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(54) Title: GENERATING POPULATIONS OF HUMAN BLOOD AND BLOOD VESSEL PROGENITORS FROM PLURIPOTENT STEM CELLS

(57) Abstract: Methods are provided for the efficient differentiation of hPSCs into hematopoietic stem cells in defined, monolayer conditions. The instant disclosure also provides systems, compositions, and kits for practicing the methods of the disclosure.



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GENERATING POPULATIONS OF HUMAN BLOOD AND BLOOD VESSEL PROGENITORS FROM PLURIPOTENT STEM CELLS

BACKGROUND

[0001] Manufacturing and engineering human blood and immune cells holds promise to treat myriad diseases, including various cancers (e.g., leukemia and lymphoma), autoimmune disorders (e.g., Type 1 Diabetes and multiple sclerosis), and genetic diseases (e.g., sickle cell anemia and inborn immunodeficiencies). These diseases are all caused by genetic mutations in the blood and immune systems, and can thus be treated or cured by replacing faulty blood or immune cells with healthy ones, or alternatively, engineering cells with new functionalities (e.g., to target cancers in the form of cancer immunotherapies). In particular, human pluripotent stem cells (hPSCs) provide a genetically-customizable platform for the large-scale *in vitro* manufacture of blood and immune cell-types (e.g., T cells, NK cells, red blood cells, and platelets) to serve a variety of applications, including cancer immunotherapy, blood transfusions, and disease modeling.

[0002] Differentiation of hPSCs into HSCs provides a foundation to generate all the aforementioned human blood and immune cell-types. However, hPSCs can generate thousands of different cell-types—including HSCs—through a sequence of branching lineage decisions. At each step of differentiation, stem cells can stray from the intended lineage path, inadvertently generating non-blood cells; this poses a formidable challenge to the goal of creating pure populations of HSCs *in vitro*. Navigating this complex and branching developmental landscape is predicated on (1) identifying the correct developmental progenitor that forms a desired cell-type and (2) discovering the combinations and timings of extracellular signals that must be turned on and off at each step along the way to guide differentiation towards desired lineages, in preference to unwanted fates. *In vitro* generation of HSCs from hPSCs therefore hinges on discovering how pluripotent cells naturally develop into HSCs *in vivo*.

[0003] The developmental precursors of blood-forming hematopoietic stem cells (HSCs)—which form all blood and immune cells for life—have been intensively studied, although important questions remain. Multiple models have been proposed for the developmental origins of HSCs. The first model suggested that blood and endothelial cells largely arise from independent precursors, on the account of single-cell lineage analyses in zebrafish, mouse, and chicken. The second model, suggests that endothelial cells (known as “hemogenic endothelium”) generate blood. This has been documented by multiple methods including live imaging, staining of fixed sections, and pseudotemporal inferences drawn from single-cell RNA-sequencing data (scRNAseq). These imaging and sequencing studies reveal that cells expressing certain HSC markers emerge from endothelial cells. Nevertheless, it is technically challenging to test whether the emergent cells are, strictly speaking, functional HSCs capable of engrafting mice and

generating all blood and immune cell-types. Taken together, multiple cellular precursors to HSCs have been suggested by various approaches, and broad consensus remains to be achieved.

[0004] These questions surrounding the embryonic origins of blood and mapping *in vivo* progenitor-progeny differentiation paths are of paramount importance when differentiating hPSCs into HSCs *in vitro*. There have been many successes to differentiate hPSCs into hematopoietic progenitors that are subsequently capable of generating a range of myeloid, erythroid and lymphoid cell-types *in vitro*. However, multiple challenges remain. First, these differentiation methods typically generate heterogeneous populations containing a mixture of blood and non-blood cells. This intimates that the signals controlling the segregation between blood vs. non-blood cells, at every step of differentiation, remain to be fully defined. Second, despite tremendous progress in differentiating hPSCs towards HSCs, the resultant hematopoietic progenitors often minimally express key HSC transcription factors (TFs) including *HLF*, *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10* (collectively referred to as "*HOXA5-10*"). *Hlf* is required to maintain functional HSCs and has emerged as one of the most specific markers of HSCs: within the blood system, it is highly restricted to HSCs, being lowly expressed by their immediate descendants (multipotent progenitors capable of short-term engraftment), but turned off in more restricted blood lineages. Likewise, *HOXA5-10* family members are required to maintain HSCs, and forced expression of *HOXA* and other genes enhances the engraftment of hPSC-derived hematopoietic progenitors. This underscores the importance of generating *HLF*⁺ *HOXA*⁺ hematopoietic progenitors from hPSCs, and discovering the extracellular signals that ignite expression of these hallmark HSC markers.

[0005] Developing methods for efficient production of HSC is therefore of great interest and is addressed herein.

SUMMARY

[0006] Methods are provided for efficient differentiation of human pluripotent stem cells (hPSC) into human hematopoietic stem cells (HSC) *in vitro* using extracellular factors to drive differentiation. The *in vitro* generated HSC can be characterized by the expression of transcription factors associated with naturally occurring HSC, including *HLF*, *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10* at levels comparable to human HSCs. Further, the *in vitro* generated HSC are shown to efficiently generate all blood lineages, including myeloid, lymphoid and erythroid lineages, e.g. B cells, T cells, NK cells, etc. The methods of the disclosure provide for a near-stoichiometric conversion of hPSCs into HSC. The ability to efficiently and rapidly generate hPSC-derived HSC cells provides a means to produce a variety of human blood and immune cells for research and therapeutic purposes.

[0007] The *in vitro* differentiation of the disclosure occurs through five sequential steps, generating posterior primitive streak, lateral mesoderm, arterial ECs, hemogenic ECs, and finally, HSC, in serum-free, monolayer culture, within 10 days. At each stage, specific factors are

provided, and in some cases, specific signaling pathways are inhibited, to achieve highly purified populations. In some embodiments greater than 80%, greater than 85%, greater than 90% of the final cell population express the HSC surface markers CD34, CD144, CD45; and the HSC transcription factors/chromatin regulators HLF, MECOM, MLLT3, RUNX1, MEIS1, and MYB.

[0008] In some embodiments, methods are provided for the use of the differentiated cell population in screening for cellular responses. In some embodiments, methods are provided for the use of the differentiated cell population in treating a subject for a condition, using the produced cell types, and/or terminally differentiated cells and tissues. In some embodiments systems and kits for producing HSC types and/or screening for cellular responses and/or treating subjects with such HSC are provided.

[0009] Aspects of the disclosure relate to methods of producing a substantially pure population of hematopoietic stem cells. As described herein, the level of purity of a particular purified population will vary depending on various factors and may be achieved through use of the cell derivation methods described herein. In some embodiments, such methods include the use of one or more binding agents used to isolate particular cell types. In other embodiments the cells are used without isolation of particular cell types.

[0010] Aspects of the disclosure relate to screening a substantially pure population of hematopoietic stem cells produced according to the methods, or a differentiated cell population derived therefrom, as described herein, for a cellular response to an agent or action of interest. In certain aspects, a method of screening a substantially pure population of hematopoietic stem cells for a cellular response comprises contacting a population of substantially pure population of hematopoietic stem cells with a pharmacological agent and evaluating the population of cells for a cellular response induced by the pharmacological agent. In certain aspects, the screening may be *in vitro* screening and the contacting may be performed *in vitro*. In certain aspects, the screening may be *in vivo* screening and the contacting may be performed by administering the pharmacological agent to a host animal that contains the population of cells.

[0011] Aspects of the disclosure relate to methods of treating a subject for a condition through the administration of a substantially pure population of hematopoietic stem cells derived or produced according to the methods described herein. In certain aspects, the method of treating a subject for a condition through administration of cells derived according to the methods as described herein may further include co-administration with at least one pro-survival or pro-engraftment factor. In certain aspects, the cells administered to a subject may be genetically modified at least one genetic locus.

[0012] Aspects of the disclosure include kits for the production, derivation, purification, and use of a substantially pure population of hematopoietic stem cells that include one or more induction compositions and/or one or more specific binding agents and/or combinations thereof. In certain aspects, such kits may or may not include one or more cell types described herein.

[0013] Aspects of the disclosure include systems for the production, derivation, purification, and use of a substantially pure population of hematopoietic stem cells that include one or more components configured to administer one or more induction compositions and/or one or more specific inducing agents and/or one or more specific binding agents and/or combinations thereof. In certain aspects, such systems are configured to administer such compositions and/or agents at specific amounts or for specific periods of time according to the methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0015] FIGS 1A-1M: Genetic lineage tracing reveals that artery endothelial cells give rise to HSCs *in vivo*. A) arrow. E: embryonic day. P: postnatal day. B) Female adult *Cx40-CreERT2/+* mice were intraperitoneally injected with (Z)-4OHT, and at different timepoints, peripheral blood was collected and liquid chromatography-tandem mass spectrometry was performed to quantify (Z)-4OHT levels. C) *In situ* hybridization of *Cx40* mRNA expression in E8.5 mouse embryos, using HCR3. Red arrows: paired dorsal aortae. D) scRNAseq of the entire E8.5 mouse embryo; data taken from a published resource. *Cx40* expression is more restricted than *VE-Cadherin* expression. E) Arteries were lineage-traced in *Cx40-CreERT2; Ai6 (zsGreen reporter)* embryos by administering 4OHT at E8.5, and immunostaining was performed on the E11.5 dorsal aorta. This revealed that CD45+ blood cells had originally derived from arteries (zsGreen+). The *Cx40-CreERT2* allele also encodes *RFP*, which was used to visualize Cx40+ cells. Scale bar: 50 μ m. F) Arteries were lineage-traced in *Cx40-CreERT2; Ai6 (zsGreen reporter)* embryos by administering 4OHT at E8.5, and flow cytometry was performed on the E11.5 dorsal aorta. This revealed that CD45+ CD144+ HSPCs had originally derived from arteries (zsGreen+). G) Arteries were lineage-traced in *Cx40-CreERT2; Ai6 (zsGreen reporter)* embryos by administering 4OHT at E8.5, and immunostaining was performed on the E11.5 yolk sac. This revealed that CD45+ blood cells had originally derived from arteries (zsGreen+). Scale bar: 50 μ m. H) Arteries were lineage-traced in *Cx40-CreERT2; Ai6 (zsGreen reporter)* embryos by administering 4OHT at E8.5, and flow cytometry was performed on the E11.5 yolk sac. This revealed that CD45+ CD144+ HSPCs had originally derived from arteries (zsGreen+). I) Arteries were lineage-traced in *Cx40-CreERT2; Ai6 (zsGreen reporter)* embryos by administering 4OHT at E8.5, and immunostaining was performed on the E16.5 fetal liver. This revealed that KIT+ (CD117+) HSPCs had originally derived from arteries (zsGreen+). Scale bar: 50 μ m. J) Arteries were lineage-traced in *Cx40-CreERT2; Ai6 (zsGreen reporter)* embryos by administering 4OHT at E8.5, and flow cytometry was performed on the E16.5 fetal liver. This revealed that CD150+

CD48- Lineage- Sca1+ Kit+ HSCs had originally derived from arteries (zsGreen+). K) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (zsGreen reporter) embryos by administering one dose of 4OHT at the indicated times (E7.5-E12.5), and flow cytometry was performed on the E14.5-E18.5 fetal liver (*left*). An example of CD150+ CD48- Lineage- Sca1+ Kit+ HSCs that had originally derived from arteries (zsGreen+) after E9.0 4OHT administration is shown (*right*). L) Arteries were lineage-traced in *Efnb2-CreERT2; Ai6* (zsGreen reporter) embryos by administering 4OHT at E8.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the E14.5-E18.5 fetal liver. M) Veins were lineage-traced in *Aplnr-CreERT2; Ai6* (zsGreen reporter) embryos by administering 4OHT at E9.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the E14.5-E18.5 fetal liver.

[0016] FIGS. 2A-2E: Artery-derived HSCs are functional *in vivo*. A) Experimental strategy. B) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (zsGreen reporter) embryos by administering 4OHT at E8.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) myeloid, erythroid, and lymphoid cells in the peripheral blood of 3-month-old adults. C) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (zsGreen reporter) embryos by administering 4OHT at either E8.0, E8.5, or E9.0, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) myeloid, erythroid, and lymphoid cells in the peripheral blood of 1- to 12-month-old adults. D) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (zsGreen reporter) embryos by administering 4OHT at E8.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the bone marrow of 6-month-old adults. E) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (zsGreen reporter) embryos by administering 4OHT at E8.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the bone marrow of 6- or 12-month-old adults.

[0017] FIGS. 3A-3F: Artery-derived HSCs are functional *in vivo*, upon transplantation. A) Experimental strategy. B) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* embryos by administering 4OHT at E8.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the E16.5 fetal liver. C) E16.5 fetal liver cells from lineage-traced *Cx40-CreERT2; Ai6* embryos (4OHT administered at E8.5) were transplanted into a lethally-irradiated primary recipient mouse. Four months post transplantation, flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the bone marrow. D) E16.5 fetal liver cells from lineage-traced *Cx40-CreERT2; Ai6* embryos (4OHT administered at E8.5) were transplanted into a lethally-irradiated primary recipient mouse. Four months post transplantation, flow cytometry was performed to quantify the percentage of artery-derived (i.e., zsGreen+) myeloid, erythroid, and lymphoid cells in the peripheral blood. E) After four months of

transplantation, primary recipient mice described in Fig. 3c,d were sacrificed, and bone marrow was transplanted into secondary recipient mice. Four months post transplantation, flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*+) CD150+ CD48-Lineage- Sca1+ Kit+ HSCs in the bone marrow. F) After four months of transplantation, primary recipient mice described in Fig. 3c,d were sacrificed, and bone marrow was transplanted into secondary recipient mice. Four months post transplantation, flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*+) myeloid, erythroid, and lymphoid cells in the peripheral blood.

[0018] FIGS. 4A-4E: Differentiation of hPSCs into posterior primitive streak competent to subsequently generate *HOXA*+ artery endothelial cells and *HOXA*+ *HLF*+ HSC-like cells. A) Overview of hPSC differentiation strategy (this study). B) qPCR (i,ii) and flow cytometry (iii) of hPSCs differentiated into anterior primitive streak (day 1, “APS”), mid primitive streak (day 1, “MPS”), posterior primitive streak (day 1, “day 1 PPS”), or posterior primitive streak (day 2, “day 2 PPS”). C) hPSCs were initially differentiated into anterior primitive streak (day 1), mid primitive streak (day 1), posterior primitive streak (day 1), or posterior primitive streak (day 2), and then further differentiated into artery endothelial cells, which were profiled by flow cytometry (i,ii), qPCR (iii), and immunostaining (iv). D) scRNAseq of undifferentiated hPSCs (day 0), posterior primitive streak (day 2), lateral mesoderm (day 3) and artery endothelial cells (day 4). Cells were colored by the day of differentiation they were profiled. E) hPSCs were initially differentiated into anterior primitive streak (day 1), mid primitive streak (day 1), posterior primitive streak (day 1), or posterior primitive streak (day 2), and then further differentiated into artery endothelial cells, which were profiled by flow cytometry (i,ii) and qPCR (iii).

[0019] FIGS. 5A-5F: Differentiation of hPSCs into hemogenic endothelium and *HOXA*+ *HLF*+ HSC-like cells. A) Flow cytometry of *RUNX1-mOrange* hPSC-derived hemogenic endothelium cells (i), or alternatively, immunostaining (ii) or scRNAseq (iii) of wild-type hPSC-derived hemogenic endothelium cells. B) Day 4 hPSC-derived artery endothelium cells were differentiated for 3 days into hemogenic endothelium cells, or alternatively, individual differentiation factors were individually withheld during hemogenic endothelium induction (i), followed by qPCR of day 7 hemogenic endothelium cells (ii) as well as qPCR of day 10 HSC-like cells derived from these hemogenic endothelium cells (iii, iv). C) Flow cytometry (i,ii) and absolute number/cell yield (iii) of day 10 hPSC-derived HSC-like cells. Each dot indicates the outcome of an independent experiment. D) Day 7 hPSC-derived hemogenic endothelium cells were differentiated for 3 days into HSC-like cells, or alternatively, individual differentiation factors were individually withheld during HSC-like cell induction (i), followed by qPCR of day 10 HSC-like cells (ii,iii). E) scRNAseq of day 10 hPSC-derived HSC-like cells. F) Bulk-population RNA-seq of day 10 hPSC-derived HSC-like cells, compared with published RNA-seq profiles of FACS-purified CD45+ CD144+ HSCs vs. CD45+ CD144- non-HSCs isolated from the aorta-gonad-mesonephros region (AGM) of a human embryo.

[0020] FIGS. 6A-H: hPSC-derived HSC-like cells generate lymphoid, myeloid, and erythroid cells *in vitro*. A) Overview of hPSC differentiation strategy (this study). B) Day 10 hPSC-derived HSC-like cells were differentiated in methylcellulose culture (i) and separately, into erythroid cells (ii) and macrophages (iii). C) Day 10 hPSC-derived HSC-like cells were differentiated into NK cells, which were profiled by flow cytometry (i,ii) and the absolute number/yield of NK cells generated per input HSC-like cell was quantified (iii). As a control, CD34⁺ human cord blood HSPCs were also differentiated into NK cells using the same protocol. hPSC-derived and cord blood-derived NK cells were mixed with fluorescent *DLL4-GFP* OP9 cells, and live imaging was performed to quantify the killing of GFP⁺ cells (iv). D) Day 10 hPSC-derived HSC-like cells were differentiated into T cells, which were profiled by flow cytometry (i,ii) and the absolute number/yield of T cells generated per input HSC-like cell was quantified (iii). Differentiation was conducted from both wild-type H7 hESCs and iLC13-F1 hiPSCs, the latter of which were originally reprogrammed from an EBV-specific T cell. E) Day 10 hPSC-derived HSC-like cells were differentiated into T cells, which were profiled by flow cytometry. Differentiation was conducted from both wild-type H7 hESCs and iLC13-F1 hiPSCs, the latter of which were originally reprogrammed from an EBV-specific T cell. F) Day 10 hPSC-derived HSC-like cells were differentiated into B cells, which were profiled by flow cytometry (i-iii) and the absolute number/yield of B cells generated per input HSC-like cell was quantified (iv). As a control, CD34⁺ human cord blood HSPCs were also differentiated into B cells using the same protocol. G) iSU223n hiPSCs (which were originally reprogrammed from patient AML cells) were differentiated into day 10 HSC-like cells, which were transplanted into NSG mice. Flow cytometry was performed of the bone marrow 3-months post-transplantation. H) *AkaLuciferase*-expressing hPSCs were differentiated day 10 HSC-like cells, which were intrafemorally transplanted into the right femur of NSG mice, followed by bioluminescent imaging to track *AkaLuciferase*-expressing cells *in vivo* (i) and flow cytometry of the bone marrow (iii). As a control, CD34⁺ human cord blood HSPCs were also transplanted into NSG mice (ii).

[0021] FIGS. 7A-7E: Marker expression and *in vivo* lineage tracing of HSC precursors. A) scRNAseq of the entire E8.5 mouse embryo; data taken from a published resource. B) scRNAseq of endothelial and hematopoietic cells within the E10 mouse embryo; data taken from a published resource. Nascent *Hlf*⁺ HSPCs still express *VE-Cadherin* (*Cdh5*) to some extent, but minimally express *Cx40*. C) scRNAseq of endothelial and hematopoietic cells within the E11 mouse embryo; data taken from a published resource. Nascent *Hlf*⁺ HSPCs still express *VE-Cadherin* (*Cdh5*) to some extent, but minimally express *Cx40*. D) Design of new *Efnb2-CreERT2* mouse allele. E) Veins were lineage-traced in *Aplnr-CreERT2*; *Ai6* (*zsGreen* reporter) embryos by administering 4OHT at E8.5, E9.0, or E9.5, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*⁺) CD150⁺ CD48⁻ Lineage⁻ Sca1⁺ Kit⁺ HSCs in the E14.5-E18.5 fetal liver.

[0022] FIGS. 8A-8D A) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (*zsGreen* reporter) embryos by administering 4OHT at E8.0, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*+) myeloid, erythroid, and lymphoid cells in the peripheral blood of 3-month-old adults. B) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (*zsGreen* reporter) embryos by administering 4OHT at E9.0, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*+) myeloid, erythroid, and lymphoid cells in the peripheral blood of 3-month-old adults. C) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (*zsGreen* reporter) embryos by administering 4OHT at E8.0, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the bone marrow of 6-month-old adults. D) Arteries were lineage-traced in *Cx40-CreERT2; Ai6* (*zsGreen* reporter) embryos by administering 4OHT at E9.0, and flow cytometry was performed to quantify the percentage of artery-derived (i.e., *zsGreen*+) CD150+ CD48- Lineage- Sca1+ Kit+ HSCs in the bone marrow of 6-month-old adults.

[0023] FIG. 9: Artery-derived HSCs are functional *in vivo*, upon primary and secondary transplantation. Flow cytometry of E16.5 fetal liver HSCs from lineage-traced *Cx40-CreERT2; Ai6* embryos (4OHT administered at E8.5), followed by peripheral blood and bone marrow analyses from primary and secondary recipient mice transplanted with lineage-traced fetal liver cells.

[0024] FIGS. 10A-10D: Stepwise differentiation of hPSCs into posterior primitive streak, lateral mesoderm, and artery endothelial cells. A) qPCR of hPSCs differentiated into anterior primitive streak (day 1, "APS"), mid primitive streak (day 1, "MPS"), posterior primitive streak (day 1, "day 1 PPS"), or posterior primitive streak (day 2, "day 2 PPS"). B) Bulk population RNA-seq and single-cell RNA-sequencing of undifferentiated hPSCs (day 0), posterior primitive streak (day 2), lateral mesoderm (day 3) and artery endothelial cells (day 4). For scRNAseq, cells were colored by the day of differentiation they were profiled. TPM: transcripts per million. C) hPSCs were initially differentiated into anterior primitive streak (day 1), mid primitive streak (day 1), posterior primitive streak (day 1), or posterior primitive streak (day 2), and then further differentiated into artery endothelial cells (i-v), or HSC-like cells (vi-x), which were profiled by flow cytometry. qPCR data were normalized to the sample with the highest expression in this experiment. D) hPSCs were differentiated into posterior primitive streak (day 2), which was subsequently differentiated into lateral mesoderm (day 3) in the presence or absence of retinoid pathway agonist TTNPB, followed by qPCR of lateral mesoderm cells (ii). Alternatively, hPSC-derived lateral mesoderm (day 3) was further differentiated into artery endothelial cells (day 4) in the presence or absence of retinoid pathway agonist TTNPB, followed by qPCR or flow cytometry of artery endothelial cells (iii-vi). qPCR data were normalized to the sample with the highest expression in this experiment.

[0025] FIGS. 11A-11G: Optimization of hPSC differentiation into hemogenic endothelium and *HOXA+ HLF+* HSC-like cells. A) Day 4 hPSC-derived artery endothelium cells were dissociated and re-plated at the indicated densities, followed by differentiation into day 7 hemogenic

endothelium cells (i) or day 10 HSC-like cells (ii,iii), which were analyzed by qPCR or flow cytometry. qPCR data were normalized to the sample with the highest expression in this experiment. B) Flow cytometry of *RUNX1-mOrange* hPSC-derived hemogenic endothelium cells, pre-gated on CD144+ endothelial cells (i), or alternatively, immunostaining (ii) or scRNAseq (iii) of wild-type hPSC-derived hemogenic endothelium cells. C) Day 4 hPSC-derived artery endothelium cells were differentiated for 3 days into hemogenic endothelium cells, or alternatively, individual differentiation factors were individually withheld during hemogenic endothelium induction, followed by continued differentiation into day 10 HSC-like cells, which were analyzed by flow cytometry. Individual differentiation factors were withheld only during hemogenic endothelium induction; the complete set of HSC-like cell induction signals were used. D) Day 7 hPSC-derived hemogenic endothelium cells were differentiated for 3 days into HSC-like cells, or alternatively, individual differentiation factors were individually withheld during HSC-like cell induction, followed by flow cytometry (i) or qPCR (ii) of day 10 HSC-like cells. E) Day 7 hPSC-derived hemogenic endothelium cells were differentiated for 3 days into HSC-like cells in the absence of PRC2 inhibitors, or alternatively, in the presence of PRC2 inhibitors UNC1999 and/or EED226. qPCR was performed on day 10 hPSC-derived HSC-like cells. qPCR data were normalized to expression in the negative control (absence of PRC2 inhibitors). F) Bulk-population RNA-seq of day 10 hPSC-derived HSC-like cells, compared with FACS-purified human cord blood HSCs (CD34+ CD90+ CD38- CD45RA-), MPPs (CD34+ CD90- CD38-) and downstream progenitors (CD34+ CD90- CD38+), as well as published RNA-seq profiles of FACS-purified CD45+ CD144+ HSCs vs. CD45+ CD144- non-HSCs isolated from the aorta-gonad-mesonephros region (AGM) of a human embryo. G) scRNAseq of day 10 hPSC-derived HSC-like cells.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0026] Methods are provided for the generation of substantially purified hematopoietic stem cells. Treatment methods making use of the generated hematopoietic stem cells are also provided. The instant disclosure also provides systems, compositions, and kits for practicing the methods of the disclosure.

[0027] Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular method or composition described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0028] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in

that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0029] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0030] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0031] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the peptide" includes reference to one or more peptides and equivalents thereof, e.g. polypeptides, known to those skilled in the art, and so forth.

[0032] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication date, which may need to be independently confirmed.

Definitions

[0033] The terms "treatment", "treating", "treat" and the like are used herein to generally refer to obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disease or symptom(s) thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. The term "treatment" encompasses any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease and/or symptom(s)

from occurring in a subject who may be predisposed to the disease or symptom(s) but has not yet been diagnosed as having it; (b) inhibiting the disease and/or symptom(s), i.e., arresting development of a disease and/or the associated symptoms; or (c) relieving the disease and the associated symptom(s), i.e., causing regression of the disease and/or symptom(s).

[0034] The terms “recipient”, “individual”, “subject”, “host”, and “patient”, are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans. “Mammal” for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, sheep, goats, pigs, camels, etc. In some embodiments, the mammal is human.

[0035] The terms “pluripotent progenitor cells”, “pluripotent progenitors”, “pluripotent stem cells”, “multipotent progenitor cells” and the like, as used herein refer to cells that are capable of differentiating into two or more different cell types and proliferating. Non limiting examples of pluripotent precursor cells include but are not limited to embryonic stem cells, blastocyst derived stem cells, fetal stem cells, induced pluripotent stem cells. Pluripotent progenitor cells may be acquired from public or commercial sources or may be newly derived. As described herein, in some instances, pluripotent progenitor cells of the subject disclosure are those cells capable of giving rise to hematopoietic stem cells.

[0036] The term “lineage bifurcation” and “lineage segregation” are used interchangeably herein and refer to a cell-fate decision where a stem cell and/or progenitor cell has the ability to differentiate into two or more cell-types.

[0037] The term “population”, e.g., “cell population” or “population of cells”, as used herein means a grouping (i.e., a population) of two or more cells that are separated (i.e., isolated) from other cells and/or cell groupings. For example, a 6-well culture dish can contain 6 cell populations, each population residing in an individual well. The cells of a cell population can be, but need not be, clonal derivatives of one another. A cell population can be derived from one individual cell. For example, if individual cells are each placed in a single well of a 6-well culture dish and each cell divides one time, then the dish will contain 6 cell populations. The cells of a cell population can be, but need not be, derived from more than one cell, i.e. non-clonal. The cells from which a non-clonal cell population may be derived may be related or unrelated and include but are not limited to, e.g., cells of a particular tissue, cells of a particular sample, cells of a particular lineage, cells having a particular morphological, physical, behavioral, or other characteristic, etc. A cell population can be any desired size and contain any number of cells greater than one cell. For example, a cell population can be 2 or more, 10 or more, 100 or more, 1,000 or more, 5,000 or more, 10^4 or more, 10^5 or more, 10^6 or more, 10^7 or more, 10^8 or more, 10^9 or more, 10^{10} or more, 10^{11} or more, 10^{12} or more, 10^{13} or more, 10^{14} or more, 10^{15} or more, 10^{16} or more, 10^{17} or more, 10^{18} or more, 10^{19} or more, or 10^{20} or more cells.

[0038] The terms “homogenous population”, as it relates to cell populations, refers to a cell population that is essentially pure and does not consist of a significant amount of undesired or contaminating cell types. By significant amount, in this context, is meant an amount of undesired or contaminating cell types that negatively impacts the use of the isolated desired cell population. As such, the actual amount of undesired or contaminating cells that defines a significant amount will vary and depend on the particular type of undesired or contaminating cells and/or the particular use of the desired cell type. For example, in a population of differentiated cells used in the treatment of a subject, a significant amount of improperly differentiated contaminating cell types will be small as such cells may have a high capacity to negatively impact the use of the generated desired cell population. In comparison, e.g., in a population of differentiated cells used in the treatment of a subject, a significant amount of contaminating progenitor cells may be relatively large as such cells may have a low capacity to negatively impact the use of the generated desired cell population. In some instances, a homogenous population may refer to a highly enriched population. Levels of homogeneity will vary, as described, and may, in some instances, be greater than 60% pure, including e.g., more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99%, more than 99.5%, more than 99.6%, more than 99.7%, more than 99.8%, and more than 99.9%.

[0039] The term “heterologous”, as it refers to a “heterologous sequence” or “heterologous nucleic acid”, means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For example, a polynucleotide introduced by genetic engineering techniques into a plasmid or vector derived from a different species is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence with which it is not naturally found linked is a heterologous promoter.

[0040] The term “high cell density”, as it relates to cells, means the cell numbers within an area or volume is high. For example, cells are in close contact with one another when cultured in high cell density. In some embodiments high cell density refers to a density of at least about 1.25×10^6 cells/cm².

Methods

[0041] Methods and compositions are provided for producing a substantially pure population of hematopoietic stem cells (HSC) in defined monolayer conditions in media comprising extracellular signaling agents to guide differentiation. Aspects of the disclosure include methods for deriving hematopoietic stem cells from pluripotent progenitor cells.

[0042] Pluripotent progenitors of the instant disclosure may be acquired from any convenient source, including but not limited to newly derived from a subject of interest or tissue specimen or other cellular sample, obtained from a public repository, obtained from a commercial vendor, and the like. In some instances, pluripotent cells of interest include human cells including but not

limited to, e.g., human embryonic stem cells, human induced pluripotent stem cells, human fetal stem cells, and the like. Exemplary pluripotent cells include H1, H7 and H9 hESCs, as known in the art. Exemplary induced pluripotent stem cells include iPSCs derived from peripheral blood mononuclear cells, fibroblasts, and other somatic cell sources, as known in the art.

[0043] In some instances, pluripotent progenitor cells of the subject disclosure may be unmodified such that the cells have not been genetically or otherwise modified from their natural state prior to modification according the methods described herein. Exemplary modified pluripotent cells include HES3 *MIXL1-GFP* hESCs, H1 *SOX17-2A-mPlum* hESCs, TkDA3-4 *RUNX1-2A-mOrange* hiPSCs and others known in the art. In other instances, pluripotent progenitor cells of the subject disclosure may be unmodified such that the cells have been genetically or otherwise modified from their natural state prior to modification according the methods described herein.

[0044] Generation of hematopoietic stem cells from pluripotent progenitors as described herein generally involves one or more lineage restriction events in which cultured pluripotent progenitor cells are subjected to one or more treatments causing the cultured cells or a population thereof to differentiate along specific pathways. Multiple lineage restriction events are required to achieve desired hematopoietic stem cells. In certain instances, lineage restriction events are performed successively such that a first cell type is achieved by a first lineage restriction event and the first cell type is subjected to a second lineage restriction event to achieve a desired second cell type; etc.

[0045] Lineage restriction events as described herein are induced by compositions of extracellular agents that act on specific signaling pathways in cultured cells, including those agents that activate or inhibit developmental signaling pathways that drive development. As will be clear from the instant disclosure, whether activation or inhibition of a particular signaling pathway is necessary to generate a particular cell type of interest will depend on a number of factors including but not limited to, e.g., the particular desired cell type, the timing of use of the particular agent and/or composition, the starting cell type to be induced, etc.

[0046] The culture of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), may be performed using methods known in the art. The cells are grown on suitable culture substrate that supports hPSC growth and maintains their undifferentiated state. Commonly used substrates include Matrigel, laminin, or synthetic polymers, with a defined and optimized culture medium to support hPSC growth and pluripotency. Commonly used media include mTeSR1, Essential 8, or other commercially available formulations, and may be supplemented with basic fibroblast growth factor (bFGF). The cells are generally passaged as clusters.

[0047] The methods of the invention may be carried out by any number of cell culture processes. In some embodiments, one or more culturing steps, for example, all culturing steps, are performed using cells plated on a substrate. Defined and xeno-free substrates are

advantageously used. Exemplary substrates include recombinant proteins, e.g. recombinant human proteins, for example, vitronectin, collagen, atelocollagen, hyaluronic acid, elastin, proteoglycan, glucosaminoglycan, fibronectin, laminin, collagen IV, heparan sulfate proteoglycan, entactin and nidogen. Commercially available basement membrane extracts, for example, containing proteins such as laminin, collagen IV, entactin, and heparin sulfate proteoglycans, for example, GELTREX(TM) substrate may be used.

