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(71) **Applicant** *(for all designated States except US)*: **OSAKA UNIVERSITY** [JP/JP]; 1-1, Yamadaoka, Suita-shi, Osaka, 5650871 (JP).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **MIYAZAKI, Junichi** [JP/JP]; c/o OSAKA UNIVERSITY, 1-1, Yamadaoka, Suita-shi, Osaka, 5650871 (JP). **TASHIRO, Fumi** [JP/JP]; c/o OSAKA UNIVERSITY, 1-1, Yamadaoka, Suita-shi, Osaka, 5650871 (JP). **MIYAZAKI, Satsuki** [JP/JP]; c/o OSAKA UNIVERSITY, 1-1, Yamadaoka, Suita-shi, Osaka, 5650871 (JP). **YAMATO, Eiji** [JP/JP]; c/o OSAKA UNIVERSITY, 1-1, Yamadaoka, Suita-shi, Osaka, 5650871 (JP).

(74) **Agent**: **IWATANI, Ryo**; KEIHAN Dojima Bldg. 3F, 1-31, Dojima 2-chome, Kita-ku, Osaka-shi, Osaka, 5300003 (JP).

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(54) **Title**: METHOD AND KIT FOR PREPARING IPS CELLS

(57) **Abstract**: The present invention provides a method for preparing iPS cells without use of such a vector that causes the integration of a foreign gene into the chromosomes of a host cell, for example, a retrovirus vector or a lentivirus vector, and a kit for preparing the same. The method for preparing iPS cells comprises a nuclear reprogramming factor introduction step which involves introducing, into somatic cells, an episomal vector into which the gene encoding a nuclear reprogramming factor is inserted in an expressible form; a cultivation step which involves culturing somatic cells having the episomal vector introduced therein; and a selection step which involves selecting iPS cells generated by reprogramming of the somatic cells.

Description

Title of Invention: METHOD AND KIT FOR PREPARING IPS CELLS

Technical Field

[0001] The present invention relates to a method and a kit for preparing iPS cells, and particularly to a method and a kit for preparing safe iPS cells free from chromosomal integration of any foreign gene, by use of an episomal vector.

Background Art

[0002] Induced pluripotent stem cells (abbreviated as iPS cells) are cells established by introducing several kinds of transcription factors (genes) into differentiated somatic cells (for example, fibroblasts). Such iPS cells have pluripotency similar to that of embryonic stem cells (ES cells). The world-first iPS cells were generated by the team of Shinya Yamanaka from Kyoto University (patent literature 1 and nonpatent literature 1). Pluripotency, that is, the ability to differentiate into various cells which constitute a living organism, is originally a special ability given to only the inner cell mass (a part of a blastocyst), ES cells cultured from the inner cell mass, hybrid cells from ES cells and somatic cells, and some kinds of cultured cells derived from reproductive cells. However, the discovery of the method for establishing iPS cells made it possible to prepare pluripotent cells without using any fertilized egg or ES cell.

[0003] The aforementioned Shinya Yamanaka et al. demonstrated that pluripotent cells (iPS cells) can be established by expression of Oct3/4, Sox2, Klf4 and c-Myc in mouse fibroblasts via a retrovirus vector-mediated gene expression system (nonpatent literatures 1 and 2). The team of Rudolf Jaenisch from Massachusetts Institute of Technology (nonpatent literature 3), the team of Konrad Hochedlinger from the Harvard Stem Cell Institute, and the team of Kathrin Plath from School of Medicine, UCLA (nonpatent literature 4), succeeded in the establishment of mouse iPS cells in the same manner as above. In addition, Rudolf Jaenisch et al. demonstrated that iPS cells can be isolated only based on observed morphological changes of cells, even without using, as a marker, gene expression specific to ES cells (nonpatent literature 5). The team of Miguel Ramalho-Santos from University of California San Francisco demonstrated that iPS cells can be established by use of a lentivirus vector, a kind of a retrovirus vector, as well (nonpatent literature 6).

[0004] Shinya Yamanaka et al. demonstrated that mouse or human iPS cells can be established, albeit inefficiently, by gene transduction of only three factors, Oct3/4, Sox2 and Klf4, excluding c-Myc, and they also successfully inhibited the transformation of iPS cells into cancer cells (nonpatent literature 7). At almost the same time, Rudolf

Jaenisch et al. also succeeded in the same experiment in mice (nonpatent literature 8).

[0005] The team of James Thomson succeeded in the establishment of human iPS cells, using the same approach as employed when Shinya Yamanaka et al. succeeded in the establishment of mouse iPS cells. That is, James Thomson et al. succeeded in the establishment of human iPS cells, by selecting 14 genes expressed specifically in human ES cells, and then introducing the following four genes of them, Oct3/4, Sox2, Nanog and Lin28, into fetal fibroblasts or newborn foreskin fibroblasts (nonpatent literature 9). Coincidentally, the team of Shinya Yamanaka also established human iPS cells individually from fibroblasts isolated from the facial skin of a 36-year-old woman, fibroblast-like synoviocytes from a 69-year-old man, and newborn foreskin fibroblasts, by gene transduction of Oct3/4, Sox2, Klf4 and c-Myc, which are the human homologue of the mouse genes used for the establishment of mouse iPS cells (nonpatent literature 10).

[0006] Theoretically, pluripotent cells are capable of differentiating into any tissue or organ which constitutes a body. If the technology of establishing iPS cells from a human patient's body is practically completed, it is anticipated that the technology will make it possible to prepare autologous tissues and organs to be used for transplantation free from immunological rejection. This technology leads to a drastic solution to ethical problems over use of fertilized eggs, which have been a matter of concern with respect to application of human ES cells. Therefore, this technology has drawn world's attention with hope for realization of regenerative medicine.

Citation List

Patent Literature

[0007] PTL 1: WO 2007/069666

Non Patent Literature

[0008] NPL 1: Takahashi K, Yamanaka S. (2006) "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell 126: 663-676.

NPL 2: Okita K, Ichisaka T, Yamanaka S. (2007) "Generation of germline-competent induced pluripotent stem cells." Nature 448: 313-317.

NPL 3: Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. (2007) "In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state." Nature 448: 318-324.

NPL 4: Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K. (2007) "Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution." Cell Stem Cell 1: 55-70.

NPL 5: Meissner A, Wernig M, Jaenisch R. (2007) "Direct reprogramming of ge-

netically unmodified fibroblasts into pluripotent stem cells." Nat Biotechnol 25: 1177-1181.

NPL 6: Blelloch R, Venere M, Yen J, Ramalho-Santos M. (2007) "Generation of induced pluripotent stem cells in the absence of drug selection." Cell Stem Cell 1: 245-247.

NPL 7: Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. (2008) "Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts." Nat Biotechnol 26: 101-106.

NPL 8: Wering M, Meissner A, Cassady JP, Jaenisch R. (2008) "c-Myc is dispensable for direct reprogramming of mouse fibroblasts." Cell Stem Cell 2: 10-12.

NPL 9: Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. (2007) "Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells." Science 318: 1917-1920.

NPL 10: Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. (2007) "Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors." Cell 131: 861-872.

Summary of Invention

Technical Problem

[0009] The success in the establishment of iPS cells allows us to avoid bioethical problems attributed to ES cells, and this success is a major step towards realization of regenerative medicine free from immunological rejection. However, conventional methods for preparing iPS cells involve gene transduction via a retrovirus vector or another similar vector, a lentivirus vector. Such a viral vector may cause mutation of an endogenous gene, or may cause activation of an endogenous oncogene since the viral vector mediates the integration of a gene into a random position of the host cell's chromosomes. In view of future use of iPS cells in regenerative medicine, use of such a viral vector has the risks of oncogenic transformation and of developing dysfunction, etc. For that reason, an extremely important issue towards future application of iPS cells is to develop a method for preparing iPS cells without any effect on the chromosomes.

[0010] Accordingly, an object of the present invention is to provide a method for preparing iPS cells without use of such a vector that causes the integration of a foreign gene into the chromosomes of a host cell, for example, a retrovirus vector or a lentivirus vector, and a kit for preparing the same.

Solution to Problem

[0011] The present inventors conducted extensive research to solve the above-mentioned issue. As a result, they found that by introducing, into fibroblasts, the gene which encodes a nuclear reprogramming factor via an episomal vector, expression of the nuclear reprogramming factor was achieved without any chromosomal integration of a foreign gene, followed by generation of iPS cells derived from somatic cells as in the case where a retrovirus vector is used. The present inventors also found that the introduced episomal vector can be eliminated from iPS cells, and thus completed the present invention.

