# DIFFERENTIATED ANUCLEATED CELLS AND METHOD FOR PREPARING THE SAME

## CROSS-REFERENCE

[0001] This application claims priority from U.S. Provisional Application No. 61/082,410, entitled Differentiated Cells and Processes for Preparing the Same, filed 21-July-2008, which is incorporated herein by reference in its entirety.

## BACKGROUND OF THE INVENTION

[0002] Anucleated cells include, but are not limited to, erythrocytes and thrombocytes. Erythrocytes and thrombocytes are derived from the differentiation of hematopoietic stem cells.

## SUMMARY ~~OF THE INVENTION~~

[0003a] In one aspect, the present invention refers to a method of preparing a plurality of anucleated cells comprising: culturing conditionally immortalized stem cells in the presence of an agent for inducing differentiation to erythrocytes; wherein the agent is IL-3 and EPO;
wherein the conditionally immortalized stem cells were generated by:

a.transfecting a plurality of stem cells with a first vector comprising: a nucleic acid sequence encoding a MYC molecule;

b.transfecting the stem cells with a second retroviral vector comprising a nucleic acid sequence encoding Bcl-2 Bcl-X, or a combination thereof; and

c.culturing stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof;
and wherein the activity of the Myc molecule has been suppressed before differentiation.

[0003] Disclosed herein, in certain examples ~~embodiments~~, is a method of preparing a plurality of anucleated cells comprising: culturing conditionally immortalized stem cells in the presence of at least one cytokine that directs differentiation toward an anucleated cell; wherein the conditionally immortalized stem cells are generated by: (a) transfecting a plurality of stem cells with a first vector comprising: a nucleic acid sequence encoding a MYC molecule; (b) transfecting the stem cells with a second retroviral vector comprising a nucleic acid sequence encoding Bcl-2, Bcl-X, or a combination thereof; and (c) culturing the stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof; and wherein the activity of the Myc molecule has been suppressed before differentiation. In some examples ~~embodiments~~, the cytokine that directs differentiation toward an anucleated cell is IL-3, EPO, or a combination thereof. In some examples ~~embodiments~~, the MYC

molecule is c-Myc, l-Myc, n-Myc, s-Myc, or a combination thereof. In some examples ~~embodiments~~, the first vector further comprises the hormone-binding domain of the human estrogen receptor. In some examples ~~embodiments~~, the method further comprises inducing translocation of the Myc molecule to a nucleus. In some examples ~~embodiments~~, the method further comprises contacting the plurality of stem cells with: estradiol (E2), 4-hydroxytamoxifen (4-OHT), or both. In some examples ~~embodiments~~, the first vector, the second vector, or both the first vector and the second vector is Murine Stem Cell Virus (MSCV), or a derivative thereof. In some examples ~~embodiments~~, the first vector, the second vector, or both the first vector and the second vector is MSCV- (IRES)-GFP, or a derivative thereof. In some examples ~~embodiments~~, the first vector, the second

vector, or both the first vector and the second vector is MSCV- (IRES)-GFP, and further comprises woodchuck hepatitis B virus RNA regulatory element (WRE).

[0004a] In one aspect, the present invention refers to a method of producing a plurality of anucleated cells, comprising: culturing conditionally immortalized stem cells in the presence of an agent for inducing differentiation to erythrocytes; wherein the agent is IL-3 and EPO;
wherein the conditionally immortalized stem cells were generated by:

a. contacting a plurality of stem cells with an exogenously synthesized first peptide comprising: a MYC molecule that translocates to a nucleus;

b. contacting the stem cells with an exogenously synthesized second peptide comprising: Bcl-2 Bcl-X, or a combination thereof; and

c. culturing stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof;
and wherein the first peptide is removed from contact with the conditionally immortalized stem cells before differentiation.

**[0004]** Disclosed herein, in certain examples ~~embodiments~~, is a method of producing a plurality of anucleated cells, comprising: culturing conditionally immortalized stem cells in the presence of at least one cytokine that directs differentiation toward an anucleated cell; wherein the conditionally immortalized stem cells are generated by: (a) contacting a plurality of stem cells with an exogenously synthesized first peptide comprising: a MYC molecule that translocates to a nucleus; (b) contacting the stem cells with an exogenously synthesized second peptide comprising: Bcl-2, BCL-X, or a combination thereof; and (c) culturing the stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof; and wherein the first peptide is removed from contact with the conditionally immortalized stem cells before differentiation. In some examples ~~embodiments~~, the first peptide further comprises the hormone-binding domain of the human estrogen receptor. In some examples ~~embodiments~~, the first peptide, the second peptide, or both the first and second peptides comprise a transporter sequence. In some examples ~~embodiments~~, the first protein, the second protein, or both the first and second proteins comprise a TAT sequence. In some examples ~~embodiments~~, the method further comprises contacting the plurality of stem cells with: estradiol (E2), 4-hydroxytamoxifen (4-OHT), or both.