[0048] In alternative embodiments, one or more cell culture steps is performed in liquid culture, for example, lacking a culture substrate.

[0049] Exemplary cell culture systems include planar vessels such as a T-flask, roller bottles, or multilayer plates, 3-dimensional cell culture systems, and liquid culture systems such as bioreactors.

[0050] Culture medium may comprise any medium suitable for cell culture, including CDM2, CDM3, Essential 6, Essential 8, Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) media. The medium in which the cells are cultured may be modified at each step to include one or more agents that promote differentiation to a target cell type.

[0051] In the methods of the invention, cells will be referred to as being contacted with one or more agents that, in combination, promote differentiation of a selected cell type to a different, target cell type. Such contacting may be achieved by addition of the one or more agents into the liquid culture medium. Alternatively, such contacting may be achieved by the presence of the one or more selected agents in the culture substrate. For illustration, in certain implementations of the invention, in various steps, the cells are contacted with a NOTCH activating agent, wherein the NOTCH activating agent is present in the culture substrate material. For example, 20 nM of the high-affinity, engineered NOTCH agonist DLL4-E12 (Luca et al., 2015) can be added to 10 µg/mL vitronectin solution prior to coating cell culture plates, therefore immobilizing the NOTCH agonist in the vitronectin substrate where it contacts the cells.

[0052] In one implementation, the scope of the invention encompasses a method of producing a population of hematopoietic stem cells from hPSCs. In one embodiment, the scope of the invention encompasses a method of producing a substantially pure population of hematopoietic stem cells in defined monolayer conditions, in media comprising extracellular signaling agents to guide differentiation, the method comprising:

- (a) differentiating human pluripotent stem cells into posterior primitive streak cells;
- (b) differentiating posterior primitive streak cells into lateral mesoderm cells;
- (c) differentiating lateral mesoderm cells into artery endothelium cells;
- (d) differentiating artery endothelium cells into hemogenic endothelium cell; and
- (e) differentiating hemogenic endothelium cells into hematopoietic stem cells.

[0053] Methods of achieving each step of the foregoing process are described next.

[0054] Posterior Primitive Streak Cell Differentiation. In one aspect, the scope of the invention comprises a method of differentiating human pluripotent stem cells to posterior primitive streak

cells. In one implementation, the posterior primitive streak cell differentiation process is performed as a first step in the differentiation of hematopoietic stem cells from hPSCs. Undifferentiated hPSCs are seeded for differentiation as single cells. In one embodiment, the undifferentiated hPSCs are cultured on a substrate and are plated at a density of 30,000-50,000 cells/cm². To induce posterior primitive streak cells, the pluripotent stem cells are induced by contacting the cells with an effective amount of: an FGF agent; a WNT agent; and a BMP agent, for a period of time sufficient to generate posterior primitive streak (PPS) cells. For example, the period of time may be about 2 days, e.g. from about 40-52 hours, or around 48 hours. In one embodiment, the contacting is achieved by culturing the cells a medium, such as CDM2 basal medium, comprising an FGF agent; a WNT agent; and a BMP agent.

[0055] Posterior primitive streak (PPS) cells, for example, day 2 cells, can be characterized by expression of HOXA5-HOXA10, for example by expression of BRACHYURY, MIXL1, HOXA5, HOXA7, HOXA9, HOXA10, CDX1, CDX2, and CDX4. A high percentage of the cells, e.g. greater than about 95%, greater than 98%, express the primitive streak marker MIXL1.

[0056] In some embodiments of the PPS cell differentiation process, exemplary FGF agents include FGF2 (for example, at 0.01-1000 ng/mL, for example at about 20 ng/mL), FGF1, FGF8, FGF10, and other FGF pathway ligands. Exemplary BMP agents include BMP4 (for example, at 0.01-1000 ng/mL, for example at about 40 ng/mL), BMP2, BMP7, GDF5, and other BMP pathway ligands, or small-molecule BMP pathway activators, such as FK506. Exemplary WNT agents include CHIR99021 (for example, at 0.001-1000 μ M, for example, at about 6 μ M), WNT3A or other pathway ligands, RSPO1, RSPO2, RSPO3, RSPO4, lithium chloride, BIO, BIO-acetoxime, and GSK3 inhibitors. In one embodiment, the medium does not include any TGF β agonist.

[0057] In one embodiment, the BMP agent comprises BMP4; the FGF agent comprises FGF2; and the WNT agent comprises CHIR99021.

[0058] Lateral Mesoderm Differentiation. In one aspect, the scope of the invention encompasses a method of differentiating posterior primitive streak cells into lateral mesoderm cells. In one implementation, the lateral mesoderm cell differentiation process is performed as a step, for example, the second step, in the differentiation of hematopoietic stem cells from hPSCs. In one embodiment, the posterior primitive streak cells are replated prior to the lateral mesoderm cell differentiation process. In one embodiment, the posterior primitive streak cells are present on a substrate and are differentiated to lateral mesoderm cells by changing the media composition as follows, without replating. In one embodiment, posterior primitive streak cells are cultured on a substrate and are present at a density of 30,000-50,000 cells/cm².

[0059] Posterior primitive streak cells may be differentiated to lateral mesoderm by contacting the cells with: an effective amount of a BMP agent; a cAMP-elevating agent; a retinoic acid pathway agonist; a VEGF agent; a TGF β inhibitor; a WNT inhibitor; and a PI3K inhibitor, for a period of time sufficient to produce lateral mesoderm cells. In some embodiments, the cells are further contacted with an effective amount of an ascorbic acid composition during the culture

period. The culture time period may be for about 1 day, e.g. from about 18-30 hours, or around 24 hours. In one embodiment, the contacting is achieved by culturing the cells in a medium, for example, a basal medium comprising CDM2 basal medium, wherein the medium comprises a VEGF agent; a TGF β inhibitor; a WNT inhibitor; and a PI3K inhibitor.

[0060] The lateral mesoderm cells will express markers of lateral mesoderm identity, for example, SCL, LMO2, FLI1, ETV2, HAND1, HOXA5, HOXA7, HOXA9, HOXA10.

[0061] In the lateral mesoderm differentiation method, exemplary BMP agents include BMP4 (for example, at 0.01-1000 ng/mL, for example at about 40 ng/mL), BMP2, BMP7, GDF5, and other BMP pathway ligands, or small-molecule BMP pathway activators, such as FK506. Exemplary cAMP-elevating agents include forskolin (for example, at 0.001-1000 μ M, for example, at about 10 μ M), adenylate cyclase agonists, activators of the cAMP/PKA pathway, NKH 477, PACAP 1-27, PACAP 1-38; cAMP or 8-bromo-cAMP (*R*)-(-)-Rolipram and other phosphodiesterase inhibitors. Exemplary retinoic acid pathway agonists include TTNPB (for example, at 0.001-10,000 nM, for example at about 0.5 nM), AM580, Bexarotene, and all-*trans* retinoic acid. Exemplary VEGF agents include VEGF (for example at about 0.01-10,000 ng/mL, for example at about 100 ng/mL), VEGF-A, VEGF-B, VEGF-C, VEGF-D and other VEGF receptor ligands, and FGF2 or other ligands for the FGF receptor, which is in the same receptor family as VEGF receptor. Exemplary TGF β inhibitors include SB-505124 (for example, at 0.001-1000 μ M, for example at about 2 μ M), LY 364947, A-83-01, RepSox or other small-molecule TGF β pathway inhibitors, Follistatin, and Lefty1, or Lefty2 or other proteins that inhibit extracellular TGF β ligands. Exemplary WNT inhibitors include XAV939 (for example at 0.001-1000 μ M, for example at about 1 μ M), C59, IWR1, LGK974 and other small-molecule WNT pathway inhibitors, or proteins such as DKK1 that inhibit WNT pathways. Exemplary and a PI3K inhibitors include GDC-0941 (for example, at 0.001-1000 μ M, for example, at about 2.5 μ M), LY294002, PI-103, and PIK90. Exemplary ascorbic acid compositions include 2-phospho-ascorbic acid (AA2P) (for example at 0.1-10,000 μ g/mL, for example at about 200 μ g/mL) and ascorbic acid.

[0062] In one embodiment the BMP agent comprises BMP4; the cAMP-elevating agent comprises Forskolin; the retinoic acid pathway activator comprises TTNPB; the VEGF agent comprises VEGF; the TGF β inhibitor comprises SB-505124; the WNT inhibitor comprises XAV939; and the PI3K inhibitor comprises GDC-0941 and the ascorbic acid agent comprises AA2P.

[0063] Artery Endothelium Differentiation. In one aspect, the scope of the invention encompasses a method of differentiating lateral mesoderm cells into artery endothelium cells. In one implementation, the artery endothelium cell differentiation process is performed as a step, for example, the third step, in the differentiation of hematopoietic stem cells from hPSCs. In one embodiment, the lateral mesoderm cells are replated prior to the artery endothelium cell differentiation process. In one embodiment, the lateral mesoderm cells are present on a substrate

and are differentiated to artery endothelium cells by changing the media composition as follows, without replating. In one embodiment, lateral mesoderm cells are cultured on a substrate and are present at a density of 30,000-50,000 cells/cm².

[0064] In one embodiment, lateral mesoderm cells are differentiated to artery endothelium cells by contacting the cells with an effective amount of: a TGF β agent; a VEGF agent; a retinoic acid pathway activator; a BMP inhibitor; a WNT inhibitor; and a PI3K inhibitor, for a period of time sufficient to produce artery endothelial cells. In some embodiments, the cells are further contacted with an effective amount of an ascorbic acid composition during the culture period. In some embodiments, the period of time is for about 1 day, e.g. from about 18-30 hours, or around 24 hours. In one embodiment, the contacting is achieved by culturing the cells in a medium, for example, a basal medium comprising CDM2 basal medium, wherein the medium comprises a TGF β agent; a VEGF agent; a retinoic acid pathway activator; a BMP inhibitor; a WNT inhibitor; and a PI3K inhibitor.

[0065] The artery endothelial cells express markers of artery endothelial cell identity, including SOX17, DLL4, EFNB2, FOXC1, JAG1, NOTCH1, NRP1, CD31/PECAM1, CD34, CD144/VE-CADHERIN, HOXA5, HOXA7, HOXA9, and HOXA10. The artery endothelium express *SOX17+* *CD144+*, with minimal expression of heart (*NKX2.5*) and vein (*APLN*) markers. At each of these steps, *HOXA5-HOXA10* genes continued to be expressed.

[0066] In the differentiation of artery endothelial cells, exemplary TGF β agents include Activin A (for example, at 0.01-1000 ng/mL, for example, at about 15 ng/mL), TGF β 1, TGF β 2, TGF β 3, NODAL, GDF8, GDF11, or TGF β pathway ligands. Exemplary VEGF agents include VEGF (for example at about 0.01-10,000 ng/mL, for example at about 100 ng/mL), VEGF-A, VEGF-B, VEGF-C, VEGF-D and other VEGF receptor ligands, and FGF2 or other ligands for the FGF receptor. Exemplary retinoic acid pathway agonists include (for example, at 0.001-10,000 nM, for example at about 0.5 nM), (, AM580, Bexarotene, and all-*trans* retinoic acid. Exemplary BMP inhibitors include DMH1 (for example, at 0.001-1000 μ M, for example, at about 250 nM), LDN193189, Dorsomorphin/Compound C, and small-molecule BMP pathway inhibitors, NOGGIN, CHORDIN, and proteins that inhibit extracellular BMP ligands. Exemplary WNT inhibitors include XAV939 (for example at 0.001-1000 μ M, for example at about 1 μ M), C59, IWR1, LGK974 and other small-molecule WNT pathway inhibitors, or proteins such as DKK1 that inhibit WNT pathways. Exemplary and a PI3K inhibitors include GDC-0941 (for example, at 0.001-1000 μ M, for example, at about 2.5 μ M), LY294002, PI-103, and PIK90. Exemplary ascorbic acid compositions include 2-phospho-ascorbic acid (AA2P) and ascorbic acid.

[0067] In one embodiment the TGF β agent comprises Activin A; the VEGF agent comprises VEGF; the retinoic acid pathway activator comprises TTNPB; the BMP inhibitor comprises DMH1; the WNT inhibitor comprises XAV939; and the PI3K inhibitor comprises GDC-0941.

[0068] Hemogenic Endothelium Differentiation. In one aspect, the scope of the invention encompasses a method of differentiating artery endothelium cells into hemogenic endothelium

cells. In one implementation, the hemogenic endothelium cell differentiation process is performed as a step, for example, the fourth step, in the differentiation of hematopoietic stem cells from hPSCs. In one embodiment, the artery endothelial cells are cultured on a substrate and are replated prior to the hemogenic endothelium cell differentiation process, for example, replated at a higher density. In one embodiment, lateral mesoderm cells are plated at a density of 250,000-750,000 cells/cm², for example, 400,000-600,000 cells/cm² for example, at about 500,000 cells/cm². In one embodiment, the lateral mesoderm cells are present on a substrate and are differentiated to artery endothelium cells by changing the media composition, without replating.

[0069] In one embodiment, artery endothelium cells are differentiated to hemogenic endothelium cells by contacting the cells with an effective amount of: a cAMP-elevating agent; a GP130 agonist; a TGFβ inhibitor; a PRC2 inhibitor; and a NOTCH agonist, for a period of time sufficient to produce hemogenic endothelial cells. The period of time may be for about 3 days, e.g. from about 48-96 hours, for example, about 60-84 hours, or around 72 hours. In one embodiment, the contacting is achieved by culturing the cells in a medium, for example, a basal medium comprising CDM3 basal medium, wherein the medium comprises a cAMP-elevating agent; a GP130 agonist; a TGFβ inhibitor; a PRC2 inhibitor; and wherein the cells are cultured on a substrate comprising a NOTCH activator.

[0070] The hemogenic endothelium cells will express markers of hemogenic endothelium identity. The majority of hemogenic endothelium cells will *CD31+* and *RUNX1+*. Hemogenic endothelium cell markers include *RUNX1*, *GFI1*, *GFI1B*, *PU.1*, *CD31/PECAM1*, *CD34*, *CD144/VE-CADHERIN*, *HOXA5*, *HOXA7*, *HOXA9*, and *HOXA10*.

[0071] In hemogenic endothelium cell differentiation, exemplary cAMP-elevating agents include forskolin (for example, at 0.001-1000 μM, for example, at about 10 μM), adenylylate cyclase agonists, activators of the cAMP/PKA pathway, NKH 477, PACAP 1-27, PACAP 1-38; cAMP or 8-bromo-cAMP (*R*)-(-)-Rolipram and other phosphodiesterase inhibitors. Exemplary GP130 agonists include OSM (for example at 0.01-10,000 ng/mL, for example at about 10 ng/mL), LIF (for example at 0.01-10,000 ng/mL, for example at about 20 ng/mL), IL6, IL11, CNTF, CT1, CLC, IL27, and other GP130 pathway ligands. Exemplary TGFβ inhibitors include SB-505124 (for example at 0.001-1000 μM, for example at about 2 μM), LY 364947, A-83-01, RepSox or other small-molecule TGFβ pathway inhibitors, Follistatin, and Lefty1, or Lefty2 or other proteins that inhibit extracellular TGFβ ligands. Exemplary PRC2 inhibitors include UNC1999 (for example at 0.001-1000 μM, for example at about 1 μM), GSK343, EED226, and A-395. Exemplary NOTCH agonists include DLL4-E12 (for example at 0.001-10,000 nM in the composition used to coat culture plate, for example at about 20 nM), DLL1, DLL3, DLL4, JAG1, JAG2, and other NOTCH receptor ligands.

[0072] In one embodiment, the GP130 agonist comprises LIF and OSM; the NOTCH agonist comprises DLL4-E12; the cAMP-elevating agent comprises Forskolin; the inhibitor of TGFβ comprises SB-505124; and the inhibitor of PRC2 comprises UNC1999.

[0073] Hematopoietic Stem Cell Differentiation. In one aspect, the scope of the invention encompasses a method of differentiating hemogenic endothelium cells into hematopoietic stem cells. In one implementation, the hematopoietic stem cell differentiation process is performed as a step, for example, the final step, in the differentiation of hematopoietic stem cells from hPSCs. In one embodiment, the hemogenic endothelial cells are cultured on a substrate and are replated prior to the hemogenic endothelium cell differentiation process. In an alternative embodiment, the cells are present on a substrate and are differentiated to hematopoietic stem cells by changing the media composition, without replating. In one embodiment, the hemogenic endothelium cells are cultured on a substrate and are present at a density of 250,000-750,000 cells/cm², for example, 400,000-600,000 cells/cm², for example, at about 500,000 cells/cm².

[0074] In one embodiment, hemogenic endothelium cells are differentiated to hematopoietic stem cells by contacting the cells with an effective amount of: a cAMP-elevating agent; a TGFβ inhibitor; a PRC2 inhibitor; a G9A/GLP inhibitor; a NOTCH agonist; and one or more compositions that maintain hematopoietic stem cells in an undifferentiated state, for a period of time sufficient to product hematopoietic stem cells. In one embodiment, the period of time may be for about 3 days, e.g. from about 48-96 hours, for example, about 60-84 hours, or around 72 hours. In one embodiment, the one or more compositions that maintain hematopoietic stem cells in an undifferentiated state comprises an aryl hydrocarbon receptor inhibitor and/or a CoREST-HDAC complex inhibitor. In one embodiment, the contacting is achieved by culturing the cells in a medium, for example a CDM3 basal medium, comprising a cAMP-elevating agent; a TGFβ inhibitor; a PRC2 inhibitor; a G9A/GLP inhibitor; and one or more compositions that maintain hematopoietic stem cells in an undifferentiated state and wherein the cells are cultured on a substrate comprising a NOTCH activator.

[0075] The hematopoietic stem cells express markers of hematopoietic stem cell identity. Markers include HSC signature genes (ATF3, HLF, RUNX1, HES1, MLLT3, HOPX, HIF3A, MEIS1, PRDM16, MECOM, NKX2.3, HMGA2, MAFF, SPINK2), HSC surface markers (CD43, CD45, CD90, KIT/CD117, CD31/PECAM1, CD34, CD144/VE-CADHERIN) and HOXA genes (HOXA5, HOXA7, HOXA9, HOXA10).

[0076] In the hematopoietic stem cell differentiation process, exemplary cAMP-elevating agents include forskolin (for example, at 0.001-1000 μM, for example, at about 10 μM), adenylyl cyclase agonists, activators of the cAMP/PKA pathway, NKH 477, PACAP 1-27, PACAP 1-38; cAMP or 8-bromo-cAMP (*R*)-(-)-Rolipram and other phosphodiesterase inhibitors. Exemplary TGFβ inhibitors include SB-505124 (for example at 0.001-1000 μM, for example at about 2 μM), LY 364947, A-83-01, RepSox or other small-molecule TGFβ pathway inhibitors, Follistatin, and Lefty1, or Lefty2 or other proteins that inhibit extracellular TGFβ ligands. Exemplary PRC2 inhibitors include UNC1999 (for example at 0.001-1000 μM, for example at about 1 μM), GSK343, EED226, and A-395. Exemplary NOTCH agonists include DLL4-E12 (for example at 0.001-10,000 nM in the composition used to coat culture plate, for example at about 20 nM), DLL1,

DLL3, DLL4, JAG1, JAG2, and other NOTCH receptor ligands. Exemplary G9A/GLP inhibitors and H3K9 methyltransferase complex inhibitors include UNC0638 (for example at 0.001-10,000 nM, for example, at about 500 nM), A 366, BIX 01294, UNC 0224, and UNC 0642. The one or more compositions that maintain hematopoietic stem cells in an undifferentiated state may include an aryl hydrocarbon receptor inhibitor. Exemplary aryl hydrocarbon receptor inhibitors include SR1 (for example at 0.001-1000 μ M, for example, at about 750 nM), CH 223191, 2-Hydroxyxanthone, Retusin 7-methyl ether, 3-Hydroxyflavone, Pratol and other aryl hydrocarbon receptor inhibitors. The one or more compositions that maintain hematopoietic stem cells in an undifferentiated state may include an a CoREST-HDAC complex inhibitor. Exemplary CoREST-HDAC complex inhibitors include UM171 (for example at 0.001-10,000 nM, for example at about 75 nM), 2-PCPA, GSK-LSD1, RN-1, Parnate/ Tranylcypromine, Valproic Acid, Scriptaid, Trichostatin A.

[0077] In one embodiment, the cAMP-elevating agent comprises Forskolin; the TGF β inhibitor comprises SB-505124; the PRC2 inhibitor comprises UNC1999; the GLP/G9A inhibitor comprises UNC0638; the NOTCH agonist comprises DLL4-E12; and the one or more agents which maintain hematopoietic stem cells in an undifferentiated state comprises SR1 and UM171.

[0078] In one implementation, differentiating pluripotent stem cells into posterior primitive streak cells into posterior primitive streak cells is achieved by plating the cells at a selected density, for example, 30,000-50,000 cells/cm²; without replating, changing the culture medium composition to differentiate the posterior primitive streak cells into lateral mesoderm cells; without replating, changing the medium composition to differentiate the lateral mesoderm cells into artery endothelium cells; dissociating and replating the artery endothelium cells at higher density, for example, about 250,000-750,000 cells/cm², for example 400,000-600,000 cells/cm², for example, about 500,000 cells/cm² and changing the culture medium composition to differentiate the arteryendothelium cells to hemogenic endothelium cells; and without replating, changing the culture medium composition to differentiate the hemogenic endothelium cells into hematopoietic stem cells.

Agents for use in the methods of the invention.

[0079] In some instances, as disclosed above, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the TGF-beta (transforming growth factor β (TGF- β)) pathway. Activators and inhibitors of the TGF-beta pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the TGF-beta pathway resulting in a corresponding activation or inhibition in cellular TGF-beta signaling. Components and downstream effectors of the TGF-beta pathway include but are not limited to, e.g., 14-3-3 e (UniProtID P62258), ark (UniProtID Q6ZNA4), axin1 (UniProtID

O15169), bambi (UniProtID Q13145), beta arrestin 2 (UniProtID P32121), beta catenin (UniProtID P35222), beta glycan (UniProtID Q03167), camkii α (UniProtID Q9UQM7), caveolin-1 (UniProtID Q03135), ctgf (UniProtID P29279), dab2 (UniProtID P98082), dapper2 (UniProtID Q5SW24), daxx (UniProtID Q9UER7), eif2 α (UniProtID Q9BY44), elf (UniProtID Q01082), endofin (UniProtID Q7Z3T8), fkbp12 (UniProtID P62942), gadd34 (UniProtID O75807), grb2 (UniProtID P62993), itch (UniProtID Q96J02), km23-1 (UniProtID Q9NP97), nedd4-2 (UniProtID Q96PU5), ocln (UniProtID Q16625), p70s6k (UniProtID P23443), par6 (UniProtID Q9NPB6), pdk1 (UniProtID O15530), pml (UniProtID P29590), ppp1ca (UniProtID P62136), ppp2ca (UniProtID P67775), ppp2cb (UniProtID P62714), ppp2r2a (UniProtID P63151), rhoa (UniProtID P61586), sara (UniProtID O95405), shc (UniProtID P29353), smad2 (UniProtID Q15796), smad3 (UniProtID P84022), smad4 (UniProtID Q13485), smad7 (UniProtID O15105), smurf1 (UniProtID Q9HCE7), smurf2 (UniProtID Q9HAU4), snon (UniProtID P12757), sos1 (UniProtID Q07889), strap (UniProtID Q9Y3F4), tab1 (UniProtID Q15750), tab2 (UniProtID Q9NYJ8), tak1 (UniProtID O43318), TGFB1 (UniProtID P01137), TGFB2 (UniProtID P61812), TGFB3 (UniProtID P10600), tgfr1 (UniProtID P36897), tgfr2 (UniProtID P37173), trap-1 (UniProtID O60466), wwp1 (UniProtID Q9H0M0), xiap (UniProtID P98170), yap65 (UniProtID P46937), and the like.

[0080] Activators of the TGF-beta pathway include but are not limited to, e.g., TGF-beta family ligands (e.g., TGF-beta proteins and other activators of TGF-beta receptors) and portions thereof, Activin A, TGF-beta1, TGF-beta2, TGF-beta3, IDE1/2 (IDE1 (1-[2-[(2-Carboxyphenyl)methylene]hydrazide]heptanoic acid), IDE2 (Heptanedioic acid-1-(2-cyclopentylidenehydrazide))), Nodal, and the like. In some instances, activation of the TGF-beta pathway may be achieved through repression of the a TGF-beta pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the TGF-beta pathway or an antibody or small molecule directed to a TGF-beta pathway inhibitor.

[0081] Inhibitors of the TGF-beta pathway include but are not limited to, e.g., A-83-01 (3-(6-Methyl-2-pyridinyl)-*N*-phenyl-4-(4-quinolinyl)-1*H*-pyrazole-1-carbothioamide), D4476 (4-[4-(2,3-Dihydro-1,4-benzodioxin-6-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide), GW 788388 (4-[4-[3-(2-Pyridinyl)-1*H*-pyrazol-4-yl]-2-pyridinyl]-*N*-(tetrahydro-2*H*-pyran-4-yl)-benzamide), LY 364947 (4-[3-(2-Pyridinyl)-1*H*-pyrazol-4-yl]-quinoline), RepSox (2-(3-(6-Methylpyridine-2-yl)-1*H*-pyrazol-4-yl)-1,5-naphthyridine), SB431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide), SB-505124 (2-[4-(1,3-Benzodioxol-5-yl)-2-(1,1-dimethylethyl)-1*H*-imidazol-5-yl]-6-methyl-pyridine), SB 525334 (6-[2-(1,1-Dimethylethyl)-5-(6-methyl-2-pyridinyl)-1*H*-imidazol-4-yl]quinoxaline), SD208 (2-(5-Chloro-2-fluorophenyl)-4-[(4-pyridyl)amino]pteridine), ITD1 (4-[1,1'-Biphenyl]-4-yl-1,4,5,6,7,8-hexahydro-2,7,7-trimethyl-5-oxo-3-quinolinecarboxylic acid ethyl ester), DAN/Fc, antibodies to TGF-beta and TGF-beta receptors, TGF-beta inhibitory nucleic acids, and the like.

[0082] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the Wnt pathway. Activators and inhibitors of the Wnt pathway

include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the Wnt pathway resulting in a corresponding activation or inhibition in cellular Wnt signaling. Components and downstream effectors of the Wnt pathway include but are not limited to, e.g., cthrc1 (UniProtID Q96CG8), dkk1 (UniProtID O94907), fzd1 (UniProtID Q9UP38), fzd10 (UniProtID Q9ULW2), fzd2 (UniProtID Q14332), fzd4 (UniProtID Q9ULV1), fzd5 (UniProtID Q13467), fzd6 (UniProtID O60353), fzd7 (UniProtID O75084), fzd8 (UniProtID Q9H461), fzd9 (UniProtID O00144), igfbp4 (UniProtID P22692), kremen 1 (UniProtID Q96MU8), kremen 2 (UniProtID Q8NCW0), lrp5 (UniProtID O75197), lrp6 (UniProtID O75581), prr (UniProtID O75787), ror2 (UniProtID Q01974), rspo1 (UniProtID Q2MKA7), ryk (UniProtID P34925), wnt inhibitory 1 (UniProtID Q9Y5W5), wnt1 (UniProtID P04628), wnt2 (UniProtID P09544), wnt3 (UniProtID P56703), wnt3a (UniProtID P56704), wnt5a (UniProtID P41221), wnt7a (UniProtID O00755), wnt7b (UniProtID P56706), CTNNB1 (UniProtID P35222), GSK3A (UniProtID P49840), GSK3B (UniProtID P49841), TNKS1 (UniProtID O95271), TNKS2 (UniProtID Q9H2K2) and the like.

[0083] Activators of the WNT pathway include but are not limited to, e.g., CHIR99021 (6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile), WNT family ligands (e.g., including but not limited to Wnt-1, Wnt-2, Wnt-2b, Wnt-3a, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7a, Wnt-7a/b, Wnt-7b, Wnt-8a, Wnt-8b, Wnt-9a, Wnt-9b, Wnt-10a, Wnt-10b, Wnt-11, Wnt-16b, etc.), RSPO co-agonists (e.g., RSPO2), lithium chloride, TDZD8 (4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione), BIO-Acetoxime ((2*Z*,3*E*)-6-Bromoindirubin-3'-acetoxime), A1070722 (1-(7-Methoxyquinolin-4-yl)-3-[6-(trifluoromethyl)pyridin-2-yl]urea), HLY78 (4-Ethyl-5,6-Dihydro-5-methyl-[1,3]dioxolo[4,5-*j*]phenanthridine), CID 11210285 hydrochloride (2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine hydrochloride), WAY-316606, (hetero)arylpyrimidines, IQ1, QS11, SB-216763, DCA, and the like. In some instances, activation of the Wnt pathway may be achieved through repression of the a Wnt pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the Wnt pathway or an antibody or small molecule directed to a Wnt pathway inhibitor.

[0084] Inhibitors of the WNT pathway include but are not limited to, e.g., C59 (4-(2-Methyl-4-pyridinyl)-N-[4-(3-pyridinyl)phenyl]benzeneacetamide), DKK1, IWP-2 (N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-*d*]pyrimidin-2-yl)thio]-acetamide), Ant1.4Br, Ant 1.4Cl, Niclosamide, apicularen, bafilomycin, XAV939 (3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4*H*-thiopyrano[4,3-*d*]pyrimidin-4-one), IWR-1 (4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2*H*-isoindol-2-yl)-N-8-quinolinyl-Benzamide), NSC668036 (N-[(1,1-Dimethylethoxy)carbonyl]-L-alanyl-(2*S*)-2-hydroxy-3-methylbutanoyl-L-Alanine-(1*S*)-1-carboxy-2-methylpropyl ester hydrate), 2,4-diamino-quinazoline, Quercetin, ICG-001 ((6*S*,9*aS*)-Hexahydro-6-[(4-hydroxyphenyl)methyl]-8-(1-naphthalenylmethyl)-4,7-dioxo-N-

(phenylmethyl)-2H-pyrazino[1,2-a]pyrimidine-1(6H)-carboxamide), PKF115-584, BML-284 (2-Amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine), FH-535, iCRT-14, JW-55, JW-67, antibodies to Wnts and Wnt receptors, Wnt inhibitory nucleic acids, and the like.

[0085] In some instances, a specific WNT inhibitor may be administered in such a manner as to result in a decrease in PAX3 expression and a promotion of FOXC2 expression.

[0086] In some instances, a Wnt activator or inhibitor useful in the methods described herein may include those described in, e.g., Dodge and Lum et al. *Annu Rev Pharmacol Toxicol.* 2011;51:289-310; Chen et al. *Am J Physiol Gastrointest Liver Physiol.* 2010 Aug;299(2):G293-300; Baker and Clevers, *Nat Rev Drug Discov.* 2006 Dec;5(12):997-1014; Meijer et al. *Trends Pharmacol Sci.* 2004 Sep;25(9):471-80; and Lepourcelet et al. *Cancer Cell.* 2004 Jan;5(1):91-102, the disclosures of which are incorporated herein by reference in their entirety.