[0012] That is, the present invention relates to a method and a kit for preparing iPS cells and comprises the following inventions:

[1] a method for preparing an iPS cell by nuclear reprogramming of a somatic cell, which comprises the following steps:

a nuclear reprogramming factor introduction step which involves introducing, into somatic cells, an episomal vector into which a gene encoding a nuclear reprogramming factor is inserted in an expressible form;

a cultivation step which involves culturing somatic cells having the episomal vector introduced therein; and

a selection step which involves selecting iPS cells generated by reprogramming of the somatic cells;

[2] the method according to the above [1], which further comprises a replication factor introduction step which involves introducing, into somatic cells, an episomal vector into which a gene encoding a replication factor is inserted in an expressible form, before the nuclear reprogramming factor introduction step;

[3] the method according to the above [1] or [2], which further comprises a vector elimination step which involves eliminating an episomal vector from iPS cells, after the selection step;

[4] the method according to the above [3], wherein the episomal vector is constituted so that expression of the gene encoding the nuclear reprogramming factor and/or the gene encoding the replication factor is driven by a retroviral promoter;

[5] the method according to the above [3], wherein the episomal vector is constituted so that expression of the gene encoding the nuclear reprogramming factor and/or the gene encoding the replication factor can be regulated by a drug;

[6] the method according to any one of the above [1] to [5], wherein the episomal vector is selected from the group consisting of a mouse polyomavirus-based episomal vector, a BK virus-based episomal vector, an Epstein-Barr virus-based episomal vector, a bovine papilloma virus-based episomal vector and an episomal vector carrying an S/MAR;

[7] the method according to any one of the above [1] to [6], wherein the nuclear re-

programming factor is selected from the group consisting of the following (1), (2) and (3):

(1) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, and iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5,

(2) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5, and iv) one kind selected from the group consisting of c-Myc, N-Myc and L-Myc, and

(3) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) Nanog and iv) Lin28;

[8] a kit for preparing an iPS cell, which comprises an episomal vector into which a gene encoding a nuclear reprogramming factor is inserted in an expressible form;

[9] the kit according to the above [8], wherein the episomal vector is selected from the group consisting of a mouse polyomavirus-based episomal vector, a BK virus-based episomal vector, an Epstein-Barr virus-based episomal vector, a bovine papilloma virus-based episomal vector and an episomal vector carrying an S/MAR; and

[10] the kit according to the above [8] or [9], wherein the nuclear reprogramming factor is selected from the group consisting of following (1), (2) and (3):

(1) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, and iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5,

(2) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5, and iv) one kind selected from the group consisting of c-Myc, N-Myc and L-Myc, and

(3) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) Nanog and iv) Lin28.

Advantageous Effects of Invention

[0013] According to the method of the present invention for preparing iPS cells, iPS cells free from chromosomal integration of any foreign gene can be prepared. For this reason, the resulting iPS cells of the present invention have extremely high safety, and are free from mutation of an endogenous gene, or activation of an endogenous oncogene, either of which results from chromosomal integration of a foreign gene. When autotransplantation is performed according to the procedures which involve preparing iPS cells from somatic cells of a patient according to such a preparation method of the present invention, inducing differentiation of the iPS cells into desired

differentiated cells or tissues, and returning thus obtained cells or tissues to the patient, the risks of oncogenic transformation and of developing dysfunction can be reduced. Thus, the present invention is highly beneficial for use in the field of regenerative medicine, and will bring a great contribution to the future development of regenerative medicine.

Brief Description of Drawings

[0014] [fig.1]Fig. 1 is a diagrammatic view illustrating, in accordance with one of the examples, a method for preparing iPS cells by use of an episomal vector.

[fig.2]Fig. 2 shows the structure of pPyCAG-Oct3-IRES-Klf4-IRES-zeo.

[fig.3]Fig. 3 shows the structure of pPyCAG-Sox2-IRES-c-Myc-IRES-puro.

[fig.4]Fig. 4 is fluorescence microscope images showing immunostaining results of BMT10 cells with pPyCAG-Oct3-IRES-Klf4-IRES-zeo introduced therein. (a) is an immunostaining image for Oct3/4, (b) is an immunostaining image for Klf4, (c) is a merged image of (a) and (b), and (d) is a merged image of a DAPI staining image on (c).

[fig.5]Fig. 5 is fluorescence microscope images showing immunostaining results of BMT10 cells with pPyCAG-Sox2-IRES-c-Myc-IRES-puro introduced therein. (a) is an immunostaining image for Sox2, (b) is an immunostaining image for c-Myc, (c) is a merged image of (a) and (b), and (d) is a merged image of a DAPI staining image on (c).

[fig.6A]Fig. 6A is a microscope image of zeocin- and puromycin-resistant cells 15 days from the start of culture on feeder cells.

[fig.6B]Fig. 6B is a microscope image of zeocin- and puromycin-resistant cells 15 days from the start of culture on feeder cells. Fig. 6B shows a different colony from that in Fig. 6A.

[fig.7A]Fig. 7A is a microscope image of zeocin- and puromycin-resistant cells which were isolated as a colony 15 days from the start of culture on feeder cells, and then were cultured for another 4 days. Fig. 7A is an image of colonies observed at low magnification.

[fig.7B]Fig. 7B is a microscope image of zeocin- and puromycin-resistant cells which were isolated as a colony 15 days from the start of culture on feeder cells, and then were cultured for another 4 days. Fig. 7B is an image of colonies observed at high magnification.

[fig.8]Fig. 8 is an image showing the results of alkaline phosphatase staining of iPS cells derived from mouse embryonic fibroblasts in Example 1.

[fig.9]Fig. 9 shows the structure of pBK-COK-CSM.

[fig.10]Fig. 10 is an image showing the results of alkaline phosphatase staining of iPS

cells derived from human keratinocytes in Example 2.

Description of Embodiments

[0015] Method for preparing iPS cells

The present invention provides a method for preparing iPS cells by nuclear reprogramming of somatic cells, the method comprising the following steps: a nuclear reprogramming factor introduction step which involves introducing, into somatic cells, an episomal vector into which the gene encoding a nuclear reprogramming factor is inserted in an expressible form; a cultivation step which involves culturing somatic cells having the episomal vector introduced therein; and a selection step which involves selecting iPS cells generated by reprogramming of the somatic cells. This method is hereinafter referred to as "the preparation method of the present invention."

[0016] The term "iPS cell" refers to a cell having properties similar to those of an ES cell, and specifically a cell having pluripotency and proliferative capacity. The iPS cell is also referred to as an "induced pluripotent stem cell."

The term "somatic cell" refers to any cell except an ES cell or other cells which retain their undifferentiated, pluripotent state. Specific examples of the somatic cell include, for example, (i) tissue stem cells (somatic stem cells), such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells and the like, (ii) tissue progenitor cells, and (iii) differentiated cells, such as lymphocytes, epithelial cells, muscle cells, fibroblasts and the like. The kind of the somatic cell used for the preparation method of the present invention is not particularly limited and any somatic cell can be suitably used.

[0017] The term "nuclear reprogramming factor" refers to a substance that has an effect of reprogramming a differentiated somatic cell into an iPS cell. In the preparation method of the present invention, after introduction of the gene encoding the nuclear reprogramming factor into somatic cells, expression of the nuclear reprogramming factor (protein) is induced in somatic cells, thus followed by generation of iPS cells derived from the somatic cells. The nuclear reprogramming factor used for the present invention is not particularly limited as long as it has the aforementioned effect. Examples of the suitable nuclear reprogramming factor include

(1) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, and iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5,

(2) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5, and iv) one kind selected from the group consisting of c-Myc, N-Myc and L-Myc, and

(3) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) Nanog and iv) Lin28.

Among the combinations described in the above (1), the combination of Oct3/4, Sox2 and Klf4 is particularly preferable (see the nonpatent literature 7). Among the combinations described in the above (2), the combination of Oct3/4, Sox2, Klf4 and c-Myc is particularly preferable (see the nonpatent literature 1). Among the combinations described in the above (3), the combination of Oct3/4, Sox2, Nanog and Lin28 is particularly preferable (see the nonpatent literature 9).

The aforementioned Klf1, Klf5, N-Myc, L-Myc, Sox1, Sox3, Sox15 and Sox18 are described in the nonpatent literature 7. In addition, n-Myc (see the nonpatent literature 6), hTERT, SV40 large T (see Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. (2007) "Reprogramming of human somatic cells to pluripotency with defined factors." Nature 451: 141-146.), and the like are known as a nuclear reprogramming factor. One or more kinds of these nuclear reprogramming factors can be further added to the aforementioned combinations (1) to (3) for use in the preparation method of the present invention. The nuclear reprogramming factor used for the preparation method of the present invention is not limited to the examples illustrated above, and any nuclear reprogramming factor that is not illustrated herein or has not yet been discovered can also be suitably used for the present invention.

[0018] The term "episomal vector" refers to a plasmid vector which autonomously replicates as a circular DNA and maintains itself in several copies to tens of copies within the nucleus of a nucleated cell. Any episomal vector that has such properties can be used for the preparation method of the present invention, without particular limitation. Specifically, examples of the episomal vector include episomal vectors based on viruses, for example, an episomal vector based on a mouse polyomavirus (Gassmann M, Donoho G, Berg P.: Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells. Proc Natl Acad Sci USA. 92: 1292-1296, 1995), an episomal vector based on a BK virus, a kind of human polyomaviruses (De Benedetti A, Rhoads RE.: A novel BK virus-based episomal vector for expression of foreign genes in mammalian cells. Nucleic Acids Res. 19: 1925-1931, 1991), an episomal vector based on an Epstein-Barr virus (Margolskee RF, Kavathas P, Berg P.: Epstein-Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. Mol. Cell. Biol. 8: 2837-2847, 1988), and an episomal vector based on a bovine papilloma virus (BPV) (Ohe Y, Zhao D, Saijo N, Podack ER.: Construction of a novel bovine papilloma virus vector without detectable transforming activity suitable for gene transfer. Hum Gene Ther. 6: 325-333, 1995). Inter alia, an episomal vector based on a BK virus and an episomal vector based on an Epstein-Barr virus are suitably used

for human cells.

[0019] Each of these vectors contains a replication origin (ori) derived from the corresponding virus. A "replication factor" binds to such a replication origin (ori), thereby triggering vector replication. The term "replication factor" as used herein refers to an indispensable factor for replication, which binds to the ori, thereby triggering nucleic acid replication. The "replication factors" corresponding to the respective virus-based episomal vectors illustrated above are the large T antigen of a mouse polyomavirus, the large T antigen of a BK virus, EBNA1 of an Epstein-Barr virus, and E1 and E2 of a bovine papilloma virus, respectively.

[0020] An episomal vector containing a base sequence called S/MAR (scaffold/matrix attachment region) can also be used suitably for the preparation method of the present invention. This episomal vector contains at least one S/MAR and at least one viral or eukaryotic replication origin (ori). Unlike other episomal vectors described above, this episomal vector does not require any replication factor corresponding to the ori for replication. Specifically, such an episomal vector is exemplified by the episomal vector containing, as an S/MAR, the upstream region (about 2 kbs) of the human interferon beta gene, which is described in "Piechaczek C, Fetzer C, Baiker A, Bode J, Lipps HJ.: A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells. Nucleic Acids Res. 27: 426-428, 1999."