**[0005]** Disclosed herein, in certain examples ~~embodiments~~, is a method of preparing a plurality of anucleated cells, comprising: (a) transfecting a plurality of stem cells (e.g., hematopoietic stem cells) with a first vector comprising a nucleic acid sequence encoding a MYC molecule; (b) transfecting

the plurality of stem cells (e.g., hematopoietic stem cells) with a second gammaretroviral vector comprising a nucleic acid sequence encoding Bcl-2, Bcl-X, or a combination thereof; (c) culturing the plurality of stem cells (e.g., hematopoietic stem cells) with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof; (d) suppressing the activity of the Myc molecule; and (e) culturing the conditionally immortalized stem cells (e.g., hematopoietic stem cells) in the presence of at least one cytokine that directs differentiation toward an anucleated cell. In some examples ~~embodiments~~, the cytokine that directs differentiation toward an anucleated cell is IL-3, EPO, or a combination thereof. In some examples ~~embodiments~~, the MYC molecule is c-Myc, l-Myc, n-Myc, s-Myc, or a combination thereof. In some examples ~~embodiments~~, the first vector further comprises the hormone-binding domain of the human estrogen receptor. In some examples ~~embodiments~~, the method further comprises inducing translocation of the Myc molecule to a nucleus. In some

examples ~~embodiments~~, the method further comprises contacting the plurality of stem cells (e.g., hematopoietic stem cells) with: estradiol (E2), 4-hydroxytamoxifen (4-OHT), or both. In some examples ~~embodiments~~, the first vector, the second vector, or both the first vector and the second vector is Murine Stem Cell Virus (MSCV), or a derivative thereof. In some examples ~~embodiments~~, the first vector, the second vector, or both the first vector and the second vector is MSCV- (IRES)-GFP, or a derivative thereof. In some examples ~~embodiments,~~ the first vector, the second vector, or both the first vector and the second vector is MSCV- (IRES)-GFP, and further comprises woodchuck hepatitis B virus RNA regulatory element (WRE).

**[0006]** Disclosed herein, in certain examples ~~embodiments~~, is a method of producing a plurality of anucleated cells (e.g., hematopoietic stem cells), comprising: (a) contacting a plurality of stem cells (e.g., hematopoietic stem cells) with: (i) an exogenously synthesized first peptide comprising: a MYC molecule that translocates to a nucleus; and (ii) an exogenously synthesized second peptide comprising: Bcl-2, BCL-X, or a combination thereof; (b) culturing the plurality of stem cells (e.g., hematopoietic stem cells) with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof; (c) removing the first peptide; and (d) culturing the conditionally immortalized stem cells (e.g., hematopoietic stem cells) in the presence of at least one cytokine that directs differentiation towards an anucleated cell. In some examples ~~embodiments~~, the first peptide further comprises the hormone-binding domain of the human estrogen receptor. In some examples ~~embodiments~~, the first peptide, the second peptide, or both the first and second peptides comprise a transporter sequence. In some examples ~~embodiments~~, the first protein, the second protein, or both the first and second proteins comprise a TAT sequence. In some examples ~~embodiments~~, the method further comprises contacting the plurality of stem cells (e.g., hematopoietic stem cells) with: estradiol (E2), 4-hydroxytamoxifen (4-OHT), or both.

**[0007]** Disclosed herein, in certain examples ~~embodiments~~, is a thrombocyte prepared according to a method disclosed herein.

**[0008]** Disclosed herein, in certain examples ~~embodiments~~, is an erythrocyte prepared according to a method disclosed herein.

