SPECIFICATION

# TITLE
# QUALITY CONTROL MARKER FOR MEGAKARYOCYTE

## [TECHNICAL FIELD]

[0001]

The present invention relates to a quality control marker for megakaryocytes. More specifically, the present invention relates to a quality control marker for megakaryocytes, consisting of a transcript or protein of the Ral gene, and the like.

## [BACKGROUND ART]

[0002]

Donor-dependent platelet transfusion has become a standard healthcare practice despite persistent challenges of supply shortages resulting from various factors including viral contamination, alloimmune transfusion refractoriness, and the recent COVID-19 pandemic. In this context, the present inventors have adopted the strategy of *ex vivo* blood products using human induced pluripotent stem cells (iPSCs). The present inventors developed immortalized MK cell lines (imMKCLs) as the starting cell source for *ex vivo* iPSC-derived platelet (iPSC-PLT) manufacturing (Non-Patent Literatures 1, 2). imMKCLs exhibit sustained proliferation over several months in the presence of doxycycline (DOX) and shed iPSC-PLTs after DOX removal, enabling the generation of over $10^{11}$ iPSC-PLTs in a turbulent flow-based bioreactor (Non-Patent Literature 3). Building upon these achievements, the present inventors have initiated the first-in-human clinical trial of iPSC-derived platelets (iPLAT1) (Non-Patent Literatures 4-6). While iPLAT1 demonstrated promising outcomes without significant adverse events, a transient increase in D-dimer levels and leukocyte count was observed after transfusion of the maximal dose (Non-Patent Literature 4). These observations indicate the potential involvement of recently identified immune-skewed MKs (Non-Patent Literature 7, 8), raising concerns about the quality control of imMKCLs as master cells. Meanwhile, the present inventors noticed considerable variation in the quality of imMKCL clones with regard to their proliferation and iPSC-PLT production capacity (Non-Patent Literature 9). Specifically, certain imMKCL clones displaying cellular senescence exhibited a diminished capacity to generate iPSC-PLTs, but the capacity could be restored through the knockdown of p53 and CDKN1A (Non-Patent Literature 9). However, the molecular factors underlying this heterogeneity, which significantly hinder the efficiency and standardization of iPSC-PLT manufacturing, remain unknown.

[NON PATENT LITERATURE]

[0003]

[NPL 1] Takayama, N. et al. J Exp Med 207, 2817-2830 (2010)

[NPL 2] Nakamura, S. et al. Cell Stem Cell 14, 535-548 (2014)

[NPL 3] Ito, Y. et al. Cell 174, 636-648.e618 (2018)

[NPL 4] Sugimoto, N. et al. Blood 140, 2398-2402 (2022)

[NPL 5] Sugimoto, N. et al. Blood Adv 6, 6056-6069 (2022)

[NPL 6] Chen, S.J., Sugimoto, N. & Eto, K. Int J Hematol 117, 349-355 (2023)

[NPL 7] Wang, H. et al. Cell Stem Cell 28, 535-549.e538 (2021)

[NPL 8] Sun, S. et al. Blood 138, 1211-1224 (2021)

[NPL 9] Sone, M. et al. Stem Cell Reports 16, 2861-2870 (2021)

[SUMMARY OF INVENTION]

[TECHNICAL PROBLEM]

[0004]

Therefore, an object of the present invention is to provide biomarkers, etc., which can control the quality of megakaryocytes by elucidating the molecular factors underlying said heterogeneity.

[SOLUTION TO PROBLEM]

[0005]

One member of the research team of the present inventors encountered an event in which reproducibility was not observed in platelet production efficiency while conducting a study using megakaryocytes (MK). The present inventors diligently investigated the cause of this lack of reproducibility and discovered that the megakaryocytes had been cultured for a long period, causing them to undergo cellular senescence. Analysis of RNA-seq data revealed that prolonged culture of megakaryocytes resulted in cellular senescence and enhanced immune characteristics. Based on this, the present inventors conceived that elucidating the mechanism by which megakaryocytes age could lead to maintaining the quality of megakaryocytes. To elucidate the above-mentioned mechanism, the present inventors focused on microRNA.

[0006]

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate the stability and translation of the target mRNAs by binding with the complementary mRNA sequences. In hematopoietic cells, miRNAs have been identified as key players in cell fate decisions and functions (Mehta, A. & Baltimore, D. Nat Rev Immunol 16, 279-294 (2016)). Based on the evidence that DNA-based genetic circuits regulate protein expression depending on miRNA

activities rather than miRNA expression (Mullokandov, G. et al. Nat Methods 9, 840-846 (2012)), the present inventors developed an innovative biotechnology method, miRNA switch, which enables the identification of specific cell types (Miki, K. et al. Cell Stem Cell 16, 699-711 (2015); Fujita, Y. et al. Sci Adv 8, eabj1793 (2022)). MiRNA switches can detect endogenous miRNA activity to distinguish heterogeneous cell populations without the need of antibody labeling. This technology has proven successful for various cell types, including iPSC-derived hepatocytes, endothelial cells, cardiomyocytes (Miki, K. et al. Cell Stem Cell 16, 699-711 (2015)), mouse embryonic stem cell (ESC)-derived neurons (Sunohara, T. et al. Front Neurosci 13, 1141 (2019)), and undifferentiated human ESCs and iPSCs (Parr, C.J. et al. Sci Rep 6, 32532 (2016)).

[0007]

The present inventors therefore hypothesized that the miRNA switch can be employed to distinguish subpopulations among imMKCLs with heterogeneous miRNA activities (i.e., a gradient variation in miRNA activity). First, the present inventors screened a library containing 269 target miRNA switches, leading to the identification of let-7a-5p and let-7g-5p miRNAs with heterogenous activity among imMKCLs. By a transcriptional analysis of imMKCL subpopulations exhibiting either high or low let-7 activity (hereafter referred to as let-7 high and let-7 low, respectively), the present inventors discovered that let-7 low imMKCLs exhibited an immune-skewed transcriptional signature. Further investigation unveiled the pivotal role of let-7a-5p and its downstream target, RAS like proto-oncogene B (*RALB*), in modulating the lineage determination of "immune" MKs within imMKCLs. Importantly, the present inventors elucidated that the dysregulation of immune properties/subpopulations within imMKCLs, along with the secretion of inflammatory cytokines, results in a decline of their quality based on their arrested proliferation and deficient iPSC-PLT generation.

[0008]

Furthermore, in exploring factors for immune-skewed imMKCLs, the present inventors focused on lysine acetyltransferase 7 (KAT7), also known as HBO1 and MYST2, which is an important member of the MYST family of histone acetyltransferases essential for chromatin modification and gene regulation. KAT7 forms a complex with proteins such as MEAF6, ING4/5, JADE, and BRPF, and functions to acetylate histone H3 at lysine 14 and histone H4 at lysines 5, 8, and 12 (Yokoyama A. et al. Biochim Biophys Acta Gene Regul Mech. 2024 Sep;1867(3):195045). Analysis of the function of KAT7 in imMKCLs revealed that KAT7 plays an important role in suppressing the immune-skewed characteristics of imMKCLs. Specifically, KAT7 is important for maintaining the cell cycle of imMKCLs and influences the number of platelets produced in the later maturation stage by maintaining the proliferative capacity of imMKCLs. In imMKCLs, platelets are produced from G1 or G2/M phase during proliferation.

However, in senescent imMKCLs, KAT7 decreases, IL-8 or TNF-α secretion increases, transition from G1 or G2/M to G0 is enhanced, and the cell cycle stops, resulting in a decrease in platelet formation derived from G1 or G2/M phase. The present inventors conducted further research based on this postulation, which led to completion of the present invention.

[0009]

Therefore, the present invention provides the following:

[1] A quality control marker for megakaryocytes, consisting of a transcript of a Ral (RAS like proto-oncogene) gene or a Ral protein.

[2] The marker according to [1], wherein the Ral gene is a RalB (RAS like proto-oncogene B) gene.

[3] A quality control marker for megakaryocytes, consisting of a transcript of a Kat7 (lysine acetyltransferase 7) gene, a Kat7 protein, or an acetylated histone protein that is a target of the Kat7 protein.

[4] A method for evaluating the quality of a megakaryocyte, comprising a step of detecting one or more of the biomarkers according to any one of [1] to [3] in the megakaryocyte.

[5] The method according to [4], comprising a step of detecting a transcript of a RalB gene or a RalB protein, and a transcript of a Kat7 gene or a Kat7 protein in the megakaryocyte.

[6] The method according to [4] or [5], comprising:

(1) a step of measuring an abundance or degree of activity of one or more of the biomarkers according to any one of [1] to [3] in a target megakaryocyte, and

(2) a step of evaluating the quality of the megakaryocyte based on the value measured in step (1).

[7] The method according to [4] or [5], characterized by detecting the marker using a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Ral gene, or an antibody that specifically recognizes the Ral protein.

[8] The method according to [4] or [5], characterized by detecting the marker using a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Kat7 gene, an antibody that specifically recognizes the Kat7 protein, or an antibody that specifically recognizes an acetylated histone protein that is a target of the Kat7 protein.

[9] The method according to any one of [4] to [7], wherein the megakaryocyte is derived from a pluripotent stem cell.

[10] A kit for quality control of megakaryocytes, comprising a nucleic acid probe and/or a nucleic acid primer that specifically recognizes a transcript of a Ral gene, or an antibody that specifically recognizes a Ral protein.

[11] A kit for quality control of megakaryocytes, comprising a nucleic acid probe and/or a nucleic acid primer that specifically recognizes a transcript of a Kat7 gene, an antibody that

specifically recognizes a Kat7 protein, or an antibody that specifically recognizes an acetylated histone protein that is a target of the Kat7 protein.

[12] A method for producing a megakaryocyte with improved quality, comprising a step of culturing a megakaryocyte in a medium containing a Ral inhibitor.

[13] The method according to [12], wherein at least one of the Ral inhibitors is RBC8 or an agent for suppressing expression of Ral.

[14] A method for producing a megakaryocyte with improved quality, comprising a step of increasing the abundance of a Kat7 protein in a megakaryocyte.

[15] The method according to any one of [12] to [14], wherein the megakaryocyte is derived from a pluripotent stem cell.

[16] A megakaryocyte evaluated by the method according to any one of [4] to [9], or obtained by the method according to any one of [12] to [15].

[17-1] A method for producing a platelet, comprising a step of maturing the megakaryocyte according to [16].

[17-2] The method according to [17-1], comprising a shaking culture step.

[17-3] The method according to [17-1] or [17-2], wherein the medium contains a Ral inhibitor.

[18] A platelet obtained by the method according to any one of [17-1] to [17-3].

[19-1] A blood product comprising the cell according to [16] or [18].

[19-2] The product according to [19-1], for use in the treatment or prevention of blood diseases or bleeding.

[20] A method for treating or preventing blood diseases or bleeding, comprising administering or transplanting an effective amount of the cell according to [16] or [18] to a mammal.

[21] The cell according to [16] or [18], for use in the treatment or prevention of blood diseases or bleeding.

[22] Use of the cell according to [16] or [18] in the manufacture of a prophylactic or therapeutic medicament for blood diseases or bleeding.

## [ADVANTAGEOUS EFFECTS OF INVENTION]

[0010]

The present invention makes it possible to control the quality of megakaryocytes and evaluate the quality of megakaryocytes. This can lead to a stable supply of high-quality platelets.

## [BRIEF DESCRIPTION OF DRAWINGS]

[0011]

[FIG. 1] A miRNA switch-based screening identifies miRNAs with endogenous activity in imMKCLs. (a) A schematic illustration of the design of miRNA switches. The mRNA is

composed of the antisense sequence of the target miRNA and a reporter transgene. In the presence of an active target miRNA, the expression of the reporter gene is repressed. (b) Design of miRNA switches for imMKCLs. A pair of mRNAs, one encoding tagBFP containing the antisense sequence of the miRNA target in the 5' UTR and the other of Azami Green protein, were synthesized *in vitro*. The two mRNAs were co-transfected into imMKCLs via lipofection. 24 hours after the transfection, the cells were analyzed by flow cytometry to identify the miRNA activity in imMKCLs through a screening. (c) Representative dot plots of target miRNA activity in imMKCLs. Active miRNA showed less TagBFP expression. (d) Flow cytometry analysis of the target miRNAs showing endogenous activity in imMKCLs. A miRNA switch-based screening was performed from a library containing 269 kinds of miRNA switches (Table 1), and 24 kinds of miRNAs were identified with endogenous activity in imMKCLs.

[FIG. 2] Let-7 miRNAs enable the identification of immune-skewed imMKCLs. (a) Heterogenous responsive activities in imMKCLs were identified with let-7a-5p or let-7g-5p miRNA switches, resulting in let-7 low-responsive and let-7 high-responsive subpopulations. (b) A schematic illustration of the bulk RNA-seq sampling workflow. Let-7a-5p and let-7g-5p switches were transfected into imMKCLs, followed by the sorting of let-7 low- and high-responsive cells by flow cytometry. Three distinct imMKCL clones (clone 7, clone 7-3, M35-1) were employed for the bulk RNA-seq analysis. Bar graphs showing the GSEA results of the top enriched immune-related gene sets in let-7 low-responsive imMKCLs in the proliferation phase (c) and maturation phase (d). GSEA plots showing typical enriched immune-related gene sets in the proliferation phase (e) and maturation phase (g). Heatmaps of the top differentially expressed TNF targets of let-7 low- or high-responsive imMKCLs in the proliferation phase (f) and maturation phase (h).

[FIG. 3] (a) Let-7a-5p and let-7g-5p expression levels were determined in let-7 high/low imMKCLs (clone 7) by RT-qPCR. The expression levels were normalized to RNU6B (U6). Sampling was performed 48 hours after sorting. Data are expressed as the mean ± SEM from three independent experiments. (b) Clone 7-3 represents an aged stock of clone 7 that shares the same genetic background. (c) iPSC-PLT production from let-7 high/low imMKCLs in static conditions. The cells proceeded directly to the DOX-OFF step after sorting. Three distinct imMKCL clones were employed. Data are expressed as the mean ± SEM from three independent experiments. Student *t* tests were used to assess statistical significance. * $P < 0.05$.

[FIG. 4] *In vitro* ESC-derived $CD34^+$ HPCs exhibited immune-skewed transcriptional signatures in let-7 low subpopulations. (a) A schematic illustration of the ESC-derived HPC induction by the Sac method. (b) GSEA plots showing the significantly enriched immune-related gene sets in let-7 low ESC-derived HPCs. (c) Heatmaps of the gene sets enriched in the same cells.

[FIG. 5] Single-cell RNA-seq reveals the presence of transcriptionally distinct immune-skewed subpopulations that are abundant in let-7a-5p low-responsive imMKCLs. (a) Visualization of imMKCLs (clone 7) by uniform manifold approximation and projection (UMAP) and colored according to let-7a-5p high/low-responsive imMKCLs (top) and imMKCL sub-clusters (bottom). (b) The distribution of let-7a-5p low- and high-responsive cells in each cluster (left), and a bar plot showing the distribution of let-7a-5p low- and high-responsive cells in each cluster (right). (c) The relative expression level of the top ten DEGs in each cluster. (d) The representative terms of GO:BP enriched in each cluster. (e) Typical immune-related GO:BP terms in each cluster. (f) Violin plots showing the expression level of typical thrombopoiesis-related and immune-related genes in each cluster.

[FIG. 6] Let-7a-5p is a functional player in the development of immune-skewed subsets in imMKCLs. (a) A schematic illustration of the experimental workflow using a specific let-7a-5p inhibitor. (b) Let-7a-5p expression levels in imMKCLs (clone 7) treated with a negative control or let-7a-5p inhibitor. The expression levels were measured by qRT-PCR and normalized to an endogenous control, RNU6B (U6). (c) Let-7a-5p activity patterns were analyzed by FACS. (d) Let-7a-5p inhibition induced a greater secretion of IL-8 from imMKCLs at the proliferation stage (DOX-ON). After transfection of the let-7a-5p inhibitor or negative control, the imMKCLs were incubated for 24 hours in the absence or presence of LPS (50 ng/mL) in the proliferation phase. Inflammatory molecules were measured by a cytometric bead array in the supernatant. (e) The inhibition of let-7a-5p in imMKCLs (clone 7) increased the mRNA expression levels of the marker genes identified in clusters 3 and 5 (Fig. 5). The mRNA expression levels were measured using qRT-PCR and normalized to GAPDH. Data are expressed as the mean ± SEM from three independent experiments. Two-tailed student's *t*-tests were used to assess statistical significance. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

[FIG. 7] RALB is a target of let-7a-5p in the development of immune-skewed imMKCLs. (a) A flow chart of the upstream regulator analysis. (b) Bar plots showing the top 10 upstream regulators for cluster 3 and cluster 5 that were identified by the IPA based on scRNA-seq datasets. (c) The identification of eight potential upstream regulators by comparing the common upstream regulators of clusters 3 and 5 and predicted has-let-7a-5p targets. (d) Violin plots showing the expression levels of identified upstream regulators in each cluster. The regulators CUX1 and RALB showed increased expression levels in let-7a-5p low cells (clusters 3 and 5) and are colored gray. (e) The let-7a-5p binding site on *CUX1* and *RALB* predicted by TargetScan. (f) Let-7a-5p inhibition induced the mRNA expression of CUX1 and RALB in imMKCLs. (g) The lentiviral-mediated overexpression of RALB elevated the mRNA expression of RALB at both the proliferation (DOX-ON) and maturation stages (DOX-OFF). (h) The overexpression of RALB induced the expression of interferon signaling genes. (i) A schematic

illustration showing how the let-7 miRNA-RALB axis modulates interferon signaling in imMKCLs. Data are expressed as the mean ± SD from three independent experiments. Two-tailed student's *t*-tests were used to assess statistical significance. **P* < 0.05, ***P* < 0.01.

