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(71) Demandeur/Applicant:
LOH, JEFFREY THOMAS, US

(72) Inventeur/Inventor:
LOH, JEFFREY THOMAS, US

(74) Agent: GOWLING WLG (CANADA) LLP

(54) Titre : PROCEDES POUR LA PRODUCTION IN VITRO DE PLAQUETTES ET COMPOSITIONS ET UTILISATIONS CORRESPONDANTES

(54) Title: METHODS FOR IN VITRO PRODUCTION OF PLATELETS AND COMPOSITIONS AND USES THEREOF

(57) Abrégé/Abstract:
The present invention encompasses methods for generating mutant j anus kinase 2 (JAK-2), mutant calreticulin (CALR), or thrombopoietin receptor (MPL) modified megakaryocytes (modified MKs) expressing a mutant Janus kinase 2 peptide, a mutant calreticulin peptide, and/or a mutant thrombopoietin receptor peptide, JAK2-, CALR-, and/or MPL-modified platelets as a composition of matter, and methods for using the generated JAK2-, CALR-, and/or MPL- modified platelets.

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(72) **Inventor; and**
(71) **Applicant** : **LOH, Jeffrey, Thomas** [US/US]; 1815 Kumakani Place, Honolulu, HI 96821 (US).

(74) **Agent**: **BRASHEARS, Sarah**; Convergent Law Group LLP, 475 N. Whisman Road, Suite 400, Mountain View, CA 94043 (US).

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(54) Title: METHODS FOR IN VITRO PRODUCTION OF PLATELETS AND COMPOSITIONS AND USES THEREOF

(57) Abstract: The present invention encompasses methods for generating mutant j anus kinase 2 (*JAK-2)*, mutant calreticulin (*CALR*), or thrombopoietin receptor *(MPL)* modified megakaryocytes (modified MKs) expressing a mutant Janus kinase 2 peptide, a mutant calreticulin peptide, and/or a mutant thrombopoietin receptor peptide, *JAK2*-, *CALR*-, and/or *MPL*-modified platelets as a composition of matter, and methods for using the generated *JAK2*-, *CALR*-, and/or *MPL*- modified platelets.

METHODS FOR IN VITRO PRODUCTION OF PLATELETS AND COMPOSITIONS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present utility claims priority to U.S. Ser. No. 62/139,931, filed March 30, 2015, which is herein incorporated by reference.

FIELD OF THE INVENTION

[0002] The field of the invention encompasses methods for generating mutant janus kinase 2 (*JAK-2*), mutant calreticulin (*CALR*), and/or mutant myeloproliferative leukemia virus (*MPL*) modified megakaryocytes (modified MKs) expressing a mutant Janus kinase 2 peptide, a mutant calreticulin peptide, and/or a mutant thrombopoietin receptor peptide; *JAK2-*, *CALR-*, and/or *MPL*-modified platelets as a composition of matter; and methods for using the generated *JAK2-*, *CALR-*, and/or *MPL*-modified platelets.

BACKGROUND OF THE INVENTION

[0003] In the following discussion certain articles and methods will be described for background and introductory purposes. Nothing contained herein is to be construed as an "admission" of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the articles and methods referenced herein do not constitute prior art under the applicable statutory provisions.

[0004] The extraordinary capabilities of stem cells to proliferate and differentiate into numerous cell types not only offers promises for changing how diseases are treated, but may also impact how transfusion medicine is practiced in the future. The possibility of growing platelets in the laboratory to supplement and/or replace standard platelet products has distinct advantages for blood banks and for patients. Due to the high utilization of platelets by patients undergoing chemotherapy or receiving stem cell transplants, platelet transfusion has steadily increased over the past decades. This trend is likely to continue as the number of adult and pediatric patients receiving stem cell transplants is also rising. As a result of increased

demand coupled with the short shelf-life of platelet concentrates, providing platelets to patients can stretch the resources of most blood centers, and on occasion platelet shortages can compromise the care of thrombocytopenic patients.

[0005] Platelets are formed from the cytoplasm of megakaryocytes (MKs), which reside in the bone marrow. MKs are the largest (50-100 μM) and also one of the rarest cells in the bone marrow, accounting for only ~0.01% of nucleated bone marrow cells. To assemble and release platelets, MKs become polyploid by endomitosis and then undergo a maturation process in which the bulk of their cytoplasm is packaged into multiple long processes called proplatelets, the nucleus is extruded, and platelets are then produced.

[0006] There is a need in the art for a universal, limitless source of platelets generated *in vitro*. The present invention provides methods and compositions that address this need.

SUMMARY OF THE INVENTION

[0007] This Summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This Summary is not intended to identify key or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter. Other features, details, utilities, and advantages of the claimed subject matter will be apparent from the following written Detailed Description, including those aspects illustrated in the accompanying drawings and defined in the appended claims.

[0008] In some embodiments, the present invention provides a method of producing mature Janus kinase 2 V617F (mutant JAK2), calreticulin-last exon (mutant CRT), and/or thrombopoeitin receptor S505N or W515L (mutant thrombopoietin receptor) modified cultured megakaryocytes (modified MKs) comprising: providing human pluripotent stem cells or human blood cells; transforming the human pluripotent stem cells or human blood cells with an expression vector expressing mutant JAK2, mutant CRT and/or mutant thrombopoietin receptor or, alternatively, creating a mutation in an endogenous *JAK2, CALR,* and/or *MPL* locus in the human pluripotent stem cells or human blood

cells to produce modified cells; culturing the modified cells to produce modified MKs; inducing platelet formation in the modified MKs; and isolating the platelets.

[0009] In some aspects of this embodiment, the human pluripotent stem cells or human blood cells are human embryonic stem cells; human embryonal carcinoma cells; human embryonic germ cells; human multipotent germline cells; human mesodermal stem cells; human mesenchymal stem cells; human induced pluripotent stem cells; human colony forming units-granulocytes, erythrocytes, monocytes and megakaryocytes (CPU-GEMMs); burst forming units-megakaryocytes (BFU-MKs); colony forming units-megakaryocytes (CFU-MKs); promegakaryoblasts, megakaryoblasts, promegakaryocytes; or megakaryocytes. In preferred aspects, the human pluripotent stem cells or human blood cells are human embryonic stem cells, human induced pluripotent stem cells, CFU-GEMMs, BFU-MKs, CFU-MKs, promegakaryoblasts or megakaryoblasts. In more preferred aspects of this embodiment, the human pluripotent stem cells or human blood cells are human embryonic stem cells, human induced pluripotent stem cells, CFU-GEMMs, BFU-MKs, CFU-MKs, promegakaryoblasts or megakaryoblasts are ABO type A, B or O and RhD negative.

[00010] In some aspects, the human pluripotent stem cells or human blood cells are transformed with a mutant JAK2, mutant CRT and/or mutant thrombopoietin receptor expression vector; in other aspects, the human pluripotent stem cells or human blood cells comprise a human synthetic chromosome expressing mutant JAK2, mutant CRT and/or mutant thrombopoietin; and yet other aspects, the endogenous *JAK2, CALR,* and/or *MPL* locus of the human pluripotent stem cells or human blood cells is replaced with mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* via homologous recombination. In preferred aspects, the mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* is under control of an inducible promoter. In yet other aspects, human pluripotent stem cells or human blood cells are taken from an individual with, e.g., thrombocythemia, such that the human pluripotent stem cells or human blood cells naturally comprise an endogenous mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* locus.

[00011] In some aspects, the modified cells are cultured in the presence of thrombopoietin (TBO) or TBO and one or more of interleukin-3 (IL-3), Flt-3 Ligand (FL), interleukin-34 (IL-34), stem cell factor (SCF), interleukin-6 (IL-6), interleukin-

9 (IL-9), and interleukin-11 (IL-11). Alternatively, the modified cells are cultured in the presence of p45NF-E2, Maf G and Maf K.

[00012] In some aspects of the method embodiment described above, platelet formation is induced by culturing the modified MKs in the presence of IL-6 and IL-11, in the presence of physical shear forces.

[00013] In other embodiments of the present invention, the *in vitro* generated platelets are produced by the methods described herein, and other embodiments provide a method of treating a human patient comprising transfusing the patient with the *in vitro* generated platelets produced by the methods of the present invention. In preferred embodiments, the *in vitro* generated platelets produced by the methods of the present invention are ABO type A, B or O and RhD negative.

