

DESCRIPTION

TITLE OF THE INVENTION

METHOD FOR PRODUCING HIGH-QUALITY iPS CELLS

Technical Field

[0001]

The present invention relates to an agent for improving quality of an iPS cell, a method for producing an iPS cell, an iPS cell produced by such a method for production, and a composition for producing an iPS cell.

Background Art

[0002]

Induced pluripotent stem (iPS) cells can be produced from somatic cells by introducing Oct3/4, Sox2, Klf4, and c-Myc (Non-patent Document 1, Patent Document 1). This can be achieved by reprogramming the parent somatic cell transcription network and epigenetic signatures. iPS cells bring various benefits for basic research, drug innovation, and regenerative medicine. However, it is still a serious problem that cell populations of produced iPS cells are more heterogeneous in quality than cell populations of embryonic-stem cells (ES cells). For example, while ES cells have small variance in property among cells and substantially any cell can be differentiated into the intended cell, iPS cells have large variance in property among cells and there have often been cells that cannot be differentiated into the intended cell. It is important for basic studies and the

clinical purpose that any iPS cell displays high quality without variance.

[0003]

Many attempts have been made to solve the problem that cell populations of iPS cells are heterogeneous in quality. For example, Patent Document 2 discloses that the production efficiency and stability of iPS cells can be improved by the predetermined number of times of introduction of a predetermined amount of an Oct3/4 gene, a Klf4 gene, a c-Myc gene, and a Sox2 gene into somatic cells. Moreover, Patent Document 3 discloses that induced pluripotent stem cells (iPS cells) excellent in quality can be produced efficiently in a short period of time by introducing into somatic cells a Prdm14 gene or a gene product thereof, an Esrrb gene or a gene product thereof, and a Sall4a gene or a gene product thereof, in addition to an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and a c-Myc gene or a gene product thereof. Furthermore, Patent Document 4 discloses that induced pluripotent stem cells (iPS cells) excellent in quality can be produced efficiently in a short period of time by introducing into somatic cells a Jarid2 mutant gene or a gene product thereof in addition to an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and a c-Myc gene or a gene product thereof. However, there has been still room for improvement in quality of iPS cells. Therefore, the development of a method for producing high-

quality iPS cells with smaller variance in quality has been demanded.

[0004]

The linker histone H1 family binds to linker DNA and generates higher-order chromatin structures to control gene expression. The members of the linker histone H1 family include histones H1a, H1b, H1c, H1d, H1e, H1foo, H1x, H1.0, H1t, H1T2, and HILS1. Most members of the linker histone family are somatic linker histones that condense chromatin. Accordingly, such structures generally repress general gene transcription activity (Non-patent Documents 2 and 3).

Prior Art Documents

Patent Documents

[0005]

Patent Document 1: Japanese Patent No. 4183742

Patent Document 2: Japanese unexamined Patent Application Publication No. 2011-004674

Patent Document 3: Japanese unexamined Patent Application Publication No. 2014-217344

Patent Document 4: Japanese unexamined Patent Application Publication No. 2014-217345

Non-patent Documents

[0006]

Non-patent Document 1: Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676 (2006)

Non-patent Document 2: Steinbach, O.C., Wolffe, A.P. & Rupp, R.A. Somatic linker histones cause loss of mesodermal competence in *Xenopus*. *Nature* 389, 395-399 (1997)

Non-patent Document 3: Hebbar, P.B. & Archer, T.K. Altered histone H1 stoichiometry and an absence of nucleosome positioning on transfected DNA. *The Journal of biological chemistry* 283, 4595-4601 (2008).

Summary of the Invention

Object to be Solved by the Invention

[0007]

An object of the present invention is to provide an agent for improving quality of an iPS cell, a method for producing an iPS cell, an iPS cell produced by such a method for production, and a composition for producing an iPS cell.

Means to Solve the Object

[0008]

The present inventors have studied diligently to improve quality of iPS cells and found as a result that in a method for producing an iPS cell by introducing a nuclear reprogramming substance into a somatic cell, introducing not only the nuclear reprogramming substance, but also "an Hlfoo gene or a gene product thereof" into the somatic cell allows the production of high-quality iPS cells with smaller variance in quality, thereby completing the present invention. It was unexpected for those skilled in the art that the combination of the "Hlfoo gene

or gene product thereof" and the nuclear reprogramming substance allowed the production of high-quality iPS cells with smaller variance in quality.

[0009]

Accordingly, the invention provides:

- (1) an agent for improving quality of an iPS cell, comprising an Hlfoo gene or a gene product thereof; and
- (2) the agent for improving quality of an iPS cell according to (1), wherein the agent comprises an expression vector comprising the Hlfoo gene.

[0010]

Moreover, the present invention provides:

- (3) a method for producing an iPS cell, comprising the step of introducing (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof into a somatic cell;
- (4) the method for producing an iPS cell according to (3), wherein the nuclear reprogramming substance comprises at least one selected from the group consisting of a gene of Oct gene family, a gene of Sox gene family, a gene of Klf gene family, a gene of Myc gene family, a gene of Lin gene family, a Nanog gene, and gene products thereof;
- (5) the method for producing an iPS cell according to (3) or (4), wherein the nuclear reprogramming substance consists of a gene of Oct gene family or a gene product thereof, a gene of Sox gene family or a gene product thereof, and a gene of Klf gene family or a gene product thereof;
- (6) the method for producing an iPS cell according to any one of (3) to (5), wherein the nuclear reprogramming

substance consists of an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, and a Klf4 gene or a gene product thereof; and

(7) the method for producing an iPS cell according to any one of (3) to (5), wherein the nuclear reprogramming substance consists of an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and an L-Myc gene or a gene product thereof.

[0011]

Furthermore, the invention provides:

(8) an iPS cell produced by the method for producing an iPS cell according to any one of (1) to (7); and

(9) a composition for producing an iPS cell, comprising (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof.

Effect of the Invention

[0012]

The present invention can provide an agent for improving quality of an iPS cell, a method for producing an iPS cell, an iPS cell produced by such a method for production, and a composition for producing an iPS cell. According to the present invention, high-quality iPS cells with smaller variance in quality can be produced.

Brief Description of Drawings

[0013]

[Figure 1] Figure 1 is a set of microscopic images of tail tip fibroblasts (hereinafter simply referred to as "mouse

fibroblasts") of a C57BL/6J mouse expressing exogenous (mouse) Hlfoo. The "BF", "DAPI", "Hlfoo", and "MERGE" images in Figure 1a indicate a phase contrast image, a DAPI fluorescent staining image, an Hlfoo fluorescent staining image, and an overlay image of the 3 images. The left and right images in Figure 1b are Hlfoo fluorescent staining 2 dimension and 2.5 dimension images, respectively. The left and right images in Figure 1c are electron microscopic images of control cells (mouse fibroblasts) and mouse fibroblasts expressing exogenous Hlfoo, respectively.

[Figure 2] Figure 2a shows the results of introducing 3 kinds of nuclear reprogramming substances (mouse Oct3/4, Sox2, and Klf4 genes, [OSK gene]), OSK genes and a linker histone H1 gene (OSK + H1c genes) or OSK genes and an Hlfoo gene (OSK + Hlfoo genes) into mouse fibroblasts and analyzing the colonization efficiency of ALP-positive ES-like cells (iPS cells). Figure 2b shows the results of analyzing the expression of 3 kinds of pluripotent markers (Oct3/4, Nanog, and SSEA1) in iPS cells produced by introducing the OSK genes or the OSK + Hlfoo genes (hereinafter referred to as "OSK-iPS cells" and "OSK + Hlfoo-iPS cells", respectively). Figure 2c shows the results of analyzing the endogenous Hlfoo expression 1 to 5 days after the introduction of the OSK genes or the OSK genes and a c-Myc gene (OSKM genes). "MEF + Hlfoo" in Figure 2c shows the results of analyzing mouse embryonic fibroblasts (MEF) in which an Hlfoo gene is introduced.

[Figure 3] Figure 3 shows the results of introducing OSKM genes, OSKM genes and an Hlfoo gene (OSKM + Hlfoo genes),

OSK genes, or OSK + Hlfoo genes into Nanog-GFP expressing fibroblasts and analyzing the percentage of Nanog-GFP-positive ES-like cells (iPS cells).

[Figure 4] Figure 4a shows the results of comparing global gene transcriptome profiles of the OSK + Hlfoo-iPS cells and ES cells. Figure 4b shows the results of comparing global gene transcriptome profiles of the OSK + Hlfoo-iPS cells and the OSK-iPS cells.

[Figure 5] Figure 5 shows the results of analyzing DNA methylation levels in IG-DMR (Figure 5a) and Gtl2-DMR (Figure 5b) in OSK-iPS cells and OSK + Hlfoo-iPS cells. "ES" and "MEF" in the figure indicate the results of analyzing ES cells and MEF cells, respectively.

[Figure 6] Figure 6 shows the results of analyzing the morphology of embryoid bodies (EBs) formed from OSK-iPS cells and OSK + Hlfoo-iPS cells.

[Figure 7] Figure 7 shows the results of analyzing the percentage of apoptotic cells in EBs formed from OSK-iPS cells and OSK + Hlfoo-iPS cells.

[Figure 8] Figure 8 shows the results of analyzing the expression of 2 cell proliferation markers (Ki67 and PCNA) in EBs formed from OSK-iPS cells and OSK + Hlfoo-iPS cells.

[Figure 9] Figure 9 shows the results of analyzing the chimera competency of OSK-iPS cells and the OSK + Hlfoo-iPS cells.

[Figure 10] Figure 10 shows the results of analyzing germline transmission potential of chimera mice derived from OSK-iPS cells and OSK + Hlfoo-iPS cells.

[Figure 11] Figure 11 shows the results of quantitative RT-PCR analysis of the SRF gene (Figure 11a) and the ACTG2

gene (Figure 11b) in OSKL + Hlfoo-iPS cells (iPS cells produced by introducing Oct3/4, Sox2, Klf4, L-Myc, and Hlfoo genes) and OSKL-iPS cells (iPS cells produced by introducing Oct3/4, Sox2, Klf4, and L-Myc genes). The numbers 1 to 4 on the abscissa of the graph indicate the clone numbers.

[Figure 12] Figure 12 shows the results of quantitative RT-PCR analysis of the Oct3/4 gene in OSKL + Hlfoo-iPS cells and OSKL-iPS cells. The numbers 1 to 4 on the abscissa of the graph indicate the clone numbers.

[Figure 13] Figure 13 shows the numbers of viable cells after 5 days of culturing OSKL + Hlfoo-iPS cells and OSKL-iPS cells with induction of differentiation. The numbers 1 to 4 on the abscissa axis of the graph indicate the clone numbers.

[Figure 14] Figure 14 shows the results of quantitative RT-PCR analysis of the Oct3/4 gene after culturing OSKL + Hlfoo-iPS cells and OSKL-iPS cells for 5 days with induction of differentiation. The numbers 1 to 4 on the abscissa axis of the graph indicate the clone numbers.

Mode of Carrying Out the Invention

[0014]

1. Method for producing iPS cell

The method for producing an iPS cell according to the present invention comprises at least the step of: introducing (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof into a somatic cell (hereinafter also referred to simply as the

"introduction step".) and may further comprise another step as needed.

[0015]

<Introduction step>

The introduction step is at least the step of introducing (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof into a somatic cell. By introducing not only a nuclear reprogramming substance, but also an Hlfoo gene or a gene product thereof into a somatic cell, a larger amount of high-quality iPS cells can be produced. As used herein, the gene product means a messenger RNA (mRNA) transcribed from a gene and/or a protein translated from the mRNA. The Hlfoo gene means a polynucleotide encoding an Hlfoo protein. The Hlfoo gene or a gene product thereof can be used as an agent for improving quality of an iPS cell. Moreover, a vector comprising an Hlfoo gene, which is described later, can be also used as an agent for improving quality of an iPS cell.

