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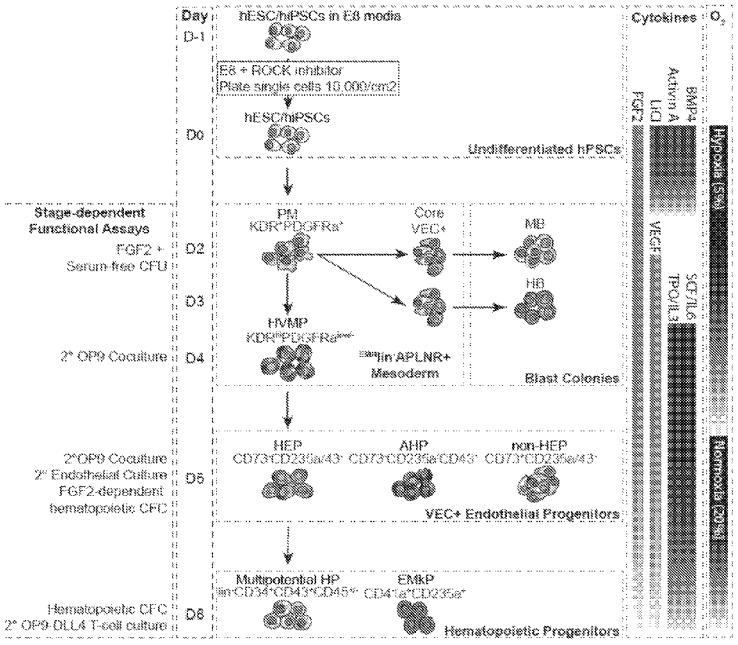
[Continued on next page]

(54) Title: METHODS AND MATERIALS FOR HEMATOENDOTHELIAL DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS UNDER DEFINED CONDITIONS

FIG. 1



(57) Abstract: Methods and compositions for differentiating pluripotent stem cells into cells of endothelial and hematopoietic lineages are disclosed.

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METHODS AND MATERIALS FOR HEMATOENDOTHELIAL DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS UNDER DEFINED CONDITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Provisional Patent Application Serial No. 61/779,564 filed on March 13, 2013.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH/DEVELOPMENT

[0002] This invention was made with government support under Grants No. HL099773 and HL116221 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] The advent of human pluripotent stem cell technologies has provided the opportunity to produce endothelial and hematopoietic cells *in vitro* for functional studies and therapies. Previously, co-culture systems using the mouse bone marrow stromal cell line, OP9, have been used to establish efficient and scalable differentiation of human pluripotent stem cells (hPSCs) into endothelial and blood lineages. However, co-culture systems that rely on mouse feeder cells and serum (*i.e.*, xenogenic sources) have limited utility for studying hPSC response to specific growth factors. Moreover, such systems have further limitations when considered in the context of manufacturing clinical grade therapeutic blood cells.

[0004] In light of the shortcomings of prior culture systems, new culture systems are needed to provide sources of endothelial and blood lineages that are suitable for use in clinical settings without the risk of introduction of xenogenic contamination.

SUMMARY OF THE INVENTION

[0005] In one aspect provided herein is a method for differentiating human pluripotent stem cells comprising: (a) providing human pluripotent stem cells; and (b) culturing the human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential.:

[0006] In some embodiments the method also includes the step of (c) exposing cells at the primitive mesoderm stage of step (b) to a mixture comprising components FGF2 and VEGF under hypoxic conditions for a period of about 1-2 days to obtain a population comprising $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($^{EMH}lin^{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) with a potential to form hematoendothelial clusters when cultured on OP9 cells. In some embodiments, the method further includes the step of: (d) exposing the cells at the hematovascular mesoderm stage of step (c) to a mixture comprising components FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to achieve formation of $CD144^{+}CD73^{+}CD235a/CD43^{-}$ non-hemogenic endothelial progenitors (non-HEP), $CD144^{+}CD73^{-}CD235a/CD43^{-}$ hemogenic endothelial progenitors (HEPs), $CD144^{+}CD73^{-}CD235a/CD43^{+}41a^{-}$ angiogenic hematopoietic progenitors (AHP), and $CD43^{+}CD41a^{+}$ hematopoietic progenitor cells. In further embodiments, the method also comprises the step of: (e) continuing to expose the HEPs and emerging hematopoietic progenitor cells to a mixture of FGF2, VEGF, IL6, SCF, TPO, IL3 under normoxia for about three days

resulting in hematopoietic expansion to obtain a population of $CD43^+$ hematopoietic progenitors composed of $CD43^+CD235a^+CD41a^+$ erythromegakaryocytic progenitors and $lin^-CD34^+CD43^+CD45^{+/-}$ multipotent hematopoietic progenitors.

[0007] In some embodiments the mixture to be used in any of the preceding methods consists essentially of the mentioned components. In some embodiments the mixture to be used is xenogen-free.

[0008] In some embodiments the human pluripotente stem cells are provided on a substrate treated with Tenascin-C.

[0009] In a further aspect provided herein is a xenogen-free culture medium for differentiating human pluripotent stem cells, comprising IF9S medium supplemented with: about 50 to about 250 ng/ml BMP4; about 10 to about 15 ng/ml Activin A; about 10 to about 50 ng/ml FGF2; and about 1 mM to about 2 mM LiCl.

[0010] In a related aspect provided herein is a xenogen-free culture medium for differentiating human pluripotent stem cells, comprising IF9S medium supplemented with: about 10 to about 50 ng/ml FGF2; and about 20 to about 50 ng/ml VEGF. In some embodiments, where the medium contains FGF2 and VEGF, the medium also includes a hematopoietic cytokine. In some embodiments, the hematopoietic cytokine comprises: about 50 to about 100 ng/ml SCF; about 50 to about 100 ng/ml TPO; about 50 to about 100 ng/ml IL-6; and about 5 to about 15 ng/ml IL-3. In some embodiments, any of the foregoing media consist essentially of the IF9S medium and the supplemented components. In some embodiments, any of the foregoing media are provided in a concentrated form.

[0011] In another aspect provided herein is a xenogen-free cell culture system for differentiating human pluripotent stem cells into mesoderm, endothelial, and hematopoietic

progenitor cells, comprising: human pluripotent stem cells seeded as a single cell suspension on a substrate comprising a layer of Tenascin C at a concentration of at least about 0.25 $\mu g/cm^2$ to 1 $\mu g/cm^{2;}$ and a xenogen-free culture medium comprising IF9S medium supplemented with: about 50 to about 250 ng/ml BMP4; about 10 to about 15 ng/ml Activin A; about 10 to about 50 ng/ml FGF2; and about 1 to about 2 mM LiCl.

[0012] In another aspect provided herein is a method of differentiating pluripotent stem cells, comprising the steps of: (a) providing human pluripotent stem cells; (b) seeding the cells as a single cell suspension on a substrate treated with Tenascin C; and (c) culturing the seeded cells in IF9S medium supplemented with BMP4, Activin A, FGF2, and LiCl under hypoxic conditions for a period of about two days to obtain about 30% $^{EMH}lin^-KDR^+APLNR^+PDGFRalpha^+$ primitive mesoderm cells with mesenchymoangioblast potential.

[0013] In some embodiments, the above method further comprises the step of culturing the cells at $^{EMH}lin^-KDR^+APLNR^+PDGFRalpha^+$ primitive mesoderm stage in IF9S medium supplemented with FGF2 and VEGF under hypoxic conditions for about 1-2 days to obtain $^{EMH}lin^-KDR^+APLNR^+PDGFRalpha^+$ primitive mesoderm with hemangioblast (HB-CFC) potential and $^{EMH}linKDR^{hi}APLNR^+PDGFRalpha^{lo/-}$ hematovascular mesodermal precursors with a potential to form hematoendothelial clusters when cultured on OP9 cells.

[0014] In a further aspect provided herein are purified populations of cells generated by any of the foregoing methods for differentiating human pluripotent stem cells, wherein the cells have not been exposed to non-human constituents.

[0015] In a related aspect provided herein is a purified population of cells created by the methods described herein, wherein the cells are greater than about 35% $^{EMH}lin^-$

$KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with a potential to form mesenchymoangioblast colonies.

[0016] In another aspect provided herein is a purified population of cells, wherein the cells are greater than about 35% of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and 30% $^{EMH}lin^{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$ hematovascular mesodermal cells with a potential to form hematoendothelial clusters when cultured on OP9 cells.

[0017] In a further aspect provided herein is a purified population of cells, wherein the population includes greater than about 40% of $CD144^{+}$ cells comprising $CD144^{+}CD73^{-}CD235a/43^{-}$ hemogenic endothelial progenitors (HEPs), $CD144^{+}CD73^{-}CD235a/CD43^{+}CD41a^{-}$ angiogenic hematopoietic progenitors, and $CD144^{+}CD73^{+}CD235a/43^{-}$ non-hemogenic endothelial progenitors (non-HEPs).

[0018] In another aspect provided herein is a population of cells, wherein the population includes greater than about 30% $CD43^{+}$ hematopoietic progenitor cells composed of $CD43^{+}CD235a+CD41a^{+}$ erythromegakaryocytic progenitors and $lin^{-}CD34^{+}CD43^{+}CD45^{+/-}$ multipotent hematopoietic progenitors.

[0019] In a further aspect provided herein is a method of producing mesenchymoangioblasts, comprising the steps of: (a) providing human pluripotent stem cells; (b) seeding the cells on a substrate treated with an effective amount of collagen; and (c) exposing the stem cells to a mixture comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for a period of about two days to form a population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential. In some embodiments the collagen to be used comprises Collagen IV.

[0020] In yet another aspect provided herein is a cell culture medium for differentiating human pluripotent stem cells, comprising: 64 mg/L L-Ascorbic Acid 2-Phosphate Mg^{2+} salt, 40 µl/L monothioglycerol, 8.4 µg/L additional sodium selenite, 10 mg/L polyvinyl alcohol, 1x GLUTAMAX, 1x non-essential amino acids, 0.1x chemically-defined lipid concentrate, 10.6 mg/L Holo-Transferrin, and 20 mg/L insulin.

[0021] In a further aspect described herein is a method for differentiating human pluripotent stem cells comprising: (a) providing human pluripotent stem cells; and (b) culturing the human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of ^{EMH}lin-KDR+APLNR+PDGFRalpha + primitive mesoderm cells with mesenchymoangioblast potential.

[0022] In some embodiments the human pluripotent stem cells are cultured on Tenascin C. In some embodiments the cell culture medium comprises an IF9S cell culture medium. In some embodiments the concentration, in the cell culture medium, of: BMP4 is about 50 ng/ml to about 250 mg/ml; Activin A is about 10 ng/ml to about 15 ng/ml; FGF2 is about 10 ng/ml to about 50 ng/ml; and LiCl is about 1 mM to about 2 mM.

[0023] In some embodiments the method further comprises (c) culturing, under hypoxic conditions, the cell population obtained in step (b) in a cell culture medium comprising FGF2 and VEGF for a period of about 1-2 days to obtain a cell population comprising ^{EMH}lin-KDR+APLNR+PDGFRalpha + primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells (^{EMH}lin-KDR^{hi}APLNR^{+}PDGFRalpha$^{lo/-}$) with a potential to form hematoendothelial clusters when cultured on OP9 cells. In some embodiments the concentration, in the cell culture medium, of FGF2 is about 10 ng/ml to about 50 ng/ml; and VEGF is about 20 ng/ml to about 50 ng/ml.

[0024] In some embodiments the method further comprises (d) culturing the hematovascular mesoderm cells of step (c), under hypoxic conditions, in a cell culture medium comprising FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to obtain a cell population comprising CD144+CD73+CD235a/CD43- non-hemogenic endothelial progenitors (non-HEP), CD144+CD73-CD235a/CD43- hemogenic endothelial progenitors (HEPs), CD144+CD73-CD235a/CD43+41a- angiogenic hematopoietic progenitors (AHP), and CD43+CD41a+ hematopoietic progenitor cells. In some embodiments the concentration, in the cell culture medium, of: FGF2 is about 10 ng/ml to about 50 ng/ml; VEGF is about 20 ng/ml to about 50 ng/ml; SCF is about 50 ng/ml to about 100 ng/ml; TPO is about 50 ng/ml to about 100 ng/ml; IL-6 is about 50 ng/ml to about 100 ng/ml, and IL-3 is about 5 ng/ml to about 15 ng/ml.

[0025] In some embodiments the method further comprises (e) culturing, under normoxia, the HEPs and hematopoietic progenitor cells in a culture medium comprising FGF2, VEGF, IL6, SCF, TPO, IL3 for about three days to obtain an expanded population of CD43+ hematopoietic progenitors comprising $CD43^{+}CD235a^{+}CD41a^{+}$ erythromegakaryocytic progenitors and $lin^{-}CD34^{+}CD43^{+}CD45^{+/-}$ multipotent hematopoietic progenitors.

[0026] In some embodiments the method further comprises further coculturing the expanded population of CD34+CD43+ hematopoietic progenitors for a period of about three weeks on OP9 cells overexpressing DLL4 to obtain a cell population comprising CD4+CD8+ double positive T cells, wherein the human pluripotent stem cells of step (b) are cultured on a Tenascin C substrate.

[0027] In a related aspect provided herein is a method of differentiating human pluripotent stem cells, comprising at least one of: (i) culturing human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive

mesoderm cells with mesenchymoangioblast potential; (ii) culturing, under hypoxic conditions, $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential in a cell culture medium comprising FGF2 and VEGF for a period of about 1-2 days to obtain a cell population comprising ^{EMH}lin-KDR+APLNR+PDGFRalpha + primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($^{EMH}lin^{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) enriched in cells with a potential to form hematoendothelial clusters when cultured on OP9 cells; (iii) culturing hematovascular mesoderm cells ($^{EMH}lin^{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) cells, under hypoxic conditions, in a cell culture medium comprising FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to achieve formation of CD144+CD73+CD235a/CD43- non-hemogenic endothelial progenitors (non-HEP), CD144+CD73-CD235a/CD43- hemogenic endothelial progenitors (HEPs), CD144+CD73-CD235a/CD43+41a- angiogenic hematopoietic progenitors (AHP), and CD43+CD41a+ hematopoietic progenitor cells; (iv) culturing, under normoxia, hemogenic endothelial progenitors (HEPs) and CD43+CD41a+ hematopoietic progenitor cells in a culture medium comprising FGF2, VEGF, IL6, SCF, TPO, IL3 for about three days to obtain an expanded population of CD43+ hematopoietic progenitors comprising CD43+CD235a+CD41a+ erythromegakaryocytic progenitors and lin-CD34+CD43+CD45+/- multipotent hematopoietic progenitors; and (v) coculturing CD34+CD43+ hematopoietic progenitors for a period of about three weeks on OP9 cells overexpressing DLL4 to obtain a cell population comprising CD4+CD8+ T cells.

[0028] In another aspect provided herein is a cell culture medium suitable for hematoendothelial differentiation of human pluripotent stem cells, comprising a base medium, L-ascorbic acid 2-phosphate Mg2+ salt, monothioglycerol, additional sodium selenite, polyvinyl

alcohol, Glutamax™, non-essential amino acids (NEAA), chemically defined lipid concentrate, Holo-Transferrin, and insulin.

[0029] In some embodiments the cell culture medium further comprises BMP4, Activin A, FGF2, and LiCl.

[0030] In other embodiments the culture medium further comprises FGF2 and VEGF.

[0031] In other embodiments the culture medium further comprises FGF2, VEGF, SCF, TPO, IL-6, and IL-3.

[0032] In some embodiments the cell culture medium comprises an IF9S medium. In some embodiments the IF9S cell culture medium has the IF9S cell culture medium formulation of Table 2.

[0033] In a related aspect provided herein is a 9S concentrated medium supplement, wherein dilution of the 9S concentrated medium supplement in an IMDM/F12 base medium yields an IF9S cell culture medium. In one embodiment is a kit comprising the 9S concentrated medium supplement, and one or more of BMP4, Activin A, FGF2, LiCl, SCF, TPO, IL-6, IL-3, and Tenascin C.

[0034] In a further aspect provided herein is a defined cell culture system for hematoendothelial differentiation of human pluripotent stem cells, comprising an IF9S cell culture medium and a Tenascin C substrate for adherent growth of human pluripotent stem cells or their differentiated progeny along the hematoendothelial lineage. In some embodiments the IF9S cell culture medium is maintained under hypoxic conditions. In some embodiments the defined cell culture system further comprises human pluripotent stem cells grown on the Tenascin C substrate.

INCORPORATION BY REFERENCE

[0035] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, and patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] **FIG. 1** shows a schematic diagram of hematopoietic differentiation showing hematopoietic development pathways, specific markers and functional assays used to identify each stage of development, and conditions used for hPSC differentiation in chemically defined medium. Main cell subsets observed in prior differentiation studies using coculture with OP9 feeders and current chemically defined cultures are shown.

[0037] **FIG. 2 Identification of a unique molecular signature of overgrown OP9 stromal cells** (**a**) Venn diagram showing the overlap between differentially expressed genes in overgrown OP9 day 8 versus freshly confluent OP9 day 4, and MS5 and S17. 21 genes marked with gray background are uniquely overexpressed in day 8 OP9 cells as compare to all other tested cell lines. (**b**) Heat map of differentially expressed overlapping genes as shown in (**a**). Tenascin-C (Tnc) is one of the top differentially overexpressed genes in over-confluent OP9 cells.