[00014] Yet other embodiments of the present invention provide a method of producing immortalized modified mutant Janus kinase 2 V617F (mutant JAK2), calreticulin-last exon (mutant CRT), and/or thrombopoeitin receptor S505N or W515L (mutant thrombopoietin) modified cultured megakaryocytes (MKs) comprising: providing human embryonic stem cells, human embryonal carcinoma cells, human embryonic germ cells, human multipotent germline cells, human mesodermal stem cells, human mesenchymal stem cells, human induced pluripotent stem cells, human colony forming units-granulocytes, erythrocytes, monocytes and megakaryocytes (CPU-GEMMs), burst forming units-megakaryocytes (BFU-MKs); colony forming units-megakaryocytes (CFU-MKs), promegakaryoblasts, megakaryoblasts, or promegakaryocytes with an expression vector expressing mutant JAK2, mutant calreticulin or mutant thrombopoietin receptor, or, alternatively, creating the Janus kinase 2 V617F mutation, calreticulin-last exon mutation (mutant CRT), and/or thrombopoeitin receptor mutation S505N or W515L (mutant thrombopoietin receptor) in an endogenous *JAK2, CALR,* and/or *MPL* locus in the cells to produce modified cells; and culturing the modified cells in nondifferentiating blood stem/blood progenitor cell culture medium. In some aspects of this embodiment, the Janus kinase 2 V617F (mutant JAK2), calreticulin-last exon (mutant CRT), and/or thrombopoeitin receptor S505N or W515L (mutant thrombopoietin receptor) modified platelets are produced from an immortalized mutant JAK2, mutant CRT or mutant thrombopoietin receptor megakaryocyte (MK)

line. In some aspects, the immortalized MKs are maintained in culture. In other aspects, the *in vitro* generated platelets are then generated from the modified MKs.

[00015] These and other aspects and uses of the invention will be described in the detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[00016] Figure 1 is a simplified flow chart of method steps for creating mutant JAK2-modified, mutant CLR-modified, and/or mutant thrombopoietin receptor-modified cultured MKs, which are then used to generate platelets *in vitro*.

DETAILED DESCRIPTION OF THE INVENTION

[00017] The methods described herein may employ, unless otherwise indicated, conventional techniques and descriptions of molecular biology (including recombinant techniques), cell biology, biochemistry, and cellular engineering technology, all of which are within the skill of those who practice in the art. Such conventional techniques include oligonucleotide synthesis, hybridization and ligation of oligonucleotides, transformation and transduction of cells, engineering of recombination systems, differentiation of cells and maintenance in cell culture, and human transfusion therapy. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (Green, et al., eds., 1999); *Genetic Variation: A Laboratory Manual* (Weiner, et al., eds., 2007); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); *Protein Methods* (Bollag et al., John Wiley & Sons 1996); *Nonviral Vectors for Gene Therapy* (Wagner et al. eds., Academic Press 1999); *Viral Vectors* (Kaplift & Loewy, eds., Academic Press 1995); *Immunology Methods Manual* (Lefkovits ed., Academic Press 1997); *Gene Therapy Techniques, Applications and Regulations From Laboratory to Clinic* (Meager, ed., John Wiley & Sons 1999); M. Giacca, *Gene Therapy* (Springer 2010); *Gene Therapy Protocols* (LeDoux, ed., Springer 2008); *Cell and Tissue Culture: Laboratory Procedures in Biotechnology* (Doyle & Griffiths, eds., John Wiley & Sons 1998); *Essential Stem*

Cell Methods, (Lanza and Klimanskaya, eds., Academic Press 2011); *Stem Cell Therapies: Opportunities for Ensuring the Quality and Safety of Clinical Offerings: Summary of a Joint Workshop* (Board on Health Sciences Policy, National Academies Press 2014); *Essentials of Stem Cell Biology*, Third Ed., (Lanza and Atala, eds., Academic Press 2013); and *Handbook of Stem Cells*, (Atala and Lanza, eds., Academic Press 2012), all of which are herein incorporated by reference in their entirety for all purposes. Before the present compositions, research tools and methods are described, it is to be understood that this invention is not limited to the specific methods, compositions, targets and uses described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

[00018] Note that as used in the present specification and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a composition" refers to one or mixtures of compositions, and reference to "an assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[00019] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned are incorporated herein by reference for the purpose of describing and disclosing devices, formulations and methodologies which might be used in connection with the present invention.

[00020] Where a range of values is provided, it is understood that each intervening value between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, subject to any specifically excluded limit in the stated range. Where the stated range includes both of the limits, ranges excluding only one of those included limits are also included in the invention.

[00021] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of ordinary skill in the art upon reading the specification that the present invention may be practiced without one or more of these specific details. In

other instances, features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention.

Definitions

[00022] Unless expressly stated, the terms used herein are intended to have the plain and ordinary meaning as understood by those of ordinary skill in the art. The following definitions are intended to aid the reader in understanding the present invention, but are not intended to vary or otherwise limit the meaning of such terms unless specifically indicated.

[00023] As used herein "blood stem cells" means stem cells having no differentiation potential to cells other than blood cells but having a differentiation potential to various types of blood cells. "Blood stem cells" are also called "hematopoietic stem cells." Blood stem cells are known to be abundantly included in cell populations separated and collected from certain tissues, such as umbilical cord blood, peripheral blood, bone marrow, or fetal liver by, e.g., flow cytometry or the like using an antibody that binds specifically to a cell surface antigen such as, e.g., CD34 on hematopoietic stem cells. The blood stem cells of the present invention can be prepared by inducing differentiation of human pluripotent stem cells. "Human pluripotent stem cells" as used herein may be any human cells that renew and can be induced to differentiate into blood stem cells. Examples of human pluripotent stem cells include human embryonic stem cells (ES cells); human embryonal carcinoma cells (EC cells); human embryonic germ cells (EG cells); human multipotent germline stem cells (mGS cells); human mesodermal stem cells; human mesenchymal stem cells. Blood stem cells that can be induced to produce megakaryocytes include human colony forming units-granulocytes, erythrocytes, monocytes and megakaryocytes (CPU-GEMMs); burst forming units-megakaryocytes (BFU-MKs); colony forming units-megakaryocytes (CFU-MKs); promegakaryoblasts, megakaryoblasts, and promegakaryocytes (collectively, "blood stem cells"). In addition, an example of human pluripotent stem cells includes cells artificially prepared in such a manner as to have differentiation pluripotency, such as induced pluripotent stem cells (iPSCs). A "megakaryocyte" ("MK) is a cell having a differentiation potential to produce platelets and no other cell. An "MK cell line"

means immortalized MKs, which can be maintained in culture through many passings.

[00024] A "blood stem/progenitor cell differentiation induction culture protocol" or "blood stem/progenitor cell differentiation induction culture medium" refers to protocols or cell culture media that are useful for inducing differentiation of human pluripotent stem cells to human blood stem cells and further to megakaryocytes.

[00025] A "coding sequence" or a sequence that "encodes" a peptide is a nucleic acid molecule that is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate control sequences. The boundaries of the coding sequence typically are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus.

[00026] The term DNA "control sequences" refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites, enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these types of control sequences need to be present so long as a selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

[00027] The term "*in vitro*" refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, etc., rather than within an organism.

[00028] The terms "heterologous DNA" or "foreign DNA" (or "heterologous RNA" or "foreign RNA") are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome in which it is present, or is found in a location or locations and/or in amounts in a genome or cell that differ from that in which it occurs in nature. Examples of heterologous DNA include, but are not limited to, DNA that encodes a gene product or gene product(s) of interest. Other examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins as well as regulatory DNA sequences.

[00029] "Operably linked" refers to an arrangement of elements where the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the

expression of the coding sequence. The control sequences need not be contiguous with the coding sequence so long as they function to direct the expression of the coding sequence. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence. In fact, such sequences need not reside on the same contiguous DNA molecule (i.e. chromosome), and may still have interactions resulting in altered regulation.

[00030] A “promoter” or “promoter sequence” is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a polynucleotide or polypeptide coding sequence such as messenger RNA, ribosomal RNAs, small nuclear or nucleolar RNAs or any kind of RNA transcribed by any class of any RNA polymerase I, II or III.

[00031] As used herein the term “selectable marker” refers to a gene introduced into a cell, particularly in the context of this invention into cells in culture that confers a trait suitable for artificial selection. General use selectable markers are well-known to those of ordinary skill in the art. In preferred embodiments, selectable markers for use to modify and/or propagate modified MKs should be non-immunogenic in the human and include, but are not limited to: human nerve growth factor receptor (detected with a monoclonal antibody (MAb), such as described in U.S. Pat. No. 6,365,373); truncated human growth factor receptor (detected with a MAb); mutant human dihydrofolate reductase (DHFR; fluorescent MTX substrate available); secreted alkaline phosphatase (SEAP; fluorescent substrate available); human thymidylate synthase (TS; confers resistance to anti-cancer agent fluorodeoxyuridine); human glutathione S-transferase alpha (GSTA1; conjugates glutathione to the stem cell selective alkylator busulfan; chemoprotective selectable marker in $CD34^+$ cells); CD24 cell surface antigen in hematopoietic stem cells; human CAD gene to confer resistance to N-phosphonacetyl-L-aspartate (PALA); human multi-drug resistance-1 (MDR-1; P-glycoprotein surface protein selectable by increased drug resistance or enriched by FACS); human CD25 (IL-2α; detectable by MAb-FITC); Methylguanine-DNA methyltransferase (MGMT; selectable by carmustine); and Cytidine deaminase (CD; selectable by Ara-C). Drug selectable markers such as puromycin, hygromycin, blasticidin, G418, tetracycline may also be

employed. In addition, using FACs sorting, any fluorescent marker gene may be used for positive selection, as may chemiluminescent markers (e.g. Halotags), and the like.