[0021] In addition, there is known a system in which two kinds of adenovirus vectors introduced into cells produce a circular episomal vector within the cells (Leblois H, Roche C, Di Falco N, Orsini C, Yeh P, Perricaudet M.: Stable transduction of actively dividing cells via a novel adenoviral/episomal vector. Mol Ther. 2000 Apr;1(4): 314-22.). The episomal vector obtained by using this system can also be used for the preparation method of the present invention. This system is based on the Cre-loxP system. More specifically, in the system, one adenovirus vector expresses Cre, and the other adenovirus vector contains a gene construct required for serving as an episomal vector, the gene construct being inserted into the region flanked by two loxP sites of the vector. The region flanked by the two loxP sites is excised by Cre, forming a circular DNA. When the region flanked by the two loxP sites is designed to carry a replication origin and a replication factor gene (for example, the oriP sequence and the gene encoding EBNA1, which are a replication origin of and a replication factor gene of an Epstein-Barr virus, respectively) in addition to an expression unit, a formed circular DNA is maintained as an episome in cells.

[0022] The episomal vector of the present invention contains an expression unit for the gene encoding a nuclear reprogramming factor and/or for the gene encoding a replication factor. The promoter used for the expression unit may be any promoter used for mammalian cells, and includes, for example, a CAG promoter, a CMV promoter, a

beta-actin promoter, an SV40 promoter, a PGK promoter and a MuLV LTR promoter. By inserting the gene (DNA) to be expressed downstream of such a promoter, expression of the gene product is achieved in cells. A plurality of genes can be expressed under the control of one promoter by using IRES (Internal Ribosome Entry Site). The IRES to be used is not particularly limited, and includes human HCV-derived IRES and picornavirus-derived IRES, for example.

[0023] The episomal vector can carry a marker gene for selecting cells having the episomal vector introduced therein. The marker gene is not particularly limited as long as it can be used for selecting cells having the episomal vector introduced therein, but a drug resistance gene is suitably used. Examples of drugs used for cell selection include, for example, neomycin (geneticin (G418)), hygromycin, puromycin, zeocin and blasticidin.

The episomal vector further contains a replication origin (ori) and a selection marker gene (an ampicillin resistance gene, etc.) which are both required for amplification of the vector in Escherichia coli.

[0024] Hereinafter, each step of the preparation method of the present invention will be explained in detail.

(1) Nuclear reprogramming factor introduction step

The nuclear reprogramming factor introduction step is to introduce, into somatic cells, an episomal vector into which the gene encoding a nuclear reprogramming factor is inserted in an expressible form. The somatic cells are previously maintained in a suitable medium and a suitable culture condition according to the kind of the somatic cell to be used. As the method for introducing an episomal vector into somatic cells, there can be used any method suitably selected from known transfection methods, depending on the episomal vector and the somatic cell to be used. Specific examples of the known transfection methods include the electroporation method, the calcium phosphate method, the lipofection method and the DEAE dextran method. In the case where the episomal vector carries a selection marker gene, cells having the episomal vector introduced therein can be selected with the aid of the marker of choice. For example, when the episomal vector carries a drug resistance gene, cells having the episomal vector introduced therein are allowed to selectively survive by addition of the corresponding drug to medium after the introduction of the episomal vector.

[0025] When the episomal vector requires a replication factor for its replication, the episomal vector may be constructed so that one vector carries two expression units or constructed by use of IRES so that one vector expresses a plurality of genes expressed under the control of one promoter. In this way, the gene encoding a replication factor and the gene encoding a nuclear reprogramming factor can be introduced simultaneously. In order to express a plurality of nuclear reprogramming factors, the genes

all may be inserted in one vector, a plurality of episomal vectors each having one gene inserted therein may be co-introduced into a cell, or a plurality of episomal vectors each having about 2 to 3 genes inserted therein may be co-introduced into a cell.

[0026] (2) Replication factor introduction step

In the case where an episomal vector carrying only the gene encoding a replication factor, not any gene encoding a nuclear reprogramming factor (hereinafter referred to as a "replication factor transfer vector") is employed, the step of introducing the replication factor transfer vector into somatic cells may be conducted before the above-mentioned nuclear reprogramming factor introduction step. It is preferable that such a replication factor transfer vector should carry a drug resistance gene as a selection marker gene. This is because, thereby, cells expected to express the replication factor through successful introduction of the above vector can be selected. Therefore, in the case where the replication factor introduction step is conducted, it is preferred to introduce the replication factor transfer vector into somatic cells by a suitable method, to select cells surviving in medium containing the corresponding drug, and then to subject the cells to the nuclear reprogramming factor introduction step. Such a replication factor introduction step makes it possible to efficiently obtain cells into which the episomal vector expressing the desired nuclear reprogramming factor is introduced. Without this step, it is essential that the episomal vector carrying the gene encoding a replication factor, and the episomal vector carrying the gene encoding a nuclear reprogramming factor should be co-introduced into the same cell. Advantageously, when the replication factor introduction step is conducted in advance, only cells expected to express a replication factor (cells selected with the aid of a selection marker, such as a drug resistance marker) can be subjected to the nuclear reprogramming factor introduction step. As a result, in the nuclear reprogramming factor introduction step, there is significantly higher probability of obtaining desired cells, that is, cells into which the episomal vector carrying the gene encoding a nuclear reprogramming factor is introduced. There is also lower possibility that the episomal vector carrying the gene encoding a nuclear reprogramming factor might drop off after introduced into cells. This is because immediately after introduced, this episomal vector can efficiently replicate with the aid of the replication factors that are already expressed in the cells.

[0027] (3) Cultivation step

The cultivation step is to culture the somatic cells having the episomal vector introduced therein in the nuclear reprogramming factor introduction step described above. Preferably, the culture is performed in accordance with the culture method for ES cells, and usually performed on feeder cells (see Gene Targeting A Practical Approach Edited by A. L. Joyner, Oxford University Press, Oxford, U.K). In the case

where the episomal vector used in the nuclear reprogramming factor introduction step contains a selection marker gene, it is preferable that cells having the episomal vector introduced therein should be selected with the aid of the selection marker and then transferred onto feeder cells for further culture. For example, when a drug resistance gene is employed as a selection marker gene, cells should be cultured in medium containing the corresponding drug after the nuclear reprogramming factor introduction step, and surviving cells should be isolated and then transferred onto feeder cells for further culture.

[0028] (4) Selection step

The selection step is to select iPS cells generated by reprogramming of somatic cells. As a method for identifying iPS cells, (a) cell and colony morphology observation, (b) confirmation of alkaline phosphatase expression, (c) confirmation of undifferentiation marker expression and the like can be used. The identification of iPS cells can be conducted using the above (a) alone, but the combination of (b) and/or (c) with (a) can be employed to achieve more accurate identification.

[0029] (a) Cell and colony morphology observation

iPS cells show the same cell and colony morphology as that of ES cells. Specifically, the colony of iPS cells is formed in a round or elliptical shape like an upside-down bowl and has a clear rim, and an iPS cell has scant cytoplasm and unclear intercellular boundary. Therefore, cells within such a morphologically characteristic colony can be identified as iPS cells.

[0030] (b) Confirmation of alkaline phosphatase expression

iPS cells express alkaline phosphatase like ES cells. Therefore, cells positively expressing alkaline phosphatase can be identified as iPS cells. The expression of alkaline phosphatase can be confirmed by staining cells (colonies), for example by use of a commercial alkaline phosphatase staining kit (for example, manufactured by Sigma-Aldrich Co.). In this case, stained cells (colonies) can be identified as iPS cells.

[0031] (c) Confirmation of undifferentiation marker expression

iPS cells can be identified by confirming the expression of a gene specifically expressed in undifferentiated cells, not expressed in differentiated cells (undifferentiation marker). Examples of the undifferentiation marker include endogenous markers such as Oct3/4, Nanog, Sox2, E-Ras, Rex1 and SSEA1. The method for confirming the expression of such an undifferentiation marker is not particularly limited, and includes the RT-PCR assay and the immunostaining method using a marker specific antibody.

[0032] (5) Vector elimination step

The vector elimination step is to eliminate episomal vectors from iPS cells. The vector elimination step is not an indispensable step because the introduced episomal

vector may spontaneously drop off from iPS cells along with cell growth, followed by its elimination from the population of iPS cells. If episomal vectors remain in iPS cells, however, undifferentiated state of the cells could become unstable or conversely the cells could lose the ability to differentiate. Therefore, it is preferred to conduct the vector elimination step. Moreover, if episomal vectors remain for a long period of time, a foreign gene could become chromosomally integrated spontaneously. Thus, the same problem as in the case where a retrovirus vector is used would arise. Therefore, it is preferred to conduct the vector elimination step also in order to reduce the possibility of spontaneous chromosomal integration of a foreign gene and thereby reduce the risks of cellular oncogenic transformation and of developing dysfunction as much as possible. The method for eliminating episomal vectors is not particularly limited, and for example, the following methods can be used.

[0033] (a) Cloning by limiting dilution

As described above, episomal vectors are expected to drop off spontaneously along with cell growth. Considering the fact, when iPS cells from which episomal vectors have completely dropped off are cloned by limiting dilution, a population consisting only of iPS cells without episomal vectors can be obtained.