[0038] **FIG. 3 Mesodermal development in hESC cultures differentiated on ColIV vs TenC for 2, 3, and 4 days in chemically defined conditions** (**a**) Flow cytometry plots and graphs comparing percentage of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ (A+P+) primitive mesodermal population on days 2 and 3. (**b**) Flow cytometry plots and graphs comparing

percentage of $KDR^{hi}CD31^{-}$ (HVMP), $CD31^{+}$, and $KDR^{lo}CD31^{-}$ populations on day 4. (**c**) Comparison of MB/HB-colony forming potential of day 2, day 3, and day 4 cultures. (**d**) Hematopoietic and endothelial potentials of $KDR^{hi}CD31^{-}$ and $KDR^{lo}CD31^{-}$ cells isolated from day 4 cells differentiated in chemically defined conditions after coculture with OP9 for 7 days. Upper panels show flow cytometry of TRA-1-85+ gated human cells and lower panels shows immunofluorescence staining of cells from OP9 cocultures with $KDR^{hi}CD31^{-}$ and $KDR^{lo}CD31^{-}$ cells. (a), (b), and (c) bars are mean + SE from 3 experiments (*$p<0.01$).

[0039] FIG. 4 Development of endothelial progenitors in cultures differentiated on ColIV or TenC for 5 days in chemically defined conditions (**a**) Flow cytometric analysis demonstrates major subsets of VE-cadherin+ (VEC; CD144+) progenitors generated after 5 days of hESC culture in chemically defined conditions on ColIV and TenC. (**b**) Percentages of VEC+ (CD144+) cells and subsets generated in ColIV and TenC cultures. Error bars are mean + SE from 3 experiments (*$p<0.01$). (**c**) CFC potential of isolated VEC+ (CD144+) subset in serum-free clonogenic medium with FGF2 and hematopoietic cytokines. (**d**) Endothelial and hematopoietic potential of day 5 VEC+ (CD144+) subsets. Progenitor subsets sorted and cultured in either endothelial conditions with subsequent tube formation assay, or on OP9 with immunofluorescent and flow cytometry results after 7 days. Scale bars, 100 μm.

[0040] FIG. 5 Development of hematopoietic progenitors in cultures differentiated on ColIV for 8 days in chemically defined conditions (**a**) Flow cytometric analysis shows major subsets of CD43+ cells generated in cultures on ColIV and TenC. (**b**) Cultures on TenC produce significantly more CD43+ cells across 3 experiments (*$p<0.01$). (**c**) Hematopoietic-CFC potential in serum-containing media is limited to the CD43+ subpopulations. (**d**) Cultures

differentiated on TenC have higher CFC potential than cultures differentiated on ColIV, statistically significant across 3 experiments (*p<0.01).

[0041] FIG. 6 T cell potential of CD43+ cells collected from H1 hESCs differentiated for 9 days in chemically defined conditions on ColIV and TenC (**a**) Flow cytometric analysis of cells collected from ColIV and TenC conditions after culture on OP9DLL4 for 3 weeks. (**b**) Analysis for T cell receptor rearrangement by genomic PCR. H1 T-Cell is the T-cells derived from differentiating H1; PB control is Peripheral Blood positive control; H1 hESC is undifferentiated H1 hESCs.

[0042] FIG. 7 The effect TGFβ inhibitor on hematopoietic development from H1 hESCs in chemically defined conditions Representative dot plots collected from flow cytometry of day 3, 4, 5, and 8 of differentiation after adding the TGFβ inhibitor, SB-431542 from day 2 to day 4 only. On day 3, SB-431542 decreases PDGFRalpha expression, but increases endothelial progenitors by days 4 and 5. By day 8, there is a significant increase in CD43+ hematopoietic progenitors.

[0043] FIG. 8 Generation of $KDR^{hi}CD31^{+}$ hematoendothelial progenitors in cultures using different basal media and matrix protein TeSR1 base medium is TeSR1 without cytokines. DF4S is DMEM/F12-based media supplemented with 4 supplements; 64 mg/L L-ascorbic Acid 2-Phosphate Mg^{2+} salt, 8.4 μg/L sodium selenite, 10.6 mg/L Holo-Transferrin, and 20 mg/L Insulin. I4S is DF4S with IMDM-based media instead of DMEM/F12-based media, but with the four previously mentioned supplements. IF4S is DF4S with IMDM/F12-based media instead of DMEM/F12-based media, but with the 4 previously mentioned supplements. VTN is vitronectin matrix; MTG is Matrigel® substrate; ColIV is Collagen IV matrix. Flow cytometry plots show percent of $KDR^{hi}CD31^{+}$ endothelial precursors of day 4 cells

differentiated in each media supplemented with 50 ng/ml FGF2, BMP4 and VEGF in hypoxic conditions

[0044] FIG. 9 Hematopoietic differentiation of iPSCs and H9 hESCs in chemically defined conditions Top panel represents the number of cells generated in cultures starting from day -1 when cells are plated on either TenC or ColIV, up to day 9 of differentiation. The numbers of $CD31^+$ and $CD43^+$ cells were calculated based on total number of cells times the percentage of positive cells based on flow cytometry. The bottom panel displays dot plots of the percentage of $CD43^+$ cells and their subsets of 19-9-7T human fibroblast iPSC line, BM19-9 human bone marrow-derived iPSC line, and H9 human ESC line differentiated for eight days on either ColIV or TenC.

[0045] FIG. 10 is a diagram of a hematopoietic differentiation timeline comparing the efficiency of differentiation on Collagen IV vs. Tenascin C.

DETAILED DESCRIPTION

[0046] Human pluripotent stem cell (hPSC) technologies provide the opportunity to study human development in vitro and develop patient-specific blood cells without relying on HLA-matched donors. Previously, an efficient protocol for the differentiation of hematopoietic stem/progenitor cells using a coculture method on the mouse stromal cell line, OP9, was developed (Vodyanik, *et al.*, 2005; Vodyanik, *et al.*, 2006). This system reproduces primitive and definitive waves of hematopoiesis and can be used to obtain $lin^-CD34^+CD38^-CD45RA^-CD90^+CD117^+CD43^+CD45^{-/+}$ multipotent definitive hematopoietic progenitors with HSC phenotype and lymphoid cells, including T and B cells (Carpenter, *et al.*, 2011; Kutlesa, *et al.*, 2009; Schmitt and Zuniga-Pflucker, 2002; Vodyanik, *et al.*, 2005).

[0047] Human pluripotent stem cell coculture with OP9 cells induces mesendodermal and hemogenic endothelial differentiation. Upon plating hPSCs onto OP9 cells, the hPSCs begin to express the mesodermal marker apelin receptor (APLNR), VEGFR2 (KDR), and PDGFRalpha and acquire mesenchymoangioblast (MB) and hemangioblast (HB) potential (days 2-3 of differentiation). With advanced maturation, $KDR^{+}APLNR^{+}$ mesodermal cells upregulate KDR expression and downregulate PDGFRα, which is enriched in cells with the potential to form hematoendothelial clusters when cultured on OP9 cells.

[0048] At day 2-3.5 of differentiation, $KDR^{+}APLNR^{+}$ cells lack the typical Endothelial (CD31, VE-cadherin (CD144)), endothelial/Mesenchymal (CD73, CD105) and Hematopoietic (CD43, CD45) markers, *i.e.* have an $^{EMH}lin^{-}$ phenotype. $^{EMH}lin^{-}$ cells lack endothelial (CD31 and CD144), mesenchymal/endothelial (CD73, CD105), and hematopoietic (CD43 and CD45) markers, whereas lin^{-} cells lack markers of differentiated hematopoietic cells including CD2, CD3, CD4, CD8, CD11b, CD11c, CD14, CD15, CD16, CD19, and CD20. By day 4, VE-$Cadherin^{+}$ (CD144) cells emerged. The emerging VE-$cadherin^{+}$ cells represent a heterogeneous population, which includes $CD144^{+}CD235a/43^{-}CD73^{+}$ non-hemogenic endothelial progenitors (non-HEPs), $CD144^{+}CD73^{-}CD235a/43^{-}$ hemogenic endothelial progenitors (HEPs) and $CD144^{+}73^{-}CD43^{+}CD235a^{+}CD41a^{-}$, angiogenic hematopoietic progenitors. HEPs have the potential to give rise to multipotent $lin^{-}CD34^{+}CD43^{+}CD45^{+/-}$ hematopoietic progenitors.

[0049] Unfortunately, the OP9 system relies on mouse feeder cells and serum, which limit its utility for studying hPSC response to specific growth factors and manufacturing clinical grade therapeutic blood cells. In addition, the OP9 coculture system is very sensitive to variations in serum quality, stromal cell maintenance, and size of PSC colonies used for differentiation.

[0050] Although other investigators have developed feeder-free differentiation protocols, these protocols rely on forming embryoid bodies (EBs) for hematopoietic differentiation. EB methods often rely on serum and also have significant drawbacks, such as asynchronous differentiation and high variability. Recently several protocols have been developed to induce hematopoiesis in serum-free conditions (Salvagiotto, *et al.*, 2011; Wang, *et al.*, 2012); however, they still require xenogenic components, serum albumin, and/or proprietary supplements. It also remains unclear whether these protocols reproduce the distinct waves of hematopoiesis as seen on OP9.

[0051] In addition, most protocols differentiate hPSCs grown on MEFs or Matrigel®. Since a new, completely chemically-defined xenogen-free medium and matrix for hPSC-derivation and maintenance has been described (Chen, *et al.*, 2011), there is a need to develop a similar chemically-defined xenogen-free directed differentiation protocol for deriving hematopoietic progenitors from hPSCs.

[0052] Unless defined otherwise in this specification, 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 and by reference to published texts.

[0053] It is to be noted that the term “a” or “an,” refers to one or more, for example, “a molecule,” is understood to represent one or more molecules. As such, the terms “a” (or “an”), “one or more,” and “at least one” are used interchangeably herein. The term “about” as used herein contemplates a range of values for a given number of +/- 10% the magnitude of that number. For example, "about 3 grams" indicates a value of 2.7 to 3.3 grams, and the like.

[0054] As referred to herein, the terms "defined conditions" or "defined medium" mean the identity and quantity of each ingredient is known. The term "ingredient," as used herein, refers to a component the molecular identity and quantity of which is known.

[0055] Disclosed herein are efficient and reproducible methods and supporting compositions that recapitulate, in a completely defined, xenogen-free system, the hematopoietic development observed in the OP9 co-culture system through early mesoderm, hematovascular mesoderm precursor, and hemogenic endothelial stages.

[0056] Table 1 provides a list of cell types, associated cell marker phenotypes, and corresponding abbreviations used herein.

Table 1 Phenotypic features and definition of subsets with angiogenic and hematopoietic potential from hPSCs presented in the current application

Abbreviation	Phenotype	Designation/Definition
PM A^+P^+	$^{EHM}lin^-APLNR^+PDGFRalpha$ GFR	Primitive posterior mesoderm (PM) enriched in cells expressing typical primitive streak and lateral plate/extraembryonic mesoderm genes. These cells have potential to form mesenchymoangioblast (MB) and hemangioblast (HB) colonies in serum-free medium in response to FGF2.
HVMP	$^{EHM}lin^-$ $APLNR^+KDR^{bright}PDGFRalpha^{low/-}$	Hematovascular mesodermal precursor lacking the expression of primitive streak genes and highly enriched in bipotential hematoendothelial cluster forming cells.
HEP	$CD144^+CD235a/CD43^-CD73^-$	Hemogenic endothelial progenitors that have primary endothelial characteristics, lacking hematopoietic CFC potential and surface markers, but are capable of generating blood and endothelial cells upon coculture with stromal cells.
Non-HEP	$CD144^+CD235a/CD43^-CD73^+$	Non-hemogenic endothelial progenitors that have all functional and molecular features of endothelial cells and form endothelial colonies on OP9.
AHP	$CD144^+CD235a/CD43^+CD73^-$ $CD41a^-$	Angiogenic blood progenitors that possess primary hematopoietic characteristics and FGF2 and hematopoietic cytokine-dependent colony-forming potential but are capable of generating endothelial cells.
	$CD43^+CD235a^+CD41a^+$	Hematopoietic progenitors enriched in erythromegakaryocytic progenitors.
	lin-CD34+CD43+CD45+/-	Multipotential hematopoietic progenitors with myelolymphoid potential

Methods for Hematoendothelial Differentiation of Human Pluripotent Stem Cells (hPSCs)

[0057] Disclosed herein are methods for the differentiation of human pluripotent stem cells (either human embryonic or human induced pluripotent stem cells) under defined conditions, and, preferably, in the absence of embryoid body formation. At least one desired outcome of this differentiation is the provision of endothelial and hematopoietic cell populations that may be a source for functional studies of these lineages as well as a source for clinical therapies.

[0058] In some embodiments the differentiation method provided herein includes the steps of (a) providing human pluripotent stem cells (*e.g.*, human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs)) and (b) culturing the human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential. Preferably, the human pluripotent stem cells are cultured without formation of embryoid bodies.

[0059] In some embodiments, the human pluripotent stem cells are plated at an initial density of about 5000 cells/cm^2 to about 15,000 cells/cm^2, *e.g.*, 6000 cells/cm^2, 7000 cells/cm^2, 8000 cells/cm^2, 9000 cells/cm^2, or another plating density from about 5000 cells/cm^2 to about 15,000 cells/cm^2, In some embodiments, the differentiation method further includes the step of (c) culturing, under hypoxic conditions, the cell population obtained in step (b) in a cell culture medium comprising FGF2 and VEGF for a period of about 1-2 days to obtain a cell population comprising $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($^{EMH}lin\text{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) enriched in cells with a potential to form hematoendothelial clusters when cultured on OP9 cells.

In further embodiments, the differentiation method further includes the step of (d) culturing the hematovascular mesoderm cells of step (c), under hypoxic conditions, in a cell culture medium comprising FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to achieve formation of $CD144^{+}CD73^{+}CD235a/CD43^{-}$ non-hemogenic endothelial progenitors (non-HEP), $CD144^{+}CD73^{-}CD235a/CD43^{-}$ hemogenic endothelial progenitors (HEPs), $CD144^{+}CD73^{-}CD235a/CD43^{+}41a^{-}$ angiogenic hematopoietic progenitors (AHP), and $CD43^{+}CD41a^{+}$ hematopoietic progenitor cells.

[0060] In some embodiments, the differentiation method further includes the step of (e) culturing, under normoxia, the HEPs and hematopoietic progenitor cells in a culture medium comprising FGF2, VEGF, IL6, SCF, TPO, IL3 for about three days to obtain an expanded population of $CD43^{+}$ hematopoietic progenitors comprising $CD43^{+}CD235a+CD41a^{+}$ erythromegakaryocytic progenitors and $lin^{-}CD34^{+}CD43^{+}CD45^{+/-}$ multipotent hematopoietic progenitors.

[0061] In some embodiments a differentiation method disclosed herein includes the step of at least one of: (i) culturing human pluripotent stem cells, without embryoid body formation, under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential; (ii) culturing, under hypoxic conditions, $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential in a cell culture medium comprising FGF2 and VEGF for a period of about 1-2 days to obtain a cell population comprising $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($EMH^{lin-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) enriched in cells with a

potential to form hematoendothelial clusters when cultured on OP9 cells; (iii) culturing hematovascular mesoderm cells (^{EMH}lin-KDR^{hi}APLNR^{+}PDGFRalpha$^{lo/-}$) cells, under hypoxic conditions, in a cell culture medium comprising FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to achieve formation of CD144^{+}CD73^{+}CD235a/CD43^{-} non-hemogenic endothelial progenitors (non-HEP), CD144^{+}CD73^{-}CD235a/CD43^{-} hemogenic endothelial progenitors (HEPs), CD144^{+}CD73^{-}CD235a/CD43^{+}41a^{-} angiogenic hematopoietic progenitors (AHP), and CD43^{+}CD41a^{+} hematopoietic progenitor cells; and (iv) culturing, under normoxia, hemogenic endothelial progenitors (HEPs) and and CD43^{+}CD41a^{+} hematopoietic progenitor cells in a culture medium comprising FGF2, VEGF, IL6, SCF, TPO, IL3 for about three days to obtain an expanded population of CD43^{+} hematopoietic progenitors comprising CD43^{+}CD235a+CD41a^{+} erythromegakaryocytic progenitors and lin^{-}CD34^{+}CD43^{+}CD45$^{+/-}$ multipotent hematopoietic progenitors.

[0062] In some embodiments, hypoxic conditionshypoxic conditions refer to a level of environmental oxygen (*e.g.*, a cell culture incubator gas mixture) of about 3% O_2 to about 10% O_2. In some embodiments, hypoxic conditions is about 5% O_2. In embodiments where a cell culture medium is allowed to equilibrate under hypoxic conditions, the cell cultue medium becomes a hypoxic cell culture medium due to the lower level of dissolved oxygen compared to a cell culture medium equilibrated under normoxic conditions (*e.g.*, a gas mixture containing about 20% oxygen)..

[0063] In some embodiments, the culture medium to be used in any of the above-described differentiation methods comprises an IF9S medium, as described herein. In one embodiment, the IF9S medium to be used is the IF9S medium having the formulation set forth in Table 2.