[00032] The terms "subject", "individual" or "patient" may be used interchangeably herein and refer to a mammal, and in preferred embodiments, a human.

[00033] As used herein, the terms "treat," "treatment," "treating," and "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down and/or stop the progression or severity of a condition associated with a disease or disorder. The terms include reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a deficiency in the number or defect in the quality of at least one blood cell type, such as platelets. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of or at least slowing of progress or worsening of symptoms that would be expected in absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. The terms "treat," "treatment," "treating," and "amelioration" in reference to a disease also include providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00034] A "vector" is a replicon, such as plasmid, phage, viral construct, cosmid, bacterial artificial chromosome, derived artificial chromosome or yeast artificial chromosome to which another heterologous DNA segment may be inserted. In some instances a vector may be a chromosome such as in the case of an arm exchange from one endogenous chromosome engineered to comprise a recombination site to a synthetic chromosome. Vectors are used to transduce and express a DNA segment, such as a mutated *JAK2* gene, mutated *CALR* gene, and/or mutant *MPL* gene in a cell.

The Invention

[00035] The *in vitro* production of platelets has recently emerged as a potential long-term alternative to the current donor-based platelet procurement system. The current donor-based system is expensive to maintain, is vulnerable to major disruption, and does not adequately serve the needs of chronically-transfused patients who often require platelets expressing rare blood groups. Production of platelets *in vitro* from stem cells fulfills the promise of changing the paradigm for transfusion medicine and overcoming dependence on the existing supply system. The use of terminally differentiated cells that no longer have the capability of proliferating allows clinical applications of human pluripotent and blood stem cells without the associated risk of tumorigenicity, as platelets lack nuclei following terminal differentiation and are highly unlikely to exhibit tumorigenicity *in vivo*. Thus, even if the original stem cells or their derivatives possessed abnormal karyotypes or genetic mutations, these cells can still be useful for clinical applications provided that such precursors can produce platelets. Another advantage is that *ex vivo*-generated platelets—like donor-based platlets—optimially should be compatible with recipient ABO and RhD antigens. Establishment of immortalized megakaryocyte lines with the genes to produce the A, B, O and/or RhD antigens would produce platelets optimized for individuals in all the most common blood groups. Moreover, it is possible to further engineer immortalized human megakaryocyte lines to negate or disable other cell surface antigens to avoid immune reactions in chronically-transfused patients.

[00036] Essential thrombocythemia is an uncommon dislorder in which an individual produces too many platelets. The condition may cause fatigue, lightheadedness, headaches and vision changes. It also increases the risk of blood clots/thrombosis. However, the megakaryocytes from these individuals may be a good source of platelets if the megakaryocytes are converted and cultured in an immortalized cell line. Alternatively, the gene mutations that lead to essential thrombocythemia can be recreated in blood stem cells—that is, megakaryocyte precursors—to create megakaryocytes that overrpoduce platelets *in vitro*. The present invention encompasses compositions and methods for producing Janus kinase 2-modified, calreticulin-modified, and/or thrombopoietin receptor-modified platelets (modified platelets).

[00037] Janus kinase 2 (JAK2) is a non-receptor tyrosine kinase that has been implicated in signaling by members of the type II cytokine receptor family (e.g., interferon receptors), the GM-CSF receptor family, the gp 130 receptor family, and single chain receptors. JAK2 signaling appears to be activated downstream from the prolactin receptor. The distinguishing feature between Janus kinase 2 and other JAK kinases is the lack of Src homology binding domains and the presence of up to seven JAK homology domains. Mutations in the Janus kinase 2 gene (herein "*JAK2*", corresponding to Entrez Gene ID:3717 and Uniprot O60674) have been implicated in essential thrombocythemia, polycythemia vera (a disorder in which the bone marrow makes too many red blood cells), myelofibrosis as well as other myeloproliferative disorders. The specific mutation, a change of valine to phenylalanine at the 617 position (V617F, herein "mutated JAK2" or "mutant JAK2" for the peptide or "mutated *JAK2*" or "mutant *JAK2*" for the gene that codes for the peptide with the change of valine to phenylalanine at the 617 position or codes for a conservative substitution therefor) appears to render hematopoietic cells more sensitive to growth factors such as erythropoietin and thrombopoietin.

[00038] Calreticulin is also known as calregulin, CRP55, CsBP3, calsequestrin-like protein, and endoplasmic reticulum resident protein 60, and is encoded by the *CALR* gene. Calreticulin is a multifuncational protein that binds Ca^{+2} ions, rendering it inactive. Calreticulin is located in storage compartments associated with the endoplasmic reticulum; however, calreticulin is also found in the nucleus, suggesting that it may have a role in transcription regulation. Calreticulin mutations have been found to be present in patients with essential thrombocythemia and primary myelofibrosis. All mutations to *CALR* associated with thrombocytosis affect the last exon, generating a reading frame shift of the resulting calreticulin protein ("mutant calreticulin" for the protein and "mutant *CALR"* for the gene), creating a novel terminal peptide.

[00039] The thrombopoietin receptor, also known as the myeloproliferative leukemia protein or CD110 protein, is a protein that in humans is encoded by *MPL*, the myeloproliferative leukemia virus oncogene, and promotes the growth and division of cells. This receptor is particularly important for the proliferation of MKs. Research suggests that the thrombopoietin receptor may also play a role in the maintenance of hematopoietic stem cells. The thrombopoietin receptor when

activated by thrombopoietin stimulates the JAK/STAT pathway. Two particular mutations in *MPL* are associated with essential thrombocythemia. An inherited condition, familial essential thrombocythemia, is caused by a mutation in *MPL* that results in the replacement of the amino acid serine with the amino acid asparagine at position 505 (thrombopoietin receptor Ser505Asn or S505N). Essential thrombocythemia that does not run in families (sporadic essential thrombocythemia) has been associated with a mutation in *MPL* that results in the replacement of the amino acid tryptophan at position 515 with another amino acid, often leucine (thrombopoietin receptor Trp515Leu or W515L). Amino acid changes at position 505 or 515 result in a thrombopoietin receptor protein that is consituitively activated, which leads to the overproduction of abnormal megakaryocytes and an increased number of platelets.

[00040] Figure 1 is a simplified flow chart of a method 100 for creating mutant *JAK2*-modified, mutant *CALR*-modified, and/or mutant *MPL*-modified *in vitro* generated platelets. First, human pluripotent stem cells or human blood stem cells are provided in step 101. In step 103, the human pluripotent stem cells or blood stem cells are transformed with mutated *JAK2* or a mutation is created in endogenous *JAK2* to create modified human pluripotent stem cells or blood stem cells. Alternatively or in addition, the human pluripotent stem cells or blood stem cells are transformed with mutated *CALR* or mutant *MPL* or a mutation is created in endogenous *CALR* and/or *MPL* to create modified human pluripotent stem cells or blood stem cells. In step 105, the modified human pluripotent stem cells or blood stem cells are maintained in culture in an undifferentiated state and later differentiated into megakaryocytes (MKs), or are differentiated into MKs and then maintained in culture. Note that it is possible to reverse steps 103 and 105; that is, the human pluripotent stem cells or blood stem cells may be differentiated into MKs before a mutation in *JAK2, CALR,* and/or *MPL* is created in the cells. Alternatively, it is possible to culture human pluripotent stem cells, blood stem cells or MKs from an individual who has a mutation in *JAK2, CALR,* and/or *MPL* naturally as a basis for the *JAK2*-modified, *CALR*-modified, and/or *MPL*-modified MKs; that is, blood stem cells that naturally comprise an endogenous *JAK2-, CALR-,* and/or *MPL*-mutation—such as blood stem cells taken from an individual with essential thrombocythemia—may be used in lieu of engineered cells. Either way, once *JAK2-, CALR-,* and/or

MPL-modified MKs are obtained, the MKs can be maintained in culture indefinitely, or platelet production from the MKs is induced (step 107), thus producing platelets at step 109. The platelets can then be used for patient transfusion, or in other uses in step 111. The details of each step outlined in the simplified flow chart are described below.

Cells

[00041] Human pluripotent stem cells, human blood stem cells, CFU-GEMMs, BFU-MKs, CFU-MKs, promegakaryoblasts, megakaryoblases, promegakaryocytes, or megakaryocytes can be used in the present invention, depending on the availability of each type of cell, and protocols that have been developed to differentiate stem cells and immortalize cell lines. Platelets have a limited life span and are the progeny of immortal self-renewing hematopoietic stems cells. Human platelets have a limited life span 10 days *in vivo* and when stored *ex vivo* progressively lose their function due to biochemical and morphological changes caused changes in their environment. Differentiation of hematopoietic stem cells into MKs involves the generation of a series of progenitors with increasingly restricted differentiation potential. That is, hemopoietic stem cells differentiate sequentially into CFU-GEMMs, BFU-MKs, CFU-MKs, promegakaryoblasts, megakaryoblases, promegakaryocytes, and megakaryocytes, which then become polyploid by endomitosis and undergo a maturation process forming proplatelets, then platelets.

