THE UNIVERSITY of EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

- This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
- A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
- This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
- The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
- When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Reprogramming peripheral blood mononuclear cells using an efficient feeder-free, non-integration method to generate iPS cells and the effect of immunophenotype and epigenetic state on HSPC fate

Jing Liu

THE UNIVERSITY
of EDINBURGH

Thesis submitted in fulfilment of
the requirements for the degree of
Doctor of Philosophy
to the
University of Edinburgh — 2014

Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted in any previous application for any other degree or professional qualification except as specified. Except where otherwise acknowledged, the work presented is entirely my own.

Jing Liu
May 2014

Abstract

Background and objectives In 2006 Shinya Yamanaka successfully reprogrammed mouse fibroblasts back to an embryonic stem cell-like state (called induced pluripotent cells, iPS cells) using retrovirus to introduce four genes that encode critical transcription factor proteins (Oct4, Sox2, KLF4, and c-Myc). This ability to reprogram has promising future applications in clinical and biomedical research for study of diseases, development of candidate drugs and to support therapeutic treatments in regenerative medicine. However, the clinical applications have to meet GMP requirements without the risk of insertional mutagenesis associated with retrovirus. Chromatin modifying agents are widely used in many protocols to generate iPS cells and culture of blood CD34$^+$ cells with chromatin-modifying agents can lead to an increase in marrow repopulating cells and in the case of valproic acid increased erythroid cell colony formation. We undertook research to help understand what effects these reagents have on mobilised peripheral blood (mPB) CD34$^+$ cells and optimised the expansion medium protocol to facilitate reprogramming work. This project aims to utilize peripheral blood mononuclear cells (MNC), one of the most easily accessible tissues to generate iPS cells using an efficient non-viral, feeder cell free methodology, with the ultimate goal of moving this methodology towards clinical use.

Materials and Methods G-CSF mobilised peripheral blood, buffy coat, cord blood and fetal liver were obtained from patients and donors under informed consent and ethics committee approval. Haematopoietic stem/progenitor cells CD34$^+$ or CD133$^+$) isolated by magnetic separation were flow cytometry sorted into CD34$^+$/CD133$^+$, CD34$^+$/CD133$^-$, and CD34$^-$/CD133$^+$ subpopulations and their lineage potential were assessed in colony forming unit assays. The effect of epigenetic modifiers valproic acid and 5-aza-2-deoxycytidine used singly or in combination with each other and with IL3 on phenotype and lineage potential of cultured CD34$^+$ cells from mobilised peripheral blood were assessed by flow cytometry and colony-forming unit assays.

Prior to reprogramming mononuclear cells from peripheral blood or $CD34^+$ cells from blood were expanded in culture medium supplemented with stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L) and Interleukin-3 (IL-3) for several days. Actively proliferating cells were reprogrammed by electroporation using episomal vectors with an oriP/EBNA-1 backbone to deliver five reprogramming genes, Oct4, Sox2, Lin28, L-Myc, and Klf4. Electroporated cells were seeded onto matrigel coated plates immediately after transfection or were reseeded after three days' culture. Subsequently, cells were cultured in specific medium on different days. When iPS colonies appeared, they were picked and cultured as for ES cells. Once established, iPS cell lines were immunophenotyped using flow cytometry and immunofluorescence and their potential to differentiate into the three germ layers was assessed *in vitro*.

Results and Conclusion The largest subpopulation of $CD34^+$ cells was $CD34^+/CD133^+$ population which was essentially committed to myeloid colony production, while much smaller $CD34^+/CD133^-$ subpopulation had a greater capacity to generate erythroid colonies. Optimised cytokine cocktail for expansion of $CD34^+$ cells included IL-3, important in improving expansion and maintaining functionality of $CD34^+$ cells. The optimised cytokine cocktail comprised 100 ng/ml SCF, 10 ng/ml Flt3L, and 20 ng/ml IL-3, which maintained $CD34^+$ cells and MNC in an active proliferating state. In addition, valproic acid and IL3 were found to act synergistically, to increase the numbers of $CD34^+/CD36^+$ positive cells. However, we found that an apparent increase in red cell colony formation actually resulted from a decrease in white cell colonies, so no overall increase in red cell colonies was seen when equivalent numbers of $CD34^+$ cells were plated.