[0064] In some embodiments, any of the above-referenced cells (*e.g.*, human pluripotent stem cells) are cultured on Tenascin C. In some embodiments, any of the referenced cells are seeded on a substrate treated with an amount of Tenascin-C sufficient to adhere 10,000 cells/cm^2 to the substrate. In some embodiments, the Tenascin-C to be used is human Tenascin C. In some embodiments, the substrated is treated with Tenascin C at a concentration of at least about 0.25 $\mu g/cm^2$ to 1 $\mu g/cm^2$, *e.g.*, 0.4 $\mu g/cm^2$, 0.5 $\mu g/cm^2$, 0.7 $\mu g/cm^2$, 0.8 $\mu g/cm^2$, or another concentration from at least about 0.25 $\mu g/cm^2$ to 1 $\mu g/cm^2$

[0065] In some embodiments, in the cell culture medium to be used in the above-described differentiation methods, the concentration of: BMP4 is about 50 ng/ml to about 250 mg/ml; Activin A is about 10 ng/ml to about 15 ng/ml; FGF2 is about 10 ng/ml to about 50 ng/ml; LiCl is about 1 mM to about 2 mM; VEGF is about 20 ng/ml to about 50 ng/ml; SCF is about 50 ng/ml to about 100 ng/ml; TPO is about 50 ng/ml to about 100 ng/ml; IL-6 is about 50 ng/ml to about 100 ng/ml, and IL-3 is about 5 ng/ml to about 15 ng/ml.

[0066] In some embodiments, any of the above-referenced cells are cultured in a xeno-free cell culture medium. Of central importance for clinical therapies is the absence of xenogenic materials in the derived cell populations, *i.e.*, no non-human cells, cell fragments, sera, proteins, and the like. Preferably, the present invention arrives at xenogen-free differentiated cells by use of Tenascin C or Collagen IV as a platform, which essentially replaces contact with OP9 cells used in earlier differentiation systems. In addition, the media disclosed herein are chemically-defined and, in some embodiments, are made xeno-free, and incorporate human proteins, which can be produced using recombinant technology or derived from placenta or other human tissues in lieu of animal-derived proteins. In some embodiments, all proteins added to the medium are recombinant proteins.

[0067] While differentiation processes include ordered, sequential events, the timing of the events may be varied by at least 20%. For example, while a particular step may be disclosed in one embodiment as lasting one day, the event may last for more or less than one day. For example, "one day" may include a period of about 18 to about 30 hours. Periods of time indicated that are multiple day periods may be multiples of "one day," such as, for example, two days may span a period of about 36 to about 60 hours, and the like. In another embodiment, time variation may be lessened, for example, where day 2 is 48 +/- 3 hours from d0; day 4 is 96 +/- 3 hours from d0, and day 5 is 120 hours +/- 3 hours from d0.

[0068] Examples of potential committed and/or differentiated lineages obtainable by the present invention include $KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with HB and MB CFC potential, $^{EMH}lin^{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$ hematovascular mesoderm cells with potential to form hematoendothelial clusters when cultured on OP9 cells, VE-Cadherin^{+} ($CD144^{+}$) subset cells, such as HEPs ($CD144^{+}CD73^{-}CD235a/43^{-}$, non-HEPS ($CD144^{+}CD73^{+}CD235a/43^{-}$, and AHPs ($CD144^{+}CD73^{-}CD235a/43^{+}41a^{-}$), $CD43^{+}$ hematopoietic progenitor cells such as $CD43^{+}CD235a^{+}41a^{+}$ erythromegakaryocytic hematopoietic progenitors, and $lin^{-}CD34^{+}CD43^{+}CD45^{+/-}$ multipotent hematopoietic progenitors. The term lineage^{-} ("lin^{-}), as used herein, refers to a hematopoietic precursor or progenitor cell that has not have committed to any of its derivative blood cell lineages as of yet, since it retains the capability to differentiate into any of them. This characteristic is monitored by looking for the absence of cell surface markers indicative of differentiation into any of the derivative lineages. A further significant advantage of the present disclosure is the ability to use clonal cell populations due to the Tenascin C platform, which removes reliance on undefined stochastic events common to cell clumps, such as embryoid bodies, to generate differentiated populations. Moreover, the clonal

cell populations exhibit greater uniformity during the differentiation process, which provides a higher yield of synchronized cells than previously seen in feeder cell systems. Therefore, the present disclosure also describes a more efficient, better scalable differentiation system than previously available.

Compositions

Defined Cell Culture Media and Concentrated Media Supplements

[0069] Some embodiments herein disclose a differentiation medium comprising a base medium, L-ascorbic acid 2-phosphate Mg^{2+} salt, monothioglycerol, sodium selenite (in addition to any present in the base medium), polyvinyl alcohol, Glutamax™, non-essential amino acids (NEAA), chemically defined lipid concentrate, Holo-Transferrin, and insulin. Suitable base media for the differentiation media described herein include, but are not limited to, Iscoves Modified Dulbecco's Medium/F12 (IMDM/F12), TeSR1 base medium, which is mTeSR1™ base medium, (Stem Cell Technologies-see Ludwig and Thomson (2007), *Curr Protoc Stem Cell Biol.*, Chapter 1:Unit 1C.2 and U.S. Patent No. 7,449,334) without FGF2 and TGF-beta; DF4S base medium, which is Essential 8™ medium (Life Technologies; also known as "E8" medium-see Chen and Thomson (2011), *Nat Methods*, 8(5):424-429 and U.S. Patent Application Publication No. 20120178166) without FGF2 and TGF-beta, I4S base medium, which is DF4S base with Iscove's modified Dulbecco's medium (IMDM) instead of DMEM/F12, and IF4S base is DF4S base with IMDM/F12 instead of DMEM/F12. Preferably, the base medium to be used is albumin-free. IMDM/F12 is a highly enriched synthetic medium suited for rapidly proliferating, high-density cell cultures with an added nutrient mixture.

[0070] In some embodiments, differentiation media used herein, referred to generically herein as "IF9S" media, comprises IMDM/F12, L-ascorbic acid 2-phosphate Mg^{2+} salt, monothioglycerol, sodium selenite (in addition to any present in the base medium), polyvinyl

alcohol, Glutamax™, non-essential amino acids (NEAA), chemically defined lipid concentrate (Life Technologies; Cat. No. 1905031), Holo-Transferrin, and insulin.

[0071] In one embodiment, an IF9S medium comprises IMDM/F12 (1x), L-ascorbic acid 2-phosphate Mg^{2+} salt (64 mg/L), monothioglycerol (50 mg/L), sodium selenite (in addition to any present in the base medium; 8.4 ug/L), polyvinyl alcohol (10 mg/L), Glutamax™ (1x), NEAA (1x), chemically defined lipid concentrate (0.1x), Holo-Transferrin (10.6 mg/L), and insulin (20 mg/L).

[0072] As described herein, at various time points/stages of hematoendothelial differentiation of hPSCs, the complete differentiation medium to be used contains various combinations of cytokines, growth factors, and/or small molecules. Depending on the stage of hematoendothelial differentiation according to the methods described herein, a suitable complete differentiation medium will be supplemented with different combinations of cytokines with concentrations within the ranges described for the complete differentiation media described herein.

[0073] In some embodiments, complete differentiation medium comprises an IF9S medium, BMP4, Activin A, FGF2, and LiCl. In other embodiments complete differentiation medium comprises an IF9S medium, FGF2, and VEGF. In further embodiments, complete differentiation medium comprises an IF9S medium, FGF2, VEGF, SCF, TPO, IL-6, and IL-3. In some embodiments, the final complete medium concentration of: BMP4 is about 50 ng/ml to about 250 mg/ml; Activin A is about 10 ng/ml to about 15 ng/ml; FGF2 is about 10 ng/ml to about 50 ng/ml; LiCl is about 1 mM to about 2 mM; VEGF is about 20 ng/ml to about 50 ng/ml; SCF is about 50 ng/ml to about 100 ng/ml; TPO is about 50 ng/ml to about 100 ng/ml; IL-6 is about 50 ng/ml to about 100 ng/ml, and IL-3 is about 5 ng/ml to about 15 ng/ml. In some embodiments all of the proteins used in the complete differentiation medium are recombinant

human proteins. In other embodiments, the complete differentiation medium comprises one or more non-human proteins (*e.g.*, recombinant non-human proteins).

[0074] In some embodiments, a complete differentiation medium comprises an IF9S medium and one of the "cytokine" combinations listed in Table 3 at the indicated concentrations. In some embodiments, the IF9S medium formulation used in the just-mentioned complete differentiation media is the IF9S medium formulation set forth in Table 2.

[0075] While the presently disclosed media may include the specific morphogens, small molecules, and hematopoietic cytokines disclosed herein, it is contemplated that additional components with the same, equivalent, or similar properties may be used in addition to or in place of those disclosed, as are known in the art.

[0076] In some embodiments, media disclosed herein may include xenogenic (*i.e.*, non-human, biologically derived) materials. For example, a xenogenic material may be a recombinant protein of xenogenic origin. Media disclosed herein may be also made in concentrated forms that are diluted prior to use, such as 2X, 10X, 100X, or 1000X concentrations.

[0077] Moreover, the replacement of certain xenogenic materials in the media of the present invention provided greater, unexpected benefits than just providing xenogen-free culture conditions. For example, it is believed that replacement of bovine serum albumin with polyvinyl alcohol led to a “thicker” medium that unexpectedly contributed to cell survival.

Table 2. Description of an exemplary embodiment of an IF9S medium.

IF9S Components	Concentration
IMDM/F12	Base Component
L-ascorbic Acid 2-Phosphate Mg^{2+} salt	64mg/L
monothiogiycerol	40ul/L
additional sodium selenite	8.4ug/L
polyvinyl alcohol	10mg/L
GLUTAMAX	1x
Non-essential amino acids	1x
Chemically defined lipid concentrate	0.1x
Holo-Transferrin	10.6mg/L
Insulin	20mg/L

Table 3. Overview of CytokineSupplement Combinations and Exemplary Concentrations at Different Days After Initiating Hematoendothelial Differentiation of hPSCs (in an IF9S medium).

Day (hours±range) (activity)	**Cytokine**	**Concentration**	**Range**	**O_2 Level**
d0 (0h) (change media)	BMP4 Activin A FGF2 LiCl	50ng/ml 12.5ng/ml 50ng/ml 2mM	50-250ng/ml 10-15ng/ml 10-50ng/ml 1-2mM	Hypoxia ($5\%O_2$, $5\%CO_2$)
d2 (48±3h) (change media)	FGF2 VEGF	50ng/ml 50ng/ml	10-50ng/ml 20-50ng/ml	Hypoxia
d4 (96±3h) (change media) d5 (120±3h) (move to normoxic incubator)	FGF2 VEGF SCF TPO IL-6 IL-3	50ng/ml 50ng/ml 50ng/ml 50ng/ml 50ng/ml 10ng/ml	10-50ng/ml 20-50ng/ml 50-100ng/ml 50-100ng/ml 50-100ng/ml 5-15ng/ml	Hypoxia (d4) Normoxia (d5) ($20\%O_2$, $5\%CO_2$)
d6 (144±3h) (add media)	FGF2 VEGF SCF TPO IL-6 IL-3	50ng/ml 50ng/ml 50ng/ml 50ng/ml 50ng/ml 10ng/ml	10-50ng/ml 20-50ng/ml 50-100ng/ml 50-100ng/ml 50-100ng/ml 5-15ng/ml	Normoxia

Concentrated Medium Supplements

[0078] Also disclosed herein is a concentrated "9S" medium supplement, comprising L-ascorbic acid 2-phosphate Mg^{2+} salt, monothioglycerol, additional sodium selenite, polyvinyl alcohol, Glutamax™ (or glutamine), non-essential amino acids (NEAA), chemically defined lipid concentrate, Holo-Transferrin, and insulin. In some embodiments, the concentrated 9S medium supplement comprises each component at a concentration 10x to 1000x of the final working concentration once diluted in a base medium. In some embodiments, the concentrations of all of the 9S components in the concentrated supplement is 10x to 1000x the concentrations listed in

Table 3. In some embodiments, the supplement is to be diluted in IMDM/F12 medium to obtain IF9S medium as described herein.

Kits

[0079] Also contemplated herein are kits useful for hematoendothelial differentiation of hPSCs. In some embodiments, a kit comprises a 9S concentrated medium supplement, as described herein, one or more of BMP4, Activin A, FGF2, LiCl, SCF, TPO, IL-6, and IL-3, and instructions for generating an IF9S medium and a method for hematoendothelial differentiation of hPSCs as described herein. In some embodiments, a kit further includes IMDM/F12 medium. In some embodiments, the kit comprises a 9S concentrated medium supplement, Activin A, FGF2, LiCl, SCF, TPO, IL-6, IL-3, and instructions for generating an IF9S medium and a method for hematoendothelial differentiation of hPSCs as described herein. In further embodiments, any of the above-mentioned kits also include Tenascin C (*e.g.*, human Tenascin C), which is used as a substrate for adhesive growth according the differentiation methods described herin.

Defined Cell Culture Systems for Hematoendothelial Differentiation of hPSCs

[0080] Also described herein is a defined cell culture system for hematoendothelial differentiation of hPSCs. Such cell culture systems include a defined differentiation culture medium as described herein, *e.g.*, an IF9S medium, a Tenascin C protein substrate for adherent growth of hPSCs or their differentiated progeny along the hematoendothelial lineage. In some embodiments, Tenascin C is used at at least 0.25 $\mu g/cm^2$ to about 1 $\mu g/cm^2$ to generate a suitable adhesive substrate.

[0081] In some embodiments, the cell culture system includes an IF9S medium supplemented with BMP4, Activin A, FGF2, and LiCl. In some embodiments, the IF9S medium is

supplemented with FGF2 and VEGF. In some embodiments, the IF9S medium utilized in the cell culture system is supplemented with FGF2, VEGF, SCF, TPO, IL6, and IL-3. In some embodiments, the IF9S medium used in the cell culture system is formulated according to the medium described in Table 2. In some embodiments, the defined cell culture system comprises a cell culture medium that is hypoxic, which is readily achieved by the use of a cell culture incubator permitting oxygen level regulation, and by equilibrating the cell culture medium in a cell culture incubator set to about 3% O_2 to about 10% O_2 (*e.g.*, 5% O_2).

[0082] In further embodiments, the defined cell culture system further includes adherent human pluripotent stem cells cultured on the Tenascin C substrate in the IF9S medium according to the methods described herein.

[0083] Cells can be grown on, *e.g.*, Tenascin C-coated cell culture dishes, multi-well cell culture plates, or microcarrier beads. Preferably, the Tenascin C protein is human Tenascin C (GenBank Accession No. CAA55309.1; available commercially, *e.g.*, Millipore Cat. No. CC065)

[0084] The use of Tenascin C and hypoxic conditions enables the generation of enriched populations of endothelial and hematopoietic cells at higher percentages than compared to cells seeded on Collagen IV or OP9 cells, such as greater than 10%, or greater than about 20%, greater than about 50%, or greater than about 60% when compared per stage per platform (see **Fig. 10** and the Examples). In one embodiment, the percentages of target populations obtainable by the present invention may be greater than about 35% for $KDR^+APLNR^+PDGFRalpha^+$ mesoderm cells or greater than about 20% of VE-$Cadherin^+CD43^-$ endothelial cells and greater than about 40% of $CD34^+CD43^+$ hematopoietic progenitor cells. Further, with respect to **Fig. 10**, the percentages of cells obtained on the Tenascin C platform are the indicated percentage or greater. Further, **Fig. 10** represents the percentage of the target population (*e.g.*, mesoderm progenitor,

endothelial progenitor, hematopoietic progenitors with the corresponding phenotypes as according to flow cytometry) of the total culture when differentiated on either Col IV or TenC.

[0085] The present methods and materials may be combined into cell culture systems to provide new differentiation platforms. In one embodiment, a basic cell culture system includes pluripotent stem cells seeded on Tenascin C. In another embodiment, a cell culture system includes stem cells seeded on Collagen IV, in a medium supplemented with Activin A. These systems have the capacity to produce cell populations enriched with hematopoietic progenitor cells.

[0086] The cell culture systems contemplated herein may be modular, in that they may incorporate additional components that alter the resulting cell populations derived from the system. For example, the cell culture system may incorporate media that are xenogen-free for certain desired outcomes. However, they may include xenogen-containing media if, for example, clinical therapies are not envisioned for the derived cell populations. Further, the cell culture systems may be based on various sized culture vessels, as are known in the art, to arrive at the desired cell population production scale.

[0087] In some cases, one can substitute some of the components of an IF9S medium. For example, ascorbic acid and monothioglycerol can be replaced with an optional supplement of a compound and/or a thiol-containing compound with antioxidant properties. GLUTAMAX™ can be replaced with an optional supplement of L-glutamine. "Non-essential amino acids (NEAA)," which is a general term for amino acids that the human body can produce from other amino acids can be replaced with an optional supplement of amino acids. "Chemically defined lipid concentrate," which is a solution specifically distributed by Life Technologies, can be replaced with an optional supplement of lipids. Additional selenite, insulin, and holo-transferrin can be

replaced with any ITS supplement. ITS stands for "Insulin-Transferrin-Selenite." Some companies (*e.g.*, Life Technologies), sells ITS solutions to be used as supplements in other basal media (DMEM, for example). However, the manufacturer does not provide concentrations for each component. Polyvinyl alcohol can be replaced with an optional supplement of a biologically inactive media thickening compound.

[0088] The following examples set forth preferred materials and methods for accomplishment of the invention. It is to be understood, however, that these examples are provided by way of illustration and nothing herein should be taken as a limitation upon the overall scope of the invention.