[FIG. 8] (a) The lentiviral-mediated overexpression (O/E) of *CUX1* elevated the mRNA expression of *CUX1* at both the proliferation (DOX-ON) and maturation (DOX-OFF) stages in imMKCLs (clone 7). (b) CUX1 overexpression did not affect the mRNA expressions of *ISG15* or *IFIT3*. The expression levels were normalized to *GAPDH*. (c) The expression of let-7a-5p was not significantly affected by the overexpression of *CUX1*. The expression levels were normalized to RNU6B. Neither the proliferation rate (d) nor the iPSC-PLT production (e) were affected by CUX1 overexpression. Data are expressed as the mean ± SEM from three independent experiments.

[FIG. 9] (a) The intracellular protein expression of RALB (mean fluorescence intensity, MFI) detected in MOCK or RALB-overexpressing imMKCLs (O/E RALB) by intracellular flow cytometry. (b) The RALB expression was compared using a histogram overlay. (c) Comparable let-7a-5p expression was observed in MOCK and RALB-overexpressing imMKCLs. (d) The overexpression of RALB did not significantly impact the expression of genes related to MK maturation. The bar graphs show FPKM values of the indicated genes. Data are expressed as the mean ± SEM from three independent experiments.

[FIG. 10] Dysregulated immune properties are associated with arrested proliferation and deficient iPSC-PLT generation of imMKCL clones. (a) A schematic illustration showing the correlation of proliferation and iPSC-PLT production by a previous study (Sone, M. et al. Stem Cell Reports 16, 2861-2870 (2021)). (b) GSEA plots showing enriched TNF signaling and interferon responsive gene sets in low-intermediate quality clones compared with high quality clones. (c) The proliferation and fold change of the cell count at Day 14 of clone 7 and of clone 7-3. (d) iPSC-PLT production of clone 7 and of clone 7-3 in static or turbulent flow conditions. (e) Representative flow cytometry plots of iPSC-PLTs generated from clone 7 and clone 7-3 in static or turbulent flow conditions. (f) GSEA plots showing enriched TNF signaling and interferon responsive gene sets in clone 7-3. (g) Clone 7-3 shows elevated RALB mRNA expression compared with clone 7. (h) The secretion of IL-8 from clone 7 and clone 7-3 at the proliferation stage. Data are expressed as the mean ± SEM from three independent experiments. Two-tailed student's *t*-tests were used to assess statistical significance. * *P* < 0.05, ***P* < 0.01.

[FIG. 11] (a) GSEA plots and (b) a heatmap showing the enrichment of senescence and aging-related gene sets in low to intermediate quality clones compared with high quality clones. (c) Heatmaps of immune-related gene sets enriched in low to intermediate quality clones. (d) *RALB* expression levels in clones with distinct qualities.

[FIG. 12] (a) A flow cytometry analysis of let-7a-5p activity patterns in clone 7 and clone 7-3 by miRNA switch technology. (b) Heatmaps of gene sets enriched in clone 7-3. The let-7 low cells from each clone were compared. (c) GSEA plots showing the enrichment of downregulated senescence TP53 targets in clone 7-3 compared with clone 7. (d) Inflammatory cytokine encoding genes exhibited increased expression levels in clone 7-3 compared with clone 7.

[FIG. 13] (a) Representative flow cytometry plots of iPSC-PLTs generated from clone 7 in the presence of human recombinant IL-8 at the indicated concentrations. (b) Flow cytometric analysis of ploidy. (c) iPSC-PLT production under the indicated conditions. (d) Molecular structure of Reparixin, a specific inhibitor of CXCR1/2. (e) iPSC-PLT production under the indicated conditions. (f) Representative flow cytometry plots of iPSC-PLTs generated from RALB-overexpressing imMKCLs (clone 7) in the presence of Reparixin at the indicated concentrations. (g) Flow cytometric analysis of ploidy. Data are expressed as the mean ± SD from three independent experiments. One-way ANOVA with multiple comparisons was used to assess statistical significance. * $P < 0.05$.

[FIG. 14] Treatment with human recombinant interferon-α2a inhibited the proliferation (a) and iPSC-PLT production (b) of imMKCLs in a dose-dependent manner. imMKCLs (clone 7) were treated with human IFN-α2a at concentrations of 0, 1, 10, or 100 ng/mL. (c) The induced mRNA expression of interferon-related genes and *CDKN2A* in imMKCLs. mRNA expression levels were measured by RT-qPCR and normalized to *GAPDH*. (d) Representative flow cytometry plots of iPSC-PLTs generated under the indicated conditions. One-way ANOVA with multiple comparisons or two-tailed student t-tests were used to assess statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

[FIG. 15] The overexpression of RALB diminishes the proliferation and iPSC-PLT generation capacity of imMKCLs, potentially by inducing the dysregulation of their immune properties. The lentiviral-mediated overexpression (O/E) of RALB leads to arrested proliferation (a) and deficient iPSC-PLT generation (b) in imMKCLs (Clone 7). (c) Representative flow cytometry plots of iPSC-PLTs generated from MOCK and O/E RALB in static or turbulent flow conditions. (d) IL-8 is secreted more by O/E RALB than by MOCK imMKCLs. (e) GSEA plots showing enriched TNF signaling and interferon responsive gene sets in O/E RALB imMKCLs. (f) A gene expression correlation analysis of RALB and immune-related genes. Data are expressed as the mean ± SEM from three independent experiments.

[FIG. 16] (a) GSEA plots and a heatmap showing the enrichment of senescence and aging-related gene sets in RALB-overexpressing imMKCLs (O/E RALB) compared with MOCK. (b) Heatmaps of the gene sets enriched in O/E RALB (clone 7).

[FIG. 17] (a) Molecular structure of RBC8, a specific inhibitor of RALA and RALB. (b) The administration of 0.1 μM RBC8 inhibited the proliferation of imMKCLs (clone 7). (c) iPSC-

PLT production under the indicated conditions. (d) Representative flow cytometry plots of iPSC-PLTs generated from RALB-overexpressing imMKCLs (clone 7) in the presence or absence of RBC8. (e) Flow cytometric analysis of ploidy. (f) siRNA-mediated *RALB* knockdown ameliorated the iPSC-PLT production of imMKCLs (clone 7). Cells were transfected with siRNA targeting *RALB* (siRALB) or non-targeting control (siNT). (g) Representative flow cytometry plots of iPSC-PLTs generated from siNT or siRALB. (h) RALB knockdown did not significantly impact IL-8 secretion at the maturation stage. (i) The relative mRNA expression of the indicated genes was assessed in imMKCLs 48 hours following the transfection procedure. mRNA expression levels were measured by RT-qPCR and normalized to *GAPDH*. Data are expressed as the mean ± SEM from three independent experiments. Two-tailed student *t*-tests were used to assess statistical significance. * $P < 0.05$.

[FIG. 18] (a) PAC-1 binding and (b) *P*-selectin expression on iPSC-PLTs generated from clone 7 or clone 7-3 with or without PMA or ADP/TRAP6 stimulation. (c) Representative flow cytometry plots of activated iPSC-PLTs. (d) Representative histograms of Annexin V bound to CD41+ iPSC-PLTs derived from clone 7 or clone 7-3. (e) PAC-1 binding and (f) *P*-selectin expression on iPSC-PLTs generated from MOCK or RALB-overexpressing imMKCLs (O/E RALB) with or without PMA or ADP/TRAP6 stimulation. (g) Representative flow cytometry plots of activated iPSC-PLTs. (h) Representative histograms of Annexin V bound to CD41+ iPSC-PLTs derived from MOCK or O/E RALB.

[FIG. 19] (a) A schematic illustration of the *in vitro* differentiation of MKs from cord blood-derived $CD34^+$ cells. The inhibition of let-7a-5p significantly impacted neither mRNA expression of the indicated genes (b) nor PLT production (c-d). (e) Significantly elevated expression levels of *RALB*, *IRF7, ISG15*, and *IFIT3* were observed in RALB-overexpressing (O/E) cells. Total RNAs were isolated from Day-16 cells. The mRNA expression levels were measured by RT-qPCR and normalized to *GAPDH*. (f-g) The overexpression of RALB also induced a significant decline of $CD41a^+CD42b^+$ platelet-like particles generated from cord blood-derived MKs at Day 20. Data are expressed as the mean ± SEM from three independent experiments. Two-tailed student's *t*-tests were used to assess statistical significance. * $P < 0.05$.

[FIG. 20] Flow cytometry gating strategies for imMKCL subpopulations distinguished by miRNA switches (a) and for iPSC-PLTs counted with the aid of Absolute Counting Beads (b).

[FIG. 21] Reduced KAT7 and H3K14ac levels in LT-C imMKCLs. (A) KAT7 protein levels as measured by the Wes Simple Western system for ST-C, LT-C, and WS clones. (B) H3K14ac protein levels as measured by the Wes Simple Western system for ST-C, LT-C, and WS clones. (C) Cell proliferation as measured by the CCK8 assay for ST-C and WS clones during the 3-day Dox-ON culture period. (D) Platelet production capacity for ST-C and WS clones after Dox-OFF maturation period. Data are presented as the mean ± SEM from more than three independent

experiments. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests or one-way ANOVA.

[FIG. 22] WM3835 impaired imMKCL proliferation and maturation by inducing cell cycle arrest. (A) WM3835 (5 μM) effectively inhibited KAT7 signaling compared to WM1119. (B) Platelet production capacity of ST-C clones after WM3835 treatment on the indicated day during the maturation period. (C) Reduced platelet production capacity in ST-C clones following WM3835 treatment during the proliferation period. (D) Cell proliferation as measured by CCK8 assay for ST-C clones during the 3-, 6-, and 9-day Dox-ON culture period with WM3835 treatment. (E) Cell cycle analyzed using the Fucci system for ST-C following WM3835 treatment. Data are presented as the mean ± SEM from more than three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests or two-way ANOVA.

[FIG.23] KAT7 loss-of-function analysis. A lentiviral vector encoding shKAT7 or a control vector (shLacZ) was introduced into imMKCL cells. After transduction, selection was performed using hygromycin. (A) KAT7 expression measured by real-time qPCR. (B) Platelet production. (C) Cell cycle analysis during the proliferation phase. Data are shown as mean ± SEM from three or more independent experiments. Statistical significance was evaluated using an unpaired two-tailed Student's t-tests or one-way ANOVA.

[FIG. 24] KAT7 gain-of-function analysis. A lentiviral vector containing the KAT7 CDS sequence or a control vector (Mock) was introduced into imMKCL cells. After transduction, selection was performed using blasticidin. (A) KAT7 expression measured by real-time qPCR. (B) H3K14ac expression detected by intracellular flow cytometry. (C) Cell proliferation measured by CCK8 assay during a 3-day DOX-ON culture period. (D) Platelet production. (E) Cell cycle analysis during the proliferation phase. Data are shown as mean ± SEM from three or more independent experiments. Statistical significance was evaluated using an unpaired two-tailed Student's t-tests.

[DESCRIPTION OF EMBODIMENTS]

[0012]

As used herein, the singular forms "a", "an" and "the" are intended to include both the singular and plural forms, unless the language explicitly indicates otherwise with words like "only", "single" and/or "one". It will be further understood that the terms "comprises", "comprising", "includes" and/or "including" when used herein, specify the presence of stated features, steps, operations, elements, ideas, and/or components, but do not themselves preclude the presence or addition of one or more other features, steps, operations, elements, components, ideas, and/or groups thereof.

[0013]

1. Quality Control Marker for Megakaryocytes

The present invention provides a quality control marker for megakaryocytes (hereinafter also referred to as "the biomarker of the present invention"). By using the biomarker of the present invention, the quality of megakaryocytes can be evaluated; therefore, this biomarker is useful for managing the quality of megakaryocytes (in particular, ensuring that the megakaryocytes have a certain quality). In the present specification, quality control of megakaryocytes may also be read as "quality evaluation of megakaryocytes." Specifically, the biomarker of the present invention includes the transcript or protein of the Ral (RAS-like proto-oncogene) gene, the transcript or protein of the Kat7 (lysine acetyltransferase 7) gene, and an acetylated histone protein that is a target of the Kat7 protein (hereinafter simply referred to as "histone").

[0014]

Ral protein is a type of small GTPase, and there are two types of Ral proteins: RalA (RAS-like proto-oncogene A) protein (also referred to as "GTPase RalA") and RalB (RAS-like proto-oncogene B) protein (also referred to as "GTPase RalB"). RalA protein and RalB protein have approximately 80% homology, and these proteins largely have the same amino acid sequence except for the hypervariable region at the C-terminus. Either RalA or RalB can be used as a biomarker of the present invention, but preferably RalB is used.

[0015]

Kat7 protein is a type of histone acetyltransferase (HAT), and it regulates chromatin structure, controls gene expression, and participates in cell division by acetylating lysine residues of histone H3 and histone H4. Histone H3, H4, etc., are histones that are targets for acetylation by Kat7 protein. Examples of the target acetylated histones of Kat7 protein include H3K14ac (histone 3 with lysine 14 acetylated), H4K5ac (histone 4 with lysine 5 acetylated), H4K8ac (histone 4 with lysine 8 acetylated), H4K12ac (histone 4 with lysine 12 acetylated), and H4K16ac (histone H4 with lysine 16 acetylated), among which H3K14ac is preferred. Here, lysine 14 means that the lysine at position 14 in the amino acid sequence of a protein is acetylated (methionine encoded by the start codon is not counted). Lysine 8, lysine 12, and lysine 16 are also similar.

[0016]

In the present specification, "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.

In one embodiment, megakaryocytes are CD34-positive CD41-positive cells. Also, in another embodiment, megakaryocytes are CD38-negative, CD90-positive, and/or CD49f-positive.

[0017]

In the present specification, "transcript" refers to RNA that encodes a protein, and unless otherwise specified, hereinafter it refers to mRNA. Complementary DNA (cDNA) synthesized from RNA by reverse transcription is also included within the scope of the term "transcript."

[0018]

The Ral gene transcript (also referred to as "Ral transcript") is a known transcript, and for example, the human RalA transcript is disclosed as NCBI Accession No.: NM_005402.4 (SEQ ID NO: 1), and the human RalB transcript is disclosed as NCBI Accession No.: NM_001369400.1 (SEQ ID NO: 2) and NCBI Accession No.: NM_002881.3 (SEQ ID NO: 3). The Kat7 gene transcript is a known transcript, and for example, the human Kat7 transcript (isoform 1) is disclosed as NCBI Accession No.: NM_007067.5 (SEQ ID NO: 4). In addition to isoform 1, other isoforms 2-6 with shorter sequences (each shown as SEQ ID NOs: 5-9 in the sequence listing) are known for the Kat7 protein, and any of these can be used as a biomarker of the present invention, but in the present specification, the Kat7 gene transcript is preferably one consisting of the sequence shown in SEQ ID NO: 4. In the present invention, each transcript may be an RNA including a base sequence represented by any of SEQ ID NOs: 1-9 (provided that T is read as U), or it may be an RNA including a base sequence substantially identical to said base sequence. The origin of each transcript is not particularly limited, but preferably it is a mammal (e.g., human, mouse, rat, monkey, cow, horse, pig, dog, etc.), and among these, one derived from humans is preferred. Each transcript may be a homolog of the corresponding human transcript in a non-human mammalian species.

[0019]

Base sequences substantially identical to any of the base sequences represented by SEQ ID NOs: 1 to 9 include, for example, base sequences that have 80% or higher, preferably 90% or higher, and more preferably 95% or higher (e.g., 96%, 97%, 98%, 99% or higher) identity thereto. Such sequences are typically sequences encoding a protein that has substantially the same activity as the Ral protein or the Kat7 protein.

[0020]

Also, base sequences substantially identical to any of the base sequences represented by SEQ ID NOs: 1 to 9 include base sequences in which one or two or more (preferably about 1 to 300, preferably about 1 to 150, more preferably about 1 to 30, and particularly preferably one to several (2, 3, 4, 5, 6, 7, 8, 9, or 10)) nucleotides are substituted, inserted, added, and/or deleted

among these base sequences. Such sequences are typically sequences encoding a protein that has substantially the same activity as the Ral protein or the Kat7 protein.

[0021]

Each transcript can be obtained, for example, by isolating and purifying it from a cell or a biological sample containing the transcript by a method known per se. In addition, it may be produced by chemical synthesis, or produced by an in vitro transcription (IVT) method.

