SPECIFICATION

TITLE
METHOD FOR PRODUCING MEGAKARYOCYTES

[TECHNICAL FIELD]

[0001]

The present invention relates to a method for producing a megakaryocyte, a megakaryocyte producible by the method, and applications of the megakaryocyte, and the like.

[BACKGROUND ART]

[0002]

A large number of blood cells are required for the treatment of blood-related diseases and surgical treatments. Among these blood cells, platelets, which are essential for blood coagulation and hemostasis, are particularly important blood cells. Platelets are in high demand in leukemia, bone marrow transplantation, cancer treatment, and other therapies, and there is a need to ensure a stable supply. To date, platelets have been obtained by collecting them from donors through blood donation, administering TPO-like structural (mimetic) formulations, or differentiating megakaryocytes from umbilical cord blood or bone marrow cells. Recently, technologies for preparing blood cells such as platelets by inducing differentiation of pluripotent stem cells such as embryonic stem (ES) cells and induced pluripotent stem cells (iPS cells) in vitro have also been developed.

[0003]

In order to meet the social demand for platelets, the present inventors have developed a technology for stably producing platelet preparations in vitro using human iPS cells (Non-Patent Literatures 1 and 2). The present inventors have established a method (artificial platelet preparation derived from human iPS cells) for introducing two factors (MYC/BMI1 or MYC/BCL-XL) or three factors (MYC/BMI1/BCL-XL) into hematopoietic progenitor cells derived from human iPS cells to establish megakaryocyte lines and then maturing the megakaryocyte lines to produce large quantities of platelets (Patent Literatures 1-4, Non-Patent Literature 1). Furthermore, culture conditions that dramatically improve platelet production in the maturation process of megakaryocytes (hydrodynamic requirements, particularly turbulent stimulation) have also been reported (Patent Literature 5, Non-Patent Literature 2).

[CITATION LIST]

[PATENT LITERATURE]

[0004]

[PTL 1] International Publication No. 2011/034073

[PTL 2] International Publication No. 2012/157586

[PTL 3] International Publication No. 2014/123242

[PTL 4] International Publication No. 2021/075568

[PTL 5] International Publication No. 2019/009364

[NON PATENT LITERATURE]

[0005]

[NPL 1] Cell Stem Cell. 2014 Apr 3; 14(4): 535-548.

[NPL 2] Cell. 2018 Jul 26; 174(3): 636-648.

[SUMMARY OF INVENTION]

[TECHNICAL PROBLEM]

[0006]

However, the present inventors have found that megakaryocytes (cell lines) induced by the above-mentioned conventional differentiation methods exhibit a decrease in platelet production capacity as they are passaged, and the degree of this decrease varies greatly depending on the pluripotent stem cell line from which the hematopoietic progenitor cells originate (Fig. 1). Therefore, an object of the present invention is to provide a method for proliferating megakaryocytes induced by conventional induction methods while maintaining their platelet production capacity.

[SOLUTION TO PROBLEM]

[0007]

The present inventors have found that in megakaryocytes in which the core proteins (UQCRC1, UQCRC2) of Complex III (ubiquinol-cytochrome c reductase), which is involved in the electron transport chain in mitochondria, are forcibly expressed, platelet production capacity is maintained even after long-term passage culture.

[0008]

Furthermore, it was found that the addition of adipogenic differentiation-inducing factors (dexamethasone, IBMX, insulin) during the proliferation phase of megakaryocytes in which UQCRC2 was forcibly expressed increased the number of platelets produced by approximately 1.5-fold, and further, that the addition of JI051 increased the number of platelets produced by approximately 2-fold. Additionally, it was found that adding an HDAC5 (histone deacetylase 5) inhibitor or an LSD1 (lysine-specific histone demethylase) inhibitor during the proliferation

phase of megakaryocytes also allows the megakaryocytes to maintain CD34/CD41 double-positive expression and maintain platelet production after long-term culture.

[0009]

The present inventors investigated the proliferative capacity of megakaryocytes derived from different iPS cell lines. As a result, it was found that in megakaryocytes induced by introducing two megakaryocyte-inducing factors (i.e., MYCdd gene and BCL2L1 gene), differences in the proliferative capacity of megakaryocytes occur depending on the origin of the iPS cells. To investigate the cause of this difference, it was discovered that a protein constituting Polycomb Repressive Complex 1.1 is involved in the proliferative capacity of megakaryocytes, and by knocking down this protein, the proliferative capacity of megakaryocytes can be improved.

[0010]

Furthermore, in order to further explore factors contributing to the proliferative capacity of megakaryocytes, several transcription factors involved in the differentiation process of the hematopoietic lineage were analyzed. As a result, surprisingly, it was found that overexpressing BACH1, which contributes to erythrocyte formation and lymphocyte formation, can improve the proliferative capacity and platelet production capacity of megakaryocytes. Based on these findings, the present inventors conducted further research, leading to the completion of the present invention.

[0011]

Therefore, the present invention provides the following:

[1] A method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of increasing the abundance of at least one protein constituting ubiquinol-cytochrome c reductase (UQCR) in a megakaryocyte.

[2] The method according to [1], wherein the at least one protein constituting UQCR is a UQCR core protein (UQCRC).

[3] The method according to [2], wherein at least one UQCRC is UQCRC2.

[4-1] The method according to any one of [1] to [3], wherein the step of increasing the abundance of the protein constituting UQCR comprises a step of introducing a nucleic acid encoding the protein into the megakaryocyte.

[4-2] The method according to any one of [1] to [4-1], wherein the nucleic acid is introduced by a viral vector.

[5-1] A method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of inhibiting the function of Polycomb Repressive Complex 1.1 in a megakaryocyte.

[5-2] The method according to [5-1], wherein the step of inhibiting the function of Polycomb Repressive Complex 1.1 comprises a step of reducing the abundance of at least one protein constituting Polycomb Repressive Complex 1.1.

[6] The method according to any one of [1] to [4-2], comprising a step of inhibiting the function of Polycomb Repressive Complex 1.1 in the megakaryocyte.

[7] The method according to [6], wherein the step of inhibiting the function of Polycomb Repressive Complex 1.1 comprises a step of reducing the abundance of at least one protein constituting Polycomb Repressive Complex 1.1.

[8] The method according to [5-2] or [7], wherein the protein constituting Polycomb Repressive Complex 1.1 is at least one selected from the group consisting of a BCOR protein, a BCORL1 protein, and a TRIM27 protein.

[9-1] The method according to any one of [1] to [8], comprising a step of increasing the abundance of a BACH1 protein in the megakaryocyte.

[9-2] The method according to any one of [1] to [9-1], comprising a step of reducing the abundance of (1) BCOR protein or BCORL1 protein, and (2) TRIM27 protein in the megakaryocyte, and increasing the abundance of (3) BACH1 protein.

[10-1] The method according to any one of [1] to [9-2], comprising a step of culturing the megakaryocyte in a medium containing an HDAC5 inhibitor.

[10-2] The method according to [10-1], wherein at least one HDAC5 inhibitor is LMK235.

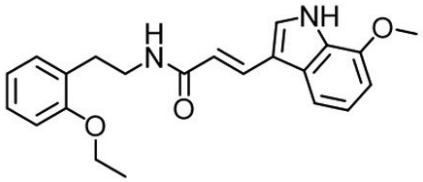
[11-1] The method according to any one of [1] to [10-2], comprising a step of culturing the megakaryocyte in a medium containing an adipogenic differentiation-inducing factor.

[11-2] The method according to any one of [1] to [11-1], wherein the adipogenic differentiation-inducing factor is a combination of dexamethasone, IBMX, and insulin.

[12] The method according to any one of [1] to [11-2], wherein a medium contains a compound (JI051) represented by the following structural formula:

[0012]

[Chemical 1]



[0013]

[13] The method according to any one of [1] to [12], comprising a step of increasing the abundance of a MYC protein and the abundance of an apoptosis-suppressing protein in the megakaryocyte.

[14-1] The method according to any one of [1] to [13], wherein the megakaryocyte is derived from a pluripotent stem cell.

[14-2] The method according to any one of [1] to [14-1], wherein the megakaryocyte expresses a CD34 gene and a CD41 gene.

[14-3] The method according to any one of [1] to [14-2], wherein the megakaryocyte is derived from a human.

[15] A megakaryocyte obtainable by the method according to any one of [1] to [14-3].

[16-1] A megakaryocyte having all of the following properties (A) to (C):

(A) having at least one exogenous protein constituting ubiquinol-cytochrome c reductase (UQCR),

(B) having platelet production capacity, and

(C) expressing a CD34 gene and a CD41 gene.

[16-2] The megakaryocyte according to [16-1], further having at least one of the following properties (D) to (F):

(D) being derived from a pluripotent stem cell,

(E) having an exogenous megakaryocyte-inducing factor, and

(F) having an expression inhibitor for a TP53 gene and/or a CDKN1A gene.

[17-1] The megakaryocyte according to [16-1] or [16-2], wherein the at least one protein constituting UQCR is a UQCR core protein (UQCRC).

[17-2] The megakaryocyte according to [17-1], wherein at least one UQCRC is UQCRC2.

[18] A method for producing a platelet, comprising a step of maturing the megakaryocyte according to any one of [15] to [17-2].

[19-1] The method according to [18], comprising a shaking culture step.

[19-2] The method according to [18] or [19-1], wherein the medium contains JI051.

[20] A platelet obtainable by the method according to any one of [18] to [19-2].

[21-1] A blood product comprising the cell according to any one of [15], [16-1], [16-2], [17-1], [17-2] and [20].

[21-2] The product according to [21-1], for treating or preventing blood diseases or bleeding.

[22-1] A method for producing a megakaryocyte, comprising a step of culturing a hematopoietic progenitor cell in a medium containing an HDAC5 inhibitor and/or an LSD1 inhibitor.

[22-2] The method according to [22-1], wherein the medium contains an HDAC5 inhibitor and an LSD1 inhibitor.

[23-1] The method according to [22-1] or [22-2], wherein at least one of the HDAC5 inhibitors is LMK235.

[23-2] The method according to any one of [22-1] to [23-1], wherein at least one of the LSD1 inhibitors is tranylcypromine.

[24] A method for treating or preventing blood diseases or bleeding, comprising administering or transplanting an effective amount of the cell according to any one of [15] to [17-2] or [20] to a mammal.

[25] The cell according to any one of [15] to [17-2] or [20], for use in the treatment or prevention of blood diseases or bleeding.

[26] Use of the cell according to any one of [15] to [17-2] or [20] in the manufacture of a prophylactic or therapeutic medicament for blood diseases or bleeding.

[ADVANTAGEOUS EFFECTS OF INVENTION]

[0014]

According to the present invention, megakaryocytes maintaining platelet production capacity over a long period can be produced, regardless of the type of pluripotent stem cell line. This makes it possible to stably supply high-quality platelets.

[BRIEF DESCRIPTION OF DRAWINGS]

[0015]

[FIG. 1] FIG. 1 shows a graph showing the time-dependent change in platelet production capacity of MBX cassette-introduced 15M41 human iPS cell-derived megakaryocytes. The horizontal axis represents the number of days after the start of culture in a medium containing doxycycline (Dox) (megakaryocyte differentiation and proliferation medium), and the vertical axis represents the number of platelets released per megakaryocyte.

[FIG. 2] FIG. 2 shows a graph showing the platelet production capacity of MBX cassette-introduced TkDN sev2 cell-derived megakaryocytes after UQCRC1 and/or UQCRC2 knockdown. The horizontal axis represents the type of shRNA introduced into the megakaryocytes, and the vertical axis represents the relative value with respect to the platelet production capacity of the control (megakaryocytes into which LacZ shRNA was introduced).

[FIG. 3] FIG. 3 shows the results of introducing UQCRC1 or UQCRC2 into megakaryocytes differentiated from MBX cassette-introduced 15M41 cells or MX cassette-introduced 15M41 cells using a lentiviral vector, and analyzing platelet production capacity after maturation. The horizontal axis represents the type of gene introduced using a lentiviral vector, and the vertical axis represents the number of platelets released per megakaryocyte cell. N=2, error bar: standard deviation.

[FIG. 4] FIG. 4 shows a graph representing the results of introducing UQCRC2 into megakaryocytes differentiated from MBX cassette-introduced 15M41 cells or MX cassette-introduced 15M41 cells using a lentiviral vector, and analyzing platelet production capacity after long-term expansion culture followed by maturation. The horizontal axis represents the number

of days after the start of culture in megakaryocyte differentiation/proliferation medium, and the vertical axis represents the number of platelets released per megakaryocyte cell.

[FIG. 5] FIG. 5 shows a graph representing the results of culturing MBX cassette-introduced 15M41 cell-derived megakaryocytes expressing exogenous UQCRC2 in differentiation/proliferation medium supplemented with dextran, IBMX, and insulin for 3 days or more, transferring to maturation medium, adding JI051 after 3 days, and analyzing platelet production capacity. The horizontal axis represents the added compound, and the vertical axis represents the number of platelets released per megakaryocyte cell.

[FIG. 6] FIG. 6 shows the results of measuring the number of cells (upper graph) and the percentage of CD34-positive cells (lower graph) by FACS 3 days after inducing differentiation into megakaryocytes using a differentiation/proliferation medium in which UM729 was replaced with LMK235. The horizontal axis represents the compound used, and the number after LMK indicates the concentration of LMK235 (unit: μM).

[FIG. 7] FIG. 7 shows the results of measuring the CD34-positive rate and CD41a-positive rate on day 3 of proliferation culture by FACS, after inducing differentiation into megakaryocytes using a differentiation/proliferation medium in which 1 μM UM729 was replaced with either 200 nM LMK235 (inhibitor of HDAC4, 5, and 6), 100 nM TMP195 (inhibitor of HDAC4, 5, 7, and 9), or 100 nM Tasquinimod (inhibitor of HDAC4). 200 nM LMK235, 100 nM TMP195, and 100 nM Tasquinimod have approximately the same inhibitory effect on HDAC.

[FIG. 8] FIG. 8 shows a graph showing platelet production capacity when differentiation induction and expansion culture are performed using a differentiation/proliferation medium in which 1 μM UM729 is replaced with 200 nM LMK235, and maturation is performed by static culture. The vertical axis represents the number of platelets released per megakaryocyte cell.

[FIG. 9] FIG. 9 shows a schematic diagram of a method for inducing differentiation from pluripotent stem cells to megakaryocytes using UM729.

[FIG. 10] FIG. 10 shows the results of transfecting iPS cell lines (15M41, MH09S01, MH15S01, MH15S02, MH23S01) with the vector shown in the figure to induce imMKCL, and measuring the CD34-positive rate and CD41a-positive rate by FACS. A) FACS results 14 days after the start of addition of SCF, TA-316, UM729, and Dox. The CD34/CD41a double-positive fraction was sorted, and then cultured in a medium containing SCF, TA-316, UM729, and Dox. B) After imMKCL induction (day 1 is the day when Dox addition starts), cells derived from all iPS cell lines maintained CD34/CD41a double-positive expression for 42 days.

