

DESCRIPTION

METHOD FOR PRODUCING RENAL PROGENITOR CELLS

TECHNICAL FIELD

[0001] The present invention relates to a method for producing renal progenitor cells (RPCs) using a cell surface marker intended for acquiring and producing a high-purity RPC population from a RPC-containing cell population differentiated from pluripotent stem cells (PSCs).

BACKGROUND ART

[0002] The kidney is an important organ that functions to maintain good physical health through filtrating and removing toxic substances and waste products generated by metabolic activity in the body from the blood. Renal failure is a serious disease that impairs the function of the kidney, but since no effective drug therapy for this disease has yet been established, this disease is at present treated by renal transplantation, dialysis or the like. However, renal transplantation is faced with the problem of severe lack of donor organs, and dialysis also has the problems of the onset of complications and a heavy burden of medical costs; thus, there is a desire to develop a new therapy for this disease.

[0003] Meanwhile, there have already been reports on pluripotent cells, such as embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) obtained by introducing a reprogramming factor(s) into somatic cells (PTLs 1 and 2). Various studies have now been conducted to develop a new therapy for renal failure which involves transplantation of renal cells obtained by inducing differentiation of such PSCs. Another focus has been placed on developing a therapeutic drug for renal failure using homogeneous renal cells derived from such PSCs.

[0004] It is known that mammalian kidneys develop through the three stages of pronephros, mesonephros and metanephros, and that among them, the metanephros develops in the posterior region of intermediate mesoderm. In previous researches, a method for inducing differentiation of mouse PSCs into intermediate mesoderm was studied (NPL 1), and Odd-

Skipped Related Transcription Factor 1 (OSR1) was identified as a characteristic marker of intermediate mesoderm. Also, SIX Homeobox 2 (SIX2) is known as one of factors characterizing RPCs (NPLs 2 and 3). As a result of the study with the human iPSCs (OSR1-GFP reporter human iPS cells) generated by introducing the green fluorescent protein (GFP) gene using a bacterial artificial chromosome (BAC) vector through homologous recombination with endogenous OSR1 allele, human PSCs were successfully induced to differentiate into intermediate mesoderm using Activin A, Wnt protein, bone morphogenetic protein (BMP) and various low-molecular compounds (NPL 3, PTL 3). Then, as a result of the study with OSR1-GFP & SIX2-tdTomato reporter human iPS cell lines generated by introducing the red fluorescent protein, tdTomato, into SIX2 loci in the OSR1-GFP reporter human iPS cell lines using the same homologous recombination procedure as adopted by Mae, *et al.* (NPL 3), a system for inducing differentiation of human PSCs into RPCs was successfully constructed, and the therapeutic efficacy of a therapy with the thus-obtained RPCs was confirmed in acute kidney injury models (NPL 4, PTL 4).

CITATION LIST

PATENT LITERATURES

- [0005] PTL 1: US 5,843,780
PTL 2: WO 2007/069666
PTL 3: WO 2012/011610
PTL 4: WO 2014/200115

NON PATENT LITERATURES

- [0006] NPL 1: Mae S, *et al.*, *Biochem. Biophys. Res. Commun.*, (2010), 393:877-882
NPL 2: Kobayashi A, *et al.*, *Cell Stem Cell*, (2008), 3:169-181
NPL 3: Mae S, *et al.*, *Nat. Commun.*, (2013), 4:1367
NPL 4: Toyohara T, *et al.*, *Stem Cells Transl. Med.*, (2015), 4:980-992

SUMMARY OF INVENTION

TECHNICAL PROBLEM

- [0007] An object of the present invention resides in providing a method for acquiring and

producing a high-purity RPC population from a RPC population into which PSCs are induced to differentiate, by identifying a cell surface marker specific to RPCs.

SOLUTION TO PROBLEM

[0008] The present inventors have made intensive studies to achieve the aforementioned object, and as a result first found that a high-purity RPC population can be acquired and produced from a RPC-containing cell population by using a cell surface marker selected from CD9-negative (CD9 (-)), CD55-negative (CD55(-)), CD106-positive (CD106(+)), CD140a-positive (CD140a(+)), CD140b-positive (CD140b(+)), CD165-positive (CD165(+)), CD271-positive (CD271(+)) and CD326-negative (CD326(-)). The present invention has been completed on the basis of this finding.

More specifically, the present invention has the characteristics defined below.

[1] A method for producing renal progenitor cells into which pluripotent stem cells are induced to differentiate, the method comprising the steps of:

- (i) culturing the pluripotent stem cells under conditions that induce differentiation into renal progenitor cells; and
- (ii) sorting a cell population from the cells obtained at step (i), by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).

[2] The method as set forth in [1], wherein at step (ii), the sorting of a cell population is performed by using at least two cell surface markers.

[3] The method as set forth in [1], wherein at step (ii), the sorting of a cell population is performed by using at least three cell surface markers.

[4] The method as set forth in [1], wherein at step (ii), the sorting of a cell population is performed by using at least four cell surface markers.

[5] The method as set forth in [1], wherein at step (ii), at least two cell surface markers selected from the group consisting of CD9(-), CD140a(+), CD140b(+) and CD271(+) are used.

[6] The method as set forth in [5], wherein at step (ii), the sorting of a cell population is

performed by using at least three cell surface markers.

[7] The method as set forth in [4], wherein at step (ii), CD9(-), CD140a(+), CD140b(+) and CD271(+) are used as the cell surface markers.

[8] The method as set forth in any of [1] to [7], wherein the pluripotent stem cells are induced pluripotent stem (iPS) cells.

[9] The method as set forth in any of [1] to [7], wherein the pluripotent stem cells are human iPS cells.

[10] A renal progenitor cell population produced by the method as set forth in any of [1] to [9].

[11] A method for sorting a cell population from a renal progenitor cell-containing cell population by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).

[12] The method as set forth in [11], wherein the sorting of a cell population is performed by using at least two cell surface markers.

[13] The method as set forth in [11], wherein the sorting of a cell population is performed by using at least three cell surface markers.

[14] The method as set forth in [11], wherein the sorting of a cell population is performed by using at least four cell surface markers.

[15] The method as set forth in [11], wherein at least two cell surface markers selected from the group consisting of CD9(-), CD140a(+), CD140b(+) and CD271(+) are used.

[16] The method as set forth in [15], wherein the sorting of a cell population is performed by using at least three cell surface markers.

[17] The method as set forth in [11], wherein CD9(-), CD140a(+), CD140b(+) and CD271(+) are used as the cell surface markers.

[18] The method as set forth in any of [11] to [17], wherein the renal progenitor cells are renal progenitor cells into which pluripotent stem cells are induced to differentiate.

[19] The method as set forth in any of [11] to [17], wherein the pluripotent stem cells are induced pluripotent stem (iPS) cells.

[20] The method as set forth in any of [11] to [17], wherein the pluripotent stem cells are human iPS cells.

[21] A cell population acquired by the method as set forth in any of [11] to [20].

ADVANTAGEOUS EFFECTS OF INVENTION

[0009] According to the present invention, it has first become possible to acquire and produce a high-purity RPC population from a RPC population into which PSCs (*e.g.*, iPSCs) are induced to differentiate, by using a cell surface marker. The RPC population acquired by the method of this invention can be used in regenerative medicine for renal diseases such as renal failure.

BRIEF DESCRIPTION OF DRAWINGS

[0010] [FIG. 1] FIG. 1 shows a set of two-dimensional scatter plots of the flow cytometric measurements of the expression of CD9, CD55, CD106, CD140a, CD140b, CD165, CD271 and CD326 versus OSR1 and SIX2. The y-axis represents the fluorescence intensity of an antibody against each of the different cell surface markers, and the x-axis represents the fluorescence intensity of a fluorescence reporter representative of OSR1 or SIX2 protein expression.

[FIG. 2] FIG. 2 shows the results of the sorting of CD9(-)CD140a(+)CD140b(+)CD271(+) cells from hiPSC-derived differentiated cell groups as performed in Example 2. FIG. 2A shows a set of flow cytograms for hiPSCs-derived differentiated cell groups stained with CD9, CD140a, CD140b and CD271. The y- and x-axes represent the fluorescence intensities of antibodies against different cell surface markers. P6 depicts the presence of a CD9(-)CD140a(+) cell population; P4 depicts the presence of a CD9(-)CD140b(+) cell population; and P3 depicts the presence of a CD9(-)CD271(+) cell population. The P3+P4+P6 cell population depicted in FIG. 2A was fractionated. FIG. 2B shows a set of two-dimensional scatter plots of the flow cytometric measurements of OSR1 and SIX2 expression in hiPSCs before differentiation induction, in hiPSC-derived differentiated cell groups, and in the P3+P4+P6 cell population sorted from the hiPSC-derived differentiated cell groups, respectively in order from left to right panels. The y- and

x-axes represent the fluorescence intensities of fluorescence reporters representative of OSR1 or SIX2 protein expression. The percent values shown represent the percentages of OSR1/SIX2 double-positive cells (OSR1(+)SIX2(+) cells; upper right fraction), OSR1-positive, SIX2-negative cells (OSR1(+)SIX2(-) cells; upper left fraction), OSR1-negative, SIX2-positive cells (OSR1(-)SIX2(+) cells; lower right fraction), and OSR1/SIX2 double-negative cells (OSR1(-)SIX2(-) cells; lower left fraction).

