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(71) Applicant *(for all designated States except US)*: **THE GEORGE WASHINGTON UNIVERSITY** [US/US]; 2300 Eye Street, N.W., Washington, DC 20037 (US).

(72) Inventors: **HAWLEY, Robert, G.**; 14027 Weeping Cherry Drive, Rockville, MD 20850 (US). **EAKER, Shannon, S.**; 20203 Spring Haven Court, Montgomery Village, MD 20886 (US). **HAWLEY, Teresa, S.**; 14027 Weeping Cherry Drive, Rockville, MD 20850 (US).

(74) Agents: **SCHIAVELLI, Alan, E.** et al.; Antonelli, Terry, Stout & Kraus, LLP, 1300 North Seventeenth Street, Suite 1800, Arlington, VA 22209 (US).

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(54) Title: CONDITIONALLY-IMMORTALIZED HEMATOPOIETIC PROGENITOR CELL LINES

(57) Abstract: A cell line comprising a cellular genome, wherein the cellular genome comprises a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, wherein the cell line is a hematopoietic progenitor cell line, and wherein the cell line is conditionally-immortalized; a method of generating a conditionally-immortalized hematopoietic progenitor cell line; the cell line generated thereby; a method of generating erythrocytes; a method of generating granulocytes; methods of generating monocytes/macrophages, granulocytes, and megakaryocytes; a method of generating monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes; a method of performing a blood transfusion in a host; a method of determining whether or not an agent influences hematopoiesis; and a method of screening a drug candidate for an effect on a hematopoietic cell.

CONDITIONALLY-IMMORTALIZED HEMATOPOIETIC PROGENITOR CELL LINES

The present application claims the benefit of the filing date of United States Provisional Application No. 60/479,415, filed June 18, 2003. The provisional application is incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0001] This invention was made in part with Government support under Grant Number R01 HL66305 awarded by the National Institutes of Health. The Government may have certain rights in this invention.

TECHNICAL FIELD

[0002] This invention pertains to conditionally-immortalized hematopoietic progenitor cell lines and methods related thereto.

BACKGROUND ART

[0003] Mouse embryonic stem (ES) cells, which are derived from the inner cell mass of blastocysts, can be maintained in an undifferentiated state *in vitro* and can contribute to tissues derived from all three germ layers when reintroduced into a developing blastocyst (Evans et al, *Nature*, *292*, 154-156 (1981); and Robertson et al., *Nature*, *323*, 445-448 (1986)). Hematopoiesis within the mouse embryo originates in the blood islands of the yolk sac (Keller et al., *Exp. Hematol.*, *27*, 777-787 (1999); and Nishikawa et al., *Curr. Opin. Cell. Bio.*, *13*, 673-678 (2001)). Mouse ES cells can be induced to differentiate *in vitro* into embryoid bodies (EBs) (Doetschman et al., *J. Embryol. Exp. Morph.*, *87*, 27-45 (1985); Wiles et al., *Development*, *111*, 259-267 (1991); and Keller et al., *Mol. Cell. Biol.*, *13*, 473-486 (1993)), multicellular spheroid structures in which hematopoiesis initiates within regions similar to yolk sac blood islands (Doetschman et al., *J. Embryol. Exp. Morph.*, *87*, 27-45 (1985); Kennedy et al., *Nature*, *386*, 488-493 (1997); and Choi et al., *Development*, *125*, 725-732 (1998)).

[0004] The homeobox gene HOX11 is not normally expressed in the hematopoietic system, but it induces T-cell leukemia as a consequence of the t(10;14)(q24;q11) and t(7;10)(q35;q24) chromosomal translocations involving the T-cell receptor δ and β loci, respectively (Dube et al., *Blood*, *78*, 2996-3003 (1991);

Hatano et al., *Science*, *253*, 79-82 (1991); Kennedy et al., *Proc. Natl. Acad. Sci. U.S.A.*, *88*, 8900-8904 (1991); and Lu et al., *EMBO J.*, *10*, 2905-2910 (1991)). HOX11 is believed to function primarily as a transcriptional regulator on the basis of its nuclear localization, the DNA binding activity of its homeodomain, and its ability to transactivate transcription of reporter genes (Owens et al., *Blood*, *101*, 4966-4974 (2003); and Dear et al., *Proc. Natl. Acad. Sci. U.S.A.*, *90*, 4431-5 (1993)). Retroviral vector-mediated expression of HOX11 in primary mouse bone marrow has been shown to give rise to immortalized myeloid precursor cell populations (Hawley et al., *Oncogene*, *9*, 1-12 (1994); and Hawley et al., *Cancer Res.*, *57*, 337-345 (1997)). Overexpression of HOX11 in differentiating mouse EBs was subsequently demonstrated to result in the establishment of primitive (erythroid) and definitive (myeloid, erythroid and megakaryocytic) hematopoietic precursor cell lines (Keller et al., *Blood*, *92*, 877-887 (1998)) and U.S. Patent Nos. 6,110,739 and 5,874,301). Thus, ectopic HOX11 expression appears to be an effective strategy to obtain immortalized cells representing a spectrum of hematopoietic precursors.

[0005] As it is shown herein, however, cells constitutively expressing the HOX11 gene are blocked from further differentiation. Therefore, there remains a need in the art for improved hematopoietic precursor cell lines conditionally-immortalized and able to fully differentiate into specific cellular lineages.

[0006] The invention provides such cell lines, as well as methods of generating the cell lines and other methods related thereto. These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

DISCLOSURE OF INVENTION

[0007] The invention is directed to a cell line comprising a cellular genome, wherein the cellular genome comprises a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, wherein the cell line is a hematopoietic progenitor cell line, and wherein the cell line is conditionally-immortalized.

[0008] The invention also provides a conditionally-immortalized hematopoietic progenitor cell line generated by a method comprising (a) providing embryonic stem cells from a mammal, wherein the embryonic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, (b) initiating embryoid body (EB) formation in the embryonic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic progenitor cells,

and (c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

[0009] Further provided by the invention are methods of generating a conditionally-immortalized hematopoietic progenitor cell line. In a first method, the method comprises (a) providing hematopoietic stem cells from a mammal, wherein the hematopoietic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, (b) initiating differentiation in the hematopoietic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic progenitor cells, and (c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

[0010] In a second method, the method comprises (a) providing embryonic stem cells from a mammal, wherein the embryonic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, (b) initiating EB formation in the embryonic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic progenitor cells, and (c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

[0011] The invention provides a method of generating erythrocytes. The method comprises (a) providing cells from the iEBHX1S-4 cell line, (b) culturing the cells in medium containing KL (c-kit ligand), IL-3 (interleukin-3), Epo (erythropoietin), and doxycycline, and (c) culturing the cells in medium that lacks doxycycline, thereby inducing the cells to differentiate into erythrocytes.

[0012] A method of generating granulocytes is provided by the invention. The method comprises (a) providing cells from the iEBHX43 cell line, (b) culturing the cells in medium containing KL, IL-3, Epo, doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises GM-CSF (granulocyte mactophage-colony stimulating factor) or G-CSF (granulocyte- colony stimulating factor), thereby inducing the cells to differentiate into granulocytes.

[0013] Also provided by the invention are methods of generating monocytes/macrophages, granulocytes, and megakaryocytes. In a first method, the method comprises (a) providing cells from the iEBHX8S-5 cell line, (b) culturing the cells in medium containing KL, IL-3, Epo, and doxycycline, and (c) culturing the

cells in medium that lacks doxycycline and comprises methylcellulose, GM-CSF, G-CSF, M-CSF (macrophage colony stimulating factor), KL, IL-3, IL-5 (interleukin-5), IL-6 (interleukin-6), IL-11, TPO (thrombopoietin), and Epo, thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, and megakaryocytes. In a second method, the method comprises (a) providing cells from the iEBHX24 cell line, (b) culturing the cells in medium containing KL, IL-3, Epo and doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises GM-CSF, G-CSF, M-CSF, and TPO, thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, and megakaryocytes.