[0016]

(Hlfoo gene)

The source of the Hlfoo gene is not particularly limited, but may be appropriately selected according to the purpose and examples include any mammal such as a human, a mouse, a rat, a cow, a sheep, a horse, and a monkey. The sequence information of the Hlfoo gene can be obtained from a publically known database and can be obtained, for example, under accession number BC047943 (human), AY158091 (human), or BC137916 (mouse) in the GenBank. The nucleotide sequence of an human Hlfoo gene

(BC047943) is set forth in SEQ ID No: 1 and the amino acid sequence of an human Hlfoo protein (the protein encoded by the Hlfoo gene of BC047943) is set forth in SEQ ID No: 2. The nucleotide sequence of an human Hlfoo gene (AY158091) is set forth in SEQ ID No: 59 and the amino acid sequence of an human Hlfoo protein (the protein encoded by the Hlfoo gene of AY158091) is set forth in SEQ ID No: 60. The nucleotide sequence of an mouse Hlfoo gene (BC137916) is set forth in SEQ ID No: 3 and the amino acid sequence of an mouse Hlfoo protein (the protein encoded by the Hlfoo gene of BC137916) is set forth in SEQ ID No: 4.

[0017]

The nucleotide sequences of the Hlfoo genes and the nucleotide sequences of the mRNAs thereof may be the same as the nucleotide sequences of the wild-type Hlfoo genes and the nucleotide sequences of the mRNAs thereof or may comprise a mutation. Examples of such a nucleotide sequence comprising a mutation include a "nucleotide sequence that is modified from a nucleotide sequence of a wild-type Hlfoo gene (for example, a nucleotide sequence set forth in SEQ ID No: 1 or 59 or 3) or the nucleotide sequence of the mRNA thereof by a deletion, a substitution, an insertion, or an addition of 1 to 30, preferably 1 to 20, more preferably 1 to 15, more preferably 1 to 10, more preferably 1 to 5, more preferably 1 to 3 nucleotides and encodes a protein having the Hlfoo activity" and a "nucleotide sequence that comprises in the part to be translated into a protein, a nucleotide sequence having 70% or more, preferably 80% or more, more preferably 85% or more, more preferably 90% or more, more preferably 95%

or more, or more preferably 98% or more sequence identity with a nucleotide sequence of a wild-type Hlfoo gene (for example, a nucleotide sequence set forth in SEQ ID No: 1 or 59 or 3) or the nucleotide sequence of the mRNA thereof, and encodes a protein having the Hlfoo activity".

[0018]

The amino acid sequences of the Hlfoo proteins may be the same as the amino acid sequences of the wild-type Hlfoo proteins (for example, an amino acid sequence set forth in SEQ ID No: 2 or 60 or 4) or may comprise a mutation. Examples of such a protein comprising a mutation include a "protein that consists of an amino acid sequence modified from an amino acid sequence of a wild-type Hlfoo protein (for example, an amino acid sequence set forth in SEQ ID No: 2 or 60 or 4) by a deletion, a substitution, an insertion, or an addition of 1 to 30, preferably 1 to 20, more preferably 1 to 15, more preferably 1 to 10, more preferably 1 to 5, or more preferably 1 to 3 amino acids and has the Hlfoo activity" and a "protein that consists of an amino acid sequence having a 70% or more, preferably 80% or more, more preferably 85% or more, more preferably 90% or more, more preferably 95% or more, or more preferably 98% or more sequence identity with an amino acid sequence of a wild-type Hlfoo protein (for example, an amino acid sequence set forth in SEQ ID No: 2 or 60 or 4) and has the Hlfoo activity". As used herein, the "protein that has the Hlfoo activity" means a protein that allows a larger amount of high-quality iPS cells to be produced when it is introduced into a somatic cell with a nuclear

reprogramming substance than the amount when only the nuclear reprogramming substance is introduced into the somatic cell.

[0019]

(Nuclear reprogramming substance)

As used herein, the "nuclear reprogramming substance" means a substance (or a group of substances) that allows the induction of a somatic cell into an iPS cell by introducing the substance singly or a combination of the substance and another substance into the somatic cell. Such a nuclear reprogramming substance may be any substance such as a gene (including one in a form of being incorporated in an expression vector) or a gene product thereof or a low molecular weight compound, as long as it is a substance (or a group of substances) that allows the induction of an iPS cell from a somatic cell. The gene that is a nuclear reprogramming substance means a polynucleotide encoding a protein that is a nuclear reprogramming substance. Examples of the nuclear reprogramming substance when it is a gene or a gene product thereof include at least one selected from the group consisting of: a gene of Oct gene family, a gene of Sox gene family, a gene of Klf gene family, a gene of Myc gene family, a gene of Lin gene family, and a Nanog gene, and gene products thereof (WO2007/69666, Japanese Patent No. 5696282; Science, 2007, 318:1917-1920); in particular, preferably 2 or more and more preferably 3 or more selected from the group, and preferably 2 to 4 and more preferably 3 or 4 selected from the group. Specific examples of genes of these families and combinations

thereof are listed below. Although only the gene names are described in the following, use of gene products thereof are also included.

[0020]

An example may be:

- (a) 1 nuclear reprogramming substance consisting of a gene of Oct gene family;
- (b) a combination of 2 nuclear reprogramming substances consisting of a gene of Oct gene family and a gene of Sox gene family;
- (c) a combination of 2 nuclear reprogramming substances consisting of a gene of Oct gene family and a gene of Klf gene family;
- (d) a combination of 2 nuclear reprogramming substances consisting of a gene of Oct gene family and a Nanog gene,
- (e) a combination of 3 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Sox gene family, and a gene of Klf gene family,
- (f) a combination of 3 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Klf gene family, and a gene of Myc gene family;
- (g) a combination of 4 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Sox gene family, a gene of Klf gene family, and a gene of Myc gene family; and
- (h) a combination of 4 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Sox gene family, a gene of Lin gene family, and a Nanog gene, or a combination of a nuclear reprogramming substance set forth in the (a) to (h) or a combination thereof and a

further added other nuclear reprogramming substance (a gene or a gene product thereof).

Specific examples include:

(a') a combination of nuclear reprogramming substances including 1 nuclear reprogramming substance consisting of a gene of Oct gene family;

(b') a combination of nuclear reprogramming substances including 2 nuclear reprogramming substances consisting of a gene of Oct gene family and a gene of Sox gene family;

(c') a combination of nuclear reprogramming substances including 2 nuclear reprogramming substances consisting of a gene of Oct gene family and a gene of Klf gene family;

(d') a combination of nuclear reprogramming substances including 2 nuclear reprogramming substances consisting of a gene of Oct gene family and a Nanog gene;

(e') a combination of nuclear reprogramming substances including 3 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Sox gene family, and a gene of Klf gene family;

(f') a combination of nuclear reprogramming substances including 3 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Klf gene family, and a gene of Myc gene family;

(g') a combination of nuclear reprogramming substances including 4 nuclear reprogramming substances consisting of a gene of Oct gene family, a gene of Sox gene family, a gene of Klf gene family, and a gene of Myc gene family;
and

(h') a combination of nuclear reprogramming substances including 4 nuclear reprogramming substances consisting of

a gene of Oct gene family, a gene of Sox gene family, a gene of Lin gene family, and a Nanog gene.

[0021]

More specific examples include, but are not limited to, the following combinations.

(1) an Oct3/4 gene, a Klf4 gene, a c-Myc gene;

(2) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene (wherein the Sox2 gene is replaceable with a Sox1 gene, a Sox3 gene, a Sox15 gene, a Sox17 gene, or a Sox18 gene; the Klf4 gene is replaceable with a Klf1 gene, a Klf2 gene, or a Klf5 gene; and the c-Myc gene is replaceable with a T58A (active form mutant) gene, a N-Myc gene, or a L-Myc gene.);

(3) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an Fbx15 gene, a Nanog gene, an Eras gene, an ECAT15-2 gene, a Tc1I gene, β -catenin (active form mutant S33Y);

(4) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an hTERT gene, an SV40 Large T antigen (hereinafter, SV40LT) gene;

(5) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an hTERT gene, an HPV16 E6 gene;

(6) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an hTERT gene, an HPV16 E7 gene;

(7) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an hTERT gene, an HPV6 E6 gene, an HPV16 E7 gene;

(8) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an hTERT gene, a Bmil gene,

(For the combinations (1) to (8), see WO2007/069666 (however, for the substitution from the Sox2 gene to the Sox18 gene and the substitution from the Klf4 gene to the

Klf1 gene or the Klf5 gene in the combination (2), see Nature Biotechnology, 26, 101-106 (2008)). For the combination of "an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene", see also Cell, 126, 663-676 (2006), Cell, 131, 861-872 (2007), and the like. For the combination of "an Oct3/4 gene, a Sox2 gene, a Klf2 (or Klf5) gene, a c-Myc gene", see also Nat. Cell Biol., 11, 197-203 (2009). For the combination of "an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, an hTERT gene, an SV40LT gene", see also Nature, 451, 141-146 (2008).)

(9) an Oct3/4 gene, a Sox2 gene, a Klf4 gene (see Nature Biotechnology, 26, 101-106 (2008));

(10) an Oct3/4 gene, a Sox2 gene, a Nanog gene, a Lin28 gene (see Science, 318, 1917-1920 (2007));

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

(12) an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene, a Nanog gene, a Lin28 gene (see Cell Research (2008) 600-603);

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

(14) an Oct3/4 gene, a Klf4 gene (see Nature 454: 646-650 (2008), Cell Stem Cell, 2: 525-528 (2008));

(15) an Oct3/4 gene, a c-Myc gene (see Nature 454: 646-650 (2008));

(16) an Oct3/4 gene, a Sox2 gene (see Nature, 451, 141-146 (2008), WO2008/118820);

- (17) an Oct3/4 gene, a Sox2 gene, a Nanog gene (see WO2008/118820);
 - (18) an Oct3/4 gene, a Sox2 gene, a Lin28 gene (see WO2008/118820);
 - (19) an Oct3/4 gene, a Sox2 gene, a c-Myc gene, an Esrrb gene (wherein the Esrrb gene is replaceable with an Esrrg gene. See Nat. Cell Biol., 11, 197-203 (2009));
 - (20) an Oct3/4 gene, a Sox2 gene, an Esrrb gene (see Nat. Cell Biol., 11, 197-203 (2009));
 - (21) an Oct3/4 gene, a Klf4 gene, an L-Myc gene;
 - (22) an Oct3/4 gene, a Nanog gene;
 - (23) an Oct3/4 gene;
 - (24) an Oct3/4 gene, a Klf4 gene, a c-Myc gene, a Sox2 gene, a Nanog gene, a Lin28 gene, an SV40LT gene (see Science, 324: 797-801 (2009));
- [0022]

In the combinations (1) to (24), other member genes of Oct gene family (for example, Oct1A, Oct6) may be used instead of an Oct3/4 gene. Moreover, other member genes of Sox gene family (for example, a Sox7 gene) can be used instead of a Sox2 gene (or a Sox1 gene, a Sox3 gene, a Sox15 gene, a Sox17 gene, a Sox18 gene). Furthermore, other member genes of Lin gene family (for example, a Lin28b gene) can be used instead of a Lin28 gene.

Moreover, a combination that is not the same as any of the combinations (1) to (24), but includes all components in any one of the (1) to (24) and further includes any other substance (preferably another nuclear reprogramming substance) can be included in the category of "nuclear reprogramming substance" in the present

invention. Moreover, under conditions in which somatic cells to be nuclear-reprogrammed endogenously expresses a part of the components in any of the combinations (1) to (24) at a level sufficient for the nuclear reprogramming, combinations of the remaining components except the expressed components may also be included in the category of "nuclear reprogramming substance" in the present invention.