[FIG. 3] FIG. 3 shows an example of the proximal renal tubule-like structures prepared in Example 3 from the CD9(-)CD140a(+)CD140b(+)CD271(+) cells fractionated from hiPSC-derived differentiated cell groups. FIG. 3A illustrates the differentiation procedure into proximal renal tubule, in which cell aggregates are formed and cocultured with Wnt4-expressing NIH3T3 fibroblasts according to the same procedure as disclosed in NPL 4. Cell aggregates composed of 1×10^5 cells were formed using a mouse ureteric bud cell (UBC)-conditioned medium supplemented with BMP7 and the Rho-kinase inhibitor Y-27632 (Wako; Cat. No. 253-00513). The next day, the culture medium was replaced with a mouse UBC-conditioned medium supplemented with BMP7, Y-27632, and the GSK-3 β inhibitor BIO (Wako; Cat. No. 029-16241), and the cells were further cultured for one day and then cocultured on top of Wnt4-expressing NIH3T3 fibroblasts treated with mitomycin C to induce the cells to differentiate into proximal renal tubule. FIG. 3B shows a microscopic image (upper left panel) of CD9(-)CD140a(+)CD140b(+)CD271(+) cell aggregates after coculture with Wnt4-expressing NIH3T3 fibroblasts, immunostaining images (upper right and lower left panels) of said cell aggregates, and a merged image of these images (lower right panel). Further, Lotus Tetragonolobus Lectin (LTL)-expressing cells are shown in enlarged views indicated by arrows. In these panels, LTL is a proximal renal tubule marker, Hoechst33342 is a cell nuclear staining dye, and the scale bar represents 100 μ m.

[FIG. 4] FIG. 4 shows the percentages of OSR1(+)SIX2(+) cells, OSR1(+)SIX2(-) cells, OSR1(-)SIX2(+) cells, and OSR1(-)SIX2(-) cells in cell populations sorted in Example 4 by all exhaustive combinations of the negative selection marker CD9 with any three of the positive selection markers. This figure also shows the percentages of OSR1(+)SIX2(+)

cells, OSR1(+)SIX2(-) cells, OSR1(-)SIX2(+) cells, and OSR1(-)SIX2(-) cells in cell populations sorted by other marker combinations in which the negative selection marker CD55 or CD326 was used instead of CD9.

[FIG. 5] FIG. 5 shows the following cell percentages: the percentage of OSR1(+)SIX2(+) cells in cell populations collected in Example 5 from an unsorted cell population using any combination of the cell surface markers CD9(-), CD140a(+), CD140b(+) and CD271(+); the percentage of cells demarcated by each of different cell marker combinations in the unsorted cell population; and the percentage of the number of OSR1(+)SIX2(+) cells collected using each of different surface marker combinations with respect to the number of unsorted cells.

[FIG. 6] FIG. 6 shows the results of inducing differentiation of a cell population sorted from a hiPSC 201B7 line-derived differentiated cell population using CD9(-)CD140a(+)CD140b(+)CD271(+) as an indicator into proximal renal tubule-like structures in Example 6. FIG. 6A shows a set of histograms of the number of cells as a function of the fluorescence intensity of each of OSR1 (detected by GFP), SIX2 (detected by tdTomato) and the different cell surface markers. FIG. 6B shows a set of scatter plots of the flow cytometric measurements of the expression of CD9 v.s. CD140a, CD140b or CD271 in an iPSC 201B7 line-derived differentiated cell population. The y- and x-axes represent the fluorescence intensities of antibodies against different cell surface markers.

CD9(-)CD140a(+)CD140b(+)CD271(+) cells were fractionated by sorting of gating (P4+P2+P3) fractions. FIG. 6C shows a merged image of the microscopic and immunostaining images of sorted CD9(-)CD140a(+)CD140b(+)CD271(+) cell aggregates after coculture with Wnt4-expressing NIH3T3 fibroblasts. In this panel, LTL is a proximal renal tubule marker (boxed), and the scale bar represents 100 μ m.

[FIG. 7] FIG. 7 shows the results of immunostaining of an iPSC 201B7 line-derived differentiated cell population with anti-SIX2 antibodies as performed in Example 7. FIG. 7A shows a set of immunostaining images of an unsorted cell population, and FIG. 7B shows a set of immunostaining images of a cell population sorted using

CD9(-)CD140a(+)CD140b(+)CD271(+) as an indicator. In these panels, the white solid arrows represent SIX2-positive cells, and the white outline arrows represent SIX2-negative cells. The scale bar represents 100 μ m. In both of FIGs. 7A and 7B, the left panel shows a light field image, the central panel shows an anti-SIX2 antibody-staining image, and the right panel shows a Hoechst33342 nuclear staining image.

DESCRIPTION OF EMBODIMENTS

[0011] Hereunder, the present invention will be described in detail.

[0012] The present invention provides a method for acquiring and producing a high-purity RPC population from a RPC population into which PSCs are induced to differentiate, by using a cell surface marker. To be specific, this invention includes the following method (hereinafter also referred to as “the production method of the present (this) invention”):

“A method for producing a renal progenitor cell population into which pluripotent stem cells are induced to differentiate, the method comprising the steps of:

- (i) culturing the pluripotent stem cells under conditions that induce differentiation into renal progenitor cells; and
- (ii) sorting a cell population from the cells obtained at step (i), by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).”

[0013] The present invention also provides a method for sorting a cell population from a RPC-containing cell population using a cell surface marker. To be specific, this invention includes the following method (hereinafter also referred to as “the sorting method of the present (this) invention”):

“A method for sorting a cell population from a renal progenitor cell-containing cell population using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).”

[0014] The present invention includes a renal progenitor cell population produced by the production method of this invention.

[0015] The present invention also includes a cell population acquired by the sorting method

of this invention.

[0016] The following provides descriptions of the present invention.

1. Step (i) of culturing PSCs under conditions that induce differentiation into a RPC population:

The procedure for inducing differentiation of PSCs into a RPC population, which can be used at this step, can be any procedure including but not limited to those disclosed in NPL 4 and PTL 4. At step (i), it is only necessary to obtain a cell population containing RPCs, since RPCs can be concentrated by the subsequent sorting at step (ii). The percent content of RPCs in the cell population obtained at step (i) is not of particular importance, and is, for example, not less than 5%, not less than 10%, not less than 15%, not less than 20%, not less than 25%, or not less than 30%.

[0017] In the present invention, RPCs are produced as a cell population of concentrated RPCs. The percent content of RPCs in the produced cell population is not particularly limited, and is, for example, not less than 50%, not less than 60%, not less than 65%, not less than 70%, not less than 71%, not less than 72%, not less than 73%, not less than 74%, or not less than 75%. Accordingly, as referred to in this invention, "sorting" means obtaining a cell population containing desired cells at a concentration of not less than 50%, not less than 60%, not less than 65%, not less than 70%, not less than 71%, not less than 72%, not less than 73%, not less than 74%, or not less than 75%.

[0018] As referred to in the present invention, the "renal progenitor cells (RPCs)" refers to cells partially transforming into renal tubule, and to OSR1/SIX2 double-positive (OSR1(+)/SIX2(+)) cells. As an example, OSR1 is a protein encoded by the human OSR1 gene (NCBI Accession No. NM_145260.2), and SIX2 is a protein encoded by the human SIX2 gene (NCBI Accession No. NM_016932.4). "OSR1-positive (OSR1(+))" means that OSR1 transcription activity is high, and more specifically means, for example, that OSR1 mRNA can be detected by a known method, that OSR1 protein can be detected by a known method (Examples 1, 2 and 4-6), or that the expression of a marker gene functionally linked to OSR1 promoter can be observed. Likewise, "SIX2-positive (SIX2(+))" means that SIX2

transcription activity is high, and more specifically means, for example, that SIX2 mRNA can be detected by a known method, that SIX2 protein can be detected by a known method (Examples 1, 2 and 4-6), or that the expression of a marker gene functionally linked to SIX2 promoter can be observed. As referred to in this invention, the “marker gene” refers to, for example, but is not limited to, a gene encoding a fluorescent protein.

[0019] As referred to in the present invention, the “pluripotent stem cells (PSCs)” refers to stem cells that not only have pluripotency, which is an ability to differentiate into many types of cells with different properties and morphologies as found in living organisms, but also have proliferative ability, and this term includes any types of cells that are able to be induced into RPCs. Examples of PSCs include, but are not particularly limited to, embryonic stem cells (ESCs), nuclear transfer embryonic stem cells (ntESCs), which are produced by using a nuclea transfer technique, germline stem cells (GSCs), embryonic germ cells (EGCs), induced pluripotent stem cells (iPSCs), and pluripotent cells derived from cultured fibroblasts or myeloid stem cells (multi-lineage differentiating stress enduring cells; Muse cells). Preferred PSCs are iPSCs, more preferably human iPSCs, from the viewpoint that such cells can be acquired without destroying the embryo, ovum or the like during the cell production process.