[0014] The invention provides a method of generating monocytes/macrophages, granulocytes, megakaryocytes and erythrocytes. The method comprises (a) providing cells from the iEBHX11-T, iEBHX12-T, or iEBHX12-T2 cell line, (b) culturing the cells in medium containing TPO and doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises methylcellulose, GM-CSF, G-CSF, M-CSF, Epo, KL, IL-3, IL-5, IL-6, IL-11 and TPO, thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, megakaryocytes and erythrocytes.

[0015] Further provided by the invention is a method of performing a blood transfusion in a host. The method comprises (a) providing conditionally-immortalized hematopoietic progenitor cells, (b) culturing the conditionally-immortalized hematopoietic progenitor cells under conditions wherein the conditionally-immortalized hematopoietic progenitor cells differentiate into erythrocytes and/or platelets, and (c) administering to the host the erythrocytes and/or platelets obtained in step (b), whereupon a blood transfusion is performed in the host.

[0016] The invention provides a method of determining whether or not an agent influences hematopoiesis. The method comprises (a) providing a conditionally-immortalized hematopoietic progenitor cell line, (b) culturing the cell line in medium comprising an agent, (c) monitoring hematopoiesis, and (d) determining whether or not the agent influences hematopoiesis.

[0017] A method of screening a drug candidate for an effect on a hematopoietic cell is also provided. The method comprises (a) providing a conditionally-immortalized hematopoietic progenitor cell line, (b) culturing the cell line in medium comprising one or more agents that induce differentiation of the cell line, (c) contacting the cell line with the drug candidate, and (d) assaying the effect of the drug candidate.

BEST MODE FOR CARRYING OUT THE INVENTION

[0018] The invention provides a cell line comprising a cellular genome, wherein the cellular genome comprises a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, wherein the cell line is a hematopoietic progenitor cell line, and wherein the cell line is conditionally-immortalized.

[0019] For purposes herein, the term "cell line" as used herein refers to a collection of cells having the same genetic origin, i.e., a collection of cells that are genetic clones of one another. In this regard, the term "hematopoietic progenitor cell line" as used herein means a cell line that is an immediate progeny of hematopoietic stem cells that results in the production of mature blood cells.

[0020] The inventive cell line comprises a cellular genome, which comprises a nucleic acid sequence encoding HOX11. The term "HOX11" as used herein refers to the homeobox gene having the nucleotide sequence found in the National Center for Biotechnology Information (NCBI) GenBank database as Accession No. S38742, which encodes the protein having the amino acid sequence found in the NCBI Genbank as Accession No. AAB19293. The encoded HOX11 protein is believed to function primarily as a transcriptional regulator (Owens et al., *Blood*, *101*, 4966-4974 (2003); and Dear et al., *Proc. Natl. Acad. Sci. U.S.A.*, *90*, 4431-5 (1993)). HOX11 is normally not expressed in the hematopoietic system, although this gene induces T-cell leukemia as a consequence of the t(10;14)(q24;q11) and t(7;10)(q35;q24) chromosomal translocations involving the T-cell receptor δ and β loci, respectively (Dube et al., *Blood*, *78*, 2996-3003 (1991); Hatano et al., *Science*, *253*, 79-82 (1991); Kennedy et al., *Proc. Natl. Acad. Sci. U.S.A.*, *88*, 8900-8904 (1991); and Lu et al., *EMBO J.*, *10*, 2905-2910 (1991)).

[0021] The nucleic acid sequence encoding HOX11 is operably linked to an inducible promoter. By "inducible promoter" is meant any promoter having the ability to control the expression of a gene upon the addition of one or more exogeneous factors. Inducible promoters are known in the art. Heat shock proteins, hormones such as glucocorticoids, estrogens, progesterones and androgens, as well as heavy metal ions have all been used to artificially control gene expression *in vitro* and *in vivo* in experimental models (Searle et al., *Mol. Cell. Biol.*, *5*, 1480-9 (1985); Brinster et al., *Nature*, *296*, 39-42 (1982); Israel et al., *Nucleic Acids Res.*, *17*, 4589-604 (1989); Mayo et al., *Cell*, *29*, 99-108 (1982); Lee et al., *Biol. Signals*, *5*, 180-91 (1996); Lee, et al., *Nature*, *294*, 228-32 (1981); Klock et al., *Nature*, *329*, 734-6 (1987); Hynes et al., *Proc. Natl. Acad. Sci. U.S.A.*, *78*, 2038-42 (1981)). Several "gene switches" (inducible promoters) have been developed by combining DNA-binding and transcriptional activation domains from

bacterial, yeast, *Drosophila* and mammalian proteins to create hybrid transcription factors for achieving regulated transgene expression. These have included systems based on the *E. coli lacI* repressor protein, the *Drosophila* ecdysone steroid hormone receptor, the drug rapamycin and the human FKBP12 and FRAP proteins, the yeast *GAL4* DNA binding domain and a mutated human progesterone receptor, and the *E. coli tet* operon (Brown et al., *Cell*, *49*, 603-12 (1987); No et al., *Proc. Natl. Acad. Sci. U.S.A.*, *93*, 3346-51 (1996); Rivera et al., *Nat. Med.*, *2*, 1028-32 (1996); Wang et al., *Gene Ther.*, *4*, 432-41 (1997); Gossen et al., *Proc. Natl. Acad. Sci. U.S.A.*, *89*, 5547-51 (1992)). Regulatable systems based on the *E. coli tet* operon offer a number of advantages compared to other expression systems. In particular, tetracycline derivatives are nontoxic, and, because the method involves a regulatory circuit not used by eukaryotic cells, transgene expression can be controlled without affecting the expression of endogenous genes (Furth et al., *Proc. Natl. Acad. Sci. U.S.A.*, *91*, 9302-6 (1994)).

[0022] Two complementary versions of the tetracycline-regulatable system have been developed — commonly referred to as "tet-off" and "tet-on". A key component of both systems is a minimal promoter, usually derived from the human cytomegalovirus (CMV) immediate early region, fused to seven copies of the *tet* operon sequence ($P_{(tetO)7\text{-}CMVmin}$). In the tet-off system, a tetracycline-regulatable transcriptional activator (tTA), a fusion protein consisting of the repressor of the *tet* operon (TetR) and the activation domain of the viral protein VP16 of herpes simplex virus, activates transcription by binding to $P_{(tetO)7\text{-}CMVmin}$ in the absence of tetracycline. In the presence of low concentrations of tetracycline, tTA is prevented from binding to $P_{(tetO)7\text{-}CMVmin}$ and transcription is abolished. In the tet-on system, a "reverse" transactivator (rtTA) with the opposite properties of tTA binds to $P_{(tetO)7\text{-}CMVmin}$ and activates transcription only in the presence of tetracycline derivatives like doxycycline (DOX) (Gossen et al., *Science*, *268*, 1766-9 (1995)). With respect to the invention, the inducible promoter preferably is responsive to doxycycline.

[0023] The inventive cell line is conditionally-immortalized. As used herein, the term "conditionally-immortalized" means that the cell line is immortalized and prevented from further differentiation under certain conditions. Without being held to a particular theory, the cell line of the invention is conditionally-immortalized due to the inducible expression of the nucleic acid sequence encoding HOX11. In particular, the expression of HOX11 confers immortalization on the cell line.