[00042] One source of human stem and progenitor cells is circulating stem and progenitor cells. Laboratory-scale methods to produce MKs from circulating stem and progenitor cells have been developed. A preferred source of cells for platelet production is pluripotent stem cells such as human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). The main advantage of these cells is that they are immortal, karyotypically stable and can be reproducibly generated from any individual using a variety of well-developed methods (see, e.g., Okita, et al., Philosophical Transaction of the Royal Society of London Biological Sciences, 366:2198-207 (2011)).

[00043] Alternatively, it is possible to culture human pluripotent stem cells, blood stem cells or MKs from an individual who has a mutation in *JAK2, CALR,* and/or *MPL* naturally as a basis for the *JAK2*-, *CALR*-, and/or *MPL* modified MKs; that is,

blood stem cells that naturally comprise an endogenous *JAK2*, *CALR*-, and/or *MPL* mutation—such as blood stem cells taken from an individual with essential thrombocythemia be used in lieu of engineered cells.

Mutant JAK2 Expression in Hematopoietic Stem Cells

[00044] Methods to introduce mutated *JAK2*, mutated *CALR*, and/or mutated *MPL* or to replace endogenous *JAK2*, *CALR*, and/or *MPL* with the mutated versions are generally known to those in the art. For example, a viral or non-viral vector engineered to express mutated *JAK2* can be introduced into the MKs, blood stem cells or human pluripotent stem cells of choice. Alternatively, a blood stem cell line, human pluripotent stem cell line or MK line can be engineered to produce a human synthetic chromosome that is engineered to express mutated *JAK2*, mutated *CALR*, and/or mutated *MPL*. In yet another alternative, endogenous *JAK2*, *CALR*, and/or *MPL* in a MK line, blood stem cell, or human pluripotent stem cell can be replaced via homologous recombination systems with mutated *JAK2*, mutated *CALR*, and/or mutated *MPL*.

[00045] In the first alternative, the choice of vector to be used in delivery of mutated *JAK2*, mutated *CALR*, and/or mutated *MPL* to the cell of choice will depend upon a variety of factors such as the type of cell in which propagation is desired. Certain vectors are useful for amplifying and making large amounts of a desired DNA sequence such as in this case, mutated *JAK2*, mutated *CALR*, and/or mutated *MPL*, while other vectors are suitable for expression in cells in culture. The choice of an appropriate vector is well within the skill of those in the art, and many vectors are available commercially. To prepare the constructs, a mutated *JAK2*, mutated *CALR*, and/or mutated *MPL* polynucleotide is inserted into a vector, typically by means of ligation into a cleaved restriction enzyme site in the vector.

[00046] Exemplary vectors that may be used include but are not limited to those derived from recombinant bacteriophage DNA, plasmid DNA or cosmid DNA. For example, plasmid vectors such as pBR322, pUC 19/18, pUC 118, 119 and the M13 mp series of vectors may be used. Bacteriophage vectors may include λgt10, λgt11, λgt18-23, λZAP/R and the EMBL series of bacteriophage vectors. Cosmid vectors that may be utilized include, but are not limited to, pJB8, pCV 103, pCV 107, pCV 108, pTM, pMCS, pNNL, pHSG274, COS202, COS203, pWE15, pWE16 and the

charomid 9 series of vectors. Additional vectors include bacterial artificial chromosomes (BACs) based on a functional fertility plasmid (F-plasmid), yeast artificial chromosomes (YACs), and P1-derived artificial chromosomes, DNA constructs derived from the DNA of P1 bacteriophage (PACS). Alternatively and preferably, recombinant virus vectors may be engineered, including but not limited to those derived from viruses such as herpes virus, retroviruses, vaccinia virus, poxviruses, adenoviruses, lentiviruses, adeno-associated viruses or bovine papilloma virus.

[00047] Whichever vector is chosen, typically an expression cassette expressing mutated *JAK2,* mutated *CALR,* and/or mutated *MPL* is employed. An expression vector provides transcriptional and translational regulatory sequences, and may provide for inducible or constitutive expression, where the coding region is operably linked under the transcriptional control of the transcriptional initiation region and a transcriptional and translational termination region. These control regions may be native to *JAK2, CALR,* and/or *MPL* or may be derived from exogenous sources, including species-specific endogenous promoters. In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In addition to constitutive and inducible promoters, strong promoters (e.g., T7, CMV, and the like) find use in the constructs described herein, particularly where high expression levels are desired in an *in vivo* (cell-based) or in an *in vitro* expression system. Other exemplary promoters include mouse mammary tumor virus (MMTV) promoters, Rous sarcoma virus (RSV) promoters, adenovirus promoters, the promoter from the immediate early gene of human CMV, and the promoter from the long terminal repeat (LTR) of RSV. Alternatively, the promoter can also be provided by, for example, a 5'UTR of a retrovirus.

[00048] In preferred embodiments, mutated *JAK2,* mutated *CALR,* and/or mutated *MPL* is under the control of an inducible promoter, such as tetracycline-controlled transcriptional activation where transcription is reversibly turned on (Tet-On) or off (Tet-Off) in the presence of the antibiotic tetracycline or a derivative thereof, such as doxycycline. In a Tet-Off system, expression of tetracycline response element-controlled genes can be repressed by tetracycline and its derivatives. Tetracycline

binds the tetracycline transactivator protein, rendering it incapable of binding to the tetracycline response element sequences, preventing transactivation of tetracycline response element-controlled genes. In a Tet-On system on the other hand, the tetracycline transactivator protein is capable of initiating expression only if bound by tetracycline; thus, introduction of tetracycline or doxycycline initiates the transcription of mutated *JAK2,* mutated *CALR,* and/or mutated *MPL.* Another inducible promoter system known in the art is the estrogen receptor conditional gene expression system. Compared to the Tet system, the estrogen receptor system is not as tightly controlled; however, because the Tet system depends on transcription and subsequent translation of a target gene, the Tet system is not as fast-acting as the estrogen receptor system.

[00049] In general, the inducible promoters of use in the present invention are not particularly limited, as long as the promoter is capable of inducing expression of the downstream gene in response to an external stimulus. An example of such a promoter includes: a promoter capable of inducing expression of the downstream gene by binding to a complex including a tetracycline antibiotic (tetracycline, doxycycline, or the like) and a tetracycline transactivator in a case where the external stimulus is the presence of the tetracycline antibiotic; a promoter capable of inducing expression of the downstream gene by release of a tetracycline repressor in a case where the external stimulus is the absence of a tetracycline antibiotic; a promoter capable of inducing expression of the downstream gene by binding of an ecdysteroid (ecdysone, muristerone A, ponasterone A, or the like) to an ecdysone receptor-retinoid receptor complex in a case where the external stimulus is the presence of the ecdysteroid; and a promoter capable of inducing expression of the downstream gene by binding of FKCsA to a complex including a Gal4 DNA binding domain fused to FKBP12 and a VP16 activator domain fused to cyclophilin in a case where the external stimulus is the presence of FKCsA.

[00050] The expression cassette may comprise, as necessary, an enhancer, a silencer, a selection marker gene (for example, a drug resistance gene such as a neomycin resistance gene), an SV40 replication origin, and the like. Further, those skilled in the art could construct an expression cassette capable of inducing expression of mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* at a desired expression level by appropriately selecting a combination of known enhancers,

silencers, selection marker genes, terminators, and so forth in consideration of the type of the promoter utilized and so on. In addition, as necessary, an expression cassette may also be introduced into the target cells that is capable of constantly expressing in the nucleus a factor (for example, tetracycline transactivator, a tetracycline repressor, an ecdysone receptor-retinoid receptor complex, a complex including a Gal4 DNA binding domain fused to FKBP12 and aVP16 activator domain fused to cyclophilin) for inducing expression of mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* in response to an external stimulus.

[00051] Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences (such as, in the present invention, a mutant *JAK2,* mutant *CALR,* and/or mutant *MPL*) encoding proteins of interest (such as mutant JAK2, mutant calreticulin, and mutant thromobopoietin receptor). A selectable marker operative in the expression host may be present to facilitate selection of cells containing the vector. In addition, the expression construct may include additional elements. For example, the expression vector may have one or two replication systems; thus allowing it to be maintained in different organisms, for example in mammalian cells for expression and in a prokaryotic host for cloning and amplification. In addition the expression construct may contain a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