EXAMPLES

[0089] Example 1 IMDM/F12 based media significantly improves differentiation efficiency of hPSCs into hematoendothelial lineage

[0090] Previously, our lab developed protocol for the efficient differentiation of hematopoietic hPSC differentiation using a coculture method on the mouse stromal cell line, OP9.[9,13] Although OP9 system supports efficient generation of HE and multilineage hematopoietic progenitors (Fig. 1), this system is very sensitive to variations in serum quality, stromal cell maintenance, and size of hPSC colonies and clumps used for differentiation.[13,14] Forming embryoid bodies (EBs) is another commonly used approach for inducing HE and blood formation from hPSCs.[7,15,16] However, EB methods often rely on serum or non-defined medium and also have significant drawbacks, such as asynchronous differentiation, high variability and dependence on initial clump size. Additionally, inconsistency in quality of hPSCs due to variations in albumin batches used for hPSC maintenance may introduce variations in efficiency of blood production.

[0091] To overcome these limitations we decided to identify chemically defined medium and matrix proteins capable to support hematoendothelial differentiation without serum from single cell suspension of H1 human embryonic stem cells (hESCs) expanded in E8 completely defined xenogene-free medium on vitronectin (VTN)[17].

Methods

[0092] *Human Pluripotent Stem Cell Maintenance*

[0093] Human pluripotent stem cells (H1 and H9 hESCs, fibroblast derived iPSC 19-9-7T, and BM119-9 iPSCs derived from bone marrow mononuclear cells) were maintained on vitronectin or matrigel in E8 media made in-house supplemented with FGF2 and TGFβ (Peprotech). Cells were passaged when they reached 80% confluency using 0.5 mM EDTA in PBS. The cells were maintained in normoxic conditions with 5% CO_2.

[0094] *Human Pluripotent Stem Cell Differentiation*

[0095] Human pluripotent stem cells were detached from vitronectin or matrigel when they reached 80% confluency using 1x TrypLE (Life Technologies) and plated at an optimized density ranging from 5000 cells/cm^2 to 15,000 cells/cm^2 depending on the cell line onto 6-well plates coated with 0.5 μg/cm^2 of ColIV (Sigma-Aldrich) or 0.5 μg/cm^2 Tenascin-C (Millipore) in E8 media supplemented with 10 μM Rho Kinase inhibitor (Tocris Y-27632). After 24 hours (day 0), the media was changed to IF9S media supplemented with 50 ng/ml BMP4 (Peprotech), 15 ng/ml Activin A (Peprotech), 50 ng/ml FGF2 (Miltenyi Biotech), 2mM LiCl (Sigma), and on occasion, 1 μM Rho Kinase inhibitor to increase cell viability. On day 2, the media was changed to IF9S media supplemented with 50 ng/ml FGF2 and 50 ng/ml VEGF, and 10 μM SB-431542 (Tocris) where mentioned. On day 4, the media was changed to IF9S media supplemented with

50 ng/ml FGF2, VEGF, TPO, SCF, IL-6, and 10 ng/ml IL-3. On day 6, additional IF9S media supplemented with the same 6 factors were added to the cultures without aspirating the old media (Table 3Table 3). IF9S (IMDM/F12 with 9 supplements) was made in-house with the following: 50% IMDM 50% F12 (Life Technologies) supplemented with 64 mg/L L-asorbic Acid 2-Phosphate Mg2+ salt (Sigma-Aldrich), 40ul/L monothioglycerol (Sigma-Aldrich), 8.4 μg/L additional sodium selenite (Sigma-Aldrich), 10mg/L polyvinyl alcohol (Sigma-Alderich), 1x glutamax (Life Technologies), 1x non-essential amino acids (Life Technologies), 0.1x chemically defined lipid concentrate (Life Technologies), 10.6 mg/L Holo-Transferrin (Sigma-Aldrich), and 20 mg/L Insulin (Sigma-Aldrich) (Table 2). Differentiation was conducted in hypoxic condition from day 0 to day 5, and transferred to normoxic condition from day 6 to day 9 (Fig. 1). The 1x TrypLE was used to dissociate and collect cells for analysis.

[0096] *Mesenchymo- (MB) and Hemangioblast (HB) Assay*

[0097] MB and HB were detected using serum-free CFC medium supplemented with FGF2 assay as previously described[11]. Day 2 or 3 cultures were dissociated and prepared in a single-cell suspension using 1x TrypLE (Life Technologies) and 5,000 cells of the total culture were plated into the CFC media. MB and HB colonies were scored 12 days after plating the single-cell suspension.

[0098] *Hematopoietic CFC assay.*

[0099] Hematopoietic CFC were detected using serum-containing H4436 Methocult™ supplemented with human recombinant SCF, G-CSF, GM-CSF, IL-3, IL-6, and EPO (Stem Cell Technologies). Hematopoietic potential of AHPs was evaluated using serum-free SF H4236 methocult with added FGF2 (20 ng/ml), SCF (20ng/mL), IL3 (10 ng/mL), IL6 (10 ng/mL), and

EPO (2U/mL) (Stem Cell Technologies) as previously described[6]. 1000-10000 differentiated cells were plated into the CFC medium and the colonies were scored after 14 days of culture.

[00100] *Flow Cytometry and FACS*

[00101] Flow Cytometry was conducted using the using a FACSCalibur flow cytometer and following antibodies: CD31-FITC (clone WM59), CD34-FITC (8G12), CD41a-FITC/APC (clone HIP8), CD43-FITC/PE/APC (clone 1G10), CD45-APC (clone HI30), CD73-FITC/PE (clone AD2), CD144-FITC/PE/AlexaFluor647 (clone 55-7H1), CD235a-FITC/PE/APC (clone GA-R2), KDR-PE/AlexaFluor647 (clone 89106), PDGFRα-PE (clone aR1) (BD Biosciences), TRA-1-85-FITC/PE (clone TRA-1-85), and APLNR-APC (clone 72133) (R&D Systems). Sorting was conducted on a FACS Aria, as described previously 46. The purity of isolated populations was 92-95%.

[00102] *Secondary Culture of Differentiated hPSCs onto OP9*

[00103] OP9 cells were maintained in α-MEM (Gibco) supplemented with 20% FBS (Hyclone) as previously described.[10] Sorted day 4 or day 5 cultures were plated on a confluent layer of OP9 cells in α-MEM (Gibco) supplemented with 10% FBS (Hyclone) supplemented with 100 μM MTG, 50 μg/ml ascorbic acid, 50 ng/ml SCF, TPO, IL-6, and 10ng/ml IL-3 at a density of 5,000 cells/well of a 6 well plate as previously described[6]. Cultures were prepared for flow cytometry 4 to 7 days later by collecting floating cells and dissociating the entire cultures using 1x TrypLE.

[00104] *T-cell Differentiation of Day 9 cultures*

[00105] An OP9 cell line (OP9-DLL4) constitutively expressing human delta-like ligand 4 (DLL4) was established by our lab using lentivirus and was maintained similarly to OP9. After human pluripotent stem cells were differentiated for 9 days, the floating cells were collected, strained through a 70 μm cell strainer (BD Biosciences) and washed. Then, they were

resuspended in T-cell differentiation media consisting of α-MEM (Gibco) supplemented with 20% FBS (Hyclone) supplemented with IL7 (5 ng/ml), Flt3L (5 ng/ml) and SCF (10ng/ml). Then, they were plated on an OP9-DLL4 and cultured at 37 °C and 5% CO2. After 4 days, the cells were harvested using collagenase IV (Gibco) solution (1 mg/ml in DMEM/F12, Gibco) and 1x TrypLE (Invitrogen), and passaged onto a fresh layer of OP9-DLL4. After 3 days, the cells are passaged again. Subsequent passages are conducted every 7 days up to 4 weeks, after which the floating cells are collected for flow analysis and genomic DNA extraction for TCR rearrangement assay.

[00106] *TCR Rearrangement Assay*

[00107] Genomic DNA was isolated using quick-gDNA MiniPrep (Zymo Research). TCRβ and TCRγ clonality was detected using a PCR amplification kit (Invivoscribe) and AmpliTaq Gold® DNA polymerase (Applied Biosystems) as previously described[33]. The PCR products were analysed using heteroduplex analysis on a 6% polyacrylamide gel stained with ethidium bromide.

[00108] *Microarray analysis of mouse stromal cell lines*

[00109] A mouse bone marrow stromal cell line, OP9, was obtained from Dr. Toru Nakano (Research Institute for Microbial Diseases, Osaka University, Japan), S17 was obtained from Dr. Kenneth Dorshkind (University of California, Los Angeles) and MS-5 was obtained from the German Tissue Culture Collection. Stromal cell lines were cultured as described[9]. DNA-free RNA was isolated using RiboPure™ RNA and DNAse using TURBO™ DNAfree reagents (Ambion). All samples were processed at the Gene Expression Center of the Biotechnology Center at the University of Wisconsin, Madison and analyzed using A4543-00-01 MM8 60mer expr Mus musculus 1-Plex Array standard arrays manufactured by NimbleGen Systems

(Madison, WI). Gene expression raw data were extracted using NimbleScan software v2.1. Considering that the signal distribution of the RNA sample is distinct from that of the gDNA sample, the signal intensities from RNA channels in all eight arrays were normalized with a Robust Multiple-chip Analysis (RMA) algorithm[47]. Separately, the same normalization procedure was performed on those from the gDNA samples. For a given gene, the median-adjusted ratio between its normalized intensity from the RNA channel and that from the gDNA channel was then calculated as follows: Ratio = intensity from RNA channel/(intensity from gDNA channel + median intensity of all genes from the gDNA channel). Genes with more than 3 fold differences in expression were considered differentially expressed. Only genes with expression level > 1 were selected for analysis.

[00110] Results

[00111] *IMDM/F12 based medium significantly improves differentiation efficiency of hPSCs into the hematoendothelial lineage*

[00112] We plated hESCs as single cells and allowed to attach over 24 hours in E8 media supplemented with 10 μM Rho kinase inhibitor on Matrigel (MTG), VTN, or Collagen IV (ColIV). Then, the media was changed to one of three basal media free of animal proteins, or growth factor-free TeSR1, supplemented with human recombinant BMP4, FGF2, and VEGF factors which commonly used to induce blood formation from hPSCs[18,19]. After 4 days of differentiation cell cultures evaluated for the presence of $KDR^{hi}CD31^{+}$ cells which are highly enriched in hematoendothelial progenitors[6]. Flow cytometry analysis showed that cells differentiated on ColIV-coated plates in IMDM/F12 media supplemented with L-asorbic Acid 2-Phosphate Mg^{2+} salt, 8.4 μg/L additional sodium selenite, Holo-Transferrin, and insulin differentiated most efficiently into $KDR^{h}iCD31^{+}$ hematoendothelial precursors (**Fig. 8**). Later,

we found that the addition of polyvinylalcohol, NEAA, Glutamax, chemically defined lipid concentrate, and monothioglycerol increased cell viability and differentiation efficiency (data not shown). The subsequent basal media is referred to as IF9S (IMDM/F12 plus 9 supplements). These results demonstrated that the selected media and supplements made it possible to obtain hematoendothelial cells in a chemically defined, xenogene-free conditions on ColIV matrix from hPSCs maintained in E8 media.

Example 2 Analysis of a unique molecular signature of hematopoiesis-supportive stromal cells identified Tenascin C as an extracellular matrix that promotes the development and maintenance of hematopoietic precursors.

[00113] Previously, we showed that OP9 is superior to other stromal cell lines such as S17, and MS5 in induction of hematopoietic differentiation [9]. It was also found that day 8 overgrown OP9 cultures are superior to day 4 freshly confluent OP9 in induction of hematopoietic-CFCs, including multipotential GEMM-CFCs[9]. Since the confluency of the stromal cells affect differentiation efficiency, this led us to believe that there is an extracellular matrix influencing hematoendothelial differentiation. In order to find the matrix protein(s) critical for hematopoiesis-supportive activity of OP9 we performed molecular profiling of S17 and MS5 stromal cell lines with low hematopoiesis-inducing potential and OP9 cells. In addition, we compared overgrown OP9 (day 8) with freshly confluent OP9 (day 4) monolayers. Transcriptome analysis revealed 21 genes differentially expressed in day 8 overgrown OP9 cells as compared to all other stromal cells (**Fig. 2a**). These included genes encoding *Ptn* (pleiotrophin), a secreted regulator of HSC expansion and regeneration,[20] *Rspo3* (R-spondin 3), an important regulator of Wnt signaling and angioblast development [21], and an extracellular matrix proteins *Postn* (periostin) required for B lymphopoiesis,[22]. Interestingly, one gene that

showed the most significant expression change in overconfluent OP9 was *Tnc* (Tenascin C) (**Fig. 2b**). TenC is expressed in mesenchymal cells underlying hematopoietic clusters in the Aorta-Gonado-Mesonephros (AGM) region and is required for intraembryonic and postnatal hematopoiesis [23-25]. It is also expressed in the bone marrow stem cell niche[25]. Because of these unique properties, we tested whether TenC could support hematopoietic differentiation more effectively than ColIV.

Example 3 Time- and dose-dependent treatment of FGF2, BMP4, Activin A, LiCl, and VEGF induces mesodermal, endothelial, and hematopoietic stages of development

[00114] Our prior studies identified distinct stages of hematoendothelial development following hPSC differentiation in coculture with OP9 (**Fig. 1**) [6,9-11,26]. Plating hPSCs onto OP9 stromal cells induces formation of primitive streak and mesodermal cells which can be detected based on expression apelin receptor (APLNR) and KDR (VEGFR2) [11] and lack of expression Endothelial (CD31, CD144(VE-cadherin)), endothelial/Mesenchymal (CD73, CD105) and Hematopoietic (CD43, CD45) markers, *i.e.* by ^{EMH}lin- phenotype. The first KDR$^+$ mesodermal cells appearing in OP9 coculture on day 2 of differentiation express APLNR and PDGFRalpha (^{EMH}lin$^-$ KDR$^+$APLNR$^+$PDGFRalpha$^+$ hereafter referred as A$^+$P$^+$ cells). These cells display mesenchymoangioblast (MB) potential, *i.e.* capacity to form colonies with both mesenchymal stem cell (MSC) and vascular potential. On day 3 of differentiation A$^+$P$^+$ cells acquire blast (BL)-CFC or hemangioblast (HB) potential [11]. Both MB and HB potentials can be detected using colony-forming assay in serum-free clonogenic medium supplemented with FGF2 [11]. With advanced maturation, mesodermal cells loss BL-CFC activity and upregulate KDR expression and downregulate PDGFRalpha, *i.e.* acquire KDR^{hi}APLNR$^+$PDGFRalpha$^{lo/-}$ hematovascular

progenitor (HVMP) phenotype which enriches in cells with the potential to form hematoendothelial clusters on OP9 [6]. The endothelial stage of development is defined by expression of endothelial-specific marker VE-cadherin (CD144). The first VE-Cadherin^{+} (CD144^{+}) cells emerge from KDR^{hi}APLNR^{+}PDGFRalpha$^{lo/-}$ mesodermal cells by day 4 of differentiation. The emerging VE-cadherin^{+} (CD144+) cells represent a heterogeneous population which include CD43^{-}CD73^{+} (CD144^{+}CD43^{-}CD73^{+}) non-hemogenic endothelial progenitors (non-HEPs) and CD43^{-}CD73^{-} (CD144^{+}CD43^{+}CD73^{-}) hemogenic endothelial progenitors (HEPs) [6]. HEPs lacking hematopoietic CFC potential, but acquire it after culture with stromal cells. The hematopoietic stage of development is defined by expression of hematopoietic-specific marker CD43 [6,10]. The first CD43^{+} cells emerge within VE-cadherin^{+} (CD144+) cells on day 4-5 of differentiation. These cells express low level CD43 and coexpress CD235a, but lack CD41a expression, *i.e.* had CD144^{+}CD43/235a^{+}41a^{-} phenotype. Because these cells have capacity to form hematopoietic colonies in presence of FGF2 and hematopoietic cytokines as well to grow endothelial cells on fibronectin, we designated them as angiogenic hematopoietic progenitors (AHPs). The first CD41a cell appears within CD235a positive cells. CD235a^{+}CD41a^{+} cells are highly enriched and erytho-megakaryocytic progenitors and lacking endothelial potential. The progenitors with broad myelolymphoid potential and lin^{-} CD34^{+}CD43^{+}CD45$^{+/-}$ phenotype can be detected in hPSC cultures shortly after emergence of CD235a^{+}CD41a^{+} cells. Acquisition of CD45 expression by lin^{-} cells is associated with progressive myeloid commitment.[10]

[00115] To reproduce the hematoendothelial program observed in OP9 coculture we decided to select the optimal combinations of morphogens for mesoderm induction and hematoendothelial specification and define specific growth factors required for step-wise progression of

differentiation toward HE and blood cells in hPSC cultures (**Fig. 1**) differentiated in chemically-defined conditions on ColIV and TenC. During embryonic development, BMP4, Wnt, and TGFβ/Nodal/Activin A signaling have been found to be critical to initiate primitive streak formation and subsequent mesoderm development [27,28]. It has been shown that activation of these signaling pathways is essential to induce the expression of brachyury and KDR (Flk-1, VEGFR2), and initiate mesodermal commitment of mouse and human PSCs [18,19,29-32]. We have found that high concentrations of BMP4 (50ng/ml) combined with low concentrations of Activin A (15 ng/ml) and a supplement of LiCl (2 mM) consistently induced expression of the mesodermal surface markers APLNR, KDR, and PDGFRalpha after 2 days of culture of single cell suspension of hESCs on ColIV in chemically-defined conditions as we described above. However, these conditions poorly supported cell survival and required the addition of FGF2 and a hypoxic conditions (5% O_2, 5% CO_2) to improve cell viability and output of mesodermal cells. Day 2 KDR^+ mesodermal cells differentiated in these conditions expressed APLNR and PDGFRalpha, *i.e.* became $APLNR^+PDGFRalpha^+$ cells and displayed MB colony-forming potential similar to $APLNR^+PDGFRalpha^+$ mesodermal cells obtained from day 2 hPSCs differentiated in OP9 coculture [11] (Fig. 3). After 2 days of differentiation, we found that only FGF2 and VEGF are required for $APLNR^+PDGFRalpha^+$ mesoderm to acquire HB potential on day 3 of differentiation and advance mesoderm specification toward HVMPs signified by increase in KDR expression and the decrease in PDGFRalpha expression in $APLNR^+$ cells ($KDR^{hi}APLNR^+PDGFRalpha^{lo/-}$ phenotype) in $CD31^-$ mesodermal cells on day 4 of differentiation. The pattern of development was similar in cells cultured on ColIV and TenC, however the later one produced significantly higher $APLNR^+PDGFRalpha^+$ cells, MB and HB colonies **(Fig. 3a, 3c)**.