[0024] Furthermore, the inventive cell line can differentiate into cells belonging to a specific cell lineage when the inducible expression of the nucleic acid sequence encoding HOX11 is terminated. By "cell lineage" is meant a specific

subset of cells within the hematopoietic system. Preferably, the inventive cell line differentiates into cells belonging to a myeloid lineage when the inducible expression of the nucleic acid sequence encoding HOX11 is terminated. Cells of a myeloid lineage include monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes. Cells of an erythroid lineage include those expressing hemoglobin and other erythroid-specific markers, the most mature of which are the enucleated erythrocytes in adult mammals. Cells of a megakaryocytic lineage include those expressing the GPIIb/IIIa and GPIb-IX-V complexes and other megakaryocytic-specific markers, and those which stop mitosis and enter endomitosis during which DNA replication proceeds in the absence of cell division giving rise to polypoid cells that eventually shed enucleated platelets. Alternatively, it is preferred that the inventive cell line differentiates into cells belonging to a lymphoid lineage when the inducible expression of the nucleic acid sequence encoding HOX11 is terminated. Cells of a lymphoid lineage include B lymphocytes, T lymphocytes, and NK (natural killer) cells of the immune system. Methods of determining whether or not a cell line differentiates into cells belonging to an erythroid lineage, a megakaryocytic lineage, a myeloid lineage, or a lymphoid lineage are known in the art. See, for instance, Beutler, *Williams Hematology* 6th ed., Lictman, Coller, Kipps, and Seligsohn eds., McGraw-Hill (2001), and Handin, *Blood: Principles and Practice of Hematology*, Lux IV and Stossel, eds., Lippincott Williams and Williams (2003), and Example 2 set forth below.

[0025] The inventive cell line can be a unipotential, bipotential, tripotential, or multipotential hematopoietic progenitor cell line. By "unipotential" as used herein is meant that the cell line is precursory to a single cell lineage. In this regard, the terms "bipotential" "tripotential" and "multipotential" as used herein refers to cell lines that are precursory to two cell lineages, three cell lineages and multiple cell lineages, respectively. For instance, the inventive cell line can be a multipotential hematopoietic progenitor cell line that is a precursor to monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes. More specifically, the cell line can be the iEBHX11-T cell line, the iEBHX12-T cell line, or the iEBHX12-T2 cell line.

[0026] The inventive cell line can be a unipotential erythrocyte precursor cell line, such as the iEBHX1S-4 cell line.

[0027] The cell line of the invention can be a unipotential granulocyte precursor, such as the iEBHX43 cell line.

[0028] The cell line of the invention can be a tripotential precursor to monocytes/macrophages, granulocytes, and megakaryocytes. Such a cell line includes, for instance, the iEBHX8S-5 cell line and the iEBHX24 cell line.

[0029] The invention further provides methods of generating a conditionally-immortalized hematopoietic progenitor cell line. In a first method, the method comprises (a) providing hematopoietic stem cells from a mammal, wherein the hematopoietic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, (b) initiating differentiation in the hematopoietic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic progenitor cells, and (c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

[0030] The term "hematopoietic stem cells" as used herein means pluripotent cells that can undergo hematopoiesis to generate the cellular elements of blood, including red blood cells (erythocytes), leukocytes (monocytes/macrophages, granulocytes, B lymphocytes, T lymphocytes, NK cells, and myeloid and lymphoid dendritic cells), and megakaryocytes/platelets. The hematopoietic stem cells can be obtained from any source of hematopoietic tissue. Preferably, the hematopoietic stem cells of step (a) are obtained from bone marrow. The hematopoietic stem cells can alternatively be obtained from peripheral blood, e.g., mobilized peripheral blood, or umbilical cord blood. The term "mobilized peripheral blood" as used herein means blood isolated from humans after administration of specific growth factors, such as G-CSF, or novel drug candidates that block a specific cellular receptor, known as the CXCR4 chemokine receptor, which induce hematopoietic stem cell release from the bone marrow into the peripheral circulation.

[0031] The hematopoietic stem cells can be obtained from any host having hematopoietic stem cells. Preferably, the host is a mammal. For purposes of the invention, mammals include, but are not limited to, the order Rodentia, such as mice, and the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

[0032] In the first method of generating a conditionally-immortalized hematopoietic progenitor cell line described herein, differentiation is initiated in the hematopoietic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated. Methods of initiating differentiation in hematopoietic stem cells are known in the art. See, for instance, McNiece et al., *Exp. Hematol.*, *29*, 3-11, (2001).

[0033] One of ordinary skill in the art realizes that the way in which the expression of the nucleic acid sequence encoding HOX11 is terminated depends upon the type of inducible promoter that is operably linked to the nucleic acid sequence encoding HOX11. For instance, if the inducible promoter is responsive to doxycycline, then the hematopoietic stem cells are cultured under conditions which lack doxycycline, in order to keep the expression of HOX11 terminated.

[0034] In a second method of generating a conditionally-immortalized hematopoietic progenitor cell line, the method comprises (a) providing embryonic stem (ES) cells from a mammal, wherein the ES cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, (b) initiating embryoid body (EB) formation in the embryonic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic progenitor cells, and (c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

[0035] As used herein, the term "embryonic stem cells" or "ES cells" refers to pluripotential cells isolated from host blastocysts. Such ES cells are known in the art and are described in references, such as Evans et al., *Nature*, *292*, 154-156, (1981) and Thomson et al., *Science*, *282*, 1145-1147 (1998). The ES cells to be used in the inventive methods can be obtained from any mammal. The mammal can be any mammal, such as those described herein. Preferably, the mammal is a human.

[0036] In the second method of generating a conditionally-immortalized hematopoietic progenitor cell line described herein, EB formation is initiated while keeping the expression of the nucleic acid sequence encoding HOX11 terminated. The term "embryoid body" as used herein refers to multicellular spheroid structures in which hematopoiesis initiates within regions similar to yolk sac blood islands. Methods of initiating EB formation are known in the art. See, for instance, Doetschman et al., *J. Embryol. Exp. Morph.*, *87*, 27-45 (1985); Kennedy et al.,

Nature, *386*, 488-493 (1997); Choi et al., *Development*, *125*, 725-732 (1998)), and Example 1 set forth below.

[0037] In the aforementioned methods of generating a conditionally-immortalized hematopoietic progenitor cell line, the step of providing ES cells or hematopoietic stem cells can comprise contacting the stem cells from a mammal with a vector comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, such that the nucleic acid sequence encoding HOX 11 and the inducible promoter operably linked thereto are integrated into the cellular genome of the embryonic stem cells.

[0038] Methods of contacting cells with a vector are also well-known in the art. Suitable methods of contacting cells with a vector include, for instance, infection with a viral vector, transfection with a lipofection reagent, DEAE, calcium phosphate, electroporation, and the like. See Sambrook et al., *supra*.

[0039] The vector comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto can be any suitable vector, and can be used to transform or transfect any cell. Suitable vectors include those designed for propagation and expansion or for expression or both, such as plasmids, liposomes, molecular conjugates (e.g., transferring), and viruses. The vector can be selected from the group consisting of the pUC series, the pBluescript series (Stratagene, LaJolla, CA), the pET series (Novagen, Madison, WI), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clontech, Palo Alto, CA). Bacteriophage vectors, such as λGT10, λGT11, λZapII (Stratagene), λ EMBL4, and λ NM1149, also can be used. Examples of plant expression vectors include pBI101, pBI101.2, pBI101.3, pBI121, and pBIN19 (Clontech). Examples of animal expression vectors include pEUK-C1, pMAM, and pMAMneo (Clontech). Preferably, the vector is ploxHOX11 when the ES cell line is Ainv15.

[0040] The selection of vectors and methods to construct them are commonly known to persons of ordinary skill in the art and are described in general technical references (see, in general, "Recombinant DNA Part D," *Methods in Enzymology*, Vol. 153, Wu and Grossman, eds., Academic Press (1987), and Example 1 set forth below).

[0041] Constructs of vectors, which are circular or linear, can be prepared to contain an entire nucleic acid sequence encoding HOX11 as described above or a portion thereof ligated to a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived from ColE1, 2 mμ plasmid, λ, SV40, bovine papilloma virus, and the like.

[0042] In addition to the replication system and the inserted nucleic acid sequence, the construct can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to antibiotics, heavy metals, etc., complementation in an auxotrophic host to provide prototrophy, and the like. Preferably, the marker gene is the neomycin-resistance gene or the green fluorescent protein gene (Clontech).