[0020] The methods for producing iPSCs are already known in the art, and iPSCs can be produced by introducing a reprogramming factor(s) into any type of somatic cells. As referred to herein, the “reprogramming factor(s)” refers to a gene(s) or gene product(s), such as Oct3/4, Sox2, Sox1, Sox3, Sox15, Sox17, Klf4, Klf2, c-Myc, N-Myc, L-Myc, Nanog, Lin28, Fbx15, ERas, ECAT15-2, Tcl1, beta-catenin, Lin28b, Sall1, Sall4, Esrrb, Nr5a2, Tbx3 or Glis1. Such reprogramming factors may be used alone or in combination. Exemplary combinations of reprogramming factors include those combinations disclosed in each of the following literatures: WO 2007/069666; WO 2008/118820; WO 2009/007852; WO 2009/032194; WO 2009/058413; WO 2009/057831; WO 2009/075119; WO 2009/079007; WO 2009/091659; WO 2009/101084; WO 2009/101407; WO 2009/102983; WO 2009/114949; WO 2009/117439; WO 2009/126250;

WO 2009/126251; WO 2009/126655; WO 2009/157593; WO 2010/009015; WO 2010/033906; WO 2010/033920; WO 2010/042800; WO 2010/050626; WO 2010/056831; WO 2010/068955; WO 2010/098419; WO 2010/102267; WO 2010/111409; WO 2010/111422; WO 2010/115050; WO 2010/124290; WO 2010/147395; WO 2010/147612; Huangfu D, *et al.*, *Nat. Biotechnol.*, (2008), 26: 795-797; Shi Y, *et al.*, *Cell Stem Cell*, (2008), 2: 525-528; Eminli S, *et al.*, *Stem Cells*, (2008), 26:2467-2474; Huangfu D, *et al.*, *Nat. Biotechnol.*, (2008), 26:1269-1275; Shi Y, *et al.*, *Cell Stem Cell*, (2008), 3: 568-574; Zhao Y, *et al.*, *Cell Stem Cell*, (2008), 3:475-479; Marson A, *Cell Stem Cell*, (2008), 3: 132-135; Feng B, *et al.*, *Nat. Cell Biol.*, (2009), 11:197-203; R. L. Judson *et al.*, *Nat. Biotechnol.*, (2009), 27:459-461; Lyssiotis C.A., *et al.*, *Proc. Natl. Acad. Sci. USA*, (2009), 106:8912-8917; Kim J.B., *et al.*, *Nature*, (2009), 461:649-643; Ichida J.K., *et al.*, *Cell Stem Cell*, (2009), 5:491-503; Heng J.C., *et al.*, *Cell Stem Cell*, (2010), 6:167-174; Han J, *et al.*, *Nature*, (2010), 463:1096-1100; Mali P, *et al.*, *Stem Cells*, (2010), 28:713-720; and Maekawa M, *et al.*, *Nature*, (2011), 474:225-229.

[0021] Non-limiting examples of somatic cells include not only all types of somatic cells from neonates and from healthy or affected individuals, but also all types of primary cultured cells, passaged cells and established cells derived from the aforementioned somatic cells. Specific examples of somatic cells include: (1) tissue stem cells (somatic stem cells), such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells, and dental pulp stem cells; (2) tissue progenitor cells; and (3) differentiated cells found in organs and tissues, such as blood cells (*e.g.*, peripheral blood cells, cord blood cells), muscle cells, skin cells, hair cells, hepatic cells, gastric mucosal cells, intestinal cells, splenic cells, pancreatic cells, brain cells, pulmonary cells, renal cells, and fat cells.

[0022] When iPSCs are used as a material for transplant cells, it is desirable to use somatic cells having the same, or substantially the same, human leucocyte antigen (HLA) genotype as a transplantation recipient, from the viewpoint that no episode of transplant rejection can occur. As referred to herein, the wording “substantially the same” in relation to human leucocyte antigen (HLA) genotype means that somatic cells have a matching in HLA

genotype to such an extent that immune response to transplanted cells can be suppressed with an immune suppressor. Examples of such somatic cells include those cells with the same HLA type at the three different loci of HLA-A, HLA-B and HLA-DR, or at the four different loci of HLA-A, HLA-B, HLA-DR and HLA-C.

[0023] In one embodiment, step (i) is performed using the procedure for inducing differentiation of PSCs into a RPC population as disclosed in NPL 4 and PTL 4. To be specific, step (i) involves the following steps (i-1) to (i-3):

(i-1) culturing PSCs in a culture medium supplemented with at least one substance selected from Activin A, GSK-3 β inhibitors, and retinoic acid derivatives;

(i-2) culturing a cell population obtained at step (i-1), in a culture medium supplemented with at least one substance selected from BMP7, GSK-3 β inhibitors, and retinoic acid derivatives; and

(i-3) culturing a cell population obtained at step (i-2), in a culture medium supplemented with a TGF β signal stimulator and a BMP inhibitor.

[0024] The following provides descriptions of steps (i-1) to (i-3).

[0025] Step (i-1) of culturing PSCs in a culture medium supplemented with at least one substance selected from Activin A, GSK-3 β inhibitors, and retinoic acid derivatives:

At this step, PSCs may be dissociated by any procedure known in the art and cultured by suspension culture or adhesion culture. Exemplary PSC dissociation procedures include mechanical dissociation, and dissociation using a dissociation solution with proteolytic and collagenolytic activities (*e.g.*, Accutase® and Accumax (Innovative Cell Technologies, Inc.)) or a dissociation solution with only collagenolytic activity. Preferred is a procedure in which PSCs are dissociated using a dissociation solution with proteolytic and collagenolytic activities and finely dispersed mechanically into single cells. The human PSCs to be preferably used at this step are PSC colonies cultured to 80% confluence per culture dish used.

[0026] The suspension culture to be used in the method of the present invention refers to culturing of cells while they are not adhered to a culture dish. The suspension culture can

be performed, but is not particularly limited to, using a vessel not artificially treated (*e.g.*, with extracellular matrix coating) to enhance the adhesion to cells, or a vessel artificially treated (*e.g.*, with poly(hydroxyethyl methacrylate) (poly-HEMA) coating) to prevent adhesion.

[0027] The adhesion culture to be used in the method of the present invention refers to culturing of cells while they are adhered to a culture dish. The adhesion culture can also be performed, but is not particularly limited to, in a coated culture dish. Exemplary coating agents include matrigel (BD Biosciences), Synthemax® (Corning), collagen, gelatin, laminin, heparan sulfate proteoglycan or entactin, and combinations thereof, with preference being given to matrigel, Synthemax® or gelatin.

[0028] The culture medium to be used at step (i-1) can be prepared by adding at least one substance selected from Activin A, GSK-3 β inhibitors, and retinoic acid derivatives to a basal medium for use in culturing animal cells. In one embodiment, the substances used at this step are a combination of Activin A and a GSK-3 β inhibitor, or a combination of a GSK-3 β inhibitor and a retinoic acid derivative. Examples of the basal medium include Iscove's modified Dulbecco's medium (IMDM), Medium 199, Eagle's minimum essential medium (EMEM), alpha-modified Eagle's minimum essential medium (α MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F12 (F12) medium, RPMI 1640 medium, Fischer's medium, and mixed media thereof. The culture medium may be supplemented with serum (*e.g.*, fetal bovine serum (FBS)) or may be serum-free. Depending on the need, the culture medium may be supplanted with at least one serum alternative such as albumin, knockout serum replacement (KSR) (Invitrogen), N2 supplement (Invitrogen), and/or B27 supplement (Invitrogen), or may also be supplemented with at least one substance such as transferrin, fatty acids, insulin, collagen precursors, trace elements, 2-mercaptoethanol, 3'-thiol glycerol, lipids, amino acids, L-glutamine, GlutaMAX (Invitrogen), non-essential amino acids (NEAAs), vitamins, growth factors, low-molecular compounds, antibiotics, antioxidants, pyruvic acid, buffers, and/or inorganic salts. In one embodiment of this step, the basal medium is a DMEM/F12 (1:1) mixed medium supplemented with GlutaMAX,

serum and an antibiotic.

[0029] Examples of Activin A that can be used at step (i-1) include Activin A proteins derived from humans and other animals, and functional variants thereof, as exemplified by Activin A products commercially available from R&D Systems and other manufacturers. The concentration of Activin A used at this step is in the range of 1 ng/mL to 1000 ng/mL, preferably 10 ng/mL to 500 ng/mL, more preferably 50 ng/mL to 200 ng/mL.