[00052] In addition to vector-based delivery of mutant *JAK2,* mutant *CALR,* and/or mutant *MPL*, it is also contemplated that MKs, blood stem cells or human pluripotent stem cells of choice can be engineered to produce human synthetic chromosomes that express mutant JAK2, mutant calreticulin, and/or mutant thrombopoietin receptor. Fully-functional human synthetic chromosomes offer several advantages over viral-based delivery systems including increased payload size, the fact that extrachromosomal maintenance avoids host-cell disruption, and transcriptional silencing of introduced genes and possible immunological complications are avoided. Currently, there are several methods for engineering human synthetic chromosomes, including the "top down" method, the "bottom up" method, creating minichromosomes, and induced *de novo* chromosome generation. The "bottom up" approach of synthetic chromosome formation relies on cell-mediated *de novo* chromosome formation following transfection of a permissive cell line with cloned α-

satellite sequences, which comprise typical host cell-appropriate centromeres and selectable marker gene(s), with or without telomeric and genomic DNA. (For protocols and a detailed description of these methods see, e.g., Harrington, et al., Nat. Genet., 15:345-55 (1997); Ikeno, et al., Nat. Biotechnol., 16:431-39 (1998); Masumoto, et al., Chromosoma, 107:406-16 (1998), Ebersole, et al., Hum. Mol. Gene., 9:1623-31 (2000); Henning, et al., PNAS USA, 96:592-97 (1999); Grimes, et al., EMBO Rep. 2:910-14 (2001); Mejia, et al., Genomics, 79:297-304 (2002); and Grimes, et al., Mol. Ther., 5:798-805 (2002).) The "top down" approach of producing synthetic chromosomes involves sequential rounds of random and/or targeted truncation of pre-existing chromosome arms to result in a pared down synthetic chromosome comprising a centromere, telomeres, and DNA replication origins. (For protocols and a detailed description of these methods see, e.g., Heller, et al., PNAS USA, 93:7125-30 (1996); Saffery, et al., PNAS USA, 98:5705-10 (2001); Choo, Trends Mol. Med., 7:235-37 (2001); Barnett, et al., Nuc. Ac. Res., 21:27-36 (1993); Farr, et al., PNAS USA, 88:7006-10 (1991); and Katoh, et al., Biochem. Biophys. Res. Commun., 321:280-90 (2004).) "Top down" synthetic chromosomes are constructed optimally to be devoid of naturally-occuring expressed genes and are engineered to contain DNA sequences that permit site-specific integration of target DNA sequences onto the truncated chromosome, mediated, e.g., by site-specific DNA integrases.

[00053] A third method of producing synthetic chromosomes known in the art is engineering of naturally occurring minichromosomes. This production method typically involves irradiation-induced fragmentation of a chromosome containing a functional, e.g., human neocentromere possessing centromere function yet lacking α-satellite DNA sequences and engineered to be devoid of non-essential DNA. (For protocols and a detailed description of these methods see, e.g., Auriche, et al., EMBO Rep. 2:102-07 (2001); Moralli, et al., Cytogenet. Cell Genet., 94:113-20 (2001); and Carine, et al., Somat. Cell Mol. Genet., 15:445-460 (1989).) As with other methods for generating synthetic chromosomes, engineered minichromosomes can be engineered to contain DNA sequences that permit site-specific integration of target DNA sequences. The fourth approach for production of synthetic chromosomes involves induced *de novo* chromosome generation by targeted amplification of specific chromosomal segments. This approach involves large-scale amplification of

pericentromeric/ribosomal DNA regions situated on acrocentric chromosomes. The amplification is triggered by co-transfection of excess DNA specific to the pericentric region of chromosomes, such as ribosomal RNA, along with DNA sequences that allow for site-specific integration of target DNA sequences and also a drug selectable marker which integrates into the pericentric regions of the chromosomes. (For protocols and a detailed description of these methods see, e.g., Csonka, et al., J. Cell Sci 113:3207-16 (2002); Hadlaczky, et al., Curr. Opini. Mol. Ther., 3:125-32 (2001); and Lindenbaum and Perkins, et al., Nuc. Ac. Res., 32(21):e172 (2004).) During this process, targeting to the pericentric regions of acrocentric chromosomes with co-transfected DNA induces large-scale chromosomal DNA amplification, duplication/activation of centromere sequences, and subsequent breakage and resolution of dicentric chromosomes resulting in a "break-off" satellite DNA-based synthetic chromosome containing multiple site-specific integration sites.

[00054] Alternatively, mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* can be inserted into endogenous *JAK2, CALR,* and/or *MPL* chromosomal sites by site-specific recombination. Site-specific recombination requires specialized recombinases to recognize specific recombination sites and catalyze recombination at these sites. A number of bacteriophage- and yeast-derived site-specific recombination systems, each comprising a recombinase and specific cognate sites, have been shown to work in eukaryotic cells for the purpose of DNA integration and are therefore applicable for use in engineering cells to express mutant JAK2, calreticulin and/or thrombopoietin receptor. Site-specific recombination systems include but are not limited to the bacteriophage P1 Cre/lox system, yeast FLP-FRT system, and the Dre system of the tyrosine family of site-specific recombinases. Such systems and methods of use are described, for example, in U.S. Pat. Nos. 7,422,889; 7,112,715; 6,956,146; 6,774,279; 5,677,177; 5,885,836; 5,654,182; and 4,959,317, which are incorporated herein by reference to teach methods for using such recombinases. Other systems of the tyrosine family such as bacteriophage lambda Int integrase, HK2022 integrase, and systems belonging to a separate serine family of recombinases such as bacteriophage phiC31, R4Tp901 integrases are known to work in mammalian cells are also applicable for use in the present invention.

[00055] The methods of the invention preferably utilize site-specific recombination sites that utilize the same recombinase, but which do not facilitate recombination

between the sites. For example, a Lox P site and a mutated Lox P site can be integrated into the genome of a host, but introduction of Cre into the host will not facilitate recombination between the two sites; rather, the LoxP site will recombine with another LoxP site, and the mutated site will only recombine with another similarly-mutated LoxP site. Examples of such mutated recombination sites include those that contain a combination of inverted repeats or those that comprise recombination sites having mutant spacer sequences. For example, two classes of variant recombinase sites are available to engineer stable Cre-loxP integrative recombination. Both exploit sequence mutations in the Cre recognition sequence, either within the 8-bp spacer region or the 13-bp inverted repeats. Spacer mutants such as lox511, lox5171, lox2272, m2, m3, m7, and m11 recombine readily with themselves but have a markedly reduced rate of recombination with the wild-type site. This class of mutants has been exploited for DNA insertion by recombinase mediated cassette exchange using non-interacting Cre-Lox recombination sites and non-interacting FLP recombination sites (see, e.g., Baer and Bode, Curr. Opin. Biotechnol., 12:473-480 (2001); Albert, et al., Plant J., 7:649-659 (1995); Seibler and Bode, Biochemistry, 36:1740-1747 (1997); and Schlake and Bode, Biochemistry, 33:12746-12751 (1994)).

[00056] Inverted repeat mutants represent the second class of variant recombinase sites. For example, LoxP sites can contain altered bases in the left inverted repeat (LE mutant) or the right inverted repeat (RE mutant). An LE mutant, lox71, has 5 bp on the 5' end of the left inverted repeat that is changed from the wild type sequence to TACCG (see Araki, et al, Nucleic Acids Res, 25:868-872 (1997)). Similarly, the RE mutant, lox66, has the five 3'-most bases changed to CGGTA. Inverted repeat mutants are used for integrating plasmid inserts into chromosomal DNA with the LE mutant designated as the "target" chromosomal loxP site into which the "donor" RE mutant recombines. Post-recombination, loxP sites are located in cis, flanking the inserted segment. The mechanism of recombination is such that post-recombination one loxP site is a double mutant (containing both the LE and RE inverted repeat mutations) and the other is wild type (see, Lee and Sadowski, Prog. Nucleic Acid Res. Mol. Biol., 80:1-42 (2005); and Lee and Sadowski, J. Mol. Biol., 326:397-412 (2003)). The double mutant is sufficiently different from the wild-type site that it is unrecognized by Cre recombinase and the inserted segment is not excised.

[00057] Introduction of the site-specific recombination sites may be achieved by conventional homologous recombination techniques. Such techniques are described in references such as e.g., Sambrook and Russell, *Molecular cloning: a laboratory manual*, 3rd ed. (2001, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press); Nagy, *Manipulating the mouse embryo: a laboratory manual*, 3rd ed. (2003, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press); and Miller, et al., *Genetic Recombination: Nucleic acid, Homology (biology), Homologous recombination, Non-homologous end joining, DNA repair, Bacteria, Eukaryote, Meiosis, Adaptive immune system, V(D)J recombination* (2009).

[00058] Specific recombination into the endogenous *JAK2, CALR,* and/or *MPL* locus can be facilitated using vectors designed for positive or negative selection as known in the art. In order to facilitate identification of cells that have undergone the replacement reaction, an appropriate genetic marker system may be employed and cells selected by, for example, use of a selection medium. However, in order to ensure that the genome sequence is substantially free of extraneous nucleic acid sequences at or adjacent to the two end points of the replacement interval, desirably the marker system/gene can be removed following selection of the cells containing the replaced nucleic acid.

[00059] In one preferred aspect of the methods of the present invention, cells in which the replacement of all or part of the endogenous *JAK2, CALR,* and/or *MPL* locus has taken place are negatively selected upon exposure to a toxin or drug. For example, cells that retain expression of HSV-TK can be selected through use of appropriate use of nucleoside analogues such as gancyclovir. In another aspect of the invention, a positive selection system that is used based on the use of two non-functional portions of a marker gene, such as HPRT, that are brought together through a recombination event. These two portions are brought into functional association upon a successful gene replacement reaction being carried out wherein the functionally reconstituted marker gene is flanked on either side by further site-specific recombination sites (which are different to the site-specific recombination sites used for the replacement reaction), such that the marker gene can be excised from the genome if desired, using an appropriate site-specific recombinase. The recombinase may be provided to the target cell as a purified protein, or a construct transiently expressed within the cell in order to provide the recombinase activity.