[0022]

The Ral protein is a known protein, and for example, the human RalA protein is disclosed as NCBI Accession No.: NP_005393.2 (SEQ ID NO: 10), and the human RalB protein is disclosed as NCBI Accession No.: NP_001356329.1 and NP_002872.1 (both of which have the same sequence (SEQ ID NO: 11)). The Kat7 protein is also a known protein, and for example, the human Kat7 protein (isoform 1) is disclosed as NCBI Accession No.: NP_008998.1 (SEQ ID NO: 12). The sequences of human Kat7 protein isoforms 2 to 6 are shown in the sequence listing as SEQ ID NOs: 13 to 17. In the present invention, each protein may be a protein containing the amino acid sequence represented by any one of SEQ ID NOs: 10 to 17, or a protein containing an amino acid sequence substantially identical to said amino acid sequence. The origin of each protein is not particularly limited, but preferably it is a mammal (e.g., human, mouse, rat, monkey, cow, horse, pig, dog, etc.), and among these, those derived from humans are preferred. Each protein may be a homolog of the corresponding human protein in mammals other than humans.

[0023]

Amino acid sequences substantially identical to any of the amino acid sequences represented by SEQ ID NOs: 10 to 17 include, for example, amino acid sequences that have 80% or higher, preferably 90% or higher, and more preferably 95% or higher (e.g., 96%, 97%, 98%, 99% or higher) identity thereto. Proteins comprising such sequences typically have substantially the same activity as the Ral protein or the Kat7 protein.

[0024]

Also, amino acid sequences substantially identical to any of the amino acid sequences represented by SEQ ID NOs: 10 to 17 include amino acid sequences in which one or two or more (preferably about 1 to 100, preferably about 1 to 50, more preferably about 1 to 10, and particularly preferably one to several (2, 3, 4, or 5)) amino acids are substituted, inserted, added, and/or deleted among these amino acid sequences. Proteins comprising such sequences typically have substantially the same activity as the Ral protein or the Kat7 protein.

[0025]

Ral proteins or Kat7 proteins can be produced according to known protein synthesis methods, for example, solid-phase synthesis, liquid-phase synthesis, and the like. The obtained

proteins can be purified and isolated by known purification methods, for example, solvent extraction, distillation, column chromatography, liquid chromatography, recrystallization, combinations thereof, and the like. In addition, they may be isolated and purified from biological samples by a method known per se. Alternatively, Ral proteins or Kat7 proteins can be produced by culturing a transformant containing a nucleic acid encoding the same and separating and purifying the protein from the resulting culture. Such nucleic acids may be DNA or RNA, or a DNA/RNA chimera, but are preferably DNA. The nucleic acid may be double-stranded or single-stranded.

[0026]

2. Quality Evaluation Method of Megakaryocytes

The present invention provides a method for evaluating the quality of a megakaryocyte, comprising a step of detecting a biomarker of the present invention in the megakaryocyte (hereinafter, referred to as "the evaluation method of the present invention"). In the present specification, "evaluating" can also be read as "determining", "inspecting", or "verifying" as appropriate. In the evaluation method of the present invention, only one type of biomarker of the present invention may be detected, or two or more types may be detected. When two or more types are detected, the combination of biomarkers to be detected is not particularly limited, and different types of transcripts or proteins may be detected (for example, a transcript of RalA and a transcript of RalB may be detected), or the same type of transcript or protein may be detected (for example, a transcript of RalB and a RalB protein may be detected). Furthermore, it is also a preferred embodiment to detect a transcript or protein (in one aspect, a transcript) of the Ral gene (in one aspect, the RalB gene) and a transcript or protein (in one aspect, a Kat7 protein) of the Kat7 gene.

[0027]

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.

[0028]

The megakaryocytes used in the present invention 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 megakaryocyte progenitor cell lines (imMKCL)." The megakaryocytes used in the present invention are typically CD34-positive CD41-positive immortalized megakaryocytes. 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.

[0029]

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. Hereinafter, these MYC protein, apoptosis-suppressing protein, and polycomb group proteins may be collectively referred to as "megakaryocyte-inducing factors."

[0030]

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 below, the gene can be said to be expressed.

[0031]

Examples of genes encoding MYC proteins (hereinafter also referred to as "MYC genes") include the c-MYC gene, N-MYC gene, and L-MYC gene. Preferably, the MYC gene is the c-MYC gene. Examples of the c-MYC gene include a gene consisting of the nucleic acid sequence represented by NCBI Accession No. NM_002467.

[0032]

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 ProteoTuner™ Shield System C, #631072).

[0033]

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.

[0034]

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.

[0035]

The origin of each gene encoding a megakaryocyte-inducing factor (hereinafter also referred to as "megakaryocyte-inducing factor gene") is not particularly limited, but is preferably a mammal (e.g., human, mouse, rat, monkey, cow, horse, pig, dog, etc.), and among them, human-derived is preferred. Alternatively, as a megakaryocyte-inducing factor gene, a homolog of the human gene in a non-human mammalian species is also preferred. Genes with high base sequence 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.

[0036]

Each megakaryocyte-inducing factor gene is preferably linked under the control of a drug-responsive promoter. A drug-responsive promoter is a promoter that expresses a gene in the presence of a corresponding drug, or whose gene expression is suppressed. Examples of drug-responsive promoters include a fusion protein of reverse tetR (rtetR) and VP16AD (rtTA), or a fusion protein of tetR and VP16AD (rTA), and a TRE promoter that can bind in the presence or absence of a corresponding drug. Such drugs include, for example, doxycycline (Dox),

tetracycline, or derivatives thereof (referred to as "Dox, etc."). Furthermore, metallothionein promoters (corresponding drug: heavy metal ions) and steroid-responsive promoters (corresponding drug: steroid hormone or derivative thereof) can also be used 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) and heat shock protein promoters (induced by heat shock) may also be used, and these promoters induced by drugs or stimuli are also referred to as inducible promoters.

[0037]

The megakaryocytes used in the present invention can be obtained by known methods. For example, a method of isolating from biological tissues (e.g., bone marrow, umbilical cord blood, peripheral blood), a method of differentiating pluripotent stem cells or hematopoietic progenitor cells, and a method of obtaining from companies such as ATCC are mentioned. Specific isolation methods and production methods are as described below.

[0038]

In the present specification, "detecting a biomarker" includes not only examining the presence or absence of the biomarker of the present invention in megakaryocytes (i.e., whether an amount of transcript or protein above the detection limit in the detection method is present), but also measuring (quantifying) its abundance or degree of activity. Therefore, the evaluation method of the present invention can be implemented by using the abundance or degree of activity of the biomarker of the present invention as an index. Accordingly, in another aspect, the evaluation method of the present invention includes:

(1) a step of measuring the abundance or degree of activity of one or more biomarkers of the present invention in a target megakaryocyte; and

(2) a step of evaluating the quality of the megakaryocyte based on the value measured in step (1).

[0039]

In one aspect, step (2) described above is the following step (2'): determining that a megakaryocyte is of high quality when the value measured in step (1) is equal to or less than a reference value. In one aspect, the reference value used in this step is based on measurements of the biomarker of the present invention in low-quality megakaryocytes, such as megakaryocytes that have undergone multiple passages and become senescent after establishment or isolation from biological tissue, or megakaryocytes exhibiting high expression levels of senescence markers such as IL-8. Such measurements are typically obtained from a cell population having the same number of megakaryocytes as the target megakaryocyte population. Examples of the reference value include the mean, mode, or median of measurements obtained from a plurality of cell populations, as well as values calculated therefrom using arithmetic operations.

[0040]

The reference value used in this step may be a value based on the measurement value of the biomarker of the present invention in megakaryocytes with high quality (for example, megakaryocytes immediately after establishment or immediately after isolation from biological tissues, or megakaryocytes with low expression of aging markers such as IL-8).

[0041]

The above-mentioned reference value may be a cutoff value. The method for calculating the cutoff value is well known in the art. For example, an ROC (Receiver Operating Characteristic) curve can be created, and the value at which diagnostic sensitivity and diagnostic specificity are as close to 100% as possible can be determined and used as the cutoff value.

[0042]

In the present specification, "quality of megakaryocytes" refers to the proliferative capacity and platelet production capacity of megakaryocytes. Therefore, "high quality of megakaryocytes" means that at least one (preferably both) of the proliferative capacity and platelet production capacity of megakaryocytes is high or predicted to be high.

[0043]

The detection or quantification of the biomarker of the present invention in megakaryocytes can be investigated by preparing an RNA fraction (e.g., total RNA, mRNA) from a population of megakaryocytes and detecting the transcript of Ral or Kat7 contained in the fraction. Therefore, in one embodiment, the evaluation method of the present invention includes detection or quantification using a nucleic acid probe or a nucleic acid primer capable of specifically recognizing the transcripts of Ral or Kat7, respectively.

[0044]

The preparation of the RNA fraction can be carried out using known methods such as guanidine-CsCl ultracentrifugation and AGPC method, and high-purity total RNA can be rapidly and easily prepared from a small amount of sample using commercially available RNA extraction kits (e.g., RNeasy Mini Kit; QIAGEN). As a means for detecting the transcripts of Ral or Kat7 in the RNA fraction, examples include methods using hybridization (Northern blot, dot blot, etc.) and methods using quantitative PCR (e.g., real-time PCR, digital PCR, etc.).

[0045]

In the case of Northern blot or dot blot hybridization, the detection or quantification of the transcripts of Ral or Kat7 can be carried out using a nucleic acid probe capable of specifically recognizing the transcripts of Ral or Kat7. Such a nucleic acid probe includes, for example, a nucleic acid containing a sequence complementary to a continuous region of 15 bases or more, preferably 16 to 100 bases, more preferably 17 to 80 bases, and even more preferably 18 to 50 bases in the known base sequence of the aforementioned transcript. The nucleic acid may be

DNA, RNA, or a DNA/RNA chimera, but is preferably DNA. Also, the nucleic acid used as a probe may be double-stranded or single-stranded. In the case of a double strand, it may be double-stranded DNA, double-stranded RNA, or a DNA:RNA hybrid. In the case of a single strand, one containing an antisense sequence can be used.

[0046]

The above nucleic acid probe is preferably labeled with a labeling agent to enable the detection of the target nucleic acid. Examples of the labeling agent include radioactive isotopes, enzymes, fluorescent substances, and luminescent substances. Examples of radioactive isotopes include [$^{32}$P], [$^{3}$H], and [$^{14}$C]. Enzymes should be stable and have high specific activity; examples include β-galactosidase, β-glucosidase, alkaline phosphatase, peroxidase, and malic acid dehydrogenase. Fluorescent substances include, for example, fluorescamine and fluorescein isothiocyanate. Luminescent substances include, for example, luminol, luminol derivatives, luciferin, and lucigenin. Furthermore, biotin-(strepto)avidin can be used to bind the probe and the labeling agent.

[0047]

The above nucleic acid probe can be obtained by using a primer set designed based on the sequence of the Ral or Kat7 transcript, amplifying a nucleic acid of a desired length by PCR using cellular cDNA or genomic DNA as a template, or cloning the gene or cDNA from the cDNA or genomic DNA library by colony or plaque hybridization, and if necessary, obtaining an appropriate fragment using restriction enzymes. Alternatively, it can also be obtained by chemical synthesis using commercially available DNA/RNA automatic synthesizers.

[0048]

In a preferred embodiment, quantitative PCR is used as a method for detecting or quantifying the Ral or Kat7 transcript. Quantitative PCR can be performed by known methods. For example, cDNA is synthesized from total RNA using reverse transcriptase, and PCR is carried out in the presence of a pair of nucleic acid primers specific to the target gene, DNA polymerase, and a dye or probe that can function as a DNA intercalator for quantifying the expression level.

[0049]

Examples of digital PCR include droplet digital PCR (ddPCR) and chip-based digital PCR (cdPCR). Digital PCR is performed, for example, by the following procedure. A reaction solution containing a probe set, a DNA sample, a PCR primer set, and DNA polymerase is set in a digital PCR apparatus. The mixing ratio of each reaction liquid component can be appropriately selected and optimized within known ranges, and can be changed as appropriate depending on the primers and probes used.

[0050]

The detection or quantification of Ral protein or Kat7 protein can be examined by preparing a protein fraction from a megakaryocyte population and detecting or quantifying the protein contained in the fraction. The detection or quantification of these proteins can be performed by an immunological assay (e.g., ELISA, FIA, RIA, Western blotting) using antibodies that specifically recognize each protein.

[0051]

An antibody that specifically recognizes Ral protein or Kat7 protein can be produced by using, as an immunogen, a partial peptide having these proteins or epitopes, and employing existing general production methods. In the present specification, antibodies include natural antibodies such as polyclonal antibodies and monoclonal antibodies (mAbs), chimeric antibodies and humanized antibodies, single-chain antibodies, and binding fragments thereof, which can be produced by using gene recombination technology, but are not limited thereto. Preferably, the antibody is a polyclonal antibody, a monoclonal antibody, or a binding fragment thereof. The binding fragment refers to a region of the aforementioned antibody that has specific binding activity, and specifically includes, for example, F(ab')2, Fab', Fab, Fv, sFv, dsFv, sdAb, and the like (Exp. Opin. Ther. Patents, Vol. 6, No. 5, p. 441-456, 1996). The class of the antibody is not particularly limited, and includes antibodies having any isotype such as IgG, IgM, IgA, IgD or IgE. Preferably, it is IgG or IgM, and more preferably IgG in consideration of ease of purification and the like. In addition, in the present invention, it is also preferable to use a commercially available antibody or a kit or array containing an antibody that specifically recognizes Ral protein or Kat7 protein as an antibody.

[0052]

When applying individual immunological detection methods or quantitative methods to the evaluation method of the present invention, no special conditions or operations need to be set. The detection or quantification system for the biomarker of the present invention can be constructed by adding ordinary technical considerations of a person skilled in the art to the normal conditions and operation methods in each method.

[0053]

The measurement of the degree of activity of the biomarker of the present invention in megakaryocytes can be performed, for example, by the following methods. Ral protein exists in two states: GTP-bound (active form) and GDP-bound (inactive form). Therefore, the degree of activity can be measured by measuring the abundance of GTP-bound Ral protein or calculating the ratio of active form to inactive form. Specifically, for example, a pull-down assay is used to pull down only the active form (GTP-bound form) of RalB using the Ral binding domain of an effector of Ral protein (for example, RalBP1), and then detect or measure the amount of protein by immunological measurement method, G-LISA (GTPase-linked Immunosorbent Assay) is

used to detect or quantify GTP-bound Ral protein, FRET (Forster Resonance Energy Transfer) assay is used to measure changes in Ral protein activity, GDP/GTP exchange assay is used to measure the GDP/GTP exchange rate of Ral protein, and so on.

[0054]

The level of Kat7 protein activity can be measured, for example, by a radioactive labeling acetyltransferase assay ($^{14}$C-acetyl CoA method), in which the amount of $^{14}$C incorporated into histones is measured; or by an immunological measurement, and the like, using an anti-acetyl histone antibody to detect or measure acetylated histones; or by mass spectrometry (LC-MS/MS) to analyze acetylated histones; or by FRET (fluorescence resonance energy transfer) assay to analyze changes in fluorescence intensity when acetylation occurs.

[0055]

Therefore, in one aspect, the evaluation method of the present invention is characterized by detecting the biomarker using a nucleic acid probe and/or a nucleic acid primer that specifically recognizes one or more Ral transcripts, or an antibody that specifically recognizes Ral protein. In another aspect, the evaluation method of the present invention is characterized by detecting the marker using a nucleic acid probe and/or a nucleic acid primer that specifically recognizes the transcript of the Kat7 gene, or an antibody that specifically recognizes Kat7 protein, or an antibody that specifically recognizes acetylated histone protein, which is the target of Kat7 protein. These evaluation methods may be used in combination, and in one embodiment, the evaluation method of the present invention includes (1) a step of detecting a Ral transcript or protein using a nucleic acid probe and/or a nucleic acid primer that specifically recognizes one or more Ral transcripts, or an antibody that specifically recognizes Ral protein (in one aspect, a nucleic acid probe and/or a nucleic acid primer that specifically recognizes the transcript of the Ral gene (particularly, the RalB gene)), and (2) a step of detecting a Kat7 transcript or protein using a nucleic acid probe and/or a nucleic acid primer that specifically recognizes the transcript of the Kat7 gene, or an antibody that specifically recognizes Kat7 protein, or an antibody that specifically recognizes acetylated histone protein, which is the target of Kat7 protein (in one aspect, an antibody that specifically recognizes Kat7 protein). These steps may be performed sequentially or simultaneously. In addition, step (1) may be performed after step (2).