[0043] An expression vector can comprise a native or nonnative promoter operably linked to the nucleic acid sequence encoding HOX11 as described above. The selection of promoters, e.g., strong, weak, inducible, tissue-specific, and developmental-specific, is within the ordinary skill in the art. The promoter is inducible, such as the minimal promoter-tet operator construct $P_{(tet0)7CMVmin}$, which consists of seven tet operators located upstream of a minimal sequence of the cytomegalovirus immediate early promoter (Gossen et al., *Science*, *268*, 1766-1769 (1995)). Similarly, the combining of a nucleic acid sequence encoding HOX11 as described above with an inducible promoter is also within the skill in the art. See Example 1 set forth below.

[0044] The vector containing the HOX11 gene can be designed for either transient expression or for stable expression. Preferably, the vector of the invention promotes stable expression, such that the vector is one that integrates into the genome of the hematopoietic progenitor cell. More preferably, the vector is ploxHOX11 when the ES cell line is Ainv15. Such vectors that integrate into the genome of a cell include, for example, non-episomal mammalian vectors and some viral vectors. For purposes of the invention, the vector is preferably a viral vector when the method comprises ES cell lines other than Ainv15 or hematopoietic stem cells as the starting cell populations.

[0045] Suitable viral vectors include, for example, retroviral vectors (such as lentiviral vectors), parvovirus-based vectors, e.g., adeno-associated virus (AAV)-based vectors, AAV-adenoviral chimeric vectors, adenovirus-based vectors, and *Herpes simplex* (HSV)-based vectors. These viral vectors can be prepared using standard recombinant DNA techniques described in, for example, Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994).

[0046] A retroviral vector is derived from a retrovirus. Retrovirus is an RNA virus capable of infecting a wide variety of host cells. Upon infection, the retroviral genome integrates into the genome of its host cell and is replicated along with host

cell DNA, thereby constantly producing viral RNA and any nucleic acid sequence incorporated into the retroviral genome. As such, long-term expression of a therapeutic factor(s) is achievable when using retrovirus. Retroviruses contemplated for use in the inventive cells are relatively non-pathogenic, although pathogenic retroviruses exist. When employing pathogenic retroviruses, e.g., human immunodeficiency virus (HIV) or human T-cell lymphotrophic viruses (HTLV), care must be taken in altering the viral genome to eliminate toxicity to the host. A retroviral vector additionally can be manipulated to render the virus replication-deficient. As such, retroviral vectors are considered particularly useful for stable gene transfer *in vivo*. Lentiviral vectors, such as HIV-based vectors, are exemplary of retroviral vectors used for gene delivery. Unlike other retroviruses, HIV-based vectors are known to incorporate their passenger genes into non-dividing cells and, therefore, can be of use in treating persistent forms of disease. It is preferable that the viral vector is an HIV-based lentiviral vector derived from pSINF (Ramezani et al., *Blood*, *101*, 4717-4724 (2003)).

[0047] The cell line produced by either of the above described methods is further provided by the invention. Namely, the invention also provides a conditionally-immortalized hematopoietic progenitor cell line generated by a method comprising (a) providing embryonic stem cells from a mammal, wherein the embryonic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, (b) initiating EB formation in the embryonic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic progenitor cells, and (c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line. Preferably, the inducible promoter is responsive to doxycycline. For purposes herein, it is preferable that the viral vector is an HIV-based lentiviral vector derived from pSINF (Ramezani et al., *Blood*, *101*, 4717-4724 (2003)).

[0048] As the inventive cell lines can differentiate into a specific cell lineage, the invention further provides methods of generating the cells of those lineages. In this regard, the invention provides, for instance, a method of generating erythrocytes. The method comprises (a) providing cells from the iEBHX1S-4 cell line, (b) culturing the cells in medium containing KL, IL-3, Epo, and doxycycline, and (c) culturing the cells in medium that lacks doxycycline, thereby inducing the cells to differentiate into erythrocytes.

[0049] A method of generating granulocytes is also provided by the invention. The method comprises (a) providing cells from the iEBHX43 cell line, (b) culturing the cells in medium containing KL, IL-3, Epo, doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises GM-CSF or G-CSF, thereby inducing the cells to differentiate into granulocytes.

[0050] Also provided by the invention are methods of generating monocytes/macrophages, granulocytes, and megakaryocytes. In a first method, the method comprises (a) providing cells from the iEBHX8S-5 cell line, (b) culturing the cells in medium containing KL, IL-3, Epo, and doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises methylcellulose, GM-CSF, G-CSF, M-CSF, KL, IL-3, IL-5, IL-6, IL-11, TPO, and Epo, thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, and megakaryocytes. In a second method, the method comprises (a) providing cells from the iEBHX24 cell line, (b) culturing the cells in medium containing doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises GM-CSF, G-CSF, M-CSF, and TPO, thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, and megakaryocytes.

[0051] The invention provides a method of generating monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes. The method comprises (a) providing cells from the iEBHX11-T, iEBHX12-T, or iEBHX12-T2 cell line, (b) culturing the cells in medium containing TPO and doxycycline, and (c) culturing the cells in medium that lacks doxycycline and comprises methylcellulose, GM-CSF, G-CSF, M-CSF, Epo, KL, IL-3, IL-5, IL-6, IL-11, and TPO, thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes.

[0052] In these inventive methods, the inventive cell lines are provided and cultured in medium containing the specified ingredients/agents. Without being bound to a particular theory, the doxycycline contained in the medium used in the methods described herein is believed to act on the inducible promoter to induce the expression of the nucleic acid sequence encoding HOX11, the expression of which leads to the immortalization of the cell line and the subsequent termination of expression of which leads to the differentiation of the cells into mature cells. Furthermore, the specified agents are believed to be necessary for cell growth and survival or differentiation and are specific to the cell lines provided and cells being generated. Methods of culturing the cell lines are known in the art. See, for instance, Keller et al., *Blood*, *92*, 877-887 (1998), and Examples 1 and 2 set forth below.

[0053] The invention provides a method of performing a blood transfusion in a host. The method comprises (a) providing conditionally-immortalized hematopoietic progenitor cells, (b) culturing the conditionally-immortalized hematopoietic progenitor cells under conditions wherein the conditionally-immortalized hematopoietic progenitor cells differentiate into cells of an erythroid lineage, a megakaryocytic lineage, a myeloid lineage, or a lymphoid lineage, and (c) administering to the host the cells provided in step (b), whereupon a blood transfusion is performed in the host.

[0054] The invention also provides a method of performing a blood transfusion in a host. The method comprises (a) providing conditionally-immortalized hematopoietic progenitor cells, (b) culturing the conditionally-immortalized hematopoietic progenitor cells under conditions wherein the conditionally-immortalized hematopoietic progenitor cells differentiate into erythrocytes and/or platelets, and (c) administering to the host the erythrocytes and/or platelets provided in step (b), whereupon a blood transfusion is performed in the host.

[0055] The transfusion can be performed in any host that, under normal conditions, has erythrocytes and platelets in the blood of the host. Preferably, the host is a mammal. More preferably, the mammal is a human.

[0056] One skilled in the art will appreciate that suitable methods of administering the erythrocytes and/or platelets of the invention are known, and, although more than one route can be used to administer a particular composition comprising the erythrocytes and/or platelets, a particular route can provide a more immediate and more effective response than another route.

[0057] Erythrocytes and/or platelets that can be used in the inventive method can be formed as a composition, such as a pharmaceutical composition. Pharmaceutical compositions containing the erythrocytes and/or platelets can comprise other cell types in addition to erythrocytes and/or platelets (such as human mesenchymal stem cells; see, for example, Angelopoulou et al., *Exp. Hematol.*, *31*, 413-420 (2003)). The pharmaceutical composition can further comprise one or more other pharmaceutically active agents or drugs, i.e., active agents other than the erythrocytes and/or platelets. Examples of pharmaceutically active agents or drugs suitable for use in the context of the inventive method include recombinant human Epo and/or recombinant human TPO.

[0058] The carrier can be any suitable carrier. Preferably, the carrier is a pharmaceutically acceptable carrier. With respect to pharmaceutical compositions, the carrier can be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the

erythrocytes and/or platelets and, if present, other pharmaceutically active agents or drugs, and by the route of administration.