[FIG. 11] FIG. 11 shows the results of transfecting the iPS cell line (QHJI14s04/AB II-KO-03) with the vector shown in the figure to induce imMKCL, and measuring the CD34-positive rate and CD41a-positive rate by FACS (the vertical axis of the graph shows the CD34 expression

level, and the horizontal axis shows the CD41a expression level). On day 19 after induction (day 1 is the day when Dox addition starts), the CD34/CD41a double-positive fraction was sorted, and cells on days 28, 32, 36, and 42 after imMKCL induction were analyzed by FACS. As a result, it became clear that CD34-negative and CD41a-positive cells appeared, and it is difficult to maintain the expression of CD34/CD41a.

[FIG. 12] FIG. 12 shows the results of transfecting the iPS cell line (15M41) with the vector shown in the figure to induce imMKCL, and measuring the CD34-positive rate and CD41a-positive rate by FACS. It has been reported that 15M41 has mutations in the BCOR gene and BRD3 gene. Left of A): shows the results 14 days after the start of addition of SCF, TA-316, UM729, and Dox. The CD34/CD41a double-positive fraction was sorted, and then cultured in a medium containing SCF, TA-316, UM729, and Dox. Right of A): After imMKCL induction (day 1 is the day when Dox addition starts), only cells derived from the 15M41 iPS cell line maintained CD34/CD41a double-positive expression.

[FIG. 13] FIG. 13 shows the results of FACS analysis measuring the CD34-positive rate and CD41a-positive rate in megakaryocyte lines derived from QHJI14s04/AB II-KO-03 (day 28 after the start of Dox addition), where BCOR or BCORL1 was knocked down using lentivirus. By knocking down BCOR (BCL6 corepressor) or BCORL1 (BCL6 corepressor like 1), it was possible to obtain megakaryocytes that maintained CD34/CD41a double-positive expression.

[FIG. 14] FIG. 14 shows a comparison of the platelet production capacity in megakaryocytes derived from QHJI14s04/AB II-KO-03 (No. 3 cell; Control), megakaryocytes with BCOR knocked down in No. 3 cells (sh BCOR), and megakaryocytes with BCORL1 knocked down in No. 3 cells (sh BCORL1). The results for No. 3 cells are from day 29 after the start of Dox addition, and the results for sh BCOR and sh BCORL1 are from day 36 after the start of Dox addition. It was shown that sh BCOR and No. 3 sh BCORL1 produced 2 to 3 times more platelets compared to No. 3 cells.

[FIG. 15] FIG. 15 shows the preparation of a plasmid incorporating BACH1 (the numbers 1-5 are attached in the figure to show an overview) and the induction of megakaryocytes by introducing the above combinations of plasmids (combination of 1, 4, and 5; combination of 2, 4, and 5; combination of 3, 4, and 5) into iPS cells (QHJI14s04/AB II-KO-03) by lipofection. The combination of 1, 4, and 5 is a combination in which c-MYCdd is overexpressed, BACH1 and BCL-XL are overexpressed, and BCOR is knocked down under Dox conditions. The combination of 2, 4, and 5 is a combination in which c-MYCdd is overexpressed, BACH1 and BCL-XL are overexpressed, and BCOR and BCORL1 are knocked down under Dox conditions. The combination of 3, 4, and 5 is a combination in which c-MYCdd is overexpressed, BACH1 and BCL-XL are overexpressed, and BCOR and TRIM27 (Tripartite Motif Containing 27) are knocked down under Dox conditions.

[FIG. 16-1] FIG. 16-1 shows the following results. A) The iPS cell line (QHJI14s04/AB II-KO-03) into which plasmids 1, 4, and 5 of FIG. 15 were introduced was induced to differentiate into megakaryocytes, CD34/CD41a double-positive cells were sorted, and the results of FACS analysis on day 58 after induction (day 1 being the start of Dox addition) are shown. B) The iPS cell line (QHJI14s04/AB II-KO-03) into which plasmids 2, 4, and 5 of FIG. 15 were introduced was induced to differentiate into megakaryocytes, CD34/CD41a double-positive cells were sorted, and the results of FACS analysis on day 58 after induction (day 1 being the start of Dox addition) are shown. C) The iPS cell line (QHJI14s04/AB II-KO-03) into which plasmids 3, 4, and 5 of FIG. 15 were introduced was induced to differentiate into megakaryocytes, CD34/CD41a double-positive cells were sorted, and the results of FACS analysis on day 58 after induction (day 1 being the start of Dox addition) are shown. By expressing BACH1, it was possible to obtain megakaryocytes that maintained CD34/CD41a double-positive expression.

[FIG. 16-2] FIG. 16-2 shows the following results. D) This figure shows a comparison of the platelet production capacity of each cell. Megakaryocytes induced with plasmids 3, 4, and 5 (BCOR/TRIM27) produced the most platelets. The leftmost graph shows the number of platelets produced by megakaryocytes into which plasmids 4 and 5 (c-MYC/BCL-XL/UQCRC2) were introduced.

[FIG. 17-1] FIG. 17-1 shows the results of transfecting iPS cells (QHJI14s04/AB II-KO-11) with plasmids 3, 4, and 5 shown schematically in the figure using lipofection to induce megakaryocytes, and measuring the CD34-positive rate and CD41a-positive rate by FACS. A) The iPS cell line (QHJI14s04/AB II-KO-11) transfected with plasmids 3, 4, and 5 was induced into megakaryocytes, and the CD34/CD41a double-positive cells were sorted. The results of FACS on day 64 after induction (day 1 being the day when Dox addition was started) are shown.

[FIG. 17-2] FIG. 17-2 shows a comparison of platelet production capacity in megakaryocytes derived from QHJI14s04/AB II-KO-03 (No. 3 cells) and megakaryocytes derived from QHJI14s04/AB II-KO-11 (No. 11 cells). The graph shows, from left to right: the number of platelets produced by No. 3 cells (c-MYC/BCL-XL/sh p21 (p21 was knocked down using shRNA)/sh p53 (p53 was knocked down using shRNA)) on day 40 after Dox addition; the number of platelets produced by No. 3 UQ2 cells (c-MYC/BCL-XL/UQCRC2/sh p21/sh p53) on day 36 after Dox addition; the number of platelets produced by No. 3 UQ2 BACH1 cells (c-MYC/BCL-XL/UQCRC2/BACH1/sh BCOR/sh TRIM27 (TRIM27 was knocked down using shRNA)) on days 26, 27, 33, 42, 48, and 50 after Dox addition (the average value ± standard deviation of the number of platelets at each time point is shown); the number of platelets produced by No. 11 cells (c-MYC/BCL-XL/sh p21/sh p53) on day 40 after Dox addition; the number of platelets produced by No. 11 UQ2 cells (c-MYC/BCL-XL/UQCRC2/sh p21/sh p53) on day 36 after Dox addition; and the number of platelets produced by No. 11 UQ2 BACH1 cells

(c-MYC/BCL-XL/UQCRC2/BACH1/sh BCOR/sh TRIM27) on days 26 and 29 after Dox addition (the average value ± standard deviation of the number of platelets at each time point is shown). It was demonstrated that No. 11 UQ2 BACH1 cells produced the largest number of platelets.

[FIG. 18-1] FIG. 18-1 shows the results of transfecting iPS cells (QHJI14s04/AB II-KO-03, QHJI14s04/AB II-KO-11) with plasmids shown schematically in the figure using lipofection to induce megakaryocytes, and measuring the CD34-positive rate and CD41a-positive rate by FACS. A) Each iPS cell line transfected with a plasmid was induced into megakaryocytes, and the CD34/CD41a double-positive cells were sorted. The results of FACS on day 48 (in the case of megakaryocytes derived from QHJI14s04/AB II-KO-03) or day 42 (in the case of megakaryocytes derived from QHJI14s04/AB II-KO-11) after induction (day 1 being the day when Dox addition was started) are shown.

[FIG. 18-2] FIG. 18-2 shows the following results. B) This figure shows the comparative results of platelet production capacity for each megakaryocyte. The graph on the left shows, from left to right, the number of platelets produced by No. 3 cells (c-MYC/BCL-XL) 40 days after the start of Dox addition, the number of platelets produced by No. 3 UQ2 cells (c-MYC/BCL-XL/UQCRC2) 36 days after the start of Dox addition, and the number of platelets produced by No. 3 UQ2 tet BACH1 cells (c-MYC/BCL-XL/UQCRC2/tet BACH1) at 33, 35, 39, 43, and 46 days after the start of Dox addition (showing the average number of platelets ± standard deviation at each time point), the number of platelets produced by No. 11 cells (c-MYC/BCL-XL) 40 days after the start of Dox addition, the number of platelets produced by No. 11 UQ2 cells (c-MYC/BCL-XL/UQCRC2) 36 days after the start of Dox addition, and the number of platelets produced by No. 11 UQ2 tet BACH1 cells (c-MYC/BCL-XL/UQCRC2/tet BACH1) at 26, 28, 32, 36, 39, and 46 days after Dox addition (showing the average number of platelets ± standard deviation at each time point). The results show that No. 11 UQ2 tet BACH1 cells produced the largest number of platelets. The graph on the right shows the results of measuring the number of platelets produced over time in No. 11 UQ2 tet BACH1 cells.

[FIG. 19] FIG. 19 shows the cell count and FACS analysis results after culturing imMKC differentiated from MX cassette-introduced 15M41 cells for 10 days with SCF, TA316, and Dox, together with UM729 (1 μM), LMK235 (200 nM), 2PCPA (trans-2-phenylcyclopropylamine) (also known as tranylcypromine) (1 μM), or a combination of LMK235 (200 nM) and 2PCPA (1 μM). Although LMK235 alone and 2PCPA alone did not show high effects in maintaining CD34/CD41a expression, the combined use of LMK235 and 2PCPA enabled maintenance of CD34/CD41a expression.

[DESCRIPTION OF EMBODIMENTS]

[0016]

1. Method for Producing Megakaryocyte

The present invention provides a method for producing a megakaryocyte maintaining platelet production capacity. Specifically, the present invention provides a method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of increasing the abundance of at least one protein constituting ubiquinol-cytochrome c reductase (UQCR) in a megakaryocyte (hereinafter, may be referred to as "the production method of the present invention"). In another embodiment, the present invention provides a method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of inhibiting the function of Polycomb Repressive Complex 1.1 (PRC1.1; also referred to as non-canonical PRC1.1) in a megakaryocyte (unless otherwise specified, this method is also included in "the production method of the present invention"). Furthermore, these methods may be combined, and specifically, a method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of increasing the abundance of at least one protein constituting UQCR in a megakaryocyte and a step of inhibiting the function of PRC1.1 in a megakaryocyte is also provided. When these steps are combined, each step may be performed sequentially or simultaneously. In addition, in the present specification, "method for producing a megakaryocyte" can be appropriately read as "method for proliferating a megakaryocyte" or "method for expansion culture of megakaryocyte."

[0017]

In the present specification, the term "cells", such as megakaryocytes, includes "cell populations", unless otherwise specified. Furthermore, unless otherwise specified, the term "cells" refers to those obtainable by cell culture. A cell population may be composed of a single type of cell or two or more different types of cells.

[0018]

Megakaryocytes can be characterized, for example, as CD41a-positive/CD42a-positive/CD42b-positive cells. In addition to these markers, megakaryocytes may further express one or more markers selected from the group consisting of CD9, CD34, CD61, CD62p, CD42c, CD42d, CD49f, CD51, CD110, CD123, CD131, and CD203c. Megakaryocytes may also express GATA1, FOG1, NF-E2, and β1-tubulin. Megakaryocytes may be multinucleated cells or mononuclear or binuclear cells. Also, megakaryocytes may be immortalized as a megakaryocyte line or may be clonal cell populations.

Megakaryocytes that are not multinucleated can be proliferated by expansion culture and may be referred to as "immortalized mega-karyocyte progenitor cell lines (imMKCL).". Immortalized megakaryocytes produce functional platelets upon maturation. Maturation of a megakaryocyte refers to the differentiation of the megakaryocyte into the necessary

multinucleated state, enabling it to produce functional platelets. Functional platelets may be, for example, CD42b-positive platelets. Megakaryocyte maturation can also be confirmed by an increase in expression of megakaryocyte maturation-related genes such as GATA1, FOG1, FLI1, NF-E2, and β1-tubulin.

In one embodiment, the megakaryocytes used in the present invention are CD34-positive CD41-positive cells. Also, in another embodiment, the megakaryocytes used in the present invention are CD38-negative, CD90-positive, and/or CD49f-positive. In the present specification, a CD41-positive cell refers to a CD41a-positive cell.

[0019]

In one embodiment of the present invention, the megakaryocytes have an exogenous gene encoding MYC protein and an exogenous gene encoding an apoptosis-suppressing protein, preferably further having an exogenous gene encoding a polycomb group protein. In the present specification, these MYC protein, apoptosis-suppressing protein, and polycomb group proteins may be collectively referred to as "megakaryocyte-inducing factors."

[0020]

Examples of genes encoding the MYC protein (hereinafter also referred to as "MYC gene") include c-MYC, N-MYC, and L-MYC. More preferably, it is the c-MYC gene. Examples of the c-MYC gene include a gene consisting of the nucleic acid sequence shown in NCBI accession number NM_002467.

[0021]

The c-MYC gene may be a c-MYC gene that encodes a protein fused with a destabilization domain. The "destabilization domain" refers to a domain that destabilizes a protein operably linked thereto. The destabilization domain can be operably linked to the N-terminus or C-terminus of the protein. Examples of destabilization domains include ubiquitin, PEST sequences (sequences rich in proline, glutamic acid, serine and threonine), cyclin degradation boxes, hydrophobic stretches of amino acids, Escherichia coli dihydrofolate reductase (ecDHFR), human estrogen receptor ligand binding domain (ERLBD), FK506-binding protein (FKBP12), and mutants thereof. Examples of destabilization domains include FKBP12 and its mutants. As FKBP12 mutants used as destabilization domains, for example, F15S, V24A, H25R, E60G, L106P, M66T, R71G, D100G, D100N, E102G and K105I mutants can be mentioned (Banaszynski et al., Cell 126:995 (2006)). The destabilization domain may be a commercially available product, for example, sold by TAKARA Bio (Clontech ProteoTunerTM Shield System C, #631072).

[0022]

The "apoptosis-suppressing gene" is not particularly limited as long as it is a gene that suppresses apoptosis, and examples include BCL2 genes, BCL2L1 genes (protein name: Bcl-

xL), Survivin, and MCL1. Preferably, the BCL2L1 gene is used. As the BCL2L1 gene, for example, a gene consisting of the nucleic acid sequence indicated by NCBI accession number NM_001191 or NM_138578 can be mentioned.

[0023]

In the present specification, a gene encoding a polycomb group protein (hereinafter also referred to as "polycomb gene") refers to a gene that is known to negatively regulate CDKN2a (cyclin-dependent kinase inhibitor 2A, INK4a/ARF) and functions to avoid cellular senescence. Specifically, examples of polycomb genes include BMI1 (Polycomb complex protein BMI-1, polycomb group RING finger protein 4 (PCGF4), RING finger protein 51 (RNF51)), Mel18 (Polycomb group RING finger protein 2), Ring1a/b, Phc1/2/3, Cbx2/4/6/7/8, Ezh2 (Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit), Eed (Embryonic Ectoderm Development), Suz12 (SUZ12 Polycomb Repressive Complex 2 Subunit), HDAC (Histone deacetylases), Dnmt1/3a/3b, and the like; preferably, it is the BMI1 gene. As the BMI1 gene, for example, a gene consisting of the nucleic acid sequence indicated by accession number NM_005180 in NCBI can be mentioned.