[0056]

3. Kit for Quality Control of Megakaryocytes

Furthermore, the present invention provides a kit for quality control of megakaryocytes (hereinafter referred to as "the kit of the present invention"). The kit of the present invention preferably includes a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of Ral, or an antibody that specifically recognizes the Ral protein. In another aspect, the present invention provides a kit for quality control of megakaryocytes (this kit is also

included in "the kit of the present invention"), which includes a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Kat7 gene, or an antibody that specifically recognizes the Kat7 protein, or an antibody that specifically recognizes an acetylated histone protein, which is a target of the Kat7 protein. These may be included as one type only, or as multiple types. These kits may be used in combination, and in one embodiment, the kit of the present invention includes (1) one or more nucleic acid probes and/or nucleic acid primers that specifically recognize transcripts of Ral, or an antibody that specifically recognizes the Ral protein (in one aspect, a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Ral gene (particularly, the RalB gene)), and (2) a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Kat7 gene, or an antibody that specifically recognizes the Kat7 protein, or an antibody that specifically recognizes an acetylated histone protein, which is a target of the Kat7 protein (in one aspect, an antibody that specifically recognizes the Kat7 protein).

[0057]

When the kit of the present invention includes the aforementioned nucleic acid probe or nucleic acid primer (also simply referred to as "nucleic acid") as a component, the same nucleic acids as those exemplified in the evaluation method of the present invention described above 2. can be used. These nucleic acids may be provided as solids in a dry state or in an alcohol precipitate, or they may be provided dissolved in water or a suitable buffer (e.g., TE buffer). When used as labeled probes, the nucleic acids may be provided in advance with any of the aforementioned labeling substances, or they may be provided separately from the labeling substance and then labeled for use. Alternatively, the nucleic acid may be provided in a state where it is fixed on a suitable substrate (also referred to as "supported" or "solid-phase"). Examples of substrates include glass, silicone, plastic, nitrocellulose, nylon, polyvinylidene fluoride, and others, but are not limited thereto. As a method for fixing, a functional group such as an amino group, aldehyde group, SH group, or biotin is introduced into the nucleic acid in advance, and on the other hand, a functional group that can react with the nucleic acid (e.g., an aldehyde group, amino group, SH group, streptavidin) is also introduced on the substrate, and the substrate and the nucleic acid are cross-linked by covalent bonds between these functional groups. Alternatively, a method in which a polyanionic nucleic acid is used, and the substrate is coated with a polycationic coating to fix the nucleic acid using electrostatic interaction can be mentioned as an example.

[0058]

When the kit of the present invention includes the aforementioned antibody as a component, the same antibodies as those exemplified in the evaluation method of the present invention described above 2. can be used.

[0059]

The kit of the present invention may include, in addition to the nucleic acids and antibodies described above, other substances required for a reaction for detecting or quantifying expression of a biomarker of the present invention. Such other substances may be provided in a state coexisting with the nucleic acids, antibodies, and the like, or may be provided together with separate reagents, so long as they do not adversely affect the reaction. For example, when the reaction for detecting or quantifying expression of a biomarker of the present invention is PCR, such other substances include, for example, a reaction buffer, dNTPs, and a thermostable DNA polymerase. When quantitative PCR is used, competitor nucleic acids and fluorescent reagents (such as the above-described intercalators and fluorescent probes) may be further included. Further, when the reaction for detecting or quantifying expression of a biomarker of the present invention is an antigen-antibody reaction, such other substances include, for example, a reaction buffer, a competitor antibody, a labeled secondary antibody (for example, where the primary antibody is a rabbit antibody, a mouse anti-rabbit IgG labeled with peroxidase, alkaline phosphatase, or the like), a blocking solution, and an ELISA plate. The kit of the present invention may further include instructions describing methods of using the kit and reagents, evaluation criteria, and the like. Furthermore, the above-described evaluation kit may further include one or more biomarkers of the present invention, for example, for use as a positive control. As for the types, specific examples, methods of use, and the like of reagents used in the kit of the present invention, all contents described in "2. Quality Evaluation Method of Megakaryocytes" are incorporated herein by reference.

[0060]

4. Method for Producing Megakaryocyte and Platelet

In another aspect, the present invention provides a method for producing a megakaryocyte with improved quality. Specifically, the present invention provides a method for producing a megakaryocyte with improved quality, including a step of culturing a megakaryocyte in a medium containing a Ral (RAS-like proto-oncogene) inhibitor (hereinafter referred to as "the production method of the present invention").

[0061]

Furthermore, as shown in the Examples described below, it has been shown that the expression level of the Kat7 gene affects the maintenance of the proliferative capacity of megakaryocytes and the amount of platelet production (yield) in subsequent maturation stages. Therefore, a method for producing a megakaryocyte with improved quality is also provided, which includes a step of increasing the abundance of Kat7 protein in a megakaryocyte (this method is also included in "the production method of the present invention"). The megakaryocytes used in this method are typically megakaryocytes with a low expression level of

the Kat7 gene. Examples of megakaryocytes with a low expression level of the Kat7 gene include, for example, megakaryocytes in which the expression level of the Kat7 gene is reduced by multiple passages compared to after establishing or isolating megakaryocytes from tissue, and megakaryocytes having a mutation in the Kat7 gene, which have a lower expression level of the Kat7 gene compared to megakaryocytes with wild-type Kat7 genes.

[0062]

These production methods may be used in combination, and in one embodiment, the production method of the present invention includes (1) a step of culturing a megakaryocyte in a medium containing a Ral inhibitor, and (2) a step of increasing the abundance of Kat7 protein in the megakaryocyte. These steps may be performed sequentially or simultaneously. Furthermore, step (1) may be performed after step (2). The megakaryocyte obtained by the production method of the present invention may also be referred to as "the megakaryocyte of the present invention." The "method for producing a megakaryocyte with improved quality" can also be read as "a method for improving the quality of a megakaryocyte."

[0063]

In the present specification, "increasing the abundance of Kat7 protein" means that the amount of the protein present in megakaryocytes is made greater compared to the amount of the protein present in the megakaryocytes before the step of increasing the abundance of Kat7 protein. The state in which the protein amount is high in the megakaryocytes may be maintained constantly, or may be made high transiently. The method for increasing the abundance of Kat7 protein in megakaryocytes is not particularly limited, but examples include: a method of forcibly expressing the protein by externally introducing a nucleic acid (DNA or RNA) encoding Kat7 protein (hereinafter also referred to as "exogenous nucleic acid") into megakaryocytes or cells having differentiation potential into megakaryocytes (e.g., hematopoietic progenitor cells, pluripotent stem cells, etc.); a method of activating the expression of an endogenous gene encoding Kat7 protein by modifying a promoter through techniques such as genome editing; and a method of delivering the protein into cells by adding Kat7 protein to the culture medium. The Kat7 protein or the nucleic acid encoding the protein may be introduced into cells only once or multiple times.

[0064]

Exogenous nucleic acids are typically introduced into cells (e.g., megakaryocytes, hematopoietic progenitor cells, pluripotent stem cells, etc.) in the form of an expression vector containing the nucleic acid. Vectors expressing exogenous nucleic acids may include, for example, viral vectors such as retrovirus, lentivirus, adenovirus, adeno-associated virus, herpes virus, and Sendai virus, and animal cell expression plasmids (e.g., pA1-11, pXT1, pRc/CMV,

pRc/RSV, pcDNAI/Neo). Since they can be implemented by a single introduction, retroviral vectors or lentiviral vectors are preferred.

[0065]

Examples of promoters used in expression vectors include EF-α promoter, CAG promoter, SRα promoter, SV40 promoter, LTR promoter, CMV (cytomegalovirus) promoter, RSV (Rous sarcoma virus) promoter, MoMuLV (Moloney mouse leukemia virus) LTR, HSV-TK (herpes simplex virus thymidine kinase) promoter, and the like. In addition to a promoter, an expression vector may contain, if desired, an enhancer, a polyA addition signal, a selection marker gene, an SV40 replication origin, and the like. Useful selection marker genes include, for example, dihydrofolate reductase gene, neomycin resistance gene, puromycin resistance gene, and the like.

[0066]

Various methods can be used to introduce exogenous nucleic acids into cells, including lipofection, liposome method, electroporation, nucleofection, calcium phosphate coprecipitation, DEAE dextran method, microinjection, and gene gun method. When the exogenous nucleic acid is contained in a viral vector, the nucleic acid-containing plasmid can be introduced into an appropriate packaging cell (e.g., Plat-E cells) or a complementary cell line (e.g., 293 cells), and the virus produced in the culture supernatant is collected and brought into contact with cells to infect them, thereby enabling introduction into cells.

[0067]

In the present specification, "improved quality" means that the proliferation capacity and/or platelet production capacity are higher in megakaryocytes after the production method of the present invention is performed compared to megakaryocytes before the production method of the present invention is performed. Furthermore, in the present specification, "platelet production capacity" means the capacity to produce platelets through proplatelet formation by transitioning the megakaryocytes into the maturation stage. The transition of megakaryocytes into the maturation stage can be performed by the "step of maturing megakaryocytes" described below. In the Examples described below, the proliferation capacity is said to be high if the proliferation rate - calculated by dividing the number of megakaryocytes on Day 14 after the start of proliferation by the number of megakaryocytes at the time proliferation was started - is higher than the proliferation rate in megakaryocytes before the production method of the present invention is performed. Also, as performed in the Examples described below, the platelet production capacity is said to be high if the ratio of platelets collected after transitioning megakaryocytes to the maturation stage and subsequently culturing them for 6 days (number of platelets / number of megakaryocytes) is higher than the ratio in megakaryocytes before the production method of the present invention is performed.

[0068]

The production method of the present invention typically includes a step of proliferating megakaryocytes in the presence of a Ral inhibitor. In one embodiment, megakaryocytes can start proliferation by increasing the abundance of MYC protein (especially c-MYC) and an apoptosis-suppressing protein (especially Bcl-xL), and preferably further increasing the abundance of a polycomb group protein (especially BMI1). 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, or a method of delivering the protein into cells by adding the protein to the culture medium. Specifically, as a method of increasing the expression level of a gene encoding the protein, examples include introducing exogenous nucleic acids (DNA or RNA) encoding the protein into megakaryocytes, or culturing megakaryocytes in the presence of a corresponding drug or stimulus using the aforementioned inducible promoter. From the perspective of efficient maturation, it is preferable that the period during which the abundance of megakaryocyte-inducing factors (especially MYC protein) is increased in the megakaryocytes is limited to the period of proliferating the megakaryocytes.

[0069]

The Ral inhibitor used in the present invention is not limited to an inhibitor specific to RalA or RalB, and may be one that inhibits both RalA and RalB, or one that has inhibitory activity against other molecules (typically, other GTPases). Examples of such Ral inhibitors include RBC6 (Cas No: 381186-64-7), RBC8 (Cas No: 361185-42-4; 6-amino-4-(2,5-dimethoxyphenyl)-1,4-dihydro-3-(2-naphthalenyl)-pyrano[2,3-c]pyrazole-5-carbonitrile), RBC10 (CAS No: 362503-73-9), BAY 293 Negative Control, BQU57, CE3F4, CID-1067700, and the like, but RBC8 is preferred. Also, an antibody, peptide, or aptamer against Ral protein is also preferred. Alternatively, the Ral inhibitor may be a Ral expression suppressor such as antisense nucleic acid, siRNA, shRNA, or heteroduplex nucleic acid that suppresses Ral expression. The Ral inhibitor may be used alone or in combination of multiple types.

[0070]

The concentration of the Ral inhibitor can be appropriately selected by a person skilled in the art, depending on the Ral inhibitor used. When the Ral inhibitor is RBC8, its concentration in the medium is typically from 1 nM to 100 μM, preferably from 10 nM to 10 μM, and more preferably from 20 nM to 1 μM (in one aspect, 100 nM).

[0071]

In the production method of the present invention, the culture may be either a suspension culture or an adherent culture, but is typically a suspension culture. Also, megakaryocytes isolated from a living body can be cultured by adherent culture. There are no particular restrictions on the culture period in the presence of the Ral inhibitor (the period for proliferating the megakaryocytes), but it is typically 3 days or more, 5 days or more, 7 days or more, or 10

days or more, and typically 20 days or less, 17 days or less, 15 days or less, or 14 days or less. During the above culture period, it is preferable to replace the medium every 3 to 4 days.

[0072]

Also, the period for culturing megakaryocytes in a medium containing the Ral inhibitor (in other words, the period for contacting the Ral inhibitor and the megakaryocytes), and the period for maintaining the state in which the abundance of Kat7 protein is increased in the megakaryocytes are not particularly limited, and may be only during the period for proliferating the megakaryocytes, but may also continue during the period for maturing the megakaryocytes. As such a period, it is typically 6 days or more, 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.

[0073]

In the present specification, "suspension culture" refers to culture performed under conditions that maintain a state in which cells or cell aggregates are floating in a culture medium, i.e., culture under conditions that do not allow a strong cell-substratum junction to be formed between the cells or cell aggregates and the culture vessel. Suspension culture may be static culture or shaking culture, and in the case of shaking culture, it can be called suspension culture as long as the condition is such that cells are not fixed in one place.

[0074]

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.

[0075]

The megakaryocyte used in the present invention can be obtained by known methods. For example, a method of isolating from biological tissues (e.g., bone marrow, umbilical cord blood, peripheral blood, etc.) using known techniques, a method of inducing differentiation of pluripotent stem cells or hematopoietic progenitor cells, and a method of obtaining from companies such as ATCC can be mentioned. From biological tissues, for example,

megakaryocytes can be isolated by methods using flow cytometry or mass cytometry based on surface antigens, magnetic cell separation, or affinity columns immobilized with the desired antigen. It is preferable that the megakaryocyte used in the present invention is obtained by a method of inducing differentiation of hematopoietic progenitor cells. Therefore, the production method of the present invention may include a step of producing megakaryocytes from pluripotent stem cells or hematopoietic progenitor cells (which can also be referred to as "differentiation induction") prior to the production of megakaryocytes with improved quality ("megakaryocyte production process"). Hematopoietic progenitor cells can be obtained, for example, by isolating from biological tissues (e.g., bone marrow, umbilical cord blood, peripheral blood, etc.) or by inducing differentiation from pluripotent stem cells.

[0076]

The megakaryocyte production process is not particularly restricted and can be implemented by 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, International Publication No. 2012/157586, and the like. In one embodiment, the megakaryocyte production process includes step (A) culturing pluripotent stem cells to produce hematopoietic progenitor cells (which can also be phrased as "differentiation induction"), and/or step (B) culturing hematopoietic progenitor cells to produce megakaryocytes.

[0077]

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., 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.

[0078]

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.

[0079]

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.).

[0080]

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.

[0081]

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.

[0082]

As induced pluripotent stem cell lines, various iPSC lines established by NIH, Riken, Kyoto University, etc., are available. For example, if human iPSC lines, HiPS-RIKEN-1A strain, HiPS-RIKEN-2A strain, HiPS-RIKEN-12A strain, Nips-B2 strain, etc. of Riken; 253G1 strain, 253G4 strain, 1201C1 strain, 1205D1 strain, 1210B2 strain, 1383D2 strain, 1383D6 strain, 201B7 strain, 409B2 strain, 454E2 strain, 606A1 strain, 610B1 strain, 648A1 strain, 1231A3 strain, FfI-01s04 strain, Ff-MH23s01 strain, 15M41 strain, and TkDN-sev2 strain of Kyoto University can be mentioned.

[0083]

The induced pluripotent stem cells used in the present invention may be cells derived from a patient with a hereditary disease (e.g., a patient with a hereditary blood disease). Since cells differentiated from pluripotent stem cells derived from a patient with a hereditary blood disease can become a disease model reflecting the pathology of the disease, they are suitable for screening therapeutic or preventive drugs for the disease. Alternatively, it is possible to use the

cells as a therapeutic agent for the disease by repairing genes using genome editing such as the CRISPR-Cas system for pluripotent stem cells derived from a patient with a hereditary blood disease and then differentiating them into the target cells.

[0084]

The species of origin of the pluripotent stem cells is not particularly limited, and may be cells of, for example, rodents such as rats, mice, hamsters, and guinea pigs; lagomorphs such as rabbits; artiodactyls such as pigs, cows, goats, and sheep; feliforms such as dogs and cats; and primates such as humans, monkeys, rhesus macaques, marmosets, orangutans, and chimpanzees. The preferred species of origin is human.

[0085]

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.

[0086]

Regarding the culture period of step (A), those skilled in the art can determine it appropriately while monitoring the number of hematopoietic progenitor cells and the like. As long as hematopoietic progenitor cells are obtained, the number of days is not particularly limited, but it is preferably 6 days or more, 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). Long culture periods are not problematic in the production of hematopoietic progenitor cells, but it is typically 30 days or less, and may be 20 days or less.

[0087]

Step (B) can also be carried out by a known method (e.g., methods described in WO 2011/034073, WO 2012/157586, WO 2014/123242, WO 2021/075568). Specifically, examples include a method of simultaneously forcibly expressing 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.

[0088]

Regarding the culture period of step (B), those skilled in the art can determine it appropriately while monitoring the number of megakaryocytes and the like. As long as megakaryocytes are obtained, 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. There is no

particular upper limit for the culture period, but it is typically 20 days or less, preferably 17 days or less, and more preferably 14 days or less.

