporter [Okita K. et al., Nature 448, 313-317(2007)] were transfected by means of retroviruses to establish iPS cells, and colonies expressing EGFP were counted by means of the Nanog reporter to evaluate the efficiency of establishment of iPS cells.

The retroviruses used for reprogramming were prepared by transferring each retrovirus expression vector [pMXs-Oct3/4, pMXs-Sox2, pMXs-Klf4, pMXs-cMyc, and pMXs vectors containing each of the candidate genes shown in Table 1] into Plat-E cells (Morita, S. et al., Gene Ther. 7, 1063-1066) seeded at 2×10^6 cells per 100 mm culture dish (Falcon) on the previous day. The culture broth used was DMEM/10% FCS [DMEM (Nacalai Tesque) supplemented with 10% fetal bovine serum], and the cells were cultured at 37°C in the presence of 5% CO_2.

Table 1

genes		chemical compounds	medium
Ecat1	Sall4	VPA	KSR
Dppa5	Rex1	5'azaC	
Fbx15	Utf1	TSA	
Nanog	Tcl1		
ERas	Dppa3		
Dnmt3L	β-cateninS33Y		
Ecat8	Stat3-C		
Gdf3	Grb2ΔSH2		
Sox15	Lin28		
Dppa4	N-Myc		
Dppa2	Fthl17		

For vector introduction, 27 µL of the FuGene6 transfection reagent (Roche) was placed in 300 µl of Opti-MEM I Reduced-Serum Medium (Invitrogen), and the medium was allowed to stand at room temperature for 5 minutes. Thereafter, 9 µg of each expression vector was added, and the medium was allowed to stand at room temperature for 15 minutes, and then added to the Plat-E culture broth. On day 2, the Plat-E supernatant was replaced with a fresh medium. On day 3, the culture supernatant was recovered and filtered through a 0.45 µm sterile filter (Whatman), polybrene (Nacalai) was added to obtain a concentration of 4 µg/mL, and this was used as the virus liquid.

Mouse embryonic fibroblasts (MEFs) were isolated from a fetus at 13.5 days after fertilization of a Nanog reporter mouse, and cultured with a medium (DMEM/10% FCS). The tail tissue fibroblasts (TTFs) used were obtained by shredding the tail tissue of a Nanog reporter mouse, placing the pieces of the tissue standing on a 6-well dish, culturing them in a primary culture cell starting medium (Toyobo Life Science Department) for 5 days, and further culturing the fibroblasts migrating from the tail tissue onto the dish with the DMEM/10% FCS medium.

Not expressing the Nanog gene, MEFs and TTFs do not express EGFP and do not emit green fluorescence. Not expressing the puromycin resistance gene as well, MEFs and TTFs are susceptible to the antibiotic puromycin. As such, MEFs and TTFs were seeded to a 6-well dish (Falcon) at $1x10^5$ cells per well. The culture broth used was DMEM/10% FCS, and the cells were cultured at 37°C and 5% CO_2. The following day, each retrovirus liquid (a total of five different genes: Oct3/4, Sox2, Klf4, c-Myc and one of the candidate genes shown in Table 1) was added to transfer the genes by overnight infection.

Two days after the viral infection, the retrovirus liquid was removed and replaced with DMEM/10% FCS, and the cells were cultured using DMEM/10% FCS until day 4 after the infection. On

day 4 after the infection, the media for the MEFs and TTFs were removed, and the cells were washed by the addition of 1 mL of PBS. After the PBS was removed, 0.25% trypsin/1 mM EDTA (Invitrogen) was added, and a reaction was allowed to proceed at 37°C for about 5 minutes. After the cells floated up, they were suspended by the addition of DMEM/10% FCS; $5x10^3$ MEFs or $1x10^4$ TTFs were seeded to a 6-well dish having feeder cells seeded thereto previously. The feeder cells used were SNL cells treated with mitomycin C to terminate the cell division thereof [McMahon, A.P. & Bradley, A. Cell 62, 1073-1085 (1990)].