[00060] The mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* expression vector can be delivered to the cells to be engineered and/or produce a synthetic chromosome by any method known in the art. The terms transfection and transformation refer to the taking up of exogenous nucleic acid, e.g., an expression vector, by a host cell whether or not any coding sequences are, in fact, expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, by Agrobacterium-mediated transformation, protoplast transformation (including polyethylene glycol (PEG)-mediated transformation, electroporation, protoplast fusion, and microcell fusion), lipid-mediated delivery, liposomes, electroporation, sonoporation, microinjection, particle bombardment and silicon carbide whisker-mediated transformation and combinations thereof (see, e.g., Paszkowski, et al., EMBO J., 3:2717-2722 (1984); Potrykus, et al., Mol. Gen. Genet., 199:169-177 (1985); Reich, et al., Biotechnology, 4:1001-1004 (1986); Klein, et al., Nature, 327:70-73 (1987); U.S. Pat. No. 6,143,949; Paszkowski, et al., in *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 6, Molecular Biology of Plant Nuclear Genes, (Schell and Vasil, eds., Academic Publishers 1989); and Frame, et al., Plant J., 6:941-948 (1994)); direct uptake using calcium phosphate (Wigler, et al., PNAS U.S.A., 76:1373-1376 (1979)); polyethylene glycol (PEG)-mediated DNA uptake; lipofection (see, e.g., Strauss, Meth. Mol. Biol., 54:307-327 (1996)); microcell fusion (Lambert, PNAS U.S.A., 88:5907-5911 (1991); U.S. Pat. No. 5,396,767; Sawford, et al., Somatic Cell Mol. Genet., 13:279-284 (1987); Dhar, et al., Somatic Cell Mol. Genet., 10:547-559 (1984); and McNeill-Killary, et al., Meth. Enzymol., 254:133-152 (1995)); lipid-mediated carrier systems (see, e.g., Teifel, et al., Biotechniques, 19:79-80 (1995); Albrecht, et al., Ann. Hematol., 72:73-79 (1996); Holmen, et al., *In Vitro* Cell Dev. Biol. Anim., 31:347-351 (1995); Remy, et al., Bioconjug. Chem., 5:647-654 (1994); Le Bolch, et al., Tetrahedron Lett., 36:6681-6684 (1995); and Loeffler, et al., Meth. Enzymol., 217:599-618 (1993)); or other suitable methods. Methods for production of synthetic chromosomes are described in U.S. application Ser. No. 09/815,979. Successful transfection is generally recognized by detection of the presence of mutant *JAK2,* mutant *CALR,* and/or mutant *MPL* within the transfected cell, such as, for example, any visualization of the heterologous nucleic acid, expression of a selectable marker or any indication of the operation of a vector within the host cell. For a description of delivery methods useful in practicing the present

invention, see U.S. Pat. No. 5,011,776; U.S. Pat. No. 5,747,308; U.S. Pat. No. 4,966,843; U.S. Pat. No. 5,627,059; U.S. Pat. No. 5,681,713; Kim and Eberwine, Anal. Bioanal. Chem. 397(8): 3173-3178 (2010).

Culturing the Modified Hematopoietic Stem Cells and Inducing Platelet Formation

[00061] The blood cell/progenitor cell differentiation induction culture protocols for culturing the human pluripotent stem cells, human blood stem cells, CPU-GEMMs, BFU-MKs. CFU-MKs, promegakaryoblasts, megakaryoblasts, promegakaryocytes or MKs used in the present invention will depend upon the cell used, how much differentiation is required, the selection methods to be employed, etc. Further, improved methods for culturing human pluripotent stem cells, human blood stem cells and MKs are being developed continually. The present invention is not dependent on any particular cell or any particular culture/differentiation methods. For general methods of blood cell/progenitor cell differentiation induction culture, see, e.g., Murphy, et al., US Pub. No. 2014/0050711; Nakamura, et al., US Pub. No. 2014/0024118; Lu, et al., Blood, DOI 10.1182/bllod-2008-05-157198 (August 19, 2008); Olivier, et al., Stem Cells Transl. Med., 1:604-14 (2012); Hiroyama, et al., Stem Cells Int'l, DOI:10.4061/2011/195780 (2011), Machlus and Italiano, JCB, 201(8):785-96 (2013); Masuda, et al., Cell Research, 23:176-78 (2013); and Reems, et al., Transfus Med Rev, 24(1):33-43 (2010), all of which are incorporated herein in their entirety.

[00062] Cytokines have been found to be the best tool to expand and control the differentiation of uncommitted CD34+ cells (e.g., human blood stem cells). Typically, serum-deprived media supplemented with different cytokines are used. Thrombopoietin (TPO) is primarily responsible for the growth and differentiation of MKs. Unlike other lineage-specific cytokines, TPO also plays a vital role in maintaining the hematopoietic stem cell population. TPO synergizes in vitro with multiple cytokines including IL-34, stem cell factor (SCF), IL-6, IL-9, and IL-11, each of which can increase the number of CFU-MKs and/or MKs. IL-6 and IL-11 particularly have been used in the last stage of culture to induce production of platelets.

[00063] There is another set of cytokines that have been identified as synergizing with TPO to support the proliferation of immature progenitors, mainly IL-3, Flt-3

Ligand (FL) and SCF. It has also been shown in vivo that there are two sets of chemokines—stromal-derived factor-1 (SDF-1) and fibroblast growth factor 4 (FGF-4)—that can promote thrombopoiesis in the absence of TPO or c-Mpl. These two chemokines facilitate the migration of MKs toward the bone marrow sinusoidal endothelial, which promotes maturation and release of platelets. Conversely, other growth factors are known to inhibit megakaryocytopoiesis, including TGF-β1, platelet factor 4 (PF4), and IFN-α, such that these factors can be used to arrest differentiation of blood stem cells at different stages (i.e., CFU-GEMM, BFU-MK, CFU-MK, promegakaryoblasts, megakaryoblasts, promegakaryocytes, megakaryocytes), thus immortalizing MKs or MK precursor cell lines.

[00064] Besides cytokines, small molecules or mimetics have been reported to modulate the growth and development of MKs and platelet biogenesis. Some of these are TPO receptor agonists, such as AKR-501 (YK477), 75 AMG531, and afungal nuclear migration protein, hNUDC. There is also the Src kinase inhibitor, SU6656, which can induce TPO-dependent polyploidization of MKs.

[00065] There are a host of variables that can be exploited to optimize cell culture conditions to maximize platelet production, For example, physical parameters such as oxygen pressure and temperature can be adjusted to maximize MK and platelet production. Elevating O2 conditions increases MK expansion and accelerate MK differentiation, maturation and proplatelet formation. Further, it has been reported that mild hypothermia can be used to favor and accelerate MK differentiation of UCB CD34+ cells.

[00066] The majority of culture systems employ at least a 2-step culture strategy to generate platelets. For most 2-step strategies, the first step is to amplify blood cell progenitors followed by a second step to differentiate MKs and support platelet biogenesis. For the 3-step strategies, the first step is to amplify the progenitors, the second step is to support MK differentiation, and the third step is to promote platelet biogenesis.

[00067] The first demonstration that functional human platelets could be generated in vitro was reported by Choi et al. using a two-step strategy (Blood, 85:402-13 (1995)). MK formation was first promoted using peripheral blood CD34+ cells cultured with aplastic canine serum. MKs were isolated from the cultures after 11-12 days and replated with fresh media supplemented with human AB plasma without

aplastic canine serum. At the peak of proplatelet formation, an average of 40% of the MKs exhibited proplatelets and platelet-sized fragments and the platelet sized fragments aggregated in the presence of agonists (i.e., thrombin and ADP). Subsequent investigators have reported the in vitro production of functional platelets using thrombin- (TPO) treated human stem cells from different sources using peripheral blood, mononuclear cells, bone marrow, umbilical cord blood, andhuman embryonic stem cells (see, e.g., Matsunaga, et al., Stem Cells, 24:2877-87 (2006); and Sullenbarger, et al., Exp Hematol., 37:101-10 (2009)).

[00068] Matsunaga and colleagues (Biochem Biophys Res Commun, 402:796-800 (2010)) have demonstrated that mouse and human fibroblasts can be directly converted into MKs (induced MKs or iMKs), from which platelets are released. The authors identified three factors as MK-inducing factors: p45NF-E2, Maf G and Maf K, using MKL1 (megakaryocyte lineage induction) medium, containing thrombopoietin (TBO)). P45NF-E2 is a gene expressed in 3T3-LI cells, but not 3T3 cells, and Maf G and Maf K are known binding partners of p45NF-E2.