[0023]

Furthermore, in addition to the nuclear reprogramming substances, one or more nuclear reprogramming substances selected from the group consisting of an Fbx15 gene, an ERas gene, an ECAT15-2 gene, a Tcl1 gene, and a β -catenin gene may be combined and/or one or more nuclear reprogramming substances selected from the group consisting of an ECAT1 gene, an Esg1 gene, a Dnmt3L gene, an ECAT8 gene, a Gdf3 gene, a Mybl2 gene, an ECAT15-1 gene, an Fthl17 gene, a Sall4 gene, a Rex1 gene, a UTF1 gene, a Stella gene, a Stat3 gene, and a Grb2 gene may be combined. These combinations are specifically described in WO2007/69666.

[0024]

Examples of preferable nuclear reprogramming substances include an Oct3/4 gene, a Sox2 gene, a Klf4 gene, a c-Myc gene (or an L-Myc gene), a Lin28 gene, and a Nanog gene and at least 1, preferably 2 or more, more preferably 3 or more selected from the group consisting of gene products thereof. Examples of particularly preferable combinations of nuclear reprogramming substances include: (1) an Oct3/4 gene or a gene product

thereof, a Sox2 gene or a gene product thereof, and a Klf4 gene or a gene product thereof; (2) an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and a c-Myc gene or a gene product thereof; and (3) an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and an L-Myc gene or a gene product thereof; in particular, preferable examples include combinations of an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, and a Klf4 gene or a gene product thereof; and combinations of an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and an L-Myc gene or a gene product thereof; and in particular, preferable examples include a combination of an Oct3/4 gene, a Sox2 gene, and a Klf4 gene; and a combination of an Oct3/4 gene, a Sox2 gene, a Klf4 gene, and an L-Myc gene.

[0025]

A c-Myc gene or a gene product thereof may be used as one of the nuclear reprogramming substances used in the present invention, but it is preferable not to use a c-Myc gene or a gene product thereof. This is because c-Myc is reported to reduce the percentage of Nanog-GFP-positive colonies and increase the carcinogenicity of cells (Nakagawa, M., et al., Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nature biotechnology 26, 101-106 (2008)).

[0026]

When the nuclear reprogramming substance is a gene or a gene product thereof, the source of such a gene is not particularly limited, but it may be selected as appropriate depending on the purpose and examples thereof include any mammal such as a human, a mouse, a rat, a cow, a sheep, a horse, and a monkey.

[0027]

The sequence information of mouse and human cDNAs of the nuclear reprogramming substances can be obtained under GenBank accession numbers described in WO2007/069666. The Nanog gene is described under the name "ECAT4" in the pamphlet. Moreover, the sequence information of mouse and human cDNAs of the particularly preferable 3 genes (the Oct3/4 gene, the Sox2 gene, the Klf4 gene) among the nuclear reprogramming substances is also described below.

Gene name	Mouse	Human
Oct3/4	NM_013633	NM_002701
Sox2	NM_011443	NM_003106
Klf4	NM_010637	NM_004235
L-Myc	NM_008506	NM_001033081

The cDNA sequence of the human Oct3/4 gene is set forth in SEQ ID No: 47, the amino acid sequence of the human Oct3/4 protein is set forth in SEQ ID No: 48, the cDNA sequence of the human Sox2 gene is set forth in SEQ ID No: 49, the amino acid sequence of the human Sox2 protein is set forth in SEQ ID No: 50, the cDNA sequence of the human Klf4 gene is set forth in SEQ ID No: 51, the amino acid sequence of the human Klf4 protein is set forth in SEQ ID No: 52, the cDNA sequence of the human L-Myc gene is set forth in SEQ ID No: 53, and the amino acid

sequence of the human L-Myc protein is set forth in SEQ ID No: 54.

[0028]

Among the nuclear reprogramming substances, sequence information of mouse and human cDNAs of the genes whose GenBank accession numbers are not described in WO2007/069666 is described below.

Gene name	Mouse	Human
Lin28	NM_145833	NM_024674
Lin28b	NM_001031772	NM_001004317
Esrrb	NM_011934	NM_004452
Esrrg	NM_011935	NM_001438

[0029]

Those skilled in the art can easily isolate cDNAs of these nuclear reprogramming substances based on the sequence information of mouse and human cDNAs of the nuclear reprogramming substances.

[0030]

When the nuclear reprogramming substances are the genes or the mRNAs thereof, the nucleotide sequences thereof are not particularly limited as long as the effect of the present invention is unimpaired and they may be only the part translated into protein in the nucleotide sequences of the genes or contain other parts. Moreover, the nucleotide sequences of the genes and the nucleotide sequences of the mRNAs thereof may be the same as the nucleotide sequences of the wild-type genes or the nucleotide sequences of the mRNAs thereof or may comprise a mutation. Examples of such a nucleotide sequence comprising a mutation include a "nucleotide sequence that

is modified from the nucleotide sequence of the wild-type gene or the nucleotide sequence of the mRNA thereof by a deletion, a substitution, an insertion, or an addition of 1 to 30, preferably 1 to 20, more preferably 1 to 15, more preferably 1 to 10, more preferably 1 to 5, more preferably 1 to 3 nucleotides and encodes a protein having the nuclear reprogramming effect of the gene product of the gene" and a "nucleotide sequence that has 70% or more, preferably 80% or more, more preferably 85% or more, more preferably 90% or more, more preferably 95% or more, more preferably 98% or more sequence identity with the nucleotide sequence of the wild-type gene or the nucleotide sequence of the mRNA thereof in the nucleotide sequence in the part to be translated into protein and encodes a protein having the nuclear reprogramming effect of the gene product of the gene".

[0031]

When the nuclear reprogramming substances are the proteins encoded by the genes (that is, the proteins translated from the mRNAs transcribed from the genes), the amino acid sequences thereof may be the same as those of the proteins encoded by the wild-type genes or may comprise a mutation. Examples of such a protein comprising a mutation include a "protein that consists of an amino acid sequence modified from the amino acid sequence of the protein encoded by the wild-type gene by a deletion, a substitution, an insertion, or an addition of 1 to 30, preferably 1 to 20, more preferably 1 to 15, more preferably 1 to 10, more preferably 1 to 5, more preferably 1 to 3 amino acids and has the nuclear

reprogramming effect" and a "protein that consists of an amino acid sequence having 70% or more, preferably 80% or more, more preferably 85% or more, more preferably 90% or more, more preferably 95% or more, more preferably 98% or more sequence identity with the amino acid sequence of the protein that the wild-type gene encodes and has the nuclear reprogramming effect".

[0032]

(Somatic cell)

The somatic cell is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a fetal somatic cell and a mature somatic cell. Specific examples of the mature somatic cell include a tissue stem cell (somatic stem cell) such as a mesenchymal stem cell, a hematopoietic stem cell, an adipose-derived stromal cell, an adipose-derived stromal stem cell, a neural stem cell, and a spermatogenic stem cell; a tissue progenitor cell, a differentiated cell such as a fibroblast, an epithelial cell, a lymphocyte, and a muscle cell.

[0033]

The species of organism from which the somatic cell is derived is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include any mammal such as a human, a mouse, a rat, a cow, a sheep, a horse, and a monkey. Moreover, the species of organism from which the somatic cell is derived and the species of organism from which the gene to be introduced into the somatic cell derived do not need to be the same, but they are preferably the same.

[0034]

The individual from which the somatic cell is harvested is not particularly limited, but may be selected as appropriate depending on the purpose and when obtained iPS cells are to be used for the regenerative medical use, the individual to be treated or another individual having the same or a substantially same MHC type is preferable from the viewpoint of the rejection. The substantially same MHC type refers to an MHC type that matches to the degree that allows the engraftment of the transplanted cells by the use of an immunosuppressive drug or the like when the cells obtained by inducing differentiation from the iPS cells derived from the somatic cell are transplanted into the individual.

[0035]

The somatic cell may be recombinant to facilitate the selection of an iPS cell. Specific examples of the recombinant somatic cell include a recombinant somatic cell in which at least either of a reporter gene and a drug resistance gene incorporated at the locus of the gene that is highly expressed specifically in pluripotent cells. Examples of the gene that is highly expressed specifically in pluripotent cells include an Fbx15 gene, a Nanog gene, and an Oct3/4 gene. Examples of the reporter gene include a green fluorescent protein (GFP) gene, a luciferase gene, and a beta-galactosidase gene. Examples of the drug resistance gene include a blasticidin gene, a hygromycin gene, a puromycin resistance gene, and a neomycin resistance gene.

[0036]

Culture conditions for the somatic cell are not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a culture temperature of approximately 37°C and a CO₂ concentration of approximately 2% to 5%. The medium to be used for culturing the somatic cells is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a minimum essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium containing 5% by mass to 20% by mass serum.

[0037]

(Method for introducing (a) nuclear reprogramming substance and (b) H1foo gene or gene product thereof into a somatic cell)

The method for introducing (a) a nuclear reprogramming substance and (b) an H1foo gene or a gene product thereof into a somatic cell is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a method involving use of an expression vector, a method involving use of mRNA, and a method involving use of a recombinant protein. In consideration of ease of the introduction into the somatic cell, it is more preferable that the nuclear reprogramming substance is introduced into the somatic cell in the form of a gene or in the form of mRNA of the gene rather than in the form of protein and it is more preferable that it is introduced into the somatic cell in the form of a gene. Such a gene may be DNA or RNA or it may be a DNA/RNA chimera, but it is preferable to be

DNA from the viewpoint of stability. The gene may be double-stranded or single-stranded, but it is preferable to be double-stranded. Examples of the preferable nuclear reprogramming substance include cDNA of the gene and, in particular, preferably include a double-stranded cDNA of the gene.

[0038]

Similarly, in consideration of ease of the introduction into the somatic cell, it is more preferable that the "Hlfoo gene or gene product thereof" is introduced into the somatic cell in the form of a gene or in the form of mRNA of the gene rather than in the form of protein and it is more preferable that it is introduced into the somatic cell in the form of a gene. Such an Hlfoo gene may be DNA or RNA, or it may be a DNA/RNA chimera, but it is preferable to be DNA from the viewpoint of stability. The Hlfoo gene may be double-stranded or single-stranded, but it is preferable to be double-stranded. Preferable aspects of the "Hlfoo gene or gene product thereof" include the cDNA of the Hlfoo gene and, in particular, preferably include the double-stranded cDNA of the Hlfoo gene.

[0039]

(Expression vector)

When the nuclear reprogramming substance is introduced into the somatic cell in the form of a gene or when the Hlfoo gene is introduced into the somatic cell, the expression vector obtained by incorporating the nuclear reprogramming substance or Hlfoo gene in a suitable expression vector containing a promoter that can

function in the somatic cell to be a host may be used preferably. The expression vector in which the nuclear reprogramming substance or H1foo gene is to be incorporated is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include an episomal vector, an artificial chromosome vector, a plasmid vector, and a virus vector.

[0040]

Examples of the promoter used in the expression vector include an SR α promoter, an SV40 early promoter, a retroviral LTR, a CMV (cytomegalovirus) promoter, an RSV (Rous sarcoma virus) promoter, an HSV-TK (herpes simplex virus thymidinekinase) promoter, an EF1 α promoter, a metallothionein promoter, and a heat shock promoter. Moreover, the enhancer of the IE gene of human CMV may be used with the promoter. As an example, the CAG promoter (containing a cytomegalovirus enhancer, a chicken β -actin promoter, and a poly A signal of the β -globin gene) may be used.