[0024]

The origin of each gene (hereinafter also referred to as "megakaryocyte-inducing factor gene") encoding a megakaryocyte-inducing factor is not particularly limited, but it is preferably a mammal (e.g., human, mouse, rat, monkey, cow, horse, pig, dog, etc.), and among these, a human-derived one is preferred. Alternatively, as the megakaryocyte-inducing factor gene, a homolog of a human gene in a non-human mammalian species is also preferred. A gene with high identity to the wild-type gene (e.g., 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identity) is also preferred.

[0025]

Each megakaryocyte-inducing factor gene is preferably operably linked to a drug-responsive promoter. A drug-responsive promoter is a promoter that allows a gene to be expressed, or suppresses expression of the gene, in the presence of a corresponding drug. Examples of drug-responsive promoters include TRE promoters to which a fusion protein of reverse tetR (rtetR) and VP16AD (rtTA), or a fusion protein of tetR and VP16AD (rTA), can bind in the presence or absence of the corresponding drug. Examples of such drugs include doxycycline (Dox), tetracycline, derivatives thereof (hereinafter abbreviated as “Dox, etc.”), and the like. Metallothionein promoters (corresponding drug: heavy metal ions) and steroid-responsive promoters (corresponding drug: a steroid hormone or a derivative thereof) may also be mentioned as drug-responsive promoters. Vectors containing these drug-responsive promoters are also referred to as drug-responsive vectors. In addition to drug-responsive promoters, light-

responsive promoters (induced by light), heat shock protein promoters (induced by heat shock), and the like may also be used, and promoters induced by these drugs or stimuli are also referred to as inducible promoters.

[0026]

In the present specification, "maintaining platelet production capacity" means not only that the platelet production capacity of megakaryocytes is maintained without decreasing over the course of proliferation culture, but also that the megakaryocytes have a higher platelet production capacity than a control (specifically, megakaryocytes cultured under the same conditions without increasing the abundance of proteins constituting the corresponding UQCR, or megakaryocytes cultured under the same conditions without inhibiting the function of PRC1.1 (in one embodiment, without reducing the abundance of proteins constituting the corresponding PRC1.1)). In the present specification, "platelet production capacity" refers to the capacity to produce platelets through proplatelet formation by transferring megakaryocytes to the maturation stage. The transfer of megakaryocytes to the maturation stage can be carried out by the "step of maturing megakaryocytes" described below. As shown in the Examples below, if the ratio of platelets collected after culturing for 6 days after transferring megakaryocytes to the maturation stage on day 28 from the start of proliferation (platelet count/megakaryocyte count) is higher than that of the control, it can be said that the platelet production capacity of the megakaryocytes is maintained.

[0027]

Ubiquinol-cytochrome c reductase (UQCR) is one of the four complexes present in the mitochondrial inner membrane, and is also referred to as "Complex III." Within mitochondria, UQCR catalyzes an electron transfer reaction from ubiquinol to cytochrome c, while simultaneously actively transporting protons from the matrix side to the cytoplasmic side. Although not intended to be bound by any particular theory, one reason why forced expression of the UQCR core protein enhances platelet production is that it promotes the proliferation capacity of megakaryocytes via enhancement of mitochondrial energy production capacity. Therefore, if a protein can exert such an effect, any protein constituting UQCR other than the UQCR core protein can be used in the present invention. As proteins constituting UQCR, for example, UQCRC1, UQCRC2, UQCRB, BCS1L, UQCRQ, CYC1, TTC19, LYRM7, UQCC2, and UQCC3 can be mentioned, but preferably the UQCR core protein (i.e., UQCRC1, UQCRC2) is used, with UQCRC2 being more preferable. These may be used alone or in combination (for example, UQCRC1 and UQCRC2 are used in combination).

[0028]

An example of the base sequence and amino acid sequence of human UQCRC1 is shown as SEQ ID NO: 1 (the coding sequence (CDS) of NCBI accession number NM_003365.3) and SEQ

ID NO: 2 (NCBI accession number NP_003356.2). Also, an example of the base sequence and amino acid sequence of human UQCRC2 is shown as SEQ ID NO: 3 (the CDS of NCBI accession number NM_003366.4) and SEQ ID NO: 4 (NCBI accession number NP_003357.2). An example of the base sequence and amino acid sequence of human BACH1 is shown as SEQ ID No: 5 (the CDS of NCBI accession number NM_001186.4) and SEQ ID NO: 6 (NCBI accession number NP_001177.1).

[0029]

PRC1.1 is a complex that mediates transcriptional repression by polycomb group proteins, and it suppresses gene expression through ubiquitination of Lys119 of histone H2A (H2AK119). PRC1.1 is involved in various biological processes, including epigenetic regulation of gene expression, cell differentiation, proliferation, and suppression of tumor formation (e.g., Nakajima-Takagi Y. et al., Elife. 12: e83004 (2023)). PRC1.1 mainly consists of PCGF1 (Polycomb group RING finger protein 1), RING1A (ring finger protein 1A) or RING1B (ring finger protein 1B), KDM2B (lysine demethylase 2B), SKP1 (S-phase kinase-associated protein 1), and BCOR or BCORL1. In addition, TRIM27 and USP7 (Ubiquitin-specific-processing protease) can also be mentioned as proteins constituting PRC1.1 (Maat H., et al., iScience. 24(5): 102435 (2021)). In the production method of the present invention, a method for inhibiting the function of PRC1.1 is not particularly limited as long as it can inhibit the ubiquitination of Lys119 of H2AK119, and examples include a method in which megakaryocytes are brought into contact with an inhibitor of PRC1.1 (typically, culturing megakaryocytes in a medium containing the inhibitor), and a method in which the abundance of at least one protein constituting PRC1.1 is reduced.

[0030]

Examples of PRC1.1 inhibitors used in the present invention include RING1B inhibitors such as PRT4165, KDM2B inhibitors such as GSK-J4, SD70, and CPI-455, BCOR inhibitors such as Lenalidomide and Pomalidomide, and PCGF1 inhibitors such as AUX-001 and Pevonedistat, but are not limited to these. These may be used alone or in combination.

[0031]

Due to a frameshift caused by a partial deletion of the BCOR gene, a mutation introduced into the PCGF1-binding site, or the like, PRC1.1 becomes unable to accumulate at the target sequence. For other proteins constituting PRC1.1, reducing the abundance of the protein by knockdown or the like can inhibit the function of PRC1.1. Therefore, the target protein whose abundance is to be reduced may be any constituent protein of PRC1.1, such as PCGF1, RING1A, RING1B, KDM2B, SKP1, BCOR, BCORL1, TRIM27, or USP7, and is preferably BCOR, BCORL1, or TRIM27. One or more of these proteins may be targeted.

[0032]

As shown in the Examples described below, platelet production capacity can be improved by overexpressing BACH1 in the production method of the present invention. Therefore, in one embodiment of the present invention, the production method of the present invention includes a step of increasing the abundance of the BACH1 protein in megakaryocytes. Accordingly, the step of increasing the abundance of the BACH1 protein in megakaryocytes may be combined with a step of increasing the abundance of at least one protein constituting the UQCR described above and/or a step of inhibiting the function of PRC1.1. When these steps are combined, each step may be performed sequentially or simultaneously. BACH1 is a type of transcription factor, and it has been reported that, together with Bach2, it promotes erythrocyte formation by regulating heme metabolism in erythrocytes (e.g., Kato H., et al., Nat Immunol. 19(10):1059-1070 (2018)).

[0033]

The UQCR core protein and the BACH1 protein used in the present invention are not particularly limited in their origin, but preferably are from mammals (e.g., humans, mice, rats, monkeys, cows, horses, pigs, dogs, etc.), with human-derived proteins being particularly preferred. Furthermore, it is also preferable that the UQCR core protein and the BACH1 protein used in the present invention are homologous proteins derived from a mammal species other than humans. Alternatively, they may be paralogous proteins. Also, the UQCR core protein and the BACH1 protein include not only wild-type proteins but also variants thereof having similar functions. Examples of variants include proteins that have high identity (e.g., 80% or higher, 85% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, or 99% or higher) with the amino acid sequence of a specific wild-type protein (e.g., the sequence shown in SEQ ID NO: 2, 4, or 6). Also preferred are variants that consist of an amino acid sequence in which one or more (two, three, four, five, six, seven, eight, nine, or ten) amino acids are deleted, substituted, inserted, and/or added in the amino acid sequence of a specific wild-type protein (e.g., the sequence shown in SEQ ID NO: 2, 4, or 6), and have the same function as the wild-type protein.

[0034]

In the present specification, "increasing the abundance of a protein" means that the abundance of the protein in megakaryocytes is greater than the abundance of the same protein existing in the megakaryocytes before the process of increasing the abundance of the specific protein. The state of increased protein abundance may be maintained constantly in the megakaryocytes, or it may be transiently present. A method for increasing the abundance of a protein constituting UQCR in megakaryocytes is not particularly limited, and examples thereof include a method of introducing an exogenous nucleic acid (DNA or RNA) encoding a protein constituting UQCR into megakaryocytes or cells having differentiation potential into

megakaryocytes (e.g., hematopoietic progenitor cells, pluripotent stem cells), thereby causing the forced expression of the protein; a method of activating the expression of an endogenous gene encoding a protein constituting UQCR by modifying a promoter using genome editing techniques and the like; and a method of adding a protein constituting UQCR to the culture medium and delivering the protein into the cells. The protein constituting UQCR, or the nucleic acid encoding the protein, may be introduced into the cells once or multiple times. A method for increasing the abundance of BACH1 protein in megakaryocytes is also similar.

[0035]

On the other hand, "reducing the abundance of a protein" means making the abundance of the specific protein present in megakaryocytes lower than the abundance of the specific protein present in megakaryocytes before the step of reducing the abundance of the specific protein. Such protein abundance is typically the abundance of wild-type (in other words, normally functioning) protein. In megakaryocytes, a state of low protein abundance may be maintained constantly, or the protein abundance may be low transiently. The method for reducing the abundance of proteins constituting PRC1.1 is not particularly limited. Examples thereof include methods of knocking down the expression of each protein by introducing antisense nucleic acids (e.g., antisense oligonucleotides (ASOs)) (including nucleic acids encoding the same), siRNA (including nucleic acids encoding the same), heteroduplex oligonucleotides (HDOs), shRNA (including nucleic acids encoding the same), or miRNA (microRNA) (including nucleic acids encoding the same), which are directed against mRNAs encoding proteins constituting PRC1.1, into megakaryocytes or cells having the potential to differentiate into megakaryocytes. The introduction of these nucleic acids into megakaryocytes may be performed once or multiple times. For example, when these nucleic acids are integrated into the genome of the cell, continuous expression of the nucleic acid in the cell can be expected even with a single introduction. In addition, mutations that cause loss of function of proteins constituting PRC1.1, mutations accompanying a decrease in the function of the protein and/or a decrease in the expression abundance of the protein may be introduced into a gene by techniques such as genome editing. Such mutations include, for example, nonsense mutations, frameshift mutations, missense mutations, mutations accompanying splicing abnormalities, mutations in gene regulatory regions (e.g., promoters, enhancers), dominant-negative mutations, and the like.

[0036]

Nucleic acids, such as antisense nucleic acids, for suppressing the expression or the like of proteins constituting PRC1.1 can be appropriately designed with reference to known nucleotide sequence information and the like. An example of the nucleotide sequence and an example of the amino acid sequence of human BCOR are shown as SEQ ID NO: 7 (the CDS of NCBI Accession No. NM_001123385.2) and SEQ ID NO: 8 (NCBI Accession No. NP_001116857.1),

respectively. An example of the nucleotide sequence and an example of the amino acid sequence of human BCORL1 are shown as SEQ ID NO: 9 (the CDS of NCBI Accession No. NM_001184772.3) and SEQ ID NO: 10 (NCBI Accession No. NP_001171701.1), respectively. An example of the nucleotide sequence and an example of the amino acid sequence of human TRIM27 are shown as SEQ ID NO: 11 (the CDS of NCBI Accession No. NM_006510.5) and SEQ ID NO: 12 (NCBI Accession No. NP_006501.1), respectively.

[0037]

In the present specification, when it is stated that the abundance of a protein is increased or decreased in megakaryocytes, it is not necessary to increase or decrease the abundance of the protein only at the megakaryocyte stage. Instead, a protein or nucleic acid encoding the protein may be introduced into cells having differentiation potential into megakaryocytes (e.g., pluripotent stem cells, hematopoietic progenitor cells), and the abundance of the protein may already be increased or decreased in the cells before they differentiate into megakaryocytes. Therefore, for example, increasing the expression level of a specific protein (i.e., BACH1 protein, any protein constituting UQCR) in pluripotent stem cells and maintaining that state, followed by inducing differentiation of the pluripotent stem cells into megakaryocytes, is also included in the process of increasing the abundance of the target protein (or decreasing it) in megakaryocytes. The same applies to the process of inhibiting the function of PRC1.1 or reducing the abundance of any protein constituting PRC1.1 in megakaryocytes.

[0038]

Exogenous nucleic acids are typically introduced into cells (e.g., megakaryocytes, hematopoietic progenitor cells, pluripotent stem cells, and the like) in the form of an expression vector containing the nucleic acid. Examples of vectors for expressing exogenous nucleic acids that may be used include viral vectors, such as retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, herpesvirus vectors, and Sendai virus vectors, and animal cell expression plasmids (e.g., pA1-11, pXT1, pRc/CMV, pRc/RSV, and pcDNAI/Neo). Retroviral vectors or lentiviral vectors are preferred in that introduction can be accomplished by a single transduction.

[0039]

Examples of a promoter used in an expression vector include an EF-α promoter, a CAG promoter, an SRα promoter, an SV40 promoter, an LTR promoter, a CMV (cytomegalovirus) promoter, an RSV (Rous sarcoma virus) promoter, an MoMuLV (Moloney murine leukemia virus) LTR, and an HSV-TK (herpes simplex virus thymidine kinase) promoter. In addition to the promoter, the expression vector may optionally include an enhancer, a poly-A addition signal, a selection marker gene, an SV40 replication origin, and the like. Examples of useful

selection marker genes include a dihydrofolate reductase gene, a neomycin resistance gene, and a puromycin resistance gene.

[0040]

In addition, the aforementioned inducible promoters may be used as promoters used in expression vectors. This allows the exogenous nucleic acids to be expressed at a desired timing and for a desired period. Furthermore, in order to excise the exogenous nucleic acids from the vector using the Cre-loxP system, an expression vector in which loxP sequences are arranged so as to flank a gene, a promoter region, or both may be used. This enables expression to be stopped when expression of the exogenous nucleic acids is no longer necessary.