**[0009]** Disclosed herein, in certain examples ~~embodiments~~, is a method of treating a disorder characterized by a deficiency of anucleated cells, comprising: administering an anucleated cell prepared according to a method disclosed herein. In some examples ~~embodiments~~, the disorder is an anemia (e.g., aplastic anemia, pernicious anemia, iron deficiency anemia, sickle cell anemia, spherocytosis, hemolytic anemia), Gaucher's disease, hemolysis, neutropenia,

thrombocytopenia, granulocytopenia, hemophilia, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, B cell chronic lymphoma, Burkitt's lymphoma, Follicular-like lymphoma, diffused large B-cell lymphoma, multiple myeloma, acute myeloid leukemia, pre-B acute lymophcytic leukemia, pre-T acute lymphocytic leukemia, acute promeylocytic leukemia, refractory leukemia, or combinations thereof In certain instances, the disorder characterized by an deficiency in anucleated cells results from (partially or fully) chemotherapy. In some examples ~~embodiments~~, an anucleated cell produced by a method disclosed herein is co-administered with chemotherapy.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0010]** The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

**[0011]** Figure 1 illustrates an approach for generation of a ctlt-hematopoietic stem cell line. Figure 1A shows a schematic representation of retroviral constructs used to transduce primary murine HSC cells with Myc-ER and Bcl-2. Figure 1B shows FACS analyses of HSCs obtained from 5-fluorouracil treated donors following the transduction. Figure 1C shows kinetics of leukemogenesis in mice transplanted with the transduced HSCs. Figure 1D shows FACS analysis of a ctlt-hematopoietic stem cell soon after recovery from the bone marrow of leukemic mice. Figure 1E shows FACS analysis of a ctlt-hematopoietic stem cell line that has been established. Figure 1F shows FACS analysis of stem cell marker in normal, unmanipulated hematopoietic stem cells from the bone marrow of C57/BL6 mice.

**[0012]** Figure 2 illustrates Analysis of clonality of ctlt-hematopoietic stem cell lines by PCR amplification of retroviral insertions.

**[0013]** Figure 3 illustrates characteristics of mature immune cells that arise following transplantation of ctlt-hematopoietic stem cells. Figure 3A shows the frequency of cells derived from ctlt-hematopoietic stem cells in lymphoid tissues after transplantation. Figure 3B shows detection of GFP+ myeloid lineage cells in the bone marrow. Figure 3C shows analysis of peripheral, mature lymphocytes in the spleen. Figure 3D shows analysis of peripheral, mature lymphocytes in transplant recipient mice following the second serial passage of ctlt-hematopoietic stem cells.

WHAT IS CLAIMED IS:

1. A method of preparing a plurality of anucleated cells comprising: culturing conditionally immortalized stem cells in the presence of an agent for inducing differentiation to erythrocytes; wherein the agent is IL-3 and EPO;
wherein the conditionally immortalized stem cells were generated by:

a. transfecting a plurality of stem cells with a first vector comprising: a nucleic acid sequence encoding a MYC molecule;

b. transfecting the stem cells with a second retroviral vector comprising a nucleic acid sequence encoding Bcl-2 Bcl-X, or a combination thereof; and

c. culturing stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof:
and wherein the activity of the Myc molecule has been suppressed before differentiation.

2. The method according to claim 1, wherein the first vector further comprises the hormone-binding domain of the human estrogen receptor.

3. The method according to claim 1 or claim 2, further comprising inducing translocation of the Myc molecule to a nucleus.

4. The method according to any of claims 1-3, wherein the first vector, the second vector, or both the first and the second vector is Murine Stem Cell Virus (MSCV), or a derivative thereof; optionally MSCV- (IRES)-GFP or a derivative thereof; optionally MSCV-(IRES)-GFP further comprising woodchuck hepatitis B virus RNA regulatory element (WRE).

5. A method of producing a plurality of anucleated cells, comprising: culturing conditionally immortalized stem cells in the presence of an agent for inducing differentiation to erythrocytes; wherein the agent is IL-3 and EPO;
wherein the conditionally immortalized stem cells were generated by:

a. contacting a plurality of stem cells with an exogenously synthesized first peptide comprising: a MYC molecule that translocates to a nucleus;

b. contacting the stem cells with an exogenously synthesized second peptide comprising: Bcl-2 Bcl-X, or a combination thereof; and

c. culturing stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof:
and wherein the first peptide is removed from contact with the conditionally immortalized stem cells before differentiation.

6. The method according to claim 5, wherein the first peptide, the second peptide, or both the first and second peptides comprise a transporter sequence.

7. The method according to any of claims 5-6, wherein the first protein, the second protein, or both the first and second proteins comprise a TAT sequence or a VPR sequence.