[0034] (b) Use of retroviral promoter

It is known that a retroviral promoter does not serve as a promoter in undifferentiated cells (Gorman CM, Rigby PW, Lane DP: Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell. 1985 Sep; 42(2): 519-26.). Many reports show that a retroviral vector becomes inactivated in undifferentiated cells (for example, Pannell D, Osborne CS, Yao S, Sukonnik T, Pasceri P, Karaiskakis A, Okano M, Li E, Lipshitz HD, Ellis J.: Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. EMBO J. 2000 Nov 1; 19(21): 5884-94.). Therefore, when an episomal vector carrying the gene encoding a replication factor downstream of a retroviral promoter is employed, for example, the expression of the replication factor stops after reprogramming of somatic cells into iPS cells. As a result, such an episomal vector loses the ability to replicate, and a population consisting only of iPS cells without the vectors is gradually formed. The gene ligated downstream of a retroviral promoter is not limited to the gene encoding a replication factor, and may be the gene encoding a nuclear reprogramming factor, but the gene encoding a replication factor is preferred from a viewpoint of suppressing vector replication.

[0035] (c) Use of drug-regulated gene expression system

There is known a system in which the expression of a target gene product can be regulated by switching medium conditions, between the presence and absence of a specific drug in medium. When this system is employed so that the expression of a

replication factor is suspended by addition of a specific drug to medium, the expression of the replication factor is inhibited, the episomal vector loses the ability to replicate, and then a population consisting only of iPS cells without the vectors is gradually formed. The drug used for the drug-regulated gene expression system is not particularly limited, and any drug can be suitably used as long as it can help to realize such a system.

[0036] For example, the Tet-Off system is known as a publicly known system. Based on the Tet-Off system, the episomal vector carrying the gene encoding a replication factor can be designed so that the expression of the replication factor is suspended by addition of tetracycline to medium. Specifically, for example, the primary vector used by the present inventors in the examples can be modified to express tTA (tet-regulated transcriptional activator) under CMV promoter control, and to carry the gene encoding a replication factor (large T antigen) downstream of the tTA-regulated tetO (Tet operator sequence) promoter. The replication origin (ori) and the neomycin resistance gene that this primary vector originally carries still remain as they are. When such an episomal vector is employed, addition of tetracycline to medium after iPS cell selection inhibits the expression of the replication factor, the episomal vector loses the ability to replicate, and then a population consisting only of iPS cells without episomal vectors is gradually formed.

[0037] It is also possible to employ the Tet-On system, which is regulated in an opposite manner to the Tet-Off system, that is, in which the expression of the replication factor is suspended by removal of tetracycline from medium. In the case of employing the Tet-On system, medium with tetracycline should be used until iPS cell selection, and this medium should be replaced with medium without tetracycline after iPS cell selection. The Tet-Off system and the Tet-On system are commercially available from Clontech.

[0038] (d) Use of herpesvirus-derived thymidine kinase

The episomal vector is prepared so as to carry the herpesvirus-derived thymidine kinase gene in addition to the gene encoding a replication factor and/or the gene encoding a nuclear reprogramming factor. In this case, iPS cells which stop expressing the thymidine kinase due to loss of episomal vectors are allowed to selectively survive by addition of ganciclovir or aciclovir to medium after iPS cell selection. Thus, a population consisting only of iPS cells without episomal vectors can be obtained.

[0039] As explained in the examples below, the present inventors prepared iPS cells using a mouse polyomavirus-based episomal vector by taking two steps for introducing episomal vectors, the replication factor introduction step and the nuclear reprogramming factor introduction step. Fig. 1 is a diagrammatic view illustrating the preparation method of the present invention in accordance with one of the examples

below. First, the primary vector carrying the gene of a large T antigen as a replication factor is transfected into somatic cells, and then the selection of cells having the primary vector introduced therein is performed (replication factor introduction step). Two kinds of secondary vectors, an episomal vector carrying the Oct3/4 gene and the Klf4 gene, and an episomal vector carrying the Sox2 gene and the c-Myc gene, are transfected into the selected cells (nuclear reprogramming factor introduction step). Since these cells already express the large T antigen via the primary vector, the secondary vectors replicate as episomes with the aid of the expressed large T antigen. Through the cultivation step, somatic cells each expressing four nuclear reprogramming factors (Oct3/4, Sox2, Klf4 and c-Myc) are dedifferentiated and reprogrammed into iPS cells. iPS cells can be selected based on colony morphology and undifferentiation marker expression as mentioned above (selection step). Through the elimination of the episomal vectors from the cells (vector elimination step), preparation of safe iPS cells which have extremely low risk of oncogenic transformation and of mutagenesis can be achieved.

[0040] The application of the iPS cells prepared according to the preparation method of the present invention is not limited, and can be used suitably for the ES cell-based tests, research, treatment of diseases, regenerative medicine and the like. The iPS cells prepared according to the preparation method of the present invention have extremely low risk of oncogenic transformation and of developing dysfunction and have high safety. Therefore, such iPS cells are highly useful especially in the autotransplantation therapy, which is performed according to the procedures which involve preparing iPS cells from the somatic cells of a patient, inducing differentiation of the iPS cells into desired differentiated cells or tissues, and returning thus obtained cells or tissues to the patient.

[0041] Preparation kit of iPS cells

The present invention provides a kit for preparing iPS cells, which comprises an episomal vector into which the gene encoding a nuclear reprogramming factor is inserted in an expressible form (hereinafter referred to as "the preparation kit of the present invention"). The episomal vector contained in the preparation kit of the present invention may be any episomal vector usable for the aforementioned preparation method of the present invention. Since such an episomal vector is already described above in detail, the repetitive description is avoided here. When a replication factor is required for replication of the episomal vector contained in the preparation kit of the present invention, that is, the episomal vector into which the gene encoding a nuclear reprogramming factor is inserted in an expressible form, it is preferable that the preparation kit of the present invention should comprise an episomal vector into which the gene encoding a replication factor is inserted in an expressible form. There is no

particular limitation on the kit components other than these episomal vectors. Any reagent, equipment or other requirements for the kit can be suitably selected and contained in the kit. The preparation kit of the present invention enables the aforementioned preparation method of the present invention to be performed simply and quickly, and iPS cells to be prepared with high reproducibility. Particularly, when the kit to be used is optimized in terms of the episomal vector system, the expression level of the nuclear reprogramming factor and the like, the reproducibility in generation of iPS cells can be further improved and the safety of the resulting iPS cells can be also secured.

[0042] The term "kit" used herein is intended to mean a package, which comprises a container holding a specific material (for example, a bottle, a plate, a tube, a dish, etc.). Preferably, the kit comprises an instruction manual for using the aforementioned material. The instruction manual may be written or printed on paper or other media, or may be provided in the form of electronic media, such as a magnetic tape, a computer-readable disk or tape, and a CD-ROM.

Examples

[0043] Hereinafter, the present invention will be illustrated in detail by way of examples, but is not limited thereto.

Example 1

[0044] Primary vector

As a primary vector expressing a large T antigen, the mouse polyomavirus-based pMGD20neo was used. The pMGD20neo plasmid carries the large T antigen gene of and the replication origin (ori) of a mouse polyomavirus, and the neomycin resistance gene, and is replicated and retained stably as an episome in mouse ES cells (Gassmann M, Donoho G, Berg P.: Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells. Proc Natl Acad Sci USA. 92: 1292-1296, 1995). The present inventors obtained a clonal mouse ES cell line which stably retains pMGD20neo as an episome (1.19 cell line), which was reported in the aforementioned publication of Gassmann et al., from Dr. Austin Smith (School of Biological Sciences, University of Edinburgh). pMGD20neo was isolated from the ES cell line, amplified in Escherichia coli and purified for later use.

[0045] Secondary vector construction

Two kinds of secondary vectors, an episomal vector expressing mouse Oct3/4 and Klf4, and an episomal vector expressing mouse Sox2 and c-Myc, were prepared in the following procedures for later use.

First, each cDNA of mouse Oct3/4, Sox2, Klf4 and c-Myc was obtained by the PCR method. The base sequence data on each gene was obtained from a known database

(GenBank), and the primers of each gene were designed based on this data. As a template for PCR, cDNA was prepared by a known method from total RNA derived from mouse ES cells or organs. The total RNA was prepared from organs of an 8-week-old C57BL6 mouse in the laboratory of the present inventors. Each cDNA fragment obtained by PCR was cloned into the pBluescriptKS (-) or pCR4 plasmid. The cDNA fragment was confirmed to have the correct base sequence of each gene by sequencing determination using ABI3100 or ABI3730. Table 1 shows the data regarding each gene, that is, GenBank Acc. No., the source of the total RNA used, primer sequences and the vector used.

[0046] [Table 1]

	GenBank Acc. No.	source	forward primer (5'-3')	reverse primer (5'-3')	vector
Oct3/4	NM_013633	mouse ES cell	GTGAGCCGTCTTTCCACCAG (SEQ ID NO: 1)	CCAGTCTTTATTTAAGAACA (SEQ ID NO: 2)	pBS KS(-)
Sox2	NM_011443	mouse brain	AGCGCCCGCATGTATAACAT (SEQ ID NO: 3)	GAAAATCTCTCCCCTTCTCC (SEQ ID NO: 4)	pCR4
Klf4	NM_010637	mouse stomach	TCTGCCATGGCTGTCAGCGA (SEQ ID NO: 5)	GTCACATCCACTACGTGGGA (SEQ ID NO: 6)	pCR4
c-Myc	NM_010849	mouse liver	ATAGGTCTCCCATGCCCCTCAACGTGAACT (SEQ ID NO: 7)	GTCAGTTTATGCACCAGAGTTTCGAAGC (SEQ ID NO: 8)	pBS KS(-)

[0047] pPyCAG-IZ carrying the mouse polyomavirus replication origin (ori) and the zeocin-resistance gene (Genes Dev. 12: 2048-60, 1998) was used for construction of an episomal vector expressing Oct3/4 and Klf4. pPyCAG-IP carrying the mouse polyomavirus replication origin (ori) and the puromycin resistance gene (Mol Cell Biol. 22: 1526-36, 2002) was used for construction of an episomal vector expressing Sox2 and c-Myc. pPyCAG-IP and pPyCAG-IZ were obtained from Dr. Hitoshi Niwa, an author of the two aforementioned publications.

