SPECIFICATION

Nuclear Reprogramming Factor

Technical Field

The present invention relates to a nuclear reprogramming factor having an action of reprogramming a differentiated somatic cell to derive an induced pluripotent stem cell.

Background Art

Embryonic stem cells (ES cells) are stem cells established from human or mouse early embryos which have a characteristic feature that they can be cultured over a long period of time while maintaining pluripotent ability to differentiate into all kinds of cells existing in living bodies. Human embryonic stem cells are expected for use as resources for cell transplantation therapies for various diseases such as Parkinson's disease, juvenile diabetes, and leukemia, taking advantage of the aforementioned properties. However, transplantation of ES cells has a problem of causing rejection in the same manner as organ transplantation. Moreover, from an ethical viewpoint, there are many dissenting opinions against the use of ES cells which are established by destroying human embryos. If dedifferentiation of patients' own differentiated somatic cells could be induced to establish cells having pluripotency and growth ability similar to those of ES cells (in this specification, these cells are referred to as "induced pluripotent stem cells (iPS cells)", though they are sometimes called "embryonic stem cell-like cells" or "ES-like cells"), it is anticipated that such cells could be used as ideal pluripotent cells, free from rejection or ethical difficulties.

As a method for reprogramming a somatic nucleus, for example, a technique of establishing an embryonic stem cell from a cloned embryo, prepared by transplanting a nucleus of a somatic cell into an egg, was reported (W.S. Hwang et al., Science, 303, pp.1669-74, 2004; W.S. Hwang et al., Science, 308, pp.1777-83, 2005: these articles were, however, proved to be fabrications and later withdrawn). However, this technique of preparing the cloned embryo only for the purpose of establishing ES cells, has rather more serious ethical problems when compared with

ordinary ES cells using surplus embryos produced in fertilization therapy. A technique of reprogramming a somatic cell nucleus by fusing a somatic cell and an ES cell was also reported (M. Tada et al., Curr. Biol., 11, pp.1553-1558, 2001; C.A. Cowan et al., Science, 309, pp.1369-73, 2005). However, this method results in the use of human ES cells, which fails to provide a solution to the ethical difficulties. Further, a technique of reprogramming a cell nucleus by reacting an extract of a cell strain, derived from a germ cell tumor generated in a human, with a differentiated cell was reported (C.K. Taranger et al., Mol. Biol. Cell, 16, pp.5719-35, 2005). However, it was completely unknown which component in the extract induced the reprogramming in this method, and therefore, this method has problems of technical reliability and safety.

A method for screening a nuclear reprogramming factor having an action of reprogramming differentiated somatic cells to derive induced pluripotent stems cell was proposed (International Publication WO2005/80598). This method comprises the steps of: contacting somatic cells containing a gene, in which a marker gene is positioned so as to receive expression control by an expression control region of the ECAT (ES cell associated transcript) genes (i.e., a class of genes specifically expressed in ES cells), with each test substance; examining presence or absence of the appearance of a cell that expresses the marker gene; and choosing a test substance inducing the appearance of said cell as a candidate of a nuclear reprogramming factor for somatic cells. A method for reprogramming a somatic cell is disclosed in Example 6 and the like of the above publication. However, this publication fails to report an actual identification of a nuclear reprogramming factor.

Patent document 1: International Publication WO2005/80598

Disclosure of the Invention

An object of the present invention is to provide a nuclear reprogramming factor. More specifically, it is an object of the present invention to provide a means for inducing reprogramming of a differentiated cell without using eggs, embryos, or ES cells, to conveniently and highly reproducibly establish an induced pluripotent stem cell having pluripotency and growth ability similar to those of ES cells.

The inventors of the present invention conducted various research to achieve the aforementioned object and attempted to identify a nuclear reprogramming factor

by using the screening method for a nuclear reprogramming factor disclosed in International Publication WO2005/80598. As a result, 24 kinds of candidate genes were found as genes relating to nuclear reprogramming, and among them, three kinds of the genes were found as essential genes for nuclear reprogramming. The present invention was achieved on the basis of the aforementioned findings.

The present invention thus provides a nuclear reprogramming factor for a somatic cell, which comprises a gene product of each of the following three kinds of genes: an Oct family gene, a Klf family gene, and a Myc family gene. According to a preferred embodiment of the invention, there is provided the aforementioned factor comprising a gene product of each of the following three kinds of genes: Oct3/4, Klf4 and c-Myc.

According to another preferred embodiment, there is provided the aforementioned factor, which further comprises a gene product of the following gene: a Sox family gene, and as a more preferred embodiment, there is provided the aforementioned factor, which comprises a gene product of Sox2.

According to still another preferred embodiment, there is provided the aforementioned factor, which comprises a cytokine together with the gene product of the Myc family gene, or alternatively, instead of the gene product of the Myc family gene. As a more preferred embodiment, there is provided the aforementioned factor, wherein the cytokine is basic fibroblast growth factor (bFGF) and/or stem cell factor (SCF).

According to particularly preferred embodiments, there are provided a nuclear reprogramming factor for a somatic cell, which comprises a gene product of the TERT gene in addition to a gene product of each of an Oct family gene, a Klf family gene, a Myc family gene, and a Sox family gene; and the aforementioned factor, which comprises a gene product or gene products of one or more kinds of genes selected from the group consisting of the following genes: SV40 Large T antigen, HPV16 E6, HPV16 E7, and Bmil, in addition to a gene product of each of the Oct family gene, the Klf family gene, the Myc family gene, the Sox family gene, and the TERT gene.

In addition to these factors, there is provided the aforementioned factor, which further comprises a gene product or gene products of one or more kinds of genes selected from the group consisting of the following: Fbx15, Nanog, ERas, ECAT15-2,

Tcl1, and ß-catenin.

According to another preferred embodiment of the aforementioned invention, there is also provided the aforementioned factor, which comprises a gene product or gene products of one or more kinds of genes selected from the group consisting of the following: ECAT1, Esg1, Dnmt3L, ECAT8, Gdf3, Sox15, ECAT15-1, Fthl17, Sall4, Rex1, UTF1, Stella, Stat3, and Grb2.

In another aspect, the present invention provides a method for preparing an induced pluripotent stem cell by nuclear reprogramming of a somatic cell, which comprises the step of contacting the aforementioned nuclear reprogramming factor with the somatic cell.

According to a preferred embodiment of the invention, there are provided the aforementioned method, which comprises the step of adding the aforementioned nuclear reprogramming factor to a culture of the somatic cell; the aforementioned method, which comprises the step of introducing a gene encoding the aforementioned nuclear reprogramming factor into the somatic cell; the aforementioned method, which comprises, the step of introducing said gene into the somatic cell by using a recombinant vector containing at least one kind of gene encoding the aforementioned nuclear reprogramming factor; and the aforementioned method, wherein a somatic cell isolated from a patient is used as the somatic cell.

In another aspect, the present invention provides an induced pluripotent stem cell obtained by the aforementioned method. The present invention also provides a somatic cell derived by inducing differentiation of the aforementioned induced pluripotent stem cell.

The present invention further provides a method for stem cell therapy, which comprises the step of transplanting a somatic cell, wherein said cell is obtained by inducing differentiation of an induced pluripotent stem cell obtained by the aforementioned method using a somatic cell isolated and collected from a patient, into said patient.

The present invention further provides a method for evaluating a physiological function or toxicity of a compound, a medicament, a poison or the like by using various cells obtained by inducing differentiation of an induced pluripotent stem cell obtained by the aforementioned method.

The present invention also provides a method for improving ability of

differentiation and/or growth of a cell, which comprises the step of contacting the aforementioned nuclear reprogramming factor with the cell, and further provides a cell obtained by the aforementioned method, and a somatic cell derived by inducing differentiation of a cell obtained by the aforementioned method.

By using the nuclear reprogramming factor provided by the present invention, reprogramming of a differentiated cell nucleus can be conveniently and highly reproducibly induced without using embryos or ES cells, and an induced pluripotent stem cell, as an undifferentiated cell having differentiation ability, pluripotency, and growth ability similar to those of ES cells, can be established. For example, an induced pluripotent stem cell having high growth ability and differentiation pluripotency can be prepared from a patient's own somatic cell by using the nuclear reprogramming factor of the present invention. Cells obtainable by differentiating said cell (for example, cardiac muscle cells, insulin producing cells, nerve cells and the like) are extremely useful, because they can be utilized for stem cell transplantation therapies for a variety of diseases such as cardiac insufficiency, insulin dependent diabetes mellitus, Parkinson's disease and spinal cord injury, thereby the ethical problem concerning the use of human embryo and rejection after transplantation can be avoided. Further, various cells obtainable by differentiating the induced pluripotent stem cell (for example, cardiac muscle cells, hepatic cells and the like) are highly useful as systems for evaluating efficacy or toxicity of compounds, medicaments, poisons and the like.