[0041]

Exogenous nucleic acids may be introduced into cells by a transposon system. Transposons are a general term for short gene sequences that cause gene transposition and have been conserved throughout the process of evolution. A transposon system causes gene transposition with a pair of a gene enzyme (transposase) and its specific recognition sequence. As a transposon system, for example, the PiggyBac™ transposon system can be used. The PiggyBac™ transposon system utilizes a transposon isolated from insects (see Fraser MJ et al., Insect Mol Biol. 1996 May;5(2):141-51.; Wilson MH et al., Mol Ther. 2007 Jan;15(1):139-45.), and enables highly efficient integration into mammalian chromosomes. The PiggyBac™ transposon system is actually used for gene introduction (see, for example, Nakazawa Y, et al., J Immunother 32:826-836, 2009; Nakazawa Y et al., J Immunother 6:3-10, 2013, etc.). The transposon system applicable to the present invention is not limited to those utilizing PiggyBac™, and systems utilizing transposons such as Sleeping Beauty (Ivics Z, Hackett PB, Plasterk RH, Izsvak Z (1997) Cell 91: 501-510.), Frog Prince (Miskey C, Izsvak Z, Plasterk RH, Ivics Z (2003) Nucleic Acids Res 31: 6873-6881.), Tol1 (Koga A, Inagaki H, Bessho Y, Hori H. Mol Gen Genet. 1995 Dec 10;249(4):400-5.; Koga A, Shimada A, Kuroki T, Hori H, Kusumi J, Kyono-Hamaguchi Y, Hamaguchi S. J Hum Genet. 2007;52(7):628-35. Epub 2007 Jun 7.), and Tol2 (Koga A, Hori H, Sakaizumi M (2002) Mar Biotechnol 4: 6-11.; Johnson Hamlet MR, Yergeau DA, Kuliyev E, Takeda M, Taira M, Kawakami K, Mead PE (2006) Genesis 44: 438-445.; Choo BG, Kondrichin I, Parinov S, Emelyanov A, Go W, Toh WC, Korzh V (2006) BMC Dev Biol 6: 5.) may also be adopted.

[0042]

Introduction operations by a transposon system may be performed by conventional methods, and publicly known documents (for example, for the PiggyBac™ transposon system, see the aforementioned Nakazawa Y, et al., J Immunother 32:826-836, 2009, Nakazawa Y et al., J Immunother 6:3-10, 2013, or Saha S, Nakazawa Y, Huye LE, Doherty JE, Galvan DL, Rooney CM, Wilson MH. J Vis Exp. 2012 Nov 5;(69): e4235, etc.) can be referred to. In one

embodiment, the above gene is introduced into cells by the PiggyBac™ transposon system. Typically, in the PiggyBac™ transposon system, a vector (transposase plasmid) containing a gene encoding PiggyBac™ transposase and a vector having a structure in which a gene encoding a target protein is flanked by PiggyBac™ inverted repeat sequences (transposon plasmid; PiggyBac™ vector) are prepared, and these two vectors are introduced into target cells. A gene encoding a megakaryocyte-inducing factor may also be introduced into cells such as pluripotent stem cells and hematopoietic stem cells using a transposon system.

[0043]

In addition, in order to introduce a plurality of genes simultaneously, the genes may be linked in tandem to obtain a polycistronic vector. To enable polycistronic expression, a 2A self-cleaving peptide of foot-and-mouth disease virus (see, for example, Science, 322, 949-953, 2008, etc.) and IRES sequences may be used by ligating them between the genes to be forcibly expressed.

[0044]

Various techniques can be used for the introduction of exogenous nucleic acids into cells, such as lipofection, liposome method, electroporation, nucleofection, calcium phosphate co-precipitation, DEAE-dextran method, microinjection, and gene gun method. When the exogenous nucleic acid is contained in a viral vector, introduction into cells is possible by introducing a plasmid containing the nucleic acid into appropriate packaging cells (e.g., Plat-E cells) or complementing cell lines (e.g., 293 cells), recovering the viruses produced in the culture supernatant, and allowing the cells to be infected by contacting them with the viruses.

[0045]

When delivering proteins to cells, this can be implemented using known methods for introducing proteins into cells. Such methods include, for example, methods using protein introduction reagents, methods using protein transduction domain (PTD) or cell-penetrating peptide (CPP) fusion proteins, and microinjection. As protein introduction reagents, those such as BioPOTER Protein Delivery Reagent (Gene Therapy Systems), Pro-Ject™ Protein Transfection Reagent (PIERCE), and ProVectin (IMGENEX) based on cationic lipids; Profect-1 (Targeting Systems) based on lipids; Penetratin Peptide (Q biogene) and Chariot Kit (Active Motif) based on membrane-permeable peptides; and GenomONE (Ishihara Sangyo) utilizing HVJ envelopes (inactivated Sendai virus) are commercially available. Introduction can be performed according to the protocols attached to these reagents.

[0046]

In one embodiment, megakaryocyte proliferation can be initiated by increasing the abundance of MYC protein (particularly c-MYC) and an apoptosis-suppressing protein (particularly Bcl-xL) (preferably, further increasing the abundance of a polycomb group protein,

particularly BMI1). Therefore, in a preferred embodiment of the present invention, the production method of the present invention includes a step of increasing the abundance of MYC protein and an apoptosis-suppressing protein (preferably, further increasing the abundance of a polycomb group protein) in megakaryocytes. The method for increasing the abundance of these proteins in megakaryocytes is not particularly limited, but examples include a method of increasing the expression level of a nucleic acid encoding the protein and a method of adding the protein to the medium and delivering it into cells. As a method for increasing the expression level of the gene encoding the protein, specific examples include a method of introducing an exogenous nucleic acid (DNA or RNA) encoding the protein into megakaryocytes and a method of culturing megakaryocytes in the presence of a corresponding drug or stimulus using the above-mentioned inducible promoter.

[0047]

The culture in the production method of the present invention may be either suspension culture or adhesion culture, but is typically suspension culture. In addition, megakaryocytes isolated from a living organism can be cultured by adhesion culture. Since megakaryocytes maintaining platelet production capacity can be proliferated for a long period of time by the production method of the present invention, there is no particular limit on the culture period, but it is typically 6 days or more, and may be 12 days or more, 18 days or more, 24 days or more, 30 days or more, 36 days or more, 42 days or more, 48 days or more, 54 days or more, or 60 days or more.

[0048]

Furthermore, in megakaryocytes, the period during which the abundance of at least one protein constituting UQCR is maintained in an elevated state, the period during which the abundance of the BACH1 protein is maintained in an elevated state, and the period during which the function of PRC1.1 is inhibited are not particularly limited. Each of these periods may be limited to the period during which megakaryocytes are caused to proliferate, or may also continue during the period during which megakaryocytes are caused to mature. Such periods are typically 6 days or more, and may be 12 days or more, 18 days or more, 24 days or more, 30 days or more, 36 days or more, 42 days or more, 48 days or more, 54 days or more, or 60 days or more. On the other hand, from the viewpoint of efficient maturation, it is preferable that, in megakaryocytes, the period during which the abundance of a megakaryocyte-inducing factor, particularly the MYC protein, is maintained in an elevated state be limited to the period during which megakaryocytes are caused to proliferate.

[0049]

In the present specification, "suspension culture" means culture carried out under conditions in which cells or cell aggregates maintain a state of floating in a culture solution, i.e., culture

under conditions in which a strong cell-substratum junction is not allowed to be formed between the cells or cell aggregates and the culture vessel. Suspension culture may be static culture or shaking culture; in the case of shaking culture, it can be said to be suspension culture as long as conditions are such that cells are not fixed in one place.

[0050]

The culture vessel used for suspension culture is not particularly limited, but examples include a flask, tissue culture flask, dish, petri dish, tissue culture dish, multidish, microplate, microwell plate, micropore, multiplate, multiwell plate, chamber slide, schale, tube, tray, culture bag, and roller bottle. However, for the shaking culture (also referred to as "stirred culture") described below, it is preferable that the culture vessel is sealed. Examples of such culture vessels include a tissue culture flask, culture bag, and roller bottle. In addition, to enable culture under non-adhesive conditions, the culture vessel is preferably non-adhesive to cells. As a non-adhesive culture vessel, a vessel whose surface is not artificially treated for the purpose of improving adhesion with cells (e.g., coating treatment with an extracellular matrix), or a vessel that has been artificially treated to suppress adhesion (e.g., coating treatment with poly(2-hydroxyethyl methacrylate) (poly-HEMA)), can be used.

[0051]

Megakaryocytes used in the present invention can be obtained by known methods. Examples include methods of isolating them from biological tissue (e.g., bone marrow, cord blood, peripheral blood, etc.) by known techniques, methods of inducing differentiation of pluripotent stem cells or hematopoietic progenitor cells, and methods of obtaining them from companies such as ATCC. Megakaryocytes can be isolated from biological tissue using, for example, methods using flow cytometry or mass cytometry using cell-surface antigens as an index, magnetic cell separation, or affinity columns having a desired antigen immobilized thereon. The megakaryocytes used in the present invention are preferably obtained by inducing differentiation of hematopoietic progenitor cells. Therefore, prior to producing megakaryocytes maintaining platelet production capacity, the production method of the present invention may include a step (hereinafter also referred to as a "megakaryocyte production step") of producing megakaryocytes from pluripotent stem cells or hematopoietic progenitor cells (also referred to as "inducing differentiation"). Hematopoietic progenitor cells can be obtained, for example, by isolating them from biological tissue (e.g., bone marrow, cord blood, peripheral blood, etc.) or by inducing differentiation from pluripotent stem cells.

[0052]

The megakaryocyte production process is not particularly limited and can be carried out using known induction methods. As a specific example, the production of megakaryocytes can be carried out using methods described in, for example, International Publication No.

2011/034073 and International Publication No. 2012/157586. In one embodiment, the megakaryocyte production process includes step (A) culturing pluripotent stem cells to produce hematopoietic progenitor cells (which can also be referred to as "differentiation induction") and/or step (B) culturing hematopoietic progenitor cells to produce megakaryocytes.

[0053]

As a method for differentiating pluripotent stem cells into hematopoietic progenitor cells in step (A), any method is acceptable as long as differentiation into hematopoietic progenitor cells can be achieved, but examples include a method of preparing them from a net-like structure (also referred to as ES-sac or iPS-sac) obtainable by culturing pluripotent stem cells on C3H10T1/2 in the presence of VEGF, according to the method described in Takayama N., et al. J Exp Med. 2817-2830 (2010). Here, "net-like structures" refer to three-dimensional sac-like (containing an internal space) structures derived from pluripotent stem cells, which are formed by endothelial cell populations and the like, and contain hematopoietic progenitor cells internally. Other examples include a method by forming embryoid bodies and adding cytokines (Chadwick et al. Blood 2003, 102: 906-15, Vijayaragavan et al. Cell Stem Cell 2009, 4: 248-62, Saeki et al. Stem Cells 2009, 27: 59-67) or a co-culture method with heterologous stromal cells (Niwa A et al. J Cell Physiol. 2009 Nov; 221(2): 367-77). Furthermore, methods described in, for example, International Publication No. 2013/075222, International Publication No. 2016/076415, International Publication No. 2017/221975, and Liu S. et al., Cytotherapy, 17 (2015); 344-358 may also be mentioned.

[0054]

The term "pluripotent stem cell" refers to a stem cell that can differentiate into tissues or cells having various different forms or functions in the body, and that can also differentiate into cells of any lineages of the three germ layers (endoderm, mesoderm, and ectoderm). Examples of pluripotent stem cells used in the present invention include induced pluripotent stem cells (iPS cells), embryonic stem cells (ES cells), embryonic stem cells from cloned embryos obtainable by nuclear transfer (nuclear transfer embryonic stem cells; ntES cells), multipotent germline stem cells ("mGS cells"), and embryonic germ cells (EG cells), of which iPS cells (and more preferably human iPS cells) are preferred. When the above pluripotent stem cells are ES cells or any cells derived from human embryos, the cells may be produced by destroying an embryo or without destroying an embryo, but from an ethical point of view, they are preferably cells produced without destroying an embryo.

[0055]

ES cells are stem cells having pluripotency and proliferation potency by auto-replication, established from the inner cell mass of an early embryo (e.g., blastocyst) of a mammal such as a human or mouse. ES cells were discovered in mice in 1981 (M.J. Evans and M.H. Kaufman

(1981), Nature 292:154-156), and subsequently, ES cell lines were established in primates such as humans and monkeys (J.A. Thomson et al. (1998), Science 282:1145-1147; J.A. Thomson et al. (1995), Proc. Natl. Acad. Sci. USA, 92:7844-7848; J.A. Thomson et al. (1996), Biol. Reprod., 55:254-259; J.A. Thomson and V.S. Marshall (1998), Curr. Top. Dev. Biol., 38:133-165). ES cells can be established by extracting the inner cell mass from the blastocyst of a fertilized egg of a target animal and culturing the inner cell mass on a fibroblast feeder. Alternatively, ES cells can be established using a single embryo blastomere alone during the cleavage stage before the blastocyst stage (Chung Y. et al. (2008), Cell Stem Cell 2: 113-117), or they can be established using a developmentally arrested embryo (Zhang X. et al. (2006), Stem Cells 24: 2669-2676.).

[0056]

As ES cell lines used in the present invention, in the case of mouse ES cells, various mouse ES cell lines established by, for example, inGenious targeting laboratory, Riken (Riken Research Institute), etc., are available; in the case of human ES cell lines, various human ES cell lines established by, for example, the University of Wisconsin, NIH, Riken, Kyoto University, the National Center for Child Health and Development, and Cellartis, etc., are available. Specifically, for example, human ES cell lines include CHB-1 to CHB-12 lines, RUES1 line, RUES2 line, HUES1 to HUES28 lines, etc., distributed by ESI Bio Co.; H1 and H9 lines, etc., distributed by WiCell Research; and KhES-1, KhES-2, KhES-3, KhES-4, KhES-5, SSES1, SSES2, and SSES3 lines, etc., distributed by Riken.

[0057]

iPS cells are cells obtainable by reprogramming mammalian somatic cells or undifferentiated stem cells by introducing specific factors (nuclear reprogramming factors). Currently, various iPS cells exist, and in addition to iPSCs established by introducing 4 factors of Oct3/4, Sox2, Klf4, and c-Myc into mouse fibroblasts by Yamanaka et al. (Takahashi K, Yamanaka S., Cell, (2006) 126: 663-676), those such as human cell-derived iPSCs established by introducing the same 4 factors into human fibroblasts (Takahashi K, Yamanaka S., et al. Cell, (2007) 131: 861-872.), Nanog-iPSCs established by selecting based on Nanog expression after introducing the above 4 factors (Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Nature 448, 313-317.), iPSCs produced by a method not including c-Myc (Nakagawa M, Yamanaka S., et al. Nature Biotechnology, (2008) 26, 101-106), and iPSCs established by introducing 6 factors by a virus-free method (Okita K et al. Nat. Methods 2011 May; 8(5):409-12, Okita K et al. Stem Cells. 31(3):458-66.), etc. may also be used. Furthermore, induced pluripotent stem cells established by introducing 4 factors of OCT3/4, SOX2, NANOG, and LIN28 by Thomson et al. (Yu J., Thomson JA. et al., Science (2007) 318: 1917-1920.), induced pluripotent stem cells produced by Daley et al. (Park IH, Daley GQ. et al., Nature (2007) 451: 141-146), and induced

pluripotent stem cells produced by Sakurata et al. (Japanese Unexamined Patent Publication No. 2008-307007), etc., may also be used.