[0087] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the FGF pathway. In some instances, an activator or inhibitor of the FGF pathway may also include activators or inhibitors of related signal transduction pathways including but not limited to, e.g., the MAPK/ERK signal transduction pathway. Activators and inhibitors of the FGF pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the FGF pathway resulting in a corresponding activation or inhibition in cellular FGF signaling. Components and downstream effectors of the FGF pathway include but are not limited to, e.g., akt1 (UniProtID P31749), beta-klotho (UniProtID Q86Z14), camkii α (UniProtID Q9UQM7), cbl (UniProtID P22681), cortactin (UniProtID Q14247), e-cadherin (UniProtID P12830), erk1 (UniProtID P27361), erk2 (UniProtID P28482), FGF1 (UniProtID P05230), FGF16 (UniProtID O60258), FGF17 (UniProtID O60258), FGF18 (UniProtID O76093), FGF19 (UniProtID O95750), FGF2 (UniProtID P09038), fgf23 (UniProtID Q9GZV9), FGF4 (UniProtID P08620), FGF6 (UniProtID P10767), FGF8 (UniProtID P55075), FGF9 (UniProtID P31371), fgfr1 (UniProtID P11362), fgfr2 (UniProtID P21802), fgfr2b (UniProtID P21802-18), FGFR2c (UniProtID P21802-5), FGFR3c (UniProtID P22607-1), FGFR4 (UniProtID P22455), fos (UniProtID P01100), frs2 (UniProtID Q8WU20), gab1 (UniProtID Q13480), grb2 (UniProtID P62993), hgf (UniProtID P14210), jun (UniProtID P05412), klotho (UniProtID Q9UEF7), mapk 14 (UniProtID Q16539), met (UniProtID P08581), mkp-3 (UniProtID Q16828), mmp9 (UniProtID P14780), n-cad-ctf1 (UniProtID P19022), n-cad-ctf2 (UniProtID P19022), n-cadherin (UniProtID P19022), ncam (UniProtID P13591), osteocalcin (UniProtID P02818), osteopontin (UniProtID P10451), p110- α (UniProtID P42336), p120ctn (UniProtID O60716), p90-rsk 1 (UniProtID Q15418), pak4 (UniProtID Q8WYL5), pak4 (UniProtID O96013), pdk1 (UniProtID O15530), pik3r1 (UniProtID P27986), plcgamma1 (UniProtID P19174), pro-e-cadherin (UniProtID P12830), pro-mmp9 (UniProtID P14780), ps1 (UniProtID gamma), pyk2 (UniProtID Q14289), runx2 (UniProtID Q13950), se-cad (UniProtID P12830), secad-ntf2 (UniProtID P12830), sef (UniProtID Q8NFM7), shc (UniProtID P29353), shp2 (UniProtID

Q06124), sn-cad (UniProtID P19022), sos1 (UniProtID Q07889), sprouty2 (UniProtID O43597), src (UniProtID P12931), stat1 (UniProtID P42224), stat3 (UniProtID P40763), stat5b (UniProtID P51692), syndecan-2 (UniProtID P34741), syndecan-4 (UniProtID P31431), upa (UniProtID P00749), upar (UniProtID Q03405),, and the like. Activators and inhibitors of the MAPK/ERK pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the MAPK/ERK pathway resulting in a corresponding activation or inhibition in cellular MAPK/ERK signaling. Components and downstream effectors of the MAPK/ERK pathway MAPK/ERK signaling include but are not limited to, e.g., a-raf (EntrezGeneID 369), ask1 (EntrezGeneID 4217), atf2 (EntrezGeneID 1386), cebpa (EntrezGeneID 1050), c-myc (EntrezGeneID 4609), creb (EntrezGeneID 1385), elk1 (EntrezGeneID 2002), erk5 (EntrezGeneID 5598), fos (EntrezGeneID 2353), grb2 (EntrezGeneID 2885), hexokinase type iv glucokinase (EntrezGeneID 2645), ikk-alpha (EntrezGeneID 1147), ikk-beta (EntrezGeneID 3551), jnk (EntrezGeneID 5599), jun (EntrezGeneID 3725), map2k1 (EntrezGeneID 5604), map2k2 (EntrezGeneID 5605), map2k4 (EntrezGeneID 6416), map2k5 (EntrezGeneID 5607), map2k6 (EntrezGeneID 5608), map2k7 (EntrezGeneID 5609), map3k1 (EntrezGeneID 4214), map3k11 (EntrezGeneID 4296), map3k12 (EntrezGeneID 7786), map3k13 (EntrezGeneID 9175), map3k14 (EntrezGeneID 9020), map3k2 (EntrezGeneID 10746), map3k3 (EntrezGeneID 4215), map3k4 (EntrezGeneID 4216), map3k7 (EntrezGeneID 6885), map3k8 (EntrezGeneID 1326), map4k1 (EntrezGeneID 11184), map4k3 (EntrezGeneID 8491), map4k5 (EntrezGeneID 11183), mapk1 (EntrezGeneID 5594), mapk10 (EntrezGeneID 5602), mapk11 (EntrezGeneID 5600), mapk12 (EntrezGeneID 6300), mapk13 (EntrezGeneID 5603), mapk14 (EntrezGeneID 1432), mapk3 (EntrezGeneID 5595), mapk9 (EntrezGeneID 5601), max (EntrezGeneID 4149), mef2 polypeptide a (EntrezGeneID 4205), mef2 polypeptide c (EntrezGeneID 4208), mef2b (EntrezGeneID 4207), mef2 polypeptide d (EntrezGeneID 4209), mek3 (EntrezGeneID 5606), mnk2 (EntrezGeneID 2872), mnk1 (EntrezGeneID 8569), msk1 (EntrezGeneID 9252), ngf r (EntrezGeneID 4804), ngfb (EntrezGeneID 4803), nik (EntrezGeneID 9448), pak1 (EntrezGeneID 5058), pak2 (EntrezGeneID 5062), pp2a (EntrezGeneID 5528), ptprr (EntrezGeneID 5801), rac1 (EntrezGeneID 5879), raf1 (EntrezGeneID 5894), ras (EntrezGeneID 3265), rps6ka1 (EntrezGeneID 6195), shc (EntrezGeneID 6464), sos1 (EntrezGeneID 6654), sp1 (EntrezGeneID 6667), src (EntrezGeneID 6714), stat1 (EntrezGeneID 6772), stat3 (EntrezGeneID 6774), tert (EntrezGeneID 7015), and the like.

[0088] Activators of the FGF pathway and/or the MAPK/ERK pathway include but are not limited to, e.g., FGF family ligands (e.g., FGF1, FGF2, FGF-3, FGF-4, FGF-5, FGF-6, KGF/FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-15, FGF-16, FGF-17, FGF-19, FGF-20, FGF-21, FGF-22, FGF-23, etc.), SUN 11602 (4-[[4-[[2-[(4-Amino-2,3,5,6-tetramethylphenyl)amino]acetyl]methylamino]-1-piperidinyl]methyl]benzamide), t-

Butylhydroquinone, U-46619, C2 Ceramide, Lactosyl Ceramide, Angiotensin II, Baicalin, and the like. In some instances, activation of the FGF pathway and/or the MAPK/ERK pathway may be achieved through repression of the a FGF pathway and/or the MAPK/ERK pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the FGF pathway and/or the MAPK/ERK pathway or an antibody or small molecule directed to a FGF pathway inhibitor and/or MAPK/ERK pathway inhibitor.

[0089] Inhibitors of the FGF pathway and/or the MAPK/ERK pathway and or the p38/JNK/MAPK cascade include but are not limited to, e.g., AP 24534 (3-(2-Imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methyl-N-[4-[(4-methyl-1-piperazinyl)methyl]-3-(trifluoromethyl)phenyl]-benzamide), PD173074 (N-[2-[[4-(Diethylamino)butyl]amino]-6-(3,5-dimethoxyphenyl)pyrido[2,3-d]pyrimidin-7-yl]-N'-(1,1-dimethylethyl)urea), FIIN 1 hydrochloride (N-(3-((3-(2,6-dichloro-3,5-dimethoxyphenyl)-7-(4-(diethylamino)butylamino)-2-oxo-3,4-dihydropyrimido[4,5-d]pyrimidin-1(2H)-yl)methyl)phenyl)acrylamide), PD 161570 (N-[6-(2,6-Dichlorophenyl)-2-[[4-(diethylamino)butyl]amino]pyrido[2,3-d]pyrimidin-7-yl]-N'-(1,1-dimethylethyl)urea), SU 5402 (2-[[1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-4-methyl-1H-pyrrole-3-propanoic acid), SU 6668 (5-[1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic acid), PD0325901 (N-[(2R)-2,3-Dihydroxypropoxy]-3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]-benzamide), BIX 02189 ((3Z)-3-[[[3-[(Dimethylamino)methyl]phenyl]amino]phenylmethylene]-2,3-dihydro-N,N-dimethyl-2-oxo-1H-indole-6-carboxamide), FR 180204 (5-(2-Phenylpyrazolo[1,5-a]pyridin-3-yl)-1H-pyrazolo[3,4-c]pyridazin-3-ylamine), Pluripotin (N-[3-[7-[(1,3-Dimethyl-1H-pyrazol-5-yl)amino]-1,4-dihydro-1-methyl-2-oxopyrimido[4,5-d]pyrimidin-3(2H)-yl]-4-methylphenyl]-3-(trifluoromethyl)benzamide), TCS ERK 11e (4-[2-[(2-Chloro-4-fluorophenyl)amino]-5-methyl-4-pyrimidinyl]-N-[(1S)-1-(3-chlorophenyl)-2-hydroxyethyl]-1H-pyrrole-2-carboxamide), TMCB (2-(4,5,6,7-Tetrabromo-2-(dimethylamino)-1H-benzo[d]imidazol-1-yl)acetic acid), XMD 8-92 (2-[[2-Ethoxy-4-(4-hydroxy-1-piperidinyl)phenyl]amino]-5,11-dihydro-5,11-dimethyl-6H-pyrimido[4,5-b][1,4]benzodiazepin-6-one), SU5402, AZD4547, BGJ398, AL 8697, AMG 548, CMPD-1, DBM 1285 dihydrochloride, EO 1428, JX 401, ML 3403, RWJ 67657, SB 202190, SB-203580, SB 239063, SB 706504, Scio-469, SKF 86002 dihydrochloride, SX 011, TA 01 (4-(2-(2,6-Difluorophenyl)-4-(fluorophenyl)-1H-imidazol-5-yl)pyridine), TA 02 (4-(2-(2-Fluorophenyl)-4-(fluorophenyl)-1H-imidazol-5-yl)pyridine), TAK 715, VX-702, VX-745, antibodies to FGF and/or MAPK pathway components including ligands and receptors, FGF and/or MAPK inhibitory nucleic acids, and the like.

[0090] In some instances, a FGF or MAPK activator or inhibitor useful in the methods described herein may include those described in, e.g., English and Cobb, Trends Pharmacol Sci. 2002 Jan;23(1):40-5, the disclosure of which is incorporated herein by reference in its entirety.

[0091] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the BMP pathway. Activators and inhibitors of the BMP pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors,

antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the BMP pathway resulting in a corresponding activation or inhibition in cellular BMP signaling. Components and downstream effectors of the BMP pathway include but are not limited to, e.g., bambi (UniProtID Q13145), bmp2 (UniProtID P12643), bmp4 (UniProtID P12644), bmp6 (UniProtID P22004), bmp7 (UniProtID P18075), bmpr1a (UniProtID P36894), bmpr1b (UniProtID O00238), bmpr2 (UniProtID Q13873), cer1 (UniProtID O95813), chrd (UniProtID Q9H2X0), chrd1l (UniProtID Q9BU40), endofin (UniProtID Q7Z3T8), erk2 (UniProtID P28482), fetua (UniProtID P02765), fs (UniProtID P19883), gadd34 (UniProtID O75807), grem1 (UniProtID O60565), gsk3beta (UniProtID P49841), nog (UniProtID Q13253), nup214 (UniProtID P35658), ppm1a (UniProtID P35813), ppp1ca (UniProtID P62136), rgma (UniProtID Q96B86), rgmb (UniProtID Q6NW40), rgmc (UniProtID Q6ZVN8), scp1 (UniProtID Q9GZU7), scp2 (UniProtID O14595), scp3 (UniProtID O15194), ski (UniProtID P12755), smad1 (UniProtID Q15797), smad4 (UniProtID Q13485), smad5 (UniProtID Q99717), smad6 (UniProtID O43541), smad7 (UniProtID O15105), smad8a (UniProtID O15198), smurf1 (UniProtID Q9HCE7), smurf2 (UniProtID Q9HAU4), tab1 (UniProtID Q15750), tab2 (UniProtID Q9NYJ8), tak1 (UniProtID O43318), usag1 (UniProtID Q6X4U4), xiap (UniProtID P98170), and the like.

[0092] Activators of the BMP pathway include but are not limited to, e.g., BMP family ligands (e.g., BMP2, BMP4, BMP7, etc.), Alantolactone, FK506, isoliquiritigenin, 4'-hydroxychalcone, and the like. In some instances, activation of the BMP pathway may be achieved through repression of the a BMP pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the BMP pathway or an antibody or small molecule directed to a BMP pathway inhibitor.

[0093] Inhibitors of the BMP pathway include but are not limited to, e.g., NOGGIN, CHORDIN, LDN-193189 (4-[6-[4-(1-Piperazinyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline hydrochloride), DMH1 (4-[6-[4-(1-Methylethoxy)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline), Dorsomorphin (6-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine dihydrochloride), K 02288 (3-[(6-Amino-5-(3,4,5-trimethoxyphenyl)-3-pyridinyl]phenol), ML 347 (5-[6-(4-Methoxyphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline), DMH-1, antibodies to BMPs and BMP receptors, BMP inhibitory nucleic acids, and the like.

[0094] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the retinoic acid signaling pathway. Activators and inhibitors of the retinoic acid signaling pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the retinoic acid signaling pathway resulting in a corresponding activation or inhibition in cellular retinoic acid signaling. Components and downstream effectors of the retinoic acid signaling pathway include but are not limited to, e.g., CRABP (e.g., Accession: NP_004369), TRAIL (e.g., Accession: NP_003801), TRAILR1 (e.g., Accession: NP_003835), TRAILR2 (e.g., Accession: NP_003833), DAP3 (e.g., Accession:

NP_001186780), FADD (e.g., Accession: CAG33019), FLIP (e.g., Accession: NP_001294972), Caspase 8 (e.g., Accession: AAD24962), BID (e.g., Accession: NP_001304162), tBID (e.g., Accession: P55957), APAF1 (e.g., Accession: ABQ59028), Caspase 9 (e.g., Accession: P55211), PARPs (e.g., Accession: AAH14206), RAR (e.g., Accession: NP_001138773 and components thereof e.g., AF2 domain, AF1 domain, DBD domain, and the like. Activators and inhibitors of the retinoic acid signaling include but are not limited to e.g., Tretinoin, Retinol palmitate, Etretinate, Isotretinoin, Adapalene, Tazarotene, Tamibarotene, Retinol acetate, Acitretin, Alitretinoin, Bexarotene, Isotretinoin anisatol, Motretinide, Vitamin A, Retinol propionate, and the like. In some instances, useful modulators of the retinoic acid signaling pathway include retinoid agonist, including but not limited to e.g., all-*trans* retinoic acid, TTNPB, AM580 and the like. In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the Hedgehog pathway. Activators and inhibitors of the Hedgehog pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the Hedgehog pathway resulting in a corresponding activation or inhibition in cellular Hedgehog signaling. Components and downstream effectors of the Hedgehog pathway include but are not limited to, e.g., akt1 (UniProtID P31749), beta arrestin2 (UniProtID P32121), boc (UniProtID Q9BWV1), cdo (UniProtID Q4KMG0), dhh (UniProtID O43323), gas1 (UniProtID P54826), gli2 (UniProtID P10070), grk2 (UniProtID P25098), hhat (UniProtID Q5VTY9), hhpf (UniProtID Q96QV1), ihh (UniProtID Q14623), Irap1 (UniProtID P30533), megalin (UniProtID P98164), p110-alpha (UniProtID P42336), pik3r1 (UniProtID P27986), ptch1 (UniProtID Q13635), ptch2 (UniProtID Q9Y6C5), pthrp (UniProtID P12272), shh (UniProtID Q15465), sil (UniProtID Q15468), smo (UniProtID Q99835), tgfbeta2 (UniProtID P61812), and the like.

[0095] Activators of the Hedgehog pathway include but are not limited to, e.g., Hedgehog family ligands (Hh, Shh, Ihh, Dhh, etc.) and fragments thereof, benzothiophene smoothened agonists, SAG (Hh-Ag1.3), SAG21k (3-chloro-4,7-difluoro-N-(4-methoxy-3-(pyridin-4-yl)benzyl)-N-((1*r*,4*r*)-4-(methylamino)cyclohexyl)benzo[*b*]thiophene-2-carboxamide), Hh-Ag1.1, Hh-Ag1.5, purmorphamine, and the like. In some instances, activation of the Hedgehog pathway may be achieved through repression of a Hedgehog pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the Hedgehog pathway or an antibody or small molecule directed to a Hedgehog pathway inhibitor.

[0096] Inhibitors of the Hedgehog pathway include but are not limited to, e.g., Hedgehog antagonists that target smoothened (SMO), Hedgehog antagonists that target patched (PTCH), Hedgehog antagonists that target Gli, cyclopamine and analogs and derivatives thereof, cyclopamine-competitive antagonists, IPI-926 (Saridegib), LDE225 (sonidegib), itraconazole, GDC-0449 (vismodegib), SANT1, KAAD-cyclopamine, LEQ506, PF-04449913, TAK-441, BMS833923 (XL-139), LY2940680, and inhibitory nucleic acids targeting SMO, inhibitory nucleic

acids targeting a Hedgehog, inhibitory nucleic acids targeting PTCH, inhibitory nucleic acids targeting Gli (e.g., siRNA targeting Gli1), arsenic trioxide, and the like.

[0097] In some instances, Hedgehog pathway activators and Hedgehog pathway inhibitors include those agents described in, e.g., Chen et al. (2002) *PNAS*. 99(22):14071-14076; Frank-Kamenetsky, et al. (2002) *J Biol.* 1(2):10; Paladini et al. (2005) *J Invest Dermatol.* 125(4):638-46; Nakamura et al. (2014) *J Cell. Physiol.* ePub, Yun et al., *Arch Pharm Res.* 2012 Aug;35(8):1317-33; the disclosures of which are incorporated herein by reference in their entirety.

[0098] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the PI3K pathway. Activators and inhibitors of the PI3K pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the PI3K pathway resulting in a corresponding activation or inhibition in cellular PI3K signaling. Components and downstream effectors of the PI3K pathway include but are not limited to, e.g., arap3 (UniProtID Q8WWN8), arf1 (UniProtID P84077), arf5 (UniProtID P84085), arf6 (UniProtID P62330), arno (UniProtID Q99418), bam32 (UniProtID Q9UN19), blk (UniProtID P51451), blk (UniProtID Q8WV28), btk (UniProtID Q06187), centa1 (UniProtID O75689), cytohesin-1 (UniProtID Q15438), fgr (UniProtID P09769), foxo3a (UniProtID O43524), fyn (UniProtID P06241), grp1 (UniProtID O43739), hck (UniProtID P08631), h-ras isoform 1 (UniProtID P01112), h-ras isoform 2 (UniProtID P01112), hsp90 (UniProtID P07900), itk (UniProtID Q08881), k-ras isoform 2a (UniProtID P01116-1), k-ras isoform 2b (UniProtID P01116-2), lat (UniProtID O43561-2), lck (UniProtID P06239), lyn (UniProtID P07948), n-ras (UniProtID P01111), p101 (UniProtID Q8WYR1), p110-alpha (UniProtID P42336), p110-beta (UniProtID P42338), p110D (UniProtID O00329), p55-gamma (UniProtID Q92569), p84 (UniProtID Q5UE93), p85-beta (UniProtID O00459), pdk1 (UniProtID O15530), PI3Kgamma (UniProtID P48736), PIK3R1 (UniProtID P27986), plcgamma1 (UniProtID P19174), plcgamma2 (UniProtID P16885), pten (UniProtID P60484), rac1 (UniProtID P63000), rap1a (UniProtID P62834), rhoa (UniProtID P61586), sgk1 (UniProtID O00141), ship (UniProtID O00145), ship2 (UniProtID O15357), src (UniProtID P12931), syk (UniProtID P43405), tapp1 (UniProtID Q9HB19), tapp2 (UniProtID Q9HB21), yes (UniProtID P07947), zap-70 (UniProtID P43403), and the like.

[0099] Activators of the PI3K pathway include but are not limited to, e.g., PI3K family ligands, 740 Y-P, Insulin receptor substrate (Tyr608) peptide (KKHTDDGYMPMSPGVA, SEQ ID NO:1), and the like. In some instances, an FGF signaling protein may serve as an activator of the PI3K pathway. In some instances, activation of the PI3K pathway may be achieved through repression of the a PI3K pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the PI3K pathway or an antibody or small molecule directed to a PI3K pathway inhibitor.

[00100] Inhibitors of the PI3K pathway include but are not limited to, e.g., AS 252424 (5-[[5-(4-Fluoro-2-hydroxyphenyl)-2-furanyl]methylene]-2,4-thiazolidinedione), AS 605240 (5-(6-Quinoxalinylmethylene)-2,4-thiazolidine-2,4-dione), AZD 6482 ((-)-2-[[[(1R)-1-[7-Methyl-2-(4-morpholinyl)-4-oxo-4H-pyrido[1,2-a]pyrimidin-9-yl]ethyl]amino]benzoic acid), BAG 956 (α,α -Dimethyl-4-[2-methyl-8-[2-(3-pyridinyl)ethynyl]-1*H*-imidazo[4,5-*c*]quinolin-1-yl]-benzeneacetonitrile), CZC 24832 (5-(2-Amino-8-fluoro[1,2,4]triazolo[1,5-*a*]pyridin-6-yl)-*N*-(1,1-dimethylethyl)-3-pyridinesulfonamide), GSK 1059615 (5-[[4-(4-Pyridinyl)-6-quinolinyl]methylene]-2,4-thiazolidinedione), KU 0060648 (4-Ethyl-*N*-[4-[2-(4-morpholinyl)-4-oxo-4H-1-benzopyran-8-yl]-1-dibenzothienyl]-1-piperazineacetamide), LY 294002 hydrochloride (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride), 3-Methyladenine (3-Methyl-3H-purin-6-amine), PF 04691502 (2-Amino-8-[trans-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxy-3-pyridinyl)-4-methyl-pyrido[2,3-*d*]pyrimidin-7(8H)-one), PF 05212384 (*N*-[4-[[4-(Dimethylamino)-1-piperidinyl]carbonyl]phenyl]-*N'*-[4-(4,6-di-4-morpholinyl-1,3,5-triazin-2-yl)phenyl]urea), PI 103 hydrochloride (3-[4-(4-Morpholinyl)pyrido[3',2':4,5]furo[3,2-*d*]pyrimidin-2-yl]phenol hydrochloride), PI 828 (2-(4-Morpholinyl)-8-(4-aminophenyl)-4H-1-benzopyran-4-one), PP 121 (1-Cyclopentyl-3-(1H-pyrrolo[2,3-*b*]pyridin-5-yl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine), Quercetin, TG 100713 (3-(2,4-Diamino-6-pteridinyl)-phenol), Wortmannin, PIK90, GDC-0941, antibodies to PI3K and PI3K receptors, PI3K inhibitory nucleic acids, and the like.

[00101] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the PDGF pathway. Activators and inhibitors of the PDGF pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the PDGF pathway resulting in a corresponding activation or inhibition in cellular PDGF signaling. Components and downstream effectors of the PDGF pathway include but are not limited to, e.g., 14-3-3 e (UniProtID P62258), abi1 (UniProtID Q8IZP0), acta2 (UniProtID P62736), afadin (UniProtID P55196), alpha actinin 4 (UniProtID O43707), alphav integrin (UniProtID P06756), arap1 (UniProtID Q96P48), arp2 (UniProtID P61160), arp3 (UniProtID P61158), arpc1b (UniProtID O15143), arpc2 (UniProtID O15144), arpc3 (UniProtID O15145), arpc4 (UniProtID P59998), arpc5 (UniProtID O15511), beta3 integrin (UniProtID P05106), blk (UniProtID P51451), braf (UniProtID P15056), c3g (UniProtID Q13905), c-abl (UniProtID P00519), caveolin-1 (UniProtID Q03135), caveolin-3 (UniProtID P56539), cbl (UniProtID P22681), ck2a1 (UniProtID P68400), cortactin (UniProtID Q14247), crk (UniProtID P46108), crkl (UniProtID P46109), csk (UniProtID P41240), dep1 (UniProtID Q12913), dock4 (UniProtID Q8N110), dynamin 2 (UniProtID P50570), elk1 (UniProtID P19419), eps8 (UniProtID Q12929), erk1 (UniProtID P27361), erk2 (UniProtID P28482), fgr (UniProtID P09769), fos (UniProtID P01100), fyn (UniProtID P06241), gab1 (UniProtID Q13480), grb10 (UniProtID Q13322), grb2 (UniProtID P62993), hck (UniProtID P08631), h-ras isoform 1 (UniProtID P01112), h-ras isoform 2 (UniProtID P01112), hspc300 (UniProtID Q8WUW1), ifn-gamma (UniProtID

P01579), iggap1 (UniProtID P46940), irsp53 (UniProtID Q9UQB8), jak1 (UniProtID P23458), jak2 (UniProtID O60674), jnk1 (UniProtID P45983), jnk2 (UniProtID P45984), jnk3 (UniProtID P53779), jun (UniProtID P05412), jund (UniProtID P17535), k-ras isoform 2a (UniProtID P01116-1), k-ras isoform 2b (UniProtID P01116-2), ksr (UniProtID Q81VT5), lck (UniProtID P06239), lrp1 (UniProtID Q07954), lyn (UniProtID P07948), mek1 (UniProtID Q02750), mek2 (UniProtID P36507), mkk4 (UniProtID P45985), mkk7 (UniProtID O14733), myc (UniProtID P01106), myocardin (UniProtID Q81ZQ8), nap1 (UniProtID Q9Y2A7), nck1 (UniProtID P16333), nck2 (UniProtID O43639), nherf1 (UniProtID O14745), nherf2 (UniProtID Q15599), n-ras (UniProtID P01111), n-wasp (UniProtID O00401), p101 (UniProtID Q8WYR1), p110-alpha (UniProtID P42336), p110-beta (UniProtID P42338), p110D (UniProtID O00329), p130 cas (UniProtID P56945), p190rhogap (UniProtID Q9NRY4), p52 shc (UniProtID P29353-2), p55-gamma (UniProtID Q92569), p62dok (UniProtID Q99704), p84 (UniProtID Q5UE93), p85-beta (UniProtID O00459), pag1 (UniProtID Q9NWQ8), pak1 (UniProtID Q13153), pdgfa (UniProtID P04085), pdgfb (UniProtID P01127), pdgfc (UniProtID Q9NRA1), pdgfd (UniProtID Q9GZP0), pdgfra (UniProtID P16234), pdgfrb (UniProtID P09619), PI3Kgamma (UniProtID P48736), pik3r1 (UniProtID P27986), pin1 (UniProtID Q13526), pkc alpha (UniProtID P17252), pkc delta (UniProtID Q05655), pkc epsilon (UniProtID Q02156), pkr (UniProtID P19525), pla2g4a (UniProtID P47712), plcgamma1 (UniProtID P19174), ppp2ca (UniProtID P67775), ppp2r1a (UniProtID P30153), ppp2r2b (UniProtID Q00005), pten (UniProtID P60484), ptp1b (UniProtID P18031), rab4a (UniProtID P20338), rab5 (UniProtID P20339), rac1 (UniProtID P63000), raf1 (UniProtID P04049), rap1a (UniProtID P62834), rap1b (UniProtID P61224), rasgap (UniProtID P20936), rhoa (UniProtID P61586), rhogdi (UniProtID P52565), rntre (UniProtID Q92738), rsk2 (UniProtID P51812), s1p1 (UniProtID P21453), shb (UniProtID Q15464), shc (UniProtID P29353), shf (UniProtID Q7M4L6), shp2 (UniProtID Q06124), slap (UniProtID Q13239), sm22 (UniProtID Q01995), sos1 (UniProtID Q07889), spa-1 (UniProtID Q96FS4), sphk1 (UniProtID Q9NYA1), sra1 (UniProtID Q96F07), src (UniProtID P12931), srf (UniProtID P11831), stat1 (UniProtID P42224), stat3 (UniProtID P40763), STAT5A (UniProtID P42229), STAT5B (UniProtID P51692), tcptp p45 (UniProtID P17706-1), vav2 (UniProtID P52735), wave2 (UniProtID Q9Y6W5), yes (UniProtID P07947), ywhab (UniProtID P31946), ywhag (UniProtID P61981), ywhah (UniProtID Q04917), ywhaq (UniProtID P27348), ywhas (UniProtID P31947), ywhaz (UniProtID P63104), and the like.

[00102] Activators of the PDGF pathway include but are not limited to, e.g., PDGF family ligands (e.g., PDGF, PDGF A, PDGF B, PDGF C, PDGF D, etc.) and fragments thereof and/or dimers thereof (e.g., PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, PDGF-AB, etc.), and the like. In some instances, activation of the PDGF pathway may be achieved through repression of the a PDGF pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the PDGF pathway or an antibody or small molecule directed to a PDGF pathway inhibitor.

[00103] Inhibitors of the PDGF pathway include but are not limited to, e.g., AG 18 ([3,4-Dihydroxyphenyl)methylene]-propenedinitrile), AG1295, AG1296, AGL2043, AP 24534 (3-(2-Imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methyl-N-[4-[(4-methyl-1-piperazinyl)methyl]-3-(trifluoromethyl)phenyl]-benzamide), CDP860, DMPQ dihydrochloride (5,7-Dimethoxy-3-(4-pyridinyl)quinoline dihydrochloride), Imatinib, PD 166285 dihydrochloride (6-(2,6-Dichlorophenyl)-2-[[4-[2-(diethylamino)ethoxy]phenyl]amino]-8-methylpyrido[2,3-d]pyrimidin-7(8H)-one dihydrochloride), SU 16f (5-[1,2-Dihydro-2-oxo-6-phenyl-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic acid), SU 6668 (5-[1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic acid), SU11248, Sunitinib malate (N-[2-(Diethylamino)ethyl]-5-[(Z)-(5-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide (2S)-2-hydroxybutanedioate salt), Toceranib (5-[(Z)-(5-Fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-N-[2-(1-pyrrolidinyl)ethyl]-1H-pyrrole-3-carboxamide), antibodies targeting PDGF and/or PDGF receptor, PDGF inhibitory nucleic acids, and the like.

[00104] In some instances, an inducing agent useful in a particular induction composition may include an activator of the NOTCH pathway. Activators of the NOTCH pathway include small molecule activators, peptide activators, antibodies against NOTCH repressors, nucleic acid activators, nucleic acid inhibitors of NOTCH repressors, and the like that activate at least one component of the NOTCH pathway resulting in a corresponding activation in cellular NOTCH signaling.

[00105] Activators of the NOTCH pathway include but are not limited to, e.g., NOTCH family ligands, including both canonical and non-canonical NOTCH family ligands, and portions or fragments thereof. Canonical and non-canonical NOTCH family ligands include but are not limited to, e.g., Delta-like ligands, Jagged ligands, homologous vertebrate proteins and polypeptides to invertebrate NOTCH ligands (e.g., delta, serrate, LAG-2, APX-1, ARG-1, DSL-1, and the like), and the like. NOTCH ligands and methods of activating NOTCH signaling are known in the art and include, e.g., those described in D'Souza et al. (Curr Top Dev Biol. 2010;92:73-129) Li et al. (J Biol Chem. 2008;283(12):8046-54), the disclosures of which is incorporated herein by reference in their entirety. In some instances, activation of the NOTCH pathway may be achieved through repression of a NOTCH pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the NOTCH pathway or an antibody or small molecule directed to a NOTCH pathway inhibitor.

[00106] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the PKA/cAMP pathway (i.e., the cAMP-dependent pathway, adenylyl cyclase pathway, PAK signaling, etc.). Activators and inhibitors of the PKA/cAMP pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the PKA/cAMP pathway resulting in a corresponding activation

or inhibition in cellular PKA/cAMP signaling. Components and downstream effectors of the PKA/cAMP pathway include but are not limited to, e.g., G-protein alpha-12 family, WASF1 (WAVE1), LBC, G-protein alpha-i family, AKAP2, ATP cytosol, PDE3B, SMAD3, Androgen receptor, KDELR, AKAP7 gamma, PCTK1, 4.6.1.1, AKAP12, SMAD4, Anaphase-promoting complex (APC), GABA-A receptor beta-2 subunit, Ryanodine receptor 1, Troponin I, cardiac, AKAP8, 3.1.4.17, AKAP11, PHK beta, GABA-A receptor beta-3 subunit, PKA-cat alpha, CREB1, cAMP, G-protein alpha-s, GSK3 alpha/beta, AKAP3, Adenylate cyclase, PDK (PDPK1), GABA-A receptor beta-1 subunit, PKA-reg (cAMP-dependent), PDE4D, PKA-cat (cAMP-dependent), DARPP-32, PKA-reg type II (cAMP-dependent), NFKBIA, Meprin A, beta, AKAP82, AMP, PDE3A, PKI, PHK gamma, PDE4A, NFKBIB, PP2A regulatory, BAD, p90RSK1, G-protein alpha-13, Phospholamban, G-protein alpha-i family, RAP-1A, Adenylate cyclase type II, cAMP, G-protein beta/gamma, Calcineurin A (catalytic), PKC, Calmodulin, GSK3 alpha/beta, Adenylate cyclase type VII, Adenylate cyclase type IV, Adenylate cyclase type VIII, CREB1, ATP cytosol, Ca⁽²⁺⁾ cytosol, 4.6.1.1, Ryanodine receptor 1, G-protein alpha-s, PKC-alpha, RAP-2A, CaMK IV, PHK alpha, PKA-reg (cAMP-dependent), Adenylate cyclase type III, cAMP-GEFII, Adenylate cyclase type V, LIPS, KDELR, cAMP-GEFI, Adenylate cyclase type VI, PKA-cat (cAMP-dependent), PHK gamma, CaMK II, PKC-zeta, PKC-delta, Adenylate cyclase type I, Adenylate cyclase type IX, and the like.

[00107] Activators of the PKA/cAMP pathway include but are not limited to, e.g., forskolin, dibutyryl-cAMP (bucladesine), 8-bromo-cAMP, 8-CPT-cAMP, taxol, Adenosine 3',5'-cyclic Monophosphate, N6-Benzoyl, Adenosine 3',5'-cyclic monophosphate, belinostat, 8-Chloroadenosine 3',5'-Cyclic Monophosphate, (S)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate), Sp-Adenosine 3',5'-cyclic monophosphorothioate, Sp-5,6-DCI-cBiMPS, Adenosine 3',5'-cyclic Monophosphorothioate, 8-Bromo-, Sp-Isomer, Sp-8-pCPT-cyclic GMPS Sodium, N6-Monobutyryl-adenosine 3':5'-cyclic monophosphate, 8-PIP-cAMP, Sp-cAMPS caffeine, theophylline, pertussis toxin and the like. In some instances, activation of the PKA/cAMP pathway may be achieved through repression of the a PKA/cAMP pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the PKA/cAMP pathway or an antibody or small molecule directed to a PKA/cAMP pathway inhibitor.