On day 5 after the infection, the medium was changed from DMEM/10% FCS to an ES cell culture medium [DMEM (Nacarai Tesque) supplemented with 15% fetal bovine serum, 2 mM L-glutamine (Invitrogen), 100 μM non-essential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Invitrogen), 50 U/mL penicillin (Invitrogen) and 50 μg/mL streptomycin (Invitrogen)]. Cultivation was continued while replacing the ES cell culture medium with a fresh supply of the same medium every two days until a visible colony emerged. On day 30 after the infection, GFP-positive colonies were counted. The means of the results of four independent experiments are shown in Fig. 1 (TTF) and Fig. 2 (MEF). A larger number of iPS cells (GFP-positive cells) tended to be established when the following genes were added, than when only the 4 genes (Oct3/4, Sox2, Klf4, c-Myc) were transferred.

In case of TTFs: Nanog, Dppa2, Sall4, Rex1, Utf1, β-catenin S33Y, Stat3-C, N-Myc

In case of MEFs: Fbx15, Nanog, ERas, Ecat8, Gdf3, Sall4, Rex1, Utf1, Dppa3, β-catenin S33Y, Stat3-C, Grb2ΔSH2, N-Myc

When the following genes were added to the 4 genes, a larger number of iPS cells tended to be established with TTFs than with MEFs.

Dppa2, Sall4, Utf1, β-catenin S33Y, Stat3-C, N-Myc

Example 2: Effects of epigenetics-related compounds and KSR on iPS cell establishment

The effects of the epigenetics-related low-molecular compounds shown in Table 1 (VPA, 5'azaC, TSA) and KSR (KnockOut™ Serum Replacement; Invitrogen) on the establishment of iPS cells from TTFs were examined. Each low-molecular compound (VPA, 5'azaC or TSA) was allowed to be present in the experimental system between day 1 and day 4 and between day 5 and day 14, after infection with the 4 genes (Oct3/4, Sox2, Klf4, c-Myc). Cultivation in the presence of KSR was performed between day 5 and day 25 after the infection. Except for these conditions, the experiments were performed in the same manner as Example 1. On day 25 after the infection, GFP-positive colonies were counted. The results are shown in Fig. 3. When 0.4 mM VPA was added to the 4 genes, the number of non-iPS cells decreased, whereas the number of iPS cells (GFP-positive cells) established increased. Similar results were obtained with the use of the KSR medium in place of the medium containing 15% fetal bovine serum.

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 No. 61/258,751 filed on November 6, 2009, the content of which is hereby incorporated by reference.

CLAIMS

[1] A method of improving iPS cell establishment efficiency, comprising bringing one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR into contact with a somatic cell in a nuclear reprogramming step.

[2] The method according to claim 1, wherein the somatic cell is a somatic cell of adult derivation.

[3] An iPS cell establishment efficiency improver comprising a factor selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR.

[4] The agent according to claim 3, wherein the improver is to be used to produce iPS cells from a somatic cell of adult derivation.

[5] A method of producing iPS cells, comprising the step of bringing one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR, and a nuclear reprogramming substance into contact with a somatic cell.

[6] The method according to claim 5, wherein the nuclear reprogramming substance is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Klf4 family, members of the Myc family, members of the Lin28 family, and Nanog, as well as nucleic acids that encode the same.

[7] The method according to claim 5, wherein the nuclear reprogramming substance includes Oct3/4 or a nucleic acid that encodes the same.

[8] The method according to claim 7, wherein the nuclear reprogramming substance consists of Oct3/4, Sox2, Klf4, and L-Myc or c-Myc, or nucleic acids that encode the same.

[9] The method according to any one of claims 5 to 8, wherein the somatic cell is a somatic cell of adult derivation.

[10] An iPS cell inducer from a somatic cell, comprising a factor selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR, and a nuclear programming substance.

5 [11] The inducer according to claim 10, wherein the nuclear reprogramming substance is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Klf4 family, members of the Myc family, members of the Lin28 family, and Nanog, as well as nucleic acids that

10 encode the same.

[12] The inducer according to claim 10, wherein the nuclear reprogramming substance includes Oct3/4 or a nucleic acid that encodes the same.

[13] The inducer according to claim 12, wherein the nuclear

15 reprogramming substance consists of Oct3/4, Sox2, Klf4, and L-Myc or c-Myc, or nucleic acids that encode the same.