Brief Explanation of the Drawings

Fig. 1 shows a screening method for reprogramming factors using embryonic fibroblasts (MEFs) of a mouse having ßgeo knockin Fbx15 gene.

Fig. 2 depicts photographs showing morphology of iPS cells obtained by introducing the 24 genes shown in Table 1. Morphologies of differentiated cells (MEF) and of normal embryonic stem cells (ES) are also shown as a reference.

Fig. 3 shows expression profiles of marker genes in iPS cells. The results of RT-PCR using total RNAs extracted from iPS cells, ES cells and MEF cells as templates are shown.

Fig. 4 shows methylation status of DNA in iPS cells. Genomic DNAs extracted from iPS cells, ES cells, and MEF cells were treated with bisulfite. The

target DNAs were amplified by PCR and then inserted into plasmid. Ten clones of plasmid were isolated for each of the genes, and sequenced. Methylated CpGs are indicated with closed circles, and unmethylated CpGs with open circles.

Fig. 5 shows colony numbers of G418 cells obtained by transduction of 24-gene group and 23-gene groups wherein each individual gene was withdrawn from the 24-gene group. The lower parts of the graph show colony numbers obtained in one week after the G418 selection, and the upper parts of the graph show numbers of clones obtained in three weeks. When each boxed gene (the reference number for each gene is the same as that indicated in Table 1) was withdrawn, no colonies were obtained at all, or only a few colonies were observed after 3 weeks.

Fig. 6 shows colony numbers of G418 cells obtained by transduction of 10-gene group and 9-gene groups wherein each individual gene was withdrawn from the 10-gene group. When each of genes #14, #15 or #20 was withdrawn, no colony was obtained. When gene #22 was withdrawn, a few G418-resistant colonies were obtained. However, the cells gave differentiated morphology which was apparently different from that of iPS cells.

Fig. 7 shows numbers of G418-resistant emerged colonies (reprogrammed colony) with 10-gene group, 4-gene group, 3-gene groups, or 2-gene groups. Typical morphology and sizes of the colonies are shown.

Fig. 8 depicts photographs showing results of hematoxylin-eosin (H & E) staining of tumors formed after subcutaneous transplantation of iPS cells derived from MEFs into nude mice. Differentiation into a variety of tissues in a triploblastic system was observed.

Fig. 9 depicts photographs of embryos prepared by transplanting iPS cells derived from adult dermal fibroblasts into mouse blastocysts and transplanting the cells into the uteri of pseudopregnant mice. It can be observed that, in the upper left embryo, cells derived from the iPS cells (emitting green fluorescence) were systemically distributed. In the lower photographs, it can be observed that almost all cells of the heart, liver, and spinal cord of the embryo were GFP-positive and were derived from the iPS cells.

Fig. 10 depicts photographs showing results of RT-PCR confirming the expression of the ES cell marker genes. In the photographs, Sox2 minus indicates iPS cells established by the transduction of 3 genes into MEFs, 4ECATs indicates iPS

cells established by the transduction of 4 genes into MEFs, 10ECATs indicates iPS cells established by the transduction of 10 genes into MEFs, 10ECATs Skin fibroblast indicates iPS cells established by the transduction of 10 genes into dermal fibroblasts, ES indicates mouse ES cells, and MEF indicates MEF cells without gene transduction. The numerical values under the symbols indicate clones numbers.

Fig. 11 shows an effect of bFGF on the establishment of iPS cells from MEFs. Four factors (upper row) or three factors except for c-Myc (lower row) were retrovirally transduced into MEFs derived from Fbx15$^{\beta geo/\beta geo}$ mice, and cultured on ordinary feeder cells (STO cells) (left) and bFGF expression vector-introduced STO cells (right). G418 selection was performed for 2 weeks, and cells were stained with crystal blue and photographed. The numerical values indicate the number of colonies.

Fig. 12 depicts explanations of the experiments using Nanog-EGFP-IRES-Puro mice. A: *E. coli* artificial chromosome (BAC) containing the mouse Nanog gene in the center was isolated, and the EGFP-IRES-Puro cassette was inserted upstream from the coding region of Nanog by recombineering. B: Transgenic mice were prepared with the modified BAC. GFP expression was observed limitedly in inner cell masses of blastocysts and gonads.

Fig. 13 depicts explanations of the experiments using Nanog-EGFP-IRES-Puro mice. From embryos of Nanog-EGFP-IRES-Puro mice (13.5 days after fertilization), heads, viscera and gonads were removed to establish MEFs. As a result of analysis with a cell sorter, almost no GFP-positive cells existed in MEFs derived from the Nanog-EGFP-IRES-Puro mice (Nanog) in the same manner as the Fbx15$^{\beta geo/\beta geo}$ mouse-derived MEFs (Fbx15) or wild-type mouse-derived MEFs (Wild).

Fig. 14 depicts photographs of iPS cells established from the Nanog-EGFP-IRES-Puro mouse MEFs (left) and the Fbx15$^{\beta geo/\beta geo}$ mouse MEFs (right). The cells were selected with puromycin and G418, respectively.

Fig. 15 shows results of growth of iPS cells. 100,000 cells of each of ES cells, iPS cells derived from the Nanog-EGFP-IRES-Puro mouse MEFs (Nanog iPS, left), and iPS cells derived from the Fbx15$^{\beta geo/\beta geo}$ mouse MEFs (Fbx iPS) were seeded on 24-well plates, and passaged every 3 days. Cell count results are shown. The numerical values represent average doubling times.

Fig. 16 shows gene expression profiles of iPS cells. Expression of the marker genes in MEFs, ES cells, iPS cells derived from Nanog-EGFP-IRES-Puro mouse MEFs

(Nanog iPS, left), and iPS cells derived from Fbx15$^{\beta geo/\beta geo}$ mouse MEFs (Fbx iPS) were analyzed by RT-PCR. The numerical values at the bottom indicate the numbers of passages.

Fig. 17 shows teratoma formation from the Nanog iPS cells. 1,000,000 cells of each of ES cells or Nanog iPS cells were subcutaneously injected into the backs of nude mice, and the appearance of tumors formed after 3 weeks (left) and tissue images (right, H & E stained) are shown.

Fig. 18 shows preparation of chimeric mice with the Nanog iPS cells. The chimeric mice that were born after transplantation of the Nanog iPS cells (clone NPMF4EK-24, passaged 6 times) into the blastocysts. Four chimeric mice were born from 90 transplanted embryos.

Fig. 19 shows germ-line transmission from the Nanog iPS cells. PCR analysis of genomic DNA of mice, born by mating of the chimeric mice shown in Fig. 18 and C57BL/6 mice, revealed the existence of transgenes of Oct3/4 and Klf4 in all of the mice, thereby confirming germ-line transmission.

Fig. 20 shows induction of differentiation into nerve cells from iPS cells. Nerve cells (top, βIII tubulin-positive), oligodendrocytes (left, O4-positive), and astrocytes (right, GFAP-positive) differentiated *in vitro* from dermal fibroblasts-derived iPS cells are shown.

Fig. 21 depicts explanations of establishment of the iPS cells without using drug selection. MEFs at 10,000 to 100,000 cells per 10 cm dish were seeded, and the 4 factors were retrovirally transduced. No colony appeared in the control (Mock, left), whilst in the dish with the transduction by the 4 factors, swelling colonies similar to those of the iPS cells were obtained (center), as well as flat transformant colonies. When the cells were passaged, cells similar to the iPS cells were obtained (right).

Fig. 22 shows gene expression profiles of cells established without using drug selection. RNA was extracted from the established cells shown in Fig. 21, and expression of the ES cell marker genes was analyzed by RT-PCR.

Fig. 23 shows iPS cell-like cells derived from human fibroblasts. The colonies obtained by retroviral transduction with human homologous genes of the 4 factors into fibroblasts derived from human embryos (left), and the cells after two passages (right) are shown.

Fig. 24 shows establishment of the iPS cells from human adult dermal

fibroblasts. The factors mentioned in the left column were transduced retrovirally into human adult dermal fibroblasts infected with the mouse retroviral receptor with lentivirus. The photographs shows phase contrast images (object x 10) on day 8 after the viral infection.