[00116] Day 4 differentiated hESCs lost capability to form HB colonies (Fig. 3c), however these cells were capable to form hematoendothelial clusters when sorted and plated onto OP9 in αMEM supplemented with 10% FBS, SCF, TPO, IL6, and IL3. The hematoendothelial cluster potential was restricted to $KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}CD31^{-}$ HVMPs (**Fig. 3d**). The $KDR^{lo}CD31^{-}$ cells only formed endothelial clusters with almost no hemogenic activity **(Fig. 3d)**, while KDR- cells fail to grow both, endothelial and blood cells (not shown). This is also consistent with differentiation in OP9 coculture [6]. The percentage of $KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$ HVMPs cells was consistently higher in TenC cultures (**Fig. 3a**).

[00117] Because formation of HVMPs in hPSC/OP9 cocultures is closely followed by development of HE and blood progenitors, we supplemented our cultures with SCF, TPO, IL-6, and IL-3 hematopoietic cytokines in addition to VEGF and FGF2 starting from day 4 of differentiation. Although we noticed that the continuous treatment of cultures with FGF2 and VEGF was sufficient for induction of endothelial progenitors and hematopoietic specification, addition of hematopoietic cytokines was essential to increase output of these cells in chemically defined cultures. On day 5 of differentiation in these conditions, the previously identified 3 major subsets of the $CD144^{+}$ populations [6] emerged: $CD144^{+}CD43^{-}CD73^{+}$, $CD144^{+}CD43^{-}CD73^{-}$ and $CD144^{+}CD43/CD235a^{+}CD41a^{-}$ (**Fig. 4**). When these subsets were sorted and plated into endothelial conditions, all of them formed a monolayer of VE-cadherin expressing cells with capacity to uptake AcLDL and form vascular tubes in the tube formation assay, consistent with OP9 coculture (**Fig. 4d**). However, hematopoietic CFC potential was mostly restricted to $CD144^{+}CD43/CD235a^{+}CD41a^{-}$ cells (**Fig. 4c**). Importantly similar to finding with day 5 $CD144^{+}$ subsets generated in coculture with OP9, the hematopoietic CFC potential of $CD144^{+}CD43/CD235a^{+}CD41a^{-}$ cells was detected only in serum-free medium in presence of

FGF2 in addition to hematopoietic cytokines, indicating that these cells essentially similar to AHP identified in hPSC/OP9 coculture [6]. We previously defined HEP as $CD144^{+}CD43^{-}CD73^{-}$ cells lacking hematopoietic CFC potential, but capable to acquire it after culture on OP9. To determine whether $CD144^{+}CD43^{-}CD73^{-}$ generated in completely defined conditions similar to OP9-induced HEPs, we sorted day 5 $CD144^{+}$ subsets and cultured with OP9 as previously described [6]. In these conditions, the HEPs formed both endothelial and hematopoietic cells with large number of HE-clusters, while AHPs formed predominantly hematopoietic cells with few endothelial cells and hematoendothelial culsters. $CD144^{+}CD43^{-}CD73^{+}$ cells formed endothelial clusters only consistent with non-HEP phenotype (**Fig. 4d**). Cultures differentiated on TenC had a larger population of total $CD144^{+}$ cells, thereby increasing the population of HEPs, non-HEPs, and AHPs compared to cultures differentiated on ColIV (**Fig. 4a, b**).

[00118] When numerous floating round hematopoietic cells became visible in cultures on day 6, the hypoxic conditions were not necessary to sustain hematopoietic development. Therefore, from day 6 of differentiation, the cultures were transferred cells to a normoxic incubator (20% O_2, 5% CO_2). By day 8 of differentiation, cultures showed development of large number of $CD43^{+}$ hematopoietic cells composed $CD235a^{+}CD41a^{+}$ cells enriched in erythro-megakaryocytic progenitors and $lin^{-}CD43^{+}CD45^{-/+}$ cells which expressed CD34 (**Fig. 5**) and lacked of other lineage markers (not shown). Consistent with cells differentiated on OP9, hematopoietic colony forming potential was limited to the $CD43^{+}$ subpopulations **(Figure 5c)**. $CD43^{+}$ hematopoetic progenitors expanded significantly more on TenC compared to ColIV (Figure 5b). In addition, the GEMM-CFC potential of cultures on TenC was significantly greater than cultures on ColIV (**Fig. 5d**).

[00119] Although the differentiation protocol was initially developed using H1 hESCs, we found that chemically defined conditions described here also supports formation of HE and blood from other hESCs (H9) and hiPSCs generated from fibroblasts or bone marrow mononuclear cells (**Fig. 9**). Previously, we found that hiPSC obtained through reprogramming of cord blood mononuclear cells (CB hiPSCs) differentiate less efficiently into the blood cells on OP9 feeders compared to fibroblast-derived (FB) hiPSCs[33]. These findings have been reproduced when we differentiated CB and FB iPSCs on ColIV. However, differentiation on TenC restored hematopoietic differentiation potential of CB hiPSCs to the level seen with hESCs and FB hiPSCs (Fig. 9), thereby confirming that TenC is superior over ColIV in promoting hematopoietic differentiation from hPSCs.

Example 4 Tenascin C uniquely supports specification of T lymphoid progenitors from hPSCs

[00120] To find out whether our culture system supports establishment of definitive hematopoietic program from hPSCs, we analyzed T cell potential of blood cells generated in our system as indicator of definitive hematopoiesis[7]. When we collected $CD43^+$ floating cells from day 9 differentiated cultures, and replated them onto OP9 cells expressing DLL-4 in α-MEM with 20% FBS, Flt3L, IL-7, and SCF, $CD7^+CD5^+$ lymphoid progenitors began to emerge by week 2 of secondary coculture. By week 3, $CD4^+CD8^+$ double positive T-cells arose (**Fig. 6a**).

[00121] Interestingly, $CD43^+$ cells generated on both ColIV and TenC matrices had a capacity to generate $CD5^+CD7^+$ lymphoid progenitors. However, progression toward $CD4^+CD8^+$ T lymphoid cells was observed only from $CD43^+$ cells generated on TenC but not on ColIV. To confirm T cell development, we analyzed genomic DNA from these cultures for the presence of TCR rearrangements. This analysis demonstrated the presence of multiple PCR products of random V-J and D-J rearrangements at the γ-locus and multiple V-J and rearrangements at the γ-

locus indicative of polyclonal T lineage repertoire (**Fig. 6b and 6c**). Overall, these findings signify that extracellular matrix Tenascin C is essential for supporting definitive hematopoiesis in completely chemically defined conditions.

Example 5 Inhibition of TGF-β promotes hematoendothelial specification in chemically defined conditions

[00122] Recent studies have shown that adding TGF-β inhibitors after mesoderm specification but before endothelial development increases definitive hematopoietic differentiation. We found that when 10 μM of SB-431542, a potent but non-specific TGFβ inhibitor, is added from day 2 to day 4, it significantly decreases the development of PDGFRalpha-positive mesoderm cells by day 3, and increases $CD31^+$ differentiation by day 4. After day 4, SB-431542 is no longer added, but the effect of the 2 day treatment continues to increase $CD43^+$ population by day 9 (**Fig. 7**).

[00123] During the last decade significant progress has been made in hematopoietic differentiation from hPSCs. Multiple protocols for hematopoietic differentiation have been developed and made it possible routinely produce blood cells for experimentation. However, generation of blood cells with long-term reconstitution potential, HSCs, from hPSCs remains significant challenge. In the embryo, hematopoietic cells and HSCs arise from specific subset of endothelium (HE) [1-5], thus the ability to interrogate signaling pathways leading to HE specification and transition into the blood cells in completely chemically defined environment is essential for identification of factors required for HSC specification and eventually development of conditions for *de novo* HSC generation. Although original protocols for hematopoietic differentiation have employed xenogenic, feeder and/or serum, several serum- and feeder-free systems for hematopoietic differentiation have been described recently [18,34,35]. However, these protocols still requires serum components (albumin), and it remains unclear whether these protocols reproduce distinct waves of hematopoiesis, including generation of HE with definitive lymphomyeloid potential, observed in the original differentiation systems. More recently Kennedy et al,[7] have developed feeder- and stroma-free conditions for EB-based

hematopoietic differentiation of hPSCs and showed that these conditions reproduced primitive and definitive waves of hematopoiesis and generate HE with T lymphoid potential. However, this protocol uses hPSCs growing on MEFs for EB-based hematopoietic differentiation in proprietary medium with non-disclosed chemical and human protein content. Here we developed for the first time protocol that enable efficient production of blood cells in completely chemically defined conditions free of serum and xenogeneic proteins from a single cell suspension of hPSCs maintained in chemically defined E8 medium [12]. This protocol eliminates variability associated with animal- or human-sourced albumins, xenogenic matrix, clump sizes and asynchronous differentiation observed in EB system and reproduces typical waves of hematopoiesis, including formation of HE and definitive hematopoietic progenitors, observed in hPSCs differentiated on OP9. Importantly, based on molecular profiling of OP9 and stromal cell lines with different hematopoiesis-inducing activity, we found that TenC matrix protein uniquely expressed in OP9 with robust hemato-inducing potential, strongly promotes hematoendothelial and T lymphoid development from hPSCs. TenC is disulfide-linked hexameric glycoprotein that is mainly expressed during embryonic development. Although TenC mostly disappear in adult organism, its expression upregulated during wound repair, neovascularization and neoplasia.[36] Interestingly TenC is found in adult bone marrow where it expressed predominantly in endosteal region [37,38] and upregulated following myeloablation [25]. TenC supports proliferation of bone marrow hematopoietic cells[39] and erythropoiesis [40]. TenC-deficient mice had lower bone marrow CFC potential [24], failed to reconstitute hematopoiesis after bone marrow ablation and showed reduced ability to support engraftment of wild type HSCs [25]. High level of TenC expression was also detected in human and chicken aorta-gonad-mesonephros (AGM) region [23,41], the site where the first HSC emerge, and hematopoietic sites in the human fetal liver [42]. Because

TenC expression is highly enriched in subaortic mesenchyme right underneath of hematopoietic clusters, it was suggested that TenC plays pivotal role in HSC development during embryogenesis [23]. TenC is also involved in regulation of angiogenesis and cardiac endothelial progenitors [43]. Our studies demonstrated the superior properties of TenC in promoting hematopoiesis from hPSCs. The positive effect of TenC was obvious at all stages of differentiation and included the enhancement of hemogenic mesoderm, HE and $CD43^+$ hematopoietic progenitors production. Importantly, TenC was able to support development of definitive hematopoietic cells with T lymphoid potential, while we were not able to obtain such cells in cultures on ColIV. TenC molecule is composed of an amino-terminal oligomerization region followed by heptad repeats, EGF-like and fibronectin type III repeats and fibrinogen globe [36]. The function of these domains is poorly understood. It is believed that effect and interaction of TenC with cells requires the integrate action of multiple domains [44], although several unique mitogenic domains capable of inducing a proliferation of hematopoietic cells were identified within this molecule [39]. Several signaling mechanisms implicated in cell interaction with TenC have been identified, including suppression of fibronectin-activated focal adhesion kinase- and Rho-mediated signaling and stimulation of Wnt signaling (reviewed in[45]). Further studies aimed to identify mechanism of TenC action on hPSCs and their hematopoietic derivatives would be of value to understand the role of this matrix protein in hematopoietic development.

[00124] In summary, the findings provided here identified TenC matrix proteins and completely chemically defined conditions free of serum/serum components and animal proteins capable of supporting the scalable production of HE and definitive blood cells from hPSCs. This differentiation system allows precise interrogation of signaling molecules implicated in

hematopoietic differentiation and provide platform for production of cGMP grade of blood cells for clinical application.

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CLAIMS

We claim:

1. A method of differentiating human pluripotent stem cells, comprising the steps of:

(a) providing human pluripotent stem cells; and

(b) exposing the stem cells to a mixture comprising components FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for a period of about two days to form a population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential.

2. The method of claim 1, additionally comprising the step of:

(c) exposing cells at the primitive mesoderm stage of step (b) to a mixture comprising components FGF2 and VEGF under hypoxic conditions for a period of about 1-2 days to obtain a population comprising $^{EMH}lin\text{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($^{EMH}lin^{-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) enriched in cells with a potential to form hematoendothelial clusters when cultured on OP9 cells.

3. The method of claim 2, additionally comprising the step of:

(d) exposing the cells at the hematovascular mesoderm stage of step (c) to a mixture comprising components FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to achieve formation of $CD144^{+}CD73^{+}CD235a/CD43^{-}$ non-hemogenic endothelial progenitors (non-HEP), $CD144^{+}CD73^{-}CD235a/CD43^{-}$ hemogenic endothelial progenitors (HEPs), $CD144^{+}CD73^{-}$

CD235a/CD43^{+}41a^{-} angiogenic hematopoietic progenitors (AHP), and CD43^{+}CD41a^{+} hematopoietic progenitor cells.

4. The method of claim 3, additionally comprising the step of:

(e) continuing to expose the HEPs and emerging hematopoietic progenitor cells to a mixture comprising components FGF2, VEGF, IL6, SCF, TPO, IL3 under normoxia for about three days resulting in hematopoietic expansion to obtain a population of CD43^{+} hematopoietic progenitors composed of CD43^{+}CD235a^{+}CD41a^{+} erythromegakaryocytic progenitors and lin-CD34^{+}CD43^{+}CD45$^{+/-}$ multipotent hematopoietic progenitors.

5. The method of claim 1, wherein the mixture consists essentially of the components.

6. The method of claim 5, wherein the components are xenogen-free.

7. The method of claim 1, wherein the cells are seeded on a substrate treated with Tenascin-C.

8. A xenogen-free culture medium for differentiating human pluripotent stem cells, comprising IF9S medium supplemented with:

about 50 to about 250 ng/ml BMP4;

about 10 to about 15 ng/ml Activin A;

about 10 to about 50 ng/ml FGF2; and

about 1 to about 2 mM LiCl.

9. A xenogen-free culture medium for differentiating human pluripotent stem cells, comprising IF9S medium supplemented with:

about 10 to about 50 ng/ml FGF2; and

about 20 to about 50 ng/ml VEGF.

10. The medium of claim 9 further comprising hematopoietic cytokines.

11. The medium of claim 10, wherein the hematopoietic cytokines comprise:

about 50 to about 100 ng/ml SCF,

about 50 to about 100 ng/ml TPO,

about 50 to about 100 ng/ml IL-6, and

about 5 to about 15 ng/ml IL-3.

12. A concentrated form of the media of claim 9.

13. A xenogen-free cell culture system for differentiating human pluripotent stem cells into mesoderm, endothelial, and hematopoietic progenitor cells, comprising;

human pluripotent stem cells seeded as a single cell suspension on a substrate comprising a layer of Tenascin C at a concentration of at least about 0.25 $\mu g/cm^2$ to about 1 $\mu g/cm^2$; and

a xenogen-free culture medium comprising IF9S medium supplemented with:

about 50 to about 250 ng/ml BMP4;

about 10 to about 15 ng/ml Activin A;

about 10 to about 50 ng/ml FGF2; and

about 1 to about 2 mM LiCl.

14. A method of differentiating human pluripotent stem cells, comprising the steps of:

(a) providing human pluripotent stem cells;

(b) seeding the cells as a single cell suspension on a substrate treated with Tenascin C; and

(c) culturing the seeded cells in IF9S medium supplemented with BMP4, Activin A, FGF2, and LiCl under hypoxic conditions for a period of about two days to obtain about 30% $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential.

15. The method of claim 14 further comprising the step of culturing the cells at the $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm stage in IF9S medium supplemented with FGF2 and VEGF under hypoxic conditions for about 1-2 days to obtain $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and

^{EMH}linKDR^{hi}APLNR^{+}PDGFRalpha$^{lo/-}$ hematovascular mesodermal precursors with a potential to form hematoendothelial clusters when cultured on OP9 cells.

16. A method of producing mesenchymoangioblasts, comprising the steps of:

(a) providing human pluripotent stem cells;

(b) seeding the cells on a substrate treated with an effective amount of collagen; and

(c) exposing the stem cells to a mixture comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for a period of about two days to form a population of ^{EMH}lin^{-} KDR^{+}APLNR^{+}PDGFRalpha^{+} primitive mesoderm cells with mesenchymoangioblast potential.