8. The method according to any of claims 1-7, wherein the MYC molecule is c-Myc, l-Myc, n-Myc, s-Myc, v-Myc or a combination thereof.

9. The method according to any of claims 1-8, further comprising contacting both the plurality of stem cells with: estradiol (E2), 4-hyrdorxytamoxifen (4-OHT), or both.

10. The method according to any of claims 1-9, wherein the conditionally immortalized stem cells further comprise one or more polypeptides of interest.

11. The method according to any of claims 1-10, wherein the MYC molecule is MYC-ER.

12. The method of any one of claims 5-10, wherein the MYC molecule is TAT-MYC.

13. The method of any one of claims 5-12, wherein the Bcl-2 molecule is TAT-Bcl-2.

14. The method according to any of claims 1-4, wherein the second retroviral vector comprises a nucleic acid sequence encoding Bcl-2.

# CLAIMS

WHAT IS CLAIMED IS:

1. A method of preparing a plurality of anucleated cells comprising: culturing conditionally immortalized stem cells in the presence of at least one cytokine that directs differentiation toward an anucleated cell;

wherein the conditionally immortalized stem cells are generated by:

a. transfecting a plurality of stem cells with a first vector comprising: a nucleic acid sequence encoding a MYC molecule;

b. transfecting the stem cells with a second retroviral vector comprising a nucleic acid sequence encoding Bcl-2, Bcl-X, or a combination thereof; and

c. culturing the stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof;

and wherein the activity of the Myc molecule has been suppressed before differentiation.

2. The method of Claim 1, wherein the cytokine that directs differentiation toward an anucleated cell is IL-3, EPO, or a combination thereof.

3. The method of Claim 1, wherein the MYC molecule is c-Myc, l-Myc, n-Myc, s-Myc, or a combination thereof.

4. The method of Claim 1, wherein the first vector further comprises the hormone-binding domain of the human estrogen receptor.

5. The method of Claim 1, further comprising inducing translocation of the Myc molecule to a nucleus.

6. The method of Claim 1, further comprising contacting the plurality of stem cells with: estradiol (E2), 4-hydroxytamoxifen (4-OHT), or both.

7. The method of Claim 1, wherein the first vector, the second vector, or both the first vector and the second vector is Murine Stem Cell Virus (MSCV), or a derivative thereof.

8. The method of Claim 1, wherein the first vector, the second vector, or both the first vector and the second vector is MSCV- (IRES)-GFP, or a derivative thereof.

9. The method of Claim 1, wherein the first vector, the second vector, or both the first vector and the second vector is MSCV- (IRES)-GFP, and further comprises woodchuck hepatitis B virus RNA regulatory element (WRE).

10. A method of producing a plurality of anucleated cells, comprising: culturing conditionally immortalized stem cells in the presence of at least one cytokine that directs differentiation toward an anucleated cell;

wherein the conditionally immortalized stem cells are generated by:

a. contacting a plurality of stem cells with an exogenously synthesized first peptide comprising: a MYC molecule that translocates to a nucleus;

b. contacting the stem cells with an exogenously synthesized second peptide comprising: Bcl-2, BCL-X, or a combination thereof; and

c. culturing the stem cells with IL-3, IL-6, stem cell factor, thrombopoietin, Flt3 ligand, or a combination thereof;

and wherein the first peptide is removed from contact with the conditionally immortalized stem cells before differentiation.

11. The method of Claim 10, wherein the first peptide further comprises the hormone-binding domain of the human estrogen receptor.

12. The method of Claim 10, wherein the first peptide, the second peptide, or both the first and second peptides comprise a transporter sequence.

13. The method of Claim 10, wherein the first protein, the second protein, or both the first and second proteins comprise a TAT sequence.

14. The method of Claim 10, further comprising contacting the plurality of stem cells with: estradiol (E2), 4-hydroxytamoxifen (4-OHT), or both.

15. A thrombocyte prepared according to the method of Claim 1.

16. A thrombocyte prepared according to the method of Claim 10.

17. An erythrocyte prepared according to the method of Claim 1.

18. An erythrocyte prepared according to the method of Claim 10.

19. A method of treating a disorder characterized by a deficiency of anucleated cells, comprising: administering an anucleated cell prepared according to Claim 1.

20. A method of treating a disorder characterized by a deficiency of anucleated cells, comprising: administering an anucleated cell prepared according to Claim 10.