Direct Differentiation of Human iPS Cells Towards the Erythroid Lineage

Thesis by

Nihal AlMuraikhi

December 2015

A thesis submitted for the degree of Doctor of Philosophy and the Diploma of membership of Imperial College London

Department of Chemical Engineering, Imperial College London,

London, SW7 2AZ

December 2015

©2015

Nihal AlMuraikhi

All right reserved.

Abstract

Pluripotent stem cells including induced pluripotent stem (iPS) cells and embryonic stem (ES) cells are known for their distinctive property of indefinite self-renewal in an undifferentiated status, with the potential to differentiate into all types of cells. Current protocols used in the differentiation of human iPS cells and human ES cells towards erythropoiesis utilize two main approaches: (1) embryoid body (EB) formation, which influences heterogeneity of the produced population, and/or (2) co-culture with mouse stromal cells, where obstacles of purification of the cells rise, which makes the xeno-free culture requirement difficult to achieve, in addition to the cytokine supplements. Moreover, these protocols reported low efficiency in number and functionality, especially with human iPS cells, and required long culture times. One of the major challenges in erythroid cell production from human ES/iPS cells is achieving full maturation and enucleation of erythroid cells in serum-free and feeder-free condition, in order to ensure a completely xeno-free culture condition suitable for clinical applications.

In this study, we have designed a novel protocol for direct differentiation of human iPS cells towards erythroid cells under serum-free conditions bypassing the EB formation step without requiring co-culture. Our protocol involves three steps: (1) hematopoietic/erythropoietic induction, followed by (2) erythroid differentiation, and finally, (3) erythroid maturation and enucleation. Differentiated cells were separated into normoxia and hypoxia conditions. As early as day 7 of culture, an early hematopoietic marker, CD34, was observed, followed by a high expression of CD45, which is a pan leukocyte marker in parallel to less expression of an early erythroid marker, CD71. Over the culture period, an increase in the expression of the late erythroid marker, CD235a, was monitored, which reached high levels by the end of the 28-day culture protocol. Further studies on functional and morphological analysis using CFU assay showed that the cell population on day 14 were able to form erythroid progenitor colonies, i.e. BFU-Es. Immunocytochemical staining showed the presence of heme-containing proteins, which was later confirmed by globin expressions by qPCR. Interestingly, staining with new methylene blue confirmed reticulocyte morphology, which indicated that partial maturation was achieved. Hypoxia condition is a key regulator for erythropoiesis and haemoglobin formation, as indicated by the BFU-Es formed under hypoxic conditions,

together with formation of adult-type haemoglobin, as shown in qPCR. Further studies on maturation of those cells are required in order to achieve fully mature and functional erythroid cells phenotype. This thesis thus presents a direct differentiation protocol toward erythroid cells using human iPS cells in serum-free and feeder-free system, bypassing EB-stage and resulting on high efficiency of erythroid cells formation within 4 weeks of culture, which include partial maturation and formation of adult-type haemoglobin.

Declaration

The work described in this thesis was carried out in the Department of Chemical Engineering, Imperial College London, United Kingdom between October 2010 and December 2015. Except where acknowledge, the material is the original work of the author and includes nothing, which is the outcome of work in collaboration, and no part of it, has been submitted for a degree at any other university.

'The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work'

Acknowledgement

All praise to God Almighty, the Merciful and the Benevolent. Had it not been due to His will and favour, the completion of this study would not have been possible.

This thesis completed in part out of 5 years of research and by that time, I have worked with a great helpful people whose contribution, in various ways, to this research and the writing of the thesis deserved special remark. It is a pleasure to convey my gratitude to each and every one of them.

First and foremost, I offer my sincerest appreciation to my supervisor, Professor. Athanasios Mantalaris for his valuable provision, guidance, support and constant encouragement towards the completion of this work with patience and knowledge whilst encouraging me to work in my own way.

While pursuing this work, I gained extraordinary skills and experiences, learnt new practices and techniques and, more notably, worked with a large group full of intellectual experts.

Special thanks go to my fellow seniors, Dr. Cristobal Aguilar, Dr. Iliana Fauzi, Dr. Spyridon Vernardis, Dr. Eirini Velliou, Dr. David Yeo, and Dr. Maria Rende, whom I have learned many talents and knowledge from. I have adored many engaging discussions with, brainstorming thoughts, and performing exciting experiments jointly with them. They have not only been wonderful colleagues but also great friends who will always be remembered.

To all the supporting staff in the Department of Chemical Engineering, in specific, a big thank you goes to Mrs. Sarah Payne, Mrs. Susi Underwood, Mrs. Vani Thuvaragan, Mr. Keith Walker and Mr. Ben Kistnah who have cooperated with me a lot during my time in Imperial College London.

To Mrs. Catherine Simpson from flow cytometry facility for her guidance on the flow cytometry analysis, thank you.

To Mr. Stephen Rothery from the facility for imaging by light microscopy, for his guidance on the imaging analysis, thank you.

To Ms. Yvoon Daniel and Ms. Katy Sanchez from haematology laboratory, Guy's Hospital for their help with O_2 Equilibrium Curve, thank you.

Not forgetting my fellow friends from the Biological System Engineering Lab group; Dr. Osama Fallata who helped me a lot with flow cytometry techniques, Dr. Chonlatep Usaku who always be my best buddy, Dr. David Garcia Munzer, Dr. Iro Tsipa, Dr. Maria Fuentes Gari, Maria Papathanasiou, Naveed Tariq, and many more, thank you for your friendship, counsels assistance, and willingness to share the positive thoughts with me, which helped me shaping up my project.

I would like to acknowledge The Ministry of Higher Education Saudi Arabia, Saudi Arabia Cultural Bureau in London, and King Saud University for their financial support.

I have been always lucky and very fortunate in receiving advices from a number of my friends whom it would not be possible to name them all. However, I wish to express my sincere thankfulness to them who are always with me to share my ups and downs.

To my life-time friends, my siblings; Abdullah, Walaa, Abdulaziz, Mohammed, Yazeed, Anas, and Layan, I cannot imagine life without them and I would like to thank them for their continues support, love, time and effort they have given to me. They will always be my sunshine; I will always love them deep in my heart.

Finally, and above all, my deepest gratitude and love goes to my parent; Msr. Naeemah AlThumairi and Mr. Ahmed AlMuraikhi, who are the greatest parent someone could ever ask for who made me the person I am today. For that, I am forever in debt to them. I thank my parent for their unwavering support, fountain of wisdom, selfless love, and ultimate belief in me. This thesis is dedicated to the love of my life, my parents.

International Conference Presentations

1. An Oral presentation titled: “Production of Red Blood Cells from Human Induced Pluripotent Stem Cells in a Bioreactor" at the XI International Congress of Saudi Society of Hematology, which took place in King Fahad Cultural Centre, Riyadh, Saudi Arabia 26-29 November 2012.

2. A poster titled: “A Novel System to enhance the Production of Erythroid Cells from Human Induced Pluripotent Stem Cells using HIF-1α" at the 43rd Annual Scientific Meeting of the ISEH, International Society for Experimental Hematology, which took place in Montréal, Canada 21-24 August 2014.

3. An oral presentation titled: “Direct differentiation of hiPSCs towards Erythroid cells under Hypoxia condition without EB formation and Co-culture system” at FIRM, Future Investigators of Regenerative Medicine, which took place in Girona, Spain, 8-11 September 2014.

4. A poster titled: “A Novel System to enhance the Production of Erythroid Cells from Human Induced Pluripotent Stem Cells using HIF-1α" at IBC, Bioprocessing International Conference and Exhibition, which took place in Boston, USA, 21-23 October 2014.

5. A poster titled: “Production of Erythroid Cells from Human Induced Pluripotent Stem Cells using Hypoxia” at ISSCR, International Society for Stem Cell Research “Global Controls in Stem Cells” Regional Forum, which took place in Singapore, 5-7 November 2014.

6. An oral presentation titled: “A Direct Differentiation of hiPSCs towards Erythroid cells under Serum-Free and Hypoxia Condition” at the 4th International Conference on Tissue Science & Regenerative Medicine, which took place in Rome, Italy, 27-29 July 2015.

Table of Content

Abstract	3
Declaration	6
Acknowledgements	7
International Conference Presentations	9
List of Figures	15
List of Tables	19
Chapters	
1. Introduction	20
2. Literature Review	25
2.1. Haematopoiesis	26
2.1.1. Hematopoietic Stem Cells and Hematopoietic Niche	26
2.1.2. Haematopoiesis Process	27
2.1.3. Factors Required For Haematopoiesis	31
2.1.4. Extracellular Matrix	33
2.2. Erythropoiesis	33
2.2.1 Erythropoiesis Process	33
2.2.2. Factors Required for Erythropoiesis	37
2.3. Therapeutic Need for an Alternative Blood Supply	41
2.4. Stem Cells	42
2.5. Generation of Mature Erythrocytes	46
2.5.1. Erythrocytes Generated from Multipotent Stem Cells	46
2.5.2. Erythrocytes Generated from Pluripotent Stem Cells	50
2.5.2.1. Erythrocytes Generated from Embryonic Stem Cells	50
2.5.2.2. Erythrocytes Generated from Induced Pluripotent Stem Cells	53
2.6. Terminal Maturation and Enucleation of Erythroid Cells	60
3. Aim and Objectives	62
4. Materials and Methods	65
4.1. Culture Media Materials	66
4.1.1. Human iPS Cells Maintenance Medium	66
4.1.2. Hematopoietic/Erythroid Induction Medium	66
4.1.3. Erythroid Differentiation Medium	66
4.1.4. Erythroid Maturation/Enucleation Medium	67

4.2. Cell Culture Methods	67
4.2.1. Human iPS Cells Maintenance	67
4.2.1.1. Aliquoting Corning® Matrigel®-hESC-Qualified Matrix	68
4.2.1.2. Thawing an Aliquot and Coating Plates with Corning® Matrigel®	68
4.2.1.3. Thawing Human iPS Cells on Corning® Matrigel® Matrix	69
4.2.1.4. Passaging Human iPS Cells	69
4.2.1.5. Freezing Human iPS Cells	70
4.2.2. Human iPS cells Differentiation towards the Erythroid Lineage	71
4.3. Cellular Analysis	71
4.3.1. Erythrosin-B Exclusion Assay for Cellular Growth	71
4.3.2. Cell Proliferation Assay (MTS Assay)	72
4.3.3. Morphological Analysis	72
4.3.3.1. Wright-Giemsa Staining	72
4.3.3.2. Reticulocyte Staining (New Methylene Blue)	73
4.3.4. Clonogenic Ability- Colony Forming Unit (CFU) Assay	73
4.3.5. Immunocytochemical Staining	74
4.3.6. Alkaline Phosphatase	76
4.3.7. Flow cytometry	76
4.3.8. Gene Expression Analysis	77
4.3.8.1. RNA Isolation and Quantification	77
4.3.8.2. cDNA Formation By Reverse Transcriptase (RT)	78
4.3.8.3. Real-time Quantitative Polymerase Chain Reaction (qPCR)	78
4.3.9. O_2 Equilibrium Curve	81
4.4. Statistical Analysis	81
5. Human iPS Cells Culture and Maintenance	82
5.1. Introduction	83
5.2. Aim and objectives	84
5.3. Experimental plan	85
5.4. Results	89
5.4.1. Morphology of Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium	89
5.4.2. Growth Proliferation Study of Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium	89
5.4.3. Immunocytochemistry for Pluripotency Markers	92

5.4.4. Alkaline Phosphatase	95
5.4.5. Flow Cytometry	96
5.4.6. Gene Expression and qPCR	98
5.4.7. Metabolic Analysis of Maintenance Culture Medium	98
5.4.7.1. Nutrient consumption rates (glucose and glutamine)	100
5.4.7.2. Metabolite concentration profiles (lactate and ammonia)	100
5.4.7.3. pH Variations Level	102
5.5. Discussion	103
5.6. Conclusion	106
6. Optimizing the Differentiation of Human iPS Cells towards the Erythroid Lineage	107
6.1. Introduction	108
6.1.1. Factors Regulating Early-Stages of Erythropoiesis	108
6.1.2. Approaches to Differentiate Human ES/iPS Cells: Single Cells, EB Formation, or Monolayer Differentiation	109
6.1.3. The Use of Extracellular Matrix for Hematopoietic/Erythropoietic Differentiation of Human ES/iPS Cells	110
6.1.4. The Role of O_2 on Erythropoiesis	110
6.2. Aim and Objectives	112
6.3. Experimental Plan	113
6.3.1. Effect of Recombinant Human FGF-basic Concentration	114
6.3.2. Effect of Recombinant Human BMP-4 Concentration	114
6.3.3. Effect of Recombinant Human VEGF-A165 Concentration	114
6.3.4. Effect of Serum	115
6.3.5. Viability of Human iPS Cells during Haematopoietic/Erythropoietic Differentiation in Colonies vs Single Cell	115
6.3.6. Efficiency of Haematopoietic/Erythropoietic Differentiation of Human iPS Cells in Colonies vs Single Cell at Different Time Points	116
6.4. Results	116
6.4.1. Effect of Recombinant Human FGF-basic Concentration	116
6.4.2. Effect of Recombinant Human BMP-4 Concentration	117
6.4.3. Effect of Recombinant Human VEGF-A165 Concentration	118
6.4.4. Effect of Serum	119
6.4.5. Viability of Human iPS Cells during Haematopoietic/Erythropoietic Differentiation in Colonies vs Single Cell	121
6.4.6. Efficiency of Haematopoietic/Erythropoietic Differentiation of Human iPS Cells in Colonies vs Single Cell at Different Time Points	122

6.5. Discussion	124
6.6. Conclusion	127
7. The Effect of O_2 on the Differentiation and Maturation of Human iPS Cells towards the Erythroid Lineage: Normoxia vs Hypoxia	129
7.1. Introduction	130
7.2. Aim and Objectives	131
7.3. Experimental Plan	132
7.3.1. Differentiation of Human iPS Cells towards the Erythroid Lineage	132
7.3.2. Maturation of Human iPS Cells-Derived Erythroid Cells	132
7.3.3. The Effect of O_2 in the Differentiation of Human iPS Cells towards Erythroid Lineage: Normoxia vs Hypoxia	132
7.4. Results	133
7.4.1. Morphology Analysis of Human iPS Cells Differentiated towards the Erythroid Lineage	133
7.4.1.1. Wright-Giemsa Staining of Differentiated Human iPS Cells	133
7.4.1.2. Reticulocyte Staining of Differentiated Human iPS Cells	134
7.4.2. Flow Cytometry Analysis of Differentiated Human iPS Cells	135
7.4.3. Clonogenic Ability of Differentiated Human iPS Cells in CFU Assay	145
7.4.4. Immunocytochemical Staining of Differentiated Human iPS Cells	149
7.4.5. Gene Expression and qPCR of Differentiated Human iPS Cells	161
7.4.6. Viability of Differentiated Human iPS Cells	163
7.4.7. Haemoglobin Analysis of Differentiated Human iPS Cells	164
7.4.7.1. Immunocytochemical Staining	164
7.4.7.2. Gene Expression and qPCR	167
7.4.8. Metabolic Analysis of Erythroid Differentiation Culture Medium	168
7.4.8.1. Nutrient consumption rates (glucose and glutamine)	168
7.4.8.2. Metabolite concentration profiles (lactate, glutamate, and ammonia)	170
7.4.8.3. pH variations level	171
7.5. Discussion	172
7.6. Conclusion	183
8. Conclusion and Future Plans	185
8.1. Conclusion	186
8.2. Future Plans	187
Appendix	190

Appendix 1. Optimizing the Concentration of Recombinant Human FGF-basic for the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	191
Appendix 2. Optimizing the Concentration of Recombinant Human BMP-4 for the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	192
Appendix 3. Optimizing the Concentration of Recombinant Human VEGF-A165 for the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	193
Appendix 4. Effect of Serum on the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	194
Appendix 5. Hematopoietic/Erythropoietic Differentiation of Human iPS Cells in Colonies vs Single Cells	195
Appendix 6. The Efficiency of Hematopoietic/Erythropoietic Differentiation of Human iPS Cells at Different Time Points	196
Appendix 7. Flow Cytometry Analysis of SSES4-PE for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	201
Appendix 8. Flow Cytometry Analysis of CD34-FITC and CD45-PE-Cy7 for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	209
Appendix 9. Flow Cytometry Analysis of CD71-APC and CD235a-PE for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	217
Appendix 10. Flow Cytometry Analysis of CD43-APC for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells	225
Appendix 11. Relative Gene Expression and qPCR of Haematopoietic Markers (CD34-CD45) in Differentiated Human iPS cells.	233
Appendix 12. Relative Gene Expression and qPCR of Erythropoietic Markers (CD71-CD235a) in Differentiated Human iPS cells.	234
Appendix 13. Relative Gene Expression and qPCR of Cellular Receptors (c-kit and EPOR) in Differentiated Human iPS cells.	235
Appendix 14. Relative Gene Expression and qPCR of CD43 in Differentiated Human iPS cells.	236
Appendix 15. Relative Gene Expression and qPCR of HIF1α in Differentiated Human iPS cells.	237
Appendix 16. Viability Analysis of Cells Differentiated in Normoxia	238
Appendix 17. Viability Analysis of Cells Differentiated in Hypoxia	239
Appendix 18. O_2 Equilibrium Curve of Differentiated iPS Cells	240
References	242

List of Figures

Figure 1.	Development of different mature blood cells from haematopoietic stem cells with the required Transcription Factors	27
Figure 2.	Sources of primitive and definitive haematopoiesis	28
Figure 3.	Developmental pattern of haemoglobin	30
Figure 4.	The cytokines effect on haematopoiesis at different stages	32
Figure 5.	Erythropoiesis	35
Figure 6.	Stages of erythrocyte maturation	36
Figure 7.	The erythroblastic island	37
Figure 8.	The differentiation potential of different stem cell types	44
Figure 9.	Derivation of hematopoietic cells from human ES cells and iPS cells	54
Figure 10.	A Schematic Diagram Summarizing Coating Plates with Corning® Matrigel® Matrix	86
Figure 11.	A Schematic Diagram Summarizing Passaging Human iPS Cells	87
Figure 12.	A Schematic Diagram Summarizing Freezing Human iPS Cells	88
Figure 13.	Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium at Days 1 to 6	90
Figure 14.	Human iPS Colony Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium at Day 6	91
Figure 15.	Area of Spontaneous Differentiation within Human iPS Colonies	91
Figure 16.	Growth Kinetics of Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium	92
Figure 17.	Immunocytochemistry Staining of Pluripotency-Specific Nuclear Proteins for Human iPS Cells	93
Figure 18.	Immunocytochemistry Staining of Pluripotency-Specific Surface Proteins for Human iPS Cells	94
Figure 19.	Negative Controls for Non-Specific Immunocytochemistry Staining of Pluripotency-Specific Primary Antibodies for Human iPS Cells	95
Figure 20.	AP Activity Staining for Human iPS Cells	96
Figure 21.	The Flow Cytometry Analysis Profile of the Cultured Human iPS Cells	97
Figure 22.	Relative Gene Expression of Pluripotency Markers Observed from Human iPS Cells	99
Figure 23.	Relative Gene Expression of Germ-Layers Markers Observed from Human iPS Cells	99
Figure 24.	Glucose Consumption during Human iPS Cells Maintenance Culture in mTeSR™1 Medium	101

Figure 25.	Glutamine Consumption during Human iPS Cells Maintenance Culture in mTeSR™1 Medium	101
Figure 26.	Accumulation of Lactate during Human iPS Cells Maintenance Culture in mTeSR™1 Medium	102
Figure 27.	Accumulation of Ammonia during Human iPS Cells Maintenance Culture in mTeSR™1 Medium	102
Figure 28.	pH Variations during Human iPS Cells Maintenance Culture in mTeSR™1 Medium	103
Figure 29.	Effect of Different Concentrations of Recombinant Human FGF-basic on the Differentiation of Human iPS Cells	116
Figure 30.	Effect of Different Concentrations of Recombinant Human BMP-4 on the Differentiation of Human iPS Cells	117
Figure 31.	Effect of Different Concentrations of Recombinant Human VEGF-A165 on the Differentiation of Human iPS Cells	118
Figure 32.	Effect of Serum on the Differentiation of Human iPS Cells	119
Figure 33.	A Flow Diagram Summarising the Optimisation Process of Some Early-Acting Cytokines and Serum Addition for the Differentiation of Human iPS Cells Towards the Erythroid Lineage	120
Figure 34.	Comparing the viability of the Differentiated Human iPS Cells in Colonies vs Single Cells	121
Figure 35.	Comparing the Expression of CD71 throughout the Erythroid Differentiation of Human iPS Cells in Colonies vs Single Cells	123
Figure 36.	Comparing the Expression of CD235a throughout the Erythroid Differentiation of Human iPS Cells in Colonies vs Single Cells	123
Figure 37.	Morphological Analysis by Wright-Giemsa staining of Differentiated Human iPS Cells at Different Time Points along the Erythroid Differentiation Protocol	133
Figure 38.	Morphological Analysis by Reticulocyte Staining of Differentiated Human iPS Cells at Different Time Points along the Erythroid Differentiation Protocol	135
Figure 39.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for SSEA4	137
Figure 40.	Representative Dot Plots Flow Cytometry Analysis of Differentiated Human iPS Cells Stained for CD34 and CD45	137
Figure 41.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34	138
Figure 42.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD45	138
Figure 43.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 and CD45 to Detect $CD34^{+}$ $CD45^{-}$ Cells	139

Figure 44.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 and CD45 to Detect $CD34^+$ $CD45^+$ Cells	139
Figure 45.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 and CD45 to Detect $CD34^-$ $CD45^+$ Cells	140
Figure 46.	Representative Dot Plots Flow Cytometry Analysis of Differentiated Human iPS Cells Stained for CD71 and CD235a	140
Figure 47.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71	141
Figure 48.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD235a	141
Figure 49.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 and CD235a to Detect $CD71^+$ and $CD235a^+$	142
Figure 50.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 and CD235a to Detect $CD71^+$ and $CD235a^-$	142
Figure 51.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 and CD235a to Detect $CD71^-$ and $CD235a^+$	143
Figure 52.	The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD43	143
Figure 53.	Clonogenic Capacity of Differentiated Human iPS Cells	147
Figure 54.	Clonogenic Capacity of Differentiated Human iPS Cells at Low Plating Density	148
Figure 55.	Matured Reddish/Brownish BFU-E Colonies Detected at Day 14 of Hypoxia Condition	148
Figure 56.	Negative Controls for Immunocytochemistry Staining of Differentiated Human iPS Cells	149
Figure 57.	Immunocytochemistry Staining for CD34 and CD45 of Differentiated Human iPS Cells under Normoxia Conditions	150
Figure 58.	Immunocytochemistry Staining for CD34 and CD45 of Differentiated Human iPS Cells under Hypoxia Conditions	151
Figure 59.	Immunocytochemistry Staining for CD71 and CD235a of Differentiated Human iPS Cells under Normoxia Conditions	152
Figure 60.	Immunocytochemistry Staining for CD71 and CD235a of Differentiated Human iPS Cells under Hypoxia Conditions	153
Figure 61.	Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD34	154

Figure 62.	Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD45	154
Figure 63.	Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD71	155
Figure 64.	Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD235a	155
Figure 65.	Negative Control for Single Cell Analysis of Immunocytochemistry Signals of Differentiated Human iPS Cells in Normoxia Condition	157
Figure 66.	Negative Control for Single Cell Analysis of Immunocytochemistry Signals of Differentiated Human iPS Cells in Hypoxia Condition	157
Figure 67.	Single Cell Analysis of Immunocytochemistry Signals of CD34 and CD45 of Differentiated Human iPS Cells in Normoxia and Hypoxia Conditions at Different Time Points	158
Figure 68.	Single Cell Analysis of Immunocytochemistry Signals of CD71 and CD235a of Differentiated Human iPS Cells in Normoxia and Hypoxia Conditions at Different Time Points	159
Figure 69.	Relative Gene Expression Analysis of Pluripotency Markers (OCT4-NANOG) in Differentiated Human iPS Cells	162
Figure 70.	Percentage of Viable Cells within Normoxia and Hypoxia Culture groups Along the Erythroid Differentiation	164
Figure 71.	Immunocytochemistry Staining of Human Haemoglobin in Differentiated Human iPS Cells	165
Figure 72.	Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for Human Haemoglobin	166
Figure 73.	Relative Gene Expression Analysis of haemoglobin in Differentiated Human iPS Cells	167
Figure 74.	Glucose Consumption during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol	169
Figure 75.	Glutamine Consumption during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol	169
Figure 76.	Accumulation of Lactate during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol	170
Figure 77.	Accumulation of Glutamate during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol	171
Figure 78.	Accumulation of Ammonia during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol	171
Figure 79.	pH Variations during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol	172
Figure 80.	A Schematic Diagram of the Direct Differentiation of Human iPS Cells Towards the Erythroid Lineage under Serum-Free and Feeder-Free Culture Condition	183

List of Tables

Table 1.	The Different Locations of Haematopoiesis with Times of Initiation of Activity during Development of Mammals	29
Table 2.	Summary of the Most Efficient Protocols in Generating Erythroid Cells from Human Pluripotent Stem Cells	59
Table 3.	List of the Critical Analytical Procedures Used for the Assessments of the Differentiated Human iPS Cells.	64
Table 4.	List of Primary and Secondary Antibodies for Immunocytochemistry Staining	75
Table 5.	List of Antibodies for Flow Cytometry Analysis	77
Table 6.	List of PCR Primers	79
Table 7.	List of Assays Included in the Customized TaqMan® Array 96-well Fast Plates	80
Table 8.	Optimizing the Concentrations of the Main Haematopoietic Inducers and Factors Required for the Haematopoietic/Erythropoietic Induction of Human iPS Cells	126
Table 9.	Comparing the Efficiency of the Haematopoietic/Erythropoietic Differentiation of Human iPS Cells at Different Cell Seeding Conditions and Time Points	127
Table 10.	Our Novel Hematopoietic/Erythropoietic Induction Protocol	128
Table 11.	Percentages of Hematopoietic/Erythroid Markers on Plated Cells for CFU Assay	146

Chapter 1.

Introduction

Blood is one of the most crucial tissues of the body. It plays essential roles in transporting dissolved gases - oxygen (O_2) and carbon dioxide (CO_2)- waste products of metabolism, nutrients, hormones, enzymes, and plasma proteins. Blood can also maintain homeostasis, fight infection, remove toxins, regulate body PH and temperature, and control body fluid electrolytes. It consists of four main components: red blood cells, white blood cells, platelets, and plasma. Blood cellular constitutes are replenished mainly in the bone marrow through a continuous process called haematopoiesis. Haematopoiesis is the process of production and development of blood cells by which they acquire defining characteristic phenotypes as a result of cell-specific gene expression. This process is needed to maintain a certain average number of blood cells in the body in order to ensure body welfare. Failure to maintain that average, owing to blood disorders, surgery, or severe blood loss, will require a blood transfusion.

Blood transfusion is a common clinical practice used to replenish the blood level after a severe loss associated with injuries, surgeries, or haematological diseases (Ganji et al., 2015). According to the World Health Organization (WHO), a principal regulator of the global blood supply, around 107 million units of all blood types are collected annually worldwide (Palmer and Intaglietta, 2014). Yet, blood shortage is one of the major global concerns with several countries reporting insufficient blood supply (Focosi et al., 2014). In developed countries, the average donation rate is 45.4 donations per 1000 population, which accounts for 5% of the population, while it is estimated that 30% of that population will need a life-saving transfusion at some point during their lives (WHO, 2011). Moreover, even with present demand being satisfied, studies predict a deficiency as early as 2050 (Whitsett et al., 2012, Focosi et al., 2014). This significant imbalance comparing to demand creates a threatening shortage. This fact, beside other drawbacks of conventional blood transfusion practices, including the cross-matching requirements, the narrow storage time, and the risk of disease transmission, have encouraged researchers to investigate and develop potential substitutes for donated blood (Lippi et al., 2011, Ebihara et al., 2012). However, the current alternatives have been dissatisfactory (Focosi et al., 2014).

These alternatives include the development of haemoglobin-based O_2 carriers, polymerized haemoglobin solutions, and many other haemoglobin modulators. Although these developments have demonstrated important benefits such as long shelf-life, lack of red blood cell (RBC) antigens, and viral and bacterial sterility, safety, efficacy, and side effects remain major concerns. Thus, research focus has been redirected towards safer options, such as the *in vitro* production of blood that can be achieved using either cellular precursor cells, or synthetic biomaterials. Moreover, *in vitro* production of blood, especially RBCs, appears to be the safest and most adequate process for the generation of group O, Rhesus D-negative RBCs - the universal donor blood (Mountford et al., 2010).

In the last decade, many attempts have been made towards producing RBCs from stem cells. Stem cells are defined as cells able to undergo self-renewal by asymmetric cell division, and, under proper conditions, can give rise to any specialized cells - a feature called potency (Ebihara et al., 2012). RBCs have been produced from different cellular progenitors such as multipotent stem cells; hematopoietic stem cells (HSCs) derived from bone marrow, cord blood, and peripheral blood, pluripotent stem cells; embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells), all of which have shown significant results and generated RBCs with similar morphology and role of the native RBCs with a comparable haemoglobin content (Ebihara et al., 2012). Nonetheless, RBC generation from different sources hold both advantages and limitations (Anstee, 2010).

Indeed, until recently, the use of human ES cells was limited by ethical concerns. These concerns were negated by the breakthrough of iPS cells that resemble human ES cells *in vitro* and *in vivo* (Dorn et al., 2015). Since then, there has been a rising excitement that human iPS cells can provide embryo-free, patient-specific cells suitable for cellular therapy and regenerative medicine, combined with reporting different methods for reprogramming adult somatic cells to pluripotency (Feng et al., 2010). These human iPS cells were first produced by Yu et al. (2007) and Takahashi et al. (2007). These cells can be differentiated into all three embryonic germ layers, as well as progenitors of many cell types (Feng et al., 2010) through a feature called plasticity (Lakshmipathy and Verfaillie, 2005).

Latest research studies have shown derivation of erythroid cells from iPS cells with similar characteristics to those derived from human ES cells (Choi et al., 2009, Lapillonne et al., 2010a, Dias et al., 2011). Thus, although the use of iPS cells still faces many challenges, this technology has demonstrated to be achievable and reproducible, and will lead to safe and efficient clinical applications. In addition, these cells can be used as models of developmental biology and genetics, as well as in the field of toxicology and pharmaceutical testing.

Toward such erythropoietic differentiation of human iPS cells, two main approaches have been developed. The first is the EB formation protocol conditioned with cytokines, with or without serum, to obtain hemangioblasts, followed by subsequent expansion and maturation of erythroid cells produced using erythropoiesis-induced cytokines (Chang et al., 2011). However, the EB formation is associated with the production of a heterogeneous cell population that can be difficult to purify (Fauzi et al., 2015), while the use of serum exposes the cell culture to undefined factors (Lebkowski et al., 1995). The second approach is the co-culture on stromal cells of animal origins to induce haematopoiesis as a step towards producing erythroid cells. However, in this approach, risks of disease transmission and immune reactions arise, thus, making the xeno-free culture requirement difficult to achieve (Dias et al., 2011).

This thesis therefore proposes to deliver an overview of some of the current challenges faced by researchers in generating erythroid cells from human iPS cells, addressing the drawbacks of the current approaches, while providing possible contributions that can be of a significant value in translating iPS cell- derived therapies into clinical applications.

The outline of this thesis will be as follows: Chapter 2 will provide a literature review, introducing the problems and clinically urgent requirements for an alternative blood supply and providing an overview of the limitations and drawbacks of the current clinical practises applied. It will then summarize the *in vivo* process of blood formation during embryogenesis, i.e. haematopoiesis, and red blood cells (erythrocytes) formation, i.e. erythropoiesis. Next will follow a discussion of the state-of-art in erythrocytes production and the different source of cells used for that purpose, as well as the relevant obstacles

and challenges. Finally, studies on terminal maturation of erythroid cells process and ways to approach these issues will be briefly addressed. Chapter 3 will then summarize the requirements of human iPS cells differentiation towards erythroid cells production. It will then present the overall aim and objectives of the thesis. Chapter 4 will present the experimental materials and methods used for human iPS cells maintenance culture, the differentiation protocols applied, as well as the methodologies employed to characterize the cells. Chapter 5 will offer a discussion on the maintenance of human iPS cells culture, as well as the approaches employed to characterize the pluripotent-undifferentiated status of the cells. Chapter 6 will form a description of the optimization of the direct haematopoietic/erythropoietic induction of human iPS cells under serum-free and feeder-free condition in hypoxia. It will then present a comparison between the efficiency of the optimized protocol in single cells and colonies as seeding conditions. Chapter 7 will be used to combine the haematopoietic/erythropoietic induction protocol described in Chapter 6 to further erythroid differentiation and maturation protocols, and assess the role of O_2 on the differentiation of human iPS cells towards the erythroid lineage. This chapter will also include the evaluation of the direct serum-free differentiation of human iPS cells towards erythroid lineage in normoxia and hypoxia conditions. Final thoughts regarding the findings from this thesis as well as future plans will be summarized in Chapter 8, forming the conclusion.

Chapter 2.

Literature Review

2.1. Haematopoiesis

2.1.1. Hematopoietic Stem Cells and Hematopoietic Niche

HSCs are multipotent cells that are at the top of a hierarchy of progenitors that give rise to blood precursors committed to uni-lineage differentiation into mature blood cells, including RBCs, megakaryocytes, monocytes, granulocytes, and lymphocytes (Figure 1). Like all other stem cells, HSCs possess two distinct properties: self-renewal, which is the ability to go through several cell divisions to produce additional HSCs while maintaining the undifferentiated state; and potency, which is the capacity to differentiate to all blood cell types. The hematopoietic niche is the anatomical site where HSCs reside and proliferate (Purton and Scadden, 2008).

Within the haematopoietic niche, HSCs undergo one of three fates: (1) symmetrical division into two cells with the same potential of the mother cell; (2) asymmetrical division into one stem cell, which stays in the niche, and another, which is a committed cell with a uni-lineage differentiation potential; or (3) cell death, which is known as apoptosis. HSCs can only start the process of differentiation to ultimately form mature blood cells outside the niche (Purton and Scadden, 2008). The bone marrow is an example of a haematopoietic site that provides physical support and maintenance to the haematopoietic tissues. It offers a low O_2 environment (hypoxia condition), the optimal condition for HSCs (Kim, 2010).

The bone marrow consists of stromal cells, macrophages, osteocytes, adipocytes, vascular endothelium, and the extracellular matrix, which interacts with haematopoietic cells, expressed and secreted proteins, as well as growth factors in order to ensure survival, proliferation, and differentiation of HSCs (Abboud and Lichtman, 2001, Panoskaltsis et al., 2005).

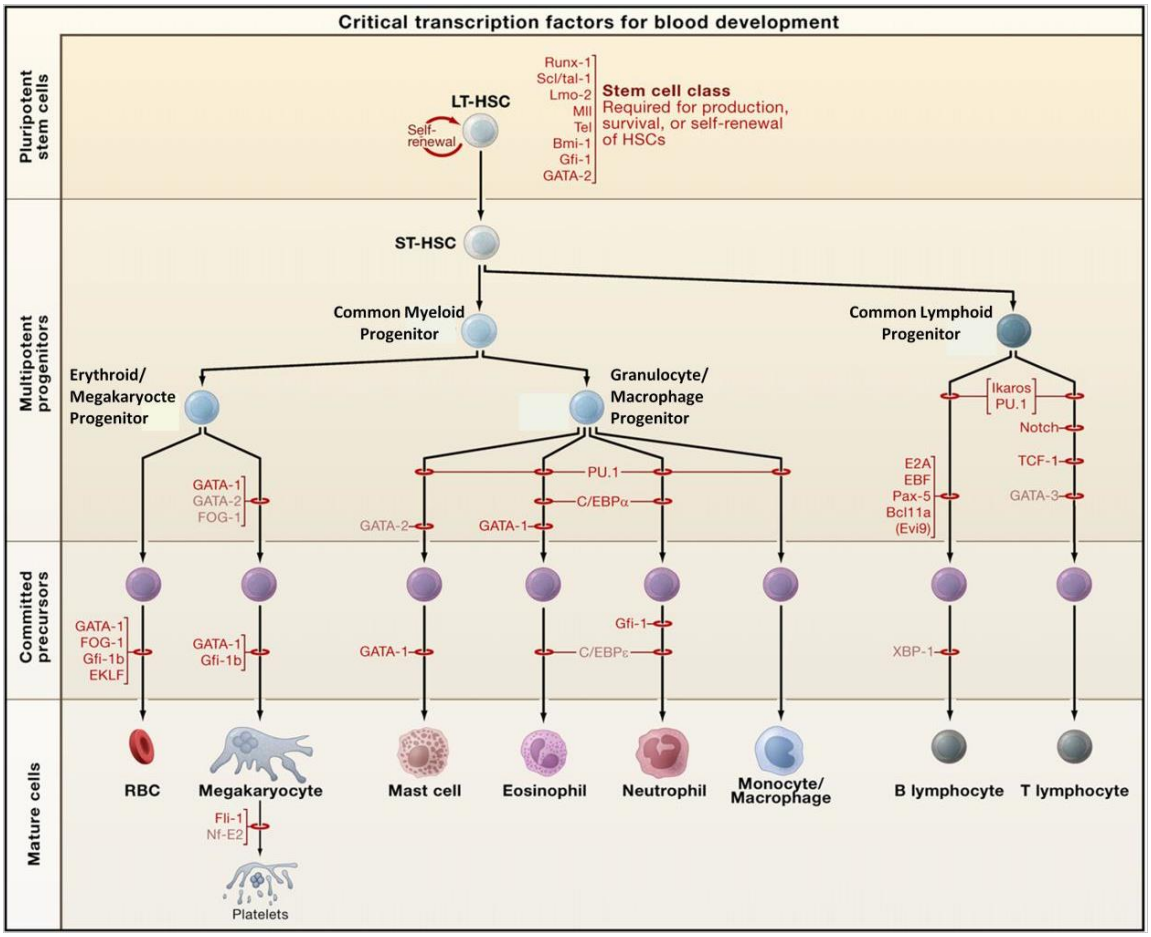


Figure 1. Development of different mature blood cells from haematopoietic stem cells with the required Transcription Factors. (Adapted from (Orkin and Zon, 2008)).

LT-HSC, long-term HSCs; ST-HSC, short-term HSCs.

2.1.2. Haematopoiesis Process

Haematopoiesis is the process of the production and development of blood cells from which they acquire defining characteristic phenotypes as a result of cell-specific gene expression (Shivdasani and Orkin, 1996). Studies in mammals reveal that hematopoietic niches and the processes involved in haematopoiesis change during mammalian development from embryo to adult, depending on age (Mikkola et al., 2005, McGrath and Palis, 2008). The sequential locations of haematopoiesis are the extra-embryonic mesoderm (yolk sac); the para-aorta splanchpleural (P-Sp) region, which later becomes

the aorta-gonad mesonephros (AGM) region; fetal liver; thymus; spleen; and, ultimately, the bone marrow, with some variations of HSCs properties in each location (Orkin and Zon, 2008). Moreover, recent studies have suggested that the placenta is an additional location for haematopoiesis.

The primary blood production is termed “primitive haematopoiesis”, which begins in mesoderm blood islands of the yolk sac soon after gastrulation (Silver and Palis, 1997), around day 7.5 (E7.5) in mice or day 18 in humans (Shivdasani and Orkin, 1996). Here, primitive erythroid colony-forming cells (EryPCFC) give rise to large and nucleated primitive erythroblasts, which express embryonic haemoglobin type ε2δ2, and differentiate into nucleated erythrocytes (Steiner and Vogel, 1973, Ma et al., 2008). Primitive erythroid cells are produced in parallel with endothelial cells from a common precursor termed “hemangioblast” (Lu et al., 2008b). However, this blood production system is transient and is then replaced by a permanent one termed “definitive haematopoiesis” in the fetal liver by the HSCs producing adult definitive erythropoiesis (Kingsley et al., 2004, Ma et al., 2008) (Figure 2).

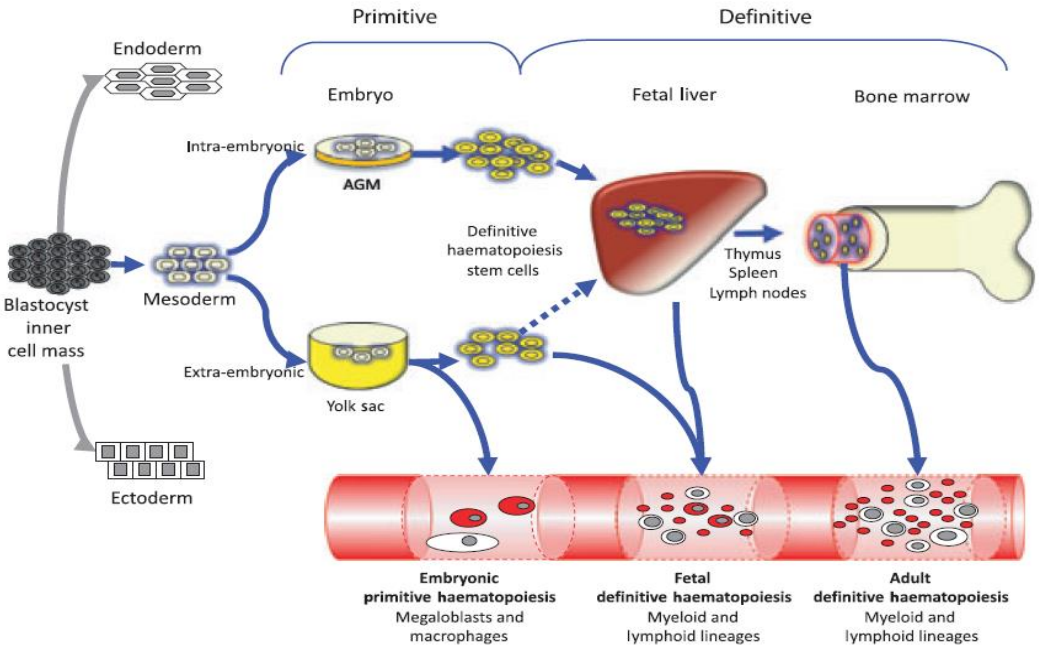


Figure 2. Sources of primitive and definitive haematopoiesis. (Adapted from (Mountford et al., 2010)).

Unlike embryonic haematopoiesis, adult haematopoiesis is considered a lasting source of mature red cells from the continuing repopulating hematopoietic multipotent progenitors in the placenta, the AGM region, then in the fetal liver, and, finally, in the bone marrow (Ema and Nakauchi, 2000, Kumaravelu et al., 2002). Definitive erythrocytes from the fetal liver and bone marrow enter the circulation as enucleated cells and the haemoglobin switches to fetal haemoglobin, haemoglobin F (α2γ2), and perinatally to adult haemoglobin, haemoglobin A (α2β2) (Figure 3) (Lu et al., 2008a, Cantu and Philipsen, 2014). Table 1 shows the different locations of haematopoiesis with times of initiation of activity during development of mammals.

Table 1. The Different Locations of Haematopoiesis with Times of Initiation of Activity during Development of Mammals. (Adapted from (Orkin and Zon, 2008)).

Initiation of Hematopoiesis or Lymphopoiesis (days)	Mouse	Human
Yolk sac	7.5	18
Aortic/AGM region	9.5–10	27
Liver	11	42
Spleen	13	48
Bone marrow	15	77
Thymus	11	40
Bursa of Fabricius	No	No
Onset of circulation	8.2	24

Conventionally, HSCs have been isolated on the basis of the expression of cell surface molecules including CD34 and CD133 (Larochelle et al., 2011). Mature erythrocytes are CD235a (glycophorin A) positive and negative for CD36 and CD71 (transferring receptor) (Marsee et al., 2010). CD71 is an early erythroblast marker, which decreases its expression over time and has a crucial role in the uptake of transferrin-iron complex (Marsee et al., 2010), while CD235a protein is a mature erythrocyte marker closely

associated with the erythrocyte membrane and mediates the expression of O-linked antigens on the erythrocyte membrane protein (Lu et al., 2009). CD36 is an adhesion molecule of monocytes, platelets, endothelial cells, and immature erythroid cells (Lee et al., 2001).

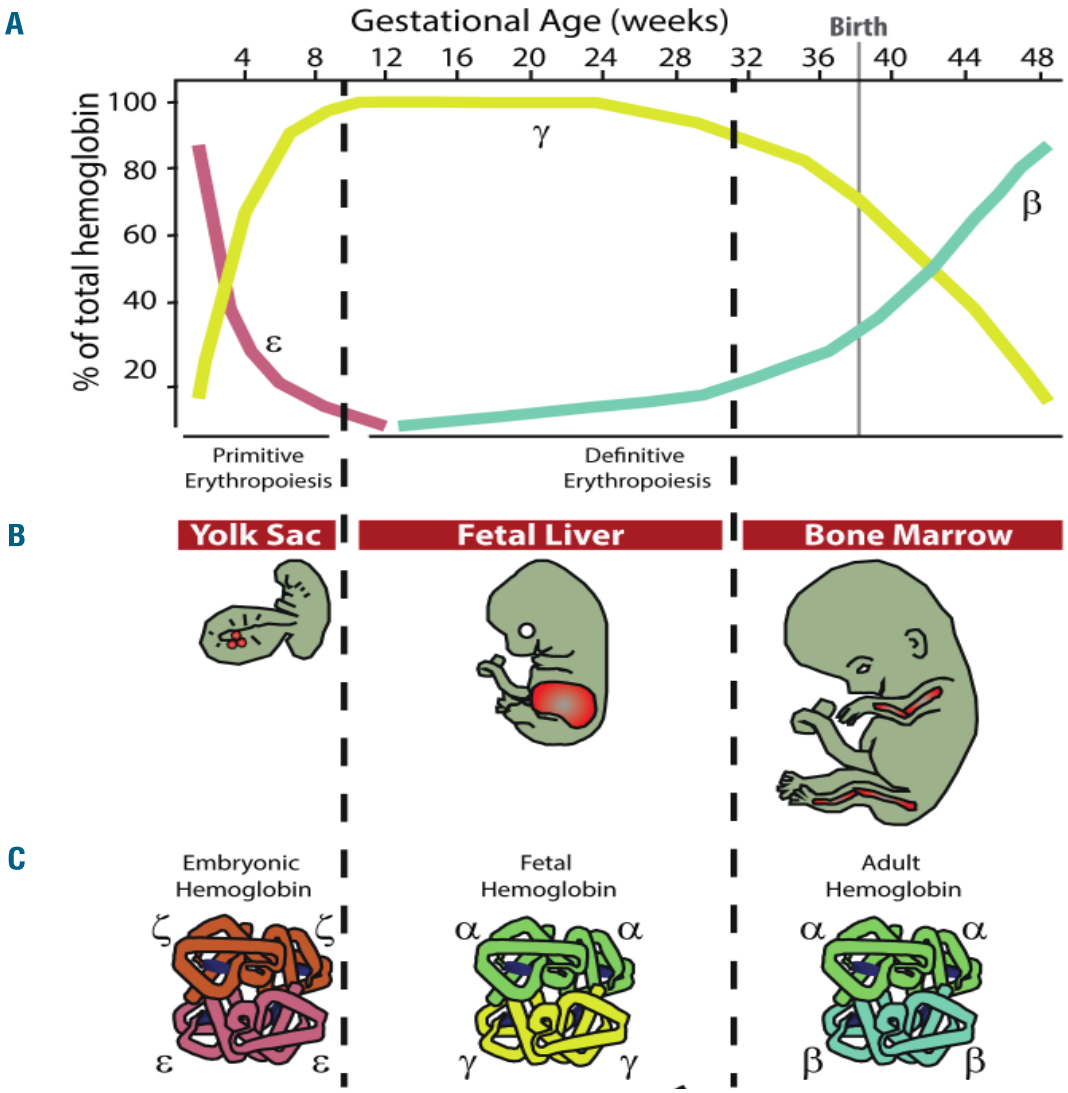


Figure 3. Developmental pattern of haemoglobin. (Adapted from(Cantu and Philipsen, 2014)).

(A) About week 6 of gestation, embryonic globin (ε) drops and fetal globin (γ) starts to express. The switch to adult globin (β) occurs during perinatal period. (B) Main anatomical sites of hematopoiesis during development. (C) Structural configuration of main human haemoglobins types expressed during development.

2.1.3. Factors Required For Haematopoiesis

Haematopoiesis is a complicated process regulated by several factors. The bone marrow niche is composed of different regulatory cells with a positive and negative effect on the process, including osteoblasts, CXC chemokine ligand 12 (CXCL12)-expressing reticular cells, and vascular endothelium cells (Kim, 2010). Various molecular signaling pathways are key controllers of the hematopoiesis, including Wnt, calcium-sensing receptors, angiopoietin 1, and Tie-2 (Kim, 2010, Murry and Keller, 2008). Within the niche, these pathways are involved in the maintenance of stem cell niche, and proliferation and differentiation of HSCs. However, the pathways' regulatory mechanisms have not yet been completely identified (Kim, 2010).

Several cytokines are also key regulators of the early hematopoietic progenitors, such as stem cell factor (SCF), notch ligands, bone morphogenic proteins (BMPs), transforming growth factor-β, thrompopoietin (TPO), fibroblast growth factors (FGFs), and insulin-like growth factors II (IGF-II) (Kim, 2010) (Figure 4). Other cytokines are key regulators of the differentiation of progenitor cells to committed cell lineages, such as granulocyte colony stimulating factors (G-CSF), granulocyte macrophage (GM)-CSF, interleukin-3 (IL-3), IL-7, erythropoietin (EPO), and macrophage (M)-CSF (Kim, 2010) (Figure 4).

In addition, activins have been identified for the activation of Nodal pathway, which is an important molecular signaling for mesoderm formation (Watabe and Miyazono, 2009). Recent studies have highlighted that BMP-4, SCF, and IL-3 facilitate the proliferation and differentiation of adult hematopoietic progenitors (Kassouf et al., 2008).

Furthermore, GATA-2 gene, a member of GATA family, expresses in HSCs and plays an important role in self-renewal and differentiation of early hematopoietic progenitors. During erythroid differentiation, a down-regulation of GATA-2 occurs together with an up-regulation of GATA-1, an erythroid-specific gene (Chen et al., 2014a). AML1/Runx1 is another key gene regulating primitive and definitive hematopoiesis; mutations of AML1/Runx1 inhibit hematopoiesis and lead to embryo death (Chen et al., 2014a). c-Myb gene controls the proliferation of HSCs and is highly expressed in early hematopoietic

cells while down-regulated as they differentiate (Chen et al., 2014a).

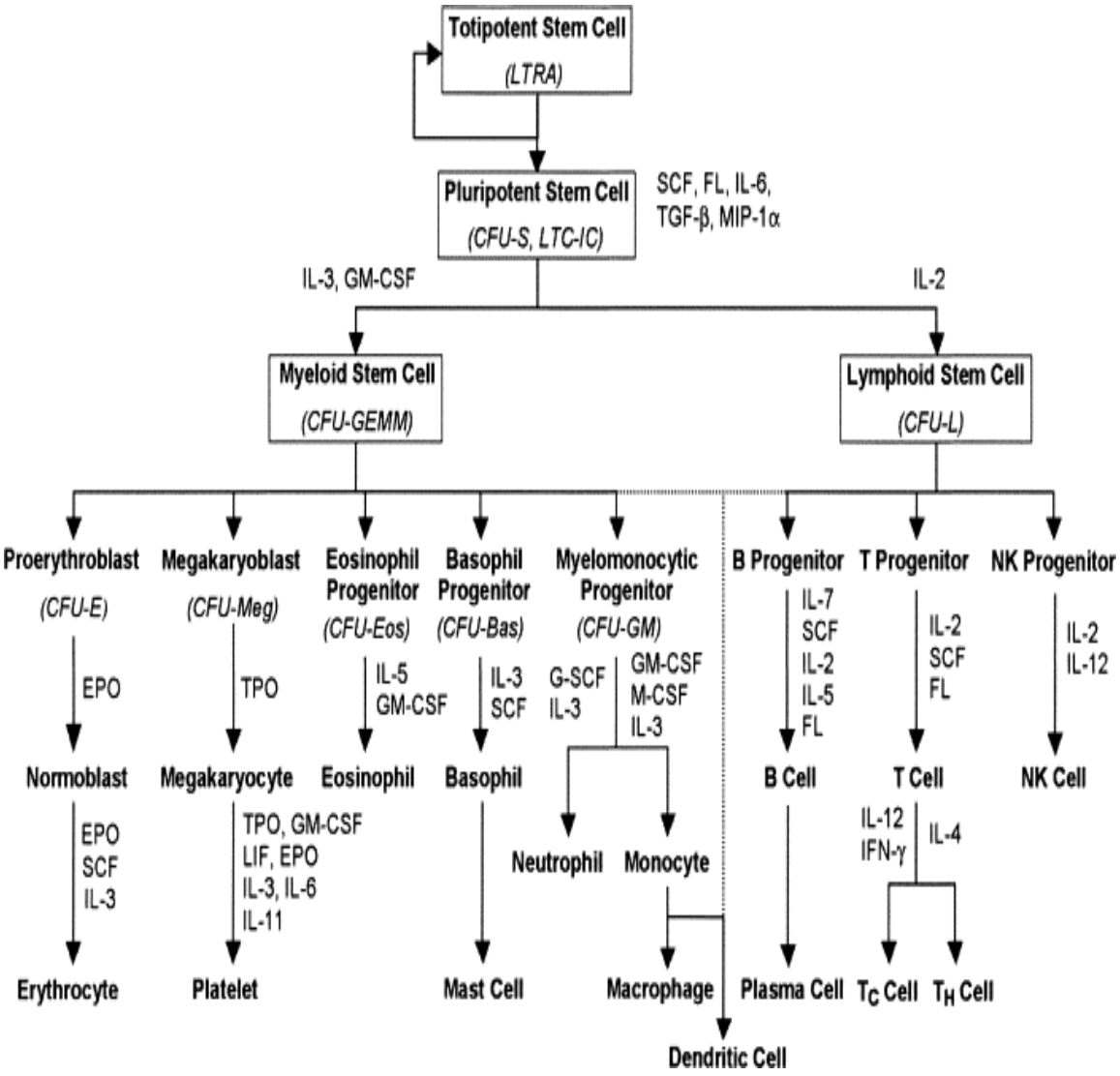


Figure 4. The cytokine effects on haematopoiesis at different stages (Adapted from (Panoskaltsis et al., 2005)).

CFU, Colony-forming unit; LTRA, long-term repopulating assay; LTC-IC, long-term culture-initiating cell; S, spleen; L, lymphocyte; GEMM, granulocyte, erythrocyte, macrophage, megakaryocyte; E, erythrocyte; Meg, megakaryocyte; Eos, eosinophil; Bas, basophil; GM, granulocyte, macrophage; TC, T cell, cytotoxic; TH, T cell, helper; SCF, stem cell factor; FL, Flt3 ligand; IL, interleukin; TGF-β, transforming growth factor-β; MIP-1α, macrophage inhibitory protein-1α; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; EPO, erythropoietin; TPO, thrombopoietin; IFNγ, interferon γ; LIF, leukaemia inhibitory factor.

2.1.4. Extracellular Matrix

The extracellular matrix, including collagen, proteoglycans, and adhesive proteins such as laminin and fibronectin, helps to structure haematopoietic microenvironments and regulate haematopoiesis by containing the haematopoietic cells within the collagen-based haematopoietic microenvironments. The extracellular matrix also regulates the propagation, differentiation, and maturation of HSCs by controlling various interactions of binding proteins, cytokines, and cells (Abboud and Lichtman, 2001, Panoskaltsis et al., 2005).

2.2. Erythropoiesis

2.2.1 Erythropoiesis Process

Erythropoiesis is a complicated process that involves a multi-step differentiation of HSCs into erythroid-committed progenitors that undergo proliferation and terminal maturation of mature red cells. Normal erythropoiesis is a continuous production of new RBCs owing to senescence or blood loss. RBCs are highly specialized and the most plenteous cells in humans (Focosi et al., 2014), and the major function of these cells is transportation of the respiratory gases, mainly transporting O_2 from the lungs to tissues of all body parts and CO_2 back for continuous exchange (Torbett and Friedman, 2009). Thus, RBCs are considered as the main antioxidant pool for the whole body (Focosi et al., 2014). A healthy adult has an average 2–3 × 10^{13} RBCs, with males and females maintaining 5–6 × 10^6/µl blood and 4–5 × 10^6 RBCs/µl blood respectively. Moreover, mature erythrocytes can live for up to 120 days in the body, with a daily substitution of 2 × 10^6 erythrocytes produced every second, in order to maintain a steady level of RBCs in the blood stream. Furthermore, production of RBCs can be elevated owing to tissue hypoxia as a result of blood loss or disease (Torbett and Friedman, 2009).

In erythropoiesis, multipotent hematopoietic progenitors differentiate into erythroid progenitor cells, which propagate and give rise to mature RBCs to compensate for lost or old cells and re-balance red cell count in the blood. The erythropoiesis process has been divided into three consecutive phases: (1) hematopoietic progenitors give rise to erythroid-committed blast cells. (2) These erythroid progenitors divide and differentiate. (3) Cellular morphological alteration and enucleation occur to produce mature RBCs (Baron and Fraser, 2005). The different cellular phases can be distinguished either by cellular markers or by *in vitro* colony-forming ability in cytokines-containing semisolid medium. Additionally, each phase has a defined morphology, with a distinguishable cytoplasm and nucleus after Wright's stain (Baron and Fraser, 2005, McGrath and Palis, 2008).

In detail, HSCs in the bone marrow give rise to erythroid-committed progenitors, which then differentiate into erythroblasts. As the hierarchy moves down towards lineage restriction, there is a loss of multipotency (Dzierzak and Speck, 2008). HSCs produce a common myeloid progenitor, which is bipotential and gives rise to either granulocyte/macrophage or erythroid/megakaryocte progenitor cells (Figure 1) (Suda et al., 1983, Akashi et al., 2000). This phase of development, as defined in semisolid medium, is the colony-forming unit–granulocyte, erythroid, macrophage, and megakaryocte (CFU-GEMM) precursor. Erythroid/megakaryocte progenitors can be then recognized by erythropoietin receptor (EPOR) expression, which is responsive to EPO (Heath et al., 1976). The first erythroid-committed progenitor cell to be developed is the burst-forming unit–erythroid (BFU–E), from which colony-forming unit–erythroid (CFU–E) originates. Unlike BFU-E, CFU-E are EPO receptive, retain high EPOR and transferrin receptors, and commence haemoglobin expression and definitive erythropoiesis (Heath et al., 1976, Stephens.Jr et al., 1971). EPO is thought to be essential for the proliferation and viability of late-stage erythroid cells (Koury and Bondurant, 1990).

During erythroid differentiation, many morphological changes take place to form reticulocytes and mature erythroids, ranging from the proerythroblast, basophilic, polychromatophilic, and orthochromatic cell structures (Figure 5-6) (Stephens.Jr et al., 1971). These are reflections of major cellular changes, including haemoglobin

accumulation, in order to switch the basophilic cytoplasm to acidophilic during maturation, cell size reduction, limited cellular expansion, nuclear condensation, and, finally, enucleation (Torbett and Friedman, 2009).

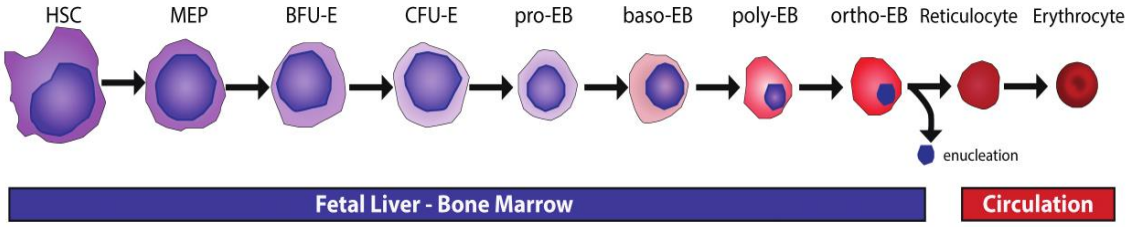


Figure 5. Erythropoiesis. (Adapted from (Cantu and Philipsen, 2014)).

Schematic overview of erythrocyte development from the HSC via several committed progenitor stages to mature erythrocyte. MEP: megakaryocyte-erythroid progenitor; BFU-E: burst forming unit erythroid; CFU-E: colony forming unit erythroid; EB: erythroblast; baso: basophilic; poly: polychromatic; ortho: orthochromatic.

Erythropoiesis takes place in the yolk sac during the first 8 weeks of gestation, then in the fetal liver between 8 and 32 weeks, and finally in the bone marrow from 32 weeks onward (Cantu and Philipsen, 2014) (Figure 3). Maturation of erythroblasts occurs in "erythroblast islands", micro-environmental niches in the bone marrow, in the presence of macrophages that function as "nurse" cells and supply iron for the heme synthesis needed for erythroid development (Rhodes et al., 2008, Manwani and Biekert, 2008). Furthermore, scientists suggest that macrophages anchor erythroblasts within island niches and offer interactions needed for their maturation and enucleation (Chasis and Mohandas, 2008) (Figure 7). Erythroids depart the marrow into the blood circulation in the shape of reticulocytes, their final stage of development, where they mature into erythrocytes. At this stage, alterations in the cytoskeleton and cellular components occur, such as loss of ribosomes, Golgi bodies, some organelles, and enucleation, to provide cells with a biconcave discoid shape (Torbett and Friedman, 2009).

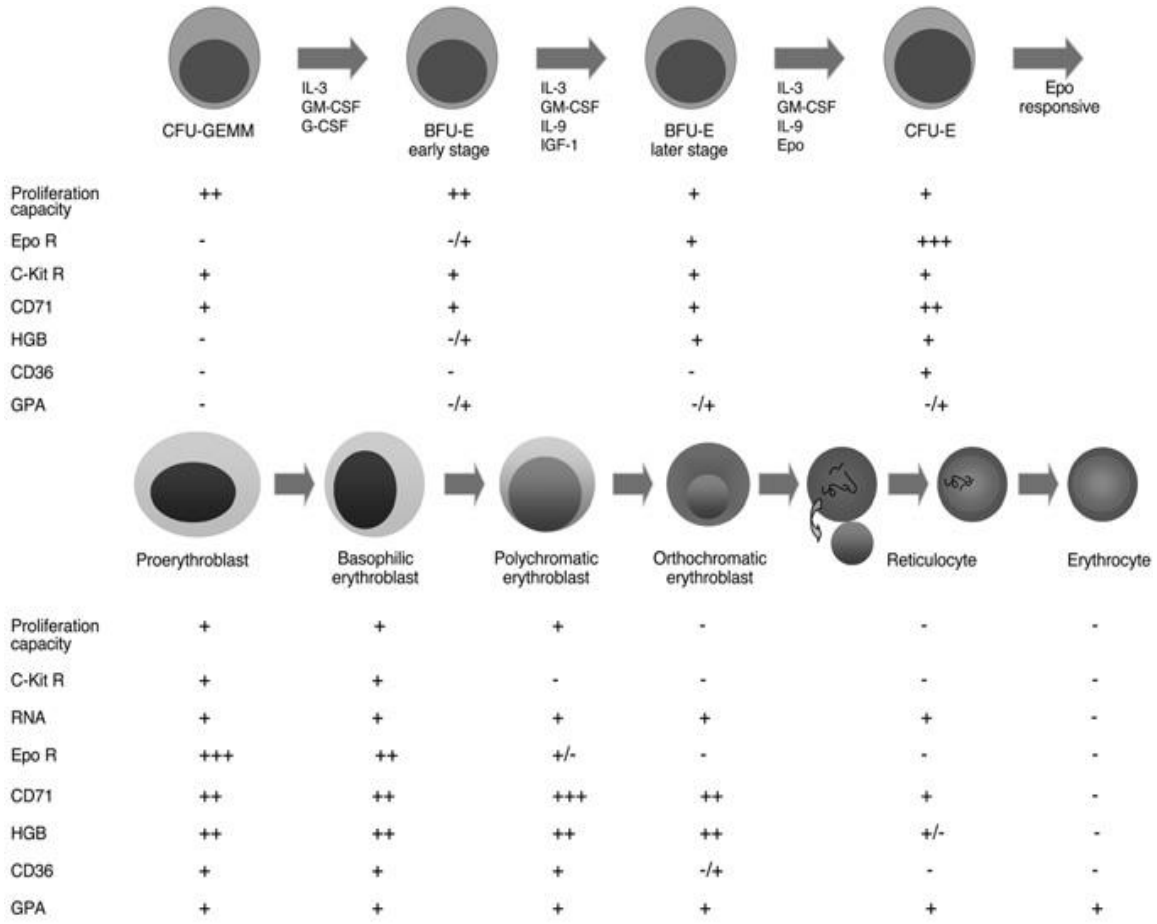


Figure 6. Stages of erythrocyte maturation. (Adapted from (Torbett and Friedman, 2009)).

CFU-GEMM: colony-forming unit–granulocyte, erythroid, macrophage, megakaryocte; BFU–E: burst-forming unit–erythroid; EPOR: erythropoietin receptor; CFU–E: colony-forming unit–erythroid; C-Kit R: stem cell factor receptor, CD117; CD71: transferrin receptor; HGB: haemoglobin synthesis; CD36: gpIIIb, thrombospondin receptor; GPA: glycophorin A present on human cells only.

As indicated above, the life span of mature erythrocytes is 120 days in circulation before they reach “senescence”, which is when cells undergo physical changes as well as surface antigen expressions that stimulate macrophages of the reticuloendothelial system to remove them (Gifford et al., 2006).

Studies in mice and human embryos propose that some immature erythroid progenitors (BFU–Es) are also generated in the yolk sac and reside in the fetal liver where they mature and form the first definitive erythroid cells in the embryo (Wong et al., 1986). Towards the end of gestation, definitive erythropoiesis starts to take place in the bone marrow (Palis, 2014).

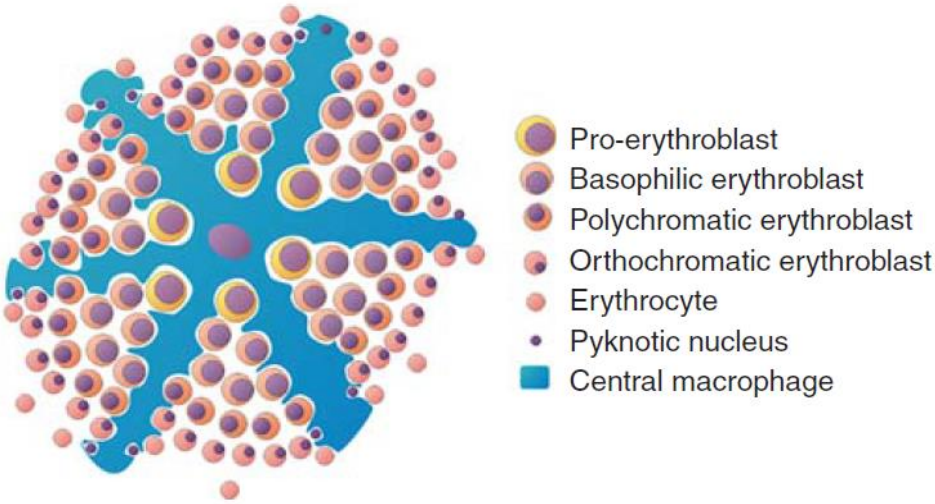


Figure 7. The Erythroblastic Island. (Adapted from (Dzierzak and Philipsen, 2013)).

2.2.2. Factors Required for Erythropoiesis

Erythropoiesis is a developmental process that can be regulated by a number of factors. The key regulator of erythropoiesis is EPO, which binds to EPO receptor (EPOR) expressed on erythroid progenitors from late BFU-E stage, in order to enhance their survival, expansion, and further differentiation (Krantz, 1991). EPO is a glycoprotein hormone produced mainly in the kidney, with a 30–34 kDa molecular weight. It is considered to be the main hematopoietic growth factor for the production of red cells in the bone marrow in association with the availability of responsive precursors, iron, and cofactors needed for heme and globin production, including folate and B12, and a microenvironment niche

suitable for erythroid development (Torbett and Friedman, 2009). EPO gene transcription can be also found in liver and many other body tissues, such as brain, but in lower quantities, which suggests some non-hematopoietic role for this hormone (Koury et al., 1991). EPO concentrations range from 10 mU/mL in healthy individuals to 10,000 mU/mL in severe anaemic subjects, which reveals an inverse relation to haemoglobin concentration (Jelkmann, 1992). Thus, erythropoiesis can increase remarkably from its base-line level in response to EPO stimulation as a result of a hypoxic stress occurs after a disruption of erythrocyte level in blood due to bleeding, destruction, disease, or senescence. In such conditions, production of RBCs in the bone marrow can increase 3-5-fold, or even 7-8-fold in severe haemolysis, as the marrow recycles iron from the lysed red cells (Hillman and Henderson, 1969). While high levels of EPOR are found only on late erythroid precursors, it is thought to be crucial for erythroid cell growth, survival, and differentiation, but not for early committed erythroid progenitors that express only low EPOR levels (Spivak et al., 1991, Sawyer and Penta, 1994), with CFU-E being the most EPO-responsive erythroid progenitor (Neildez-Nguyen et al., 2002).

EPOR is expressed on erythroid-committed precursor cells as a homodimeric transmembrane protein with an extracellular domain for ligand-binding, and a cytoplasmic domain related to the Janus-type tyrosine kinase (JAK)-2 (Witthuhn et al., 1993). Upon EPO binding to its receptor, EPOR endures structural modifications, which activate JAK2 and phosphorylate multiple tyrosines of the EPOR cytoplasmic tail, leading to the stimulation of several pathways that are essential for the survival, proliferation, and maturation of erythroid progenitors, including STAT5, RAS/raf/MAP-Kinase (MAPK), and phosphatidylinositol 3-kinase (PI3-K)/AKT (Wilson and Jolliffe, 1999, Richmond et al., 2005, Ingley, 2012a).

The erythropoiesis process is also influenced by many extrinsic factors such as SCF, TPO, FLT-3 ligand, GM-CSF, vascular endothelial growth factor (VEGF), IL-3, BMP-4, glucocorticoids, and hypoxia. BMP-4, a mesoderm inducer, strongly promotes embryonic, fetal, and adult hematopoietic differentiation, acting together with other hematopoietic cytokines (Chadwick et al., 2003a). SCF is a hematopoietic growth factor that can enhance the sensitivity of cultured erythroid progenitor cells to Epo and IL-3

(Broudy et al., 1992). SCF and IL-3 both enhance the development of many hematopoietic lineages, exhibiting main roles in erythropoiesis in the survival, proliferation, and differentiation of erythroid progenitors (Mcniece et al., 1991, Koller et al., 1993). SCF binds to its tyrosine kinase receptor c-KIT and activates several pathways, including PI-3 kinase, Src kinases, and PLC-γ. During later stages of erythropoiesis, SCF is needed together with EPO for the proliferation of erythroblasts produced, as it plays a major role in the phosphorylation process of EPOR (Dzierzak and Philipsen, 2013). Furthermore, IL-3 has contradictory effects in the proliferation and differentiation of HSCs and mature hematopoietic cells, depending on its concentration, the presence of serum and other cytokines, culture conditions, and type of cells (Robin et al., 2006). In addition, FLT-3 ligand promotes the activation of FLT-3, a receptor tyrosine kinase expressed mainly on early haematopoietic cells. The activated receptor then activates the PI3-K and RAS signal-transduction pathways controlling their proliferation, differentiation, and apoptosis (Stirewalt and Radich, 2003, Chung et al., 2005).

Early-acting cytokines, involving FLT-3 ligand, TPO, and IL-3, in addition to other cytokines, including IL-6, encourage the survival, expansion, and division of HSCs *in vitro* (Antonchuk et al., 2004, Jenkins et al., 2007). Additionally, it has been shown that supplementing the serum-free media with growth factors SCF, TPO, and FLT-3 ligand, enhances the isolation of hematopoietic progenitors for further erythroid differentiation (Tian et al., 2004). In addition, in the differentiation of human ES cells towards erythropoiesis, VEGF-A165 showed a major role in enhancing the *in vitro* self-renewal potential of primitive hematopoietic cells, promoting erythropoietic development in the presence of other hematopoietic/erythropoietic cytokines, BMP-4 and EPO, and increasing the frequency of cells expressing CD34 and erythroid markers. Most importantly, VEGF-A165 induces the expression of embryonic globins zeta (ζ) and epsilon (ε) (Cerdan et al., 2004).

Other factors have also shown to have a secondary role in the growth of the erythroid progenitors. In particular, BMP-4/SMAD5 and STAT5, are known to support the self-renewal of the early erythroid progenitors (Dzierzak and Philipsen, 2013). STAT5 transcription factor is the major downstream signaling of EPOR to reduce erythropoietic

response (Dzierzak and Philipsen, 2013). In addition, FAS/FASL death receptor system is known to regulate erythroid homeostasis (Dzierzak and Philipsen, 2013).

Some studies suggested that some nuclear hormones/receptors may also affect and regulate erythropoiesis, such as insulin, glucocorticoids receptor, estrogen receptor, dexamethasone, and estradiol (Singer and Adamson, 1976, Golde et al., 1976, Ingley, 2012b). Dexamethasone and estradiol are believed to act by holding up the erythroid progenitors at the proerythroblasts stage and delaying their maturation in order for them to proliferate into a higher number of colonies (Wessely et al., 1999). Glucocorticoids have a very significant role in the maintenance and development of the BFU-Es, whereas it has no effect on erythroid maturation stages (Ingley, 2012b). Iron is also critical during erythropoiesis, mainly for the haemoglobin synthesis (Koury and Ponka, 2004). As CFU-Es develop into proerythroblasts, the surface expression of transferrin receptors is up-regulated, allowing the uptake of transferrin-iron complex for haemoglobin production along their differentiation, where levels of transferrin are progressively reduced, until they disappear at reticulocyte stage. Transferrin-iron complex also triggers the PI3-K/Akt and MAPK pathways (Ingley, 2012b). In addition, erythroblasts require folate and vitamin B_{12} for DNA synthesis and cell divisions during their differentiation, as their deficiencies lead to hematopoietic cell death as a result of impaired DNA synthesis (Koury and Ponka, 2004).

GATA-1, another member of the GATA gene family, also appears to highly support the development of erythroid phenotype while suppressing other lineage progenitors (Chiba et al., 1991). Expression of GATA1 is highly restricted to erythroid cells, suggesting that it has an important role during late stage differentiation of the erythroblasts (Pan et al., 2005). Mice with a GATA-1-/- mutation will die from anemia. Such a mutation would allow only primitive erythroid cells arrested at a proerythroblast stage to be found in peripheral blood, which then undergo apoptosis (Chen et al., 2014a). GATA-1 is also said to be important during erythroid maturation, as it starts the expression of erythroid lineage-affiliated genes such as β-globin, Alas2, and Gata-1, and suppresses Gata-2, c-Kit, c-Myb, and c-Myc, which are responsible for the proliferation of hematopoietic progenitors (Chen et al., 2014a).

The EKLF gene is weakly expressed in HSCs and early progenitors and it is up-regulated in myeloid/erythroid-committed progenitors toward erythropoiesis, while down-regulated toward megakaryopoiesis (Chen et al., 2014a). EKLF also plays an important role in globin switching. Its expression is weak in embryo and fetus, resulting in a weak expression of adult β-globin, while in adults it is highly expressed leading to a high level of adult β-globin in definitive RBCs (Chen et al., 2014a). Finally, Rbtn2 is a nuclear protein found in erythroid lineage, which is needed for erythroid development. Mutations of rbtn2 prevent erythropoiesis and lead to death (Chen et al., 2014a).

Erythropoiesis is thus a complex, multistage developmental process that requires cell–cell interactions in addition to intrinsic and extrinsic factors, which bind to the cell surface receptors and trigger signalling pathways.

2.3. Therapeutic Need for an Alternative Blood Supply

Blood transfusion is a regular main practice in modern medicine, performed in clinics worldwide to replace blood lost after an injury or an operation, or to treat patients with leukaemia, cancer, or hemolytic disorders (Slukvin, 2010). Blood transfusion is usually defined as the process of transferring blood, mainly RBCs, to increase or restore the O_2 transport capability of the blood to all body parts (Lippi et al., 2011). To date, transfused blood supplies are obtained through voluntary donations, which make them subject to periodic shortages (Ganji et al., 2015). Although a significant amount of blood is donated and collected daily in the world, there are still donor supply shortages. Furthermore, poor screening for infectious diseases and difficulty in finding compatible blood, especially for chronically transfused patients, remain major concerns. In addition, haemolytic reactions are common immunological complications associated with incompatible transfusion- the main cause of mortality (Ganji et al., 2015).

In response to these concerns, scientists have been experimenting with the *in vitro* generation of blood products from stem cells to ensure quality and quantity for safe transfusions as an international priority (Douay and Andreu, 2007). This generation refers

to the concept of culturing stem cells as an alternative source of blood. HSCs of the bone marrow, peripheral blood, cord blood, ES cells, and iPS cells are used to examine the feasibility of this approach (Neildez-Nguyen et al., 2002, Giarratana et al., 2005). RBCs derived from these cells showed some similarities in morphology, haemoglobin content, and function to their native counterparts. Nonetheless, these RBCs-production methods hold both advantages and limitations (Lippi et al., 2011). In this context, pluripotent stem cells are shown to be the best candidate for their unlimited source of cells. Several groups have explored the hematopoietic potential of pluripotent cells, with a focus on erythroid differentiation using many complicated protocols (Kaufman et al., 2001, Vodyanik et al., 2006).

Human ES cells and iPS cells can be proposed as the best candidates for the generation of RBCs because of their indefinite self-renewal property. They are considered as infinite sources of cells that are able to derive large quantities of functional, mature RBCs. However, all techniques used so far for that purpose possess some drawbacks, require critical quality control procedures, and are associated with several risks of malignancy formation and pathogen transmission, as well as the risk correlated to the insertion of vectors-containing oncogenes for reprogramming induced pluripotent cells (Mountford et al., 2010, Lippi et al., 2011).

2.4. Stem Cells

Stem cells are unspecialized cells, defined by their unique characteristics of indefinite self-renewal and ability to give rise to terminally differentiated functional cells of any type (Eisenberg and Eisenberg, 2003). Based on their differentiation potential, they are classified as, firstly, totipotent, which refers to cells that are capable of giving rise to all embryonic and extra-embryonic cell types in order to form a fully developed human. The zygote, the fertilized egg, is thought to be the only totipotent stem cell. Secondly, they are pluripotent, which refers to cells that are able to give rise to all cell types of the embryo but not the extra-embryonic membranes. The third type, multipotent, refers to

cells that are able to differentiate into many types of specialized cells within their family, but with less capability than pluripotent stem cells such as neural stem cells of the brain, which give rise to different neural cells and glia, and HSCs, which can differentiate into many blood cell types, but not neural cells. Next, they can be classified as oligopotent, referring to cells that are able to give rise to few cell types. The most restricted type of stem cells are, finally, unipotent, which are able to contribute to one mature cell type (Temple, 2001, Eisenberg and Eisenberg, 2003, Wagers and Weissman, 2004) (Figure 8).

Based on their origin, stem cells can be broadly categorized into: ES cells, which are isolated from embryos; adult stem cells, which are found in various body tissues; and umbilical cord stem cells. In an embryo, stem cells develop all the specialized cells of the three germ layers, ectoderm, endoderm, and mesoderm. While in adults, they act as a repair system and maintain the normal turnover of tissues and cells (Poulsom et al., 2002). Umbilical cord stem cells are stem cells isolated from umbilical cord blood after birth (Wang et al., 2004). Umbilical cord stem cells are less mature than adult HSCs, with similar features and more differentiation potential (Giarratana et al., 2011).

Stem cells are advantageous in tissue repair and regeneration therapies. However, there are advantages and disadvantages associated with their potential usage. For instance, adult and ES cells differ in the number and type of differentiated cells they can produce. ES cells are pluripotent; they can become any body cell type, while adult stem cells are multipotent, and thus can produce only cell types of their tissue of origin. In addition, ES cells can be isolated from the inner cell mass of blastocysts (Evans and Kaufman, 1981, Martin, 1981) and grown easily in culture, whereas adult stem cells are hard to isolate from adult tissues, and expanding them *in vitro* has not yet been identified. Scientists have lately used the term "somatic" stem cell instead of adult stem cell, referring to cells of the body, but not germ cells, sperm, or eggs.

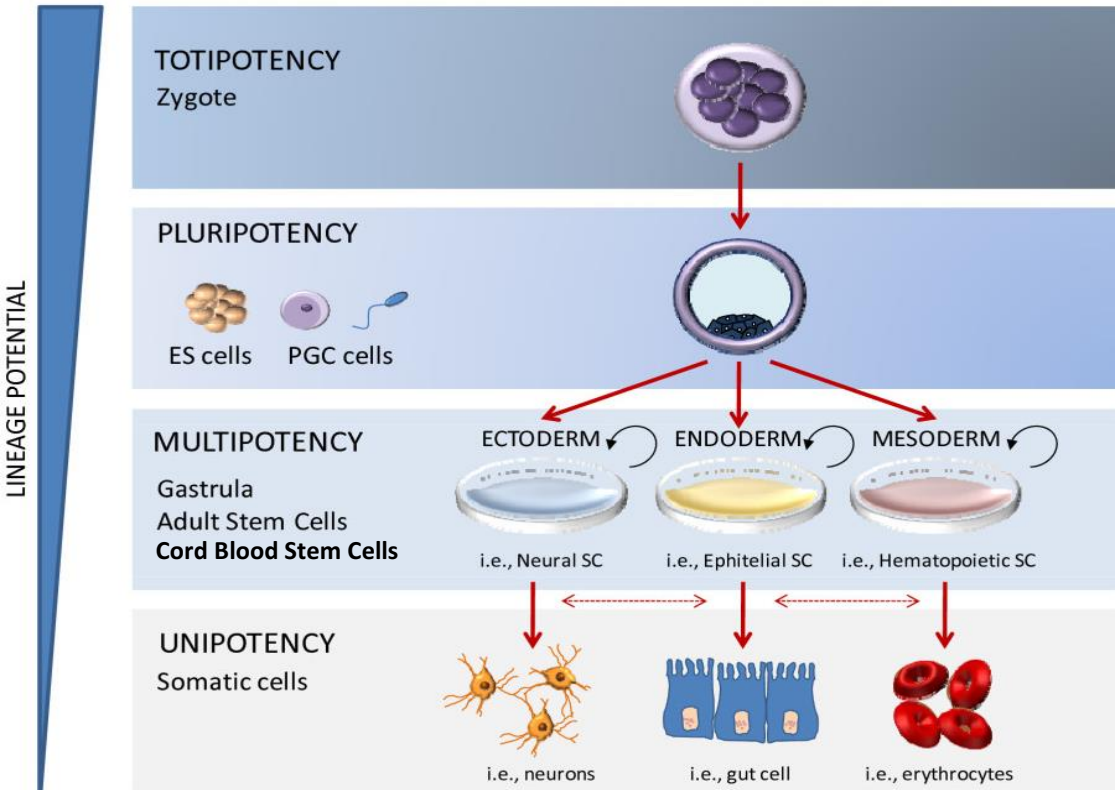


Figure 8. The differentiation potential of different stem cell types (Adapted from http://dezian3.wix.com/regenerativemedicine).