[0030] The GSK-3 β inhibitor to be used at step (i-1) is not particularly limited as long as it is capable of inhibiting GSK-3 β functions such as kinase activity. Exemplary GSK-3 β inhibitors include: the indirubin derivative BIO (also named as GSK-3 β Inhibitor IX; 6-bromoindirubin-3'-oxime); the maleimide derivative SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione); the phenyl α -bromomethyl ketone compound GSK-3 β Inhibitor VII (2,4'-dibromoacetophenone); the cell membrane-permeable phosphorylation peptide L803-mts (also named as GSK-3 β Peptide Inhibitor; Myr-N-GKEAPPAPPQSpP-NH₂ (SEQ ID NO:15)); and the highly selective GSK inhibitor CHIR99021 (6-[[[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile) (*Nature*, (2008), 453: 519-523). The compounds listed above are available from Stemgent, Calbiochem, Biomol and other manufacturers, or may be prepared on one's own. A preferred example of the GSK-3 β inhibitor to be used at this step is CHIR99021. The concentration of the GSK-3 β inhibitor used at this step can be selected by one skilled in the art as appropriate depending on the type of the GSK-3 β inhibitor to be used. For example, when CHIR99021 is used as a GSK-3 β inhibitor, the concentration of this inhibitor is in the range of 0.01 μ M to 100 μ M, preferably 0.1 μ M to 10 μ M, more preferably 1 μ M to 3 μ M.

[0031] The retinoic acid derivative to be used at step (i-1) is an optionally artificially modified retinoic acid that maintains the functions of naturally occurring retinoic acid. Exemplary retinoic acid derivatives include retinoid compounds and vitamin A compounds. Examples of retinoid compounds include retinoic acids, 3-dehydroretinoic acid, 4-[[[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbonyl]amino]-benzoic acid (AM580)

(Tamura K, *et al.*, *Cell Differ. Dev.*, (1990), 32: 17-26), 4-[(1E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl]-benzoic acid (TTNPB) (Strickland S, *et al.*, *Cancer Res.*, (1983), 43: 5268-5272), those retinoid compounds disclosed in Takenaga, K. *et al.*, *Cancer Res.*, (1980), 40: 914-919, retinol palmitate, retinol, retinal, 3-dehydroretinol, and 3-dehydroretinal. Retinoic acid compounds refer to retinoid compounds having a carboxyl group, as exemplified by retinoic acids, 3-dehydroretinoic acid, AM580, and TTNPB. In one embodiment of this step, the retinoic acid derivative is a retinoid compound or a vitamin A compound. In another embodiment of this step, the retinoic acid derivative is a retinoic acid compound. In yet another embodiment of this step, the retinoic acid derivative is a vitamin A compound. A preferred example of the retinoic acid derivative to be used at this step is AM580 or TTNPB. The concentration of the retinoic acid derivative used at this step can be selected by one skilled in the art as appropriate depending on the type of the retinoic acid derivative to be used. For example, when AM580 or TTNPB is used as a retinoic acid derivative, the concentration of this derivative is in the range of 0.01 μ M to 100 μ M, preferably 0.1 μ M to 10 μ M, more preferably 0.5 μ M to 2 μ M.

[0032] The culture medium used at step (i-1) may be further supplemented with a ROCK inhibitor. In particular, when this step involves dispersing PSCs into single cells, it is preferred that the culture medium be supplemented with a ROCK inhibitor.

[0033] The type of a ROCK inhibitor is not particularly limited as long as it is capable of inhibiting the functions of Rho-kinase (ROCK). Exemplary ROCK inhibitors include Y-27632 (*e.g.*, Ishizaki *et al.*, *Mol. Pharmacol.*, (2000), 57, 976-983; Narumiya *et al.*, *Methods Enzymol.*, (2000), 325, 273-284), Fasudil/HA1077 (*e.g.*, Uehata *et al.*, *Nature*, (1997), 389: 990-994), H-1152 (*e.g.*, Sasaki *et al.*, *Pharmacol. Ther.*, (2002), 93: 225-232), Wf-536 (*e.g.*, Nakajima *et al.*, *Cancer Chemother. Pharmacol.*, (2003), 52(4): 319-324) and derivatives thereof, as well as antisense nucleic acids, RNA interference-triggering nucleic acids (*e.g.*, siRNA) and dominant-negative variants, which target ROCK, and expression vectors thereof. Other known low-molecular compounds can also be used as ROCK inhibitors (refer to, *e.g.*, U.S. Patent Application Publication Nos. US 2005/0209261, US

2005/0192304, US 2004/0014755, US 2004/0002508, US 2004/0002507, US 2003/0125344 and US 2003/0087919, and International Patent Publication Nos. WO 2003/062227, WO 2003/059913, WO 2003/062225, WO 2002/076976 and WO 2004/039796). In the present invention, one or two or more ROCK inhibitors can be used. A preferred example of the ROCK inhibitor to be used at this step is Y-27632. The concentration of the ROCK inhibitor used at this step can be selected by one skilled in the art as appropriate depending on the type of the ROCK inhibitor to be used. For example, when Y-27632 is used as a ROCK inhibitor, the concentration of this inhibitor is in the range of 0.1 μ M to 100 μ M, preferably 1 μ M to 50 μ M, more preferably 5 μ M to 20 μ M.

[0034] The cell culture at step (i-1) is performed at a culture temperature of, but not limited to, about 30 to 40°C, preferably about 37°C, and in an atmosphere of CO₂-containing air. The CO₂ concentration is in the range of about 2 to 5%, preferably about 5%. The culture period at this step is for example not longer than 2 days, preferably 2 days.

[0035] Step (i-2) of culturing a cell population obtained at step (i-1), in a culture medium supplemented with at least one substance selected from BMP7, GSK-3 β inhibitors, and retinoic acid derivatives:

At this step, the population of suspension cultured cells obtained at step (i-1) described above may be adhesion cultured, as it is, in a given culture medium in a coated culture dish, or the population of adhesion cultured cells obtained at step (i-1) may be continued to be cultured through replacement of a culture medium.

[0036] The culture medium to be used at step (i-2) can be prepared by adding at least one substance selected from BMP7, GSK-3 β inhibitors, and retinoic acid derivatives to a basal medium for use in culturing animal cells. In one embodiment, the substance(s) used at this step is(are) a combination of BMP7 and a GSK-3 β inhibitor, or a retinoic acid derivative. Examples of the basal medium include IMDM, Medium 199, EMEM, α MEM, DMEM, Ham's F12 medium, RPMI 1640 medium, Fischer's medium, and mixed media thereof. The culture medium may be supplemented with serum (*e.g.*, FBS) or may be serum-free. Depending on the need, the culture medium may be supplanted with at least one serum

alternative such as albumin, KSR (Invitrogen), N2 supplement (Invitrogen), and/or B27 supplement (Invitrogen), or may also be supplemented with at least one substance such as transferrin, fatty acids, insulin, collagen precursors, trace elements, 2-mercaptoethanol, 3'-thiol glycerol, lipids, amino acids, L-glutamine, GlutaMAX (Invitrogen), non-essential amino acids (NEAAs), vitamins, growth factors, antibiotics, antioxidants, pyruvic acid, buffers, inorganic salts and equivalents thereto. In one embodiment of this step, the basal medium is a DMEM/F12 medium supplemented with GlutaMAX, KSR, NEAAs, 2-mercaptoethanol and an antibiotic.

[0037] At step (i-2), for example, the cell population obtained at step (i-1) may be cultured in a culture medium supplemented with at least one substance selected from BMP7 and GSK-3 β inhibitors, and then further cultured in a culture medium supplemented with a retinoic acid derivative. Preferably, step (i-2) involves culturing the cell population obtained at step (i-1), in a culture medium supplemented with at least one substance selected from BMP7 and GSK-3 β inhibitors, and then further culturing the cell population in a culture medium supplemented with a retinoic acid derivative and a TGF β signal stimulator. In other words, step (i-2) may be performed by taking the following two separate steps (i-2-a) and (i-2-b): (i-2-a) culturing the cell population in a culture medium supplemented with at least one substance selected from BMP7 and GSK-3 β inhibitors; and (i-2-b) culturing the cell population in a culture medium supplemented with a retinoic acid derivative and a TGF β signal stimulator.

More preferably, step (i-2) involves the step (i-2-a) of culturing the cell population in a culture medium supplemented with BMP7 and a GSK-3 β inhibitor, and the step (i-2-b) of culturing the cell population in a culture medium supplemented with a retinoic acid derivative and a TGF β signal stimulator.

[0038] Examples of BMP7 that can be used at step (i-2) include human BMP7 (NCBI Accession No. NM_001719.2) and BMP7 proteins derived from other animals, and functional variants thereof (variants that maintain a differentiation induction ability), as exemplified by BMP7 products commercially available from Invitrogen, R&D Systems and

other manufacturers. The concentration of BMP7 used at this step is in the range of 1 ng/mL to 1000 ng/mL, preferably 10 ng/mL to 500 ng/mL, more preferably 50 ng/mL to 200 ng/mL.

[0039] Examples of the GSK-3 β inhibitor that can be used at step (i-2) include those inhibitors mentioned above in relation to step (i-1). A preferred example of the GSK-3 β inhibitor is CHIR99021. The concentration of the GSK-3 β inhibitor used at this step can be selected by one skilled in the art as appropriate depending on the type of the GSK-3 β inhibitor to be used. For example, when CHIR99021 is used as a GSK-3 β inhibitor, the concentration of this inhibitor is in the range of 0.01 μ M to 100 μ M, preferably 0.1 μ M to 10 μ M, more preferably 1 μ M to 3 μ M.