Best Mode for Carrying out the Invention

The nuclear reprogramming factor of the present invention is characterized in that it comprises a gene product of each of the following three kinds of genes: an Oct family gene, a Klf family gene, and a Myc family gene; and according to a preferred embodiment, it is characterized in that it comprises a gene product of a Sox family gene in addition to the aforementioned three kinds of genes.

As a means for confirming the nuclear reprogramming factor of the present invention, for example, the screening method for nuclear reprogramming factors disclosed in International Publication WO 2005/80598 can be used. The entire disclosure of the aforementioned publication is incorporated into the disclosure of the specification by reference. By referring to the aforementioned publication, those skilled in the art can perform screening of nuclear reprogramming factors to confirm the existence and the action of the reprogramming factor of the present invention.

For example, as an experimental system enabling observation of the reprogramming phenomenon, a mouse can be used in which the βgeo (a fusion gene of the β galactosidase gene and the neomycin resistance gene) is knocked into the Fbx15 locus. The details are described in the examples of the specification. The mouse Fbx15 gene is a gene specifically expressed in differentiation pluripotent cells such as ES cells and early embryos. In a homomutant mouse in which βgeo is knocked into the mouse Fbx15 gene so as to be deficient in the Fbx15 function, abnormal phenotypes including those relating to differentiation pluripotency or generation are not generally observed. In this mouse, the expression of the βgeo is controlled by the enhancer and promoter of the Fbx15 gene, and differentiated somatic cells in which βgeo is not expressed have sensitivity to G418. In contrast, βgeo knockin homomutant ES cells have resistance against G418 at an extremely high concentration (higher than 12 mg/ml). By utilizing this phenomenon, an experimental system can be constructed to visualize reprogramming of somatic cells.

By applying the aforementioned experimental system, fibroblasts

(Fbx15$^{\beta geo/\beta geo}$ MEFs) can be first isolated from an embryo of the βgeo knockin homomutant mouse (13.5 days after fertilization). The MEFs do not express the Fbx15 gene, and accordingly also do not express βgeo to give sensitivity to G418. However, when the MEFs are fused with genetic manipulation-free ES cells (also have sensitivity to G418), βgeo is expressed and the cells become G418-resistant as a result of reprogramming of nuclei of MEFs. Therefore, by utilizing this experimental system, the reprogramming phenomenon can be visualized as G418 resistance.

Nuclear reprogramming factors can be selected by using the aforementioned experimental system. As candidates of genes relevant to nuclear reprogramming factors, a plurality of genes can be selected which show specific expression in ES cells or of which important roles in the maintenance of pluripotency of ES cells are suggested, and it can be confirmed whether or not each candidate gene can induce nuclear reprogramming alone or in an appropriate combination thereof. For example, a combination of all of the selected primary candidate genes is confirmed to be capable of inducing the reprogramming of differentiated cells into a state close to that of ES cells. Combinations are then prepared by withdrawing each individual gene from the aforementioned combination, and the same actions of the combinations are confirmed in order to select each secondary candidate gene whose absence causes a reduction of the reprogramming induction ability or loss of the reprogramming induction ability. By repeating similar steps for the secondary candidate genes selected as described above, an essential combination of nuclear reprogramming genes can be selected, and it can be confirmed that a combination of gene products of each of the three kinds of genes, an Oct family gene, a Klf family gene, and a Myc family gene, acts as a nuclear reprogramming factor. It can be further confirmed that a combination of a gene product of a Sox family gene additionally with the gene products of the aforementioned three kinds of genes has extremely superior characteristics as a nuclear reprogramming factor. Specific examples of the selection method for the nuclear reprogramming factors are demonstrated in the examples of the specification. Therefore, by referring to the above general explanations and specific explanations of the examples, those skilled in the art can readily confirm that the combination of these three kinds of genes induces the reprogramming of somatic cells, and that the combination of these three kinds of gene products is essential for nuclear reprogramming.

The nuclear reprogramming factor provided by the present invention comprises at least a combination of gene products of an Oct family gene, a Klf family gene, and a Myc family gene, for example, a combination of gene products of Oct3/4, Klf4, and c-Myc. Examples of the Oct family gene include, for example, Oct3/4, Oct1A, Oct6, and the like. Oct3/4 is a transcription factor belonging to the POU family, and is reported as a marker of undifferentiated cells (K. Okamoto et al., Cell, 60, pp461-72, 1990). Oct3/4 is also reported to participate in the maintenance of pluripotency (J. Nichols et al., Cell, 95, pp379-91, 1998). Examples of the Klf family gene include Klf1, Klf2, Klf4, Klf5 and the like. Klf4 (Kruppel like factor-4) is reported as a tumor repressing factor (A.M. Ghaleb et al., Cell Res., 15, pp92-6, 2005). Examples of the Myc family gene include c-Myc, N-Myc, L-Myc and the like. c-Myc is a transcription control factor involved in differentiation and proliferation of cells (S. Adhikary, M. Eilers, Nat. Rev. Mol. Cell Biol., 6, pp.635-45, 2005), and is also reported to be involved in the maintenance of pluripotency (P. Cartwright et al., Development, 132, pp.885-96, 2005). The NCBI accession numbers of the genes of the families other than Oct3/4, Klf4 and c-Myc are as follows:

Table 1

		Mouse	Human
Klf1	Kruppel-like factor 1 (erythroid)	NM_010635	NM_006563
Klf2	Kruppel-like factor 2 (lung)	NM_008452	NM_016270
Klf5	Kruppel-like factor 5	NM_009769	NM_001730
c-Myc	myelocytomatosis oncogene	NM_010849	NM_002467
N-Myc	v-Myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	NM_008709	NM_005378
L-Myc	v-Myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	NM_008506	NM_005376
Oct1A	POU domain, class 2, transcription factor 1	NM_198934	NM_002697
Oct6	POU domain, class 3, transcription factor 1	NM_011141	NM_002699

All of these genes are those commonly existing in mammals including human, and for use of the aforementioned gene products in the present invention, genes

derived from arbitrary mammals (those derived from mammals such as mouse, rat, bovine, ovine, horse, and ape) can be used. In addition to wild-type gene products, mutant gene products including substitution, insertion, and/or deletion of several (for example, 1 to 10, preferably 1 to 6, more preferably 1 to 4, still more preferably 1 to 3, and most preferably 1 or 2) amino acids and having similar function to that of the wild-type gene products can also be used. For example, as a gene product of c-Myc, a stable type product (T58A) may be used as well as the wild-type product. The above explanation may be applied similarly to the other gene products.

The nuclear reprogramming factor of the present invention may comprise a gene product other than the aforementioned three kinds of gene products. An example of such gene product includes a gene product of a Sox family gene. Examples of the Sox family gene include, for example, Sox1, Sox3, Sox7, Sox15, Sox17 and Sox18, and a preferred example includes Sox2. A nuclear reprogramming factor comprising at least a combination of the gene products of four kinds of genes, an Oct family gene (for example, Oct3/4), a Klf family gene (for example, Klf4), a Myc family gene (for example, c-Myc), and a Sox family gene (for example, Sox2) is a preferred embodiment of the present invention from a viewpoint of reprogramming efficiency, and in particular, a combination of a gene product of a Sox family gene is sometimes preferred to obtain pluripotency. Sox2, expressed in an early development process, is a gene encoding a transcription factor (A.A. Avilion et al., Genes Dev., 17, pp.126-40, 2003). The NCBI accession numbers of Sox family genes other than Sox2 are as follows.

Table 2

		Mouse	Human
Sox1	SRY-box containing gene 1	NM_009233	NM_005986
Sox3	SRY-box containing gene 3	NM_009237	NM_005634
Sox7	SRY-box containing gene 7	NM_011446	NM_031439
Sox15	SRY-box containing gene 15	NM_009235	NM_006942
Sox17	SRY-box containing gene 17	NM_011441	NM_022454
Sox18	SRY-box containing gene 18	NM_009236	NM_018419

Further, a gene product of a Myc family gene may be replaced with a cytokine.

As the cytokine, for example, SCF, bFGF or the like is preferred. However, cytokines are not limited to these examples.