[00108] In some instances, an inducing agent useful in a particular induction composition may include an activator or inhibitor of the VEGF pathway. Activators and inhibitors of the VEGF pathway include small molecule activators, small molecule inhibitors, peptide activators, peptide inhibitors, antibodies, nucleic acid activators, nucleic acid inhibitors, and the like that activate or inhibit at least one component of the VEGF pathway resulting in a corresponding activation or inhibition in cellular VEGF signaling. Components and downstream effectors of the VEGF pathway include but are not limited to, e.g., VEGFA, KDR, SH2D2A, PLCG1, PLCG2, PRKCA, PRKCB, PRKCG, SPHK1, SPHK2, HRAS, KRAS, NRAS, RAF1, MAP2K1, MAP2K2, MAPK1, MAPK3, PLA2G4E, PLA2G4A, JMJD7-PLA2G4B, PLA2G4B, PLA2G4C, PLA2G4D, PLA2G4F,

PPP3CA, PPP3CB, PPP3CC, PPP3R1, PPP3R2, NFATC2, PTGS2, PTK2, SHC2, PXN, CDC42, MAPK11, MAPK12, MAPK13, MAPK14, MAPKAPK2, MAPKAPK3, HSPB1, SRC, PIK3CA, PIK3CD, PIK3CB, PIK3CG, PIK3R1, PIK3R5, PIK3R2, PIK3R3, RAC1, RAC2, RAC3, AKT1, AKT2, AKT3, NOS3, CASP9, BAD, and the like.

[00109] Modulators of the VEGF signaling pathway include but are not limited to e.g., Aspirin, Naproxen, Sulindac, Ibuprofen, Piroxicam, Diflunisal, Ketoprofen, Indometacin, Mefenamic acid, Tolmetin sodium, Meclofenamate sodium, Etodolac, Flurbiprofen, Nabumetone, Sasapyrine, Oxaprozin, Phenylbutazone, Sodium salicylate, Celecoxib, Rofecoxib, Axitinib, Bosutinib, Dasatinib, Doramapimod, Pegaptanib sodium, Ranibizumab, Semaxanib, Sorafenib tosilate, Vatalanib, Sunitinib malate, Vandetanib, Bevacizumab, Dasatinib hydrate, Motesanib, Dexketoprofen, Ketoprofen sodium, Meclofenamate sodium, Piketoprofen, Piketoprofen hydrochloride, Toceranib, Sorafenib, Toceranib phosphate, Sunitinib, Bevasiranib sodium, Brivanib alaninate, Cediranib, Cediranib maleate, Motesanib phosphate, Pamapimod, Ramucirumab, Talmapimod, Aflibercept, Dilmapimod, Dilmapimod tosylate, Foretinib, Linifanib, Losmapimod, Saracatinib, Saracatinib difumarate, Tivozanib, Bosutinib hydrate, Pegdinetanib, Naproxen etemesil, Cabozantinib, Tivozanib hydrochloride, Golvatinib, Pimasertib, Pimasertib hydrochloride, and the like. In some instances, VEGF activators useful in the subject methods include but are not limited to e.g., a VEGF polypeptide and/or a nucleic acid encoding a VEGF polypeptide.

[00110] In some instances, activation of the PKA/cAMP pathway may be achieved through repression of a PKA/cAMP pathway inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of the PKA/cAMP pathway or an antibody or small molecule directed to a PKA/cAMP pathway inhibitor.

[00111] In some instances, an inducing agent useful in a particular induction composition may include a SCF agonist. SCF activators (i.e., SCF agonists) with vary and may include small molecule activators, peptide activators, agonist antibodies, nucleic acid activators, and the like that activate a molecule that responds to SCF or promotes the expression or functional bioactivity of SCF. In some instances, activation of SCF may be achieved through repression of a SCF inhibitor, e.g., including but not limited to the use of an inhibitory nucleic acid targeting an inhibitor of SCF or an antibody or small molecule directed to a SCF inhibitor. SCF agonists include but are not limited to, e.g., a SCF protein or polypeptide, an agonistic SCF peptide, a nucleic acid encoding a SCF protein or polypeptide, a nucleic acid encoding an agonistic SCF peptide, and the like.

[00112] In some instances, an inducing agent useful in a particular induction composition may include a gp130/IL6 superfamily agonist. Gp130/IL6 superfamily agonists will vary and may include small molecules, peptides, nucleic acids, and the like that activate Gp130/IL6 signaling or promotes the functional bioactivity of Gp130/IL6. In some instances, activation of Gp130/IL6 may be achieved through repression of a Gp130/IL6 inhibitor, e.g., including but not limited to the

use of an inhibitory nucleic acid targeting an inhibitor of Gp130/IL6 or an antibody or small molecule directed to a Gp130/IL6 inhibitor.

[00113] Gp130/IL6 agonists will include a gp130/IL6 agonist binding-pair where such a binding-pair includes a first binding partner and a second binding partner that, when both binding partners are present in the culture medium function as a gp130/IL6 agonist. In some instances, one or more component of the gp130/IL6 agonist binding-pair may be added to the culture media. In other instances, one or more component of the gp130/IL6 agonist binding-pair may be expressed from a cell of the culture. The first and second binding partners of the a gp130/IL6 agonist binding-pair may be ligand receptor pairs, including soluble ligand and soluble receptor pairs that are capable of functioning as a gp130/IL6 agonist including extracellularly activating gp130/IL6.

[00114] Exemplary gp130/IL6 agonist binding-pairs include but are not limited to e.g., soluble IL6 and soluble IL6 receptor or a portion thereof (including e.g., soluble IL6 receptor alpha (IL6RA) ectodomain), soluble IL11 and soluble IL11 receptor (IL11R) or a portion thereof, soluble LIF and soluble LIF receptor (LIFR) or a portion thereof, soluble OSM and soluble OSM receptor (OSMR) or a portion thereof, soluble CNTF and soluble CNTF receptor (CNTFR) or a portion thereof, soluble CT1 and soluble CT1 receptor (i.e., LIF receptor (LIFR)) or a portion thereof. In some instances, gp130/IL6 agonist binding-pairs include those pairs containing component parts selected from those described in Taga & Kishimoto (Annu Rev Immunol. 1997;15:797-81), the disclosure of which is incorporated herein by reference in its entirety.

[00115] In some instances, pathway modulating agents, as described above and including pathway activators and pathway inhibitors include, e.g., those that are commercially available, e.g., from such suppliers such as Tocris Bioscience (Bristol, UK), Sigma-Aldrich (St. Louis, MO), Santa Cruz Biotechnology (Santa Cruz, CA), and the like.

[00116] Pluripotent progenitors and derivatives thereof may be contacted with induction agents by any convenient means. Generally an induction agent is added to culture media, as described herein, within which cells of the instant disclosure are grown or maintained, such that the induction agent is present, in contact with the cells, at an effective concentration to produce the desired effect, e.g., induce a desired lineage restriction event. In other instances, e.g., where the existing culture media is not compatible with a particular induction agent, the culture media in which the cells are being grown is replaced with fresh culture media containing the particular induction agent present in the fresh media at an effective concentration to produce the desired effect. In instances where fresh or specific culture media is provided with a particular induction agent the culture agent may, in some instances, be specifically formulated for the particular induction agent, e.g., containing one or more specific additional reagents to, e.g., aid in the delivery of the induction agent, aid in the solubility of the induction agent, aid in the stability of the induction agent, etc.

[00117] In instances where a particular induction agent may consists of two or more parts, e.g., in the instance of a specific binding pair including but not limited to e.g., a gp130/IL6 agonist binding

pair, both components may be administered simultaneously or the components may be added sequentially provided both components are present together in an effective concentration in the culture medium at the time necessary to perform the desired induction.

[00118] The effective concentration of a particular induction agent will vary and will depend on the agent. In addition, in some instances, the effective concentration may also depend on the cells being induced, the culture condition of the cells, other induction agents co-present in the culture media, etc. As such, the effective concentration of induction agents will vary and may range from 1 ng/mL to 10 µg/mL or more, including but not limited to, e.g., 1 ng/mL, 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, 10 ng/mL, 11 ng/mL, 12 ng/mL, 13 ng/mL, 14 ng/mL, 15 ng/mL, 16 ng/mL, 17 ng/mL, 18 ng/mL, 19 ng/mL, 20 ng/mL, 21 ng/mL, 22 ng/mL, 23 ng/mL, 24 ng/mL, 25 ng/mL, 26 ng/mL, 27 ng/mL, 28 ng/mL, 29 ng/mL, 30 ng/mL, 31 ng/mL, 32 ng/mL, 33 ng/mL, 34 ng/mL, 35 ng/mL, 36 ng/mL, 37 ng/mL, 38 ng/mL, 39 ng/mL, 40 ng/mL, 41 ng/mL, 42 ng/mL, 43 ng/mL, 44 ng/mL, 45 ng/mL, 46 ng/mL, 47 ng/mL, 48 ng/mL, 49 ng/mL, 50 ng/mL, 1-5 ng/mL, 1-10 ng/mL, 1-20 ng/mL, 1-30 ng/mL, 1-40 ng/mL, 1-50 ng/mL, 5-10 ng/mL, 5-20 ng/mL, 10-20 ng/mL, 10-30 ng/mL, 10-40 ng/mL, 10-50 ng/mL, 20-30 ng/mL, 20-40 ng/mL, 20-50 ng/mL, 30-40 ng/mL, 30-50 ng/mL, 40-50 ng/mL, 1-100 ng/mL, 50-100 ng/mL, 60-100 ng/mL, 70-100 ng/mL, 80-100 ng/mL, 90-100 ng/mL, 10-100 ng/mL, 50-200 ng/mL, 100-200 ng/mL, 50-300 ng/mL, 100-300 ng/mL, 200-300 ng/mL, 50-400 ng/mL, 100-400 ng/mL, 200-400 ng/mL, 300-400 ng/mL, 50-500 ng/mL, 100-500 ng/mL, 200-500 ng/mL, 300-500 ng/mL, 400 to 500 ng/mL, 0.001-1 µg/mL, 0.001-2 µg/mL, 0.001-3 µg/mL, 0.001-4 µg/mL, 0.001-5 µg/mL, 0.001-6 µg/mL, 0.001-7 µg/mL, 0.001-8 µg/mL, 0.001-9 µg/mL, 0.001-10 µg/mL, 0.01-1 µg/mL, 0.01-2 µg/mL, 0.01-3 µg/mL, 0.01-4 µg/mL, 0.01-5 µg/mL, 0.01-6 µg/mL, 0.01-7 µg/mL, 0.01-8 µg/mL, 0.01-9 µg/mL, 0.01-10 µg/mL, 0.1-1 µg/mL, 0.1-2 µg/mL, 0.1-3 µg/mL, 0.1-4 µg/mL, 0.1-5 µg/mL, 0.1-6 µg/mL, 0.1-7 µg/mL, 0.1-8 µg/mL, 0.1-9 µg/mL, 0.1-10 µg/mL, 0.5-1 µg/mL, 0.5-2 µg/mL, 0.5-3 µg/mL, 0.5-4 µg/mL, 0.5-5 µg/mL, 0.5-6 µg/mL, 0.5-7 µg/mL, 0.5-8 µg/mL, 0.5-9 µg/mL, 0.5-10 µg/mL, and the like.

[00119] In some instances, the effective concentration of an induction agent in solution, e.g., cell culture media, may range from 1 nM to 100 µM or more, including but not limited to, e.g., 1 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 7 nM, 8 nM, 9 nM, 10 nM, 11 nM, 12 nM, 13 nM, 14 nM, 15 nM, 16 nM, 17 nM, 18 nM, 19 nM, 20 nM, 21 nM, 22 nM, 23 nM, 24 nM, 25 nM, 26 nM, 27 nM, 28 nM, 29 nM, 30 nM, 31 nM, 32 nM, 33 nM, 34 nM, 35 nM, 36 nM, 37 nM, 38 nM, 39 nM, 40 nM, 41 nM, 42 nM, 43 nM, 44 nM, 45 nM, 46 nM, 47 nM, 48 nM, 49 nM, 50 nM, 1-2 nM, 1-3 nM, 1-4 nM, 1-5 nM, 1-6 nM, 1-7 nM, 1-8 nM, 1-9 nM, 1-10 nM, 1.5 nM, 1.5-2 nM, 1.5-3 nM, 1.5-4 nM, 1.5-5 nM, 1.5-6 nM, 1.5-7 nM, 1.5-8 nM, 1.5-9 nM, 1.5-10 nM, 2-3 nM, 2-4 nM, 2-5 nM, 2-6 nM, 2-7 nM, 2-8 nM, 2-9 nM, 2-10 nM, 3-4 nM, 3-5 nM, 3-6 nM, 3-7 nM, 3-8 nM, 3-9 nM, 3-10 nM, 4-5 nM, 4-6 nM, 4-7 nM, 4-8 nM, 4-9 nM, 4-10 nM, 5-6 nM, 5-7 nM, 5-8 nM, 5-9 nM, 5-10 nM, 6-7 nM, 6-8 nM, 6-9 nM, 6-10 nM, 7-8 nM, 7-9 nM, 7-10 nM, 8-9 nM, 8-10 nM, 9-10 nM, 5-15 nM, 5-20 nM, 5-25 nM, 5-30 nM, 5-35 nM, 5-40 nM, 5-45 nM, 5-50 nM, 10-15 nM, 10-20 nM, 10-25 nM,

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[00120] In some instances, the effective concentration of an induction agent will be below a critical concentration such that the induction produces the desired effect essentially without undesirable effects. As used herein, the term "critical concentration" refers to a concentration of induction agent above which undesirable effects are produced. Undesirable effects that may be the result of a concentration exceeding the critical concentration include but are not limited to, e.g., off-target effects (off-target activation of signaling, off-target inhibition of signaling), reduction or loss of function (e.g., loss of desired activator function, loss of desired inhibitor function) reduction of cell viability, increase in cell mortality, lineage restriction towards an undesired cell type, differentiation into an undesired cell type, loss of expression of a particular desired marker, etc. Whether a particular induction agent will have a critical concentration and what the critical

concentrations of those agents having a critical concentration are will depend on the agent and the specific conditions in which the agent is used.

[00121] In some instances, cells of the instant disclosure may be contacted with multiple induction agents and/or multiple induction compositions in order achieve a desired cell type of derivative thereof. In some instances, a particular induction composition will contain two or more induction agents such that a particular cell culture is simultaneously contacted with multiple induction agents. In some instances, a particular series of induction compositions may be used, one at a time, in generating a desired cell type such that a particular cell culture is successively contacted with multiple induction agents.

[00122] The duration of contact of a particular induction composition with a particular cell type will vary and will depend, e.g., on the desired cell type, the cell type being induced, and the components of the induction composition. In some instances, a particular induction composition may be introduced for different exposure times depending on the context of use, e.g., cell type X may be contacted with induction composition Y for time Z whereas cell type A may be contacted with induction composition Y for time B, wherein cell type X is different than cell type A and time Z is different than time B. As such, the time cells are contacted with a particular induction composition may vary, e.g., when being used on different cells, when being used to generate different cells, or when being used at different steps of a differentiation process.

[00123] The duration of contact of a particular induction composition with a particular cell type, in some instances, may be referred to as the "exposure time" and exposure times may range from a day to weeks or more, including but not limited to e.g., 1 day, 1.5 days, 2 days, 2.5 days, 3 days, 3.5 days, 4 days, 4.5 days, 5 days, 5.5 days, 6 days, 6.5 days, 7 days, 7.5 days, 8 days, 8.5 days, 9 days, 9.5 days, 10 days, 11 days, 12, days, 13, days, 14 days, 15, days, etc. As used herein, exposure times are, in some instances, referred as consisting essentially of, e.g., 24 hours, indicating that the exposure time may be longer or shorter than that specified including those exposure times that are longer or shorter but do not materially affect the basic outcome of the particular exposure. As such, in some instances where a particular exposure is more time sensitive such that under or over exposure, e.g., of more or less than 1 hour, materially affects the outcome of the exposure, a time period consisting essentially of, e.g., 24 hours, will be interpreted to refer to a time period ranging from about 23 hours to about 25 hours. In some other instances where a particular exposure is less time sensitive such that under or over exposure, e.g., of more than 12 hours, does not materially affect the outcome of the exposure, a time period consisting essentially of, e.g., 24 hours will mean a time period ranging from about 12 hours or less to about 36 hours or more. In some instances, depending on the context, an exposure period consisting essentially of 24 hours may refer to an exposure time of 22-26 hours, 21-27 hours, 20-28 hours, 19-29 hours, 18-30 hours, etc.

[00124] In some instances, time periods of exposure may be pre-determined such that cells are contacted with an induction composition according to a schedule set forth prior to the contacting.

In some instances, the time period of exposure, whether pre-determined or otherwise, may be modulated according to some feature or characteristic of the cells and/or cell culture, including but not limited to, e.g., cell morphology, cell viability, cell appearance, cellular behaviors, cell number, culture confluence, marker expression, etc.

[00125] In some instances, cells are grown in densities that may range from but not limited to 100 cells/cm², 10³ cells/cm², 10⁴ cells/cm², 10⁵ cells/cm², 10⁶ cells/cm², 10⁷ cells/cm², 10⁸ cells/cm², 10⁹ cells/cm², 10¹⁰ cells/cm².

Markers

[00126] Aspects of the present disclosure include identifying cells based on the presence or absence or relative amount of one or more markers. In some instances, markers of interest include cell surface markers that may be detected, e.g., on live cells. In other instances, markers of interest include expression markers, e.g., cellular expression markers indicative of cell type.

[00127] Markers may be detected or measured by any convenient means as such marker detection is well-known in the art and may make use of one or more detection reagents including but not limited to, e.g., antibodies, antibody fragments, binding partners (e.g., ligands, binding pairs, etc), hybridizable nucleic acids, aptamers, etc. In some instances, a marker may be a cell surface marker and detection of the marker may be performed based on the use of one or more detection reagents that specifically bind to the marker. Detection reagents, e.g., antibodies, may be detectably labeled (e.g., fluorescently labeled through the attachment of a fluorescent molecule, fluorescent bead, or other fluorescent label) or may be detected through the use of a second detectably labeled detection reagent that specifically binds to the first detection reagent (e.g., a fluorescently labeled secondary antibody). In some instances, a detection agent, e.g., having a detectable label or having been bound by a second agent having a detectable label, can be visualized or otherwise observed or detected based on the visual characteristics of the label, including e.g., fluorescent detection, colorimetric detection, and the like. Detectable labels useful in detection reagents need not be visually detectable and may, in some instances, be detected by a detection device configured to detect a non-visual detectable label including but not limited to, e.g., a magnetic label, a radioactive label, etc. In some instances, detectable labels may be detected through the use of one or more detection reactions, including but not limited to, e.g., enzymatic detection reactions (enzymatic reactions generating a detectable substrate, e.g., a fluorescent or colorimetric substrate), amplification reactions (PCR amplification, fluorescent signal amplification (e.g., tyramide signal amplification, etc.), etc.)

[00128] In certain aspects of the instant disclosure, methods described make use of cell surface markers detectable on the surface of cells using one or more appropriate detection reagents. Cell surface markers of interest may vary and depend on the type of cell to be detected or the desired cell type being derived.

[00129] In some instances, identification and/or selection for sorting of cells may be performed using a combination of markers. Such combinations may include but combinations of positive selection markers, combinations of negative selection markers or mixed combinations of positive and negative selection markers.

[00130] In certain embodiments marker detection and/or measurement of marker level is performed using flow cytometry. Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multi-parametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. FACS provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, generally one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. The flow cytometer and the FACS machine are useful scientific instruments as they provide fast, objective and quantitative recording of signals, e.g., fluorescent signals, and/or detection of cellular characteristics, e.g., size, granularity, viability, etc., from individual cells as well as physical separation of cells of particular interest. Fluorescent signals used in flow cytometry, for instance when quantifying and/or sorting cells by any marker present on or in the cell, typically are fluorescently-tagged antibody preparations or fluorescently-tagged ligands for binding to antibodies or other antigen-, epitope- or ligand-specific agent, such as with biotin/avidin binding systems or fluorescently-labeled and optionally addressable beads (e.g. microspheres or microbeads). The markers or combinations of markers detected by the optics and/or electronics of a flow cytometer vary and in some cases include but are not limited to: cell surface markers, intracellular and nuclear antigens, DNA, RNA, cell pigments, cell metabolites, protein modifications, transgenic proteins, enzymatic activity, apoptosis indicators, cell viability, cell oxidative state, etc.

[00131] In certain instances, flow cytometry is performed using a detection reagent, e.g., a fluorochrome-labeled antibody, e.g., a monoclonal antibody, with specific avidity against a cell surface marker of interest. A cellular sample is contacted with a detection reagent under conditions sufficient to allow the detection reagent to bind the cell surface marker and the cells of the sample are loaded into the flow cytometer, e.g., by first harvesting the cells from a cell culture using methods known in the art or described herein and re-suspending the isolated cells in a suitable buffer, e.g., running buffer. The cells loaded into the flow cytometer are run through the flow cytometer, e.g., by flowing cell containing buffer or liquid sample through the flow cell of the flow cytometer. The flow cytometer detects events as the cell passes one or more detection areas of the flow cytometer. For example, the flow cytometer may detect fluorescence emitted from a fluorochrome of a detection reagent upon excitation of the fluorochrome with a particular wavelength of light. In some instances, the flow cytometer detects the relative intensity of a particular signal, e.g., fluorescence of a particular detection reagent, of a particular cell, e.g., to quantify the level of a marker present on the surface of the cell and/or to qualitatively categorize

the cell, e.g., as a cell that is positive for a particular marker or a cell that is negative for a particular marker. Detected events are counted or otherwise evaluated by the flow cytometer with or without input from an operator and used to determine, e.g., the total number of cells, the number or proportion of cells bound to a particular detection reagent, etc. In instances where FACS is utilized cells may be sorted, e.g., into separate containers, based on the detection or measurement of a particular marker. In some instances, cell sorting, e.g., by FACS, may be utilized to generate a purified population of a desired cell type.

[00132] In some instances, a threshold level of a particular detectable marker is used to categorize cells for sorting by FACS. Threshold levels may be used to categorize cells as “positive”, “negative”, “high”, “low”, etc. for a particular marker based on the level of detection of the marker. In some instances, a marker threshold level is determined by making a comparison of the levels of marker within a population of cells, e.g., a population of cells of unknown expression levels of Marker X or a population of cells suspected of containing subpopulations of cells having different expression levels of Marker X. For example, the expression level of Marker X is measured on a flow cytometer of at least a sufficient number of cells such that the measurements may be plotted, e.g., on a histogram, and separation between two or more subpopulations of cells is revealed based on individual cell expression levels of Marker X. Accordingly, the flow cytometer operator may then determine a threshold level between the subpopulations that may be used to categorize cells as belonging to a particular subpopulation, e.g., a subpopulation having a low level of expression of Marker X or a subpopulation having high level of expression of Marker X.

[00133] In other instances, a threshold is predetermined based on a known or expected difference in marker level between cells of different populations. In some instances, a threshold is pre-calibrated or saved, e.g., in computer readable form, in a device, e.g., a flow cytometer, used in detecting or measuring a marker and/or sorting cells based on marker detection and/or measurement.

[00134] In some instances, the marker threshold is based on the limit of detection of the flow cytometer. For example, cells of a population of cells may be identified as expressing a particular marker (i.e. being positive for a particular marker) if the cells have any detectable level of a particular marker. Likewise, cells of a population of cells may be identified as not expressing a particular marker (i.e. being negative for a particular marker) if the cells do not have a detectable level of a particular biomarker. Accordingly, the detection level of the flow cytometer may be used to determine the biomarker threshold.

[00135] Expression markers of interest may be used to identify a particular cell type or verify that a derived cell type expresses a characteristic component of the derived cell type. In some instances, detection of expression markers may allow for optimization of a particular differentiation protocol, e.g., to optimize production of a desired cell type based on detection of one or more expression markers. Expression markers will vary depending on the type of cell to be identified or verified and/or desired downstream uses of the cell following identification or

verification with the expression marker. Types of expression markers will include but are not limited to, e.g., gene expression marker, protein expression markers, expressed reporters, and the like. Expression marker detection and/or measurement may be detrimental to cell viability (e.g., wherein detection requires lysing or fixing a cell of interest) or may be essentially neutral to cell viability (e.g., wherein detection does not require lysing or fixing a cell of interest and may be performed on live cells).

[00136] Gene expression markers include but are not limited to the presence, absence, and/or relative amounts of a particular gene transcript that is indicative of particular cell type. Protein expression markers include but are not limited to the presence, absence, and/or relative amounts of a particular expression product that is indicative of particular cell type. Protein expression markers may be intercellular proteins, intracellular proteins or cell surface proteins. In some instances, a gene expression marker and a protein expression marker derived from the same gene may be indicative of a particular cell type.

[00137] Methods of detecting and/or measuring gene expression and/or protein expression are well-known in the art and include but are not limited to, e.g., Northern blot, Western blot, ELISA, PCR, quantitative PCR, in situ hybridization, fluorescent in situ hybridization, immunohistochemistry, immunofluorescence, microarray, quantitative sequencing, RNAseq, quantitative mass spectrometry, and the like.

[00138] Gene and protein expression markers useful in characterizing and/or identifying arterial endothelial cells, e.g., derived as described herein, include but are not limited to, e.g., CD31, CD34, CD144 (VE-cadherin), SCL, LMO2, FLI1, AA4.1, ESAM1, artery markers (SOX17, DLL4, JAG1, EFNB2), hemogenic markers (RUNX1, MYB), and the like. In some instances, the measurement of one or more such arterial endothelial markers above a particular threshold is indicative of an increased likelihood that an analyzed cell or cell population is an arterial endothelial cell or are arterial endothelial mesoderm cells. Markers useful in characterizing hematopoietic stem cells include the HSC surface markers CD34, CD144, CD45; and the HSC transcription factors/chromatin regulators HLF, MECOM, MLLT3, RUNX1, MEIS1, and MYB. Generally, the detection and/or measurement of more such markers increases confidence in such determinations. In certain instances, measurement of one or more markers above a particular threshold indicates that a cell is the desired cell type.

[00139] Expressed markers useful in identifying the above cell types as well as other cell types described herein are not limited to those specifically disclosed as other markers are known in the art may be deployed either independently to identify or characterize a particular cell type or in combination with one or more markers described herein. Furthermore, expressed markers are not limited to those gene products that produce a polypeptide and may include e.g., non-coding RNAs, non-coding transcripts, microRNAs, and the like. For example, in some instances identification and/or characterization of a cell type of interest may make use of one or more differentially expressed long noncoding RNAs as described herein.

[00140] In some instances, cells may be identified based on an expressed reporter wherein the expressed reporter may be heterologous sequence introduced into a cell. For example, in some instances, heterologous sequence encoding a detectable reporter may be introduced into a cell such that upon differentiation and/or lineage restriction to a cell type of interest the reporter, e.g., a fluorescent molecule, becomes alternatively active or inactive. As describe herein, heterologous sequence may be stably or transiently introduced. Such introduced heterologous sequence may be configured to be responsive to activation of a marker, e.g., a marker of a particular cell type as described herein or known in the art, such that upon expression of the marker the reporter is activated. Alternatively, such introduced heterologous sequence may be configured to be responsive to activation of a marker, e.g., a marker of a particular cell type as described herein or known in the art, such that the reporter is active independent of expression of the marker but upon expression of the marker the reporter is deactivated. Methods of creating and using expression reporters are well-known in art.

Cell Modification

[00141] Methods of modification of cells, including modification of pluripotent cells and modification of hematopoietic stem cells are well-known in the art and include but are not limited to e.g., genetic modification (e.g., through deletion mutagenesis, through substitution mutagenesis), through insertional mutagenesis (e.g., through the introduction of heterologous nucleic acid into the pluripotent cell, etc.), non-mutagenic genetic modification (e.g., the non-mutagenic insertion of heterologous nucleic acid, etc.), epigenetic modification (e.g., through the treatment with one or more specific or general epigenetic modifying agents (e.g., methylation inhibitors, methylation activators, demethylases, etc.), other modifications (e.g., non-genetic labeling, etc.).

[00142] Modifications of cells may be transient or stable. In some instances, a modification of a particular pluripotent cell or progenitor cell may be stable such that the modification persists through derivation of a desired cell type from the pluripotent cell or progenitor cell as described herein. In some instances, stable modifications may persist through introduction of a cell type into a host. In some instances, stable modifications may persist through proliferation of the cell such that all progenitors of a particular modified cell also contain the subject modification. In some instances, a modification of a particular pluripotent cell or progenitor cell may be transient such that the modification is lost after derivation of a cell type of interest from the transiently modified pluripotent cell. In certain instances, transient modifications may persist through one or more rounds of proliferation of the modified cell such that some but not all of the progeny of the modified cell contain the subject modification. In some instances, a transient modification will not persist during proliferation such that none of the progeny of a modified cell will contain the subject modification. In some instances, a transiently modified cell may be configured such that the modification persists through certain aspects of derivation of the cell type of interest, e.g., through

derivation of a particular cell type of interest, but is lost prior to introduction of the derived cell into a host.

Screening

[00143] Aspects of the instant disclosure include methods of screening pharmacological agents using hematopoietic stem cells derived according to the methods described herein. In some instances, a plurality of cell populations derived according to the methods as described herein are contacted with a plurality of pharmacological agents in order to screen for agents producing a cellular response of interest. A cellular response of interest may be any cellular response including but not limited to, e.g., cell death, cell survival, cell self-renewal, proliferation, differentiation, expression of one or more markers, loss of expression of one or more markers, change in morphology, change in cellular physiology, cellular engraftment, change in cell motility, change in cell migration, production of a particular cellular component, cease of production of a particular cellular component, change in metabolic output, response to stress, and the like.

[00144] Screening pharmacological agents using cells described herein may be performed *in vitro*, e.g., in a tissue culture chamber, on a slide, etc., or may be performed *in vivo*, e.g., in an animal host, etc. Cells used in such screening assays may be genetically altered or may be unaltered. In some instances, cells generated according to the methods as described herein are used in multiplexed *in vitro* pharmacological screening. Methods for evaluating cellular responses during *in vitro* screening are well-known in the art and include but are not limited to, e.g., microscopic methods (e.g., light microscopy, electron microscopy, etc.), expression assays, enzymatic assays, cytological assays (e.g., cellular staining), genomics, transcriptomics, metabolomics, and the like.

[00145] In some instances, cells generated according to the methods as described herein are introduced into a host animal and the host animal may be administered a pharmacological agent in order to screen for a response from the introduced cells. In some instances, the cells of the *in vivo* assay may be directly evaluated, e.g., for an intrinsic response to a pharmacological agent. In some instances, the host animal of the *in vivo* assay may be evaluated as an indirect measurement of the response of the cells to the pharmacological agent.

[00146] In certain embodiments, the subject disclosure includes screening cells derived according to the methods described herein as a method of therapy of an animal model of disease and/or a human disease. Methods of screening cells derived according to the methods described herein as a method of therapy may be, in some instances, performed according to those methods described below regarding using such cells in therapeutic protocols.

[00147] In certain embodiments, the subject disclosure includes screening cells derived according to the methods described herein introduced to a host animal as a method of directly evaluating the cells or particular cellular behaviors, e.g., due to an introduced genetic modification or a naturally derived mutation. In one embodiment, genetically modified cells, e.g., having at least

one modified genomic locus, derived according to the methods described herein may be introduced into a host animal and the ability of the cells to differentiate into a particular tissue or cell type may be evaluated. In another embodiment, genetically modified cells derived according to the methods described herein may be introduced into a host animal and the behavior of the cells within the host animal and/or within a tissue of the host animal may be evaluated. In another embodiment, cells derived from a donor organism having a particular mutation or phenotype and lineage restricted according to the methods described herein may be introduced into a host animal and the behavior of the cells within the host animal and/or within a tissue of the host animal may be evaluated, including, e.g., the ability of the cells to differentiate into one or more tissue or cell types. The cells may be introduced into the host animal in an autologous graft, an allograft, or a xenograft such that the introduced cells may be derived from the host animal, a separate donor of the same species as the host animal, or a separate donor of a different species as compared to the host animal, respectively.

Therapy

[00148] The hematopoietic stem cells of the invention may be further differentiated to produce any number of cell types, including blood cells, including red blood cells, immune cells, and other cells derived from hematopoietic stem cells. The scope of the invention encompasses a cell differentiated from a hematopoietic stem cell, wherein the hematopoietic stem cell was produced by the methods of the invention. Cell types that may be produced from the hematopoietic stem cells of the invention include red blood cells, platelets, lymphoid cells, myeloid cells, macrophages, T-cells, B-cells, and others.