Human ES are pluripotent cells, derived from embryos, and can be expanded indefinitely *in vitro*, providing a potentially unlimited source of cells for clinical therapy (Lu et al., 2008b). Human ES cells were first grown by Thomas et al. (1998). They were isolated from the inner cell mass of 3–5 day old blastocysts. Human ES cells are undifferentiated pluripotent cells that can give rise to cells of all three germ layers, endoderm, mesoderm, or ectoderm. They have normal karyotype with high telomerase activity that can allow them to maintain their pluripotent state indefinitely *in vitro* when cultured on feeder layer or supplemented with condition media and basic-FGF (bFGF) (Itskovitz-Eldor et al., 2000). Moreover, these cells express specific pluripotent nuclear markers, including transcription factors Oct-3/4, Nanog, and Sox2, as well as stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumour rejection antigen (Tra)-1–60, and Tra-1–81 (Mountford

et al., 2010). Owing to these unique properties, they seem to be the best candidate cells for cellular therapy.

However, ES cell-derived products, including hematopoietic progenitors, express immune antigens such as human leucocyte antigens, which can stimulate immunological rejection upon transfusion or transplantation (Drukker et al., 2002). Thus, recently, an alternative method of making pluripotent stem cells has been discovered using adult somatic cells, such as skin fibroblast cells. These iPS cells were first produced 2006 in mice (Takahashi and Yamanaka, 2006), followed by human iPS cell production in 2007 (Takahashi et al., 2007).

Human iPS cells can be generated by reprogramming adult somatic cells using viral or other vectors to express pluripotent genes (Nishikawa et al., 2008). The group of Yamanaka used Oct4, Sox2, Klf4, and c-Myc to form iPS cells of both origins, mouse and human (Takahashi and Yamanaka, 2006, Takahashi et al., 2007), while the group of Thomson used Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). Subsequently, different groups were able to replace the proto-oncogene c-Myc with safer genes because of transformation fears (Chiou et al., 2013). Recent protocol modifications showed that iPS cells can be successfully generated with as few as 1 or 2 genes that can be transferred transiently without genome integration (Kaufman, 2009). In order to enhance the transfection efficiency, different vectors, retrovirus and lentivirus, have been used to introduce pluripotency genes into cells. However, the insertional mutagenesis linked with these vectors detracts slightly from the overall success of this approach (Focosi et al., 2014). Many attempts have thus been made to improve the delivery methods and to provide stable expression systems of the reprogrammed factors (Focosi et al., 2014). Indeed, further advancements showed that derivation of iPS cells can be achieved using protein transduction of transcription factors rather than viral transduction (Zhou et al., 2009).

Human iPS cells have been successfully produced from skin fibroblasts, peripheral blood, and cord blood using episomal vectors (Okita et al., 2011, Okita et al., 2013). These iPS cells have similar properties of gene expression patterns and differentiation potential as

ES cells. When iPS cells are injected into mouse blastocytes, they form viable chimeras and all germline differentiation (Wernig et al., 2007). Teratoma formation is another pluripotency feature that has been added to iPS cells, with contributions of all three germ layers, endoderm, mesoderm, and ectoderm (Lowry et al., 2008). The teratoma formation feature of the iPS cells has been employed in the generation of hematopoietic progenitors produced after an injection of human iPS cells into immunocompromised mice. Teratoma-derived hematopoietic progenitors have been isolated, approved functional, and transplanted into immunodeficient mice. That study has provided evidence that patient-specific cells can be generated *in vivo*, providing materials that are useful for transfusion and transplantation (Amabile et al., 2013).

Although these studies support the similarity in differentiation potential between human iPS cells and human ES cells, it is not yet clear whether human iPS cells follow cellular differentiation similarly to human iPS cells to specific lineages (Choi et al., 2009), and whether they are able to proliferate into homogeneous cell populations appropriate for use in drug discovery and clinical applications (Feng et al., 2010). Moreover, the complication of genetic reprograming and limited expansion capacity of differentiated cells produced from iPS substantially constraints improvements of this technology (Dias et al., 2011). However, in comparison with ES cells, the use of iPS cells is not subjected to ethical issues, as no embryonic or fetal materials are used (Park et al., 2008), and are not associated with any immunological fears as they are autologous somatic cells, which render them compatible. Yet, iPS cells show the distinctive features of ES cells of self-renewal and potency (Ganji et al., 2015).

2.5. Generation of Mature Erythrocytes

2.5.1. Erythrocytes Generated from Multipotent Stem Cells

The urgent need for an alternative source of transfusable RBCs has encouraged scientists to explore the ability of HSCs of different sources, including cord blood, peripheral blood, and bone marrow, to derive mature RBCs (Cantu and Philipsen, 2014). Huge progress has

been made using these sources in the *ex vivo* generation of human RBCs, with high expansion rates and full maturation into enucleated reticulocytes (Dorn et al., 2015). $CD34^+$ HSCs can be isolated from these sources and differentiated into erythrocytes using EPO, SCF, and IL-3. Enucleation of erythrocytes derived from these progenitors can be achieved by co-culturing them with a mouse stromal cell line or human mesenchymal stem cells. This protocol can yield up to 95% pure erythrocytes in culture, with similar features and functions to typical RBCs, together with comparable enzymatic and haemoglobin content (Giarratana et al., 2005). $CD34^+$ HSCs consist of approximately 1.5% of human bone marrow-derived mononuclear cells, while only 0.5% of umbilical cord blood and 0.1% of adult peripheral blood (Civin et al., 2004). However, stem cells of the cord blood have more expansion capacity than those from peripheral blood and bone marrow. HSCs from cord blood can generate up to ten times higher RBCs than that generated from adult peripheral blood (Cantu and Philipsen, 2014). Conversely, stem cells from peripheral blood and bone marrow are adult cells that can easily express adult haemoglobin upon erythroid maturation, while those from cord blood can only contain fetal globins (Lu et al., 2008a).

Furthermore, early studies in this field used the semi-solid colony assays containing growth factors in order to develop erythroid precursors from hematopoietic $CD34^+$ progenitors, followed by liquid cultures containing EPO under low O_2 conditions for a further erythroid differentiation (Malik et al., 1998). However, this experimental system is limited in the number of cells produced, which hinders further proliferation and analysis. Moreover, liquid cultures for HSCs are usually associated with the production of heterogeneous mixtures of erythroid and myeloid cells, which therefore requires high concentrations of EPO in order to suppress myeloid cells. In addition, this system does not support enucleation (Neildez-Nguyen et al., 2002). Further modifications have been applied to avoid such limitation.

Two-phase liquid culture systems were thus then used to allow for selective propagation and differentiation. This procedure was exploited to generate erythroid cells with adult pattern of haemoglobin (β-globin) from human $CD34^+$ progenitors isolated from bone marrow, peripheral blood, and umbilical cord. The first phase was to propagate erythroid

colony-forming cells (BFU-E and CFU-E) in a semi-solid media conditioned with growth factors. The second phase was to isolate enucleated erythrocytes by flow cytometry. Using this method has also yielded erythroblastic islands followed by enucleation of some cells with adult haemoglobin (Hb A) and insignificant fetal globin (Hb F) (Malik et al., 1998).

Douay and Andreu then claimed the generation mature RBCs *in vitro* from $CD34^+$ HSCs isolated from cord blood, bone marrow, and peripheral blood (Douay and Andreu, 2007), using a protocol comprising three steps: first, liquid culture medium in the presence of SCF, IL-3, and EPO; second, culture with stromal cells and EPO supplements; third, culture with stromal cells alone without growth factors (Douay and Giarratana, 2009). Erythroid cells production was confirmed morphologically and by the loss of transferrin receptor (CD71) expression as final termination evidence. 90–100% enucleation was reported. Functionally, these cells were examined for their enzyme levels of glucose-6-phosphate-dehydrogenase (G6PD) and pyruvate kinase (PK), as high G6PD activity in erythroid cells proposes high protection against oxidative damage (Ma et al., 2008). Moreover, they showed similar O_2 dissociation features and survival to native erythroid cells (Douay and Giarratana, 2009).

In addition, a high number of functional erythroid cells have been derived from cord blood HSCs under serum-free condition by a sequential supplementation of growth factors using a 3-phases protocol: a proliferation hematopoietic progenitors, a stimulation of erythroid progenitor production using erythroid induction cytokines cocktail, and, finally, an ultimate erythroid differentiation. A terminal maturation into mature functional enucleated erythrocytes was achieved *in vivo* after injecting the erythroid precursors, containing mainly fetal haemoglobin, into NOD/SCID mice (Neildez-Nguyen et al., 2002).

Recently, a protocol of generating erythrocytes from cord blood $CD34^+$ cells has been reported using a serum-free 4-phase protocol through culture on human stromal cells supplemented with cytokines to expand the cells, followed by a liquid-culture medium conditioned with cytokines to develop erythroblasts differentiation. These erythroblasts

were then co-cultured with human macrophages and supplemented with EPO to allow the differentiation into erythrocytes, which were purified and tested. It was shown that this method was able to produce a high number of functional RBCs with haemoglobin content and surface markers similar to adult peripheral blood (Fujimi et al., 2008).

It has thus been generally believed that enucleation of erythroid cells depends on cell interactions with other cells within their local environment, such as macrophages. However, nearly 80% of production of enucleated erythrocytes has been achieved from cord blood $CD34^+$ cells in the presence of 0.5% serum, proving that interactions with other cells is not required and that humoral factors, such as mifepristone, a glucocorticoid antagonist, seem to be sufficient for that purpose (Miharada et al., 2006).

Together with this finding, the first injection of homogenous reticulocytes into humans has been reported and tested for the *in vivo* behaviour (Giarratana et al., 2011). Erythroid cells were autologous peripheral blood $CD34^+$ HSCs cultured under good manufacturing practice. The viability pattern of the cultured cells was similar to native ones. The functionality of the cells was tested and they reported acceptable O_2 binding capacity for gas transport function; normal contents of glucose-6-phosphate dehydrogenase and pyruvate kinase for the affinity of haemoglobin; and deformability, which facilitates the survival of the cells *in vivo* (Mel et al., 1977, Giarratana et al., 2011). Peripheral HSCs are interesting for providing autologous RBCs. However, cord blood–derived HSCs generate higher proliferation rate with comparable enucleation capacity, which make them a better source (Mel et al., 1977, Giarratana et al., 2011).

In conclusion, isolation of HSCs from bone marrow, cord blood, and adult peripheral blood is a standard and safe procedure with low risk of infections. Nevertheless, these progenitors are difficult to expand *in vitro*. Moreover, because they have a limited proliferation capacity, they exhaust quickly, preventing their application to large-scale production of RBCs (Cantu and Philipsen, 2014). In addition, there is their immune incompatibility issue to be considered, which makes them no better than the conventional blood transfusion practices. Thus, HSCs fail to meet the requirements to satisfy the world demand of blood (Lippi et al., 2011, Ganji et al., 2015). Furthermore,

cord blood-derived RBCs showed similarity to fetal RBCs, which will probably restrict their usage for transfusion purposes. Additionally, since cord blood can only be collected once after birth, it is cannot be considered as a sustainable, unlimited cell source for large-scale production of RBCs (Cantu and Philipsen, 2014).

2.5.2. Erythrocytes Generated from Pluripotent Stem Cells

2.5.2.1. Erythrocytes Generated from Embryonic Stem Cells

Owing to the fact that erythroid cells in the mammalian embryo originated from the mesoderm of yolk sac and placenta, ES cells have been proposed to be good source for *in vitro* erythroid production. The challenges of this approach have led scientists to undertake two protocols for the *in vitro* production of haematopoietic progenitor cells from human ES cell: (1) to utilize a wide array of cytokines to generate hemangioblasts via EB formation as a step toward erythrocytes production (Lu et al., 2008b), and (2) to co-culture human ES cells on a stromal cell line (Olivier et al., 2006, Ma et al., 2008) (Figure 9).

Ma et al. (2008) explained the generation of definitive hematopoietic progenitors by co-culturing human ES cells on murine fetal liver stromal cells after an initial EB formation stage, and enhancing erythropoiesis by adding a cytokine cocktail including EPO, TPO, SCF, IL3, IL6, and TPO. Continuous presence of the stromal layer was needed for the maturation of definitive progenitors and erythroid progenitors were produced and determined by expression of CD71, glycophorin A, and CD81. The group suggested that expression of CD81 indicates embryonic erythropoiesis, as it was gradually down-regulated over the culture period and disappeared when cells contained adult type haemoglobin. Along the differentiation, reticulocyte formation was observed in addition to enucleation (Ma et al., 2008). Haemoglobin contained at the beginning was of the embryonic and fetal types, but later it was switched into adult-type beta globins ($\alpha 2\beta 2$) in at least in 50–60% of cells demonstrating definitive haematopoiesis. Progenitors derived from this protocol showed comparable O_2-carrying capacity, and G6PD activity

to cord blood-derived progenitors rather than adult erythrocytes. Nevertheless, the quantity of mature erythrocytes produced were insufficient for clinical applications and further improvements are needed for scale-up production (Mountford et al., 2010).

Another study by Lu et al. (2008) followed the hemangioblast-generating protocol under serum-free conditions by supplementing the human ES cell culture medium with SCF, TPO, and FLT-3 ligand in the presence of BMP-4, VEGF, and bFGF. Hemangioblasts were then sub-cultured to expand in the presence of bFGF and HOXB4 protein, followed by a sequential exposure to EPO and SCF to allow erythroid differentiation and enrichment. This protocol produced large nucleated erythrocytes with expression of embryonic and fetal haemoglobin. Upon RBCs maturation, size was gradually reduced, the majority of cells were $CD71^{+}$, glycophorin A expression was increased (30%), and chromatin and nuclear condensation was observed. Adult globin was detected in some cells on further maturation. Enucleation was reported in 10-30% of cells, while it was enhanced and noticed in almost 30–65% of cells at day 42 after co-culture on mesenchymal stem cells (MSCs) stromal cells and OP9 stromal cell layer. Functionally, mature RBCs showed similar O_2 equilibrium curves and response to pH change to their native counterparts (Lu et al., 2008a). The use of serum creates variability and inconsistency in ES cells differentiation, as it contains a variable collection of inducers and inhibitors (Lu et al., 2008a).

It has also been reported that BMP-4 can stimulate haematopoietic differentiation from human ES cells when combined with other haematopoietic cytokines such as SCF, IL-3, IL-6, FLT-3 ligand, and G-CSF (Chadwick et al., 2003a). Additionally, VEGF-A165 has been found to selectively stimulate erythropoiesis from human ES cells when combined with BMP-4 and augmented by EPO (Cerdan et al., 2004). To attain high number of expanded cells in culture during differentiation, one study reported the addition of a recombinant HOXB4, a homeobox gene involved in the enhancement of haematopoiesis from ES cells (Bowles et al., 2006), although another study was able to obtain and expand RBCs without it (Dias et al., 2011).

Unfortunately, none of these above reported studies show a complete development of mature biconcave erythrocytes from human ES cells. In addition, no functional evaluation

was detailed (Anstee, 2010). A common problem in all erythrocytes derived from these protocols was the detection of embryonic and fetal haemoglobin rather than adult phenotype (Chang et al., 2006, Qiu et al., 2008). However, the derivation of mature erythroid cells expressing adult globins, α and β, from mouse ES cells has been obtained by the co-culture on the stromal cell line and the stimulation by mouse VEGF and mouse IGF-II, followed by mouse SCF, human EPO, mouse IL-3, and dexamethasone. These cells were transplanted into anaemic mice to proliferate and differentiate into functional, mature RBCs, which resulted in a marked improvement of anaemia (Hiroyama et al., 2008).

On the other hand, differentiation of mouse ES cells to erythroid progenitors can also be achieved with no additional exogenous cytokines. Erythroid cell production can be accomplished by co-culturing these cells on OP9, a mouse stromal cell line deficient in macrophage colony stimulating factor (M-CSF), as M-CSF has an inhibitory role on the differentiation of mouse ES cells to hematopoietic cells other than macrophages (Nakano, 1996).

From the literature we can summarize that the EB-based culture system showed the best erythroid differentiation results with mouse ES cells. EBs are three-dimensional (3D) cell aggregates that mimic the *in vivo* environment and can be formed when plating ES cells in ultra-low binding plates, which prevent cell attachment to the surface and thus force cells to cluster in 3D forms. Maturation can be then enhanced using erythroid cytokines to finally produce erythrocytes with adult haemoglobin. Enucleation can be achieved by exposing mouse ES cells-derived erythrocytes to a high concentration of transferrin, EPO, and insulin.

From a clinical prospective, one group was able to produce all ABO and Rhesus blood types, but not the antigen-free universal donor blood (O RH negative blood type), for the reason that none of the available approved human ES cells lines encode the O-Rhesus negative gene (Lu et al., 2008a, Dorn et al., 2015). Therefore, ES cells appear to have constraints in generating blood with a wide range of transfusion compatibility (Lapillonne et al., 2010b). Moreover, it is been found that the quality of human ES cells is a crucial

factor for an efficient differentiation (Lu et al., 2008a). It seems doubtful that human ES cells will rise above cord blood cells, HSCs, and other biomaterials as a source of RBCs (Anstee, 2010). Hence, the reprogrammed iPS cells into an embryonic-like property appears to be the most promising substitute.

2.5.2.2. Erythrocytes Generated from Induced Pluripotent Stem Cells

iPS cell-derived RBCs are strong candidates to combat the global shortage of O Rh-negative "universal donor" RBCs. Nevertheless, transfusion of these cells suffers from two main limitations: short half-life and need for frequent lifetime transfusions (Focosi et al., 2014). Results of recent studies support the use of iPS cells to generate haematopoietic cells in order to obtain patient-specific RBCs. Such success would be beneficial for patients with rare blood groups or complicated cross-matching transfusion, when compatible blood cannot be found in a blood bank. To date, RBC generation from iPS cells is less efficient than the available protocols for adult stem cells and recapitulating the physiological erythropoiesis *in vitro* remains a challenge (Dorn et al., 2015).

Toward erythropoietic differentiation, similar to human ES cells, scientists have carried out experiments to differentiate human iPS cells using two approaches. The first is the EB formation protocol conditioned with cytokines, with or without serum, to obtain hemangioblasts, followed by subsequent expansion and maturation of erythroid cells produced using erythropoiesis-induced cytokines. The second is the co-culture on stromal cells to induce haematopoiesis as a step toward producing RBCs (Dias et al., 2011) (Figure 9). Significant drawbacks are associated with the usage of iPS cell-derived erythroid cells for physiological applications, including poor expansion rate, incomplete maturation and enucleation of the produced cells, inefficient switching to adult type haemoglobin, and the lack of the required number of RBCs equivalent to one unit (Focosi et al., 2014, Dorn et al., 2015).

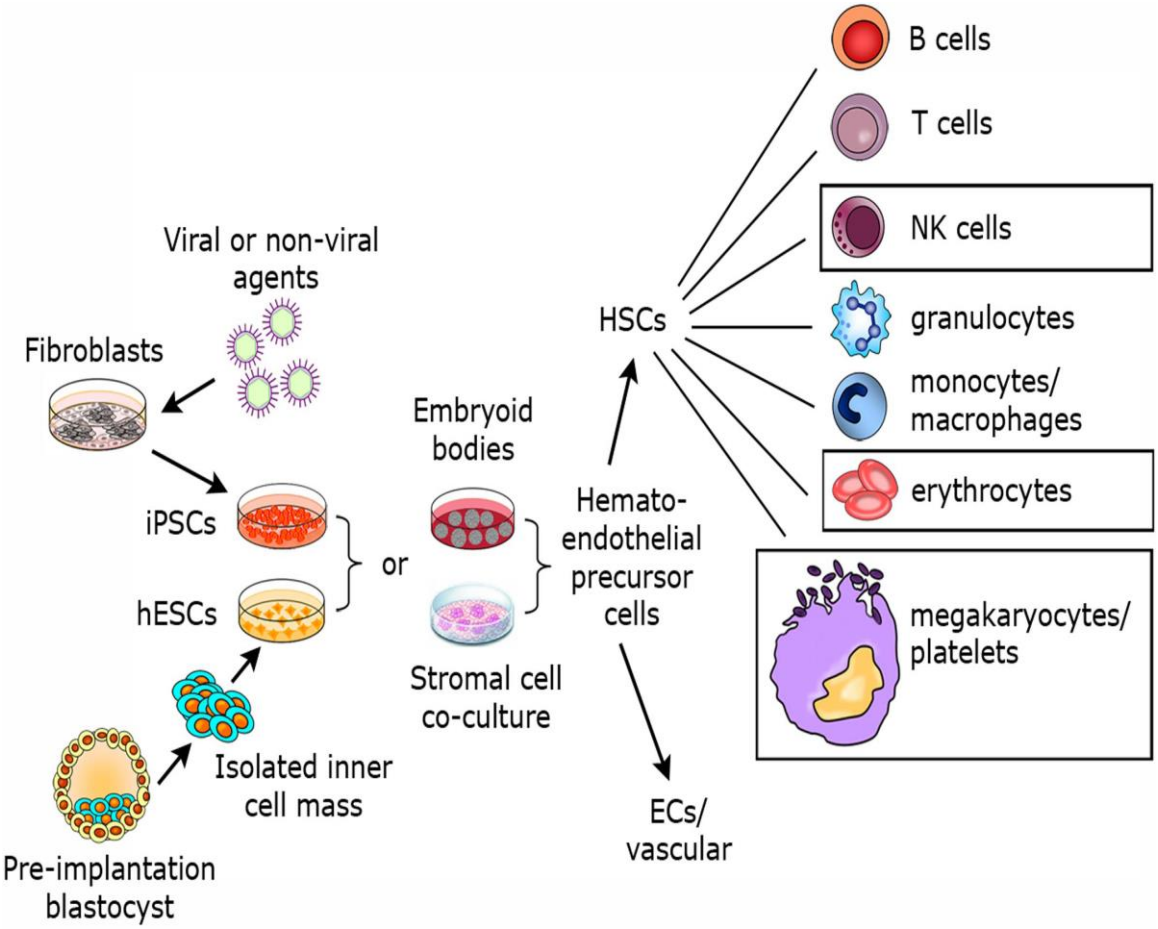


Figure 9. Derivation of hematopoietic cells from human ES cells and iPS cells. (Adapted from (Kaufman, 2009)).

Both human ES cells and iPS cells have the potential to produce hematopoietic cells. Two main methods have been used for this purpose: EB formation and stromal cell co-culture.

Many studies have used the EB formation protocol to generate erythroid cells with the addition of cytokines (Lengerke et al., 2009, Anstee, 2010). A recent study claimed a complete differentiation of human iPS cells into definitive enucleated RBCs containing fetal haemoglobin. Erythroid commitment was obtained via EB formation in the presence of erythropoiesis cytokines and human plasma, followed by differentiation and maturation into enucleated RBC contained fetal (a2γ2) that was switched in only few cells into adult (a2β2) haemoglobin (Lapillonne et al., 2010b). This protocol has the advantage

of dispensing with the co-culture on stromal cells, as well as the use of animal products (Lapillonne et al., 2010b). However, human plasma was used, which exposed the cell culture to undefined factors (Lebkowski et al., 1995). No difference was observed between human iPS cells and ES cells in terms of erythropoiesis recapitulating potential and expression of erythroid markers, or type and functionality of haemoglobin. However, differences in expansion rate and percentage of enucleated RBC were still reported (Lapillonne et al., 2010b).

In another relevant study, Feng et al. (2010) compared the efficiency of generating hemangioblasts and hematopoietic cells from human iPS cells via EB formation to the results generated from human ES cells. They reported similarity in morphology and phenotype, but with a lower efficiency (Lu et al., 2007, Lu et al., 2008b, Feng et al., 2010). In contrast with human ES cell-derived hemangioblasts, higher apoptosis, slower growth, and limited erythroid lineage expansion and differentiation capabilities were observed in human iPS cell-derived hemangioblasts. Moreover, functional differences were also noted after the dramatically decreased hematopoietic colony-forming capability and O_2-carrying capacity were observed with human iPS cell-derived erythroid cells (Feng et al., 2010).

The reasons behind the lower expansion rate and early senescence associated with iPS cell differentiation are unknown. One study has suggested that contribution of p53/p21 pathways in the reprogramming of somatic cells may be responsible (Hong et al., 2009). Another study has proposed a tumorigenic impact of Lin28, one of the transcription factors used for the reprogramming of human iPS cells (Viswanathan et al., 2009). Alternatively, the intrinsic properties of the human iPS cell lines used and their maintenance protocol might have an impact (Dias et al., 2011). As it has been recently proposed that different cytokines concentrations are needed to induce mesodermal differentiation by different human iPS cell lines (Kattman et al., 2011), scientists have suggested that EB formation might be optimum for erythroid differentiation from human ES cells only, but suboptimum for human iPS cells, whereas OP9 co-culture supports this approach for both human ES cells and iPS cells (Dias et al., 2011).

In contrast, a study conducted by Chang et al. (2011) has revealed that differentiated iPS cells produced from different somatic cell types and ages into RBCs using the co-culture method on stromal cells sequentially expressed embryonic and fetal haemoglobins, recapitulating early human erythropoiesis. They are undistinguishable from those derived from human ES cells (Chang et al., 2011). Another study induced haematopoietic/endothelial differentiation from several human iPS cell lines of different origins by co-culture on OP9 mouse bone marrow stromal cells with supplementation of 10% fetal bovine serum. The pattern of the hematopoietic differentiation among the different lines was similar and comparable to the differentiation potential of human ES cells (Vodyanik et al., 2006, Choi et al., 2009). However, no expansion capabilities were compared on the hematopoietic progenitors produced (Feng et al., 2010).

This outcome was supported by another study by Dias et al. (2011), who reported the generation of long life-span RBCs from transgenic and transgene-free iPS cells after co-culturing with OP9 mouse bone marrow stromal cells with an efficiency and haemoglobin profile similar to human ES cells. The RBCs produced expressed mainly fetal γ and embryonic ε globins and a trace amount of adult β globin (Dias et al., 2011). Hematopoietic differentiation of human ES cells and iPS cells was induced using an OP9 co-culture system, followed by selective expansion of erythroid cells in serum-free media supplemented with cytokines needed for erythropoiesis (Dias et al., 2011). Fibroblast-derived iPS cells used in this study were transgenically reprogramed, using lentivirus-based vectors and transgene-free human iPS cells reprogramed using episomal vectors (Dias et al., 2011), as viral-based reprogramming methods are associated with permanent genomic integration of transgenes that can be still detected in differentiated cells and affect their differentiation manner (Park et al., 2008, Choi et al., 2009). Thus, it is suggested that irrespective of their reprogramming strategy or source of tissue, human iPS cells can be differentiated on OP9 stromal cells towards a multipotent $CD34^+$ haematopoietic progenitor that can contribute to lymphoid and myeloid lineage (Kim et al., 2011, Stadtfeld et al., 2012, Yang et al., 2014). Moreover, Yang et al. (2014) has described the ability of human iPS cell-derived $CD34^+$ HSCs to undergo definitive erythropoiesis, giving rise to erythroblasts that contain mainly fetal haemoglobin (HbF),

with a sub-fraction of erythroblasts co-expressing adult haemoglobin (HbA) after co-culturing on OP9 stromal cells (Yang et al., 2014).

Recently, the production of antigen-free universal donor RBCs has been achieved with dermal fibroblasts of a person with Bombay blood type reprogrammed into iPS cells using Klf4, Oct4, Sox2, and c-Myc transcription factors. The Bombay phenotype is known to lack ABH antigens that exist in the ABO blood group system. The detection of this rare blood group is very significant for the field of immunohematology. Accordingly, it is suggested that erythroid cells derived from Bombay phenotype iPS might be histocompatible erythroid cells that can pave the way toward attaining the universal non patient-specific blood source, and can be of great value for transfusion to patients with different blood types-A, B, AB, and O (Seifinejad et al., 2010). Another attempt was tested by Ganji group to derive human iPS cells from donor fibroblasts with a Bombay phenotype. They have shown that the differentiation of iPS cells into erythroid cells follows the same expression patterns as human ES cells in a sequential differentiation via hemangioblast colonies. Pluripotent cells were differentiated into erythroid lineage in a feeder-free serum-free suspension culture supplemented with several cytokines via aggregates formation. However, the erythroid cells produced expressed haemoglobin F ($\alpha 2\gamma 2$), rather than adult A ($\alpha 2\beta 2$), and no enucleation was reported (Ganji et al., 2015).

One recent study claimed that iPS cells maintain an epigenetic memory from their original donor cell type due to incomplete elimination of tissue-specific methylation, which can affect their differentiation performance (Dorn et al., 2015). Accordingly, this study compared iPS cells generated from $CD34^+$ HSC derived from human cord blood to human neural stem cell-derived iPS cells and human fibroblasts-derived iPS cells for erythroid differentiation. All iPS cell lines showed similarity and gave rise to erythroid cells expressing CD235a (glycophorin A) (>96%), fetal haemoglobin (89%-96%), and some enucleation (21%-29%). However, the expansion rate of the erythroid cells and frequency of erythroid colony formation was slightly higher in the $CD34^+$ iPS cells (Dorn et al., 2015).

In conclusion, the field of erythroid cell formation from ES and iPS cells has expanded and different methods have been used that show impressive results in terms of

reproducibility of the method, quantity of cells produced, and functional activity of these cells (Figure 9) (Kaufman, 2009). However, significant enhancements have to be made before translating pluripotent stem cell-based therapies into clinics, including the development of completely defined serum-free and xeno-free culture conditions for derivation, expansion, and differentiation; bioreactor-based technology suitable for large-scale production; and full assessments of the therapeutic effect and safety of produced cellular products in animal models (Dias et al., 2011).

Furthermore, because the use of ES cells is associated with a risk of stimulating immunological reaction upon transfusion or transplantation as the express immune antigen such as human leucocyte antigens (Drukker et al., 2002), the need to use iPS cells as substitute pluripotent stem cells arises. These iPS cells can be created by introducing specific genes encoding transcription factors into adult somatic cells using viral or other vectors (Nishikawa et al., 2008).

In an attempt to evaluate the best source of pluripotent cells suitable for large-scale erythroid cell production and best protocol to be used, successful techniques used with pluripotent cells are summarized and compared for their efficiency in terms of generation of mature, functional, enucleated RBCs and haemoglobin content (Table 2). All the listed protocols followed either the EB formation protocol, which influences heterogeneity of the produced population, and/or the co-culture with mouse stromal cells, where obstacles of purification of the cells rise, which makes the xeno-free culture requirement difficult to achieve. Furthermore, significant disadvantages are associated with the generated erythroid cells, including poor expansion rate, incomplete maturation and enucleation, and inefficient switching to adult-type haemoglobin.

Table 2. Summary of the Most Efficient Protocols in Generating Erythroid Cells from Human Pluripotent Stem Cells.

Type of pluripotent cells	Human ES cells	Human ES cells	Human ES/iPS cells	Human ES/iPS cells
Protocol used	Co-cultured on murine fetal liver stromal cells in the presence of a cytokine cocktail and 15-30% serum	• EB formation and hemangioblasts generation by supplementing media with cytokines • Co-culture • Serum-free	• EB formation in a medium supplemented with cytokines and 10% human plasma • Dissociated EBs were cultured in a liquid medium+ cytokines	• Co-culture on OP9 then on MS5 cells+ cytokines • 10%serum
Length in culture	34 days	36-42 days	45 days	20–90 days
Number of erythrocytes produced	$5x10^5$- $1x10^6$ cells/ E bursts	10^{10} to 10^{11} cells/6-well plate	$1x10^6$ hiPSC gave $2.5x10^8$ erythroid cells	$2x10^5$ of RBCs from 1 ES cell
Maturation events:				
• Size reduction	---------	Observed	Observed	---------
• Chromatin and nuclear condensation	---------	Observed	---------	---------
• CD235a	Detected	>30% of cells	90-93%	>97% of cells
• CD71	Up-regulated	>75% of cells	80-84%	---------
Enucleation	Observed	10-30%, enhanced to 30–65% of cells after co-culture	4–10% iPS cells 52-66% ES cells	2–10%
Haemoglobin expressed	Embryonic (ε) and fetal (γ) haemoglobin followed by adult β-globin in 50–60% of cells	Mainly fetal haemoglobin (65%) but they can express adult β-globin upon further maturation	Fetal haemoglobin, no β-globin was detected	Predominately fetal and embryonic hemoglobin
Haemoglobin function	• O_2 dissociation curve similar to that of cord blood • High G6PD activity	O_2 dissociation curve displaced left compared to normal adult RBCs	The rebinding kinetics of haemoglobin with carbon monoxide (CO) was identical to cord blood	---------
References	(Ma et al., 2008)	(Lu et al., 2008b)	(Lapillonne et al., 2010a)	(Dias et al., 2011)

2.6. Terminal Maturation and Enucleation of Erythroid Cells

Signs of the terminal maturation of erythroid cells mainly include loss of the expression of transferrin receptor CD71, expression of glycophorin A, enucleation, and acquisition of biconcave shape (Lu et al., 2008a, Giarratana et al., 2011). Enucleation *in vitro* remains difficult to achieve. It has been suggested that *in vitro* co-culturing pluripotent cells with stromal cells can trigger hematopoietic cell differentiation, owing to the fact that haematopoiesis in bone marrow highly depends on the presence of mesenchymal stem cells to excrete soluble factors needed for adult haematopoiesis. In addition, this method provides direct cell–cell contact needed for the expansion of hematopoietic cells, which cannot be offered by stromal-conditioned media. Even more importantly, the stromal cells might be responsible for engulfing organelles and nuclei of the erythroblasts on maturation (Lu et al., 2008a). Scientists have proposed that MS-5 stromal cells might mimic the *in vivo* role played macrophages, a surface where erythroids can roll over during enucleation in order to acquire the biconcave discoid shape and increase the iron uptake (Giarratana et al., 2005, Lu et al., 2008a). Moreover, MS-5 has been shown to support haematopoiesis by secreting soluble factors such as IL-6. OP9 stromal cell layer has been suggested as the best co-culture enucleation system for human ES cells-derived erythroid cells, as described above, where Lu et al. (2008) demonstrated enucleation in 65% of erythroid cells after using this system (Lu et al., 2008a).

Another way to enucleate erythroblasts without using stromal cells is to prime the cells with VEGF and IGF-II to extrude nucleus, in addition to enucleation medium containing disodium hydrogenphosphate dodecahydrate, Plasmanate, D-mannitol, adenine, and mifepristone (Miharada et al., 2006). Yet the cellular mechanisms involved in the erythroid enucleation are poorly understood. Some studies have proposed that enucleation occurs after asymmetric cell division to extrude nucleus enclosed by the plasma membrane, which is thought to be promoted by the direct contact with macrophages (Rhodes et al., 2008). Further studies are needed to reach a full maturation of *in vitro*-generated RBCs for clinical applications. Indeed, it has been recently shown that enucleation and synthesis of adult haemoglobin are possible in human iPS cells-derived erythroblasts, *in vivo* (Kobari et al., 2012). This study has suggested that terminal

maturation of blasts can occur under the influence of an adult hematopoietic microenvironment (Kobari et al., 2012).

As described above, erythroid cells are released from the bone marrow in the form of reticulocytes into the circulation where the final maturation takes place in 48-72 hr (Giarratana et al., 2011). Remnants of mitochondria, Golgi complex, and ribosomes are eradicated and erythrocytes start to adopt the biconcave disk shape for effective gas exchange (Giarratana et al., 2011). The transition of reticulocytes into mature erythrocytes is associated with remodelling of the plasma membrane, which results in extensive changes in structure, properties, and protein content of that membrane (Liu et al., 2010). The erythrocyte membrane is composed of three layers: a carbohydrate-rich layer called the glycocalyx, a lipid bilayer with transmembrane proteins, and an inner structure called the membrane skeleton or cytoskeleton (Doherty and McMahon, 2008). Several dynamic processes in a mature erythrocyte require a cross-linking between the membrane and the cytoskeleton, including cell motility, cell-cell interactions, cell adhesion, and membrane deformability. Moreover, this cytoskeleton-membrane interaction seems to govern the diffusion of membrane integral proteins and phospholipids into the erythrocytes (Doherty and McMahon, 2008).

To date, these attempts cannot replace conventional blood transfusion, but at least offer a hope for patients with rare blood groups or polyimmunized blood (1%-3% of the transfused patients) (Giarratana et al., 2011). Moreover, the cost of RBCs production should be considered. Thus, the challenge is to provide an automated, cost-effective, and efficient erythroid differentiation system including purification and packaging of sufficient transfusable RBCs equivalent to one unit of conventional blood unit ~10^{12} RBC (Dorn et al., 2015).

Chapter 3.

Aim and Objectives

Blood transfusion is a common clinical procedure used to treat the need for blood cells. However, there is a global threat of insufficient blood supply for transfusion, where the shortage is predicted to be felt as early as 2050 (Shah et al., 2014). Much focus has been directed toward this problem in order to find a replacement for a continuous supply of blood cells, where cellular therapy attempts has arisen to generate RBC *in vitro* from stem cells (Mazurier et al., 2011).

Since ES cells and iPS cells possess two distinct properties -unlimited self-renewal and high differentiation potential (Lakshmipathy and Verfaillie, 2005)- they have been suggested as cell sources the *in vitro* generation of RBCs. Moreover, human iPS cells are advantageous in comparison with ES cells, as they are not ethically controversial in regards to their cell origin, and they can be precisely selected for their phenotypes, which can be useful for generating RBCs of a blood group of interest (Mazurier et al., 2011).

Two methodologies or combinations have been identified so far for this approach: the co-culture on stromal cells in the presence of cytokine cocktail, and the production of hemangioblasts, via EB formation, which creates heterogenicity and produces cells of three germ layers (Anstee, 2010). However, the current protocols are complicated, long, multi-steps, and associated with many restraints, including low efficiency, incomplete maturation and enucleation of the produced cells, poor functional evaluation, production of embryonic and fetal haemoglobin and inefficient switching to adult-type haemoglobin, compulsory passage through a hematopoietic progenitor- hemangioblasts, and poor expansion rate and Lack of required number of RBCs equivalent to one unit. In addition, the use of serum expose the culture to undefined factors and confines the standardization needed for clinical applications, while the co-culture on animal cells presents animal-derived materials that can trigger immune reactions upon transfusion (Anstee, 2010, Mazurier et al., 2011).

Therefore, for the safe use of RBCs generated from iPS cells in clinics, an optimized direct erythrocyte differentiation and maturation protocol under serum-free and feeder-free conditions is needed (Mazurier et al., 2011).

Thus, the overall aim of this project is to develop a robust system for a direct and efficient production of functional erythroid cells *in vitro* from human iPS cells in a serum-free and feeder-free conditions. In order to achieve this aim, certain key objectives have been pinpointed along the way. These include:

3.1. Adapt the human iPS cells to our serum-free and feeder-free maintenance culture conditions to ensure pluripotency.

3.2. Identify the important factors for the differentiation of human iPS cells towards the erythroid lineage.

3.3. Optimize a direct haematopoietic/erythropoietic induction protocol of human iPS cells based on the factors identified in (3.2) under hypoxic condition.

3.4. Compare the efficiency of the haematopoietic/erythropoietic induction protocol identified in (3.3) in colonies vs single cells seeding conditions.

3.5. Combine the haematopoietic/erythropoietic induction protocol with further erythroid differentiation and maturation steps.

3.6. Assess the role of O_2 on the differentiation of human iPS cells towards the erythroid lineage by comparing the protocol identified in (3.5) in normoxia and hypoxia using critical analytical procedures (Table 3.):

Table 3. List of the Critical Analytical Procedures Used for the Assessments of the Differentiated Human iPS Cells.

Morphology	• Wright-Giemsa Staining • Reticulocyte Staining
Cellular Markers	Flow Cytometry
Clonogenic Ability	CFU Assay
Protein Analysis	Immunocytochemical Staining
Gene Expression	qPCR
Viability	DAPI Staining
Haemoglobin Analysis	• Immunocytochemical Staining • Gene Expression and qPCR
Metabolic Analysis	• Nutrient consumption rates • Metabolite concentration profiles

Chapter 4.

Materials and Methods

4.1. Culture Media Materials

4.1.1. Human iPS cells Maintenance Medium

The maintenance and expansion of human iPS cells was performed under feeder-free conditions using mTeSR™1 Medium (StemCell Technologies™, Canada), a defined and serum-free medium.

4.1.2. Hematopoietic/Erythroid Induction Medium

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12-GlutaMAX™-I (DMEM/F-12-GlutaMAX™-I) (Gibco® by Life Technologies™, UK) was supplemented with 10 mg/mL bovine serum albumin (BSA) (sigma, UK) in the presence of 100 ng/mL human SCF, 100 ng/mL human TPO, 100 ng/mL human FLT-3 ligand, 50 ng/mL recombinant human BMP-4, 50 ng/mL recombinant human VEGF-A165, 5 ng/mL IL-3, 5 ng/mL IL-6 (all supplied by PeproTech, USA), 50 ng/mL recombinant human FGF-basic (Life Technologies™, UK), 3 U/mL human EPO (R&D Systems), in addition to 100 units/mL penicillin and 100 µg/ml streptomycin (both supplied by Gibco® by Life Technologies, UK).

4.1.3. Erythroid Differentiation Medium

StemLine II hematopoietic stem cell expansion medium (Sigma, UK) was supplemented with 40 µg/mL *myo*-inositol (Sigma, UK), 10 µg/mL folic acid (Sigma, UK), 160 µM monothioglycerol (Sigma, UK), 120 µg/mL holo-human transferrin (Sigma, UK), 10 µg/mL insulin (Sigma, UK), 90 ng/mL iron (III) nitrate (Sigma, UK), 900 ng/mL iron (II) sulphate (Sigma, UK), 10 mg/mL BSA (Sigma, UK), 4 mM Glutamax™-1 (Gibco® by Life Technologies™, UK), 1µM hydrocortisone (Sigma, UK), 100ng/mL SCF (PeproTech, USA), 5ng/mL IL-3 (PeproTech, USA), 3U/ml human EPO (R&D Systems), and 100 units/mL

penicillin and 100 µg/mL streptomycin (both supplied by Gibco® by Life Technologies, UK).

4.1.4. Erythroid Maturation/Enucleation Medium

StemLine II hematopoietic stem cell expansion medium (Sigma, UK) was supplemented with 40 µg/mL *myo*-inositol (Sigma, UK), 10 µg/mL folic acid (Sigma, UK), 160 µM monothioglycerol (Sigma, UK), 120 µg/mL holo-human transferrin (Sigma, UK), 10 µg/mL insulin (Sigma, UK), 90 ng/mL iron (III) nitrate (Sigma, UK), 900 ng/mL iron (II) sulphate (Sigma, UK), 10 mg/mL BSA (Sigma, UK), 4 mM Glutamax™-1 (Gibco® by Life Technologies™, UK), 3U/mL human EPO (R&D Systems), and 100 units/mL penicillin and 100 µg/mL streptomycin (both supplied by Gibco® by Life Technologies, UK).

4.2. Cell Culture Methods

4.2.1. Human iPS Cells Maintenance

Human iPS cell line used throughout experiments included in this thesis was IMR90-1 at passage 39 purchased from WiCell bank, University of Wisconsin, Laboratory of Dr. James Thomson (Yu et al., 2007). This iPS line was generated from IMR90 fetal lung fibroblasts and reprogrammed via viral transfection of four pluripotent genes: Oct4, Sox2, Nanog, and Lin28, using Lentivirus. This commercially available iPS cell line has a normal karyotype, demonstrate telomerase activity and express pluripotent surface markers. Thus, it is considered as the best iPS cell line and is favoured for researches including our aim (Anstee, 2010). Human iPS cells were cultured on 6-well tissue culture plates (VWR International Ltd., Leicestershire, UK) coated with Corning® Matrigel® hESC-qualified Matrix (Corning®, Fisher scientific, UK) under serum-free and feeder-independent protocol (Lannon et al., 2008), according to the following steps:

4.2.1.1. Aliquoting Corning® Matrigel®- hESC-Qualified Matrix

Corning® Matrigel® Matrix was always kept at -20°C upon arrival, but once aliquoted, these aliquots were transferred immediately at -80°C to avoid clotting, as Matrigel is known to be frozen at <-20°C, liquid at 4°C, and gels quickly at room temperature. Corning® Matrigel® Matrix hESC-qualified Matrix vial was submerged in ice in a 4°C refrigerator overnight to thaw. Once thawed, the vial was swirled to ensure that Corning® Matrigel® Matrix was evenly mixed before dispensing it into aliquots in cold, sterile tubes placed in a cold container using cold pipette tips. The aliquoting procedure was always performed in a sterile biosafety cabinet, with changing of pipette tips every 5-7 tubes to prevent clotting of the Corning® Matrigel® Matrix. The volume/aliquote was prepared according to the dilution factor provided on Certificate of Analysis, which was usually between 270-350µl. The dilution factor was calculated based on the protein concentration of each lot (0.5mg/6-well plate and 2.0mg/4x6-well plates).

4.2.1.2. Thawing an Aliquot and Coating Plates with Corning® Matrigel®

This step needed to be performed in the sterile biosafety cabinet. A 50ml sterile conical tube containing 25ml of cold DMEM/F-12-GlutaMAX™ medium was used to dilute one 2.0 mg Corning® Matrigel® Matrix aliquot taken directly from the -80°C freezer. The diluted mixture was pipetted gently up and down to dissolve the Corning® Matrigel® Matrix before transferring 1ml/well of a 6-well plate. This dilution was enough for 4 complete 6-well plates. Plates were allowed to set for 1hr at room temperature before use. These coated plates were then used to culture human iPS cells. If the plates were not used immediately, an additional 1ml of DMEM/F-12-GlutaMAX™ medium was added to each well before storing to prevent dryness, as drying wells cannot be used to culture cells. Plates were always wrapped in parafilm and stored at 2-8°C. Coated plates were used within 7-10 days after preparation.

4.2.1.3. Thawing Human iPS Cells on Corning® Matrigel® Matrix

Excess coating medium from prepared Corning® Matrigel® Matrix was removed from wells and 1.5ml mTeSR™1 Medium/well was added. Plates were labelled with the cell line name (IMR90-1), passage number, date, and name of the person performing the procedure. A vial of IMR90-1 human iPS cells to be cultured was carefully removed from the liquid nitrogen tank using forceps. Appropriate protective cryo gloves and eye googles were worn while handling frozen vials stored in the liquid nitrogen in consideration of the risk of vial explosion when warmed. The vial was rolled between the hands to remove the coating frost before swirling it in a 37°C water bath until only small ice crystals could be seen. The vial was then sprayed with 70% ethanol, air-dried, and transferred to the sterile biosafety cabinet. Cells were gently pipetted into a sterile 15ml conical tube and 11ml of mTeSR™1 Medium was added drop-wise, using a 5ml glass pipette to reduce osmotic shock to the cells. Cells were then centrifuged at 200 x g for 5 min. Supernatant was aspirated using a sterilized Pasteur pipette, and the cell pellet was re-suspended in 0.5ml mTeSR™1 Medium for each well that was to be seeded with cells. The cell suspension was very gently pipetted up and down to avoid making single cells, and 0.5ml was added drop-wise into each well. Seeded plates were gently shaken and placed in a humidified water-based incubator set at 37° C, 5% CO_2. The medium was changed every day by aspirating old medium and adding 2ml of mTeSR™1 Medium into each well until cells were ready to be passaged.

4.2.1.4. Passaging Human iPS Cells

Human iPS cells (passage 40-45) were split every 3-6 days, whenever colonies looked too dense or too large under the microscope, or when signs of differentiation were noticed. Split ratio was adjustable, generally between 1:2 and 1:6. Before passaging the cells, all areas of differentiation were marked on the plate and removed by suction using the Pasteur pipette tip. Passaging cells was made enzymatically by adding Dispase solution (Gibco® by Life Technologies™, UK). A 2mg/ml Dispase solution was prepared, filter

sterilized, labelled, and kept at 4°C for up to 14 days. To start splitting the cells, human iPS cells were removed from the incubator and placed in the biosafety cabinet. The spent medium was aspirated from the wells to be passaged, and plates were incubated for 7 min at 37° C with 1ml Dispase solution/well. Wells were then washed gently three times using DMEM/F-12-GlutaMAX™, without peeling off the attached cell layer, before adding 1-2ml of mTeSR™1 Medium/well. In order to passage the cells, a 5ml pipette was held perpendicularly to the surface of the plate with a gentle scrape while simultaneous pipetting of the medium slowly up and down to wash the cells off and break-up the large colonies took place. The cell suspension was then dispensed into new Corning® Matrigel® Matrix-coated wells (~0.5ml/well), with a total of 2.0ml of medium and cells/well (0.5ml of cell suspension + 1.5ml of fresh mTeSR™1 medium). Seeded plates were placed in the incubator for the cells to attach, with quick, short, back-and-forth and side-to-side moves to disperse cells across the surface of the wells.

4.2.1.5. Freezing Human iPS Cells

Cryovials were labelled with the cell line (IMR90-1), passage number, the freeze date, and initial of the person performing the freezing procedure. All wells to be frozen were examined under the microscope to exclude any contaminated plates and to mark the differentiation areas as explained in 4.2.1.4. Spent medium was removed and wells were incubated with 1ml Dispase solution 5-7 min at 37°C. Wells were then carefully washed twice with 1ml/well of DMEM/F-12-GlutaMAX™ before adding 1ml of mTeSR™1 medium. The cells layers were detached by scraping the surface of the well with 5ml pipette while slowly pipetting the mTeSR™1 Medium up and down to break clumps as little as possible. Cell suspensions were pooled in a sterile 50ml centrifuge tube and centrifuged at 200 x g for 5 min. The cell pellet was then re-suspended in 1ml mFreSR™1 Medium (StemCell Technologies™, Canada)/well slowly and drop-wise and the resulting cell suspension was distributed into labelled cryovials. Cryovials were then quickly placed into an isopropanol-containing freezing container and left in a -80°C freezer overnight before they were transferred to a liquid nitrogen storage tank.

4.2.2. Human iPS Cells Differentiation towards the Erythroid Lineage

The differentiation protocol addressed in this context is feeder-independent and completely serum-free. Undifferentiated human iPS cells were cultured on the coated 6-well tissue culture plates with Matrigel and maintained in mTeSR™1 Medium, as described in 4.2.1. Undifferentiated human iPS cells were treated as the day 0 control group of all analysis. When undifferentiated human iPS cells reached 50% confluence, they underwent differentiation. At this point, the medium in the plates was replaced with Hematopoietic Induction Medium (as described in 4.1.2.) for 7 days, followed by 7 days in Erythroid Differentiation Medium (as described in 4.1.3.), and 14 days in Erythroid Maturation Medium (as described in 4.1.4.). Steps of the differentiation process occurred either under normoxia or hypoxia conditions. Plates containing cells cultured under normoxia conditions were incubated in a humidified water-based incubator set at 37°C and 5% CO_2, while plates containing cells cultured under hypoxia conditions were placed in humidified, water-based incubator, set at 37°C, 5% CO_2, and 5% O_2. Media was changed every two days and cells were harvested and analysed every 7 days.

4.3. Cellular Analysis

4.3.1. Erythrosin-B Exclusion Assay for Cellular Viability

Cellular viability and manual count for cell density were determined throughout the culture using a haemacytometer (Fisher scientific, UK), under the Leica DM-IL inverted phase microscope (Leica). Cellular viability was assessed using Erythrosin-B stain solution (ATCC, Middlesex, UK), a dye-exclusion method where only dead cells are stained reddish-pink.

4.3.2. Cell Proliferation Assay (MTS Assay)

The dynamic profile of metabolic activity of human iPS cells to proliferate and expand in mTeSR™1 Medium in a specific time was assessed using the Cell Titer 96 Aqueous One Solution Reagent MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega, UK) as per manufacturer's instructions. This reagent converts into a coloured product in metabolically active cells by NADPH or NADH produced by dehydrogenase enzymes. The assay was run in 24-well tissue culture polystyrene plates (VWR International Ltd., Leicestershire, UK) coated with Corning® Matrigel® hESC-qualified Matrix (Corning®, Fisher scientific, UK) and incubated for at least 1 hr. Then the plates were seeded with similar number and size of colonies (5 colonies/well) and cultured for 1, 2, 3, 4, and 5 days in mTeSR™1 medium. Well-containing cells from each day of culture were washed and incubated with MTS solution for 3 hr. Finally, 100mL of the solution from each treated well was transferred into a 96-well plate (Thermo Scientific™, UK) and absorbance was read at 490nm using an enzyme-linked immunosorbent assay (ELISA) reader (ELx808, BIO-TEK, USA). The average absorbance readings of each day were normalized to a blank reading and plotted on a graph.

4.3.3. Morphological Analysis

4.3.3.1. Wright-Giemsa Staining

Changes in morphology of the cultured cells were observed using the following analysis on a slide. A cell suspension of $5x10^5$cells/ml was prepared in Phosphate-buffered saline (PBS) (Gibco® by Life Technologies™, UK) containing 0.1% BSA and 100µl of the cell suspension was loaded on a slide. The prepared slides were air-dried for 10 min and then stained with Wright-Giemsa stain (Sigma, UK) by dipping for 10 sec in a Coplin jar containing Wright-Giemsa stain for nuclear-cytoplasmic differentiation. The slides were subsequently washed in deionized (DI) water for approximately 15 min. Cells were

visualized on the Olympus BX51 microscope (Olympus UK Ltd) and images were captured using the DP50 camera (Olympus UK Ltd).

4.3.3.2. Reticulocyte Staining (New Methylene Blue)

Morphology of the cultured cells at different time points was observed in order to examine the formation of reticulocytes. A cell suspension of $5x10^5$cells/ml was prepared in PBS+0.1% BSA and 100µl of the cell suspension was loaded onto a slide. Slides were air-dried for 10 min then stained with Reticulocyte stain (Sigma, UK) for 5 min for precipitation of ribosomal RNA. Slides were subsequently washed in DI water for approximately 15 min. Cells were visualized on the Olympus BX51 microscope (Olympus UK Ltd) and images were captured using the DP50 camera (Olympus UK Ltd).

4.3.4. Clonogenic Ability- Colony Forming Unit (CFU) Assay

The clonogenic ability for the erythroid progenitors (CFU-E and BFU-E), granulocyte-macrophage progenitors (colony-forming unit granulocyte-macrophage; CFU-GM), and multi-lineage progenitors (CFU-GEMM) cells, produced along human iPS differentiation process, was assessed *in vitro* using MethoCult™ H4034 Optimum (StemCell Technologies™, Canada), a methylcellulose-based media. Differentiated cells were harvested at different time points to prepare cell suspensions of $3x10^3$cells/300µl in StemLine II hematopoietic stem cell expansion medium (Sigma, UK) supplemented with 3% fetal bovine serum (FBS) (Gibco® by Life Technologies™, UK). The cell suspensions were mixed with 1ml of MethoCult media, and plated into 35 mm Petri dishes (VWR International Ltd., Leicestershire, UK). The seeded dishes were then cultured for 14-16 days at 37°C and 5% CO_2 in a humidified incubator. All assays were performed in duplicates and the resultant colonies were counted and identified according to Stem Cell Technologies Hematopoietic Manual as BFU-E, CFU-E, CFU-GM, and CFU-GEMM.

4.3.5. Immunocytochemical Staining

The following immunocytochemical staining protocol was followed throughout all experiments. Samples for this procedure were cell suspensions either loaded directly onto slides (VWR International Ltd., Leicestershire, UK) or grown in 4-or 8-well chamber slides (Lab-Tek™, Thermo Scientific™, UK). For slide samples, cultured cells at specific time points were harvested using UltraPure™ 0.5M EDTA (Invitrogen™ by Life Technologies™, UK) or TrypLE™ (Gibco® by Life Technologies™, UK), and a cell suspension of $5x10^5$cells/ml was prepared in PBS+0.1% BSA, then 100µl of that cell suspension was loaded onto a slide. For chamber slides, cultured cells at specific time points were grown overnight in 4- or 8-well chamber slides (Lab-Tek™, Thermo Scientific™, UK) in order to attach. Samples were fixed in 4% paraformaldehyde (PFA; VWR International Ltd., Leicestershire, UK) at room temperature for 10 min, followed by washing 3 times in cold PBS. Fixed cells were then permeabilised with PBST (0.1% Triton-X-100 (VWR International Ltd., Leicestershire, UK) in PBS) at room temperature for another 10 min. This step was followed by a blocking step by incubating the permeabilised cells with PBST+ 0.1% BSA (Sigma, UK) (blocker solution) for 1 hr at room temperature. Prior to staining, the blocker solution was used to dilute the primary and secondary antibodies to prepare working solutions. Cells were then incubated with primary antibody listed in Table 4 at 4^0 C overnight. The following day, cells were washed 3 times with the blocker solution and then incubated with secondary antibodies listed in Table 4 for 1 hr at room temperature in the dark. Slides were then washed, mounted using Vectashield with 1.5 mg/mL 40,6 diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK), and covered with coverslips (Corning®, Fisher scientific. UK). Negative controls were taken through the whole process except the addition of the primary antibodies in order to exclude unspecific background bindings. Slides were visualized to prove expressions and distribution of cellular antigens using either Widefield- Zeiss Axiovert 200 inverted microscope equipped with a highly sensitive 1300x1000 pixel camera, or Confocal- Zeiss LSM-510 inverted microscope with a high resolution cooled CCD camera. Data obtained were then analysed and processed using either velocity or FIJI.

Table 4. List of Primary and Secondary Antibodies for Immunocytochemistry Staining.

Antigen	Primary Antibody	Secondary Antibody
Pluripotency Antibodies		
Oct4	Anti-Oct4 (Applied StemCell, Inc.)	Goat-anti-rabbit (Alexa Fluor 594), Human ES/iPS Cell Characterization kit (Applied StemCell, Inc.)
Sox2	Anti-Sox2 (Applied StemCell, Inc.)	
SSEA-4	Anti-SSEA-4 (Applied StemCell, Inc.)	Goat-anti-mouse (Alexa Fluor 594), Human ES/iPS Cell Characterization kit (Applied StemCell, Inc.)
TRA-1-60	Anti-TRA-1-60 (Applied StemCell, Inc.)	
TRA-1-81	Anti-TRA-1-81 (Applied StemCell, Inc.)	
Nanog	1/300 Rabbit polyclonal to Nanog (abcam)	1/500 Alexa Fluor® 488 goat anti-rabbit IgG (H+L)
Differentiation Antibodies		
CD34	1/300 Mouse monoclonal [9B10D4] to CD34 (abcam)	1/500 Donkey Anti-Mouse IgG H&L (Alexa Fluor® 647) preadsorbed
CD45	1/100 Rabbit polyclonal to CD45 (abcam)	1/500 Donkey Anti-Rabbit IgG H&L (FITC) preadsorbed
CD71	5ug/ml Rabbit polyclonal to Transferrin Receptor (abcam)	1/500 Donkey Anti-Rabbit IgG H&L (FITC) preadsorbed
CD235a	1/500 Mouse monoclonal [HIR2] to Glycophorin A + B (abcam)	1/500 Donkey Anti-Mouse IgG H&L (Alexa Fluor® 647) preadsorbed
Human hemoglobin	1/500 Goat polyclonal to Hemoglobin (FITC) (abcam)	N/A
Nuclear Staining		
DAPI	DNA staining solution (Applied StemCell, Inc.)	N/A

FITC, fluorescein isothiocyanate.

4.3.6. Alkaline Phosphatase

Alkaline Phosphatase (AP) is a highly sensitive and simple colorimetric assay used to measure AP activity, which is associated with the pluripotency status of cells. To perform this assay, cells were grown in 4- or 8-well chamber slides (Lab-Tek™, Thermo Scientific™, UK), fixed in 4% paraformaldehyde (PFA; VWR International Ltd., Leicestershire, UK) at room temperature for 10 min, followed by washing 3 times in cold PBS. Fixed cells were then treated with AP test solution (Human ES/iPS Cell Characterization kit (Applied StemCell, Inc.) for 2 hr before pictures were taken under the microscope. Cells were visualized on the Olympus BX51 microscope (Olympus UK Ltd) and images were captured using the DP50 camera (Olympus UK Ltd).

4.3.7. Flow Cytometry

Along the culture, cells at specific time points were stained with antibodies as listed in Table 5 and were analysed using BD FORTESSA (Becton Dickinson (BD), UK) flow cytometry instrument, equipped with five lasers: Argon 488nm laser (3 colours), Red HeNe 640nm (3 colours), UV 355nm laser (2 colours), Violet diode 405nm laser (6 colours) and Yellow-Green 561nm laser (4 colours). Prior to antibodies staining, cells were harvested using UltraPure™ 0.5M EDTA (Invitrogen™ by Life Technologies™, UK) or TrypLE™ (Gibco® by Life Technologies™, UK) in order to obtain single cells, and a cell suspension of 0.5-1.0x10^6 cells/mL was prepared in PBS+0.1% BSA. The samples were directly incubated with antibodies at 4°C for 30 min. After staining, cells were washed twice with PBS+0.1% BSA prior to analysis. Data acquisition and colour compensation for the fluorescent panels using the BD FACSDiva™ software was normally performed immediately following staining, and a total of 10,000 ungated events were acquired for each sample. Subsequent data analysis was performed using FlowJo 10.0.8 (©FlowJo, LLC).

Table 5. List of Antibodies for Flow Cytometry Analysis.

Antigen	Antibody	Isotype controls
Pluripotency Antibodies		
SSEA-4	1/60 PE Mouse anti-SSEA-4 (BD biosciences)	1/60 PE Mouse IgG3, κ Isotype Control (BD biosciences)
Differentiation Antibodies		
CD34	1/3 FITC Mouse Anti-Human CD34 (BD biosciences)	1/3 FITC Mouse IgG1, κ Isotype Control (BD biosciences)
CD45	1/60 PE-Cy™7 Mouse Anti-Human CD45 (BD biosciences)	1/60 PE-Cy™7 Mouse IgG1 κ Isotype Control (BD biosciences)
CD71	1/60 APC Mouse Anti-Human CD71 (BD biosciences)	1/60 APC Mouse IgG2a, κ Isotype Control (BD biosciences)
CD235a	1/120 PE Mouse Anti-Human CD235a (BD biosciences)	1/120 PE Mouse IgG2b κ Isotype Control (BD biosciences)
CD43	1/60 APC Mouse anti-Human CD43 (BD biosciences)	1/60 APC Mouse IgG1, κ Isotype Control (BD biosciences)
Viability Antibodies		
Dapi	DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) (BioLegend)	N/A

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phyco-erythrin; PeCy7, phyco-erythrin Cyno-7

4.3.8. Gene Expression Analysis

4.3.8.1. RNA Isolation and Quantification

RNAs from cultured human iPS cells were extracted using RNeasy kit (Qiagen, West Sussex, UK), according to the kit manual. Harvested cell pellets were disrupted and lysed using buffer RLT+1% β-Mercapthoethanol (Sigma-Aldrich, UK). The resulting lysates were then homogenized using a QIAshredder spin column and centrifuged at maximum speed

for 2 min before adding one volume of 70% ethanol in order to promote binding of RNA to RNeasy spin column. Total RNA samples in the membrane columns were washed with Buffer RW1 before the treatment with DNase I for 15 min to eliminate genomic DNA contamination. Purified RNA samples were washed once again with Buffer RW1 and twice with buffer RPE. RNAs were then eluted in a 1.5 ml collection tube using RNase-free water. The quantity of RNA extracted was quantified using a BioPhotometer (Eppendorf UK, Cambridge, UK), where the A260/A280 ratio of greater than 1.70 is required to confirm the purity of RNA sample. The RNA samples were adjusted to a working concentration of 10 µg/ml for the final one-step polymerase chain reaction (PCR).

4.3.8.2. cDNA Formation By Reverse Transcriptase (RT)

Total mRNA was reversed transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, West Sussex, UK), according to the kit manual. Initially, genomic DNA was eliminated by incubating the 1 µg RNA samples with gDNA Wipeout Buffer at 42 °C for up to 10 min. During the incubation period, the mastermix for the RT reaction was prepared, which consisted of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. Then, the incubated RNA samples were mixed with the prepared mastermix, and placed in the G-STROM thermal cycler (G-STROM™, UK) for the RT reaction. The reaction was executed by firstly incubating the samples at 42 °C for 15 min, then at 95 °C for 3 min, and, finally, a cooling step to 4 °C. Samples were stored at -20 °C before starting the qPCR.

4.3.8.3. Real-time Quantitative Polymerase Chain Reaction (qPCR)

Relative gene expression was analysed by conducting real-time quantitative polymerase chain reaction (qPCR) on the genes of interest to amplify cDNA using StepOnePlus™ Real-time PCR instrument (Applied Biosystems™, UK).

Initial qPCR analysis was performed on human iPS cells to ensure pluripotency status of the cells using QuantiTect SYBR Green PCR Master Mix kit (Qiagen, West Sussex, UK), based on manufacturer's guidelines. The kit guidelines are briefly summarized: 1-2µg cDNA sample was mixed with different reagents within the same reaction: 2× QuantiTect SYBR Green PCR Master Mix, 10x QuantiTect Primer Assay, with RNase-free water comprising a 25µl volume PCR reaction. The reaction started with a PCR initial activation step to activate DNA polymerase at 95°C for 15 min, followed by A 3-step cycling condition involving 40 cycles with 15sec of denaturation at 94°C, 30sec of annealing at 55°C, and 34sec of extension at 72°C. Primers were all bought from Qiagen, UK, as detailed in Table 6.

Table 6. List PCR Primers.

Gene symbol	Gene name	Assay name	Amplicon Length	Species
Housekeeping Gene				
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs_GAPDH_2_SG	119 bp	Human
Pluripotent Genes				
POU5F1	POU class 5 homeobox 1 (Oct4)	Hs_POU5F1_1_SG	77 bp	Human
NANOG	Nanog homeobox	Hs_NANOG_2_SG	164 bp	Human
SOX2	SRY(sex determining region Y)-box2	Hs_SOX2_1_SG	64 bp	Human
ZFP42	ZFP42 zinc finger protein (REX-1)	Hs_ZFP42_1_SG	89 bp	Human
Germ Layers-Markers				
Ectodermal Marker				
PAX6	paired box 6	Hs_PAX6_1_SG	113 bp	Human
Mesodermal Marker				
T	T, brachyury homolog	Hs_T_1_SG	91 bp	Human
Endodermal Marker				
AFP	alpha-fetoprotein	Hs_AFP_1_SG	142 bp	Human

Subsequent qPCR analysis was performed on differentiated human iPS cells at specific time points along the differentiation protocols to monitor changes in gene expression. Gene expressions were analysed using The TaqMan Universal Master Mix II (Life Technologies™, UK), according to the manufacturer's instructions, together with Customized TaqMan® Array 96-well Fast Plates (Life Technologies™, UK), ready-to-use gene expression assays pre-plated with 16 optimized primers and probes designed to target sequences relevant to my experiments, as shown in Table 7. A 5µl TaqMan master mix was prepared/samples with 1-2µg cDNA sample/test and RNase-free water comprising a 10µl volume/well. The reaction started with a PCR initial holding stage at 50°C for 2 min, followed by 10min at 95°C, before the 40 cycles-cycling stage began at 95°C for 15sec, and finished at 60°C for 1min.

Table 7. List of Assays Included in the Customized TaqMan® Array 96-well Fast Plates.

Gene symbol	Gene name	Assay ID	Species
18s	18s	Hs99999901_s1	Human
GAPDH	GAPDH	Hs99999905_m1	Human
NANOG	Nanog	Hs04260366_g1	Human
POU5F1	Oct4	Hs04260367_gH	Human
CD34	CD34	Hs00990732_m1	Human
PTPRC	CD45	Hs04189704_m1	Human
TFRC	Transferrin Receptor (CD71)	Hs00951083_m1	Human
GYPA	Glycophorin A (CD235a)	Hs00266777_m1	Human
KIT	c-kit (CD117)	Hs00174029_m1	Human
EPOR	EPO receptor	Hs00959427_m1	Human
HBA2	Hemoglobin subunit alpha-2	Hs00361191_g1	Human
HBB	Hemoglobin subunit beta	Hs00758889_s1	Human
HBG2	Hemoglobin subunit gamma-2	Hs00361131_g1	Human
HBE1	Hemoglobin subunit epsilon	Hs00362216_m1	Human
SPN	CD43	Hs00174604_m1	Human
HIF1A	Hypoxia-inducible factor 1-alpha	Hs00153153_m1	Human

A threshold level was calculated using the installed software, i.e. StepOne™ Software v2.3, for each "gene of interest" to determine Ct (threshold cycle) values. Quantitation-Comparative gene expression analysis was calculated using the routinely used $2^{-\Delta\Delta CT}$ method. The expression of each gene of interest was normalized with the housekeeping gene i.e. GAPDH expression and a following normalization with the difference in expression (gene of interest and housekeeping gene) for pluripotent human iPS cells. Duplicate or triplicate values were obtained for all analysis. Negative control consisted of samples without cDNA.

4.3.9. O_2 Equilibrium Curve

The functional analysis of the haemoglobin produced in the human iPS-derived erythroid cells was perfomed using O_2 equilibrium curve. Following the 28-day differentiation of human iPS cells towards erythroid lineage, cells were collected, washed with PBS, and sent to the haematology laboratory, Guy's Hospital, for analysis. O_2 equilibrium curves were determined using a Hemox-Analyzer (TCS Scientific, New Hope, PA). The analysis was performed as described by Lu et al. (2008).

4.4. Statistical Analysis

Samples for the quantitative analyses were pooled cells from 2-5 wells. Error bars on all graphs represent the standard deviation (SD) of the mean. Comparable values from each group were statistically analysed: multiple group analysis of a single factor using one-way analysis of variance (ANOVA) (Turkey Test), and multiple group analysis of two factors using two-way ANOVA (Holm-Sidak method), at a level of significance of $p<0.050$ (single asterisks) using the SigmaStat 3.5 software (Systat Software, Inc., London, UK).

Chapter 5.

Human iPS Cells Culture and Maintenance

5.1. Introduction

Despite the advancement in improving human ES/iPS cell culture methods, there are still many issues that need to be resolved prior to their clinical applications, including: (1) an absence of defined standardized maintenance and differentiation protocols in serum-free and xeno-free conditions, (2) a lack of proper analysis to monitor cellular stress, poor growth conditions, spontaneous differentiation, and apoptosis during cell processing, and (3) the heterogeneity of cell populations (Chen et al., 2014b).

The ultimate aim for clinical use of human ES/iPS cells is to develop a serum-free, xeno-free, and defined protocol, able to support their propagation. The conventional medium used to maintain pluripotent stem cells involved xenogenic materials, i.e. FBS-containig medium or medium conditioned with components secreted from mouse embryonic fibroblasts (MEFs) (Chen et al., 2014b). Lately, scientists have been optimizing replacement xeno-free defined media. For instance, Thomson et al. (2006) have developed a defined culture medium, the TeSR1 family, to culture pluripotent stem cells in feeder-free conditions (Ludwig et al., 2006).

MEFs are usually used as feeder cells as they support the self-renewal of human ES/iPS cells (Thomson et al., 1998, Mallon et al., 2006). Nonetheless, MEFs are undefined xeno-derived materials. Human cells have been used instead as feeder cells including human fibroblast cells (Richards et al., 2002), fallopian tube, foreskin, and stromal cells (Mallon et al., 2006). However, beside the fact that preparation of the feeder layer cells is a time-consuming effort, they cannot be used to support human pluripotent stem cells intended for clinical application, as they introduce undefined factors into cultures. Thus, extensive work has been done to develop feeder-free conditions for the maintenance of human ES/iPS cells (Wang et al., 2005). Thus, scientists have been studying the extracellular matrices as substitutes of feeder cells to support the growth of human ES/iPS cells, including hydrogels, matrix proteins of human and animal origins (Braam et al., 2008, Rodin et al., 2010), and synthetic surfaces (Derda et al., 2007, Li et al., 2011).

Matrigel is the most frequently used extracellular matrix for research purposes in feeder-free culture of human ES/iPS cells (Chen et al., 2014b). Corning® Matrigel® Matrix is a reconstituted basement membrane isolated from the Engelbreth-Holm-Swarm mouse sarcoma tumour, which is rich in extracellular proteins: around 56% laminin, 31% collagen IV, and 8% entactin. It is also includes heparan sulfate proteoglycan, TGF-β, EGF, IGF, FGF, and tissue plasminogen activator (Osiecka-Iwan et al., 2008). Although Matrigel can maintain high quality human ES/iPS cells, it cannot be used to culture cells designed for therapeutic use as it is undefined and xenogeneic material.

Furthermore, although there are defined, animal-free proteins that are also known to support long-term human ES/iPS cell culture suitable for future therapeutic use such as recombinant vitronectin (Braam et al., 2008) and Laminin-511 (Rodin et al., 2010), their uses are limited due to the high cost related to the protein purification needed.

Hence, good quality human ES/iPS cells, as starting materials, cultured in completely defined serum-free and xeno-free conditions, are required for all subsequent derivatives and different applications.

5.2. Aim and Objectives

The aim of this chapter is to maintain and expand human iPS cells (IMR90-1) in a feeder-free and serum-free culture condition approached by mTeSR™1 Medium on Corning® Matrigel® Matrix. In order to achieve this aim, the following objectives were followed:

1. Reproduce the simple feeder-free and serum-free protocol to maintain and expand human iPS cells in mTeSR™1 Medium on Corning® Matrigel® Matrix, and adapt the cells to our laboratory conditions
2. Evaluate the pluripotency status for the human iPS cells cultured in our laboratory after few passages.

5.3. Experimental Plan

Human iPS cells were at passage 39 when obtained from WiCell bank. Cells were cultured and maintained as described in 4.2.1. 6-well tissue culture plates were coated with Corning® Matrigel® hESC-qualified Matrix, left at room temperature for 1 hr before seeding Human iPS cells, or stored in a humidified water-based incubator set at 37°C and 5% CO_2 (Figure 10). These plates were then used to culture human iPS cells in mTeSR™1 Medium with a final seeding volume 2ml/well. The plates were labelled and incubated in a humidified water-based incubator set at 37°C and 5% CO_2 overnight, with a daily microscope examination and medium change with fresh mTeSR™1 Medium. Undifferentiated stages of human iPS cells (passages 40-45) were passaged every 3 or 5 days, whenever iPS colonies became dense or large, or when any signs of differentiation were noticed, with a split ratio between 1:2 and 1:4. Plates to be passaged were examined microscopically in order to remove differentiation. Passaging cells was enzymatically made by adding Dispase solution after medium aspiration in order to detach cells. The cells were treated for 7 min at 37°C before the Dispase solution was removed to stop the detachment process. Cells were then washed gently 3 times with 1ml/ well of DMEM-F12 before 2ml/well of mTeSR™1 Medium was dispensed by holding a 5ml pipette perpendicularly to the well and scraping the surface to peel off the colonies. The cell suspension was then pipetted up and down to wash the well before distributing the small colonies into new wells. There should have been a total of 2.0ml of medium and cells/well (0.5ml of cell suspension + 1.5ml of fresh mTeSR™1 medium) (Figure 11). Plates were then moved gently back-and-forth and side-to-side to distribute cells across the surface of the wells. The plates were then incubated overnight for the cells to attach.

To freeze the human iPS cells, cryovials were labelled with the cell line (IMR90-1), passage number, the freeze date, and initial of the person performing the freezing procedure. The spent medium was removed and wells were incubated with 1ml Dispase solution 5 min at 37°C. Wells were then carefully washed before adding 1ml of mTeSR™1 Medium. The cell layers were detached by scraping the surface of the well with 5ml pipette while slowly pipetting the mTeSR™1 Medium up and down to break clumps as little as possible. Cell suspensions were pooled in a sterile 50ml centrifuge tube and centrifuged at 200xg for 5

min. The cell pellet was then re-suspended in 1ml mFreSR™1 Medium/well slowly and drop-wise, and the resulting cell suspension was distributed into labelled cryovials. Cryovials were then quickly placed into an isopropanol containing freezing container and left in a -80°C freezer overnight, before they were transferred to a liquid nitrogen storage tank (Figure 12).

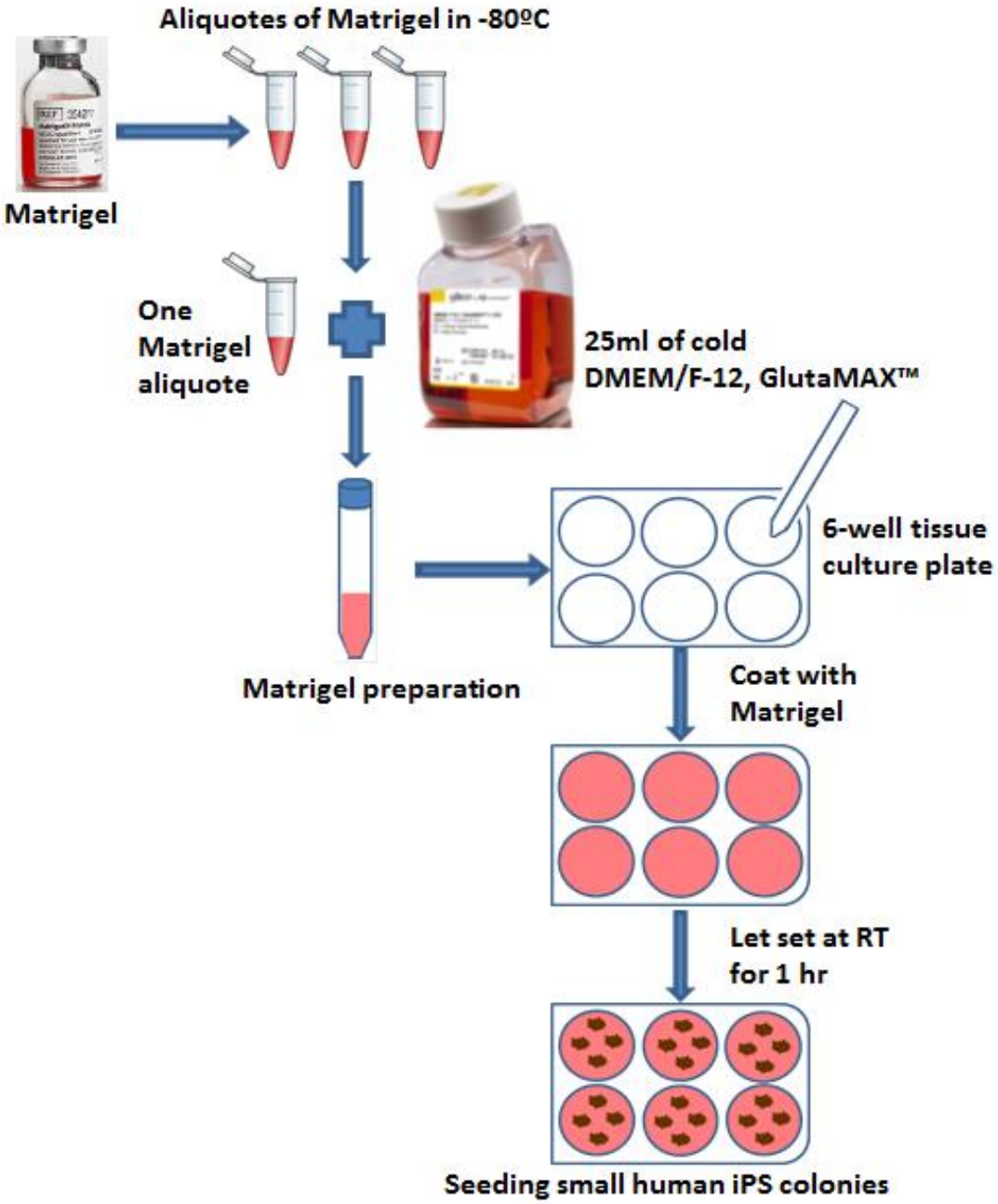


Figure 10. A Schematic Diagram Summarizing Coating Plates with Corning® Matrigel® Matrix.

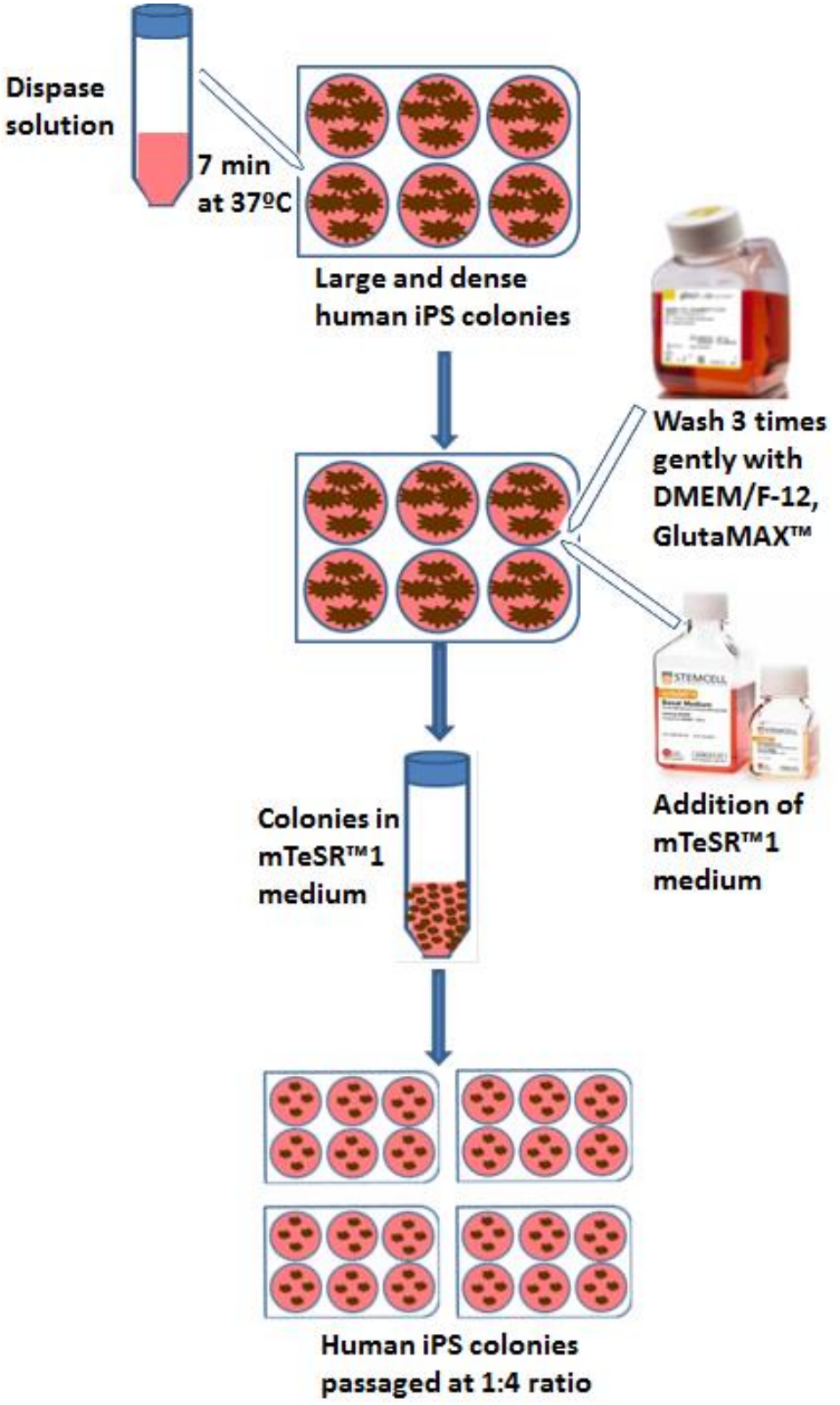


Figure 11. A Schematic Diagram Summarizing Passaging Human iPS Cells.