[14] The inducer according to any one of claims 10 to 13, wherein the somatic cell is a somatic cell of adult derivation.

[15] An iPS cell containing an exogenous nucleic acid that

20 encodes Dppa2, Sall4, Utf1, β-catenin, Stat3 or N-Myc.

[16] The iPS cell according to claim 15, wherein the exogenous nucleic acid is integrated in the genome.

[17] A method of producing a somatic cell, comprising performing a differentiation induction treatment on the iPS

25 cell according to claim 15 or 16 to cause the iPS cell to differentiate into a somatic cell.

[18] A method of producing a somatic cell, comprising the steps of:

(1) producing an iPS cell by the method according to any one

30 of claims 5 to 9, and

(2) performing a differentiation induction treatment on the iPS cell obtained through the step (1) to cause the iPS cell to differentiate into a somatic cell.

[19] A use of one or more factors selected from the group

35 consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc,

nucleic acids that encode the same, VPA and KSR for improving the efficiency of establishment of iPS cells.

[20] The use according to claim 19, wherein the use is for producing an iPS cell from a somatic cell of adult derivation.

5 [21] A use of one or more factors selected from the group consisting of Dppa2, Sall4, Utf1, β-catenin, Stat3, N-Myc, nucleic acids that encode the same, VPA and KSR for producing an iPS cell, wherein the factor(s), along with a nuclear programming substance, is(are) brought into contact with a 10 somatic cell.

[22] The use according to claim 21, wherein the nuclear reprogramming substance is selected from the group consisting of members of the Oct family, members of the Sox family, members of the Klf4 family, members of the Myc family, members 15 of the Lin28 family, and Nanog, as well as nucleic acids that encode the same.

[23] The use according to claim 21, wherein the nuclear reprogramming substance includes Oct3/4 or a nucleic acid that encodes the same.

20 [24] The use according to claim 23, wherein the nuclear reprogramming substance consists of Oct3/4, Sox2, Klf4, and L-Myc or c-Myc, or nucleic acids that encode the same.

[25] The use according to any one of claims 21 to 24, wherein the somatic cell is a somatic cell of adult derivation.

25 [26] A use of the iPS cell according to claim 15 or 16 in producing a somatic cell.

[27] The iPS cell according to claim 15 or 16 as a source of cells for producing a somatic cell.