[0048] The encephalomyocarditis virus-derived IRES sequence was excised from the pCITE-1 plasmid (Novagen). The Oct3/4 cDNA containing the translation initiation codon ATG and the stop codon was ligated upstream of the IRES. Then, the Klf4 cDNA containing the stop codon was ligated downstream of the IRES so that ATG located immediately downstream of the IRES served as the translation initiation codon ATG for Klf4. In this way, an Oct3/4-IRES-Klf4 fragment was prepared. This Oct3/4-IRES-Klf4 fragment was inserted into pPyCAG-IZ between the CAG promoter and the IRES-zeo, resulting in pPyCAG-Oct3-IRES-Klf4-IRES-zeo (see Fig. 2).

Similarly, a Sox2-IRES-c-Myc fragment was prepared. Namely, the Sox2 cDNA containing the translation initiation codon ATG and the stop codon was ligated upstream of another IRES excised from the pCITE-1 plasmid. Then, the c-Myc cDNA containing the stop codon was ligated so that ATG located immediately downstream of the IRES served as the translation initiation codon ATG for c-Myc. Subsequently, this

Sox2-IRES-c-Myc fragment was inserted into pPyCAG-IP between the CAG promoter and the IRES-puro, resulting in pPyCAG-Sox2-IRES-c-Myc-IRES-puro (see Fig. 3).

[0049] Confirmation of Oct3/4, Klf4, Sox2 and c-Myc expression

The two resulting plasmids were introduced into BMT10 cells, and then the expression of each protein was confirmed. BMT10 is a monkey cell line. It is known that this cell line expresses the SV40 T antigen gene (Gerard RD, Gluzman Y.: New host cell system for regulated simian virus 40 DNA replication. Mol Cell Biol. 1985 Nov; 5(11): 3231-40.), and that extremely strong expression of a desired gene can be achieved in this cell line by use of the chicken beta actin promoter-driven expression vector (Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, Yamamura K.: Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. Gene. 1989 Jul 15; 79(2):269-77). For these reasons, the present inventors decided to use BMT10 cells. BMT10 cells were received from Dr. R.D. Gerard, an author of the aforementioned publication.

In accordance with the method described in the aforementioned publication of Miyazaki et al., pPyCAG-Oct3-IRES-Klf4-IRES-zeo or pPyCAG-Sox2-IRES-c-Myc-IRES-puro was transfected into BMT10 cells. At 2 days post-transfection, the cells were fixed with 4% paraformaldehyde, immunostained using the primary and secondary antibodies described in Table 2, and observed and photographed with a fluorescence microscope.

[0050] [Table 2]

	primary antibody	secondary antibody
Oct3/4	anti-Oct3/4 antibody (SC-8629, SANTA CRUZ)	Alexa Fluor 488-conjugated donkey anti-goat IgG
Klf4	anti-Klf4 antibody (SC-20691, SANTA CRUZ)	Alexa Fluor 594-conjugated donkey anti-rabbit IgG
c-Myc	anti-c-Myc antibody (SC-764, SANTA CRUZ)	Alexa Fluor 568-conjugated goat anti-rabbit IgG
Sox2	anti-Sox2 antibody (245610, R&D)	Alexa Fluor 488-conjugated goat anti-mouse IgG

[0051] The results are shown in Figs. 4 and 5. Fig. 4 is fluorescence microscope images showing immunostaining results of BMT10 cells with pPyCAG-Oct3-IRES-Klf4-IRES-zeo introduced therein. (a) to (d) cover the same field of view. (a) is an immunostaining image for Oct3/4, (b) is an immunostaining image for Klf4, (c) is a merged image of (a) and (b), and (d) is a merged image of a DAPI staining image (DAPI stains nucleic acids to fluoresce blue) on (c). As clearly shown in Figs. 4 (a) to (d), the cells expressing Oct3/4 and the cells expressing Klf4 were identical. The cells not expressing the transgenes when observed, due to transience of their expression, are stained with DAPI and shown in blue in (d). These results demonstrated that the BMT cells with pPyCAG-Oct3-IRES-Klf4-IRES-zeo introduced therein expressed both Oct3/4 and Klf4 simultaneously.

[0052] Fig. 5 is fluorescence microscope images showing immunostaining results of BMT10

cells with pPyCAG-Sox2-IRES-c-Myc-IRES-puro introduced therein. (a) to (d) cover the same field of view. (a) is an immunostaining image for Sox2, (b) is an immunostaining image for c-Myc, (c) is a merged image of (a) and (b), and (d) is a merged image of a DAPI staining image on (c). As clearly shown in Figs. 5 (a) to (d), the cells expressing Sox2 and the cells expressing c-Myc were identical. The cells not expressing the transgenes when observed, due to transience of their expression, are stained with DAPI and shown in blue in (d). These results demonstrated that the BMT cells with pPyCAG-Sox2-IRES-c-Myc-IRES-puro introduced therein expressed both Sox2 and c-Myc simultaneously.

[0053] Preparation of iPS cells

(1) Culture of fibroblasts

MEFs (mouse embryonic fibroblasts) were isolated from a mouse 13-day-old embryo and then cultured. As culture medium for the MEFs, DMEM + 10% FBS was used. The MEFs were cultured in the culture medium in a CO_2 incubator (37°C, 5% CO_2). One third of the MEFs were passaged every 3 days.

[0054] (2) Primary vector introduction and cell selection

Twenty-four microgram of pMGD20neo was added to 1.5 ml of serum-free DMEM. Twenty microliter of lipofectamine 2000 was added to 1.5 ml of serum-free DMEM. Both of them were mixed and let stand at room temperature for 20 minutes. Meanwhile, MEFs grown to about 80% confluency in a 10-cm-diameter culture dish were removed by treatment with trypsin/EDTA, and then suspended in 15 ml of the culture medium (DMEM+10% FBS). The mixed solution of pMGD20neo and lipofectamine 2000 was added to this cell suspension, which was then seeded onto a 10-cm-diameter culture dish. On the following day, the culture medium was exchanged. Two days later, the MEFs started to be cultured in culture medium supplemented with 350 microgram/ml G418. After 3-week culture, G418 resistant MEFs were replated onto a culture dish for further culture.

[0055] (3) Secondary vector introduction and cell selection

The secondary vectors were transfected on the day after the G418-resistant MEFs were replated.

Ten microgram of pPyCAG-Oct3-IRES-Klf4-IRES-zeo and 10 microgram of pPyCAG-Sox2-IRES-c-Myc-IRES-puro were added to 1.5 ml of serum-free DMEM. Twenty microliter of lipofectamine 2000 was added to 1.5 ml of serum-free DMEM. Both of them were mixed and let stand at room temperature for 20 minutes. Meanwhile, the G418-resistant MEFs seeded onto a 10-cm-diameter culture dish on the previous day were removed by treatment with trypsin/EDTA, and then suspended in 15 ml of the culture medium (DMEM+10%FBS). The mixed solution of two kinds of plasmids and lipofectamine 2000 was added to this cell suspension, which was then

seeded onto a 10-cm-diameter culture dish. On the following day, the culture medium was exchanged. Two days later, the MEFs started to be cultured in culture medium supplemented with zeocin (10 microgram/ml) and puromycin (1.5 microgram/ml), and maintained for 3 days. Surviving zeocin- and puromycin-resistant MEFs were seeded onto feeder cells, and continued to be cultured.

The feeder cells were prepared by treatment of MEFs with mitomycin C. Specifically, mitomycin C was added in a concentration of 15 microgram/ml to a dish of confluent MEFs, which was further maintained in a CO_2 incubator at 37°C for 3 hours. After the cells were removed by treatment with trypsin/EDTA, they were counted and then 3 x 10^6 cells/10 ml per dish were seeded and cultured in 10 cm-diameter gelatin-coated dishes. On the following day or later, these cells were used as feeder cells.

[0056] (4) Cell morphology observation

At 15 days after zeocin- and puromycin-resistant cells started to be cultured on feeder cells, it was shown that some of zeocin- and puromycin-resistant cells piled up and formed colonies. The microscope images of the formed colonies are shown in Figs. 6A and 6B.

The colonies were isolated, seeded onto feeder cells, and continued to be cultured. At 4 days after the colony isolation, colonies morphologically similar to ES cell colonies were formed. That is, each of the colonies was formed in a round or elliptical shape like an upside-down bowl and had a clear rim. The microscope images of the colonies are shown in Figs. 7A and 7B.

Further, these colonies were isolated, seeded onto feeder cells, and continued to be cultured. After 4 days, alkaline phosphatase staining was performed. The alkaline phosphatase staining kit manufactured by Sigma-Aldrich Co. was used for staining. The results are shown in Fig. 8. A and B are staining results of the cells of this example, C is a staining result of feeder cells alone, and D is a staining result of mouse ES cells (derived from E14 cells). As clearly shown in Fig. 8, the cells of this example were stained red like ES cells. These results demonstrated that the cells of this example were reprogrammed into iPS cells.

Example 2

[0057] BK virus-based episomal vector construction

A DNA fragment containing the BK virus ori sequence and T antigen gene was excised from pRBK plasmid (Invitrogen) by ScaI digestion. A HindIII-KpnI DNA fragment including the MC1 promoter and the HSV Tk gene was excised from pMC1-tk plasmid (Hogan B, Beddington R, Costantini F, Lacy E. Manipulating the Mouse Embryo: A Laboratory Manual. Vol. 2. Plainview, NY: Cold Spring Harbor Lab. Press; 1995. Pp. 217-253). These fragments were inserted into the BamHI and

SalI sites, respectively, of the pPyCAG-Sox2-IRES-c-Myc-IRES-puro plasmid described above. The CAG-Oct3/4-IRES-Klf4-IRES-zeo-polyA sequence was excised from pPyCAG-Oct3-IRES-Klf4-IRES-zeo described above by SpeI and BamHI digestion. This fragment was inserted into the SalI site of the above BK-based plasmid, resulting in pBK-COK-CSM (Fig. 9).