17. The method of claim 16, wherein the collagen comprises Collagen IV.

18. A cell culture medium for differentiating human pluripotent stem cells, comprising:

64 mg/L L-Ascorbic Acid, 2-Phosphate Mg^{2+} salt, 40 µl/L monothioglycerol, 8.4 µg/L additional sodium selenite, 10 mg/L polyvinyl alcohol, 1x GLUTAMAX, 1x non-essential amino acids, 0.1x chemically-defined lipid concentrate, 10.6 mg/L Holo-Transferrin, and 20 mg/L insulin.

19. A method for differentiating human pluripotent stem cells comprising:

(a) providing human pluripotent stem cells; and

(b) culturing the human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two

days to form a cell population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential.

20. The method of claim 19, wherein the human pluripotent stem cells are cultured on Tenascin C.

21. The method of claim 19, wherein the concentration, in the cell culture medium, of: BMP4 is about 50 ng/ml to about 250 mg/ml; Activin A is about 10 ng/ml to about 15 ng/ml; FGF2 is about 10 ng/ml to about 50 ng/ml; and LiCl is about 1 mM to about 2 mM.

22. The method of claim 19, further comprising (c) culturing, under hypoxic conditions, the cell population obtained in step (b) in a cell culture medium comprising FGF2 and VEGF for a period of about 1-2 days to obtain a cell population comprising $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($EMH^{lin-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) enriched in cells with a potential to form hematoendothelial clusters when cultured on OP9 cells.

23. The method of claim 22, wherein the concentration, in the cell culture medium, of FGF2 is about 10 ng/ml to about 50 ng/ml; and VEGF is about 20 ng/ml to about 50 ng/ml.

24. The method of claim 21, further comprising (d) culturing the hematovascular mesoderm cells of step (c), under hypoxic conditions, in a cell culture medium comprising FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to obtain a cell population comprising

$CD144^+CD73^+CD235a/CD43^-$ non-hemogenic endothelial progenitors (non-HEP), $CD144^+CD73^-CD235a/CD43^-$ hemogenic endothelial progenitors (HEPs), $CD144^+CD73^-CD235a/CD43^+41a^-$ angiogenic hematopoietic progenitors (AHP), and $CD43^+CD41a^+$ hematopoietic progenitor cells.

25. The method of claim 24, wherein the concentration, in the cell culture medium, of: FGF2 is about 10 ng/ml to about 50 ng/ml; VEGF is about 20 ng/ml to about 50 ng/ml; SCF is about 50 ng/ml to about 100 ng/ml; TPO is about 50 ng/ml to about 100 ng/ml; IL-6 is about 50 ng/ml to about 100 ng/ml, and IL-3 is about 5 ng/ml to about 15 ng/ml.

26. The method of claim 24, further comprising (e) culturing, under normoxia, the HEPs and hematopoietic progenitor cells in a culture medium comprising FGF2, VEGF, IL6, SCF, TPO, IL3 for about three days to obtain an expanded population of $CD43^+$ hematopoietic progenitors comprising $CD43^+CD235a/CD41a^+$ erythromegakaryocytic progenitors and $lin^-CD34^+CD43^+CD45^{+/-}$ multipotent hematopoietic progenitors.

27. The method of claim 26, further comprising coculturing the expanded population of $CD34^+CD43^+$ hematopoietic progenitors for a period of about three weeks on OP9 cells overexpressing DLL4 to obtain a cell population comprising $CD4^+CD8^+$ double positive T-cells, wherein the human pluripotent stem cells of step (b) are cultured on a Tenascin C substrate.

28. The method of claim 19, wherein the cell culture medium comprises an IF9S cell culture medium.

29. A method of differentiating human pluripotent stem cells, comprising at least one of:

(i) culturing human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential;

(ii) culturing, under hypoxic conditions, $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast potential in a cell culture medium comprising FGF2 and VEGF for a period of about 1-2 days to obtain a cell population comprising $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm with hemangioblast (HB-CFC) potential and hematovascular mesoderm cells ($EMH^{lin-}KDR^{hi}APLNR^{+}PDGFRalpha^{lo/-}$) enriched in cells with a potential to form hematoendothelial clusters when cultured on OP9 cells;

(iii) culturing hematovascular mesoderm cells (EMHlin-KDRhiAPLNR+PDGFRalpha$^{lo/-)}$ cells, under hypoxic conditions, in a cell culture medium comprising FGF2, VEGF, IL6, SCF, TPO, and IL3 for about one day to achieve formation of $CD144^{+}CD73^{+}CD235a/CD43^{-}$ non-hemogenic endothelial progenitors (non-HEP), $CD144^{+}CD73^{-}CD235a/CD43^{-}$ hemogenic endothelial progenitors (HEPs), $CD144^{+}CD73^{-}$

CD235a/CD43^{+}41a^{-} angiogenic hematopoietic progenitors (AHP), and CD43^{+}CD41a^{+} hematopoietic progenitor cells;

(iv) culturing, under normoxia, hemogenic endothelial progenitors (HEPs) and and CD43^{+}CD41a^{+} hematopoietic progenitor cells in a culture medium comprising FGF2, VEGF, IL6, SCF, TPO, IL3 for about three days to obtain an expanded population of CD43^{+} hematopoietic progenitors comprising CD43^{+}CD235a/CD41a^{+} erythromegakaryocytic progenitors and lin^{-}CD34^{+}CD43^{+}CD45$^{+/-}$ multipotent hematopoietic progenitors; and

(v) coculturing CD34^{+}CD43^{+} hematopoietic progenitors for a period of about three weeks on OP9 cells overexpressing DLL4 to obtain a cell population comprising CD4^{+}CD8^{+} T-cells.

30. A cell culture medium suitable for hematoendothelial differentiation of human pluripotent stem cells, comprising a base medium, L-ascorbic acid 2-phosphate Mg^{2+} salt, monothioglycerol, sodium selenite, polyvinyl alcohol, Glutamax™, non-essential amino acids (NEAA), chemically defined lipid concentrate, Holo-Transferrin, and insulin.

31. The cell culture medium of claim 30, further comprising BMP4, Activin A, FGF2, and LiCl.

32. The cell culture medium of claim 30, further comprising FGF2 and VEGF.

33. The cell culture medium of claim 30, further comprising FGF2, VEGF, SCF, TPO, IL-6, and IL-3.

34. The cell culture medium of claim 30, wherein the cell culture medium comprises an IF9S medium.

35. The cell culture medium of claim 34, wherein the IF9S cell culture medium has the IF9S cell culture medium formulation of Table 2.

36. A 9S concentrated medium supplement, wherein dilution of the 9S concentrated medium supplement in an IMDM/F12 base medium yields an IF9S cell culture medium.

37. A kit comprising the 9S concentrated medium supplement of claim 36, and one or more of BMP4, Activin A, FGF2, LiCl, SCF, TPO, IL-6, IL-3, and Tenascin C.

38. A defined cell culture system for hematoendothelial differentiation of human pluripotent stem cells, comprising an IF9S cell culture medium and a Tenascin C substrate for adherent growth of human pluripotent stem cells or their differentiated progeny along the hematoendothelial lineage.

39. The defined cell culture system of claim 38, wherein the IF9S cell culture medium is maintained under hypoxic conditions.

The defined cell culture system of claim 39, further comprising human pluripotent stem cells grown on the Tenascin C substrate.

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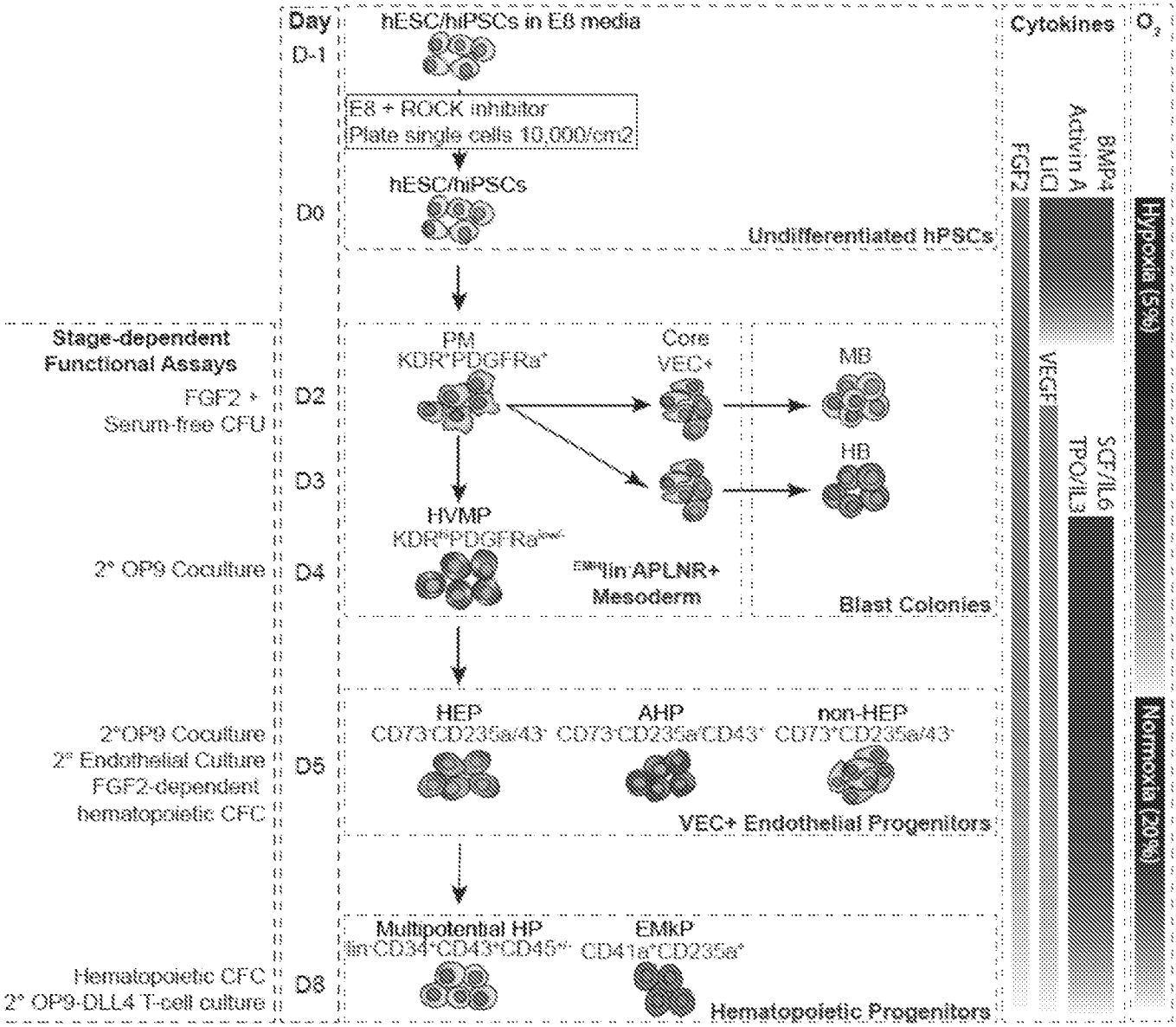
FIG. 1


Day
D-1
hESC/hiPSCs in E8 media
E8 + ROCK inhibitor
Plate single cells 10,000/cm2
hESC/hiPSCs
D0
Undifferentiated hPSCs
Cytokines
O2
FGF2
LiCl
Activin A
BMP4
VEGF
SCF/IL6
TPO/IL3
Hypoxia (5%)
Normoxia (20%)
Stage-dependent
Functional Assays
FGF2 +
Serum-free CFU
D2
D3
2° OP9 Coculture
D4
PM
KDR+PDGFRa+
Core
VEC+
MB
HB
HVMP
KDR+PDGFRa
lin-APLNR+
Mesoderm
Blast Colonies
2°OP9 Coculture
2° Endothelial Culture
FGF2-dependent
hematopoietic CFC
D5
HEP
CD73-CD235a/43-
AHP
CD73-CD235a/CD43+
non-HEP
CD73+CD235a/43-
VEC+ Endothelial Progenitors
Hematopoietic CFC
2° OP9-DLL4 T-cell culture
D8
Multipotential HP
lin-CD34+CD43+CD45+/-
EMkP
CD41a+CD235a+
Hematopoietic Progenitors

2/10

FIG. 2

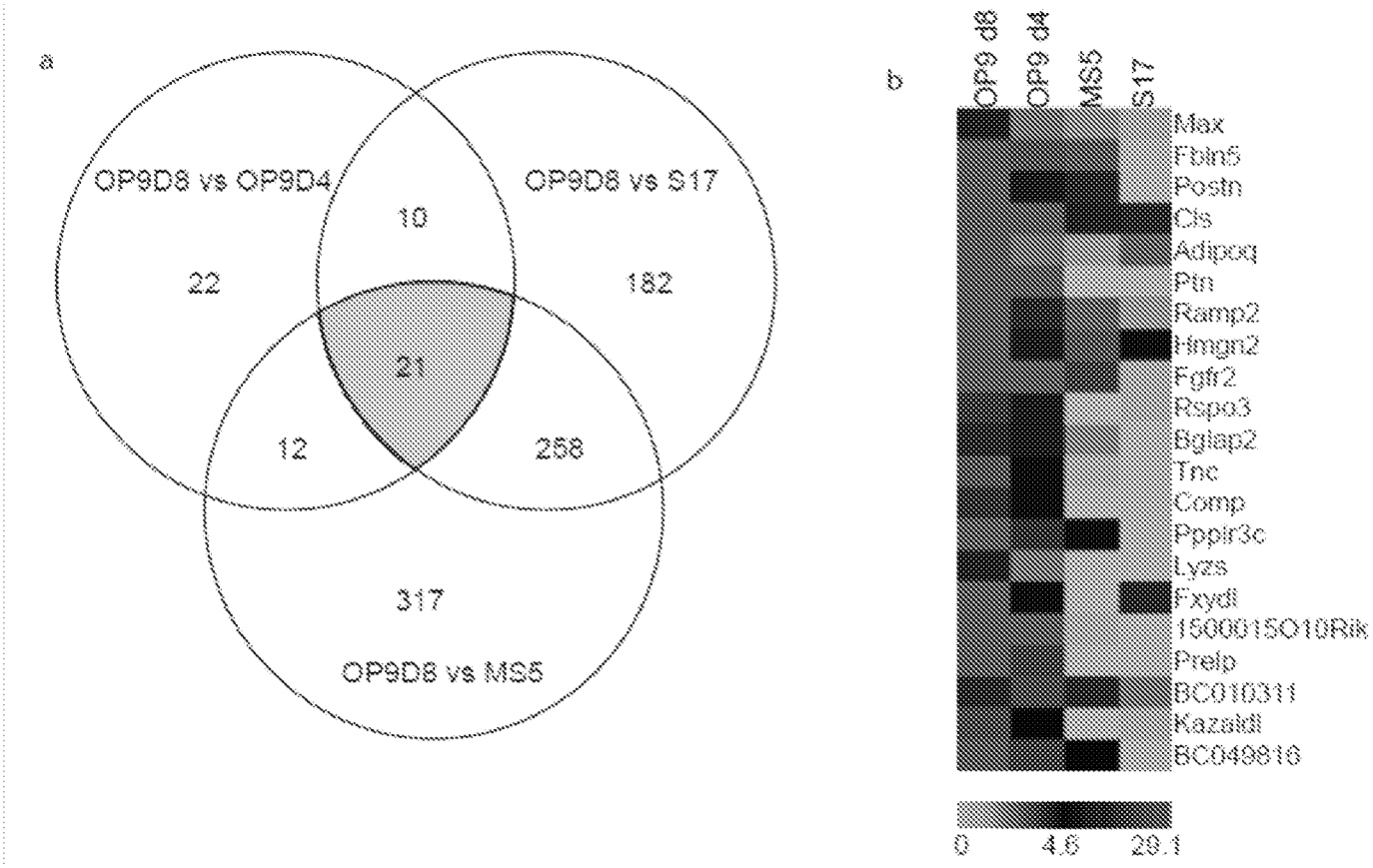

a
OP9D8 vs OP9D4
OP9D8 vs S17
OP9D8 vs MS5
22
10
182
21
12
258
317
b
OP9 d8
OP9 d4
MS5
S17
Max
Fbln5
Postn
Cls
Adipoq
Ptn
Ramp2
Hmgn2
Fgfr2
Rspo3
Bglap2
Tnc
Comp
Ppplr3c
Lyzs
Fxyd1
1500015O10Rik
Prelp
BC010311
Kazald1
BC049816
0
4.6
20.1