Fig. 1

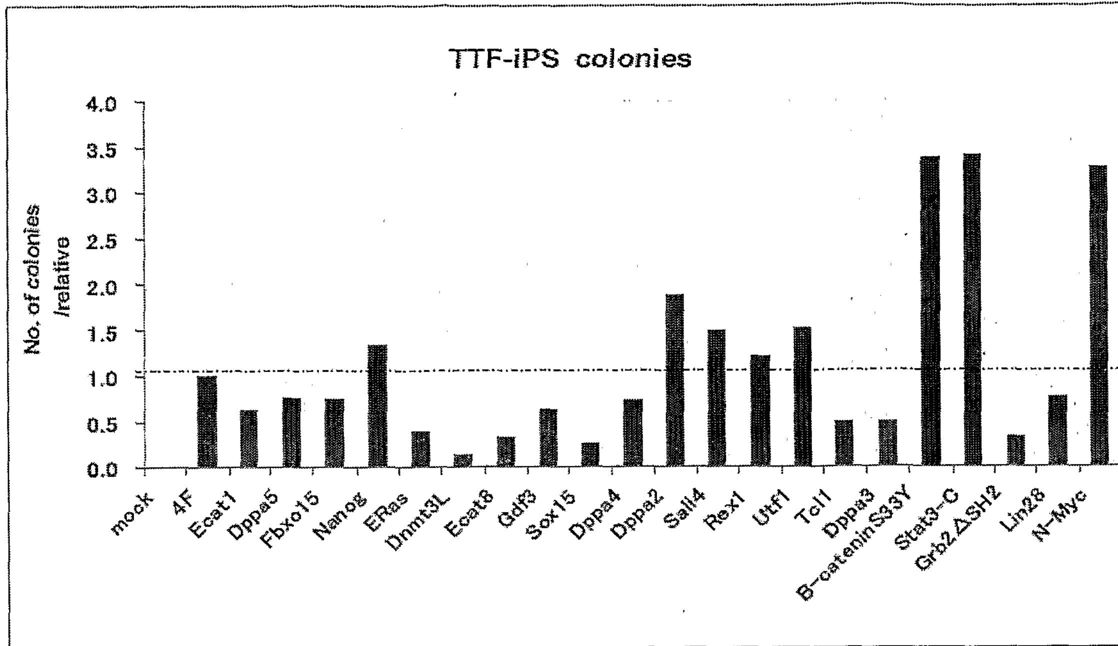


Fig. 2

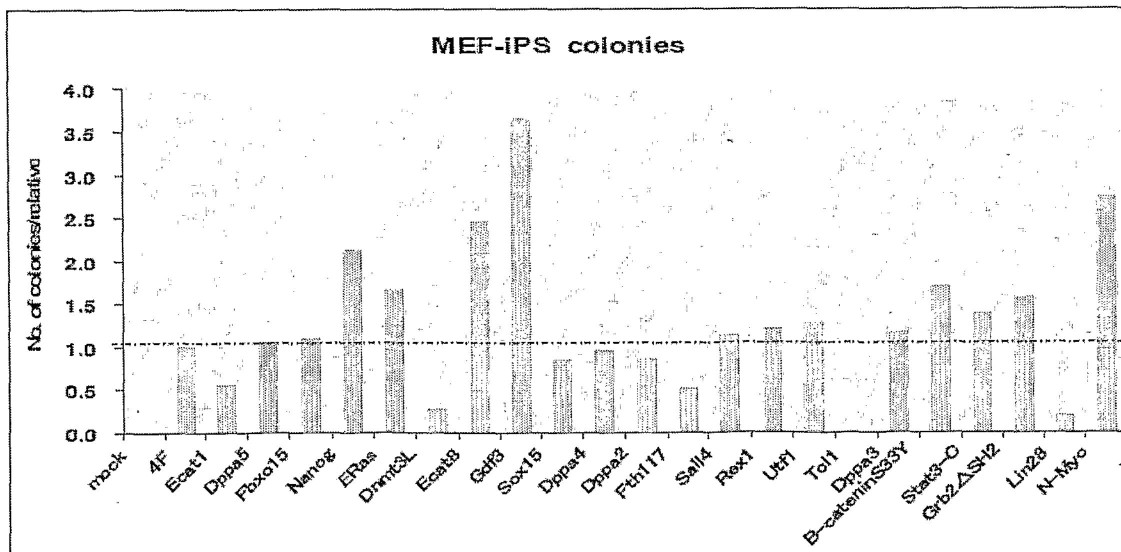
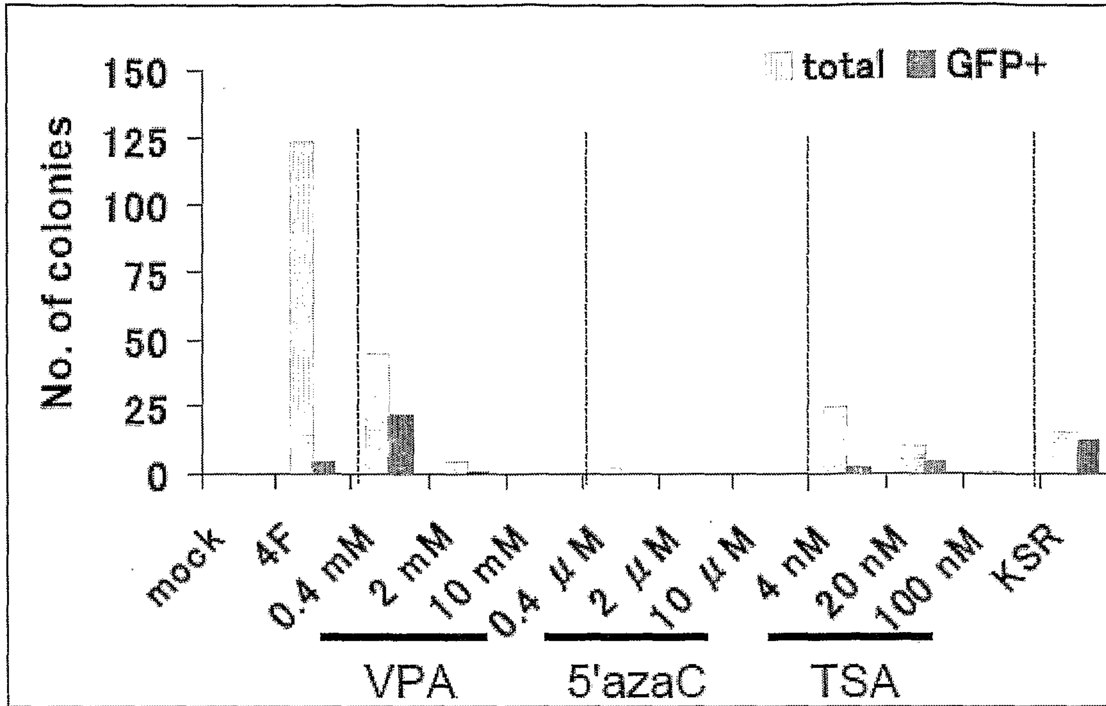