[0040] Examples of the retinoic acid derivative that can be used at step (i-2) include those derivatives mentioned above in relation to step (i-1). A preferred example of the retinoic acid derivative is AM580 or TTNPB. The concentration of the retinoic acid derivative used at this step can be selected by one skilled in the art as appropriate depending on the type of the retinoic acid derivative to be used. For example, when AM580 or TTNPB is used as a retinoic acid derivative, the concentration of this derivative is in the range of 0.01 μ M to 100 μ M, preferably 0.1 μ M to 10 μ M, more preferably 0.5 μ M to 2 μ M.

[0041] The type of the TGF β signal stimulator to be used at step (i-2) is not particularly limited as long as it is capable of activating TGF β signal pathway. Exemplary TGF β signal stimulators include proteins such as TGF β 1, TGF β 2 and TGF β 3 (available from Peprotech, R&D, and other manufacturers), and compounds such as IDE1 (1-[2-[(2-carboxyphenyl)methylene]hydrazide]heptanoic acid) and IDE2 (1-(2-cyclopentylidenehydrazide)-heptanedioic acid) (Borowiak M, *et al.*, *Cell Stem Cell*, (2009), 4: 348-358). IDE1 and IDE2 are available from Stemgent, Tocris and other manufacturers. A preferred example of the TGF β signal stimulator is TGF β 1. The concentration of the TGF β signal stimulator used at this step can be selected by one skilled in the art as appropriate depending on the type of the TGF β signal stimulator to be used. For example, when any of proteins such as TGF β 1, TGF β 2 and TGF β 3 is used as a TGF β signal

stimulator, the concentration of this stimulator is in the range of 0.1 ng/mL to 100 ng/mL, preferably 1 ng/mL to 10 ng/mL, more preferably 5 ng/mL to 10 ng/mL. When any of IDE1 and IDE2 is used as a TGF β signal stimulator, the concentration of this stimulator is in the range of 1 μ M to 100 μ M, preferably 25 μ M to 75 μ M, more preferably 40 μ M to 60 μ M. [0042] The cell culture at step (i-2) is performed at a culture temperature of, but not limited to, about 30 to 40°C, preferably about 37°C, and in an atmosphere of CO₂-containing air. The CO₂ concentration is in the range of about 2 to 5%, preferably about 5%. The culture period at this step is for example not shorter than 3 days, preferably not shorter than 3 days and not longer than 12 days, more preferably not shorter than 3 days and not longer than 9 days. During this step, it is desirable to replace a culture medium every 3 days. When step (i-2) involves steps (i-2-a) and (i-2-b), the total culture period at step (i-2) is as described above, and more particularly, the culture period at step (i-2-a) is for example not shorter than 1 day, preferably not shorter than 2 days and not longer than 11 days, more preferably not shorter than 2 days and not longer than 6 days, and the culture period at step (i-2-b) is for example not shorter than 1 day, preferably not shorter than 2 days and not longer than 11 days, more preferably not shorter than 3 days and not longer than 6 days. During these steps, it is desirable to replace a culture medium every 3 days.

[0043] By taking the steps (i-1) and (i-2) as described above, PSCs can be induced into intermediate mesodermal cells. OSR1 is known as a marker characterizing intermediate mesodermal cells. In one embodiment, a cell population induced by following steps (i-1) and (i-2) contains a large number of OSR1-positive SIX2-negative (OSR1(+)/SIX2(-)) intermediate mesodermal cells, but may also contain OSR1/SIX2 double-positive (OSR1(+)/SIX2(+)) renal progenitor cells. Accordingly, step (i) may be completed by taking the steps (i-1) and (i-2) as described above, but from the viewpoint of increasing the content of RPCs, it is desirable to take not only the above two steps but also step (i-3).

[0044] Step (i-3) of culturing the cell population obtained at step (i-2), in a culture medium supplemented with a TGF β signal stimulator and a BMP inhibitor:

At this step, the (intermediate mesodermal) cell population obtained at steps (i-1)

and (i-2) as described above can be cultured by suspension or adhesion culture as they are, or after being dissociated by any procedure known in the art. Exemplary cell dissociation procedures include mechanical dissociation, and dissociation using a dissociation solution with proteolytic and collagenolytic activities (*e.g.*, Accutase® and Accumax (Innovative Cell Technologies, Inc.)) or a dissociation solution with only collagenolytic activity.

[0045] The culture medium to be used at step (i-3) can be prepared by adding a TGFβ signal stimulator and a BMP inhibitor to a basal medium for use in culturing animal cells. Examples of the basal medium include IMDM, Medium 199, EMEM, αMEM, DMEM, Ham's F12 medium, RPMI 1640 medium, Fischer's medium, and mixed media thereof. The culture medium may be supplemented with serum (*e.g.*, FBS) or may be serum-free. Depending on the need, the culture medium may be supplanted with at least one serum alternative such as albumin, KSR (Invitrogen), N2 supplement (Invitrogen), and/or B27 supplement (Invitrogen), or may also be supplemented with at least one substance such as transferrin, fatty acids, insulin, collagen precursors, trace elements, 2-mercaptoethanol, 3'-thiol glycerol, lipids, amino acids, L-glutamine, GlutaMAX (Invitrogen), non-essential amino acids (NEAAs), vitamins, growth factors, antibiotics, antioxidants, pyruvic acid, buffers, inorganic salts and equivalents thereto. In one embodiment of this step, the basal medium is a DMEM/F12 medium supplemented with GlutaMAX, KSR, NEAAs, 2-mercaptoethanol and an antibiotic.

[0046] The type of the TGFβ signal stimulator to be used at step (i-3) is not particularly limited as long as it is capable of activating TGFβ signal pathway. Exemplary TGFβ signal stimulators include proteins such as TGFβ1, TGFβ2 and TGFβ3, and compounds such as IDE1 and IDE2. A preferred example of the TGFβ signal stimulator is TGFβ1. The concentration of the TGFβ signal stimulator used at this step can be selected by one skilled in the art as appropriate depending on the type of the TGFβ signal stimulator to be used. For example, when any of proteins such as TGFβ1, TGFβ2 and TGFβ3 is used as a TGFβ signal stimulator, the concentration of this stimulator is in the range of 0.1 ng/mL to 100 ng/mL, preferably 1 ng/mL to 10 ng/mL, more preferably 5 ng/mL to 10 ng/mL. When any of

IDE1 and IDE2 is used as a TGF β signal stimulator, the concentration of this stimulator is in the range of 1 μ M to 100 μ M, preferably 25 μ M to 75 μ M, more preferably 40 μ M to 60 μ M.

[0047] The type of the BMP inhibitor to be used at step (i-3) is not particularly limited as long as it is capable of activating BMP signal pathway. Exemplary BMP inhibitors include proteinaceous inhibitors such as chordin, noggin and follistatin, dorsomorphin (6-[4-(2-piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine) and derivatives thereof (Yu *et al.*, *Circulation*, (2007), 116:II_60; Yu *et al.*, *Nat. Chem. Biol.*, (2008), 4:33-41; J. Hao *et al.*, *PLoS ONE*, (2008), 3:e2904), DMH1 (4-[6-(4-isopropoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline, 4-[6-[4-(1-methylethoxy)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]quinoline), and LDN193189 (4-(6-(4-(piperidin-1-yl)phenyl)pyrazolo[1,5-a]pyrimidin-3-yl)quinoline). The compounds listed above are available from Stemgent, Tocris Bioscience, Merck Life Science, Wako, and other manufacturers, or may be prepared on one's own. Preferred examples of the BMP inhibitor include DMH1, LDN193189, noggin, and dorsomorphin, and a more preferred example thereof is DMH1. The concentration of the BMP inhibitor used at this step can be selected by one skilled in the art as appropriate depending on the type of the BMP inhibitor to be used. For example, when any of proteinaceous inhibitors such as chordin, noggin and follistatin is used as a BMP inhibitor, the concentration of this inhibitor is in the range of 0.1 ng/mL to 1000 ng/mL, preferably 1 ng/mL to 500 ng/mL, more preferably 10 ng/mL to 100 ng/mL. When any of dorsomorphin, LDN193189 and DMH1 is used as a BMP inhibitor, the concentration of this inhibitor is in the range of 0.01 μ M to 100 μ M, preferably 0.1 μ M to 10 μ M, more preferably 0.5 μ M to 1 μ M.

[0048] In one embodiment, the combination of a TGF β signal stimulator and a BMP inhibitor to be used at step (i-3) is a combination of TGF β 1 and DMH1.

[0049] At the cell culture step of (i-3), the basal medium may be further supplemented with any, or any combination, of fibroblast growth factor (FGF) 9, FGF20, BMP7, a retinoic acid derivative, and a GSK-3 β inhibitor.

[0050] There is no upper limit on the number of days of cell culture at step (i-3), since no

particular influence is exerted by long-term cell culture on the efficiency of RPC population production. For example, the number of days of cell culture is not less than 2 days, not less than 4 days, not less than 6 days, not less than 8 days, not less than 10 days, not less than 11 days, not less than 12 days, not less than 13 days, not less than 14 days, not less than 15 days, not less than 16 days, not less than 17 days, not less than 18 days, not less than 19 days, or not less than 20 days.