[00149] Aspects of the disclosure include methods for lessening the symptoms of and/or ameliorating a dysfunction in hematopoietic stem cells and cells derived therefrom. Treatment methods described herein include therapeutic treatments, in which the subject is inflicted prior to administration, and prophylactic treatments, in which the subject is not inflicted prior to administration. In some embodiments, the subject has an increased likelihood of becoming inflicted or is suspected of having an increased likelihood of becoming inflicted (e.g., relative to a standard, e.g., relative to the average individual, e.g., a subject may have a genetic predisposition to dysfunction or disorder and/or a family history indicating increased risk of dysfunction or disorder), in which case the treatment can be a prophylactic treatment. Any and all forms of dysfunction, whether treated or untreated, or resulting from any primary condition, whether treated or untreated, are suitable dysfunctions or disorders to be treated by the subject methods described herein.

[00150] In some instances, the treatment methods described herein include the alleviation or reduction or prevention of one or more symptoms of dysfunction or disorder. Symptoms of dysfunction or disorder will vary, may be infrequent, occasional, frequent, or constant.

- [00151] Conditions that may be treated with HSC transplantation include, for example, multiple myeloma, Non-Hodgkin lymphoma, Hodgkin disease, acute myeloid leukemia, neuroblastoma, germ cell tumors, autoimmune disorders - Systemic lupus erythematosus (SLE), systemic sclerosis, amyloidosis, etc..
- [00152] Allogeneic or genetically modified autologous HSC may be used, for example, in the treatment of: Acute myeloid leukemia, Acute lymphoblastic leukemia, Chronic myeloid leukemia; Chronic lymphocytic leukemia, Myeloproliferative disorders, Myelodysplastic syndromes, Multiple myeloma, Non-Hodgkin lymphoma, Hodgkin disease, Aplastic anemia, Pure red cell aplasia, Paroxysmal nocturnal hemoglobinuria, Fanconi anemia, Thalassemia major, Sickle cell anemia, Severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome, Hemophagocytic lymphohistiocytosis (HLH), Inborn errors of metabolism - Eg, mucopolysaccharidosis, Gaucher disease, metachromatic leukodystrophies, and adrenoleukodystrophies, Epidermolysis bullosa, Severe congenital neutropenia, Shwachman-Diamond syndrome, Diamond-Blackfan anemia, Leukocyte adhesion deficiency, and the like.
- [00153] Embodiments of the invention include transplantation into a patient suffering from a genetic blood disorder, where hematopoietic stem cells of a normal phenotype generated by the methods of the disclosure are transplanted into the patient. Such diseases include, without limitation, the treatment of anemias caused by defective hemoglobin synthesis (hemoglobinopathies).
- [00154] Sickle cell diseases include HbS Disease; drepanocytic anemia; meniscocytosis. Chronic hemolytic anemia occurring almost exclusively in blacks and characterized by sickle-shaped RBCs caused by homozygous inheritance of Hb S. Homozygotes have sickle cell anemia; heterozygotes are not anemic, but the sickling trait (sickleemia) can be demonstrated *in vitro*. In Hb S, valine is substituted for glutamic acid in the sixth amino acid of the beta chain. Deoxy-Hb S is much less soluble than deoxy-Hb A; it forms a semisolid gel of rodlike tactoids that cause RBCs to sickle at sites of low PO₂. Distorted, inflexible RBCs adhere to vascular endothelium and plug small arterioles and capillaries, which leads to occlusion and infarction. Because sickled RBCs are too fragile to withstand the mechanical trauma of circulation, hemolysis occurs after they enter the circulation. In homozygotes, clinical manifestations are caused by anemia and vaso-occlusive events resulting in tissue ischemia and infarction. Growth and development are impaired, and susceptibility to infection increases. Anemia is usually severe but varies highly among patients. Anemia may be exacerbated in children by acute sequestration of sickled cells in the spleen.
- [00155] Thalassemias are a group of chronic, inherited, microcytic anemias characterized by defective Hb synthesis and ineffective erythropoiesis, particularly common in persons of Mediterranean, African, and Southeast Asian ancestry. Thalassemia is among the most common inherited hemolytic disorders. It results from unbalanced Hb synthesis caused by decreased production of at least one globin polypeptide chain (β , α , γ , δ).

- [00156] Aplastic anemia results from a loss of RBC precursors, either from a defect in stem cell pool or an injury to the microenvironment that supports the marrow, and often with borderline high MCV values. The term aplastic anemia commonly implies a panhypoplasia of the marrow with associated leukopenia and thrombocytopenia.
- [00157] Combined immunodeficiency is a group of disorders characterized by congenital and usually hereditary deficiency of both B- and T-cell systems, lymphoid aplasia, and thymic dysplasia. The combined immunodeficiencies include severe combined immunodeficiency, Swiss agammaglobulinemia, combined immunodeficiency with adenosine deaminase or nucleoside phosphorylase deficiency, and combined immunodeficiency with immunoglobulins (Nezelof syndrome). Most patients have an early onset of infection with thrush, pneumonia, and diarrhea. If left untreated, most die before age 2. Most patients have profound deficiency of B cells and immunoglobulin. The following are characteristic: lymphopenia, low or absent T-cell levels, poor proliferative response to mitogens, cutaneous anergy, an absent thymic shadow, and diminished lymphoid tissue. Pneumocystis pneumonia and other opportunistic infections are common.
- [00158] The methods of treatment described herein include administering a therapeutically effective amount of an HSC population, e.g., an essentially homogenous population, of hematopoietic stem cells to a subject in need thereof in order to treat the subject for a dysfunction or deficiency.
- [00159] The effective amount administered varies depending upon the goal of the administration, the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (e.g., human, non-human primate, primate, etc.), the degree of resolution desired (e.g., the amount of alleviation or reduction of symptoms), the formulation of the cell composition, the treating clinician's assessment of the medical situation, and other relevant factors.
- [00160] A "therapeutically effective dose" or "therapeutic dose" is an amount sufficient to effect desired clinical results (i.e., achieve therapeutic efficacy) or reduce, alleviate, or prevent symptoms to a desired extent as determined by the patient or the clinician. A therapeutically effective dose can be administered in one or more administrations. For purposes of this disclosure, a therapeutically effective dose of cells is an amount that is sufficient, when administered to (e.g., transplanted into) the individual, to palliate, ameliorate, stabilize, reverse, prevent, slow or delay the progression of the disease state by, for example, inducing stabilization, repair, or regeneration.
- [00161] In some embodiments, a therapeutically effective dose of cells is one cell or more (e.g., 1×10^2 or more, 5×10^2 or more, 1×10^3 or more, 5×10^3 or more, 1×10^4 cells, 5×10^4 or more, 1×10^5 or more, 5×10^5 or more, 1×10^6 or more, 2×10^6 or more, 5×10^6 or more, 1×10^7 cells, 5×10^7 or more, 1×10^8 or more, 5×10^8 or more, 1×10^9 or more, 5×10^9 or more, or 1×10^{10} or more).

[00162] In some embodiments, a therapeutically effective dose of cells is in a range of from 1×10^3 cells to 1×10^{10} cells (e.g., from 5×10^3 cells to 1×10^{10} cells, from 1×10^4 cells to 1×10^{10} cells, from 5×10^4 cells to 1×10^{10} cells, from 1×10^5 cells to 1×10^{10} cells, from 5×10^5 cells to 1×10^{10} cells, from 1×10^6 cells to 1×10^{10} cells, from 5×10^6 cells to 1×10^{10} cells, from 1×10^7 cells to 1×10^{10} cells, from 5×10^7 cells to 1×10^{10} cells, from 1×10^8 cells to 1×10^{10} cells, from 5×10^8 cells to 1×10^{10} , from 5×10^3 cells to 5×10^9 cells, from 1×10^4 cells to 5×10^9 cells, from 5×10^4 cells to 5×10^9 cells, from 1×10^5 cells to 5×10^9 cells, from 5×10^5 cells to 5×10^9 cells, from 1×10^6 cells to 5×10^9 cells, from 5×10^6 cells to 5×10^9 cells, from 1×10^7 cells to 5×10^9 cells, from 5×10^7 cells to 5×10^9 cells, from 1×10^8 cells to 5×10^9 cells, from 5×10^8 cells to 5×10^9 , from 5×10^3 cells to 1×10^9 cells, from 1×10^4 cells to 1×10^9 cells, from 5×10^4 cells to 1×10^9 cells, from 1×10^5 cells to 1×10^9 cells, from 5×10^5 cells to 1×10^9 cells, from 1×10^6 cells to 1×10^9 cells, from 5×10^6 cells to 1×10^9 cells, from 1×10^7 cells to 1×10^9 cells, from 5×10^7 cells to 1×10^9 cells, from 1×10^8 cells to 1×10^9 cells, from 5×10^8 cells to 1×10^9 , from 5×10^3 cells to 5×10^8 cells, from 1×10^4 cells to 5×10^8 cells, from 5×10^4 cells to 5×10^8 cells, from 1×10^5 cells to 5×10^8 cells, from 5×10^5 cells to 5×10^8 cells, from 1×10^6 cells to 5×10^8 cells, from 5×10^6 cells to 5×10^8 cells, from 1×10^7 cells to 5×10^8 cells, from 5×10^7 cells to 5×10^8 cells, or from 1×10^8 cells to 5×10^8 cells).

[00163] In some embodiments, the concentration of cells to be administered is in a range of from 1×10^5 cells/ml to 1×10^9 cells/ml (e.g., from 1×10^5 cells/ml to 1×10^8 cells/ml, from 5×10^5 cells/ml to 1×10^8 cells/ml, from 5×10^5 cells/ml to 5×10^7 cells/ml, from 1×10^6 cells/ml to 1×10^8 cells/ml, from 1×10^6 cells/ml to 5×10^7 cells/ml, from 1×10^6 cells/ml to 1×10^7 cells/ml, from 1×10^6 cells/ml to 6×10^6 cells/ml, or from 2×10^6 cells/ml to 8×10^6 cells/ml).

[00164] In some embodiments, the concentration of cells to be administered is 1×10^5 cells/ml or more (e.g., 1×10^5 cells/ml or more, 2×10^5 cells/ml or more, 3×10^5 cells/ml or more, 4×10^5 cells/ml or more, 5×10^5 cells/ml or more, 6×10^5 cells/ml or more, 7×10^5 cells/ml or more, 8×10^5 cells/ml or more, 9×10^5 cells/ml or more, 1×10^6 cells/ml or more, 2×10^6 cells/ml or more, 3×10^6 cells/ml or more, 4×10^6 cells/ml or more, 5×10^6 cells/ml or more, 6×10^6 cells/ml or more, 7×10^6 cells/ml or more, or 8×10^6 cells/ml or more).

[00165] A therapeutically effective dose of cells may be delivered or prepared and any suitable medium, including but not limited to, e.g., those described herein. Suitable medium for the delivery of a therapeutically effective dose of cells will vary and may depend on, e.g., the type of pluripotent cells from which the effective dose of cells is derived or the type of derived cells of the effective dose. In some instances, a suitable medium may be a basal medium. "Cell medium" as used herein are not limited to liquid media may, in some instances, include non-liquid components or combinations of liquid media and non-liquid components. Non-liquid components that may find use a delivery or preparation medium include those described herein and those known in the art. In some instances, non-liquid components include natural or synthetic extra cellular matrix components including but not limited to, e.g., basement membrane matrix components and the like.

[00166] In some instances, an effective dose of the cells described herein may be co-administered with one or more additional agents (e.g., prepared in a suitable medium). Additional agents useful in such co-administration include agents that improve the overall effectiveness of the effective dose of cells or decrease the dose of cells necessary to achieve an effect essentially equal to administration of an effective dose of the cells without the additional agent. Non-limiting examples of additional agents that may be co-administered include: conventional agents for treating diseases, pro-survival factors, pro-engraftment factors, functional mobilization agents, and the like.

[00167] By pro-survival factors is meant a factor or agent that may be added to the medium, culture media, delivery excipient, or storage solution that promotes the survival of a desired cell type. Such pro-survival factors may be general pro-survival factors that generally promote the survival of most cell types or may be specific pro-survival factors that only promote the survival of certain specific cell types. In some instances, pro-survival factors of the subject disclosure include but are not limited to, e.g., Rho-associated kinase (ROCK) inhibitor, pinacidil, allopurinol, uricase, cyclosporine (e.g., low dose, i.e., sub-immunosuppressive dose, cyclosporine), ZVAD-fmk, pro-survival cytokines (e.g., insulin-like growth factor-1 (IGF-1)), extra cellular matrix (ECM) components, hydrogels, matrigel, collagen, gelatin, agarose, alginate, poly(ethylene glycol), hyaluronic acid, etc.

[00168] By pro-engraftment factors is meant a factor or agent that may be added to the administered dose or the delivery excipient or the cell storage solution that, upon delivery of the cells into a subject for treatment, increase the engraftment of the administered cells into the tissue targeted for engraftment and therapy. In some instances, pro-engraftment factors include factors that physically retain the administered cells at the delivery site, e.g., the injection site in the case of direct injection to the affected area, including but not limited to, e.g., gels, polymers, and highly viscous liquids that have physical properties that prevent the administered cells from freely diffusing. Such gels, polymers, and highly viscous liquids include but are not limited to e.g., ECM components, hydrogels, matrigel, collagen, gelatin, agarose, alginate, poly(ethylene glycol), and the like.

[00169] The terms "co-administration" and "in combination with" include the administration of two or more therapeutic agents either simultaneously, concurrently or sequentially within no specific time limits. In one embodiment, the agents are present in the cell or in the subject's body at the same time or exert their biological or therapeutic effect at the same time. In one embodiment, the therapeutic agents are in the same composition or unit dosage form. In other embodiments, the therapeutic agents are in separate compositions or unit dosage forms. In certain embodiments, a first agent can be administered prior to (e.g., minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours,

6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapeutic agent.

[00170] The cells may be introduced by injection, catheter, intravenous perfusion, or the like. The cells may be frozen at liquid nitrogen temperatures and stored for long periods of time, being capable of use upon thawing. Once thawed, the cells may be expanded by use of growth factors and/or feeder cells or in feeder-free conditions associated with progenitor cell proliferation and differentiation. In some instances, the cells may be administered fresh such that the cells are expanded and differentiated and administered without being frozen.

[00171] The cells of this disclosure can be supplied in the form of a pharmaceutical composition, comprising an isotonic excipient or buffer or media prepared under sufficiently sterile conditions for human administration. For general principles in medicinal formulation, the reader is referred to *Cell Therapy: Stem Cell Transplantation, Gene Therapy, and Cellular Immunotherapy*, by G. Morstyn & W. Sheridan eds, Cambridge University Press, 1996; and *Hematopoietic Stem Cell Therapy*, E. D. Ball, J. Lister & P. Law, Churchill Livingstone, 2000. Choice of the cellular excipient and any accompanying elements of the composition will be adapted in accordance with the route and device used for administration. The composition may also comprise or be accompanied with one or more other ingredients that facilitate the engraftment or functional mobilization of the cells. Suitable ingredients include matrix proteins that support or promote adhesion of the cells, or complementary cell types.

[00172] Cells of the subject methods may be autologously derived. By autologously derived it is meant that the cells are derived from the subject that is to be treated with the cells. The cells may be derived from a tissue sample obtained from the subject including but not limited to, e.g., a blood sample (e.g., a peripheral blood sample), a skin sample, a bone marrow sample, and the like. In some instances, the sample from which cells are derived may be a biopsy or swab, e.g., a biopsy or swab collected to diagnose, monitor, or otherwise evaluate the subject, e.g., diagnose the subject for a dysfunction or deficiency for example a hematologic disorder. In some instances, the autologous sample from which the cells are derived may be a previously collected and stored sample, e.g., a banked tissue sample, from the subject to be treated, including but not limited to e.g., banked cardiac tissue or cells, banked musculoskeletal tissue or cells, banked reproductive tissue or cells, banked skin tissue or cells, banked bone tissue or cells, banked bone marrow tissue or cells, banked vascular tissue or cells, banked umbilical cord blood tissue or cells, and the like.

[00173] In some instances, cells of the subject methods are non-autologously derived. By non-autologously derived it is meant that the cells are not derived from the subject that is to be treated with the cells. In some instances, non-autologously derived cells may be xeno-derived (i.e., derived from a non-human animal) or allo-derived (i.e. derived from a human donor other than the subject to be treated). Non-autologously derived cells or tissue may be derived from any convenient source of cells or tissue collected by any convenient means.

[00174] Whether to use autologously derived or non-autologously derived cells may be determined according to the discretion of the subject's clinician and may depend on, e.g., the health, age, genetic predisposition or other physical state of the subject. In some instances, autologous cells may be preferred, including, e.g., to decrease the risk of immune rejection of the transplanted cells. In some instances, non-autologous cells may be preferred, including, e.g., when the subject has a genetic defect that affects hematologic cells.

[00175] Methods of derivation of pluripotent progenitor cells from an autologous or non-autologous tissue useful in the methods described herein include but are not limited to, e.g., methods of embryonic stem cell derivation and methods of induced pluripotent stem cell derivation. In some instances, methods as described herein may be performed using non-autologous pluripotent progenitor cells previously derived including, e.g., those publically or available or commercially available (e.g., from Biotime, Inc., Alameda, CA). In some instances, methods as described herein may be performed using newly derived non-autologous pluripotent progenitor cells or newly derived autologous pluripotent progenitor cells including but not limited to, e.g., newly derived embryonic stem cells (ESC) (including, e.g., those derived under xeno-free conditions as described in, e.g., Lei et al. (2007) *Cell Research*, 17:682-688) and newly derived induced pluripotent stem cells (iPS). General methods of inducing pluripotency to derive pluripotent progenitor cells are described in, e.g., Rodolfa KT, (2008) Inducing pluripotency, StemBook, ed. The Stem Cell Research Community, doi/10.3824/stembook.1.22.1 and Selvaraj et al. (2010) *Trends Biotechnol*, 28(4):214-23, the disclosures of which are incorporated herein by reference. In some instances, pluripotent progenitor cells, e.g., iPS cells, useful in the methods described herein are derived by reprogramming and are genetically unmodified, including e.g., those derived by integration-free reprogramming methods, including but not limited to those described in Goh et al. (2013) *PLoS ONE* 8(11): e81622; Awe et al (2013) *Stem Cell Research & Therapy*, 4:87; Varga (2014) *Exp Cell Res*, 322(2):335-44; Jia et al. (2010) *Nat Methods*, 7(3):197-9; Fusaki et al. (2009) *Proc Jpn Acad Ser B Phys Biol Sci*. 85(8):348-62; Shao & Wu, (2010) *Expert Opin Biol Ther*. 10(2):231-42; the disclosures of which are incorporated herein by reference.

[00176] In some instances, the derived or obtained pluripotent progenitor cells are prepared, dissociated, maintained and/or expanded in culture prior to being differentiated and/or lineage restricted as described herein.

[00177] In some instances, before differentiation or lineage restriction of the pluripotent progenitor cells the pluripotent progenitor cells are dissociated, e.g., to generate a single-cell suspension. In some instances, the dissociation of the pluripotent progenitors is chemical, molecular (e.g., enzyme mediated), or mechanical dissociation. Methods of chemical, molecular, and/or enzyme mediated dissociation will vary and in some instances may include but are not limited to the use of, e.g., trypsin, TrypLE Express™, TrypLE Select™, Accutase®, StemPro® (Life Technologies, Inc., Grand Island, NY), calcium and magnesium free media, low calcium and magnesium

medium, and the like. In some instances the dissociation media may further include pro-survival factors including but not limited to, e.g., Rho-associated kinase (ROCK) inhibitor, pinacidil, allopurinol, uricase, cyclosporine (e.g., low dose, i.e., sub-immunosuppressive dose, cyclosporine), ZVAD-fmk, pro-survival cytokines (e.g., insulin-like growth factor-1 (IGF-1)), Thiazovivin, etc.

[00178] In some instances, methods of culturing pluripotent stem cells include xeno-free culture conditions wherein, e.g., human cells are not cultured with any reagents derived from non-human animals. In some instances, methods culturing of pluripotent stem cells include feeder-free culture conditions, wherein the pluripotent stem cells are cultured under conditions that do not require feeder cells and/or in feeder cell free medium, including e.g., commercially available feeder-free mediums, such as, e.g., those available from STEMCELL Technologies, Inc. (Vancouver, BC). In some instances, methods culturing of pluripotent stem cells include culture conditions that include supplemental serum, including e.g. supplement of autologously derived serum, e.g., as described in Stute et al. (2004) *Exp Hematol*, 32(12):1212-25. In some instances, methods of culturing of pluripotent cells or derivatives thereof include culture conditions that are serum-free, meaning the culture media does not contain animal, mammal, or human derived serum. Serum-free culture conditions may be performed for only a portion of the life of the culture or may performed for the entire life of the culture. In some instances, serum-free culture conditions are used for a particular method step or procedure, e.g., during differentiation, during lineage restriction, prior to or during harvesting, etc. As is known in the art, in some instances, cells may be cultured in two dimensional or three dimensional formats (e.g., on non-coated or coated surfaces or within a solid or semi-solid matrix). Instances where two dimensional or three dimensional culture is appropriate for use in the methods as described herein, e.g., to promote survival or differentiation of a desired cell type, will be readily apparent to the ordinary skilled artisan. In some instance the pluripotent progenitor cell media includes one or more pro-survival factors, e.g., including those described herein. General methods of culturing human pluripotent progenitor cells are described in, e.g., Freshney et al. (2007) Culture of human stem cells, Wiley-Interscience, Hoboken, NJ and Borowski et al. (2012) Basic pluripotent stem cell culture protocols, StemBook, ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook, the disclosures of which are incorporated herein by reference.

[00179] In some instances, the pluripotent progenitor cells used according to the methods described herein may be genetically unmodified. By “genetically unmodified” is meant that essentially no modification of the genome of the cells transplanted into the subject has been performed. Encompassed within the term genetically unmodified are instances wherein transient genetic modification is performed at some point during the derivation of the cells but essentially no genetic modification persists in the cells that are eventually transplanted into the subject (i.e. the cells are essentially indistinguishable before the transient genetic modification and after the course of the transient modification). Also encompassed within the term genetically unmodified

are instances wherein the genome of the cells is not transiently or stably modified, e.g., where the cells are manipulated, e.g., pluripotent progenitors are derived or cells are transformed, without genetic modification (e.g., modification of the nucleotide sequence of the genome) of the cells.

[00180] In some instances, the cells used according to the methods described herein may be genetically modified. By “genetically modified” is meant that at least one nucleotide is added to, changed within, or deleted from of the genome of the cell. In some instances, the genetic modification may be an insertion of a heterologous sequence, e.g., a sequence that encodes a tag, a label sequence, a reporter, a selectable marker, a gene encoding a protein from a species different from that of the host cell, etc. In some instances, the genetic modification corrects a defect or a mutation within the cell, e.g., corrects an anomalous mutation that confers a tissue dysfunction or deficiency. In some instances, the genetic modification deletes or renders inoperable an endogenous gene of the host cell. In some instances, the genetic modification enhances an endogenous gene of the host cell. In some instances, the genetic modification represents a change that enhances survival, control of proliferation, and the like. Cells may be genetically altered by transfection or transduction with a suitable vector, homologous recombination, or other appropriate technique, so that they express a heterologous sequence or have altered expression of an endogenous gene.

[00181] For further elaboration of general techniques useful in the practice of this disclosure, the practitioner can refer to standard textbooks and reviews in cell biology, tissue culture, and embryology. With respect to tissue culture and stem cells, the reader may wish to refer to Teratocarcinomas and embryonic stem cells: A practical approach (E. J. Robertson, ed., IRL Press Ltd. 1987); Guide to Techniques in Mouse Development (P. M. Wasserman et al. eds., Academic Press 1993); Embryonic Stem Cell Differentiation in Vitro (M. V. Wiles, Meth. Enzymol. 225:900, 1993); Properties and uses of Embryonic Stem Cells: Prospects for Application to Human Biology and Gene Therapy (P. D. Rathjen et al., Reprod. Fertil. Dev. 10:31, 1998).

Systems

[00182] Also provided are systems for use in practicing the subject methods. Systems of the subject disclosure may include a cell production system, e.g., for the production of a homogenous or highly pure population of hematopoietic stem cells from pluripotent progenitor cells.

[00183] In some instances, the cell production system includes a cell culture chamber or cell culture vessel for the culture of desired cell types. Such cell culture chambers may be configured for the expansion of pluripotent progenitor cells and for the differentiation and/or lineage restriction of such pluripotent progenitor cells into desired cell types. In some instances, the cell culture chamber is also configured for the expansion of hematopoietic stem cells. In certain embodiments, the cell culture chamber or cell culture vessel may be an open culture system, including but not limited to e.g., tissue culture dishes, tissue culture plates, tissue culture multi-

well plates, tissue culture flasks, etc. In certain embodiments, the cell culture chamber or cell culture vessel may be a closed culture system, including e.g., a bioreactor, a stacked tissue culture vessel (e.g., CellSTACK Culture Chambers available from Corning, Inc. Corning, NY). In some instances, culture media and or other factors or agents may be exchanged in and out of the cell culture chamber through the use of one or more pumps (e.g., syringe pumps, peristaltic pumps, etc.) or gravity flow devices. In instances where the cells are cultured under sterile conditions the culture system may allow for the sterile exchange of culture media, e.g., through the use of sterile tubing connected, sealed, and reconnected through the use of a sterile devices, including but not limited to, e.g., a sterile tube welder and/or a sterile tube sealer. The cell culture system may be configured to control certain environmental conditions, including but not limited to e.g., temperature, humidity, light exposure, air composition (e.g., oxygen levels, carbon dioxide levels, etc.) to achieve the conditions necessary for expansion and/or differentiation of desired cell types. In some instances, the cell culture chamber may include a cell culture vessel that includes one or more patterned cell culture substrates or one or more arrays of patterned cell culture substrates as described herein.

[00184] The cell culture chamber may be configured for the production of cells for clinical use, e.g., according to current good manufacturing practice (cGMP) compliant cell culture practices, including the methods and configurations described in e.g., Fekete et al. *PLoS ONE* (2012) 7(8): e43255; Pham et al. (2014) *J Trans Med* 12:56; Gastens et al. (2007) *Cell Transplant* 16(7):685-96; Fernandes et al. (2013) Stem Cell Bioprocessing: For Cellular Therapy, Diagnostics and Drug Development, Burlington, Oxford: Elsevier Science: Woodhead Publishing, the disclosures of which are incorporated herein by reference.

[00185] The cell production system may, in some instances, be computer controlled and/or automated. Automated and/or computer-controlled cell production systems may include a “memory” that is capable of storing information such that it is accessible and retrievable at a later time or date by a computer. Any convenient data storage structure may be chosen, based on the means used to access the stored information. In certain aspects, the information may be stored in a “permanent memory” (i.e. memory that is not erased by termination of the electrical supply to a computer or processor) or “non-permanent memory”. Computer hard-drive, CD-ROM, floppy disk, portable flash drive and DVD are all examples of permanent memory. Random Access Memory (RAM) is an example of non-permanent memory. A file in permanent memory may be editable and re-writable.

[00186] In certain instances, a computer controlled and/or automated cell culture system may include a module or program stored in memory for production of cells according to the methods described herein. Such a module may include instructions for the administration of induction agent and/or induction compositions, e.g., at particular timing intervals or according to a particular schedule, in order to generate a desired cell type. In some instances, such a computer module

may further include additional modules for routine cell culture tasks including but not limited to, e.g., monitoring and record keeping, media changes, environmental monitoring, etc.

[00187] Systems of the present disclosure include components and/or devices for delivering cells produced according to the methods described herein to a subject in need thereof. For example, in some instances a system for treating a subject with a dysfunction or deficiency includes a cell injection system for delivering cells in a carrier, with or without optional adjuvants, to a desired injection site, including diseased tissue, adjacent to diseased tissue, and/or within, on or near a dysfunctioning organ. Such systems utilize known injection devices (e.g., including but not limited to needles, bent needles, cannulas, syringes, pumps, infusion devices, diffusion devices, etc.) and techniques (e.g., including but not limited to intramuscular injection, subcutaneous injection, device-guided injection, etc.). In some instances, a device or technique used for the delivery of a cell scaffold or other bioengineered device may be configured or adapted for use in a cell delivery system for use in delivering cells derived according to the methods described herein

[00188] In addition to the above-described components systems of the subject disclosure may include a number of additional components, such as data output devices, e.g., monitors and/or speakers, data input devices, e.g., interface ports, keyboards, etc., fluid handling components, power sources, controllers, etc.

Compositions and Kits

[00189] Also provided are compositions and kits for use in the subject methods. The subject compositions and kits include any combination of components for performing the subject methods. In some embodiments, a composition can include, but is not limited to and does not require, the following: cell dissociation agents and/or media, cell reprogramming agents and/or media, pluripotent progenitor cells, cell culture agents and/or media, cell differentiation agents and/or media; lineage restriction agents (e.g., induction agents) and/or media; conventional agents for treating diseases and/or dysfunctions, pro-survival factors, pro-engraftment factors, functional mobilization agents and any combination thereof.

[00190] In some embodiments, a kit can include, but is not limited to and does not require, the following: any of the above described composition components, a sample collection container, a sample collection device (e.g., a sample collection container that includes a sample enrichment mechanism including, e.g., a filter), a tissue collection device (e.g., a biopsy device), a tissue dissociation device, a cell culture vessel, a cell production system; and any combination thereof.

[00191] In some embodiments, a kit can include, but is not limited to and does not require, a cell delivery system and/or a cell injection system configured for delivery of cells derived according to the methods described herein. For example, a kit may include a cell injection system configured for injection or delivery of cells into a desired area of the subject in order to effectively treat the subject for a dysfunction or deficiency, e.g., through delivery of cells to the tissue. Such kits may include a cell delivery or injection system, as described herein, including individual components

of such systems in assembled or unassembled form. In some instances, cells derived according to the methods described herein may be “preloaded” into a cell injection or delivery system such that the system is provided in a “ready-to-use” configuration. In other instances, a cell injection or delivery system may be provided in an “unloaded” configuration such that cells derived according to the methods described herein must be loaded into the system, with any desired carrier or vehicle, prior to use.

[00192] In addition to the above components, the subject kits may further include (in certain embodiments) instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, and the like. Yet another form of these instructions is a computer readable medium, e.g., diskette, compact disk (CD), flash drive, and the like, on which the information has been recorded. Yet another form of these instructions that may be present is electronic, e.g., a website address which may be used via the internet to access the information at a removed site.

EXAMPLES

[00193] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., room temperature (RT); base pairs (bp); kilobases (kb); picoliters (pl); seconds (s or sec); minutes (m or min); hours (h or hr); days (d); weeks (wk or wks); nanoliters (nl); microliters (ul); milliliters (ml); liters (L); nanograms (ng); micrograms (ug); milligrams (mg); grams ((g), in the context of mass); kilograms (kg); equivalents of the force of gravity ((g), in the context of centrifugation); nanomolar (nM); micromolar (uM), millimolar (mM); molar (M); amino acids (aa); kilobases (kb); base pairs (bp); nucleotides (nt); intramuscular (i.m.); intraperitoneal (i.p.); subcutaneous (s.c.); and the like.

Example 1

Lineage-tracing hematopoietic stem cell origins *in vivo* to efficiently and rapidly reconstruct human HSC-like cells from pluripotent stem cells

[00194] The developmental origin of blood-forming hematopoietic stem cells (HSCs) is a longstanding question. Here our non-invasive genetic lineage-tracing confirms that artery endothelial cells generate HSCs *in vivo*. Arteries are transiently competent to generate HSCs for 2 days (~E8.5-E10.5), but subsequently cease, delimiting a narrow window for *de novo* HSC formation. Guided by the arterial origins of blood, we efficiently and rapidly differentiate human pluripotent stem cells (hPSCs) into artery endothelium, hemogenic endothelium, and >90% pure HSC-like cells within 10 days. We generate HSC-like cells with nearly-complete efficiency by blocking formation of unwanted lineages at each step of differentiation. These hPSC-derived HSC-like cells generate T, B, NK, erythroid and myeloid cells *in vitro* and, critically, express hallmark HSC transcription factors *HLF* and *HOXA5-HOXA10*, which were previously challenging to upregulate. We thus convert hPSCs into nearly-pure *HLF+* *HOXA+* HSC-like cells *in vitro* with almost stoichiometric precision, empowering basic research and cellular therapies.