[0089]

In addition, the expression or the function of the expression product of the TP53 gene (protein name: p53) and/or the CDKN1A gene (protein name: p21) may be suppressed in hematopoietic progenitor cells. As the TP53 gene, for example, a gene consisting of the nucleic acid sequence shown in NCBI accession number NM_000546.6 can be mentioned. As the CDKN1A gene, for example, a gene consisting of the nucleic acid sequence shown in NCBI accession number NM_001291549.3 can be mentioned. The origin of these genes is not particularly limited, but is preferably a mammal (e.g., human, mouse, rat, monkey, cow, horse, pig, dog, etc.), and among them, human-derived is preferred. In one embodiment, these genes are human genes or homologs of human genes in other mammalian species. Genes with high base sequence 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.

[0090]

The megakaryocytes of the present invention are suitable as starting cells for platelet production. Therefore, in another embodiment, a method for producing platelets including a step of maturing the megakaryocytes of the present invention is provided (hereinafter may be referred to as "the platelet production method of the present invention"; also, platelets obtained by the production method of the present invention may be referred to as "the platelets of the present invention").

[0091]

The platelet production method of the present invention can be carried out by known methods. For example, when megakaryocytes have one or more (preferably at least the MYC gene) or all of the megakaryocyte-inducing factors forcibly expressed, megakaryocytes can be matured by reducing or stopping the forced expression of the megakaryocyte-inducing factors. For example, if the megakaryocyte-inducing factors are 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, if megakaryocyte-inducing factor genes are introduced into cells using a vector containing LoxP, it may be achieved by introducing Cre recombinase into the cells. Furthermore, if a transient expression vector and the introduction of RNA or protein are used, it may be achieved by stopping contact with the vector or the like.

[0092]

It is also a preferred embodiment that 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.

[0093]

The culture medium used in the platelet production method of the present invention may also contain the aforementioned Ral inhibitor. In one embodiment, the culture medium used in the platelet production method of the present invention contains a Ral inhibitor. The specific types of compounds and their concentrations in the medium are all incorporated by reference to the description in the production method of the present invention. As a Ral inhibitor, RBC8 is preferred, and the concentration of RBC8 in the medium is preferably 20 nM to 1 μM (in one embodiment, 100 nM).

[0094]

The mature megakaryocytes induced by the platelet production method of the present invention can be CD34-positive CD41-positive cells. The mature megakaryocytes induced by this method may also be CD38-negative, CD90-positive, and/or CD49f-positive.

[0095]

The culture period in the platelet production method of the present invention is not particularly limited as long as the function of platelets is maintained, but it is, for example, 2 days to 9 days (in one embodiment, 6 days). When a Ral inhibitor is used, the culture in the platelet production method of the present invention may be performed in the presence of a Ral inhibitor throughout the entire culture period (in one embodiment, 6 days), or during a part of the period. In the above culture period, the medium may be exchanged at an appropriate time, or may not be exchanged; in one embodiment, the medium is not exchanged.

[0096]

Culture in the platelet production method of the present invention is typically suspension culture. In addition, it is preferable to perform the culture while shaking. Therefore, in one embodiment, the platelet production method of the present invention includes a shaking culture step. Shaking culture can be implemented by stirring the culture liquid by the rotational movement of a stirring blade using, for example, a commercially available rotary shaking culture machine (e.g., VerMES reactor (Shatake Multimix Co., Ltd.), etc.). The stirring speed may be 50 to 200 rpm, and for example, approximately 100 rpm.

[0097]

Shaking culture in the platelet production method of the present invention may be carried out using the apparatus and method described in WO 2019/009364. Specifically, for example, it includes a step of stirring the medium in a culture container using a stirring blade, 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 stirring blade is reciprocated to satisfy one or more of these indices.

[0098]

Furthermore, as a culture apparatus, for example, there is provided an apparatus that includes a container containing a medium including megakaryocytes and a stirring blade that reciprocates within the container, wherein the medium is stirred by reciprocating the stirring blade 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.

[0099]

The turbulent energy, shear stress, and Kolmogorov scale in the medium within the container, which is the object of stirring, can be calculated by simulation based on the basic equations of turbulence. For example, they can be calculated using thermal fluid analysis software FLUENT (ANSYS), but it is not limited to specific software. More specifically, when the stirring blade is configured to reciprocate, the turbulent energy varies depending on the stroke of the reciprocal movement of the stirring blade, the speed of the reciprocal movement, the frequency of the reciprocal movement, and the like. When configured using a plurality of stirring blades, the number of stirring blades can also be a factor that causes change. The shear stress and Kolmogorov scale also change due to changes in the same factors.

[0100]

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 medium, Eagle's Minimum Essential Medium (EMEM) medium, αMEM medium, Dulbecco's modified Eagle's Medium (DMEM) medium, 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.

[0101]

In the present invention, culture conditions for cells are not particularly restricted, and ordinary culture conditions can be adopted. As specific examples, the culture temperature is, for example, approximately 35 to 42°C, approximately 36 to 40°C, or approximately 37 to 39°C. The $CO_2$ concentration is, for example, approximately 5 to 15%. The $O_2$ concentration is, for example, approximately 15 to 25%.

[0102]

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.

[0103]

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 cells can proliferate. Typically, it is $1.0\times10^2$ to $1.0\times10^7$ cells/cm$^2$, preferably $1.0\times10^3$ to $1.0\times10^6$ cells/cm$^2$, and more preferably $1.0\times10^4$ to $1.0\times10^5$ cells/cm$^2$.

[0104]

When a substance used in the present invention is a low-molecular-weight compound, the compound is intended to encompass not only the free form thereof, but also pharmacologically acceptable salts and hydrates thereof. Although suitable pharmacologically acceptable salts vary depending on the type of compound, examples thereof include base addition salts and acid addition salts. Examples of base addition salts include salts with inorganic bases, such as alkali metal salts (e.g., sodium salts and potassium salts), alkaline earth metal salts (e.g., calcium salts and magnesium salts), aluminum salts, and ammonium salts; and salts with organic bases, such as trimethylamine, triethylamine, pyridine, picoline, ethanolamine, diethanolamine, triethanolamine, dicyclohexylamine, and N,N′-dibenzylethylenediamine. Examples of acid addition salts include salts with inorganic acids, such as hydrochlorides, hydrobromides, sulfates, hydroiodides, nitrates, and phosphates; and salts with organic acids, such as citrates, oxalates, acetates, formates, propionates, benzoates, trifluoroacetates, maleates, tartrates, methanesulfonates, benzenesulfonates, and p-toluenesulfonates.

[0105]

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

[0106]

The production method of the present invention and the platelet production method of the present invention may include a step of collecting target cells or tissues obtained in each step. The collected cells may be cryopreserved using a cell cryopreservation solution. In addition, the obtained cells may be counted using a cell counter, and may be sorted or purified using flow cytometry, mass cytometry, a magnetic cell sorting method, or the like by labeling with an antibody against cell surface markers.

[0107]

5. Use of Megakaryocyte and Platelet

In another aspect of the present invention, a megakaryocyte (i.e., the megakaryocyte of the present invention) and platelet (i.e., the platelet of the present invention), which have been

evaluated by the evaluation method of the present invention (typically, those evaluated as having high quality), or obtained by the production method of the present invention and the platelet production method of the present invention (where "obtained" can be read as "obtainable"), are provided. Hereinafter, the megakaryocyte and platelet of the present invention may be collectively referred to as "the cells of the present invention."

[0108]

Since the cells of the present invention can be used in medical applications such as blood transfusions, 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 megakaryocytes of the present invention can be used, for example, by administering or transplanting them into a living organism to produce functional platelets in vivo. In addition, the platelets 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.

[0109]

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.).

[0110]

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.

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.

[0111]

The cells of the present invention can be manufactured as a parenteral preparation, such as an injectable agent, a suspension, or an infusion agent, by mixing with a pharmaceutically acceptable carrier according to common practice. Therefore, in one embodiment, a method for producing a blood product comprising a step of formulating the cells of the present invention is provided. Such a method may include a step of preparing the cells of the present invention. Furthermore, it may include a step of preserving the cells of the present invention.

[0112]

As pharmaceutically acceptable carriers that can be contained in the parenteral preparation, examples include aqueous solutions for injection such as physiological saline, isotonic solutions containing glucose and other auxiliary agents (e.g., D-sorbitol, D-mannitol, sodium chloride, etc.). The cells of the present invention may be combined with, for example, human plasma, infusion solutions, buffers (e.g., phosphate buffer, sodium acetate buffer), anesthetics (e.g., benzalkonium chloride, procaine hydrochloride, etc.), stabilizers (e.g., human serum albumin, polyethylene glycol, etc.), preservatives, antioxidants, and the like.

[0113]

The blood product of the present invention is provided in a frozen state under conditions normally used for cryopreservation of cells, and can be thawed and used when needed. In this case, it may further contain serum or its substitute, organic solvents (e.g., DMSO), etc. In this case, the concentration of the serum or its substitute is not particularly limited but may be about 1 to about 30% (v/v), preferably about 5 to about 20% (v/v). The concentration of the organic solvent is not particularly limited but may be 0 to about 50% (v/v), preferably about 5 to about 20% (v/v).

[0114]

Furthermore, the cells of the present invention can be used in a method for screening candidate drugs that are useful for the treatment or prevention of blood diseases. Therefore, in a further embodiment of the present invention, a screening method for therapeutic or preventive agents for blood diseases is provided, comprising a step of culturing the cells of the present invention in the presence or absence of a test substance. For example, by using, as the cells, a disease model that reflects the pathology of a blood disease, if the pathology is improved in the presence of a test substance, the test substance can be selected as a candidate substance for a therapeutic or preventive agent for the blood disease. Examples of such blood diseases include those similar to the blood diseases that are the targets of treatment or prevention by the blood product of the present invention.

[0115]

The step of contacting the test substance with the cell of the present invention can typically be carried out by adding the test substance to a culture medium in which the cell of the present invention is cultured, or by transferring the cell of the present invention to a medium to which the test substance has been added in advance. The duration of the step of contacting the test substance with the cell of the present invention is not particularly limited, but typically it is from 1 minute to 5 days, preferably from 1 hour to 1 day.

[0116]

Examples of the test substances used in the present invention include, for example, cell extracts, cell culture supernatants, microbial fermentation products, extracts derived from marine organisms, plant extracts, purified proteins or crude proteins, peptides, non-peptide compounds, synthetic low molecular weight compounds, and natural compounds. The test substance may be an existing component or a candidate component of pharmaceuticals, nutritional foods, and the like.

[0117]

The invention will be more fully understood by reference to the following examples, which provide illustrative non-limiting embodiments of the invention.

[EXAMPLES]

[0118]

<Results>

Identification of miRNAs with endogenous activity in imMKCL

MiRNA switch technology describes synthetic mRNA-based genetic circuits that regulate transgene expressions through miRNA activity (Miki, K. et al. Cell Stem Cell 16, 699-711 (2015); Fujita, Y. et al. Sci Adv 8, eabj1793 (2022)). The present inventors hypothesized that miRNA switches can identify imMKCL subsets with distinct miRNA activities, providing a valuable tool for exploring the heterogeneity within imMKCLs. Fig. 1a depicts a schematic illustration of miRNA switch technology. The present inventors designed synthetic mRNAs that contain complementary sequences of target miRNAs in the 5′ untranslated region (UTR) and encode a reporter fluorescent protein to monitor endogenous miRNA activity. A pair of mRNAs were synthesized and co-transfected into imMKCLs, including Azami-Green 1 (AG1)-coding mRNA as a transfection control and TagBFP-coding mRNA containing the target miRNA sequence (Fig. 1b). TagBFP expression levels were measured by flow cytometry in cells with endogenous activity for a specific miRNA (Fig. 1c). A miRNA switch-based screening was conducted using a library containing 269 miRNA switches (Table 1) and resulted in the identification of 24 types of miRNAs with endogenous activity in imMKCLs (Fig. 1d).

[0119]

[Table 1-1]

The target miRNAs included in the miRNA switch screening library.

| | | | | | |
|---|---|---|---|---|---|
| miR-1234-5p | miR-185-3p | miR-301a-5p | miR-425-3p | miR-497-5p | miR-10b-3p |
| miR-329 | miR-144-3p | miR-211-3p | miR-485-5p | miR-450-5p | miR-379-5p |
| miR-629-5p | miR-362-3p | miR-589-5p | miR-671-5p | miR-506-3p | miR-125b-1-3p |
| miR-30b-3p | miR-296-3p | miR-506-5p | miR-361-3p | miR-582-3p | miR-422a |
| miR-98-3p | miR-499a-5p | miR-24-2-5p | miR-105-5p | miR-381-3p | miR-181b-3p |
| miR-122-5p | miR-27a-5p | miR-505-5p | miR-454-3p | miR-301a-3p | miR-510 |
| miR-411-5p | miR-29c-5p | miR-148b-5p | miR-204-3p | miR-486-3p | miR-125b-2-3p |

| | | | | | |
|---|---|---|---|---|---|
| miR-212-5p | miR-582-5p | miR-197-5p | miR-222-5p | miR-93-3p | miR-483-5p |
| miR-576-5p | miR-146a-3p | miR-548-3p | miR-34a-3p | miR-190a | miR-383 |
| miR-140-5p | miR-326 | miR-576-3p | miR-219-5p | miR-342-5p | miR-590-3p |
| miR-133a | miR-17-3p | miR-17-5p | miR-1 | miR-206 | miR-21-5p |
| miR-367-3p | miR-373-5p | miR-92a-3p | miR-16-5p | miR-197-3p | miR-24-3p |
| miR-339-5p | miR-224-5p | miR-127-3p | miR-365a-3p | miR-183-5p | miR-331-3p |
| miR-203a | miR-214-3p | miR-137 | miR-298 | miR-325 | miR-449a |
| miR-498 | miR-661 | miR-429 | miR-1182 | miR-208a-3p | miR-320b |
| miR-423-5p | miR-140-3p | miR-378c | miR-185-5p | miR-101-3p | miR-378b |
| miR-128 | miR-509-5p | miR-340-5p | miR-25-5p | miR-92b-3p | miR-320c |
| miR-508-3p | miR-744-5p | miR-3180-3p | miR-30d-3p | miR-215 | miR-4510 |
| miR-1260a | miR-151a-5p | miR-584-5p | miR-503-5p | miR-34c-5p | miR-3180 |
| miR-514a-3p | miR-374b-5p | miR-28-3p | miR-1307-5p | miR-21-3p | miR-193a-5p |
| miR-1269a | miR-365b-3p | miR-4286 | miR-4454 | miR-138-5p | miR-1307-3p |
| miR-652-3p | miR-502-3p | miR-92b-5p | miR-501-3p | miR-1285-3p | miR-126-3p |

[0120]

[Table 1-2]

| | | | | | |
|---|---|---|---|---|---|
| miR-4531 | miR-193b-5p | miR-181a-2-3p | miR-1301 | miR-877-5p | miR-4443 |
| miR-425-5p | miR-320d | let-7a-3p | miR-92a-1-5p | miR-365b-5p | miR-142-3p |

| | | | | | |
|---|---|---|---|---|---|
| miR-320e | miR-106b-3p | miR-548 | miR-1261 | miR-9-3p | miR-129-1-3p |
| miR-424-3p | miR-760 | miR-365a-5p | miR-374a-5p | miR-873-5p | miR-30c-2-3p |
| miR-99b-3p | miR-4532 | 20b-3p | miR-4448 | miR-363-5p | miR-145-3p |
| miR-574-5p | miR-223-3p | miR-4521 | miR-22-5p | miR-339-3p | miR-16-2-3p |
| miR-374a-3p | miR-542-3p | miR-452-5p | miR-513a-3p | miR-532-39 | miR-548a-3p |
| miR-27b-5p | miR-3180-5p | miR-4324 | let-7d-3p | miR-184 | miR-95 |
| miR-664a-5p | let-7a-5p | let-7g-5p | let-7i-5p | miR-191-5p | miR-125b-5p |
| miR-100-5p | miR-342-3p | miR-210 | miR-193b-3p | miR-99b-5p | miR-106b-5p |
| miR-27a-3p | miR-30a-3p | miR-423-3p | miR-409-3p | miR-186-5p | miR-324-3p |
| miR-22-3p | miR-378a-3p | miR-196b-5p | miR-296-5p | miR-99a-5p | miR-34a-5p |
| miR-501-5p | miR-500a-3p | miR-155-5p | miR-182-5p | miR-10a-5p | miR-345-5p |
| miR-335-5p | miR-362-5p | miR-193a-5p | miR-134 | miR-195-5p | miR-26a-5p |
| miR-151a-3p | miR-200c-3p | miR-212-3p | miR-143-3p | miR-149-5p | miR-28-5p |
| miR-505-3p | miR-192-5p | miR-187-3p | miR-129-5p | miR-106a-5p | miR-299-3p |
| miR-483-3p | miR-18a-3p | miR-330-3p | miR-200b-3p | miR-154-5p | miR-150-5p |
| miR-145-5p | miR-199a-5p | miR-135a-5p | miR-200a-3p | miR-133b | miR-141-3p |

| | | | | | |
|---|---|---|---|---|---|
| miR-148a-3p | miR-9-5p | miR-520c-3p | miR-328 | miR-361-5p | miR-375 |
| miR-378a-5p | miR-382-5p | miR-484 | miR-486-5p | miR-7-5p | miR-124-3p |
| miR-302a-5p | miR-370 | miR-373-3p | miR-492 | miR-509-3p | miR-512-5p |
| miR-516b-5p | miR-518b | miR-518c-3p | miR-518c-5p | miR-519d | miR-520f |
| miR-520g | miR-523-3p | miR-525-5p | miR-526a | miR-98-5p | |

[0121]

Enrichment of Immune-skewed imMKCL by Let-7 miRNA Switch

Among the active miRNAs, the present inventors focused on let-7a-5p and let-7g-5p, as they showed heterogeneous activity among imMKCLs (Fig. 2a). The present inventors sorted subpopulations with high or low let-7 activity, with the let-7 low subset representing less than 5% of the total population. The present inventors confirmed that the let-7 activity levels in these populations correlated with their expression levels in imMKCL subpopulations (Fig. 3a). To characterize molecular differences between the let-7 high and let-7 low subpopulations, The present inventors performed a bulk RNA-seq analysis using three different imMKCL clones (M35-1, clone 7, clone 7-3) in both the proliferation and maturation phases. The properties of clone 7 imMKCLs and the functionality of the derived iPSC-PLTs were reported previously (Non-Patent Literature 2, 3). Besides, clone 7 has been employed by multiple research groups for the investigation of megakaryopoiesis and thrombopoiesis (Lo, R.W. et al. Blood 136, 715-725 (2020); Ver Donck, F. et al. Blood (2023); Seo, H. et al. Blood Adv 2, 2262-2272 (2018)), highlighting its importance as a research tool. Clone 7-3, which is derived from clone 7 and displays an aged phenotype characterized by reduced proliferation and diminished iPSC-PLT production following repeated culture, was also selected (Fig. 3b). M35-1 was established from patient iPSCs for the iPLAT1 clinical trial due to its relatively superior expandability and iPSC-PLT productivity when compared with other competent patient-derived imMKCL clones (Non-Patent Literature 5). Comparable iPSC-PLTs production was observed in the three clones for the let-7 low or let-7 high subpopulations, respectively (Fig. 3c).