[0058] Plasmid construction

The SV40 virus large T antigen gene was excised from pSV40 by BglI digestion (Miyazaki j, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K.: Establishment of a pancreatic beta cell line that retains glucose inducible insulin secretion: Special reference to expression of glucose transporter isoforms. Endocrinology 127: 126-132, 1990). This DNA fragment was inserted into pAc-lacZ plasmid (Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, Yamamura K: Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. Gene 79: 269-277, 1989) in place of the lacZ gene, resulting in pAc-Tag plasmid. The mouse Nanog cDNA was PCR-amplified from mouse ES cell cDNA and was inserted into pPyCAG plasmid, resulting in pPyCAG-Nanog. The sequences of the primers used to amplify the Nanog cDNA were as follows: 5'-ACTGACATGAGTGTGGGTCTT-3' and 5'-GCGTAAGTCTCATATTTCACCT-3'

[0059] Cell culture and transfection

Human neonatal epidermal keratinocytes (Cascade Biologics) were cultured in EpiLife medium (Cascade Biologics), supplemented with EDGS (Cascade Biologics). For reprogramming, a combination of episomal and nonepisomal plasmids (pBK-COK-CSM, pAc-Tag, and pPyCAG-Nanog) was cotransfected into human keratinocytes in 10-cm dishes via FuGENE 6 transfection reagent (Roche). On the next day, transfected keratinocytes (up to 1.0×10^6 cells per dish) were directly plated onto two 10-cm MEF (mitomycin C-treated mouse embryonic fibroblast)-seeded dishes in keratinocyte culture medium supplemented with 2 mM valproic acid (VPA). Culture medium was exchanged every other day. On day 4 posttransfection, the keratinocyte culture medium was replaced with human ES cell culture medium (DMEM/F12 culture medium supplemented with 20% KnockOut serum replacer (KSR; Invitrogen, Carlsbad, CA), 0.1 mM non-essential amino acids (Invitrogen), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 10 ng/ml human basic fibroblast growth factor (Wako)) containing VPA. Human ES cell culture medium conditioned with MEFs (CM) was used to sustain the reprogramming culture from 8 days after plating.

[0060] Alkaline phosphatase staining

Colonies with morphology similar to iPS colonies were visible on day 20 post-transfection. These colonies were replated onto 10-cm MEF-seeded dishes. To

examine the presence or absence of human iPS colonies, these dishes were stained with alkaline phosphatase (Sigma-Aldrich Co.). The result showed that these colonies were stained red (Fig. 10). Therefore, the cells obtained from human keratinocytes in this example were considered to be iPS cells.

[0061] The present invention is not limited to the aforementioned embodiments and examples, and various modifications can be made within the scope of the appended claims. Other embodiments provided by suitably combining the different technical means disclosed in the different embodiments are also within the technical scope of the present invention. All the academic publications and cited patent literatures in the above description are incorporated herein by reference.

Industrial Applicability

[0062] The iPS cells prepared according to the preparation method of the present invention have extremely low risk of cellular oncogenic transformation and of developing dysfunction, and therefore are highly useful for generation of tissues and organs used for regenerative medicine.

Claims

[Claim 1] A method for preparing an iPS cell by nuclear reprogramming of a somatic cell, which comprises the following steps:
a nuclear reprogramming factor introduction step which involves introducing, into somatic cells, an episomal vector into which a gene encoding a nuclear reprogramming factor is inserted in an expressible form;
a cultivation step which involves culturing somatic cells having the episomal vector introduced therein; and
a selection step which involves selecting iPS cells generated by reprogramming of the somatic cells.

[Claim 2] The method according to Claim 1, which further comprises a replication factor introduction step which involves introducing, into somatic cells, an episomal vector into which a gene encoding a replication factor is inserted in an expressible form, before the nuclear reprogramming factor introduction step.

[Claim 3] The method according to Claim 1 or 2, which further comprises a vector elimination step which involves eliminating an episomal vector from iPS cells, after the selection step.

[Claim 4] The method according to Claim 3, wherein the episomal vector is constituted so that expression of the gene encoding the nuclear reprogramming factor and/or the gene encoding the replication factor is driven by a retroviral promoter.

[Claim 5] The method according to Claim 3, wherein the episomal vector is constituted so that expression of the gene encoding the nuclear reprogramming factor and/or the gene encoding the replication factor can be regulated by a drug.

[Claim 6] The method according to any one of Claims 1 to 5, wherein the episomal vector is selected from the group consisting of a mouse polyomavirus-based episomal vector, a BK virus-based episomal vector, an Epstein-Barr virus-based episomal vector, a bovine papilloma virus-based episomal vector and an episomal vector carrying an S/MAR.

[Claim 7] The method according to any one of Claims 1 to 6, wherein the nuclear reprogramming factor is selected from the group consisting of the following (1), (2) and (3):
(1) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, and iii) one kind

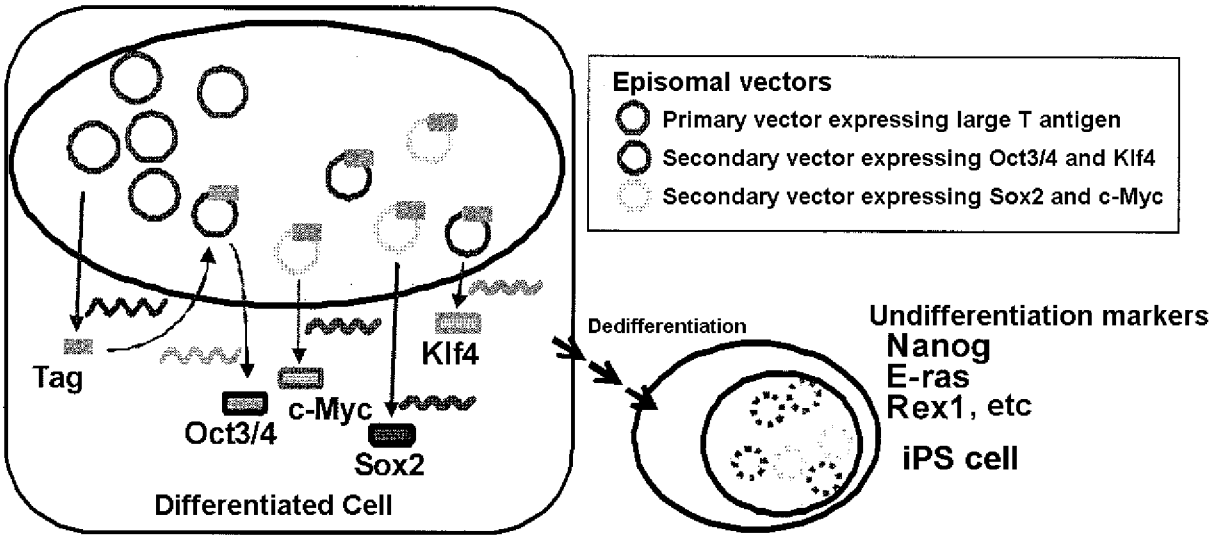
selected from the group consisting of Klf1, Klf4 and Klf5,
(2) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5, and iv) one kind selected from the group consisting of c-Myc, N-Myc and L-Myc, and
(3) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) Nanog and iv) Lin28.

[Claim 8] A kit for preparing an iPS cell, which comprises an episomal vector into which a gene encoding a nuclear reprogramming factor is inserted in an expressible form.

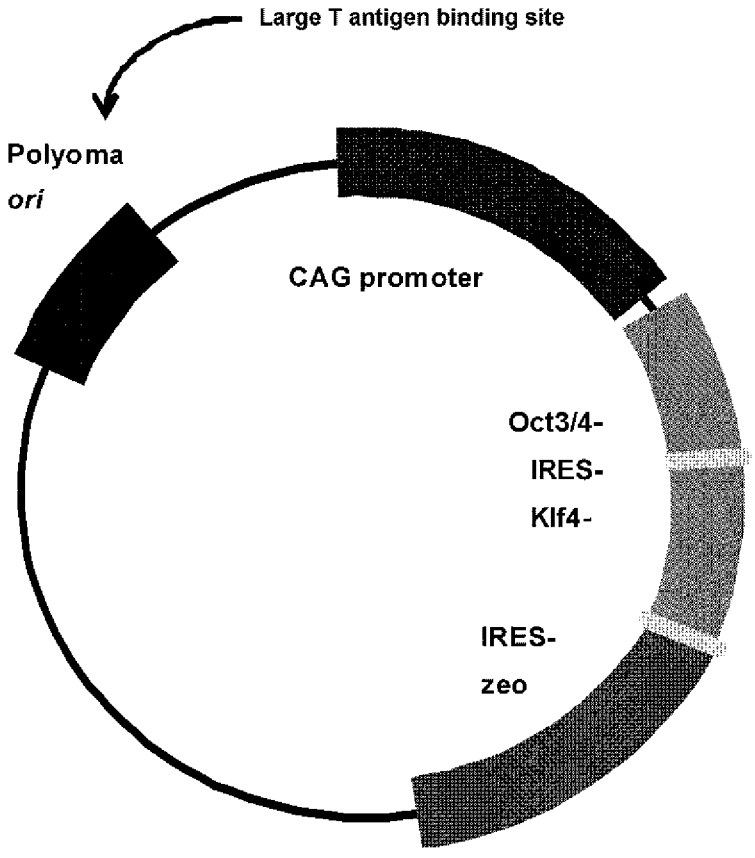
[Claim 9] The kit according to Claim 8, wherein the episomal vector is selected from the group consisting of a mouse polyomavirus-based episomal vector, a BK virus-based episomal vector, an Epstein-Barr virus-based episomal vector, a bovine papilloma virus-based episomal vector and an episomal vector carrying an S/MAR.