As a more preferred embodiment, an example includes a factor which induces immortalization of cells, in addition to the aforementioned three kinds of gene products, preferably, the four kinds of gene products. For example, an example includes a combination of a factor comprising a gene product of TERT gene with a factor comprising a gene product or gene products of one or more kinds of genes selected from the group consisting of the following genes: SV40 Large T antigen, HPV16 E6, HPV16 E7, and Bmil. TERT is essential for the maintenance of the telomere structure at the end of chromosome at the time of DNA replication, and the gene is expressed in stem cells or tumor cells in humans, whilst it is not expressed in many somatic cells (I. Horikawa, et al., Proc. Natl. Acad. Sci. USA, 102, pp.18437-442, 2005). SV40 Large T antigen, HPV16 E6, HPV16 E7, or Bmil was reported to induce immortalization of human somatic cells in combination with Large T antigen (S. Akimov et al., Stem Cells, 23, pp.1423-1433, 2005; P. Salmon et al., Mol. Ther., 2, pp.404-414, 2000). These factors are extremely useful particularly when iPS cells are induced from human cells. The NCBI accession numbers of TERT and Bmi1 genes are as follows.

Table 3

		Mouse	Human
TERT	telomerase reverse transcriptase	NM_009354	NM_198253
Bmi1	B lymphoma Mo-MLV insertion region 1	NM_007552	NM_ 005180

Furthermore, a gene product or gene products of one or more kinds of genes selected from the group consisting of the following: Fbx15, Nanog, ERas, ECAT15-2, Tcl1, and β-catenin may be combined. As a particularly preferred embodiment from a viewpoint of reprogramming efficiency, an example includes a nuclear reprogramming factor comprising a total of ten kinds of gene products, wherein gene products of Fbx15, Nanog, ERas, ECAT15-2, Tcl1, and β-catenin are combined with the aforementioned four kinds of gene products. Fbx15 (Y. Tokuzawa et al., Mol. Cell Biol., 23, pp.2699-708, 2003), Nanog (K. Mitsui et al., Cell, 113, pp.631-42, 2003), ERas (K. Takahashi, K. Mitsui, S. Yamanaka, Nature, 423, pp.541-5, 2003), and

ECAT15-2 (A. Bortvin et al., Development, 130, pp.1673-80, 2003) are genes specifically expressed in ES cells. Tcl1 is involved in the activation of Akt (A. Bortvin et al., Development, 130, pp.1673-80, 2003), and β-catenin is an important factor constituting the Wnt signal transmission pathway, and also reported to be involved in the maintenance of pluripotency (N. Sato et al, Nat. Med., 10, pp.55-63, 2004).

Further, the nuclear reprogramming factor of the present invention may comprise, for example, a gene product or gene products of one or more kinds of genes selected from the group consisting of the following: ECAT1, Esg1, Dnmt3L, ECAT8, Gdf3, Sox15, ECAT15-1, Fthl17, Sall4, Rex1, UTF1, Stella, Stat3, and Grb2. ECAT1, Esg1, ECAT8, Gdf3, and ECAT15-1 are genes specifically expressed in ES cells (K. Mitsui et al., Cell, 113, pp.631-42, 2003). Dnmt3L is a DNA methylating enzyme-related factor, and Sox15 is a class of genes expressed in an early development process and encoding transcription factors (M. Maruyama et al., J. Biol. Chem., 280, pp.24371-9, 2005). Fthl17 encodes ferritin heavy polypeptide-like 17 (A. colLoriot, T. Boon, C. De Smet, Int. J. Cancer, 105, pp.371-6, 2003), Sall4 encodes a Zn finger protein abundantly expressed in embryonic stem cells (J. Kohlhase et al., Cytogenet. Genome Res., 98, pp.274-7, 2002), and Rex1 encodes a transcription factor locating downstream from Oct3/4 (E. Ben-Shushan, J.R. Thompson, L.J. Gudas, Y. Bergman, Mol. Cell Biol., 18, pp.1866-78, 1998). UTF1 is a transcription cofactor locating downstream from Oct3/4, and it is reported that the suppression of the proliferation of ES cells is induced when this factor is suppressed (A. Okuda et al., EMBO J., 17, pp.2019-32, 1998). Stat3 is a signal factor for proliferation and differentiation of cells. The activation of Stat3 triggers the operation of LIF, and thereby the factor plays an important role for the maintenance of pluripotency (H. Niwa, T. Burdon, I. Chambers, A. Smith, Genes Dev., 12, pp.2048-60, 1998). Grb2 encodes a protein mediating between various growth factor receptors existing in cell membranes and the Ras/MAPK cascade (A.M. Cheng et al., Cell, 95, pp.793-803, 1998).

However, the gene products which may be included in the nuclear reprogramming factor of the present invention are not limited to the gene products of the genes specifically explained above. The nuclear reprogramming factor of the present invention may contain one or more factors relating to differentiation, development, proliferation or the like and factors having other physiological activities, as well as other gene products which can function as a nuclear reprogramming factor.

It is understood that such embodiments fall within the scope of the present invention. By using somatic cells in which only one or two genes among the three kinds of the gene Oct3/4, Klf4, and c-Myc are expressed, the other gene products which can function as a nuclear reprogramming factor can be identified by, for example, performing screening for a gene product which can induce nuclear reprogramming of said cells. According to the present invention, the aforementioned screening method is also provided as a novel method for screening for a nuclear reprogramming factor.

The gene products contained in the nuclear reprogramming factor of the present invention may be, for example, a protein, per se, produced from the aforementioned gene, or alternatively, in a form of a fusion gene product of said protein with another protein, peptide or the like. For example, a fusion protein with green fluorescence protein (GFP) or a fusion gene product with a peptide such as a histidine tag can also be used. Further, by preparing and using a fusion protein with the TAT peptide derived form the virus HIV, intracellular uptake of the nuclear reprogramming factor through cell membranes can be promoted, thereby enabling induction of reprogramming only by adding the fusion protein to a medium thus avoiding complicated operations such as gene transduction. Since preparation methods of such fusion gene products are well known to those skilled in the art, skilled artisans can easily design and prepare an appropriate fusion gene product depending on the purpose.

By using the nuclear reprogramming factor of the present invention, the nucleus of a somatic cell can be reprogrammed to obtain an induced pluripotent stem cell. In the specification, the term "induced pluripotent stem cells" means cells having properties similar to those of ES cells, and more specifically, the term encompasses undifferentiated cells having pluripotency and growth ability. However, the term should not be construed narrowly in any sense, and should be construed in the broadest sense. The method for preparing induced pluripotent stem cells by using a nuclear reprogramming factor is explained in International Publication WO2005/80598 (the term "ES-like cells" is used in the publication), and a means for isolating induced pluripotent stem cells is also specifically explained. Therefore, by referring to the aforementioned publication, those skilled in the art can easily prepare induced pluripotent stem cells by using the nuclear reprogramming factor of the present invention.

The method for preparing induced pluripotent stem cells from somatic cells by using the nuclear reprogramming factor of the present invention is not particularly limited. Any method may be employed as long as the nuclear reprogramming factor can contact with somatic cells under an environment in which the somatic cells and induced pluripotent stem cells can proliferate. For example, a gene product contained in the nuclear reprogramming factor of the present invention may be added to a medium. Alternatively, by using a vector containing a gene that is capable of expressing the nuclear reprogramming factor of the present invention, a means of transducing said gene into a somatic cell may be employed. When such vector is used, two or more kinds of genes may be incorporated into the vector, and each of the gene products may be simultaneously expressed in a somatic cell. When one or more of the gene products contained in the nuclear reprogramming factor of the present invention are already expressed in a somatic cell to be reprogrammed, said gene products may be excluded from the nuclear reprogramming factor of the present invention. It is understood that such embodiment falls within the scope of the present invention.

In the preparation of induced pluripotent stem cells by using the nuclear reprogramming factor of the present invention, types of somatic cells to be reprogrammed are not particularly limited, and any kinds of somatic cells may be used. For example, matured somatic cells may be used, as well as somatic cells of an embryonic period. When induced pluripotent stem cells are used for therapeutic treatment of diseases, it is desirable to use somatic cells isolated from patients. For example, somatic cells involved in diseases, somatic cells participating in therapeutic treatment of diseases and the like can be used. A method for selecting induced pluripotent stem cells that appear in a medium according to the method of the present invention is not particularly limited, and a well-known means may be suitably employed, for example, a drug resistance gene or the like can be used as a marker gene to isolate induced pluripotent stem cells using drug resistance as an index. Various media that can maintain undifferentiated state and pluripotency of ES cells and various media which cannot maintain such properties are known in this field, and induced pluripotent stem cells can be efficiently isolated by using a combination of appropriate media. Differentiation and proliferation abilities of isolated induced pluripotent stem cells can be easily confirmed by those skilled in the art by using

confirmation means widely applied to ES cells.