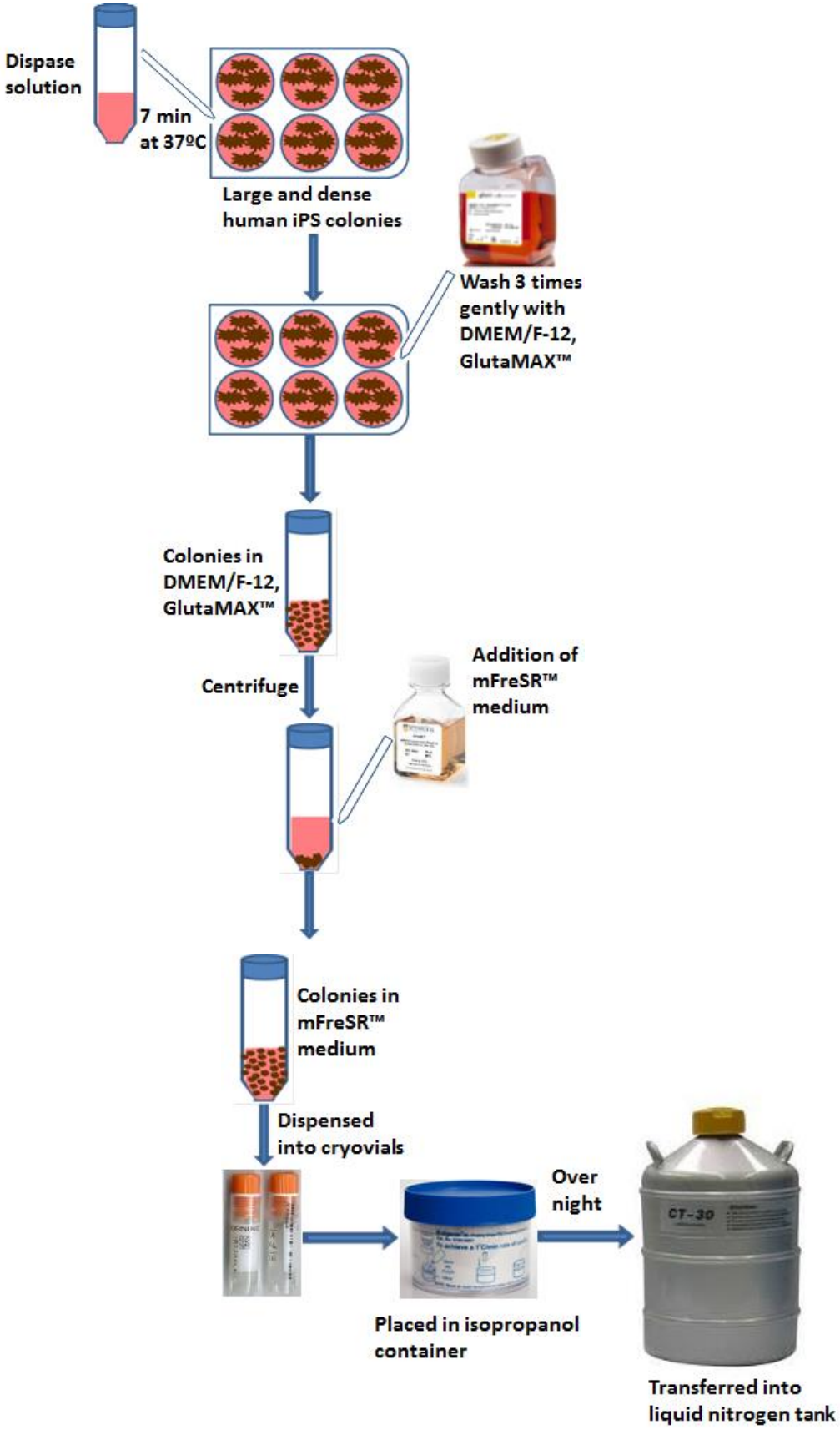


Figure 12. A Schematic Diagram Summarizing Freezing Human iPS Cells.

5.4. Results

5.4.1. Morphology of Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium

Morphology of human iPS cells cultured in our feeder-free and serum-free system Corning® Matrigel® Matrix in mTeSR™1 Medium was examined by light microscopy analysis. Undifferentiated human iPS cells usually expand as healthy condensed and round multicellular colonies with distinct edges (Courtot et al., 2014). Light microscopy showed that human iPS colonies appeared to grow over the culture period (Figure 13 (A-F)). Observation on day 1 of culture (Figure 13 (A)) exhibited small and scattered colonies attached to the Matrigel layer. Multicellular colonies of day 6 of culture (Figure 13 (F)) appeared tightly packed with distinct borders, and were large enough to be split. Each cell revealed high nuclear-to-cytoplasm ratio with prominent nucleoli (Figure 14). Spontaneous differentiation within the colonies was characterized by areas of irregular cellular morphology and loss of border integrity as shown in Figure 15 (A-B) and indicated by the arrows.

5.4.2. Growth Proliferation Study of Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium

The growth kinetics and capacity of human iPS colonies to proliferate in mTeSR™1 Medium were examined using MTS colorimetric assay over the culture period as explained in 4.3.2. The assay was run in 24-well tissue culture plates coated with Corning® Matrigel® hESC-qualified Matrix, seeded with similar number and size of colonies (5 colonies/well), and cultured for 1, 2, 3, 4, and 5 days in mTeSR™1 medium. Well-containing cells from each day of culture were washed and incubated with MTS solution for 3 hr. Finally, 100mL of the solution from each treated well was transferred into a 96-well plate and absorbance was read at 490nm. The average absorbance readings were normalized to a blank reading and plotted on a graph (Figure 16). The human iPS cells showed an increase in the absorbance reading over the culture period, which reflected a

higher growth proliferation rate achieved by maintaining cells in mTeSR™1 Medium. Statistical analysis were done by one way ANOVA between all groups ($p<0.001$).

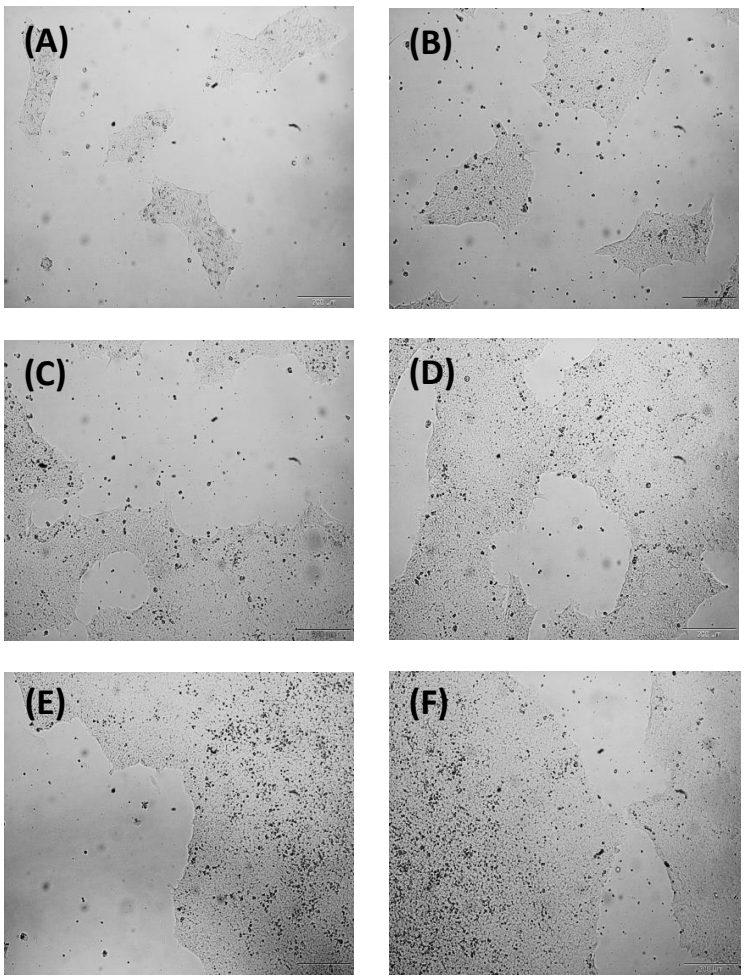


Figure 13. Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium at Days 1 to 6 (A-F, respectively).

Images of human iPS cells (IMR90-1) growing over the culture period using a magnification of 10x (Scales for each image: 200µm). (A) Day 1 of the maintenance culture showing small and scattered human iPS colonies attached to the Matrigel layer. (B) Day 2 of the maintenance culture when colonies started to become larger than they were on day 1. (C) Day 3 of the maintenance culture. (D) Day 4 of the maintenance culture. (E) Day 5 of the maintenance culture showing human iPS colonies covering a large area of the well. (F) Day 6 of the maintenance culture showing large and tightly packed human iPS multicellular colonies with distinct borders, which was the optimal time for passaging.

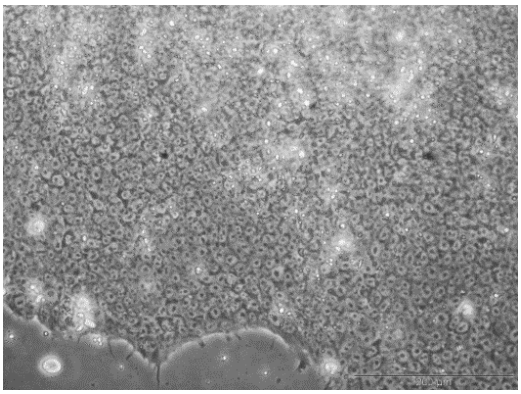


Figure 14. Human iPS Colony Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium at Day 6.

Images of human iPS colony (IMR90-1) using a magnification of 20x (Scale for image: 200µm) showing individual cells with high nuclear-to-cytoplasm ratio and prominent nucleoli within a large colony at day 6 of maintenance culture.

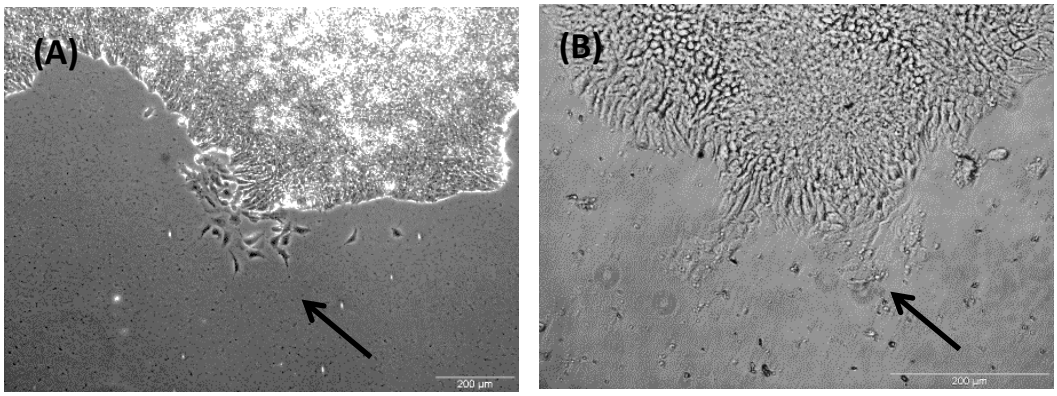


Figure 15. Area of Spontaneous Differentiation within Human iPS Colonies.

Images of spontaneous differentiation at the border of an undifferentiated human iPS colony, as indicated by the arrows, taken using 10x (A) and 20x (B) (Scales for each image: 200µm).

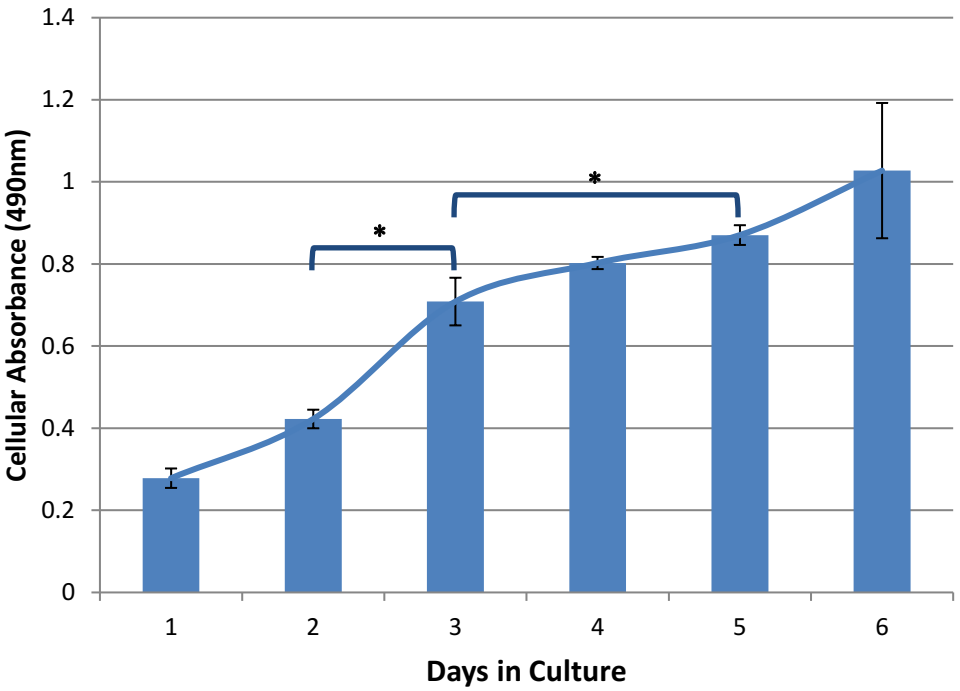


Figure 16. Growth Kinetics of Human iPS Cells Cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium. Values are mean ± standard deviation (SD) of n=4 ($p \leq 0.001$).

5.4.3. Immunocytochemistry for Pluripotency Markers

Immunocytochemistry was performed on confluent cultured human iPS cells at passage 45 at day 5 of culture in order to confirm the pluripotency status of the cells. Cells showed high expression of the common pluripotency proteins: (1) nuclear markers, Oct4, Sox2, and Nanog (Figure 17), in addition to (2) surface markers, SSEA4, TRA-1-81, and TRA-1-60 (Figure 18). Negative controls were cells stained with the secondary antibodies only: Goat-anti-rabbit (Alexa Fluor 594), Goat-anti-mouse (Alexa Fluor 594), and Goat anti-rabbit (Alexa Fluor 488), to exclude any unspecific background staining (Figure 19). Octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (Sox2), and Nanog are transcription factors associated with stemness highly expressed in undifferentiated human ESCs and iPS cells and involved in the self-renewal feature (Loh et al, 2006). Stage-specific embryonic antigen 4 (SSEA-4), TRA-1-60, and TRA-1-81 are antigens present on the surface of human ESC (Aoki et al, 2010).

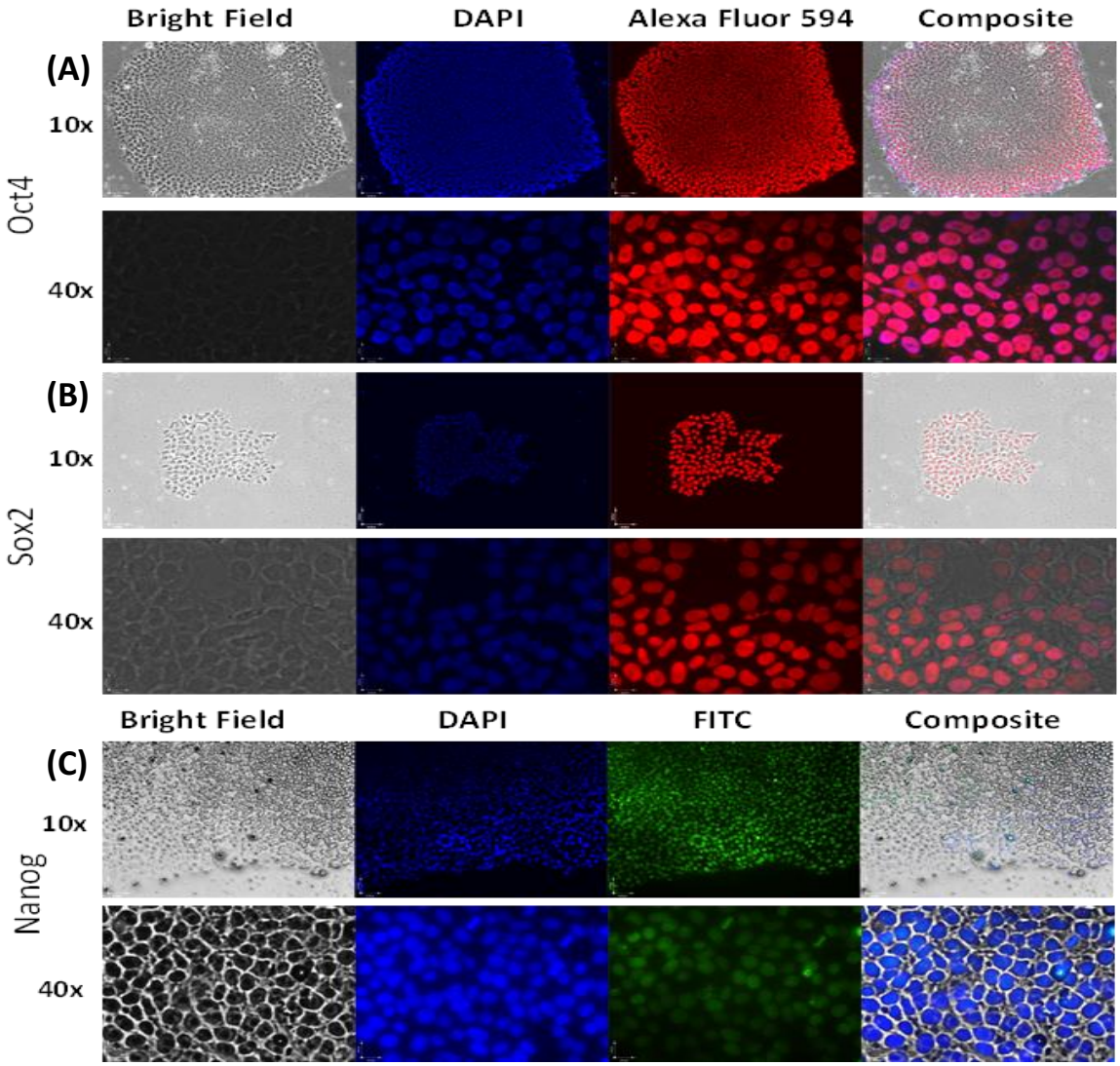


Figure 17. Immunocytochemistry Staining of Pluripotency-Specific Nuclear Proteins for Human iPS Cells. Expressions of pluripotency nuclear markers (A) Oct4 (Alexa Fluor 594-red), (B) Sox2 (Alexa Fluor 594-red), and (C) Nanog (Alexa Fluor® 488-FITC-green), were assessed by immunofluorescent staining in confluent human iPS cells cultured at day 5. The first column shows the bright field images. The second column shows the nuclear staining with DAPI. The third column shows the expression of the nuclear markers either in red or green, as indicated in the figure. The forth column shows the composite images. Images were captured at 10x (scale for images: 60 µm) and 40x (Scale for images: 15µm) magnification, as indicated in the figures, under fluorescent microscopy.

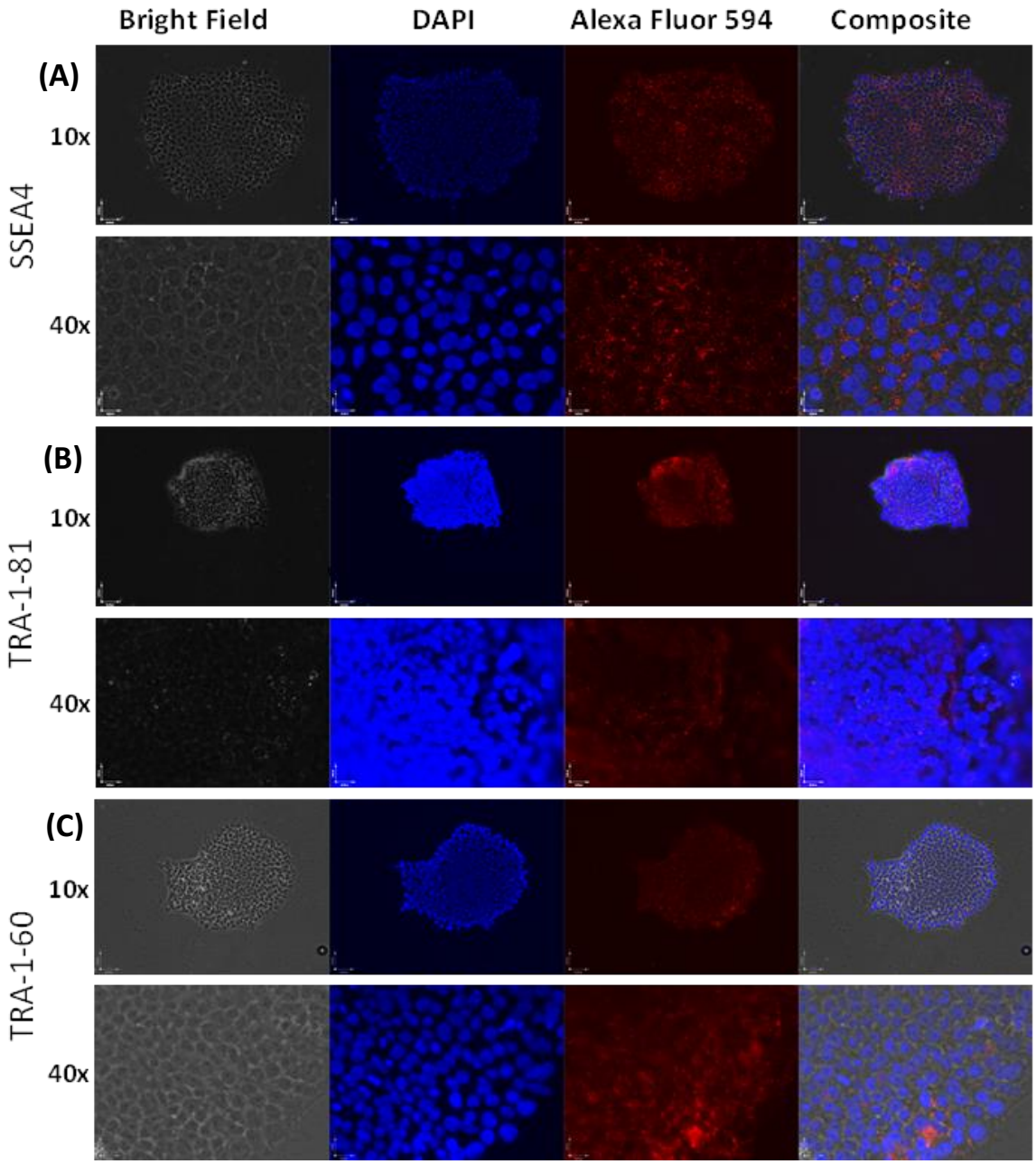


Figure 18. Immunocytochemistry Staining of Pluripotency-Specific Surface Proteins for Human iPS Cells. Expressions of pluripotency surface markers (A) SSEA (Alexa Fluor 594-red), (B) TRA-1-81 (Alexa Fluor 594-red), and (C) TRA-1-60 (Alexa Fluor 594-red), were assessed by immunofluorescent staining in confluent human iPS cells cultured at day 5. The first column shows the bright field images. The second column shows the nuclear staining with DAPI. The third column shows the expression of the surface markers in red. The forth column shows the composite images. Images were captured at 10x (Scale for images: 60µm) and 40x (Scale for images: 15µm) magnification, as indicated in the figures, under fluorescent microscopy.

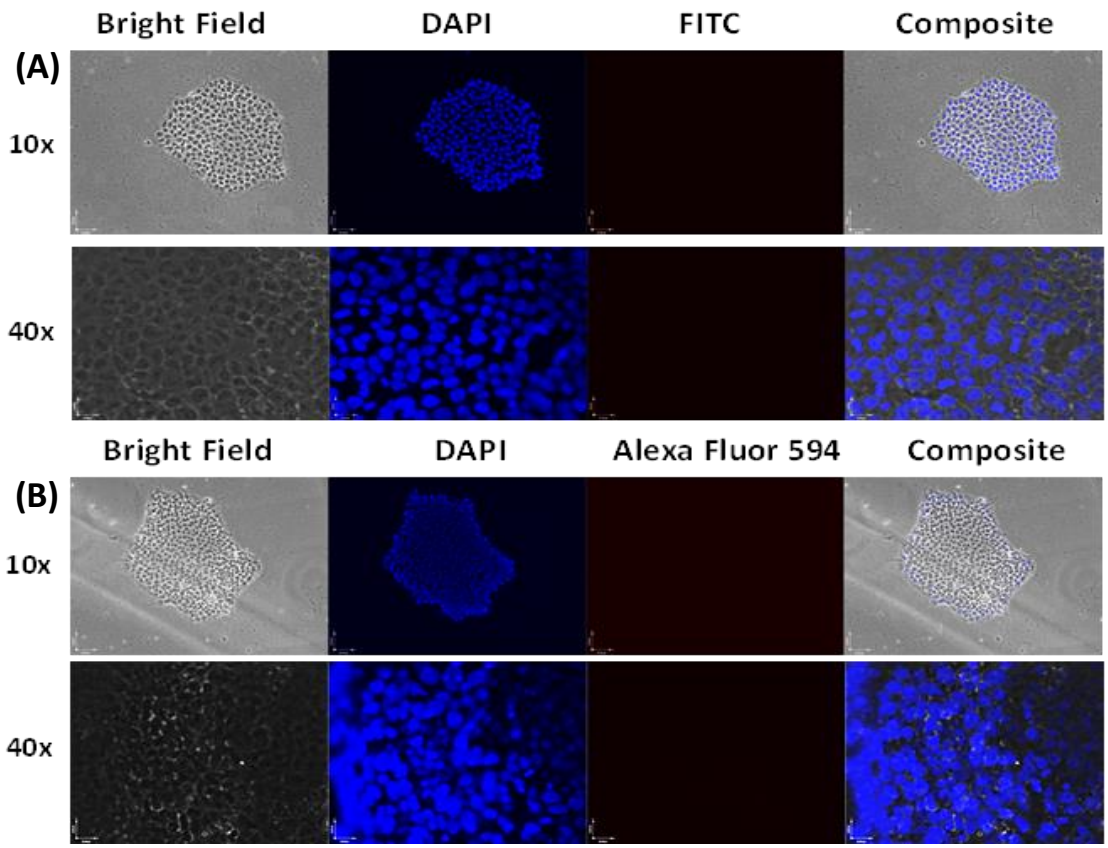


Figure 19. Negative Controls for Non-Specific Immunocytochemistry Staining of Pluripotency-Specific Primary Antibodies for Human iPS Cells. (A) Human iPS cells stained only with the secondary antibody Alexa Fluor® 488 goat anti-rabbit (FITC-green); (B) Human iPS cells stained only with the secondary antibodies: Goat-anti-rabbit (Alexa Fluor 594-red) and Goat-anti-mouse (Alexa Fluor 594-red). The first column shows the bright field images. The second column shows the nuclear staining with DAPI. The third column shows any non-specific binding either in red or green, as indicated in the figure. The forth column shows the composite images. Images were captured at 10x (Scale for images: 60µm) and 40x (Scale for images: 15µm) magnification, as indicated in the figures, under fluorescent microscopy.

5.4.4. Alkaline Phosphatase

AP activity staining was performed on confluent cultured human iPS cells at passage 45 at day 5 of culture for further confirmation of the undifferentiated status of the human iPS cells cultured in our laboratory. Almost all cells within the colony showed high AP activity, as demonstrated in Figure 20.

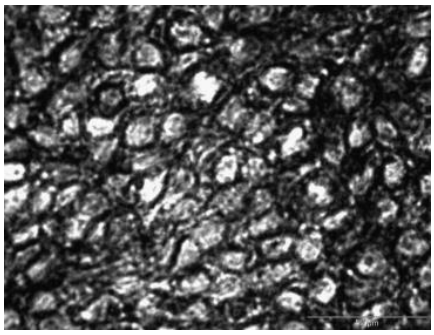


Figure 20: AP Activity Staining for Human iPS Cells. (50x) (Scales for image: 50µm)

5.4.5. Flow Cytometry

Following the high expression of the pluripotency proteins showed by the immunocytochemistry analysis, cultured human iPS cells were analysed using flow cytometry technique for phenotypic expression of a surface marker specific for pluripotency, SSEA4, within the viable population defined by the nucleic acid staining, DAPI. In this study, the confluent cultured human iPS cells at passage 45 were harvested and stained with a specific pluripotency monoclonal antibody (PE-Mouse anti-SSEA-4) and DAPI before being examined with flow cytometry machine. SSEA4 is a cell surface marker used to identify human pluripotent cells, while DAPI is used to identify viable cells.

Unstained human iPS cells sample and an isotype control sample (PE Mouse IgG3, κ Isotype Control) were used as negative controls to set the gates (Figure 21). The high expression of SSEA4 pluripotency marker (~98%) within the viable population, i.e. negative for DAPI (91%), indicates that the undifferentiation status of human iPS cells was successfully maintained using our culture protocol.

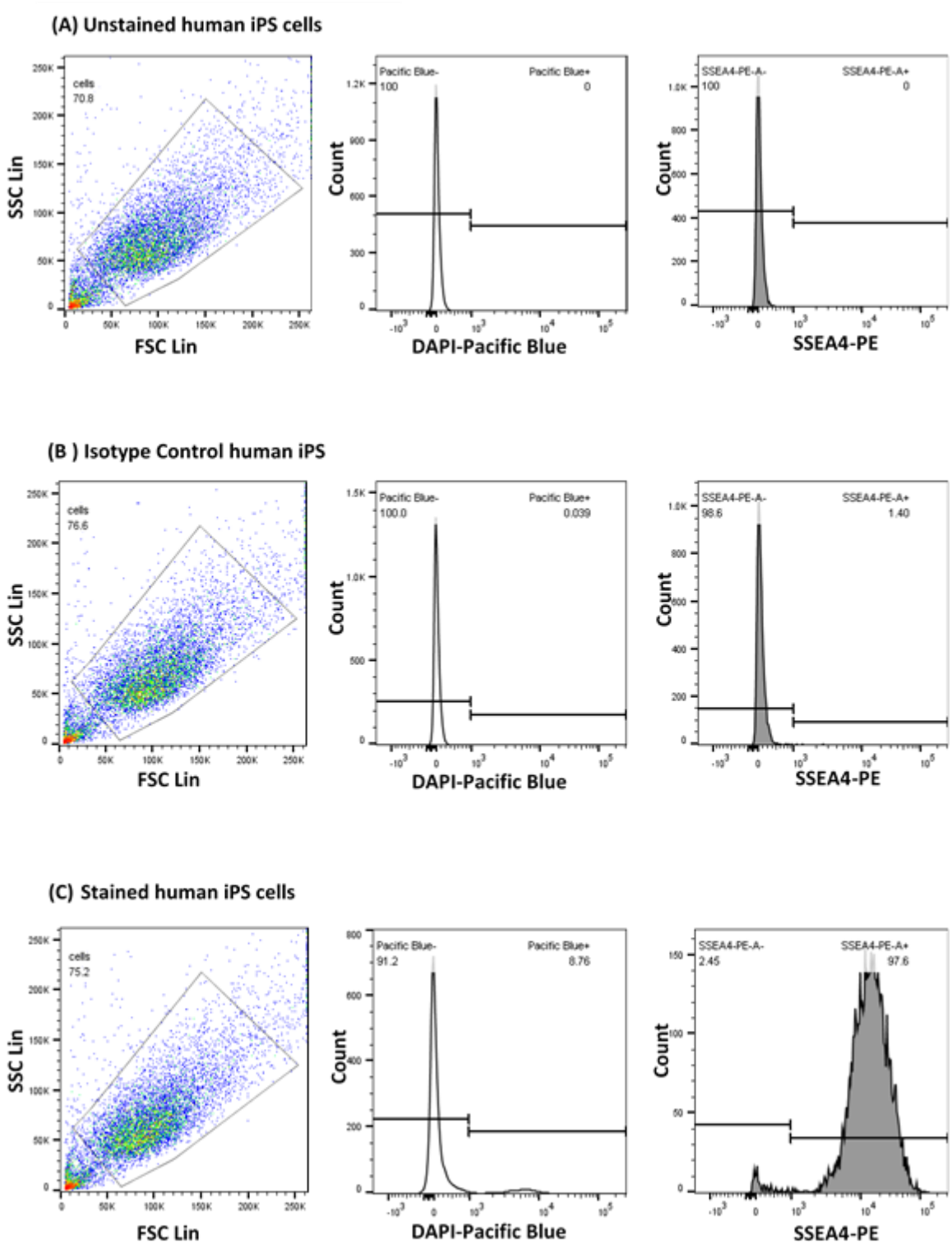


Figure 21. The Flow Cytometry Analysis Profile of the Cultured Human iPS Cells. (A) Flow cytometry plots of unstained human iPS cells; (B) Flow cytometry plots of the isotope control; (C) Flow cytometry plots of human iPS cells stained with DAPI and SSEA4-PE.

5.4.6. Gene Expression and qPCR

Following the evaluation of the pluripotency status of the human iPS cells by the previously listed analysis, further confirmation was achieved by analysing gene expression using qPCR. Pluripotency gene expression was performed on human iPS cells using QuantiTect SYBR Green PCR Master Mix kit (Qiagen, West Sussex, UK), based on the manufacturer's guidelines, to observe the relative expression of pluripotency gene markers at different passages, an early passage (40) vs a late passage (45). Negative control consisted of samples without cDNA. The expression of each gene of interest was normalized to an earlier passage (passage 40) and to the housekeeping gene GAPDH.

As shown in Figure 22, the high expression of pluripotency gene markers OCT4, NANOG, SOX2, and reduced expression 1 (REX-1) were clearly maintained after a number of passages were performed using our culture protocol, as no statistical differences were demonstrated between passage 40 and 45 (Figure 22). Rex-1 is another marker critical for maintaining pluripotency (Shi et al., 2006). In contrast, low expression of germ layer-associated genes were also maintained with no statistical differences between the two passages (Figure 23). Paired box protein (Pax6) is an early ectodermal marker, acting as a "master control" gene for the development of tissues derived from ectoderm lineage (Li et al., 1994). Alpha-fetoprotein (AFP) is an early endoderm-specific gene (Abe et al., 1996). Brachyury (T) is a transcription gene defining the mesoderm during gestation (Vidricaire et al., 1994).

5.4.7. Metabolic Analysis of Maintenance Culture Medium

In order to evaluate the metabolic needs of human iPS cells cultured on Corning® Matrigel® Matrix in mTeSR™1 Medium, the consumption of nutrients in the mTeSR™1 Medium was determined by measuring time-course concentration of glucose, glutamine, lactate, ammonium, in addition to changes of pH of mTeSR™1 Medium during the maintenance culture period. In this study, the mTeSR™1 Medium was refreshed every day and the spent medium was used to measure the concentration of nutrients

consumed by human iPS cells using a bio-profiler. Day 4 represents the last day of expansion before human iPS cells were split. Statistical analysis was done for time points by one way ANOVA ($p > 0.05$).

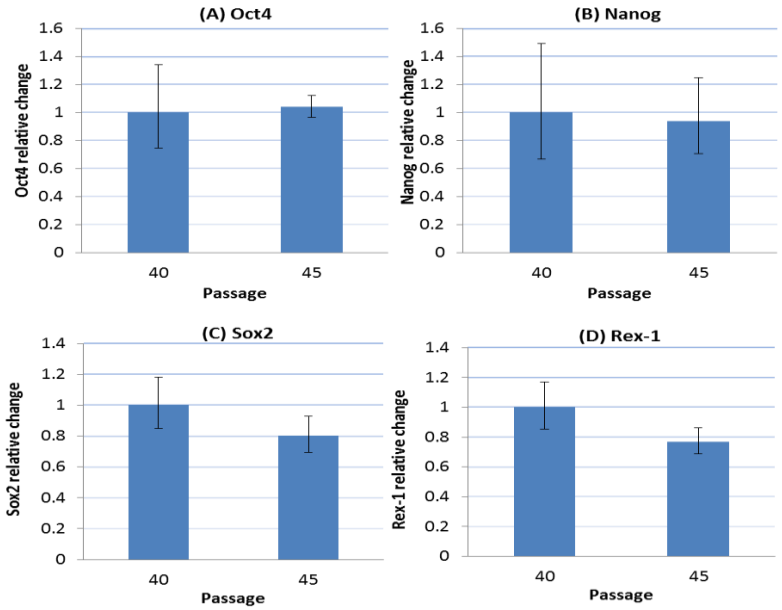


Figure 22. Relative Gene Expression of Pluripotency Markers Observed from Human iPS Cells. Values are mean ± standard deviation (SD) of n=3 biological samples**.**

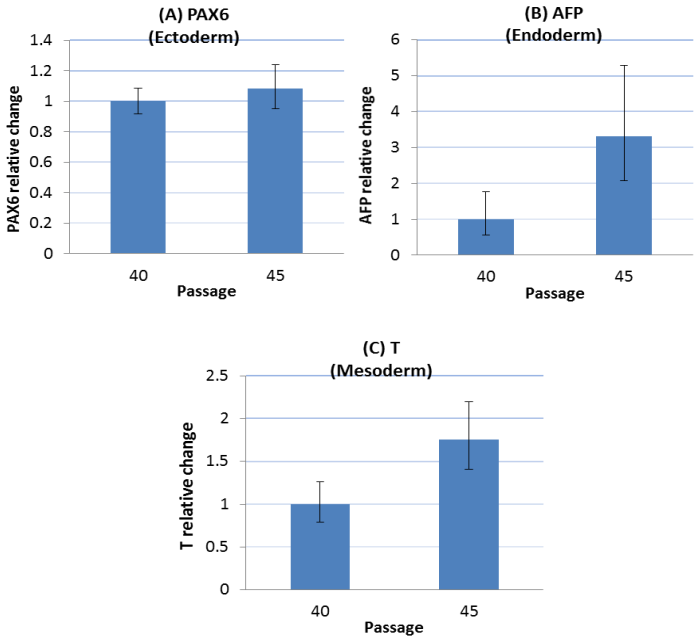


Figure 23. Relative Gene Expression of Germ-Layers Markers Observed from Human iPS Cells. Values are mean ± standard deviation (SD) of n=3 biological samples.

5.4.7.1. Nutrient consumption rates (glucose and glutamine)

Glucose concentration was measured from control fresh mTeSR™1 Medium and consumed mTeSR™1 Medium from every day in the maintenance culture, which showed similar levels along the maintenance culture. The culture maintained high levels of glucose throughout the culture days, indicating that the cultured cells did not consume all the glucose supplied in the medium (Figure 24).

A similar pattern was seen with glutamine concentrations along the maintenance culture from day 1 to day 4, which was the optimal day for splitting, thus indicating that cells consumed some of the glutamine supplied in medium (Figure 25).

5.4.7.2. Metabolite concentration profiles (lactate and ammonia)

In an opposing pattern to glucose and glutamine, lactate accumulation was increased from 0 mM to approximately 10.45 mM when cells became ready for passaging (Figure 26).

In terms of ammonia (NH_4^+) accumulation, a similar kinetic pattern with lactate concentration was observed along the maintenance culture period starting from 0 mM to approximately 0.7 mM (Figure 27).

The increase pattern of lactate and ammonia levels along the maintenance and expansion culture indicated that the two metabolites were released through metabolism of human iPS cells into the medium, which reflects high consumption rates of glucose and glutamine profiles as described above. However, since one molecule of glucose can only produce 2 molecules of lactate, Figures 24-26 are suggesting that lactate might have been produced from glutamine via the glutaminolysis pathway (Zielke et al., 1980) or from pyruvate that might have been contained in the media (was not measured) (Adeva-Andany et al., 2014), which can explain the high lactate production in the maintenance culture.

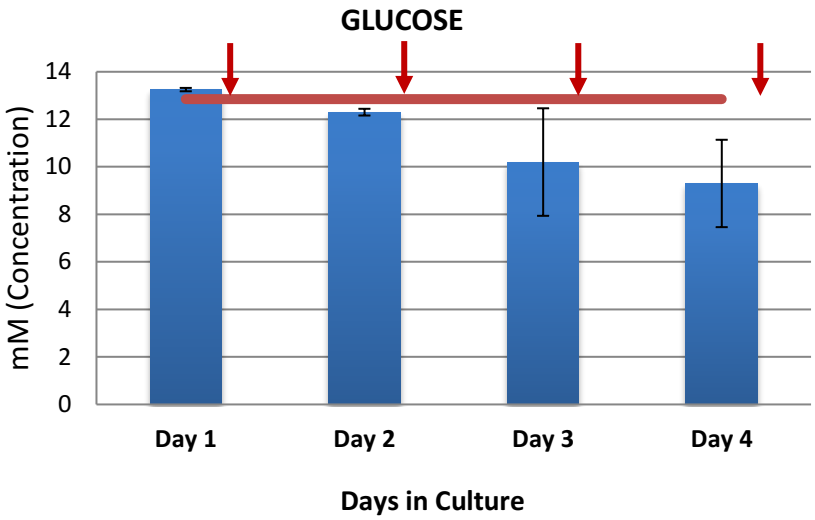


Figure 24. Glucose Consumption during Human iPS Cells Maintenance Culture in mTeSR™1 Medium. The red horizontal line indicates the glucose concentration in fresh mTeSR™1 media. The red arrows indicate the daily replacement of mTeSR™1 media. (n=2).

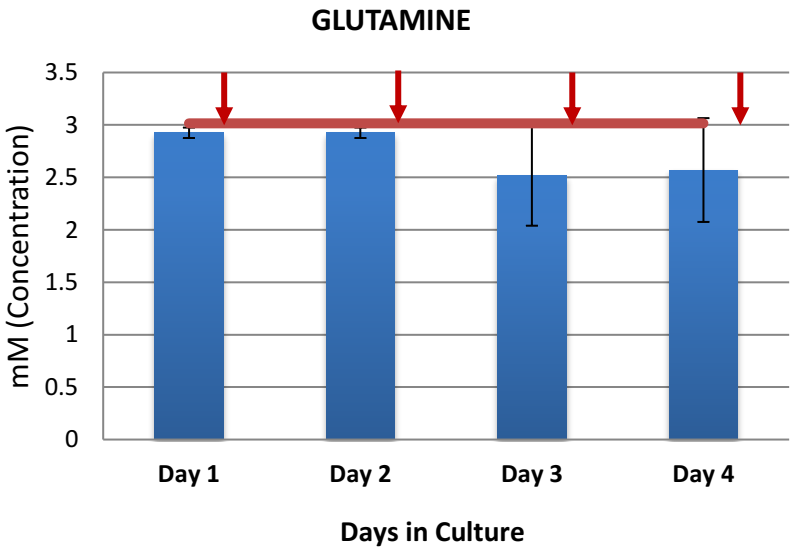


Figure 25. Glutamine Consumption during Human iPS Cells Maintenance Culture in mTeSR™1 Medium. The red horizontal line indicates the glutamine concentration in fresh mTeSR™1 media. The red arrows indicate the daily replacement of mTeSR™1 media. (n=2).

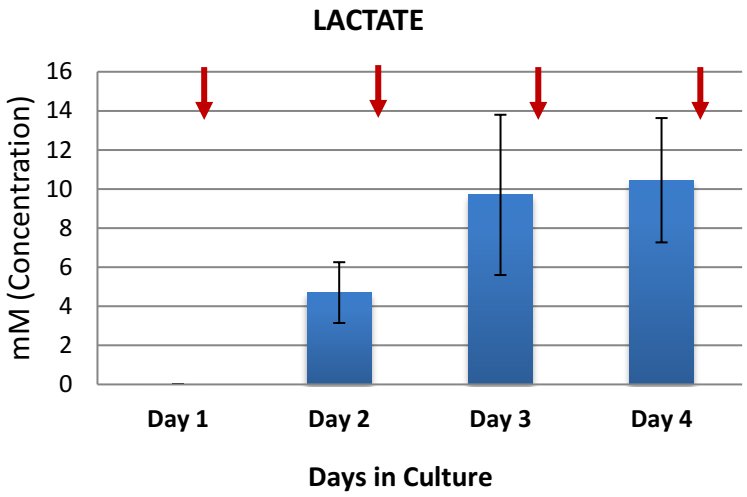


Figure 26. Accumulation of Lactate during Human iPS Cells Maintenance Culture in mTeSR™1 Medium. The red arrows indicate the daily replacement of mTeSR™1 media. (n=2).

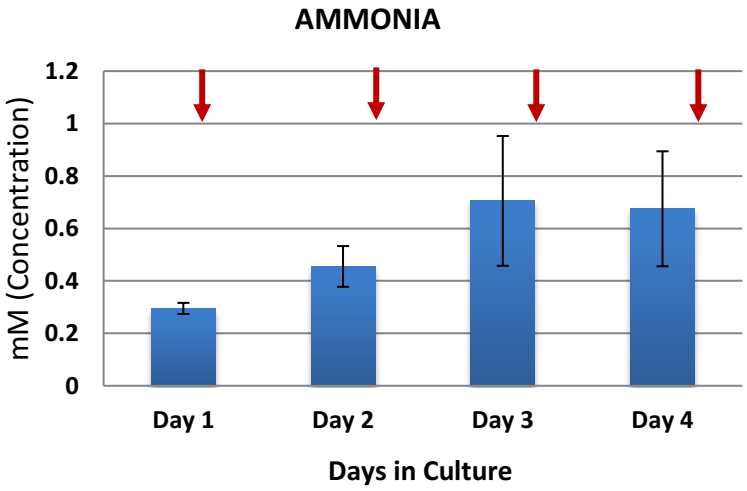


Figure 27. Accumulation of Ammonia during Human iPS Cells Maintenance Culture in mTeSR™1 Medium. The red arrows indicate the daily replacement of mTeSR™1 media. (n=2).

5.4.7.3. pH Variations Level

The pH of the fresh mTeSR™1 Medium was measured at about 7.2, which was gradually decreased as the culture time passed even though fresh medium was supplied every day.

Overall, the pH levels were maintained at lower levels, approximately 6.93 to 7.23, without large differences (Figure 28). These measurements do not reflect the accurate pH level of the medium at the corresponding time point as samples were not freshly analysed. However, they can still provide good indications about pH variations level during the maintenance period.

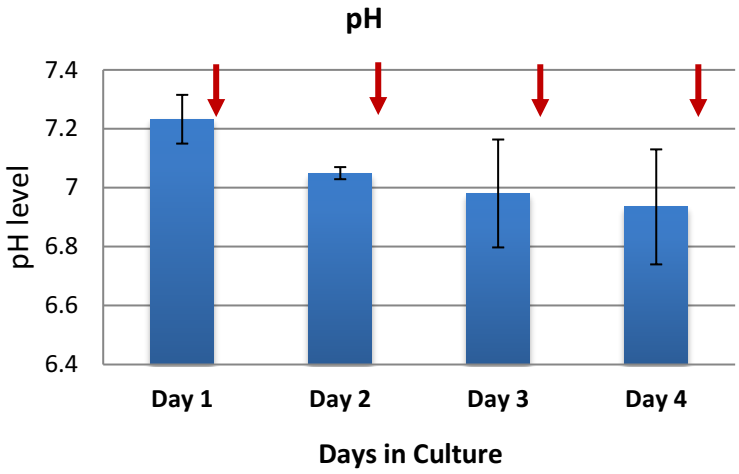


Figure 28. pH Variations during Human iPS Cells Maintenance Culture in mTeSR™1 Medium. The red arrows indicate the daily replacement of mTeSR™1 media. (n=2).

5.5. Discussion

An intense focus has been recently on improving the reproducibility of human iPS cell culture in simple defined feeder-free methods from all aspects of the culture system, such as media, coating matrix, and passaging method (Totonchi et al., 2010). However, some studies reported an elevated expression of lineage-specific genes in human ES/iPS cells cultured under feeder-free conditions, which may affect any consequent attempt to differentiate these cells towards different lineages (Badger et al., 2012). Additionally, changes in karyotype of human ES/iPS cells cultured under feeder-free conditions have been also reported (Badger et al., 2012). Another concern accompanied with adapting human ES/iPS cells to feeder-free culture methods is the decrease in the proliferation rate as cells are exposed to fewer growth factors that would originally be released from

MEFs. The capability of different human ES/iPS cell lines to attach to feeder-free matrix varies, owing to different containment of integrins, cell-attachment proteins (Badger et al., 2012). However, the use of feeder-free conditions eliminates the undefined biological variability component of the feeder layer from the culture system (Nakagawa et al., 2014). For this reason, Matrigel has been used as a matrix to culture human iPS cells, which is easy to use and reproducible (Lam and Longaker, 2012).

Furthermore, a complete elimination of serum and animal-derived materials is essential for clinical approaches. Thus, it is significant to move toward a feeder-free and serum-free cultural system for maintaining, expanding, and differentiating human iPS cells. Hence, ensuring the quality of pluripotent cells is important for their differentiation efficiency. Recent publications showed the feasibility of using a defined serum-free and feeder-free media such as mTeSR™1 for a high quality maintenance of human ES and iPS cells (Lannon et al., 2008). Human ES and iPS cells cultured in mTeSR™1 Medium maintain pluripotency status, which was measured by the expression of multiple pluripotency genes such as OCT4, NANOG, SSEA-3, SSEA4, TRA-1-60, and TRA-1-81 (Lannon et al., 2008), normal karyotype, and the ability to form teratomas containing derivatives of all germ layers (Yu et al., 2007, Lowry et al., 2008).

This chapter addresses the successful maintenance and propagation of human iPS cells generated from fetal lung fibroblasts (IMR90-1) by viral transduction of four of Thomson's factors: OCT4, SOX2, NANOG, and LIN28 (Yu et al., 2007, Lannon et al., 2008), following the serum-free and feeder-free protocol reported by (Lannon et al., 2008, Ludwig et al., 2006). These conditions were accomplished by culturing the human iPS cells on Corning® Matrigel® Matrix in mTeSR™1 Medium. Under these conditions, human iPS colonies formed on Matrigel resembled human ES cell colonies, and cells were maintained in a naïve undifferentiated status, confirmed by the high expression of pluripotency markers.

The human ES cell-like colonies produced (Figure 13) were analysed via immunocytochemistry for the expression of Oct4, Nanog, Sox2, Oct4, SSEA4, Tra-1-81, and Tra-1- 60 (Figure 17-18). All markers showed high expression comparable to hESCs

(Adewumi et al., 2007). Human iPS cells also showed a high AP activity (Figure 20). In addition, human iPS cells were dissociated into single cells and stained against SSEA4 and DAPI for a further confirmation of their viability and pluripotency using flow cytometry (Figure 21). Human iPS cells culture under our serum-free and feeder-free culture conditions showed a high expression of SSEA4 pluripotency marker (~98%) within the viable population (91%), indicating that the viability and undifferentiation status of human iPS cells were successfully maintained. Different gene expressions were also analysed through qPCR analysis, and it was noted that expressions of pluripotency markers Oct4, Nanog, Sox2, and Rex-1 continued to be high over the expansion culture period (Figure 22). On the other hand, expressions of genes associated with the three germ layers were consistently low throughout the culture (Figure 23). Oct4, Nanog, and Sox2 are important regulators of the plasticity of pluripotent stem cells (Loh et al., 2006). Oct4, encoded by Pou5f1, is a POU domain–containing transcription factor found in the ES cells (Pan et al., 2002). Sox2, part of the SoxB1 transcription factor family, plays important roles in pluripotent stem cells maintenance and neural differentiation (Zhang and Cui, 2014). Nanog is a homeodomain–containing protein, a pluripotency factor required at a later stage than Oct4 during development (Loh et al., 2006). Rex-1 is a known marker for undifferentiated ES cells critical for maintaining stemness (Shi et al., 2006).

Along the human iPS cells maintenance culture, cells became confluent and metabolically active, which was evaluated by measuring pH change, glucose and glutamine consumption, and lactates and ammonia production in mTeSR™1 Medium during cultures. Glucose and glutamine provided in the culture medium are the main energy sources for mammalian cells. Glycolysis is the main pathway for energy metabolism of cells on the presence of O_2, and plays a central role in cell growth (Varum et al., 2011, Madonna et al., 2013). Glycolysis consumes glucose and releases lactate as a by-product (Varum et al., 2011). Active proliferating cells exhibit active glycolysis, which is reflected by the lactate levels (Varum et al., 2011, Madonna et al., 2013). Human iPS cells have been reported to predominantly utilize glucose through glycolysis at undifferentiated state (Li and Yang, 2013). Glutamine is an amino acid important as an energy substrate

that decomposes upon utilization into ammonia and pyrrolidonecarboxylic acid (Heeneman et al., 1993). Ammonia has been described as toxic for cell culture and hence it was important to be monitored over the culture period (Heeneman et al., 1993). The pH measurements and metabolites profiles can also reflect the cell metabolism within the culture medium (Zhao et al., 2005). Decreasing the pH of media along the culture corresponds to increasing lactate and ammonia concentration (Barngrover et al., 1985b). However, the pH change reported in our culture was within the normal range (6.93 to 7.23). Thus, it is suggested achieving an optimal nutrient profile is necessary for cellular growth and metabolism.

5.6. Conclusion

Conventional protocols for maintaining human pluripotent stem cells used animal feeder cells, where obstacles of disease transmission and immune reactions arise, thus making the xeno-free culture requirement difficult to achieve. Moreover, maintenance medium used to be supplemented with serum, which contains undefined factors and is subjected to lot-to-lot variation. Thus, the use of a feeder-free and serum-free human iPS cells culture system was originally incorporated to overcome those limitations. In addition, procedures followed to maintain, expand, and passage cells should be simple, reproducible, efficient, animal-free, defined, and scalable, to establish good manufactory practices able to maintain clinical-grade human iPS cells and their derivatives. Our findings supported the protocol reported previously that culturing human iPS cells on Corning® Matrigel® Matrix in mTeSR™1 Medium maintained stemness with high expansion rates (Lannon et al., 2008). As compared to the conventional protocols, this culture method is simple, straightforward, and reproducible. Thus, it shows value for maintaining and expanding human iPS cells proposed for drug screening and therapeutics or research applications.

Chapter 6.

Optimizing the Differentiation of Human iPS Cells towards the Erythroid Lineage

6.1. Introduction

6.1.1. Factors Regulating Early-Stages of Erythropoiesis

The key regulator of erythropoiesis process is EPO (Krantz, 1991). However, many other factors play major roles at specific stages of the process. The early stages of erythropoietic development *in vivo* and *in vitro* are influenced by some early acting growth factors such as BMP-4, VEGF, and FGF-basic (Keller et al., 1993, Kennedy et al., 1997, Levenstein et al., 2006, Pick et al., 2007, Pearson et al., 2008), but not highly influenced by other late acting factors, including IL-3, IL-6, and EPO (Keller et al., 1993). The role of BMP-4 during embryogenesis has been widely reported in the commitment of cells to mesodermal lineages and the expression of mesoderm-associated genes (Pick et al., 2007, Pearson et al., 2008). The addition of this factor can induce a rapid differentiation of human ES cells even within a proliferation culture for the maintenance of pluripotency status (Levenstein et al., 2006). A study stated that BMP-4 alone, or mixed with other factors such as FGF-basic and VEGF, can significantly trigger the generation of some blood precursors (Pearson et al., 2008), as FGF-basic can direct the differentiation of mesodermal cells into haematopoietic fate, while VEGF is vital for the creation of fully committed hematopoietic precursors (Pick et al., 2007, Pearson et al., 2008). However, FGF influence is dose-dependent since the application of high doses (100ng/mL), on the other hand, can indeed sustain the maintenance and proliferation of human ES cell (Amit et al., 2000, Xu et al., 2005a, Xu et al., 2005b, Levenstein et al., 2006). VEGF is known to play a main function in vascular-related cellular activities generating endothelial precursors (Breier et al., 1992). In addition, it has a clear role in haematopoiesis development and haematopoiesis-related cellular responses leading into erythroid differentiation (Nakayama et al., 2000, Cerdan et al., 2004). Moreover, a study reported a selective function of VEGF-A165 in the differentiation of human ES cells into erythroid progenitors but not myeloid progenitors (Cerdan et al., 2004). Thus, for the generation of erythroid cells *in vitro*, many factors need to be considered and added to the media.

6.1.2. Approaches to Differentiate Human ES/iPS Cells: Single Cells, EB Formation, or Monolayer Differentiation

Main approaches used to differentiate human ES/iPS cells are: (1) EB formation, (2) single cell dissociation, (3) and/or co-culture on mesenchymal cells. However, cells within EBs give rise to different types of derivatives of the three primary germ layers (Doetschman et al., 1985). Also, EB aggregates are heterogeneous in size, structure, and composition, which make the reproducibility of EB-formation protocols problematic. Thus, critical parameters need to be optimized, including: the starting number of cells used to form EBs, their plating time, and the medium and serum used (Mummery et al., 2012), as all cultures involving xeno-serum or cells frequently show dependency on their lots (Niwa et al., 2011).

Moreover, unlike mouse ES cells, human ES cells are sensitive for enzymatic dissociation into single cells, as they undergo apoptosis or show low viability (Amit et al., 2000). Thus, researchers avoid the single cells culture of pluripotent cells.

Few studies showed new approaches of differentiating human ES/iPS cells as monolayers instead of single cells or EB formation (Laflamme et al., 2007, Mummery et al., 2012, Zhang et al., 2012). The monolayer differentiation method has the advantages of relatively uniform cells, and easy control of growth factors and cytokines application, which makes this approach comparatively convenient and reproducible alternative. Another important advantage of the monolayer protocols is the direct differentiation without the replating step required for EB or single cells dissociation protocols (Mummery et al., 2012, Shi, 2013). Combining the monolayer system with xeno-free and serum-free differentiation culture would be the most suitable approach for future clinical applications (Lim et al., 2013).

In terms of including an energy source in the media, GlutaMAX, also known as L-Alanyl-L-glutamine, is widely used as a more stable form of L-glutamine in cell culture, not only as an essential source of energy, but also as nitrogen source for mammalian cell cultures (Kromer et al., 2011). Catabolism and decomposition of glutamine leads to ammonia build-up, obstructing cell growth. Thus, the use of GlutaMAX instead of glutamine in

many commercial cell culture media can reduce the amount of ammonia generated (Kromer et al., 2011).

6.1.3. The Use of Extracellular Matrix for Hematopoietic/Erythropoietic Differentiation of Human ES/iPS Cells

Extracellular matrixes-coated dishes are usually used for monolayer cultures to maintain and differentiate ES/iPS cells in feeder-independent protocols (Lim et al., 2013). The use of collagen IV-coated dishes directed the mesodermal differentiation of mouse ES cells and enhanced differentiation into hematopoietic cells (Nishikawa et al., 1998). Likewise, the presence of main matrix constituents, such as Matrigel, including laminin, collagen, entactin and heparin-sulfate proteoglycan, as well as growth factors, could enhance the generation of hematopoietic progenitors and accelerate the differentiation towards functional blood cells (Lim et al., 2013).

6.1.4. The Role of O_2 on Erythropoiesis

Studies have approved that the environment surrounding the preimplantation embryo is approximately 1.5–5.3% O_2 (Thomson et al., 1998, Harvey, 2007, Badger et al., 2012). Nonetheless, the routine protocols followed in culturing ES cells in the laboratories are usually under normoxic O_2 conditions (20% O_2). This higher O_2 level exposes the cells to an oxidative stress and reactive oxygen species (ROS), which may lead to chromosomal abnormalities (Badger et al., 2012). Hence, some researchers have reported improved expansion and purity of human ES/iPS cells with less spontaneous differentiation when cultured at low O_2 concentration, which is called hypoxia (Badger et al., 2012). However, hypoxia conditions may reduce aerobic respiration and ATP energy production by the mitochondria needed for cellular processes (Badger et al., 2012, Nelson and Cox, 2013). Some recent studies claim that culturing human ES cells under hypoxic conditions supports the upregulation of genes associated with chondrogenesis, angiogenesis, and

vasculogenesis (Prado-Lopez et al., 2010). However, low efficiency was always a critical issue (Prado-Lopez et al., 2010).

Hypoxia inducible factors (HIFs) are activated by low O_2 concentrations and play central roles during embryogenesis by regulating transcriptional genes of angiogenesis, erythropoiesis, endothelial cells, and glycolysis (Hakim et al., 2014). HIF-1, a transcriptional activator comprising α subunit (O_2-regulated) and β subunit, controls erythropoiesis by regulating the high production of renal EPO and the expression of the EPOR (Semenza, 2009). Moreover, HIF-1 controls the uptake of iron needed for haemoglobin synthesis of RBCs via activating genes encoding transferrin and transferrin receptor (Semenza, 2009). Hydroxylation of HIF-1α or HIF-2α by the prolyl hydroxylase PHD2 is required for ubiquitination and proteasomal degradation, while in hypoxia conditions, hydroxylation is repressed, resulting in accumulation of HIF-1 that stimulates transcription of set of target genes (Semenza, 2009, Badger et al., 2012).

Hypoxia is a major extrinsic factor affecting erythropoiesis by controlling the expansion of committed erythroid progenitors and their transcriptions (Ingley, 2012b). Specifically, in an interaction with glucocorticoids, hypoxia stabilizes HIF-1α within erythroid progenitors and enhances their expansion (Ingley, 2012b). Polymeric IgA1 (pIgA1) is another extrinsic factor controlling erythropoiesis that is up-regulated in hypoxia conditions to interact with EPO and activate the Akt and MAPK pathways via transferrin receptors on mature erythroid cells (Ingley, 2012b).

In this study, we sought to optimize a protocol for a direct hematopoietic/erythroid differentiation from human iPS cells under hypoxia condition without EB formation or co-culture step. This action was taken in order to induce hematopoietic/erythropoietic differentiation, looking at the application of different concentrations of some early hematopoietic/erythropoietic-induced cytokines and growth factors, and following the standard serum-based protocol, with high (10%) and low (5%) levels of serum concentration as compared to the serum-free group. The study was performed in single cells vs monolayer differentiation, and looked at the best time point to get the most yield

of the phenotype of interest, and present an efficient method for the development of the haematopoietic/erythropoietic lineage.

6.2. Aim and Objectives

The focus of this chapter is to establish a new direct protocol that could enhance the induction of haematopoietic/erythropoietic differentiation from human iPS cells in standard 2 dimensional (2D) culture system, optimized from previous protocols reported in the literature. Herein, we exploited the low O_2 level (hypoxia) to support our goal as hypoxia serves as an enhancer for erythropoiesis as it promotes EPO production, iron uptake (Semenza, 2009), erythroid cell haemostasis, proliferation and maturation of erythroid progenitors through restructuring the hematopoietic niche, as well as up-regulating the expression of GATA1 important for the survival of those progenitors (Lesinski et al., 2012). More important, the bone marrow, the main hematopoietic/erythropoietic site, offers low oxygen level (0-4%) (Ivanovic, 2009, Kim, 2010). In order to achieve our aim, the following objectives were followed:

1. To optimise the concentrations required for the production of hematopoietic/erythroid progenitor colonies of some early growth factors/cytokines, including recombinant human BMP-4, recombinant human FGF-basic, and recombinant human VEGF-A165, that showed wide ranges in the literature, one at a time. The initial concentrations examined were obtained from Lu et al. (2008) where a serum-free condition was described (Lu et al., 2008a, Ma et al., 2008, Feng et al., 2010, Lapillonne et al., 2010b, Dias et al., 2011, Dorn et al., 2015). This optimisation was done in a serum-free condition and was measured by flow cytometry analysis of CD71 and CD235a to compare the efficiency of haematopoietic/erythropoietic differentiation at different concentrations.
2. To assess the effect of serum addition by comparing high (10%) and low (5%) concentrations of serum for the differentiation of human iPS cells towards

hematopoietic/erythropoietic lineages, to the optimised serum-free condition using flow cytometry analysis of CD71 and CD235a.

3. To compare the viability and haematopoietic/erythropoietic differentiation efficiency in colonies with different seeding densities of single cells.
4. To compare four different time points of our differentiation protocols in order to select the favoured time to switch to the following differentiation step.

6.3. Experimental Plan

Here we have attempted to differentiate human iPS cells towards the hematopoietic/erythropoietic lineages by testing a range of different growth factors and cytokines, described elsewhere in the literature, and the effect of serum addition. Thereafter, we compared the efficiency of the differentiation in colonies vs single cells at different time points. Many studies have previously demonstrated differentiation of human ES/iPS cells into hematopoietic/erythropoietic lineages using different conditions, as described in Chapter 2. In this study, the main factors inducing the differentiation towards the haematopoietic/erythropoietic lineages were examined separately and the best condition was added to the main protocol concluded at the end of this chapter. The cellular outputs were analysed using flow cytometry for one or more of the following haematopoietic/erythropoietic-associated markers:

1. CD71 (transferrin receptor): an integral membrane protein that facilitates the uptake of transferrin-iron complexes (Aisen, 2004, Sargent et al., 2005). CD71 is an early erythroid marker (Zambidis et al., 2005), highly expressed as a cell-surface marker on erythroid lineage at normoblast phase, which decreases at the reticulocyte phase while mature erythrocytes loss the expression of CD71, which make them easy to be identified (Lawrence et al., 1999, Marsee et al., 2010).
2. CD235a (Glycophorin A): a late erythroid-specific membrane sialoglycoprotein that forms the antigens of the MN blood group of RBCs (Dupont et al., 1995). This protein was isolated at different stages of erythropoiesis in normal bone marrow (Gahmberg et al., 1984). Glycophorin A consists of three dispersed domains: a hydrophilic segment at the

external surface, a hydrophobic segment embedded within the lipid bilayer, and a COOH-terminal in the cytoplasm (Andersson et al., 1981, Gahmberg et al., 1984).

6.3.1. Effect of Recombinant Human FGF-basic Concentration

Human iPS colonies, cultured on Matrigel coated 6-well tissue culture plates in mTeSR™1 medium, were differentiated towards the haematopoietic/erythropoietic lineages. The media was changed into the hematopoietic/erythroid induction medium (4.1.2.) (3ml/well). The media was supplemented with three different concentrations of recombinant human FGF-basic: low (20ng/mL), medium (50ng/mL), and high (100ng/mL). The differentiation culture was run for 14 days and the media was refreshed every two days. Cells were placed in humidified hypoxia chamber.

6.3.2. Effect of Recombinant Human BMP-4 Concentration

Human iPS colonies, cultured on Matrigel coated 6-well tissue culture plates in mTeSR™1 medium, were differentiated towards the haematopoietic/erythropoietic lineages. The media was changed into the hematopoietic/erythroid induction medium (4.1.2.) (3ml/well). The media was supplemented with two concentrations of recombinant human BMP-4: low (10ng/mL) and high (50ng/mL). The differentiation culture was run for 14 days and the media was refreshed every two days. Cells were placed in humidified hypoxia chamber.

6.3.3. Effect of Recombinant Human VEGF-A165 Concentration

Human iPS colonies, cultured on Matrigel coated 6-well tissue culture plates in mTeSR™1 medium, were differentiated towards the haematopoietic/erythropoietic lineages. The media was changed into the hematopoietic/erythroid induction medium (4.1.2.) (3ml/well). The media was supplemented with two different concentrations of

recombinant human VEGF-A165: low (5ng/mL) and high (50ng/mL). The differentiation culture was run for 14 days and the media was refreshed every two days. Cells were placed in humidified hypoxia chamber.

6.3.4. Effect of Serum

Human iPS colonies, cultured on Matrigel coated 6-well tissue culture plates in mTeSR™1 medium, were differentiated towards the haematopoietic/erythropoietic lineages. The media was changed into the hematopoietic/erythroid induction medium (4.1.2.) (3ml/well). The media was supplemented with three different concentrations of FBS: serum-free (0%), low (5%), and high (10%). The differentiation culture was run for 14 days and the media was refreshed every two days. Cells were placed in humidified hypoxia chamber.

6.3.5. Viability of Human iPS Cells during Haematopoietic/Erythropoietic Differentiation in Colonies vs Single Cell

Human iPS colonies, cultured on Matrigel coated 6-well tissue culture plates in mTeSR™1 medium, were differentiated towards the haematopoietic/erythropoietic lineages in three different seeding densities: colonies and single cells ($50x10^3$/cm2 and $100x10^3$/cm2). For the colonies culture group, human iPS colonies at 50% confluency were directly differentiated by changing the media into the haematopoietic/erythroid induction medium (4.1.2.). For single cells differentiation, undifferentiated human iPS colonies were dissociated as single cells using TrypLE^{TM}, washed with PBS+0.1% BSA, counted, and seeded on Matrigel coated 6-well tissue culture plates at two seeding densities: $50x10^3$/cm2, and $100x10^3$/cm2, and supplemented with haematopoietic/ erythroid induction medium (4.1.2.). The media was refreshed every two days (3ml/well) for 14 days. Cells were placed in humidified hypoxia chamber. The viability of the cells in each culture group was assessed and compared.

6.3.6. Efficiency of Haematopoietic/Erythropoietic Differentiation of Human iPS Cells in Colonies vs Single Cell at Different Time Points

Human iPS colonies, cultured on Matrigel-coated 6-well tissue culture plates in mTeSR™1 medium, were differentiated towards the haematopoietic/erythropoietic lineages in three different seeding densities: colonies and single cells ($50x10^3$/cm2 and $100x10^3$/cm2) as explained in 6.3.5. The differentiation efficiency was compared at different time points throughout the differentiation: days 4, 7, 10, and 14, in order to detect the best time point with the optimal cellular yield of the phenotype of interest.

6.4. Results

6.4.1. Effect of Recombinant Human FGF-basic Concentration

Hematopoietic/erythropoietic differentiation at each recombinant human FGF-basic concentration, 20, 50, and 100ng/mL, was assessed by flow cytometry analysis. Differentiated human iPS cells were harvested at the end of the experiment, i.e. day 14, stained for CD71 and CD235a, and compared.

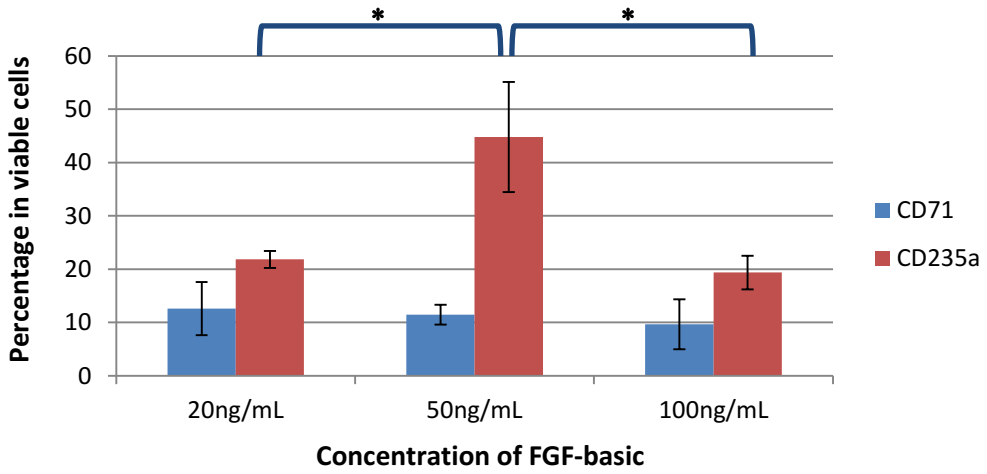


Figure 29. Effect of Different Concentrations of Recombinant Human FGF-basic on the Differentiation of Human iPS Cells.

The flow cytometry analysis profile of differentiated human iPS cells stained with conjugated specific antibodies for CD71 and CD235a (P = 0.005) to compare between 20ng/mL, 50ng/mL, and 100ng/mL concentration of recombinant human FGF-basic. Negative controls were unspecific isotype antibodies conjugated with the same dyes (n=3) (Appendix 1).

All concentrations showed percentages of $CD71^+$ cells and $CD235a^+$ cells (Figure 29). With CD71 analysis, there was no significant difference between the different concentrations. However, with the late main erythroid marker analysis, CD235a, a significant rise in $CD235a^+$ cells with the 50ng/mL FGF-basic concentration was observed in comparison to the other concentrations (P=0.005), which suggests that this concentration might be preferential for the differentiation of human iPS cells towards erythroid lineage (Figure 33).

6.4.2. Effect of Recombinant Human BMP-4 Concentration

Hematopoietic/erythropoietic differentiation at each recombinant human BMP-4 concentration, 10 and 50ng/mL, was assessed by flow cytometry analysis. Differentiated human iPS cells were harvested at the end of the experiment, i.e. day 14, stained for CD71 and CD235a, and compared.

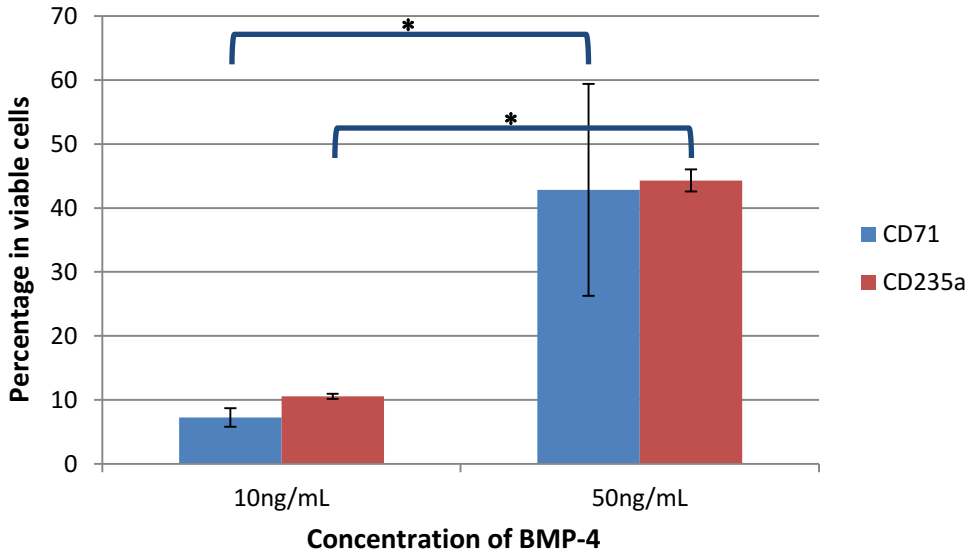


Figure 30. Effect of Different Concentrations of Recombinant Human BMP-4 on the Differentiation of Human iPS Cells.

The flow cytometry analysis profile of differentiated human iPS cells stained with conjugated specific antibodies for CD71 (P = 0.021) and CD235a (P = <0.001) to compare between 10ng/mL and 50ng/mL concentration of recombinant human BMP-4. Negative controls were unspecific isotype antibodies conjugated with the same dyes (n=3) (Appendix 2).

There were significant differences in percentages of $CD71^+$ cells and $CD235a^+$ cells between the different concentrations of BMP-4 (P = 0.021 and P = <0.001, respectively) (Figure 30). Although $CD71^+$ and $CD235a^+$ cells were observed at 10ng/mL BMP-4, the percentage was not significant, which suggests that 50ng/mL might be the favoured concentration for the differentiation of human iPS cells towards erythroid lineage (Figure 33).

6.4.3. Effect of Recombinant Human VEGF-A165 Concentration

Hematopoietic/erythropoietic differentiation at each recombinant human VEGF-A165 concentration, 5 and 50ng/mL, was assessed by flow cytometry analysis. Differentiated human iPS cells were harvested at the end of the experiment, i.e. day 14, stained for CD235a, and compared.

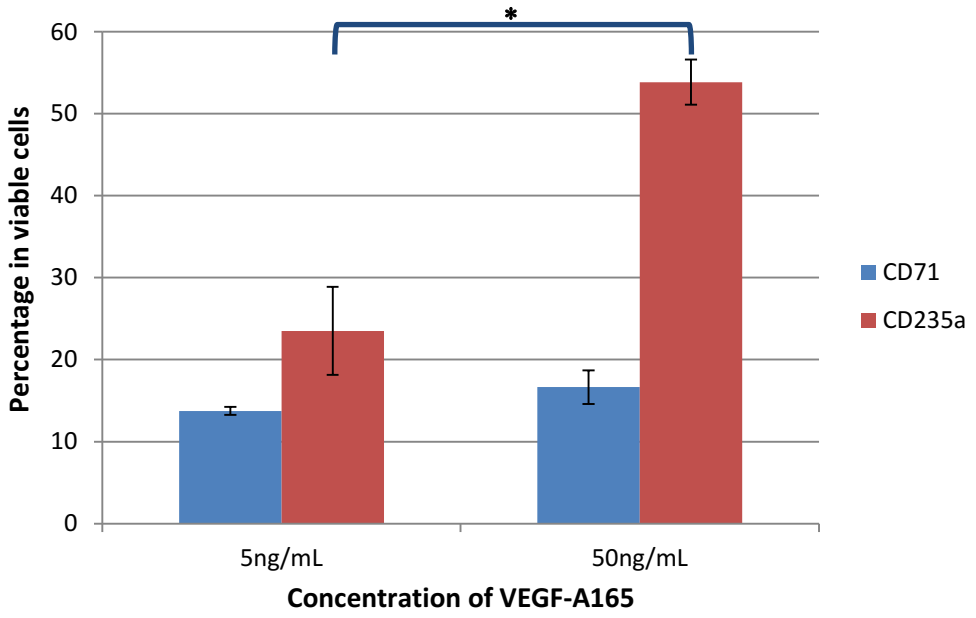


Figure 31. Effect of Different Concentrations of Recombinant Human VEGF-A165 on the Differentiation of Human iPS Cells.

The flow cytometry analysis profile of differentiated human iPS cells stained with conjugated specific antibody for CD71 and CD235a (P=0.019) to compare between 5ng/mL and 50ng/mL concentration of recombinant human VEGF-A165. Negative controls were unspecific isotype antibody conjugated with the same dye (n=2) (Appendix 3).

Both concentrations showed cells positive for CD71 and CD235a (Figure 31). With CD71 analysis, there was no significant difference between the cultures of different VEGF-A165 concentrations. However, there is a significant difference in the percentage of $CD235a^{+}$ cells (P = 0.019) at 50ng/mL VEGF-A165 concentration than at 5ng/mL, which suggests that the former concentration might be better for the differentiation of human iPS cells towards erythroid lineage (Figure 33).

6.4.4. Effect of Serum

Hematopoietic/erythropoietic differentiation at different serum concentrations, 0%, 5%, and 10%, was assessed by flow cytometry analysis. Differentiated human iPS cells were harvested at the end of the experiment, i.e. day 14, stained for CD71 and CD235a, analysed, and compared.

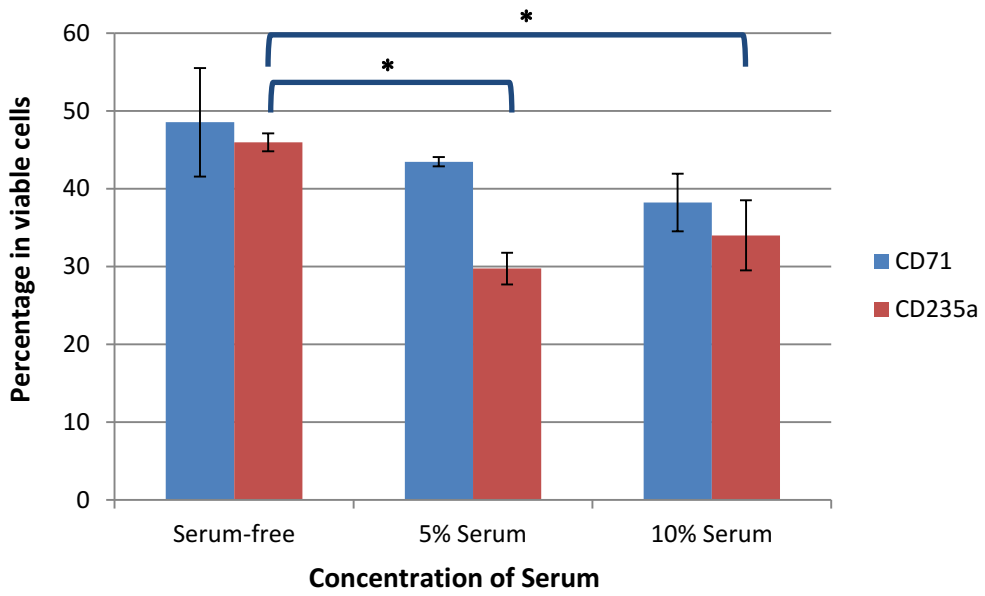


Figure 32. Effect of Serum on the Differentiation of Human iPS Cells towards the Hematopoietic/Erythropoietic Lineages.

The flow cytometry analysis profile of differentiated human iPS cells stained with conjugated specific antibodies for CD71 and CD235a (P = 0.001) to compare between 0%, 5%, and 10% serum concentrations. Negative controls were unspecific isotype antibodies conjugated with the same dyes (n=3) (Appendix 4).

All conditions showed significant presence of $CD71^+$ cells and $CD235a^+$ cells (Figure 32). With CD71 analysis, there was no significant difference between the different serum concentrations. However, the serum-free condition (0%) showed the highest percentage of $CD71^+$ cells. In comparison, CD235a analysis showed significant rise in $CD235a^+$ cells with the serum-free condition in comparison to the serum-containing conditions (P = 0.001), which suggests that serum might not support the differentiation of human iPS cells towards hematopoietic/erythropoietic lineages (Figure 33).