[00195] Here, we revisited the developmental precursors of HSCs *in vivo* to efficiently reconstitute the developmental pathway leading from hPSCs to *HLF+* *HOXA+* human HSCs *in vitro*. First, we performed non-invasive genetic lineage tracing using three independent systems, and precisely identified artery endothelial cells as the *in vivo* precursor to HSCs. This inducible lineage tracing system permitted us to determine the temporal dynamics of HSC development: arteries were competent to generate HSCs for approximately 2.5 days (~E8.5-E11), thereby delimiting a narrow time window for *de novo* HSC generation *in vivo*. Second, we exploited this knowledge to sequentially differentiate hPSCs into artery endothelial cells, hemogenic endothelium, and nearly-pure (>90%) populations of HSC-like cells, which expressed HSC hallmark TFs *HLF* and *HOXA5-10* at levels comparable to primary human HSCs. We defined the extracellular signals that control each step of differentiation, and found they had to be turned on and off: for instance, TGF β had to be activated to specify arteries, but 24 hours later, repressed to generate hemogenic endothelium. In contrast, OSM and LIF specified hemogenic endothelium, but then had to be sharply repressed to generate HSC-like cells. The resultant hPSC-derived HSC-like cells could generate T, NK, B, myeloid and erythroid cells *in vitro*. We further found that while multiple types of arteries could generate HSC-like cells *in vitro*, in our experimental conditions only arteries derived from a specific upstream precursor (posterior primitive streak) were instilled with the competence to generate *HLF+* *HOXA+* HSC-like cells. Taken together, our *in vivo* and *in vitro* studies demonstrate that not all endothelial cells can generate HSCs. Rather, specialized subtypes of endothelial cells over brief embryonic time intervals are competent to generate HSCs, and we define extracellular signals that can convert hPSC-derived arteries into HSC-like cells with near-complete efficiency. The ability to produce nearly-pure populations of hPSC-derived HSC-like cells provides a powerful foundation for basic research and regenerative medicine, and could avail the quest to generate fully-functional HSCs *in vitro*.

RESULTS

[00196] *Genetic lineage-tracing reveals that artery endothelial cells give rise to HSCs in vivo.* To stringently test whether arteries form HSCs *in vivo*, we performed non-invasive genetic lineage tracing with an artery-specific, tamoxifen-inducible *Cx40-CreERT2* driver crossed to a Cre-dependent *zsGreen* reporter (Fig. 1a). In this approach, *Cx40*⁺ arteries—and all of their progeny cells, even if they downregulate *Cx40*—are permanently labeled with fluorescent protein *zsGreen*. To initiate lineage tracing, we employed a tamoxifen metabolite ((*Z*)-4-hydroxytamoxifen [4OHT]), which has a shorter half-life *in vivo* compared to tamoxifen, allowing for a restricted labeling period. Mass spectrometry showed the *in vivo* half-life of 4OHT in plasma was less than 3 hours, with 4OHT declining ~10.6-fold to almost undetectable levels by 12 hours post-injection (Fig. 1b). Taken together, 4OHT acutely labels target cells within a ~12 hour window. This circumvents a limitation of tamoxifen, which remains active for several days *in vivo*. Tamoxifen was previously used to label presumed HSC precursors, but owing to its several day perdurance, incurs the risk of inadvertently labeling emerging HSCs as well.

[00197] We exploited *Cx40-CreERT2* for arterial lineage tracing, because *Cx40* (*Connexin 40*) encodes a gap-junction protein that is highly specific to arterial ECs. The earliest artery ECs in the ~E8.5 dorsal aorta express *Cx40* (Fig. 1c). Single-cell RNA-sequencing (scRNAseq) of the entire E8.5 mouse embryo revealed *Cx40* is exquisitely specific to artery ECs: it is not expressed by other cell-types in the body, including other EC subtypes or blood cells, such as E10-E11 HSPCs (Fig. 1d, FIG. 7a-c). Thus, *Cx40-CreERT2* affords an advantage compared to previous *VE-Cadherin-CreERT2* lineage tracing models. *VE-Cadherin* (CD144) is expressed by both HSCs and ECs, and indeed, *VE-Cadherin-CreERT2* directly labels embryonic HSCs.

[00198] *Cx40*⁺ arterial ECs were lineage-traced by administering 4OHT at E8.5, leading to the emergence of arterially-derived (i.e., *zsGreen*⁺) CD45⁺ CD144⁺ blood progenitor cells in the E11.5 dorsal aorta (Fig. 1e-f) and yolk sac (Fig. 1g-h). Of note, the first adult-engrafting HSCs within the dorsal aorta are CD45⁺ CD144⁺. Subsequently, arterially-derived Lineage⁻ Sca1⁺ Kit⁺ (LSK) CD150⁺ CD48⁻ HSCs⁷⁰ arose in the fetal liver between E14.5 and E18.5 (Fig. 1i-j). Taken together, these results indicate that cell surface marker-defined HSCs arise from arteries; below, we demonstrate that these HSCs are in fact functional.

[00199] *Artery endothelial cells are competent to generate HSCs for a brief period (E8.5-E11.0) in vivo.* The time-restricted nature of 4OHT labeling allows us to delineate precisely *when* arteries are competent to generate HSCs *in vivo*, by injecting 4OHT at 12-hour increments between E7.5-E12.5 (Fig. 1k). Do HSCs continuously emerge from arteries, or are they instead produced in a brief burst? Strikingly, arteries were only competent to form HSCs during a narrow developmental window: 4OHT injection between E8.5-E10.0 led to steadily-increasing percentages of *zsGreen*⁺ fetal liver HSCs, but arteries labeled from E11.5-onwards failed to generate appreciable numbers of HSCs (Fig 1k). Additionally, labeling peaked at ~93.7%, suggesting that most (if not all) HSCs originated from a *Cx40*⁺ arterial precursor (Fig. 1k). As expected, 4OHT administration at E7.5

and E8.0 did not lead to labeled HSCs (Fig. 1k), as *Cx40*⁺ arterial ECs only arise at E8.5; this reiterates the extremely narrow labeling window of 4OHT, which does not seemingly perdure beyond 12 hours. In summary, arteries are briefly competent to form HSCs for ~2.5 days (E8.5-E10.0), thus delimiting a narrow time window for *de novo* HSC generation.

[00200] To independently confirm the arterial origin of HSCs using a different arterial marker, we engineered a new *Efnb2-CreERT2* driver line (Fig. 1l, FIG. 7d), exploiting the well-known arterial specificity of *Efnb2* expression. 4OHT labeling of E8.5 arterial ECs using *Efnb2-CreERT2* likewise revealed they subsequently gave rise to fetal liver HSCs (Fig. 1l). This provides further evidence for the arterial origin of HSCs.

[00201] Finally, we found that vein ECs minimally give rise to HSCs, underscoring that artery ECs uniquely serve as HSC precursors. To this end, we genetically labeled vein ECs using *Aplnr-CreERT2* (Fig. 1m): at E8.5, *Aplnr* (*Apj*) marks mesoderm, but is restricted to vein ECs by E9.5 and thereafter, allowing its use as a vein-specific marker at this timepoint. Vein ECs labeled with *Aplnr-CreER* at E9.5 gave rise to few, if any, fetal liver HSCs (<1%) (Fig. 1m, FIG. 7e). We conclude that arteries, but not veins, are the dominant source of HSCs *in vivo*.

[00202] *Artery-derived HSCs are functional in vivo.* We further showed that *Cx40*⁺ artery-derived HSCs are functional: they could self-renew to generate additional HSCs, and produced all major blood and immune cell-types *in vivo* over prolonged amounts of time. This thus fulfills the functional definition of HSCs. First, we performed long-term lineage tracing by genetically labeling *Cx40*⁺ arteries at E8.5 using 4OHT, and found that as these embryonic mice developed into adulthood (Fig. 2a), artery-derived (zsGreen⁺) cells were found in all major blood and immune lineages (including B cells, T cells, macrophages/granulocytes, red blood cells, and platelets; Fig. 2b,c) and HSCs (Fig. 2d,e). This suggests long-term self-renewal and differentiation of artery-derived HSCs. Additionally, artery-derived cells contributed to all major blood lineages at similar frequencies (Fig. 2b,c), indicating balanced production of all major blood lineages.

[00203] Additionally, artery-derived HSCs were capable of reconstituting a blood and immune system upon transplantation into lethally-irradiated recipient mice (Fig. 3a). *Cx40*⁺ artery-derived fetal liver HSCs (Fig. 3b, FIG. 9ai) were capable of engrafting lethally-irradiated recipient mice, whereupon they regenerated the bone marrow HSC compartment (Fig. 3c, FIG. 9aii), as well as myeloid and lymphoid cells for 4 months (Fig. 3d, FIG. 9aiii). Similar results were observed upon serial transplantation into lethally-irradiated secondary recipient mice for 4 months (Fig. 3e,f, FIG. 9aiv,v). Taken together, upon both primary and secondary transplantation, artery-derived HSCs generated all major blood and immune cell-types within the peripheral blood, and reconstituted the HSC pool within the bone marrow, of recipient mice. Our genetic lineage tracing strongly supports the hypothesis that artery endothelial cells generate HSCs *in vivo*.

[00204] *Differentiation of hPSCs into posterior primitive streak is critical to ignite HOXA5-HOXA10 expression at the beginning of differentiation.* Our lineage tracing provides evidence for a model in which cells traverse an arterial intermediate before forming HSCs *in vivo*. We therefore sought

to recapitulate this developmental trajectory *in vitro* with the goal of producing HSCs from hPSCs. We developed a method to sequentially differentiate hPSCs into primitive streak, lateral mesoderm, artery endothelial cells (ECs), hemogenic ECs, and subsequently HSC-like cells within 10 days of differentiation with high efficiency. While we previously differentiated hPSCs into mid primitive streak and subsequently, lateral mesoderm and nearly-pure artery ECs, our attempts to differentiate these artery ECs into *HLF*⁺ *HOXA*⁺ HSC-like cells were not successful (as detailed below). To this end, we first revisited the very first step of differentiation: the primitive streak.

[00205] *In vivo*, there exist multiple types of primitive streak (anterior, mid and posterior) that each generate different mesodermal subtypes. *In vivo*, primitive streak is induced by FGF and WNT, which act alongside anteriorizing TGFβ and posteriorizing BMP gradients. Armed with this knowledge, we generated four different types of *BRACHYURY*⁺ primitive streak, including anterior, mid and posterior primitive streak (within 1 day of hPSC differentiation) as well prolonged posterior primitive streak (within 2 days of hPSC differentiation) (Fig. 4bi, FIG. 10a). Only day 2 posterior primitive streak (PPS) expressed *HOXA5-HOXA10* (Fig. 4bii, FIG. 10bix), consistent with how *HOX* genes are expressed in posterior primitive streak and are expressed later in development. Furthermore, production of Day 2 posterior primitive streak was remarkably efficient: by flow cytometry, over 98% of cells expressed the primitive streak marker *MIXL1* (Fig. 4biii), as assessed by *MIXL1-GFP* reporter hPSCs. scRNAseq revealed that during posterior primitive streak induction, pluripotency markers (*NANOG*, *SOX2*) were synchronously downregulated and primitive streak markers (*BRACHYURY*, *MIXL1*) became uniformly expressed (Fig. 4di-iii, FIG. 10bi,ii).

[00206] These four different types of primitive streak gave rise to fundamentally different types of artery ECs and hematopoietic progenitors later during differentiation. We previously showed that hPSC-derived day-1 mid primitive streak could generate artery ECs, but here we found these ECs minimally expressed *HOXA5-HOXA10* (Fig. 4ei,iii, FIG. 10c). Importantly, day-2 posterior primitive streak generated artery ECs that expressed *HOXA5-HOXA10* (Fig. 4ei-iii), which could further differentiate into CD34⁺ CD45⁺ HSC-like cells that expressed the hallmark HSC transcription factors *HLF* and *HOXA5-HOXA10* (Fig. 4e). Interestingly, while all four types of primitive streak could subsequently form CD34⁺ CD45⁺ hematopoietic progenitors, day 2 posterior primitive streak was solely capable of producing *HLF*⁺ HSC-like cells in these conditions (Fig. 4eiii, FIG. 10c), reiterating the importance of creating the appropriate type of primitive streak at the very first step of differentiation.

[00207] While several manipulations, including modulating the RA pathway at intermediate steps of differentiation, can transiently elevate *HOXA* expression, these genes were often turned off upon later differentiation into hematopoietic progenitors. Starting differentiation through the appropriate type of primitive streak—which is when *HOX* gene expression is first ignited during development and stably persists thereafter—appears critical to generate *HOXA5-HOXA10*⁺

HLF+ HSC-like cells *in vitro*. Below, we detail the extracellular signals that needed to be turned on and off at each step of differentiation to convert day-2 posterior primitive streak into HSC-like cells.

[00208] *Efficient differentiation of hPSC-derived artery ECs into hemogenic ECs, and subsequently, HLF+ HOXA+ HSC-like cells.* Next, we next differentiated day 2 posterior primitive streak into day 3 lateral mesoderm, and subsequently day 4 artery ECs. Generation of each of these cell-types required explicit inhibition of signals that would instead generate unwanted cell-types. To differentiate posterior primitive streak into lateral mesoderm, we activated BMP, PKA, RA and VEGF pathways, while simultaneously inhibiting TGF β , WNT, and PI3K for 24 hours. In particular, we explicitly blocked TGF β and WNT signaling, which instead specify endoderm and lateral mesoderm, respectively. At this stage, RA activation promoted *HOXA1-HOXA4* expression (FIG. 10di,ii). scRNAseq revealed that this combination of lateral mesoderm-inducing signals generated an enriched population of *SCL+* *KDR+* lateral mesoderm, with minimal expression of endoderm (*FOXA2*) and paraxial mesoderm (*MSGN1*) markers, reiterating the precision of lateral mesoderm induction (Fig. 4div, FIG. 10bvii,x).

[00209] This day-3 lateral mesoderm was further differentiated into day-4 artery ECs, by activating TGF β , VEGF and RA, while simultaneously inhibiting BMP, WNT, and PI3K, for 24 hours. We blocked BMP and PI3K, which respectively induced heart and vein ECs at this stage of differentiation, thus consolidating artery specification. At this stage, RA also promoted *HOXA1-HOXA4* expression (FIG. 10diii-vi). scRNAseq revealed efficient generation of *SOX17+* *CD144+* artery ECs, with minimal expression of heart (*NKX2.5*) and vein (*APLNR*) markers; the minority of remaining of non-ECs correspond to mesenchymal cells (Fig. 4dv, FIG. 10bviii,x,xi). Taken together, we efficiently differentiated hPSCs into posterior primitive streak, lateral mesoderm, and artery ECs, while inhibiting differentiation into alternate fates at each step of differentiation. At each of these steps, *HOXA5-HOXA10* genes were continuously expressed (Fig. 4bix).

[00210] Next, we drove *HOXA+* artery ECs out of an arterial state and further differentiated them into hemogenic ECs within 3 additional days, by activating the GP130, NOTCH and PKA pathways, while simultaneously inhibiting TGF β and PRC2. This yielded >80% pure *RUNX1+* hemogenic ECs (Fig. 5ai,ii, FIG. 11bi,ii), as assessed using *RUNX1-mOrange* reporter hPSCs; *Runx1* expression in ECs *in vivo* signifies their future hematopoietic potential. scRNAseq of day-7 hemogenic EC populations reaffirmed the large majority of cells were *CD31+* *RUNX1+* hemogenic EC, with a small minority of mesenchymal cells (Fig. 5aiii, FIG. 11biii,iv).

[00211] Withholding GP130 agonists (OSM and LIF), PKA agonist, or TGF β inhibitor revealed that they were combinatorially critical to specify *RUNX1+* hemogenic ECs with the future potential to generate blood (Fig. 5b, FIG. 11c). This underscores the combinatorial importance of these signals; individual signals were insufficient. We thus define an important role for OSM/GP130 signaling in human HE specification, paralleling its role in mouse and zebrafish hematopoietic development. Additionally, we discover that PKA agonist induces human hemogenic ECs: in

model organisms, PKA induces *Runx1* downstream in response to prostaglandin E2⁹⁸ and blood-induced shear stress. Therefore our use of PKA agonist may partly alleviate the requirement for shear stress in blood development. Finally, high cell density was crucial for hemogenic EC specification (Fig. 5a). In summary, we discovered temporally-dynamic signals to efficiently convert artery ECs into hemogenic ECs: while VEGF and TGFβ initially induced arterial fate, subsequently withholding these signals and providing others (e.g., GP130 and PKA) drove cells out an arterial fate and induced hemogenic ECs.

[00212] Finally, we differentiated RUNX1+ hemogenic ECs into HSC-like cells within 3 additional days (Fig. 5c). To this end, we activated the PKA pathway, while inhibiting TGFβ and two distinct chromatin complexes (PRC2 and G9A/GLP); additionally, we provided UM171 and SR1 to stabilize incipiently-arising hPSC-derived HSC-like cells in an undifferentiated state and to reduce their spontaneous differentiation into downstream progeny. Withholding any of these factors individually reduced *HLF* (Fig. 4c), attesting to their combined significance (Fig. 5d, FIG. 11d). These combined signals yielded 91.5±1.0% pure CD34+ CD45+ and 91.0±1.0% pure CD144+ CD45+ HSC-like cells (*N*=20 independent experiments; Fig. 5c). In particular, the first adult-engrafting HSCs within the human and mouse embryo are CD144+ CD45+, and CD45 distinguishes blood cells from ECs. Consistent with our use of chromatin modulators (PRC2 and G9A/GLP inhibitors) to enhance human blood progenitor specification *in vitro* (FIG. 11e), inhibition of PRC2/EZH1 leads to precocious HSC emergence *in vivo* and G9A/GLP inhibitors stabilize primary HSPCs in an undifferentiated state. Finally, each 1 input hPSC yielded 1.01±0.1 output HSC-like cells (*N*=8 independent experiments), indicating near-stoichiometric conversion (Fig. 5ciii).

[00213] hPSC-derived HSC-like cells express hallmark HSC transcription factors, including *HLF* and *HOXA5-10*. scRNAseq revealed that the large majority of day-10 cells were HSC-like cells that expressed HSPC surface markers (*CD34*, *CD144*, *CD45*) and HSC transcription factors/chromatin regulators (*HLF*, *MECOM*, *MLLT3*, *RUNX1*, *MEIS1*, *MYB*); there were also a few remaining mesenchymal cells (Fig. 5e, FIG. 11g). At this stage of differentiation, we did not find evidence for *IL7R*+ lymphoid and *GATA1*+ erythroid progenitors (data not shown), which are present alongside *HLF*+ HSPCs in the human dorsal aorta.

[00214] RNA-seq revealed that hPSC-derived HSC-like cells and CD144+ CD45+ human dorsal aorta expressed comparable levels of HSC TFs, including *HLF*, *HOPX* and *PRDM16* (Fig. 5f, FIG. 11f). Indeed, six previously-reported “HSC signature” genes—*RUNX1*, *MLLT3*, *HOXA9*, *MECOM*, *HLF*, and *SPINK2*—showed largely comparable expression between hPSC-derived HSC-like cells and human dorsal aorta HSCs (Fig. 5f, FIG. 11f). As a negative control, we found that human dorsal aorta CD144- CD45+ non-HSCs, minimally expressed these HSC signature markers (Fig. 5f, FIG. 11f). Taken together, our hPSC-derived HSC-like cells expressed *HLF*, *HOXA5-10*, and other signature HSC markers that were previously challenging to upregulate in other hPSC differentiation protocols.

[00215] *hPSC-derived HSC-like cells can generate T, B, NK, myeloid and erythroid cells in vitro.*

Finally, day 10 hPSC-derived HSC-like cells harbored the ability to generate all major types of blood and immune cells *in vitro*: lymphoid, myeloid and erythroid cells (Fig. 7a). First, hPSC-derived HSC-like cells differentiated into granulocytes, erythroid cells, monocytes and megakaryocytes in methylcellulose cultures (Fig. 7bi). Second, hPSC-derived HSC-like cells could differentiate into erythroid cells, Fig. 7bii) and macrophages (Fig. 7biii).

[00216] Third, hPSC-derived HSC-like cells could differentiate into NK cells, which expressed archetypic NK cell markers including CD56, NKp46, NKp44, CD94, CD16 and NKG2D (Fig. 7cii-ii). There was a 99.4-fold increase in cell numbers during NK cell differentiation (Fig. 7ciii), hinting at the ability to generate large numbers of hPSC-derived NK cells. Live imaging revealed that hPSC-derived NK cells were functional, achieving near-complete destruction of target cells within 12 hours (Fig. 7civ). In our experimental system, generation of NK cells from hPSCs was more efficient than from CD34+ human cord blood HSPCs, and NK cells produced from the two cell sources were comparably efficient at killing target cells (Fig. 7cii,iv).

[00217] Fourth, hPSC-derived HSC-like cells could differentiate into T cells in feeder-free conditions (Fig. 7d). Starting from wild-type HSC-like cells, CD5+ CD7+ lymphoid progenitors emerged within 2 weeks (Fig. 7di). By 4 weeks, CD4+ CD8+ double positive T cells that expressed the T-cell receptor (TCR α/β) and the T-cell coreceptor (CD3) were produced (Fig. 7dii, Fig. 7ei), followed by CD8+ single positive TCR α/β + CD3+ T cells by 5 weeks (Fig. 7eii). Additionally, we generated a "T-hiPSC" line (iLC13-F1) from patient-derived T cells that recognized Epstein-Barr virus (EBV) antigens. These T-hiPSCs could be re-differentiated into HSC-like cells, and subsequently, CD8+ TCR α/β + CD3+ T cells using our approach, suggesting the feasibility of producing antigen-specific T cells (Fig. 7d,e). During T-cell differentiation, there was also a massive (502.5- to 711.6-fold) increase in cell numbers, attesting to the potential scalability of hPSC-derived T cell manufacturing (Fig. 7diii).

[00218] Fifth, hPSC-derived HSC-like cells could also differentiate into CD10+ CD19+ B cells (Fig. 7f). Taken together, hPSC-derived HSC-like cells provide a platform to generate multiple human blood and immune cell-types, therefore building on the progress on generating these various cell-types from hPSCs. However, because *HLF*+ *HOXA*+ hPSC-derived HSC-like cells can be generated with high efficiency and speed, they offer a standardized platform for the production of these lineages. Additionally, the lymphoid potential of these hPSC-derived HSC-like cells implies that they approximate the definitive phase of hematopoiesis, as earlier-arising primitive blood lineages often lack lymphoid potential *in vivo*.

[00219] Next, we asked whether our blood differentiation protocol could be used to model a hematologic cancer, acute myeloid leukemia (AML). AML cells from a patient bearing multiple mutations (including an *MLL1-MLLT3* fusion) were reprogrammed into hiPSCs, which were further differentiated into HSC-like cells using our approach and transplanted into NOD-SCID

Il2rg^{-/-} (NSG) mice. These AML-hiPSC-derived HSC-like cells robustly engrafted, yielding almost exclusively myeloid (CD33⁺) blood cells *in vivo* (Fig. 6g).

[00220] However, wild-type hPSC-derived HSC-like cells exhibited very limited ability to engraft NSG mice. To track hPSC-derived HSC-like cells *in vivo* using bioluminescent imaging, first we engineered them to constitutively express an *AkaLuciferase* reporter. Bioluminescent imaging revealed that hPSC-derived HSC-like cells transplanted directly into the femur subsequently spread to multiple bones throughout the mouse, as expected for HSPCs (**Fig. 6hi**). 6 months post-transplantation, there were very low levels of human blood cells (<0.1% CD45⁺), the majority of which were myeloid (CD33⁺) in lineage (**Fig. 6hiii**).

[00221] Through non-invasive genetic lineage tracing, we definitively confirm that artery ECs give rise to HSCs *in vivo*, and we define the time when arteries are competent to do so. Equipped with this developmental knowledge, we rapidly and efficiently differentiate hPSCs into >90% pure HSC-like cells, which express HSC signature TFs including *HLF*, *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10* at levels comparable to human HSCs. This differentiation occurs through five sequential steps (posterior primitive streak, lateral mesoderm, arterial ECs, hemogenic ECs, and finally, HSC-like cells) in serum-free, monolayer culture, within 10 days. At each step of differentiation, we illuminated the extracellular signals that had to be turned on or off to efficiently segue from one step to the next. Consequently, we achieved near-stoichiometric conversion of hPSCs into HSC-like cells (1.01 blood progenitor produced for each input hPSC). The resultant hPSC-derived HSC-like cells could generate a wide range of blood and immune cells, including T cells, B cells, NK cells, myeloid and erythroid cells. This provides a foundation to reliably and efficiently derive human blood and immune cell-types for a variety of applications, including regenerative medicine, cancer immunotherapy, and disease modeling.

[00222] *Arteries generate HSCs in vivo, and a narrow timeframe for HSC production.* Our genetic lineage tracing provides firm evidence that artery endothelial cells are the dominant source of HSCs *in vivo*. We find that arteries generate up to ~90% of HSCs, implying they are the dominant, if not exclusive, source of HSCs.

[00223] Using *Cx40-CreERT2* lineage tracing, we definitively show that artery endothelial cells give rise to HSCs: the advantage of *Cx40* is that it is exquisitely artery-specific and is not expressed by HSCs. We observed similar findings using another artery lineage tracing system (*Efnb2-CreERT2*). These approaches afford greater genetic specificity compared to a previously-used *VE-Cadherin-CreERT2* system, which labels both endothelial cells as well as HSCs themselves. Additionally, non-invasive lineage tracing *in vivo* affords multiple advantages. We directly demonstrate that artery-derived HSCs are functional, thus expanding beyond live-imaging and scRNAseq studies showing the emergence of cells expressing HSC markers, but which could not interrogate them functionally. Moreover, we show that artery endothelial cells

generate HSCs *in vivo*, which provides a more physiological system than *ex vivo* explant culture systems previously used to investigate HSC origins.

[00224] *Importance of the earliest primitive streak stage in blood differentiation.* Despite considerable past successes in differentiating hPSCs into hematopoietic progenitors, it has long been recognized that certain HSC transcription factors, including *HLF* and *HOXA* family members, have proven more difficult to upregulate, thus raising the question of how and when to turn them on. In particular, human HSCs express *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10*. Numerous experimental manipulations (including RA modulation) have been tested at various differentiation stages, but often only transiently upregulate *HOXA*. Other pioneering studies have instead emphasized the importance of modulating specific signaling pathways (e.g., WNT activation and TGF β inhibition) on days 2-4 of hPSC differentiation towards blood lineages.

[00225] During embryonic development, *HOX* genes are first turned on in the primitive streak at the very beginning of gastrulation, with posterior *HOX* genes activated in the posterior primitive streak, even prior to the emergence of mesoderm. We therefore revisited the very first step of differentiation: primitive streak induction. By generating four different types of primitive streak *in vitro*, we reveal that day 2 posterior primitive streak expresses *HOXA5-HOXA10*, and is uniquely competent to differentiate into *HLF+* *HOXA5-HOXA10+* HSC-like cells. In our *in vitro* differentiation system, once *HOXA* gene expression is activated within the primitive streak, these genes are continuously expressed throughout differentiation, consistent with how *HOX* genes encode a “persistent positional identity” in developmental biology. Interestingly, other types of primitive streak, including mid primitive streak, can generate artery endothelial cells, but these express more anterior *HOXA* genes and cannot produce *HLF+* *HOXA5-10+* HSC-like cells. In fact, these hPSC-derived HSC-like cells obtained from other primitive streak regions, which express certain blood progenitor markers (CD45+ CD144+) but lack *HLF* and *HOXA* genes, may approximate hPSC-derived hematopoietic progenitors produced by previous differentiation protocols. On the basis of *HOX* codes, it appears that our hPSC-derived anterior, mid and posterior primitive streak can respectively produce anterior, mid and posterior endothelial cells. Why are posterior endothelial cells uniquely capable of upregulating *HLF* in our system? This may presage mechanistic investigations of how anterior-posterior positional identity is encoded within incipiently-emerging endothelial cells and how these different identities exert far-reaching effects on the hematopoietic progenitors they can subsequently produce.

[00226] *Temporally dynamic signals drive consecutive steps of blood development.* One of our main findings is that key extracellular signaling pathways must be turned on and off every 24 hours to effect differentiation; even closely-related cell-types (e.g., arteries and hemogenic endothelium) are specified by diametrically opposed signals. For instance, on day 4 of differentiation, TGF β is required to specify artery endothelial cells, but 24 hours later, we show that it must be repressed for artery cells to segue into hemogenic endothelium. Conversely, GP130 (OSM and LIF) signaling is required to upregulate *RUNX1* and generate hemogenic

endothelium on days 5-7, but subsequently they must be withdrawn on days 8-10; their continued activation blocks the production of *HLF*⁺ HSC-like cells. We must therefore understand the temporal dynamics with which these signals act, and manipulate them with equal dynamism, to effect differentiation. Prolonged activation or inhibition of these signals instead generates heterogeneous cell populations. We also find that certain signals (e.g., OSM and LIF) enhance production of CD45⁺ CD144⁺ HSC, but decrease *HLF* expression, emphasizing that there are multiple routes to produce hematopoietic progenitors and care must be taken to specifically induce *HLF*⁺ HSC.

[00227] Our work also clarifies the exact lineage relationships between hPSC-derived artery and hemogenic endothelium. In our *in vitro* system, artery endothelium differentiates into hemogenic endothelium, which subsequently generates HSC-like cells, congruent with scRNAseq-based pseudotemporal inferences from human and mouse embryos.

[00228] *HLF*⁺ *HOXA*⁺ HSC: a platform to efficiently produce human blood and immune cells from hPSCs. By sequentially generating >90% pure populations of artery ECs, and subsequently, HSC-like cells, we provide a standardized, efficient and reproducible platform to create a range of human blood and immune cells from hPSCs, including T cells, NK cells and macrophages, as shown here. Starting from nearly-pure populations of *HLF*⁺ *HOXA*⁺ HSC-like cells can enhance the speed or efficiency of protocols to generate downstream blood and immune cells, therefore providing a boon for regenerative medicine, cancer immunotherapy, disease modeling, and a range of other applications. We also demonstrate a proof-of-principle for genetically engineering the resultant downstream immune cells, by differentiating “T-hiPSCs” carrying a rearranged T-cell receptor specific for viral (EBV) antigens.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

[00229] *Cell culture.* All cells in this study were cultured in standard incubator conditions (20% O₂, 5% CO₂ and 37 °C).

[00230] *Human pluripotent stem cell lines.* Wild-type H1, H7 and H9 hESCs have been described previously. HES3 *MIXL1-GFP* hESCs have been described previously. HES3 hESCs were genetically engineered to partially replace the coding sequence of the endogenous *MIXL1* gene with a *GFP* reporter. This approach did not preserve the coding sequence of the endogenous *MIXL1* gene. H1 *SOX17-2A-mPlum* hESCs have been described previously. H1 hESCs were genetically engineered to replace the stop codon of the endogenous *SOX17* gene with an *2A-mPlum* reporter. This approach theoretically preserved the coding sequence of the *SOX17* gene. TkDA3-4 *RUNX1-2A-mOrange* hiPSCs have been described previously. Human dermal fibroblasts were retrovirally transduced with the reprogramming factors to yield TkDA3-4 hiPSCs, which were subsequently genetically engineered to replace the stop codon of the endogenous *RUNX1* gene with an *2A-mOrange* reporter. iSU223n hiPSCs have been described previously. They were reprogrammed from a SU223, a 20-year-old female patient with relapsed acute

myeloid leukemia (AML), which carried a t(9;11)(p22;q23) chromosomal rearrangement (which encodes a *MLL1-MLLT3* fusion protein), in addition to *FLT3*^{ITD}, *NRAS*^{G12D}, *SEMA4A*^{Y5589H} and *WT1*^{T390fs} ⁷. They were subsequently engineered to express *BFP* as well as a fluorescent marker of the G0 cell-cycle phase (Venus-P27K-). iLC13-F1 hiPSCs were generated as part of this study. They were generated from a human adult T cell that carried a T cell receptor (TCR) recognizing an Epstein-Barr virus (EBV) peptide. After delivery of the reprogramming factors, the resultant “T-hiPSC” encode a rearranged EBV-specific TCR. Wild-type SUN hiPSCs have been described previously. Human adult peripheral blood mononuclear cells (PBMCs) were infected with non-integrating, replication-deficient Sendai viruses carrying reprogramming factors, thereby yielding SUN hiPSCs.

[00231] Feeder cells for lymphoid differentiation and NK cell killing assays. OP9-DLL4 feeder cells were maintained in aMEM (Thermo Fisher) supplemented with 20% FBS (R&D Systems) and 1% penicillin/streptomycin (Thermo Fisher). MS5 feeder cells (DSMZ) were maintained in aMEM (Thermo Fisher) supplemented with 10% FBS (R&D Systems) and 1% penicillin/streptomycin (Thermo Fisher).

[00232] *Human cord blood hematopoietic stem and progenitor cells.* Human cord blood CD34+ Stem/Progenitor cells were obtained from StemExpress.

[00233] *Mouse models.* *Cx40-CreERT2* mice have been described previously, and were provided by Lucile Miquerol's laboratory. In these mice, the endogenous coding sequence of the *Cx40* gene was replaced by a *CreERT2-IRES-RFP-PGK-NeomycinR* cassette. These mutant mice were maintained heterozygously (i.e., *Cx40*^{CreERT2/+}) on a CD1 background. These mice were also maintained with homozygosity for CD45.2 antigen.

[00234] *Efnb2-CreERT2* mice were generated as part of this study, by Hong Zeng, Charlene Wang, and the Stanford Transgenic, Knockout and Tumor Model Center. In these mice, the endogenous *Efnb2* gene was edited to replace the *Efnb2* stop codon with a *GSG-P2A-CreERT2-F5* cassette. A GSG-P2A linker was chosen, owing to the high translational skipping efficiency afforded by this linker; the GSG sequence (preceding P2A) was published previously. A single F5 site was also inserted downstream of *CreERT2*. This approach theoretically preserves the coding sequence of the endogenous *Efnb2* gene. These mutant mice were maintained heterozygously (i.e., *Efnb2*^{CreERT2/+}).