Uses of the induced pluripotent stem cells prepared by the method of the present invention are not particularly limited. The cells can be used for any experiments and research conducted with ES cells, therapeutic treatments utilizing ES cells and the like. For example, desired differentiated cells (e.g., nerve cells, cardiac muscle cells, hemocyte cells and the like) can be derived by treating induced pluripotent stem cells obtained by the method of the present invention with retinoic acid, growth factors such as EGF, glucocorticoid or the like, and stem cell therapy based on cellular auto-transplantation can be achieved by returning the differentiated cells obtained as described above to the patient. However, uses of the induced pluripotent stem cells of the present invention are not limited to the aforementioned specific embodiments.

Examples

The present invention will be more specifically explained with reference to examples. However, the scope of the present invention is not limited to the following examples.

Example 1: Selection of reprogramming factor

In order to identify reprogramming factors, an experimental system for easy observation of the reprogramming phenomenon is required. As an experimental system, a mouse in which βgeo (a fusion gene of β-galactosidase gene and neomycin resistance gene) was knocked into the Fbx15 locus was used. The mouse Fbx15 gene is a gene specifically expressed in differentiation pluripotent cells such as ES cells and early embryos. However, in a homomutant mouse in which βgeo was knocked into the mouse Fbx15 gene so as to delete the function of Fbx15, no abnormal phenotypes including those concerning differentiation pluripotency or development were observed. In this mouse, expression control of βgeo is attained by the enhancer and promoter of the Fbx15 gene. Specifically, βgeo is not expressed in differentiated somatic cells, and they have sensitivity to G418. In contrast, the βgeo knockin homomutant ES cells have resistance against G418 at an extremely high concentration (higher than 12 mg/ml). By utilizing the above phenomenon, an experimental system for visualizing the reprogramming of somatic cells was constructed.

In the aforementioned experimental system, fibroblasts (Fbx15$^{\beta geo/\beta geo}$ MEFs) were first isolated from an embryo of the βgeo knockin homomutant mouse (13.5 days after fertilization). Since MEFs do not express the Fbx15 gene, the cells also do not express βgeo and thus have sensitivity to G418. Whist, when the MEFs are fused with ES cells that have not been gene-manipulated (also having sensitivity to G418), the nuclei of MEFs are reprogrammed, and as a result, βgeo is expressed to give G418-resistance. The reprogramming phenomenon can thus be visualized as G418 resistance by using this experimental system (International Publication WO2005/80598). Searches for reprogramming factors were performed by using the aforementioned experimental system (Fig. 1), and total 24 kinds of genes were selected as candidate reprogramming factors, including genes showing specific expression in ES cells and genes suggested to have important roles in the maintenance of differentiation pluripotency of ES cells. These genes are shown in Tables 4 and 5 below. For β-catenin (#21) and c-Myc (#22), active type mutants (catenin: S33Y, c-Myc: T58A) were used.

Table 4

Number	Name of Gene	Explanation of Gene
1	ECAT1	ES cell associated transcript 1 (ECAT1)
2	ECAT2	developmental pluripotency associated 5 (DPPA5), ES cell specific gene 1 (ESG1)
3	ECAT3	F-box protein 15 (Fbx15),
4	ECAT4	homeobox transcription factor Nanog
5	ECAT5	ES cell expressed Ras (ERas),
6	ECAT7	DNA (cytosine-5-)-methyltransferase 3-like (Dnmt3l), valiant 1
7	ECAT8	ES cell associated transcript 8 (ECAT8)
8	ECAT9	growth differentiation factor 3 (Gdf3),
9	ECAT10	SRY-box containing gene 15 (Sox15),
10	ECAT15-1	developmental pluripotency associated 4 (Dppa4), variant 1
11	ECAT15-2	developmental pluripotency associated 2 (Dppa2),
12	Fthl17	ferritin, heavy polypeptide-like 17 (Fthl17),
13	Sall4	sal-like 4 (Drosophila) (Sall4), transcript variant a

Table 4 (Cont.)

14	Oct3/4	POU domain, class 5, transcription factor 1 (Pou5f1),
15	Sox2	SRY-box containing gene 2 (Sox2),
16	Rex1	zinc finger protein 42 (Zfp42),
17	Utf1	undifferentiated embryonic cell transcription factor 1 (Utf1)
18	Tcl1	T-cell lymphoma breakpoint 1 (Tcl1),
19	Stella	developmental pluripotency-associated 3 (Dppa3),
20	Klf4	Kruppel-like factor 4 (gut) (Klf4),
21	β-catenin	catenin (cadherin associated protein), beta 1, 88kDa (Ctnnb1)
22	c-Myc	myelocytomatosis oncogene (Myc),
23	Stat3	signal transducer and activator of transcription 3 (Stat3), transcript variant 1
24	Grb2	growth factor receptor bound protein 2 (Grb2),

Table 5

			NCBI accession number	
Number	Name of Gene	Characteristic Feature	Mouse	Human
1	ECAT1	Gene specifically expressed in ES cell	AB211060	AB211062
2	ECAT2	Gene specifically expressed in ES cell	NM_025274	NM_001025290
3	ECAT3	Gene specifically expressed in ES cell	NM_015798	NM_152676
4	ECAT4	Transcription factor having homeodomain, essential factor for differentiation pluripotency maintenance	AB093574	NM_024865

Table 5 (Cont.)

5	ECAT5	Ras family protein, ES cell growth promoting factor	NM_181548	NM_181532
6	ECAT7	DNA methylation enzyme-related factor, essential for imprinting	NM_019448	NM_013369
7	ECAT8	Gene specifically expressed in ES cell, having Tudor domain	AB211061	AB211063
8	ECAT9	Gene specifically expressed in ES cell, belonging to TGFβ family	NM_008108	NM_020634
9	ECAT10	Gene specifically expressed in ES cell, SRY family transcription factor	NM_009235	NM_006942
10	ECAT15-1	Gene specifically expressed in ES cell	NM_028610	NM_018189
11	ECAT15-2	Gene specifically expressed in ES cell	NM_028615	NM_138815
12	Fthl17	Gene specifically expressed in ES cell, similar to ferritin heavy chain	NM_031261	NM_031894
13	Sall4	Gene specifically expressed in ES cell, Zn finger protein	NM_175303	NM_020436
14	Oct3/4	POU family transcription factor, essential for pluripotency maintenance	NM_013633	NM_002701
15	Sox2	SRY family transcription factor, essential for pluripotency maintenance	NM_011443	NM_003106
16	Rex1	Gene specifically expressed in ES cell, Zn finger protein	NM_009556	NM_174900
17	Utf1	Transcription regulation factor highly expressed in ES cell, promoting growth of ES	NM_009482	NM_003577

Table 5 (Cont.)

18	Tcl1	Oncogene activating AKT, abundantly expressed in ES cell	NM_009337	NM_021966
19	Stella	Gene specifically expressed in ES cell	NM_139218	NM_199286
20	Klf4	Abundantly expressed in ES cell, both actions as antioncogene and oncogene are reported	NM_010637	NM_004235
21	ß-catenin	Transcription factor activated by Wnt signal, involvement in pluripotency maintenance is reported	NM_007614	NM_001904
22	c-Myc	Transcription control factor participating in cell proliferation and differentiation and oncogene, involvement in pluripotency maintenance is reported	NM_010849	NM_002467
23	Stat3	Transcription factor activated by LIF signal, considered essential for pluripotency maintenance of mouse ES cells	NM_213659	NM_139276
24	Grb2	Adapter protein mediating growth factor receptors and Ras/MAPK cascade	NM_008163	NM_002086

cDNAs of these genes were inserted into the retroviral vector pMX-gw by the Gateway technology. First, each of the 24 genes was infected into Fbx15$^{\beta geo/\beta geo}$ MEFs, and then G418 selection was performed under ES cell culture conditions. However, no G418-resistant colony was obtained. Next, the retroviral vectors of all of the 24 genes were simultaneously infected into Fbx15$^{\beta geo/\beta geo}$ MEFs. When G418 selection was performed under ES cell culture conditions, a plurality of drug resistant colonies were obtained. These colonies were isolated, and cultivation was continued. It was found that cultivation of these cells over a long period of time could be performed, and

that these cells had morphology similar to that of ES cells (Fig. 2). In the figure, iPS cells represent induced pluripotent stem cells (also called "ES like cells", "ES-like cells", or "ESL cells"), ES represents embryonic stem cells, and MEF represents differentiated cells (embryonic fibroblasts).