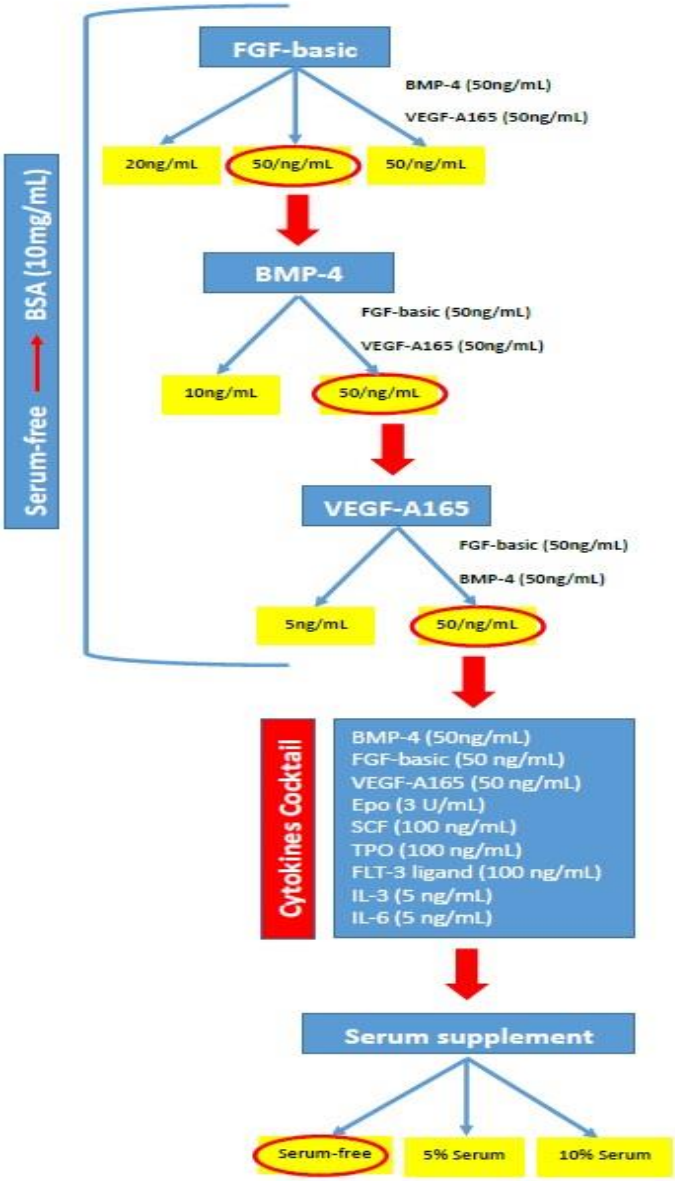


Figure 33. A Flow Diagram Summarising the Optimisation Process of Some Early-Acting Cytokines and Serum Addition for the Differentiation of Human iPS Cells Towards the Erythroid Lineage.

6.4.5. Viability of Human iPS Cells during Haematopoietic/Erythropoietic Differentiation in Colonies vs Single Cell

The viability of human iPS cells during the hematopoietic/erythropoietic differentiation was compared in colonies vs single cells at different seeding densities; 50 x10^3/cm2 and 100x10^3/cm2. Differentiated human iPS cells were harvested at the end of the experiment, i.e. day 14, stained for DAPI, and compared.

The viability of human iPS cells in all cell seeding conditions were maintained at a good percentage (>90%), with no significant statistical difference (Figure 34).

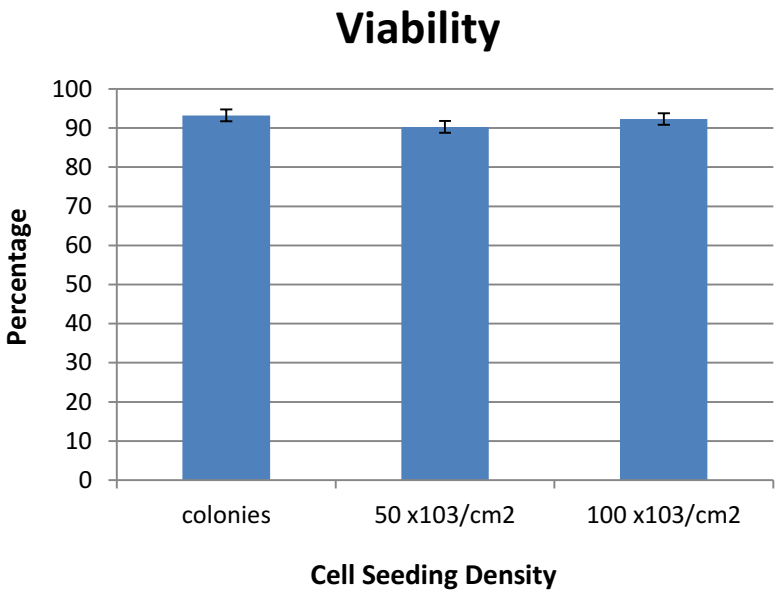


Figure 34. Comparing the viability of the Differentiated Human iPS Cells in Colonies vs Single Cells.

The flow cytometry analysis profile of differentiated human iPS cells stained with DAPI to compare the viability in colonies differentiation vs single cell differentiation at different seeding densities, 50 x10^3/cm2 and 100 x10^3/cm2 (n=2) (Appendix 5).

6.4.6. Efficiency of Haematopoietic/Erythropoietic Differentiation of Human iPS Cells in Colonies vs Single Cell at Different Time Points

The efficiency of the hematopoietic/erythropoietic differentiation of human iPS cells was compared in colonies vs single cells at different seeding densities, 50 $x10^3$/cm2 and 100 $x10^3$/cm2, throughout the differentiation process. Differentiated human iPS cells were harvested at different time points, days 4, 7, 10, and 14, stained for CD71 and CD235a, and compared.

It was evident that the highest expression of CD71 was seen in colonies, followed by single cell seeding density at $100x10^3$/cm2, and the lowest expression was observed at $50x10^3$/cm2 seeding density (P=0.006) (Figure 35). The expression of CD71 in colony differentiation was compared between time points, and there was no significant difference. Moreover, colonies differentiation also showed the highest percentage of $CD235a^+$ cells among the other seeding densities, although there was no statistical difference in mean values among cell seeding densities (Figure 36). Day 7 of erythroid differentiation in colonies showed the highest expression among the other time points. Thus, we pursued the analysis further looking at day 7 time point, which was statistically higher than the previous time point (P=0.025). Also, we compared between the three cell seeding densities at that specific time point, which showed a statistically significant rise in $CD235a^+$ cells in colonies compared to single cells differentiation (P=0.002). This finding suggests that this condition at that time point might be the ideal condition for the differentiation of human iPS cells towards erythroid lineage. Thus, it was concluded that this hematopoietic/erythropoietic differentiation protocol gives the highest yield of erythroid cells at day 7.

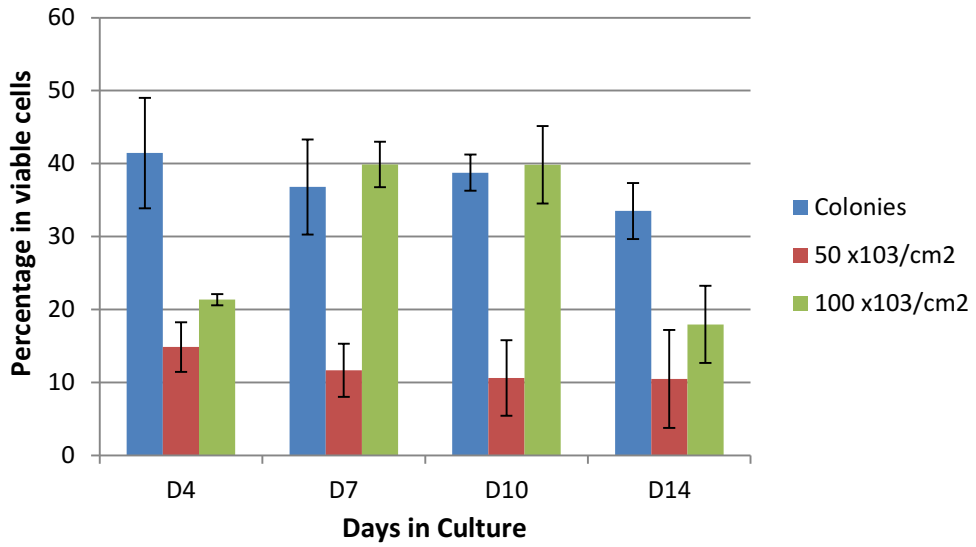


Figure 35. Comparing the Expression of CD71 throughout the Erythroid Differentiation of Human iPS Cells in Colonies vs Single Cells.

The flow cytometry analysis profile of differentiated human iPS cells stained with conjugated specific antibody for CD71 (P = 0.006) to compare the efficiency of erythroid differentiation at different time points throughout the differentiation in colonies and single cells at different seeding densities, $50x10^3$/cm2 and $100x10^3$/cm2. Negative controls were unspecific isotype antibody conjugated with the same dye (n=3) (Appendix 6).

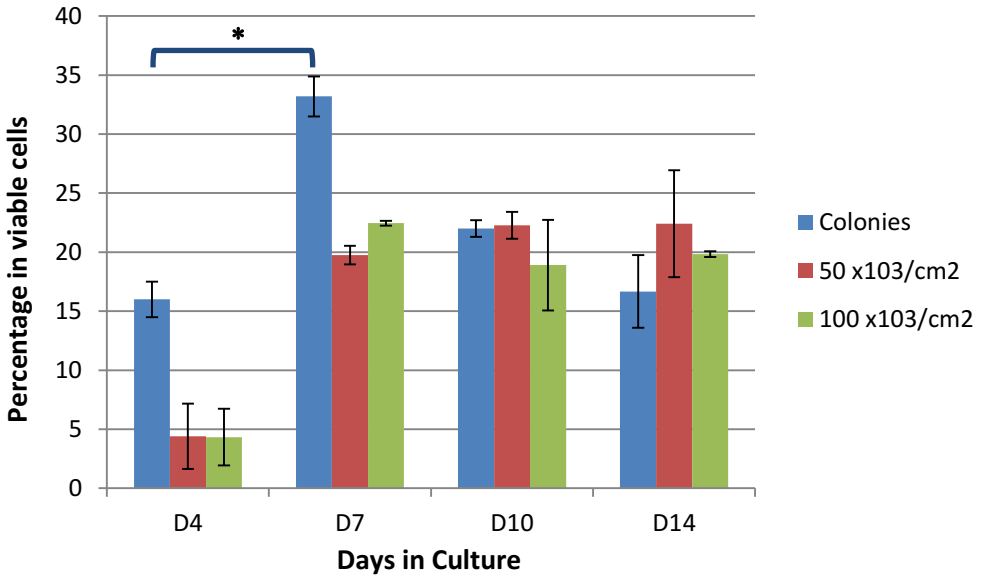


Figure 36. Comparing the Expression of CD235a throughout the Erythroid Differentiation of Human iPS Cells in Colonies vs Single Cells.

The flow cytometry analysis profile of differentiated human iPS cells stained with conjugated specific antibody for CD235a (P = 0.289) to compare the efficiency of erythroid differentiation at different time points throughout the differentiation in colonies and single cells at different seeding densities, $50x10^3$/cm2 and $100x10^3$/cm2. Negative controls were unspecific isotype antibody conjugated with the same dye (n=3) (Appendix 6).

6.5. Discussion

The early stages of erythropoietic development *in vivo* and *in vitro* are influenced by some early acting growth factors such as BMP-4, VEGF, and FGF-basic (Keller et al., 1993, Kennedy et al., 1997, Levenstein et al., 2006, Pick et al., 2007, Pearson et al., 2008). In looking at the literature and considering the influence of the hypoxia condition and the above-mentioned factors, we attempted in this chapter to optimize their levels of application in our culture media in order to induce an efficient haematopoietic/erythropoietic differentiation of human iPS cells in a serum-free condition under hypoxia condition. The concentrations examined were obtained from previously reported studies of the same interest (Lu et al., 2008a, Ma et al., 2008, Feng et al., 2010, Lapillonne et al., 2010b, Dias et al., 2011, Dorn et al., 2015).

The effect of BMP-4 on the haematopoietic/erythropoietic differentiation of human iPS cells was assessed using two different concentrations: low (10ng/mL) and high (50ng/mL). There were significant increases in $CD71^+$ cells and $CD235a^+$ cells at the 50ng/mL concentration in comparison to 10ng/mL, which suggests that 50ng/mL might be favourable for the differentiation of human iPS cells towards erythroid lineage (Table 8). Moreover, testing a higher concentration of BMP-4 for our purpose was not preferable, as higher concentrations have been shown to induce trophectoderm differentiation of human ES cells (Xu et al., 2002, Vallier et al., 2005, D'Amour et al., 2005). Moreover, many studies have emphasized the role of FGF-basic in the haematopoietic differentiation (Pick et al., 2007, Lu et al., 2008b, Lee et al., 2009, Feng et al., 2010). Thus, we have compared the effect of three different concentrations of FGF-basic: low (20ng/mL), medium (50ng/mL), and high (100ng/mL), on the haematopoietic/erythroid differentiation of human ES/iPS cells. Our findings suggest that 50ng/mL might be favourable for our purpose as it showed a significant rise in $CD235a^+$ cells in comparison to the other two concentrations, although there was no significant difference in CD71 expression among the three concentrations (Table 8). Furthermore, the effect of VEGF-A165 on the haematopoietic/erythropoietic differentiation of human iPS cells was also assessed using two different concentrations: low (5ng/mL) and high (50ng/mL). Both concentrations showed cells positive for CD71 and CD235a. With CD71 analysis, there

was no significant difference between the cultures of different VEGF-A165 concentrations. However, there is a significant increase in CD235a^{+} cells at 50ng/mL VEGF-A165 concentration in comparison to 5ng/mL, which suggests that this concentration might be better for the differentiation of human iPS cells towards erythroid lineage (Table 8). Moreover, testing a higher concentration of VEGF-A165 for our purpose was not preferable, as higher concentrations have shown to induce vascular endothelium differentiation of human ES cells (Sone et al., 2007).

The preferred concentration of FGF-basic observed in our study is higher than what has been reported previously in the literature, while with BMP-4 and VEGF-A165 concentrations, our results support those reported by Lu et al. (2008) (Lu et al., 2008a) and Feng et al. (2010) (Feng et al., 2010) (Table 8). These factors, together with other late-acting growth factors and cytokines, including FLT-3, TPO, SCF, IL-3, IL-6, and the main erythroid lineage inducer, EPO, at their routinely used concentrations, led to the optimization of our novel haematopoietic/erythropoietic induction media under serum-free condition (4.1.2.).

Furthermore, the effect of serum addition on the haematopoietic/erythroid induction of human iPS cells using our optimized cocktail was assessed by comparing different concentrations of serum (0%, 5%, and 10%). All conditions showed significant expression of CD71 and CD235a. With CD71 analysis, there was no significant difference between the three serum concentrations. However, the serum-free condition (0%) showed the highest percentage of CD71^{+} cells, which was also accompanied by a significant increase in CD235a expression in comparison to the other concentrations. Accordingly, these results suggest that serum might not support the differentiation of human iPS cells towards the hematopoietic/erythropoietic lineages using our differentiation protocol. Moreover, the use of serum creates variability and inconsistency in cell differentiation that confines the need for standardization in clinical applications (Lu et al., 2008b). Thus, it is crucial to move to serum-free differentiation condition.

Table 8. Optimizing the Concentrations of the Main Haematopoietic Inducers and Factors Required for the Haematopoietic/Erythropoietic Induction of Human iPS Cells.

Factor	Condition	CD71	CD235a
Serum	10% Serum	38.2%	34%
	5% Serum	43.5%	29.7%
	Serum-free*	48.5%	45.9%
Recombinant Human BMP-4	10ng/mL	7.24%	10.5%
	50ng/mL*	42.83%	44.3%
Recombinant Human FGF-basic	20ng/mL	12.6%	21.8%
	50ng/mL*	11.45%	44.8%
	100ng/mL	9.65%	19.4%
Recombinant Human VEGF-A165	5ng/mL	13.75%	23.5%
	50ng/mL*	16.65%	53.85%

* Indicates the favoured concentration of the factors tested that resulted in the highest percentage of CD71 and/or CD235a expressing cells (highlighted in darker shades).

Since human iPS cells are social cells, in which cell-to-cell interactions are necessary for their survival, proliferation and differentiation, the viability and efficiency of our direct haematopoietic/erythropoietic induction of human iPS cells was compared between different seeding densities: colonies and single cells at different seeding densities: $50x10^3$/cm2 and $100x10^3$/cm2. No significant difference was seen in viability. However, it was important to differentiate them as colonies rather than single-cells that can lead into physical damages and apoptosis (Bajpai et al., 2008, Heng et al., 2007). In terms of differentiation efficiency, a significant increase was observed in CD71 expression when differentiating the cells as colonies. A significant increase was also observed in the expression of CD235a at day 7 in colonies in comparison to other seeding densities. Thus, we concluded that the highest yield of the phenotype of interest was achieved at an early

time point, day 7, which is the optimum time point to start a further erythroid differentiation phase (Table 9).

Table 9. Comparing the Efficiency of the Haematopoietic/Erythropoietic Differentiation of Human iPS Cells at Different Cell Seeding Conditions and Time Points.

Time point	Condition	CD71	CD235a
Day 4	Colonies	41.45%	16%
	50 x10^3/cm2	14.87%	4.41%
	100 x10^3/cm2	21.35%	4.3%
Day 7*	Colonies *	36.8%	33.2%
	50 x10^3/cm2	11.66%	19.75%
	100 x10^3/cm2	39.9%	22.45%
Day 10	Colonies	38.75%	22%
	50 x10^3/cm2	10.6%	22.3%
	100 x10^3/cm2	39.85%	18.9%
Day 14	Colonies	33.5%	16.6%
	50 x10^3/cm2	10.47%	22.4%
	100 x10^3/cm2	17.97%	19.83%

*Indicates the ideal time point and seeding density that resulted in the highest percentage of CD71 and/or CD235a expressing cells (highlighted in darker shades) under our Haematopoietic/erythropoietic induction protocol.

6.6. Conclusion

In this chapter we have described an efficient and direct method to induce a haematopoietic/ erythropoietic differentiation from human iPS cells in a serum-free

system under hypoxia conditions that can be suitable for future clinical applications. It was concluded, using our differentiation protocol, that the addition of serum does not support the haematopoietic/ erythropoietic differentiation of human iPS cells. The main haematopoietic inducers and factors were gathered from previous studies, tested for their optimum concentrations, and adapted to our cell line and culture conditions. Then, the viability of the differentiated cells and efficiency of the haematopoietic/erythropoietic induction at different time points were compared between different cell seeding densities, colonies vs single cells, in order to define the ideal seeding condition and time point where the highest expressions of CD71 and/or CD235a were found. In this chapter we thus defined our novel haematopoietic/ erythropoietic induction strategy (Table 10), which will be followed by further erythroid differentiation and maturation in the following chapter in order to describe a fully defined protocol for the direct generation of erythroid cells from human iPS cells under hypoxia environment in serum-free and feeder-free culture conditions.

Table 10. Our Novel Hematopoietic/Erythropoietic Induction Protocol.

Media Components		Seeding Density	Days in Culture	O_2 Level
DMEM/F-12- GlutaMAX™	-	**Colonies**	**7 Days**	**Hypoxia**
BSA	10 mg/mL			
Human EPO	3 U/mL			
Human SCF	100 ng/mL			
Human TPO	100 ng/mL			
Human FLT-3	100 ng/mL			
Human BMP-4	50 ng/mL			
Human FGF-basic	50 ng/mL			
Human VEGF-A165	50 ng/mL			
Human IL-3	5 ng/mL			
Human IL-6	5 ng/mL			
Penicillin/ Streptomycin	100 units/mL			

Chapter 7.

The Effect of O_2 on the Differentiation and Maturation of Human iPS Cells towards the Erythroid Lineage:

Normoxia vs Hypoxia

7.1. Introduction

Erythropoiesis is a multi-step process, including the differentiation of early erythroid progenitors (BFU-E) into late erythroid progenitors (CFU-E), and then into erythroblasts. Erythroblasts undergo a series of differentiation and maturation events, including nuclear and chromatin condensation, and size reduction (Lu et al., 2008a). Signs of the terminal maturation of erythroid cells mainly include loss of the expression of CD71, expression of CD235a, enucleation to acquire the typical biconcave discoid shape, and the haemoglobin switching into adult-type globins (Lu et al., 2008a, Giarratana et al., 2011).

The *in vitro* generation of adult erythroid cells presents significant challenges. A critical issue is whether erythroblasts generated *in vitro* from human ES/iPS cells can mature into mature enucleated erythrocytes expressing adult-type haemoglobin, since ~97% of haemoglobin in adult erythrocytes is adult haemoglobin (Cantu and Philipsen, 2014).

In vitro enucleation of erythroid cells thus remains a difficult stage to achieve, and the cellular mechanisms involved are weakly understood. Studies proposed that enucleation occurs after asymmetric cell division to extrude nucleus enclosed by the plasma membrane, which is thought to be promoted by the direct contact with macrophages (Rhodes et al., 2008). Moreover, co-culturing erythroid cells with stromal cells has shown to enhance erythropoiesis by excreting soluble factors and providing direct cell–cell contact needed for the erythroid maturation, in addition to engulfing nuclei upon enucleation (Lu et al., 2008a).

10% erythroid enucleation has been achieved using a serum-free HSCs expansion medium Stemline II supplemented with cytokines without stromal layers (Giarratana et al., 2005, Lu et al., 2010), while combining the co-culture step further improved the enucleation (Giarratana et al., 2005). The resulted enucleated erythrocytes showed comparable morphology and size to normal erythrocytes (Lu et al., 2008a). The expression of CD71 decreased while the expression of CD235a increased dramatically upon maturation (Lu et al., 2008a). However, erythroid cells cultured in haematopoietic expansion Stemline II medium expressed mainly embryonic and fetal globin chains, and

the expression of the adult β-globin chain started to express at low levels only after the stromal co-culture (Lu et al., 2008a).

In order to develop a potential clinical transfusion product, a development of a serum-free and feeder-free system thus has to be achieved to enable the generation of mature enucleated adult erythrocytes as close as possible to the normal deformable adult erythrocytes in the haemoglobin type, enzyme content, and O_2 dissociation features.

In this chapter, we sought to apply our serum-free and feeder-free haematopoietic/erythroid induction protocol optimized in chapter 6 and combine it with an erythroid differentiation medium then erythroid maturation/enucleation medium optimized from the erythroid differentiation protocol reported by Lu et al. (2008) (Giarratana et al., 2005, Lu et al., 2008a). Then we sought to compare the efficacy of the overall differentiation in hypoxia and normoxia to assess the role of O_2 on erythropoiesis.

7.2. Aim and Objectives

The aim of this chapter is to evaluate the role of O_2 in the differentiation and maturation of human iPS cells towards the erythroid lineage. In order to achieve this aim, the following objectives were followed:

1. To induce the hematopoietic/erythropoietic differentiation in human iPS cells, as described in chapter 6, in normoxic and hypoxic conditions.
2. To pursue with a further erythroid differentiation and an erythroid maturation protocols in normoxia and hypoxia conditions
3. To compare the experimental outcomes in normoxia and hypoxia in order to assess the role of O_2 on the differentiation of human iPS cells towards the erythroid lineage.

7.3. Experimental Plan

7.3.1. Differentiation of Human iPS Cells towards the Erythroid Lineage

Human iPS cells cultured on Matrigel coated 6-well tissue culture plates in mTeSR™1 medium were differentiated as monolayer of colonies. Media were changed into haematopoietic/erythroid induction medium (4.1.2.) for 7 days (3ml/well), with refreshing of media every two days. Cells undergoing differentiation were separated into two groups: normoxia (20% O_2) and hypoxia group (5% O_2).

7.3.2. Maturation of Human iPS Cells-Derived Erythroid Cells

Differentiated human iPS cells at day 7 of hematopoietic/erythropoietic induction started the erythroid differentiation step, where media were changed into erythroid differentiation medium (4.1.3.) for 7 days. This stage was followed by 14 days in erythroid maturation/enucleation medium (4.1.4.), with refreshing of media every two days. Cells undergoing differentiation were separated into two groups: normoxia (20% O_2) and hypoxia group (5% O_2).

7.3.3. The Effect of O_2 in the Differentiation of Human iPS Cells towards Erythroid Lineage: Normoxia vs Hypoxia

The differentiated cells were harvested at different time points, days 7, 14, 21, and 28, and analysed using a wide range of techniques such as CFU assay, immunocytochemical characterization, flow cytometry, qPCR, and O_2 dissociation curve. The cellular outputs of both conditions, normoxia and hypoxia, were compared in order to assess the effect of O_2 on our differentiation protocol.

7.4. Results

7.4.1. Morphology Analysis of Human iPS Cells Differentiated towards the Erythroid Lineage

7.4.1.1. Wright-Giemsa Staining of Differentiated Human iPS Cells

Differentiated human iPS cells of both culture groups, normoxia and hypoxia, were stained with Wright-Giemsa staining at different time points, days 7, 14, 21, and 28, and evaluated under the microscope. Wright-Giemsa staining is used to study the different morphology of blood cells and nuclear-cytoplasmic differentiation under the microscope.

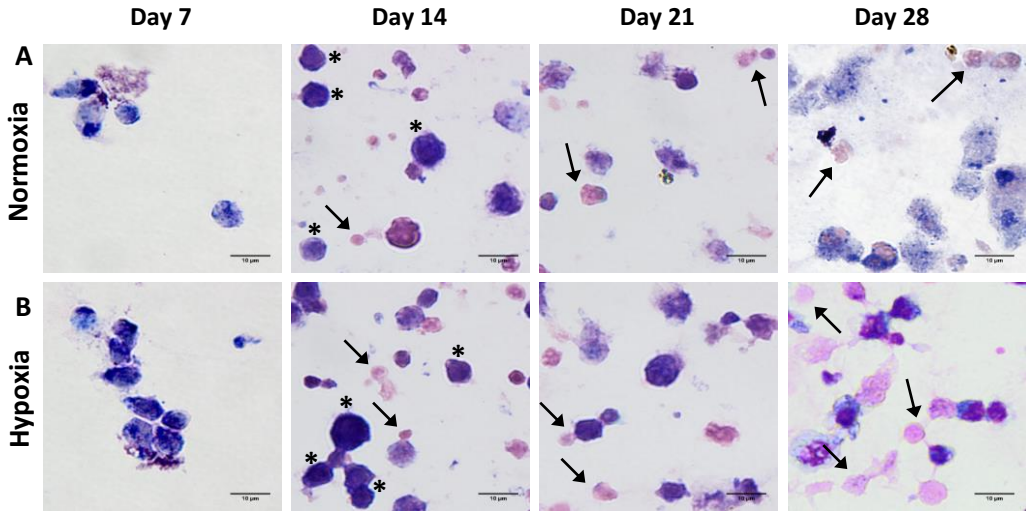


Figure 37. Morphological Analysis by Wright-Giemsa staining of Differentiated Human iPS Cells at Different Time Points along the Erythroid Differentiation Protocol.

A) Normoxia; B) Hypoxia. The first column are day7 images. The second column are day14 images, which show mainly nucleated erythroblasts in both conditions, indicated by asterisks (*), and few enucleated erythroid cells, indicated by arrows. The third column are day21 images, where size reduction is noticed in both conditions, in addition to a lesser erythroblasts count. The forth column are day 28 images, which show more enucleated erythroid cells. (Scales for each image: 10µm).

As shown in Figure 37, the Wright-Giemsa staining confirmed erythroid maturation along the differentiation process. Nucleated erythroblasts were observed as early as day 14 in both conditions. They were identified by their relative large size (10-15µm) and high nuclear to cytoplasmic ratio, with a round nucleus occupying almost three-fourths of the cell that stain violet blue, and surrounded by a narrow ring-shaped cytoplasm stain light purple colour. Few enucleated erythroid cells have been observed as early as day 14, which, according to their size (7-10µm), could be reticulocytes. The number of enucleated cells increased towards day 28 in both conditions, in parallel to a size reduction (7-8µm), which can be explained as a further maturation into enucleated erythrocyte (Blann and Holtom, 2010). Day 28 cells of hypoxia condition show a higher number of healthy enucleated cells, while cells at the same time point in normoxia look fragile and exhausted.

7.4.1.2. Reticulocyte Staining of Differentiated Human iPS Cells

Differentiated human iPS cells of both culture groups, normoxia and hypoxia, were stained with Reticulocyte staining at different time points, days 7, 14, 21, and 28, and evaluated under the microscope. Reticulocytes can be identified by the loss of nucleus, their size (7-10µm), and the RNA ribosomal remnant of haemoglobin detectable by supravital stain like Reticulocyte staining (new methylene blue), which is used to observe the precipitation of ribosomal RNA under the microscope (Blann and Holtom, 2010).

As shown in Figure 38, the reticulocyte staining confirmed the formation of reticulocytes in both conditions, which is one stage before the final formation of the mature erythrocytes (Blann and Holtom, 2010). Reticulocytes were detected as early as day 21 of erythroid differentiation, with no clear difference between the two conditions.

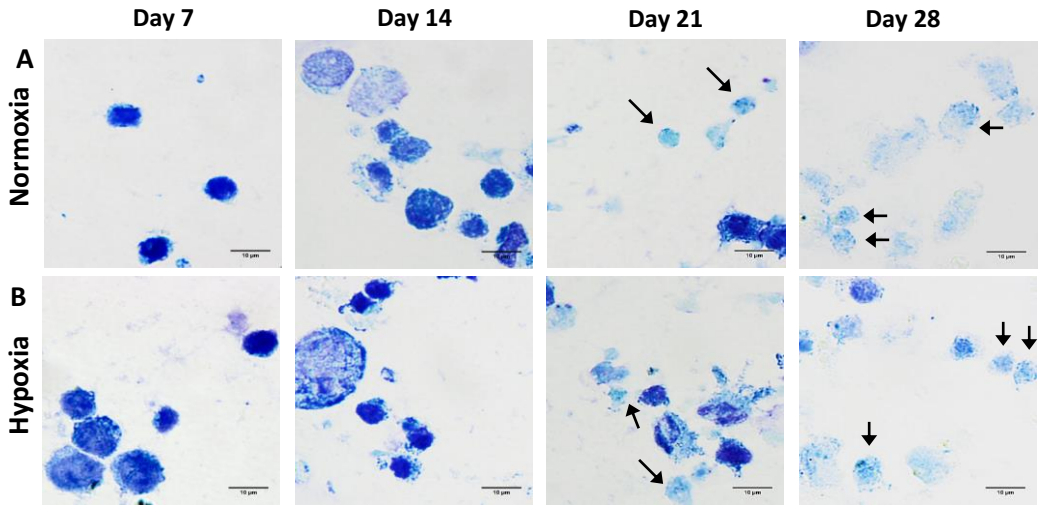


Figure 38. Morphological Analysis by Reticulocyte Staining of Differentiated Human iPS Cells at Different Time Points along the Erythroid Differentiation Protocol. A) Normoxia; B) Hypoxia. The first column are day7 images and the second column are day14 images, where reticulocytes were not observed in both conditions. The third column are day21 images, which show some reticulocytes-like cells, as indicated by the arrows. The forth column are day28 images, which show the majority of reticulocytes in both conditions. (Scales for each image: 10μm).

7.4.2. Flow Cytometry Analysis of Differentiated Human iPS Cells

Erythropoietic differentiation was first assessed by distinct cellular phenotype of specific pluripotency-, haematopoiesis-, and erythropoiesis-associated markers. Samples of differentiated human iPS cells of each culture group, normoxia and hypoxia, were harvested at different time points, days 7, 14, 21, and 28, stained for the following markers, and compared to day 0 undifferentiated human iPS cells as the control sample.

1. SSEA4: a glycolipid antigen composed of a globoseries carbohydrate core, expressed by pluripotent human ES cells, while downregulated upon differentiation. This antigen together with SSEA3 plays a central role during embryogenesis as it belongs to the P blood group system (Kannagi et al., 1983, Henderson et al., 2002).
2. CD34: an early hematoendothelial surface antigen expressed on hematopoietic stem and progenitor cells and vascular endothelial cells (Tavian et al., 1996, Yin et al., 1997, Matsuoka et al., 2001, Zambidis et al., 2005). CD34 antigen is widely used as a marker

for the purification of HSCs in transplantation or gene therapy (Krause et al., 1994, Tavian et al., 1996). Hematopoietic precursor cells derived from ES cells express the cell surface antigen CD34 (Kaufman et al., 2001). However, the expression of CD34 decreases during hematopoietic differentiation and it disappears in terminally differentiated cells (Krause et al., 1994).

3. CD45: a panhematopoietic marker (Zambidis et al., 2005), a membrane-associated tyrosine phosphatase that controls T- and B-cell through dephosphorylation of Src family kinases and JAKs (Harashima et al., 2002). CD45 is expressed on most hematopoietic cells except mature RBCs and platelets (Harashima et al., 2002).
4. CD71 (transferrin receptor): an integral membrane protein that facilitates the uptake of transferrin-iron complexes (Aisen, 2004, Sargent et al., 2005). CD71 is an early erythroid marker (Zambidis et al., 2005), highly expressed as a cell-surface marker on erythroid lineage at normoblast phase, which decreases at the reticulocyte phase. Mature erythrocytes loss the expression of CD71, which make them easily identifiable (Lawrence et al., 1999, Marsee et al., 2010).
5. CD235a (Glycophorin A): a late erythroid-specific membrane sialoglycoprotein that forms the antigens of the MN blood group of RBCs (Dupont et al., 1995). This protein was isolated at different stages of erythropoiesis in normal bone marrow (Gahmberg et al., 1984). Glycophorin A consists of three dispersed domains: a hydrophilic segment at the external surface, a hydrophobic segment embedded within the lipid bilayer, and a COOH-terminal in the cytoplasm (Andersson et al., 1981, Gahmberg et al., 1984).
6. CD43 (Leukosialin): also known as sialophorin, a leukocyte transmembrane sialoglycoproteins expressed on hematopoietic progenitors and differentiated haematopoietic cells, except mature erythrocytes and B-cells. During the *in vitro* haematopoietic differentiation of human ES cells, the surface expression of CD43 was detected before expression of CD45, and distinguishes hematopoietic from endothelial cells (Vodyanik et al., 2006). CD43 has two domains: a cytoplasmic domain that interrelates with cytoskeletal proteins and controls intracellular signal pathways in cell proliferation and survival, and a negative charge extracellular glycosylated domain with anti-adhesive (Vodyanik et al., 2006).

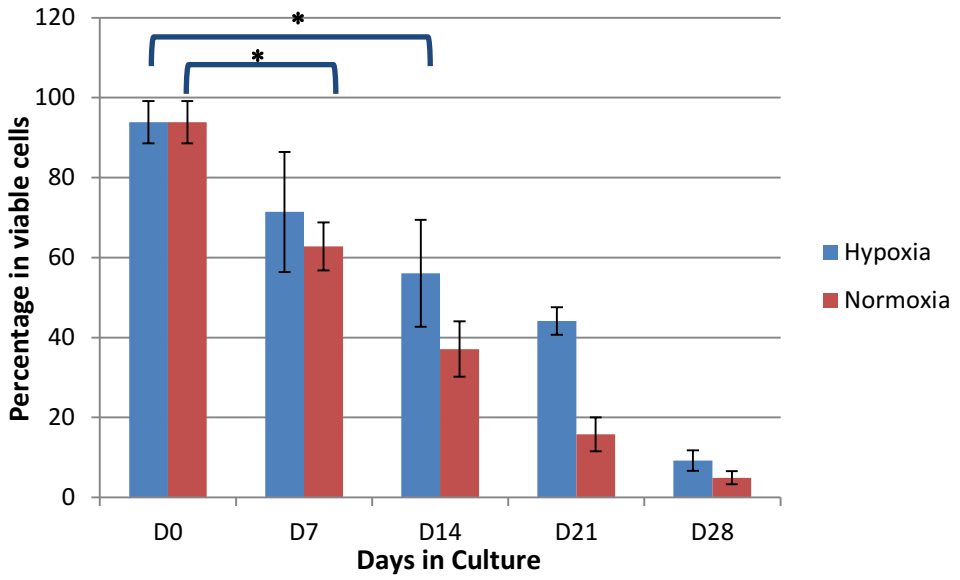


Figure 39. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for SSEA4 (Appendix 7). Negative controls were unspecific isotype antibodies conjugated with the same dyes. The expression of SSEA4 decreased significantly by day7 in normoxia (P=<0.001) and day14 in hypoxia (P=<0.001) (n≥3).

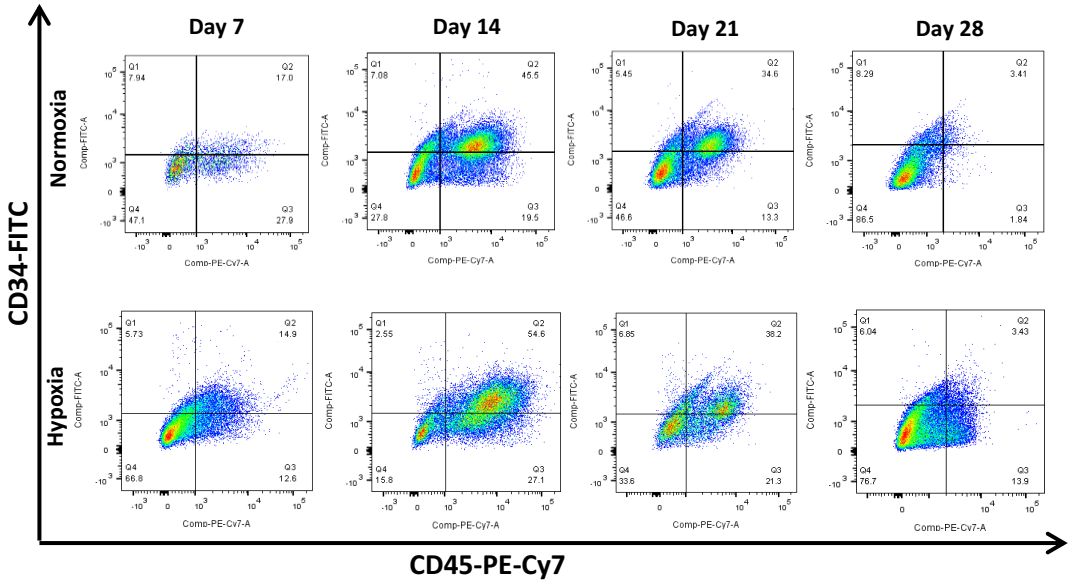


Figure 40. Representative Dot Plots Flow Cytometry Analysis of Differentiated Human iPS Cells Stained for CD34 and CD45 (Appendix 8).

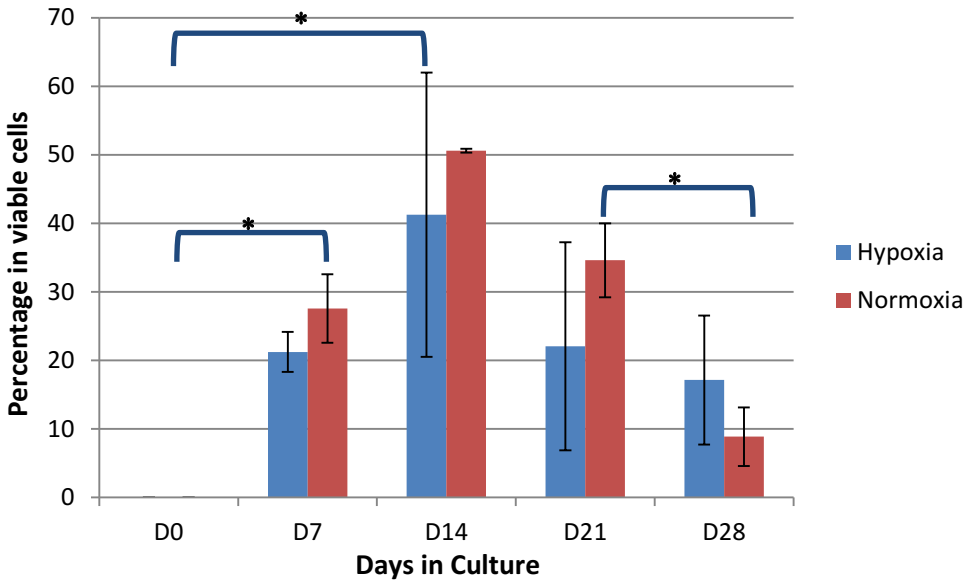


Figure 41. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 (Appendix 8). Negative controls were unspecific isotype antibodies conjugated with the same dyes. A significant increase in CD34 expression at day7 of normoxia (P=<0.001), and day14 in hypoxia (P = 0.027). The expression decreased significantly at day 28 of Normoxia (n≥3).

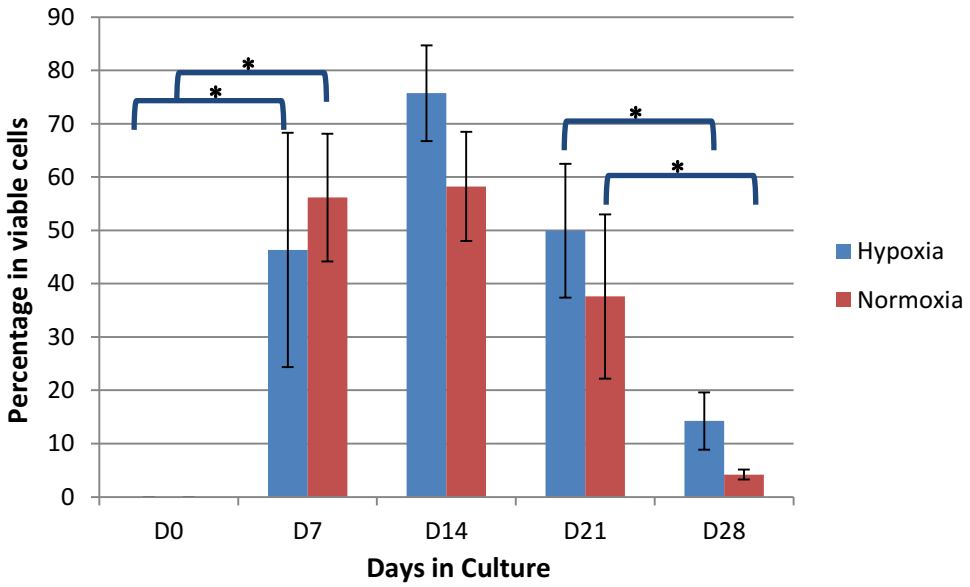


Figure 42. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD45 (Appendix 8). Negative controls were unspecific isotype antibodies conjugated with the same dyes. A significant increase in CD45 expression at day7, followed by a significant decrease at day 28 of both conditions, normoxia (P=<0.001) and hypoxia (P=<0.001) (n≥3).

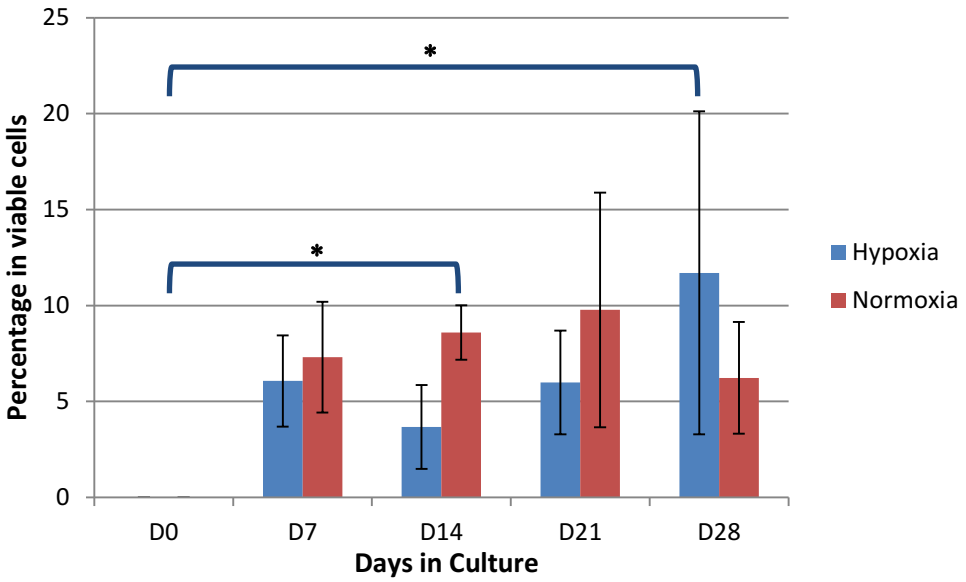


Figure 43. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 and CD45 to Detect $CD34^{+}$ $CD45^{-}$ Cells (Appendix 8). Negative controls were unspecific isotype antibodies conjugated with the same dyes. Normoxia (P= 0.028). Hypoxia (P = 0.038) (n≥3).

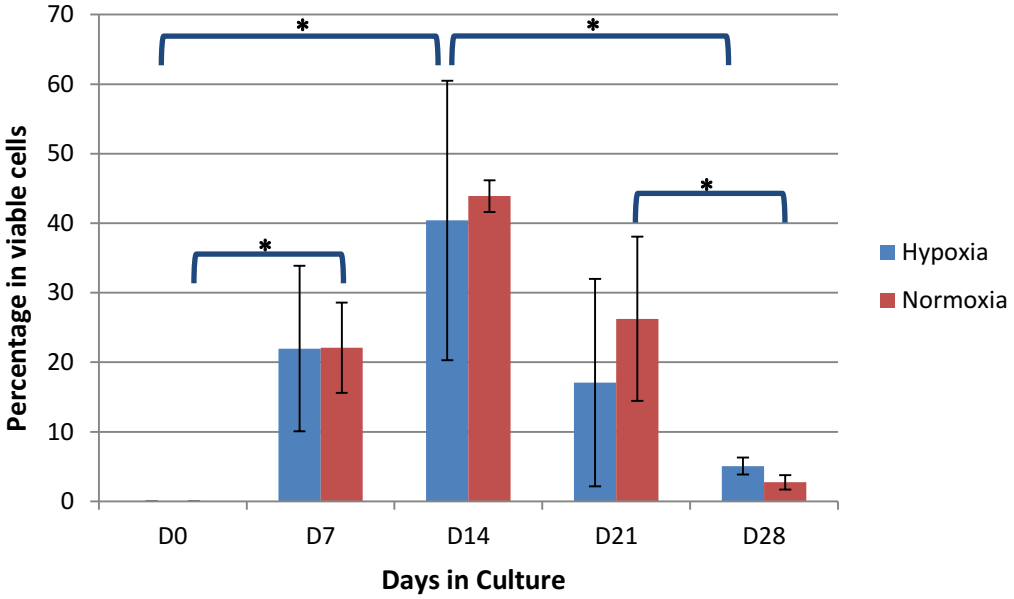


Figure 44. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 and CD45 to Detect $CD34^{+}$ $CD45^{+}$ Cells (Appendix 8). Negative controls were unspecific isotype antibodies conjugated with the same dyes. Normoxia (P=<0.001). Hypoxia (P = 0.012) (n≥3).

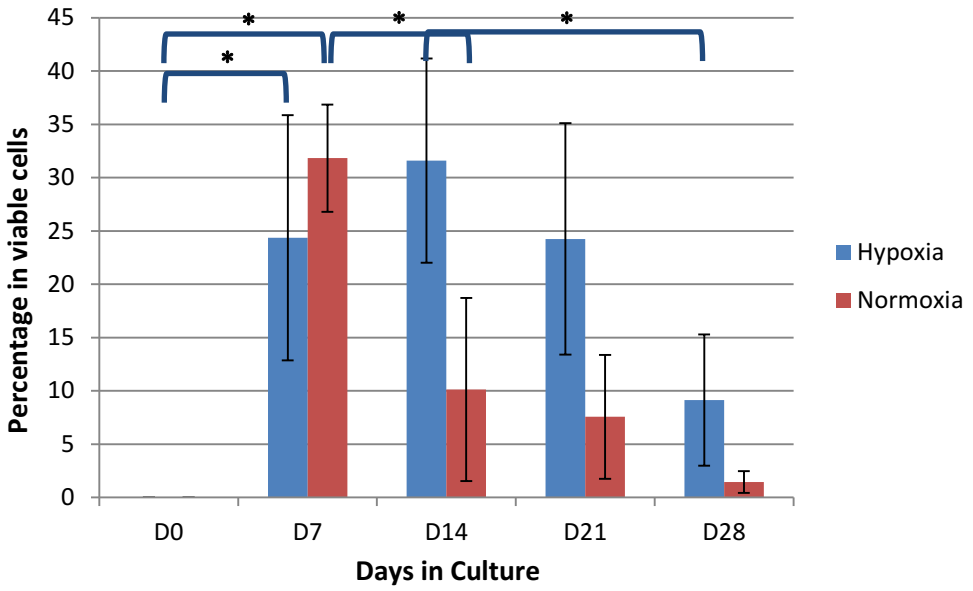


Figure 45. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD34 and CD45 to Detect $CD34^{-}$ $CD45^{+}$ Cells (Appendix 8). Negative controls were unspecific isotype antibodies conjugated with the same dyes. Normoxia (P=<0.001). Hypoxia (P = 0.004) (n≥3).

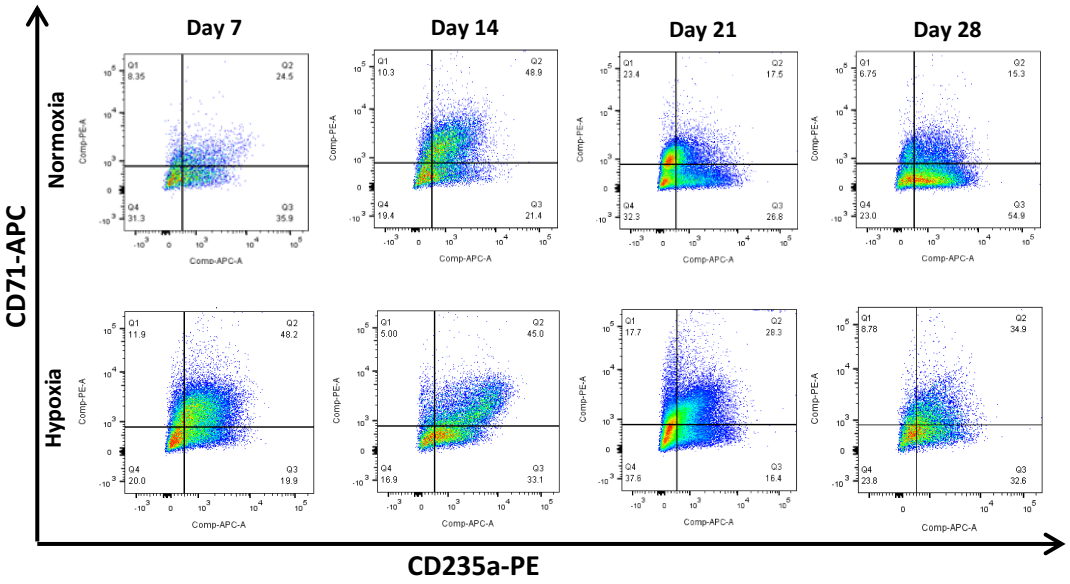


Figure 46. Representative Dot Plots Flow Cytometry Analysis of Differentiated Human iPS Cells Stained for CD71 and CD235a (Appendix 9).

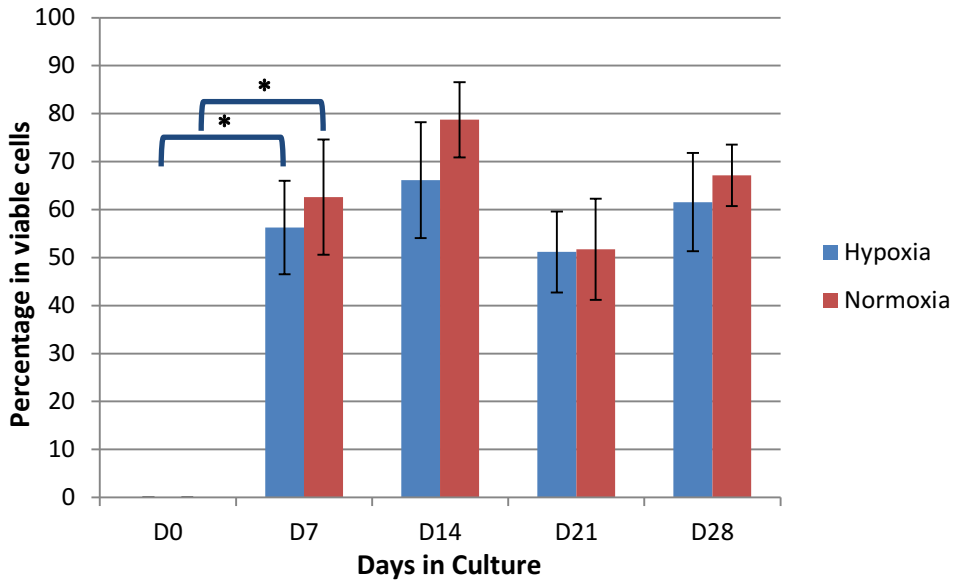


Figure 47. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 (Appendix 9). Negative controls were unspecific isotype antibodies conjugated with the same dyes. A significant increase in the expression of CD71 at day 7 of both conditions, normoxia (P=<0.001) and hypoxia (P=<0.001) (n≥3).

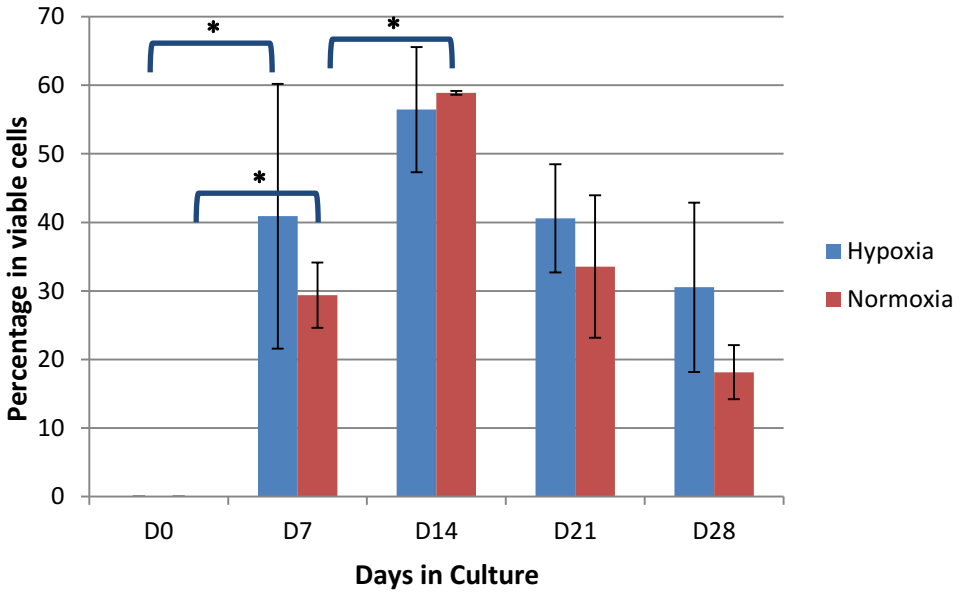


Figure 48. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD235a (Appendix 9). Negative controls were unspecific isotype antibodies conjugated with the same dyes. A significant increase in the expression of CD235a at day 7 of both conditions, normoxia (P=<0.001) and hypoxia (P = 0.003) (n≥3).

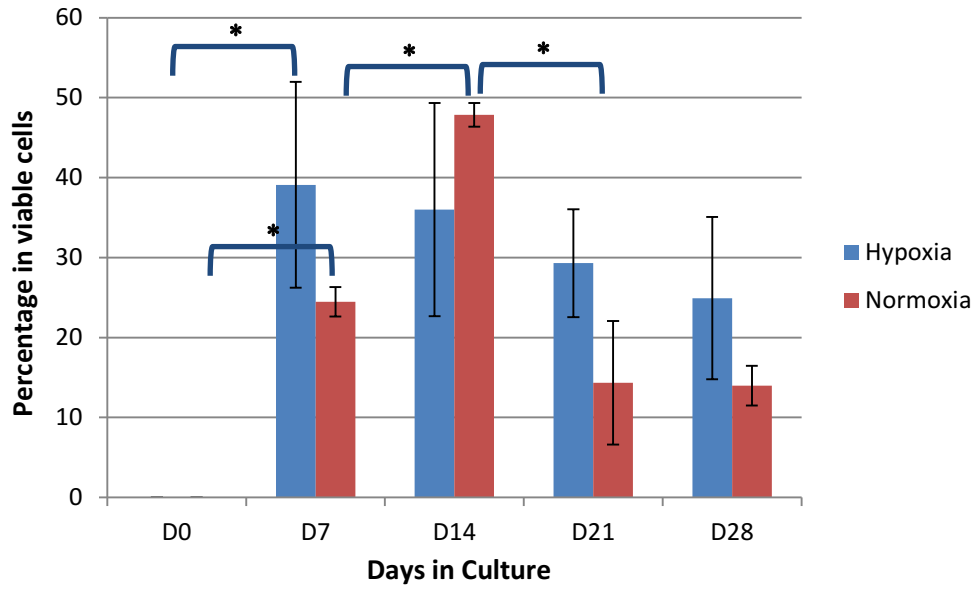


Figure 49. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 and CD235a to Detect $CD71^{+}$ and $CD235a^{+}$ (Appendix 9). Negative controls were unspecific isotype antibodies conjugated with the same dyes. Normoxia (P=<0.001). Hypoxia (P = 0.005) (n≥3).

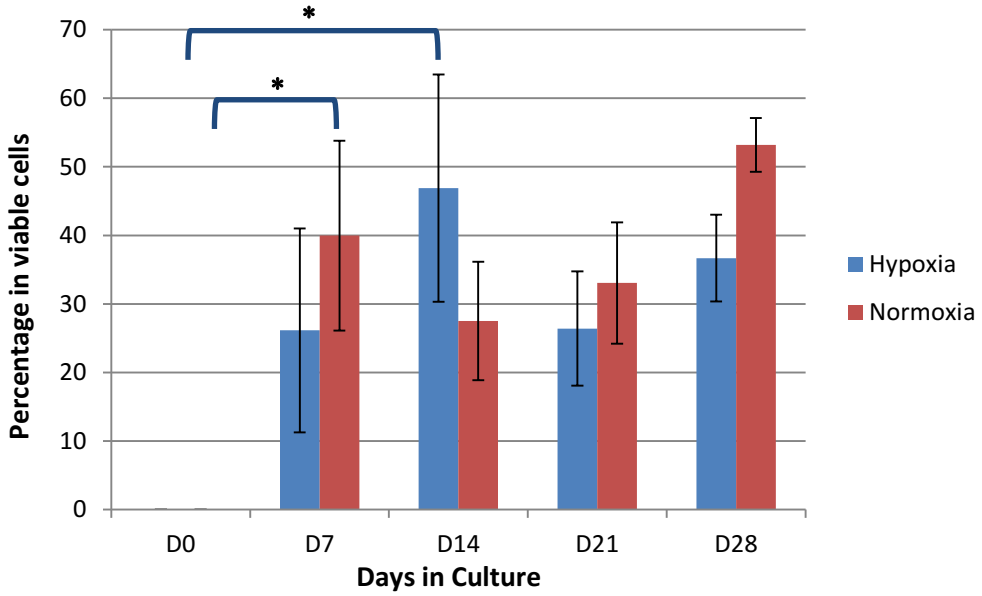


Figure 50. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 and CD235a to Detect $CD71^{+}$ and $CD235a^{-}$ (Appendix 9). Negative controls were unspecific isotype antibodies conjugated with the same dyes. Normoxia (P=<0.001). Hypoxia (P=0.002) (n≥3).

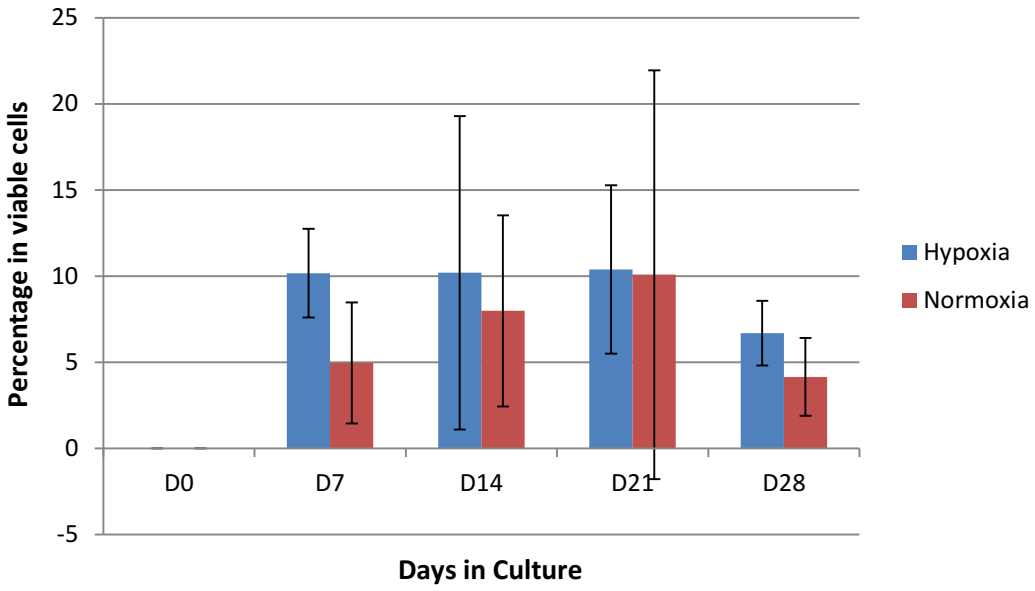


Figure 51. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD71 and CD235a to Detect $CD71^-$ and $CD235a^+$ (Appendix 9). Negative controls were unspecific isotype antibodies conjugated with the same dyes (n≥3).

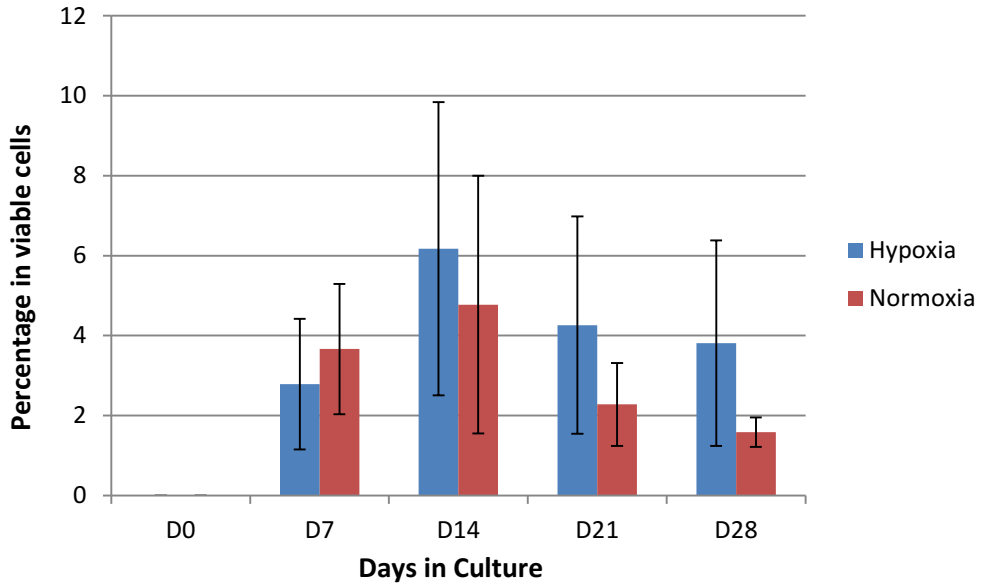


Figure 52. The Flow Cytometry Analysis Profile of Differentiated Human iPS Cells Stained with Conjugated Specific Antibodies for CD43 (Appendix 10)**.** Negative controls were unspecific isotype antibodies conjugated with the same dyes (n≥3).

Both conditions showed a significant drop in SSEA4 early in the differentiation culture, indicating loss of pluripotency (Figure 39). CD34, the HSCs marker, was observed at day 7 in both conditions, peaked at day 14 (50% in normoxia and 41% in hypoxia), and decreased gradually toward the end of the differentiation (Figure 41). Normoxia condition showed higher percentage of $CD34^+$ cells (HSCs) at days 7, 14 and 21, and a sharper drop by day28 in comparison to hypoxia (Figure 40-41) (summarized in Table 11). However, the difference was not significant in comparison to hypoxic condition. CD45, also started to express significantly by day 7, peaked at day 14 (58% in normoxia and 76% in hypoxia), and dropped significantly at day28, in both conditions (Figure 42) (summarized in Table 11). However, hypoxia showed higher numbers of $CD45^+$ cells at all time points. Representative dot plots flow cytometry analysis of the differentiated cells stained for CD34 and CD45 are shown in Figure 40. $CD34^+CD45^-$ phenotype is usually associated with HSCs and hematopoietic progenitor cells. This phenotype was found at low percentages along the culture period in both conditions, suggesting that cells might differentiate to a further mature cellular phenotype by the first analysed time point (Figure 43). On the other hand, CD34+CD45+ phenotype is usually associated with megakaryocyte–erythrocyte progenitors and proerythroblasts, and this phenotype peaked at day 14 in both conditions but decreased at the following time points, thus indicating further erythroid maturations (Figure 44). BFU-E and CFU-E cells are erythroid progenitors characterized by $CD45^+CD235a^-CD34^+CD36^-$IL-$3R^-CD71^{low}$ and $CD45^+CD235a^-CD34^-CD36^+$IL-$3R^-CD71^{high}$ phenotypes, respectively (Li et al., 2014). Thus, Figures 44 and 45 suggest that the culture might have had BFU-E and CFU-E progenitors that differentiated further into erythroblasts towards day28. Moreover, Figures 47 and 48 showed high percentage expressions of an early and a late erythroid markers, CD71 and CD235a, respectively (summarized in Table 11), and representative dot plots flow cytometry analysis of the differentiated cells stained for CD71 and CD235a are shown in Figure 46. The expression of CD71 reached 79% in normoxia and 66% in hypoxia in 14 days only, while the expression of CD235a reached 59% in normoxia and 56% in hypoxia at the same time point. Both markers were maintained high throughout the differentiation, which reflects the formation of erythroblasts (Attar, 2014). The coexpression of CD71 and CD235a in both conditions showed the typical profile expected

in erythropoiesis at proerythroblast, basophilic erythroblast, polychromatic erythroblast, and reticulocyte stages (Figure 49). $CD71^{+}CD235a^{+}$ coexpression decreased toward the end as CD71 expression is expected to decline at later erythropoiesis stages (Attar, 2014). Nevertheless, the desired phenotype of $CD71^{-}CD235a^{+}$ cells did not show a significant expression (Figure 51). CD43 was analysed, as some researchers claim that it has been detected during hematopoietic differentiation of human ES cells in OP9 co-culture prior to CD45 expression on early hematopoietic progenitors (Vodyanik et al., 2006). As demonstrated in Figure 52, negligible expressions of this marker was witnessed in both conditions.

7.4.3. Clonogenic Ability of Differentiated Human iPS Cells in CFU Assay

Cells suspension at different time points from both culture groups were plated on MethoCult medium in order to assess the ability of differentiated human iPS cells to produce haematopoietic/erythropoietic progenitor colonies.

Incubation to the standard 14 days resulted in the formation of distinct erythroid progenitor colonies, CFU-E and BFU-E (Figure 53), in addition to the haemaglobinization observed in hypoxia BFU-Es (pink/brownish colour) (Figure 55). Day 14 of both normoxia and hypoxia conditions are hypothesized to be the ideal time-frame for hematopoietic and erythroid differentiation, based on flow cytometry analysis, with the highest number of cells expressing hematopoietic and erythroid markers (Table 11). It seemed that a large proportion of day 14 cells were colony-forming progenitors based on the images of the resulted CFU assays. The colonies were abundantly observed and quantified, which yielded >200 colonies on day14. BFU-E colonies formed in normoxia were smaller, while BFU-E colonies of hypoxia condition were more uniform in shape (Figure 53). However, the colonies may not had developed optimally, not as large or as red as typically seen, perhaps due to overcrowding (1.5-3x 10^{3}cells/1ml), which resulted in limited nutrients and growth factors in the dish. It was also hypothesized that towards the end of the differentiation, cells will differentiate further and will start to mature and lose their clonogenic ability. Accordingly, it seemed that hypoxia condition supports maturation

earlier than normoxia, as day 28 cells in normoxia condition were still colony-forming progenitor cells able to produce colonies, while cells of hypoxia condition were differentiated and unable to form colonies.

The actual number of colonies formed in dishes was very high and difficult to quantify, due to the overcrowding. However, the crowdedness and high number of colony formation was observed even when the plating density was reduced to 1.5x 10^3cells/1ml. Given that a very large proportion of the plated cells were expressing CD34 and other hematopoietic markers, overcrowding in the CFU assay is not surprising. Nevertheless, there were no colonies observed when the plating density was reduced to 0.7x10^3cells/1ml (Figure 54).

The haemoglobinized BFU-E colonies were observed only at day 14 of hypoxia condition (Figure 55), which suggests that differentiating the cells under hypoxic condition might support haemoglobinization more than normoxia condition. However, it is worth noticing that colonies derived from more "primitive" BFU-E may not appear red, as it can take more than two weeks of culture for haemoglobinization to occur in some cord blood derived-BFU-E (Chadwick et al., 2003a).

Table 11. Percentages of Hematopoietic/Erythroid Markers on Plated Cells for CFU Assay. Day14 is highlighted as it shows the largest proportion of cells expressing hematopoietic and erythroid markers.

	Normoxia				**Hypoxia**			
	CD34	**CD45**	**CD71**	**CD235a**	**CD34**	**CD45**	**CD71**	**CD235a**
Day7	27.57%	56.17%	62.6%	29.37%	21.23%	46.33%	56.25%	40.9%
Day14	50.6%	58.25%	78.73%	58.9%	41.25%	75.75%	66.13%	56.45%
Day21	34.6%	37.6%	51.75%	33.55%	22.07%	49.93%	51.18%	40.57%
Day28	8.88%	4.19%	67.13%	18.13%	17.13%	14.23%	61.55%	30.52%

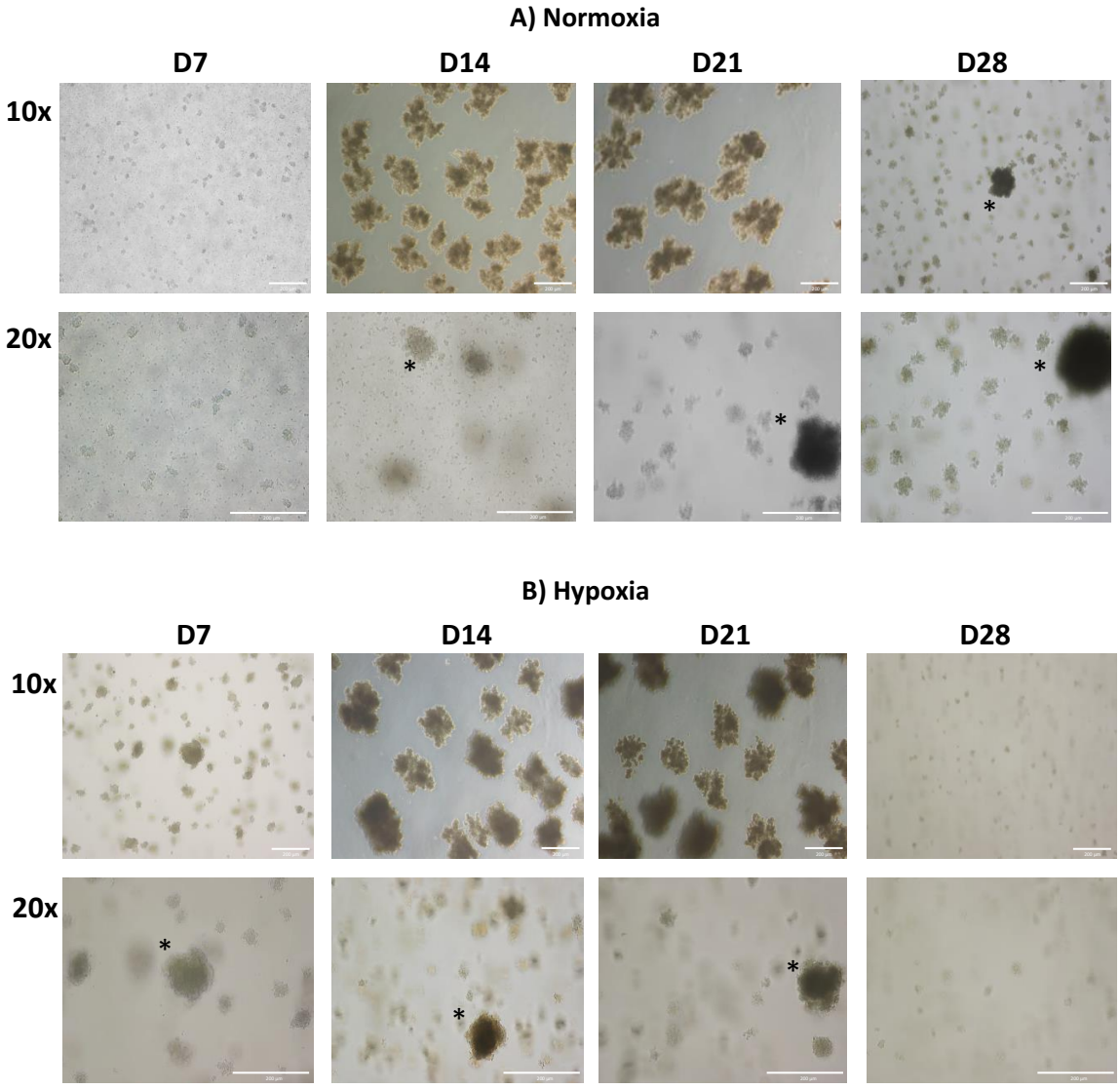


Figure 53. Clonogenic Capacity of Differentiated Human iPS Cells. A) Normoxia; B) Hypoxia. The first column are day7 images, the second column are day14 images, the third column are day21 images, and the forth column are day28 images. Both culture groups showed the formation of erythroid progenitors, identifiable by the formation of BFU-E, indicated by asterisk (*), and CFU-E colonies that were most prominently detected (Scales for each image: 200µm). (n=3).

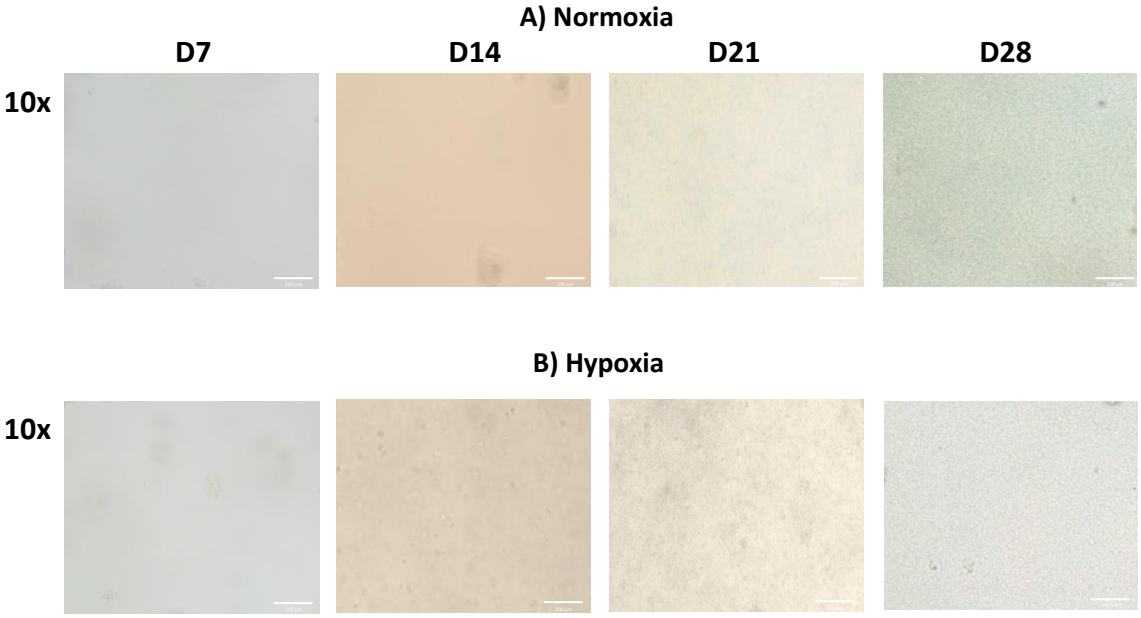


Figure 54. Clonogenic Capacity of Differentiated Human iPS Cells at Low Plating Density. A) Normoxia; B) Hypoxia. The first column are day7 images, the second column are day14 images, the third column are day21 images, and the forth column are day28 images. No colonies were formed in both culture groups at any time point (Scales for each image: 200µm). (n=3).

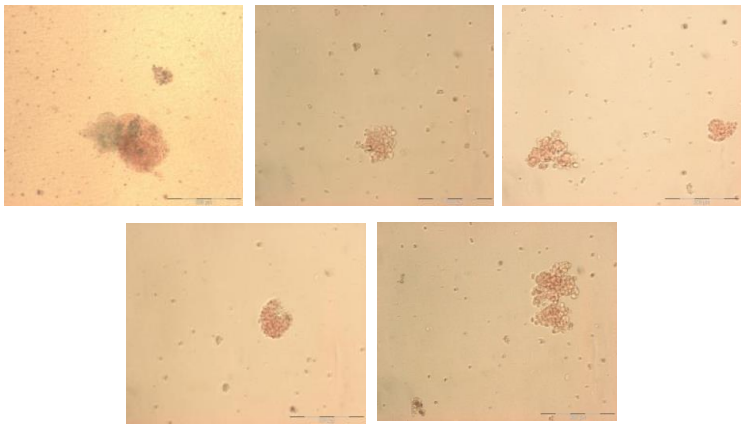


Figure 55. Matured Reddish/Brownish BFU-E Colonies Detected at Day 14 of Hypoxia Condition. (Scales for each image: 200µm)**.**

7.4.4. Immunocytochemical Staining of Differentiated Human iPS Cells

Immunocytochemistry was performed in both normoxia and hypoxia groups at days 7, 14, 21, and 28 for further confirmation of erythropoiesis in our differentiation culture. The expressions of CD34, CD45, CD71, and CD235a were assessed in Figures 56-60.

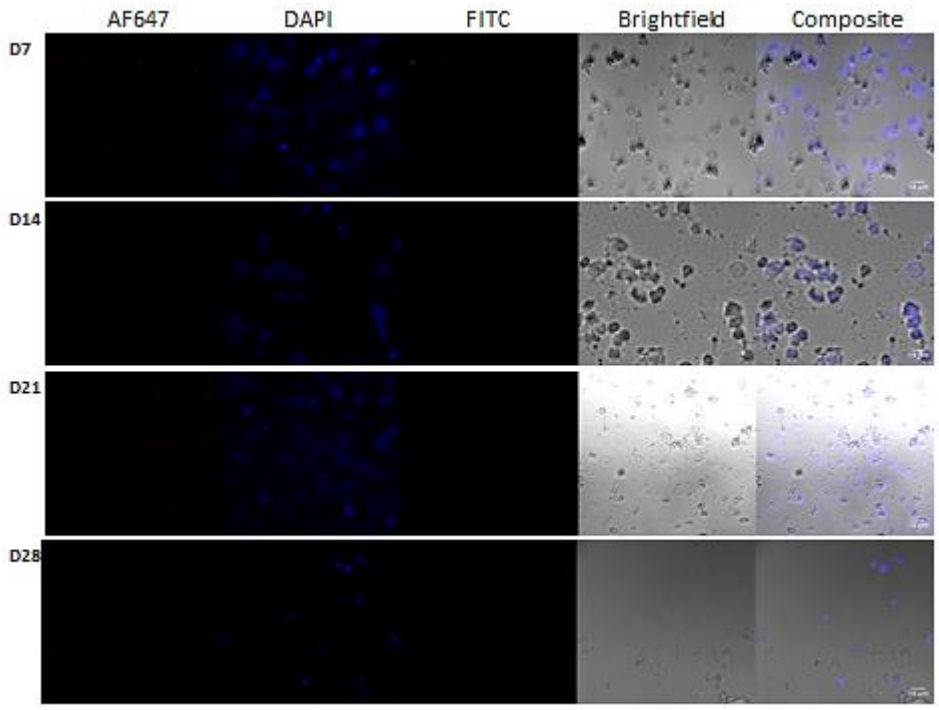


Figure 56. Negative Controls for Immunocytochemistry Staining of Differentiated Human iPS Cells. As labelled on the figure, the first column are images taken under the red channel, the second column are images taken under the blue channel, the third column are images taken under the green channel, the forth column are images taken under the bright field, and the fifth column are the composite images. The first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. Negative controls of the secondary antibodies showed no unspecific signals. The nuclear staining with DAPI is shown with blue staining. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 14µm).

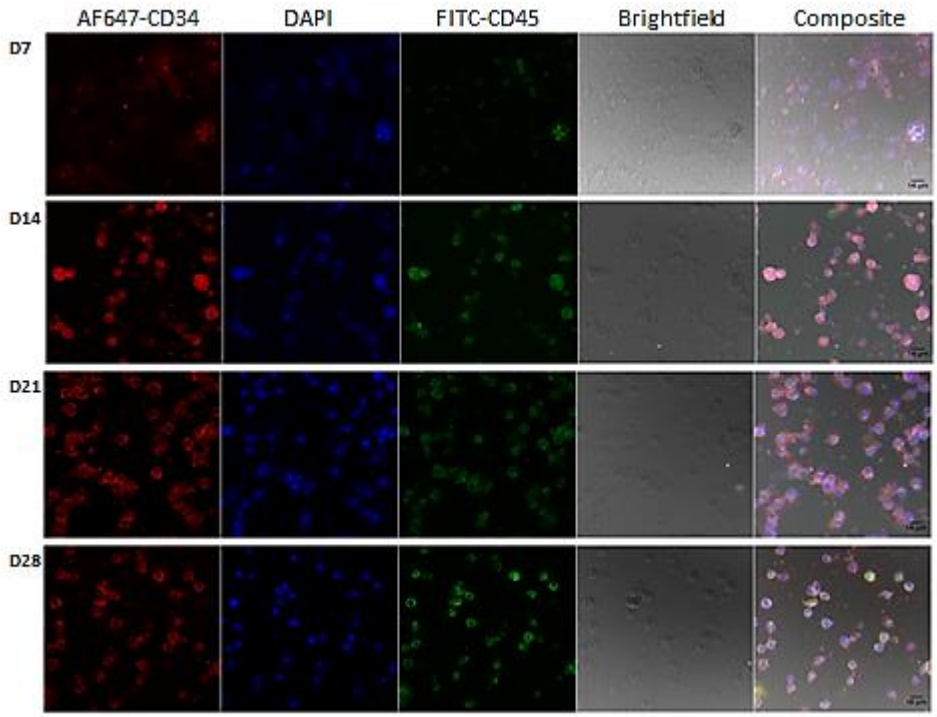


Figure 57. Immunocytochemistry Staining for CD34 and CD45 of Differentiated Human iPS Cells under Normoxia Conditions. As labelled on the figure, the first column are images taken under the red channel, the second column are images taken under the blue channel, the third column are images taken under the green channel, the forth column are images taken under the bright field, and the fifth column are the composite images. The first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. Positive expressions of haematopoietic markers: anti-CD45 (FITC-green) and anti-CD34 (Alexa Fluor® 647-red) were both observed by day 7 of culture and persisted throughout the differentiation. The nuclear staining with DAPI is shown with blue staining. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 14µm).