[00235] *Apj-CreERT2* mice have been described previously, and were provided by Kristy Red-Horse's laboratory. In these mice, a bacterial artificial chromosome (BAC) containing *Apj* (otherwise known as *Aplnr*) was genetically edited to replace the *Apj* gene with *CreERT2*, and the resultant BAC was randomly integrated into the mouse genome. *Apj-CreERT2* mice were maintained on a FVB/NJ background with regular genotyping performed to confirm presence of *CreERT2*.

[00236] *Rosa26-CAG-LoxP-Stop-LoxP-zsGreen* mice (otherwise known as “Ai6”) have been described previously, and were provided by the Allen Brain Institute through the intermediacy of

The Jackson Laboratory (JAX 007906). In these mice, a *CAG-LoxP-Stop-LoxP-zsGreen* allele was inserted into the endogenous *Rosa26* safe harbor locus, such that Cre-dependent recombination leads to the stable expression of the zsGreen fluorescent reporter.

[00237] *C57BL/6-CD45.2* and *C47BL/6-CD45.1* (Pepboy) mice were purchased from The Jackson Laboratory (JAX# 000664 and JAX#002014 respectively) and bred in-house. *C57BL/6-CD45.2* and *C47BL/6-CD45.1* were crossed to yield heterozygous mice (CD45.2+ CD45.1). For mouse HSC transplant experiments, 6–12-week-old mice were used as recipients.

[00238] *NSG* and *NBSGW* mice were purchased from The Jackson Laboratory (JAX# 005557 and JAX# 026622) and bred in-house. For human HSC transplant experiments, 6-12-week-old mice were used as recipients.

METHOD DETAILS

[00239] *Basement membrane matrices.* hPSCs were maintained and differentiated on cell culture plates that been pre-coated with either Geltrex or vitronectin basement membrane matrices, largely as described previously. To coat cell culture plates, a volume of basement membrane matrix solution was added, roughly equivalent to half the working volume of the well or dish (e.g., 1 mL or 0.5 mL of basement membrane matrix solution was added per well of a 6-well or 12-well plate, respectively).

[00240] Geltrex (Thermo Fisher) was diluted 1:100 in DMEM/F12 (Thermo Fisher) and was used to coat tissue culture plastics for at least 1 hour at 37 °C. Recombinant human truncated vitronectin (Gibco, A14700; “VTN-N”) was diluted to a 10 µg/mL stock in PBS (lacking Ca²⁺ or Mg²⁺) and was used to coat tissue culture plastics overnight or for at least 1 hour at 37 °C. For hemogenic endothelium induction, 20 nM of the high-affinity, engineered NOTCH agonist DLL4-E12 was added to 10 µg/mL vitronectin solution prior to coating cell culture plates, therefore immobilizing NOTCH agonist and vitronectin to these cell culture plates. After coating with either Geltrex or vitronectin, the basement membrane solution was aspirated, leaving behind a thin film; subsequently, cells were plated on the Geltrex- or vitronectin-coated cell culture plastics. Prior to plating cells, DLL4-E12 coated plates were gently washed three times to remove any soluble DLL4-E12, which would instead strongly inhibit NOTCH signaling.

[00241] *Culture of undifferentiated hPSCs.* Undifferentiated hPSCs were routinely propagated, as described previously, in mTeSR1 (STEMCELL Technologies) + 1% penicillin/streptomycin (Thermo Fisher) or alternatively, mTeSR Plus (STEMCELL Technologies) + 1% penicillin/streptomycin. For the sake of brevity, we henceforth refer to mTeSR1 and mTeSR Plus interchangeably as “mTeSR”. Undifferentiated hESCs were passaged for maintenance by treating them for 7 minutes with EDTA (Versene, Thermo Fisher) at room temperature, after which EDTA was removed, mTeSR was added, and then hESCs were manually scraped off the plate to generate clumps. hESC clumps were then seeded onto new plates that had been

precoated with Geltrex basement membrane matrix (diluted 1:100 in DMEM/F12, Thermo Fisher).

[00242] *Preparation of CDM2 and CDM3 basal media for differentiation.* The composition of CDM2 has been described previously: 50% IMDM + GlutaMAX (Thermo Fisher, 31980-097) + 50% F12 + GlutaMAX (Thermo Fisher, 31765-092) + 1 mg/mL polyvinyl alcohol (Sigma, P8136-250G) + 1% v/v chemically defined lipid concentrate (Thermo Fisher, 11905-031) + 450 μ M 1-thioglycerol (Sigma, M6145-100ML) + 0.7 μ g/mL recombinant human insulin (Sigma, 11376497001) + 15 μ g/mL human transferrin (Sigma, 10652202001) + 1% v/v penicillin/streptomycin (Thermo Fisher, 15070-063).

[00243] The composition of CDM3 has been described previously: 45% IMDM + GlutaMAX (Thermo Fisher, 31980-097) + 45% F12 + GlutaMAX (Thermo Fisher, 31765-092) + 10% KnockOut Serum Replacement (Thermo Fisher, 10828028) + 1 mg/mL polyvinyl alcohol (Sigma, P8136-250G) + 1% v/v chemically defined lipid concentrate (Thermo Fisher, 11905-031) + 1% v/v penicillin/streptomycin (Thermo Fisher, 15070-063).

[00244] For both CDM2 and CDM3, polyvinyl alcohol was brought into suspension by gentle warming and magnetic stirring, and the media was sterilely filtered (through a 0.22 μ m filter) prior to use.

[00245] *hPSC differentiation into HSC.* hPSC differentiation into HSC-like cells. In brief, undifferentiated hPSCs were dissociated using Accutase (Thermo Fisher) and sparsely seeded as single cells prior to commencing differentiation. Sparse seeding of single cells was crucial for efficient differentiation. To reiterate, undifferentiated hPSCs were maintained by passaging as cellular clumps (with EDTA; to maintain normal karyotype [as described above]), but were seeded for differentiation as single cells (to enable efficient differentiation). These initial steps of differentiation (posterior primitive streak, lateral mesoderm, and artery endothelium induction) were conducted in CDM2 basal media.

[00246] Seeding hESCs for differentiation (Step 0). Largely-confluent hPSCs were dissociated into single cells (Accutase, Thermo Fisher) and plated into recipient wells in mTeSR supplemented with thiazovivin (1 μ M, Tocris; a ROCK inhibitor, to enhance hPSC survival after passaging) onto plates precoated with either Geltrex or recombinant vitronectin basement membrane matrix (as described above), thus plating ~30,000-50,000 hPSCs/cm² (i.e., ~1.2-2e5 hPSCs/well of a 12-well plate or ~1.5-2.75e6 hPSCs/well of a 10cm-dish). Seeding density may need to be optimized for different hPSC lines with the day 4 artery purity (% CD144+ DLL4+) used to assess the best day 0 seeding density. Freshly-seeded hPSCs were allowed to adhere and recover for 24 hours in mTeSR + 1 μ M thiazovivin prior to initiating differentiation, during which the hPSCs re-formed small clumps.

[00247] Day 1-2 (posterior primitive streak induction, 48 hours) (Step 1). Day 0 hPSCs were briefly washed (DMEM/F12, Thermo Fisher) to remove all traces of mTeSR + thiazovivin. Then, they were differentiated towards posterior primitive streak in CDM2 media supplemented with BMP4

(40 ng/mL, R&D Systems), CHIR99021 (6 μ M, Tocris), FGF2 (20 ng/mL, Thermo Fisher) for 48 hours. Posterior primitive streak induction media was refreshed every 24 hours.

[00248] Day 3 (lateral mesoderm induction, 24 hours) (Step 2). Day 2 posterior primitive streak cells were briefly washed (DMEM/F12) and then differentiated towards lateral mesoderm in CDM2 media supplemented with BMP4 (40 ng/mL), GDC-0941 (2.5 μ M, Cellagen Technology), Forskolin (10 μ M, Tocris), SB-505124 (2 μ M, Tocris), VEGF (100 ng/mL, R&D Systems), XAV939 (1 μ M, Tocris), AA2P (200 μ g/mL) and TTNPB (0.5 nM) for 24 hours.

[00249] Day 4 (artery endothelium induction, 24 hours) (Step 3). Day 3 lateral mesoderm cells were briefly washed (DMEM/F12) and then differentiated towards artery endothelial cells in CDM2 media supplemented with Activin A (15 ng/mL, R&D Systems), DMH1 (250 nM, Tocris), GDC-0941 (2.5 μ M), VEGF (100 ng/mL), XAV939 (1 μ M), AA2P (200 μ g/mL) and TTNPB (0.5 nM, Tocris) for 24 hours.

[00250] Subsequently, hPSC-derived day 4 artery endothelial cells were dissociated into single cells (Accutase) and then re-seeded at high density ($\sim$ 500,000 cells/cm²; i.e., $\sim$ 1x10⁶ cells/well of a 24-well plate) on plates pre-coated with 10 μ g/mL vitronectin + 20 nM of the high-affinity NOTCH agonist DLL4-E12 for continued differentiation. At this stage, high cellular seeding density was critical to subsequently achieve efficient blood differentiation, and the seeding density used may need to be optimized for different hPSC lines. The next steps of differentiation (hemogenic endothelium and blood progenitor [HSC-like cell] induction) were conducted in CDM3 basal media.

[00251] Day 5-7 (hemogenic endothelium induction, 72 hours) (Step 4). Day 4 artery endothelial cells were dissociated into a single-cell suspension (Accutase); densely re-seeded at 500,000 cells/cm² onto plates precoated with 10 μ g/mL Vitronectin + 20 nM of high-affinity NOTCH agonist DLL4-E12; and then further differentiated towards hemogenic endothelium in CDM3 media supplemented with Forskolin (10 μ M), LIF (20 ng/mL, R&D Systems), OSM (10 ng/mL, R&D Systems), SB505124 (2 μ M) and UNC1999 (1 μ M, Tocris) for 24 hours. Hemogenic endothelium induction media was refreshed every 24 hours with a complete media change.

[00252] Day 8-10 (HSC-like cell induction, 72 hours [Step 5b]). Day 7 hemogenic endothelium cells were briefly washed (DMEM/F12, Thermo Fisher) and then differentiated towards HSC-like cells (otherwise known as blood progenitors) in CDM3 media supplemented with Forskolin (10 μ M), SB505124 (2 μ M), SR1 (750 nM, Cellagen Technology) and UM171 (75 nM, ApexBio), UNC0638 (500 nM, Tocris) and UNC1999 (1 μ M) for 72 hours. HSC-like cell induction media was refreshed every 24 hours with a complete media change for day 8 and 9, but on the last day (day 10), media was supplemented only.

[00253] Day 10 HSC-like cells were collected by gentle mixing and dissociation using TrypLE (Thermo Fisher) for 3-5 minutes at 37C or for a gentler dissociation, Papain (0.5mg/mL,

Worthington Biochemical) for 30-45 minutes at 37C. HSC-like cells were then counted, washed (DMEM/F12, Thermo Fisher) and prepared as needed for downstream assays.

[00254] *Differentiation of hPSC-derived HSC-like cells into myeloid and erythroid cells in methylcellulose culture.* HSC- were collected and cultured in Methylcellulose (MethoCult H4435 Enriched, STEMCELL Technologies, #04435) with 1.6e3 cells per 35mm dish prepared in triplicate. Cultures were incubated at 37C for 14 days and then manually counted and scored for colony formation and identification of a colony forming unit (CFU) containing -granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), -granulocyte, macrophage (CFU-GM), - erythroid (CFU-E) and burst forming unit -erythroid (BFU-E).

[00255] *Differentiation of hPSC-derived HSC into erythroid cells.* Erythroid differentiation was performed using the StemSpan Erythroid Expansion Supplement (STEMCELL Technologies, #02692) following manufacturer recommendations. Briefly, HSC-like cells were collected, counted and seeded in StemSpan SFEM II supplemented with the StemSpan Erythroid Expansion Supplement at a density of 1e4-1e5 cells/mL. On day 3, cells were supplemented with an equal volume of complete media. On day 7 and 10, cells were harvested are replated in complete media at 1e5 cells/mL. On day 14, cells were harvested and processed for flow cytometry.

[00256] *Differentiation of hPSC-derived HSC into macrophages.* HSC-like cells were collected, counted and seeded in StemPro-34 base media supplemented with the the following cytokines: Day 0-5: SCF (50 ng/mL, R&D Systems), TPO (10ng/mL, R&D Systems), IL-3 (50ng/mL, R&D Systems), FLT3L (50ng/mL, R&D Systems), M-CSF (50ng/mL, R&D Systems), ITS-X (Thermo Fisher). Day 6-10: FLT3L (50ng/mL, R&D Systems), M-CSF (50ng/mL, R&D Systems), GM-CSF (25ng/mL, R&D Systems), ITS-X (Thermo Fisher). Day 10-17: M-CSF (100ng/mL, R&D Systems), GM-CSF (50ng/mL, R&D Systems), ITS-X (Thermo Fisher). At day 17, cells were collected. Using TrypLE (Thermo Fisher) and processed for flow cytometry.

[00257] *Differentiation of hPSC-derived HSC into T cells.* T cell differentiation was performed using the StemSpan T Cell Generation Kit (STEMCELL Technologies, #09940) following manufacturer recommendations. Briefly, HSC-like cells were collected, counted and seeded in StemSpan Lymphoid Progenitor Expansion Medium at a density of 1-2e4 cells cells/mL (1e4 cells/mL for CD34+ cord blood HSPCs) in plates pre-coated with Lymphoid Differentiation Coating Material. On day 3, cells were supplemented with an equal volume of StemSpan Lymphoid Progenitor Expansion Medium. On days 7 and 10, half medium changes were performed. On day 14, cells were harvested, counted and reseeded in StemSpan T Cell Progenitor Maturation Medium at a density of 1e5-1e6 cells/mL in plates pre-coated with Lymphoid Differentiation Coating Material. On day 17, cells were supplemented with an equal volume of StemSpan T Cell Progenitor Maturation Medium. On days 21 and 24, hald media

changes were performed. On day 28, cells were collected, counted and processed for flow cytometry.

[00258] For further maturation into CD8 SP T cells, T cells from day 28 were harvested, counted and reseeded in StemSpan T Cell Progenitor Maturation Medium supplemented with IL-15 (10ng/mL, R&D Systems) and 12.5uL/mL ImmunoCult Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies, #10970) at a density of 1e6 cells/mL in plates pre-coated with Lymphoid Differentiation Coating Material. On day 31, cells were supplemented with an equal volume of StemSpan T Cell Progenitor Maturation Medium supplemented with IL-15 (10ng/mL, R&D Systems). On day 35, cells were collected, counted and processed for flow cytometry.

[00259] *Differentiation of hPSC-derived HSC into NK cells.* NK cell differentiation was performed using co-culture with OP9-DLL4 feeder cells. HSC-like cells were collected, counted and reseeded onto a confluent layer of OP9-DLL4 feeder cells at a concentration of 1e4-1e5 cells/mL (1e3 cells/mL for CD34+ cord blood HSPCs) in aMEM (Thermo Fisher) supplemented with 20% FBS (R&D Systems), 1% penicillin/streptomycin (Thermo Fisher), SCF (30ng/mL, R&D Systems), FLT3L (5ng/mL, R&D Systems), IL7 (5ng/mL, R&D Systems), IL15 (10ng/mL, R&D Systems), and for the first week of culture only, IL3 (5ng/mL). Cells were supplemented with an equal volume of media on days 3 and 10, and cells were collected and reseeded onto fresh OP9-DLL4 feeders on day 7. On day 14, cells were collected, counted, and processed for flow cytometry.

[00260] *NK cell killing assay.* On day -1, 1e3 OP9-DLL4-GFP feeder cells were seeded per well of a 96 well plate in aMEM (Thermo Fisher) supplemented with 20% FBS (R&D Systems), 1% penicillin/streptomycin (Thermo Fisher). On day 0, NK cells derived from human CD34+ cord blood HSPCs or HSC-like cells were collected, counted and MACS enriched for CD56+ cells. 1e4 CD56+ NK cells were seeded onto the OP9-DLL4-GFP feeders per well of a 96 well plate in aMEM (Thermo Fisher) supplemented with 20% FBS (R&D Systems), 1% penicillin/streptomycin (Thermo Fisher). Cells were cultured in an Incucyte Live-Cell Analysis System and imaged every hour for 72 hours. Total GFP+ area was measured and used to determine NK cell killing efficiency over time.

[00261] *Differentiation of hPSC-derived HSC into B cells.* B cell differentiation was performed using co-culture with MS5 feeder cells (DSMZ). HSC-like cells were collected, counted and reseeded onto a confluent layer of MS5 feeder cells at a concentration of 1-5e5 cells/mL (1e3-1e4 cells/mL for CD34+ cord blood HSPCs) in aMEM (Thermo Fisher) supplemented with 10% FBS (R&D Systems), 1% penicillin/streptomycin (Thermo Fisher), SCF (100ng/mL, R&D Systems), G-CSF (10ng/mL, R&D Systems). Cells were supplemented with equal volume of media on days 3, 10, and 17, and cells were collected and reseeded onto fresh MS5 feeders on days 7 and 14. On day 21, cells were collected, counted, and processed for flow cytometry.

[00262] *Culture of human cord blood stem and progenitor cells.* Human cord blood CD34+ Stem/Progenitor cells (StemExpress) were thawed and cultured in StemSpan II (STEMCELL

Technologies) supplemented with SCF (20ng/mL, R&D Systems), TPO (50ng/mL, R&D Systems), FLT3L (20ng/mL, R&D Systems), IL6 (20ng/mL, R&D Systems), UM171 (75nM, ApexBio), SR1 (750nM, Cellagen Technology). Cells were seeded at 2e5 cells/mL in U-bottom tissue culture treated plates. Each day, cells were gently trituated and half were transferred to a new well with all wells supplemented with fresh media to maintain the culture at low density. CD34+ cells were either used immediately from thaw or cultured 3 days or less for all assays.

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[00411] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

THAT WHICH IS CLAIMED IS:

1. A method of producing a substantially pure population of hematopoietic stem cells from a population of pluripotent stem cells, the method comprising:

- (a) differentiating the pluripotent stem cells into posterior primitive streak cells;
- (b) differentiating the posterior primitive streak cells into lateral mesoderm cells;
- (c) differentiating the lateral mesoderm cells into artery endothelium cells;
- (d) differentiating the artery endothelium cells into hemogenic endothelium cells; and
- (e) differentiating the hemogenic endothelium cells into hematopoietic stem cells.

2. The method of claim 1, wherein greater than 80% of the final hematopoietic stem cell population express the hematopoietic stem cell surface markers CD34, CD144, CD45; and the HSC transcription factors/chromatin regulators HLF, HOXA5, HOXA9, HOXA10, MECOM, MLLT3, RUNX1, MEIS1, and MYB.

3. The method of claim 1 or claim 2, wherein there is a substantially stoichiometric conversion of pluripotent into hematopoietic stem cells.

4. The method of any of the previous claims, wherein pluripotent cells are contacted with an effective amount of: a BMP agent; an FGF agent; and a WNT agent, for a period of time sufficient to generate a population of primitive streak cells.

5. The method of claim 4, wherein the period of time is about 2 days.

6. The method of claim 4, wherein the BMP agent comprises BMP4; the FGF agent comprises FGF2; and the WNT agent comprises CHIR99021.

7. The method of any of the previous claims, wherein the primitive streak cells are contacted with an effective amount of: a BMP agent; a cAMP-elevating agent; a retinoic acid pathway activator; a VEGF agent; a TGF β inhibitor; a WNT inhibitor; and a PI3K inhibitor for a period of time sufficient to generate a population of lateral mesoderm cells.

8. The method of Claim 7, wherein the primitive streak cells are additionally contacted with an ascorbic acid agent.

9. The method of claim 7 or claim 8, wherein the period of time sufficient to generate a population of lateral mesoderm cells is about 1 day.

10. The method of any of claims 7-9, wherein the BMP agent comprises BMP4; the cAMP-elevating agent comprises Forskolin; the retinoic acid pathway activator comprises TTNPB; the VEGF agent comprises VEGF; the TGF β inhibitor comprises SB-505124; the WNT inhibitor comprises XAV939; and the PI3K inhibitor comprises GDC-0941.

11. The method of any of claims 7-10 wherein the primitive streak cells are further contacted with AA2P.

12. The method of any of the preceding claims, wherein the lateral mesoderm cells are contacted with an effective amount of: a TGF β agent; a VEGF agent; a retinoic acid pathway activator; a BMP agent; a WNT inhibitor; and a PI3K inhibitor, for a period of time sufficient to produce a population of artery endothelium cells.

13. The method of claim 12, wherein the lateral mesoderm cells are further contacted with an ascorbic acid agent.

14. The method of claim 12 or claim 13, wherein the period of time sufficient to produce a population of artery endothelium cell is about 1 day.

15. The method of any of claims 12-14, wherein the TGF β agent comprises Activin A; the VEGF agent comprises VEGF; the retinoic acid pathway activator comprises TTNPB; the BMP inhibitor comprises DMH1; the WNT inhibitor comprises XAV939; and the PI3K inhibitor comprises GDC-0941.

16. The method of any of claims 12-15, wherein the lateral mesoderm cells are contacted with AA2P.

17. The method of any of the preceding claims, wherein the artery endothelium cells are contacted with: a GP130 agonist; a NOTCH agonist; a cAMP-elevating agent; an inhibitor of TGF β ; and an inhibitor of PRC2 for a period of time sufficient to produce a population of hemogenic endothelium cells.

18. The method of claim 17, wherein the period of time sufficient to produce a population of hemogenic endothelium cells is about 3 days.

19. The method of claim 17 or 18, wherein the artery endothelium cells are plated at a density of about 500,000 cells/cm².

20. The method of any of claims 17-19, wherein the GP130 agonist comprises LIF and OSM; the NOTCH agonist comprises DLL4-E12; the cAMP-elevating agent comprises Forskolin; the inhibitor of TGF β comprises SB-505124; and the inhibitor of PRC2 comprises UNC1999.

21. The method of any of the previous claims, wherein the hemogenic endothelium cells contacted with: a cAMP-elevating agent; a TGF β inhibitor; a PRC2 inhibitor; a GLP/G9A inhibitor; and one or more agents which maintain hematopoietic stem cells in an undifferentiated state; for a period of time sufficient to generate a population of hematopoietic stem cells.

22. The method of claim 21, wherein the one or more agents which maintain hematopoietic stem cells in an undifferentiated state comprises an aryl hydrocarbon receptor inhibitor and/or a CoREST-HDAC complex inhibitor.

23. The method of claim 21 or claim 22, wherein the period of time sufficient to generate a population of hematopoietic stem cells is from about 2 to about 4 days.

24. The method of any of claims 21-23, wherein the cells are cultured on a substrate at a density of about 500,000 cells/cm².

25. The method of any of claims 21-24, wherein the cAMP-elevating agent comprises Forskolin; the TGF β inhibitor comprises SB-505124; the PRC2 inhibitor comprises UNC1999; the GLP/G9A inhibitor comprises UNC0638; and the one or more agents which maintain hematopoietic stem cells in an undifferentiated state comprises SR1 and UM171.

26. A substantially pure population of hematopoietic stem cells produced by the method according to any of claims 1-25.

27. A cell, wherein the cell is a cell differentiated from hematopoietic stem cells produced by the method according to any of claims 1-25.

28. A method of treatment, comprising administering to an individual the cell according to claim 26 or 27.

29. A method of screening a substantially pure population of hematopoietic stem cells for a cellular response, comprising contacting a population of substantially pure population of hematopoietic stem cells of claim 26 or cell derived therefrom with a pharmacological agent and evaluating the population of cells for a cellular response induced by the pharmacological agent.

25. A kit or system for use in the methods of any of claims 1-25.

FIG. 1

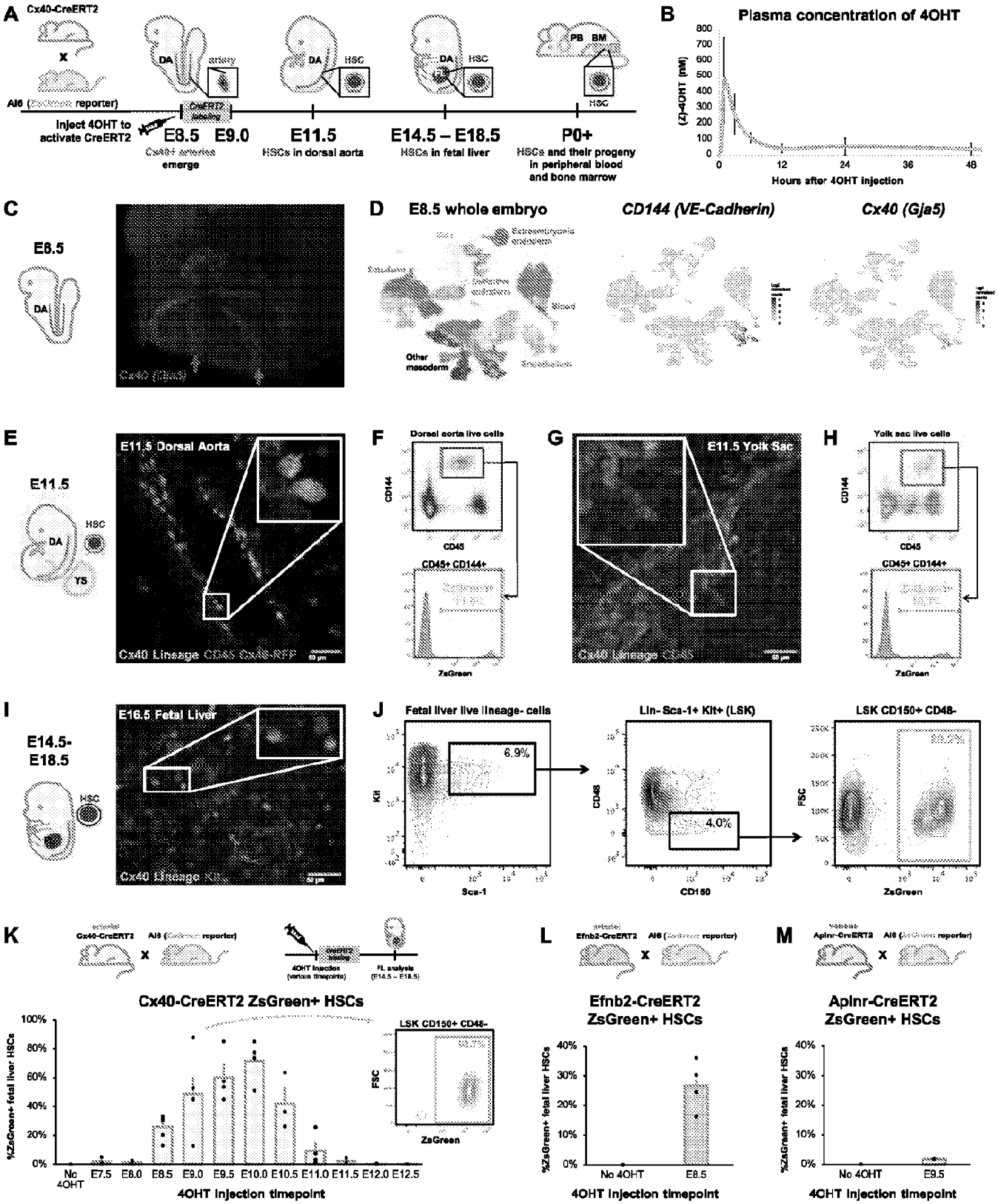


FIG. 2

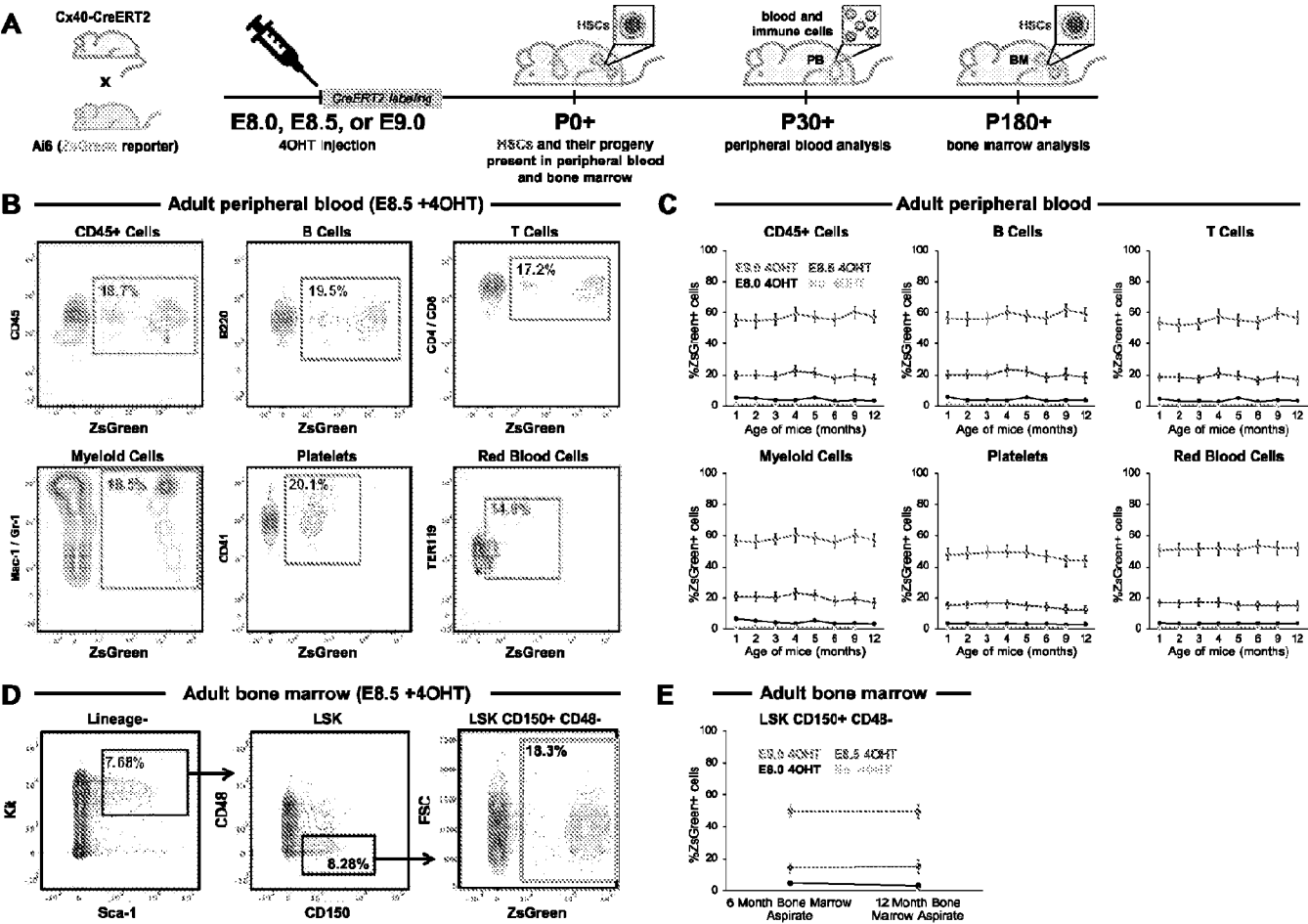


FIG. 3

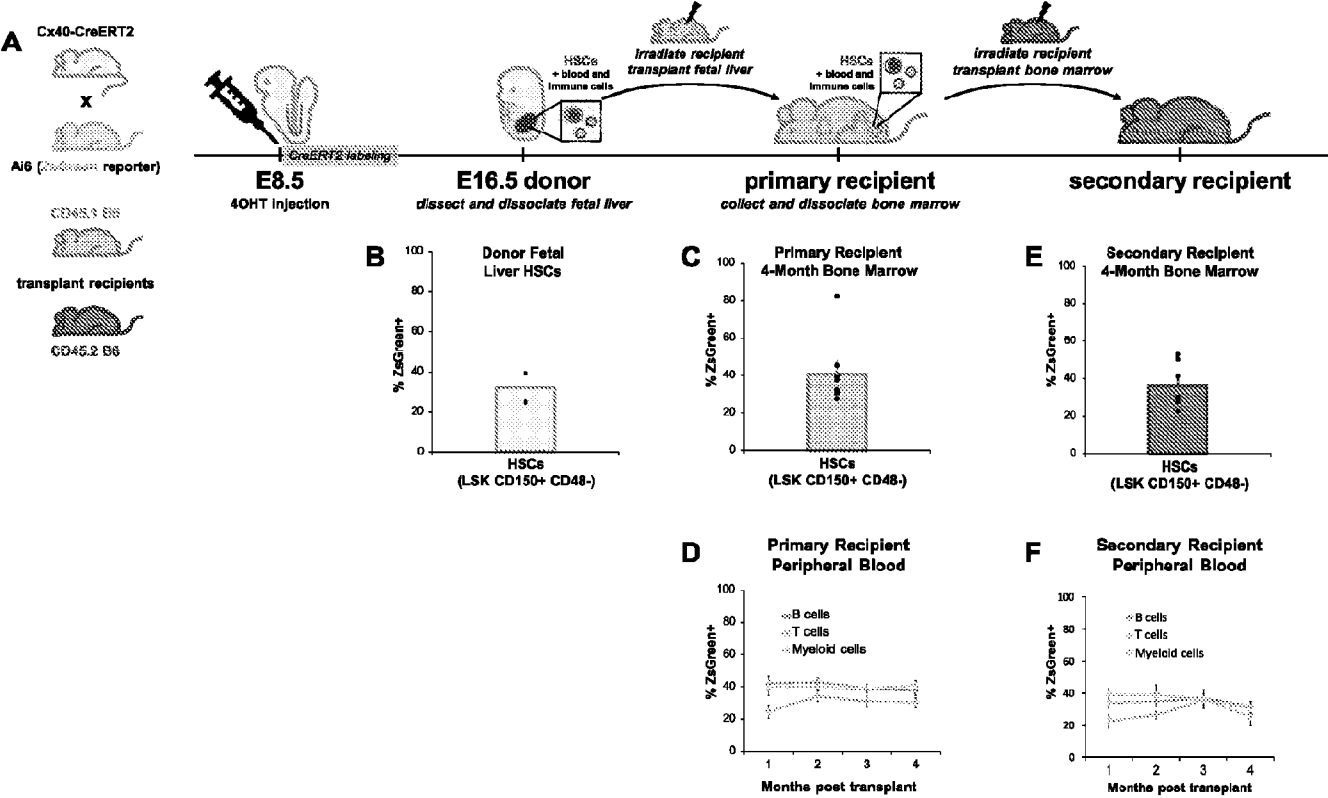
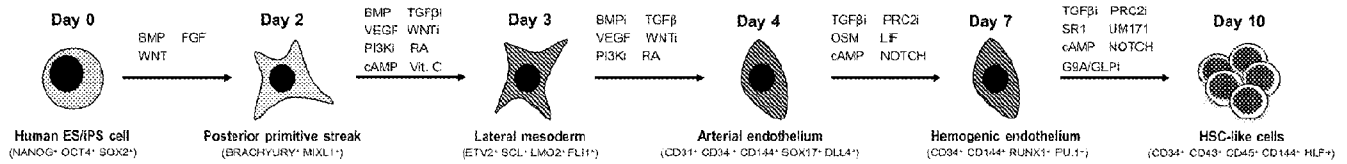
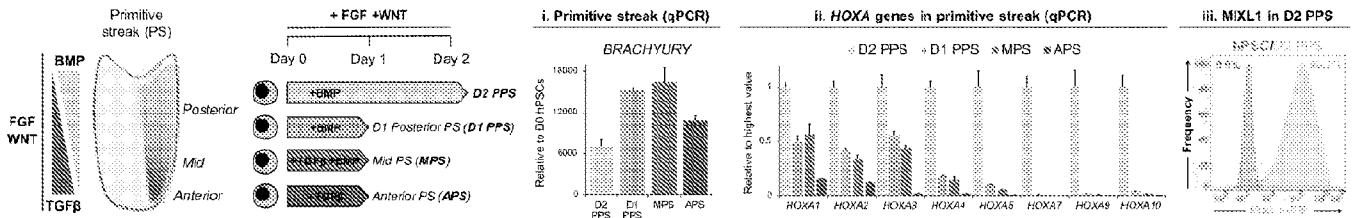