Fig. 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/070152

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N15/09(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N15/09

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

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TSUBOOKA,N et al., Roles of Sall4 in the generation of pluripotent stem cells from blastocysts and fibroblasts. Genes Cells. 2009 Jun, vol.14(6), pp.683-94. Epub 2009 May 19.	1-27
X	WO 2009/057831 A1(KYOTO UNIVERSITY) 2009.05.07 & US 2009/0047263 A1 & US 2009/0227032 A1 & US 2010/0062533 A1 & US 2010/0210014 A1 & US 2010/0216236 A1 & US 2009/0068742 A1 & EP 2096169 A1 & EP 2206724 A1 & EP 2206778 A1 & EP 2208786 A1	1-27

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28.01.2011	08.02.2011

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/070152

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAKAGAWA,M et al., Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol. 2008 Jan, vol.26(1), pp.101-106	1-27
X	HUANGFU,D et al., Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol. 2008 Jul, vol.26(7), pp.795-797	1-27
Y	TAKAHASHI,K et al., Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006 Aug 25, vol.126(4), pp.663-676	1-27
Y	DU,J et al., Dppa2 knockdown-induced differentiation and repressed proliferation of mouse embryonic stem cells. J Biochem. 2010 Feb, vol.147(2), pp.265-271. Epub 2009 Oct 20. Erratum in: J Biochem. 2010 Jun, vol.147(6), p.929	1-27
Y	LLUIS,F et al., Somatic cell reprogramming control: signaling pathway modulation versus transcription factor activities. Cell Cycle. 2009 Apr 15, vol.8(8), pp.1138-1144. Epub 2009 Apr 16.	1-27
Y	TAN,SM et al., A UTF1-based selection system for stable homogeneously pluripotent human embryonic stem cell cultures. Nucleic Acids Res. 2007, vol.35(18), p.e118	1-27
Y	KIDDER,BL et al., Stat3 and c-Myc genome-wide promoter occupancy in embryonic stem cells. PLoS One. 2008, vol.3(12), p.e3932	1-27
Y	GARCIA-GONZALO,FR et al., Albumin-associated lipids regulate human embryonic stem cell self-renewal. PLoS One. 2008 Jan 2, vol.3(1), p.e1384	1-27
P,X	OKADA,M et al., Effective culture conditions for the induction of pluripotent stem cells. Biochim Biophys Acta. 2010 Sep;1800(9):956-63. Epub 2010 Apr 22	1-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/070152

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FENG,B et al., Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol. 2009 Feb, vol.11(2), pp.197-203. Epub 2009 Jan 11	1-27
A	SHI,Y et al., Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell. 2008 Nov 6, vol.3(5), pp.568-574	1-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2010/070152

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

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

D1-4 disclose that Sall4, N-Myc and VPA improve iPS cell establishment efficiency, respectively. Therefore, 8 iPS cell establishment efficiency improvers in the claims 1-27 involve neither the same nor corresponding special technical features. Thus, there are 8 inventions corresponding to each of the iPS cell establishment efficiency improvers in this application.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☑ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.