In addition, any induced pluripotent stem cells publicly known in the field, as described in any published paper (for example, Shi Y., Ding S., et al., Cell Stem Cell, (2008) Vol. 3, Issue 5, 568-574; Kim JB., Scholer HR., et al., Nature, (2008) 454, 646-650; Huangfu D., Melton, DA., et al., Nature Biotechnology, (2008) 26, No. 7, 795-797) or patent gazette (for example, Japanese Unexamined Patent Publication No. 2008-307007, Japanese Unexamined Patent Publication No. 2008-283972, US2008-2336610, US2009-047263, WO2007-069666, WO2008-118220, WO2008-124133, WO2008-151058, WO2009-006930, WO2009-006997, WO2009-007852), may be used.

[0058]

As induced pluripotent stem cell lines, various iPS cell lines established by, for example, NIH, Riken, Kyoto University, and CiRA Foundation, etc., are available. For example, in the case of human iPS cell lines, HiPS-RIKEN-1A, HiPS-RIKEN-2A, HiPS-RIKEN-12A, Nips-B2, etc., by Riken; 253G1, 253G4, 1201C1, 1205D1, 1210B2, 1383D2, 1383D6, 201B7, 409B2, 454E2, 606A1, 610B1, 648A1, 1231A3, and TkDN-sev2, etc., by Kyoto University; and FfI-01s04, Ff-MH23s01, 15M41 (Ff-I 01s01), and QHJI14s04, etc., established by CiRA Foundation, are examples. Human iPS cell lines established other than the above may also be used, such as TC-1133 line from RONZA. In addition, iPS cell lines having a mutation in each protein constituting PRC1.1 (especially BCOR), such that the function of PRC1.1 is reduced, are also preferred.

Furthermore, cell lines that have undergone genetic modification of iPS cell lines, for example, cell lines in which gene disruption or modification has been performed to reduce antigenicity, may also be suitably used. Examples of such iPS cell lines include QHJI14s04-AB II-KO-03, QHJI14s04-AB II-KO-11, and QHJI14s04-AB II-KO-12 lines established by CiRA Foundation, and Universal Donor Cells established by Healios, etc.

[0059]

The induced pluripotent stem cells used in the present invention may be cells derived from patients with hereditary diseases (for example, patients with hereditary blood diseases, etc.). Since cells induced to differentiate from pluripotent stem cells derived from patients with hereditary blood diseases can become disease models reflecting the pathology of the disease, they are suitable for the screening of therapeutic or preventive drugs for the disease, etc. Alternatively, by repairing a gene using genome editing such as the CRISPR-Cas system on pluripotent stem cells derived from patients with hereditary blood diseases and then differentiating them into the target cells, it becomes possible to use the cells as a therapeutic agent for the disease.

[0060]

The species from which the pluripotent stem cells are derived is not particularly limited, and the pluripotent stem cells may be derived from, for example, rodents such as rats, mice, hamsters, and guinea pigs; lagomorphs such as rabbits; ungulates such as pigs, cattle, goats, and sheep; carnivorans such as dogs and cats; or primates such as humans, monkeys, rhesus monkeys, marmosets, orangutans, and chimpanzees. The preferred species of origin is human.

[0061]

In the present specification, "hematopoietic progenitor cell" refers to a cell having the capacity to differentiate into hematopoietic cells such as lymphocytes, eosinophils, neutrophils, basophils, erythrocytes, and megakaryocytes. In the present specification, hematopoietic progenitor cells and hematopoietic stem cells are not distinguished and indicate the same cell unless otherwise specified. Hematopoietic progenitor cells typically express the CD34 gene and/or the CD43 gene.

[0062]

The culture period in step (A) can be determined as appropriate by those skilled in the art while monitoring the number of hematopoietic progenitor cells, and the like. As long as hematopoietic progenitor cells are obtainable, the number of days is not particularly limited, but it is typically 6 days or more, preferably 7 days or more, 8 days or more, 9 days or more, 10 days or more, 11 days or more, 12 days or more, 13 days or more, or 14 days or more (particularly 14 days). Although a long culture period does not pose a problem in the production of hematopoietic progenitor cells, it is typically 30 days or less, and may be 20 days or less.

[0063]

Step (B) can also be carried out using known methods (e.g., those described in Patent Literatures 1 to 4). Specifically, a method of simultaneously forcing the expression of an apoptosis-suppressing gene (e.g., BCL2L1 gene) and a MYC gene (e.g., c-MYC gene) in hematopoietic progenitor cells and culturing the cells can be mentioned.

[0064]

The culture period in step (B) can be determined as appropriate by those skilled in the art while monitoring the number of megakaryocytes, and the like. As long as megakaryocytes are obtainable, the number of days is not particularly limited, but it is typically 5 days or more, preferably 6 days or more, and more preferably 7 days or more. The upper limit of the culture period is also not particularly limited, but it is typically 20 days, preferably 17 days or less, and more preferably 14 days or less.

[0065]

In one embodiment of the present invention, any step in the production method (e.g., a megakaryocyte production step) is carried out in the presence of a pyrimidoindole derivative.

Examples of the pyrimidoindole derivative include UM171 ((1r,4r)-N1-(2-benzyl-7-(2-methyl-2H-tetrazol-5-yl)-9H-pyrimido[4,5-b]indol-4-yl)cyclohexane-1,4-diamine) and UM729 (methyl 4-(3-(1-piperidinyl)propylamino)-9H-pyrimido[4,5-b]indole-7-carboxylate). A pyrimido[4,5-b]indole derivative is preferred as the pyrimidoindole derivative, and UM171 or UM729 is more preferred. The pyrimidoindole derivative may be used alone or in combination of multiple types.

[0066]

The concentration of the pyrimidoindole derivative can be appropriately selected by those skilled in the art depending on the pyrimidoindole derivative to be used. When the pyrimidoindole derivative is UM729, its concentration in the medium is typically 1 nM to 100 μM, preferably 10 nM to 100 μM, and more preferably 100 nM to 10 μM (in one embodiment, 1 μM).

[0067]

The pyrimidoindole derivative may be added to the medium when culturing hematopoietic progenitor cells, or it may be added to the medium during culture of cells at any stage from pluripotent stem cells to hematopoietic progenitor cells. For example, pluripotent stem cells or cells at any stage from pluripotent stem cells to hematopoietic progenitor cells can be cultured in a medium containing the pyrimidoindole derivative.

[0068]

Any step in the production method of the present invention (e.g., megakaryocyte production step, megakaryocyte proliferation step) may be performed in the presence of an HDAC (histone deacetylase) 5 inhibitor and/or an LSD1 inhibitor. Therefore, in one embodiment, the production method of the present invention includes culturing megakaryocytes in a medium containing an HDAC5 inhibitor and/or an LSD1 inhibitor (preferably, a medium containing both an HDAC5 inhibitor (e.g., LMK235) and an LSD1 inhibitor (e.g., tranylcypromine)). In another embodiment, the present invention provides a method for producing a megakaryocyte, including culturing a hematopoietic progenitor cell in a medium containing an HDAC5 inhibitor and/or an LSD1 inhibitor.

[0069]

The HDAC5 inhibitor used in the present invention is not limited to an inhibitor specific to HDAC5, and may have inhibitory activity against other molecules (typically, other HDACs). Examples of HDAC5 inhibitors include LMK235, TMP195, Quisinostat (JNJ-26481585) 2HCl, CUDC-101, Pracinostat (SB939), TMP269, Domatinostat (4SC-202), and the like, but preferably LMK235 and TMP195, with LMK235 being more preferred. Also preferred are antibodies, peptides, or aptamers against HDAC5. Alternatively, the HDAC5 inhibitor may be an expression suppressor of HDAC5, such as antisense nucleic acid, siRNA, shRNA, or a heteroduplex nucleic acid that suppresses the expression of HDAC5. The HDAC5 inhibitor may be used alone or in

combination. It is also preferred to combine the HDAC5 inhibitor with a pyrimidoindole derivative.

[0070]

The concentration of the HDAC5 inhibitor can be appropriately selected by those skilled in the art depending on the HDAC5 inhibitor used. When the HDAC5 inhibitor is LMK235, its concentration in the medium is typically 1 nM to 100 μM, preferably 10 nM to 100 μM, and more preferably 20 nM to 1 μM (in one embodiment, 50 nM to 200 nM).

[0071]

The LSD1 inhibitor is not particularly limited as long as it can inhibit the enzymatic activity of LSD1 (i.e., demethylation activity of histone H3K4me1/2 and H3K9me1/2), but examples include tranylcypromine (TCP), ORY-1001 (Iadademstat), GSK2879552, IMG-7289 (Bomedemstat), SP-2509, HCI-2509, RN-1, MC3324, SP-2577 (Seclidemstat), Corin, and the like, but preferably tranylcypromine. Also preferred are antibodies, peptides, or aptamers against LSD1. Alternatively, the LSD1 inhibitor may be an expression suppressor of LSD1, such as antisense nucleic acid, siRNA, shRNA, or a heteroduplex nucleic acid that suppresses the expression of LSD1. The LSD1 inhibitor may be used alone or in combination. It is also preferred to combine the LSD1 inhibitor with a pyrimidoindole derivative.

[0072]

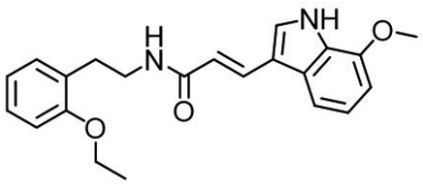
The concentration of the LSD1 inhibitor can be appropriately selected by those skilled in the art depending on the LSD1 inhibitor used. When the LSD1 inhibitor is tranylcypromine, its concentration in the medium is typically 5 nM to 500 μM, preferably 50 nM to 500 μM, and more preferably 500 nM to 5 μM (in one embodiment, 1 μM).

[0073]

As shown in the Examples below, based on insights gained from phenomena occurring in the bone marrow, the present inventors succeeded in enhancing the platelet production capacity of megakaryocytes by culturing the megakaryocytes in the presence of an adipogenic differentiation-inducing factor. Therefore, any step in the production method of the present invention (e.g., the megakaryocyte production step) may be carried out in the presence of an adipogenic differentiation-inducing factor. Examples of adipogenic differentiation-inducing factors include insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and indomethacin. In one embodiment, the production method of the present invention includes a step of culturing megakaryocytes in a medium containing dexamethasone, IBMX, and insulin. Furthermore, it is also preferable that the medium used in the megakaryocyte production step contains JI051. In one embodiment, the medium used in the megakaryocyte production step contains dexamethasone, IBMX, insulin, and JI051. The structural formula of JI051 is shown below.

[0074]

[Chemical 2]



[0075]

Furthermore, expression or function of the TP53 gene (protein name: p53) and/or CDKN1A gene (protein name: p21) may be suppressed in hematopoietic progenitor cells. As the TP53 gene, for example, a gene consisting of a nucleic acid sequence represented by accession number NM_000546.6 in NCBI can be mentioned. As the CDKN1A gene, for example, a gene consisting of a nucleic acid sequence represented by accession number NM_001291549.3 in NCBI can be mentioned. The origins of these genes are not particularly limited, but are preferably mammals (e.g., humans, mice, rats, monkeys, cattle, horses, pigs, dogs, and the like), and among these, genes derived from humans are preferred. Furthermore, in one embodiment, these genes are human genes or homologs of human genes in other mammalian species. Genes with high identity to the wild-type gene (e.g., 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identity) are also preferred.

[0076]

Since the megakaryocyte of the present invention maintains platelet production capacity, they are suitable as starting cells for producing platelets. Therefore, in another embodiment, a method for producing a platelet (hereinafter, may be referred to as "the platelet production method of the present invention") is provided, which includes a step of maturing the megakaryocyte of the present invention.

[0077]

The platelet production method of the present invention can be carried out by known methods. For example, when megakaryocytes are in a state where one or more (preferably at least the MYC gene) or all of the megakaryocyte-inducing factors are being forcibly expressed, the megakaryocytes can be matured by reducing or stopping the forced expression of the megakaryocyte-inducing factor(s). For example, when the megakaryocyte-inducing factor is being forcibly expressed using a drug, the forced expression can be reduced or stopped by culturing the megakaryocytes in the absence of the drug. In addition to this, in the case of using a vector containing LoxP, it may also be achieved by introducing Cre recombinase into the cells. Furthermore, in the case of transient expression vectors, RNA introduction, and protein transduction, it can be achieved by stopping contact with the vector or the like.

[0078]

In one embodiment, the culture medium used in the platelet production method of the present invention contains at least one (preferably all) of an ADAM17 inhibitor, an AhR (aryl hydrocarbon receptor) inhibitor, and a ROCK inhibitor. Examples of ADAM17 inhibitors include KP-457, DPC333, GW280264X, Aderbasib, TMI-1, and JG26, with KP-457 being preferred. Examples of AhR inhibitors include stemregenin 1 (SR-1), SR-1 analogs (e.g., those described in US2014/0369973), stilbene derivatives (e.g., (E)-1-(4'-trifluoromethylphenyl)-2-(3,5-bis(trifluoromethyl)phenyl)-ethene, (E)-1-(4'-methoxyphenyl)-2-(3,5-dichlorophenyl)-ethene, (E)-1-(4'-chlorophenyl)-2-(3,5-dichlorophenyl)-ethene, 3,5,4'-trihydroxystilbene, etc.), and CH-223191, with SR-1 being preferred. Examples of ROCK inhibitors include Y-27632 (e.g., Ishizaki et al., Mol. Pharmacol. 57, 976-983 (2000); Narumiya et al., Methods Enzymol. 325, 273-284 (2000)), fasudil/HA1077 (e.g., Uenata et al., Nature 389: 990-994 (1997)), SR3677 (e.g., Feng Y et al., J Med Chem. 51: 6642-6645 (2008)), GSK269962 (e.g., Stavenger RA et al., J Med Chem. 50: 2-5 (2007) or WO2005/037197), GSK429286A, H1152 (e.g., Sasaki et al., Pharmacol. Ther. 93: 225-232 (2002)), Wf-536 (e.g., Nakajima et al., Cancer Chemother Pharmacol. 52(4): 319-324 (2003)), thiazovivin, and derivatives thereof, with Y-27632 being preferred.

[0079]

The culture medium used in the platelet production method of the present invention may also contain the above-mentioned adipogenic differentiation-inducing factor and/or JI051. In one embodiment, the culture medium used in the platelet production method of the present invention contains JI051. The specific types of compounds are all incorporated by reference to the description in the method of the present invention.

[0080]

The mature megakaryocyte produced by the platelet production method of the present invention may be a CD34-positive and CD41-positive cell. The mature megakaryocyte produced by this method may also be CD38-negative, CD90-positive, and/or CD49f-positive.

[0081]

The culture period in the platelet production method of the present invention is not particularly limited as long as the function of the platelet is maintained, but it is, for example, 2 days to 9 days (in one embodiment, 6 days).

[0082]

The culture in the platelet production method of the present invention is typically a suspension culture. Furthermore, it is preferable that the culture is carried out with shaking. Therefore, in one embodiment, the platelet production method of the present invention includes a shaking culture step. The shaking culture can be performed by stirring the culture medium with the swirling motion of an impeller using, for example, a commercially available rotary shaker

(e.g., VerMES reactor manufactured by Satsuki Multimix Co., Ltd.). The stirring speed may be from 50 to 200 rpm, for example, approximately 100 rpm.