[0041]

The expression vector may contain, if desired, an enhancer, a poly A addition signal, a marker gene, an origin of replication, and a gene encoding a protein that binds to an origin of replication and controls the reproduction, besides the promoter. The marker gene refers to a gene that enables sorting or selection of a cell by introducing the marker gene into the cell. Specific examples of the marker gene include a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, and a chromogenic enzyme gene. These may be

used singly or in combination of 2 or more. Specific examples of the drug resistance gene include a neomycin resistance gene, a tetracycline resistance gene, a kanamycin resistance gene, a zeocin resistance gene, and a hygromycin resistance gene. Specific examples of the fluorescent protein gene include a green fluorescent protein (GFP) gene, a yellow fluorescent protein (YFP) gene, and a red fluorescent protein (RFP) gene. Specific examples of the luminescent enzyme gene include a luciferase gene. Specific examples of the chromogenic enzyme gene include a β -galactosidase gene, a β -glucuronidase gene, and an alkaline phosphatase gene.

[0042]

The episomal vector is a vector capable of autonomously replicating extrachromosomally. Specific means for using the episomal vector is disclosed in Yu et al., Science, 324, 797-801 (2009). In a particularly preferable embodiment of the present invention, an episomal vector in which loxP sequences are placed in the same direction in 5' and 3' of the vector elements necessary for the replication of the episomal vector may be used. Since episomal vectors are capable of autonomously replicating extrachromosomally, they can provide a stable expression in host cells without being incorporated into genome. However, it is desirable that the vector is removed promptly after an iPS cell is established. By having 2 loxP sequences flanking the vector elements necessary for the replication of the episomal vector and the recombinase Cre acting on it to cut the vector elements out, the autonomous replication

competence of the episomal vector can be lost and the vector can be removed early from the iPS cell.

[0043]

Examples of the episomal vector used in the present invention include a vector containing sequences necessary for the autonomous replication derived from EBV, SV40, or the like as the vector elements. The vector elements necessary for the autonomous replication are specifically an origin of replication and a gene encoding a protein that binds to the origin of replication and controls the replication and examples thereof include the origin of replication oriP and the EBNA-1 gene in EBV and the origin of replication ori and the SV40LT gene in SV40.

[0044]

Moreover, examples of the artificial chromosome vector include a yeast artificial chromosome (YAC) vector, a bacterial artificial chromosome (BAC) vector, and a P1-derived artificial chromosome (PAC) vector.

[0045]

Moreover, the plasmid vector is not particularly limited as long as it is a plasmid vector that can be expressed in the somatic cell into which it is to be introduced and examples thereof include a plasmid vector for expression in animal cells, such as pAl-11, pXT1, pRc/CMV, pRc/RSV, and pCDNAI/Neo, when the somatic cell is of a mammal.

[0046]

Examples of the virus vector include a retrovirus (including lentivirus) vector, an adenovirus vectors, an adeno-associated virus vector, a Sendai virus vector, a

herpes virus vector, a vaccinia virus vector, a pox virus vector, a poliovirus vector, a Sindbis virus vector, a rhabdovirus vector, a paramyxovirus vector, and an orthomyxovirus vector.

[0047]

The method for introducing the expression vector into the somatic cell is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include the lipofection, the microinjection, a DEAE dextran method, a gene gun method, the electroporation, and a calcium phosphate method.

[0048]

When a virus vector is used as the expression vector for the introduction into the somatic cell, viral particles obtained by using a packaging cell may be used. Such a packaging cell is a cell in which the genes encoding the structural proteins of the virus are introduced and that, when a recombinant virus vector having a target gene incorporated therein is introduced in the cell, produces recombinant viral particles in which the target gene is incorporated. The packaging cell is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a packaging cell based on an HEK293 cell derived from a human kidney and an NIH3T3 cell derived from a mouse fibroblast, a PLAT-E cell, which is designed to express the envelope glycoproteins derived from Ecotropic virus, a PLAT-A cell, which is designed to express the envelope glycoproteins derived from Amphotropic virus, and a PLAT-GP cell, which is designed to express the envelope

glycoproteins derived from vesicular stomatitis virus. In particular, the PLAT-A cell and the PLAT-GP cell are preferable in terms of the host tropism when a recombinant virus vector is introduced into the human somatic cell. The method for introducing the virus vector into the packaging cell is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include the lipofection, the electroporation, and a calcium phosphate method. The method for infecting the somatic cell with the obtained viral particles is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a polybrene method.

[0049]

When the nuclear reprogramming substance or the Hlfoo gene is introduced into the somatic cell using the expression vector, one gene or 2 or more genes may be incorporated into one expression vector. The incorporation of 2 or more genes in one vector enables simultaneous expression (which may hereinafter be referred to as "co-expression") of the 2 or more genes. Moreover, all nuclear reprogramming substances to be introduced into the somatic cell and an Hlfoo gene may be incorporated into one expression vector.

[0050]

The method for incorporating the 2 or more genes into the one vector is not particularly limited, but may be selected as appropriate depending on the purpose, but it is preferable to incorporate the 2 or more genes with a linker sequence. Such a linker sequence is not

particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include a gene sequence encoding a 2A peptide derived from foot-and-mouth disease virus (*Picornaviridae Aphthovirus*) and an internal ribosome entry site (IRES).

[0051]

The method for producing an iPS cell according to the present invention may involve introducing (a) a nuclear reprogramming substance and (b) a gene product of an Hlfoo gene in the form of mRNA into a somatic cell. The method for introducing the mRNA (messenger RNA) into the somatic cell is not particularly limited and a known method may be selected and used as appropriate. For example, a commercially available RNA transfection reagent such as Lipofectamine (R) MessengerMAX (manufactured by Life Technologies Corporation) or the like may be used.

[0052]

The method for producing an iPS cell according to the present invention may involve introducing (a) a nuclear reprogramming substance and (b) a gene product of an Hlfoo gene in the form of protein into a somatic cell. The method for introducing such a protein into the somatic cell is not particularly limited and a known method may be selected and used as appropriate. Examples of such a method include a method involving use of a protein transfection reagent, a method involving use of a protein transduction domain (PTD)-fusion protein, and the microinjection. Commercially available protein transfection reagents include the cationic lipid-based reagents BioPOTER (R) Protein Delivery Reagent

(manufactured by Gene Therapy Systems, Inc.) and Pro-Ject (TM) Protein Transfection Reagent (manufactured by PIERCE), the lipid-based reagent Profect-1 (manufactured by Targeting Systems), the membrane permeable peptide-based reagents Penetratin Peptide (manufactured by Q biogene Inc.) and Chariot Kit (manufactured by Active Motif), and GenomONE (manufactured by Ishihara Sangyo Kaisha, Ltd.), which uses an HVJ envelope (inactivated Sendai virus). The introduction may be conducted according to protocols attached to these reagents, but a common procedure is as follows. "(a) A nuclear reprogramming substance" and/or "(b) an Hlfoo gene or a gene product thereof" that are in the form of protein are diluted in a suitable solvent (for example, a buffer solution such as PBS or HEPES) and incubated at room temperature for about 5 to 15 minutes after the addition of a transfection reagent to form a complex. This is added to cells that have been transferred to a serum-free medium and the resultant culture may be incubated at 37°C for from 1 hour to several hours. The medium may be removed subsequently and replaced to a serum-containing medium.

[0053]

Examples of the PTD include that developed by using a cell penetrating domain of a protein such as AntP derived from *Drosophila*, TAT derived from HIV, or VP22 derived from HSV. Such a PTD may be used for introduction by constructing a fusion protein-expression vector in which cDNA of "(a) a nuclear reprogramming substance" and/or "(b) a gene product of an Hlfoo gene" and the PTD sequence is incorporated, recombinantly expressing these,

and collecting the fusion protein. The introduction of such a fusion protein may be conducted as described above except that the protein transfection reagent is not added.
[0054]

The microinjection is a method involving placing a protein solution in a glass needle having a tip diameter of about 1 μm and puncturing a cell to introduce the solution into the cell and enables reliable introduction of the protein into the cell. Methods for establishing iPS cells by introducing a nuclear reprogramming substance in the form of protein with a cell penetrating peptide (CPP) such as polyarginine or TAT has been developed in mouse and in human and these techniques may be used (Cell Stem Cell, 4: 381-384 (2009)).

[0055]

In the method for producing an iPS cell according to the present invention, the introduction of "(a) a nuclear reprogramming substance" and "(b) an Hlfoo gene or a gene product thereof" into a somatic cell may be once or two or more times. The timing of introduction is not particularly limited, but may be selected as appropriate depending on the purpose and "(a) a nuclear reprogramming substance" and "(b) an Hlfoo gene or a gene product thereof" to be introduced may all be introduced in the same period of time or a part or all of them may be introduced at a different period(s) of time. The "Hlfoo gene or gene product thereof" may be an aspect in which only the gene is used, an aspect in which only the gene product thereof is used, or an aspect in which both of the gene and the gene product thereof are used. When the

nuclear reprogramming substance is a gene or a gene product thereof, it may be an aspect in which only the gene is used, an aspect in which only the gene product thereof is used, or an aspect in which both of the gene and the gene product thereof are used. Moreover, the nuclear reprogramming substance is a gene(s) or a gene product(s) thereof and 2 or more "genes or gene products thereof" are used in combination, it may be an aspect in which as to a certain gene(s) the gene product(s) is used and as to the other gene(s) the gene(s) is used.

[0056]

The amount of introduction of an Hlfoo gene or a gene product thereof into a somatic cell is not particularly limited as long as higher-quality iPS cells with smaller variance in quality can be produced when (a) a nuclear reprogramming substance and (b) the Hlfoo gene or gene product thereof are introduced into the somatic cell. Moreover, when the nuclear reprogramming substance is a gene(s) or a gene product(s) thereof, the amount(s) of introduction of the gene(s) or gene product(s) thereof into a somatic cell is not particularly limited as long as the nucleus of such a somatic cell can be reprogrammed and all gene(s) or gene product(s) thereof to be used may be introduced at the same amount or they may be introduced at different amounts. In an example in which the gene or gene product thereof which is used is a gene, an Oct3/4 gene is preferably introduced at a large amount, for example at approximately 3 times amount, relative to a Sox2 gene, a Klf4 gene, or a c-Myc gene (PNAS 106 (31):

12759-12764 (2009); J.Biol.Chem.287(43): 36273-36282 (2012)).

[0057]

When the nuclear reprogramming substance to be used in the method for producing an iPS cell according to the present invention is a low molecular weight compound, such a low molecular weight compound may be brought into contact with the somatic cell by dissolving the low molecular weight compound into an aqueous or non-aqueous solvent at an appropriate concentration, adding the solution of the low molecular weight compound into a medium suitable for the culture of the somatic cell (for example, a minimum essential medium (MEM), Dulbecco modified Eagle medium (DMEM), RPMI1640 medium, 199 medium, F12 medium containing approximately 5 to 20% of fetal bovine serum) so that the concentration of the low molecular weight compound is sufficient to cause the nuclear reprogramming in the somatic cell and in the range in which the cytotoxicity is not found, and culturing the cell for a certain period of time. The concentration of the low molecular weight compound that is the nuclear reprogramming substance depends on the kind of the low molecular weight compound to be used, but it may be selected as appropriate in a range of about 0.1 nM to about 100 nM. The duration of the contact is not particularly limited as long as it is a period of time that is long enough to cause the nuclear reprogramming of the cell, but they may usually be left together in the medium until a positive colony appears.

[0058]

<Other step>

As described above, the method for producing an iPS cell according to the present invention comprises at least the step of introducing (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof into a somatic cell ("introduction step") and may further comprise another step(s) as needed. The other step(s) is not particularly limited as long as the effect of the present invention is unimpaired, but may be selected as appropriate depending on the purpose and examples thereof include a step of culturing the somatic cell in which (a) the nuclear reprogramming substance and (b) the Hlfoo gene or gene product thereof are introduced (hereinafter also referred to simply as "transfected cell".) (hereinafter also referred to simply as "transfected cell-culturing step".).