[0059] The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those ordinarily skilled in the art and are readily available to the public. It is preferred that the pharmaceutically acceptable carrier be one which is chemically inert to the erythrocytes and/or platelets and, if present, other pharmaceutically active agent(s) or drugs, and one which has no detrimental side effects or toxicity under the conditions of use.

[0060] The choice of carrier will be determined in part by the particular method used to administer the erythrocytes and/or platelets. Accordingly, there are a variety of suitable formulations of the pharmaceutical composition of the present inventive methods. The following formulations for parenteral, subcutaneous, intravenous, intramuscular, and interperitoneal administration are exemplary and are in no way limiting.

[0061] Injectable formulations are among those formulations that are preferred in accordance with the invention. The requirements for effective pharmaceutical carriers for injectable compositions are well-known to those of ordinary skill in the art (see, e.g., *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986)).

[0062] Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The erythrocytes and/or platelets can be administered in a physiologically acceptable diluent in a pharmaceutical carrier, such as a sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol, isopropanol, or hexadecyl alcohol, glycols, such as propylene glycol or polyethylene glycol, dimethylsulfoxide, glycerol ketals, such as 2,2-dimethyl-1,3-dioxolane-4-methanol, ethers, such as poly(ethyleneglycol) 400, an oil, a fatty acid, a fatty acid ester or glyceride, or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant, such as a soap or a detergent, suspending agent, such as pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agents and other pharmaceutical adjuvants.

[0063] Oils, which can be used in parenteral formulations include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils include peanut, soybean, sesame, cottonseed, corn, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

[0064] Suitable soaps for use in parenteral formulations include fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents include (a) cationic detergents such as, for example, dimethyl dialkyl ammonium halides, and alkyl pyridinium halides, (b) anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylenepolypropylene copolymers, (d) amphoteric detergents such as, for example, alkyl-b-aminopropionates, and 2-alkyl-imidazoline quaternary ammonium salts, and (e) mixtures thereof.

[0065] The parenteral formulations will typically contain at least about 5×10^9 cells/ml. Preservatives and buffers may be used. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations will typically range from about 5% to about 15% by weight. Suitable surfactants include polyethylene sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

[0066] For purposes of the inventive transfusion method, the amount or dose of the erythrocytes and/or platelets administered to the mammal should be sufficient to perform a blood transfusion in a host, i.e., replace the erythrocytes and/or platelets of the host. In other words, the amount of erythrocytes and/or platelets administered should be sufficient to effect a therapeutic response in the host over a reasonable time frame. Particularly, the dose of the erythrocytes and/or platelets should be sufficient to perform a blood transfusion in the host within about 24 to about 72 hours, if not 1 - 5 days, from the time of administration. The dose will be determined by the efficacy of the particular erythrocytes and/or platelets and the condition of the animal (e.g., human), as well as the body weight of the animal (e.g., human) to be treated. Many assays for determining an administered dose are known in the art. For purposes of the invention, an assay, which comprises comparing the extent to which a desired post-transfusion erythrocyte or platelet

count is achieved within a clinically relevant period upon administration of a given dose of erythrocytes and/or platelets to a mammal among a set of mammals that are each given a different dose of the erythrocytes and/or platelets, could be used to determine a starting dose to be administered to a mammal. The extent to which a desired post-transfusion erythrocyte or platelet count is achieved within a clinically relevant period upon administration of a certain dose can be assayed as described in Heaton *Transfus. Med. Rev.*, *6*, 153-169 (1992), and Schlossberg et al., *Transfus. Apheresis. Sci.*, *28*, 221-226 (2003).

[0067] The size of the dose also will be determined by the existence, nature, and extent of any adverse side effects that might accompany the administration of particular erythrocytes and/or platelets. Ultimately, the attending physician will decide the dosage of the erythrocytes and/or platelets of the invention with which to treat each individual patient, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, the severity of the condition being treated, and the route of administration.

[0068] The invention also provides a method of determining whether or not an agent influences hematopoiesis. The method comprises (a) providing a conditionally-immortalized hematopoietic progenitor cell line, (b) culturing the cell line in medium comprising an agent, (c) monitoring hematopoiesis, and (d) determining whether or not the agent influences hematopoiesis.

[0069] The term "hematopoiesis" as used herein is meant the generation of the cellular elements of blood, including red blood cells, (erythrocytes), leukocytes, (monocytes/macrophages, granulocytes, B lymphocytes, T lymphocytes, NK cells, and myeloid and lymphoid dendritic cells), and megakaryocytes/platelets. The term "agent" as used herein refers to any chemical entity, naturally-occurring or synthetic, such as a drug, a protein, a peptide, a nucleic acid, a carbohydrate or a lipid.

[0070] A method of screening a drug candidate for an effect on a hematopoietic cell is also provided. The method comprises (a) providing a conditionally-immortalized hematopoietic progenitor cell line, (b) culturing the cell line in medium comprising one or more agents that induce differentiation of the cell line, (c) contacting the cell line with the drug candidate, and (d) assaying the effect of the drug candidate. The effect can be either a toxic effect or a therapeutic effect.

[0071] For purposes of the method of performing a blood transfusion in a host, the method of determining whether or not an agent influences hematopoiesis, and the method of screening a drug candidate for an effect on a hematopoietic cell, all of which are described herein, the conditionally-immortalized hematopoietic

progenitor cells preferably comprise a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto. Preferably, the inducible promoter is responsive to doxycycline.

EXAMPLES

[0072] These examples illustrate the invention but, of course, should not be construed as in any way limiting its scope.

[0073] For convenience, the following abbreviations are used herein: KL, c-kit ligand; IL-3, interleukin-3; Epo, erythropoietin; GM-CSF, granulocyte macrophage-colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; IL-5, interleukin-5; IL-6, interleukin-6; IL-11, interleukin-11, TPO, thrombopoietin; DOX, doxycycline; ES, embryonic stem; PMEFs, primary mouse embryonic fibroblasts; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; LIF, leukemia inhibiting factor; MTG, monothioglycerol; PCR, polymerase chain reaction; IMDM, Iscove's Modified Dulbecco's Medium; iEBHX, inducible embryoid body-derived HOX11; FITC, fluorescein isothiocyanate; PMA, phorbol 12-myristate 13-acetate; FACS, fluorescence activated cell sorting; and DCFDA, dichlorodihydrofluorescein diacetate.

[0074] Example 1

[0075] This example demonstrates the use of the invention to generate a conditionally-immortalized hematopoietic progenitor cell line.

[0076] *Embryonic Stem (ES) Cell Culture:* Mouse embryonic stem (ES) cells were co-cultured with G418-resistant primary mouse embryonic fibroblasts (PMEFs; Specialty Media, Phillipsburg, NJ) on gelatinized 35 mm tissue culture dishes. ES maintenance medium consisted of Dulbecco's modified Eagle medium (DMEM), 15% fetal bovine serum (FBS; BioWhittaker), 10% leukemia inhibitory factor (LIF)-conditioned medium from Chinese hamster ovary cells transfected with a LIF expression vector, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µM monothioglycerol (MTG, Sigma), all commercially available from Invitrogen unless otherwise noted. Cells were cultured at 37° C in a humidified incubator containing a 5% CO_2/air mixture and passaged every 3 days, maintaining between 8 x 10^4 and 1.5 x 10^6 cells per dish (Doetschman, et al., *J. Embryol. Exp. Morph.*, *87*, 27-45 (1985); Wiles et al., *Development*, *111*, 259-267 (1991); Keller et al., *Mol Cell Biol.*, *13*, 473-486 (1993)).

