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 ~~aspect~~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$^+$PDGFRα-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 ~~- a bone morphogenic protein.~~ BMP4, - ~~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~~$VEGF_{165}$; 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
- $VEGF_{165}$

~~and wherein a GSK-3 inhibitor or a Wnt pathway activator is added during a stage of mesoderm induction~~.

~~2. The method of claim 1, wherein the method comprises use of a cell culture media during the mesoderm induction stage, the cell culture media comprising~~

- ~~a bone morphogenic protein,~~
- ~~GSK3 inhibitor, or a Wnt pathway activator,~~
- ~~Activin A, and~~
- ~~a vascular endothelial growth factor.~~

~~3. The method of any of the preceding claims, wherein the GSK3 inhibitor is selected from the group consisting of valproic acid sodium salt, staurosporine, KT 5720 (CAS 108068-98-0), GSK-3 Inhibitor IX (CAS 667463-62-9), Ro 31-8220 (CAS 138489-18-6), SB 216763 (CAS 280744-09-4), CID 755673 (CAS 521937-07-5), Kenpaullone (CAS 142273-20-9), lithium chloride, GSK-3beta Inhibitor XII (TWS119; CAS 601514-19-6), GSK-3 Inhibitor XVI (CAS252917-06-9), 10Z-Hymenialdisine (CAS 82005-12-7), Indirubin (CAS 479-41-4), CHIR-98014 (CAS 252935-94-7), GSK-3beta Inhibitor VI (CAS 62673-69-2), Manzamine A (CAS 104196-68-1), Indirubin-3prime-monoxime (CAS 160807-49-8), GSK-3 Inhibitor X (CAS 740841-15-0), GSK-3 Inhibitor XV, SB-415286 (CAS 264218-23-7), 1-Azakenpaullone (CAS 676596-65-9), TWS-119-ditrifluoroacetate (CAS 601514-19-6), 5-Iodo-indirubin-3' monoxime, GSK-3beta Inhibitor I (CAS 327036-89-5), 9-Cyanopaullone, Indirubin-5-sulfonic acid sodium salt, GSK-3beta inhibitor VII (CAS 99-73-0), Cdk1/5 inhibitor (CAS 40254-90-8), Hymenidin (CAS 107019-95-4), bisindolylmaleimide X hydrochloride (CAS 131848-97-0), 3F8 (CAS 159109-11-2), isogranulatimide (CAS 244148-46-7), CR8, (R) isomer (CAS 294646-77-8) L-779,450 (CAS 303727-31-3), indirubin-3prime-monoxime-5-sulphonic acid (CAS 331467-05-1), GSK-3 Inhibitor II (CAS 478482-75-6), GSK-3beta Inhibitor VIII (CAS 487021-52-3), Aloisine A (CAS 496864-16-5), GSK-3beta Inhibitor XI (CAS 626604-39-5), GSK-3 Inhibitor IX (CAS 710323-61-8), Alsterpaullone, 2-Cyanoethyl (CAS 852529-97-0), TCS 2002 (CAS 1005201-24-0), TCS 21311 (CAS 1260181-14-3), A 1070722 (CAS 1384424-80-9), Ro-31-8220 (CAS 138489-18-6), Enzastaurin (CAS 138489-18-6), MeBIO (CAS 667463-95-8), Cdk2/9 Inhibitor (CAS 507487-89-0), Cdk1/2 Inhibitor III (CAS 443798-55-8), PHA~~

~~767491 hydrochloride (CAS 845714-00-3), AR-AO 14418 d3, Indole-3-acetamide (CAS 879-37-8), Hymenialdisine Analogue 1 (CAS 693222-51-4), CHIR-99021 (also known as 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl] amino]-3-pyridinecarbonitrile and CT99021; CAS 252917-06-9), CHIR-98014 (CAS 556813-39-9), (2'Z,3'E)-6-Bromoindirubin-3'-oxime (Bio; CAS 667463-62-9), Bio-Acetoxime (CAS 667463-85-6), SB216763 (CAS 280744-09-4) and combinations thereof.~~

~~4. The method of any of the preceding claims, wherein the GSK3 inhibitor is CHIR-99021.~~

~~5. The method of any of the preceding claims, wherein the Wnt pathway activator is selected from the group consisting of IQ-1 and Wnt3a.~~

~~6. The method of any one of claims 2 to 5, wherein the bone morphogenic protein is BMP4.~~

~~7. The method of any one of claims 2 to 6, wherein the vascular endothelial growth factor is $VEGF_{165}$.~~

~~8~~2. The method of claim ~~any of claims 2 to 7~~ 1, ~~wherein the cell culture media comprises BMP4, Activin A, CHIR99021 and $VEGF_{165}$; optionally,~~ 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.

~~9~~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

- ~~a bone morphogenic protein~~BMP4,
- ~~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~~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.

~~10. The method of claim 9, wherein cell culture medium of step a is the cell culture medium as defined in any one of claims 2 to 8.~~

~~11. The method of any one of claims 9 to 10, wherein for the cell culture medium of step a: i) the bone morphogenic protein of the cell culture media of step a is BMP4; ii) wherein the Wnt pathway activator of the cell culture media of step a is selected from the group consisting of IQ-1 and Wnt3a; and/or iii) wherein the vascular endothelial growth factor of the cell culture media of step a is VEGF$_{165}$.~~

~~12. The method of any one of claims 9 to 11, wherein the cell culture media of step a comprises BMP4, Activin A, CHIR99021 and VEGF$_{165}$.~~

~~13~~4. The method of claim~~s~~ 3 ~~9 to 12~~, 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.

~~14~~5. The method of claim ~~9~~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 $VEGF_{165}$.

~~15~~6. The method of claims ~~9~~ 3 and ~~14~~5, wherein the cell culture media of step b comprises BMP4, Activin A, and $VEGF_{165}$.~~;~~

~~16~~7. The method of claim~~s~~ ~~9~~ 3 and claims ~~14~~ 5 to ~~15~~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.

~~17~~8. The method of claim ~~9~~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 $VEGF_{165}$.

~~18~~9. The method of claims ~~9~~ 3 and ~~17~~8, wherein the cell culture media of step c comprises BMP4, Activin A, bFGF, beta-estradiol, SCF and $VEGF_{165}$.

~~19~~10. The method of claim~~s~~ ~~9~~ 3 and claims ~~17~~ 8 to ~~18~~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.