[0083]

The shaking culture in the platelet production method of the present invention may be performed using the apparatus and method described in Patent Literature 5. Specifically, for example, it includes a step of stirring the medium in a culture container using an impeller, wherein the stirring step includes the following:

(a) turbulent energy of approximately 0.0005 m^2/s^2 to approximately 0.02 m^2/s^2;

(b) shear stress of approximately 0.2 Pa to approximately 6.0 Pa; and

(c) Kolmogorov scale of approximately 100 μm to approximately 600 μm,

wherein the impeller is reciprocated to satisfy one or more of these indices.

[0084]

Furthermore, as a culture apparatus, for example, there is provided an apparatus that includes a container containing a medium including megakaryocytes and an impeller that reciprocates within the container, wherein the medium is stirred by reciprocating the impeller in the medium so as to satisfy one or more of the following indices:

(a) turbulent energy of approximately 0.0005 m^2/s^2 to approximately 0.02 m^2/s^2;

(b) shear stress of approximately 0.2 Pa to approximately 6.0 Pa; and

(c) Kolmogorov scale of approximately 100 μm to approximately 600 μm,

and platelets are produced from megakaryocytes in the stirred state.

[0085]

The turbulent energy, shear stress, and Kolmogorov scale in the medium within the container, which is the object to be stirred, can be calculated by simulation based on the basic equations of turbulence. For example, it can be calculated using computational fluid dynamics software FLUENT (manufactured by ANSYS), but is not limited to a specific software. More specifically, the turbulent energy changes depending on the stroke, reciprocating speed, and reciprocating frequency of the impeller when the impeller is configured to reciprocate. When a plurality of impellers are used, the number of impellers can also be a factor that causes a change. The shear stress and Kolmogorov scale also change with changes in similar factors.

[0086]

The medium used in the present invention is not particularly limited, but can be prepared using a medium commonly used for culturing animal cells as a base medium. Examples of the base medium include IMDM medium, Medium 199, Eagle's Minimum Essential Medium (EMEM), αMEM medium, Dulbecco's modified Eagle's Medium (DMEM), Ham's F12 medium, RPMI 1640 medium, Fischer's medium, Neurobasal Medium (Life Technologies), and mixtures thereof. The medium may contain serum or may be serum-free. If necessary, the medium may

also contain one or more substances such as albumin, insulin, transferrin, selenium, fatty acids, trace elements, 2-mercaptoethanol, thioglycerol, lipids, amino acids, L-glutamine, non-essential amino acids, vitamins, growth factors, low molecular weight compounds, antibiotics, antioxidants, pyruvic acid, buffers, inorganic salts, and cytokines. Cytokines are proteins that promote hematopoietic cell differentiation, and examples thereof include VEGF, TPO, and SCF. It is also preferable to use TA-316 ((2E)-2-[1-[5-(4-Bromophenyl)-4-hydroxy-3-thienyl]ethylidene]hydrazide-5-[[[[4-[[(2-hydroxyethyl)amino]carbonyl]phenyl]methyl]amino]carbonyl]-2-thiophenecarboxylic acid) in place of TPO. In one embodiment, the medium used in the production method of the present invention contains SCF and TA-316. Furthermore, in one embodiment, the medium used in the platelet production method of the present invention contains SCF, TA-316, SR-1, KP-457, and Y27632. Also, when megakaryocytes have a drug-responsive promoter, it is preferable to include the corresponding drug, for example, tetracycline or doxycycline, in the medium when expressing the gene under that promoter.

[0087]

In the present invention, the culture conditions of the cells are not particularly limited, and conventional culture conditions can be employed. As a specific example, the culture temperature is, for example, about 35 to about 42°C, about 36 to about 40°C, or about 37 to about 39°C. The CO_2 concentration is, for example, about 5 to about 15%. The O_2 concentration is, for example, about 15 to about 25%.

[0088]

In each step of the production method of the present invention and the platelet production method of the present invention, cells may be cultured under feeder-free conditions and/or xeno-free conditions. In the production method of the present invention and the platelet production method of the present invention, all steps may be carried out under feeder-free and xeno-free conditions. Herein, "feeder-free" means a medium or culture condition that does not contain another cell type (i.e., feeder cells) that serves as an auxiliary to adjust the culture conditions of the target cells. Also, "xeno-free" means a medium or culture condition that does not contain components derived from organisms different from the species of the target cells.

[0089]

The seeding density of cells in each step of the production method of the present invention and the platelet production method of the present invention is not particularly limited as long as the cells can proliferate. Typically, it is 1.0×10^2 to 1.0×10^7 cells/cm^2, preferably 1.0×10^3 to 1.0×10^6 cells/cm^2, and more preferably 1.0×10^4 to 1.0×10^5 cells/cm^2.

[0090]

When the substance used in the present invention is a low molecular weight compound, the compound includes not only the free form but also pharmaceutically acceptable salts and hydrates thereof. Pharmaceutically acceptable salts vary depending on the type of compound, but include, for example, inorganic base salts such as alkali metal salts (sodium salts, potassium salts, etc.), alkaline earth metal salts (calcium salts, magnesium salts, etc.), aluminum salts, and ammonium salts; base addition salts such as trimethylamine, triethylamine, pyridine, picoline, ethanolamine, diethanolamine, triethanolamine, dicyclohexylamine, and N,N'-dibenzylethylenediamine; and acid addition salts such as inorganic acid salts including hydrochloride, hydrobromide, sulfate, hydroiodide, nitrate, and phosphate, and organic acid salts including citrate, oxalate, acetate, formate, propionate, benzoate, trifluoroacetate, maleate, tartrate, methanesulfonate, benzenesulfonate, and para-toluenesulfonate.

[0091]

Each substance used in the present invention may be synthesized by a known method or may be a commercially available product. In addition, when a protein or peptide is used, the protein or peptide may be obtained from cells expressing the same. A cell expressing a target protein or peptide can be produced by inserting DNA encoding the target protein or peptide into a known expression vector and introducing the obtained expression vector into an appropriate host cell.

[0092]

In the production method of the present invention and the platelet production method of the present invention, a step of recovering target cells or tissues obtained in each step may be included. The recovered cells may be cryopreserved using a cell cryopreservation solution. In addition, the obtained cells may be cell-counted using a cell counter, and may be sorted or purified by labeling with an antibody against a cell-surface marker and using flow cytometry, mass cytometry, or magnetic cell separation.

[0093]

Furthermore, in another embodiment, a method for maintaining the platelet production capacity of a megakaryocyte, comprising a step of increasing the abundance of at least one protein constituting ubiquinol-cytochrome c reductase (UQCR) in a megakaryocyte is also provided. With respect to this method, all matters described regarding the production method of the present invention are incorporated by reference.

[0094]

2. Megakaryocyte and Platelet

In another embodiment of the present invention, a megakaryocyte (hereinafter, may be referred to as "megakaryocyte of the present invention") and platelet (hereinafter, may be referred to as "platelet of the present invention") obtainable (where "obtainable" may be read as

"obtained" as appropriate) by the production method of the present invention and the platelet production method of the present invention are provided. Hereinafter, the megakaryocyte and platelet of the present invention may be collectively referred to as "cells of the present invention."

[0095]

The megakaryocyte of the present invention typically has all of the properties (A) to (C) described below:

(A) having at least one exogenous protein constituting UQCR,

(B) having platelet production capacity, and

(C) expressing a CD34 gene and a CD41 gene.

[0096]

Furthermore, the megakaryocyte of the present invention further has any one, two or three of the following properties (D) to (F):

(D) being derived from a pluripotent stem cell,

(E) having an exogenous megakaryocyte-inducing factor, and

(F) having an expression inhibitor for a TP53 gene and/or a CDKN1A gene.

[0097]

In another embodiment, the present invention provides a megakaryocyte having all of the above properties (A) to (C). Furthermore, it also provides a megakaryocyte having at least one, two or three of the above properties (D) to (F). Such megakaryocytes may be obtainable by the production method of the present invention, or they may be obtainable by another method. Hereinafter, the megakaryocytes are sometimes referred to as "megakaryocyte of the present invention."

[0098]

In the present specification, the term "exogenous" means that a substance is not present in megakaryocytes existing in vivo in a mammal unless the substance is externally introduced. Furthermore, in the present specification, the terms "expressing" a gene and being "positive" for a gene mean, unless otherwise specified, that a protein encoded by the gene is produced. Therefore, when a protein of interest is detected by FACS used in the Examples described below, the gene can be said to be expressed.

[0099]

In the above property (A), at least one of the proteins constituting the exogenous UQCR is preferably a UQCR core protein, and particularly UQCRC2 is preferred. In the above property (B), "having platelet production capacity" means that platelet production can be observed by causing the megakaryocytes to transition to the maturation stage. The method for causing the transition to the maturation stage is not limited, and if platelet production is confirmed by at least

one method, it can be said that the megakaryocyte "has platelet production capacity." In the above property (C), it is sufficient to have at least one of the megakaryocyte-inducing factors, but it is preferable to have MYC protein (particularly c-MYC) and an apoptosis-suppressing protein (particularly Bcl-xL), or to have MYC protein (particularly c-MYC) and an apoptosis-suppressing protein (particularly Bcl-xL) and a polycomb group protein (particularly BMI1). As the expression inhibitor of the above property (F), for example, antisense nucleic acid, siRNA, shRNA, heteroduplex nucleic acid, or the like for the transcript of the TP53 gene and/or CDKN1A gene can be mentioned.

[0100]

3. Use of Megakaryocyte and Platelet

The cells of the present invention can be used in medical applications such as blood transfusions; therefore, in another embodiment, a blood product (hereinafter, may be referred to as "the blood product of the present invention") comprising the cells of the present invention is provided. In the present specification, a blood product means a composition containing blood cells such as megakaryocytes and platelets. The megakaryocyte of the present invention can be used, for example, by administering or transplanting it into a living organism to produce functional platelets in vivo. In addition, the platelet of the present invention can be used, for example, for the prevention of bleeding due to thrombocytopenia or platelet dysfunction, for the treatment of bleeding, or for the treatment of blood diseases. A method for treating or preventing blood diseases or bleeding, comprising administering or transplanting an effective amount of the cells of the present invention to a mammal (e.g., a human, mouse, rat, monkey, cow, horse, pig, or dog) in need of treatment or prevention, is also included in the present invention. In the present specification, unless otherwise specified, a therapeutic or preventive agent (or a therapeutic or preventive method) for diseases includes an agent (or a method) capable of both treating and preventing the diseases.

[0101]

Examples of blood diseases to be treated or prevented include thrombocytopenia and platelet function disorders. Causes of thrombocytopenia include, for example, pancytopenia due to bone marrow failure, thrombocytopenia due to decreased platelet lifespan caused by platelet destruction, thrombocytopenia due to increased platelet consumption, drug-induced thrombocytopenia, and thrombocytopenia due to abnormal platelet distribution in internal organs. Examples of causes of platelet function disorders include, for example, drug-induced platelet dysfunction and hereditary platelet function disorders (e.g., thrombasthenia, Bernard-Soulier syndrome, MYH9 abnormality, Wiskott-Aldrich syndrome, etc.).

[0102]

When the cells of the present invention are used as a blood product, from the viewpoint of preventing rejection, it is desirable to use cells derived from iPS cells established from somatic cells having the same or substantially the same HLA genotype as the individual to which the cells are administered or transplanted. Here, "substantially the same" means that the HLA genotype matches to an extent at which an immune reaction against the administered or transplanted cells can be suppressed with an immunosuppressive agent; for example, it is a somatic cell having an HLA type that matches at 3 loci of HLA-A, HLA-B, and HLA-DR, or at 4 loci including HLA-C. When sufficient cells cannot be obtained due to age or body constitution, etc., it is also possible to administer or transplant the cells in a state of avoiding rejection by encapsulating them in a capsule made of a material such as polyethylene glycol or silicone, or a porous container.

Cells derived from iPS cells in which genes encoding components of MHC class I or II (e.g., HLA, B2M, etc.) and/or genes encoding transcription factors of said genes (e.g., CIITA, RFX5, RFXAP, RFXANK, etc.) are destroyed or modified are also preferred. In addition, HLA-A protein, HLA-B protein, and HLA-C protein are highly related to rejection when cell transplantation is performed. Therefore, it is also preferred that only the HLA-A gene and the HLA-B gene are destroyed, or that all three loci of the HLA-A gene, the HLA-B gene, and the HLA-C gene are destroyed, and it is more preferred that the HLA-E gene is also destroyed. For example, by producing genetically modified cells in which the HLA-A gene and the HLA-B gene, or the HLA-A gene, the HLA-B gene, and the HLA-C gene (and optionally, further the HLA-E gene) are destroyed in pluripotent stem cells or any cell at a differentiation stage from pluripotent stem cells to platelets (e.g., hematopoietic progenitor cells, megakaryocytes, etc., especially megakaryocytes), and by producing platelets through a differentiation induction step and/or a maturation step from said cells, it becomes possible to eliminate the expression of HLA proteins on the surface of platelets. This is expected to reduce antigenicity during platelet transplantation. In addition, a blood product such as a transplantable platelet preparation can be provided even for patients who have antibodies against HLA class I.

[0103]

The cells of the present invention may be formulated into a parenteral formulation, such as an injection, suspension, or infusion, by mixing the cells with a pharmaceutically acceptable carrier according to a conventional method. Therefore, in one aspect, a method for producing a blood product is provided, which includes a step of formulating the cells of the present invention. Such a production method may include a step of preparing the cells of the present invention. Furthermore, it may include a step of storing the cells of the present invention.

[0104]

Examples of a pharmaceutically acceptable carrier that may be contained in the parenteral formulation include aqueous solutions for injection, such as physiological saline and isotonic solutions containing dextrose and other auxiliary agents (e.g., D-sorbitol, D-mannitol, sodium chloride, etc.). The cells of the present invention may be blended with, for example, human plasma, infusion agents, buffers (e.g., phosphate buffer, sodium acetate buffer), soothing agents (e.g., benzalkonium chloride, procaine hydrochloride, etc.), stabilizers (e.g., human serum albumin, polyethylene glycol, etc.), preservatives, and antioxidants.

[0105]

The blood product of the present invention may be supplied in a state of being cryopreserved under conditions normally used for cryopreservation of cells and may be thawed before use. In this case, the blood product may further contain serum or its replacement, an organic solvent (e.g., DMSO), and the like. In this case, the concentration of serum or its replacement is not particularly limited, but may be from about 1% to about 30% (v/v), and preferably from about 5% to about 20% (v/v). The concentration of the organic solvent is not particularly limited, but may be from 0% to about 50% (v/v), and preferably from about 5% to about 20% (v/v).

[0106]

The cells of the present invention can also be used in a method for screening candidate medicaments useful for the treatment or prevention of blood diseases. Therefore, in yet another aspect, a screening method for a prophylactic or therapeutic medicament for blood diseases is provided, which comprises a step of culturing the cells of the present invention in the presence or absence of a test substance. For example, when cells serving as a disease model that reflects the pathology of a blood disease are used, improvement of the pathology in the presence of a test substance indicates that the test substance can be selected as a candidate for a prophylactic or therapeutic medicament for the blood disease. Such blood diseases include the same blood diseases as those described above that are intended to be treated or prevented with the blood product of the present invention.