FIG. 3

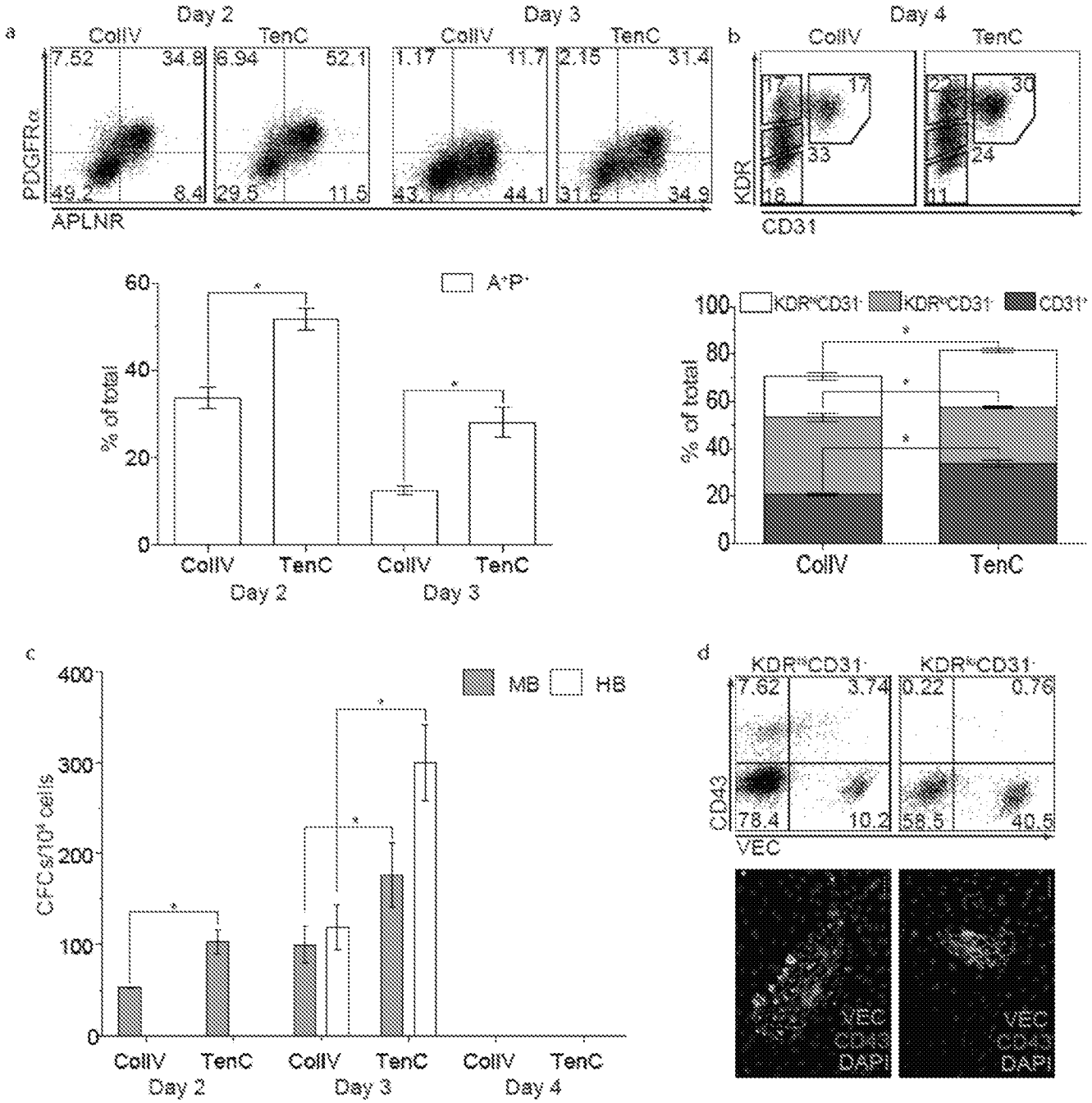
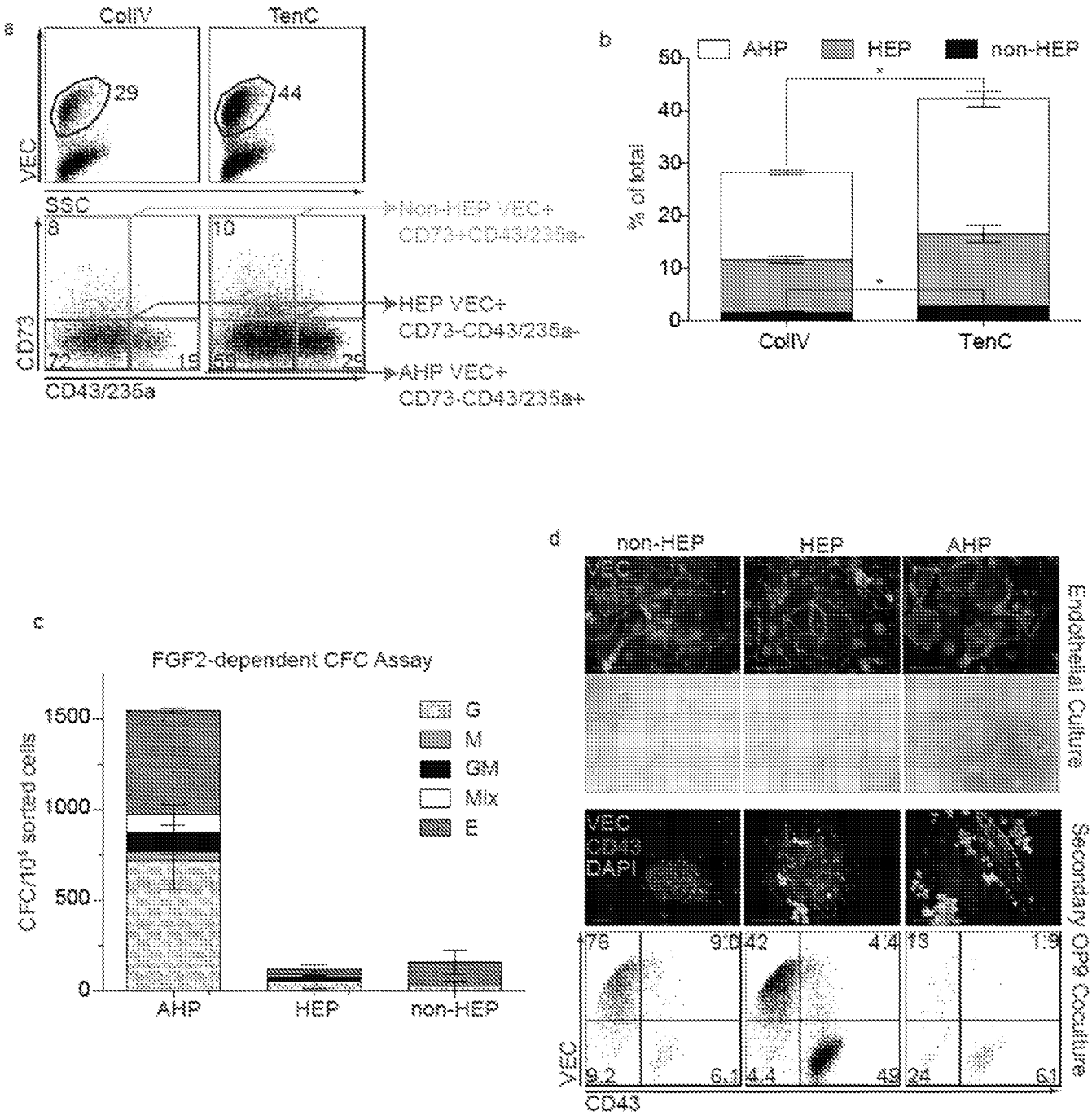

a
Day 2
Day 3
CollV
TenC
7.52
34.8
49.2
8.4
6.94
52.1
29.5
11.5
1.17
11.7
43.1
44.1
2.15
31.4
31.6
34.9
PDGFRα
APLNR
b
Day 4
CollV
TenC
17
17
33
18
30
24
11
KDR
CD31
A+P+
% of total
CollV
TenC
Day 2
Day 3
KDR+CD31-
KDR+CD31+
CD31+
c
MB
HB
CFCs/10⁴ cells
Day 2
Day 3
Day 4
d
KDR+CD31-
KDR+CD31+
7.62
3.74
76.4
10.2
0.22
0.76
58.5
40.5
CD43
VEC
VEC
CD43
DAPI

FIG. 4


a
ColIV
TenC
29
44
VEC
SSC
CD73
CD43/235a
Non-HEP VEC+
CD73+CD43/235a-
HEP VEC+
CD73-CD43/235a-
AHP VEC+
CD73-CD43/235a+
b
AHP
HEP
non-HEP
% of total
ColIV
TenC
c
FGF2-dependent CFC Assay
CFC/10³ sorted cells
G
M
GM
Mix
E
AHP
HEP
non-HEP
d
non-HEP
HEP
AHP
Endothelial Culture
VEC
CD43
DAPI
Secondary OP9 Coculture
VEC
CD43

5/10

FIG. 5

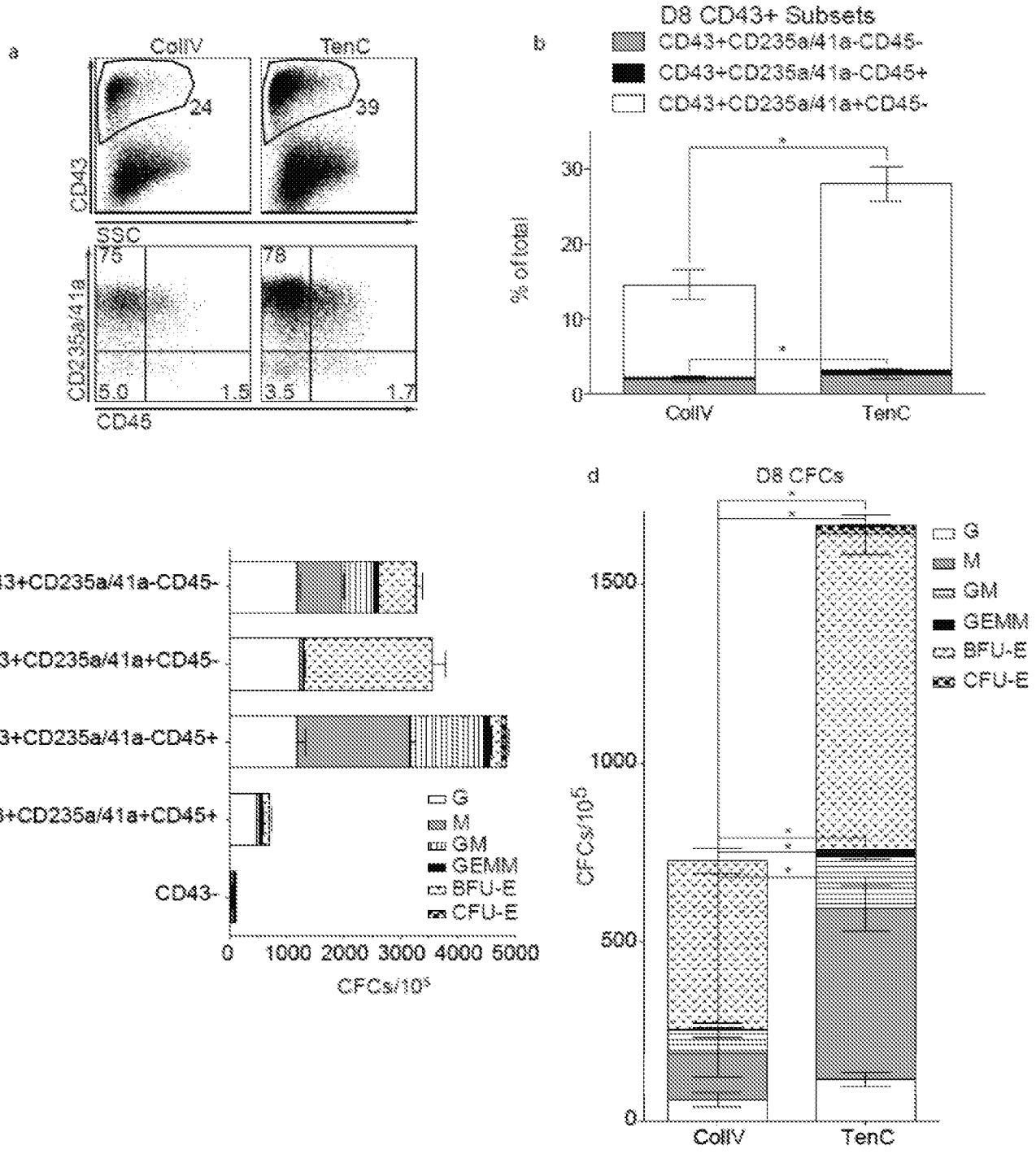

a
ColIV
TenC
24
39
CD43
SSC
75
78
CD235a/41a
5.0
1.5
3.5
1.7
CD45
b
D8 CD43+ Subsets
CD43+CD235a/41a-CD45-
CD43+CD235a/41a-CD45+
CD43+CD235a/41a+CD45-
% of total
30
20
10
0
ColIV
TenC
c
CD43+CD235a/41a-CD45-
CD43+CD235a/41a+CD45-
CD43+CD235a/41a-CD45+
CD43+CD235a/41a+CD45+
CD43-
G
M
GM
GEMM
BFU-E
CFU-E
0
1000
2000
3000
4000
5000
CFCs/10^5
d
D8 CFCs
1500
1000
500
0
CFCs/10^5
G
M
GM
GEMM
BFU-E
CFU-E
ColIV
TenC

FIG. 6

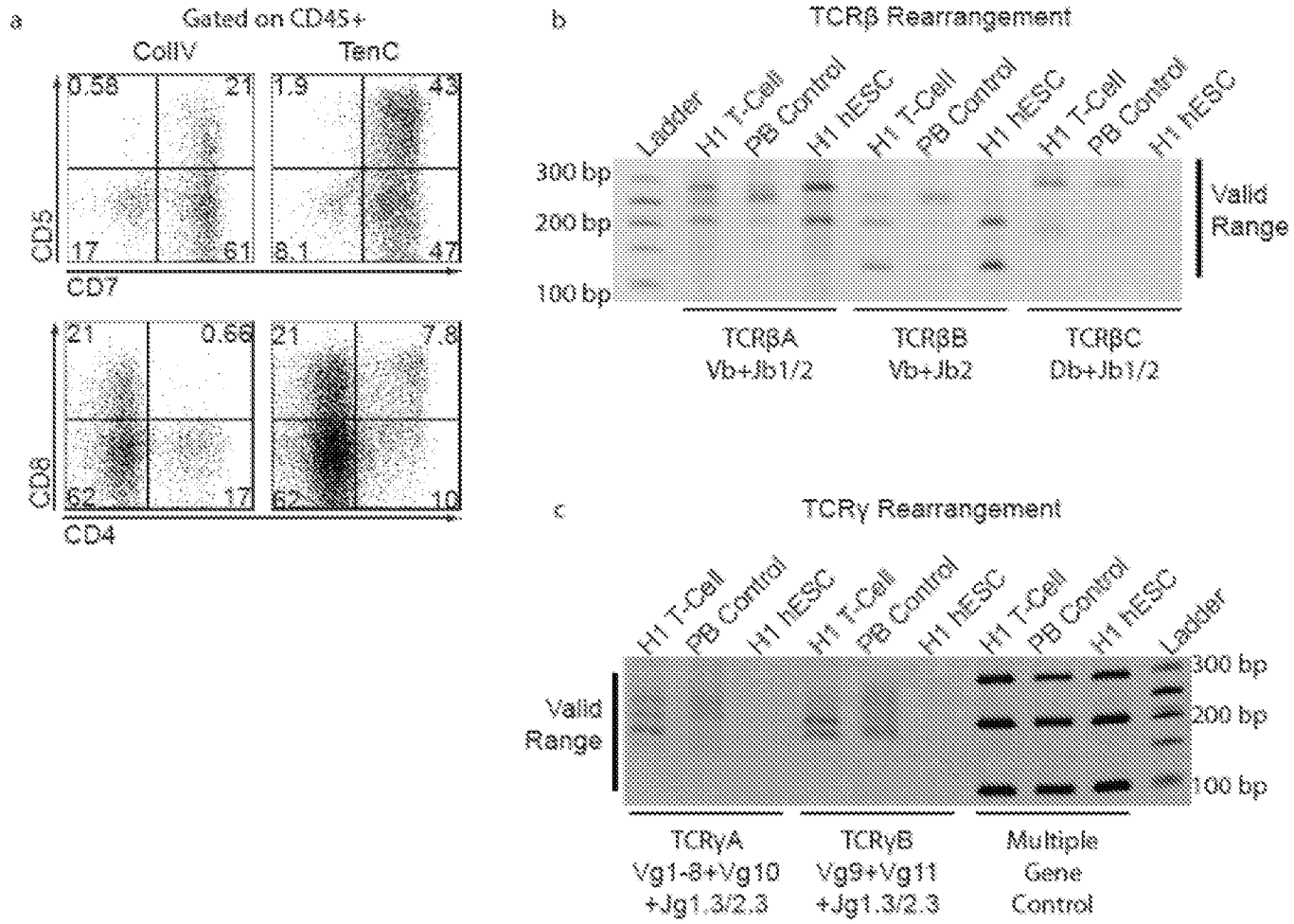

a
Gated on CD45+
CollV
TenC
0.58
21
1.3
43
17
61
8.1
47
CD5
CD7
21
0.68
21
7.8
62
17
62
10
CD8
CD4
b
TCRβ Rearrangement
Ladder
H1 T-Cell
PB Control
H1 hESC
H1 T-Cell
PB Control
H1 hESC
H1 T-Cell
PB Control
H1 hESC
300 bp
200 bp
100 bp
Valid Range
TCRβA Vb+Jb1/2
TCRβB Vb+Jb2
TCRβC Db+Jb1/2
c
TCRγ Rearrangement
H1 T-Cell
PB Control
H1 hESC
H1 T-Cell
PB Control
H1 hESC
H1 T-Cell
PB Control
H1 hESC
Ladder
Valid Range
300 bp
200 bp
100 bp
TCRγA Vg1-8+Vg10 +Jg1.3/2.3
TCRγB Vg9+Vg11 +Jg1.3/2.3
Multiple Gene Control