[0051] The cell culture at step (i-3) is performed at a culture temperature of, but not limited to, about 30 to 40°C, preferably about 37°C, and in an atmosphere of CO₂-containing air. The CO₂ concentration is in the range of about 2 to 5%, preferably about 5%.

[0052] 2. Step (ii) of sorting a cell population from the cells obtained at step (i), by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-), as well as the sorting method of the present invention:

At step (ii) and in the sorting method of the present invention (hereinafter in this section referred to collectively as “step (ii)”), a more highly purified cell population is sorted from the cells obtained at step (i), by using as an indicator the presence or absence of at least one cell surface marker selected from the group consisting of CD9, CD55, CD106, CD140a, CD140b, CD165, CD271 and CD326. More specifically, cell sorting is performed using an indicator requiring that cells be positive for CD106, CD140a, CD140b, CD165 and CD271, and negative for CD9, CD55 and CD326. In the present specification, the plus (+) symbol used for certain cell surface markers means that cells are positive for the certain antigens, and CD106(+), CD140a(+), CD140b(+), CD165(+) and CD271(+) are referred to as “positive selection markers”. In this specification, the minus (-) symbol used for certain cell markers means that cells are negative for the certain antigens, and CD9(-), CD55(-) and CD326(-) are referred to as “negative selection markers”. Also, the positive and negative selection markers may be referred to collectively as “cell surface markers”.

[0053] In a preferred mode, the combination of cell surface markers to be used at step (ii) can be a combination of at least two, three or four cell surface markers selected from the

group consisting of CD9, CD55, CD106, CD140a, CD140b, CD165, CD271 and CD326. For example, the combination of at least two cell surface markers preferably comprises a combination of one negative selection marker selected from the group consisting of CD9(-), CD55(-) and CD326(-) (preferably CD9(-)) and one positive selection marker selected from the group consisting of CD106(+), CD140a(+), CD140b(+), CD165(+) and CD271(+) (preferably the group consisting of CD140a(+), CD140b(+) and CD271(+)). The combination of at least three cell surface markers preferably comprises: a combination of one negative selection marker selected from the group consisting of CD9(-), CD55(-) and CD326(-) (preferably CD9(-)) and two positive selection markers selected from the group consisting of CD106(+), CD140a(+), CD140b(+), CD165(+) and CD271(+) (preferably the group consisting of CD140a(+), CD140b(+) and CD271(+)); or a combination of three positive selection markers selected from the group consisting of CD106(+), CD140a(+), CD140b(+), CD165(+) and CD271(+). The combination of at least four cell surface markers preferably comprises a combination of one negative selection marker selected from the group consisting of CD9(-), CD55(-) and CD326(-) (preferably CD9(-)) and three positive selection markers selected from the group consisting of CD106(+), CD140a(+), CD140b(+), CD165(+) and CD271(+). In one embodiment, the cell surface markers to be used at step (ii) are at least two or three cell surface markers selected from the group consisting of CD9(-), CD140a(+), CD140b(+) and CD271(+), preferably a combination of CD9(-), CD140a(+), CD140b(+) and CD271(+).

[0054] The cell surface markers for use at step (ii) can be used for sorting a cell population of RPCs derived from mammals including, but not limited to, humans. In the case of sorting a cell population of human RPCs, the NCBI accession numbers of the eight different human cell surface markers are as follows.

CD9: NM_001769.3 (SEQ ID NO:1)

CD55: NM_000574.4 (SEQ ID NO:3)

CD106: NM_001078.3 (SEQ ID NO:5)

CD140a: NM_006206.4 (SEQ ID NO:7)

CD140b: NM_002609.3 (SEQ ID NO:9)

CD165: geneID_23449

CD271: NM_002507.3 (SEQ ID NO:11)

CD326: NM_002354.2 (SEQ ID NO:13)

[0055] The different human cell surface markers include genes having the nucleotide sequences of the corresponding accession numbers mentioned above, proteins encoded by said genes, and naturally occurring variants thereof.

[0056] Human CD9 is encoded by the gene having the nucleotide sequence of SEQ ID NO:1 (nucleotides 185 to 871), and has the amino acid sequence of SEQ ID NO:2. Human CD55 is encoded by the gene having the nucleotide sequence of SEQ ID NO:3 (nucleotides 295 to 1440), and has the amino acid sequence of SEQ ID NO:4. Human CD106 is encoded by the gene having the nucleotide sequence of SEQ ID NO:5 (nucleotides 222 to 2441), and has the amino acid sequence of SEQ ID NO:6. Human CD140a is encoded by the gene having the nucleotide sequence of SEQ ID NO:7 (nucleotides 332 to 3601), and has the amino acid sequence of SEQ ID NO:8. Human CD140b is encoded by the gene having the nucleotide sequence of SEQ ID NO:9 (nucleotides 470 to 3790), and has the amino acid sequence of SEQ ID NO:10. Human CD271 is encoded by the gene having the nucleotide sequence of SEQ ID NO:11 (nucleotides 126 to 1409), and has the amino acid sequence of SEQ ID NO:12. Human CD326 is encoded by the gene having the nucleotide sequence of SEQ ID NO:13 (nucleotides 359 to 1303), and has the amino acid sequence of SEQ ID NO:14. Human CD165 is a 37-42 kDa membrane surface protein, also known as AD2 or gp37. As an antibody specifically binding to CD165, the monoclonal antibody SN2 has been identified (Seon, B. K., *et al.*, *J. Immunol.*, (1984), 132:2089-2095), and is now commercially available.

[0057] The naturally occurring variants of the aforementioned cell surface markers, in particular, CD9, CD55, CD106, CD140a, CD140b, CD271 and CD326, refer to cell surface markers encoded by genes whose nucleotide sequences have an identity of at least 80%, preferably at least 85%, at least 90%, at least 95%, or at least 98%, to the aforementioned

nucleotide sequences, or cell surface markers comprising amino acid sequences having an identity of at least 80%, preferably at least 85%, at least 90%, at least 95%, or at least 98%, to the aforementioned amino acid sequences.

[0058] The “identity”, as referred to herein with regard to nucleotide sequences or amino acid sequences, refers to an Identity value obtained by searching with the NEEDLE program (Needleman S. B., *et al.*, *J. Mol. Biol.*, (1970), 48: 443-453) using the parameters available by default. The default parameters are as defined below.

Gap penalty = 10

Extend penalty = 0.5

Matrix = EBLOSUM62

[0059] Specific antibodies binding to the human cell surface antigens mentioned above are commercially available. Examples of such antibodies include those mentioned below in the Examples section. Cell surface markers maintaining the characteristic in that they are bound by commercial antibodies capable of specifically binding to each of the aforementioned cell surface markers are also included by the cell surface markers that can be used in sorting a cell population in the present invention.

[0060] The sorting of a cell population using a cell surface marker at step (ii) can be performed by any of various procedures known in the art. For example, cell sorting is performed using an antibody specifically binding to a cell surface marker, based on the binding of said antibody to the cells. Examples of such antibody-based sorting procedures include cell sorting with a cell sorter using a fluorescently labeled antibody (*e.g.*, FACS® (BD Biosciences)), magnetic cell sorting using antibody-labeled magnetic beads (*e.g.*, MACS® (Miltenyi Biotec)), and cell sorting using an antibody-immobilized carrier (*e.g.*, cell enrichment column).

[0061] 3. Therapeutic agent for renal diseases, renal disease treatment method, and method for producing cells for renal disease treatment:

The RPC population acquired by the method of the present invention can be used as a therapeutic agent for renal diseases. Accordingly, this invention provides the following

method for producing cells for renal disease treatment and the following method for sorting a cell population for renal disease treatment:

“A method for producing cells for renal disease treatment, the method comprising the steps of:

- (i) culturing pluripotent stem cells under conditions that induce differentiation into renal progenitor cells; and
- (ii) sorting a cell population from the cells obtained at step (i), by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).”

[0062] “A method for sorting a cell population for renal disease treatment from a renal progenitor cell-containing cell population using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).”

[0063] As referred to in the present invention, the cells for renal disease treatment are cells characterized by at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-), preferably cells characterized by CD9(-), CD140a(+), CD140b(+) and CD271(+).

[0064] The present invention provides a therapeutic agent for renal diseases comprising a RPC population produced by the production method of this invention or a RPC population sorted by the sorting method of this invention, as well as a method for treating a renal disease, the method comprising the step of administering to a patient a RPC population produced by the production method of this invention or a RPC population sorted by the sorting method of this invention. Exemplary procedures for administering a RPC population or a therapeutic agent to a patient include: application of a cell sheet made from an acquired RPC population onto the kidney of a patient; transplantation of an acquired RPC population suspended in physiological saline, etc. into the kidney of a patient directly or through blood vessels; transplantation of RPC aggregates obtained by three-dimensional culture on a scaffold formed of matrigel, etc.; loading a RPC population into a dialysis column; and administration

of microcapsules encapsulating a RPC population. Exemplary renal diseases include chronic renal diseases including conditions not reaching chronic renal failure.