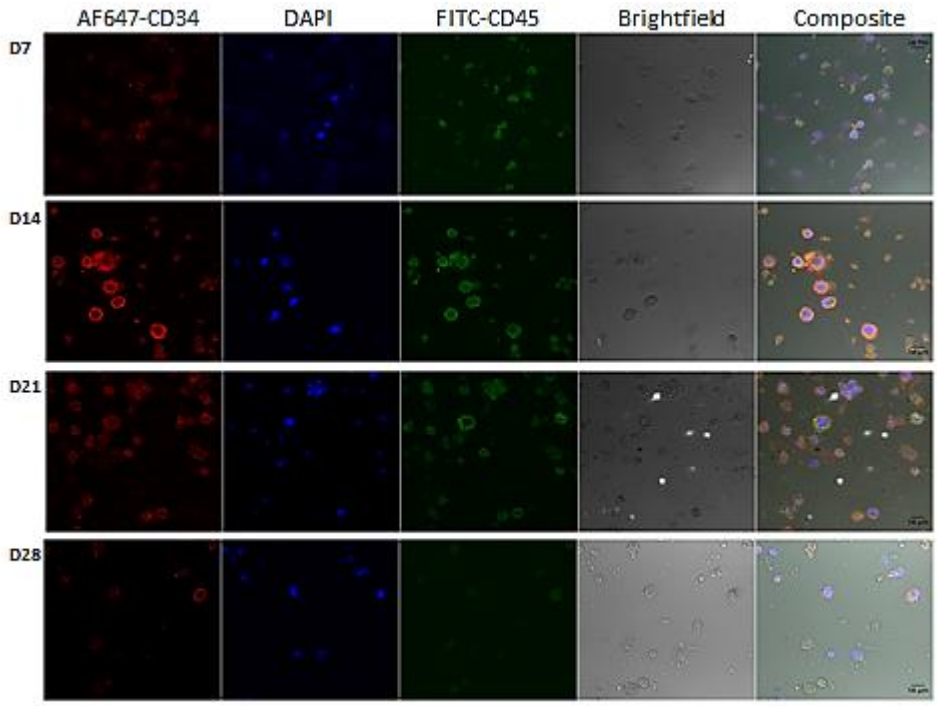


Figure 58. Immunocytochemistry Staining for CD34 and CD45 of Differentiated Human iPS Cells under Hypoxia Conditions. As labelled on the figure, the first column are images taken under the red channel, the second column are images taken under the blue channel, the third column are images taken under the green channel, the forth column are images taken under the bright field, and the fifth column are the composite images. The first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. Positive expressions of haematopoietic markers: anti-CD45 (FITC-green) and anti-CD34 (Alexa Fluor® 647-red) were observed by day 7. CD34 was strong at day 14 while CD45 gave the strongest signal by day21.The nuclear staining with DAPI is shown with blue staining. Both markers gave weak signals by day 28 of the differentiation culture. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 14µm).

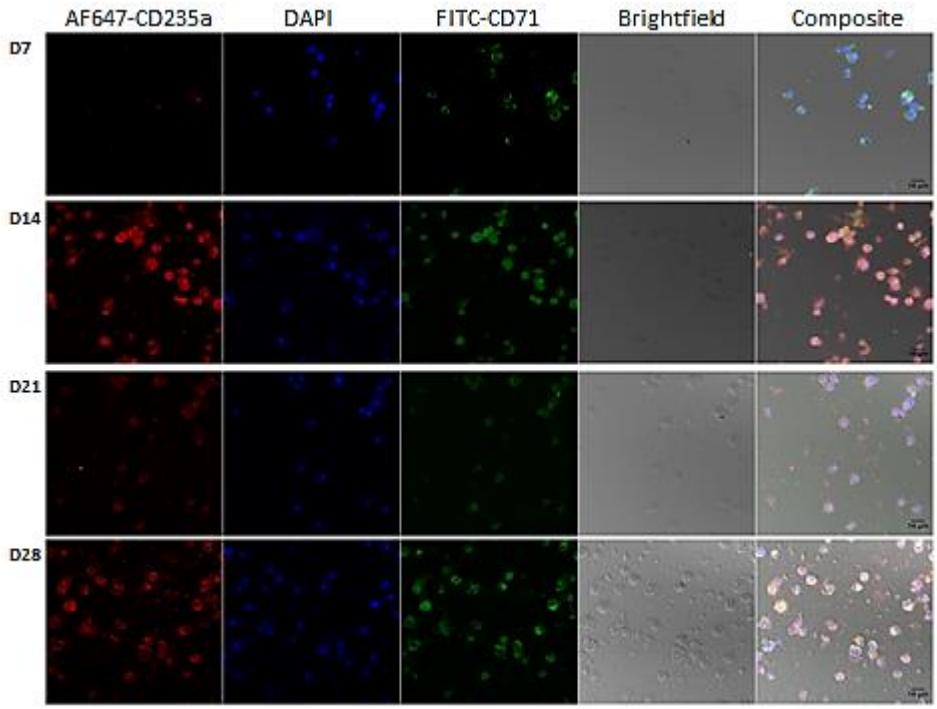


Figure 59. Immunocytochemistry Staining for CD71 and CD235a of Differentiated Human iPS Cells under Normoxia Conditions. As labelled on the figure, the first column are images taken under the red channel, the second column are images taken under the blue channel, the third column are images taken under the green channel, the forth column are images taken under the bright field, and the fifth column are the composite images**.** The first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. There are positive expressions of erythropoietic markers: anti-CD71 (FITC-green) and anti-CD235a (Alexa Fluor® 647-red). CD71 started to give a weak staining as early as day 7 of culture, which gradually amplified throughout the differentiation. CD235a was observed as strong at day 14 and was maintained during differentiation. The nuclear staining with DAPI is shown with blue staining. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 14µm).

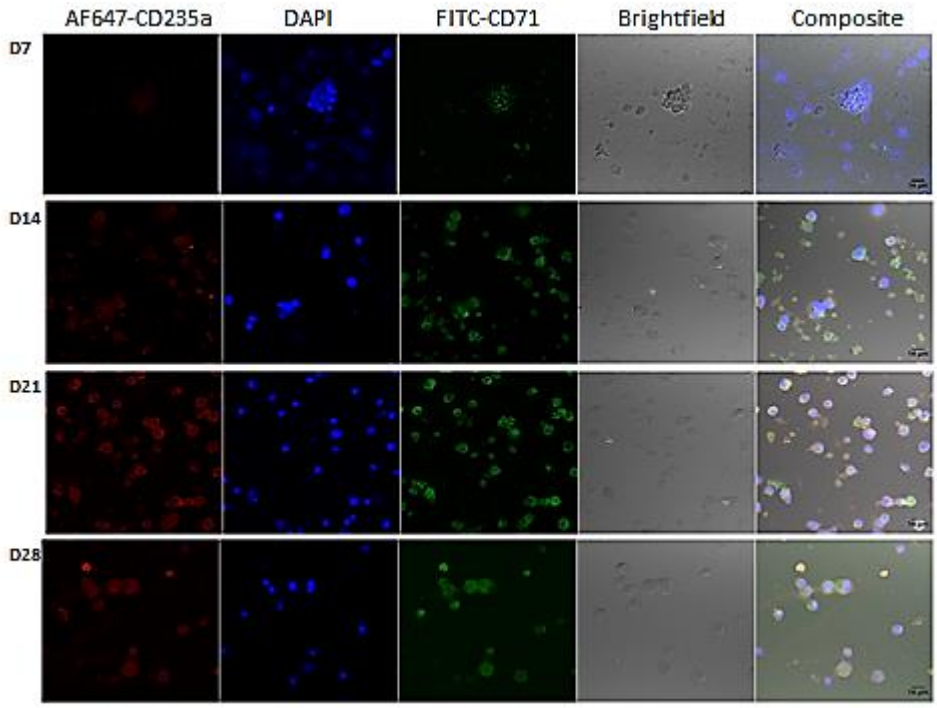


Figure 60. Immunocytochemistry Staining for CD71 and CD235a of Differentiated Human iPS Cells under Hypoxia Conditions. As labelled on the figure, the first column are images taken under the red channel, the second column are images taken under the blue channel, the third column are images taken under the green channel, the forth column are images taken under the bright field, and the fifth column are the composite images. The first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. Positive expressions of erythropoietic markers: anti-CD71 (FITC-green) and anti-CD235a (Alexa Fluor® 647-red) were observed by day 14 and maintained strong throughout the culture. The nuclear staining with DAPI is shown with blue staining. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 14µm).

The intensity of the fluorescent signals given by the different markers was compared between the two conditions; normoxia and hypoxia (Figures 61-64). Five images were selected randomly for each time point of both conditions, analysed, and plotted in the following graphs.

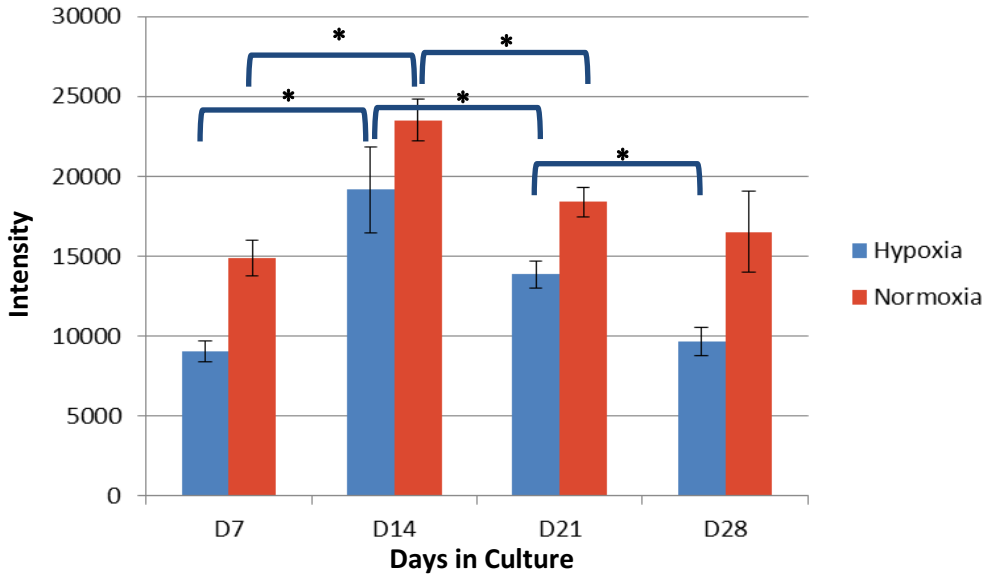


Figure 61. Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD34. Signals of CD34 increased significantly by day14 of both conditions and decreased significantly by day21 in normoxia and day28 in hypoxia. Normoxia (P=<0.001). Hypoxia (P=<0.001) (n≥3).

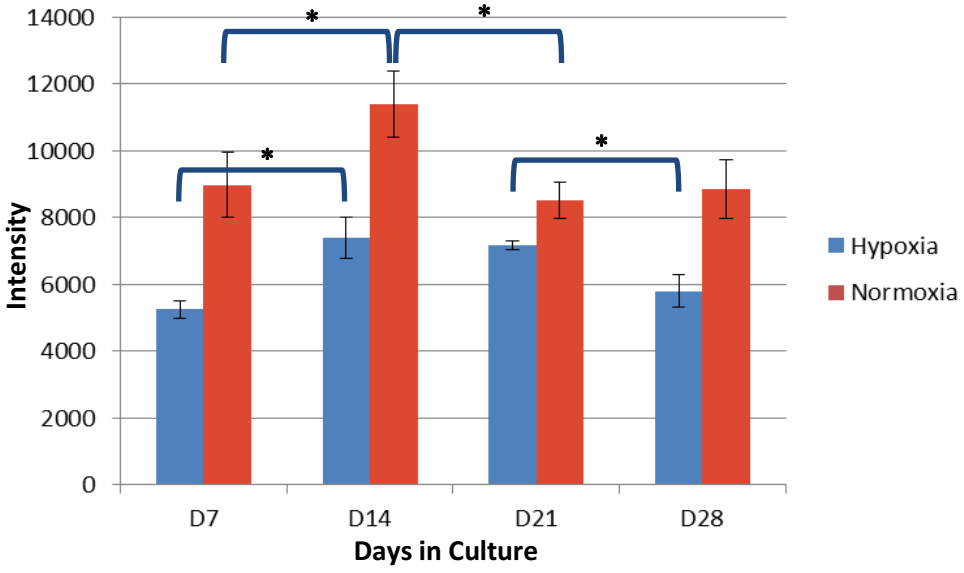


Figure 62. Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD45. Signals of CD45 increased significantly by day14 of both conditions, and decreased significantly by day21 in normoxia and day28 in hypoxia. Normoxia (P = <0.001). Hypoxia (P = <0.001) (n≥3).

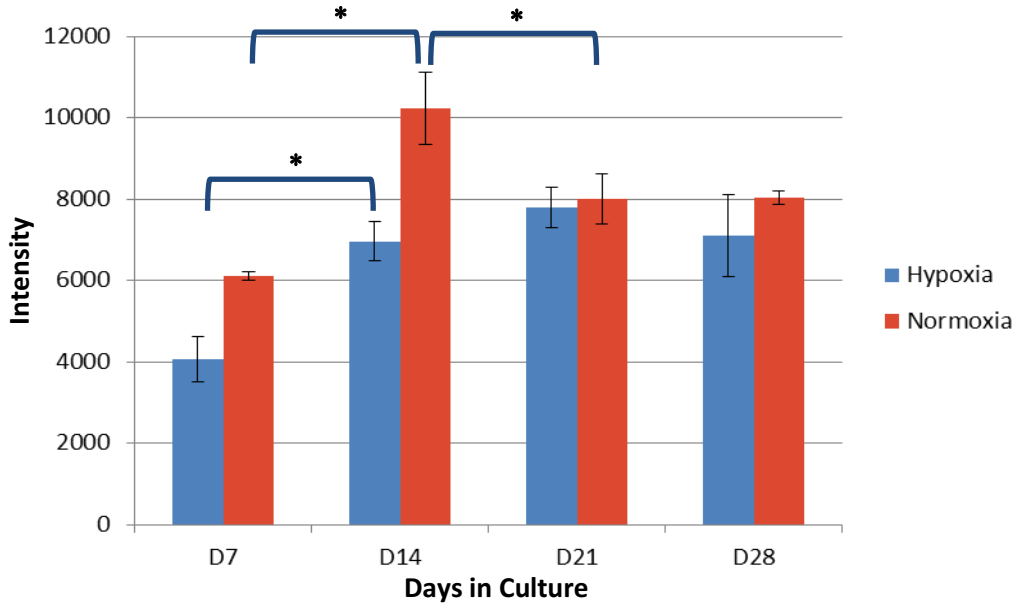


Figure 63. Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD71. Signals of CD71 increased significantly by day14 of both conditions, and decreased significantly by day21 in normoxia. Normoxia (P = <0.001). Hypoxia (P = <0.001) (n≥3).

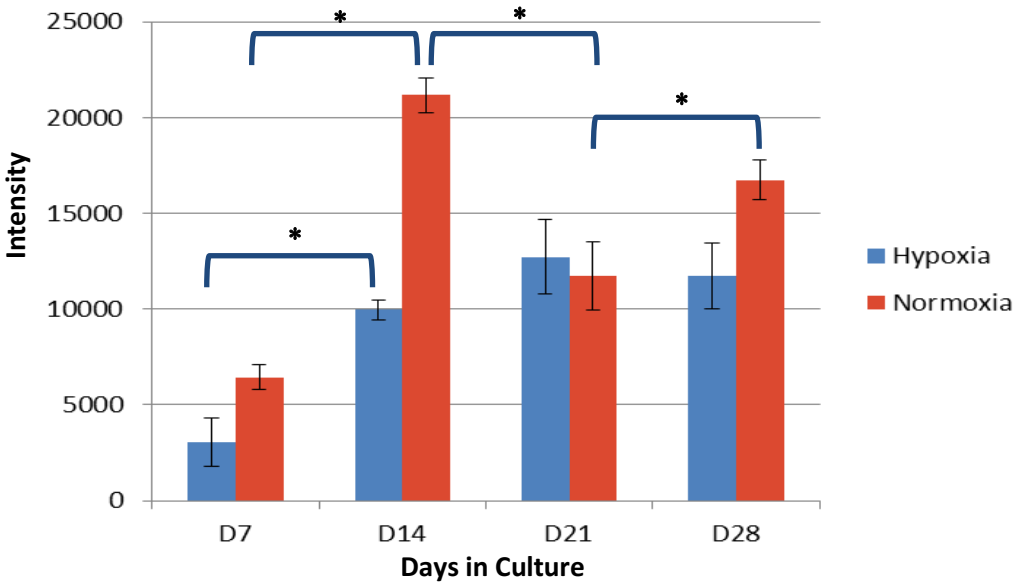


Figure 64. Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for CD235a. Signals of CD235a increased significantly by day14 of both conditions. The signals decreased significantly by day21 in normoxia and increased again by day 28. In hypoxia, the signal remained high along the culture. Normoxia (P = <0.001). Hypoxia (P = <0.001) (n≥3).

Figures 57-60 were compared to Figure 56, the negative control, to determine the positivity and strength of fluorescent signals. In normoxia, CD34, CD45, and CD71 signals were high at day7, elevated significantly at day14, then decreased substantially by days 21 and 28, with CD34 giving the strongest signal and CD71 being the weakest. CD235a signals showed a similar trend of tremendous escalation at day 14, followed by a significant drop by day21, followed by a significant increase by day28. In hypoxia, the trend of the cellular markers was comparable to their typical profile during erythropoiesis (Figures 61-64). CD34 was high at day7, significantly higher at day14, and then gradually reduced by days 21 and 28. CD45 showed less intensity at days 7 and 14, but maintained a similar level at day 21 before the signal dropped at day 28 towards the formation of erythroid committed precursors. On the other hand, erythroid markers, CD71 and CD235a, peaked later, at day 21, and maintained high, which reflects the beginning of erythroid maturation. It is worth mentioning that fluorescent signals of all analysed cellular markers was higher in cells cultured under normoxia condition in comparison to hypoxia condition.

Furthermore, it was interesting to evaluate the intensity and location of each fluorescence signal at a single cell level (Figures 65-68). Five cells from images of each time point in both conditions were selected randomly, analysed, and one representative image is shown here. These plots also indicate the size of the analysed cells.

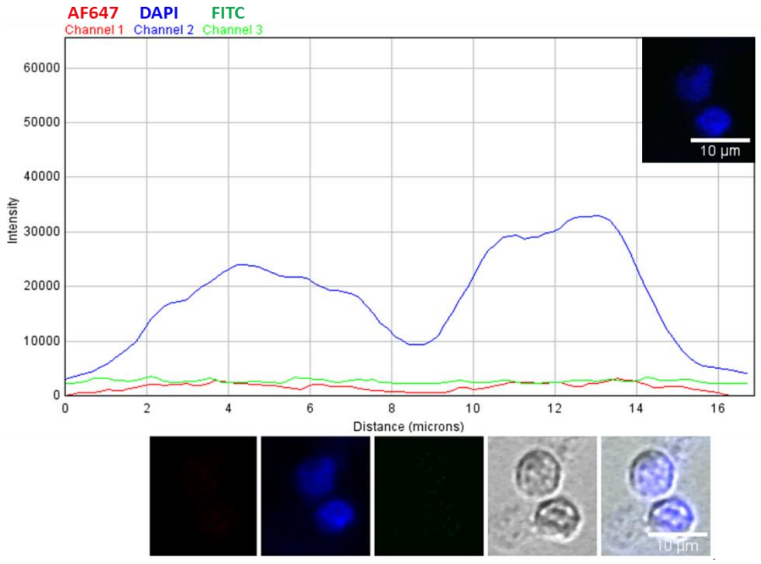


Figure 65. Negative Control for Single Cell Analysis of Immunocytochemistry Signals of Differentiated Human iPS Cells in Normoxia Condition. Negative control showing no unspecific binding of the secondary antibodies. The nuclear staining with DAPI is shown with blue staining. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 10µm).

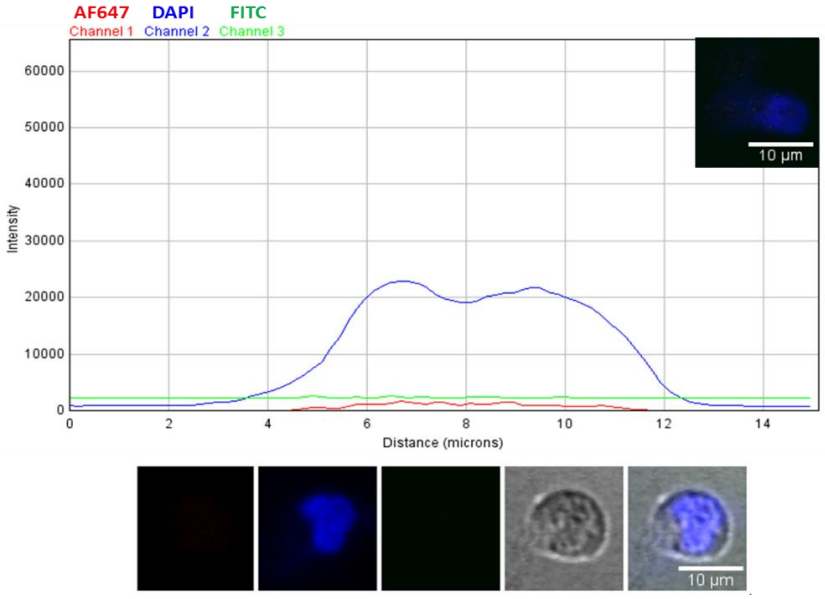


Figure 66. Negative Control for Single Cell Analysis of Immunocytochemistry Signals of Differentiated Human iPS Cells in Hypoxia Condition. Negative control showing no unspecific binding of the secondary antibodies. The nuclear staining with DAPI is shown with blue staining. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 10µm).

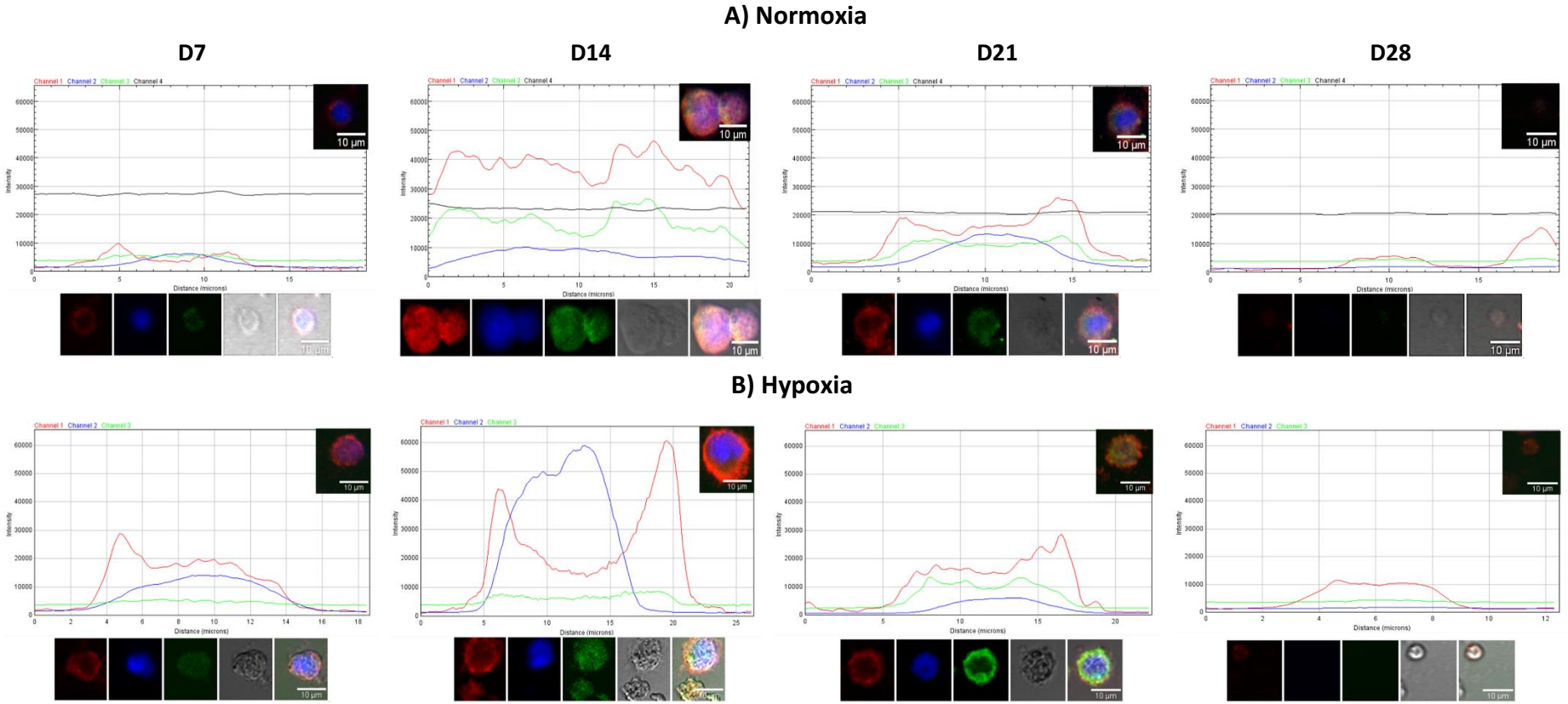


Figure 67. Single Cell Analysis of Immunocytochemistry Signals of CD34 and CD45 of Differentiated Human iPS Cells in Normoxia and Hypoxia Conditions at Different Time Points. As labelled on the figure, the first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. Images shown are taken under the red channel (CD34- Alexa Fluor® 647), the blue channel (DAPI), the green channel (CD45-FITC), the bright field, and the composite images. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 10µm).

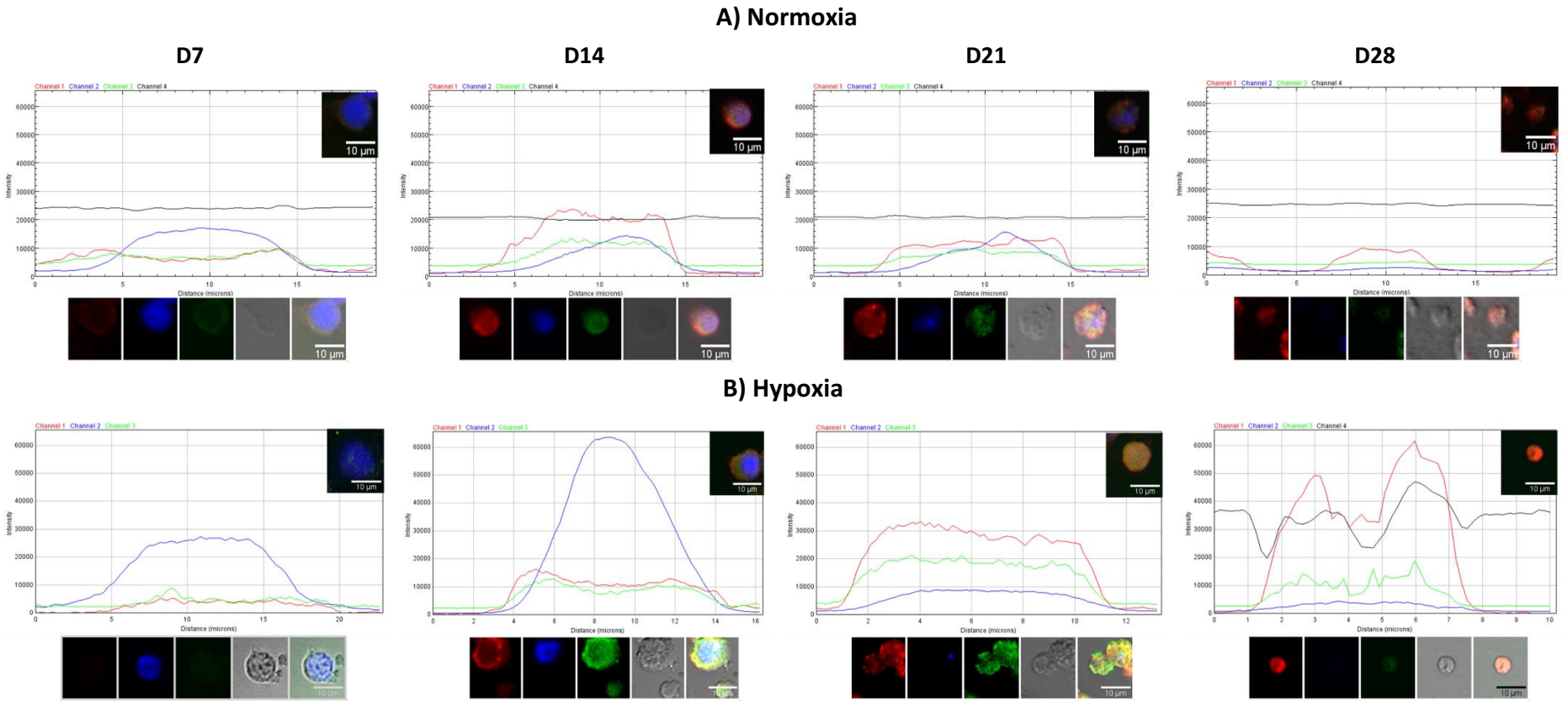


Figure 68. Single Cell Analysis of Immunocytochemistry Signals of CD71 and CD235a of Differentiated Human iPS Cells in Normoxia and Hypoxia Conditions at Different Time Points. As labelled on the figure, the first row are day7 images, the second row are day14 images, the third row are day 21 images, and the forth row are day 28 images. Images shown are taken under the red channel (CD235a- Alexa Fluor® 647), the blue channel (DAPI), the green channel (CD71-FITC), the bright field, and the composite images. Images were captured at 20x magnification under confocal microscopy. (Scales for each image: 10µm).

The single cell analysis of immunocytochemistry signals of CD34 and CD45 of differentiated human iPS cells (Figure 67) at day 7 in normoxia condition showed low expression signals of CD45 and CD34 at the cell surface, with a cell size of ~12µm. Whereas in hypoxia condition, it showed a high expression signal of CD34, a low expression signal of CD45, with a cell size of ~14µm. Analysis of day 14 showed high expression signals of CD34 in both conditions, while signals of CD45 were high only in normoxia. Images of normoxia condition at day 21 showed lower expressions of CD45 and CD34 in comparison to day14. While images of hypoxia condition at day21 showed a higher expression of CD45 together with a lower expression of CD34 in comparison to days 7 and 14. Moreover, a low nuclear staining was observed with DAPI staining. Images of day28 of both conditions presented very low expressions of CD45 and CD34, in addition to size reduction (~7µm). Likewise, no nuclear staining was observed at this time point in both conditions.

On the other hand, the single cell analysis of immunocytochemistry signals of CD71 and CD235a differentiated human iPS cells (Figure 68) at day 7 showed low expressions of CD71 and CD235a in both conditions. In comparison, analysis of day 14 showed higher expressions of both markers, which persisted in normoxia condition. While very high expressions of CD71 and CD235a were observed at that time point in hypoxia condition, together with a low nuclear staining. Images of day 28 exhibited very low expressions of CD71 and high expressions of CD235a in both conditions, in addition to cell size reduction (~7µm). Furthermore, no nuclear staining was observed which might indicate enucleation. Additionally, day 28 images showed cells with the typical biconcave discoid shape of mature erythrocyte.

Thus, figures 67-68 of the single cell level analysis confirmed the size reduction feature, which is one of the main maturation signs during erythropoiesis, from large nucleated erythroblasts (10-15µm), to reticulocytes (7-10µm), and, finally, mature enucleated erythrocytes (6-8µm) (Blann and Holtom, 2010). Moreover, DAPI stain, which stains the nucleus, was dramatically decreased at day21 and day28, confirming losing of nucleus, another main feature of mature erythrocytes. Additionally, day 28 showed cells with the typical biconcave discoid shape of mature erythrocyte. In both conditions, CD34 and

CD45 signals showed highest expression at day 14 then started to decrease. CD71 signals, however, gradually elevated until day 21 and reduced by day 28, as the mature erythrocytes are $CD71^-$. CD235a also slowly increased until the maximum expression was reached at day 28 of both conditions.

7.4.5. Gene Expression and qPCR of Differentiated Human iPS Cells

Erythropoietic differentiation was also characterized by distinct cellular phenotype of specific pluripotent-, haematopoietic-, and erythroid-transcription factor gene expression profiles. Samples from each culture group were tested at days 7, 14, 21, and 28, and compared with day 0 undifferentiated human iPS cells as the control sample.

TaqMan Universal Master Mix II was used, based on manufacturer's guidelines, to observe the relative expression of genes related to different haemoglobin types. Quantitation-Comparative gene expression analysis was calculated using the routinely used $2^{-\Delta\Delta CT}$ method. The expression of each gene of interest was normalized to day0 undifferentiated human iPS cells and to the housekeeping gene GAPDH.

Oct4 and Nanog are important regulators of the plasticity of pluripotent stem cells (Loh et al., 2006). Oct4, encoded by Pou5f1, is a POU domain–containing transcription factor found in the ES cells (Pan et al., 2002). Nanog, a homeodomain–containing protein, is a pluripotency factor required at a later stage than Oct4 during development (Loh et al., 2006).

C-kit (SCF receptor): is a proto-oncogene, a member of the family of growth factor receptors that possesses an intrinsic tyrosine kinase activity (Ashman et al., 1991). C-kit is found on erythroid progenitors, as ~70% of CD34-positive BM cells coexpress the c-kit protein. Erythroid progenitors can be stimulated when SCF binds to c-kit on cell surface (Broudy et al., 1992).

HIF-1α: is an O_2-dependent transcriptional activator that targets genes related to angiogenesis, cell proliferation, survival, and glucose and iron metabolism. HIF-1α subunit is degraded, triggering ubiquitin-proteasome pathways under normoxia conditions. While in hypoxia conditions, it becomes stable and stimulates its transcriptional activity to regulate many hypoxia-inducible genes (Lee et al., 2004).

EPOR: is a member of the cytokine receptor superfamily that is highly expressed on erythroid progenitor cells at the CFU-E and proerythroblast level, while drops upon differentiation and maturation until it disappears at reticulocytes (Winter et al., 1996). When EPOR binds to EPO, it can activate phosphorylates tyrosine residues in the cytoplasmic, which lead to activation of several signalling pathways, including PI3-K, mitogen-activated protein kinase, and the signal transducer and activator of transcription 5 (Stat5), the main regulator of cellular proliferation, differentiation, and apoptosis, which translocates to the nucleus and triggers transcription (Grebien et al., 2008).

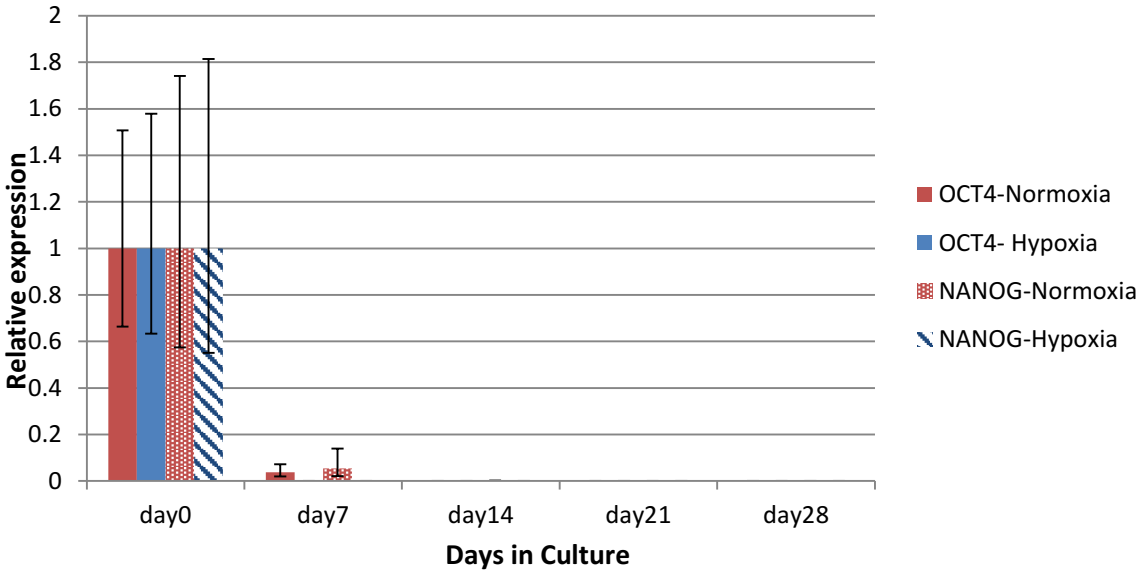


Figure 69. Relative Gene Expression Analysis of Pluripotency Markers (OCT4-NANOG) in Differentiated Human iPS Cells.

The Oct4 and Nanog gene expressions of cells cultured in both conditions, normoxia and hypoxia, were decreased by day7, indicationg that cells lost their pluripotency by that time point and were ready to differentiate. (n≥2 biological samples).

The PCR results were preliminary data only due to small sample size (n≥2). However, it shows some promising results at the gene level supporting the results obtained from the other analysis.

The expressions of two pluripotency genes, OCT4 and NANOG, in cells of both conditions, normoxia and hypoxia, appeared to decrease by day7, suggesting that cells might lost their pluripotency by that time point and were ready to differentiate (Figure 69).

The PCR results of CD34, CD45, CD71, CD235a, c-kit, EPOR, CD43, and HIF1α (Appendices 11-15) showed unexpected trends and thus no conclusions can be drawn. Many reasons might lead to the unexpected results including, technical errors while performing the test, low RNA extract as the cells started to lose their nuclei towards day 28, and the heterogenecity within the analysed population. Moreover, those results are not reliable because of the absence of technical replicates. Thus, other analysis were described instead, mainly the protein expression that reflects the gene transcription and translation.

7.4.6. Viability of Differentiated Human iPS Cells

Cell viability along the erythroid culture was evaluated at different time points in both culture groups. Cell viability was determined using DAPI, a blue fluorescent DNA stain that is used to identify dead cells (Tanious et al., 1992, Kapuscinski, 1995). However, since some of the differentiated erythroid cells are believed to have undergone enucleation, DAPI was not the most appropriate assay. Yet, it can still give an indication of the viability within our culture conditions. The cell viability is expressed as a percentage of cells treated with DAPI (Figure 70), where a good viability was maintained along the culture in both culture groups, with no significant difference between the two conditions. It is worth noting that these percentages were among the attached cells only while the culture had increasing numbers of floating dead cells towards day 28 in both conditions as observed after staining the supernatants with Erythrosin-B dye.

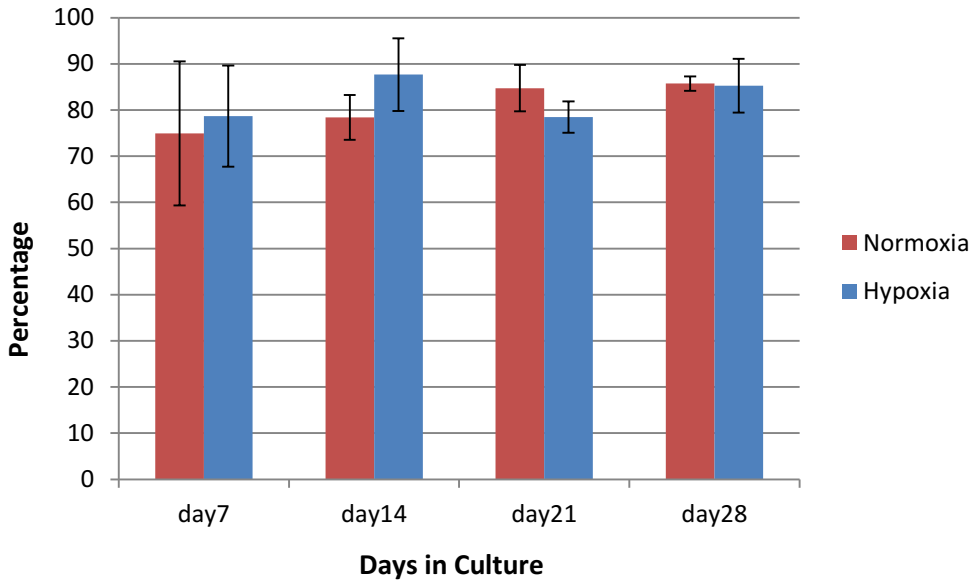


Figure 70. Percentage of Viable Cells within Normoxia and Hypoxia Culture groups Along the Erythroid Differentiation. (n>5) (Appendix 16-17).

7.4.7. Haemoglobin Analysis of Differentiated Human iPS Cells

All human haemoglobin consists of 4 globin chains, 2 α and 2 non-α, each containing a heme ring. Each type of haemoglobin has a different composition of the 4 globin chains. The main embryonic-type haemoglobin that exists during embryonic life is HbE Gower 1, which consists of $\zeta_2\varepsilon_2$; the fetal-type haemoglobin (HbF) is α2γ2, while the main adult type haemoglobin (HbA) is a tetramer of α2β2. Production of HbF remains during gestation and switches 6 months after birth into mostly HbA. The α chain production is controlled by genes on chromosome 16, while γ, β, and δ chains are controlled by a linked cluster of genes on chromosome 11 (Mavilio et al., 1983, Galanello, 2012).

7.4.7.1. Immunocytochemical Staining

Our differentiated cells were stained with goat polyclonal antibody to Haemoglobin (FITC) at different time points, days 7, 14, 21, and 28, which reacts with all human haemoglobin chains to evaluate the presence of haemoglobin.

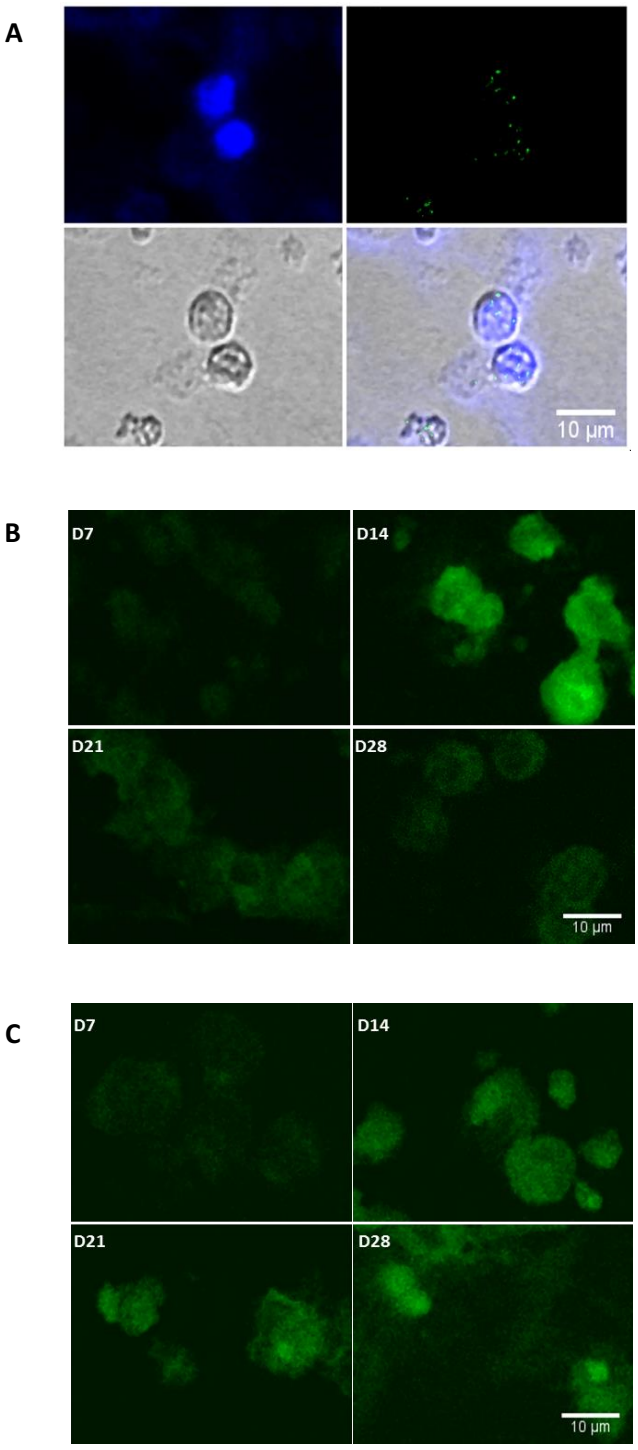


Figure 71. Immunocytochemistry Staining of Human Haemoglobin in Differentiated Human iPS Cells. (A) Negative control for unspecific binding of the antibodies using human iPS cells. The nuclear staining with DAPI is shown with blue staining. (B) Normoxia. (C) Hypoxia. As labelled on each figure, days 7 (top left), 14 (top right), 21 (lower left), and 28 (lower right). Images were captured at 40x magnification under confocal microscopy. (Scales for each image: 10µm).

As demonstrated in Figure 71, by comparing cells of the normoxia condition and hypoxia condition to the negative control, we can confirm the presence of haemoglobin. In normoxia condition, day14 showed the highest intensity, followed by an unexpected gradual decrease. Conversely, hypoxia condition showed the expected trend: low expression at day 7, followed by an escalation for the duration of the culture period, accompanied with a size reduction of cells of day28.

The overall intensity of the fluorescent signals given by the human haemoglobin staining was evaluated and compared between the two conditions, normoxia and hypoxia (Figure 72).

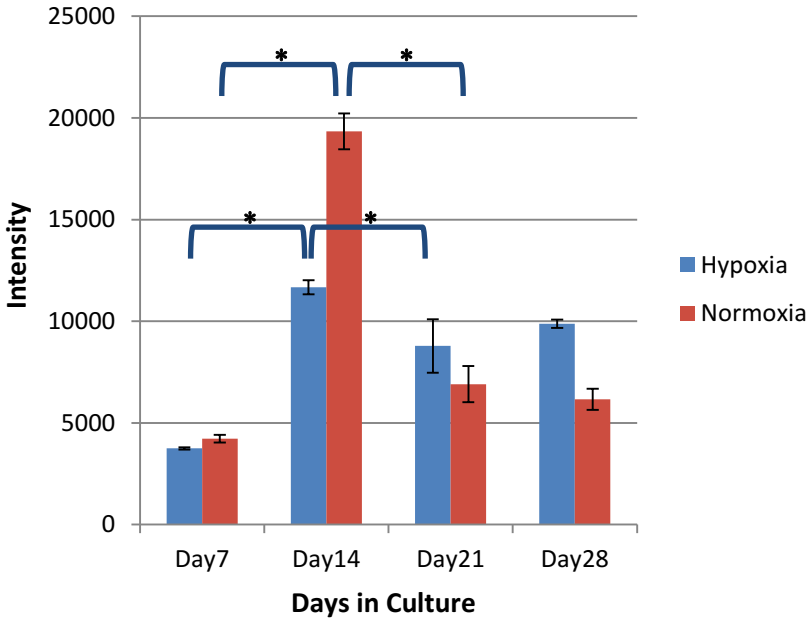


Figure 72. Intensity of Immunocytochemistry Staining of Differentiated Human iPS Cells for Human Haemoglobin. The intensity of haemoglobin signal increased significantly by day 14, and decreased significantly by day 21 of both conditions. Normoxia ($P = <0.001$). Hypoxia ($P = <0.001$) ($n \geq 3$).

Day14 of normoxia condition showed the peak intensity, followed by an unexpected reduction. Conversely, hypoxia condition showed the predictable development: low expression at day 7 followed by a significant rise for the duration of the culture period.

7.4.7.2. Gene Expression and qPCR

Following the confirmation of haemoglobin formation in our differentiated cells, the type of the haemoglobin was further analysed using qPCR. Samples from each culture group were analysed at days 7, 14, 21, and 28, and compared with day 0 undifferentiated human iPS cells as the control sample. TaqMan Universal Master Mix II was used, based on manufacturer's guidelines, to observe the relative expression of genes related to different haemoglobin types. Quantitation-Comparative gene expression analysis was calculated, using the routinely used $2^{-\Delta\Delta CT}$ method. The expression of each gene of interest was normalized to day0 undifferentiated human iPS cells and to the housekeeping gene GAPDH.

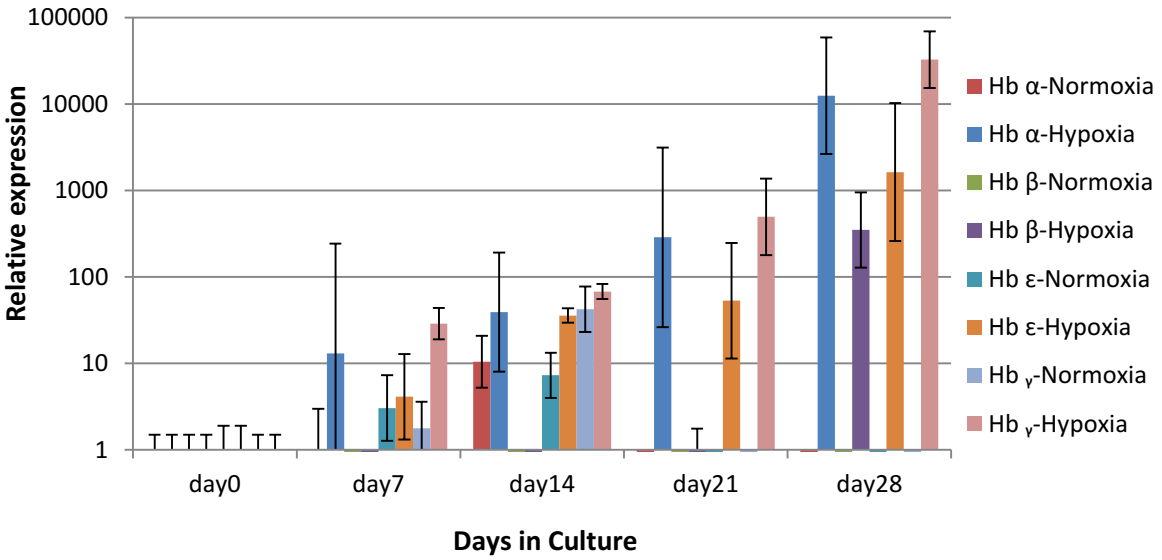


Figure 73. Relative Gene Expression Analysis of haemoglobin in Differentiated Human iPS Cells.

Hbα globin appeared at day 14 of normoxia condition, but disappeared at the rest of the time points during the differentiation. Conversely, the expression gradually increased in hypoxia condition, showing the desired trend. Hbβ globin was not detected in normoxia at all time point. However, it was detected at day28 of hypoxia. Hbε and Hbγ globins were detected at low levels in cells of day7 and day14 cultured in normoxia condition, while in hypoxia condition, the expression levels of the two globin types were increasing gradually throughout the differentiation period (n≥2 biological samples).

Figure 73 shows only preliminary PCR data due to small sample size (n≥2). However, it shows some encouraging results at the gene level, as it gives indications that at day 14, cells cultured under normoxia conditions contained trace amounts of embryonic- and fetal- type haemoglobin, but not adult-type, while cells at the time points thereafter did not show expression of any globin type. On the other hand, cells cultured under hypoxia condition showed an increase of haemoglobin, starting with only embryonic- and fetal-types, and showed some adult-type by day 28 of culture.

7.4.8. Metabolic Analysis of Erythroid Differentiation Culture Medium

In order to evaluate the growth kinetics of human iPS cells differentiated in our serum-free erythroid differentiation protocol, starting with erythroid induction media followed by the maturation and enucleation media indirectly, the consumption of nutrients in each media was determined by measuring time-course concentration of glucose, glutamine, lactate, glutamate, and ammonia, as well as changes of pH during the differentiation culture period. In this study, the medium was refreshed every day and the spent medium was used to measure the concentration of nutrients consumed by cells using a bio-profiler. Day 28 represents the last day of our erythroid differentiation protocol before harvesting the cells for the analysis. Statistical analysis was done for time points by one way ANOVA ($p > 0.001$).

7.4.8.1. Nutrient consumption rates (glucose and glutamine)

Glucose concentration was measured in consumed mediums at different time points during the erythroid differentiation culture, which showed a slight drop at day4. However, the culture maintained high levels of glucose throughout the culture days which means that the cultured cells did not consume all the glucose supplied in the medium (Figure 74). In terms of glutamine concentrations, glutamine levels decreased

along the erythroid differentiation culture, indicating that cells consumed some, but not all, of the glutamine supplied in medium (Figure 75).

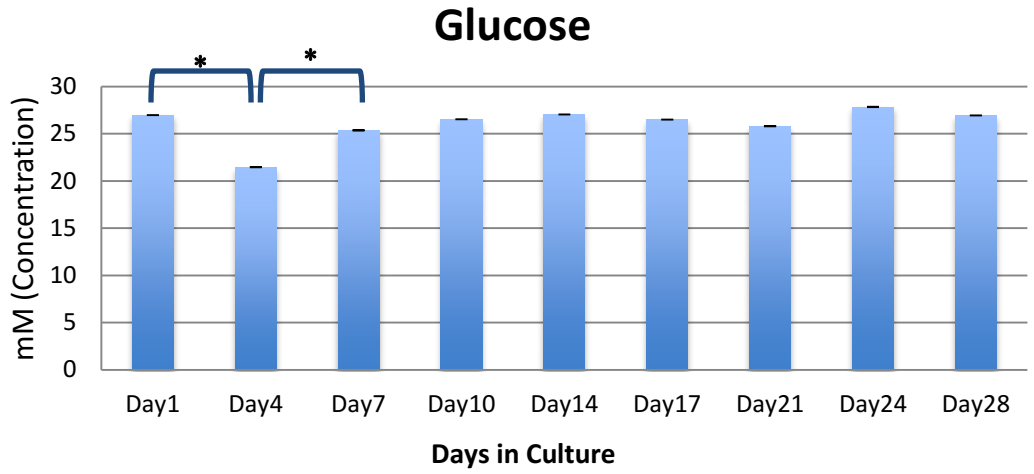


Figure 74. Glucose Consumption during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol. (n=3) (P = <0.001).

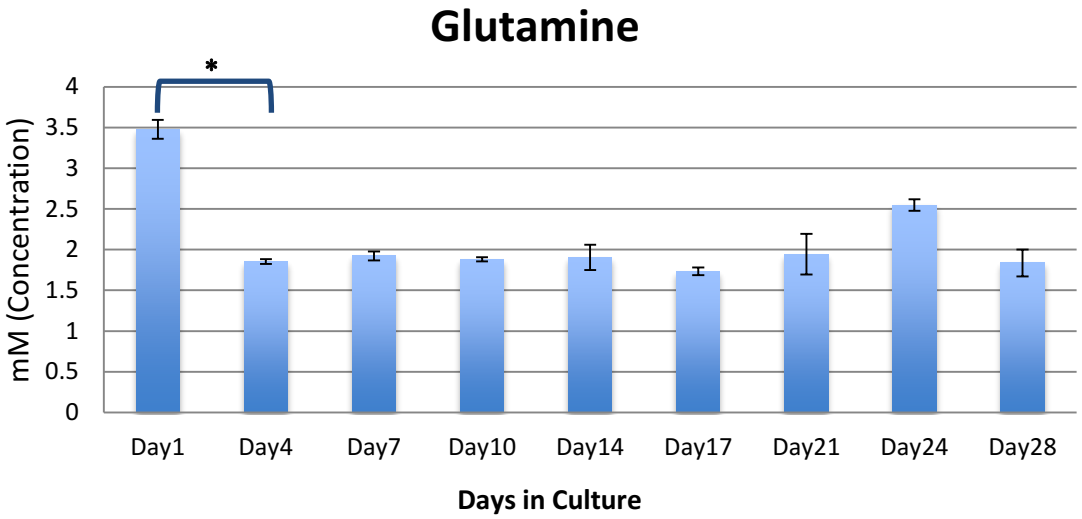


Figure 75. Glutamine Consumption during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol. (n=3) (P = <0.001).

7.4.8.2. Metabolite concentration profiles (lactate, glutamate, and ammonia)

In an opposing pattern to glucose and glutamine, lactate accumulation increased significantly from 0 mM to approximately ~3.4 mM and dropped gradually again to 0 level until the end of the experiment (Figure 76). However, the level was maintained below the toxic lactate level (>20mM). The increase in lactate level at days 4, 7, and 10 can be justified by high consumption rates of glucose at those time points.

Glutamate, produced by the glutaminolysis of glutamine, was maintained at low levels until day 10, where a significant increase was detected which persisted until the end of the experiment (Figure 77). This finding can be justified by the possibility that the cells started to differentiate into erythroid cells, where high concentrations of glutamate are usually found (Whillier et al., 2011). In terms of ammonia accumulation, a similar kinetic pattern with glutamate concentration was observed along the culture period, starting from 0 mM to approximately <0.7 mM, with a sudden increase at day10 of the culture period (Figure 78).

The increase pattern of glutamate and ammonia levels along the culture indicated that the two metabolites were released through metabolism of cells into medium, which reflects high consumption rates of glutamine profiles, as described above.

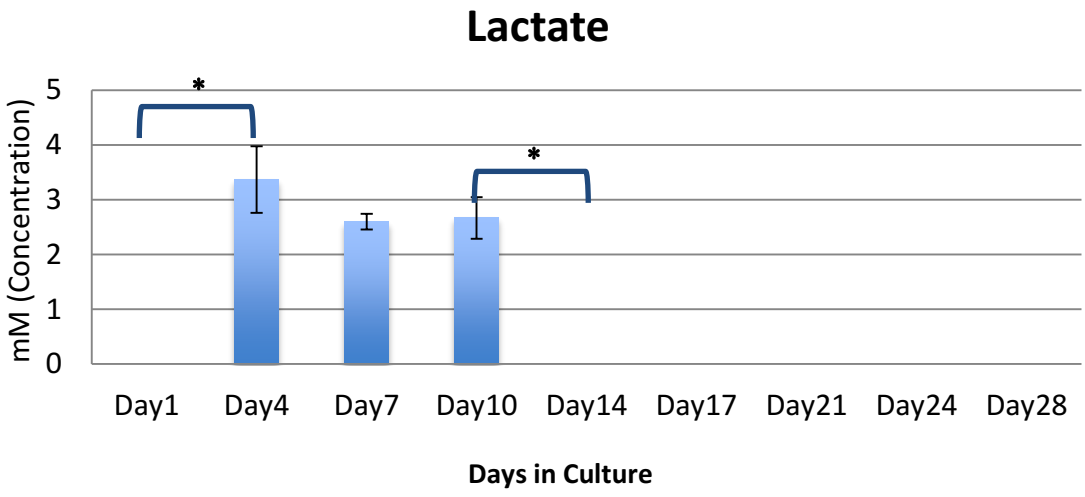


Figure 76. Accumulation of Lactate during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol. (n=3) (P = <0.001).

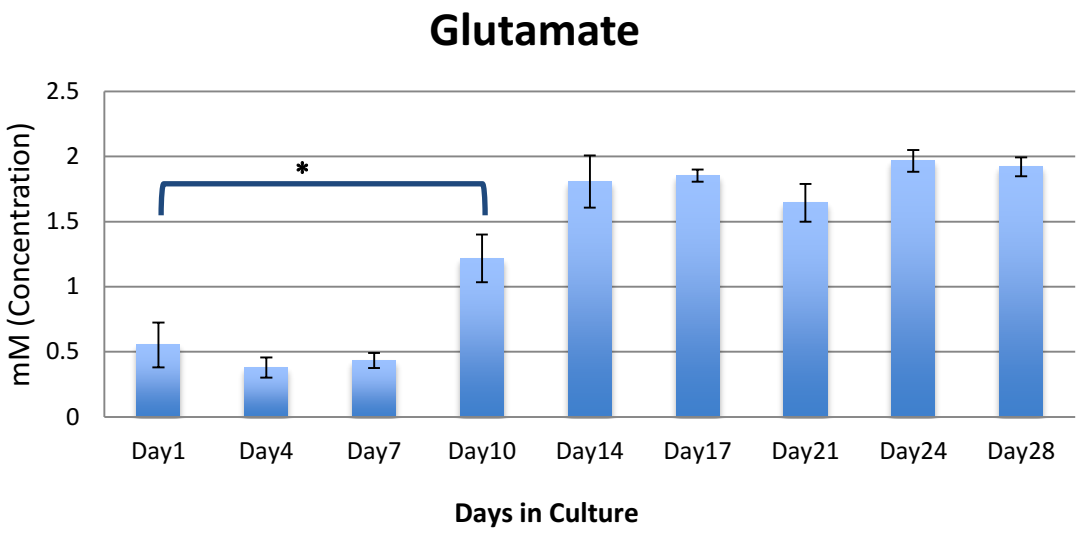


Figure 77. Accumulation of Glutamate during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol. (n=3) (P = <0.001).

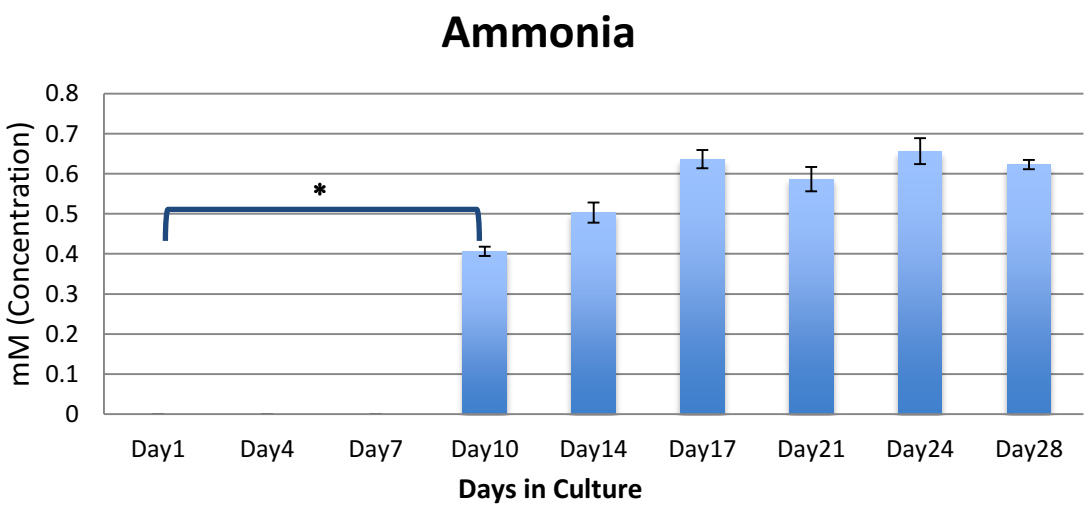


Figure 78. Accumulation of Ammonia during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol. (n=3) (P = <0.001).

7.4.8.3. pH variations level

The pH level was gradually decreased as the differentiation culture time passed, even though a fresh medium was supplied every other day. Overall, the pH levels were

maintained at lower levels, approximately 7.24 to 7.36, without large differences (Figure 79). These measurements do not reflect the accurate pH level of the medium at the corresponding time point, as samples were not freshly analysed. However, they can still provide good indications of the pH level variations along the differentiation culture.

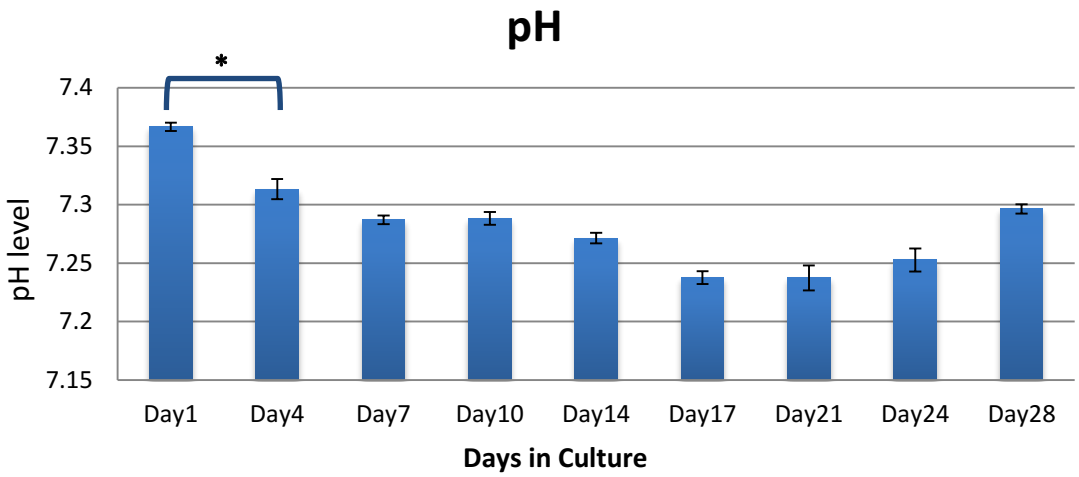


Figure 79. pH Variations during Human iPS Cells Differentiation in Serum-free Erythroid Differentiation Protocol. (n=3) (P = <0.001).

7.5. Discussion

In vitro blood production has been developed from different cell resources with modification on the conventional culture system. Success in this approach would provide a platform for a controlled *in vitro* blood production. To date, many research efforts have been extensively focused on providing safe, efficient, and scalable protocols for blood production that can be used in clinics (Mountford et al., 2010). In the last decade, the focus has been redirected toward the usage of stem cells as an unlimited cellular resource. Stem cells are defined as cells able to undergo self-renewal by asymmetric cell division, and, under proper conditions, can give rise to any specialized cells. This feature is called potency (Ebihara et al., 2012). Blood components have been produced from different types of stem cells such as HSCs, cord blood stem cells, and peripheral blood stem cells, iPS and ES cells, all of which have demonstrated significant results and created

blood cells with comparable morphology and function of their natural compartments (Ebihara et al., 2012, Anstee, 2010).

The relevant natural process, erythropoiesis, is a tightly controlled process in the fetal liver of embryos and bone marrow and spleen of adults, which produces a high number of erythrocytes in hypoxia or blood loss (Grebien et al., 2008). Erythroid maturation progresses through BFU-E stage, which requires SCF and Epo to differentiate into CFU-E stage that divide 4 to 5 times to reach the mature erythrocyte stage in response to EPO only, which is required for late maturation (Grebien et al., 2008, Li et al., 2014).

Recently, there have been various research approaches on the production of erythroid cells using human ES and iPS cells, EB-formation, stromal co-culture, and appropriate growth factors in the culture system, showing great potential for blood cells manufactured *in vitro* (Cerdan et al., 2004, Olivier et al., 2006, Lu et al., 2007, Ma et al., 2008, Lu et al., 2008a, Lengerke et al., 2009, Feng et al., 2010, Dias et al., 2011, Lapillonne et al., 2010b, Focosi et al., 2014, Dorn et al., 2015). However, in spite of these successful approaches, attempts for direct differentiations into the desired cell type in feeder-free and serum-free conditions have been limited. Likewise, achieving the terminal maturation of the produced cells has been hardly reported. In other words, little is known about the process guiding the development of erythropoiesis. Thus, our culture system was designed to develop a better and efficient culture system for *in vitro* blood cells manufacture.

In this study, human iPS cells were differentiated towards erythroid cells through three different stages, a haematopoietic/erythropoietic induction phase (7 days), followed by an erythroid differentiated phase (7 days), and, finally, an erythroid maturation phase (14 days) (Figure 80). The cellular outputs were characterized by cellular markers, protein and gene expression studies, along the differentiation stages of 28 days culture period.

To the best of our knowledge, our study is the first one demonstrating production of erythroid cells from human iPS cells under a complete serum-free and feeder-free condition, achieving strong indications of partial maturation and enucleation within such

a short time-frame, based on the analysis performed. Thus, the outcomes of this culture protocol needs a further confirmation before it can be used potentially to improve the production of RBCs manufacture *in vitro* widely.

Correspondently with the *in vivo* condition, human iPS cells in our culture groups under our three-phase differentiation protocol resulted in a fast formation of haemoglobinized erythroid cells, scaled from nucleated erythroblasts to enucleated erythroid cells.

In terms of morphology, cells cultured under both conditions, normoxia and hypoxia, showed a progressive size reduction and nucleus to cytoplasm ration before enucleation occurred in some cells. Nucleated erythroblasts were observed as early as day 14 in both conditions. Hypoxia condition also started to show some enucleated erythroblasts at day 14, which, according to the size (7-10µm), could be reticulocytes. The number of enucleated cells increased toward the end of the experiment in both conditions, with a size reduction (6-8µm) and a pale pink color, all of which can be explained as a further maturation into enucleated erythrocyte (Blann and Holtom, 2010). The gradual progression demonstrated by Wright-Giemsa stains from a blue to purple to pink stain suggests the production of pronormoblast to polychromatic erythroblasts to orthochromatic normoblast and, finally, to enucleated erythrocytes (Lu et al., 2008a). The reticulocyte formation was further confirmed in both conditions, which is one stage before the final formation of the mature erythrocytes. Reticulocytes were identified by the first loss of nucleus, their size, and the RNA ribosomal remnant of haemoglobin detected by the reticulocyte staining (Blann and Holtom, 2010).

The expression of representative markers for the evaluation of the pluripotency status such as Oct4, Nanog, and SSEA4 were relatively compared in all culture groups at different time points based on the expression level observed in day 0 undifferentiated cells. Oct4 is a key regulator for maintenance of pluripotency in ES cells (Niwa et al., 2000, Pan et al., 2002). Nanog is another pluripotency factor associated with stemness highly expressed in undifferentiated human ESCs and iPS cells and involved in the self-renewal feature (Loh et al., 2006). SSEA4 is a cell surface marker used to identify human pluripotent cells (Aoki et al., 2010). In terms of pluripotency, the pluripotency-associate

markers decreased over the culture period, suggesting that cells from both culture groups were shifted from the pluripotent stage, which could be effectively followed by the specific lineage differentiation. Flow cytometry results showed a significant drop in SSEA4 by the day7 time point, with no significant difference between the two conditions. qPCR results also showed drops in OCT4 and NANOG expression in both conditions, normoxia and hypoxia, as there was no expression by day7, suggesting that cells might lost their pluripotency by that time point and were ready to differentiate.

Early and high expressions of two hematopoietic markers, CD34 and CD45, were observed, supporting the early inducement of differentiation towards haematopoietic lineage. The expressions of both markers were evaluated using flow cytometry. CD34 was observed at day7 in both conditions, peaked at day 14, and decreased gradually toward the end of the differentiation. Normoxia condition showed higher percentage of $CD34^{+}$cells (HSCs) at days 7, 14 and 21, and a sharper decrease by day28. However, the difference was not significant in comparison to hypoxia condition. This trend was comparable to the *in vivo* trend as expression of CD34 decreases during hematopoietic differentiation and it disappears in terminally differentiated cells (Krause et al., 1994). CD45 also begun to be observed by day7, peaked at day14, and dropped towards day28 in both conditions. However, hypoxia condition showed higher number of $CD45^{+}$ cells at all time points.

$CD34^{+}CD45^{-}$ phenotype is usually associated with HSCs and hematopoietic progenitor cells. This phenotype was found at low percentages along the culture period in both conditions, suggesting that cells might differentiate to a further mature cellular phenotype by the first analysed time point. However, $CD34^{+}CD45^{+}$ phenotype is usually associated with megakaryocyte–erythrocyte progenitors and proerythroblasts (Attar, 2014). This phenotype peaked at day14 in both conditions but decreased throughout the following time points, indicating further erythroid maturations. BFU-E and CFU-E cells are erythroid progenitors characterized by CD45+ $CD235a^{-}CD34^{+}CD36^{-}$IL-$3R^{-}CD71^{low}$ and $CD45^{+}CD235a^{-}CD34^{-}CD36^{+}$IL-$3R^{-}CD71^{high}$ phenotypes, respectively (Li et al., 2014). Thus, our findings suggested that both culture groups might have had BFU-E and CFU-E progenitors that differentiated further into erythroblasts towards day28.

The expressions of both markers were also supported by immunocytochemistry staining. In normoxia, CD34 and CD45 signals were high at day7, elevated significantly at day14, then decreased substantially by day 21 and day28. In hypoxia, the trend of the cellular markers was comparable to their typical profile during erythropoiesis. CD34 was high at day7, significantly higher at day14, and then gradually reduced by days 21 and 28. CD45 showed less intensity at days 7 and 14, but maintained a similar level at day 21, before the signal dropped at day 28, towards the formation of erythroid committed precursors.

Moreover, using flow cytometry, the expression of CD43 haematopoietic marker was evaluated. However, in our study, CD43 showed negligible expressions in both conditions.

Following to the above interesting findings in this study of the expression of haematopoietic markers, which potentially could be related to the erythroid lineage development, the expressions of erythroid-specific markers were measured to track erythroid differentiation from the culture. Thus, early and late erythroid markers, CD71 and CD235a respectively, were evaluated throughout the differentiation culture period. The expressions of both erythroid markers were assessed at different time points along the culture period using flow cytometry, immunocytochemistry, and qPCR.

The expressions of CD71 and CD235a were successfully achieved using flow cytometry analysis during both culture groups, indicating that maturation process occurred. Both markers peaked later than the hematopoietic markers and were maintained at a high level throughout the differentiation, which reflects the formation of erythroblasts (Attar, 2014). The coexpression of CD71 and CD235a in both conditions, as seen in flow cytometry results, shows the typical profile expected in erythropoiesis at proerythroblast, basophilic erythroblast, polychromatic erythroblast, and reticulocyte stages. $CD71^{+}CD235a^{+}$ coexpression decreased toward the end, as CD71 expression is supposed to decline at mature erythrocyte stage (Attar, 2014). Nevertheless, the desired phenotype of $CD71^{-}CD235a^{+}$, typical mature erythrocyte phenotype, did not show significant expression.

CD71 gave high immunocytochemistry signals at day 7 of normoxia culture group, which elevated significantly at day14, then decreased substantially by days 21 and 28. CD235a signals showed a similar trend of tremendous escalation at day 14, followed by a significant drop by day21. However, this decrease was followed by a significant increase by day28. In the hypoxia culture group**,** the trend of the cellular markers was comparable to their typical profile during erythropoiesis. CD71 and CD235a peaked later, at day 21, and maintained high, which reflects the beginning of erythroid maturation. It is worth noting that the fluorescent signals of all analysed cellular markers were higher in cells cultured under normoxia condition than hypoxia condition.

The single cell level analysis using immunocytochemistry staining confirmed the size reduction feature, which is one of the main maturation signs during erythropoiesis from large nucleated erythroblasts (10-15µm), to reticulocytes (7-10µm), and, finally, mature enucleated erythrocytes (6-8µm) (Blann and Holtom, 2010). Moreover, DAPI stain, which stains the nucleus, was dramatically decreased at day21 and day28, confirming losing of nucleus, another main feature of mature erythrocytes. Additionally, day 28 showed cells with the typical biconcave discoid shape of mature erythrocyte. In both conditions, CD34 and CD45 signals showed their highest expression at day 14, then started to decrease, whereas CD71 signals gradually elevated until day 21 and reduced by day 28, as the mature erythrocytes are $CD71^{-}$. CD235a also slowly increased until the maximum expression was reached at day 28 of both conditions.

A further confirmation for the results obtained from the presented methods was attempted at the gene level using qPCR. However, this procedure gave unexpected results as explained earlier in (7.4.5.). Enucleated erythrocytes lose their nucleus and organelles upon maturation and thus do not contain any DNA and cannot produce any RNA. Therefore, their immature forms, reticulocytes, which retain residual RNA contribute in mRNA synthesized and used for qPCR (Moschandreou, 2012). This process might be the reason behind some unexpected qPCR results reported in this study, as we were able to observe enucleation as early as day 14. Moreover, due to little RNA extracted samples, there were no technical replicates tested, thus, no reliable results could be concluded.

Influence of cytokine cocktails in the haematopoietic differentiation of pluripotent stem cells are well elucidated in the literature (Zhang et al., 2005). SCF, IL-3, and IL-6 control the development of early haematopoietic progenitors (Muench et al., 1992, Ivanovic, 2004, Clanchy and Hamilton, 2013). When SCF binds its receptor, c-kit, its hematopoietic and erythropoietic lineage differentiation stimulation begins (Ashman et al., 1991, Broudy et al., 1992), while EPO/EPOR signaling controls the erythroid progenitor differentiation and maturation (Grebien et al., 2008, Li et al., 2014). The gene expressions of both receptors were unsuccessfully investigated (Appendix 13).

The clonogenic ability of the produced cells was also assessed by exposing the differentiated human iPS cells to methylcellulose media, which showed a significant increase on the erythroid progenitor colonies formation, CFU-E and BFU-E, and some haemoglobinization (pink/brownish colour), especially from hypoxia group. However, the colonies formed are an interesting finding that needs to be studied further to identify the phenotype in detail.

In details, day 14 of both normoxia and hypoxia conditions is hypothesized to be the ideal time-frame for hematopoietic and erythroid differentiation based on flow cytometry for marker expression, as the largest proportion of cells expressed hematopoietic and erythroid markers. The results were supportive to flow cytometry figures, as it seemed as though like a large proportion of these cells were colony-forming progenitor cells based on the images of the resulted CFU assays. The colonies were abundantly observed and quantified, and yielded >200 colonies on day14. BFU-E colonies formed in normoxia were smaller, while BFU-E colonies of hypoxia condition were more uniform in shape. However, the colonies may not have had developed optimally. They were not as large or as red as typically seen, perhaps due to overcrowding that resulted in limited nutrients and growth factors in the dish. It was also hypothesized that towards the end of the differentiation, cells will differentiate further and will start to mature and lose their clonogenic ability. Accordingly, it seemed that hypoxia condition supports maturation earlier than normoxia, as day 28 cells in normoxia condition were colony-forming progenitor cells able to produce colonies, while cells of hypoxia condition were differentiated and unable to form colonies.

The haemoglobinized BFU-E colonies were observed only at day14 of hypoxia condition (Chadwick et al., 2003b), which suggests that differentiating the cells under hypoxia condition might support haemoglobinazation more than normoxia condition. However, it is worth noting that colonies derived from more "primitive" BFU-E may not appear red, as it can take more than two weeks of culture for haemoglobinization to occur in some cord blood-derived BFU-E.

$CD34^+$ cells in the BM exhibit CFU-blast initiating cells such as multipotential CFU-GEMM and committed progenitor cells such as CFU, CFU-GM, BFU-E, and CFU-megakaryocte, as confirmed by *in vitro* CFU assays (Krause et al., 1994). In our assays, the resulted colonies of both conditions, normoxia and hypoxia, were mainly erythroid progenitors and there was no other hematopoietic progenitor colonies observed such as CFU-GM or CFU-GEMM. The expression of CD71 and CD235a on the majority of the plated cells also suggests that they were already erythroid-committed progenitors. However, it is difficult to confirm that hypothesis without further analysis of markers associated with granulocytic/monocytic lineages.

One of the major challenges in the *in vitro* RBCs production is the haemoglobin formation and globin-switching into adult-type haemoglobin. Switching of globin expression is a sequential event. In the primitive haematopoiesis, blood islands in the yolk sac transiently generate nucleated RBCs containing embryonic-type haemoglobin: Gower-1 (ξ,ε), Gower-2 (α,ε), and Portland (ξ,γ). In definitive haematopoiesis, HSCs produced enucleated RBCs in fetal liver with fetal-type Hb (Hb F; α,γ) before it shifts to bone marrow with adult-type Hb (Hb A; α,β) (Ma et al., 2008).

In normoxia condition, the presence of haemoglobin was confirmed by immunocytochemistry staining. In measuring the average intensity, day14 showed the highest signal, followed by an unexpected gradual decrease. Conversely, hypoxia condition showed the expected desired trend: low expression at day 7, followed by an escalation for the duration of the culture period, accompanied with a size reduction of cells of day28.

Moreover, the type of haemoglobin presented was examined at gene level using qPCR. Although the results were preliminary data only due to small sample size, it can still shows some encouraging results at the gene level. findings suggessted that at day 14, cells cultured under normoxia conditions contained trace amounts of embryonic- and fetal-type haemoglobin, but not adult-type, while cells at the time points thereafter did not show expression of any globin type. In contrast, cells cultured under hypoxia condition showed an increase of haemoglobin, starting with only embryonic- and fetal- types, and showed some adult-type by day 28 of culture, indicating that enhancement towards adult-type haemoglobin development might be attained in hypoxia. Linking these findings to the haemoglobinization observed in CFU results, a collective conclusion can be drawn that hypoxia condition might support haemoglobin formation, including adult-type, more than normoxia condition.

Moreover, our results suggest that the haemoglobin content in the produced cells resemble human primitive erythrocytes, although β globin was observed toward the end of the culture period in hypoxia group, suggesting that our cultured cells contained a mixture of haemoglobin types. Therefore, further improvements are required in our culture environment to promote definitive erythropoiesis and further maturation in our human iPS cells-derived erythroid cells.

In addition to providing insight into the type of haemoglobin produced in these iPS cells-derived erythrocytes, this current study had a critical focus on their functional role in order to use them in future clinical applications. Thus, it was evaluated whether or not these cells were able to function as O_2 carriers. Unfortunately, our human iPS cells-derived erythrocytes failed to exhibit an O_2 dissociation pattern similar to cord blood or adult blood (Appendix 18). However, the unsuccessfulness may be due to the low number of cells tested, which can be further improved and optimized (Ma et al., 2008).

Since we were relating the role of O_2 on our erythroid differentiation culture protocol, it was necessary to measure HIF-1α, the O_2-dependent transcriptional activator of angiogenesis, cell proliferation, survival, and glucose and iron metabolism. HIF-1α subunit is degraded under normoxia conditions, while in hypoxia conditions, it becomes

stable and stimulates its transcriptional activity to regulate many hypoxia-inducible genes (Lee et al., 2004). As expected, HIF1α expression was not detected in normoxia condition, while there was a rise only at day 28 in hypoxia condition (Appendix 15). This observation confirms the effect of hypoxia on our differentiation pathway, although the expression was expected at earlier time point (Yehia et al., 2015). The unexpected absence of HIF1α gene expression might be due to unsuccessful PCR analysis.

Furthermore, as the feeder-free differentiation methods of human iPS cells affect the viability (Firas et al., 2014), it was crucial to evaluate the viability within our protocol. A good viability was maintained along the culture in both culture groups, as confirmed by DAPI (Tanious et al., 1992, Kapuscinski, 1995). However, since some of the differentiated erythroid cells are believed to have undergone enucleation, DAPI was not the most appropriate assay. Yet, it can still give an indication of the viability within our culture conditions.

Differentiated cells were thus viable and metabolically active cells, which was reflected by pH change, glucose and glutamine consumption, lactates, glutamate, and ammonia production in cultural mediums. Glucose and glutamine in the media are the major energy sources for mammalian cells (Ozturk and Palsson, 1991). Glucose is utilized by glycolysis process and the lactate is the by-product under anaerobic condition (Glacken et al., 1986). Ammonia is the by-product of glutamine through the tricarboxylic acid (TCA) cycle, which make it an indicator of glutamine consumption (Miller et al., 1987, Kromer et al., 2011).

It has been reported that the *in vivo* glucose concentration in blood vessels is around 1000mg/l. However, a higher glucose level (4500 mg/l) has shown a better maintenance of pluripotent stem cells *in vitro*, while the lower glucose level has demonstrated improved differentiation of pluripotent cells (Ogawa et al., 2001, Kim et al., 2009, Khoo et al., 2005).