When expression profiles of the marker genes were examined by RT-PCR, undifferentiation markers such as Nanog and Oct3/4 were found to be expressed (Fig. 3). It was found that the expression of Nanog was close to that of ES cells, whereas the expression of Oct3/4 was lower than that of ES cells. When DNA methylation status was examined by the bisulfite genomic sequencing, it was found that the Nanog gene and Fbx15 gene were highly methylated in MEFs, whereas they were demethylated in the iPS cells (Fig. 4). About 50% of IGF2 gene, an imprinting gene, was methylated both in the MEF and iPS cells. Since it was known that the imprinting memory was deleted and the IGF2 gene was almost completely demethylated in the primordial germ cells at 13.5 days after fertilization, from which the $Fbx15^{\beta geo/\beta geo}$ MEFs were isolated, it was concluded that iPS cells were not derived from primordial germ cells contaminated in the $Fbx15^{\beta geo/\beta geo}$ MEFs. The above results demonstrated that reprogramming of the differentiated cells (MEFs) into a state close to that of ES cells was able to be induced with the combination of the 24 kinds of factors.

Then, studies were made as to whether or not all of the 24 kinds of genes were required for the reprogramming. With withdrawal of each individual gene, 23 genes were transfected into the $Fbx15^{\beta geo/\beta geo}$ MEFs. As a result, for 10 genes, colony formation was found to be inhibited with each withdrawal thereof (Fig. 5, the gene numbers correspond to the gene numbers shown in Table 4, and the genes are the following 10 kinds of genes: #3, #4, #5, #11, #14, #15, #18, #20, #21, and #22). When these ten genes were simultaneously transfected into the $Fbx15^{\beta geo/\beta geo}$ MEFs, G418-resistant colonies were significantly more efficiently obtained as compared to simultaneous transfection with the 24 genes.

Furthermore, 9 genes, with withdrawal of each individual gene from the 10 genes, were transfected into $Fbx15^{\beta geo/\beta geo}$ MEFs. As a result, it was found that G418-resistant iPS cell colonies were not formed when each of 4 kinds of genes (#14, #15, #20, or #22) was withdrawn (Fig. 6). Therefore, it was suggested that these four kinds of genes, among the ten genes, had particularly important roles in the induction

of reprogramming.

Example 2: Induction of reprogramming with combination of 4 kinds of genes

It was examined whether or not induction of reprogramming of somatic cells was achievable with the four kinds of genes of which particular importance was suggested among the 10 genes. By using the combination of the aforementioned 10 kinds of genes, the combination of the aforementioned 4 kinds of genes, combinations of only 3 kinds of genes among the 4 kinds of genes, and combinations of only 2 kinds of genes among the 4 kinds of genes, these sets of genes were retrovirally transduced into the MEF cells as somatic cells in which βgeo was knocked into the Fbx15 gene. As a result, when the 4 kinds of genes were transduced, 160 G418-resistant colonies were obtained. Although this result was almost the same as that obtained by the transduction with the 10 kinds of genes (179 colonies), the colonies obtained by the 4-gene transduction were smaller than those by the 10-gene transduction. When these colonies were passaged, the numbers of colonies having iPS cell morphology was 9 clones among 12 clones in the case of the 10-gene transduction, whereas there was a somewhat lower tendency of 7 clones among 12 clones in the case of the 4-gene transduction. As for the 4 genes, almost the same numbers of iPS cells were obtained with either of those derived from mouse or those derived from human.

When 3 genes selected from the aforementioned 4 genes were transduced, 36 flat colonies were obtained with one combination (#14, #15, and #20). However, iPS cells were not observed when they were passaged. With another combination (#14, #20, and #22), 54 small colonies were obtained. When 6 of the relatively large colonies from among those colonies were passaged, cells similar to ES cells were obtained for all these 6 clones. However, it seemed that adhesion of the cells between themselves and to the culture dish was weaker than that of ES cells. The proliferation rate of the cells was also slower than that observed in the case of the transduction with the 4 genes. Further, one colony each was formed with each of the other two kinds of combinations of 3 genes among the 4 genes. However, proliferation of the cells was not observed when the cells were passaged. With any of combinations of 2 genes selected from the 4 genes (6 combinations), no G418-resistant colonies were formed. The above results are shown in Fig. 7.

Further, the results of observation of expression profiles of the ES cell marker

genes by RT-PCR are shown in Fig. 10. The details of the method are as follows. From iPS cells established by transducing 3 genes (Oct3/4, Klf4, and c-Myc: represented as "Sox2 minus"), 4 genes (Sox2 was added to the three genes: represented as "4ECAT"), and 10 genes (#3, #4, #5, #11, #18, and #21 in Table 1 were added to the four genes: represented as "10ECAT") into Fbx15$^{\beta geo/\beta geo}$ MEFs, iPS cells established by transducing 10 genes into fibroblasts established from tail tip of an adult mouse in which βgeo was knocked into the Fbx15 gene (represented as "10ECAT Skin fibroblast"), mouse ES cells, and MEF cells with no gene transduction, total RNAs were purified, and treated with DNaseI to remove contamination of genomic DNA. First strand cDNAs were prepared by a reverse transcription reaction, and expression profiles of the ES cell marker genes were examined by PCR. For Oct3/4, Nanog, and ERas, PCR was performed by using primers which only amplified a transcript product from an endogenous gene, not from the transduced retrovirus. The primer sequences are shown in Table 6.

Table 6

ECAT1	ECAT1-RT-S	TGT GGG GCC CTG AAA GGC GAG CTG AGA T
	ECAT1-RT-AS	ATG GGC CGC CAT ACG ACG ACG CTC AAC T
Esg1	pH34-U38	GAA GTC TGG TTC CTT GGC AGG ATG
	pH34-L394	ACT CGA TAC ACT GGC CTA GC
Nanog	6047-S1	CAG GTG TTT GAG GGT AGC TC
	6047-AS1	CGG TTC ATC ATG GTA CAG TC
ERas	45328-S118	ACT GCC CCT CAT CAG ACT GCT ACT
	ERas-AS304	CAC TGC CTT GTA CTC GGG TAG CTG
Gdf3	Gdf3-U253	GTT CCA ACC TGT GCC TCG CGT CTT
	GDF3 L16914	AGC GAG GCA TGG AGA GAG CGG AGC AG

Table 6 (Cont.)

Fgf4	Fgf4-RT-S	CGT GGT GAG CAT CTT CGG AGT GG
	Fgf4-RT-AS	CCT TCT TGG TCC GCC CGT TCT TA
Cripto	Cripto-S	ATG GAC GCA ACT GTG AAC ATG ATG TTC GCA
	Cripto-AS	CTT TGA GGT CCT GGT CCA TCA CGT GAC CAT
Zfp296	Zfp296-S67	CCA TTA GGG GCC ATC ATC GCT TTC
	Zfp296-AS350	CAC TGC TCA CTG GAG GGG GCT TGC
Dax1	Dax1-S1096	TGC TGC GGT CCA GGC CAT CAA GAG
	Dax1-AS1305	GGG CAC TGT TCA GTT CAG CGG ATC
Oct3/4	Oct3/4-S9	TCT TTC CAC CAG GCC CCC GGC TC
	Oct3/4-AS210	TGC GGG CGG ACA TGG GGA GAT CC
NAT1	NAT1 U283	ATT CTT CGT TGT CAA GCC GCC AAA GTG GAG
	NAT1 L476	AGT TGT TTG CTG CGG AGT TGT CAT CTC GTC

The results shown in this figure revealed that, by transduction of the 3 genes, expression of each of ERas and Fgf4 was efficiently induced, but expression of each of Oct3/4 and Nanog, essential factors for the maintenance of pluripotency, was not induced, or was very weak even when induced. However, when the 4 genes were transduced, there was one clone (#7) in which Oct3/4 and Nanog were relatively strongly induced among 4 clones examined. Further, when the 10 genes were transduced, strong induction of each of Oct3/4 and Nanog was observed in 3 clones among 5 clones examined.

These results revealed that a combination of at least 3 genes (#14, #20, and #22) was essential for reprogramming, and in the cases of the 4-gene group and 10-gene group including the 3 kinds of genes, the reprogramming efficiency was increased in proportion to the increasing number of genes.

