METHOD FOR DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELL LINES IN SUSPENSION CULTURE

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of priority of SG provisional application No. 10201800488W, filed 18 January 2018, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[002] The present invention relates generally to the field of molecular biology. In particular, the present invention relates to a method of cell differentiation.

BACKGROUND OF THE INVENTION

[003] Erythroid differentiation of human induced pluripotent stem cells (hiPSCs) has been proposed as a means for generating limitless supply of red blood cells (RBCs). For this to be a reality, scalable suspension culture differentiation methods have to be developed. The erythroid differentiation of human pluripotent stem cells (hPSCs) expanded in agitation microcarrier (MC) suspension culture using a bone morphogenetic protein-4 (BMP4)-based differentiation protocol had been previous shown, wherein mesoderm induction and erythroblast expansion were done in static condition. However, repeated attempts to implement this process to differentiate multiple hPSC lines demonstrated variability in erythroid differentiation.

[004] Universal O-negative (neg) red blood cells (RBCs) can be derived from differentiation of human induced pluripotent stem cells (hiPSCs) generated from donors with O-negative blood type. Red blood cells generated from human induced pluripotent stem cells can potentially serve as a limitless source of cells to supplement the emergency transfusion needs of healthcare industry. Given that each unit of blood requires one trillion red blood cells, there is a need to develop efficient differentiation and bioprocesses that could allow for the generation of increased numbers of red blood cells. While many means to differentiate human induced pluripotent stem cells into erythroid cells have been described, these have not yet been demonstrated to be capable of up-scaling.

SUMMARY

[005] In one example, the present disclosure refers to a method of differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under suspension agitation, and wherein a GSK-3-inhibitor or a Wnt pathway activator is added during a stage of mesoderm induction. In one aspect, the present disclosure refers to a method of differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under continuous suspension agitation, wherein the method comprises use of a cell culture media during the mesoderm induction stage, the cell culture media comprising

- BMP4,
- CHIR99021,
- Activin A, and
- $VEGF_{165}$.

[006] In another example, the present disclosure refers to a cell culture media for differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB), the cell culture media comprising a

bone morphogenic protein, a GSK-3 kinase inhibitor, wherein the inhibitor is selected from the group consisting of CHIR99021, (2'Z,3'E)-6-Bromoindirubin-3'-oxime (Bio; CAS 667463-62-9), Kenpaullone (CAS 142273-20-9), GSK-3beta Inhibitor XII (TWS119; CAS 601514-19-6), Bio-Acetoxime (CAS 667463-85-6), CHIR-98014, SB216763 (CAS 280744-09-4), GSK-3beta Inhibitor VIII (CAS 487021-52-3) and combinations thereof, or a Wnt pathway activator, Activin A, and a vascular endothelial growth factor.

[007] In yet another example, the present disclosure refers to a cell culture media for differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB) or pluripotent stem cells, the cell culture media comprising a bone morphogenic protein, Activin A, and a vascular endothelial growth factor.

[008] In a further example, the present disclosure refers to a cell culture media for differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB) or pluripotent stem cells, the cell culture media comprising a bone morphogenic protein, Activin A, Bfgf (basic fibroblast growth factor) or variant thereof, a hormone, a cytokine, and a vascular endothelial growth factor.

[009] In yet another example, the present disclosure refers to a method for differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under suspension agitation, the method comprising a. (optionally) providing pluripotent stem cells; b. exposing the cells of step a. to the cell culture medium as defined herein for 24 hours (day 0 to day 1), thereby resulting in T-Brachyury (T-Bra; primitive streak/early mesoderm marker) positive cells; c. exposing the cells of step b. to the cell culture medium as disclosed herein for 24 hours (day 1 to day 2); d. exposing the micro-carrier attached cells of step c. to the cell culture medium as disclosed herein for 48 hours (day 2 to day 4), whereby steps b. to d. induce mesoderm induction; e. removing the cell culture medium, and isolating the resulting KDR+PDGFRα-hematopoietic precursor cells of step d. In another aspect, the present disclosure refers to a method for differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under continuous suspension agitation, the method comprising a. exposing pluripotent stem cells to a cell culture medium for 24 hours (day 0 to day 1), thereby resulting in T-Brachyury (T-Bra; primitive streak/early mesoderm marker) positive cells; b. exposing the cells of step a. to a further cell culture medium for 24 hours (day 1 to day 2); c. exposing the micro-carrier attached cells of step b. to another further cell culture medium for 48 hours (day 2 to day 4), whereby steps a. to b. induce mesoderm induction; d. removing the cell culture medium, and isolating the resulting $KDR^{+}PDGFR\alpha$-hematopoietic precursor cells of step c; wherein the cell culture medium of step a is for initiating differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB), the cell culture media comprising - BMP4, - CHIR99021,

- Activin A, and - $VEGF_{165}$; wherein the cell culture medium of step b is for initiating differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB) or pluripotent stem cells, the cell culture media comprising - a bone morphogenic protein, - Activin A, and - a vascular endothelial growth factor; wherein the cell culture medium of step c is for differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB) or pluripotent stem cells, the cell culture media comprising - a bone morphogenic protein, - Activin A, - bFGF (basic fibroblast growth factor) or variant thereof, - a hormone, - a cytokine, and - a vascular endothelial growth factor.