[0077] *Construction of the ploxHOX11 Plasmid and Generation of the HOX11-inducible ES Cell Line:* The ploxHOX11 targeting plasmid was generated by cloning a HOX11-IRES-EGFP EcoRI-SalI fragment into an EcoRI-SalI fragment of plox (Kyba et al., *Cell*, 109, 29-37 (2002)). This removed the neo-EGFP stuffer region from plox and inserted HOX11-IRES-EGFP. The doxycycline-inducible parental ES cell line Ainv15 (provided by George Daley, Harvard Medical School, Boston, MA) was electroporated in the presence of 100 µg ploxHOX11 targeting plasmid and 20 µg pSalk-Cre Cre recombinase expression plasmid (provided by George Daley) in electroporation buffer (Specialty Media, Phillipsburg, NJ). The electroporation conditions were: 270 V, 960 µF using a Gene Pulser II (BioRad, Hercules, CA). Electroporated ES cells were co-cultured with G418-resistant PMEFs in ES cell maintenance media supplemented with 500 µg/ml G418 (Invitrogen). Individual G418-resistant ES cell colonies were picked and expanded and examined by polymerase chain reaction (PCR) for confirmation of successful integration of the ploxHOX11 targeting plasmid. The primers used for PCR consisted of sequences within the ploxHOX11 targeting plasmid (*PGK* region) and within the *neo* gene in the parental Ainv15 ES cell line that would generate a 450 bp band. Primers: LoxinF 5'-CTAGATCTCGAAGGATCTGGAG-3' (SEQ ID NO: 1), LoxinR 5'-ATACTTTCTCGGCAGGAGCA-3' (SEQ ID NO: 2). PCR conditions: 10 minutes at 95° C, 40 cycles of (15 seconds at 95° C, 40 seconds at 58° C, 1 minute at 72° C), followed by 5 minutes at 72° C. Antibodies against SSEA-1 and Flk1-PE (BD Biosciences, San Jose, CA) were used to determine the differentiation status of iHOX11 ES cells. The MC-480 (SSEA-1) hybridoma developed by Solter and Knowles was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences at The University of Iowa (Iowa City, IA).

[0078] *Embryoid Body (EB) Formation, Hematopoietic Cell Differentiation, and iEBHX Hematopoietic Precursor Cell Line Derivation:* Two days prior to EB formation, ES cells were placed in pre-differentiation medium consisting of the ES maintenance medium with Iscove's modified Dulbecco's medium (IMDM) instead of DMEM. To induce EB formation, the protocol provided by StemCell Technologies (Vancouver, BC, Canada) was used. ES cells were mixed with ES-Cult™ methylcellulose (StemCell) supplemented with 40 ng/ml KL (PeproTech, Rocky Hill, NJ) and 150 µM MTG at a density of 5,000 cells/ml, and placed in 35 mm suspension dishes (StemCell) at 37° C in a humidified incubator containing a 5% CO_2/air mixture. Doxycycline in 0.5 ml ES-CULT was added to the dishes at 1 µg/ml at day 4 of differentiation. Embryoid bodies were recovered on day 6 of

differentiation in IMDM/2% FBS, centrifuged at 1200 rpm for 10 minutes, then disaggregated for 3 minutes in the presence of 0.05% Trypsin/EDTA (Invitrogen). Disaggregated EBs were passed through a 21G1.5 needle (Becton Dickinson) to facilitate the production of a single-cell suspension.

[0079] Single day-6 EB cells were plated at $4x10^4$ cells in methylcellulose-based MethoCult™ GF M3434 medium (StemCell) containing 50 ng/ml KL, 10 ng/ml IL-3, 10 ng/ml IL-6, 3 U/ml Epo, and 1 µg/ml doxycycline. After 6 days, 44 colonies were picked by pipette, washed free of methylcellulose, and placed in liquid cultures containing IMDM/10% FBS, 5.5 µM ß-mercaptoethanol (Invitrogen), 5% c-Kit ligand (KL)-conditioned medium from Chinese hamster ovary cells transfected with a KL expression vector, 5% IL-3-conditioned medium from X630-rIL3 cells, 3 U/ml Epo, and 1 µg/ml doxycycline (hereafter referred to as iEBHX media).

[0080] In order to obtain hematopoietic precursor cell lines that are conditionally immortalized by HOX11, the tet-on ES cell system was employed (Kyba et al., *Cell, 109*, 29-37 (2002)). The ploxHOX11-targeting plasmid was generated by cloning a HOX11-IRES-EGFP EcoRI-SalI fragment into corresponding EcoRI and SalI sites of the plasmid plox as described above. The doxycycline (DOX)-inducible HPRT target ES cell line Ainv15 was electroporated with the ploxHOX11 and pSalk-Cre (Cre recombinase) expression plasmids. Electroporated ES cells were selected for growth in the presence of G418, colonies were selected, and genomic DNA was analyzed for proper Cre recombinase-mediated homologous recombination of the HOX11 plasmid into the HPRT homing site. This strategy placed the HOX11-IRES-GFP sequence downstream of the tet-responsive element, thus producing ES cell lines with inducible HOX11 expression (hereafter referred to as iHOX11). The iHOX11 cell line chosen for further study has been shown to be SSEA-1 positive, indicative of an undifferentiated state, and gives rise to hematopoietic cells efficiently *in vitro* during EB formation.

[0081] Thirty four continuously growing cell lines that were established from these colonies were designated iEBHX (inducible embryoid body-derived HOX11 cell line). Of these, the iEBHX1, iEBHX8, iEBHX11, iEBHX12, iEBHX24, and iEBHX43 cell lines were selected for further study. The iEBHX1 and iEBHX8 cell lines ($2x10^6$ cells) were single-cell sorted based on GFP expression into 96-well plates containing iEBHX media, from which clones iEBHX1S-4 and iEBHX8S-5 were obtained (the suffix S denotes "sorted"). The iEBHX11 and iEBHX12 cell lines ($1x10^5$) were transferred into IMDM/10%FBS, 5.5 µM ß-mercaptoethanol, 1 µg/ml doxycycline, and 50 ng/ml thrombopoietin (TPO, PeproTech, Rocky Hill, NJ) as the

only growth factor, from which clones iEBHX11-T, iEBHX12-T and iEBHX12-T2 were obtained (the suffix T denotes "TPO-dependent").

[0082] *Flow cytometry, in situ and Western blot analysis:* Fluorescence activated cell sorting (FACS) analysis was detected using a BD LSR flow cytometer (BD Biosciences, San Jose, CA). 2.4G2-conditioned medium was used to block Fc-receptors. Mac-1, Gr-1, CD18, -CD62-L/L-selectin and CD49d/VLA-4 were purchased from BD Biosciences. For Western blot analysis, an antibody against HOX11 was used (Santa Cruz Biotech, Santa Cruz, CA). Blots were transferred onto nitrocellulose membranes, washed in PBST (PBS+0.1% Tween 20), blocked in PBST+5% non-fat dry milk (BioRad), then visualized using a goat anti-rabbit IgG-AP antibody (Santa Cruz), processed using the EFC Western Blotting kit (Amersham Biosciences., Piscataway, NJ) and analyzed using a STORM 860 imager (Molecular Dynamics).

[0083] To determine whether HOX11/GFP expression was reversibly regulated, the iHOX11 ES cell line was cultured in the presence or absence of 1 μg/ml DOX. When cultured in the presence of DOX for 3 days, HOX11 and GFP expression were detected by Western blot analysis and FACS (flow cytometric) analysis, respectively. More importantly, the expressions of HOX11 and GFP were down-regulated after iHOX11 cells were subsequently cultured in the absence of DOX for 3 days. These results show that HOX11 expression is tightly regulated by the addition or removal of DOX in the iHOX11 ES cell line.