Example 3: Analysis of pluripotency of reprogrammed cells

In order to evaluate the differentiation pluripotency of the established iPS cells, the iPS cells established with 24 factors, 10 factors, and 4 factors were subcutaneously transplanted into nude mice. As a result, tumors having a size similar to that observed with ES cells were formed in all animals. Histologically, the tumors consisted of a plurality of kinds of cells, and cartilaginous tissues, nervous tissues, muscular tissues, fat tissues, and intestinal tract-like tissues were observed (Fig. 8), which verified pluripotency of the iPS cells. In contrast, although tumors were formed when the cells established with the 3 factors were transplanted into nude mice, they were formed histologically only from undifferentiated cells. Therefore, it was found that a Sox family gene was essential for the induction of differentiation pluripotency.

Example 4: Reprogramming of fibroblasts derived from tails of adult mice

The 4 factors identified in the mouse embryonic fibroblasts (MEFs) were transduced into fibroblasts derived from tails of βgeo knockin Fbx15 adult mice systemically expressing green fluorescence protein (GFP). Then, the cells were cultured on feeder cells under the same conditions as ES cell culture conditions, and G418 selection was performed. In about two weeks after the start of the drug selection, a plurality of colonies of iPS cells were obtained. When these cells were subcutaneously transplanted to nude mice, teratomas consisting of a variety of all three germ layer tissues were formed. Further, when the iPS cells derived from adult dermal fibroblasts were transplanted to the blastocysts, and then transplanted into the uteri of pseudopregnant mice, embryos in which the GFP-positive cells were systemically distributed were observed among those at 13.5 days after fertilization (Fig. 9), demonstrating that the iPS cells had pluripotency and were able to contribute to mouse embryogenesis. These results indicate that the identified class of factors had an ability to induce reprogramming of not only somatic cells in an embryonic period, but also somatic cells of mature mice. Practically, it is extremely important that the reprogramming can be induced in cells derived from adult skin.

Example 5

An effect of cytokine on iPS cell establishment was investigated. Expression

vector (pMX retroviral vector) for basic fibroblast growth factor (bFGF) or stem cell factor (SCF) was transduced into feeder cells (STO cells) to establish cells permanently expressing the cytokines. MEFs derived from the Fbx15$^{\beta geo/\beta geo}$ mouse (500,000 cells/100 mm dish) were cultured on these STO cells and transduced with the 4 factors, and then subjected to G418 selection. As a result, the number of formed colonies increased 20 times or higher on the STO cells producing bFGF (Fig. 11) or SCF (data not shown), as compared with the culture on normal STO cells. Further, although no iPS cell colony was formed on the normal STO cells when the 3 factors other than c-Myc were transduced, colony formation was observed on the STO cells producing bFGF (Fig. 11) or SCF (data not shown). These results revealed that stimulation with the cytokine increased the efficiency of the establishment of iPS cells from MEFs, and the nuclear reprogramming was achievable by using a cytokine instead of c-Myc.

Example 6

Family genes exist for all of the Oct3/4, Klf4, c-Myc, and Sox2 genes (Tables 1 and 2). Accordingly, studies were made as to whether iPS cells could be established with the family genes instead of the 4 genes. In Table 7, combined experimental results in duplicate are shown. With regard to the Sox family, Sox1 gave almost the same number of G418-resistant colonies formed and iPS cell establishment efficiency as those with Sox2. As for Sox3, the number of G418-resistant colonies formed was about 1/10 of that with Sox2, however, iPS cell establishment efficiency of the colonies picked up was in fact higher than that with Sox2. As for Sox15, both the number of G418-resistant colonies formed and iPS cell establishment efficiency were lower than those with Sox2. As for Sox17, the number of G418-resistant colonies formed was almost the same as that with Sox2, however, iPS cell establishment efficiency was low. With regard to the Klf family, Klf2 gave a smaller number of G418-resistant colonies than Klf4, however, they gave almost the same iPS cell establishment efficiency. With regard to the Myc family, it was found that wild-type c-Myc was almost the same as a T58A mutant both in the number of G418-resistant colonies formed and iPS cell establishment efficiency. Further, each of N-Myc and L-Myc (each wild type) was almost the same as c-Myc in both of the number of G418-resistant colonies formed and iPS cell establishment efficiency.

Table 7

Transduced gene	Number of formed colonies	Number of picked colonies	Number of established iPS cell strain	iPS cell establishment efficiency (%)
4 Factors (cMycT58A)	85	12	5	42
Sox1	84	12	7	58
Sox3	8	8	7	92
Sox15	11	11	1	8
Sox17	78	12	2	17
Klf2	11	10	5	50
c-MycWT	53	11	8	72
N-MycWT	40	12	7	58
L-MycWT	50	12	11	92
3 Factors (-Sox2)	6	6	2	17

Example 7

Studies were made as to whether iPS cells could be established with a reporter other than Fbx15-βgeo. *Escherichia. coli* artificial chromosome (BAC) containing the Nanog gene in the center was isolated, and then the GFP gene and the puromycin resistance gene were knocked in by recombination in *E. coli* (Fig. 12A). Subsequently, the above modified BAC was introduced into ES cells to confirm that the cells became GFP-positive in an undifferentiated state specific manner (data not shown). Then, these ES cells were transplanted in mouse blastocysts to create transgenic mice via chimeric mice. In these mice, GFP-positive cells were specifically observed in inner cell masses of the blastocysts or gonads of embryos at 13.5 days after fertilization (Fig. 12B). The gonads were removed from the embryos at 13.5 days after fertilization (hybrid of DBA, 129, and C57BL/6 mice), and MEFs were isolated. The isolated MEFs were confirmed to be GFP-negative (Fig. 13) by flow cytometry. These MEFs were retrovirally transduced with the 4 factors and subjected to puromycin selection, and as a result, a plural number of resistant colonies were obtained. Only about 10 to 20% of the colonies were GFP-positive. When the

GFP-positive colonies were passaged, they gave morphology (Fig. 14) and proliferation (Fig. 15) similar to those of ES cells. Examination of the gene expression pattern revealed that the expression pattern was closer to that of ES cells as compared to the iPS cells isolated from Fbx15$^{\beta geo/\beta geo}$ MEFs by G418 selection (Fig. 16). When these cells were transplanted to nude mice, teratoma formation was induced, thereby the cells were confirmed to be iPS cells (Fig. 17). Further, chimeric mice were born by transplanting the iPS cells obtained by Nanog-GFP selection to the blastocysts of C57BL/6 mice (Fig. 18). When these chimeric mice were mated, germ-line transmission was observed (Fig. 19). In these iPS cells established by Nanog-GFP selection, which were closer to ES cells, the expressions of the 4 factors from the retroviruses were almost completely silenced, suggesting that self-replication was maintained by endogenous Oct3/4 and Sox2.

Example 8

iPS cells in 10 cm confluent were trypsinized and suspended in ES cell medium (the STO cells were removed by adhesion to a gelatin-coated dish for 10 to 20 minutes after the suspension). 2 x 10^6 cells were cultured for four days in a HEMA (2-hydroxyethyl methacrylate) coated *E. coli* culture dish as a suspension culture to form embryoid bodies (EBs) (day 1 to 4). On the 4th day of EB formation (day 4), all of the EBs were transferred to a 10-cm tissue culture dish, and cultured in ES cell medium for 24 hours to allow adhesion. After 24 hours (day 5), the medium was changed to an ITS/fibronectin-containing medium. The culture was performed for 7 days (medium was exchanged every 2 days), and nestin-positive cells were selected (cells of other pedigrees were dying to some extent in a culture under serum-free condition)(day 5 to 12). A2B5-positive cells were then induced. After 7 days (day 12), the cells were separated by trypsinization, and the remaining EBs were removed. 1 x 10^5 cells were seeded on a poly-L-ornithine/fibronectin-coated 24-well plate, and cultured for 4 days in an N2/bFGF-containing medium (medium was exchanged every 2 days)(day 12 to 16). After 4 days (day 16), the medium was changed to an N2/bFGF/EGF-containing medium, and the culture was continued for 4 days (medium was exchanged every 2 days)(day 16 to 20). After 4 days (day 20), the medium was changed to an N2/bFGF/PDGF-containing medium, and the culture was continued for 4 days (medium was exchanged every 2 days)(day 20 to 24). During this period (day

12 to 24), when the cells had increased excessively and reached confluent, they were passaged at appropriate times, and 1 to 2 x 10^5 cells were seeded (the number of the cells varied depending on the timing of the passage). After 4 days (day 24), the medium was changed to an N2/T3 medium, and the culture was continued for 7 days (day 24 to 31) with medium exchange every 2 days. On day 31, the cells were fixed and subjected to immunostaining. As a result, differentiation of the iPS cells into βIII tubulin-positive nerve cells, O4-positive oligodendrocytes, and GFAP-positive astrocytes was observed (Fig. 20).