[0059]

(Transfected cell-culturing step)

The transfected cell-culturing step is a step of culturing a somatic cell in which (a) the nuclear reprogramming substance and (b) the Hlfoo gene or gene product thereof are introduced. The culture conditions for the transfected cell are not particularly limited and examples thereof include conditions suitable for culturing ES cells. Examples of such conditions include a culture temperature of approximately 37°C and a CO₂ concentration of approximately 2% to 5%. Moreover, the medium to be used for culturing the transfected cell is not particularly limited, but may be selected as appropriate depending on the purpose. Mouse cells are cultured in a

normal medium to which Leukemia Inhibitory Factor (LIF) is added as a differential inhibitory factor. For human cells, basic fibroblast growth factor (bFGF) and/or stem cell factor (SCF) is desirable to be added instead of LIF. Moreover, cells are usually cultured in the presence of mouse embryonic fibroblasts (MEFs) treated with a radiation or an antibiotic to be arrested in cell division, as feeder cells. As MEFs, STO cells are usually used often, but SNL cells (McMahon, A. P. & Bradley, A. Cell 62, 1073-1085 (1990)) or the like are used often for the induction of iPS cells. The co-culture with feeder cells may be started prior to the introduction of (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof, at the time of the introduction, or after the introduction (for example, 1 to 10 days later).

[0060]

The duration of the transfected cell-culturing step is not particularly limited, but may be selected as appropriate depending on the purpose.

[0061]

2. iPS cell

The iPS cell according to the present invention prepared by the method for producing an iPS cell has the pluripotency and the self-renewal capacity. The pluripotency means the ability to differentiate into all three germ-layer lineages. The self-renewal capacity means the ability to replicate while maintaining an undifferentiated state.

[0062]

The method for confirming that the cell produced by the method for producing an iPS cell is an iPS cell is not particularly limited, but may be selected as appropriate depending on the purpose. For example, when the cell used as the somatic cell is a recombinant somatic cell in which at least either of a reporter gene and a drug resistance gene is incorporated at the locus of the gene highly expressed specifically in pluripotent cells (for example, Fbx15, Nanog, or Oct3/4, preferably Nanog or Oct3/4), the confirmation can be made using the reporter gene or drug resistance gene. Specific examples when the gene used as the reporter gene is a green fluorescent protein (GFP) gene include a method involving confirming a GFP-positive cell with a flow cytometer and, when the gene used as the drug resistance gene is a puromycin resistance gene, the confirmation can be made by adding puromycin to the cell.

[0063]

As used herein, the term "high-quality iPS cells" means iPS cells whose quality is higher than iPS cells produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell. Moreover, as used herein, the term "iPS cells with smaller variance in quality" means iPS cells that have variance in quality smaller than that of iPS cells produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell. The quality in these may be of one kind or of 2 or more kinds. Specific examples of the "high-quality iPS cells" or the "iPS cells with smaller variance in quality"

include iPS cells having one or more properties selected from the following (a) to (q).

(a) iPS cells that form embryoid bodies increased in number by 5% or more or preferably 10% or more in comparison with iPS cells (preferably "OSK-iPS cells" or "OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell when cultured for 5 days by a method of [Embryoid body (EB) formation] described below.

(b) iPS cells that form 78 or more or preferably 80 or more embryoid bodies when cultured for 5 days by a method of [Embryoid body (EB) formation] described below.

(c) iPS cells that form embryoid bodies with size (μm^2) variance (σ^2) decreased by 25% or more or preferably 40% or more in comparison with iPS cells (preferably "OSK-iPS cells" or "OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell when cultured for 5 days by a method of [Embryoid body (EB) formation] described below.

(d) iPS cells that form embryoid bodies with size (μm^2) variance (σ^2) of 10000 (μm^2) or less or preferably 8000 (μm^2) or less when cultured for 5 days by a method of [Embryoid body (EB) formation] described below.

(e) iPS cells in which the percentage of viable cells (annexin V (-)/PI (-) cells) as measured by a method of [Apoptosis assay] described below is increased to 1.1 times or more or preferably 1.2 times or more in comparison with iPS cells (preferably "OSK-iPS cells" or

"OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(f) iPS cells in which the percentage of viable cells (annexin V (-)/PI (-) cells) as measured by a method of [Apoptosis assay] described below is 68% or more or preferably 73% or more.

(g) iPS cells in which the percentage of apoptotic cells (the sum of annexin V (+)/PI (-) cells and annexin V (+)/PI (+) cells) as measured by a method of [Apoptosis assay] described below is decreased to 0.75 times or less or preferably 0.67 times or less in comparison with iPS cells (preferably "OSK-iPS cells" or "OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(h) iPS cells in which the percentage of apoptotic cells (the sum of annexin V (+)/PI (-) cells and annexin V (+)/PI (+) cells) as measured by a method of [Apoptosis assay] described below is 30% or less or preferably 25% or less.

(i) iPS cells in which the expression level of the Ki67 gene or the PCNA gene (both genes are known as a cell proliferation marker) as measured by the method described in the section of [Quantitative RT-PCR analysis] below is increased to 1.7 times or preferably 2.2 times in comparison with iPS cells (preferably "OSK-iPS cells" or "OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(j) iPS cells in which the expression level of the Ki67 gene or the PCNA gene (both genes are known as a cell proliferation marker) as measured by the method described in the section of [Quantitative RT-PCR analysis] described below is 0.65 times or more or preferably 0.73 times or more of that of ES cells.

(k) iPS cells with higher chimera competency in comparison with iPS cells (preferably "OSK-iPS cells" or "OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(l) iPS cells with higher germline transmission potential in comparison with iPS cells (preferably "OSK-iPS cells" or "OSKL-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(m) iPS cells in which the expression level of the SRF gene (the SRF gene is known as a chromosomal abnormality marker whose expression level decreases when there is chromosomal abnormality) as measured by the method described in the section of [Quantitative RT-PCR analysis] described below is increased to 1.1 times or preferably 1.2 times in comparison with iPS cells (preferably "OSKL-iPS cells" or "OSK-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(n) iPS cells in which the expression level of the ACTG2 gene (the ACTG2 gene is known as a chromosomal abnormality marker whose expression level decreases when there is

chromosomal abnormality) as measured by the method described in the section of [Quantitative RT-PCR analysis] described below is increased to 1.2 times or preferably 1.5 times in comparison with iPS cells (preferably "OSKL-iPS cells" or "OSK-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(o) iPS cells in which the variance (σ^2) of expression levels of the Oct3/4 gene (an undifferentiated state marker of stem cells) as measured by the method described in the section of [Quantitative RT-PCR analysis] described below is decreased by 10% or more or preferably 20% or more in comparison with iPS cells (preferably "OSKL-iPS cells" or "OSK-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(p) iPS cells in which the number of viable cells as measured by the method described in the section of [Comparison of cell survival rates in early stage after induction of differentiation] described below is increased to 1.2 times or preferably 1.5 times in comparison with iPS cells (preferably "OSKL-iPS cells" or "OSK-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

(q) iPS cells in which the variance (σ^2) of expression levels of the Oct3/4 gene (an undifferentiated state marker of stem cells) as measured by the method described

in the section of [Comparison of residual amounts of undifferentiated state marker in early period after induction of differentiation of iPS cells] described below is decreased by 10% or more or preferably 20% or more in comparison with iPS cells (preferably "OSKL-iPS cells" or "OSK-iPS cells" in Examples described below) produced in the same way except that neither Hlfoo gene nor gene product thereof is introduced into the somatic cell.

[0064]

The species of organism from which the iPS cells are derived is not particularly limited, but may be selected as appropriate depending on the purpose and examples thereof include any mammal such as a human, a mouse, a rat, a cow, a sheep, a horse, and a monkey.

[0065]

3. Agent for improving quality of an iPS cell

The Agent for improving quality of an iPS cell according to the present invention comprises at least an Hlfoo gene or a gene product thereof and may further comprise another configuration as needed. The Hlfoo gene or gene product thereof is similar to the one described for the method for producing an iPS cell. Moreover, the Hlfoo gene or gene product thereof may comprise a similar mutation described for the method for producing an iPS cell. Moreover, the Hlfoo gene incorporated in the expression vector containing a promoter that can function in the somatic cell to be a host may be used.

[0066]

4. Composition for producing iPS cell

The composition for producing an iPS cell according to the present invention comprises at least (a) a nuclear reprogramming substance and (b) Hlfoo gene or a gene product thereof and may further comprise another configuration as needed.

[0067]

<(a) nuclear reprogramming substance and (b) Hlfoo gene or gene product thereof>

The (a) nuclear reprogramming substance and (b) Hlfoo gene or gene product thereof are similar to those described for the method for producing an iPS cell. Moreover, the Hlfoo gene or gene product thereof and a gene or a gene product thereof that is the nuclear reprogramming substance may comprise a similar mutation described for the method for producing an iPS cell.

[0068]

Examples of the preferable form of the nuclear reprogramming substance in the composition for producing an iPS cell include a form of a gene incorporated in a vector, a form of synthetic mRNA, and a form of protein (preferably recombinant protein). Examples of the vector include that similar to those described in the method for producing an iPS cell. The synthetic mRNA and the protein (preferably recombinant protein) may be produced by a known method.

[0069]

The composition for producing an iPS cell may be packed such that the (a) nuclear reprogramming substance and the (b) Hlfoo gene or gene product thereof are contained separately in individual containers or together

in one container or in group of any number per container in containers.

[0070]

The amounts of the (a) nuclear reprogramming substance and the (b) Hlfoo gene or gene product thereof in the composition for producing an iPS cell are not particularly limited and all of the nuclear reprogramming substance(s) and/or the Hlfoo gene or gene product thereof may be contained in the same amount or in different amounts.

[0071]

The composition for producing an iPS cell may comprise, besides the (a) nuclear reprogramming substance and the (b) Hlfoo gene or gene product thereof, a gene or a gene product thereof other than these. Moreover, when the virus vector is used, the composition may comprise, for example, a packaging cell. Examples of such a gene or a gene product thereof other than the genes or gene products thereof and such a packaging cell include those described for the method for producing an iPS cell.

[0072]

The present invention also comprises, as other aspects,

"a method for improving quality of an iPS cell, comprising the step of introducing an Hlfoo gene or a gene product thereof into a somatic cell",

"use of an Hlfoo gene or a gene product thereof in the manufacture of an agent for improving quality of an iPS cell", and

"use of (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof in the manufacture of a composition for producing an iPS cell".

[0073]

The step of introducing an Hlfoo gene or a gene product thereof into a somatic cell in the "method for improving quality of an iPS cell" is the same as the introduction step in the method for producing an iPS cell according to the present invention. Comprising such a step enables improving quality of an iPS cell and obtaining a high-quality iPS cell.

[0074]

The present invention will be described by means of Examples below in detail, but the present invention is not limited to these Examples. All experiments described below were conducted according to the Guidelines on Animal and DNA Experimentation at Keio University, approved by the Ethical Committee at Keio University, and based on the Guide for the Care and Use of Laboratory Animals at the National Institute of Health.

Examples

[0075]

1. Materials and Method

[Plasmid construction]

Hlfoo cDNA and Hlc cDNA (Teranishi, T., et al., Rapid replacement of somatic linker histones with the oocyte-specific linker histone Hlfoo in nuclear transfer. Developmental Biology 266, 76-86 (2004).) were inserted respectively into the restriction enzyme BamH1-Sall and

EcoR1-Sall sites in the pMXs plasmid and it was confirmed by DNA sequencing that the Hlfoo cDNA and the Hlc cDNA were inserted.