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FIG. 7

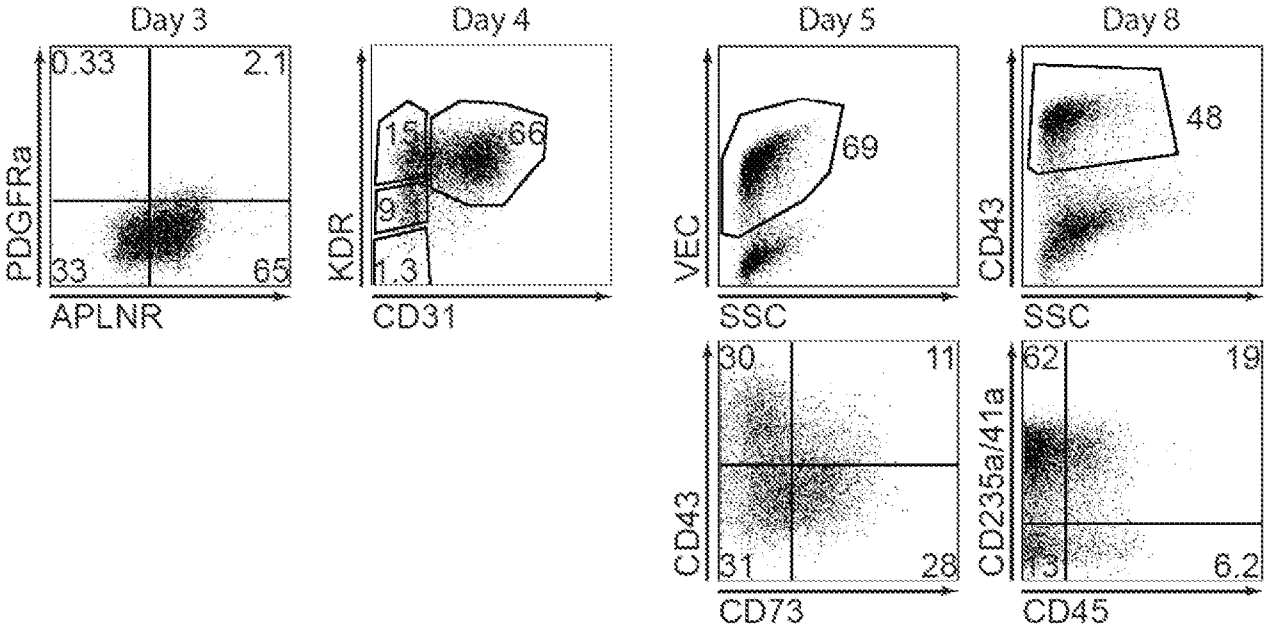
Day 3
Day 4
Day 5
Day 8
PDGFRa
APLNR
0.33
2.1
33
65
KDR
CD31
15
66
9
1.3
VEC
SSC
69
CD43
SSC
48
CD43
CD73
30
11
31
28
CD235a/41a
CD45
62
19
13
6.2

FIG. 8

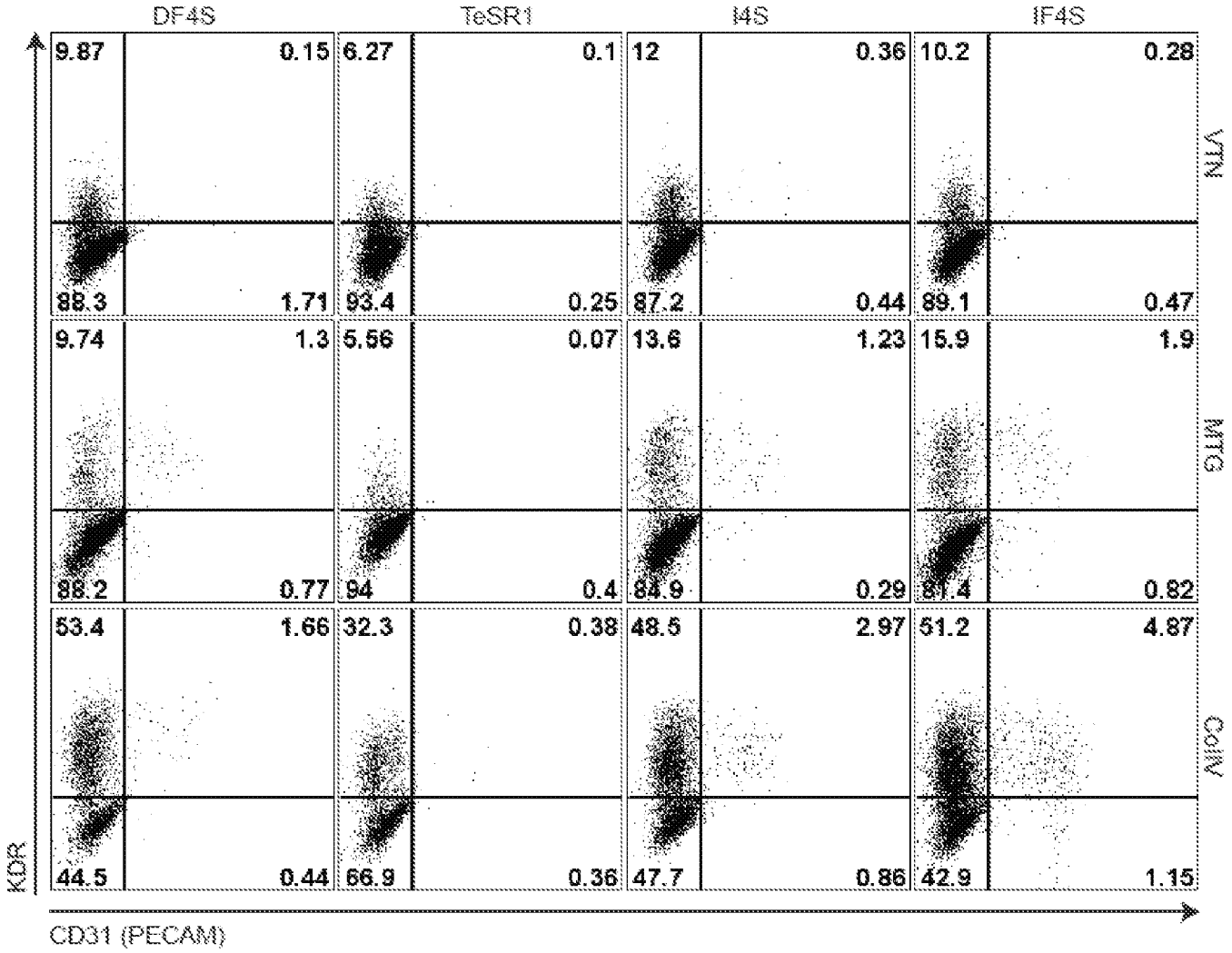
DF4S
TeSR1
I4S
IF4S
VTN
MTG
ColIV
KDR
CD31 (PECAM)
9.87
0.15
88.3
1.71
6.27
0.1
93.4
0.25
12
0.36
87.2
0.44
10.2
0.28
89.1
0.47
9.74
1.3
88.2
0.77
5.56
0.07
94
0.4
13.6
1.23
84.9
0.29
15.9
1.9
81.4
0.82
53.4
1.66
44.5
0.44
32.3
0.38
66.9
0.36
48.5
2.97
47.7
0.86
51.2
4.87
42.9
1.15

FIG. 9

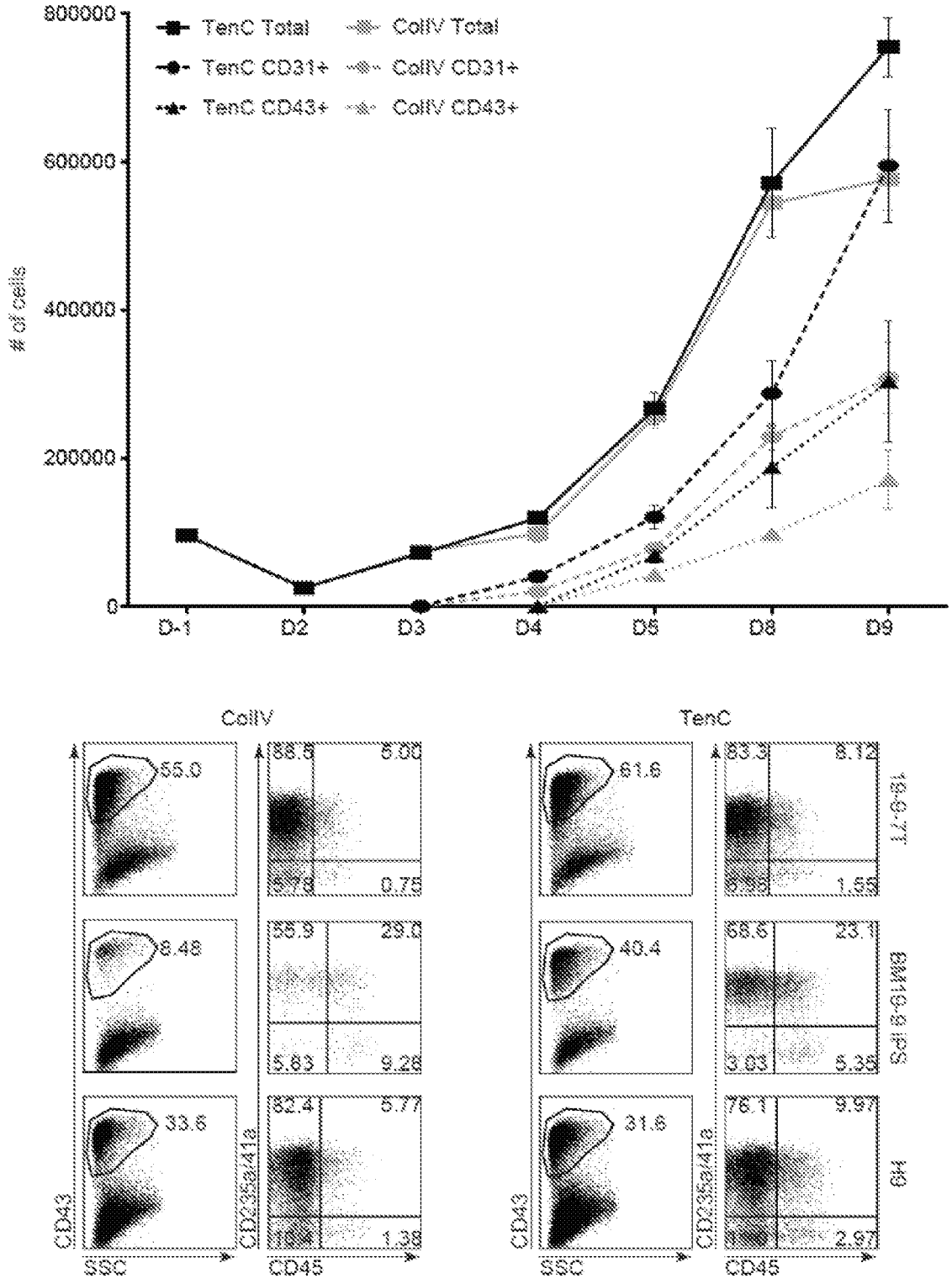
800000
600000
400000
200000
0
# of cells
TenC Total
TenC CD31+
TenC CD43+
ColIV Total
ColIV CD31+
ColIV CD43+
D-1
D2
D3
D4
D5
D8
D9
ColIV
TenC
55.0
8.48
33.6
88.5
5.00
0.75
56.9
29.0
5.83
9.28
82.4
5.77
1.38
61.6
40.4
31.6
83.3
8.12
1.55
68.6
23.1
3.03
5.35
76.1
9.97
2.97
CD43
CD235a/41a
SSC
CD45
19-9-7T
BM19-9 IPS
H9

FIG. 10

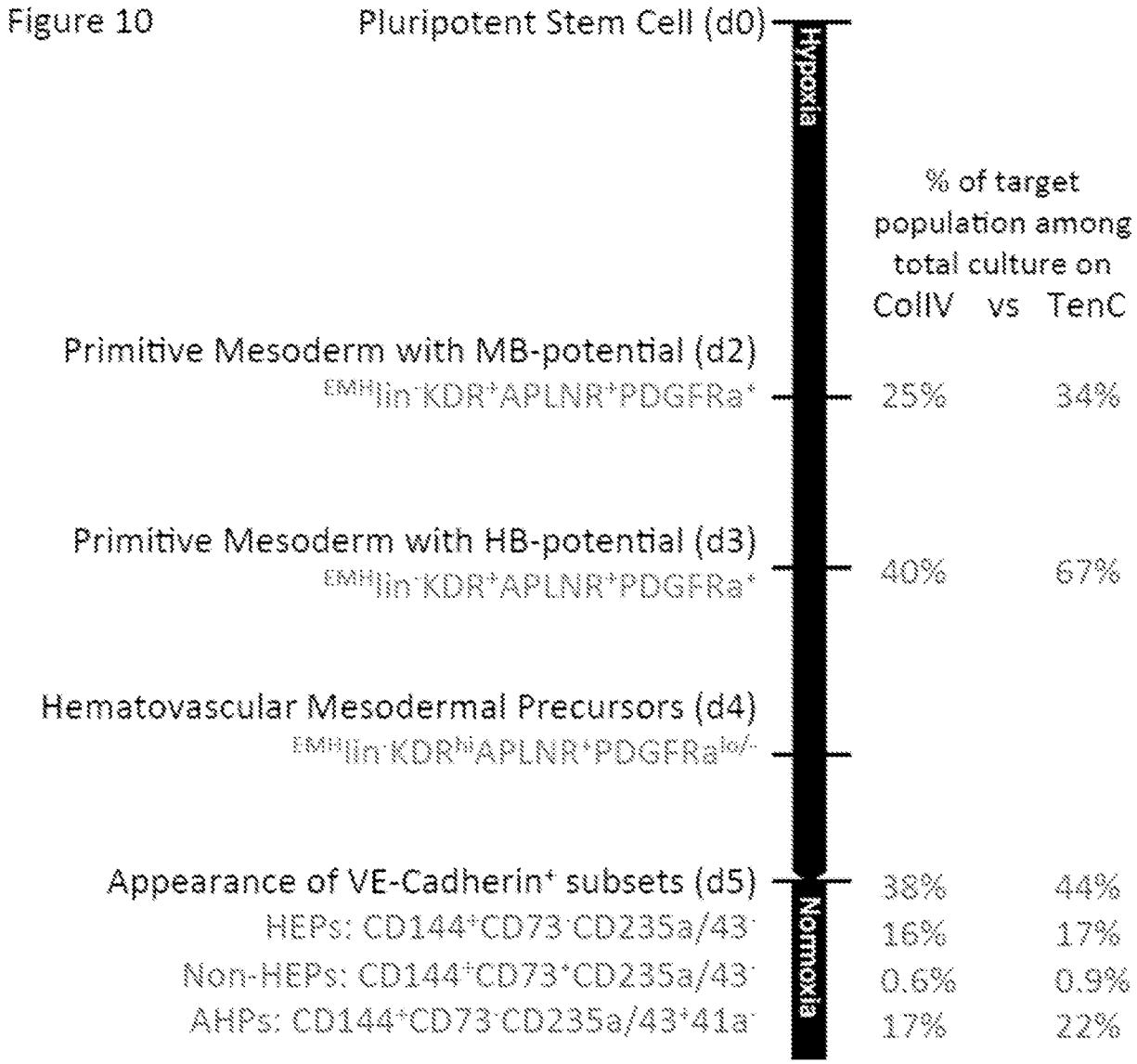
Figure 10
Pluripotent Stem Cell (d0)
Hypoxia
% of target population among total culture on ColIV vs TenC
Primitive Mesoderm with MB-potential (d2)
EMHlin-KDR+APLNR+PDGFRa+
25%
34%
Primitive Mesoderm with HB-potential (d3)
EMHlin-KDR+APLNR+PDGFRa+
40%
67%
Hematovascular Mesodermal Precursors (d4)
EMHlin-KDRMAPLNR+PDGFRalo/-
Appearance of VE-Cadherin+ subsets (d5)
38%
44%
HEPs: CD144+CD73-CD235a/43-
16%
17%
Non-HEPs: CD144+CD73+CD235a/43-
0.6%
0.9%
AHPs: CD144+CD73-CD235a/43+41a-
17%
22%
Normoxia

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024518

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0789 C12N5/0775 C12N5/071 C12N5/00 C12N5/095
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KYUNG-DAL CHOI ET AL: "Identification of the Hemogenic Endothelial Progenitor and Its Direct Precursor in Human Pluripotent Stem Cell Differentiation Cultures", CELL REPORTS, vol. 2, no. 3, 1 September 2012 (2012-09-01), pages 553-567, XP55047737, ISSN: 2211-1247, DOI: 10.1016/j.celrep.2012.08.002 page 558, column 1, last paragraph; table 1 ----- -/--	1-40

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2014	24/07/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Novak-Giese, Sabine

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024518

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	AKIRA NIWA ET AL: "A Novel Serum-Free Monolayer Culture for Orderly Hematopoietic Differentiation of Human Pluripotent Cells via Mesodermal Progenitors", PLOS ONE, vol. 6, no. 7, 27 July 2011 (2011-07-27), page e22261, XP055128572, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0022261 page 22261, paragraph 2; figures 1a,b	1-40

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/024518

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PEARSON STELLA ET AL: "The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF", DEVELOPMENT, COMPANY OF BIOLOGISTS, CAMBRIDGE, GB, vol. 135, no. 8, 1 April 2008 (2008-04-01) , pages 1525-1535, XP008092701, ISSN: 0950-1991, DOI: 10.1242/DEV.011767 page 1526, column 2, paragraph 3; figure 1	1-40
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2014/024518

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		JP 2013521806 A	13-06-2013
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