[0084] iHOX11 ES cells were differentiated as previously described by formation of EBs (Keller et al., *Mol. Cell. Biol.*, *13*, 473-486 (1993); Dang et al., *Biotechnol. Bioeng.*, *78*, 442-453 (2002)), with the exception of the addition of DOX at 1 μg/ml at day 4 of differentiation. Whether or not HOX11 expression remains regulatable after differentiation into EBs and EB-derived cells was tested first. The iHOX11 cell line was differentiated in 1% methylcellulose with 40 ng/ml c-Kit ligand (KL, also known as stem cell factor). At day 4 of differentiation, DOX was added at 1 μg/ml to the EBs. After 2 days (6 days total EB formation), HOX11 and GFP expression were detected by Western blot analysis and fluorescence microscopy, respectively. EB-derived hematopoietic cell cultures were propagated for 8 weeks in the presence of IMDM/10% fetal bovine serum (FBS), KL, interleukin (IL-3), and erythropoietin (Epo). DOX was included in the culture medium to maintain differentiation arrest, then removed for three days in a subset of cells prior to Western blot analysis. The results demonstrated that, following differentiation of iHOX11 ES cells into EBs, efficient HOX11 regulation can still be achieved. The EB-derived hematopoietic cell cultures that continued to grow by 8 weeks in the

presence of DOX were designated iEBHX (inducible embryoid body-derived HOX11 cell line). By comparison, EB-derived hematopoietic cell cultures from parental Ainv15 ES cells and from iHOX11 ES cells that were passaged in the absence of DOX failed to continue to propagate after 4 weeks in culture.

[0085] This example demonstrated that conditionally-immortalized hematopoietic progenitor cell lines having the potential to differentiate can be generated using the methods of the invention described herein.

[0086] Example 2

[0087] This example characterizes the differentiation potential of the hematopoietic progenitor cell lines produced in Example 1.

[0088] In order to examine the differentiation potential of each iEBHX cell population that was derived in Example 1, a variety of different growth factors were evaluated, including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), Epo, KL, IL-3, IL-5, IL-6, IL-11, thrombopoietin (TPO), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF). Methylcellulose was also used in combination with the above growth factors, allowing assay of clonal differentiation. Upon withdrawal of DOX from the media of the iEBHX lines, cytopsin preparations, immunofluorescent analyses, and replating in secondary methylcellulose (all without DOX) were carried out to determine the full developmental potential of each line.

[0089] Phagocytosis of fluorescein isothiocyanate (FITC)-labeled *E. coli* was measured using the Vybrant Phagocytosis Assay Kit (Molecular Probes, Eugene, OR) as described by the manufacturer. $1.2x10^5$ cells in 300 μl PBS were added to 100 μl mouse serum (1:10 dilution of whole mouse serum in PBS). 100 μl FITC-labeled bacteria was added to each sample and cultured for 2 hours at 37° C. Prior to FACS analysis and microscopy, 150 μl trypan blue was added to each sample.

[0090] Superoxide anion formation was measured using the OxyBurst/DCFDA reagent (Molecular Probes) as described by the manufacturer. $1x10^5$ cells in 100 μl PBS were placed in a 96-well plate in the presence or absence of phorbol 12-myristate 13-acetate (PMA) at 100 ng/ml. 50 μl dichlorodihydrofluorescein diacetate (DCFDA) (final concentration of 5 μM) was added to each well, and fluorescent emission at 530 nm was measured every 5 minutes up to 1 hour.

[0091] Benzidine staining was performed by incubating $2x10^4$ cells in 200 μl for various time points. 20 μl of benzidine staining solution (500 μl 0.2% benzidine in

3% acetic acid added to 50 µl H_2O_2 immediately prior to staining) was added to the 200 µl cell suspension for 8 minutes, then visualized and scored by microscopy.

[0092] Acetylcholinesterase staining was performed by a 6 hour incubation of cytospin preparations with acetylcholine substrate solution (0.5 mg/ml acetylcholine (Sigma), 75 mM dibasic sodium phosphate, 5 mM sodium citrate, 3 mM copper sulfate, 0.5 mM potassium ferricyanide). Slides were then rinsed in distilled water, and fixed in 95% ethanol for 10 minutes. Slides were washed in distilled water, counterstained with May-Grunwald stain for 10 seconds, allowed to air-dry, then mounted with Permount (Fisher Scientific, Pittsburgh, PA) for visualization of positive (brownish red) staining within the cytoplasm.

[0093] iEBHX1S-4 cells required KL (2.5 % conditioned medium), IL-3 (5% conditioned medium), and Epo (3 U/ml) for optimal growth in culture, and represent unipotential erythroid precursors. Culture conditions consisted of IMDM plus 10% FBS, 5.5 µM ß-mecaptoethanol supplemented with the above cytokines at 37° C in a humidified incubator containing a 5% CO_2/air mixture. When DOX was removed for 3 days, 70% of the cells were positive by benzidine staining, indicative of hemoglobin production. To the contrary, when DOX was present for 3 days, only 14% of cells were positive for benzidine staining, demonstrating that continual expression of HOX 11 prevents differentiation of the iEBHX cell. In order to determine whether KL, IL-3, or Epo is required for erythroid differentiation, DOX was removed from iEBHX1S-4 cultures, and the cells were maintained in the presence of various combinations of these growth factors for 3 days. The results showed that Epo, the physiologic regulator of red blood cell production, is essential for effective erythroid differentiation of iEBHX1S-4 cells. However, mature enucleated red cells were not identified, probably reflecting the fact that existing culture conditions do not yet recapitulate the *in vivo* situation (Neildez-Nguyen et al., *Nat Biotechnol.*, *20*, 467-72 (2002)).

[0094] iEBHX43 cells exhibited a blast-like nuclear morphology (high nucleus-to-cytoplasmic ration) when maintained in KL (2.5% conditioned medium), IL-3 (5% conditioned medium), and Epo (3 Um/l) in the presence of DOX. Culture conditions consisted of IMDM plus 10% FBS and 5.5 µM ß-mecaptoethanol supplemented with the above cytokines at 37° C in a humidified incubator containing a 5% CO_2/air mixture. However, when DOX was removed and iEBHX43 cells were cultured for 7 days in the presence of GM-CSF, granulocytic differentiation occurred. Granulocyte differentiation was determined by cytospin analysis, and FACS analysis using antibodies against Gr-1 and Mac-1, along with neutrophil activation-specific antibodies (CD62-L, CD49d/VLA-4, CD18). It was determined that G-CSF

can also drive this differentiation, whereas, when DOX was removed and neither of these growth factors was present, cell death occurred. The granulocytes derived from iEBHX43 expressed the Mac-1 (monocyte- and granulocyte-specific) and Gr-1 (granulocyte-specific) antigens following differentiation, and were capable of phagocytosis and superoxide anion production. Because no other cell types have been observed under any of the other differentiation conditions examined, the iEBHX43 cell population represented an immortalized unipotential granulocytic precursor.

[0095] The iEBHX8S-5 and iEBHX24 cell populations were maintained in KL (2.5% conditioned medium), IL-3 (5% conditioned medium), and Epo (3 Um/l) in the presence of DOX, and formed cells that morphologically resemble monocyte/macrophages, granulocytes, and megakaryocytes when plated in methylcellulose (iEBHX8S-5) containing GM-CSF, G-CSF, M-CSF, Epo, KL, IL-3, IL-5, IL-6, IL-11, and TPO or maintained in suspension cultures (iEBHX24) containing GM-CSF, G-CSF, M-CSF and TPO in the absence of DOX for 7 and14 days respectively. Culture conditions consisted of IMDM plus 10% FBS and 5.5 µM ß-mecaptoethanol supplemented with the above cytokines at 37° C in a humidified incubator containing a 5% CO_2/air mixture. Consistent with the notion that iEBHX8S-5 and iEBHX24 cells were capable of differentiating into monocyte/macrophages, granulocytes, and megakaryocytes, cells expressing the Mac-1 and Gr-1 surface antigens, or which stained positive for acetylcholinesterase were detected.

[0096] The cell populations iEBHX11-T, iEBHX12-T, and iEBHX12-T2 were propagated in TPO (50 ng/ml) alone, the physiologic regulator of platelet production. Culture conditions consisted of IMDM plus 10% FBS and 5.5 µM ß-mecaptoethanol supplemented with the above cytokines, at 37° C in a humidified incubator containing a 5% CO_2/air mixture. When DOX was removed and the cell populations were cultured for 7 days in methylcellulose containing a variety of growth factors (GM-CSF, G-CSF, M-CSF, Epo, KL, IL-3, IL-5, IL-6, IL-11, and TPO), monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes were detected by cytochemical and FACS analyses. Interestingly, both the undifferentiated and differentiated cells from each population tested positive for the CD41/CD61 surface antigen (GPIIb/IIIa), a marker found on megakaryocytes and certain multipotential hematopoietic precursors (Mitjavila-Garcia et al., *Development, 129*, 2003-2013 (202); and Mikkola et al., *Blood, 101*, 508-516 (2003)).