[0076]

[Production of mouse iPS cells and method for culturing the same]

1) The production of mouse iPS cells was conducted following a protocol described in literature (Takahashi, K., Okita, K., Nakagawa, M., & Yamanaka, S. Induction of pluripotent stem cells from fibroblast cultures. Nature protocols 2, 3081-3089 (2007)). In this study, however, the Hlfoo gene was also used in addition to the genes described in the document. More specifically, iPS cells were produced from mouse fibroblasts or tail tip fibroblasts (hereinafter simply referred to as "Nanog-GFP expressing fibroblasts") in a Nanog-GFP-IRES-puro transgenic mouse (Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. Nature 448, 313-317 (2007)) using the retrovirus vector pMXs containing mouse Oct3/4, Sox2, Klf4, and Hlfoo genes (nucleotide sequence set forth in SEQ ID No: 3: BC137916). As a control, DsRed gene was used instead of the Hlfoo gene. The management of laboratory mice was conducted following the Guidelines on Animal and DNA Experimentation at Keio University.

[0077]

2) Mouse ES cells (B6J-23^{UTR}) (Tanimoto, Y., et al. Embryonic stem cells derived from C57BL/6J and C57BL/6N mice. Comparative medicine 58, 347-352 (2008)) were obtained from the Laboratory Animal Resource Center at the

University of Tsukuba and used following the Guidelines on the Distribution and Utilization of Human Embryonic Stem Cells of Ministry of Education, Culture, Sports, Science and Technology of Japan.

[0078]

3) Mouse iPS and ES cell lines were cultured and maintained on X-irradiated mouse embryonic fibroblasts (iMEF feeder cells) derived from a wild-type ICR mouse in DMEM (manufactured by Sigma Aldrich Co. LLC.) culture medium containing 20% KnockOut Serum Replacement (manufactured by Gibco), in 1 mM GlutaMAX (manufactured by Gibco), 1 mM nonessential amino acids (manufactured by Sigma Aldrich Co. LLC.), 0.1 mM 2-mercaptoethanol, 50 U penicillin, 50 mg/ml streptomycin (manufactured by Gibco), and mouse Leukemia Inhibitory Factor (LIF) (hereinafter referred to as "iPS cell culture medium"). The mouse iPS cell culture medium was replaced every 2 to 3 days and the cells were subcultured using 0.5 mM trypsin-EDTA (manufactured by Gibco) every 2 to 3 days.

[0079]

[Embryoid body (EB) formation]

The EB formation from iPS cells was conducted by seeding 5×10^4 iPS cells obtained with 1 mg/ml collagenase IV on a 100 mm low attachment plate (manufactured by AGC Techno Glass Co. Ltd.) and culturing the cells for 5 days in the presence of Minimum Essential Medium Alpha Medium (manufactured by Gibco) containing 20% FBS (manufactured by Gibco), 2 mM GlutaMAX (manufactured by Gibco), 0.1 mM nonessential amino acids (manufactured by Sigma Aldrich Co. LLC.), 0.1 mM 2-mercaptoethanol, 50

U/ml penicillin, and 50 mg/ml streptomycin (manufactured by Gibco) (hereinafter referred to as "culture medium for EB formation"). The culture medium for EB formation was replaced every 2 to 3 days. As a control, ES cells were used.

[0080]

[Teratoma formation]

The teratoma-forming potential of the iPS cells were confirmed by hematoxylin eosin (HE) staining after injecting the iPS cells into the testis in SCID mice (manufactured by CLEA Japan, Inc.) anesthetized with a mixture of ketamine (50 mg/kg), xylazine (10 mg/kg), and chlorpromazine (1.25 mg/kg), sacrificing the mice by cervical vertebra dislocation approximately 8 weeks later, fixing the tissue sections in which iPS cells were injected in 10% paraformaldehyde (PFA) overnight and embedding the sections in paraffin. The anesthesia of mice was conducted appropriately by monitoring the heart rate, muscle relaxation, and sensual reflection response (that is, unresponsive to tail pinch) of the mice.

[0081]

[Immunohistochemistry staining]

The iPS cells and fibroblasts seeded on a glass bottom dish (product made in AGC Techno Glass Co. Ltd) were washed with PBS once and fixed with 4% paraformaldehyde (manufactured by Muto Pure Chemicals Co., Ltd.) at 4°C for 15 minutes. Fixed cells were treated for permeabilization in 0.5% triton X-100 in PBS at room temperature for 10 minutes, then blocked in ImmunoBlock (manufactured by DS Pharma Biomedical Co. Ltd.) for 20

minutes, incubated in the presence of 4 primary antibodies (an anti-Oct3/4 antibody [Oct3/4 antibody sc-8629, manufactured by Santa Cruz Biotechnology, Inc.], an anti-Nanog antibody [RCAB0001P, manufactured by ReproCELL Inc.], an anti-SSEA1 antibody [sc-21702, manufactured by Santa Cruz Biotechnology, Inc.], and an anti-H1foo antibody [HPA037992, manufactured by Sigma Aldrich Co. LLC.]) at room temperature for 60 minutes, washed with ImmunoBlock (manufactured by DS Pharma Biomedical Co. Ltd.), and incubated with secondary antibodies (anti-rabbit IgG and anti-mouse IgG or IgM antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 568 [manufactured by Life Technologies Corporation]) corresponding to the primary antibodies at room temperature for 60 minutes. After staining the nucleus with 6-diamidino-2-phenylindole (DAPI, manufactured by Life Technologies Corporation) the fluorescence observation was conducted using a fluorescence laser microscope equipped with a color charge-coupled device camera (BZ-9000, manufactured by Keyence Corporation), and an optical microscope (IX71, manufactured by Olympus Corporation), and a laser confocal microscope (LSM 510 META, manufactured by Carl Zeiss Jena GmbH).

[0082]

[Quantitative RT-PCR analysis]

Total RNA samples were isolated with a TRIZOL reagent (manufactured by Life Technologies Corporation) according to a manual attached to the product. The concentration and the purity of RNA samples were measured with an ND-1000 spectrophotometer (manufactured by Thermo

Fisher Scientific Inc.) and the preparation of cDNA was performed using ReverTra Ace qPCR RT Master Mix (manufactured by Toyobo Co., Ltd.). Quantitative PCR (QT-PCR) was performed by 7500 Real Time PCR System (manufactured by Life Technologies Corporation) using SYBR Premix ExTaq (manufactured by Takara Bio Inc.). The amount of mRNA was normalized with amount of the GAPDH mRNA. The nucleotide sequences of the primer sets used for the quantitative PCR are set forth in Table 1 below.

[0083]

[Table 1]

Gene name	Forward primer	Reverse primer
Nanog	AGGGTCTGCTACTGAGATGCT (SEQ ID No: 5)	CAACACCTGGTTTTTCTGCCACCG (SEQ ID No: 6)
Oct3/4 (Endogenous)	TCTTTCCACCAGGCCCCCGGCTC (SEQ ID No: 7)	TGCGGGCGGACATGGGGAGATCC (SEQ ID No: 8)
Sox2 (Endogenous)	TAGAGATAGACTCCGGGCGATGA (SEQ ID No: 9)	TTGCCTTAAACAAGACCACGAAA (SEQ ID No: 10)
Oct3/4 (Transgene)	CCCCAGGGCCCCATTTTGGTACC (SEQ ID No: 11)	CCCTTTTTCTGGAGACTAATAAA (SEQ ID No: 12)
Sox2 (Transgene)	GGCACCCCTGGCATGGCTCTTGGCTC (SEQ ID No: 13)	TTATCGTCGACCACTGTGCTGCTG (SEQ ID No: 14)
Rex1	ACGAGTGGCAGTTTCTTCTTGGGA (SEQ ID No: 15)	TATGACTCACTTCCAGGGGGCACT (SEQ ID No: 16)
Sall4	CCCTGGGAAGTGCATGAAG (SEQ ID No: 17)	TCAGAGAGACTAAAGAACTCGGC (SEQ ID No: 18)
H1foo	GGCACAGGCTTTCTTTGTCT (SEQ ID No: 19)	TCCAACACAAGTACCCGACA (SEQ ID No: 20)
Foxa2	AGCACCATTACGCCTTCAAC (SEQ ID No: 21)	CCTTGAGGTCCATTTTGTGG (SEQ ID No: 22)
Cxcr4	CGGGATGAAAACGTCCATTT (SEQ ID No: 23)	ATGACCAGGATCACCAATCCA (SEQ ID No: 24)
Col6a2	CCACCACTGAAAGGAACAACAA (SEQ ID No: 25)	TCCAACACGAAATACACGTTGAC (SEQ ID No: 26)
T	TGTCCTCCCTTGTTGCCTTA (SEQ ID No: 27)	ATGTTCCAAGGGCAGAACAG (SEQ ID No: 28)
β 3-Tubulin	CCCAGCGGCAACTATGTAGG (SEQ ID No: 29)	CCAGACCGAACACTGTCCA (SEQ ID No: 30)
Tubb3	CCAAATCTACCCACCAAGGA (SEQ ID No: 31)	AGAGCTTCCAGAACGTCGAG (SEQ ID No: 32)
Ki67	TCTGATGTTAGGTGTTTGAG (SEQ ID No: 33)	CACTTTTCTGGTAACTTCTTG (SEQ ID No: 34)
PCNA	TAAAGAAGAGGAGGCGGTAA (SEQ ID No: 35)	TAAGTGTCCTATGTCAGCAA (SEQ ID No: 36)
GAPDH	TTCAACGGCACAGTCAAGG (SEQ ID No: 37)	CATGGACTGTGGTCATGAG (SEQ ID No: 38)
SRF	ACCCATAAGCCCCAGCTATC (SEQ ID No: 55)	GGTGCCAAGATGGAAGTCAC (SEQ ID No: 56)
ACTG2	CGGTGGTCTCCTCTTCACAC (SEQ ID No: 57)	CACTGGGAGAAGAACCTCTCAT (SEQ ID No: 58)

[0084]

[Apoptosis assay]

iPS cells at 1 day after the start of culturing in the culture medium for EB formation were trypsinized and suspended into Annexin A5 Binding Buffer (manufactured by Takara Bio Inc.) and then early apoptotic cells and late apoptotic cells were stained with annexin-A5-FITC and

iodinated propidium (PI) (manufactured by Becton, Dickinson and Company), respectively, following the manual attached to the products. The cells were filtered through a nylon membrane with a pore size of 70 mm and the fluorescently stained cells were analyzed with the flow cytometry FACS Aria3 (manufactured by Becton, Dickinson and Company) using the software CellQuest (manufactured by Becton, Dickinson and Company) to determine the percentage of apoptotic cells using the software FlowJo (manufactured by Tree Star, Inc.).

[0085]

[DNA methylation analysis]

To analyze the DNA methylation level in the promoter regions of the Oct3/4 and Nanog genes using bisulfite sequencing, genomic DNA was isolated and purified from cell samples using SV Genomic DNA Purification kit (manufactured by Promega Corporation). Unmethylated cytosine (C) in the purified genomic DNA was converted into uracil (U) using EZ DNA Methylation-Gold Kit (manufactured by ZYMO RESEARCH) following a manual attached to the product and input DNA for bisulfite PCR was prepared. Using such input DNA, primer sets, and TaKaRa EpiTaq HS (manufactured by TaKaRa Bio Inc.), bisulfite PCR was performed under the PCR reaction conditions (1 cycle of denaturing at 98°C for 10 minutes; 40 cycles of amplification at 95°C for 20 seconds, at 55°C for 30 seconds, and at 72°C for 60 seconds; and 1 cycle of the final extension at 72°C for 5 minutes). The nucleotide sequences of the primer sets used for the bisulfite PCR are set forth in Table 2 below.