Proliferating MNC maintained in optimised cytokine cocktail were amenable to electroporation for the effective delivery of episomal transcription factors (Oct4, Sox2, Klf4, L-Myc, and Lin28) within a backbone of oriP/EBNA-1. We successfully developed an efficient and simple method for reprogramming MNC from fresh or frozen samples to generate induced pluripotent cells using episomal vectors in a feeder-free system without any requirement for small molecules and the highest reprogramming efficiency is 0.033% (65 colonies from 2×10^5 seeding MNC). The cytokine cocktail and reprogramming methods work better in $CD34^+$ cells from cord blood or fetal liver, and we obtained 148 iPS colonies from 10^5 seeding cells (0.148%) at most. In addition, fibroblasts from adult and fetal liver can be successfully reprogrammed using the same reprogramming method. The use of episomal vectors with an oriP/EBNA-1 backbone to deliver reprogramming genes, and efficient electroporation were the most important factors in efficiency of the reprogramming process. In addition, it is pivotal to initiate transfection when cells are actively proliferating. The iPS cell lines we generated maintained

the successful expression of ES markers including Oct4, Nanog, SSEA3. SSEA4, TRA-1-60 and TRA-1-81, and had the capacity to successfully differentiate into cell types of ectoderm, mesoderm and endoderm layers *in vitro*.

Key Words iPS cells, MNC, CD34$^+$ cells, feeder-free, episomal vectors, efficient reprogramming

Acknowledgements

I wish my very sincere thanks to my supervisors Dr. Ronald C.J. Gallagher and Professor James Ross for their advice and assistance. Without their enlightening instruction, impressive kindness and patience, I could not have finished my experiments and completed my thesis. I would also like thank Kay Samuel and Joanna Brzeszczynska for the excellent advice and help in my experiments. I really appreciate their outstanding assistance and support when I met with difficulties. Thanks must go to James Black and Kathryn Sangster for their help in the lab. Thanks also should go to NHS Scottish blood transfusion service for providing me with many of the samples used in the thesis and my colleagues in the lab.
In addition, I would like to thank my family and all my friends for their encouragement and support. Finally I sincerely thank China Scholarship Council (CSC) for the funding to support me.

Publications

Liu, J., Samuel, K., Turner, M. L. and Gallagher, R. C. J. (2014), Use of IL3 and chromatin-modifying reagents valproic acid and 5-aza-2-deoxycytidine to affect mobilized peripheral blood CD34+ cell fate decisions. Vox Sanguinis. doi: 10.1111/vox.12124

Contents

Declaration iii

Abstract v

Acknowledgements ix

Publications xi

Contents xiii

Abbreviations xix

1 Introduction 1
1.1 Introduction to stem cells 1
1.1.1 Stem cells 2
1.1.2 Adult stem cells 3
1.1.3 Applications 4
1.2 Adult stem cells-haematopoietic stem cells 5
1.2.1 Haematopoiesis: development in the human 5
1.2.2 Definition and isolation of haematopoietic stem cells 11
1.2.3 Hierarchy of haematopoietic stem/progenitor cells 14
1.2.4 Key transcription factors of haematopoietic stem cells . . . 19
1.2.5 Application of haematopoietic stem cells in the laboratory 24
1.2.6 Clinical application of haematopoietic stem cells in regenerative medicine 25
1.3 iPS introduction 27
1.3.1 Origins of iPS cells 27
1.3.2 Definition and characteristics of iPS cells 30
1.3.3 Current methods of producing iPS cells 30
1.3.4 Significance of small molecules in signalling pathways in generating iPS cells 43
1.3.5 Engineering the Stem Cell Niche and the related Micro- and Macro-environment 53
1.3.6 The reduced use of transcription factors in reprogramming process 58

1.3.7 Types of different cells which are reprogrammed into iPS cells 61
1.3.8 Application of iPS cells 63
1.4 Hypothesis and Aims 66