[0097] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0098] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0099] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

04-06-18-43757X00-SEQ-LIST.txt
SEQUENCE LISTING

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Eaker, Shannon S.
Hawley, Teresa S

<120> CONDITIONALLY-IMMORTALIZED HEMATOPOIETIC PROGENITOR CELL LINES

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WHAT IS CLAIMED IS:

1. A cell line comprising a cellular genome, wherein the cellular genome comprises a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto, wherein the cell line is a hematopoietic progenitor cell line, and wherein the cell line is conditionally-immortalized.

2. The cell line of claim 1, wherein, when the inducible expression of the nucleic acid sequence encoding HOX11 is terminated, the cell line differentiates into cells belonging to myeloid lineage.

3. The cell line of claim 1, wherein, when the inducible expression of the nucleic acid sequence encoding HOX11 is terminated, the cell line differentiates into cells belonging to a lymphoid lineage.

4. The cell line of claim 1, wherein the inducible promoter is responsive to doxycycline.

5. The cell line of claim 1, wherein the cell line is a multipotential hematopoietic progenitor cell line.

6. The cell line of claim 5, wherein the cell line is a progenitor to monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes.

7. The cell line of claim 6, wherein the cell line is iEBHX11-T.

8. The cell line of claim 6, wherein the cell line is iEBHX12-T.

9. The cell line of claim 6, wherein the cell line is iEBHX12-T2.

10. The cell line of claim 1, wherein the cell line is a unipotential erythrocyte progenitor.

11. The cell line of claim 10, wherein the cell line is iEBHX1S-4.

12. The cell line of claim 1, wherein the cell line is a unipotential granulocyte progenitor.

13. The cell line of claim 12, wherein the cell line is iEBHX43.

14. The cell line of claim 1, wherein the cell line is a tripotential progenitor to granulocytes, monocytes/macrophages, granulocytes, and megakaryocytes.

15. The cell line of claim 14, wherein the cell line is iEBHX8S-5.

16. The cell line of claim 14, wherein the cell line is iEBHX24.

17. A method of generating a conditionally-immortalized hematopoietic progenitor cell line, which method comprises:

(a) providing embryonic stem cells from a mammal, wherein the embryonic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto,

(b) initiating embryoid body (EB) formation in the embryonic stem cells while keeping the expression of the nucleic acid encoding HOX11 terminated, thereby generating hematopoietic progenitor cells, and

(c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic progenitor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

18. The method of claim 17, wherein the step of providing embryonic stems cells comprises contacting embryonic stem cells from a mammal with a vector comprising a nucleic acid sequence encoding HOX11and an inducible promoter operably linked thereto, such that the nucleic acid sequence encoding HOX 11 and the inducible promoter operably linked thereto are integrated into the cellular genome of the embryonic stem cells.

19. The method of claim 17, wherein the inducible promoter is responsive to doxycycline.

20. A method of generating erythrocytes, which method comprises:

(a) providing cells from the iEBHX1S-4 cell line,

(b) culturing the cells in medium containing KL, IL-3, Epo, and doxycycline, and

(c) culturing the cells in medium that lacks doxycycline,

thereby inducing the cells to differentiate into erythrocytes.

21. A method of generating granulocytes, which method comprises:

(a) providing cells from the iEBHX43 cell line,

(b) culturing the cells in medium containing KL, IL-3, Epo, doxycycline, and

(c) culturing the cells in medium that lacks doxycycline and comprises GM-CSF or G-CSF,

thereby inducing the cells to differentiate into granulocytes.

22. A method of generating monocytes/macrophages, granulocytes, and megakaryocytes, which method comprises:

(a) providing cells from the iEBHX8S-5 cell line,

(b) culturing the cells in medium containing KL, IL-3, Epo, and doxycycline, and

(c) culturing the cells in medium that lacks doxycycline and comprises methylcellulose, GM-CSF, G-CSF, M-CSF, KL, IL-3, IL-5, IL-6, IL-11, TPO, and Epo,

thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, and megakaryocytes.

23. A method of generating monocytes/macrophages, granulocytes, and megakaryocytes, which method comprises:

(a) providing cells from the iEBHX24 cell line,

(b) culturing the cells in medium containing KL, IL-3, Epo, doxycycline, and

(c) culturing the cells in medium that lacks doxycycline and comprises GM-CSF, G-CSF, M-CSF, and TPO,

thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, and megakaryocytes.

24. A method of generating monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes, which method comprises:

(a) providing cells from the iEBHX11-T, iEBHX12-T, or iEBHX12-T2 cell line,

(b) culturing the cells in medium containing TPO and doxycycline, and

(c) culturing the cells in medium that lacks doxycycline and comprises methylcellulose, GM-CSF, G-CSF, M-CSF, Epo, KL, IL-3, IL-5, IL-6, IL-11 and TPO,

thereby inducing the cells to differentiate into monocytes/macrophages, granulocytes, megakaryocytes, and erythrocytes.

25. A method of performing a blood transfusion in a host, which method comprises

(a) providing conditionally-immortalized hematopoietic progenitor cells,

(b) culturing the conditionally-immortalized hematopoietic progenitor cells under conditions wherein the conditionally-immortalized hematopoietic progenitor cells differentiate into erythrocytes and/or platelets, and

(c) administering to the host the erythrocytes and/or platelets obtained in step (b), whereupon a blood transfusion is performed in the host.

26. The method of claim 25, wherein the host is a mammal.

27. The method of claim 26, wherein the mammal is a human.

28. The method of claim 25, wherein the conditionally-immortalized hematopoietic progenitor cells comprise a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto.

29. The method of claim 28, wherein the inducible promoter is responsive to doxycycline.

30. A method of determining whether or not an agent influences hematopoiesis, which method comprises:

(a) providing a conditionally-immortalized hematopoietic progenitor cell line,

(b) culturing the cell line in medium comprising an agent,

(c) monitoring hematopoiesis, and

(d) determining whether or not the agent influences hematopoiesis.

31. The method of claim 30, wherein the conditionally-immortalized hematopoietic progenitor cells comprise a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto.

32. The method of claim 31, wherein the inducible promoter is responsive to doxycycline.

33. A method of screening a drug candidate for an effect on a hematopoietic cell, which method comprises:

(a) providing a conditionally-immortalized hematopoietic progenitor cell line,

(b) culturing the cell line in medium comprising one or more agents that induce differentiation of the cell line,

(c) contacting the cell line with the drug candidate, and

(d) assaying the effect of the drug candidate.

34. The method of claim 33, wherein the effect is a toxic effect.

35. The method of claim 33, wherein the effect is a therapeutic effect.

36. The method of claim 33, wherein the conditionally-immortalized hematopoietic progenitor cells comprise a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto.

37. The method of claim 36, wherein the inducible promoter is responsive to doxycycline.

38. A method of generating a conditionally-immortalized hematopoietic progenitor cell line, which method comprises:

(a) providing hematopoietic stem cells from a mammal, wherein the hematopoietic stem cells comprise a cellular genome comprising a nucleic acid sequence encoding HOX11 and an inducible promoter operably linked thereto,

(b) initiating differentiation in the hematopoietic stem cells while keeping the expression of the nucleic acid sequence encoding HOX11 terminated, thereby generating hematopoietic precursor cells, and

(c) inducing the expression of the nucleic acid sequence encoding HOX11 in the hematopoietic precursor cells, thereby generating a conditionally-immortalized hematopoietic progenitor cell line.

39. The method of claim 38, wherein the hematopoietic stem cells of step (a) are obtained from bone marrow, peripheral blood, or umbilical cord blood.