In this study, human iPS cells were differentiated in low glucose DMEM-based media and Stemline® Hematopoietic Stem Cell Expansion Medium. It was shown in the results of the

medium profiles in this study that glucose dropped slightly at day 4 but maintained high levels throughout the entire differentiation period, which means that the cultured cells did not consume all the glucose supplied in the medium. This situation can be justified, as differentiated cells showed high dependency on glutamine instead of glucose.

The bioprofiler results showed glutamine levels decreased along the erythroid differentiation culture in comparison to the beginning of the differentiation, indicating that cells consumed some, but not all, of the glutamine supplied in medium, which might support our claim above. Corresponding to the result of glutamine consumption, ammonium and glutamate production during differentiation period were also higher than in the early time points during differentiation culture phases. Moreover, glutamate is the by-product of glutamine through nicotinamide-adenine dinucleotide (NAD) synthesis in erythrocytes, a cellular antioxidation system and a mediator for energy metabolism and calcium homeostasis (Niihara et al., 1997, Ying, 2008). Glutamate and ammonium profiles showed increases in their production at day 10, which can also support the arguments for the formation of erythroid cells.

Additionally, the pH of the medium is another main key parameter in cell culture, affecting cellular viability, growth, and metabolic activity. The pH level of our differentiation media was gradually decreased with culture time, which might be attributed to an elevation in lactate and ammonia (Barngrover et al., 1985a). Nevertheless, the pH level ranged from 7.24 to 7.36, which is within the suitable pH range, suggesting that there was no main impact on the cellular activities.

We have thus described an efficient protocol for the direct differentiation of human iPS towards the erythroid lineages, under a serum-free and feeder-free condition, in 28 days only (Figure 80). The formation of erythroid cells was achieved under normoxia and hypoxia conditions. However, a better haemoglobin formation, including the synthesis of adult-type haemoglobin, was provisionally achieved under hypoxia.

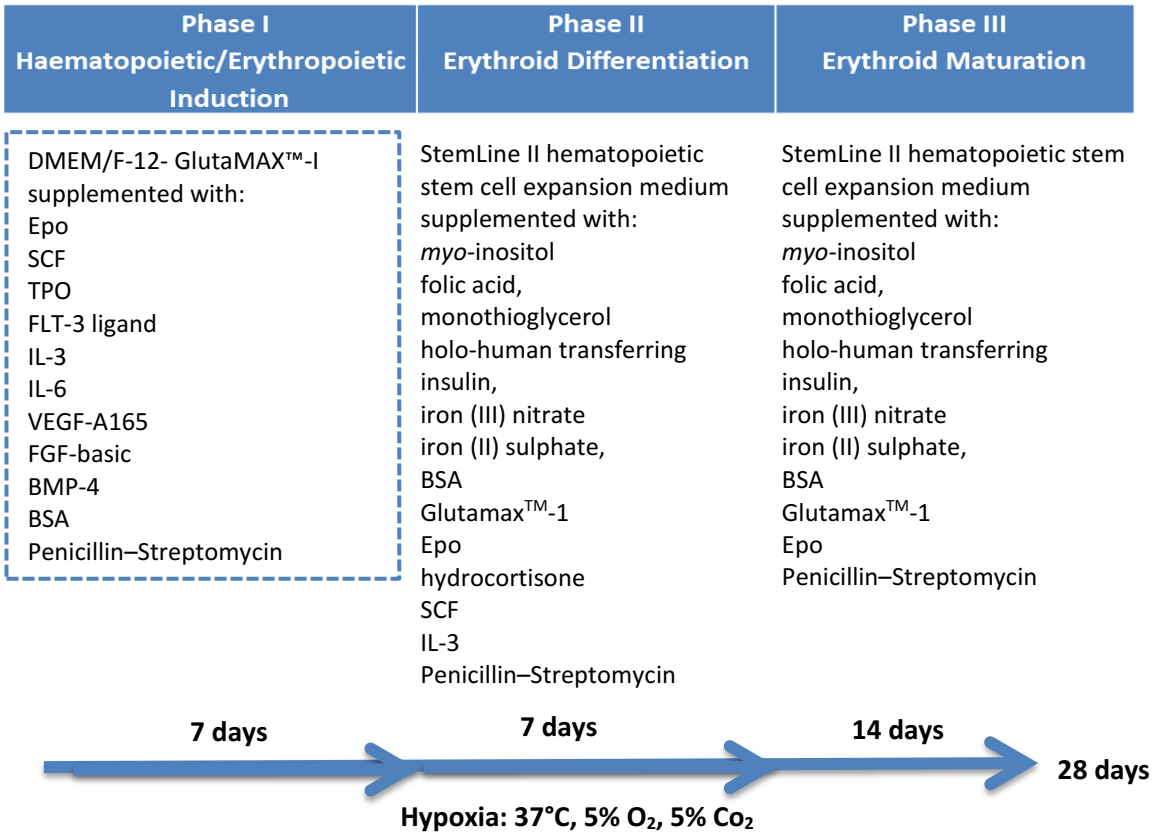


Figure 80. A Schematic Diagram of the Direct Differentiation of Human iPS Cells Towards the Erythroid Lineage under Serum-Free and Feeder-Free Culture Condition.

7.6. Conclusion

In this chapter we have described an efficient method to generate functional erythroid cells from human iPS in 28 days under conditions suitable for clinical applications. We have demonstrated the potential of human iPS cells to progressively differentiate into mature enucleated erythrocytes, in a completely serum-free and feeder-free culture condition. Haemoglobin formation was also confirmed within the produced cells. However, the globin switch cannot be concluded as the adult-type haemoglobin was provisionally observed in small group only. Our method suggests that it might be a novel source of patient-specific erythrocytes for clinical transfusions. Yet, significant improvements are still needed to improve maturation and homogenecity, in addition to better analysis methods to analyse the produced cells. Improving this system to generate

human erythrocytes can deliver experimental models to study human erythropoiesis, including maturation and globin-switching, and to examine therapeutic drugs for erythroid disorders (Ma et al., 2008).

Chapter 8.

Conclusion and Future Plans

8.1. Conclusion

Human ES/iPS cells are good candidates for providing an indefinite number of cells for clinical applications and disease modelling. Therefore, researchers have shown great interest in utilizing these sources in blood formation. Existing protocols used in the differentiation of human ES/iPS cells toward erythropoiesis utilize two main approaches: EB formation, which influences heterogeneity of the produced population, and/or co-culture with xeno-stromal cells, where obstacles of purification of the cells arise, which makes the xeno-free culture requirement difficult to achieve, in addition to the cytokines supplements. Moreover, these protocols reported low efficiency in number and functionality, especially with human iPS cells, and required long time in culture. However, iPS cells hold an invaluable promise of providing patient-specific iPS cells needed for transfusion and to cure many blood-related diseases. Current protocols, therefore, have focused on providing fully defined cultures in serum- and feeder-free conditions.

In this thesis, we presented the sequential production of erythroid cells from human iPS cells within 28 days in a novel, direct, serum-free, feeder-free monolayer culture condition, bypassing the EB formation step and the co-culture system. Simple manipulation of the combinations and concentrations of cytokines and culture supplements facilitated reproducible and directed stepwise commitment to specific haematopoietic lineages of erythroid cells. Exploiting the hypoxia condition, as a key enhancer, further improved the erythroid maturation. Our monolayer culture has showed a stepwise differentiation of iPS cells into mature erythroid cells via haematopoietic progenitors and erythroid precursors. Additionally, our defined, direct, and less labour intensive protocol enables easy and orderly harvest of hematopoietic progenitors, erythroid precursors up to erythroid cells, along the culture without a contamination by undefined serum components or co-culture stromal cell layers.

Human iPS cells lost their pluripotency as early as day 7 of our culture protocol in both culture conditions, normoxia and hypoxia, when hematopoietic progenitors expressing CD34 and CD45 were efficiently generated. Normoxia condition showed faster expressions of these markers, although the statistical analysis showed no significant

differences between the two conditions. Further culture period showed further differentiation of the hematopoietic progenitors into cells expressing erythroid markers CD71 and CD235a, along with decreasing the expression of CD34 and CD45, suggesting the formation of erythroblasts and erythroid cells, including both reticulocytes and mature enucleated erythrocytes. This finding was also confirmed by the microscopical examination for the formation of riposomal RNA, size reduction, loss of nucleus, and the biconcave shape formation.

Enhancement of the erythroid colonies formation, especially the BFU-Es in the methoCult media, reflects the functional clonogenic ability of the produced cells in normoxia and hypoxia, particularly day 14 cells, indicating that cells at the following time points were further differentiated. Furthermore, the redness in the BFU-E colonies noticed in hypoxic cells only suggests that this condition supports the haemoglobinization better than the normoxia condition.

Finally, maturation and enucleation of the erythroid cells produced was achieved in both conditions. However, it was observed more efficiently under hypoxic O_2 levels, as the switch into adult type haemoglobin was detected only with cells cultured in hypoxia environment.

Although the existence of mixed cells of haematopoietic and immature erythroid cells were observed within our differentiation culture, it needs to be improved further. This novel protocol has the potential to provide an efficient, direct, scalable, automatable, and fast culture system for application on *in vitro* RBCs manufacture, which could be potentially used in clinical and therapeutic practice.

8.2. Future Plans

This thesis presented a novel differentiation culture for deriving *in vitro* blood formation established from human iPS cell. However, from a clinical therapeutic point of view, application of this novel strategy requires further enhancement and definition.

First, our IMR90-1 cells were maintained in mTeSR™1 media and culture on Corning® Matrigel® hESC-qualified Matrix. The mTeSR™1 Medium is a completely defined serum-free medium, however, it contains bovine serum albumin. In addition, Corning® Matrigel® hESC-qualified Matrix is a reconstituted basement membrane isolated from the Engelbreth-Holm-Swarm mouse sarcoma tumour, which is rich in extracellular proteins; around 56% laminin, 31% collagen IV, and 8% entactin. It is also includes heparan sulfate proteoglycan, TGF-β, EGF, IGF, FGF, tissue plasminogen activator, and other undefined components (Osiecka-Iwan et al., 2008, Rodin et al., 2014). The involvement of undefined products of xeno origin may compromise the use of our culture protocol in clinics, as their foreign pathogens could lead to cross-species contamination (Choumerianou et al., 2008). Thus, it is crucial to move to xeno-free media. TeSR™2 and TeSR™-E8™ are good alternatives, as they are more defined xeno-free modified formulas of mTeSR™1 Medium that can be used for long-term feeder-free maintenance of human ES/iPS cells (Chen et al., 2011). This media is an ideal option, offering high quality, scalable, and reproducible system for stem cell research and clinical applications. Furthermore, it can be used with Vitronectin XF™, instead of Corning® Matrigel®, as a xeno-free adherent matrix to support the proliferation of human ES/iPS cells and ensure a completely xeno-free culture system (Braam et al., 2008, Prowse et al., 2010). In addition, recent research reports describe the use of Laminin-521 for the maintenance of human ES/iPS cells in a chemically defined, xeno-free and feeder-free culture condition (Lu et al., 2014, Rodin et al., 2014).

Second, cell sorting may be required in order to obtain a purified mature erythroid cell population from the heterogeneous cell population of haematopoietic and erythroid cells attained at day 28 of our culture protocol. By applying Fluorescence Activated Cell Sorting (FACS) technique, we can isolate the cell phenotype of interest from the mixed population using suitable monoclonal antibodies for the desired markers.

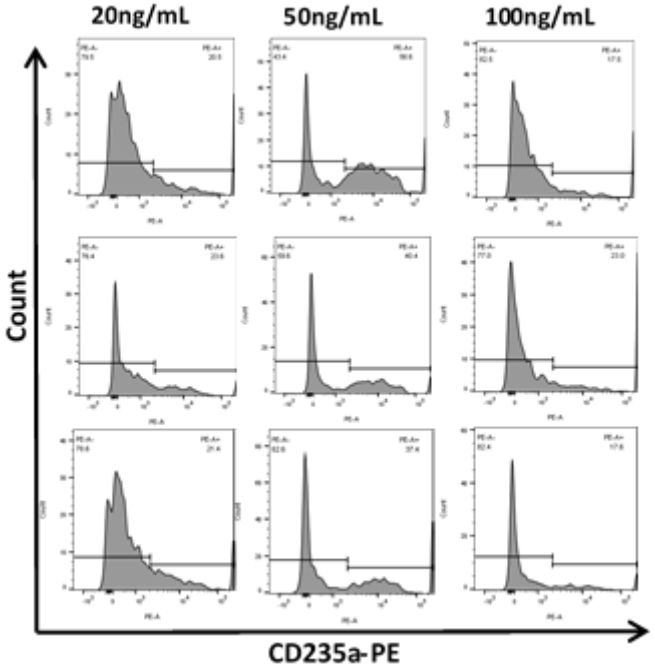
Third, it is extremely important to functionally characterize the yielded cells in terms of enzyme content of glucose-6-phosphate dehydrogenase and pyruvate kinase, their deformability profile, and the functionality of the haemoglobin content. This

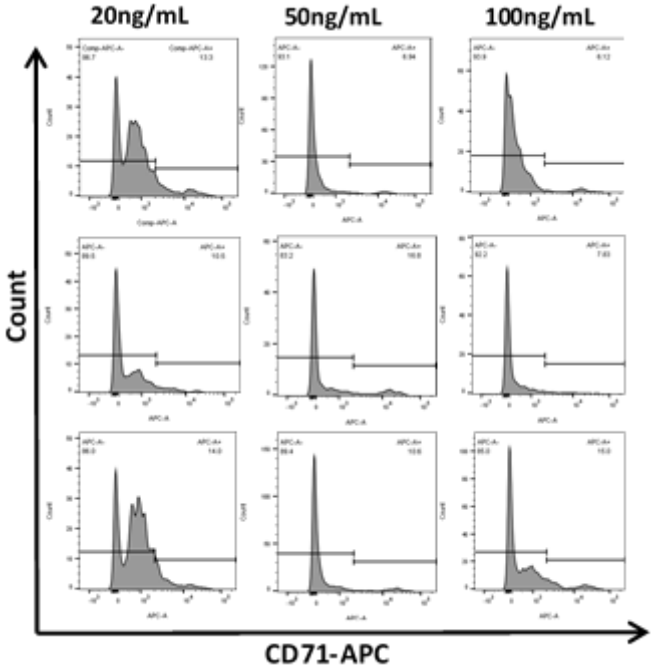
characterization may be done by checking the O_2 equilibrium curves, thus rendering their usage clinically.

Finally, in order to ensure the solidity and reproducibility of our novel erythroid cells production protocol, it is essential to test its efficacy with other human iPS cell lines in parallel with human ES cell lines. This task must be done before our protocol can be transmitted into scientific and therapeutic organizations for pre-clinical and clinical practice.

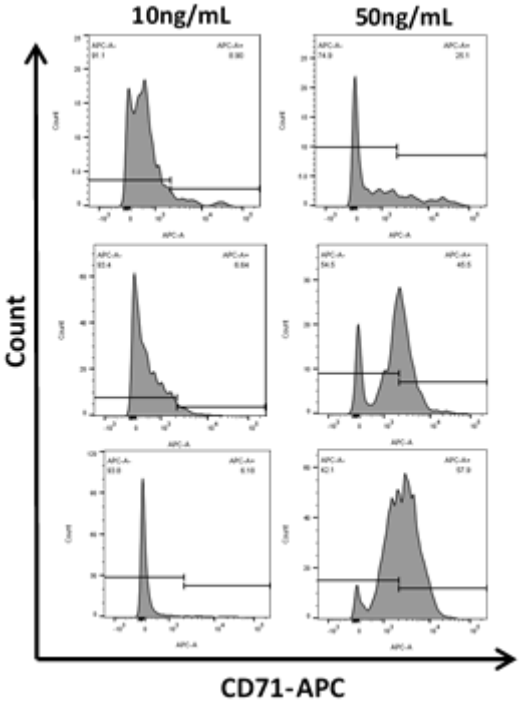
Appendix

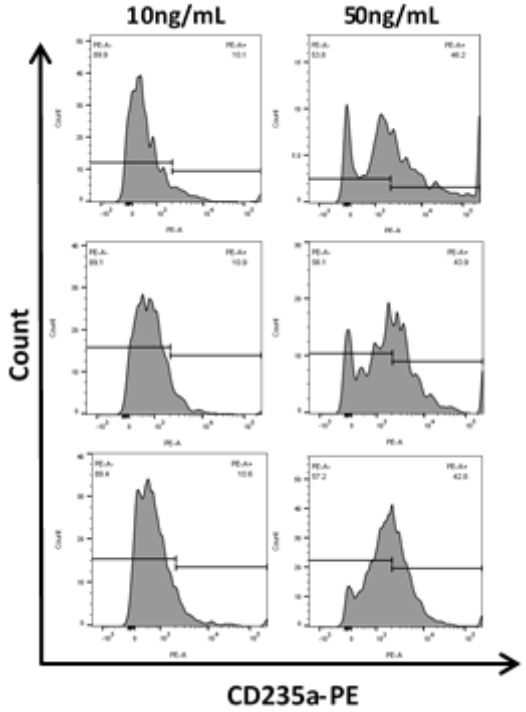
Appendix 1. Optimizing the Concentration of Recombinant Human FGF-basic for the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.



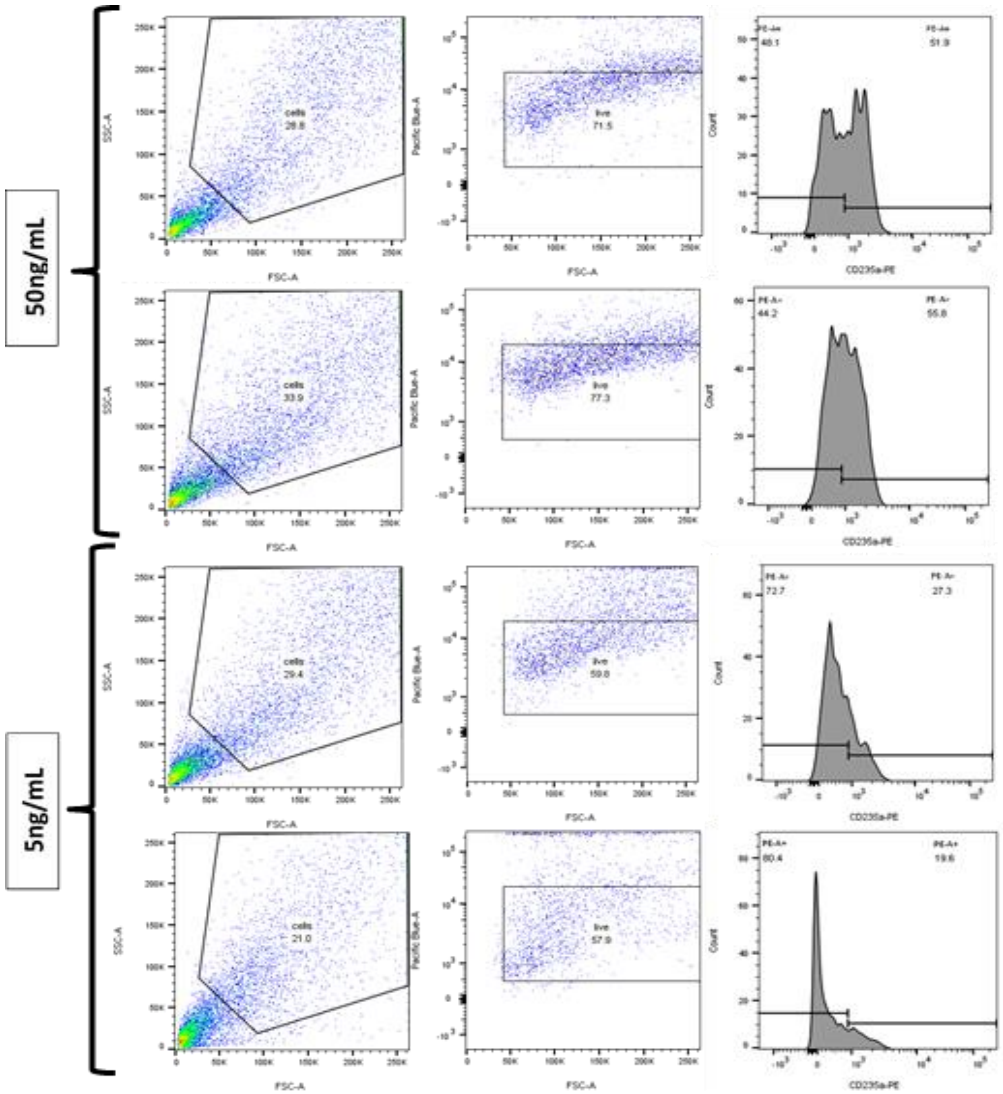


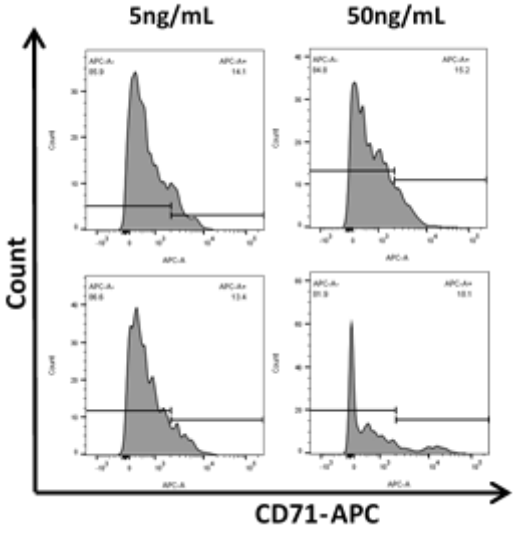
Appendix 2. Optimizing the Concentration of Recombinant Human BMP-4 for the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.



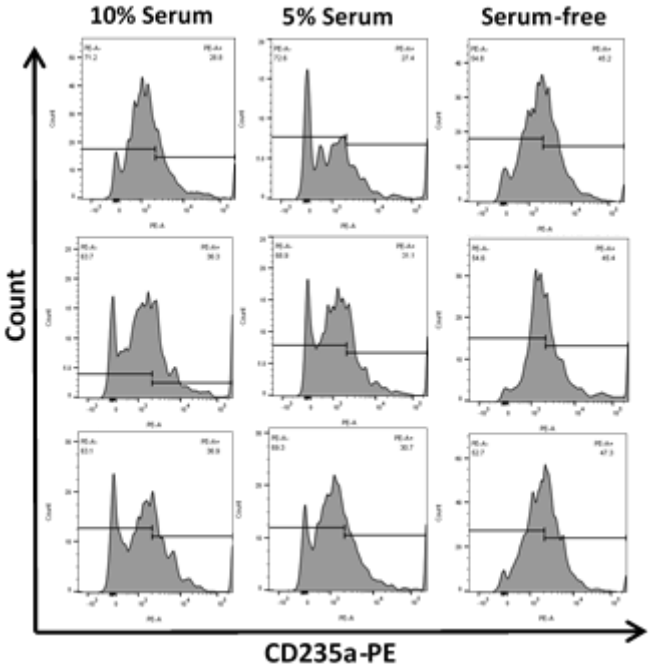


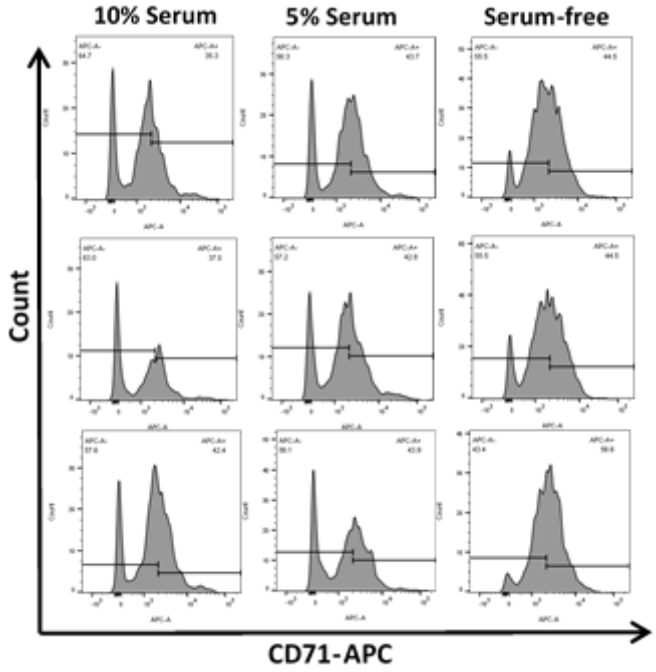
Appendix 3. Optimizing the Concentration of Recombinant Human VEGF-A165 for the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.



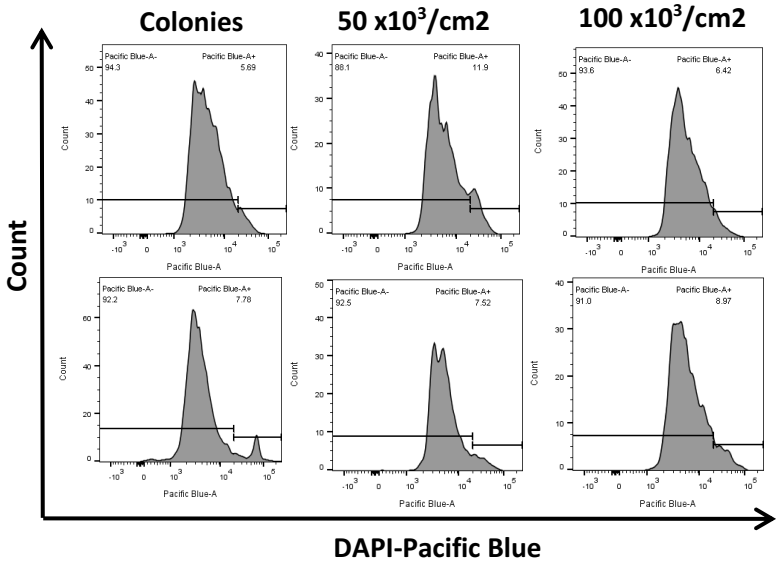


Appendix 4. Effect of Serum on the Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.



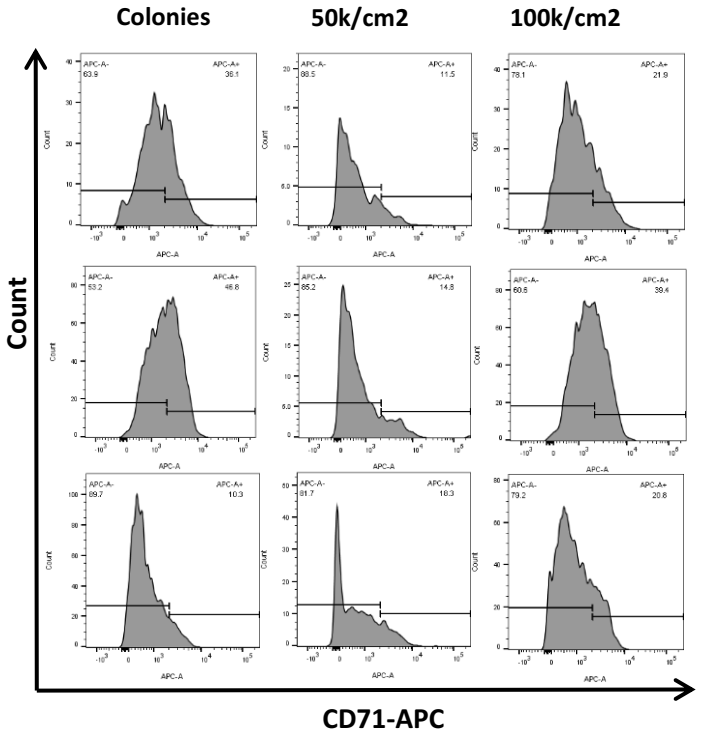


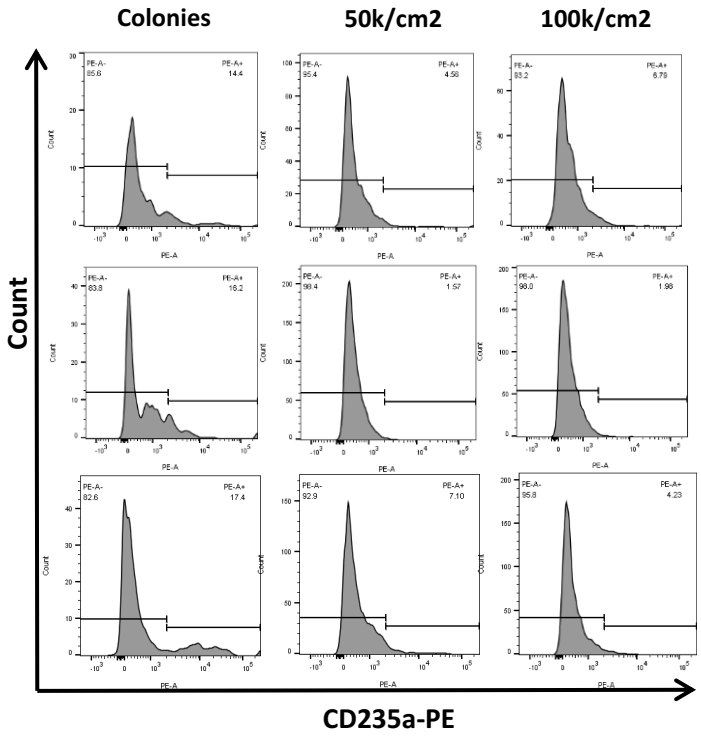
Appendix 5. Hematopoietic/Erythropoietic Differentiation of Human iPS Cells in Colonies vs Single Cells.



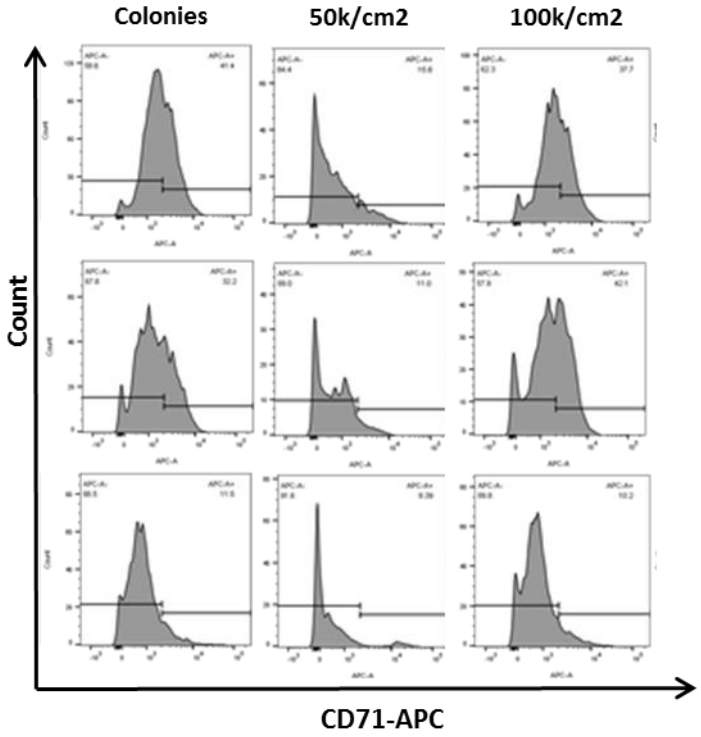
Appendix 6. The Efficiency of Hematopoietic/Erythropoietic Differentiation of Human iPS Cells at Different Time Points.

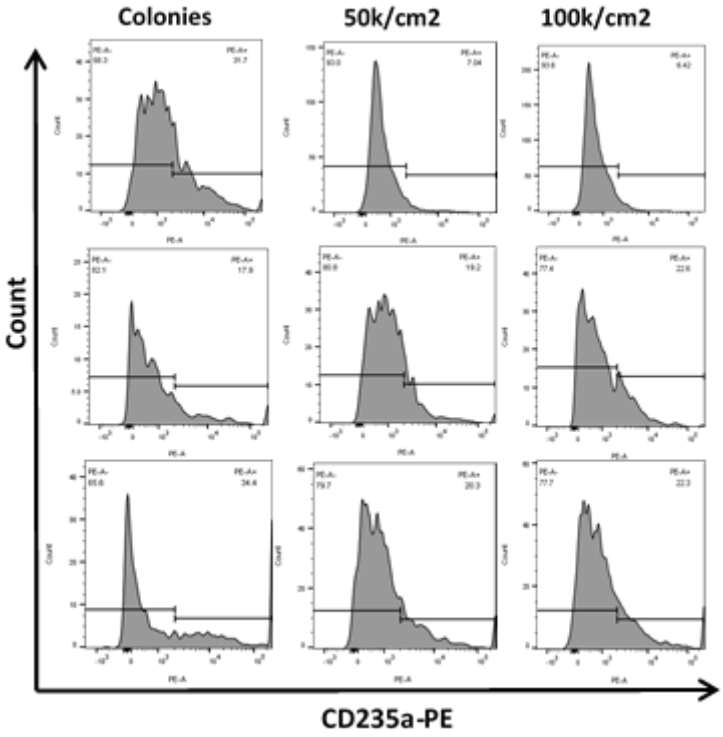
Day4:



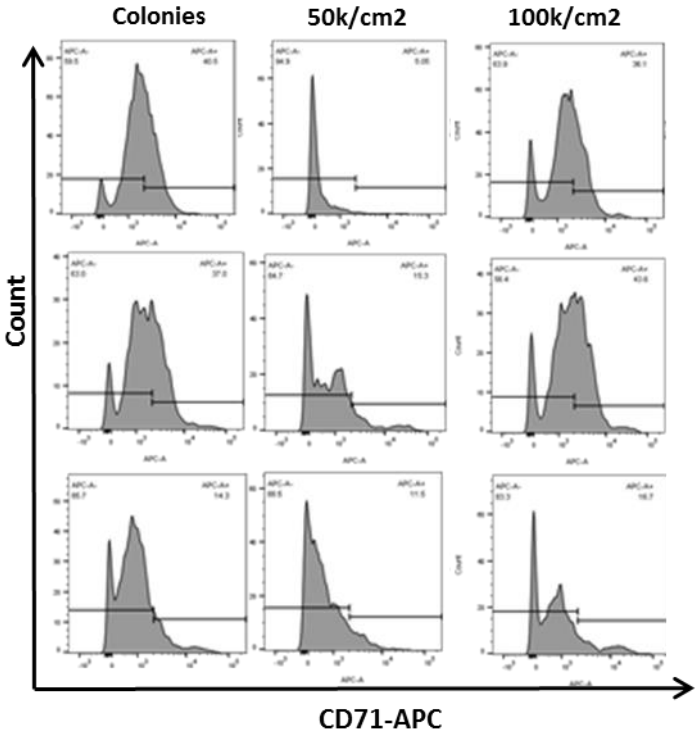


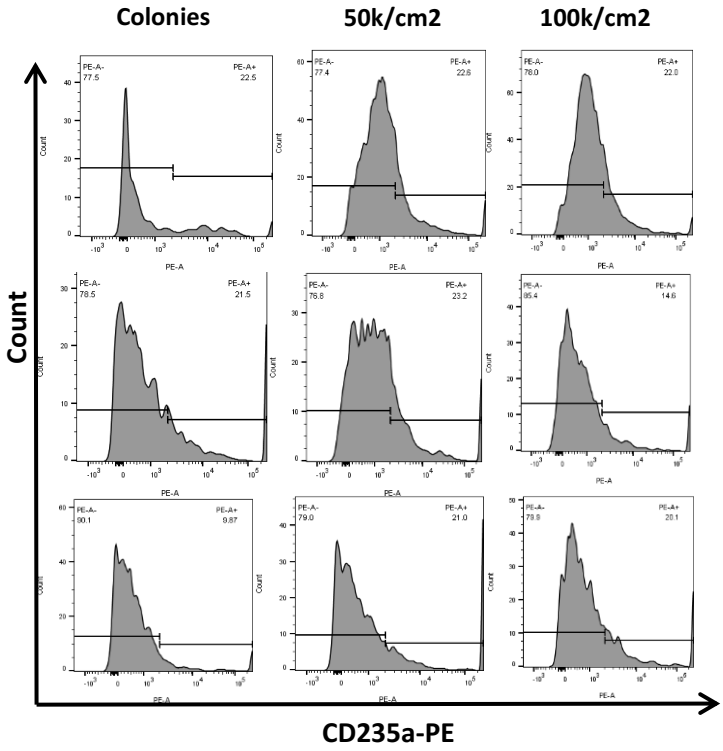
Day7:


Colonies
50k/cm2
100k/cm2
Count
CD71-APC

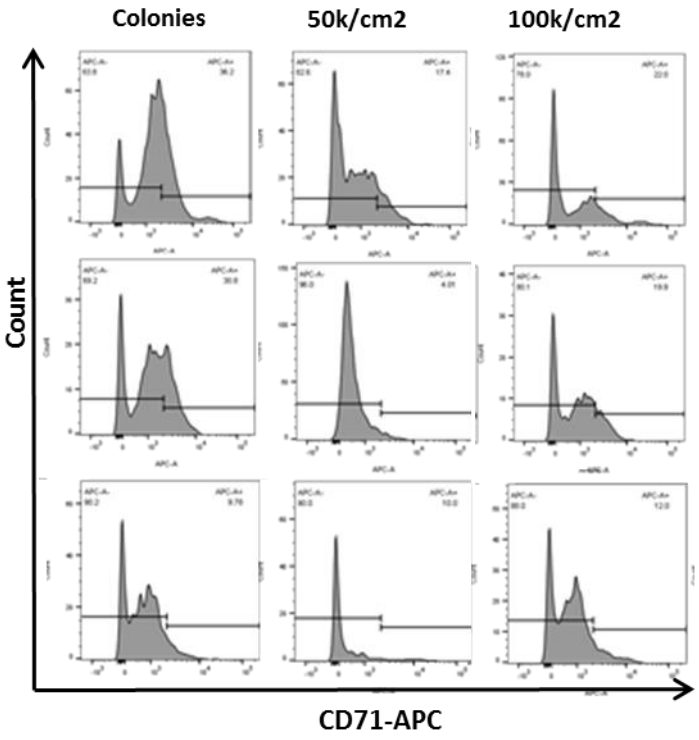

Colonies
50k/cm2
100k/cm2
Count
CD235a-PE

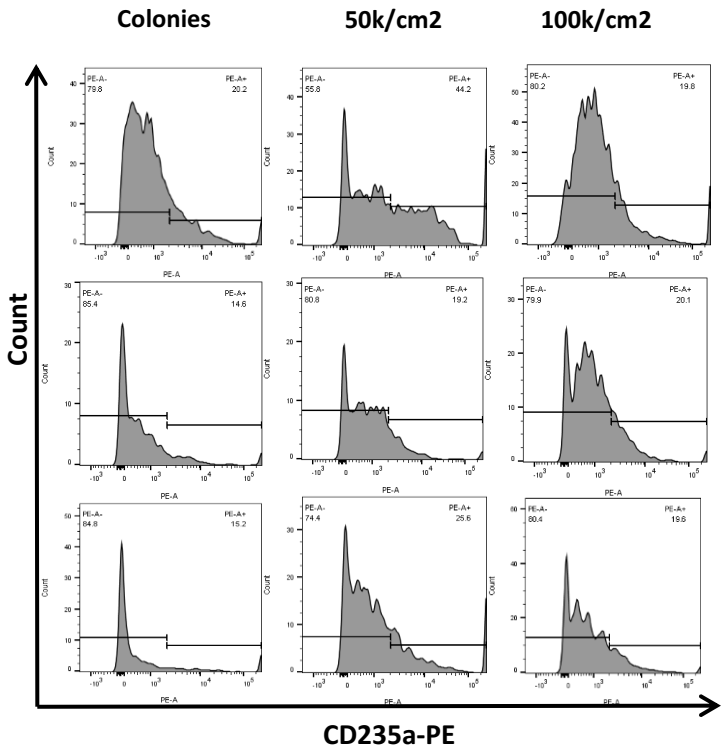
Day10:


Colonies
50k/cm2
100k/cm2
Count
CD71-APC


Colonies
50k/cm2
100k/cm2
Count
CD235a-PE

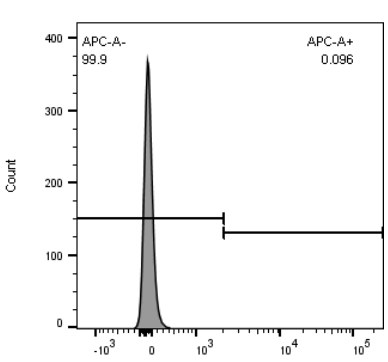
Day14:


Colonies
50k/cm2
100k/cm2
Count
CD71-APC

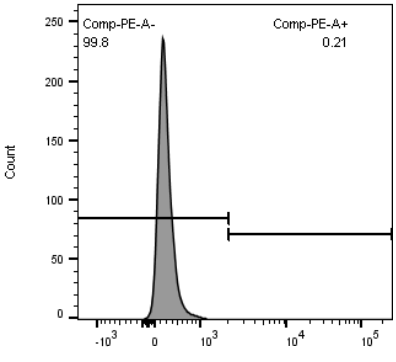

Colonies
50k/cm2
100k/cm2
Count
CD235a-PE

Flow Cytometry Controls for CD71 and CD235a.

Isotype Controls

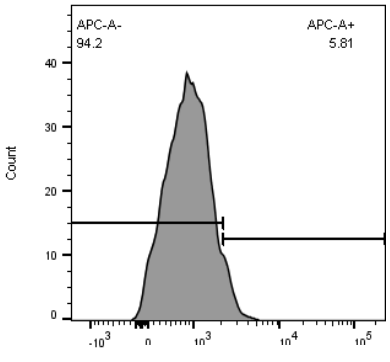


CD71-APC

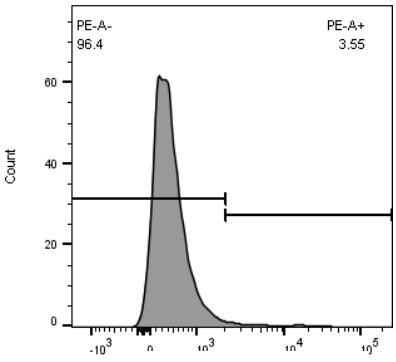


CD235a-PE

Negative Controls



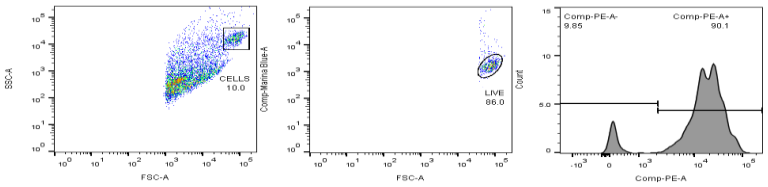
CD71-APC



CD235a-PE

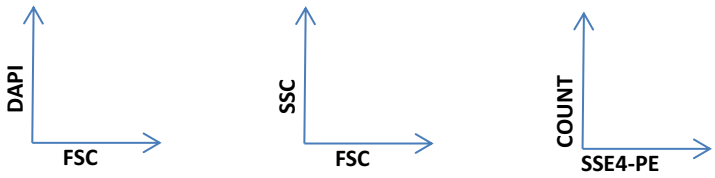
Appendix 7. Flow Cytometry Analysis of SSES4-PE for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.

Day 0 iPS Cells

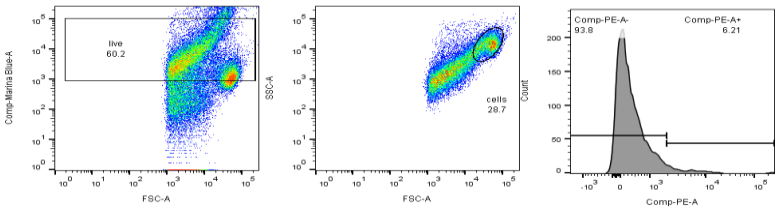


SSEA4-PE

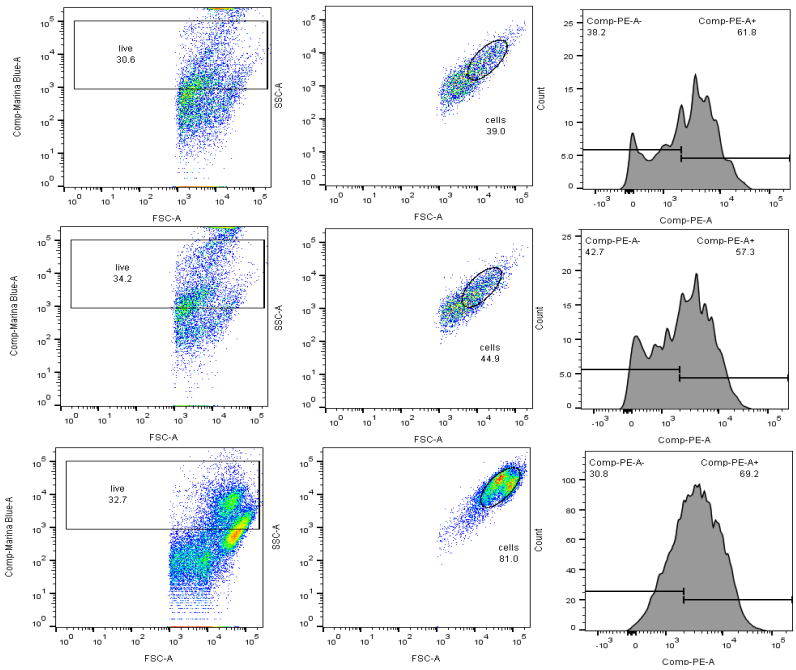
Day 7:



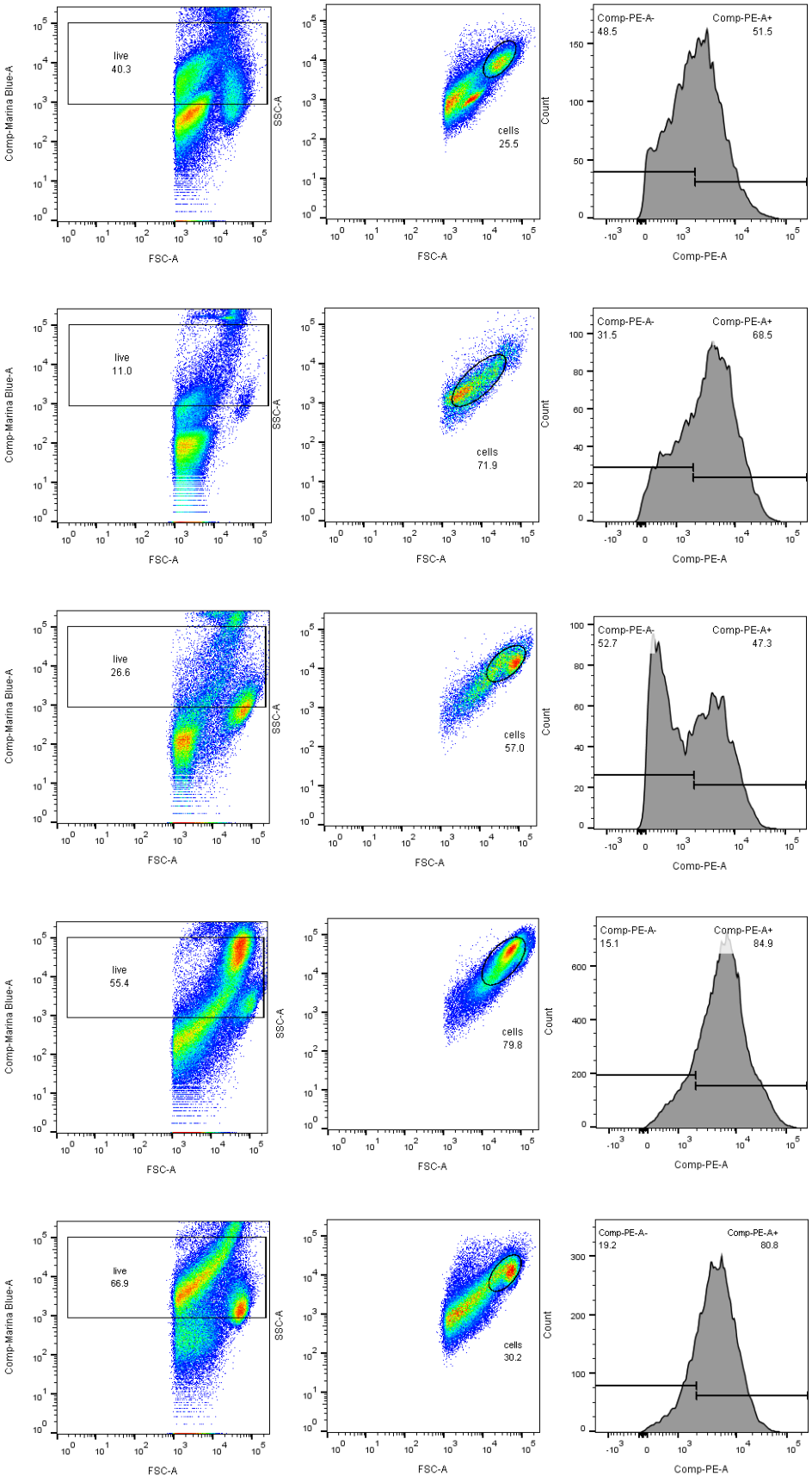
Isotype control



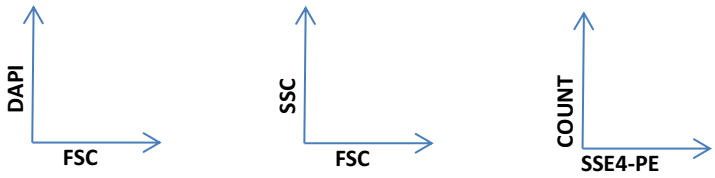
Normoxia Day 7 Samples



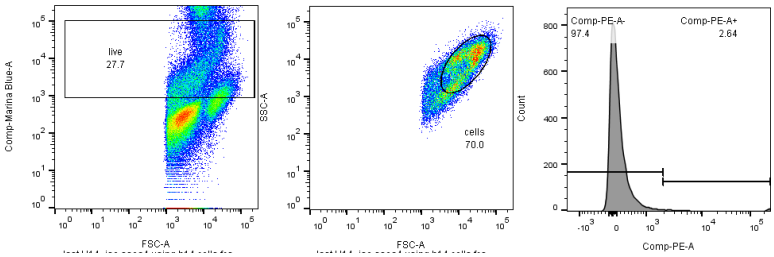
Hypoxia Day 7 Samples



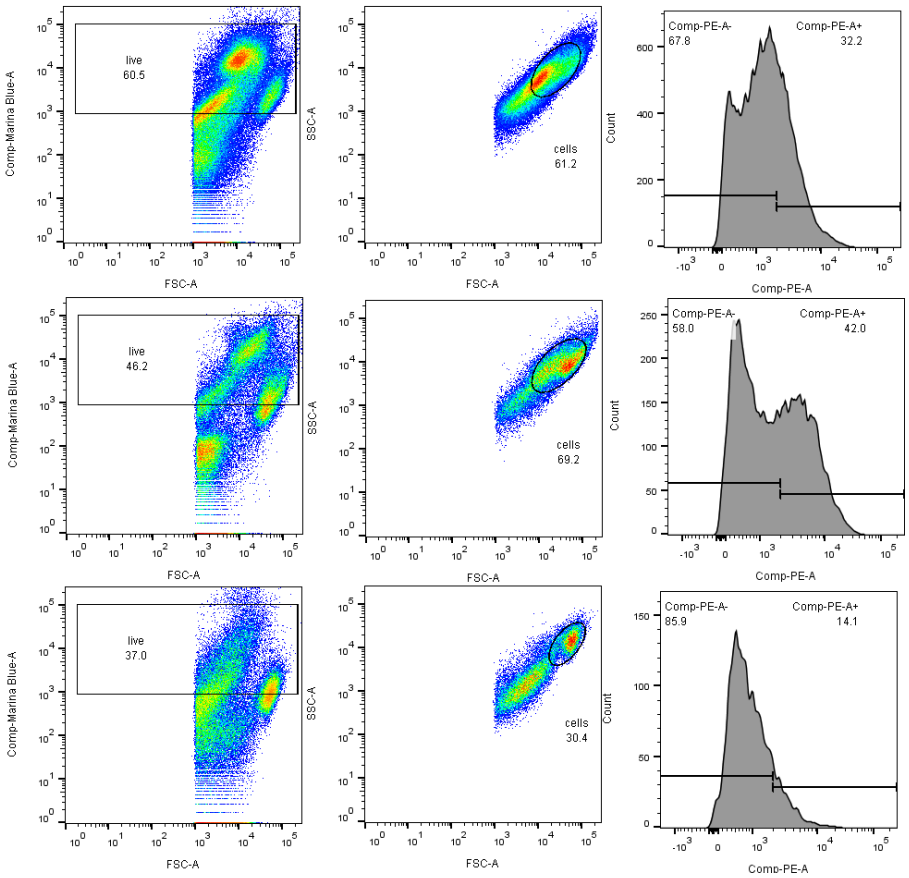
Day 14:



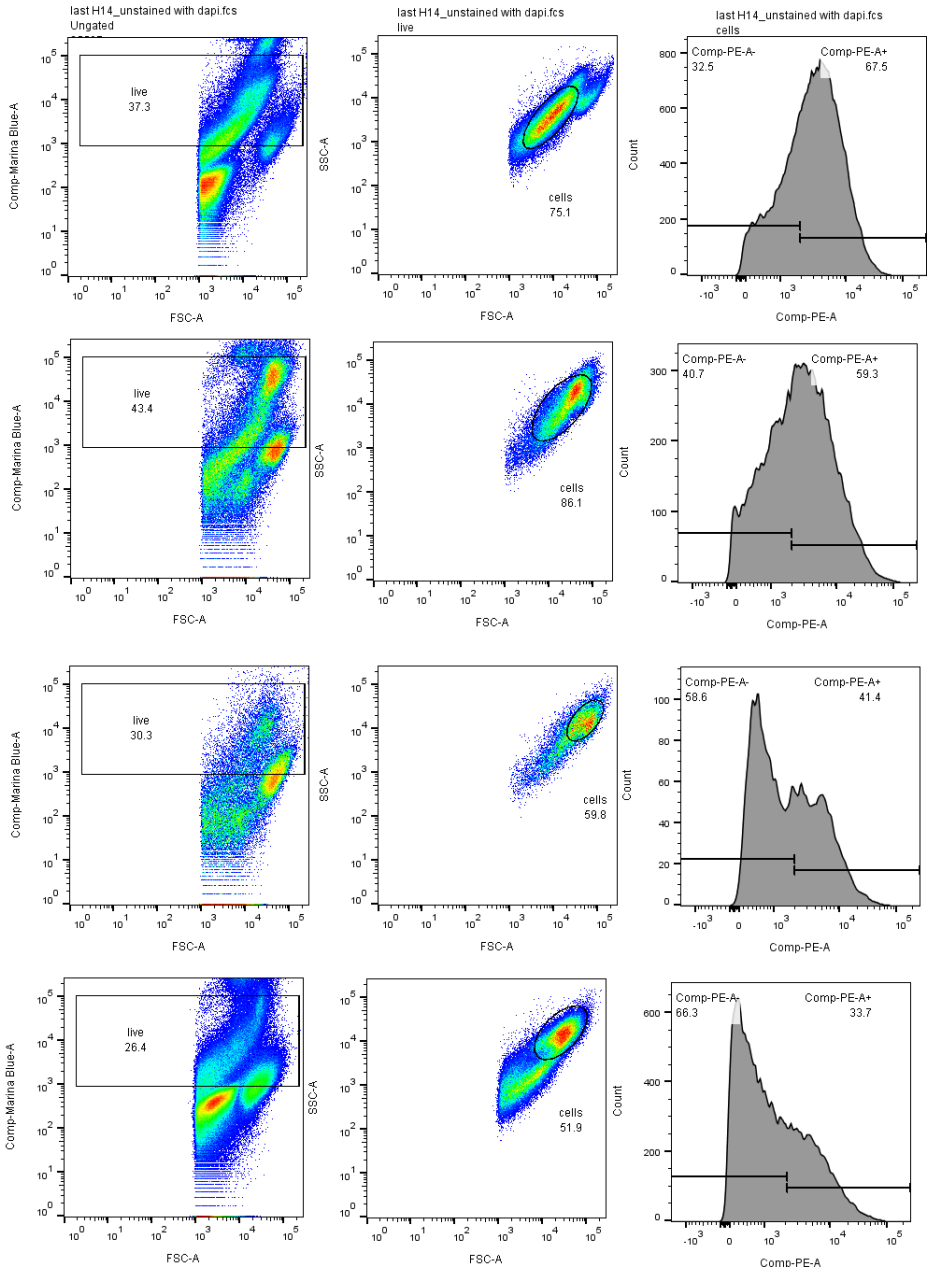
Isotype control



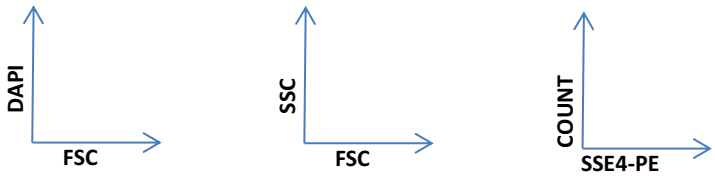
Normoxia Day 14 Samples



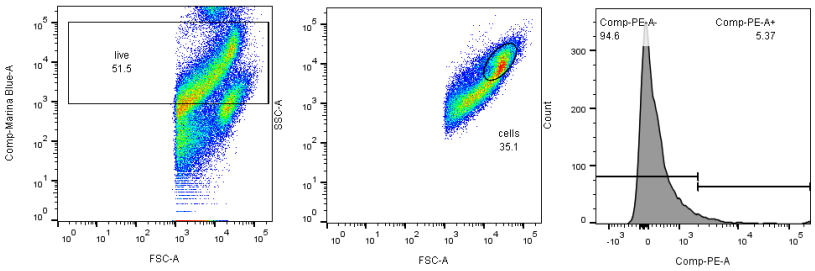
Hypoxia Day 14 Samples



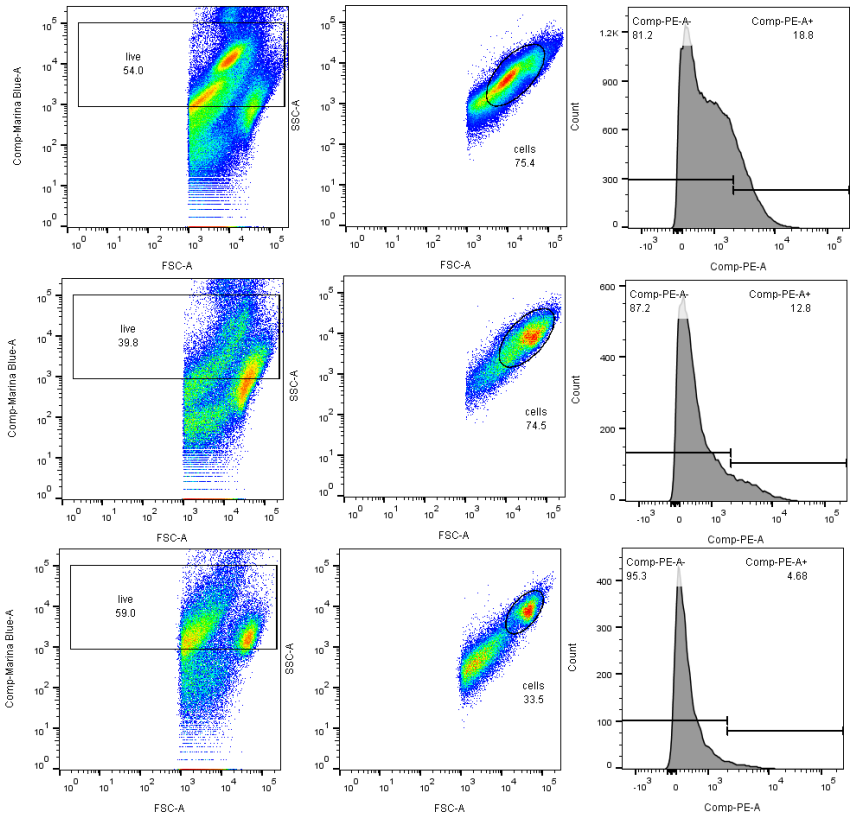
Day 21:



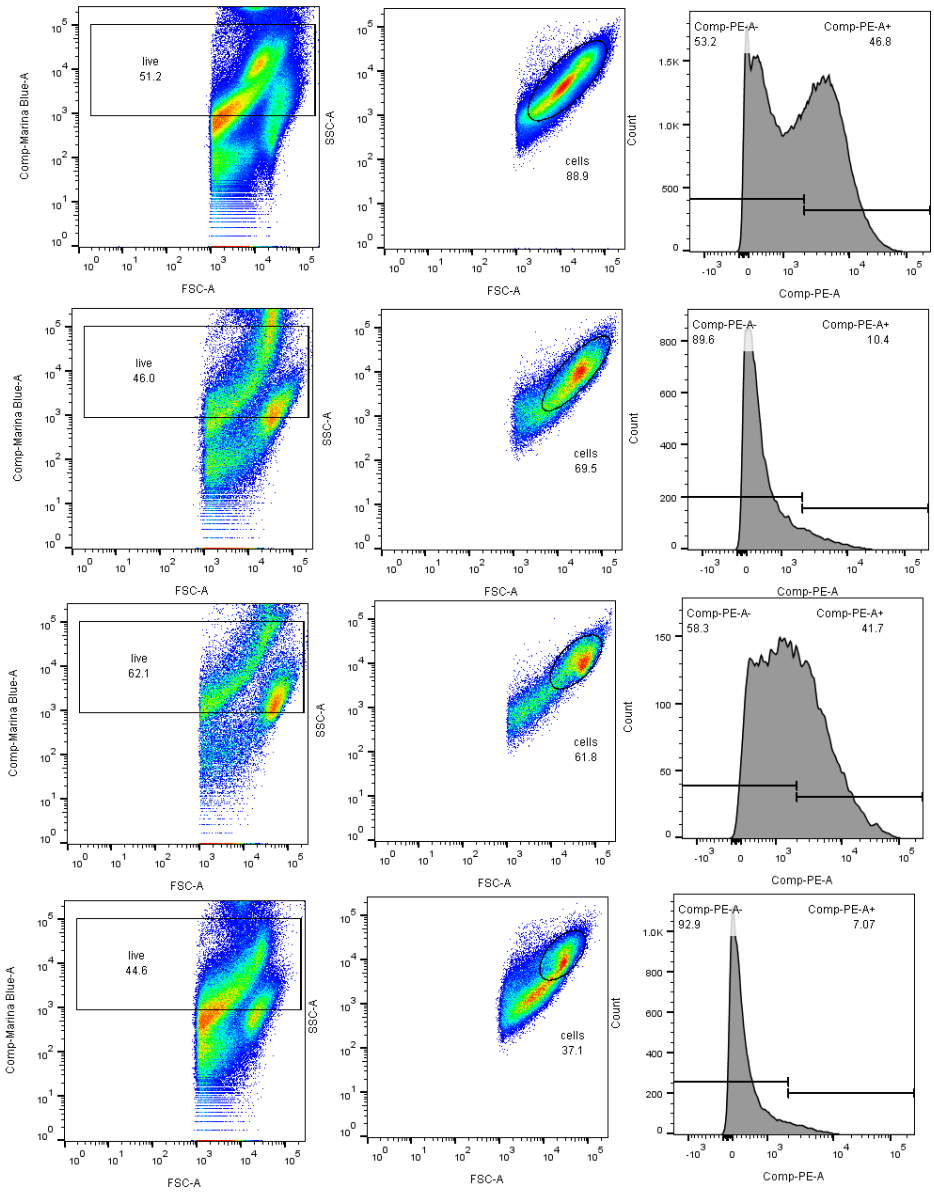
Isotype control



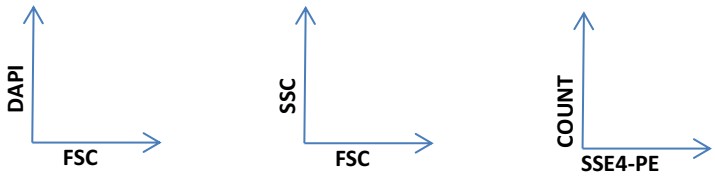
Normoxia Day 21 Samples



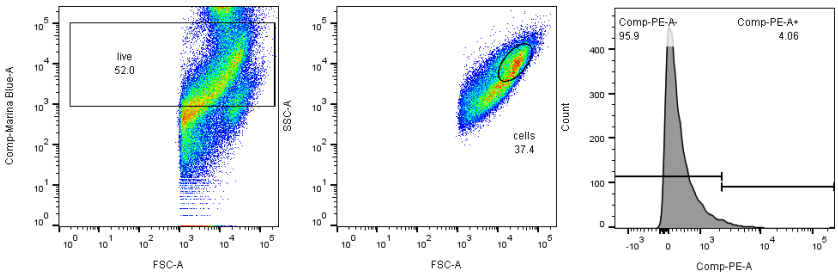
Hypoxia Day 21 Samples



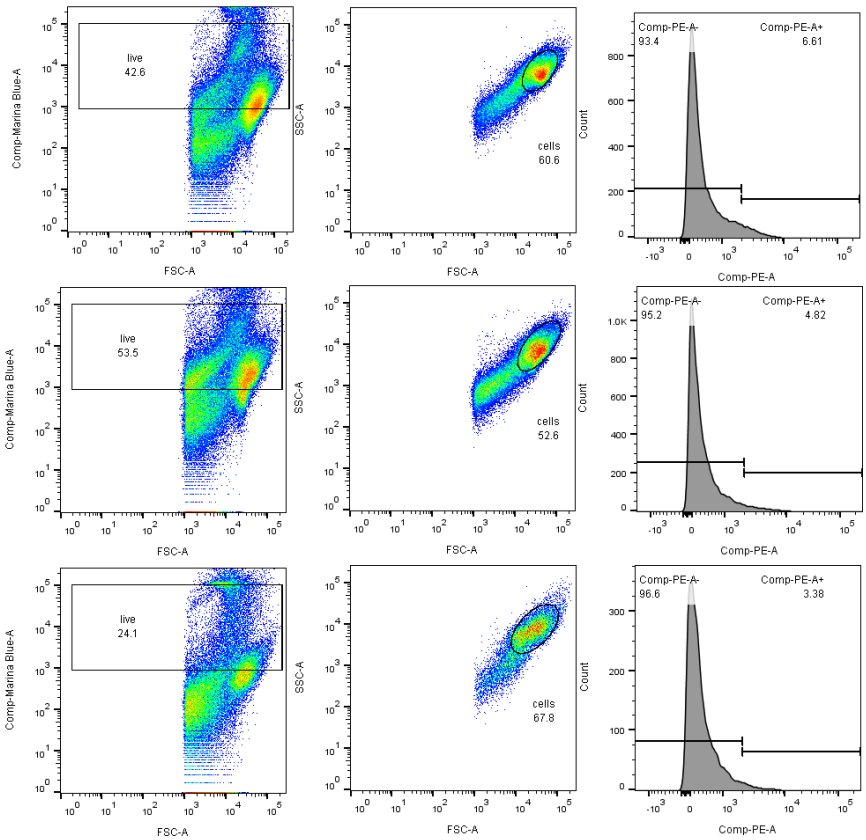
Day 28:



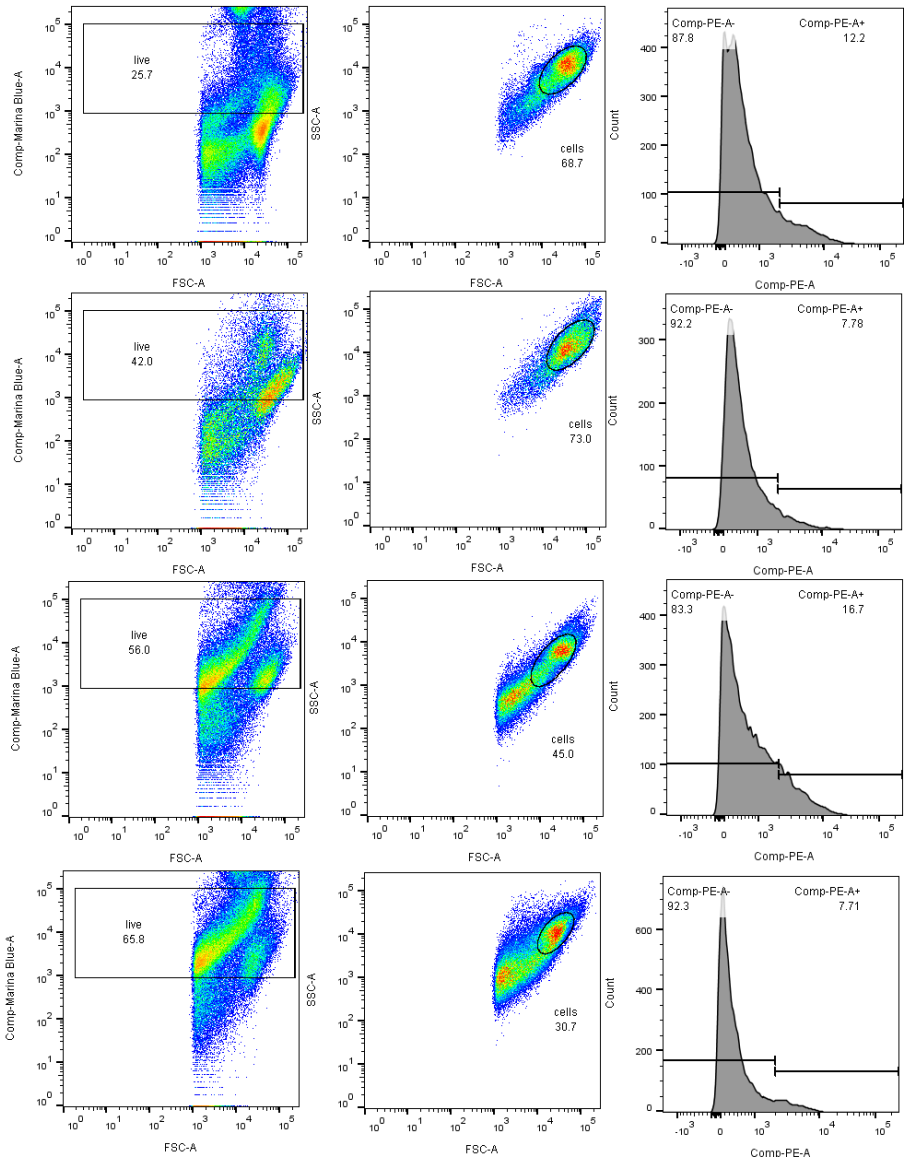
Isotype control



Normoxia Day 28 Samples

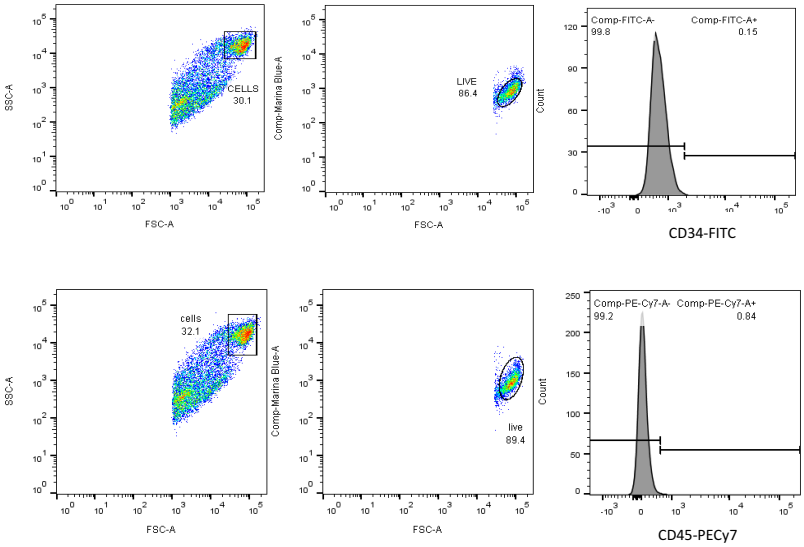


Hypoxia Day 28 Samples

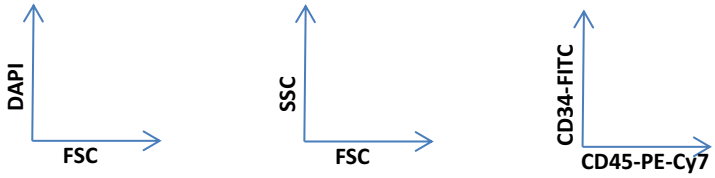


Appendix 8. Flow Cytometry Analysis of CD34-FITC and CD45-PE-Cy7 for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.