[0107]

The step of contacting the test substance with the cells of the present invention can typically be performed by adding the test substance to a medium in which the cells of the present invention are cultured, or by transferring the cells of the present invention to a medium to which the test substance has been previously added. The period for the step of contacting the test substance with the cells of the present invention is not particularly limited, but is typically from 1 minute to 5 days, and preferably from 1 hour to 1 day.

[0108]

Examples of the test substance used in the present invention include a cell extract, a cell culture supernatant, a microbial fermentation product, an extract derived from a marine organism, a plant extract, a purified or crude protein, a peptide, a non-peptide compound, a synthetic low-molecular-weight compound, and a natural compound. The test substance may be an existing component or a candidate component of pharmaceuticals, nutritional foods, or the like.

[0109]

In another embodiment of the present invention, a kit for producing a megakaryocyte maintaining platelet production capacity and/or a platelet is provided. The kit includes a protein constituting UQCR or a nucleic acid encoding the protein, but may also include a megakaryocyte-inducing factor or a nucleic acid encoding the factor, an expression vector necessary for expressing these nucleic acids in cells, reagents, and other components such as a medium for cell culture, serum, supplements such as growth factors (e.g., TA-316, TPO, EPO, SCF, Heparin, IL-6, IL-11, etc.), and antibiotics. It may also include starting cells (e.g., pluripotent stem cells, hematopoietic progenitor cells, etc.). In addition, for example, when using cells derived from pluripotent cells, it may also include antibodies for identifying net-like structures prepared from these cells (e.g., antibodies against Flk1, CD31, CD34, UEA-I lectin, etc.). Furthermore, the reagents and antibodies included in the kit are supplied in a container of any kind that ensures that the components maintain their activity for a long period of time, are not adsorbed by the material of the container, and do not undergo degradation.

[0110]

The present invention will be described more specifically with reference to the following examples. It is to be noted, however, that the present invention is not limited to these examples by any means.

[EXAMPLES]

[0111]

Unless otherwise specified, the following materials and methods were used in the Examples.

1. iPS cell

The following human iPS cell lines were obtained from CiRA Foundation and used: 15M41 (also known as Ff-I 01s01), Ff-MH23s01 (abbreviated as MH23S01), TkDN-sev2, QHJI14s04, QHJI 14s04-AB II-KO-03, and QHJI14s04-AB II-KO-11. Among these, 15M41 and QHJI14s04 are derived from peripheral blood of the most frequent HLA homozygous donors, and MH23S01 is derived from peripheral blood of the second most frequent HLA homozygous donor. TkDN-sev2 was established from human fetal skin fibroblasts using Sendai virus. QHJI 14s04-AB II-

KO-03 and QHJI14s04-AB II-KO-11 are cell lines in which HLA-A, HLA-B, and CIITA were knocked out by genome editing in QHJI14s04.

For differentiation induction into megakaryocytes, the following cell lines were used, in which a megakaryocyte-inducing factor was introduced into the aforementioned iPS cell lines. These cell lines differentiate into megakaryocytes after being differentiated into hematopoietic progenitor cells, by adding tetracycline or its derivative (doxycycline in this example) to the culture medium, and doxycycline or similar compounds are then removed from the medium, allowing for maturation and production of platelets.

[0112]

• MBX Cassette-Introduced 15M41 Cells

This is a 15M41 human iPS cell line in which a construct encoding destabilization domain-fused c-MYC (MYCdd), BMI1, and BCL2L1 (three factors: MYCdd/BMI1/BCLxL) under the control of a tetracycline-inducible promoter, and encoding p21 shRNA and p53 shRNA under the control of the H1 promoter, was inserted into the genome using the piggyBac™ system (see Sone M. et al., "Silencing of p53 and CDKN1A Establishes Sustainable Immortalized Megakaryocyte Progenitor Cells from Human iPSCs," Stem Cell Reports, 16(12), 2861-2870, 2021). In the present specification, the construct may be referred to as the [c-MYC/BMI1/BCLXL/p21 KD/p53 KD] cassette or simply MBX cassette. Hereinafter, those in which the MBX cassette is introduced into iPS cell lines other than the 15M41 line and MBX cassette-introduced 15M41 cells are collectively referred to as "MBX cassette-introduced iPS cell lines."

[0113]

• MX Cassette-Introduced 15M41 Cells

This is a 15M41 human iPS cell line in which a construct encoding two genes, namely, the MYCdd gene and the BCL2L1 gene (two factors: MYCdd/BCLxL), under the control of a tetracycline-inducible promoter, and encoding p21 shRNA and p53 shRNA under the control of the H1 promoter, was inserted into the genome using the piggyBac™ system. In the present specification, the construct may be referred to as the [c-MYC/BCLXL/p21 KD/p53 KD] cassette or simply as the MX cassette.

[0114]

• MBX Cassette-Introduced TkDN sev2 Cells

This is a TkDN sev2 human iPS cell line in which the MBX cassette has been inserted into the genome.

[0115]

<u>2. Differentiation Induction of Hematopoietic Progenitor Cells from iPS cells</u>

The method described in Takayama N., et al. J Exp Med. 2817-2830 (2010) was modified and used.

iPS cells treated with GSK-3β inhibitor CHIR-99021 and Y-27632 for 3 days were detached as cell mass fragments, and the iPS cell mass fragments were added onto feeder cells (human fibroblast strain C3H/10T1/2 cells subjected to mitomycin treatment (ATCC number: CCL-226)).

[0116]

As a basal medium, IMDM (Iscove's Modified Dulbecco's Medium) (Sigma-Aldrich, Code No. I3390) containing 15% Fetal Bovine Serum (FBS) (Life Technologies, Code No. 10270-106), 2 mM Glutamine (Life Technologies, Code No. 25030-081), 1% Insulin / Transferrin / Selenium Solution (ITS-G) (Life Technologies, Code No. 41400-045), 0.45 mM 1-Thioglycerol (Sigma-Aldrich, Code No. A6145), and 50 μg/mL L-Ascorbic Acid (Sigma-Aldrich, Code No. A4544) was prepared.

[0117]

As a medium at the start of iPS cell culture, a basal medium containing 20 ng/mL VEGF (Fujifilm Wako Pure Chemical, Code No. 226-01786) was used, and culture was started under culture conditions of 37°C, 5-8% O_2, and 5% CO_2.

[0118]

On Day 4 after the start of culture, the medium was replaced with a basal medium containing 20 ng/mL VEGF, 10 U/mL Heparin (A.W. Pharma), 50 ng/mL bFGF (Wako Pure Chemical, Code No. 068-04544), and 10 μM SB431542 (Wako Pure Chemical, Code No. 037-24293), and culture was performed under culture conditions of 37°C, 5-8% O_2, and 5% CO_2.

On Day 7 after the start of culture, the medium was replaced with a basal medium containing 20 ng/mL VEGF and 10 U/mL Heparin, and culture was performed under culture conditions of 37°C, 20% O_2, and 5% CO_2.

On Day 11 after the start of culture, a basal medium containing 20 ng/mL VEGF and 0.75 μM SR-1 (Stemregenin-1) was added or replaced, and culture was performed under culture conditions of 37°C, 20% O_2, and 5% CO_2.

From Day 7 to 14 after the start of culture, floating cells were collected as hematopoietic progenitor cells and used in subsequent differentiation induction experiments.

[0119]

<u>3. Differentiation Induction from Hematopoietic Progenitor Cells to Megakaryocytes, Expansion Culture, and Maturation</u>

The hematopoietic progenitor cells at Day 7 after the start of culture in Method 2 were cultured in a basal medium (which may be referred to as megakaryocyte differentiation/proliferation medium or simply differentiation/proliferation medium) containing

0.2 μg/mL TPO mimetic compound, 50 ng/mL SCF, 1 μM UM729 (MERCK Code No. ATEH97ECEE61), and 1 μg/mL doxycycline under feeder-free conditions at 37°C, 20% O_2, and 5% CO_2, thereby inducing differentiation into megakaryocytes. In the Examples, the day on which culture was started in the megakaryocyte differentiation/induction medium is designated as Day 0, and subsequent culture days are represented by "Day." In this method, a CD34-positive, CD41-positive cell population generally appears around Day 7. Megakaryocytes were expanded by continuing subculture using the same medium. Note that in the following examples, "differentiating hematopoietic progenitor cells from iPS cells and then differentiating the hematopoietic progenitor cells into megakaryocytes" may be abbreviated as "differentiating megakaryocytes from iPS cells."

[0120]

The forced expression of the three factors (MYCdd/BMI1/BCLxL) or two factors (MYCdd/BCLxL) in the aforementioned cassette was terminated by switching the medium from doxycycline-containing medium to a doxycycline-free medium, thereby inducing maturation of megakaryocytes. The composition of the doxycycline-free medium used (megakaryocyte maturation medium) is as follows:

・Megakaryocyte maturation medium (or maturation medium)

5% human plasma, 2 mM Glutamine, 1% ITS-G, 0.45 mM 1-Thioglycerol, 50 μg/mL L-Ascorbic Acid, 50 ng/mL SCF, 0.2 μg/mL TPO mimetic compound, 15 μM KP-457 (Koei Chemical), 10 μM Y-27632 (Nacalai Tesque, Code No. 18188-04), and 0.75 μM SR-1 (Stemregenin-1) or 0.1 μM AhR inhibitor (final concentration).

The megakaryocyte maturation process was carried out by seeding the megakaryocytes in a 125 mL culture flask at a seeding density of 1 x 10^5 cells/mL, 25 mL/flask, and culturing under shaking conditions. The shaking culture was performed using LT-X (Lab-Therm) (Kuhner) or S41i (Eppendorf) at 37°C and 5% CO_2 with a shaking speed of 100 rpm.

[0121]

4. Evaluation of Megakaryocyte Proliferation

From Day 0 onward, cell counts were measured periodically, and detection of the megakaryocyte-specific cell-surface marker CD41 was performed by flow cytometry.

Cell counts were determined using trypan blue staining, and the cumulative proliferation rate from Day 0 was calculated based on the seeding cell count. Simultaneously, the megakaryocyte proliferation rate was calculated by multiplying this value by the ratio of CD41-positive cells, which was determined from the measurement results. The cumulative proliferation rate of megakaryocytes in the n^{th} passage was calculated using the following formula:

[0122]

[Mathematical 1]

$$\text{Cumulative proliferation rate of megakaryocytes at the n-th passage} = \left(\prod_{k=1}^{n} \frac{\text{Harvested viable cell density at the k-th passage}}{\text{Seeding density at the (k-1)-th passage}} \right) \times \text{CD41 positive rate at the n-th passage}$$

[0123]

However, the seeding density for passage 0 is defined as the seeding density on Day 0.

[0124]

5. Evaluation of Platelet Production Capacity and Platelet Function

Megakaryocytes were cultured in megakaryocyte maturation medium under shaking conditions, and after 6 days, a portion of the cell suspension was collected, and flow cytometry analysis was performed using BD FACSVerse™ (BD Biosciences) to measure the number of produced platelets and evaluate platelet function according to the following procedure.

[0125]

To measure the number of platelets produced, the cells were stained with the following antibodies:

APC-labeled anti-CD41 antibody (BioLegend, Code No. 303710)

PE-labeled anti-CD42b antibody (BioLegend, Code No. 303906)

eFluor™ 450-labeled anti-CD42a antibody (Thermo Fisher, Code No. 48-0428-42)

30 minutes after staining, the number of platelets, defined as CD41/CD42b double-positive or CD41/CD42a/CD42b triple-positive small particles gated based on FSC/SSC, was measured using TruCOUNT™ (BD Biosciences, Code No. 340334). FlowJo™ Version 10 (BD Biosciences) was used to analyze the flow cytometry data for calculating the number of platelets produced. The data are presented as the number of platelets produced per megakaryocyte, based on the number of megakaryocytes seeded at the start of platelet production culture.

[0126]

To evaluate platelet function, stimulation was performed under two types of stimulation conditions:

Stimulus 1: 0.02-0.4 μM PMA

Stimulus 2: 100 μM ADP / 40 μM Thrombin Receptor Activator Peptide 6 (TRAP6)

After addition, staining was performed with the following antibodies:

APC-labeled anti-CD41 antibody (BioLegend, Code No. 303710)

PE-labeled anti-CD62P antibody (BioLegend, Code No. 304906)

FITC-labeled anti-PAC-1 antibody (BD Biosciences, Code No. 34507)

After 30 minutes, measurement was performed using BD FACSVerse™.

[0127]

Example 1: Verification of the effect of megakaryocyte passage on platelet production capacity

MBX cassette-introduced 15M41 cells were differentiated into hematopoietic progenitor cells, and these progenitor cells were further differentiated into megakaryocytes. The results of analyzing the platelet production capacity of cells from Day 21 to Day 78 after megakaryocyte induction are shown in Fig. 1. On Day 21, each megakaryocyte had the capacity to produce more than 200 platelets; however, this production capacity decreased thereafter, and after Day 64, it decreased to approximately 1/10 of the production capacity on Day 21.

In addition, a decrease in platelet production capacity associated with passage culture was observed in megakaryocytes differentiated by the same method from other human induced pluripotent stem cell lines (e.g., MH23S01, TkDN-sev2) (data not shown).

Therefore, it was confirmed that megakaryocytes differentiated from human iPS cells tend to have a decrease in platelet production capacity as they are passaged.

[0128]

Example 2: Search for proteins that promote platelet production

The present inventors had identified six types of proteins released into the medium from megakaryocytes matured by shaking culture under conditions that dramatically improve platelet production, and among these, focused on NRDC with an endopeptidase domain (Non-Patent Literature 2). To investigate the role of NRDC in platelet production, a megakaryocyte-specific NRDC knockout mouse was produced, and the abundance of platelets in vivo was analyzed. However, contrary to expectations, no significant decrease in the abundance of platelets was observed in the NRDC knockout mice compared to control mice (data not shown).

Therefore, in order to identify factors that can truly control platelet production efficiency, the aforementioned shaking culture was performed on megakaryocytes, and proteins whose expression levels were increased compared to those in megakaryocytes matured by static culture were analyzed. As a result, it was found that knockdown of the expression of UQCRC1 and UQCRC2, which are localized in mitochondria and, like NRDC, have an M16 endopeptidase domain, reduces the platelet production capacity of megakaryocytes (Fig. 2). MBX cassette-introduced TkDN sev2 cells were differentiated, and shRNA for UQCRC1 and/or UQCRC2 was introduced using lentivirus on Day 45, and the results of measuring platelet production capacity on Days 66-81 are shown in Fig. 2. Compared to the control (megakaryocytes into which LacZ shRNA was introduced), megakaryocytes into which UQCRC1 shRNA or UQCRC2 shRNA was introduced showed a decrease in platelet production capacity to about 30%, and furthermore, megakaryocytes into which both UQCRC1 shRNA and UQCRC2 shRNA were introduced showed a further decrease in platelet production capacity to about 20%.

From these results, it is strongly suggested that UQCRC1 and UQCRC2 play a role in positively regulating the platelet production capacity of megakaryocytes.

[0129]

Example 3: Verification of Platelet Production Capacity by UQCRC1 and UQCRC2

The effects of forced expression of UQCRC1 and UQCRC2 on platelet production were analyzed.