[Claim 10] The kit according to Claim 8 or 9, wherein the nuclear reprogramming factor is selected from the group consisting of following (1), (2) and (3):
(1) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, and iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5,
(2) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) one kind selected from the group consisting of Klf1, Klf4 and Klf5, and iv) one kind selected from the group consisting of c-Myc, N-Myc and L-Myc, and
(3) a combination of i) Oct3/4, ii) one kind selected from the group consisting of Sox1, Sox2, Sox3, Sox15 and Sox18, iii) Nanog and iv) Lin28.

[Fig. 1]

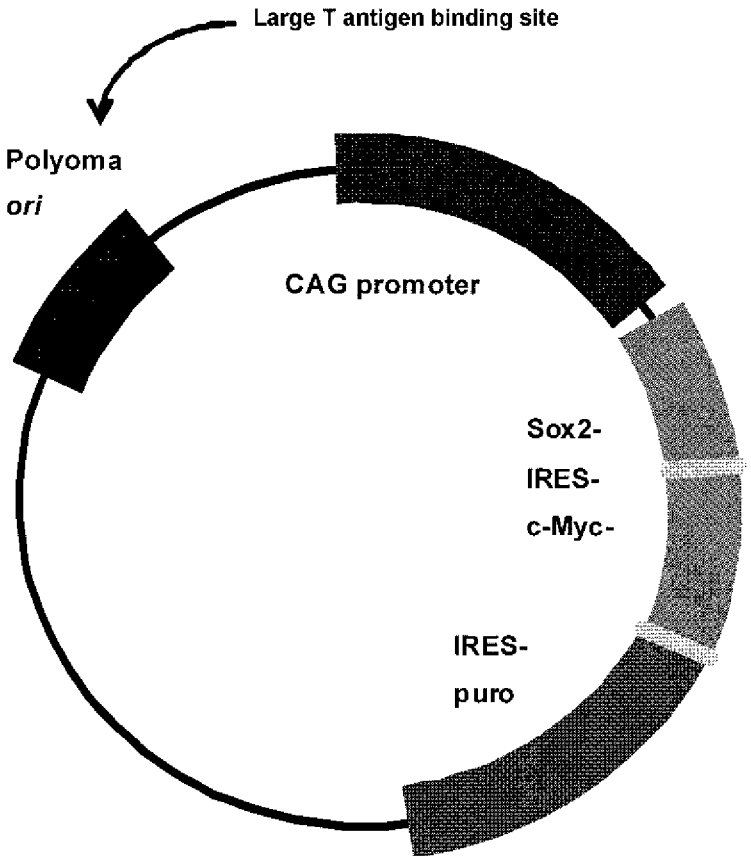


[Fig. 2]



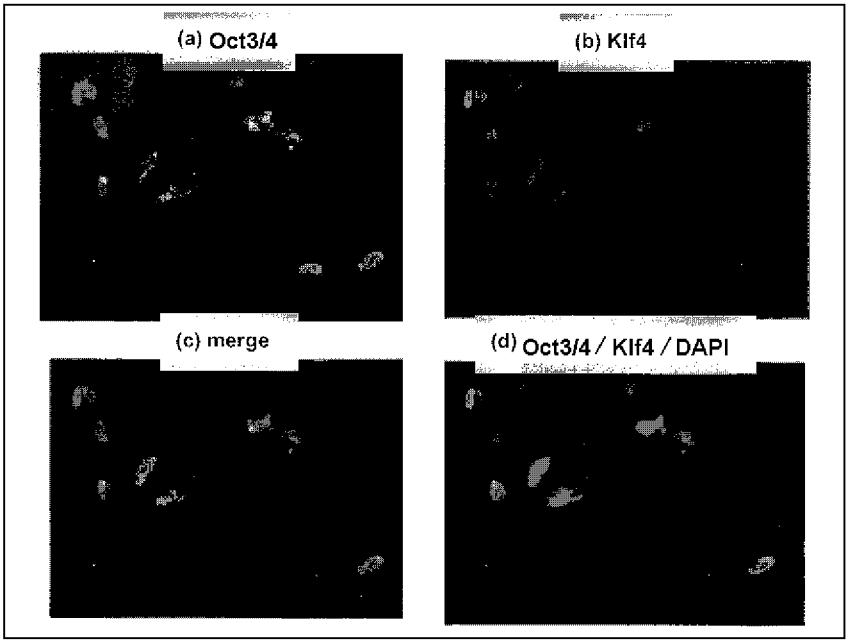
pPyCAG-Oct3-IRES-Klf4-IRES-zeo

[Fig. 3]

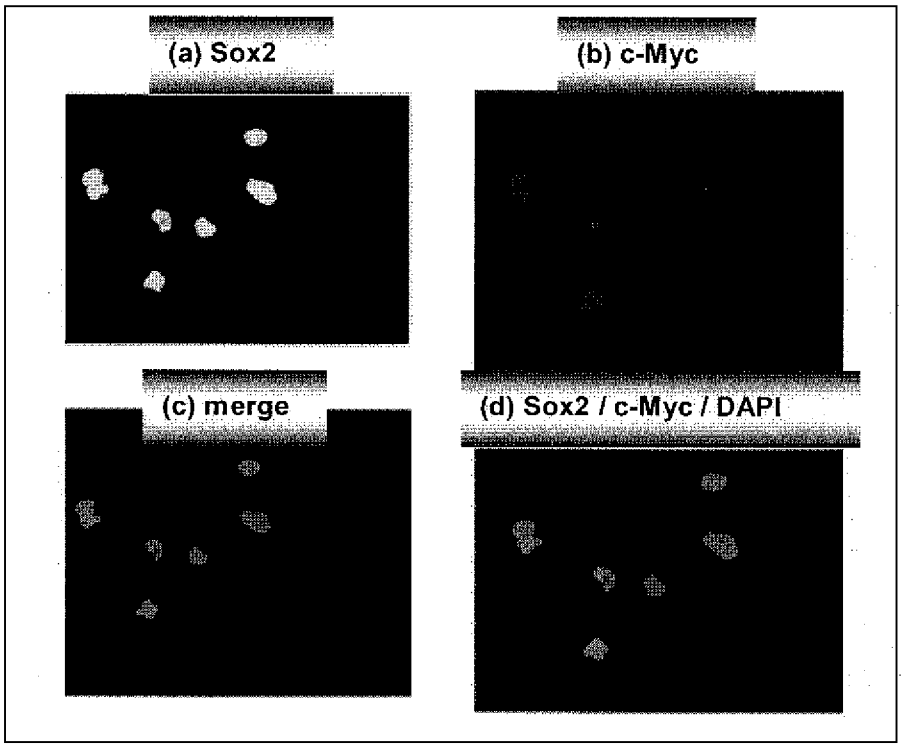


pPyCAG-Sox2-IRES-c-Myc-IRES-puro

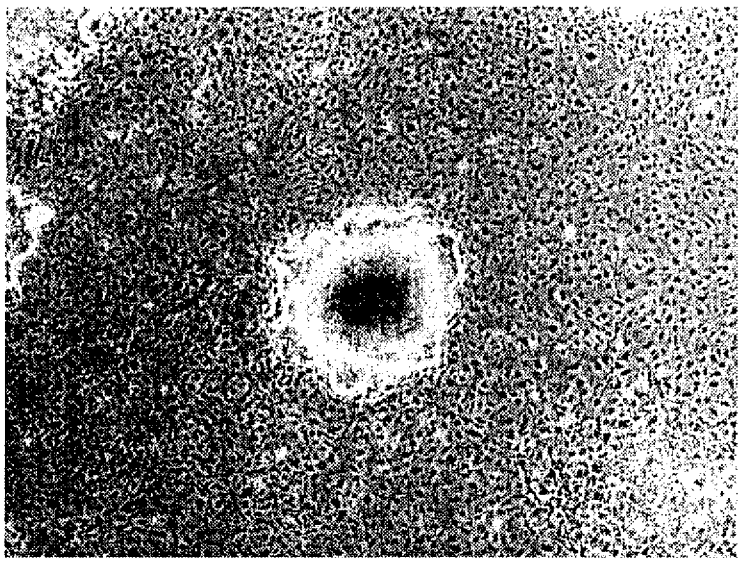
[Fig. 4]



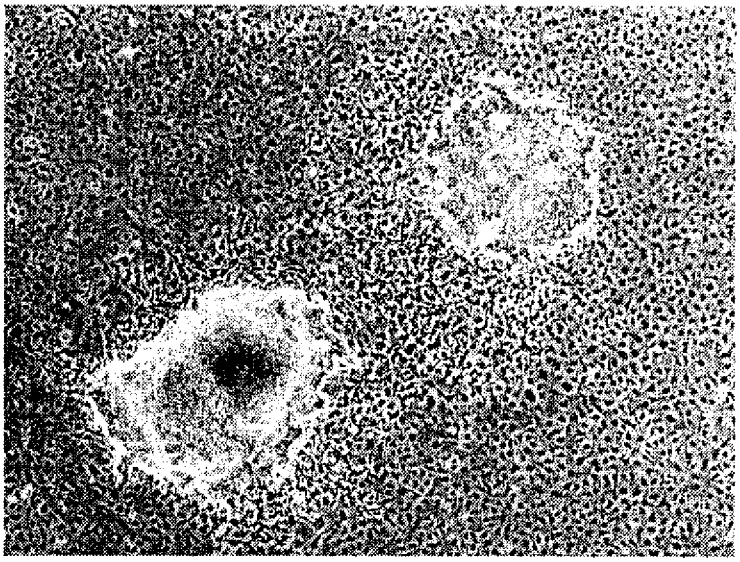
[Fig. 5]