[0086]

[Table 2]

Oct3/4- DMR	GGTTTTTTAGAGGATGGTTGAGTG (SEQ ID No: 39)	TCCAACCCTACTAACCCATCACC (SEQ ID No: 40)
Nanog- DMR	GATTTTGTAGGTGGGATTAATTGTGAATTT (SEQ ID No: 41)	ACCAAAAAAACCCACACTCATATCAATATA (SEQ ID No: 42)

[0087]

The PCR products were cloned into the vector pGEM-T (manufactured by Promega Corporation) by TA-cloning after purification and sequenced. To analyze the methylation status of the intergenic differentially methylated region (DMR) (IG-DMR) located between the Dlk1 and Dlk2 genes and the Gtl2 differentially methylated region (Gtl2-DMR), genomic DNA was purified as described above, input DNA for bisulfite PCR was prepared, and then pyrosequencing was conducted using PyroMark Q24 (manufactured by QIAGEN) following the manual attached to the product. The nucleotide sequences of primer sets used for the pyrosequencing are set forth in Table 3 below.

[0088]

[Table 3]

IG-DMR	GTGGTTTGTATGGGTAAGTTT (SEQ ID No: 43)	CCCTTCCCTCACTCCAAAAATTAA (SEQ ID No: 44)
Gtl2-DMR	AGTTATTTTTTGTGTTGAAAGGATGTGTA (SEQ ID No: 45)	CTAACTTTAAAAAAAATCCCCAACACT (SEQ ID No: 46)

[0089]

[Co-culture aggregation of ES cell and iPS cell]

Embryos at the 2-cell stage were collected from superovulated and naturally mated female ICR (CD-1 (R)) mice and then cultured to the blastocyst stage. ES cells and the iPS cells were dissociated with 0.25% trypsin just

before co-culture aggregation and 15 to 20 cells were aggregated with blastomeres at the 8-cell stage from which the zonae pellucidae had been removed. Chimeric embryos at the blastocyst stage were transferred into the uterine horns of ICR pseudopregnant mice at 2.5 dpc (days of post-coitus).

[0090]

[Global gene expression analysis]

Total RNA was isolated from iPS cells and an ES cells. Cyanine-labeled antisense RNA was amplified using the Quick Amp Labeling Kit (manufactured by Agilent Technologies), hybridized onto a Whole Mouse Genome Oligo Microarray (manufactured by Agilent Technologies) with a gene expression hybridization kit, and analyzed with the Agilent Microarray Scanner. The data was analyzed with GeneSpring GX12.0 software (manufactured by Agilent Technologies).

[0091]

[Statistical analysis]

The values in bar graph with error bars in figures are reported as the mean $\pm$ standard deviation (SME). The data was analyzed using StatView J-4.5 software. The comparison between 2 groups was performed with Student's t-test. The comparison among groups was performed by one-way ANOVA with Bonferroni's post hoc test. "*" and "***" in the figures indicate significance ($P < 0.05$ and $P < 0.01$, respectively).

[0092]

2. Results

[Production of iPS cells using exogenous Hlfoo gene]

The effect of Hlfoo on somatic cell reprogramming was examined.

First, the method described in the section [Immunohistochemistry staining] above was conducted to confirm that exogenous Hlfoo is exclusively expressed in the nucleus (Figure 1a) and that it is mostly located peripherally in the nucleus (Figure 1b). Nuclear swelling and the decrease of the region having high electron density inside of the nuclear membrane (heterochromatin) by the expression of the exogenous Hlfoo were not observed (Figure 1c).

[0093]

Then, according to the method described in the section of [Production of murine iPS cells and method for culturing the same] above, 3 nuclear reprogramming substances (the Oct3/4, Sox2, and Klf4 genes [OSK genes]) as well as the Hlfoo gene (OSK + Hlfoo genes) were introduced into murine fibroblasts, which are somatic cells, using a retrovirus vector and alkaline phosphatase (ALP) staining was conducted using TRACP & ALP double-stain Kit (manufactured by Takara Bio Inc.) on Day 20 to determine the number of colonies that are ALP-positive and consist of ES-like cells. ALP is known as a stem cell marker. As control, experiments of introducing only the OSK genes or introducing the linker histone H1 gene (OSK + H1c genes) instead of Hlfoo in the OSK + Hlfoo genes were also conducted. As a result, it was shown that the introduction of OSK + Hlfoo genes into murine fibroblasts results in marked increase of the number of ALP-positive ES-like cell (iPS cell) colonies as compared with the

introduction of the OSK genes or the OSK + H1c genes (Figure 2a).

[0094]

According to the method described in the section of [Immunohistochemistry staining] above, the expression of the 3 pluripotency markers (Oct3/4, Nanog, and SSEA1) was analyzed. The analysis demonstrated that the OSK + Hlfoo-iPS cells express the pluripotent markers as OSK-iPS cells do (Figure 2b). In contrast, the expression of endogenous Hlfoo was observed neither during the induction process to iPS cells nor in the produced iPS cells (Figure 2b, c).

[0095]

Moreover, the OSK + Hlfoo genes were introduced into Nanog-GFP expressing fibroblasts to examine the quality of the produced iPS cells (Figure 3a) using the signal of GFP as an indicator. The Nanog-GFP-positive cells are known as a marker for high-quality stem cells. Interestingly, it was shown that the introduction of the Hlfoo gene in addition to the OSK genes into Nanog-GFP expressing fibroblasts increases the number of colonies of Nanog-GFP-positive ES-like cells (iPS cells) to approximately 8 times as compared with the introduction of the OSK genes (Figure 3b). Furthermore, the ratio of the Nanog-GFP-positive ES-like cells (iPS cells) to the produced iPS cell population was as high as 90% or more (Figure 3c). In contrast, the introduction of the OSK genes and the c-Myc gene (OSKM genes) or the introduction of the Hlfoo gene as well as the OSKM genes (OSKM + Hlfoo genes) into Nanog-GFP expressing fibroblasts was shown to result in an increased number of Nanog-GFP-positive colonies themselves

(Figure 3b), but the ratio of Nanog-GFP-positive cells to the produced iPS cell population is low as compared with the introduction of the OSK + Hlfoo genes (Figure 3c). These results indicate that the introduction of the OSK + Hlfoo genes into somatic cells enables the production of higher-quality iPS cell populations as compared with the introduction of the OSK genes or the introduction of the OSKM genes in the conventional art.

[0096]

[Properties of OSK + Hlfoo-iPS cells]

Properties of the OSK + Hlfoo-iPS cells were analyzed in detail. The analysis of the expression of 4 pluripotency markers (Oct3/4, Sox2, Rex1, and Sall4) according to the method described in the section of [Quantitative RT-PCR analysis] above demonstrated that the OSK + Hlfoo-iPS cells express the pluripotency markers as the OSK-iPS cells do (Table 4). The expression of the transgenes including the Hlfoo gene was suppressed (Table 5). The cell proliferation rate was also analyzed and the analysis indicated that the proliferation level of OSK + Hlfoo-iPS cells was equivalent to that of OSK-iPS cells.

[0097]

[Table 4]

Cell	Nanog	Oct3/4	Sox2	Rex1	Sall4
ES	1.00	1.00	1.00	1.00	1.00
OSK-iPS1	0.500	0.704	0.841	0.720	1.11
OSK-iPS2	0.726	0.935	1.19	0.925	0.796
OSK-iPS3	0.906	0.731	1.02	1.07	1.29
OSKH-iPS1	0.764	0.796	1.39	0.897	0.548
OSKH-iPS2	0.990	0.704	1.06	1.05	1.33
OSKH-iPS3	0.689	0.917	1.22	0.869	0.903
MEF	0.00943	0.00	0.0341	0.00935	0.00

[0098]

Table 4 shows the results of analysis of the expression of 5 pluripotency markers (Nanog, Oct3/4, Sox2, Rex1, and Sall4) in ES cells (ES), 3 clones of OSK-iPS cells (OSK-iPS 1 to 3), 3 clones of OSK + H1foo-iPS cells (OSKH-iPS 1 to 3), and MEF cells (MEF). The expression levels of pluripotency markers are expressed as values relative to the result of the ES cells, which is 1.

[0099]

[Table 5]

Cell	Oct3/4 (Exogenous)	Sox2 (Exogenous)	Hlfoo (Exogenous)
ES	0.00	0.00	0.00
MEF+OSKH	1.00	1.00	1.00
OSK-iPS1	0.01	0.03	0.00
OSK-iPS2	0.00	0.06	0.00
OSK-iPS3	0.00	0.01	0.00
OSKH-iPS1	0.00	0.01	0.03
OSKH-iPS2	0.00	0.01	0.05
OSKH-iPS3	0.00	0.01	0.06
MEF	0.00	0.00	0.00

[0100]

Table 5 shows the results of analysis of the expression of 3 exogenous proteins (Oct3/4, Sox2, and Hlfoo) in ES cells (ES), OSKH gene-transduced MEF cells (MEF + OSKH), 3 clones of OSK-iPS cells (OSK-iPS 1 to 3), 3 clones of OSK + Hlfoo-iPS cells (OSKH-iPS 1 to 3), and MEF cells (MEF). The expression levels of pluripotency markers are expressed as values relative to the result of the OSKH gene-transduced MEF cells, which is taken as 1.

[0101]

Global gene-transcriptome profiles between the OSK + Hlfoo-iPS cells and the ES cells or the OSK-iPS cells were compared (Figures 4a and b). As a result, it was demonstrated that the gene expression pattern in OSK + Hlfoo-iPS cells and the gene expression pattern in the ES

cells and the OSK-iPS cells are very similar (a correlation coefficient [R^2] of 0.99).

[0102]

Methylation analysis of genomic DNA was conducted according to the method described in the section of [DNA methylation analysis]. The results demonstrated that DNA in the promoter regions of 2 pluripotency marker (Oct3/4 and Nanog) genes, IG-DMR, and Gtl2-DMR were demethylated in the OSK + H1foo-iPS cells as in ES cells and OSK-iPS cells (Figures 5a and b). While the demethylation level was found to be heterogeneous among clones (cell groups) of the OSK-iPS cells, such heterogeneity was smaller among the OSK + H1foo-iPS cells (Figures 5a and b).

[0103]

[Properties of EBs derived from OSK + H1foo-iPS cells]

To induce the differentiation from iPS cells to various cells, usually, EBs are formed at first and then further differentiation is induced by a method for inducing differentiation specific to the type of cells. Since homogeneity of the number of EBs formed and the EB size has been considered to have an influence on the efficiency of subsequent induction of differentiation, iPS cells that can form a large number of EBs having homogenous sizes have been considered to be desirable. Therefore, the EB formation potency of OSK + H1foo-iPS cells was analyzed.

[0104]

When the formation of EBs from the OSK + H1foo-iPS cells according to the method described in the section of [embryoid body (EB) formation] above, the EBs derived from

such OSK + Hlfoo-iPS cells are larger in EB number (increase of approximately 12% in percentage) and in EB size (μm^2) as compared with EBs derived from the OSK-iPS cells (Figures 6a to c) and smaller in EB size (μm^2) variance (σ^2) (approximately 50% decrease in percentage) (Figures 6d and e).

[0105]

The analysis of the percentage of apoptotic cells according to the method described in the section of [Apoptosis assay] above indicated that the EBs derived from the OSK + Hlfoo-iPS cells have smaller percentages of early apoptotic cells (annexin V-positive (+)/PI-negative (-) cells) and late apoptotic cells (annexin V (+)/PI (+) cells and annexin V (-)/PI (+) cells) and a higher percentage of viable cells (annexin V (-)/PI (-) cells) as compared with the EBs derived from the OSK-iPS cells (Figures 7a and b). The EBs derived from the OSK + Hlfoo-iPS cells had a percentage of viable cells (annexin V (-)/PI (-) cells) increased to approximately 1.2 times as compared with the EBs derived from the OSK-iPS cells. Moreover, the EBs derived from the OSK + Hlfoo-iPS cells had a percentage of apoptotic cells (the sum of annexin V (+)/PI (-) cells and annexin V (+)/PI (+) cells) decreased to approximately 0.65 times as compared with the EBs derived from the OSK-iPS cells.