[00069] It also has been shown that MKs can be derived from human embryonic stem cells (hESCs) by culturing undifferentiated hESCs in the presence of sub-confluent OP9 stromal cell monolayers or other stromal cells (Gaur, et al., J. Thromb Haemost, 4:436-442 (2006)). iPSC- or ESC-mediated generation of platelets has also been demonstrated by Eto, et al., (Blood, 207:2817-30 (2010) and Blood, 118:2 (2011)). Transient activation of c-Myc during megakaryopoiesis was identified as critical for efficient platelet production from human iPSCs; that is, reactivation of c-Myc after reprogramming should be followed by reduction of c-Myc expression for further maturation. Eto, et al. have also established an immortalized MK cell line derived from human iPSCs (Blood, 118:2 (2011)).

[00070] Another protocol employs a three step in vitro culture system that more closely mimics the in vivo process of MK/platelet development. First CD34+ cells are expanded for 14 days to amplify hematopoietic stem/progenitor cells. Next, the cells are transferred to a culture environment to differentiate and expand MKs for another 14 days. This is followed by a 5 day culture period to support the maturation of MKs to produce platelets that exhibited normal morphology and function. In yet another system, umbilical cord blood CD34+ cells are expanded for three days prior to placement in a 3-dimensional bioreactor continuously perfused with media and

free of stromal cells. The advantage of this system is that it permits the continuous collection of platelets, while allowing for an independent control of media and gas flow.

[00071] Additionally, it may be desired to culture the human stem cells or blood stem cells on a 2D or 3D cell support structure as reported by Laskey, et al., in US Pub. No. 2010/0248361 or a layered material as reported by Peukert, et al., in US Pub. No. 2012/0220198.

[00072] The understanding on how platelets are formed and released from MKs in vivo has greatly been enhanced in recent years, and it appears as though the primary mechanism of platelet release is via utilization of mechanical forces to sever platelets from proplatelets processes. Thus, low yields of platelets from culture-derived MKs are not surprising given the static nature of the majority of the current culture protocols that are being explored. Even though the precise mechanism used by MKs to sense and respond to shear forces is not clearly understood, shear force potentially could be used to promote *in vitro* platelet production. This is evident from recent work which shows that when mature human MKs are exposed to high shear forces, proplatelet processes become apparent and platelets are released within 20 minutes. Thus, the present invention envisions increasing platelet formation by using shear forces in the final culturing of MKs. Additionally, Phipps, et al., report use of electrophilic compounds for inducing platelet production or for maintaining platelet function in US Pub. No. 2011/0027223.

Isolating the In Vitro Generated Platelets

[00073] The *in vitro* generated platelets may be enriched using any convenient method known in the art, including fluorescence activated cell sorting (FACS), magnetically activated cell sorting (MACS), density gradient centrifugation and the like. Parameters employed for enriching certain cells from a mixed population include, but are not limited to, physical parameters (e.g., size, shape, density, etc.) and molecule expression (e.g., expression of cell surface proteins or carbohydrates, reporter molecules, e.g., green fluorescent protein, etc.). Because platelets float in media, density gradient centrifugation is particularly cost effective, and can be used safely to separate platelets from any nucleated blood cells likely to be present. Thus, separation by density is a preferred method of isolating the *in vitro* generated

platelets of the invention. In alternative embodiments, an affinity purification method may be utilized to isolate platelets that have cell-surface antibodies that bind to a specific antigen, either naturally, or platelets that have been engineered to do so. The antigen used to immobilize the platelets may be immobilized on a solid phase and used to selectively retain the platelets, while nucleated cells are washed away. The retained platelets may then be eluted by a variety of methods, such as by chaotropic agents, changing the pH, salt concentration, etc. Any of the well-known methods for immobilizing or coupling an antigen to a solid phase may be used. In the instances where the antigen is a protein, the protein may be covalently attached to a solid phase, for example, sepharose beads, by well-known techniques, etc.

[00074] Alternatively, a labeled antigen may be used to specifically label platelets that express an antibody that binds to the antigen and the labeled platelets may then be isolated by cell sorting (e.g., by FACS). In certain cases, methods for antibody purification may be adapted to isolate antibody presenting platelets. Such methods are well known and are described in, for example, Sun, et al., J. Immunol. Methods, 282(1-2):45-52 (2003); Roque et al., J. Chromatogr A., 1160(1-2):44-55 (2007); and Huse, et al., J. Biochem. Biophys. Methods, 51 (3):217-31 (2002). The platelets may also be isolated using magnetic beads or by any other affinity solid phase capture method, protocols for which are known. In some embodiments, antigen-specific antibody presenting platelets may be obtained by flow cytometry using the methods described in Wrammert, Nature, 453: 667-72 (2008), Scheid, Nature, 458: 636-40 (2009), Tiller, J. Immunol. Methods, 329 112-24 (2008); or Scheid, PNAS, 105: 9727-32 (2008), for example, all of which are incorporated by reference for disclosure of those methods. Exemplary antibody-presenting platelet enrichment methods include performing flow cytometry (FACS) of platelets, e.g., through incubating the platelets with labeled antigens and sorting the labeled platelets using a FACSVantage SE cell sorter (Becton-Dickinson, San Jose, Calif Optical sorting offers an alternative to FACs or MACs, both of which require labelling of the cells (see, e.g., Ashkin, et al., Am. Soc. Of Graviational and Space Biol., 4(2):133-46 (1991)); and MacDonald, et al., Nature, 426:421-24 (2003)).

[00075] Further, the isolated platelets can be sterilized by irradiation. Because platelets are enucleated, they remain functional after irradiation.

Use of the In Vitro Generated Platelets

[00076] A primary purpose for generating platelets *in vitro* is for transfusions in humans. As mentioned above, *in vitro* generated platelets have the advantages that reliance on the current volunteer-based collection system is not necessary; thus the supply of transfusable platelets is not vulnerable to supply chain disruptions; the platelets of the present invention can be chosen to exhibit particular phenotypes such as being, e.g., AB/ RhD- or O/RhD-, or with additional, more precise matching for chronically-infused patients; and the risk of contamination by pathogens is greatly reduced. Further, use of *in vitro* generated platelets has the additional advantage that the platelets transfused are homogenous in age or nearly so. The *in vitro* generated platelets of the invention can be used in surgical and chemotherapy settings.

[00077] Yet another application of the *in vitro* generated platelets of the present invention is the production of reagent *JAK2*-modified, *CALR*-modified, and/or *MPL*-modified platelets. Reagent *JAK2*-modified, *CALR*-modified, and/or *MPL*-modified platelets are panels of platelets with known antigen profiles that may be used prior to transfusion to test the serum of the recipient patient for the presence of antibodies that may react with the transfused platelets. Panels of reagent mutant *JAK2*-modified, mutant *CALR*-modified, and/or mutant *MPL*-modified platelets may represent antigen profiles found primarily in common populations, or in rare or uncommon phenotypes.

[00078] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventor to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The

scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments described herein. Rather, the scope and spirit of present invention is embodied by the appended claims. In the claims that follow, unless the term "means" is used, none of the features or elements recited therein should be construed as means-plus-function limitations pursuant to 35 U.S.C. §112, ¶6.

I claim:

1. A method of producing an *in vitro* generated platelet expressing one or more of a mutant Janus kinase 2 V617F protein (mutant *JAK2*), a mutant calreticulin-last exon protein (mutant *CALR*), and/or a thrombopoietin receptor protein S505N or W515L (mutant *MPL*) comprising:

providing human pluripotent stem cells or human blood cells;

transforming the human pluripotent stem cells or human blood cells with of an expression vector expressing one or more of mutant JAK2, mutant calreticulin-last exon, and/or mutant myeloproliferative leukemia protein S505N or W515L, or creating one or more of a V617F mutation in an endogenous *JAK2* locus, a mutation in the last exon of an endogenous *CALR* locus, or a S505N or W515L mutation in an endogenous *MPL* locus in the human pluripotent stem cells or human blood cells to produce JAK2, calreticulin, and/or thrombopoietin receptor modified cells;

culturing the modified cells;

inducing platelet production from the modified cells; and

isolating the platelets.

2. The method of claim 1, wherein the human pluripotent stem cells or human blood cells are human embryonic stem cells; human embryonal carcinoma cells; human embryonic germ cells; human multipotent germline cells; human mesodermal stem cells; human mesenchymal stem cells; human induced pluripotent stem cells; human colony forming units-granulocytes, erythrocytes, monocytes and megakaryocytes (CPU-GEMMs); burst forming units-megakaryocytes (BFU-MKs); colony forming units-megakaryocytes (CFU-MKs); promegakaryoblasts; megakaryoblasts; promegakaryocytes; or megakaryocytes.
3. The method of claim 2, wherein the human pluripotent stem cells are human embryonic stem cells.
4. The method of claim 2, wherein the human pluripotent stem cells are human induced pluripotent stem cells.
5. The method of claim 2, wherein the human blood cells are human colony forming units-granulocytes, erythrocytes, monocytes and megakaryocytes (CPU-GEMMs); burst forming units-megakaryocytes (BFU-MKs); colony forming units-megakaryocytes (CFU-MKs); promegakaryoblasts; megakaryoblasts; or promegakaryocytes.