FIG. 4

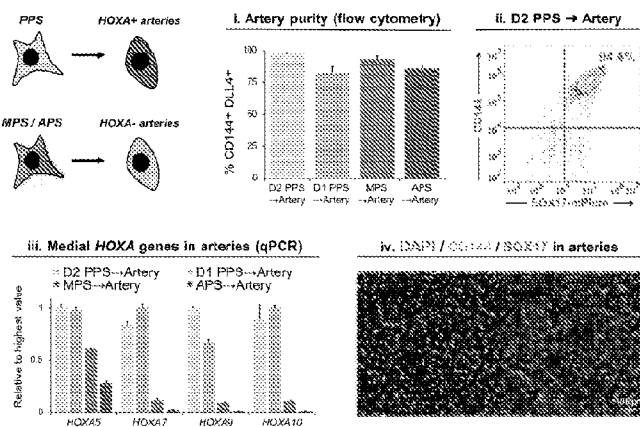
A Generation of HSC-like blood progenitor cells from human pluripotent stem cells within 10 days of differentiation



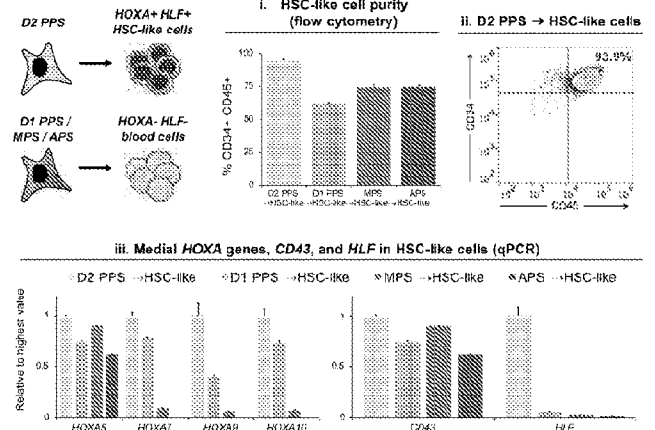
B Generating different types of primitive streak (PS) *in vitro*



C Posterior primitive streak produces *HOXA*⁺ arteries



E Day 2 posterior primitive streak produces *HOXA*⁺ *HLF*⁺ HSC-like cells



D Stepwise generation of posterior primitive streak, lateral mesoderm, and artery cells

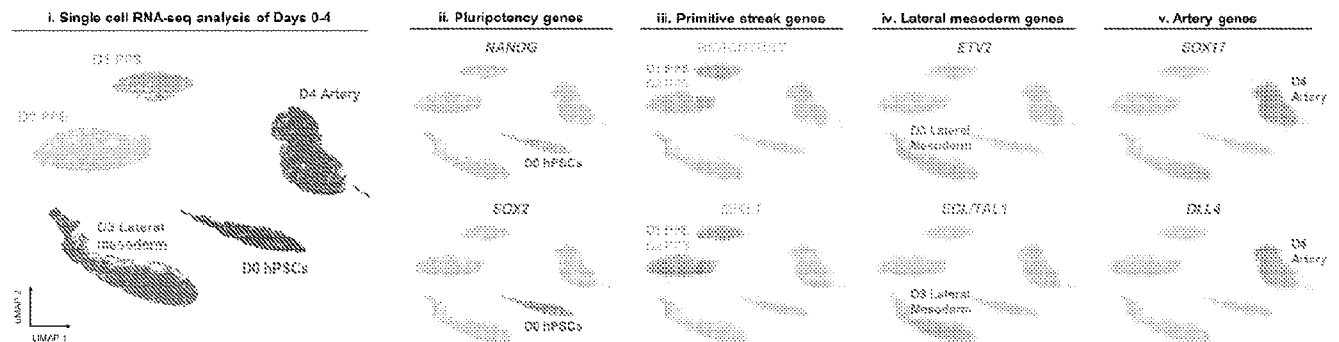


FIG. 5

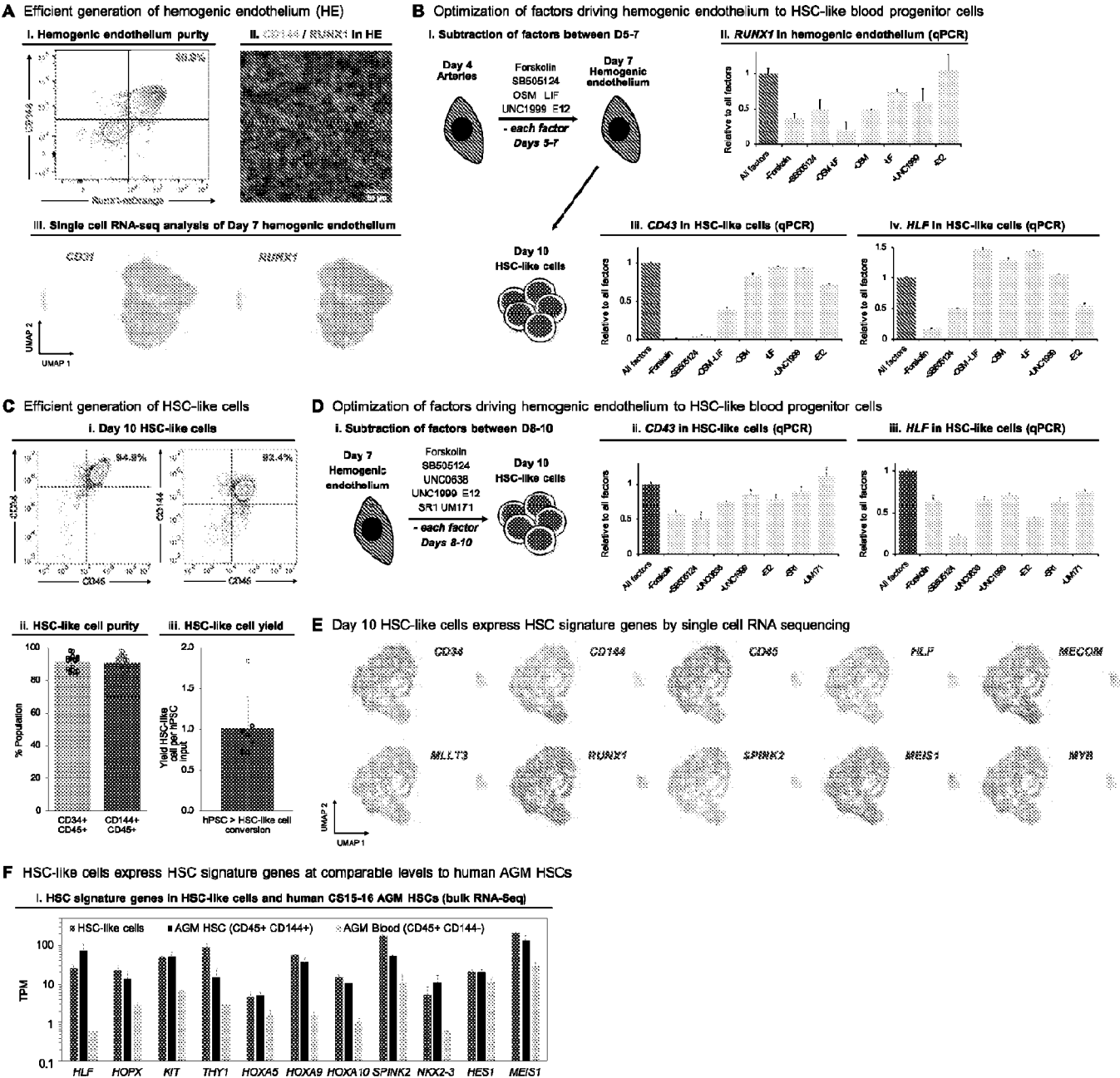


FIG. 6

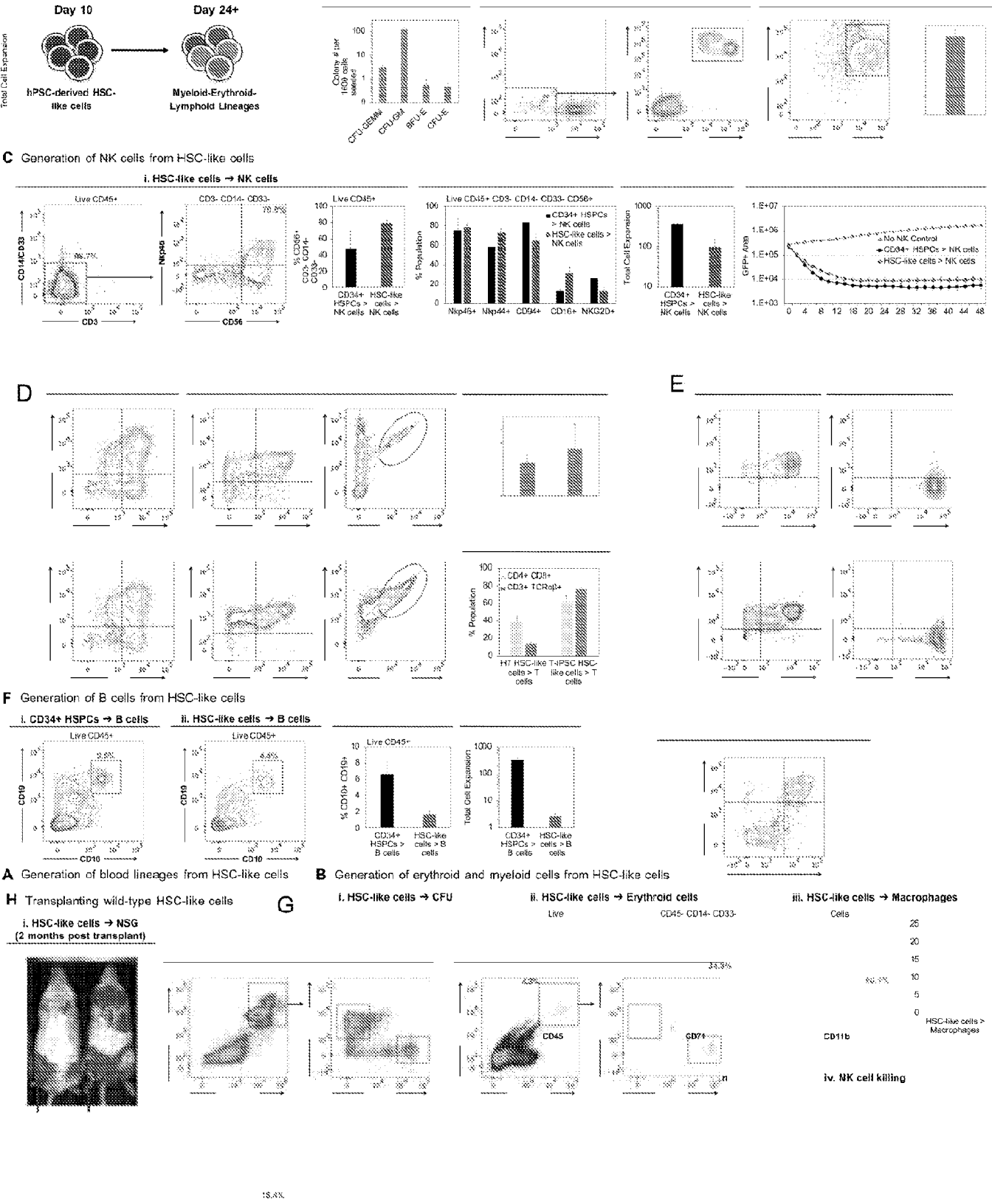


FIG. 7

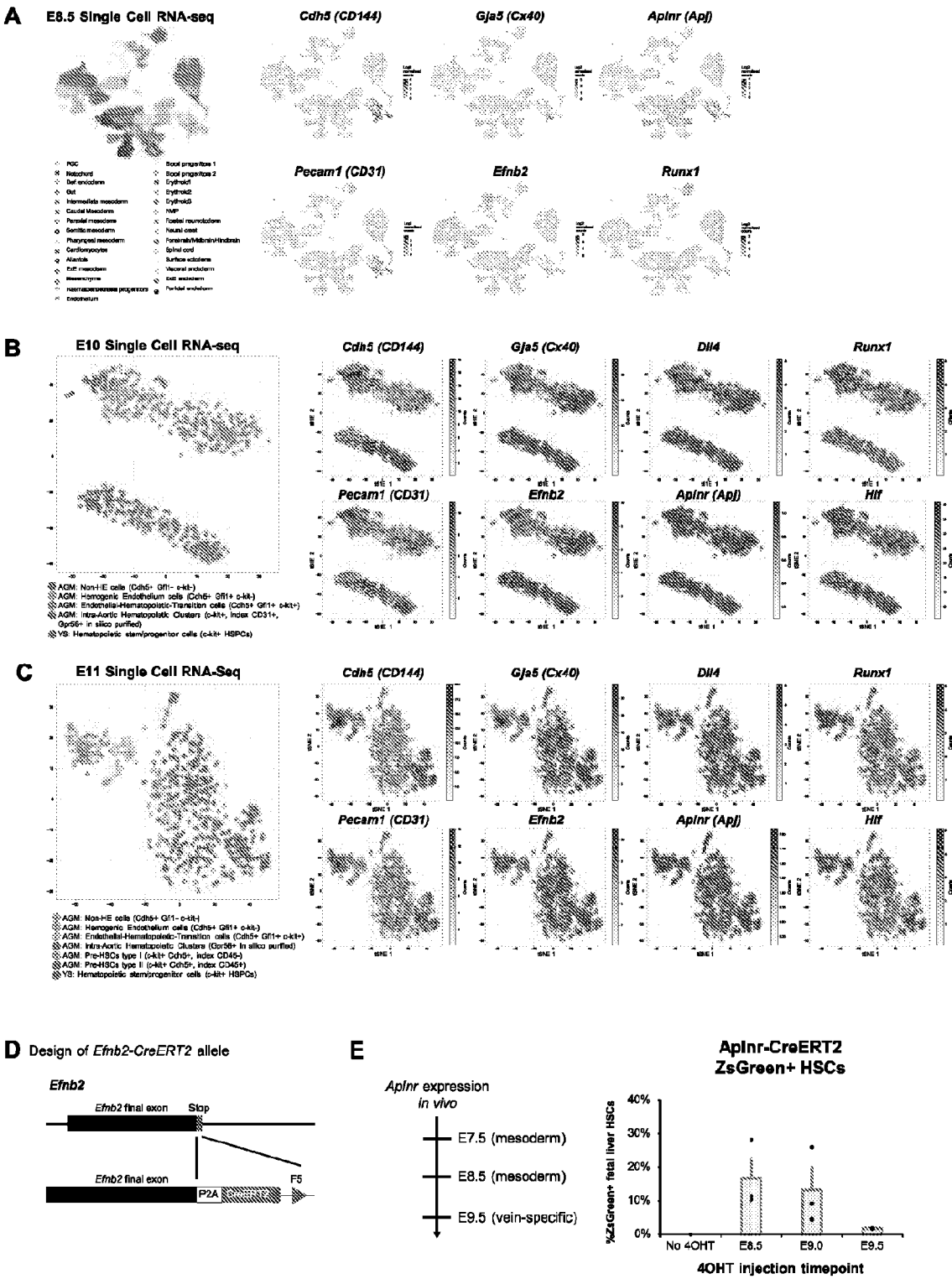


FIG. 8

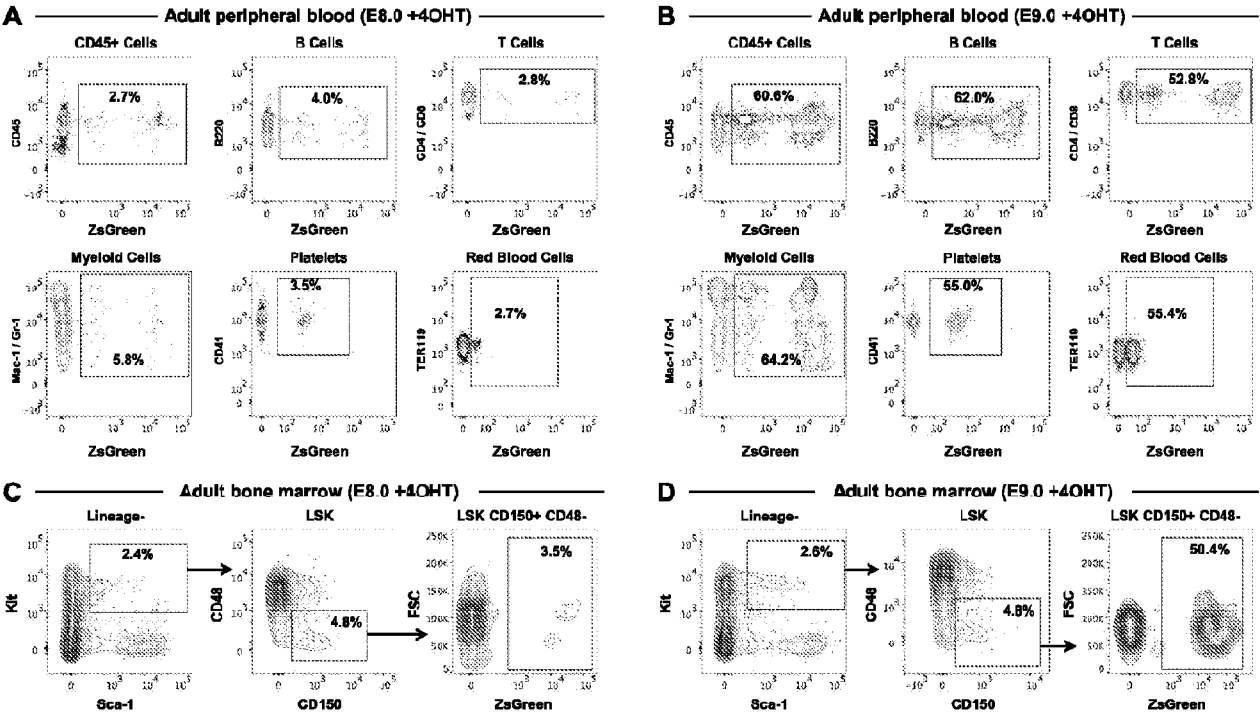
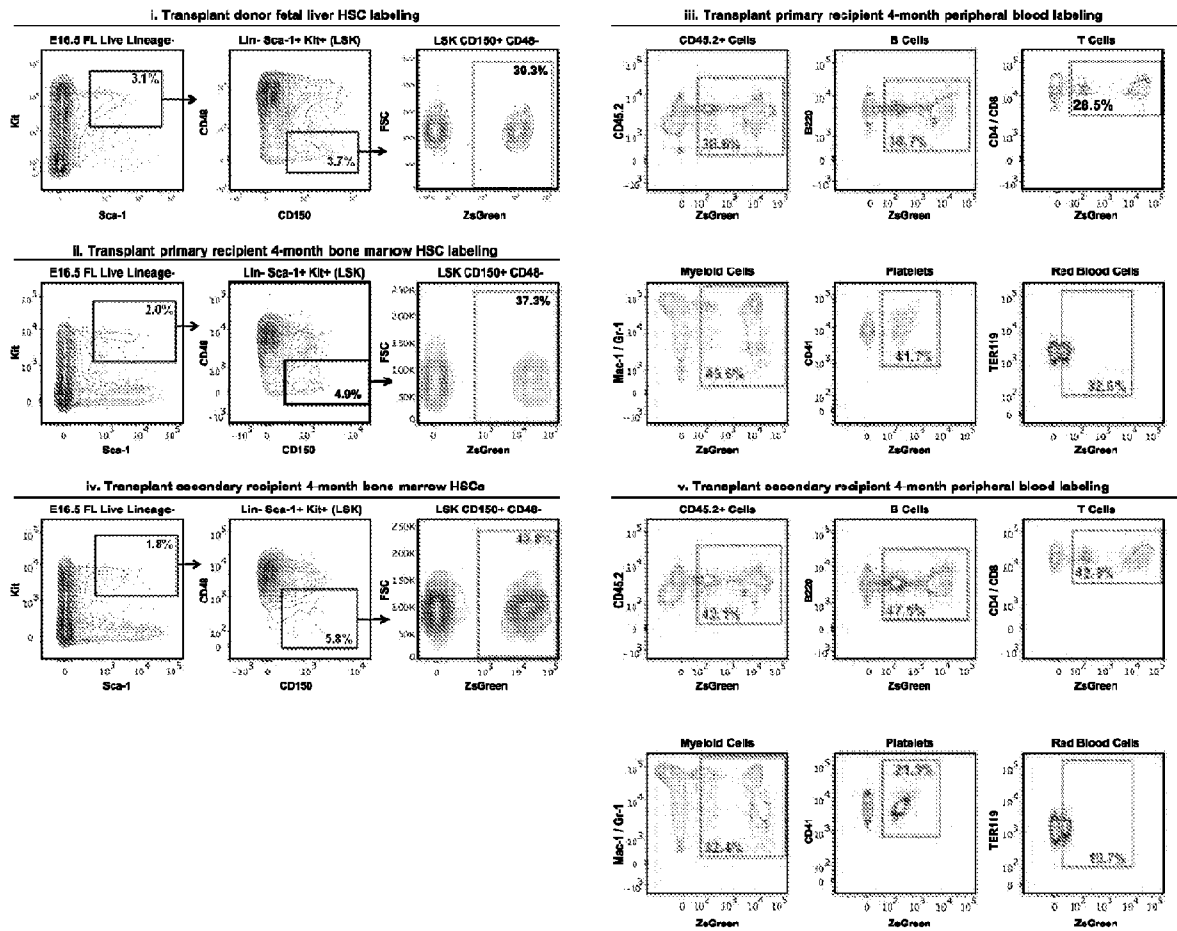


FIG. 9

A Representative plots of HSC transplant



F

A Generating four different types of primitive streak from hPSCs

FIG. 10

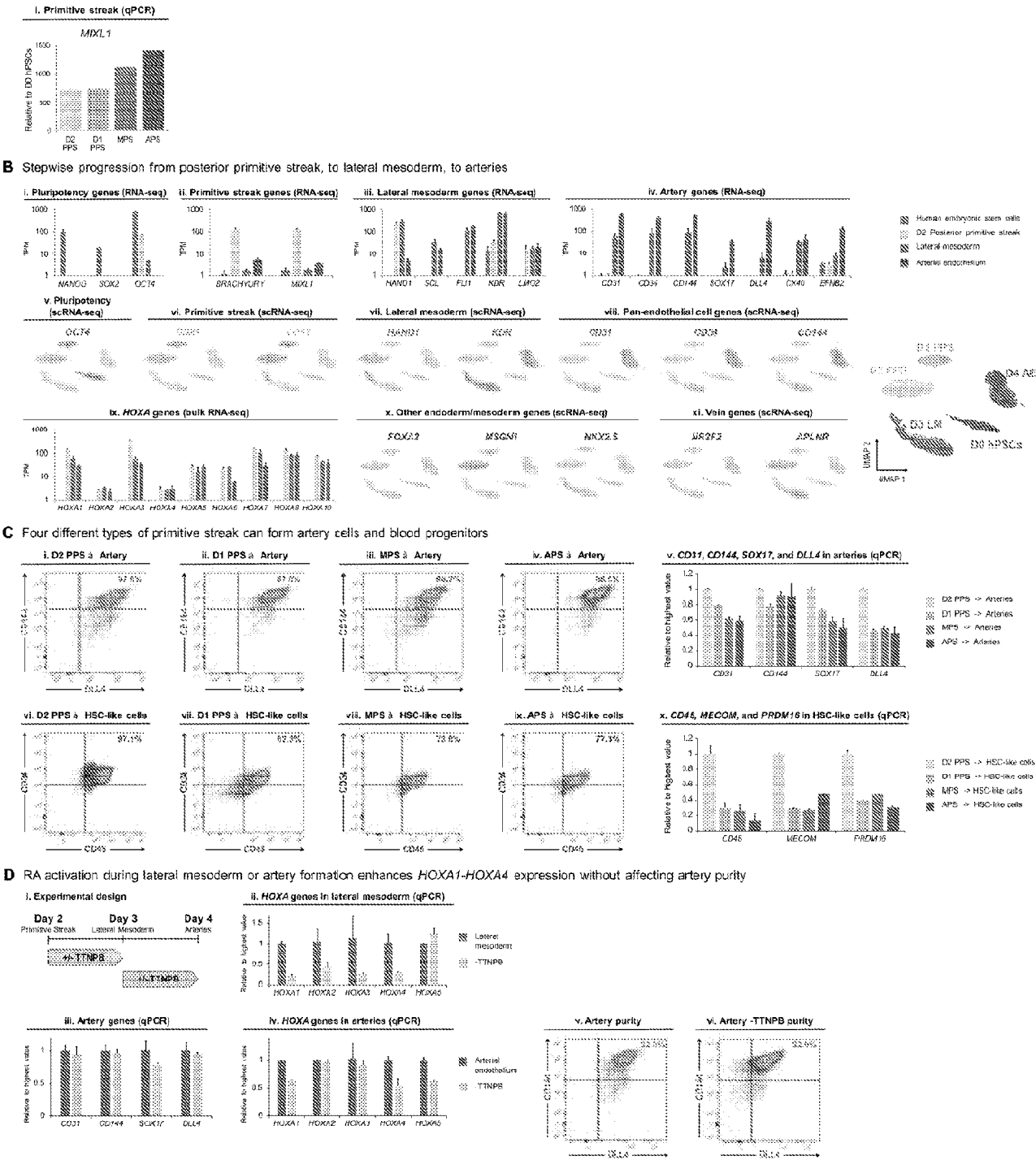
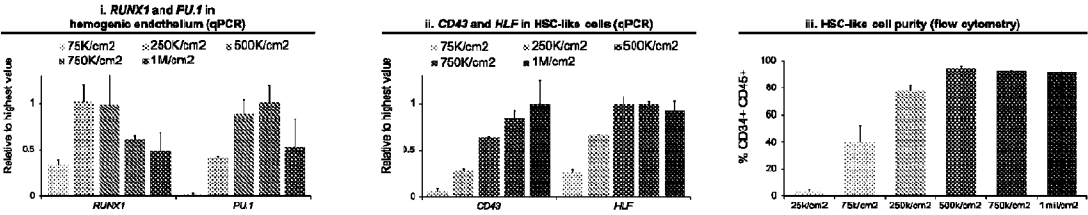


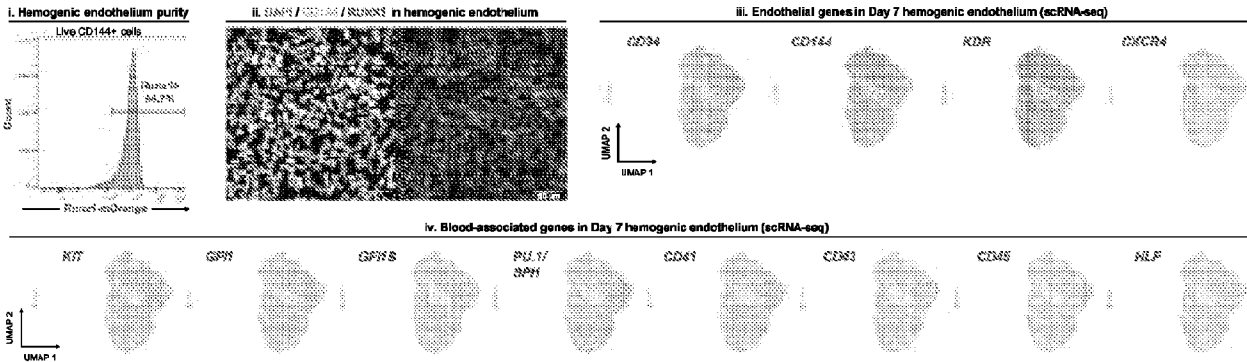
FIG. 11

F

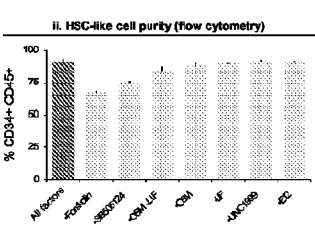
A Generation of hemogenic endothelium and HSC-like blood progenitor cells from arterial endothelium requires high density



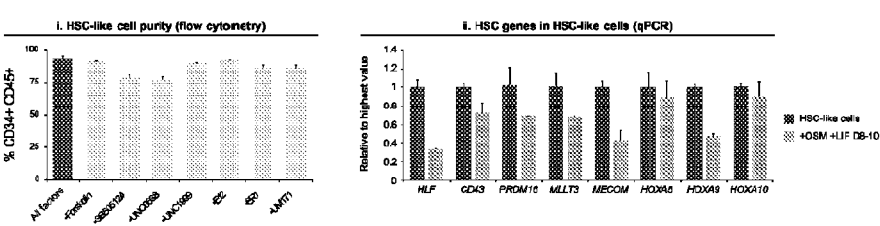
B Efficient generation of hemogenic endothelium



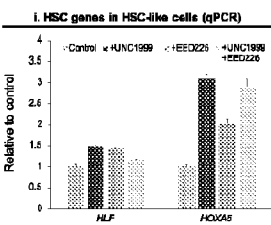
C Optimization of factors driving arteries to hemogenic endothelium



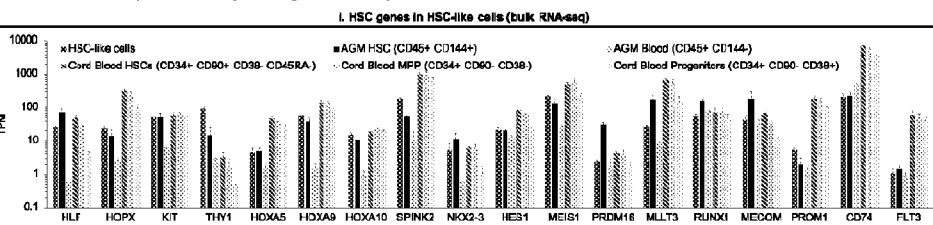
D Optimization of factors driving hemogenic endothelium to HSC-like cells



E PRC2 inhibitors during differentiation



F HSC-like cells express HSC signature genes at comparable levels to human AGM HSCs and human Cord Blood HSCs



G Day 10 HSC-like cells express HSC signature genes by single cell RNA sequencing