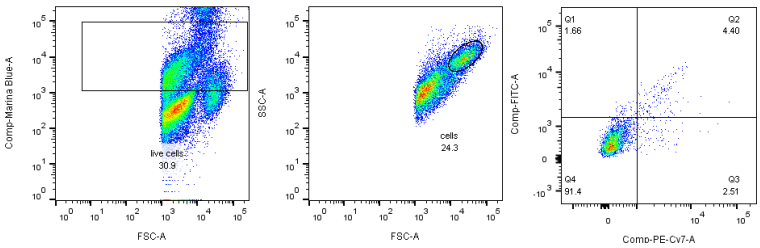
Day 0 iPS Cells



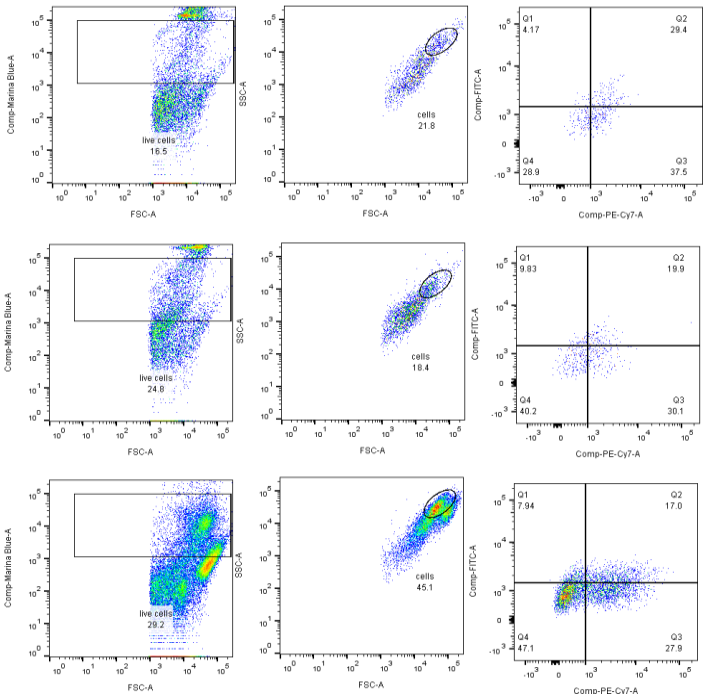
Day 7:



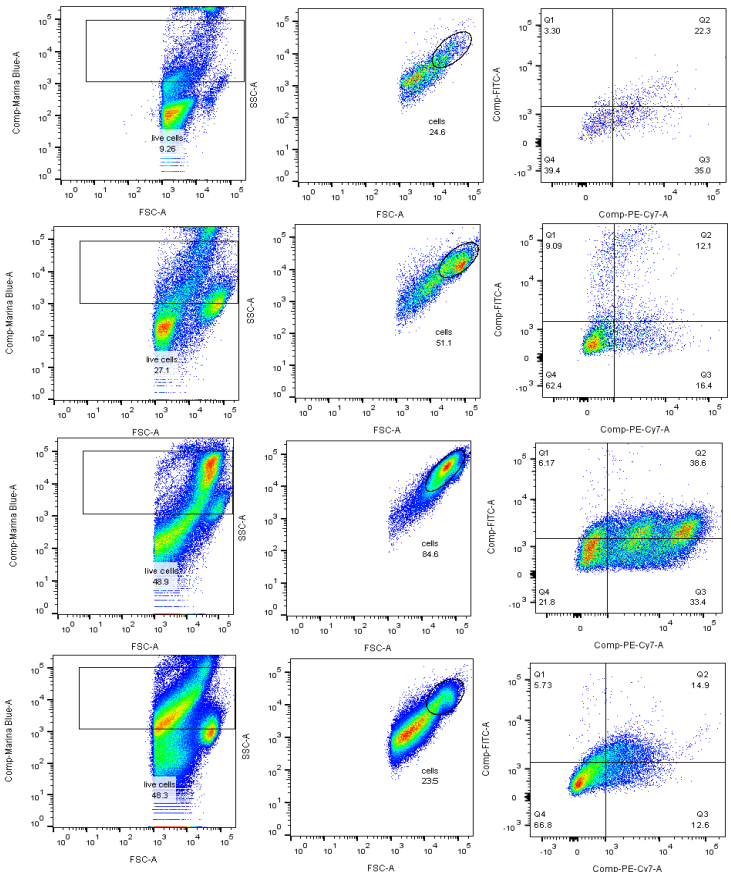
Isotype control



Normoxia Day 7 Samples



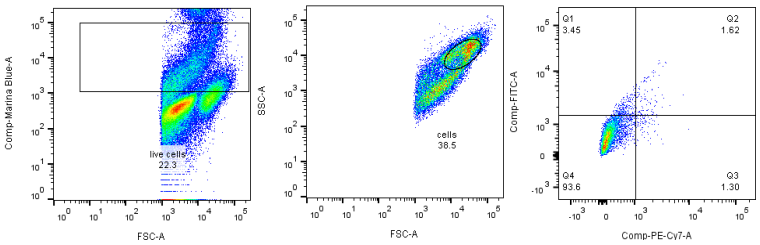
Hypoxia Day 7 Samples:



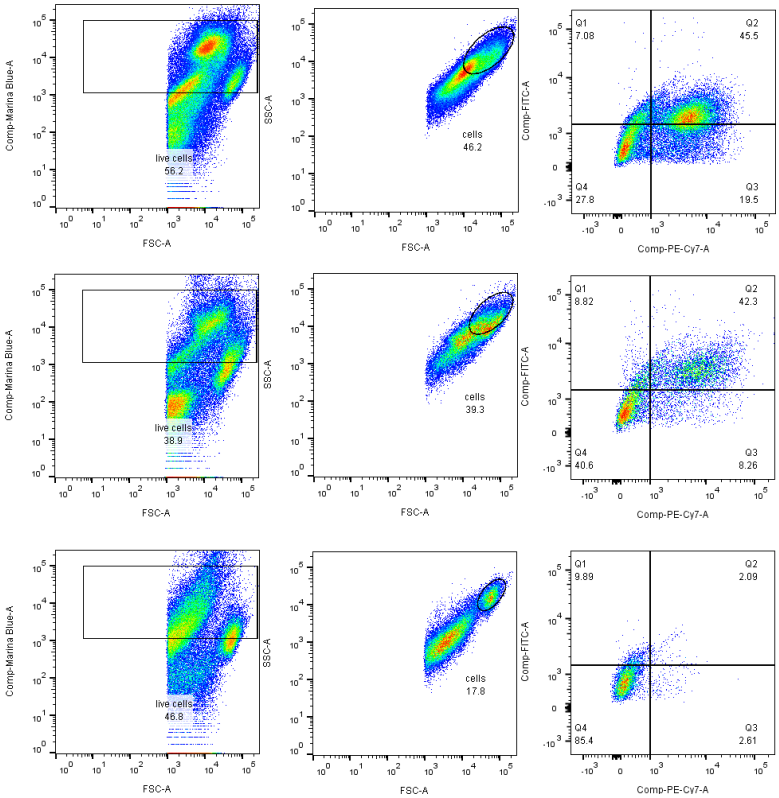
Day 14:



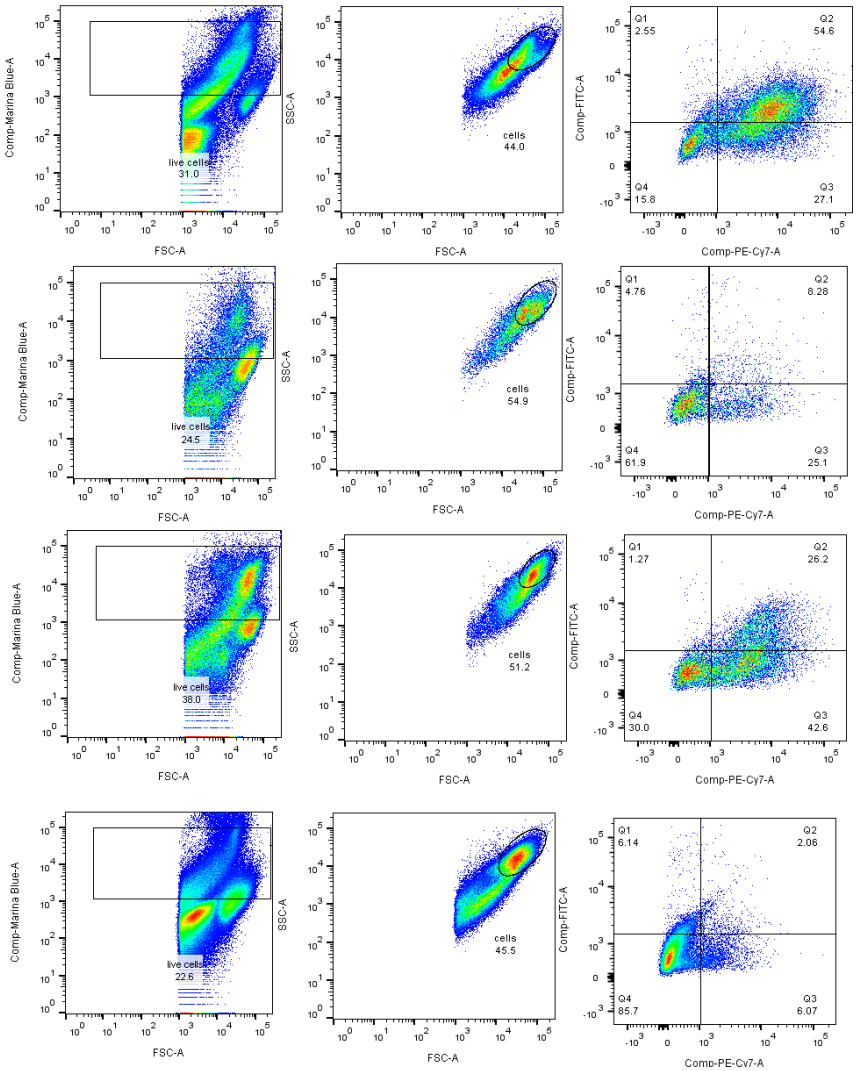
Isotype control



Normoxia Day 14 Samples:



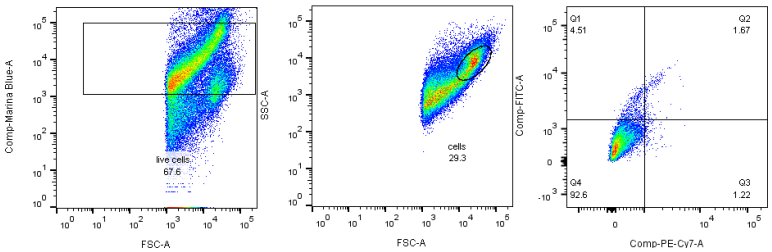
Hypoxia Day 14 Samples:



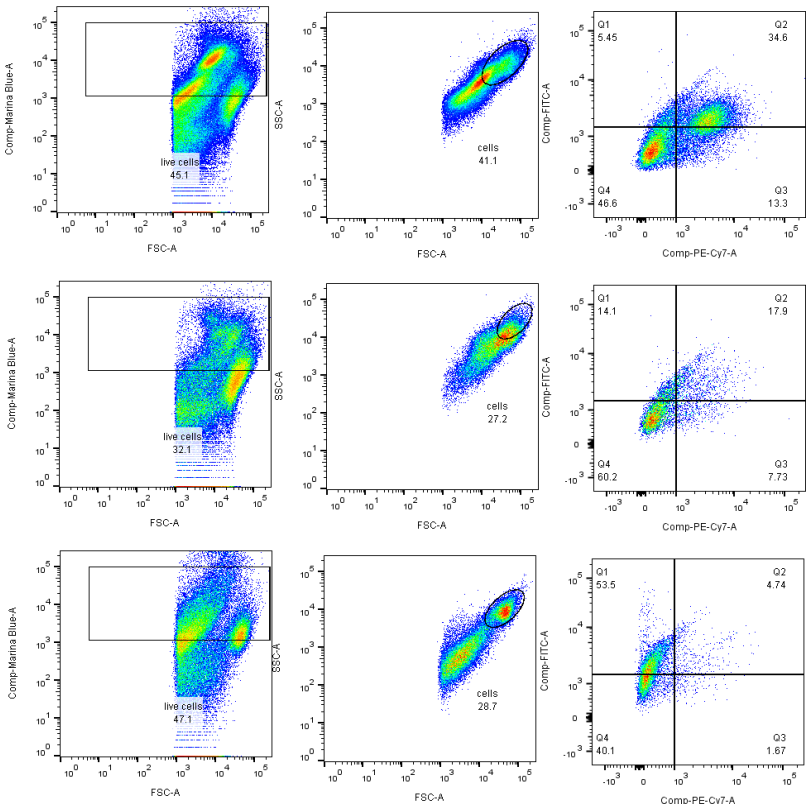
Day 21:



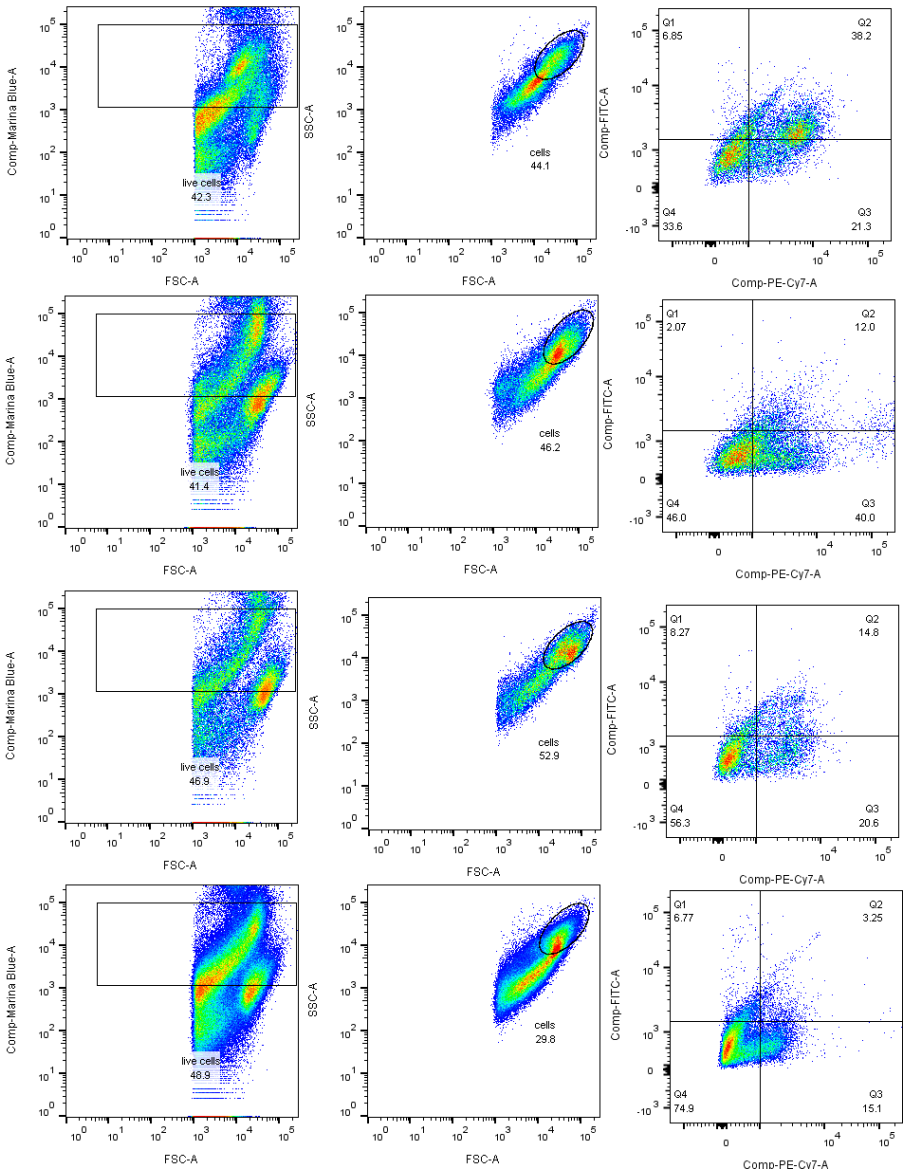
Isotype control



Normoxia Day 21 Samples:



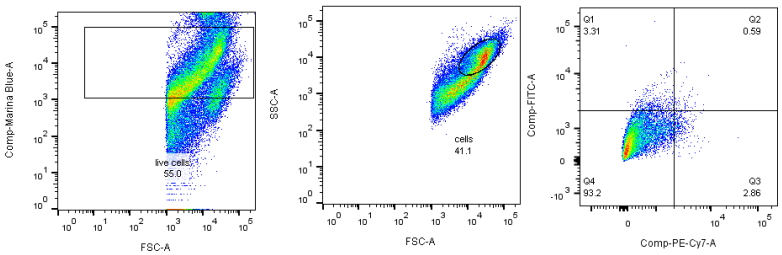
Hypoxia Day 21 Samples:



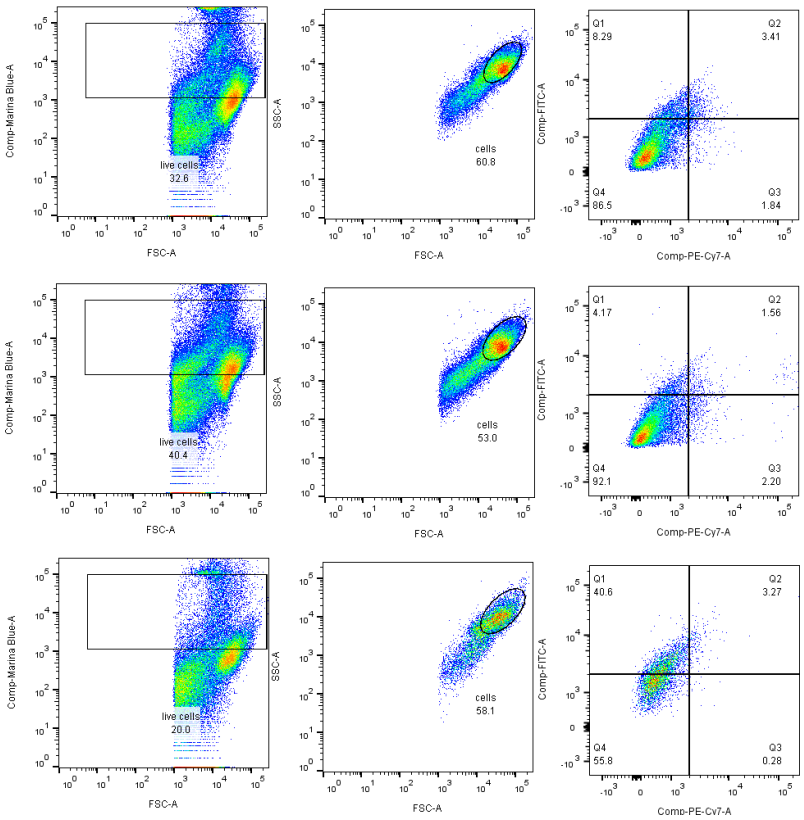
Day 28:



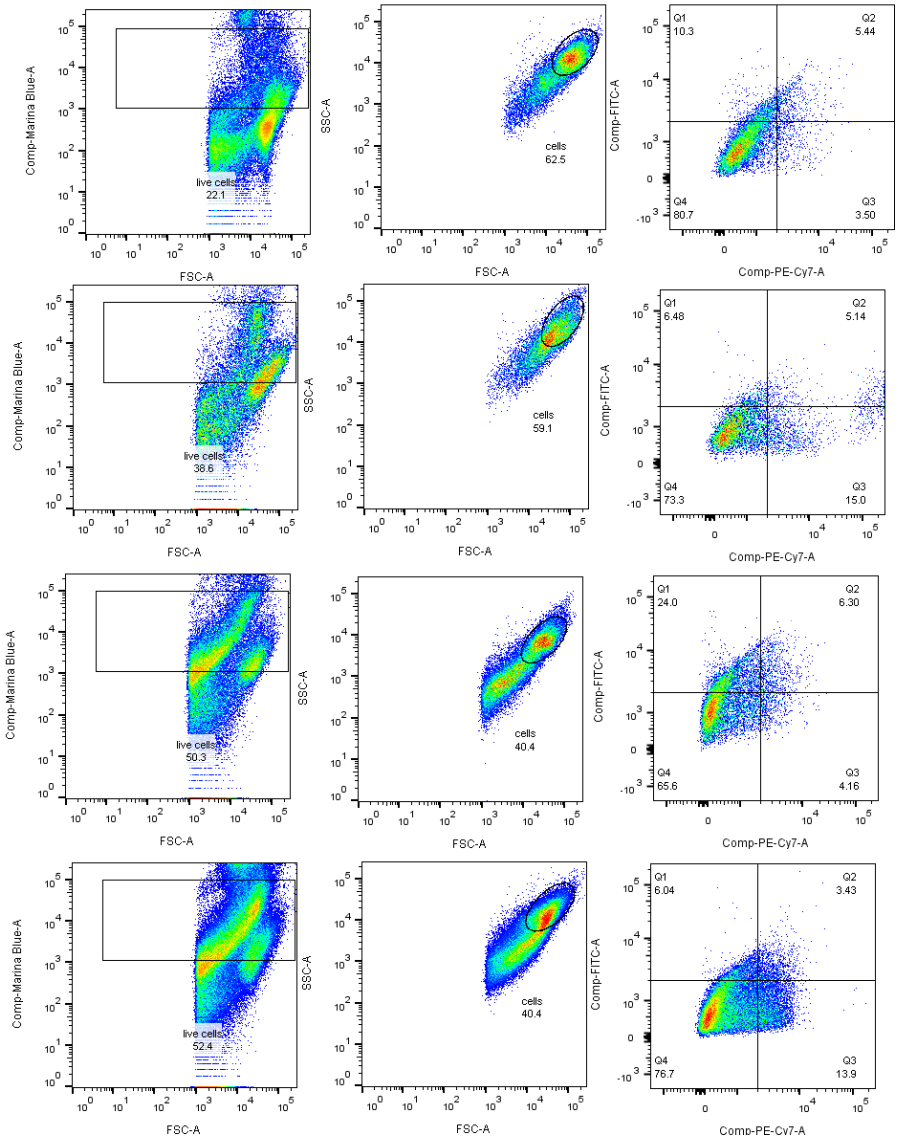
Isotype control



Normoxia Day 28 Samples:

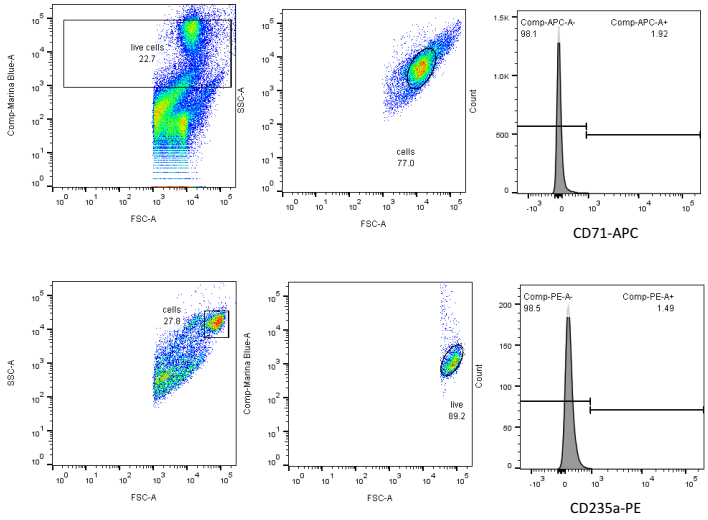


Hypoxia Day 28 Samples:

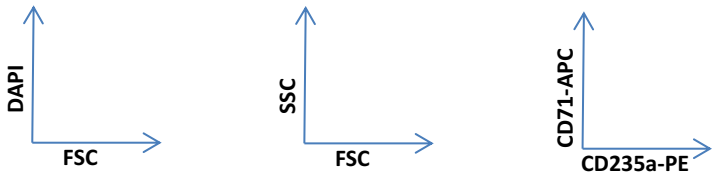


Appendix 9. Flow Cytometry Analysis of CD71-APC and CD235a-PE for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.

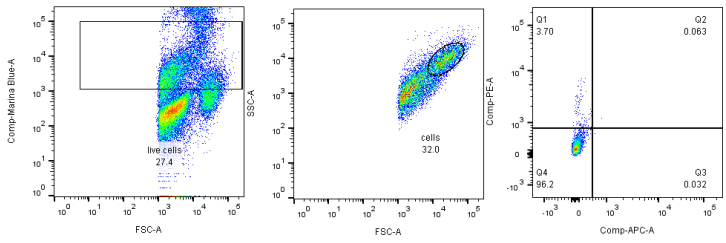
Day 0 iPS Cells



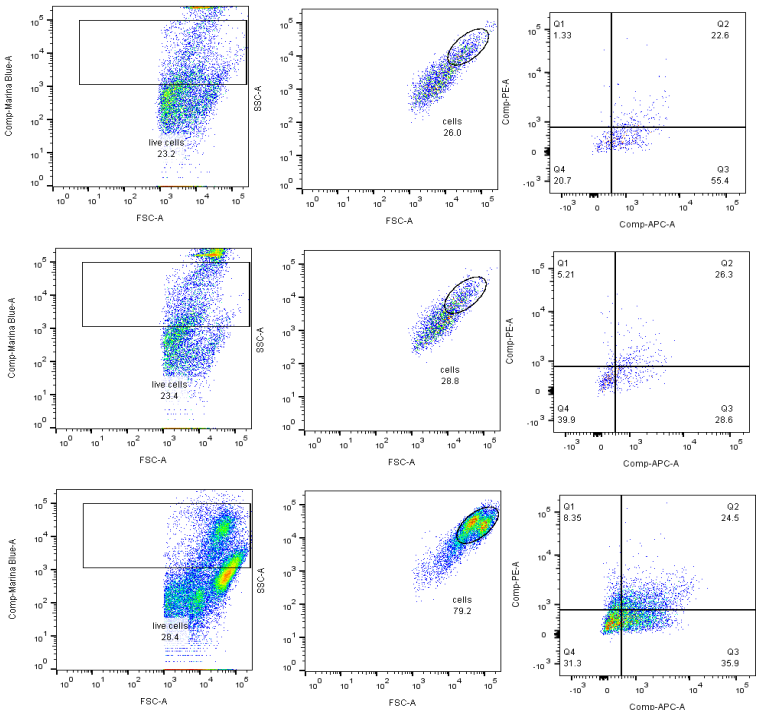
Day 7:



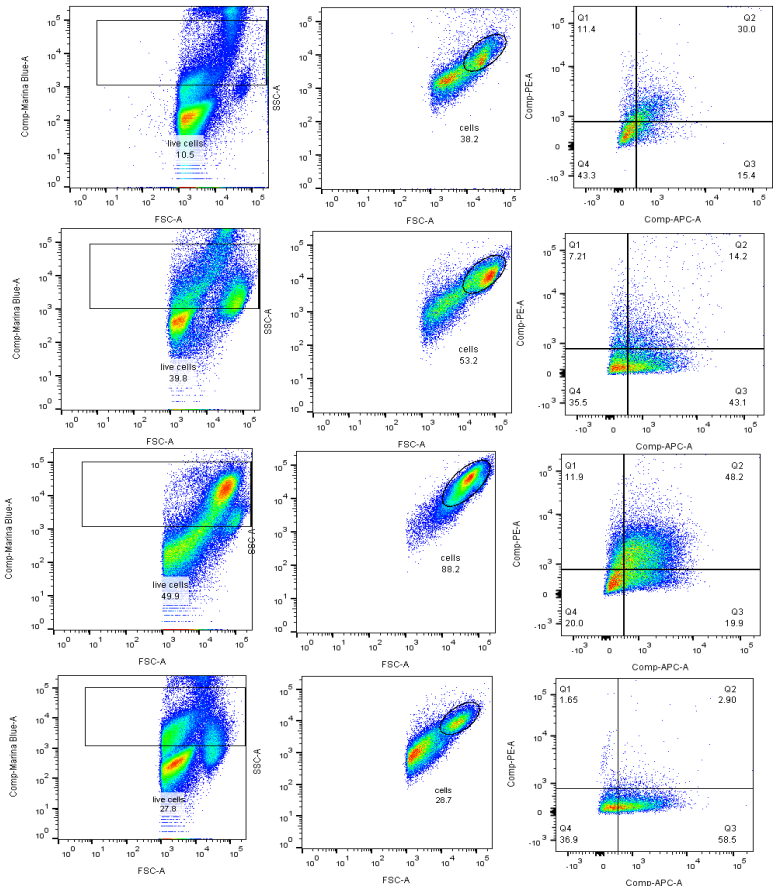
Isotype control



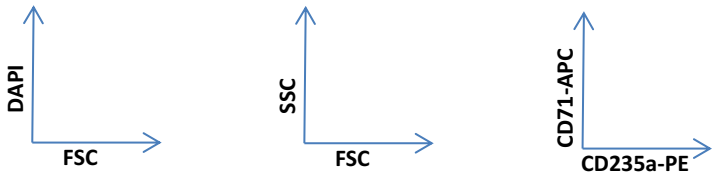
Normoxia Day 7 Samples:



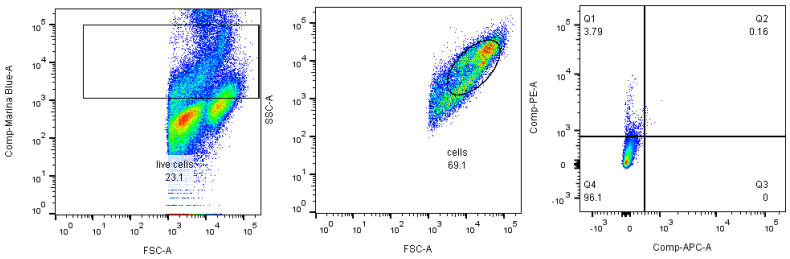
Hypoxia Day 7 Samples:



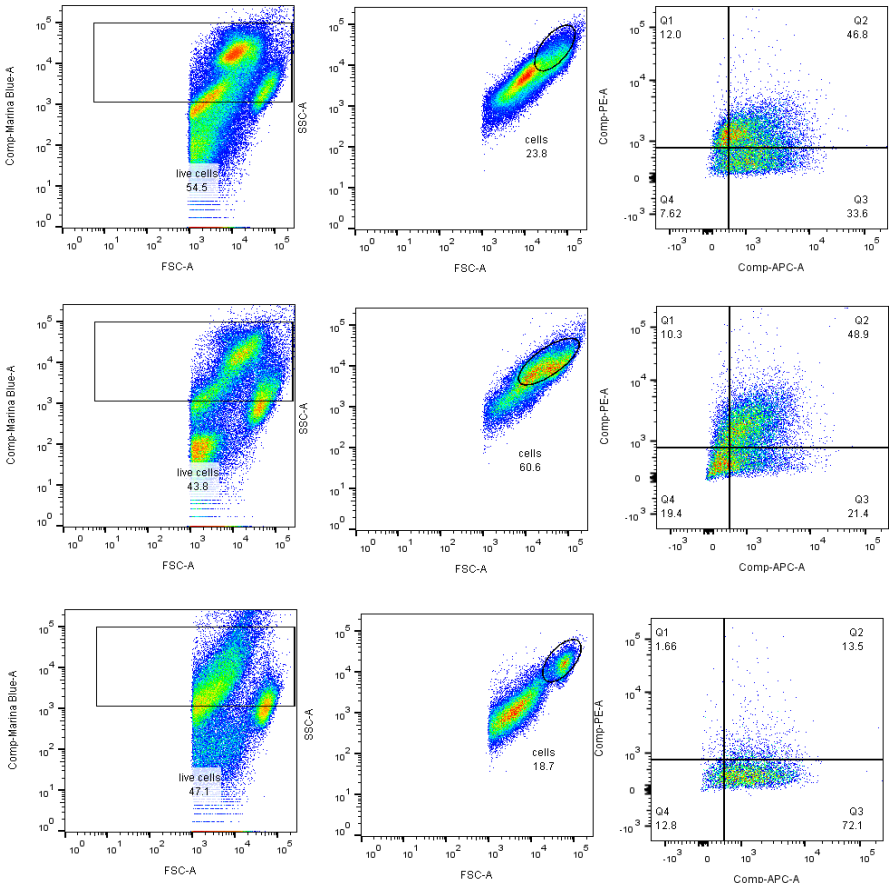
Day 14:



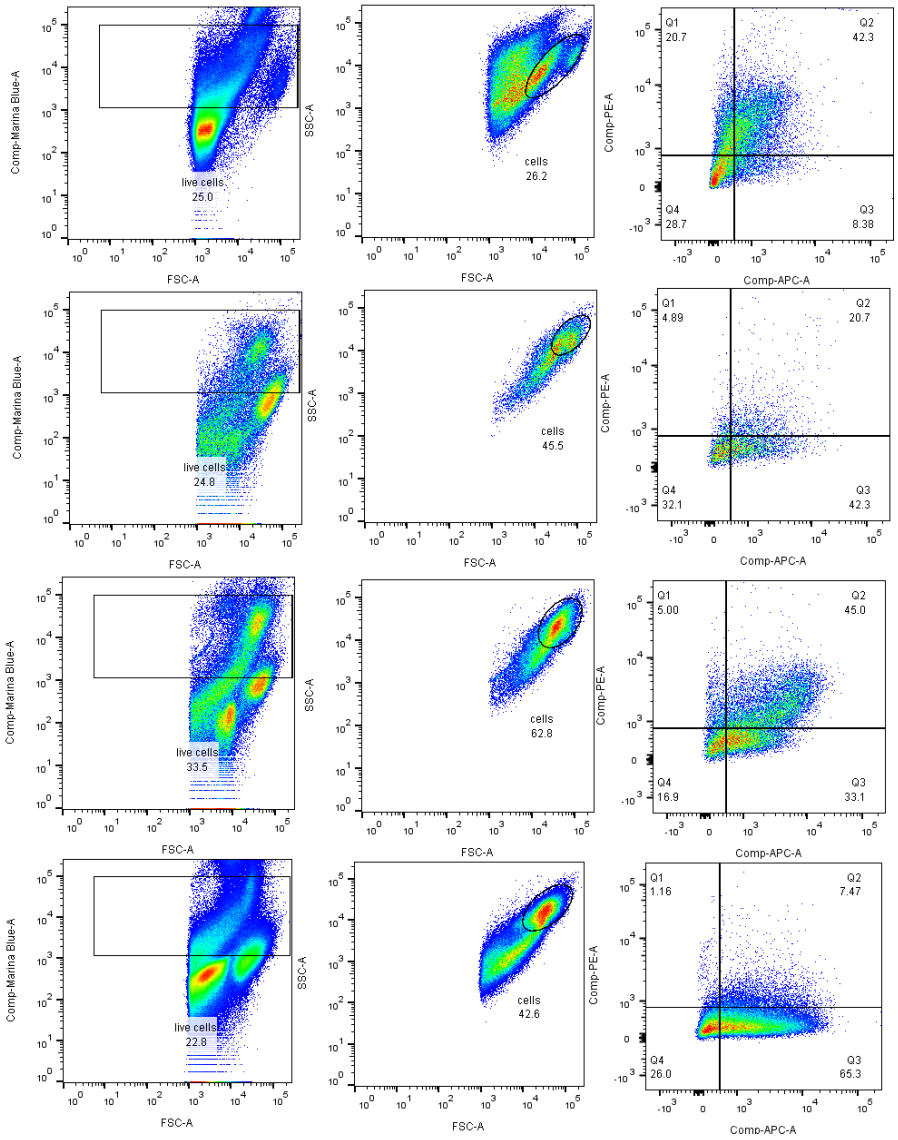
Isotype control



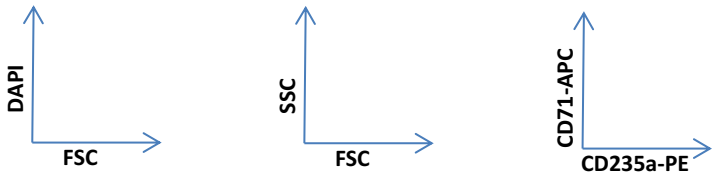
Normoxia Day 14 Samples:



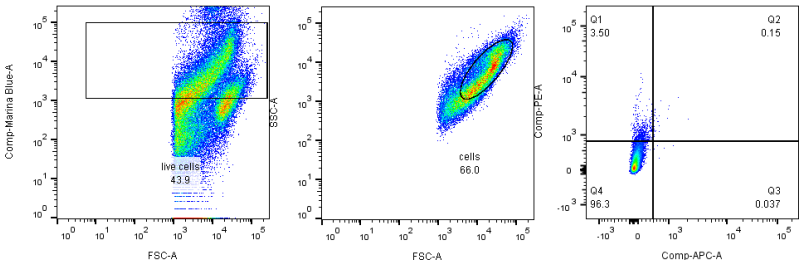
Hypoxia Day 14 Samples:



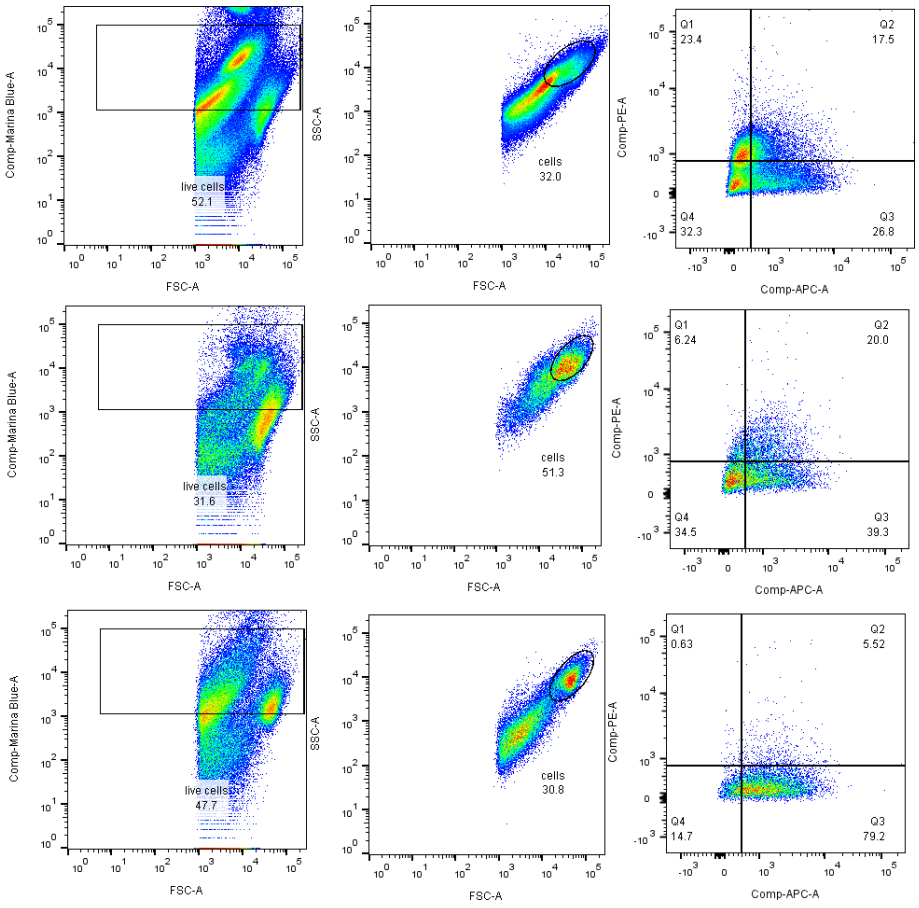
Day 21:



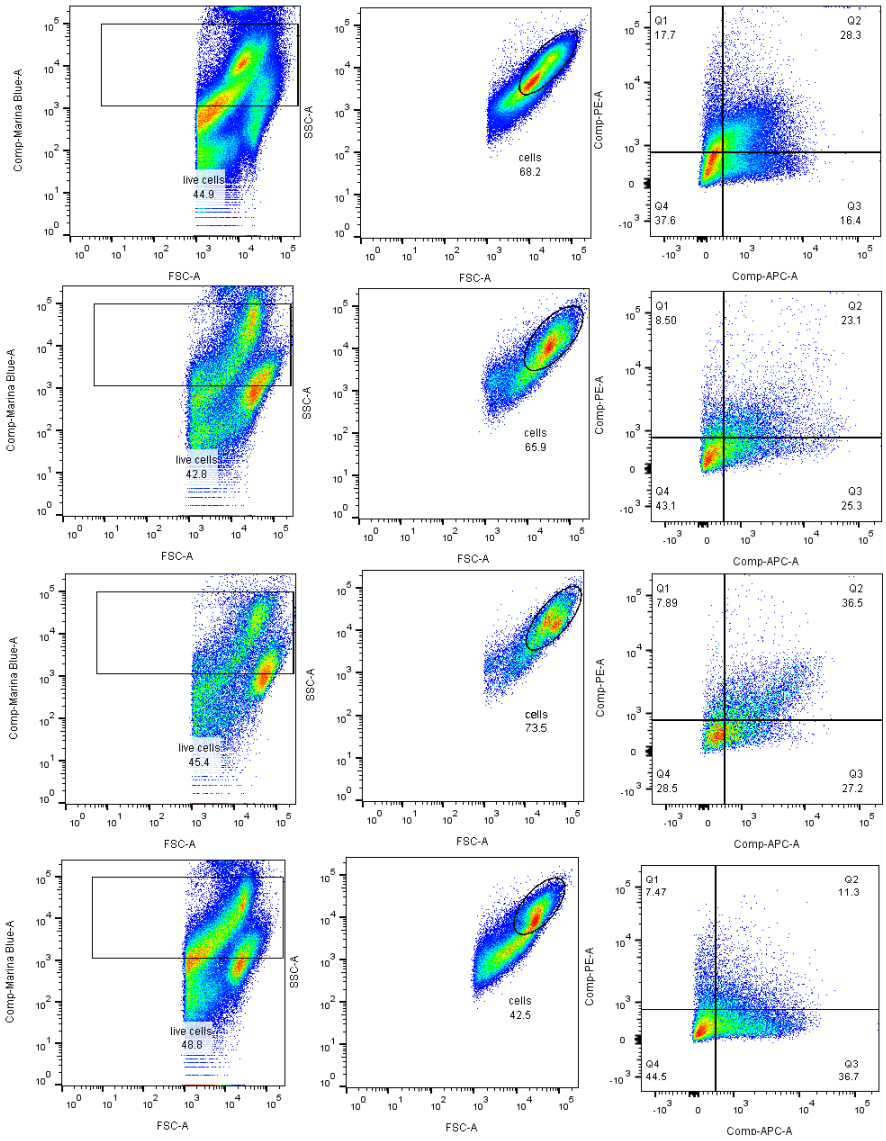
Isotype control



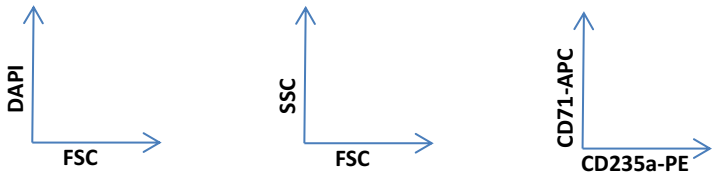
Normoxia Day 21 Samples:



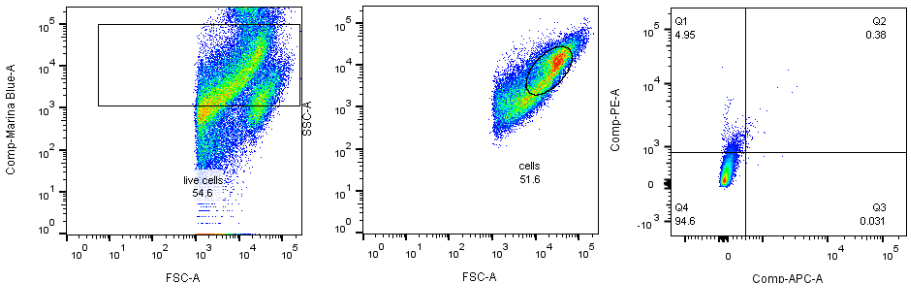
Hypoxia Day 21 Samples:



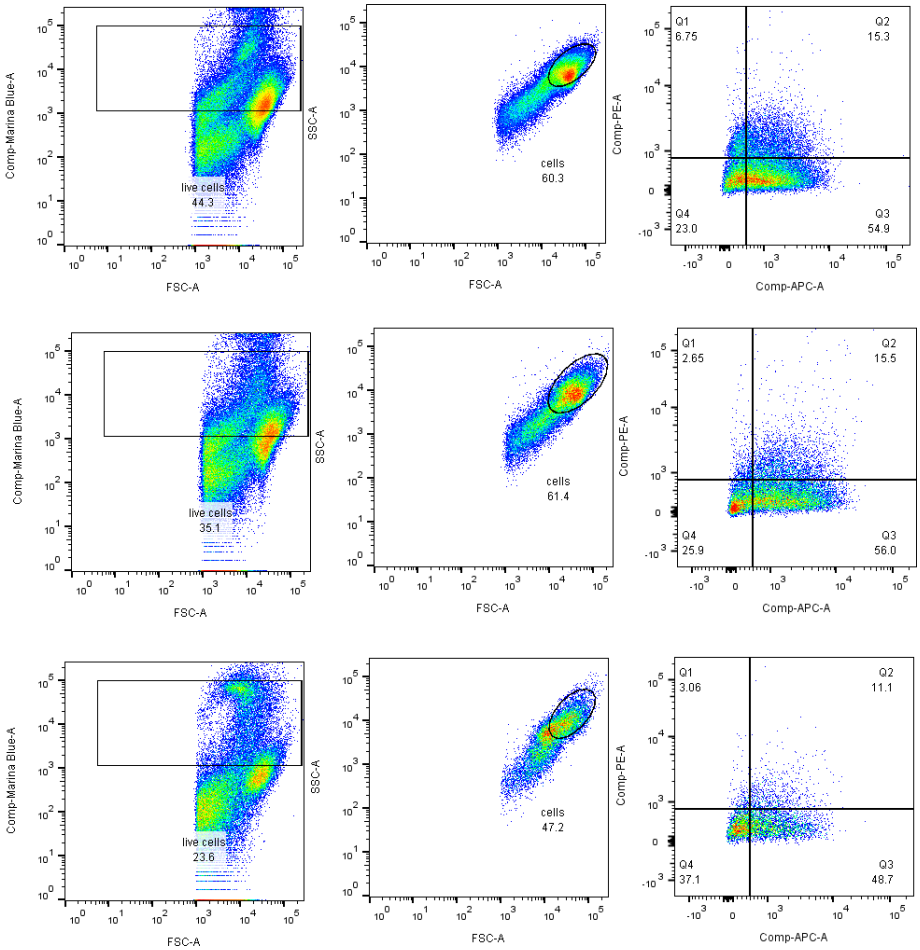
Day 28:



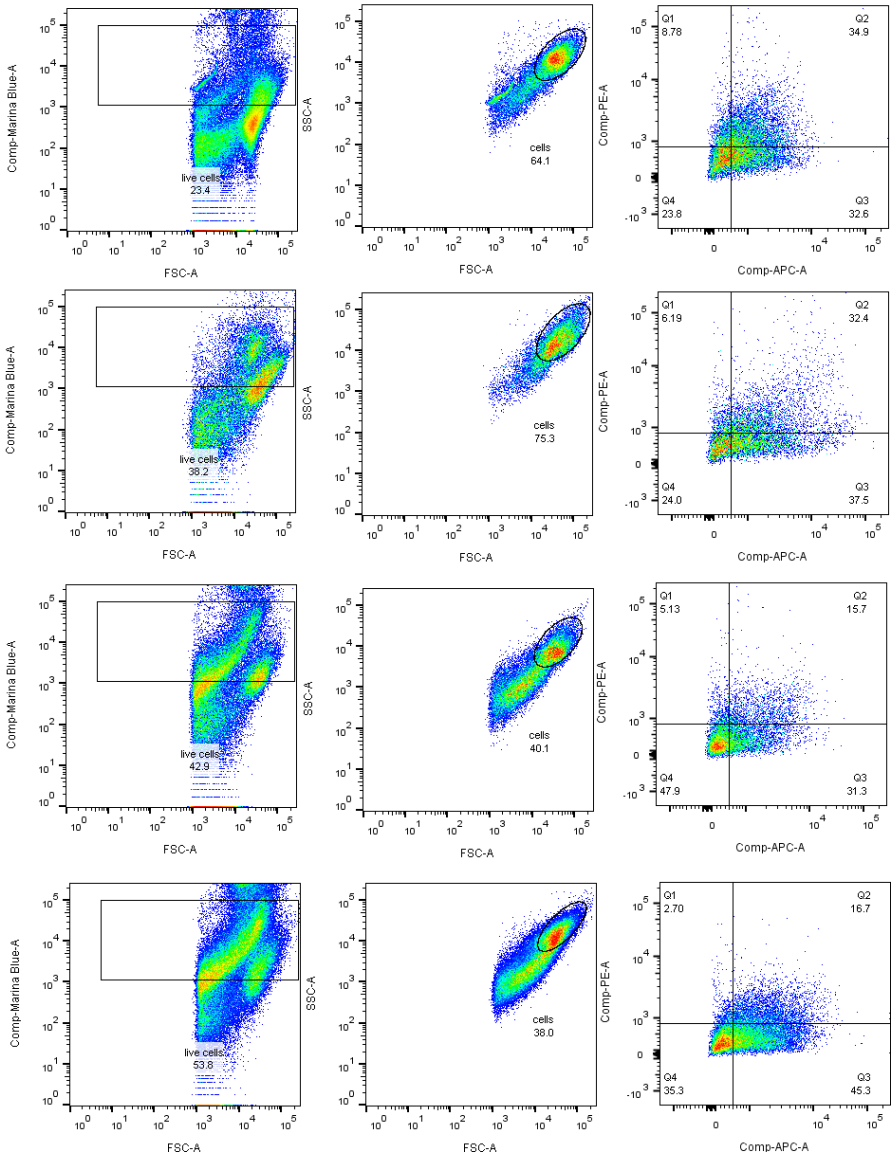
Isotype control



Normoxia Day 28 Samples:

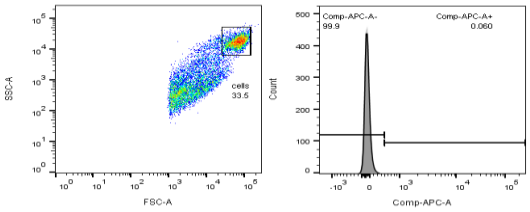


Hypoxia Day 28 Samples:



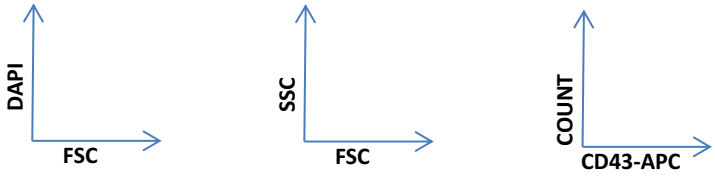
Appendix 10. Flow Cytometry Analysis of CD43-APC for Hematopoietic/Erythropoietic Differentiation of Human iPS Cells.

Day 0 iPS Cells

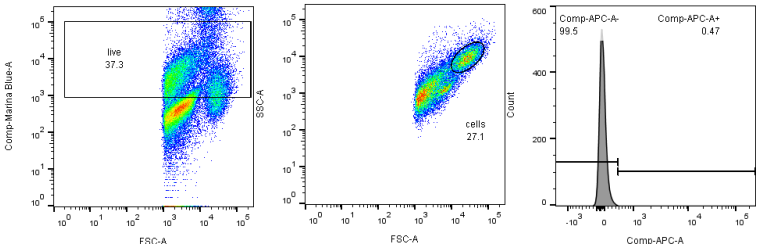


CD43-APC

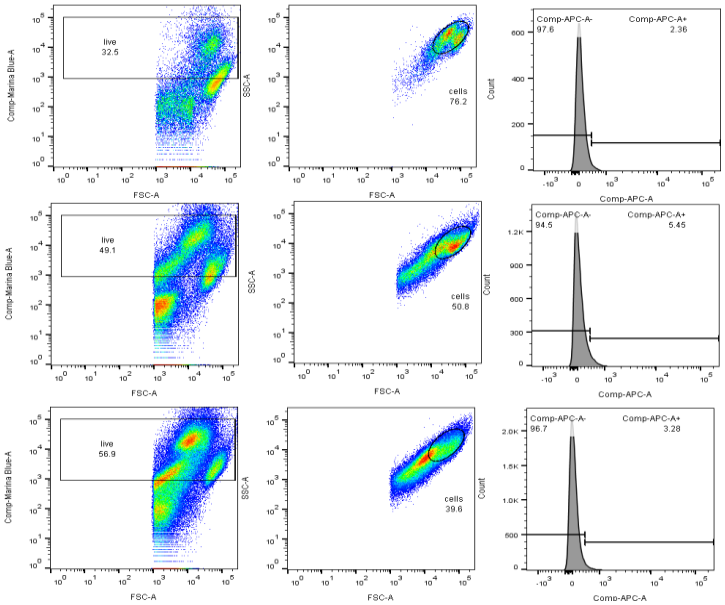
Day 7:



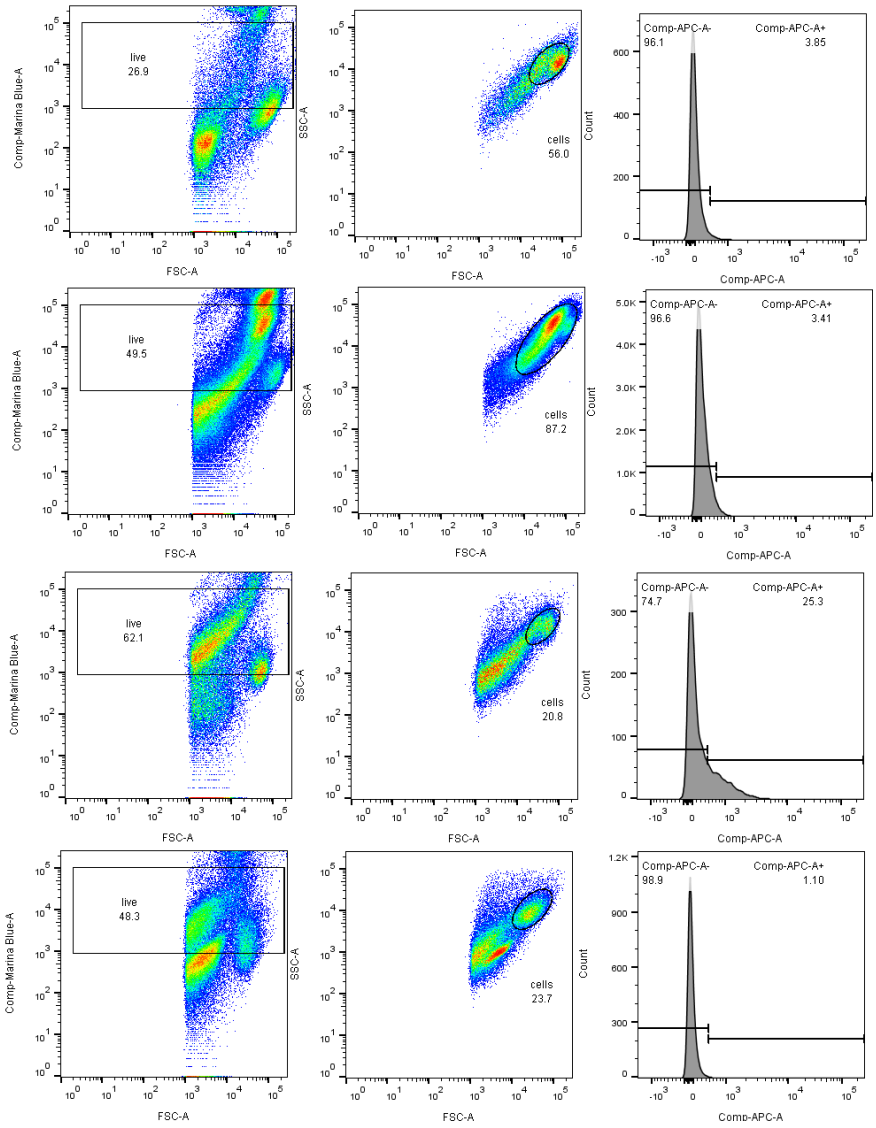
Isotype control



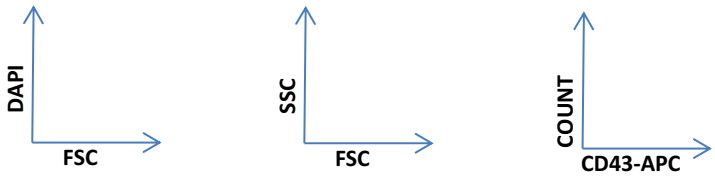
Normoxia Day 7 Samples



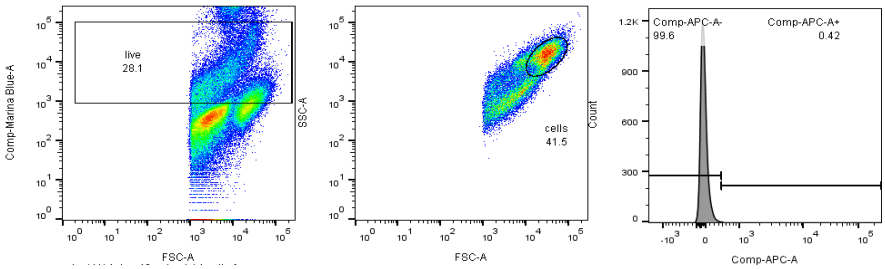
Hypoxia Day 7 Samples



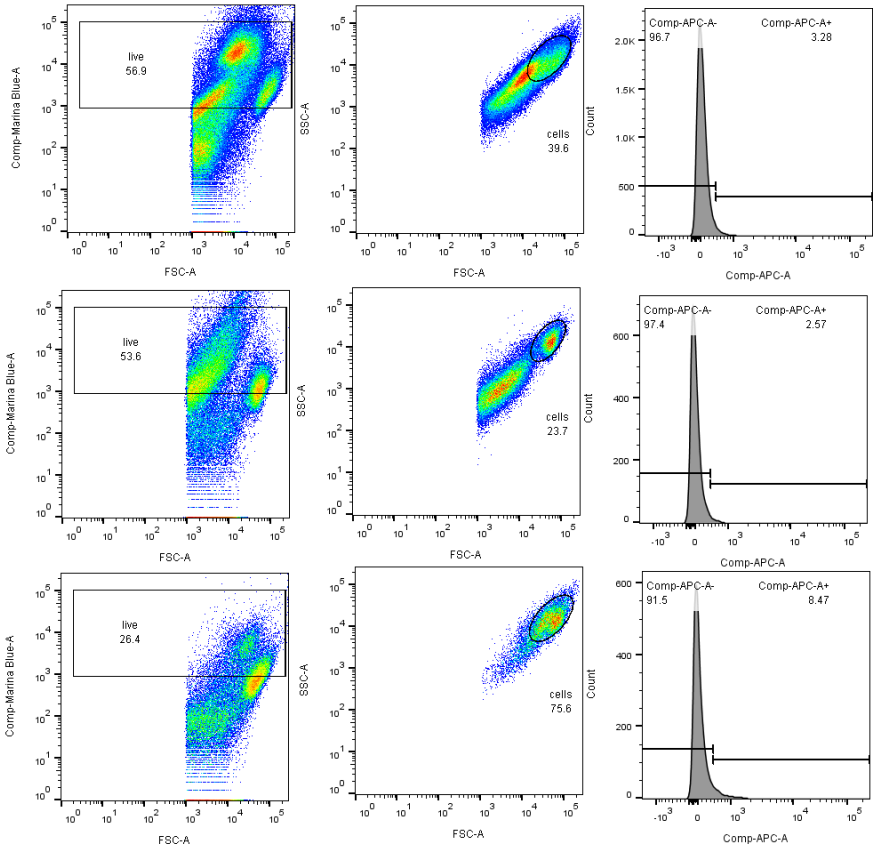
Day 14:



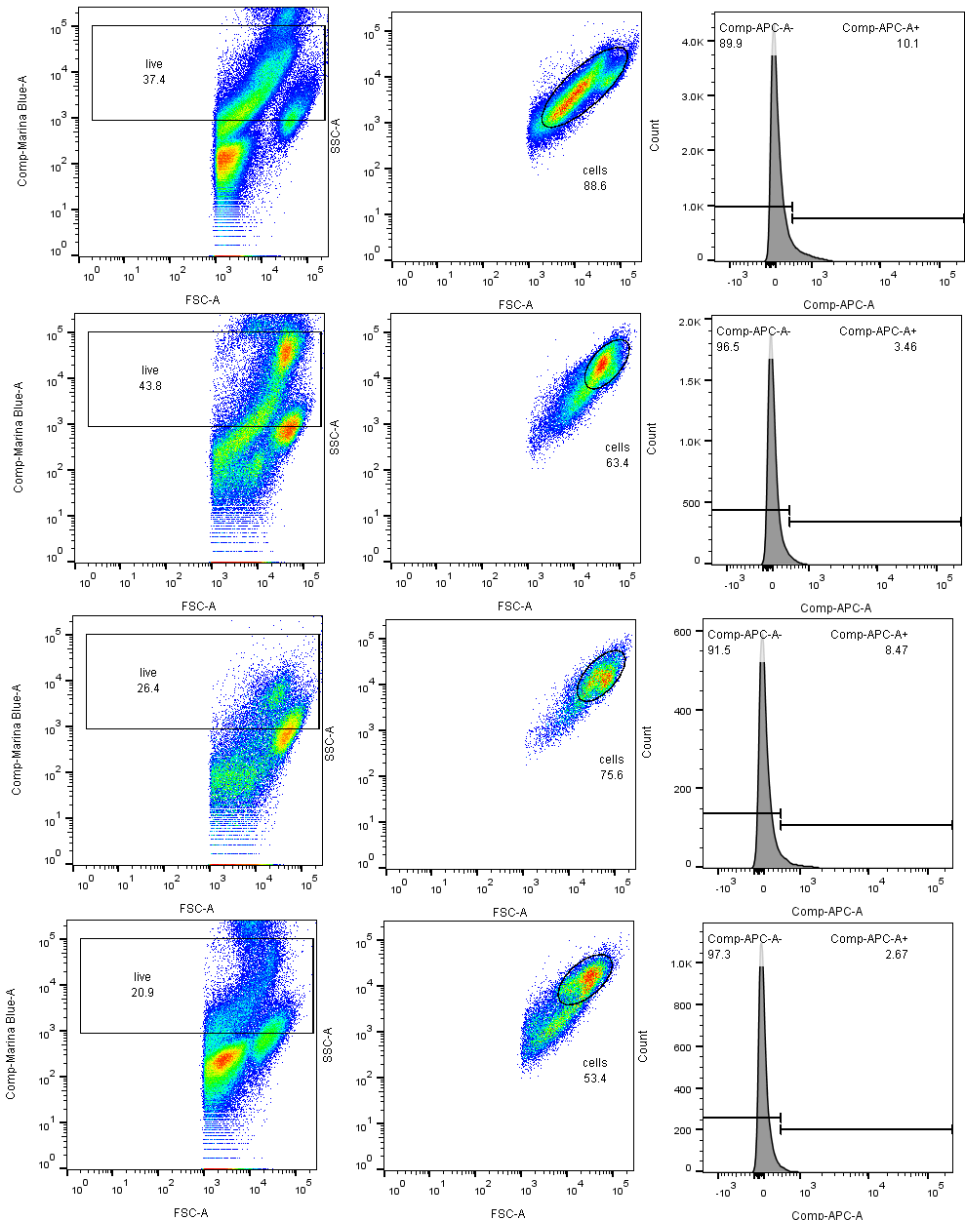
Isotype control



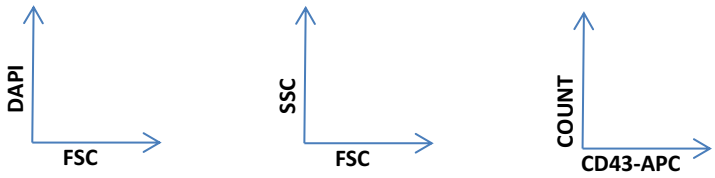
Normoxia Day 14 Samples



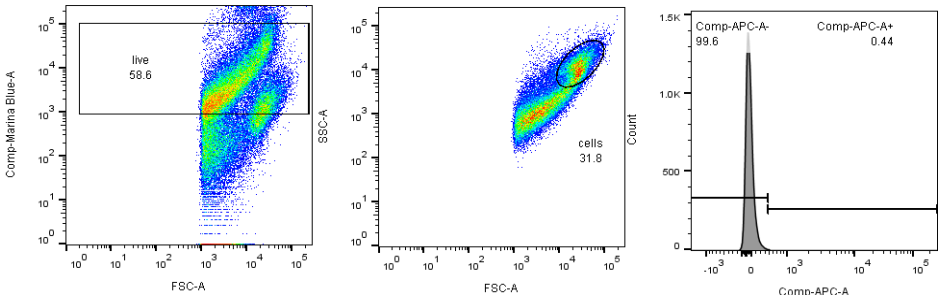
Hypoxia Day 14 Samples



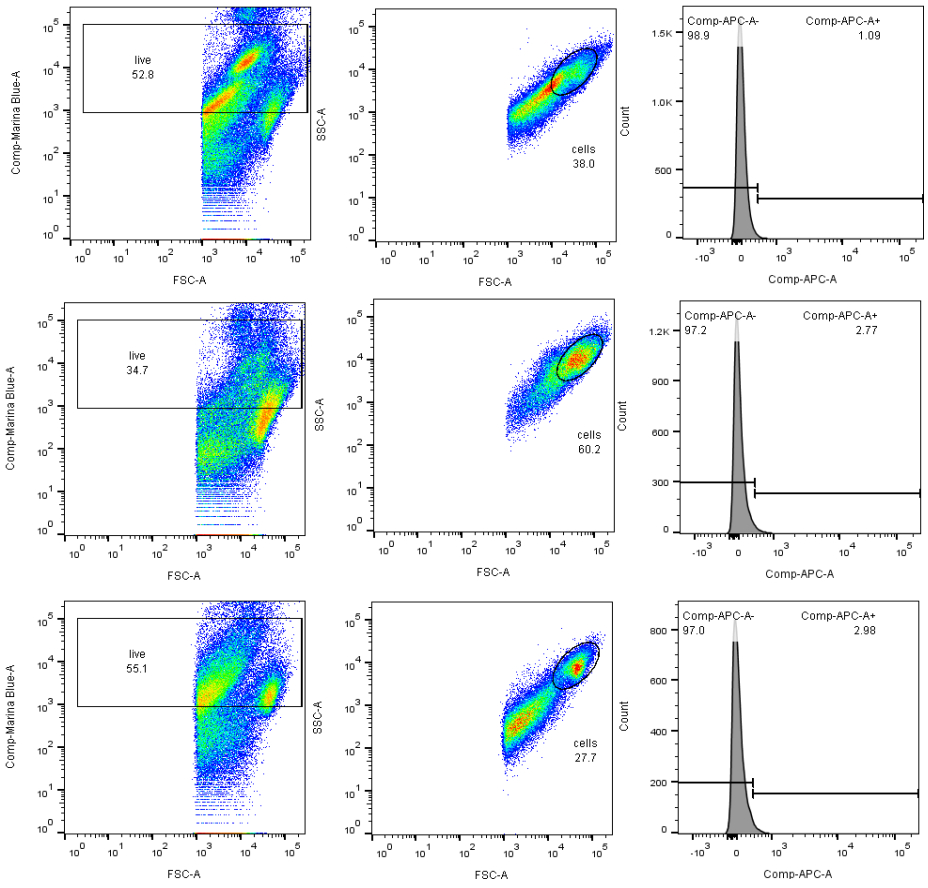
Day 21:



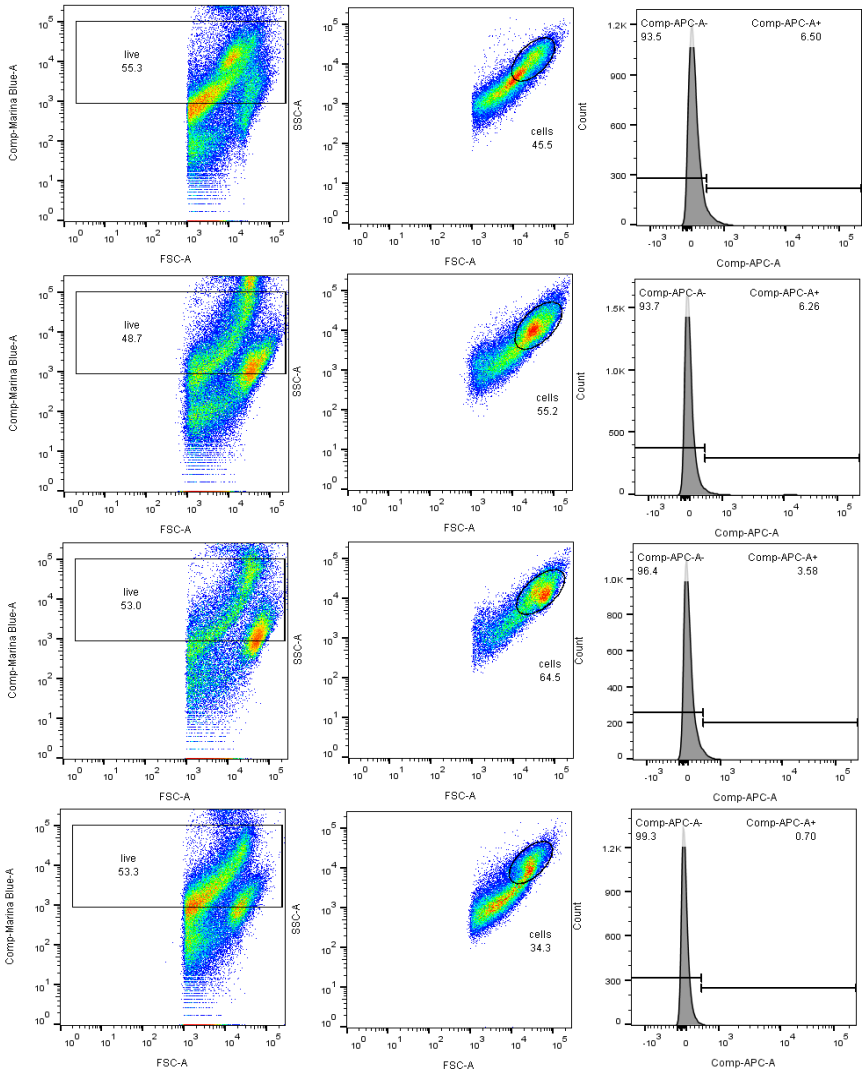
Isotype control



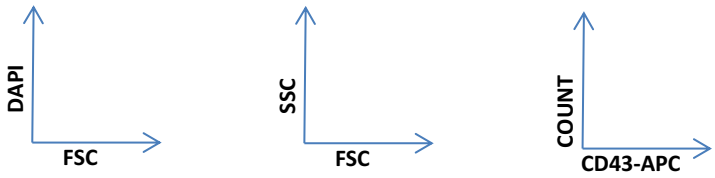
Normoxia Day 21 Samples



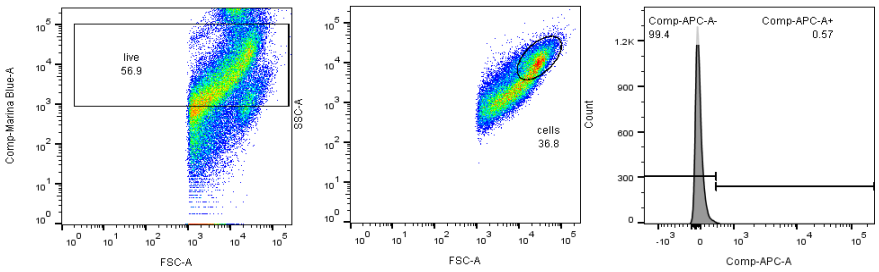
Hypoxia Day 21 Samples



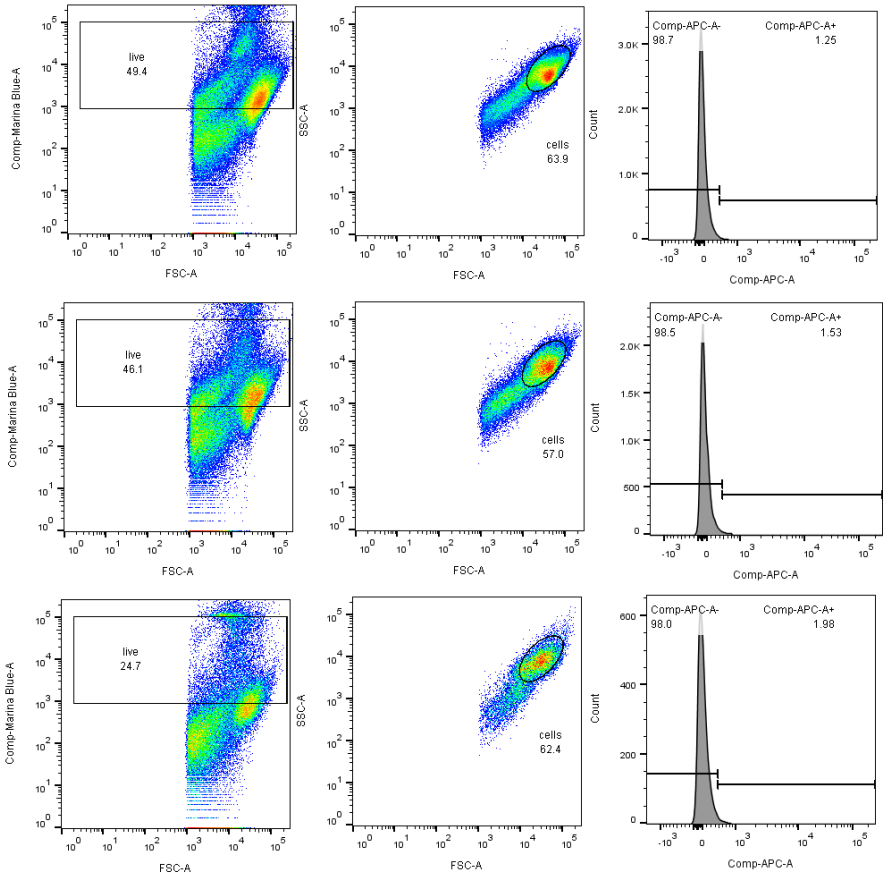
Day 28:



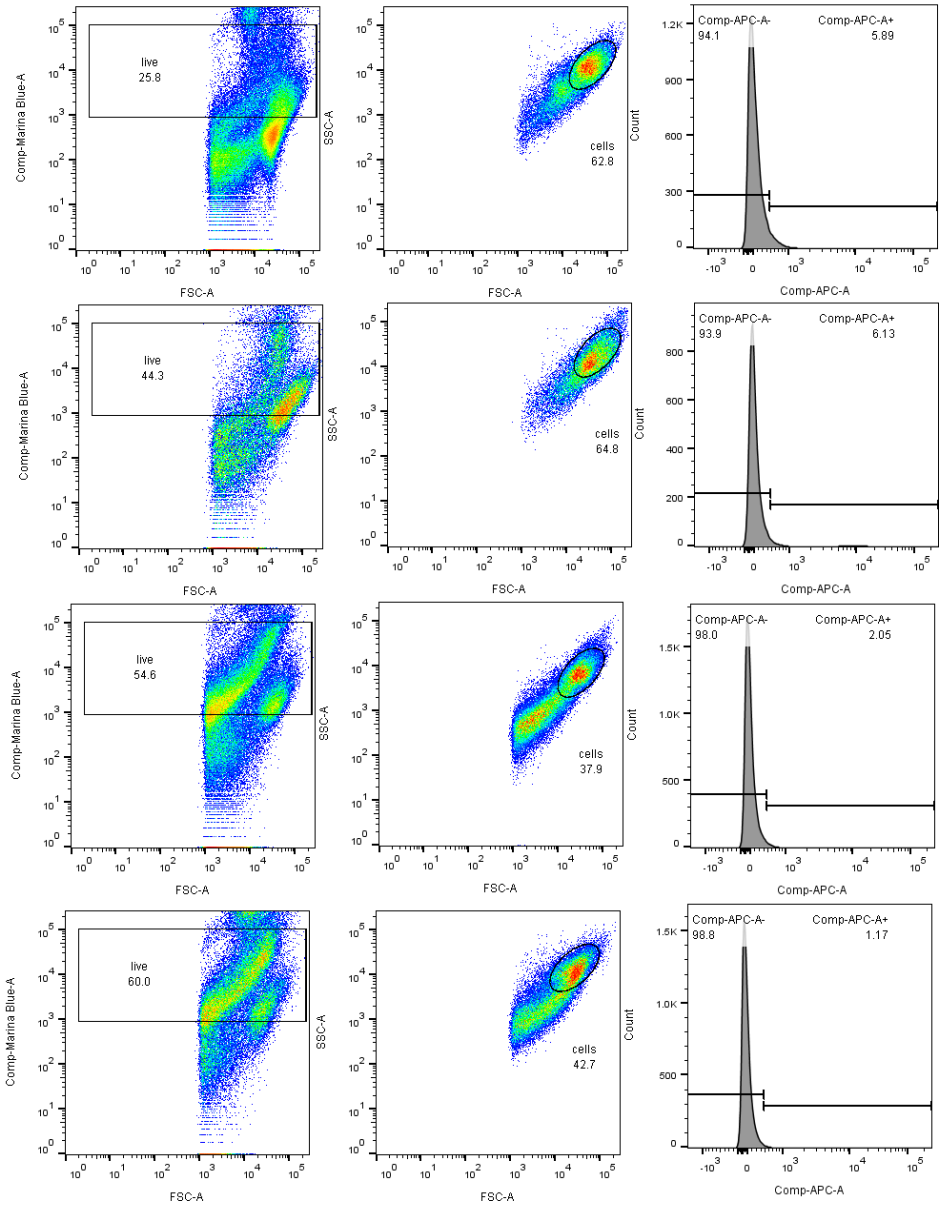
Isotype control



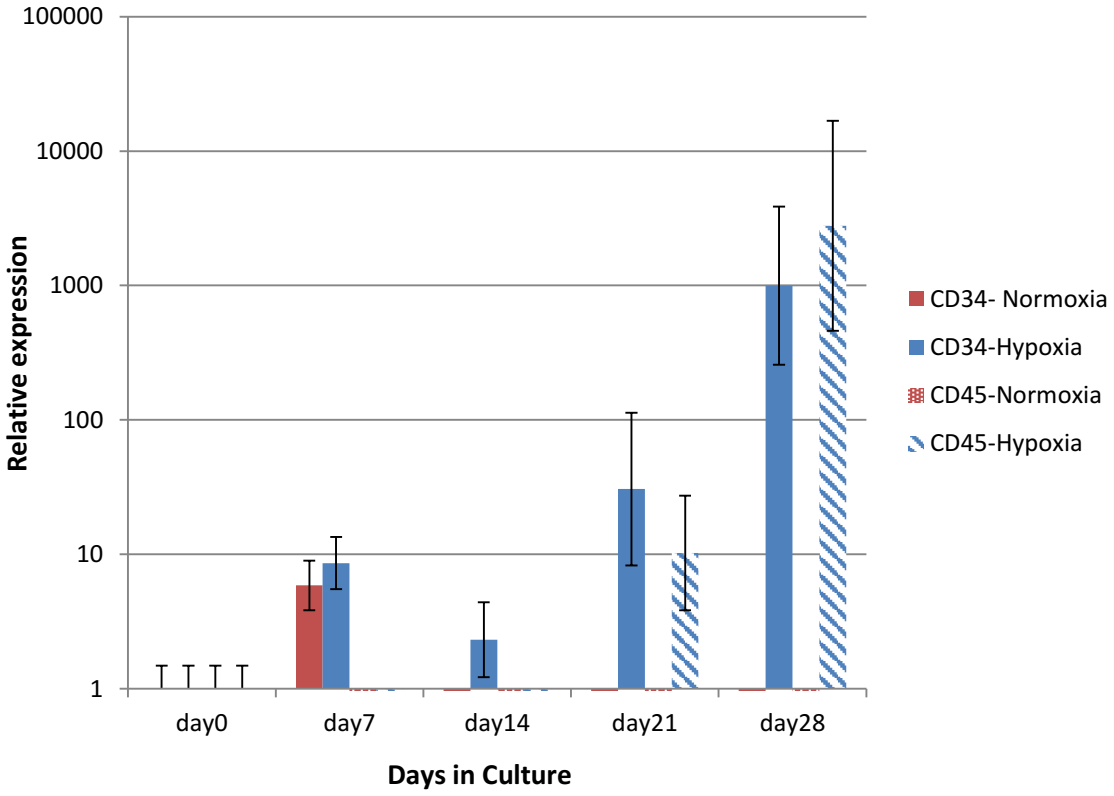
Normoxia Day 28 Samples



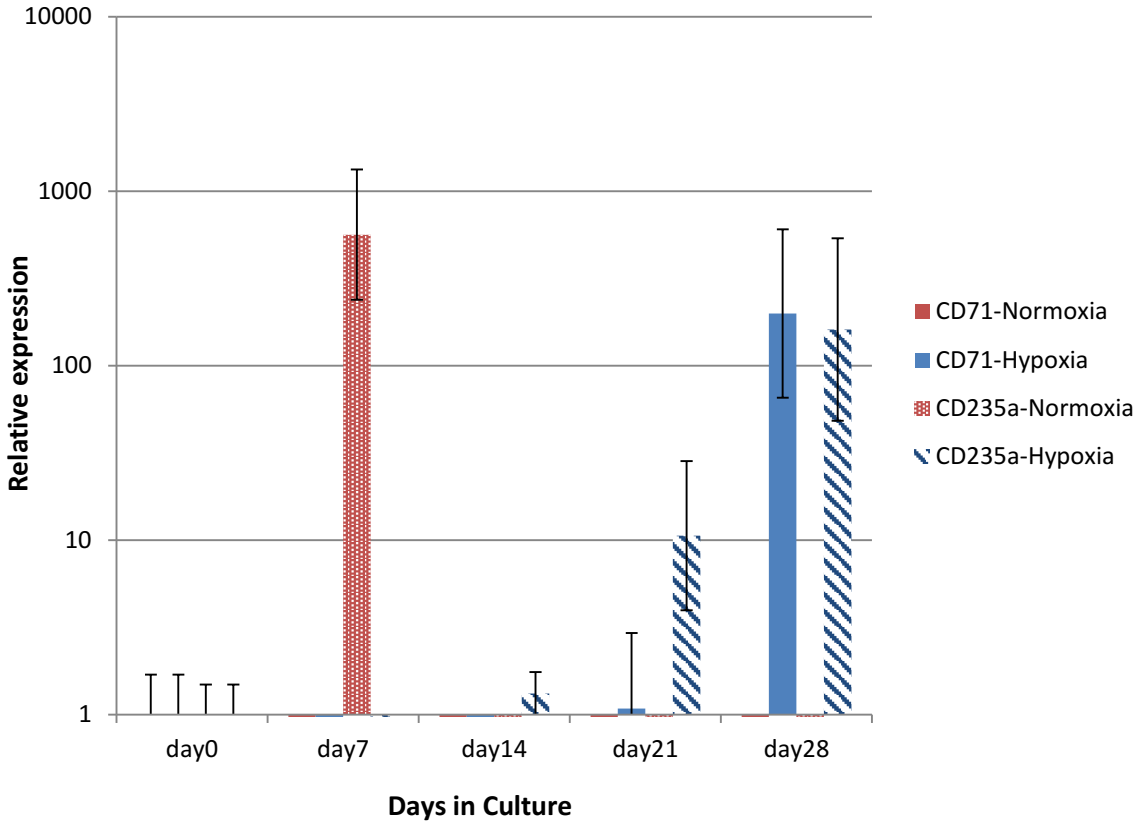
Hypoxia Day 28 Samples



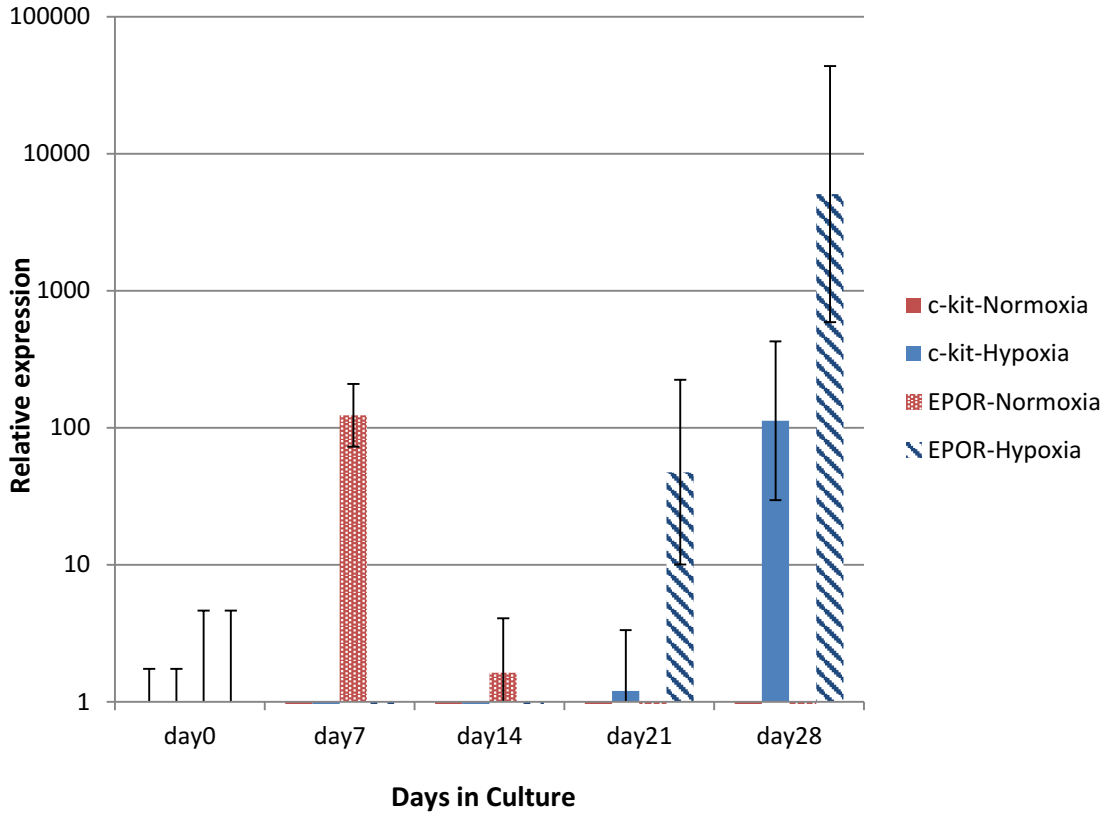
Appendix 11. Relative Gene Expression and qPCR of Haematopoietic Markers (CD34-CD45) in Differentiated Human iPS cells. (n≥2).



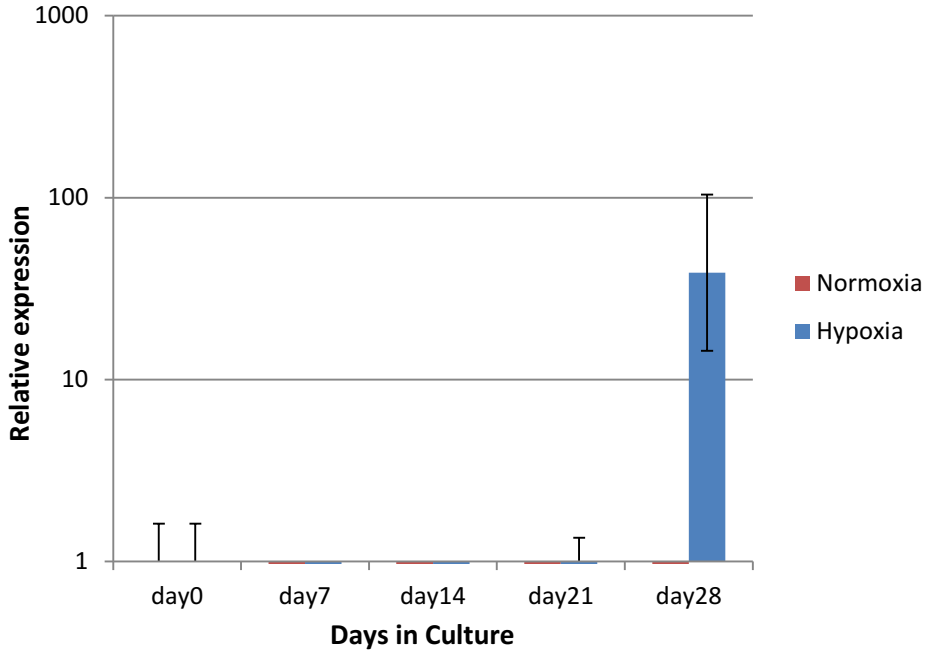
Appendix 12. Relative Gene Expression and qPCR of Erythropoietic Markers (CD71-CD235a) in Differentiated Human iPS cells. (n≥2).



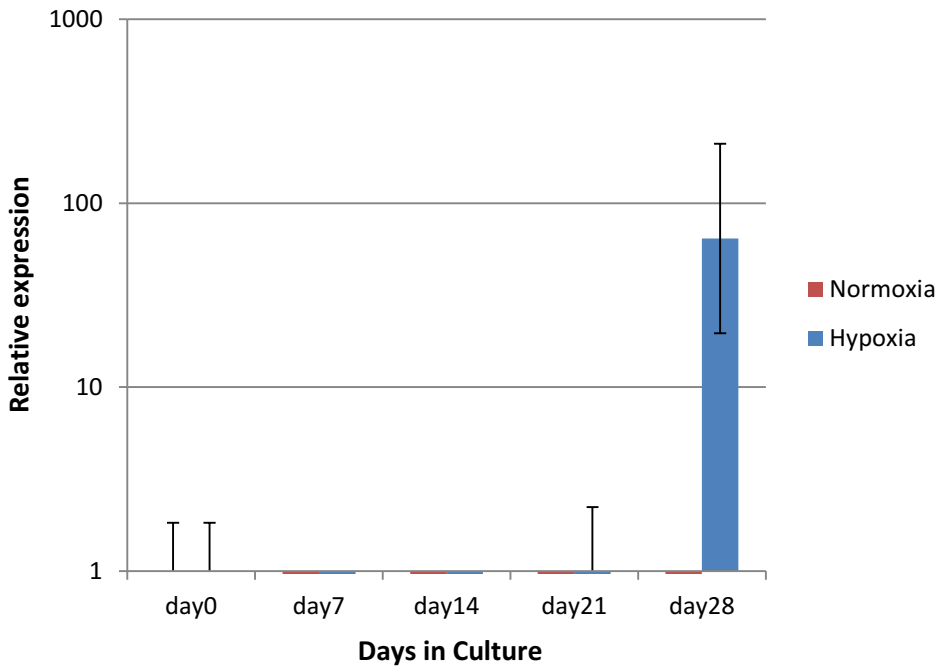
Appendix 13. Relative Gene Expression and qPCR of Cellular Receptors (c-kit and EPOR) in Differentiated Human iPS cells. (n≥2).



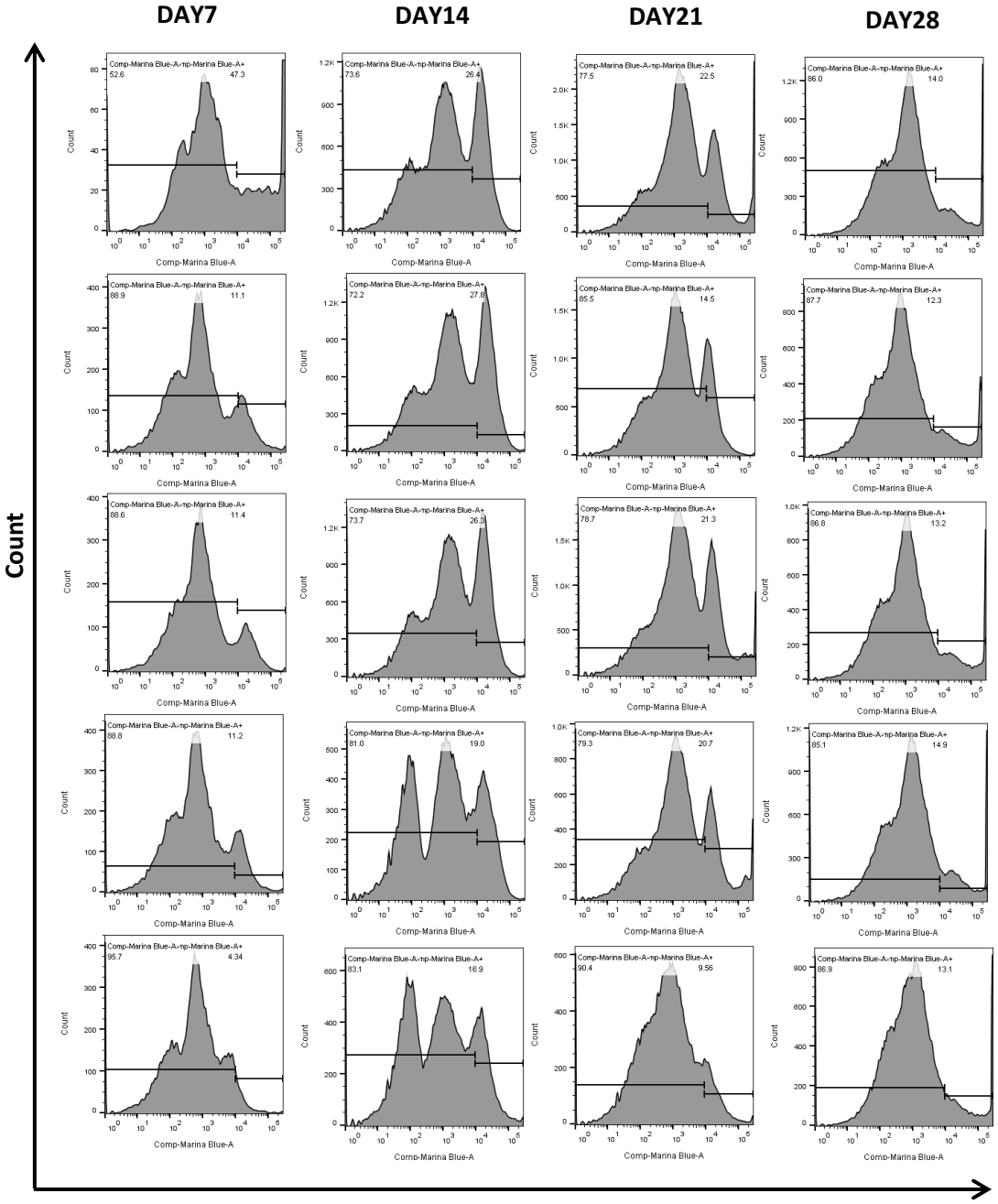
Appendix 14. Relative Gene Expression and qPCR of CD43 in Differentiated Human iPS cells. (n≥2).