Example 9

In order to establish iPS cells from arbitrary mouse somatic cells other than those derived from the Fbx15-βgeo knockin mouse, a method for the establishment without using drug selection was developed. Mouse embryo fibroblasts (MEFs) were cultured on a 10-cm dish (on STO feeder cells) in a number smaller than those used above (10,000, 50,000, or 100,000 cells), and a control DNA or the 4 factors were retrovirally transduced. When culture was performed for 2 weeks in the ES cell medium (without G418 selection), no colony formation was observed in the dish in which the control DNA was transduced, whilst in the dish in which the 4 factors were transduced, a plurality of compact colonies were formed as well as flat colonies considered to be transformed (Fig. 21). When 24 colonies were picked up from these colonies and culture was continued, ES cell-like morphology was observed. Gene expression profiles thereof were examined by RT-PCR, and as a result, the expression of Esg1, an ES cell marker, was observed in 7 clones. Induction of many ES cell markers such as Nanog, ERas, GDF3, Oct3/4, and Sox2 was observed in clone 4, and therefore the cells were considered to be iPS cells (Fig. 22). The above results demonstrated that drug selection using Fbx15-βgeo knockin or the like was not indispensable for iPS cell establishment, and iPS cells could be established from arbitrary mouse-derived somatic cells. This also suggested the possibility that iPS cells could be established from somatic cells of a disease model mouse by the aforementioned technique.

Example 10

As cells from which iPS cells were induced, hepatocytes and gastric mucous

cells being cells other than fibroblasts were examined. Hepatocytes were isolated from the liver of the Fbx15$^{\beta geo/\beta geo}$ mice by perfusion. These hepatocytes were retrovirally introduced with the 4 factors, and then subjected to G418 selection to obtain plural iPS cell colonies. As a result of gene expression pattern analysis using a DNA microarray, the iPS cells derived from the liver were found to be more similar to ES cells than the iPS cells derived from dermal fibroblasts or embryonic fibroblasts. iPS cells were obtained also from gastric mucous cells in the same manner as those from hepatocytes.

Example 11

PD98059 is an inhibitor of MAP kinase which suppresses proliferation of various differentiated cells. However, it is known to promote maintenance of undifferentiated status and proliferation of ES cells. Effects of PD98059 on iPS cell establishment were thus examined. MEFs established from a mouse having the selective markers of Nanog-EGFP-IRES-Puro were retrovirally introduced with the 4 factors and subjected to puromycin selection. When PD98059 was not added, the percentage of GFP-positive colonies was 8% of the iPS cell colonies obtained. However, in the group to which PD98059 (final concentration: 25 μM) was continuously added from the next day of the retroviral transfection, 45% of the colonies obtained were GFP-positive. The results were interpreted to be due to PD98059 promoting the proliferation of the GFP-positive iPS cells, which are closer to ES cells, whilst PD98059 suppressing the proliferation of the GFP-negative iPS cells or differentiated cells. From these results, PD98059 was demonstrated to be able to be used for establishment of the iPS cells closer to ES cells or establishment of iPS cells without using drug selection.

Example 12

A plasmid, containing the red fluorescence protein gene downstream from the mouse Oct3/4 gene promoter and the hygromycin resistance gene downstream from the PGK promoter, was introduced by nucleofection into embryonic human dermal fibroblasts (HDFs) in which solute carrier family 7 (Slc7a1, NCBI accession number NM_007513) as a mouse ecotropic virus receptor was expressed by lentiviral transduction. Hygromycin selection was performed to establish strains with stable

expression. 800,000 cells were seeded on the STO cells treated with mitomycin, and on the next day, Oct3/4, Sox2, Klf4, and c-Myc (each derived from human) were retrovirally transduced into the cells. 24 colonies were picked up from those obtained after 3 weeks (Fig. 23, left), and transferred on a 24-well plate on which the STO cells were seeded and then cultured. After 2 weeks, one grown clone was passaged on a 6-well plate on which the STO cells were seeded and cultured. As a result, cells morphologically similar to ES cells were obtained (Fig. 23, right), suggesting that the cells were iPS cells. The mouse ES cell medium was used as every medium.

Example 13

Human adult dermal fibroblasts (adult HDFs) were transduced with Slc7a1 (mouse retroviral receptor) by using lentivirus, and the resulting cells were seeded on 800,000 feeder cells (mitomycin-treated STO cells). The genes were retrovirally transduced as the following combinations.

1. Oct3/4, Sox2, Klf4, c-Myc, TERT, and SV40 Large T antigen
2. Oct3/4, Sox2, Klf4, c-Myc, TERT, HPV16 E6
3. Oct3/4, Sox2, Klf4, c-Myc, TERT, HPV16 E7
4. Oct3/4, Sox2, Klf4, c-Myc, TERT, HPV16 E6, HPV16 E7
5. Oct3/4, Sox2, Klf4, c-Myc, TERT, Bmi1

(Oct3/4, Sox2, Klf4, c-Myc and TERT were derived from human, and Bmi1 was derived from mouse)

The culture was continued under the culture conditions for mouse ES cells without drug selection. As a result, colonies considered to be those of iPS cells emerged on the 8th day after the virus transfection on the dish in which the factors were introduced according to Combination 1 (Fig. 24). iPS cell-like colonies also emerged with the other combinations (2 to 5), although they were not as apparent when compared to Combination 1. When only the 4 factors were transduced, no colonies emerged.

Industrial Applicability

By using the nuclear reprogramming factor provided by the present invention, reprogramming of differentiated cell nuclei can be conveniently and highly reproducibly induced without using embryos or ES cells, and induced pluripotent stem

cells as undifferentiated cells having differentiation ability, pluripotency and growth ability similar to those of ES cells can be established.

What is claimed is:

1. A nuclear reprogramming factor for a somatic cell, which comprises a gene product of each of the following three kinds of genes: an Oct family gene, a Klf family gene, and a Myc family gene.

2. The factor according to claim 1, which comprises a gene product of each of the following three kinds of genes: Oct3/4, Klf4, and c-Myc.

3. The factor according to claim 1 or 2, which further comprises a gene product of the following gene: a Sox family gene.

4. The factor according to claim 3, which comprises a gene product of Sox2.

5. The factor according to any one of claims 1 to 4, which comprises a cytokine together with the gene product of the Myc family gene or instead of the gene product of the Myc family gene.

6. The factor according to claim 5, wherein the cytokine is bFGF and/or SCF.

7. The factor according to any one of claims 1 to 6, which further comprises a gene product of the following gene: TERT gene.

8. The factor according to any one of claims 1 to 7, which further comprises a gene product or gene products of one or more kinds of genes selected from the group consisting of the following genes: SV40 Large T antigen, HPV16 E6, HPV16 E7, and Bmil.

9. The factor according to any one of claims 1 to 8, which further comprises a gene product or gene products of one or more kinds of genes selected from the group consisting of Fbx15, Nanog, ERas, ECAT15-2, Tcl1, and β-catenin.

10. The factor according to any one of claims 1 to 9, which further comprises a gene product or gene products of one or more kinds of genes selected from the group consisting of ECAT1, Esg1, Dnmt3L, ECAT8, Gdf3, Sox15, ECAT15-1, Fthl17, Sall4, Rex1, UTF1, Stella, Stat3, and Grb2.

11. A method for preparing an induced pluripotent stem cell by nuclear reprogramming of a somatic cell, which comprises a step of contacting the nuclear reprogramming factor according to any one of claims 1 to 10 with the somatic cell.

12. The method according to claim 11, wherein the somatic cell is a human cell.

13. An induced pluripotent stem cell obtained by the method according to

claim 11 or 12.

14. A somatic cell derived by inducing differentiation of the induced pluripotent stem cell according to claim 13.

15. A method for improving differentiation ability and/or growth ability of a cell, which comprises the step of contacting the nuclear reprogramming factor according to any one of claims 1 to 10 with the cell.

16. The method according to claim 15, wherein the cell is a human cell.

17. A cell obtained by the method according to claim 15 or 16.

18. A somatic cell derived by inducing differentiation of the cell according to claim 17.