[0122]

The sampling procedure for the bulk RNA-seq is illustrated in Fig. 2b. A differential gene expression analysis revealed many genes with altered expression profiles between let-7 high and let-7 low imMKCLs. To identify enriched transcriptional pathways in each subpopulation, the

present inventors performed a gene set enrichment analysis (GSEA). Notably, the present inventors observed a series of immune-related gene sets that were significantly enriched in let-7 low imMKCLs in both the proliferation and maturation phases (Fig. 2c, d). Specifically, in the proliferation phase, gene sets in response to tumor necrosis factor (TNF), lipopolysaccharide (LPS), and enterotoxin were enriched in let-7 low cells, consistent with the reported properties of naïve mouse and human MKs with regard to sensing invading pathogens (Pariser, D.N. et al. J Clin Invest 131 (2021); Campbell, R.A. et al. Blood 133, 2013-2026 (2019)). TNF-driven inflammation has also been suggested to induce platelet hyperreactivity in aging (Davizon-Castillo, P. et al. Blood 134, 727-740 (2019)). In the maturation phase, gene sets related to interferon signaling were significantly enriched in let-7 low cells. Some immune related properties were retained from the proliferation phase to maturation phase (Fig. 2e-h, Table 2). These findings suggest that imMKCLs with lower let-7 activity preferentially enrich an immune-skewed MK phenotype. Our bulk RNA-seq analysis also identified the upregulation of several genes encoding chemokines and cytokines, including C-X-C motif chemokine ligand 10 (*CXCL10*), C-C motif chemokine ligand 2 (*CCL2*), and *CCL3*, in the immune-skewed let-7 low subpopulations of imMKCLs (Fig. 2f, h), indicating the consistency of multiple immune responses through the secretion of these molecules in human MKs (Cunin, P. & Nigrovic, P.A. J Leukoc Biol 105, 1111-1121 (2019)). Interestingly, elevated mRNA levels of these molecules have been reported in patients with severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS) (Channappanavar, R. & Perlman, S. Semin Immunopathol 39, 529-539 (2017)). Moreover, in COVID-19 patients, increased mRNA expression levels of *CXCL10*, *CCL2*, and *TNF* were correlated with an increased number of MKs (Ren, X. et al. Cell 184, 5838 (2021); Bernardes, J.P. et al. Immunity 53, 1296-1314.e1299 (2020)).

[0123]

[Table 2]

Significantly enriched immune-related gene sets in let-7 low imMKCLs at the proliferation or differentiation stages and in let-7 low ESC-derived $CD34^+$ cells

| GSEA Gene Sets | NES | *P* value | FDR |
|---|---|---|---|
| **imMKCLs (Dox-ON)** | | | |
| Immune response to enterotoxin | 2.471 | 0.000 | 0.000 |
| TNF signaling via NFKB | 2.295 | 0.000 | 0.000 |
| NFKB targets | 2.172 | 0.000 | 0.000 |
| IFNB1 targets | 2.304 | 0.000 | 1.54E-04 |
| Inflammatory response to LPS | 2.279 | 0.000 | 2.42E-04 |
| Interferon responsive genes | 2.215 | 0.000 | 0.003 |
| **imMKCLs (Dox-OFF)** | | | |
| Immune response to enterotoxin | 2.665 | 0.000 | 3.41E-04 |
| TNF signaling via NFKB | 2.578 | 0.000 | 0.000 |
| NFKB targets | 2.214 | 0.000 | 0.008 |
| IFNB1 targets | 2.843 | 0.000 | 0.000 |
| Inflammatory response to LPS | 2.041 | 0.000 | 0.002 |
| Interferon responsive genes | 2.931 | 0.000 | 0.000 |
| **ESC-derived CD34+ HPCs** | | | |
| Immune response to enterotoxin | 1.926 | 0.000 | 0.005 |
| TNF signaling via NFKB | 1.870 | 0.000 | 0.009 |
| NFKB targets | 1.719 | 0.003 | 0.032 |
| IFNB1 targets | 2.317 | 0.000 | 0.000 |
| Inflammatory response to LPS | 1.665 | 0.000 | 0.045 |
| Interferon responsive genes | 2.408 | 0.000 | 0.000 |

[0124]

Identification of Immune-type MK using a let-7 miRNA Switch

The present inventors further investigated whether immune MKs could be identified by let-7 miRNA switches in MKs directly differentiated from human ESCs. The present inventors utilized CD34+ hematopoietic progenitor cells (HPCs) generated by Sac. method (Takayama, N. et al. Blood 111, 5298-5306 (2008); Yuzuriha, A. et al. Stem Cell Res 53, 102287 (2021)) and performed a bulk RNA-seq analysis on two separate cell populations with different let-7a-5p and let-7g-5p activities (Fig. 4a). A GSEA revealed similar enriched immune-related gene sets in let-7 low ESC-derived HPCs (Fig. 4b, c, Table 2), suggesting that the fate decision of immune-skewed MKs in MK development may occur as early as the hematopoietic progenitor stage.

[0125]

ScRNA-seq Reveals the Heterogeneity and Functional Diversity of imMKCL

To further characterize the cellular heterogeneity of imMKCLs and explore the mechanism(s) underlying the enrichment of "immune" imMKCLs by let-7 miRNA switches, the present inventors performed a single cell (sc)RNA-seq analysis on let-7 low and high imMKCLs in the proliferation phase. Since both let-7a-5p and let-7g-5p miRNA switches demonstrated similar behaviors according to the bulk RNA-seq analysis (Fig. 2), the present inventors focused their investigation on let-7a-5p. The present inventors identified five transcriptionally heterogeneous subpopulations of imMKCLs (Fig. 5a), with let-7 low imMKCLs being relatively concentrated in clusters 3 and 5 (Fig. 5b). The present inventors further determined the expression of the top differentially expressed genes (DEGs) and characterized the gene ontology (GO) terms enriched in each cluster (Fig. 5c, d).

[0126]

Cluster 1 exhibited an enrichment of GO:BP (Biological Process) terms associated with oxidative phosphorylation, indicating their role in energy supply. Conversely, clusters 2 and 4 represented subsets of cycling MK progenitors with enriched GO:BP terms related to cell cycle and mitotic nuclear division. The identification of proliferating cell nuclear antigen (*PCNA*) and tropomyosin 4 (*TPM4*), two marker genes of cycling MKs, is consistent with recent studies on human bone marrow (BM) cycling MKs (Liu, C. et al. Adv Sci (Weinh) 8, e2100921 (2021)). Cluster 3 highly expressed genes associated with "platelet activation" and "blood coagulation", suggesting a subset of MK progenitors dedicated to platelet emergence. In particular, thrombopoiesis-associated genes, such as thrombospondin 1 (*THBS1*) (Non-Patent Literature 7), von Willebrand factor (*VWF*) (Ruggeri, Z.M. Von Willebrand factor, platelets and endothelial cell interactions. J ThrombHaemost 1, 1335-1342 (2003)), and platelet glycoprotein IX (*GP9*) (Romo, G.M. et al. J Exp Med 190, 803-814 (1999)), were highly expressed in cluster 3 compared to the other clusters (Fig. 5f). Moreover, cluster 3 was enriched with genes related to the immune system process, suggesting that it may represent a subset of MK progenitors with functional duality. For instance, platelet factor 4 (*PF4*), one of the top DEGs enriched in cluster 3, promotes blood coagulation (Fig. 5c) (Kowalska, M.A., Rauova, L. & Poncz, M. Thromb Res 125, 292-296 (2010)) and is a crucial regulator in innate immunity through its activity on macrophages (Scheuerer, B. et al. Blood 95, 1158-1166 (2000)) and neutrophils (Xiao, Z., Visentin, G.P., Dayananda, K.M. & Neelamegham, S. Blood 112, 1091-1100 (2008)). Additionally, pro-platelet basic protein (*PPBP*), an activator of neutrophils against bacteria (Laarman, A.J. et al. EMBO J 31, 3607-3619 (2012)), was also significantly upregulated in cluster 3.

[0127]

Both clusters 3 and 5 may represent subpopulations of MK progenitors with highly upregulated potential immune-related genes. The annotated immune genes in clusters 3 and 5 are

involved in immune system process and immune response according to the GO analysis (Fig. 5e). Cluster 5 highly expressed gene sets in response to type I interferon, cytokine, and virus, whereas cluster 3 appeared to be responsive to myeloid leukocyte mediated immunity. CCL5, a key proinflammatory chemokine (Marques, R.E., Guabiraba, R., Russo, R.C. & Teixeira, M.M. Expert Opin Ther Targets 17, 1439-1460 (2013)), was one of the top DEGs enriched in cluster 5 (Fig. 5f). Notably, *CCL5* has been reported to promote proplatelet formation in thrombopoiesis (Machlus, K.R. et al. Blood 127, 921-926 (2016)), and its expression was upregulated in cluster 3 compared to clusters 1, 2, or 4 (Fig. 5c, f). The interferon-stimulated gene for interferon-stimulated 15 (ISG15), a ubiquitin-like protein that can be covalently bound to host and viral proteins (Perng, Y.C. & Lenschow, D.J. Nat Rev Microbiol 16, 423-439 (2018)), was identified as the top DEG in cluster 5. Given that the secretion of type 1 interferons from virus-infected cells is a hallmark of antiviral immunity (Grandvaux, N., tenOever, B.R., Servant, M.J. & Hiscott, J. Curr Opin Infect Dis 15, 259-267 (2002)), our results indicated that cluster 5 represents the representative "immune" MKs involved in antiviral functions. Recent *in vivo* models of naïve MKs have demonstrated the presence of "immune" MKs, the hematopoietic stem cell (HSC) niche supporting MKs, and thrombopoiesis-biased MKs (Non-Patent Literatures 7, 8; Liu, C. et al. Adv Sci (Weinh) 8, e2100921 (2021)). Notably, our results suggest that imMKCLs have a thrombopoiesis-biased subset (cluster 3) and a representative immune subset (cluster 5). Although the present inventors did not observe a niche-supporting subset, imMKCLs should contain an immune-biased population similar to that found in endogenous MKs.

[0128]

Inhibition of let-7a-5p activity drives the development of "immune" imMKCL

The present inventors next investigated whether let-7 functionally drives the development of "immune" subsets in imMKCLs by conducting loss-of-function experiments (Fig. 6a). The present inventors inhibited let-7a-5p expression/activity using a let-7a-5p inhibitor (Fig. 6b, c) and found enhanced gene expressions of several immune-related molecules identified in clusters 3 and 5, including *PF4*, *PPBP, ISG15*, and interferon induced protein with tetratricopeptide repeats 3 *(IFIT3)* (Fig. 6e). The present inventors also investigated whether the inhibition of let-7a-5p activity in imMKCLs alters the response to immune stimuli. The cells were stimulated with the pathogen receptor agonist LPS or control buffer. The supernatants were analyzed using a cytometric bead array kit by flow cytometry. The present inventors found that the inhibition of let-7a-5p in imMKCLs promoted the secretion of interleukin-8 (IL-8) (Fig. 6d), a critical proinflammatory chemokine (David, J.M., Dominguez, C., Hamilton, D.H. & Palena, C. Vaccines (Basel) 4 (2016)). IL-8 and its receptors have been shown to control MK proliferation and maturation (Emadi, S. et al. Blood 105, 464-473 (2005)). These results are consistent with a previous report that found human cord blood-derived MKs produce IL-8 even in the absence of

stimuli (Higuchi, T. et al. Br J Haematol 99, 509-516 (1997)). Collectively, the present inventors concluded that let-7a-5p is a functional player that modulates the development of "immune MKs" among imMKCLs.

[0129]

RALB is a functional target of let-7a-5p in the development of immune-skewed imMKCL

The preceding experiments revealed the critical role of let-7a-5p in immune-skewed imMKCLs. To further determine the underlying factors that contribute to immune-related outcomes, the present inventors conducted an ingenuity pathway analysis (IPA) to investigate the upstream regulators of cluster 3 and cluster 5 (let-7 low imMKCLs). Fig. 7a shows a flowchart depicting the overall analysis design. IPA analysis identified the MK transcription factor *GATA1* (Orkin, S.H., Shivdasani, R.A., Fujiwara, Y. & McDevitt, M.A. Stem Cells 16 Suppl 2, 79-83 (1998)) and the myeloid development regulator *KLF2* as potential transcription activators in cluster 3 (Fig. 7b). Notably, KLF2 was shown to regulate host innate immune responses to polymicrobial infections (Mahabeleshwar, G.H. et al. Immunity 34, 715-728 (2011)). On the other hand, known activators of virus-inducible cellular genes, such as *IRF*7 (Ning, S., Pagano, J.S. & Barber, G.N. Genes Immun 12, 399-414 (2011)) and *IRF3* (Kubota, T. et al. J Biol Chem 283, 25660-25670 (2008)), were identified as potential upstream regulators in cluster 5 (Fig. 7b). The present inventors also compared common upstream regulators of clusters 3 and 5 with the predicted let-7a-5p targets (Fig. 7c). Our analysis identified twenty candidate upstream regulators, of which eight candidates showed detectable expression levels in imMKCLs (Fig. 7d). Based on their increased expression levels in the enriched let-7 low clusters (cluster 3 and 5), the present inventors suspected that cut-like homeobox 1 (*CUX1*) and RAS like proto-oncogene B (*RALB*) are targets of let-7a-5p (Fig. 7e) and may contribute to the observed immune-related outcomes. Accordingly, the inhibition of let-7a-5p upregulated the mRNA expression levels of *CUX1* and *RALB* in both the proliferation and maturation phases (Fig. 7f). While the overexpression of *CUX1* did not cause significant effects (Fig. 8), *RALB* overexpression upregulated the mRNA expression of *IRF7*, *ISG15*, and *IFIT3* (Fig. 7g, h, Fig. 9a, b), but not the expression level of let-7a-5p (Fig. 9c). On the other hand, despite being predicted as an upstream regulator of both clusters 3 and 5, *RALB* overexpression did not influence the expression of thrombopoiesis-related genes identified in Cluster 3 (Fig. 9d), indicating that distinct let-7 targets may be responsible for regulating immune pathways and thrombopoietic pathways in imMKCLs. Combining our findings with the IPA (Fig. 7b), the present inventors propose that let-7a-5p tunes the immune properties of imMKCLs by targeting *RALB* (Fig. 7i).

