[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, there is provided 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. Disclosed~~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, there is 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 another~~a related~~ aspect, there is 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 another aspect, there is provided a concentrated form of the media described herein. In some embodiments, any of the foregoing media are provided in a concentrated form.

[0011] In another aspect, there is 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 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.

[0012] In another aspect, there is provided ~~herein is~~ 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.

[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] Disclosed ~~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] Disclosed ~~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] Disclosed~~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] Disclosed~~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] Disclosed~~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 another~~a further~~ aspect, there is 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 another aspect, there is provided 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. Disclosed~~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 another~~a further~~ aspect, there is provided~~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 anothera ~~related~~ aspect, there is 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, there is provided 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, sodium selenite, polyvinyl alcohol, Glutamax™, non-essential amino acids (NEAA), chemically defined lipid concentrate, Holo-Transferrin, and insulin. Disclosed~~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 another~~a related~~ aspect, there is 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 another aspect, there is provided a kit comprising the 9S concentrated medium supplement described herein, and one or more of BMP4, Activin A, FGF2, LiCl, SCF, TPO, IL-6, IL-3, and Tenascin C. 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 another~~further~~ aspect, there is 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.