[0010] In another example, the present disclosure refers to a method for differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under suspension agitation, the method comprising optionally providing pluripotent stem cells; inducing mesoderm induction in the pluripotent stem cells isolated from step a. according to the method as disclosed herein, thereby resulting in KDR+PDGFRα- hematopoietic precursor cells; inducing hematopoietic induction in the cells isolated from step b, thereby resulting in CD34/CD43/CD45 hematopoietic progenitor cells; inducing erythroblast expansion in the cells isolated from step c, thereby resulting in CD235a+CD71+ erythroblast cells; inducing erythroblast maturation in the cells isolated from step d, thereby resulting in CD235a+ DRAQ5-ve enucleated erythroblast cells; removing the cell culture medium, and isolating the resulting CD235a+ DRAQ5-ve enucleated erythroblast cells of step e.

[0011] In a further example, the present disclosure refers to a kit comprising micro-carriers and the cell culture media as disclosed herein.

CLAIMS

1. A method of differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under continuous suspension agitation, wherein the method comprises use of a cell culture media during the mesoderm induction stage, the cell culture media comprising

- BMP4,
- CHIR99021,
- Activin A, and
- $VEGF_{165}$.

2. The method of claim 1, wherein BMP4 is present in a concentration of between 26 to 36 ng/ml, wherein Activin A is present in a concentration of between 35 to 46 ng/ml, wherein CHIR-99021 is present in a concentration of between 8 µM to 14 µM, and wherein $VEGF_{165}$ is present in a concentration of between 48 ng/ml to 51 ng/ml.

3. A method for differentiation of pluripotent stem cells into hematopoietic precursor cells, wherein the method is carried out under continuous suspension agitation, the method comprising

a. exposing pluripotent stem cells to a cell culture medium for 24 hours (day 0 to day 1), thereby resulting in T-Brachyury (T-Bra; primitive streak/early mesoderm marker) positive cells;

b. exposing the cells of step a. to a further cell culture medium for 24 hours (day 1 to day 2);

c. exposing the micro-carrier attached cells of step b. to another further cell culture medium for 48 hours (day 2 to day 4), whereby steps a. to b. induce mesoderm induction;

d. removing the cell culture medium, and isolating the resulting $KDR^{+}PDGFR\alpha^{-}$ hematopoietic precursor cells of step c;

wherein the cell culture medium of step a is for initiating differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB), the cell culture media comprising

- BMP4,
- CHIR99021,
- Activin A, and
- $VEGF_{165}$;

wherein the cell culture medium of step b is for initiating differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB) or pluripotent stem cells, the cell culture media comprising

- a bone morphogenic protein,
- Activin A, and
- a vascular endothelial growth factor;

wherein the cell culture medium of step c is for differentiation of pluripotent stem cells into hematopoietic precursor cells, thereby generating hematopoietic precursor cells from pluripotent stem cells using microcarrier embryoid bodies (EB) or pluripotent stem cells, the cell culture media comprising

- a bone morphogenic protein,
- Activin A,
- bFGF (basic fibroblast growth factor) or variant thereof,
- a hormone,
- a further cytokine, and
- a vascular endothelial growth factor.

4. The method of claim 3, wherein BMP4 is present in a concentration of between 26 ng/ml to 36 ng/ml, wherein Activin A is present in a concentration of between 35 to 46 ng/ml, wherein CHIR99021 is present in a concentration of between 8 µM to 14 µM, and wherein $VEGF_{165}$ is present in a concentration of between 48 ng/ml to 51 ng/ml.

5. The method of claim 3, wherein for the cell culture medium of step b: i) the bone morphogenic protein of the cell culture media of step b is BMP4; and/or ii) wherein the vascular endothelial growth factor of the cell culture media of step b is $VEGF_{165}$.

6. The method of claims 3 and 5, wherein the cell culture media of step b comprises BMP4, Activin A, and $VEGF_{165}$.

7. The method of claim 3 and claims 5 to 6, wherein BMP4 is present in a concentration of between 26 to 36 ng/ml, wherein Activin A is present in a concentration of between 35 to 46 ng/ml, and wherein $VEGF_{165}$ is present in a concentration of between 48 ng/ml to 51 ng/ml.

8. The method of claim 3, wherein for the cell culture medium of step c: i) the bone morphogenic protein of the cell culture media of step c is BMP4; ii) wherein the hormone is beta-estradiol; iii) wherein the basic fibroblast growth factor (bFGF), or variant thereof, is a heat-stable chimeric variant of bFGF or a stable chimeric fibroblast growth factor (FGF); iv) wherein the further cytokine is stem cell factor (SCF); and/or v) wherein the vascular endothelial growth factor is $VEGF_{165}$.

9. The method of claims 3 and 8, wherein the cell culture media of step c comprises BMP4, Activin A, bFGF, beta-estradiol, SCF and $VEGF_{165}$.

10. The method of claim 3 and claims 8 to 9, wherein BMP4 is present in a concentration of between 18 ng/ml to 27 ng/ml, wherein Activin A is present in a concentration of between 3 ng/ml to 7 ng/ml, wherein the bFGF is present in a concentration of between 5 ng/ml to 14 g/ml, wherein the beta-estradiol is present in a concentration of between 0.2 ng/ml to 0.8 ng/ml, wherein the SCF is present in a concentration of between 26 ng/ml to 36 g/ml, and wherein $VEGF_{165}$ is present in a concentration of between 32 to 38 ng/ml.