[0130]

The dysregulated immune properties/subsets are associated with the quality of imMKCL

The present inventors previously reported that certain imMKCL clones exhibiting slower proliferation rates at the proliferation stage are less capable of generating iPSC-PLTs (Fig. 10a) (Non-Patent Literature 9). However, the factor(s) responsible for inducing the low quality of imMKCLs, along with the cellular senescence/aging transcriptional signature (Fig. 11a, b), remain unclear. Therefore, the present inventors reexamined our GSEA of the different qualities of MKCLs utilized in our previous study (Non-Patent Literature 9). Similar to the GSEA of the let-7 low and high populations (Fig. 2), the present inventors found TNF signaling and interferon responsive gene sets were significantly enriched in intermediate-to-low (low-intermediate) quality MKCLs (Fig. 10b, Fig. 11c). The present inventors also found that clone 7 and clone 7-3, despite being derived from the same iPSC clone with the same genetic background (Non-Patent Literatures 2, 3), display different characteristics with regards to proliferation and iPSC-PLT production in both static and turbulent flow conditions (Fig. 10c-e) and let-7a-5p activity patterns (Fig. 12a). Clone 7-3 showed enriched TNF signaling and interferon responsive signaling compared to clone 7 (Fig. 10f, Fig. 12b). These results suggest that the induced immune-related pathways are associated with low-quality imMKCLs. The GSEA further demonstrated that downregulated senescent tumor protein 53 (TP53) targets are enriched in clone 7 (Fig. 12c), indicating the cellular senescence of clone 7-3. The mRNA expression of *RALB* and IL-8 secretion of clone 7-3 was higher compared with clone 7 (Fig. 10g, h). It is known that inflammatory cytokines are secreted by senescent cells with persistent DNA damage (Rodier, F. et al. Nat Cell Biol 11, 973-979 (2009)). In addition to IL-8 secretion (Fig. 10h), genes encoding inflammatory cytokines (*IFNB1*, *CXCL8*, *CXCL10*, *CXCL11*) also showed higher expression levels in clone 7-3 (aged clone) compared with clone 7 (younger clone) (Fig. 12d). Notably, the induction of recombinant IL-8 in imMKCLs resulted in diminished iPSC-PLT production (Fig. 13a-c). Blocking IL-8 signaling with Reparixin (Gorio, A. et al. J Pharmacol Exp Ther 322, 973-981 (2007)), a specific CXCR1/2 inhibitor, improved iPSC-PLT production (Fig. 13d-g) without affecting proliferation (data not shown). Furthermore, the administration of recombinant interferon-α2a led to lower proliferation rates (Fig. 14a) as well as impaired iPSC-PLT production in a dose-dependent manner (Fig. 14b, d). Interferon treatment elevated mRNA expression of the senescence marker *CDKN2A* as well as the interferon-responsive genes *ISG15* and *IFIT3* in imMKCLs (Fig. 14c). Taken together, these finding suggest that the dysregulation of immune properties/subpopulation within imMKCLs, along with the secretion of inflammatory cytokines, leads to arrested proliferation and deficient platelet generation of the whole imMKCL population, potentially due to the upregulation of RALB levels. Moreover, these results emphasize the effect of immune cytokines on *ex vivo* iPSC-PLT manufacturing, thus bearing implications for clinical applications.

[0131]

Intriguingly, our investigation of RALB revealed that the upregulation of *RALB* levels induced arrested proliferation, deficient iPSC-PLT production, and increased IL-8 secretion (Figs. 15a-d) as well as being accompanied by a cellular senescence/aging transcriptional signature (Fig. 16a). The present inventors assumed that this mechanism could recapitulate the characteristics of low-quality imMKCLs. A bulk RNA-seq analysis revealed enriched TNF signaling and interferon response in *RALB*-overexpressing imMKCLs (Fig. 15e and Fig. 16b), providing further support for our assumption. Additionally, RALB expression levels correlated with the expression levels of several interferon responsive genes in multiple imMKCL clones (Fig. 15f).

[0132]

The present inventors employed two additional approaches to validate the effects of RALB. The small molecule inhibitor RBC8 (Fig. 17a), which is described as a selective inhibitor against the GTPase Ral (Yan, C. et al. Nature 515, 443-447 (2014)), at 0.1 μM resulted in accelerated proliferation (Fig. 17b) and ameliorated iPSC-PLT production (Fig. 17c-e). siRNA-mediated *RALB* knockdown also enhanced iPSC-PLT production (Fig. 17f, g). Although no significant difference was observed in IL-8 secretion (Fig. 17h), RALB knockdown induced a decline in the mRNA expression levels of *IRF7*, *ISG15*, *IFIT3* (Fig. 17i). Finally, the iPSC-PLTs generated from low quality clones showed decreased PAC-1 binding and *P*-selectin expression (Fig. 18). These results collectively suggest that dysregulated immune properties/subsets within imMKCLs are associated with diminished imMKCL proliferation and iPSC-PLT generation, a phenotype potentially attributed to the upregulation of RALB expression.

[0133]

Lastly, the present inventors verified our findings in an *in vitro* differentiated MK model derived from cord blood $CD34^+$ cells (Fig. 19a). The inhibition of let-7 had no significant effect on interferon signaling or platelet production (Fig. 19b-d). However, the overexpression of *RALB* resulted in the upregulation of interferon-responsive genes and a reduction in platelet production (Fig. 19e-g). While our results suggest imMKCL and primary MKs respond differently to let-7 inhibition, they indicate a common effect of RALB, highlighting the pivotal role of RALB in determining immune properties/subsets in MK development.

[0134]

<u>LT-C imMKCL has reduced levels of KAT7 and H3K14ac</u>

Cell cycle arrest is also an indicator of cellular senescence (Kumari, R., Jat, P., 2021. Front. Cell Dev. Biol. 9, 645593; Mohamad Kamal, N.S. et al. 2020. Eur. J. Cell Biol. 99, 151108). And we previously reported low/intermediate clones showed senescence signature (Sone, M. et al. Stem Cell Reports 16, 2861-2870 (2021)). Recently, Wang et al conducted a genome-wide CRISPR-based screen in Werner syndrome mesenchymal precursor cells and identified KAT7 as

a novel driver of cellular senescence (Wang, W. et al, 2021. Sci. Transl. Med. 13, eabd2655). In that context, we studied whether KAT7 expression levels in ST-C and LT-C imMKCLs are associated with the proliferation rate and subsequent platelet productivity. Notably, western blot results revealed that both LT-C imMKCLs and imMKCLs derived from iPSCs of Werner syndrome patient (WS imMKCLs) exhibited lower expression levels of KAT7 and downstream H3K14ac proteins compared to ST-C imMKCLs (Paul, S.K., et al. 2024. Nat. Commun. 15, 4772) (Figs 21A, B). Furthermore, WS imMKCLs showed decreased proliferation as measured by the CCK8 assay and reduced platelet productivity compared to ST-C imMKCLs (Fig. 21C, D). Those results suggest that KAT7 is the key regulator of DOX-ON stage in imMKCLs, whereby aging-dependent KAT7 levels might influence the status of cell cycle populations.

[0135]

Pharmacological inhibition of KAT7 causes cell cycle arrest, impairing imMKCL proliferation and maturation

To investigate the role of KAT7 in imMKCL, H3K9 acetylation activity was inhibited using WM3835 and WM1119, which are KAT6/KAT7 inhibitors. Initial experiments revealed that 5 μM WM3835 significantly reduced H3K14ac levels compared to WM1119, suggesting that it is suitable as a selective KAT7 inhibitor in this Example (MacPherson, L., et al. 2020. 266-270) (Fig. 22A). WM3835 administration on any culture day (Days 1 to 5 of the DOX-OFF stage) did not influence PLT producibility, suggesting that KAT7 is independent of maturation phase in imMKCLs (Fig. 22B). By contrast, pre-treatment with WM3835 for 9 days, but not 3 or 6 days, at Dox-on, followed by Dox removal and platelet maturation for another 6 days, significantly reduced platelet yield (Figs. 22C, D). Fucci system again allowed to confirm that KAT7 inhibition increased G0 and decreased G1 and G2/M phases (Fig. 22E).

[0136]

To avoid off-target effects of WM3835, a shRNA-mediated KAT7 knockdown (shKAT7) experiment was also performed, and a significant decrease in KAT7 mRNA expression was confirmed by RT-qPCR (Fig. 23A). In shKAT7 imMKCL, both cell proliferation and platelet production capacity were significantly reduced (Fig. 23B). Furthermore, the proportion of cells in G0 phase increased twofold in shKAT7 imMKCL, suggesting that KAT7 knockdown resulted in cell cycle arrest at the G0 phase (Fig. 23C). These results demonstrated a phenotype similar to that observed with WM3835 treatment. On the other hand, overexpression of KAT7 did not change the proliferation rate, PLT production, or cell cycle state (Figs. 23A-E). These results indicate that KAT7 is important for maintaining the cell cycle of imMKCL in the Dox-ON phase, and its role in maintaining the proliferative capacity of imMKCL and its subsequent effect on platelet yield during maturation are highlighted.

[0137]

<Materials and Methods>

Cells

The human iPSC lines TkDN-Sev2 and T-1 were established in the laboratory of the present inventors. The imMKCLs (clone 7, clone 7-3, and M35-1) were generated from human iPSCs using doxycycline (DOX)-inducible defined factors and were used in previous studies (Non-Patent Literatures 2 and 3). The human ESC line KhES-3 was obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan). The WS imMKCL was provided by the Graduate School of Medicine, Chiba University (Innovation in Regenerative Medicine), where Koji Eto, one of the present inventors, holds a concurrent appointment with Kyoto University. The use of all cell lines was approved by the ethics committees of Kyoto University and Chiba University.

[0138]

Cell Culture

The imMKCLs (clone 7, clone 7-3, M35-1) were cultured as described before (Non-Patent Literature 3). Doxycycline was used to control the proliferation and differentiation stages.

[0139]

Cell Proliferation Assay

Cell proliferation was evaluated using a CCK-8 kit according to the manufacturer's instructions. imMKCLs, which had been cultured for 3 days in the Dox-ON phase, was seeded into a 96-well plate at a density of $5 \times 10^3$ cells/well. Absorbance at 450 nm was measured using a microplate reader (Envision 2104, PerkinElmer).

[0140]

MicroRNA Switch

miRNA-responsive mRNAs (miRNA switches) were generated using a MegaScript T7 kit (Ambion) as described previously (Miki, K. et al. Cell Stem Cell 16, 699-711 (2015)). The miRNA switches were encoded on modified mRNA to post-transcriptionally regulate TagBFP, a blue fluorescent protein, in response to the activity of an arbitrary miRNA (miR-X) expressed in imMKCLs. TagBFP-coding mRNA includes the target miR-X sequence in 5′ UTR such that the expression level of TagBFP is suppressed in response to increased miR-X activity. Azami Green 1 (hmAG1)-coding mRNA served as the transfection control (Fig. 1B).

[0141]

MiRNA switch-based screening

The miRNA switch-based screening was performed using an original constructed library (total of 269 miRNA switches). Two different mRNAs encoding TagBFP and AG1, respectively, were co-transfected into the cells. The transfection of miRNA switches was performed as previously described (Miki, K. et al. Cell Stem Cell 16, 699-711 (2015)). Briefly, in the

proliferation phase of imMKCLs, the reverse transfection of 150 ng mRNAs (75 ng each) was done with StemFect mRNA (Stemgent) in 50 μL of the cell suspension ($1\times10^6$ cells/mL) for 30 min, followed by dilution to 200 μL using culture medium in 96-well plates. After 24 hours, flow cytometry was performed using a LSRFortessa (BD Biosciences, San Jose, CA, USA).

Candidate miRNA switches were selected based on the active responsive pattern of imMKCLs to the target miR-X ("active" in Fig. 1c). MiRNAs that did not show strong responsive activities in imMKCLs were defined as "inactive" (Fig. 1c). For let-7a-5p and let-7g-5p, the present inventors individually transfected the candidate switches with control hmAG1-coding mRNA into imMKCLs. Following a 24-hour incubation, the cells were sorted using a BD FACSAria II and subjected to further investigation.

[0142]

Cell sorting and flow cytometry

Cells were suspended in staining medium, incubated for 30 min with appropriate antibodies on ice in the dark, and sorted or analyzed using a BD FACSAria II. Platelet count was determined as previously described (Non-Patent Literatures 2, 3). The following antibodies were used for the flow cytometry: allophycocyanin (APC)-conjugated anti-CD41a (integrin αIIbβ3 complex: HIP8 clone) (Biolegend, San Diego, CA), phycoerythrin (PE)-conjugated anti-CD42b (GPIba) (eBioscience, San Diego, CA), and PE-conjugated anti-CD41a (HIP8 clone) (Biolegend). Alternatively, imMKCL was sorted using a FACS S6 device equipped with a 100 μm nozzle, and operated using the DIVA software package (version 8.0.2).

[0143]

Antibody-based quantitative protein analysis (Simple WES)

The cell lysate was prepared using the EpiQuik Total Histone Extraction Kit (Epigentek). Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The lysate was adjusted to a final concentration of 0.5 or 1 μg/μL and analyzed using the Wes automated western blotting system (ProteinSimple). Antibodies used were mouse anti-β-actin (1:500, Sigma), rabbit anti-histone H3 (1:50, Cell Signaling Technology), rabbit anti-acetyl histone H3 (Lys14) (D4B9) (1:25, Cell Signaling Technology), and rabbit anti-MYST2 (D4N3F) (1:50, Cell Signaling Technology).

[0144]

Reverse transcription and real-time PCR

Total RNA was extracted using the microRNeasy Micro Kit or microRNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed using SuperScript VILO™ Master Mix (Thermo Fisher Scientific) or ReverTraAce (registered trademark) qPCR RT Master Mix with gDNA Remover (Toyobo). qPCR was carried out with SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using a StepOnePlus system (Thermo Fisher

Scientific). Alternatively, real-time PCR was performed using the QuantStudio3 system (Applied Biosystems) with THUNDERBIRD (registered trademark) Next SYBR™ qPCR Mix (Toyobo). GAPDH was used as an internal control. The primer sets used are shown in Table 3. The sequences in Table 3 are shown as SEQ ID NOs: 18 to 39, in order from top to bottom, in the Sequence Listing. Let-7a-5p and let-7g-5p miRNA reverse transcription was performed using a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and miRNA specific stem-loop RT primers according to the manufacturer's instructions. qPCR was carried out with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) using the StepOnePlus system. The let-7 expression levels were determined relative to RNU6B using specific TaqMan probes.

[0145]

[Table 3]

Primer pairs used in qRT-PCR

| Gene symbol | Primer | Sequence (5'→3') |
|---|---|---|
| ***ISG15*** | Forward | GAGAGGCAGCGAACTCATCT |
| | Reverse | CTTCAGCTCTGACACCGACA |
| ***KAT7*** | Forward | TCAGGATGCCCACTGTATCATAAC |
| | Reverse | ATCTGCTTATCCCGGCTCTGT |
| ***CCL5*** | Forward | ATCCTCATTGCTACTGCCCTC |
| | Reverse | GCCACTGGTGTAGAAATACTCC |
| ***IFIT3*** | Forward | GAAGGAACTGGGCCGCCTGCTAAG |
| | Reverse | GCCCTGGCCCATTTCCTCACTACC |
| ***PF4*** | Forward | GTGTGAAGACCACCTCCCAG |
| | Reverse | GCTTGCAGGTCCAAGCAAAT |
| ***PPBP*** | Forward | TTGTAGGCAGCAACTCACCC |
| | Reverse | TGCAAGGCATGAAGTGGTCT |
| ***RALB*** | Forward | GACTACGCAGCCATTCGAGA |
| | Reverse | CCGCTCCTCTAGGTCAGACT |
| ***CUX1*** | Forward | ACAGACACTGAAGAACCAAGCCGA |
| | Reverse | TGTGAGGCCAGCTGGAGGGA |
| ***IRF7*** | Forward | CTTCCAAGAAGAGCTGGTGGAAT |
| | Reverse | TTCCAGCTTCACCAGGAC |
| ***CDKN2A*** | Forward | GCCCAACGCACCGAATAGTT |
| | Reverse | AATCGGGGATGTCTGAGGGA |
| ***GAPDH*** | Forward | GTTGCCATCAATGACCCCTTC |
| | Reverse | CATTGATGACAAGCTTCCCG |

[0146]

Analysis of cytokine secretion by imMKCL

Cytokine secretion in culture supernatants was measured using the human inflammatory cytokine cytometric bead array kit (BD Biosciences) according to the manufacturer's guidelines. Briefly, bead populations with distinct fluorescence intensities coated with capture antibody proteins were mixed with PE-conjugated detection antibodies and recombinant standards or test samples, and then incubated to form sandwich complexes. After acquiring sample data by flow cytometry, the cytokine concentrations were calculated using FCAP Array™ software (BD Biosciences).

[0147]

Transfection of let-7a-5p inhibitor

An mirVana let-7a-5p-specific inhibitor and negative control were purchased from Thermo Fisher Scientific and used according to the manufacturer's instructions. The transfection procedure was performed using a Stemfect RNA Transfection Kit (ReproCell, Yokohama, Japan).

[0148]

Lentiviral production

The use of viral vectors was approved by committees at Kyoto University and Chiba University. The full-length coding sequences of human CUX1 and RALB were cloned into the lentiviral vector CS2-Ubic-IG-GFP or CS2-Ubic-IB. For overexpression experiments, the sequence encoding the full-length human KAT7 was cloned into the CS2-Ubic-IB lentiviral vector. For knockdown experiments, shRNA oligonucleotides targeting KAT7 or LacZ (control) were inserted into the FG12-HYG lentiviral vector plasmid. The Fucci vector was obtained from TAKARA Bio. Lentiviral production using 293T cells was performed as previously reported (Non-Patent Literature 1). The oligonucleotide sequence of shKAT7 is as follows: GCCCTTCCTGTTCTATGTTAT (SEQ ID NO: 40).