UQCRC1 or UQCRC2 was introduced using a lentiviral vector into megakaryocytes differentiated from MBX cassette-introduced 15M41 cells on Day 21 or into megakaryocytes differentiated from MX cassette-introduced 15M41 cells on Day 28. Maturation was induced on Day 47 or Day 56 for the MBX cassette-introduced cells and on Day 56 or Day 63 for the MX cassette-introduced cells, and the platelet production capacity was analyzed. The results are shown in Fig. 3. Megakaryocytes differentiated from the MBX cassette-introduced 15M41 cells produced a very large number of platelets when either UQCRC1 or UQCRC2 was forcibly expressed, as compared with the control megakaryocytes infected with a lentiviral vector not encoding UQCRC1 or UQCRC2 (right graph in Fig. 3). Comparison with Fig. 1 shows that forced expression of UQCRC1 or UQCRC2 very effectively suppressed the reduction in platelet production capacity associated with long-term passage culture.

Similarly, in megakaryocytes differentiated from the MX cassette-introduced 15M41 cells, forced expression of UQCRC1 or UQCRC2 maintained a high platelet production capacity at a level comparable to that of megakaryocytes differentiated from the MBX cassette-introduced 15M41 cells (left graph in Fig. 3).

Fig. 4 shows the results of an analysis of the platelet production capacity on and after Day 53 of megakaryocytes into which UQCRC2 was introduced using a lentivirus. In megakaryocytes differentiated from both the MX cassette-introduced 15M41 cells and the MBX cassette-introduced 15M41 cells, the platelet production capacity was well maintained over a long period, until Day 67 or Day 60, respectively.

These results demonstrate that, at least in proliferating megakaryocytes, increasing the expression level of UQCRC1 and/or UQCRC2 markedly ameliorated the reduction in platelet production capacity associated with passage culture, thereby producing megakaryocytes in which the platelet production capacity was maintained over a long period.

[0130]

Example 4: Search for Compounds that Promote Platelet Production

Next, an attempt was made to promote platelet production using the compound. It is known that megakaryocytes in bone marrow promote platelet production by taking up fatty acids released from adipocytes (Adipocyte Fatty Acid Transfer Supports Megakaryocyte Maturation. Cell reports. 2020;32(1):107875). Furthermore, it is also known that lipid synthesis and the uptake of exogenous fatty acids are important for megakaryocyte maturation and proplatelet formation (Critical shifts in lipid metabolism promote megakaryocyte differentiation and

proplatelet formation Nat Cardiovasc Res. 2023 Sep; 2: 835-852). Based on this, a hypothesis was established that using a drug that induces differentiation into adipocytes which perform lipid metabolism and synthesis would lead to more active lipid synthesis in megakaryocytes. Therefore, the hypothesis was tested by culturing megakaryocytes in the presence of an adipogenic differentiation-inducing factor.

Dextran (1 μM), IBMX (50 μM), and insulin (10 μg/ml) were added to the medium (differentiation-proliferation medium) of megakaryocytes expressing exogenous UQCRC2 (megakaryocytes derived from MBX cassette-introduced 15M41 cells) between Day 1 and Day 3. The values in parentheses represent the final concentration. After transferring to a megakaryocyte maturation medium, JI051 (10 μM) was added after 3 days, and the platelet production capacity was analyzed, with the results shown in Fig. 5. Compared to the control without the addition of JI051, the platelet production capacity of megakaryocytes treated with JI051 increased approximately twofold.

Therefore, it became clear that when megakaryocytes are expanded by adding dextran, IBMX, and insulin, and then matured by adding JI051 in a subsequent maturation step, the platelet production capacity of the megakaryocytes is further improved.

[0131]

Furthermore, LMK235 was identified as an alternative compound to UM729 in the megakaryocyte differentiation-proliferation medium. Megakaryocytes were induced to differentiate using a differentiation-proliferation medium in which UM729 was replaced with LMK235, and the cell count (upper graph in Fig. 6) and CD34-positive cell ratio (lower graph in Fig. 6) after 3 days were analyzed, with the results shown in Fig. 6. In this experiment, forced expression of UQCRC2 was not performed. When LMK235 was used at a final concentration of 0.2 μM, both the cell count and CD34-positive cell ratio increased compared to when UM729 was used.

Since LMK235 is known as an inhibitor of HDAC4, 5, and 6, similar analyses were performed on other HDAC inhibitors. Fig. 7 shows the results of analyzing the CD34 and CD41a positive cell ratios in megakaryocytes derived from MBX cassette-introduced 15M41 cells or MX cassette-introduced 15M41 cells that have forced expression of UQCRC2. LMK235 and TMP195 (an inhibitor of HDAC4, 5, 7, and 9) maintained expression of CD34, but Tasquinimod (an inhibitor of HDAC4) reduced the expression of CD34. From these results, it is suggested that HDAC5 contributes to the maintenance of CD34 in proliferating megakaryocytes.

Previously, shaking culture was performed during the maturation process; however, we analyzed the effects of UM729 and LMK235 when switching to static culture. MX cassette-introduced 15M41 cells with forced expression of UQCRC2 were used, and megakaryocytes were obtained. Differentiation induction and expansion culture were performed using a standard

differentiation/proliferation medium containing 1 μM UM729 or a differentiation/proliferation medium in which UM729 was replaced with 200 nMLMK235. Subsequently, maturation was carried out under static culture conditions, and platelet production capacity was compared (Fig. 8). Although the number of platelets produced is smaller during maturation in static culture compared to shaking culture, even when expansion culture was performed in a medium containing LMK235, the number of platelets produced was not significantly different from that obtained with expansion culture in a medium containing UM729.

Therefore, as one embodiment of the present invention, it has become clear that the pyrimidoindole derivative (e.g., UM729) added to the medium during the differentiation induction from hematopoietic progenitor cells to megakaryocytes and subsequent expansion culture can be replaced with an HDAC5 inhibitor such as LMK235 or TMP195.

[0132]

Example 5: Verification of proliferation capacity of megakaryocytes depending on the origin of iPS cell lines

It was investigated whether differences in the origin of iPS cell lines affect the proliferation capacity of induced megakaryocytes. First, various iPS cell lines (15M41, MH09S01, MH15S01, MH15S02 and MH23S01) were introduced with an MBX cassette to create MBX cassette-introduced iPS cell lines (three types of megakaryocyte-inducing factors were introduced). This MBX cassette-introduced iPS cell line was induced into imMKCL, and the CD34/CD41a double-positive fraction was sorted and cultured. As a result, imMKCLs derived from all of the iPS cell lines were shown to maintain a CD34/CD41a double-positive phenotype for 42 days (Fig. 10).

[0133]

Next, verification was performed using two types of megakaryocyte-inducing factors instead of three. A construct encoding two genes (two factors: MYCdd/BCLxL), namely, the MYCdd gene under the control of a tetracycline-inducible promoter and the BCL2L1 gene under the control of the UbiC promoter, and also encoding p21 shRNA and p53 shRNA under the control of the H1 promoter, was introduced into various iPS cell lines (15M41 and QHJI14s04-AB II-KO-03). Megakaryocyte differentiation was induced from the resulting iPS cells, the CD34/CD41a double-positive fraction was sorted, and culture was continued. Expression of CD34 and CD41 was evaluated by FACS. Megakaryocytes were differentiated from these iPS cells, and the CD34/CD41a double-positive fraction was sorted and cultured. The expression of CD34 and CD41 was evaluated by FACS. As a result, in the QHJI14s04-AB II-KO-03-derived megakaryocyte population, many cells that could not maintain CD34/CD41a co-expression were observed (Fig. 11). On the other hand, 15M41-derived megakaryocytes maintained CD34/CD41a co-expression (Fig. 12).

[0134]

Example 6: Verification of the relationship between Polycomb Repressive Complex 1.1 and the proliferation capacity of megakaryocytes

It has been reported that 15M41 has mutations in the BCOR (BCL6 corepressor) gene and BRD3 gene, while QHJI14s04 does not have these mutations. Accordingly, it was considered that the presence or absence of mutations in the BCOR or BRD3 gene might account for differences among iPS cell lines in their ability to maintain CD34/CD41a co-expression. First, we focused on the BCOR gene and its homolog, the BCORL1 (BCL6 corepressor like 1) gene.

[0135]

Therefore, to determine whether suppressing the expression of BCOR or BCORL1 in megakaryocytes derived from QHJI14s04/AB II-KO-03 would result in an expression profile equivalent to that of megakaryocytes derived from 15M41, experiments were conducted. In QHJI14s04/AB II-KO-03-derived megakaryocyte cells, BCOR or BCORL1 expression was knocked down using lentivirus targeting the transcripts of these genes (referred to as shBCOR and shBCORL1, respectively) 28 days after the start of Dox addition. As a result, it was possible to obtain a megakaryocyte line that maintained CD34/CD41a co-expression (Fig. 13). Furthermore, it was demonstrated that megakaryocytes with knocked down BCOR or BCORL1 expression had higher platelet production capacity compared to megakaryocytes without knockdown (Fig. 14).

[0136]

BCOR and BCORL1 are proteins that constitute Polycomb Repressive Complex 1.1 (PRC1.1), and are known as transcription factors involved in the regulation of embryogenesis, mesenchymal stem cell function, hematopoiesis, and lymph system development (e.g., Sportoletti P., Sorcini D. and Falini B., Blood. 138(24):2455-2468 (2021)). In addition, the present inventors previously identified TRIM27 (tripartite motif containing 27) as one of the genes that showed significantly different methylation levels among iPS cells with largely different induction efficiencies in megakaryocyte induction using three factors (MYCdd/BMI1/BCLxL) (data not shown). It has also been reported that TRIM27 is a component of PRC1.1 (Maat H., et al., iScience. 24(5):102435 (2021)). Furthermore, the present inventors considered the possibility that there exists a transcription factor involved in the hematopoietic lineage differentiation process that also contributes to megakaryocyte proliferation. As a result of intensive investigation, they focused on BACH1, which contributes to erythrocyte formation and lymphocyte formation, and conducted further analysis.

[0137]

When iPS cells were induced to differentiate into megakaryocytes by introducing the combination of vectors shown in Fig. 15, it was found that overexpression of UQCRC2 and

BACH1, combined with knockdown of BCOR, could maintain megakaryocytes co-expressing CD34/CD41a and also maintain high platelet production capacity. Furthermore, it was shown that knocking down BCORL1 or TRIM27 further increased the platelet production capacity (Figs. 16 and 17). In addition, even when none of BCOR, BCORL1, and TRIM27 were knocked down, it was demonstrated that simply overexpressing BACH1 in megakaryocytes could maintain high platelet production capacity (Fig. 18).

[0138]

Example 7: Search for compounds contributing to megakaryocyte maintenance

In addition to HDAC5 inhibitors, a search was conducted for compounds that promote megakaryocyte maintenance and platelet production capacity. As a result, it was shown that an inhibitor of lysine-specific histone demethylase 1 (LSD1) (tranylcypromine) had the effect of maintaining CD34/CD41a co-expression in megakaryocytes. In particular, it was demonstrated that the combined use of an HDAC5 inhibitor and an LSD1 inhibitor maintained CD34/CD41a co-expression at a high level in megakaryocytes (Fig. 19).

[INDUSTRIAL APPLICABILITY]

[0139]

The present invention provides a method for producing a megakaryocyte (high-quality master cell) that maintains high quality for a long period of time, regardless of the type of iPS cell. This makes it possible to stably supply high-quality platelets (platelets that maintain hemostasis and circulatory function). Since this can be achieved easily and at low cost, it may be very useful from the viewpoint of social implementation of artificial platelets.

[0140]

The present application claims priority based on Japanese Patent Application No. 2024-020854 filed in Japan (filing date: February 15, 2024), the disclosure of which is incorporated herein in its entirety by reference.

CLAIMS

[Claim 1]

A method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of increasing the abundance of at least one protein constituting ubiquinol-cytochrome c reductase (UQCR) in a megakaryocyte.

[Claim 2]

The method according to claim 1, wherein the at least one protein constituting UQCR is a UQCR core protein (UQCRC).

[Claim 3]

The method according to claim 2, wherein at least one UQCRC is UQCRC2.

[Claim 4]

The method according to any one of claims 1 to 3, wherein the step of increasing the abundance of the protein constituting UQCR comprises a step of introducing a nucleic acid encoding the protein into the megakaryocyte.

[Claim 5]

A method for producing a megakaryocyte maintaining platelet production capacity, comprising a step of inhibiting the function of Polycomb Repressive Complex 1.1 in a megakaryocyte.

[Claim 6]

The method according to any one of claims 1 to 4, comprising a step of inhibiting the function of Polycomb Repressive Complex 1.1 in the megakaryocyte.

[Claim 7]

The method according to claim 6, wherein the step of inhibiting the function of Polycomb Repressive Complex 1.1 comprises a step of reducing the abundance of at least one protein constituting Polycomb Repressive Complex 1.1.

[Claim 8]

The method according to claim 7, wherein the protein constituting Polycomb Repressive Complex 1.1 is at least one selected from the group consisting of a BCOR protein, a BCORL1 protein, and a TRIM27 protein.

[Claim 9]

The method according to any one of claims 1 to 8, comprising a step of increasing the abundance of a BACH1 protein in the megakaryocyte.

[Claim 10]

The method according to any one of claims 1 to 9, comprising a step of culturing the megakaryocyte in a medium containing an HDAC5 inhibitor.

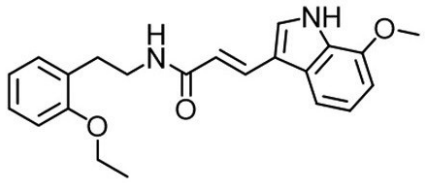
[Claim 11]

The method according to any one of claims 1 to 10, comprising a step of culturing the megakaryocyte in a medium containing an adipogenic differentiation-inducing factor.

[Claim 12]

The method according to any one of claims 1 to 11, wherein a medium contains a compound represented by the following structural formula:

[Chemical 1]



[Claim 13]

The method according to any one of claims 1 to 12, comprising a step of increasing the abundance of a MYC protein and the abundance of an apoptosis-suppressing protein in the megakaryocyte.

[Claim 14]

The method according to any one of claims 1 to 13, wherein the megakaryocyte is derived from a pluripotent stem cell.

[Claim 15]

A megakaryocyte obtainable by the method according to any one of claims 1 to 14.

[Claim 16]

A megakaryocyte having all of the following properties (A) to (C):

(A) having at least one exogenous protein constituting ubiquinol-cytochrome c reductase (UQCR),

(B) having platelet production capacity, and

(C) expressing a CD34 gene and a CD41 gene.

[Claim 17]

The megakaryocyte according to claim 16, wherein the at least one protein constituting UQCR is a UQCR core protein.

[Claim 18]

A method for producing a platelet, comprising a step of maturing the megakaryocyte according to any one of claims 15 to 17.

[Claim 19]

The method according to claim 18, comprising a shaking culture step.

[Claim 20]

A platelet obtainable by the method according to claim 18 or 19.

[Claim 21]

A blood product comprising the cell according to any one of claims 15, 16, 17 and 20.

[Claim 22]

A method for producing a megakaryocyte, comprising a step of culturing a hematopoietic progenitor cell in a medium containing an HDAC5 inhibitor and/or an LSD1 inhibitor.

[Claim 23]

The method according to claim 22, wherein at least one HDAC5 inhibitor is LMK235.