[0106]

Furthermore, the analysis of iPS cells on the second day from the start of culture in the culture medium for EB formation on the expression of 2 cell proliferation markers (Ki67 and PCNA) according to the method described

in the section of [Quantitative RT-PCR analysis] above indicated that the EBs derived from the OSK + Hlfoo-iPS cells have a higher expression level (increased to approximately 2.4 to 2.6 times) of the cell proliferation markers as compared with the EBs derived from the OSK-iPS cells (Figures 8a and b).

The foregoing results indicate that OSK + Hlfoo-iPS cells form a larger number of EBs with relatively similar sizes and can form EBs with a smaller percentage of dead cells (high-quality EBs) as compared with OSK cells

[0107]

[Pluripotency of OSK + Hlfoo-iPS cells]

The pluripotency of the OSK + Hlfoo-iPS cells was analyzed. Chimera mice were generated from 2 clones (OSKH1 and 3) of OSK + Hlfoo-iPS cells that have the normal chromosome number according to the method described in the section of [Co-culture aggregation of ES cells and iPS cells] above. Moreover, as control, chimera mice were generated from 2 clones (OSK1 and 2) of OSK-iPS cells that have the normal chromosome number according to the same method. Since the iPS cells and ES cells used in this experiment were prepared from somatic cells of black mice, the higher chimera competency of iPS or ES cells results in the generation of blacker mice at higher percentage. As a result of the chimera mice generation study, it was demonstrated that the 2 clones (OSKH1 and 3) of OSK + Hlfoo-iPS cells have chimera competency equivalent to or higher than the 2 clones (OSK1 and 2) of OSK-iPS cells (Figures 9a and b). In particular, OSKH3 was demonstrated

to have the chimera competency at the level same as ES cells (Figures 9a and b).

[0108]

Germline transmission potential refers to the potential of pluripotent stem cells such as ES cells and iPS cells to differentiate into germ cells and transmit genes derived from the pluripotent stem cells to the next generation via chimera. This germline transmission potential is one of the most stringent hallmarks of pluripotent stem cells and one of the hallmarks indicating that the pluripotent stem cells are of high quality. Therefore germline transmission potential from 100% chimeric mice was examined by in vitro fertilization (IVF). As a result, the 4 chimeric mice (OSKH1, OSKH3-1, OSKH3-2, and OSKH3-3) derived from OSK + H1foo-iPS cells exhibited higher reproductive potential than the 2 chimeric mice (OSK2-1 and OSK2-2) derived from OSK-iPS cells (Figure 10a) and generated many pups with colored coats (Figures 10b and c). No phenotypic abnormalities were found in these pups.

The foregoing results demonstrated that OSK + H1foo-iPS cells have high pluripotency.

[0109]

3. Method

[Production of human iPS cells and method for culturing the same]

Human iPS cells were produced according to a protocol described in literature (Seki, T., Yuasa, S., Fukuda, K. Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a

combination of activated T cells and Sendai virus, Nature Protocols 7, 718-728, 2012). iPS cells (OSKL + Hlfoo-iPS cells) were produced from human peripheral blood lymphocytes or human skin fibroblasts using a Sendai virus vector containing the human Oct3/4, Sox2, Klf4, L-Myc, and Hlfoo genes (a nucleotide sequence set forth in SEQ ID No: 1: BC047943). As control, iPS cells (OSKL-iPS cells) were produced using the Azami-Green gene instead of the Hlfoo gene. This experiment was conducted following the guidelines on gene recombination experimentation at Keio University.

[0110]

Human iPS cell lines were cultured and maintained on culture plates coated with iMatrix-511 (manufactured by Nippi, Incorporated) in StemFit AK02N (manufactured by Ajinomoto Co., Inc.) culture medium (hereinafter referred to as "human iPS cell culture medium"). The human iPS cell culture medium was replaced every 2 days and the cells were subcultured using StemPro Accutase (manufactured by Gibco) every 5 to 7 days.

[0111]

4. Results

[Comparison of expression level of genes reflecting chromosomal abnormality in iPS cells]

To study the genome instability of the established human iPS cells by comparison according to the method described in the section of [Production of human iPS cells and method for culturing the same] above, the expression level of the SRF and ACTG2 genes (Lamm, N., Kerem, B., Genomic Instability in Human Pluripotent Stem Cells Arises

from Replicative Stress and Chromosome Condensation Defects Cell Stem Cell 18 (2) 253-261 2016), whose expression level decreases when the cell has chromosomal abnormality, was studied by comparison by quantitative RT-PCR analysis. The quantitative RT-PCR analysis was performed following the method described in the section of [Quantitative RT-PCR analysis] above using 4 clones of OSKL + Hlfoo-iPS cells and 4 clones of OSKL-iPS cells (control). Figure 11a shows the results of quantitative RT-PCR analysis on the SRF gene and Figure 11b shows the results of quantitative RT-PCR analysis on the ACTG2 gene. [0112]

As seen from the results of Figures 11a and b, the OSKL + Hlfoo-iPS cell populations also exhibited significantly higher expression level of either of the SRF and ACTG2 genes than the OSKL-iPS cell population (control). These results have demonstrated that use of the Hlfoo gene, in addition to the nuclear reprogramming substance, suppresses the chromosomal abnormality that may result from the establishment of iPS cells and enables the production of higher-quality iPS cell population. [0113]

[Comparison of expression level of undifferentiated state marker of iPS cells]

To examine whether iPS cells established according to the method described in the section of [Production of human iPS cells and method for culturing the same] above maintain a stable undifferentiated state, the expression level of the Oct3/4 gene (an undifferentiated state marker of stem cells) was studied by comparison by quantitative

RT-PCR analysis. The quantitative RT-PCR analysis was conducted using 4 clones of the OSKL + Hlfoo-iPS cells and 4 clones of the OSKL-iPS cells (control) following the method described in the section of [Quantitative RT-PCR analysis] above. Figure 12 shows the results of the quantitative RT-PCR analysis.

[0114]

As seen from the results of Figure 12, the OSKL + Hlfoo-iPS cell population had significantly smaller variance of the expression level of the Oct3/4 gene (undifferentiated state marker in stem cells) among clones as compared with the OSKL-iPS cell population (control). The result indicated that use of the Hlfoo gene, in addition to the nuclear reprogramming substance, results in the maintenance of a more stable undifferentiated state and enables the production of iPS cell populations with less variance in quality.

[0115]

[Comparison of cell survival rate in early stage after induction of differentiation]

To study the number of viable cells in the early stage after the induction of differentiation of human iPS cells established according to the method described in the section of [Production of human iPS cells and method for culturing the same] above, a certain number of cells (1×10^6 cells/well) were seeded and then medium was replaced from iPS cell culture medium to RPMI-1640 (manufactured by Sigma Aldrich Co. LLC.) + B27 Supplement (manufactured by Gibco) culture medium, the cells were collected the next day, and the number of viable cells was calculated using a

trypan blue solution and compared. Figure 13 shows the results of such calculation of the number of germ cells conducted for 4 clones of OSKL + Hlfoo-iPS cells and 4 clones of OSKL-iPS cells (control).

[0116]

As seen from the results of Figure 13, the OSKL + Hlfoo-iPS cell population contained a significantly higher number of viable cells after the induction of differentiation and had a tendency to have higher adaptability to the environment for the induction of differentiation as compared with the OSKL-iPS cell population (control). The result demonstrated that use of the Hlfoo gene in addition to the nuclear reprogramming substance results in more number of viable cells after the induction of differentiation and allows the production of higher-quality iPS cell populations in that they have higher adaptability to the environment for the induction of differentiation.

[0117]

[Comparison of residual amounts of undifferentiated state marker in early stage after induction of differentiation of iPS cells]

In the early stage after the induction of differentiation of human iPS cells established according to the method described in the section of [Production of human iPS cells and method for culturing the same] above, the residual amounts of the Oct3/4 gene (undifferentiated state marker of stem cells) were compared. Specifically, a certain number of cells (1×10^6 cells/well) were seeded in wells, then medium was replaced from iPS cell culture

medium to culture medium for induction of differentiation [DMEM F12 HAM (manufactured by Sigma Aldrich Co. LLC.) + FBS (manufactured by Gibco) + GlutaMAX (manufactured by Gibco) + PenStrep (manufactured by Gibco) + 2-mercaptoethanol (manufactured by Sigma Aldrich Co. LLC.)], and cells were collected on the 5th day after the culture medium change to compare the expression level of the Oct3/4 gene, which is an undifferentiated state marker, by quantitative RT-PCR analysis. The quantitative RT-PCR analysis was conducted following the method described in the section of [Quantitative RT-PCR analysis] above using 4 clones of the OSKL + Hlfoo-iPS cells and 4 clones of the OSKL-iPS cells (control). Figure 14 shows the results of the quantitative RT-PCR analysis.

[0118]

As seen from the results of Figure 14, while the OSKL-iPS cell population (control) had a plurality of clones (OSKL-iPS 2 and OSKL-iPS 4) in which the undifferentiated state marker (Oct3/4) remains high despite being cultured in the culture medium for the induction of differentiation for 5 days, the OSKL + Hlfoo-iPS cell population had clones any of which expressed the undifferentiated marker at a low level and the variance of expression of the undifferentiated state marker among clones was significantly smaller. The results demonstrated that the expression of the undifferentiated state marker in the iPS cells produced using the Hlfoo gene in addition to the nuclear reprogramming substance disappears rapidly when the iPS cells are cultured in the culture medium for the induction of differentiation and

the variance of the disappearance is small. Thus, it was demonstrated that use of the Hlfoo gene in addition to the nuclear reprogramming substance enables the production of iPS cell populations with smaller variance in quality.

Industrial Applicability

[0119]

The present invention can provide an agent for improving quality of an iPS cell, a method for producing an iPS cell, an iPS cell produced by such a method for production, and a composition for producing an iPS cell. According to the present invention, higher-quality iPS cells with smaller variance in quality can be produced.

CLAIMS

1. An agent for improving quality of an iPS cell, comprising an Hlfoo gene or a gene product thereof.
2. The agent for improving quality of an iPS cell according to claim 1, wherein the agent comprises an expression vector comprising the Hlfoo gene.
3. A method for producing an iPS cell, comprising the step of introducing (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof into a somatic cell.
4. The method for producing an iPS cell according to claim 3, wherein the nuclear reprogramming substance comprises at least one selected from the group consisting of a gene of Oct gene family, a gene of Sox gene family, a gene of Klf gene family, a gene of Myc gene family, a gene of Lin gene family, a Nanog gene, and gene products thereof.
5. The method for producing an iPS cell according to claim 3 or 4, wherein the nuclear reprogramming substance consists of a gene of Oct gene family or a gene product thereof, a gene of Sox gene family or a gene product thereof, and a gene of Klf gene family or a gene product thereof.
6. The method for producing an iPS cell according to any one of claims 3 to 5, wherein the nuclear reprogramming

substance consists of an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, and a Klf4 gene or a gene product thereof.

7. The method for producing an iPS cell according to any one of claims 3 to 5, wherein the nuclear reprogramming substance consists of an Oct3/4 gene or a gene product thereof, a Sox2 gene or a gene product thereof, a Klf4 gene or a gene product thereof, and an L-Myc gene or a gene product thereof.

8. An iPS cell produced by the method for producing an iPS cell according to any one of claims 1 to 7.

9. A composition for producing an iPS cell, comprising (a) a nuclear reprogramming substance and (b) an Hlfoo gene or a gene product thereof.