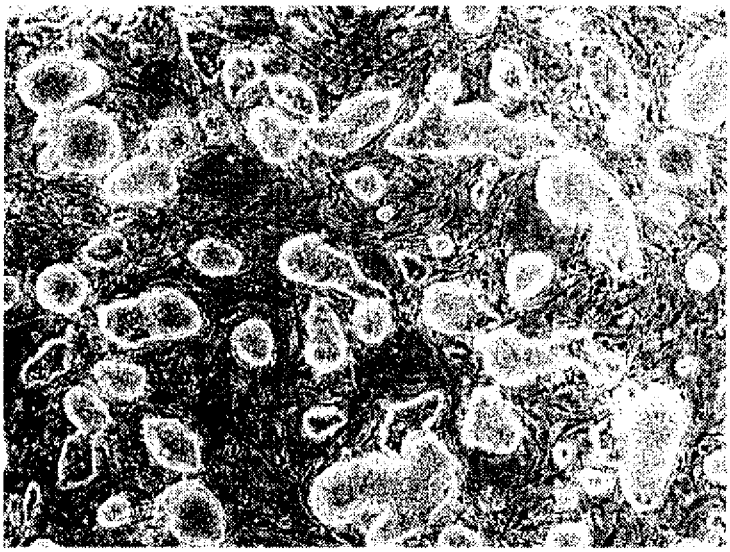
[Fig. 6A]



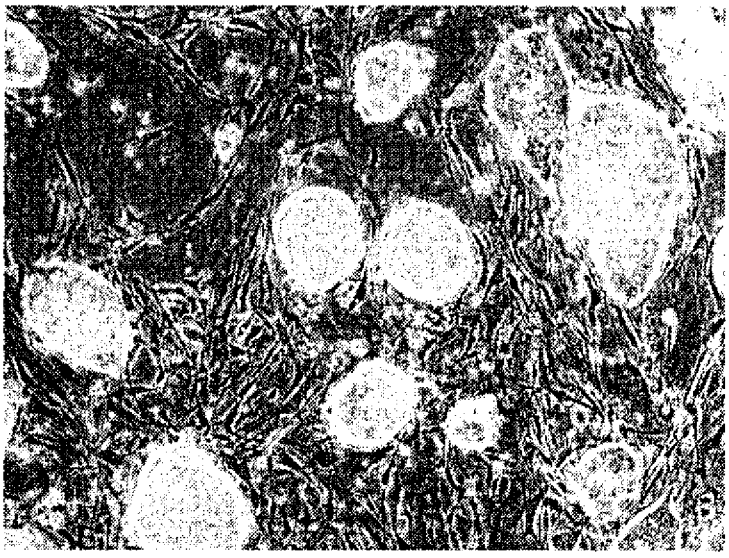
[Fig. 6B]



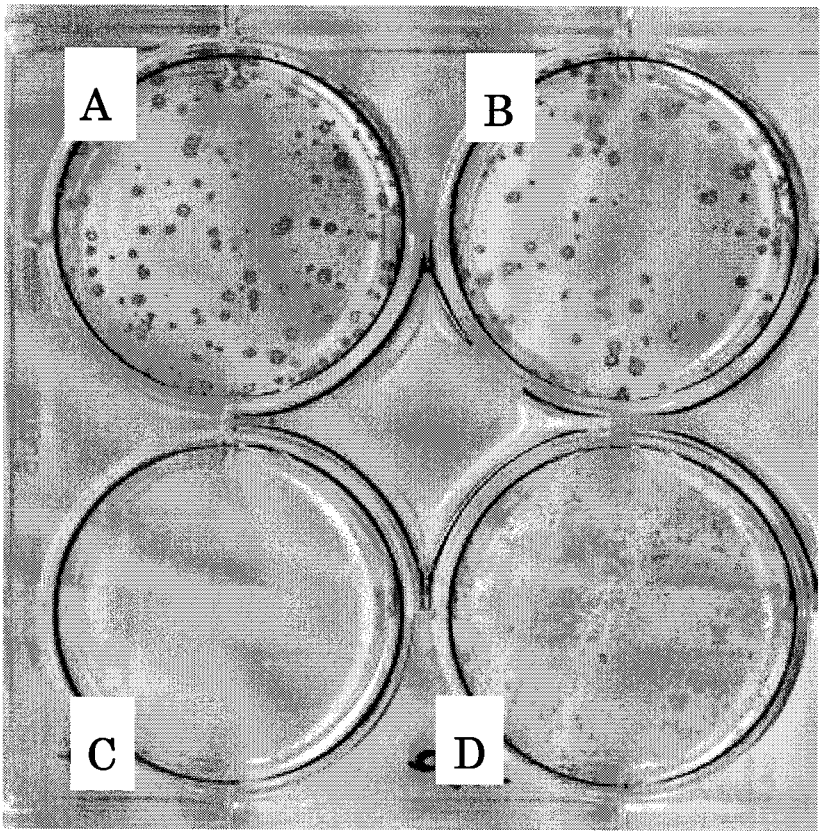
[Fig. 7A]



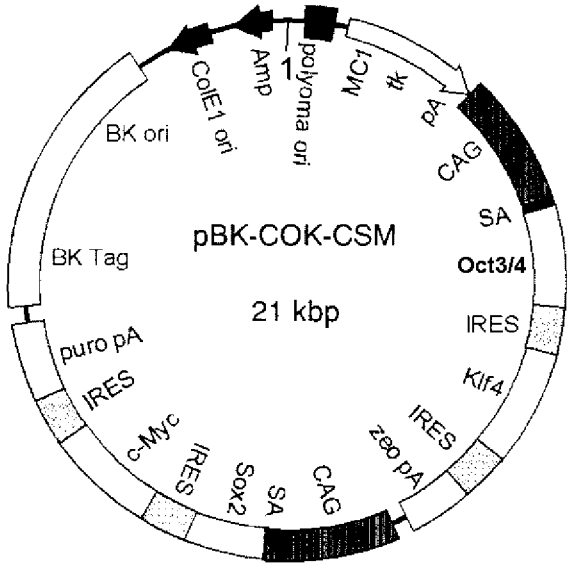
[Fig. 7B]



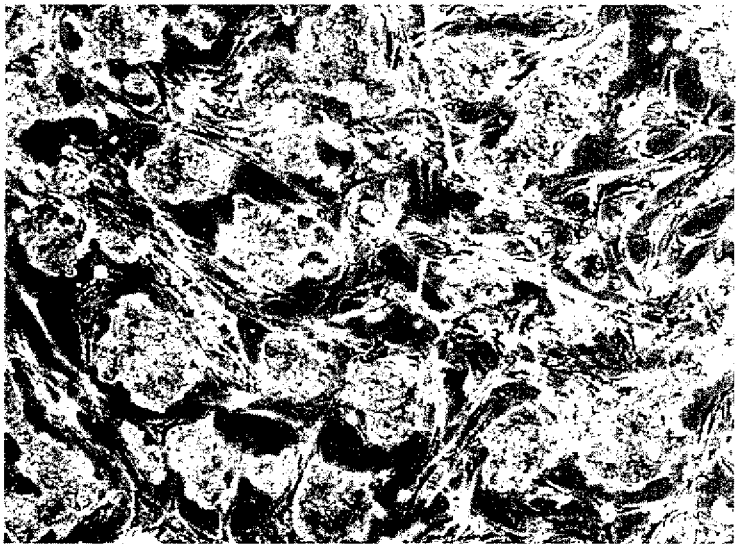
[Fig. 8]


A
B
C
D

[Fig. 9]


pBK-COK-CSM
21 kbp
Amp
ColE1 ori
polyoma ori
MCI
tk
pA
CAG
SA
Oct3/4
IRES
Klf4
IRES
zeo pA
CAG
SA
Sox2
IRES
c-Myc
IRES
puro pA
BK Tag
BK ori

[Fig. 10]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/002914

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N5/10(2006.01)i, C12N15/09(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N5/10, C12N15/09

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

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2009
Registered utility model specifications of Japan 1996-2009
Published registered utility model applications of Japan 1994-2009

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

CA/BIOSIS/MEDLINE (STN), JSTPlus/JMEDPlus/JST7580(JDreamII), PubMed, WPI, CiNii

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Okita K., et al., Generation of germline-competent induced pluripotent stem cells., Nature, 448(2007), p.313-317	1-10
Y	Yu J., et al., Induced pluripotent stem cell lines derived from human somatic cells., Science, 318(2007), p.1917-1920	1-10
Y	Nakagawa M., et al., Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts., Nat. Biotechnol., 26(1)(2008 Jan), p.101-106	1-10

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

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17.07.2009	28.07.2009

Name and mailing address of the ISA/JP	Authorized officer
Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	MUKASA Noriko Telephone No. +81-3-3581-1101 Ext. 3448

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/002914

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2007/069666 A1 (KYOTO UNIVERSITY) 2007.06.21, & JP 2008-283972 A & US 2009/0047263 A & US 2009/0068742 A & EP 1970446 A1 & WO 2009/057831 A & CA 2632142 A & KR 10-2008-0095852 A & CN 101356270 A & CA 2660123 A	1-10
Y	Gassmann M., et al., Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells, Proc. Natl. Acad. Sci. USA, 92(5)(1995), p.1292-1296	1-10
PX	Yu J., et al., Human induced pluripotent stem cells free of vector and transgene sequences., Science, (2009 May), 324, p.797-801	1-10
PA	Okita K., et al., Generation of mouse induced pluripotent stem cells without viral vectors., Science, 322(2008 Nov), p.949-953	1-10
PA	Kaji K., et al., Virus-free induction of pluripotency and subsequent excision of reprogramming factors., Nature, 458(2009 Apr), p.771-775	1-10