2 Materials and Methods 67
2.1 Cell source and sampling 67
2.2 Cell isolation and purification 68
2.2.1 MNC isolation 68
2.2.2 HSPC purification by Magnetic-activated cell sorting MACS 69
2.2.3 Mesenchymal stem cells (MSC) isolation 70
2.2.4 FACS sorting of different populations 71
2.3 Cell culture 72
2.3.1 HSC cells *ex vivo* culture 72
2.3.2 Haematopoietic cell line culture 72
2.3.3 MSC culture 73
2.3.4 iPS culture 75
2.4 Reprogramming procedures 75
2.4.1 Generation of iPS cells from MSC cells by mRNA transfection and small molecules 75
2.4.2 Generation of iPS cells from fibroblast cells by episomal reprogramming vectors 76
2.4.3 Generation of iPS cells from blood CD34 enriched cells or mononuclear cells by episomal reprogramming vectors . . . 77
2.5 Preparation of MEF- Conditioned Medium (MEF-CM) 79
2.6 *In vitro* differentiation of iPS cells into three germ layer cells . . . 79
2.7 Plasmids construction 80
2.8 Isolation of total RNA 81
2.9 Synthesis of cDNA 81
2.10 Quantitative polymerase chain reaction (qPCR) 82
2.11 Digestion of pcDNA3.1 EGFP-poly(A83), pcDN3.1 Oct4-poly(A83) and pcDN3.1 Sox2-poly(A83) 84
2.12 Purification of plasmid DNA 84
2.13 Agarose gel electrophoresis 85
2.13.1 Pouring a standard 1% agarose gel 85
2.13.2 Loading samples and running an agarose gel 85
2.14 mRNA synthesis using mMESSAGE mMACHINE® T7 kit-high yield capped RNA transcription kit 86
2.15 Transfection of mRNA and plasmids 87
2.15.1 mRNA transfection using Mirus TransIT®-mRNA Transfection Kit 87
2.15.2 Transfection of plasmids using TransIT®-2020 reagent . . . 87
2.15.3 Transfection of plasmids using nucleofector 4D 88
2.16 Flow cytometry and analysis 88
2.17 *In vitro* colony assay 89

2.18 iPS colonies live staining 91
2.19 *In situ* immunofluorescence staining 91
2.20 Statistical analysis 93

3 The selection of HSPC based on CD34 and CD133 markers affects haematopoietic functional heterogeneity 95
3.1 Introduction 95
3.2 Experimental strategy 98
3.3 Results 98
3.3.1 The expression of HSPC markers (CD34 or CD133) on MNC from different sources 98
3.3.2 Assessment of Cell isolation method by MACS 101
3.3.3 Assessment of myeloid and erythroid CFU from $CD34^+$ enriched cells population (CD34e) and $CD34^+$-depleted MNC (CD34d) 105
3.3.4 CD34e cells have reduced potential to generate CFU whose cells express glycophorin A, while CD34d cells retain capacity to generate colonies whose cells express glycophorin A 107
3.3.5 Isolation of HSPC ($CD34^+$ or $CD133^+$) subpopulations and corresponding functional heterogeneity 110
3.4 Discussion 118
3.4.1 Colony forming units assay is the basic method to measure the functional reconstituting potential of cells from different sources 118
3.4.2 CD34d cells show potential to generate a much higher proportion of erythroid colonies than CD34e cells. 119
3.4.3 The candidate progenitor cells existing in CD34d cells responsible for producing a much higher erythroid forming proportion 120
3.4.4 The relationship of functional heterogeneity to haematopoietic stem/progenitor cell phenotype heterogeneity 123
3.5 Concluding remarks 124

4 The relationship of cytokines and Chromatin modification reagents in *ex vivo* proliferation of haematopoietic stem/progenitor cells 125
4.1 Introduction 125
4.2 Experimental strategy 128
4.3 Results 129
4.3.1 The effect of different cytokine combinations in cell proliferation 129
4.3.2 The immunophenotype variance of $CD34^+$ cells after different treatments 130

4.3.3 The number of $CD34^+$ cells in the medium supplemented with different cytokine combinations and chromatin modification reagents . . . 132
4.3.4 Comparison of haematopoietic stem cell functionality of CD34 enriched cells after treating with SCF+Flt3L or SCF+Flt3L+IL3 . . . 138
4.3.5 Does the addition of drugs including VPA and 5-Aza exert an effect on cultured cells? . . . 139
4.4 Discussion . . . 142
4.4.1 The effect of IL3 and chromatin modification reagents (VPA, 5-Aza) in cell proliferation . . . 142
4.4.2 The functionality of HSPCs after expansion with SF or SF+IL3 . . . 145
4.4.3 The effect of epigenetic altering reagents in functionality of expanded HSPC . . . 145
4.5 Concluding remarks . . . 148