[0149]

Immunofluorescence confocal microscopy

Cells were fixed with 4% paraformaldehyde (Wako) for 10 minutes and permeabilized with 0.1% Triton X-100 (Sigma) for 5 minutes. Samples were blocked with 10% goat serum (Sigma) and incubated for 1 hour with mouse anti-CD41a antibody (eBioscience). The cells were then treated with a secondary antibody conjugated with Alexa Fluor 647 (Thermo Fisher Scientific) for 30 minutes. Nuclear staining was performed using DAPI (Vector). Images were acquired using a Zeiss LSM900 confocal microscope equipped with a 63×/1.40 numerical aperture oil immersion objective lens.

[0150]

Bulk RNA-sequencing analysis

Total RNA was extracted using the microRNeasy Micro Kit. RNA-seq libraries were prepared according to the manufacturer's protocol. Briefly, approximately 10 ng of total RNA was used for the cDNA synthesis using a SMART-Seq v4 Ultra Low Input RNA Kit for sequencing (Takara Bio). cDNA was fragmented using an S220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). The cDNA library was then generated using a NEBNext (registered trademark) Ultra™ DNA Library Prep Kit for Illumina (New England BioLabs, Beverly, MA, USA). Finally, the NEBnext library size was estimated using a bioanalyzer with an Agilent High Sensitivity DNA Kit. Sequencing was performed using a HiSeq2500 (Illumina) or a NextSeq 500 (Illumina) platform with a single-read sequencing length of 60 bp. TopHat (version 2.1.1) was used to map to the reference genome (UCSC/hg19) with annotation data from iGenomes (Illumina). Gene expression levels were quantified using Cuffdiff (Cufflinks version 2.2.1) and expressed as fragments per kilobase of exon per million mapped sequence reads (FPKM).

[0151]

Gene set enrichment analysis (GSEA)

A GSEA was applied to screen pathways enriched in (1) let-7 (let-7a-5p and let-7g-5p) low or high imMKCLs (M35-1, clone 7, clone 7-3), (2) let-7 low imMKCLs of clone 7-3 or of clone 7, and (3) MOCK or RALB-overexpressing imMKCLs (clone 7) based on bulk RNA-seq datasets. The plots were generated using GSEA software (version 4.2.3) from the Broad Institute. The present inventors also re-analyzed bulk RNA-seq datasets from high-, intermediate- and low-quality MKCL clones (Non-Patent Literature 9). Alternatively, a Python GSEApy package was used with the MsigDB Hallmarks library to identify pathways enriched in bulk RNA-seq data from imMKCLs treated with WM3835 and DMSO. The normalized gene expression matrix was input for the GSEA, and FDR and $P$ values $< 0.05$ were considered statistically significant.

[0152]

Chromium 10x single-cell RNA-seq library construction

imMKCLs (clone 7) with distinct let-7a-5p activity were sorted separately and resuspended at 1000 cells/μL in PBS with 0.4% BSA (Fig. 5). The cells were loaded onto a Chromium Next Gel Beads-in-EMulsion (GEMs) Chip G Single Cell Kit (10x Genomics, USA). GEM generation and barcoding, reverse transcription, and cDNA generation and library construction were performed following the manufacturer's protocol (Chromium Next GEM Single Cell 3' Reagent Kits v3.1 Dual Index, 10x Genomics). Dual-indexed, single-cell libraries were pooled and sequenced in paired-end reads on a Novaseq6000 (Illumina).

[0153]

Bioinformatics analysis

10x Genomics-derived datasets were collected, and quality control was performed to filter out low-quality and contaminated cells. Generally, reads were pre-processed with the Cell Ranger pipeline v.3.0.2 (10x Genomics). Downstream analysis and visualization were done using Seurat (version 4.0.5) implemented in R (version 4.1.1). After inspection of the quality control metrics, cells with > 15% of mitochondrial content or < 2500 detected genes were excluded from the downstream analysis. The present inventors normalized and scaled the unique molecular identifier (UMI) counts using regularized negative binomial regression. Afterward, the present inventors performed linear dimensionality reduction (principal component analysis) and used the top 20 principal components to perform the unsupervised Uniform Manifold Approximation and Projection (UMAP) and clustering, which were computed at a range of resolutions from 1.2 to 0.05. The let-7a-5p high and low imMKCL populations were combined for all subsequent analyses. Cell clusters were identified using the FindClusters function from Seurat. Five clusters for DOX-ON samples were identified (resolution 0.2). The FindAllMarkers function implemented in Seurat was used to identify DEGs between different clusters. The Wilcoxon test was performed on each gene, and the *P* values and adjusted *P* values for statistical significance were computed. Genes with adjusted *P* values less than 0.01 were considered significant. The g:Profiler was used for the identification of enriched functional terms from the GO.

[0154]

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Data are expressed as the mean ±standard error of the mean (SEM). Values of $P < 0.05$ were considered significant. Details of the sample size used, statistics, and statistical significance are indicated in each figure legend. The thresholds for statistical significance were set at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

[0155]

Differentiation of $CD34^+$ cell from human ESC

The human ESC line KhES-3 was obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan), and maintained in AK02N medium (Ajinomoto, Tokyo, Japan) on iMatrix-511 silk (Takara Bio, Shiga, Japan)-precoated 6-well plates. $CD34^+$ HPC differentiation was performed using our previously established Sac method (Takayama, N. et al. Blood 111, 5298-5306 (2008); Yuzuriha, A. et al. Stem Cell Res 53, 102287 (2021)). Briefly, small clumps of equivalent cell numbers (1.5 to $1.6\times10^5$ cells) were transferred onto a 10-cm dish coated with mitomycin C-treated C3H10T1/2 mouse feeder cells (Riken Bio-Resource Center, Tsukuba, Ibaraki, Japan) and cultured in basal medium (IMDM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with L-glutamine (Thermo Fisher Scientific, Waltham, MA,

USA), insulin-transferrin-selenium (Thermo Fisher Scientific), 50 μg/mL ascorbic acid (Sigma-Aldrich), 450 μM α-monothioglycerol (Sigma-Aldrich), and 20 ng/mL recombinant human VEGF (Wako, Osaka, Japan). Then, 50 ng/mL basic FGF (Wako) and 10 μM SB431542 (Wako) were added on days 4 to 7, and 10 U/mL Heparin (AY pharmaceuticals, Tokyo, Japan) on Days 4 to 10. Medium changes were performed on Days 4, 7, and 11 (Fig. 4a). On Day 14, the cells were collected with a cell scraper and filtered with a cell strainer for further investigation.

[0156]

Purification of human cord blood-derived $CD34^+$ cell

Fresh umbilical cord blood (UCB) samples were obtained from healthy donors sourced from the Japanese Red Cross Society Kanto-Koshinetsu Block Blood Center. Mononuclear cells (MNC) were isolated through density centrifugation using lymphocyte separation medium (Ficoll). The cells were subsequently incubated with the immunomagnetic beads (CD34) (Miltenyi Biotech) and selected using a QuadroMACS (registered trademark) separator according to manufacturer's instructions. The efficiency for the purification was verified by flow cytometry. The resulting $CD34^+$ fraction was aliquoted and stored in liquid nitrogen until use.

[0157]

Differentiation of megakaryocyte from cord blood-derived $CD34^+$ cell

The *in vitro* differentiation of cord blood-derived $CD34^+$ cells was performed in accordance with a previously published protocol with minor modifications (Kaushansky, K. et al. Proc Natl Acad Sci U S A 92, 3234-3238 (1995); Bruno, S. et al. Haematologica 88, 379-387 (2003)). Briefly, $CD34^+$ cells were expanded in X-VIVO 10 media (Lonza), supplemented with 1% bovine serum albumin (BSA, StemCell Technologies), 100 ng/mL human SCF (R&D Systems), 100 ng/mL human Flt-3 ligand (PeproTech EC Ltd.), 50 ng/mL human thrombopoietin (TPO, R&D Systems), 10 ng/mL human IL-6 (PeproTech EC Ltd.), and 10 ng/mL human IL-3 (PeproTech EC Ltd.). Following a 9-day culture period, the cells were harvested and counted. $4\times10^5$ cells were seeded and cultured for an additional 11 days in a total volume of 1 mL X-VIVO 10 media supplemented with 1% BSA, SCF, TPO, and IL-6. RNA was isolated on Day 16, and flow cytometry analysis was conducted on Day 20 to evaluate $CD41a^+CD42b^+$ platelet-like particles. The media was changed every two days across the differentiation period (Fig. 18a).

[0158]

siRNA-mediated gene knockdown

The Dharmacon™ siGENOME siRNA SMARTpool designed against human RALB (siRALB), which is comprised of four siRNAs targeting different sites, as well as a non-targeting control siRNA (siNT) were purchased from Horizon Discovery Ltd. The gene silencing procedure was performed based on the Dharmacon reverse transfection protocol with minor

modification. Briefly, siRNA at 10 nM final concentration was diluted in transfection buffer from the Stemfect RNA Transfection Kit (ReproCELL), and transfection reagent was diluted in an equal volume of buffer. The diluted siRNA and transfection reagent were combined and incubated at room temperature for 15 min. Subsequently, 12.5 μL/well of transfection mixture was added to the wells of a 96-well plate, followed by the addition of $2\times10^5$ cells in 100 μL medium to each well. After a 4-hour incubation period, the cells were washed with PBS and subjected to either proliferation or differentiation culture conditions. To validate the gene knockdown efficiency, total RNA was isolated and analyzed 48 hours after the transfection.

[0159]

Addition of recombinant human interferon-α2a or IL-8 in imMKCL culture

To examine the potential involvement of interferons or IL-8 in the proliferation and iPSC-PLT production of imMKCLs, cells were cultured under either proliferation or differentiation culture conditions with the addition of recombinant human interferon-α2a (Miltenyi Biotec) or IL-8 (R&D systems). Concentrations ranging from 0 to 100 ng/mL were utilized as working concentrations. The proliferation and iPSC-PLT production capacity of imMKCLs were analyzed accordingly.

[0160]

Addition of small molecules in imMKCL culture

The impact of two small molecules on the proliferation and iPSC-PLT production of imMKCLs was investigated: (1) Reparixin (Selleckchem), a specific inhibitor of CXCL8 receptor CXCR1/2 that is commonly used to inhibit the IL-8 signaling pathway; and (2) RBC8 (Selleckchem), a selective inhibitor of the GTPases RALA and RALB. imMKCLs were cultured under either proliferation or differentiation culture conditions in the absence or presence of Reparixin or RBC8 at working concentrations ranging from 0 to 10 μM. Under proliferation culture conditions, imMKCLs were treated with Reparixin or RBC8 for 14 days. Medium changes were performed every 3 or 4 days, imMKCLs were counted during the medium changes, and growth curves thereof were prepared. Under differentiation culture conditions, imMKCLs were treated with Reparixin or RBC8 for 6 days, and iPSC-PLT production thereof was evaluated. For these studies, dimethyl sulfoxide (DMSO) was added in control wells. Notably, the application of a high concentration (10 μM) of either molecule arrested the proliferation.

[0161]

Intracellular flow cytometry

Cells were fixed with 4% PFA for 15 minutes and permeabilized with methanol (15 minutes on ice). After PBS washing, cells were stained with anti-RalB antibody (Merck Millipore, 1:3000) on ice for 1 hour. Then, staining was performed using a secondary antibody Alexa

Fluor™ 647 (Invitrogen, 1:1000), and analyzed by flow cytometry. Cells were fixed in 4% paraformaldehyde (PFA) for 15 mins at room temperature, followed by methanol permeabilization (15 mins on ice). After two washes with PBS, the cells were incubated with anti-RalB antibody (Merck Millipore, 1:3000) for 1 hour on ice. Subsequently, the cells were washed and incubated with a goat anti-mouse secondary antibody, Alexa Fluor™ 647 (Invitrogen, 1:1000) on ice. The stained cells and non-staining controls were then analyzed by flow cytometry.

[0162]

imMKCL cells were fixed and stained using a PerFix-nc kit (Beckman Coulter) according to the manufacturer's instructions. Acetyl histone H3 (Lys14) (D4B9) rabbit antibody (Cell Signaling Technology) was used as the primary antibody, followed by Alexa Fluor 647-conjugated goat secondary antibody (Thermo Fisher Scientific). Samples were analyzed using a FACSLyric instrument (BD Biosciences).

[0163]

<u>PAC-1 binding and *P*-selectin expression of iPSC-PLTs</u>

The measurement of PAC-1 binding and *P*-selectin expression levels adhered to the protocol outlined in our previous study (Sugimoto, N. et al. Blood Adv 6, 6056-6069 (2022)). Briefly, the culture suspensions (iPSC-PLTs) were stimulated with or without phorbol-12-myristate-13-acetate (PMA, 0.2 μM) or adenosine triphosphate plus thrombin receptor activator peptide 6 (ADP/TRAP6, 100 μM and 40 μM, respectively). Subsequently, the mixtures were incubated with BV421 mouse anti-human CD62P (BD Biosciences), APC-anti-human CD41a (Bio Legend), and FITC-PAC-1 (BD Biosciences) antibodies. Following a 30-min incubation at room temperature, the samples were diluted with Hepes-Tyrode buffer and analyzed by flow cytometry.

[0164]

The present application claims priority based on U.S. Provisional Patent Application No. 63/558,394 filed in the U.S. (filing date: February 27, 2024), the disclosure of which is incorporated herein in its entirety by reference.

# CLAIMS

[Claim 1]

A quality control marker for megakaryocytes, consisting of a transcript of a Ral (RAS like proto-oncogene) gene or a Ral protein.

[Claim 2]

The marker according to claim 1, wherein the Ral gene is a RalB (RAS like proto-oncogene B) gene.

[Claim 3]

A quality control marker for megakaryocytes, consisting of a transcript of a Kat7 (lysine acetyltransferase 7) gene, a Kat7 protein, or an acetylated histone protein that is a target of the Kat7 protein.

[Claim 4]

A method for evaluating the quality of a megakaryocyte, comprising a step of detecting one or more of the biomarkers according to any one of claims 1 to 3 in the megakaryocyte.

[Claim 5]

The method according to claim 4, comprising a step of detecting a transcript of a RalB gene or a RalB protein, and a transcript of a Kat7 gene or a Kat7 protein in the megakaryocyte.

[Claim 6]

The method according to claim 4 or 5, comprising:

(1) a step of measuring an abundance or degree of activity of one or more of the biomarkers according to any one of claims 1 to 3 in a target megakaryocyte, and

(2) a step of evaluating the quality of the megakaryocyte based on the value measured in step (1).

[Claim 7]

The method according to claim 4 or 5, characterized by detecting the marker using a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Ral gene, or an antibody that specifically recognizes the Ral protein.

[Claim 8]

The method according to claim 4 or 5, characterized by detecting the marker using a nucleic acid probe and/or nucleic acid primer that specifically recognizes a transcript of the Kat7 gene, an antibody that specifically recognizes the Kat7 protein, or an antibody that specifically recognizes an acetylated histone protein that is a target of the Kat7 protein.

[Claim 9]

The method according to any one of claims 4 to 7, wherein the megakaryocyte is derived from a pluripotent stem cell.

[Claim 10]

A kit for quality control of megakaryocytes, comprising a nucleic acid probe and/or a nucleic acid primer that specifically recognizes a transcript of a Ral (RAS like proto-oncogene) gene, or an antibody that specifically recognizes a Ral protein.

[Claim 11]

A kit for quality control of megakaryocytes, comprising a nucleic acid probe and/or a nucleic acid primer that specifically recognizes a transcript of a Kat7 (lysine acetyltransferase 7) gene, an antibody that specifically recognizes a Kat7 protein, or an antibody that specifically recognizes an acetylated histone protein that is a target of the Kat7 protein.

[Claim 12]

A method for producing a megakaryocyte with improved quality, comprising a step of culturing a megakaryocyte in a medium containing a Ral (RAS like proto-oncogene) inhibitor.

[Claim 13]

The method according to claim 12, wherein at least one of the Ral inhibitors is RBC8 or an agent for suppressing expression of Ral.

[Claim 14]

A method for producing a megakaryocyte with improved quality, comprising a step of increasing the abundance of a Kat7 protein in a megakaryocyte.

[Claim 15]

The method according to any one of claims 12 to 14, wherein the megakaryocyte is derived from a pluripotent stem cell.

[Claim 16]

A megakaryocyte evaluated by the method according to any one of claims 4 to 9, or obtained by the method according to any one of claims 12 to 15.

[Claim 17]

A method for producing a platelet, comprising a step of maturing the megakaryocyte according to claim 16.

[Claim 18]

A platelet obtained by the method according to claim 17.

[Claim 19]

A blood product comprising the cell according to claim 16 or 18.