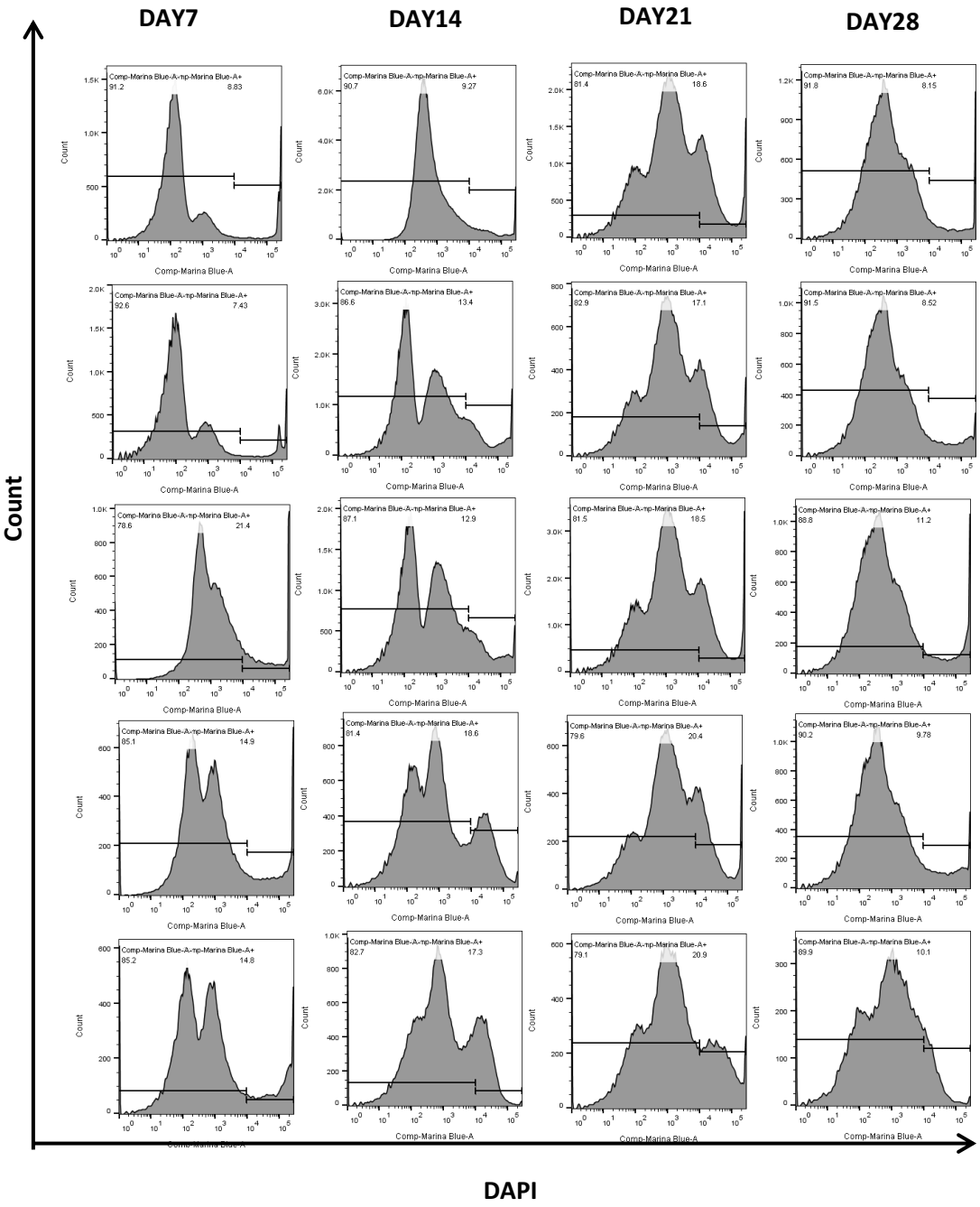
Appendix 15. Relative Gene Expression and qPCR of HIF1α in Differentiated Human iPS cells. (n≥2).



Appendix 16. Viability Analysis of Cells Differentiated in Normoxia.



Appendix 17. Viability Analysis of Cells Differentiated in Hypoxia.



Appendix 18. O_2 Equilibrium Curve of Differentiated iPS Cells.

There were noises in all curves and the reason is either the instrument is not set up correctly for research samples or there are very little haemoglobin in the sample hence S1/S2 is very low

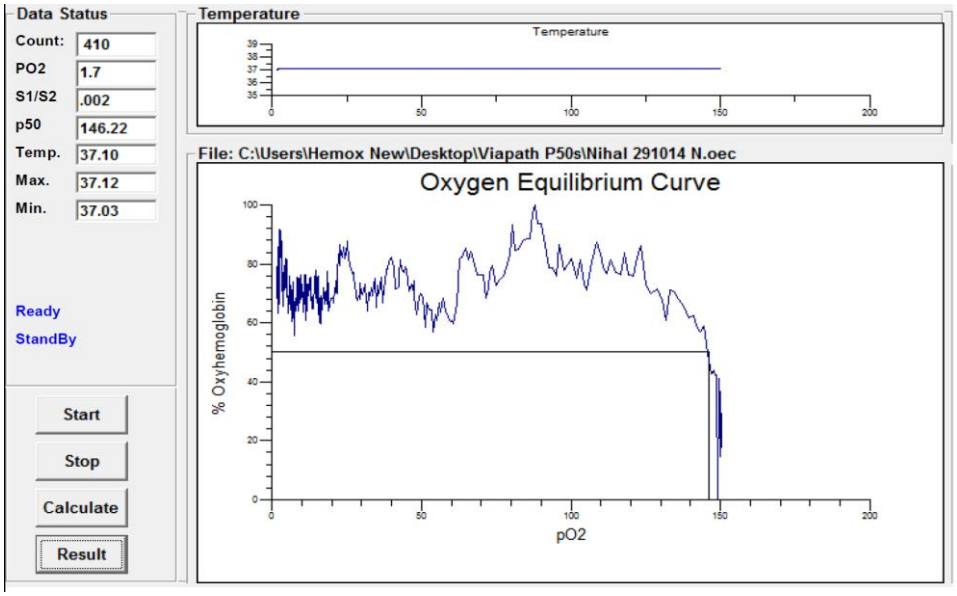
Analysis condition:

The HEMOX Analyzer

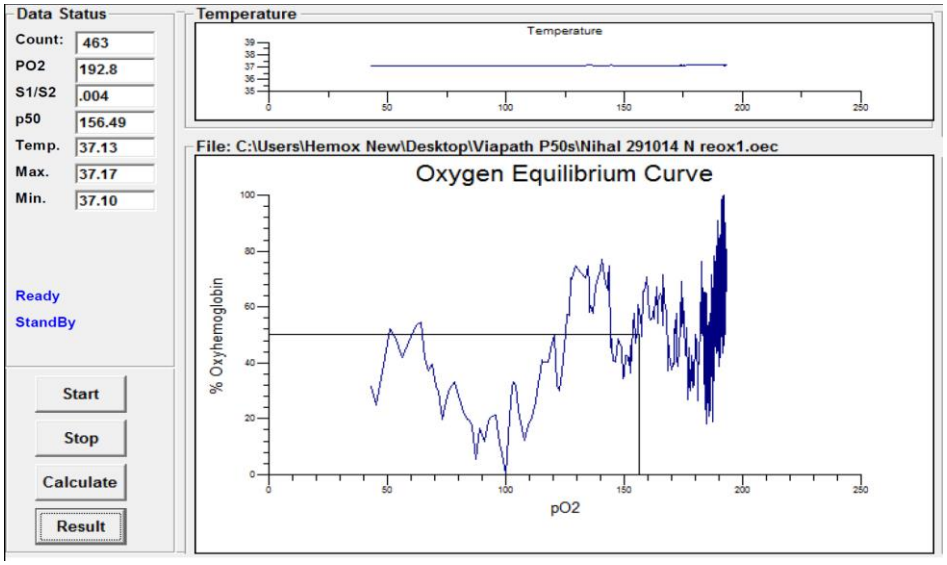
The starting PO_2: <1, HV: 170, balance: 0.0, temperature: 37°C

Normoxia:

De-oxygenation:

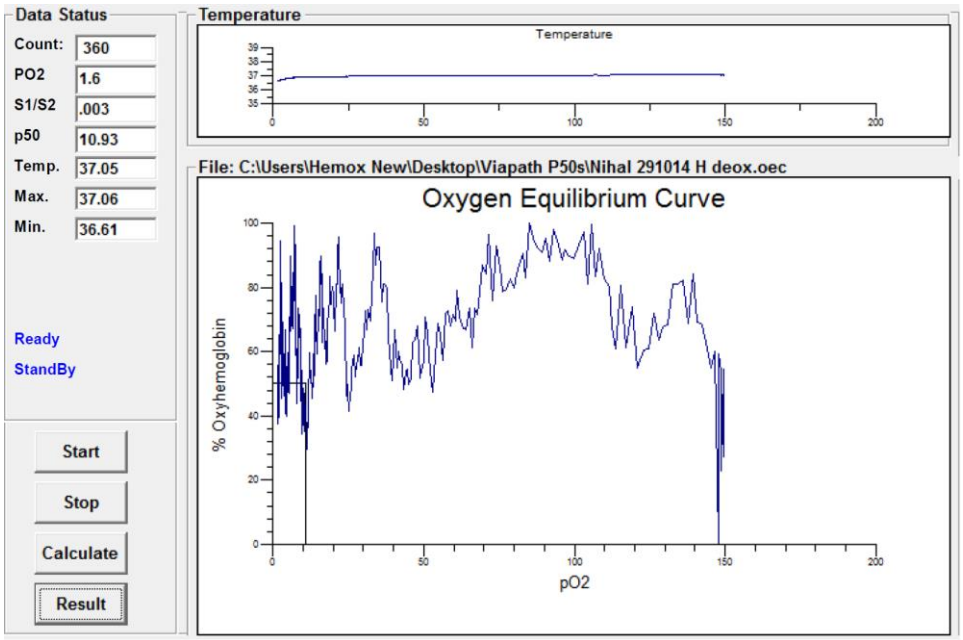


Re-oxygenation:

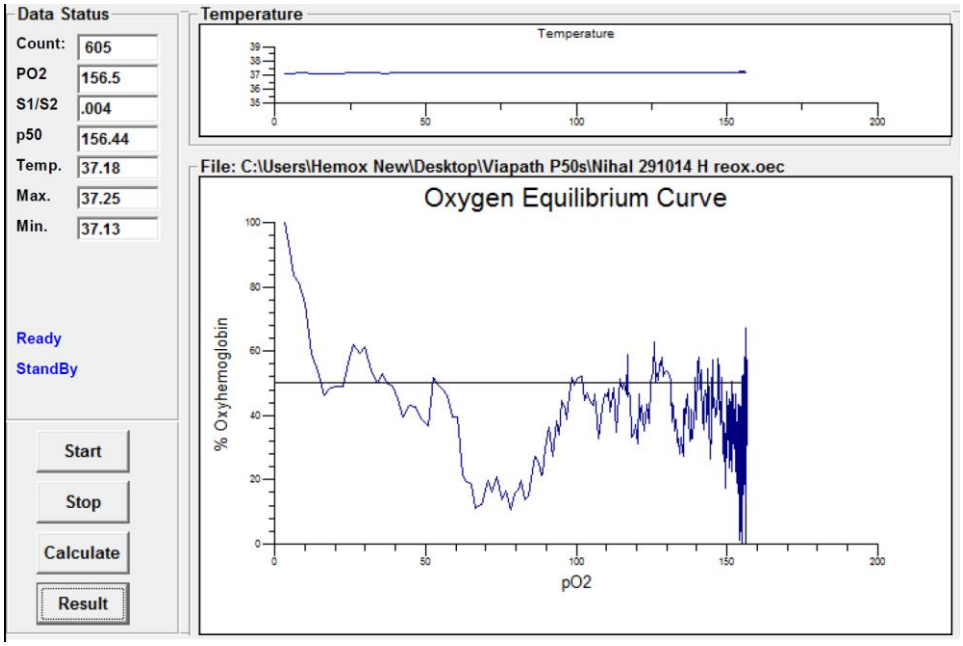


Hypoxia:

De-oxygenation:



Re-oxygenation:



References

Regenerative Medicine The cure of the future [Online]. Available: http://dezian3.wix.com/regenerativemedicine.
ABBOUD, C. & LICHTMAN, M. 2001. Structure of the marrow and the hematopoietic microenvironment. *In:* E, B., MA, L., BS, C., TJ, K. & U, S. (eds.) *Williams Hematology.* 6th ed.
ABE, K., NIWA, H., IWASE, K., TAKIGUCHI, M., MORI, A., ABE, S., ABE, K. & YAMAMURA, K. 1996. Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies. *Experimental Cell Research,* 229**,** 27-34.
ADEVA-ANDANY, M., LOPEZ-OJEN, M., FUNCASTA-CALDERON, R., AMENEIROS-RODRIGUEZ, E., DONAPETRY-GARCIA, C., VILA-ALTESOR, M. & RODRIGUEZ-SEIJAS, J. 2014. Comprehensive review on lactate metabolism in human health. *Mitochondrion,* 17**,** 76-100.
ADEWUMI, O., AFLATOONIAN, B., AHRLUND-RICHTER, L., AMIT, M., ANDREWS, P. W., BEIGHTON, G., BELLO, P. A., BENVENISTY, N., BERRY, L. S., BEVAN, S., BLUM, B., BROOKING, J., CHEN, K. G., CHOO, A. B. H., CHURCHILL, G. A., CORBEL, M., DAMJANOV, I., DRAPER, J. S., DVORAK, P., EMANUELSSON, K., FLECK, R. A., FORD, A., GERTOW, K., GERTSENSTEIN, M., GOKHALE, P. J., HAMILTON, R. S., HAMPL, A., HEALY, L. E., HOVATTA, O., HYLLNER, J., IMREH, M. P., ITSKOVITZ-ELDOR, J., JACKSON, J., JOHNSON, J. L., JONES, M., KEE, K., KING, B. L., KNOWLES, B. B., LAKO, M., LEBRIN, F., MALLON, B. S., MANNING, D., MAYSHAR, Y., MCKAY, R. D. G., MICHALSKA, A. E., MIKKOLA, M., MILEIKOVSKY, M., MINGER, S. L., MOORE, H. D., MUMMERY, C. L., NAGY, A., NAKATSUJI, N., O'BRIEN, C. M., OH, S. K. W., OLSSON, C., OTONKOSKI, T., PARK, K. Y., PASSIER, R., PATEL, H., PATEL, M., PEDERSEN, R., PERA, M. F., PIEKARCZYK, M. S., PERA, R. A. R., REUBINOFF, B. E., ROBINS, A. J., ROSSANT, J., RUGG-GUNN, P., SCHULZ, T. C., SEMB, H., SHERRER, E. S., SIEMEN, H., STACEY, G. N., STOJKOVIC, M., SUEMORI, H., SZATKIEWICZ, J., TURETSKY, T., TUURI, T., VAN DEN BRINK, S., VINTERSTEN, K., VUORISTO, S., WARD, D., WEAVER, T. A., YOUNG, L. A., ZHANG, W. D. & INITIATIVE, I. S. C. 2007. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nature Biotechnology,* 25**,** 803-816.
AISEN, P. 2004. Transferrin receptor 1. *Int J Biochem Cell Biol,* 36**,** 2137-43.
AKASHI, K., TRAVER, D., MIYAMOTO, T. & WEISSMAN, I. L. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature,* 404**,** 193-197.
AMABILE, G., WELNER, R. S., NOMBELA-ARRIETA, C., D'ALISE, A. M., DI RUSCIO, A., EBRALIDZE, A. K., KRAYTSBERG, Y., YE, M., KOCHER, O., NEUBERG, D. S., KHRAPKO, K., SILBERSTEIN, L. E. & TENEN, D. G. 2013. In vivo generation of transplantable human hematopoietic cells from induced pluripotent stem cells. *Blood,* 121**,** 1255-1264.
AMIT, M., CARPENTER, M. K., INOKUMA, M. S., CHIU, C. P., HARRIS, C. P., WAKNITZ, M. A., ITSKOVITZ-ELDOR, J. & THOMSON, J. A. 2000. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Developmental Biology,* 227**,** 271-278.
ANDERSSON, L. C., VON WILLEBRAND, E., JOKINEN, M., KARHI, K. K. & GAHMBERG, C. G. 1981. Glycophorin A as an erythroid marker in normal and malignant hematopoiesis. *Haematol Blood Transfus,* 26**,** 338-44.
ANSTEE, D. J. 2010. Production of erythroid cells from human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPS). *Transfusion Clinique Et Biologique,* 17**,** 104-109.
ANTONCHUK, J., HYLAND, C. D., HILTON, D. J. & ALEXANDER, W. S. 2004. Synergistic effects on erythropoiesis, thrombopoiesis, and stem cell competitiveness in mice deficient in thrombopoietin and steel factor receptors. *Blood,* 104**,** 1306-1313.
AOKI, T., OHNISHI, H., ODA, Y., TADOKORO, M., SASAO, M., KATO, H., HATTORI, K. & OHGUSHI, H. 2010. Generation of Induced Pluripotent Stem Cells from Human Adipose-Derived Stem Cells Without c-MYC. *Tissue Engineering Part A,* 16**,** 2197-2206.

ASHMAN, L. K., CAMBARERI, A. C., TO, L. B., LEVINSKY, R. J. & JUTTNER, C. A. 1991. Expression of the YB5.B8 antigen (c-kit proto-oncogene product) in normal human bone marrow. *Blood,* 78**,** 30-7.

ATTAR, A. 2014. Changes in the Cell Surface Markers During Normal Hematopoiesis: A Guide to Cell Isolation *Global Journal of Hematology and Blood Transfusion,* 1**,** 20-28

BADGER, J. L., BYRNE, M. L., VERAITCH, F. S., MASON, C., WALL, I. B. & CALDWELL, M. A. 2012. Hypoxic culture of human pluripotent stem cell lines is permissible using mouse embryonic fibroblasts. *Regenerative Medicine,* 7**,** 675-683.

BAJPAI, R., LESPERANCE, J., KIM, M. & TERSKIKH, A. V. 2008. Efficient propagation of single cells accutase-dissociated human embryonic stem cells. *Molecular Reproduction and Development,* 75**,** 818-827.

BARNGROVER, D., THOMAS, J. & THILLY, W. G. 1985a. High-Density Mammalian-Cell Growth in Leibovitz Bicarbonate-Free Medium - Effects of Fructose and Galactose on Culture Biochemistry. *Journal of Cell Science,* 78**,** 173-189.

BARNGROVER, D., THOMAS, J. & THILLY, W. G. 1985b. High density mammalian cell growth in Leibovitz bicarbonate-free medium: effects of fructose and galactose on culture biochemistry. *J Cell Sci,* 78**,** 173-89.

BARON, M. H. & FRASER, S. T. 2005. The specification of early hematopoiesis in the mammal. *Curr Opin Hematol,* 12**,** 217-21.

BLANN, A. & HOLTOM, P. 2010. Peripheral Blood Cells in Health and Disease. *In:* MOORE, G., KNIGHT, G. & BLANN, A. (eds.) *Haematology.*

BOWLES, K. M., VALLIER, L., SMITH, J. R., ALEXANDER, M. R. J. & PEDERSEN, R. A. 2006. HOXB4 overexpression promotes hematopoietic development by human embryonic stem cells. *Stem Cells,* 24**,** 1359-1369.

BRAAM, S. R., ZEINSTRA, L., LITJENS, S., WARD-VAN OOSTWAARD, D., VAN DEN BRINK, S., VAN LAAKE, L., LEBRIN, F., KATS, P., HOCHSTENBACH, R., PASSIER, R., SONNENBERG, A. & MUMMERY, C. L. 2008. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alpha V beta 5 integrin. *Stem Cells,* 26**,** 2257-2265.

BREIER, G., ALBRECHT, U., STERRER, S. & RISAU, W. 1992. Expression of Vascular Endothelial Growth-Factor during Embryonic Angiogenesis and Endothelial-Cell Differentiation. *Development,* 114**,** 521-532.

BROUDY, V. C., LIN, N., ZSEBO, K. M., BIRKETT, N. C., SMITH, K. A., BERNSTEIN, I. D. & PAPAYANNOPOULOU, T. 1992. Isolation and Characterization of a Monoclonal-Antibody That Recognizes the Human C-Kit Receptor. *Blood,* 79**,** 338-346.

CANTU, I. & PHILIPSEN, S. 2014. Flicking the switch: adult hemoglobin expression in erythroid cells derived from cord blood and human induced pluripotent stem cells. *Haematologica,* 99**,** 1647-1649.

CERDAN, C., ROULEAU, A. & BHATIA, M. 2004. VEGF-A(165) augments erythropoietic development from human embryonic stem cells. *Blood,* 103**,** 2504-2512.

CHADWICK, K., WANG, L., LI, L., MENENDEZ, P., MURDOCH, B., ROULEAU, A. & BHATIA, M. 2003a. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood,* 102**,** 906-15.

CHADWICK, K., WANG, L. S., LI, L., MENENDEZ, P., MURDOCH, B., ROULEAU, A. & BHATIA, M. 2003b. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood,* 102**,** 906-915.

CHANG, C. J., MITRA, K., KOYA, M., VELHO, M., DESPRAT, R., LENZ, J. & BOUHASSIRA, E. E. 2011. Production of Embryonic and Fetal-Like Red Blood Cells from Human Induced Pluripotent Stem Cells. *Plos One,* 6.

CHANG, K. H., NELSON, A. M., CAO, H., WANG, L. L., NAKAMOTO, B., WARE, C. B. & PAPAYANNOPOULOU, T. 2006. Definitive-like etythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin. *Blood,* 108**,** 1515-1523.

CHASIS, J. A. & MOHANDAS, N. 2008. Erythroblastic islands: niches for erythropoiesis. *Blood,* 112**,** 470-478.

CHEN, B., MAO, B., HUANG, S., ZHOU, Y., TSUJI, K. & MA, F. 2014a. Human Embryonic Stem Cell-Derived Primitive and Definitive Hematopoiesis. *In:* ATWOOD, C. S. & MEETHAL, S. V. (eds.) *Pluripotent Stem Cell Biology - Advances in Mechanisms, Methods and Models.* InTech.

CHEN, G. K., GULBRANSON, D. R., HOU, Z. G., BOLIN, J. M., RUOTTI, V., PROBASCO, M. D., SMUGA-OTTO, K., HOWDEN, S. E., DIOL, N. R., PROPSON, N. E., WAGNER, R., LEE, G. O., ANTOSIEWICZ-BOURGET, J., TENG, J. M. C. & THOMSON, J. A. 2011. Chemically defined conditions for human iPSC derivation and culture. *Nature Methods,* 8**,** 424-U76.

CHEN, K. G., MALLON, B. S., MCKAY, R. D. & ROBEY, P. G. 2014b. Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. *Cell Stem Cell,* 14**,** 13-26.

CHIBA, T., IKAWA, Y. & TODOKORO, K. 1991. Gata-1 Transactivates Erythropoietin Receptor Gene, and Erythropoietin Receptor-Mediated Signals Enhance Gata-1 Gene-Expression. *Nucleic Acids Research,* 19**,** 3843-3848.

CHIOU, S. H., JIANG, B. H., YU, Y. L., CHOU, S. J., TSAI, P. H., CHANG, W. C., CHEN, L. K., CHEN, L. H., CHIEN, Y. & CHIOU, G. Y. 2013. Poly(ADP-ribose) polymerase 1 regulates nuclear reprogramming and promotes iPSC generation without c-Myc. *Journal of Experimental Medicine,* 210**,** 85-98.

CHOI, K. D., YU, J., SMUGA-OTTO, K., SALVAGIOTTO, G., REHRAUER, W., VODYANIK, M., THOMSON, J. & SLUKVIN, I. 2009. Hematopoietic and Endothelial Differentiation of Human Induced Pluripotent Stem Cells. *Stem Cells,* 27**,** 559-567.

CHOUMERIANOU, D. M., DIMITRIOU, H. & KALMANTI, M. 2008. Stem cells: Promises versus limitations. *Tissue Engineering Part B-Reviews,* 14**,** 53-60.

CHUNG, K. Y., MORRONE, G., SCHURINGA, J. J., WONG, B., DORN, D. C. & MOORE, M. A. S. 2005. Enforced expression of an Flt3 internal tandem duplication in human CD34(+) cells confers properties of self-renewal and enhanced erythropoiesis. *Blood,* 105**,** 77-84.

CIVIN, C., HANDGRETINGER, R. & URBANO-ISPIZUA, A. 2004. Use of purified stem and progenitor cells for transplantation. *In:* ATKINSON, K., CHAMPLIN, R., RITZ, J., FIBBE, W., LJUNGMAN, P. & BRENNER, M. (eds.) *Clinical Bone Marrow and Blood Stem Cell Transplantation.* 3RD ed.: Cambridge Universiy Press.

CLANCHY, F. I. L. & HAMILTON, J. A. 2013. The development of macrophages from human CD34(+) haematopoietic stem cells in serum-free cultures is optimized by IL-3 and SCF. *Cytokine,* 61**,** 33-37.

COURTOT, A. M., MAGNIEZ, A., OUDRHIRI, N., FERAUD, O., BACCI, J., GOBBO, E., PROUST, S., TURHAN, A. G. & BENNACEUR-GRISCELLI, A. 2014. Morphological analysis of human induced pluripotent stem cells during induced differentiation and reverse programming. *Biores Open Access,* 3**,** 206-16.

D'AMOUR, K. A., AGULNICK, A. D., ELIAZER, S., KELLY, O. G., KROON, E. & BAETGE, E. E. 2005. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nature Biotechnology,* 23**,** 1534-1541.

DERDA, R., LI, L. Y., ORNER, B. P., LEWIS, R. L., THOMSON, J. A. & KIESSLING, L. L. 2007. Defined substrates for human embryonic stem cell growth identified from surface arrays. *Acs Chemical Biology,* 2**,** 347-355.

DIAS, J., GUMENYUK, M., KANG, H., VODYANIK, M., YU, J. Y., THOMSON, J. A. & SLUKVIN, I. I. 2011. Generation of Red Blood Cells from Human Induced Pluripotent Stem Cells. *Stem Cells and Development,* 20**,** 1639-1647.

DOETSCHMAN, T. C., EISTETTER, H., KATZ, M., SCHMIDT, W. & KEMLER, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol,* 87**,** 27-45.

DOHERTY, G. J. & MCMAHON, H. T. 2008. Mediation, modulation, and consequences of membrane-cytoskeleton interactions. *Annual Review of Biophysics,* 37**,** 65-95.

DORN, I., KLICH, K., ARAUZO-BRAVO, M. J., RADSTAAK, M., SANTOURLIDIS, S., GHANJATI, F., RADKE, T. F., PSATHAKI, O. E., HARGUS, G., KRAMER, J., EINHAUS, M., KIM, J. B., KOGLER, G., WERNET, P., SCHOLER, H. R., SCHLENKE, P. & ZAEHRES, H. 2015. Erythroid differentiation of human induced pluripotent stem cells is independent of donor cell type of origin. *Haematologica,* 100**,** 32-41.

DOUAY, L. & ANDREU, G. 2007. Ex vivo production of human red blood cells from hematopoietic stem cells: What is the future in transfusion? *Transfusion Medicine Reviews,* 21**,** 91-100.

DOUAY, L. & GIARRATANA, M. C. 2009. Ex vivo generation of human red blood cells: a new advance in stem cell engineering. *Methods Mol Biol,* 482**,** 127-40.

DRUKKER, M., KATZ, G., URBACH, A., SCHULDINER, M., MARKEL, G., ITSKOVITZ-ELDOR, J., REUBINOFF, B., MANDELBOIM, O. & BENVENISTY, N. 2002. Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci U S A,* 99**,** 9864-9.

DUPONT, B. R., GRANT, S. G., OTO, S. H., BIGBEE, W. L., JENSEN, R. H. & LANGLOIS, R. G. 1995. Molecular Characterization of Glycophorin-a Transcripts in Human Erythroid-Cells Using Rt-Pcr, Allele-Specific Restriction, and Sequencing. *Vox Sanguinis,* 68**,** 121-129.

DZIERZAK, E. & PHILIPSEN, S. 2013. Erythropoiesis: Development and Differentiation. *Cold Spring Harbor Perspectives in Medicine,* 3.

DZIERZAK, E. & SPECK, N. A. 2008. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol,* 9**,** 129-36.

EBIHARA, Y., MA, F. & TSUJI, K. 2012. Generation of red blood cells from human embryonic/induced pluripotent stem cells for blood transfusion. *International Journal of Hematology,* 95**,** 610-616.

EISENBERG, L. M. & EISENBERG, C. A. 2003. Stem cell plasticity, cell fusion, and transdifferentiation. *Birth Defects Res C Embryo Today,* 69**,** 209-18.

EMA, H. & NAKAUCHI, H. 2000. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood,* 95**,** 2284-8.

EVANS, M. J. & KAUFMAN, M. H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292**,** 154-6.

FAUZI, I., PANOSKALTSIS, N. & MANTALARIS, A. 2015. In Vitro Differentiation of Embryonic Stem Cells into Hematopoietic Lineage: Towards Erythroid Progenitor's Production. *Methods Mol Biol*.

FENG, Q., LU, S. J., KLIMANSKAYA, I., GOMES, I., KIM, D., CHUNG, Y., HONIG, G. R., KIM, K. S. & LANZA, R. 2010. Hemangioblastic Derivatives from Human Induced Pluripotent Stem Cells Exhibit Limited Expansion and Early Senescence. *Stem Cells,* 28**,** 704-712.

FIRAS, J., LIU, X. D., NEFZGER, C. M. & POLO, J. M. 2014. GM-CSF and MEF-conditioned media support feeder-free reprogramming of mouse granulocytes to iPS cells. *Differentiation,* 87**,** 193-199.

FOCOSI, D., AMABILE, G., DI RUSCIO, A., QUARANTA, P., TENEN, D. G. & PISTELLO, M. 2014. Induced pluripotent stem cells in hematology: current and future applications. *Blood Cancer Journal,* 4.

FUJIMI, A., MATSUNAGA, T., KOBUNE, M., KAWANO, Y., NAGAYA, T., TANAKA, I., IYAMA, S., HAYASHI, T., SATO, T., MIYANISHI, K., SAGAWA, T., SATO, Y., TAKIMOTO, R., TAKAYAMA, T., KATO, J., GASA, S., SAKAI, H., TSUCHIDA, E., IKEBUCHI, K., HAMADA, H. & NIITSU, Y. 2008. Ex vivo large-scale generation of human red blood cells from cord blood CD34+ cells by co-culturing with macrophages. *Int J Hematol,* 87**,** 339-50.

GAHMBERG, C. G., EKBLOM, M. & ANDERSSON, L. C. 1984. Differentiation of Human Erythroid-Cells Is Associated with Increased O-Glycosylation of the Major Sialoglycoprotein, Glycophorin-A. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences,* 81**,** 6752-6756.

GALANELLO, R. 2012. Recent advances in the molecular understanding of non-transfusion-dependent thalassemia. *Blood Reviews,* 26**,** S7-S11.

GANJI, F., ABROUN, S., BAHARVAND, H., AGHDAMI, N. & EBRAHIMI, M. 2015. Differentiation Potential of O Bombay Human-Induced Pluripotent Stem Cells and Human Embryonic Stem Cells into Fetal Erythroid-Like Cells. *Cell Journal,* 16**,** 426-439.

GIARRATANA, M. C., KOBARI, L., LAPILLONNE, H., CHALMERS, D., KIGER, L., CYNOBER, T., MARDEN, M. C., WAJCMAN, H. & DOUAY, L. 2005. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nature Biotechnology,* 23**,** 69-74.

GIARRATANA, M. C., ROUARD, H., DUMONT, A., KIGER, L., SAFEUKUI, I., LE PENNEC, P. Y., FRANCOIS, S., TRUGNAN, G., PEYRARD, T., MARIE, T., JOLLY, S., HEBERT, N., MAZURIER, C., MARIO, N., HARMAND, L., LAPILLONNE, H., DEVAUX, J. Y. & DOUAY, L. 2011. Proof of principle for transfusion of in vitro-generated red blood cells. *Blood,* 118**,** 5071-5079.

GIFFORD, S. C., DERGANC, J., SHEVKOPLYAS, S. S., YOSHIDA, T. & BITENSKY, M. W. 2006. A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. *British Journal of Haematology,* 135**,** 395-404.

GLACKEN, M. W., FLEISCHAKER, R. J. & SINSKEY, A. J. 1986. Reduction of Waste Product Excretion Via Nutrient Control - Possible Strategies for Maximizing Product and Cell Yields on Serum in Cultures of Mammalian-Cells. *Biotechnology and Bioengineering,* 28**,** 1376-1389.

GOLDE, D. W., BERSCH, N. & CLINE, M. J. 1976. Potentiation of erythropoiesis in vitro by dexamethasone. *J Clin Invest,* 57**,** 57-62.

GREBIEN, F., KERENYI, M. A., KOVACIC, B., KOLBE, T., BECKER, V., DOLZNIG, H., PFEFFER, K., KLINGMULLER, U., MULLER, M., BEUG, H., MULLNER, E. W. & MORIGGL, R. 2008. Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2. *Blood,* 111**,** 4511-4522.

HAKIM, F., KAITSUKA, T., RAEED, J. M., WEI, F. Y., SHIRAKI, N., AKAGI, T., YOKOTA, T., KUME, S. & TOMIZAWA, K. 2014. High Oxygen Condition Facilitates the Differentiation of Mouse and Human Pluripotent Stem Cells into Pancreatic Progenitors and Insulin-producing Cells*. *Journal of Biological Chemistry,* 289**,** 9623-9638.

HARASHIMA, A., SUZUKI, M., OKOCHI, A., YAMAMOTO, M., MATSUO, Y., MOTODA, R., YOSHIOKA, T. & ORITA, K. 2002. CD45 tyrosine phosphatase inhibits erythroid differentiation of umbilical cord blood CD34+ cells associated with selective inactivation of Lyn. *Blood,* 100**,** 4440-5.

HARVEY, A. J. 2007. The role of oxygen in ruminant preimplantation embryo development and metabolism. *Animal Reproduction Science,* 98**,** 113-128.

HEATH, D. S., AXELRAD, A. A., MCLEOD, D. L. & SHREEVE, M. M. 1976. Separation of the erythropoietin-responsive progenitors BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation. *Blood,* 47**,** 777-92.

HEENEMAN, S., DEUTZ, N. E. & BUURMAN, W. A. 1993. The concentrations of glutamine and ammonia in commercially available cell culture media. *J Immunol Methods,* 166**,** 85-91.

HENDERSON, J. K., DRAPER, J. S., BAILLIE, H. S., FISHEL, S., THOMSON, J. A., MOORE, H. & ANDREWS, P. W. 2002. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells,* 20**,** 329-337.

HENG, B. C., LIU, H., GE, Z. G. & CAO, T. 2007. Mechanical dissociation of human embryonic stem cell colonies by manual scraping after collagenase treatment is much more detrimental to cellular viability than is trypsinization with gentle pipetting. *Biotechnology and Applied Biochemistry,* 47**,** 33-37.

HILLMAN, R. S. & HENDERSON, P. A. 1969. Control of marrow production by the level of iron supply. *J Clin Invest,* 48**,** 454-60.

HIROYAMA, T., MIHARADA, K., SUDO, K., DANJO, I., AOKI, N. & NAKAMURA, Y. 2008. Establishment of Mouse Embryonic Stem Cell-Derived Erythroid Progenitor Cell Lines Able to Produce Functional Red Blood Cells. *Plos One,* 3.

HONG, H., TAKAHASHI, K., ICHISAKA, T., AOI, T., KANAGAWA, O., NAKAGAWA, M., OKITA, K. & YAMANAKA, S. 2009. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature,* 460**,** 1132-U95.

INGLEY, E. 2012a. Integrating novel signaling pathways involved in erythropoiesis. *Iubmb Life,* 64**,** 402-410.

INGLEY, E. 2012b. Integrating novel signaling pathways involved in erythropoiesis. *IUBMB Life,* 64**,** 402-10.

ITSKOVITZ-ELDOR, J., SCHULDINER, M., KARSENTI, D., EDEN, A., YANUKA, O., AMIT, M., SOREQ, H. & BENVENISTY, N. 2000. Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Molecular Medicine,* 6**,** 88-95.

IVANOVIC, Z. 2004. Interleukin-3 and ex vivo maintenance of hematopoietic stem cells: facts and controversies. *European Cytokine Network,* 15**,** 6-13.

IVANOVIC, Z. 2009. Hypoxia or in situ normoxia: The stem cell paradigm. *J Cell Physiol,* 219**,** 271-5.

JELKMANN, W. 1992. Erythropoietin: structure, control of production, and function. *Physiol Rev,* 72**,** 449-89.

JENKINS, B. J., ROBERTS, A. W., GREENHILL, C. J., NAJDOVSKA, M., LUNDGREN-MAY, T., ROBB, L., GRAIL, D. & ERNST, M. 2007. Pathologic consequences of STAT3 hyperactivation by IL-6 and IL-11 during hematopoiesis and lymphopoiesis. *Blood,* 109**,** 2380-2388.

KANNAGI, R., COCHRAN, N. A., ISHIGAMI, F., HAKOMORI, S., ANDREWS, P. W., KNOWLES, B. B. & SOLTER, D. 1983. Stage-Specific Embryonic Antigens (Ssea-3 and Ssea-4) Are Epitopes of a Unique Globo-Series Ganglioside Isolated from Human Teratocarcinoma Cells. *Embo Journal,* 2**,** 2355-2361.

KAPUSCINSKI, J. 1995. Dapi - a DNA-Specific Fluorescent-Probe. *Biotechnic & Histochemistry,* 70**,** 220-233.

KASSOUF, M. T., CHAGRAOUI, H., VYAS, P. & PORCHER, C. 2008. Differential use of SCL/TAL-1 DNA-binding domain in developmental hematopoiesis. *Blood,* 112**,** 1056-1067.

KATTMAN, S. J., WITTY, A. D., GAGLIARDI, M., DUBOIS, N. C., NIAPOUR, M., HOTTA, A., ELLIS, J. & KELLER, G. 2011. Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. *Cell Stem Cell,* 8**,** 228-240.

KAUFMAN, D. S. 2009. Toward clinical therapies using hematopoietic cells derived from human pluripotent stem cells. *Blood,* 114**,** 3513-3523.

KAUFMAN, D. S., HANSON, E. T., LEWIS, R. L., AUERBACH, R. & THOMSON, J. A. 2001. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 10716-10721.

KELLER, G., KENNEDY, M., PAPAYANNOPOULOU, T. & WILES, M. V. 1993. Hematopoietic Commitment during Embryonic Stem-Cell Differentiation in Culture. *Molecular and Cellular Biology,* 13**,** 473-486.

KENNEDY, M., FIRPO, M., CHOL, K., WALL, C., ROBERTSON, S., KABRUN, N. & KELLER, G. 1997. A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature,* 386**,** 488-493.

KHOO, M. L. M., MCQUADE, L. R., SMITH, M. S. R., LEES, J. G., SIDHU, K. S. & TUCH, B. E. 2005. Growth and differentiation of embryoid bodies derived from human embryonic stem cells: Effect of glucose and basic fibroblast growth factor. *Biology of Reproduction,* 73**,** 1147-1156.

KIM, C., AMANO, T., PARK, J., CARTER, M. G., TIAN, X. C. & YANG, X. Z. 2009. Improvement of Embryonic Stem Cell Line Derivation Efficiency with Novel Medium, Glucose Concentration, and Epigenetic Modifications. *Cloning and Stem Cells,* 11**,** 89-99.

KIM, C. H. 2010. Homeostatic and pathogenic extramedullary hematopoiesis. *J Blood Med,* 1**,** 13-9.

KIM, K., ZHAO, R., DOI, A., NG, K., UNTERNAEHRER, J., CAHAN, P., HUO, H. G., LOH, Y. H., ARYEE, M. J., LENSCH, M. W., LI, H., COLLINS, J. J., FEINBERG, A. P. & DALEY, G. Q. 2011. Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. *Nature Biotechnology,* 29**,** 1117-1119.

KINGSLEY, P. D., MALIK, J., FANTAUZZO, K. A. & PALIS, J. 2004. Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood,* 104**,** 19-25.

KOBARI, L., YATES, F., OUDRHIRI, N., FRANCINA, A., KIGER, L., MAZURIER, C., ROUZBEH, S., EL-NEMER, W., HEBERT, N., GIARRATANA, M. C., FRANCOIS, S., CHAPEL, A., LAPILLONNE, H., LUTON, D., BENNACEUR-GRISCELLI, A. & DOUAY, L. 2012. Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model. *Haematologica-the Hematology Journal,* 97**,** 1795-1803.

KOLLER, M. R., BENDER, J. G., MILLER, W. M. & PAPOUTSAKIS, E. T. 1993. Expansion of Primitive Human Hematopoietic Progenitors in a Perfusion Bioreactor System with Il-3, Il-6, and Stem-Cell Factor. *Bio-Technology,* 11**,** 358-363.

KOURY, M. J. & BONDURANT, M. C. 1990. Erythropoietin Retards DNA Breakdown and Prevents Programmed Death in Erythroid Progenitor Cells. *Science,* 248**,** 378-381.

KOURY, M. J. & PONKA, P. 2004. New insights into erythropoiesis: The roles of folate, vitamin B-12, and iron. *Annual Review of Nutrition,* 24**,** 105-131.

KOURY, S. T., BONDURANT, M. C., KOURY, M. J. & SEMENZA, G. L. 1991. Localization of cells producing erythropoietin in murine liver by in situ hybridization. *Blood,* 77**,** 2497-503.

KRANTZ, S. B. 1991. Erythropoietin. *Blood,* 77**,** 419-434.

KRAUSE, D. S., ITO, T., FACKLER, M. J., SMITH, O. M., COLLECTOR, M. I., SHARKIS, S. J. & MAY, W. S. 1994. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. *Blood,* 84**,** 691-701.

KROMER, J. O., DIETMAIR, S., JACOB, S. S. & NIELSEN, L. K. 2011. Quantification of L-alanyl-L-glutamine in mammalian cell culture broth: Evaluation of different detectors. *Analytical Biochemistry,* 416**,** 129-131.

KUMARAVELU, P., HOOK, L., MORRISON, A. M., URE, J., ZHAO, S., ZUYEV, S., ANSELL, J. & MEDVINSKY, A. 2002. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development,* 129**,** 4891-9.

LAFLAMME, M. A., CHEN, K. Y., NAUMOVA, A. V., MUSKHELI, V., FUGATE, J. A., DUPRAS, S. K., REINECKE, H., XU, C., HASSANIPOUR, M., POLICE, S., O'SULLIVAN, C., COLLINS, L., CHEN, Y., MINAMI, E., GILL, E. A., UENO, S., YUAN, C., GOLD, J. & MURRY, C. E. 2007. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol,* 25**,** 1015-24.

LAKSHMIPATHY, U. & VERFAILLIE, C. 2005. Stem cell plasticity. *Blood Reviews,* 19**,** 29-38.

LAM, M. T. & LONGAKER, M. T. 2012. Comparison of several attachment methods for human iPS, embryonic and adipose-derived stem cells for tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine,* 6**,** s80-s86.

LANNON, C., MOODY, J., KING, D., THOMAS, T., EAVES, A. & MILLER, C. 2008. A defined, feeder-independent medium for human embryonic stem cell culture. *Cell Research,* 18.

LAPILLONNE, H., KOBARI, L., MAZURIER, C., TROPEL, P., GIARRATANA, M.-C., ZANELLA-CLEON, I., KIGER, L., WATTENHOFER-DONZE, M., PUCCIO, H., HEBERT, N., FRANCINA, A., ANDREU, G., VIVILLE, S. & DOUAY, L. 2010a. Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine. *Haematologica,* 95**,** 1651-1659.

LAPILLONNE, H., KOBARI, L., MAZURIER, C., TROPEL, P., GIARRATANA, M. C., ZANELLA-CLEON, I., KIGER, L., WATTENHOFER-DONZE, M., PUCCIO, H., HEBERT, N., FRANCINA, A., ANDREU, G., VIVILLE, S. & DOUAY, L. 2010b. Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine. *Haematologica-the Hematology Journal,* 95**,** 1651-1659.

LAROCHELLE, A., SAVONA, M., WIGGINS, M., ANDERSON, S., ICHWAN, B., KEYVANFAR, K., MORRISON, S. J. & DUNBAR, C. E. 2011. Human and rhesus macaque hematopoietic stem cells cannot be purified based only on SLAM family markers. *Blood,* 117**,** 1550-1554.

LAWRENCE, C. M., RAY, S., BABYONYSHEV, M., GALLUSER, R., BORHANI, D. W. & HARRISON, S. C. 1999. Crystal structure of the ectodomain of human transferrin receptor. *Science,* 286**,** 779-82.

LEBKOWSKI, J. S., SCHAIN, L. R. & OKARMA, T. B. 1995. Serum-free culture of hematopoietic stem cells: a review. *Stem Cells,* 13**,** 607-12.

LEE, G., PAPAPETROU, E. P., KIM, H., CHAMBERS, S. M., TOMISHIMA, M. J., FASANO, C. A., GANAT, Y. M., MENON, J., SHIMIZU, F., VIALE, A., TABAR, V., SADELAIN, M. & STUDER, L. 2009. Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature,* 461**,** 402-U100.

LEE, J. W., BAE, S. H., JEONG, J. W., KIM, S. H. & KIM, K. W. 2004. Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. *Experimental and Molecular Medicine,* 36**,** 1-12.

LEE, K., GANE, P., ROUDOT-THORAVAL, F., GODEAU, B., BACHIR, D., BERNAUDIN, F., CARTRON, J. P., GALACTEROS, F. & BIERLING, P. 2001. The nonexpression of CD36 on reticulocytes and mature red blood cells does not modify the clinical course of patients with sickle cell anemia. *Blood,* 98**,** 966-71.

LENGERKE, C., GRAUER, M., NIEBUHR, N. I., RIEDT, T., KANZ, L., PARK, I. H. & DALEY, G. Q. 2009. Hematopoietic Development from Human Induced Pluripotent Stem Cells. *Hematopoietic Stem Cells Vii,* 1176**,** 219-227.

LESINSKI, D. A., HEINZ, N., PILAT-CAROTTA, S., RUDOLPH, C., JACOBS, R., SCHLEGELBERGER, B., KLUMP, H. & SCHIEDLMEIER, B. 2012. Serum- and Stromal Cell-Free Hypoxic Generation of Embryonic Stem Cell-Derived Hematopoietic Cells In Vitro, Capable of Multilineage Repopulation of Immunocompetent Mice. *Stem Cells Translational Medicine,* 1**,** 581-591.

LEVENSTEIN, M. E., LUDWIG, T. E., XU, R. H., LLANAS, R. A., VANDENHEUVEL-KRAMER, K., MANNING, D. & THOMSON, J. A. 2006. Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells,* 24**,** 568-574.

LI, H. S., YANG, J. M., JACOBSON, R. D., PASKO, D. & SUNDIN, O. 1994. Pax-6 Is First Expressed in a Region of Ectoderm Anterior to the Early Neural Plate - Implications for Stepwise Determination of the Lens. *Developmental Biology,* 162**,** 181-194.

LI, J., HALE, J., BHAGIA, P., XUE, F. M., CHEN, L. X., JAFFRAY, J., YAN, H. X., LANE, J., GALLAGHER, P. G., MOHANDAS, N., LIU, J. & AN, X. L. 2014. Isolation and transcriptome analyses of human erythroid progenitors: BFU-E and CFU-E. *Blood,* 124**,** 3636-3645.

LI, L. Y., KLIM, J. R., DERDA, R., COURTNEY, A. H. & KIESSLING, L. L. 2011. Spatial control of cell fate using synthetic surfaces to potentiate TGF-beta signaling. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 11745-11750.

LI, Y. & YANG, S.-T. 2013. Metabolic Regulation of Pluripotent Stem Cell Expansion, Differentiation and Reprogramming. *J Tissue Sci Eng* 4:e125.
LIM, W. F., INOUE-YOKOO, T., TAN, K. S., LAI, M. I. & SUGIYAMA, D. 2013. Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells. *Stem Cell Res Ther,* 4**,** 71.
LIPPI, G., MONTAGNANA, M. & FRANCHINI, M. 2011. Ex-vivo red blood cells generation: A step ahead in transfusion medicine? *European Journal of Internal Medicine,* 22**,** 16-19.
LIU, J., GUO, X. H., MOHANDAS, N., CHASIS, J. A. & AN, X. L. 2010. Membrane remodeling during reticulocyte maturation. *Blood,* 115**,** 2021-2027.
LOH, Y. H., WU, Q., CHEW, J. L., VEGA, V. B., ZHANG, W., CHEN, X., BOURQUE, G., GEORGE, J., LEONG, B., LIU, J., WONG, K. Y., SUNG, K. W., LEE, C. W., ZHAO, X. D., CHIU, K. P., LIPOVICH, L., KUZNETSOV, V. A., ROBSON, P., STANTON, L. W., WEI, C. L., RUAN, Y., LIM, B. & NG, H. H. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet,* 38**,** 431-40.
LOWRY, W. E., RICHTER, L., YACHECHKO, R., PYLE, A. D., TCHIEU, J., SRIDHARAN, R., CLARK, A. T. & PLATH, K. 2008. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 2883-2888.
LU, H. F., CHAI, C., LIM, T. C., LEONG, M. F., LIM, J. K., GAO, S. J., LIM, K. L. & WAN, A. C. A. 2014. A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells. *Biomaterials,* 35**,** 2816-2826.
LU, S.-J., PARK, J. S., FENG, Q. & LANZA, R. 2009. Red Blood Cells. *In:* LANZA, R. (ed.) *Essentials of Stem Cell Biology.* 2nd ed.
LU, S. J., FENG, Q., CABALLERO, S., CHEN, Y., MOORE, M. A. S., GRANT, M. B. & LANZA, R. 2007. Generation of functional hemangioblasts from human embryonic stem cells. *Nature Methods,* 4**,** 501-509.
LU, S. J., FENG, Q., PARK, J. S. & LANZA, R. 2010. Directed differentiation of red blood cells from human embryonic stem cells. *Methods Mol Biol,* 636**,** 105-21.
LU, S. J., FENG, Q., PARK, J. S., VIDA, L., LEE, B. S., STRAUSBAUCH, M., WETTSTEIN, P. J., HONIG, G. R. & LANZA, R. 2008a. Biologic properties and enucleation of red blood cells from human embryonic stem cells. *Blood,* 112**,** 4475-4484.
LU, S. J., LUO, C. M., HOLTON, K., FENG, Q., IVANOVA, Y. & LANZA, R. 2008b. Robust generation of hemangioblastic progenitors from human embryonic stem cells. *Regenerative Medicine,* 3**,** 693-704.
LUDWIG, T. E., BERGENDAHL, V., LEVENSTEIN, M. E., YU, J. Y., PROBASCO, M. D. & THOMSON, J. A. 2006. Feeder-independent culture of human embryonic stem cells (vol 3, pg 637, 2006). *Nature Methods,* 3**,** 867-867.
MA, F., EBIHARA, Y., UMEDA, K., SAKAI, H., HANADA, S., ZHANG, H., ZAIKE, Y., TSUCHIDA, E., NAKAHATA, T., NAKAUCHI, H. & TSUJI, K. 2008. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 13087-13092.
MADONNA, R., GORBE, A., FERDINANDY, P. & DE CATERINA, R. 2013. Glucose Metabolism, Hyperosmotic Stress, and Reprogramming of Somatic Cells. *Molecular Biotechnology,* 55**,** 169-178.
MALIK, P., FISHER, T. C., BARSKY, L. L. W., ZENG, L. H., IZADI, F., HITI, A. L., WEINBERG, K. I., COATES, T. D., MEISELMAN, H. T. J. & KOHN, D. B. 1998. An in vitro model of human red blood cell production from hematopoietic progenitor cells. *Blood,* 91**,** 2664-2671.
MALLON, B. S., PARK, K. Y., CHEN, K. G., HAMILTON, R. S. & MCKAY, R. D. 2006. Toward xeno-free culture of human embryonic stem cells. *Int J Biochem Cell Biol,* 38**,** 1063-75.
MANWANI, D. & BIEKERT, J. J. 2008. The erythroblastic Island. *Red Cell Development,* 82**,** 23-+.

MARSEE, D. K., PINKUS, G. S. & YU, H. 2010. CD71 (transferrin receptor): an effective marker for erythroid precursors in bone marrow biopsy specimens. *Am J Clin Pathol,* 134**,** 429-35.
MARTIN, G. R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A,* 78**,** 7634-8.
MATSUOKA, S., EBIHARA, Y., XU, M., ISHII, T., SUGIYAMA, D., YOSHINO, H., UEDA, T., MANABE, A., TANAKA, R., IKEDA, Y., NAKAHATA, T. & TSUJI, K. 2001. CD34 expression on long-term repopulating hematopoietic stem cells changes during developmental stages. *Blood,* 97**,** 419-25.
MAVILIO, F., GIAMPAOLO, A., CARE, A., MIGLIACCIO, G., CALANDRINI, M., RUSSO, G., PAGLIARDI, G. L., MASTROBERARDINO, G., MARINUCCI, M. & PESCHLE, C. 1983. Molecular Mechanisms of Human-Hemoglobin Switching - Selective Undermethylation and Expression of Globin Genes in Embryonic, Fetal, and Adult Erythroblasts. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences,* 80**,** 6907-6911.
MAZURIER, C., DOUAY, L. & LAPILLONNE, H. 2011. Red blood cells from induced pluripotent stem cells: hurdles and developments. *Current Opinion in Hematology,* 18**,** 249-253.
MCGRATH, K. & PALIS, J. 2008. Ontogeny of erythropoiesis in the mammalian embryo. *Red Cell Development,* 82**,** 1-+.
MCNIECE, I. K., LANGLEY, K. E. & ZSEBO, K. M. 1991. Recombinant Human Stem-Cell Factor Synergizes with Gm-Csf, G-Csf, Il-3 and Epo to Stimulate Human Progenitor Cells of the Myeloid and Erythroid Lineages. *Experimental Hematology,* 19**,** 226-231.
MEL, H. C., PRENANT, M. & MOHANDAS, N. 1977. Reticulocyte Motility and Form - Studies on Maturation and Classification. *Blood,* 49**,** 1001-1009.
MIHARADA, K., HIROYAMA, T., SUDO, K., NAGASAWA, T. & NAKAMURA, Y. 2006. Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells. *Nat Biotechnol,* 24**,** 1255-6.
MIKKOLA, H. K. A., GEKAS, C., ORKIN, S. H. & DIETERLEN-LIEVRE, F. 2005. Placenta as a site for hematopoietic stem cell development. *Experimental Hematology,* 33**,** 1048-1054.
MILLER, W. M., WILKE, C. R. & BLANCH, H. W. 1987. Effects of Dissolved-Oxygen Concentration on Hybridoma Growth and Metabolism in Continuous Culture. *Journal of Cellular Physiology,* 132**,** 524-530.
MOSCHANDREOU, T. 2012. RBC-ATP Theory of Regulation for Tissue Oxygenation-ATP Concentration Model *In:* MOSCHANDREOU, T. (ed.) *Blood Cell - An Overview of Studies in Hematology.* InTech.
MOUNTFORD, J., OLIVIER, E. & TURNER, M. 2010. Prospects for the manufacture of red cells for transfusion. *British Journal of Haematology,* 149**,** 22-34.
MUENCH, M. O., SCHNEIDER, J. G. & MOORE, M. A. 1992. Interactions among colony-stimulating factors, IL-1 beta, IL-6, and kit-ligand in the regulation of primitive murine hematopoietic cells. *Exp Hematol,* 20**,** 339-49.
MUMMERY, C. L., ZHANG, J. H., NG, E. S., ELLIOTT, D. A., ELEFANTY, A. G. & KAMP, T. J. 2012. Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells to Cardiomyocytes A Methods Overview. *Circulation Research,* 111**,** 344-358.
MURRY, C. E. & KELLER, G. 2008. Differentiation of embryonic stem cells to clinically relevant populations: Lessons from embryonic development. *Cell,* 132**,** 661-680.
NAKAGAWA, M., TANIGUCHI, Y., SENDA, S., TAKIZAWA, N., ICHISAKA, T., ASANO, K., MORIZANE, A., DOI, D., TAKAHASHI, J., NISHIZAWA, M., YOSHIDA, Y., TOYODA, T., OSAFUNE, K., SEKIGUCHI, K. & YAMANAKA, S. 2014. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. *Scientific Reports,* 4.
NAKANO, T. 1996. In vitro development of hematopoietic system from mouse embryonic stem cells: A new approach for embryonic hematopoiesis. *International Journal of Hematology,* 65**,** 1-8.

NAKAYAMA, N., LEE, J. & CHIU, L. 2000. Vascular endothelial growth factor synergistically enhances bone morphogenetic protein-4-dependent lymphohematopoietic cell generation from embryonic stem cells in vitro. *Blood,* 95**,** 2275-2283.

NEILDEZ-NGUYEN, T. M. A., WAJCMAN, H., MARDEN, M. C., BENSIDHOUM, M., MONCOLLIN, V., GIARRATANA, M. C., KOBARI, L., THIERRY, D. & DOUAY, L. 2002. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nature Biotechnology,* 20**,** 467-472.

NELSON, D. L. & COX, M. M. 2013. *Lehninger Principles of Biochemistry*, W.H. Freeman.

NIIHARA, Y., ZEREZ, C. R., AKIYAMA, D. S. & TANAKA, K. R. 1997. Increased red cell glutamine availability in sickle cell anemia: Demonstration of increased active transport, affinity, and increased glutamate level in intact red cells. *Journal of Laboratory and Clinical Medicine,* 130**,** 83-90.

NISHIKAWA, S., GOLDSTEIN, R. A. & NIERRAS, C. R. 2008. The promise of human induced pluripotent stem cells for research and therapy. *Nat Rev Mol Cell Biol,* 9**,** 725-9.

NISHIKAWA, S., NISHIKAWA, S., HIRASHIMA, M., MATSUYOSHI, N. & KODAMA, H. 1998. Progressive lineage analysis by cell sorting and culture identifies FLK1(+)VE-cadherin(+) cells at a diverging point of endothelial and hemopoietic lineages. *Development,* 125**,** 1747-1757.

NIWA, A., HEIKE, T., UMEDA, K., OSHIMA, K., KATO, I., SAKAI, H., SUEMORI, H., NAKAHATA, T. & SAITO, M. K. 2011. A novel serum-free monolayer culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors. *PLoS One,* 6**,** e22261.

NIWA, H., MIYAZAKI, J. & SMITH, A. G. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics,* 24**,** 372-376.

OGAWA, Z., KANASHIMA, M. & NISHIOKA, H. 2001. Improvement of the quantitative method for glucose determination using hexokinase and glucose 6-phosphate dehydrogenase. *Clinical Chemistry and Laboratory Medicine,* 39**,** 396-400.

OKITA, K., MATSUMURA, Y., SATO, Y., OKADA, A., MORIZANE, A., OKAMOTO, S., HONG, H., NAKAGAWA, M., TANABE, K., TEZUKA, K., SHIBATA, T., KUNISADA, T., TAKAHASHI, M., TAKAHASHI, J., SAJI, H. & YAMANAKA, S. 2011. A more efficient method to generate integration-free human iPS cells. *Nature Methods,* 8**,** 409-U52.

OKITA, K., YAMAKAWA, T., MATSUMURA, Y., SATO, Y., AMANO, N., WATANABE, A., GOSHIMA, N. & YAMANAKA, S. 2013. An Efficient Nonviral Method to Generate Integration-Free Human-Induced Pluripotent Stem Cells from Cord Blood and Peripheral Blood Cells. *Stem Cells,* 31**,** 458-466.

OLIVIER, E. N., QIU, C. H., VELHO, M., HIRSCH, R. E. & BOUHASSIRA, E. E. 2006. Large-scale production of embryonic red blood cells from human embryonic stem cells. *Experimental Hematology,* 34**,** 1635-1642.

ORKIN, S. H. & ZON, L. I. 2008. Hematopoiesis: An evolving paradigm for stem cell biology. *Cell,* 132**,** 631-644.

OSIECKA-IWAN, A., HYC, A., NIDERLA-BIELINSKA, J. & MOSKALEWSKI, S. 2008. Chondrocyte-associated antigen and matrix components in a 2-and 3-dimensional culture of rat chondrocytes. *Molecular Medicine Reports,* 1**,** 881-887.

OZTURK, S. S. & PALSSON, B. O. 1991. Growth, metabolic, and antibody production kinetics of hybridoma cell culture: 1. Analysis of data from controlled batch reactors. *Biotechnol Prog,* 7**,** 471-80.

PALIS, J. 2014. Primitive and definitive erythropoiesis in mammals. *Front Physiol,* 5**,** 3.

PALMER, A. F. & INTAGLIETTA, M. 2014. Blood Substitutes. *Annual Review of Biomedical Engineering, Vol 16,* 16**,** 77-101.

PAN, G. J., CHANG, Z. Y., SCHOLER, H. R. & PEI, D. Q. 2002. Stem cell pluripotency and transcription factor Oct4. *Cell Research,* 12**,** 321-329.

PAN, X. Q., OHNEDAL, O., OHNEDA, K., LINDEBOOM, F., IWATA, F., SHIMIZU, R., NAGANO, M., SUWABE, N., PHILIPSEN, S., LIM, K. C., ENGEL, J. D. & YAMAMOTO, M. 2005. Graded levels of GATA-1 expression modulate survival, proliferation, and differentiation of erythroid progenitors. *Journal of Biological Chemistry,* 280**,** 22385-22394.

PANOSKALTSIS, N., MANTALARIS, A. & WU, J. H. D. 2005. Engineering a mimicry of bone marrow tissue ex vivo. *Journal of Bioscience and Bioengineering,* 100**,** 28-35.

PARK, I. H., ZHAO, R., WEST, J. A., YABUUCHI, A., HUO, H. G., INCE, T. A., LEROU, P. H., LENSCH, M. W. & DALEY, G. Q. 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature,* 451**,** 141-U1.

PEARSON, S., SROCZYNSKA, P., LACAUD, G. & KOUSKOFF, V. 2008. The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development,* 135**,** 1525-1535.

PICK, M., AZZOLA, L., MOSSMAN, A., STANLEY, E. G. & ELEFANTY, A. G. 2007. Differentiation of human embryonic stem cells in serum-free medium reveals distinct roles for bone morphogenetic protein 4, vascular endothelial growth factor, stem cell factor, and fibroblast growth factor 2 in Hematopoiesis. *Stem Cells,* 25**,** 2206-2214.

POULSOM, R., ALISON, M. R., FORBES, S. J. & WRIGHT, N. A. 2002. Adult stem cell plasticity. *J Pathol,* 197**,** 441-56.

PRADO-LOPEZ, S., CONESA, A., ARMINAN, A., MARTINEZ-LOSA, M., ESCOBEDO-LUCEA, C., GANDIA, C., TARAZONA, S., MELGUIZO, D., BLESA, D., MONTANER, D., SANZ-GONZALEZ, S., SEPULVEDA, P., GOTZ, S., O'CONNOR, J. E., MORENO, R., DOPAZO, J., BURKS, D. J. & STOJKOVIC, M. 2010. Hypoxia promotes efficient differentiation of human embryonic stem cells to functional endothelium. *Stem Cells,* 28**,** 407-18.

PROWSE, A. B. J., DORAN, M. R., COOPER-WHITE, J. J., CHONG, F., MUNRO, T. R., FITZPATRICK, J., CHUNG, T. L., HAYLOCK, D. N., GRAY, P. P. & WOLVETANG, E. J. 2010. Long term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media. *Biomaterials,* 31**,** 8281-8288.

PURTON, L. E. & SCADDEN, D. T. 2008. The hematopoietic stem cell niche. *StemBook.* Cambridge (MA).

QIU, C., OLIVIER, E. N., VELHO, M. & BOUHASSIRA, E. E. 2008. Globin switches in yolk sac-like primitive and fetal-like definitive red blood cells produced from human embryonic stem cells. *Blood,* 111**,** 2400-8.

RHODES, M. M., KOPSOMBUT, P., BONDURANT, M. C., PRICE, J. O. & KOURY, M. J. 2008. Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin. *Blood,* 111**,** 1700-1708.

RICHARDS, M., FONG, C. Y., CHAN, W. K., WONG, P. C. & BONGSO, A. 2002. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol,* 20**,** 933-6.

RICHMOND, T. D., CHOHAN, M. & BARBER, D. L. 2005. Turning cells red: signal transduction mediated by erythropoietin. *Trends in Cell Biology,* 15**,** 146-155.

ROBIN, C., OTTERSBACH, K., DURAND, C., PEETERS, M., VANES, L., TYBULEWICZ, V. & DZIERZAK, E. 2006. An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells. *Developmental Cell,* 11**,** 171-180.

RODIN, S., ANTONSSON, L., NIAUDET, C., SIMONSON, O. E., SALMELA, E., HANSSON, E. M., DOMOGATSKAYA, A., XIAO, Z. J., DAMDIMOPOULOU, P., SHEIKHI, M., INZUNZA, J., NILSSON, A. S., BAKER, D., KUIPER, R., SUN, Y., BLENNOW, E., NORDENSKJOLD, M., GRINNEMO, K. H., KERE, J., BETSHOLTZ, C., HOVATTA, O. & TRYGGVASON, K. 2014. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. *Nature Communications,* 5.

RODIN, S., DOMOGATSKAYA, A., STROM, S., HANSSON, E. M., CHIEN, K. R., INZUNZA, J., HOVATTA, O. & TRYGGVASON, K. 2010. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol,* 28**,** 611-5.

SARGENT, P. J., FARNAUD, S. & EVANS, R. W. 2005. Structure/function overview of proteins involved in iron storage and transport. *Curr Med Chem,* 12**,** 2683-93.

SAWYER, S. T. & PENTA, K. 1994. Erythropoietin Cell Biology. *Hematology-Oncology Clinics of North America,* 8**,** 895-&.

SEIFINEJAD, A., TAEI, A., TOTONCHI, M., VAZIRINASAB, H., HASSANI, S. N., AGHDAMI, N., SHAHBAZI, E., YAZDI, R. S., SALEKDEH, G. H. & BAHARVAND, H. 2010. Generation of human induced pluripotent stem cells from a Bombay individual: Moving towards "universal-donor" red blood cells. *Biochemical and Biophysical Research Communications,* 391**,** 329-334.

SEMENZA, G. L. 2009. Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis. *Blood,* 114**,** 2015-2019.

SHAH, S., HUANG, X. S. & CHENG, L. Z. 2014. Concise Review: Stem Cell-Based Approaches to Red Blood Cell Production for Transfusion. *Stem Cells Translational Medicine,* 3**,** 346-355.

SHI, G. 2013. Differentiation of human pluripotent stem cells into functional cells. *OA Stem Cells,* 1**,** 2.

SHI, W., WANG, H., PAN, G., GENG, Y., GUO, Y. & PEI, D. 2006. Regulation of the pluripotency marker Rex-1 by Nanog and Sox2. *J Biol Chem,* 281**,** 23319-25.

SHIVDASANI, R. A. & ORKIN, S. H. 1996. The transcriptional control of hematopoiesis. *Blood,* 87**,** 4025-4039.

SILVER, L. & PALIS, J. 1997. Initiation of murine embryonic erythropoiesis: A spatial analysis. *Blood,* 89**,** 1154-1164.

SINGER, J. W. & ADAMSON, J. W. 1976. Steroids and Hematopoiesis .2. Effect of Steroids on Invitro Erythroid Colony Growth - Evidence for Different Target-Cells for Different Classes of Steroids. *Journal of Cellular Physiology,* 88**,** 135-143.

SLUKVIN, I. I. 2010. Generation of mature blood cells from pluripotent stem cells. *Haematologica-the Hematology Journal,* 95**,** 1621-1623.

SONE, M., ITOH, H., YAMAHARA, K., YAMASHITA, J. K., YURUGI-KOBAYASHI, T., NONOGUCHI, A., SUZUKI, Y., CHAO, T. H., SAWADA, N., FUKUNAGA, Y., MIYASHITA, K., PARK, K., OYAMADA, N., SAWADA, N., TAURA, D., TAMURA, N., KONDO, Y., NITO, S., SUEMORI, H., NAKATSUJI, N., NISHIKAWA, S. I. & NAKAO, K. 2007. Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. *Arteriosclerosis Thrombosis and Vascular Biology,* 27**,** 2127-2134.

SPIVAK, J. L., PHAM, T., ISAACS, M. & HANKINS, W. D. 1991. Erythropoietin Is Both a Mitogen and a Survival Factor. *Blood,* 77**,** 1228-1233.

STADTFELD, M., APOSTOLOU, E., FERRARI, F., CHOI, J., WALSH, R. M., CHEN, T. P., OOI, S. S. K., KIM, S. Y., BESTOR, T. H., SHIODA, T., PARK, P. J. & HOCHEDLINGER, K. 2012. Ascorbic acid prevents loss of Dlk1-Dio3 imprinting and facilitates generation of all-iPS cell mice from terminally differentiated B cells (vol 44, pg 398, 2012). *Nature Genetics,* 44**,** 831-831.

STEINER, R. & VOGEL, H. 1973. On the kinetics of erythroid cell differentiation in fetal mice. I. Microspectrophotometric determination of the hemoglobin content in erythroid cells during gestation. *J Cell Physiol,* 81**,** 323-38.

STEPHENS.JR, AXELRAD, A. A., MCLEOD, D. L. & SHREEVE, M. M. 1971. Induction of Colonies of Hemoglobin-Synthesizing Cells by Erythropoietin in-Vitro - (Fetal Mouse Liver/Erythropoietin/Erythroid Cells/Fe-59/Granulocytes). *Proceedings of the National Academy of Sciences of the United States of America,* 68**,** 1542-&.

STIREWALT, D. L. & RADICH, J. P. 2003. The role of FLT3 in haematopoietic malignancies. *Nature Reviews Cancer,* 3**,** 650-U1.

SUDA, T., SUDA, J. & OGAWA, M. 1983. Single-cell origin of mouse hemopoietic colonies expressing multiple lineages in variable combinations. *Proc Natl Acad Sci U S A,* 80**,** 6689-93.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-872.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-676.

TANIOUS, F. A., VEAL, J. M., BUCZAK, H., RATMEYER, L. S. & WILSON, W. D. 1992. Dapi (4',6-Diamidino-2-Phenylindole) Binds Differently to DNA and Rna - Minor-Groove Binding at at Sites and Intercalation at Au Sites. *Biochemistry,* 31**,** 3103-3112.

TAVIAN, M., COULOMBEL, L., LUTON, D., SANCLEMENTE, H., DIETERLENLIEVRE, F. & PEAULT, B. 1996. Aorta-associated CD34(+) hematopoietic cells in the early human embryo. *Blood,* 87**,** 67-72.

TEMPLE, S. 2001. Stem cell plasticity - Building the brain of our dreams (vol 2, pg 513, 2001). *Nature Reviews Neuroscience,* 2**,** 670-670.

THOMSON, J. A., ITSKOVITZ-ELDOR, J., SHAPIRO, S. S., WAKNITZ, M. A., SWIERGIEL, J. J., MARSHALL, V. S. & JONES, J. M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science,* 282**,** 1145-1147.

TIAN, X. H., MORRIS, J. K., LINEHAN, J. L. & KAUFMAN, D. S. 2004. Cytokine requirements differ for stroma and embryoid body-mediated hematopoiesis from human embryonic stem cells. *Experimental Hematology,* 32**,** 1000-1009.

TORBETT, B. E. & FRIEDMAN, J. S. 2009. ERYTHROPOIESIS: AN OVERVIEW. *ERYTHROPOIETINS, ERYTHROPOIETIC FACTORS, AND ERYTHROPOIESIS*

Birkhäuser Basel.

TOTONCHI, M., TAEI, A., SEIFINEJAD, A., TABEBORDBAR, M., RASSOULI, H., FARROKHI, A., GOURABI, H., AGHDAMI, N., HOSSEINI SALEKDEH, G. & BAHARVAND, H. 2010. Feeder- and serum-free establishment and expansion of human induced pluripotent stem cells. *International Journal of Developmental Biology,* 54**,** 877-886.

VALLIER, L., ALEXANDER, M. & PEDERSEN, R. A. 2005. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *Journal of Cell Science,* 118**,** 4495-4509.

VARUM, S., RODRIGUES, A. S., MOURA, M. B., MOMCILOVIC, O., EASLEY, C. A., RAMALHO-SANTOS, J., VAN HOUTEN, B. & SCHATTEN, G. 2011. Energy Metabolism in Human Pluripotent Stem Cells and Their Differentiated Counterparts. *Plos One,* 6.

VIDRICAIRE, G., JARDINE, K. & MCBURNEY, M. W. 1994. Expression of the Brachyury gene during mesoderm development in differentiating embryonal carcinoma cell cultures. *Development,* 120**,** 115-22.

VISWANATHAN, S. R., POWERS, J. T., EINHORN, W., HOSHIDA, Y., NG, T. L., TOFFANIN, S., O'SULLIVAN, M., LU, J., PHILLIPS, L. A., LOCKHART, V. L., SHAH, S. P., TANWAR, P. S., MERMEL, C. H., BEROUKHIM, R., AZAM, M., TEIXEIRA, J., MEYERSON, M., HUGHES, T. P., LLOVET, J. M., RADICH, J., MULLIGHAN, C. G., GOLUB, T. R., SORENSEN, P. H. & DALEY, G. Q. 2009. Lin28 promotes transformation and is associated with advanced human malignancies. *Nature Genetics,* 41**,** 843-U109.

VODYANIK, M. A., THOMSON, J. A. & SLUKVIN, I. I. 2006. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. *Blood,* 108**,** 2095-2105.

WAGERS, A. J. & WEISSMAN, I. L. 2004. Plasticity of adult stem cells. *Cell,* 116**,** 639-648.

WANG, G., ZHANG, H., ZHAO, Y., LI, J., CAI, J., WANG, P., MENG, S., FENG, J., MIAO, C., DING, M., LI, D. & DENG, H. 2005. Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem Biophys Res Commun,* 330**,** 934-42.

WANG, H. S., HUNG, S. C., PENG, S. T., HUANG, C. C., WEI, H. M., GUO, Y. J., FU, Y. S., LAI, M. C. & CHEN, C. C. 2004. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells,* 22**,** 1330-7.

WATABE, T. & MIYAZONO, K. 2009. Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Research,* 19**,** 103-115.

WERNIG, M., MEISSNER, A., FOREMAN, R., BRAMBRINK, T., KU, M. C., HOCHEDLINGER, K., BERNSTEIN, B. E. & JAENISCH, R. 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature,* 448**,** 318-U2.

WESSELY, O., BAUER, A., QUANG, C. T., DEINER, E. M., VON LINDERN, M., MELLITZER, G., STEINLEIN, P., GHYSDAEL, J. & BEUG, H. 1999. A novel way to induce erythroid progenitor self renewal: Cooperation of c-Kit with the erythropoietin receptor. *Biological Chemistry,* 380**,** 187-202.

WHILLIER, S., GARCIA, B., CHAPMAN, B. E., KUCHEL, P. W. & RAFTOS, J. E. 2011. Glutamine and alpha-ketoglutarate as glutamate sources for glutathione synthesis in human erythrocytes. *Febs Journal,* 278**,** 3152-3163.

WHITSETT, C., VAGLIO, S. & GRAZZINI, G. 2012. Alternative Blood Products and Clinical Needs in Transfusion Medicine. *Stem Cells International*.

WHO. 2011. *Key facts and figures* [Online]. Available: http://www.who.int/worldblooddonorday/media/Global_Blood_Safety_and_Availability_Key_facts_figures_2010.pdf [Accessed june 2011.

WILSON, I. A. & JOLLIFFE, L. K. 1999. The structure, organization, activation and plasticity of the erythropoietin receptor. *Current Opinion in Structural Biology,* 9**,** 696-704.

WINTER, S. S., HOWARD, T. & WARE, R. E. 1996. Regulation of expression of the human erythropoietin receptor gene. *Blood Cells Molecules and Diseases,* 22**,** 214-224.

WITTHUHN, B. A., QUELLE, F. W., SILVENNOINEN, O., YI, T. L., TANG, B., MIURA, O. & IHLE, J. N. 1993. Jak2 Associates with the Erythropoietin Receptor and Is Tyrosine-Phosphorylated and Activated Following Stimulation with Erythropoietin. *Cell,* 74**,** 227-236.

WONG, P. M., CHUNG, S. W., REICHELD, S. M. & CHUI, D. H. 1986. Hemoglobin switching during murine embryonic development: evidence for two populations of embryonic erythropoietic progenitor cells. *Blood,* 67**,** 716-21.

XU, C. H., ROSLER, E., JIANG, J. J., LEBKOWSKI, J. S., GOLD, J. D., O'SULLIVAN, C., DELAVAN-BOORSMA, K., MOK, M., BRONSTEIN, A. & CARPENTER, M. K. 2005a. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells,* 23**,** 315-323.

XU, R. H., CHEN, X., LI, D. S., LI, R., ADDICKS, G. C., GLENNON, C., ZWAKA, T. P. & THOMSON, J. A. 2002. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nature Biotechnology,* 20**,** 1261-1264.

XU, R. H., PECK, R. M., LI, D. S., FENG, X. Z., LUDWIG, T. & THOMSON, J. A. 2005b. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nature Methods,* 2**,** 185-190.

YANG, C. T., FRENCH, A., GOH, P. A., PAGNAMENTA, A., METTANANDA, S., TAYLOR, J., KNIGHT, S., NATHWANI, A., ROBERTS, D. J., WATT, S. M. & CARPENTER, L. 2014. Human induced pluripotent stem cell derived erythroblasts can undergo definitive erythropoiesis and co-express gamma and beta globins. *British Journal of Haematology,* 166**,** 435-448.

YEHIA, L., BOULOS, F., JABBOUR, M., MAHFOUD, Z., FAKHRUDDIN, N. & EL-SABBAN, M. 2015. Expression of HIF-1alpha and Markers of Angiogenesis Are Not Significantly Different in Triple Negative Breast Cancer Compared to Other Breast Cancer Molecular Subtypes: Implications for Future Therapy. *PLoS One,* 10**,** e0129356.

YIN, A. H., MIRAGLIA, S., ZANJANI, E. D., ALMEIDAPORADA, G., OGAWA, M., LEARY, A. G., OLWEUS, J., KEARNEY, J. & BUCK, D. W. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood,* 90**,** 5002-5012.

YING, W. H. 2008. NAD(+)/ NADH and NADP(+)/NADPH in cellular functions and cell death: Regulation and biological consequences. *Antioxidants & Redox Signaling,* 10**,** 179-206.
YU, J., VODYANIK, M. A., SMUGA-OTTO, K., ANTOSIEWICZ-BOURGET, J., FRANE, J. L., TIAN, S., NIE, J., JONSDOTTIR, G. A., RUOTTI, V., STEWART, R., SLUKVIN, II & THOMSON, J. A. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science,* 318**,** 1917-20.
ZAMBIDIS, E. T., PEAULT, B., PARK, T. S., BUNZ, F. & CIVIN, C. I. 2005. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood,* 106**,** 860-870.
ZHANG, J. H., KLOS, M., WILSON, G. F., HERMAN, A. M., LIAN, X. J., RAVAL, K. K., BARRON, M. R., HOU, L. Q., SOERENS, A. G., YU, J. Y., PALECEK, S. P., LYONS, G. E., THOMSON, J. A., HERRON, T. J., JALIFE, J. & KAMP, T. J. 2012. Extracellular Matrix Promotes Highly Efficient Cardiac Differentiation of Human Pluripotent Stem Cells The Matrix Sandwich Method. *Circulation Research,* 111**,** 1125-U78.
ZHANG, S. & CUI, W. 2014. Sox2, a key factor in the regulation of pluripotency and neural differentiation. *World J Stem Cells,* 6**,** 305-11.
ZHANG, W. J., PARK, C., ARENTSON, E. & CHOI, K. 2005. Modulation of hematopoietic and endothelial cell differentiation from mouse embryonic stem cells by different culture conditions. *Blood,* 105**,** 111-114.
ZHAO, F., PATHI, P., GRAYSON, W., XING, Q., LOCKE, B. R. & MA, T. 2005. Effects of oxygen transport on 3-d human mesenchymal stem cell metabolic activity in perfusion and static cultures: experiments and mathematical model. *Biotechnol Prog,* 21**,** 1269-80.
ZHOU, H. Y., WU, S. L., JOO, J. Y., ZHU, S. Y., HAN, D. W., LIN, T. X., TRAUGER, S., BIEN, G., YAO, S., ZHU, Y., SIUZDAK, G., SCHOLER, H. R., DUAN, L. X. & DING, S. 2009. Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins (vol 4, pg 381, 2009). *Cell Stem Cell,* 4**,** 581-581.
ZIELKE, H. R., SUMBILLA, C. M., SEVDALIAN, D. A., HAWKINS, R. L. & OZAND, P. T. 1980. Lactate: a major product of glutamine metabolism by human diploid fibroblasts. *J Cell Physiol,* 104**,** 433-41.