[0065] In the present invention, the number of RPCs contained in a therapeutic agent for renal diseases can be controlled to be increased or decreased as appropriate depending on the severity of disease, the size of an affected site, and/or body size.

EXAMPLES

[0066] Hereunder, the present invention will be described more specifically by way of examples, but the scope of this invention is not limited to these examples.

[0067] [Example 1]

<Two-dimensional scatter plotting of a RPC population by flow cytometry of OSR1 and SIX2 and other different cell surface markers>

The iPS cells used in this example were OSR1-GFP & SIX2-tdTomato reporter human iPS cell lines generated by the procedure disclosed in NPL 3, which are capable of expressing GFP in conjunction with endogenous OSR1 gene expression and expressing tdTomato in conjunction with endogenous SIX2 gene expression. The human iPSC cell lines were induced to differentiate into a RPC-containing cell population by following the procedure disclosed in NPL 4. As a result of searching for cell surface markers specifically expressed in RPCs using Human Cell Surface Marker Screening Panel (BD Biosciences, Cat. No. 560747), CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-) were identified as cell surface markers that enable clear fractionation of the RPC-containing cell population of interest (FIG. 1).

[0068] [Example 2]

<CD9(-)CD140a(+), CD140b(+), CD271(+) cell population contains at least 70% RPCs>

In this example, it was studied whether the percentage of RPCs present in cells fractionated from a cell population differentiated from human iPSCs can be increased by using a combination of some of the cell surface markers identified in Example 1.

[0069] First, it was studied whether the percentage of RPCs present in a cell population

differentiated from human iPSCs can be increased by using one negative selection marker and three positive selection markers. The cell surface markers selected were CD9, CD140a, CD140b and CD271, which provided clear images of a discrete cell population in the two-dimensional scatter plots drawn as a function of OSR1 and SIX2 in Example 1. As antibodies against these cell surface markers, APC-H7 Mouse Anti Human CD9 (BD Biosciences; Cat. No. 655409), Alexa Fluor® 647 Mouse Anti-Human CD140a (BD Biosciences; Cat. No. 562798), BV421 Mouse Anti-Human CD140b (BD Biosciences; Cat. No. 564124), and BV510 Mouse Anti-Human CD271 (BD Biosciences; Cat. No. 563451) were used. The antibodies were used at 20-fold dilution, and cells were reacted with the antibodies at room temperature for 15 minutes. After the reaction, the reaction mixture was washed twice with PBS supplemented with 2% FBS, and analyzed using FACS Aria™ III (BD Biosciences). Based on the resulting two-dimensional scatter plots, the CD9(-)CD140a(+)CD140b(+)CD271(+) cell fraction was demarcated to thereby fractionate said cell population. The fractionated cell population was analyzed again using FACS Aria™ III, and the results showed that OSR1(+)SIX2(+) cells were present at a concentration of more than 70% in the entire cell population (FIG. 2B). FIG. 2A shows a set of flow cytograms for hiPSCs-derived differentiated cell groups stained with CD9, CD140a, CD140b and CD271. FIG. 2B shows a set of two-dimensional scatter plots of the flow cytometric measurements of OSR1 and SIX2 expression in hiPSCs before differentiation induction, in hiPSC-derived differentiated cell groups, and in a cell population after P3+P4+P6 cell sorting, respectively in order from left to right panels.

[0070] [Example 3]

<Formation of renal tubule-like structures positive for LTL (proximal renal tubule marker) from the cell population sorted by CD9(-), CD140a(+), CD140b(+) and CD271(+)>

RPCs are characterized by not only their expression of RPC markers but also by their ability to maintain differentiation potential into renal tubular cells. Accordingly, for the purpose of confirming whether the CD9(-)CD140a(+)CD140b(+)CD271(+) cells obtained in Example 2 were RPCs, the obtained cells were tested in a system for inducing

differentiation into renal tubule. Differentiation into renal tubular cells and formation of renal tubule-like structures were determined based on expression of the proximal renal tubule marker LTL and morphological characteristics.

[0071] According to the procedure disclosed in NPL 4, the RPC-containing cell population into which the hiPSC lines had been induced to differentiate were immunostained with APC-H7 Mouse Anti Human CD9, Alexa Fluor® 647 Mouse Anti-Human CD140a, BV421 Mouse Anti-Human CD140b, and BV510 Mouse Anti-Human CD271 to fractionate the CD9(-)CD140a(+)CD140b(+)CD271(+) cells on FACS Aria™ III. The fractionated cells were seeded at 1.0×10^5 cells/well on 96-well low-cell-adhesion spindle-bottom plates (Sumitomo Bakelite; Cat. No. MS-9096M) containing a UBC-conditioned medium (see below) supplemented with 50 ng/mL BMP7 (R&D; Cat. No. 354-BP-010) and 10 μ M Y-27632 (Wako; Cat. No. 253-00513), and were cultured for 24 hours at 37°C in an atmosphere of 5% CO₂-containing air. Next, the cells were cultured for another 24 hours, with the culture medium being replaced with a UBC-conditioned medium supplemented with 50 ng/mL BMP7, 0.5 μ M BIO (Calbiochem; Cat. No. 361552) and 10 μ M Y-27632. Then, the cells were cocultured with Wnt4-expressing NIH3T3 fibroblasts according to the procedure disclosed in NPL 4. The Wnt4-expressing NIH3T3 fibroblasts were used after they had been seeded at 4.0×10^5 cells/well on 24-well plates and treated with mitomycin C. The coculture was performed using a UBC-conditioned medium. After 2 weeks of coculture, cell staining was done. During the cell staining process, LTL-Biotin Conjugate (Vector Laboratories; Cat. No. B-1325) was used for primary reaction; Streptavidin-Alexa Fluor® 546 Conjugate (Life Technologies; Cat. No. S-11225) was used for secondary reaction; and Hoechst 33342 (Life Technologies; Cat. No. H3570) was used for nuclear staining. LTL was used at 200-fold dilution, and the reaction with LTL was done at 4°C overnight. Streptavidin-Alexa Fluor® 546 Conjugate was used at 200-fold dilution, and the reaction with this conjugate was done at room temperature for one hour. As a result, it was observed that LTL-positive luminal structures were formed from the CD9(-)CD140a(+)CD140b(+)CD271(+) cell population (FIG. 3B). FIG. 3A illustrates the

differentiation procedure into proximal renal tubule, in which cell aggregates are formed and cocultured with Wnt4-expressing NIH3T3 fibroblasts according to the same procedure as disclosed in NPL 4. FIG. 3B shows the appearances of the aggregates of CD9(-)CD140a(+)CD140b(+)CD271(+) cells cocultured with Wnt4-expressing NIH3T3 fibroblasts.

<UBC-conditioned medium>

The ureteric bud cell (UBC)-conditioned medium was prepared by a modified version of the procedure disclosed in the literature (Barasch *et al.*, *Am. J. Physiol.*, (1997), 273, F757-767). UBCs (gifted from Dr. Barasch, Columbia University; *Proc. Natl. Acad. Sci. USA*, (1997), 94, 6279-6284) were cultured in a minimum essential medium (MEM; Invitrogen) supplemented with 10% FBS. After reaching 80% confluence, the cells were washed with PBS, and the culture medium was replaced with a DMEM/F12 (1:1) mixed medium supplemented with GlutaMAX, 10% KSR, 0.1 mM NEAAs, 0.55 mM 2-mercaptoethanol and 500 U/mL penicillin/streptomycin. Then, the cells were cultured for 3 days to produce a culture supernatant. The culture supernatant was filtrated through a 0.22 µm filter before use.

[0072] [Example 4]

<Percentages of RPCs (OSR1(+)/SIX2(+) cells) in cell populations sorted by different combinations of CD9(-) with three positive selection markers>

The percentages of OSR1(+)/SIX2(+) cells in cell populations sorted by all (10) exhaustive combinations of CD9(-) with any three of the positive selection markers extracted in Example 1 were investigated using FACS Aria™ Fusion (BD Biosciences). Also, with regard to the particular combination of CD9(-), CD140a(+), CD140b(+) and CD271(+), which had been confirmed in Example 3 to allow fractionation of a RPC-containing cell population, it was investigated whether CD55(-) and CD326(-) can serve as negative selection markers alternative to CD9(-).

[0073] The antibodies used were: APC-H7 Mouse Anti Human CD9 (BD Biosciences; Cat. No. 655409); Alexa Fluor® 647 Mouse Anti-Human CD140a (BD Biosciences; Cat. No.