6. The method of claim 2, wherein the human pluripotent stem cells or human blood cells are ABO type A and RhD negative.
7. The method of claim 2, wherein the human pluripotent stem cells or human blood cells are ABO type B and RhD negative.
8. The method of claim 2, wherein the human pluripotent stem cells or human blood cells are ABO type O and RhD negative.
9. The method of claim 1, wherein the human pluripotent stem cells or human blood cells are transformed with one or more of a mutant JAK2 expression vector, a mutant calreticulin expression vector, and/or a mutant thrombopoietin receptor expression vector.
10. The method of claim 9, where in the gene expressing the mutant JAK2, mutant calreticulin, and/or mutant thrombopoietin receptor is under control of an inducible promoter.
11. The method of claim 1, wherein the human pluripotent stem cells or human blood cells comprise a human synthetic chromosome expressing one or more of mutant JAK2, mutant calreticulin, and mutant thrombopoietin receptor.
12. The method of claim 11, wherein the gene expressing the mutant JAK2, mutant calreticulin, and/or mutant thrombopoietin receptor is under control of an inducible promoter.
13. The method of claim 1, wherein one or more of the endogenous *JAK2* locus is replaced with mutant *JAK2* via homologous recombination, the endogenous *CALR* locus is replaced with mutant *CALR* via homologous recombination, or the endogenous *MPL* locus is replaced with mutant *MPL* via homologous recombination.
14. The method of claim 1, wherein the modified cells are cultured in the presence of one or more of thrombopoietin (TBO).
15. The method of claim 13, wherein the modified cells are cultured in TBO and one or more of interleukin-3 (IL-3), Flt-3 Ligand (FL), interleukin-34 (IL-34), stem cell factor (SCF), interleukin-6 (IL-6), interleukin-9 (IL-9), interleukin-11 (IL-11), p45NF-E2, Maf G or Maf K.
16. The method of claim 1, wherein the modified cells are cultured in the presence of feeder cells.
17. The method of claim 14, wherein the feeder cells are OP9 cells, MEF, SNL76/7 cells, PA6 cells, NIH3T3 cells, M15 cells, or 10T1/2 cells.
18. The method of claim 1, wherein platelet production is induced by culturing modified cells in the presence of shear forces
19. The *in vitro* generated platelets produced by the method of claim 1.

20. A method of treating a human patient comprising transfusing the patient with the *in vitro* generated platelets of claim 19.
21. A method of producing an immortalized mutant Janus kinase 2 V617F (mutant JAK2) megakaryocyte line comprising:

providing human pluripotent stem cells or human blood cells;

transforming the human pluripotent stem cells or human blood cells with an expression vector expressing V617F mutant JAK2 or creating the Janus kinase 2 V617F mutation in an endogenous *JAK2* locus in the human pluripotent stem cells or human blood cells to produce modified cells; and

culturing the modified cells in nondifferentiating blood stem/blood progenitor cell culture medium.

22. A method for producing *in vitro* generated Janus kinase 2 V617F (mutant JAK2) modified platelets from the immortalized modified mutant Janus kinase 2 V617F (mutant JAK2) megakaryocyte line of claim 21, further comprising the steps of inducing platelet formation; and isolating the platelets.
23. A method of producing an immortalized mutant calreticulin megakaryocyte line comprising:

providing human pluripotent stem cells or human blood cells;

transforming the human pluripotent stem cells or human blood cells with an expression vector expressing mutant calreticulin-last exon or creating a mutation in a last exon of in an endogenous *CALR* locus in the human pluripotent stem cells or human blood cells to produce modified cells; and

culturing the modified cells in nondifferentiating blood stem/blood progenitor cell culture medium.

24. A method for producing *in vitro* generated mutant calreticulin modified platelets from the immortalized modified mutant calreticulin megakaryocyte line of claim 21, further comprising the steps of inducing platelet formation; and isolating the platelets.
25. A method of producing an immortalized mutant thrombopoiein receptor megakaryocyte line comprising:

providing human pluripotent stem cells or human blood cells;

transforming the human pluripotent stem cells or human blood cells with an expression vector expressing mutant thrombopoietin receptor protein S505N or W515L or creating a mutation in an endogenous *CALR* locus in the human pluripotent stem cells or human blood cells to produce modified cells; and

culturing the modified cells in nondifferentiating blood stem/blood progenitor cell culture medium.

26. A method for producing *in vitro* generated mutant thrombopoietin modified platelets from the immortalized modified mutant thrombopoietin receptor megakaryocyte line of claim 21, further comprising the steps of inducing platelet formation; and isolating the platelets.

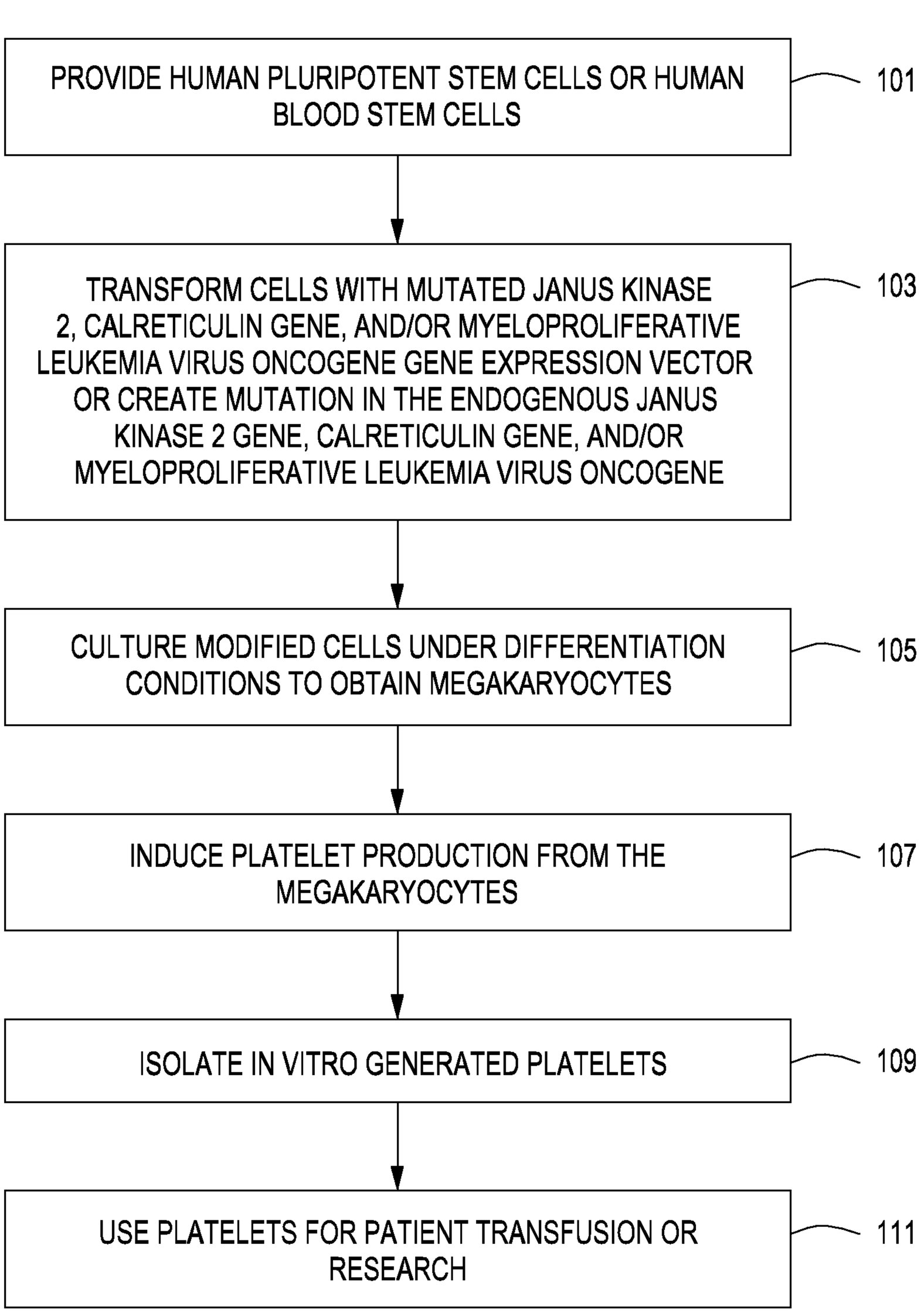
100
PROVIDE HUMAN PLURIPOTENT STEM CELLS OR HUMAN BLOOD STEM CELLS
101
TRANSFORM CELLS WITH MUTATED JANUS KINASE 2, CALRETICULIN GENE, AND/OR MYELOPROLIFERATIVE LEUKEMIA VIRUS ONCOGENE GENE EXPRESSION VECTOR OR CREATE MUTATION IN THE ENDOGENOUS JANUS KINASE 2 GENE, CALRETICULIN GENE, AND/OR MYELOPROLIFERATIVE LEUKEMIA VIRUS ONCOGENE
103
CULTURE MODIFIED CELLS UNDER DIFFERENTIATION CONDITIONS TO OBTAIN MEGAKARYOCYTES
105
INDUCE PLATELET PRODUCTION FROM THE MEGAKARYOCYTES
107
ISOLATE IN VITRO GENERATED PLATELETS
109
USE PLATELETS FOR PATIENT TRANSFUSION OR RESEARCH
111

FIG. 1