562798); BV421 Mouse Anti-Human CD140a (BD Biosciences; Cat. No. 562799); BV421 Mouse Anti-Human CD140b (BD Biosciences; Cat. No. 564124); BV510 Mouse Anti-Human CD271 (BD Biosciences; Cat. No. 563451); APC Mouse Anti-Human CD106 (BD Biosciences; Cat. No. 551147); BV605 Mouse Anti-Human CD106 (BD Biosciences; Cat. No. 563307); CD165-Biotin, Human (Miltenyi Biotec; Cat. No. 130-098-536); CD165-APC, Human (Miltenyi Biotec; Cat. No. 130-098-542); Anti-Human CD55 Biotin (eBioscience; Cat. No. 13-0559); and BV605 Mouse Anti-Human CD326 (BD Biosciences; Cat. No. 563182). The antibodies were used at 20-fold dilution, and cells were reacted with the antibodies at room temperature for 15 minutes, washed with PBS three times, and then analyzed. In the case of using the biotinylated antibodies, secondary staining was performed with BV605 Streptavidin (BD Biosciences; Cat. No. 563260) at 500-fold dilution. During the secondary staining process, cells were reacted with the indicated antibodies at room temperature for 15 minutes, washed with PBS three times, and then analyzed. The hiPSC-derived differentiated cell populations were analyzed using FACS Aria™ Fusion, and based on the resulting sets of two-dimensional scatter plots, cell fractions stained with 12 different combinations of antibodies were demarcated to thereby fractionate the respective fractions of cells. The fractionated cell populations were analyzed again using FACS Aria™ Fusion, to thereby compute the percentages of OSR1(+)SIX2(+) cells, OSR1(+)SIX2(-) cells, OSR1(-)SIX2(+) cells, and OSR1(-)SIX2(-) cells present in the whole cell populations (FIG. 4). The results found that high percentages of OSR1(+)SIX2(+) cells were found in the whole cell populations fractionated with all the combinations of markers.

[0074] [Example 5]

<Investigation of cell surface marker combinations that allow fractionation of RPCs>

Cell populations were analyzed by FACS Aria™ Fusion using the antibodies against any combinations of the cell surface markers CD9, CD140a, CD140b and CD271 as adopted in Example 4, to thereby compute the percentage of OSR1(+)SIX2(+) cells present in each of the cell populations fractionated with different combinations of the negative selection marker

CD9(-) with any positive selection markers selected from CD140a(+), CD140b(+) and CD271(+). Also, the percentage of cells demarcated by each of different cell surface marker combinations in an unsorted cell population was investigated, and on that basis, the percentage of the number of OSR1(+)/SIX2(+) cells collected using each of desired surface marker combinations with respect to the number of unsorted cells was computed (FIG. 5). The results revealed that condensation of OSR1(+)/SIX2(+) cells was possible with the use of any of the different cell surface marker combinations, and that the percentages of OSR1(+)/SIX2(+) cells condensed with different combinations of two or three cell surface markers were comparable to the percentage of OSR1(+)/SIX2(+) cells condensed with a combination of four cell surface markers.

[0075] [Example 6]

<Investigation of cell surface marker combinations that allow fractionation of RPCs>

With the view to confirming that a high-purity RPC population can be fractionated even from hiPSC lines carrying no reporter gene by the use of the cell surface markers discussed above, hiPSCs (201B7 strain, gifted from Kyoto University) were tested in a differentiation induction system. The hiPSCs (201B7) were kept in maintenance culture by a conventional procedure (Takahashi K, *et al.*, (2007), *Cell*. 131:861-72), and induced to differentiate into a RPC-containing cell population by following the procedure disclosed in NPL 4. Then, according to the same procedure as in Example 3, CD9(-)CD140a(+)/CD140b(+)/CD271(+) cells were fractionated using FACSARIA™ Fusion to form cell aggregates, and the cell aggregates were cocultured with Wnt4-expressing NIH3T3 fibroblasts, whereby it was confirmed whether a cell population maintaining differentiation potential into renal tubule was successfully fractionated. Differentiation into renal tubule was determined based on expression of the proximal renal tubule marker LTL and morphological characteristics. As a result, it was confirmed that LTL-positive luminal structures can be formed from the CD9(-)CD140a(+)/CD140b(+)/CD271(+) cell population. In other words, it was demonstrated that a high-purity RPC-containing cell population can be

fractionated even from hiPSCs (201B7)-derived differentiated cells by the use of CD9, CD140a, CD140b and CD271 (FIG. 6C). FIG. 6A shows a set of histograms of the number of cells as a function of fluorescence intensity of each of OSR1 (GFP), SIX2 (tdTomato) and the different cell surface markers. FIG. 6B shows a set of two-dimensional scatter plots of the flow cytometric measurements of CD9, CD140a, CD140b or CD271 expression in an iPSC 201B7 line-derived differentiated cell population. FIG. 6C shows an image of cell aggregates formed by sorting P4+P2+P3 cell populations and cocultured with Wnt4-expressing NIH3T3 fibroblasts.

[0076] [Example 7]

<Investigation of cell surface marker combinations that allow fractionation of RPCs (2)>

According to the same procedure as in Example 6, RPC-containing cell populations into which hiPSCs (201B7) had been induced to differentiate were subjected to immunostaining of surface antigens using APC-H7 Mouse Anti Human CD9, Alexa Fluor® 647 Mouse Anti-Human CD140a, BV421 Mouse Anti-Human CD140b, and BV510 Mouse Anti-Human CD271, and then to cell sorting with FACSARIA™ Fusion to fractionate CD9(-)CD140a(+)CD140b(+)CD271(+) cells. The fractionated cells were smeared on slide glasses using Smear Gell® (GenoStaff; Cat. No. SG-01) and immunostained for intranuclear transcription factors. Anti-SIX2, Rabbit Poly (Proteintech; Cat. No. 11562-1-AP) was used as a primary antibody; Donkey Anti-Rabbit IgG (H+L) Secondary Antibody Alexa Fluor® 488 Conjugate (Life Technologies; Cat. No. A21206) was used as a secondary antibody; and Hoechst 33342 (Life Technologies; Cat. No. H3570) was used for nuclear staining. As a result of observation with a microscope (KEYENCE; Cat. No. BZ-9000), it was observed that the CD9(-)CD140a(+)CD140b(+)CD271(+) cell population contains a higher percentage of SIX2-positive cells, which is one of the characteristics of RPCs, as compared with an unsorted differentiated cell population (FIG. 7).

INDUSTRIAL APPLICABILITY

[0077] As detailed hereinabove, the present invention provides a method for acquiring and

producing high-purity RPCs from a RPC population into which PSCs are induced to differentiate, using a cell surface marker. The RPCs acquired by the method of this invention can be used in regenerative medicine for renal diseases such as renal failure.

SEQUENCE LISTING FREE TEXT

[0078] SEQ ID NO:15: L803-mts/GSK-3 β peptide inhibitor; the 1st amino acid, glycine, is attached to myristic acid via an amide bond at the N terminus; the 11th amino acid, serine, is phosphorylated; and the 12th amino acid, proline, is amidated at the C terminus.

CLAIMS

1. A method for producing renal progenitor cells into which pluripotent stem cells are induced to differentiate, the method comprising the steps of:
 - (i) culturing the pluripotent stem cells under conditions that induce differentiation into renal progenitor cells; and
 - (ii) sorting a cell population from the cells obtained at step (i), by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).
2. The method according to claim 1, wherein at step (ii), the sorting of a cell population is performed by using at least two cell surface markers.
3. The method according to claim 1, wherein at step (ii), the sorting of a cell population is performed by using at least three cell surface markers.
4. The method according to claim 1, wherein at step (ii), the sorting of a cell population is performed by using at least four cell surface markers.
5. The method according to claim 1, wherein at step (ii), at least two cell surface markers selected from the group consisting of CD9(-), CD140a(+), CD140b(+) and CD271(+) are used.
6. The method according to claim 5, wherein at step (ii), the sorting of a cell population is performed by using at least three cell surface markers.
7. The method according to claim 4, wherein at step (ii), CD9(-), CD140a(+), CD140b(+) and CD271(+) are used as the cell surface markers.

8. The method according to any of claims 1 to 7, wherein the pluripotent stem cells are induced pluripotent stem (iPS) cells.
9. The method according to any of claims 1 to 7, wherein the pluripotent stem cells are human iPS cells.
10. A renal progenitor cell population produced by the method according to any of claims 1 to 9.
11. A method for sorting a cell population from a renal progenitor cell-containing cell population by using at least one cell surface marker selected from the group consisting of CD9(-), CD55(-), CD106(+), CD140a(+), CD140b(+), CD165(+), CD271(+) and CD326(-).
12. The method according to claim 11, wherein the sorting of a cell population is performed by using at least two cell surface markers.
13. The method according to claim 11, wherein the sorting of a cell population is performed by using at least three cell surface markers.
14. The method according to claim 11, wherein the sorting of a cell population is performed by using at least four cell surface markers.
15. The method according to claim 11, wherein at least two cell surface markers selected from the group consisting of CD9(-), CD140a(+), CD140b(+) and CD271(+) are used.
16. The method according to claim 15, wherein the sorting of a cell population is performed by using at least three cell surface markers.

17. The method according to claim 11, wherein CD9(-), CD140a(+), CD140b(+) and CD271(+) are used as the cell surface markers.
18. The method according to any of claims 11 to 17, wherein the renal progenitor cells are renal progenitor cells into which pluripotent stem cells are induced to differentiate.
19. The method according to any of claims 11 to 17, wherein the pluripotent stem cells are induced pluripotent stem (iPS) cells.
20. The method according to any of claims 11 to 17, wherein the pluripotent stem cells are human iPS cells.
21. A cell population acquired by the method according to any of claims 11 to 20.