5 Assessment of methods for delivering mRNAs and plasmid DNA into cells 149
5.1 Introduction . . . 149
5.2 Experimental strategy . . . 150
5.3 Results . . . 151
5.3.1 The persistence of *in vitro* synthesized mRNA measured indirectly by the expression of corresponding protein . . . 151
5.3.2 mRNA delivery . . . 153
5.3.3 Plasmid DNA delivery . . . 159
5.4 Discussion . . . 163
5.4.1 Synthesized mRNAs can work *in vitro*, but not the priority tool for hierarchy regulation process . . . 163
5.4.2 The delivery of Plasmid DNA into cells through electroporation can be more efficient than using chemical methods . 164
5.5 Concluding remarks . . . 164

6 iPS production 167
6.1 Introduction . . . 167
6.2 Experimental strategy . . . 172
6.3 Results . . . 173
6.3.1 Partially reprogrammed iPS-like colonies generated by Oct3/4 mRNA transfected cells cultured in hES medium with small molecules . . . 173
6.3.2 The generation of iPS colonies from $CD34^+$ cells from cord blood and fetal liver using episomal vectors . . . 175
6.3.3 The generation of iPS colonies from mononuclear cells using episomal vectors . . . 182

6.3.4 The generation of iPS colonies from fibroblasts using episomal vectors . . . 183
6.4 Discussion . . . 187
6.4.1 The successful establishment of an efficient non-integration method to reprogram mononuclear cells in a feeder-free system 187
6.4.2 The significant points in our episomal vectors reprogramming system . . . 188
6.4.3 An analysis for the failure of the mRNA-reprogramming process . . . 190
6.4.4 The combination of small molecules CHALP did not improve reprogramming efficiency . . . 193
6.5 Concluding remarks . . . 194

7 The identity of pluripotency of derived iPS cell lines 197
7.1 Introduction . . . 197
7.2 Experimental strategy . . . 199
7.3 Results . . . 199
7.3.1 The pluripotent markers expressed by iPS cells . . . 199
7.3.2 Pluripotent gene expression of established iPS cell lines . . . 201
7.3.3 *In vitro* differentiation into three germ layer cells . . . 210
7.4 Discussion . . . 210
7.4.1 Pluripotent markers can identify iPS cells . . . 210
7.4.2 Pluripotent gene expression of established iPS cell lines . . . 216
7.4.3 *In vitro* differentiation test verifies the pluripotency of iPS cells . . . 217
7.5 Concluding remarks . . . 218

8 Conclusion 219

Bibliography 223

Abbreviations

Ad (adenoviruses)
AGM (aorta-gonad-mesonephros)
AKT (protein kinase B)
ALDH (aldehyde dehydrogenase)
AMH (anti-müllerian hormone)
ANOVA (analysis of variance)
AOF1 (Aliases for Lysine (K)-Specific Demethylase 1B)
AOF2 (Aliases for Lysine (K)-Specific Demethylase 1A)
APC (allophycocyanin)
5-Aza (5-aza-2'-deoxycytidine, Decitabine)
BCL11A (B-cell lymphoma/leukemia 11A)
BMP (bone morphogenetic proteins)
BFU-E (erythroid progenitors)
bFGF (basic fibroblast factor)
CAR (coxsackie virus and adenovirus receptor)
C6VVT (CHIR99021, 616542, ascorbic acid, VPA, tranylcypromine)
CB (cord blood)
CBP (CREB-binding protein)
CD34e ($CD34^+$ enriched cells population)
CD34d ($CD34^+$-depleted MNC)
CFU (colony-forming unit)
CHALP (CHIR33021, PD0325901, A83-01, HA100, LIF)
CK1α (casein kinase 1α)
CLP (common lymphoid progenitors)
CMP (common myeloid progenitors)
CSC (China Scholarship Council)
3D (3 dimentional)
DCM (dilated cardiomyopathy)
DNMT (DNA methyltransferase)
Dsh (Dishevelled)
EBNA-1 (Epstein-Barr nuclear antigen-1)
EC (embryonal carcinoma)
EGF (epidermal growth factor)
EGFR (epidermal growth factor receptor)

EIAV (equine infectious anemia virus)
EKLF (Erythroid Krüppel-like Factor)
EMT (epithelial-mesenchymal transition)
EPC (endothelial cell progenitors)
EPO (erythropoietin)
ERBB2 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2)
ERK (extracellular signal-regulated kinases)
EryD (definitive erythroid)
EryP (primitive erythroid)
ES cells (embryonic stem cells)
EtBr (ethidium bromide)
FACS (fluorescence activated cell sorting)
FCS (fetal calf serum)
FGFR (fibroblast growth factor receptor)
FITC (fluorescein isothiocyanate)
FIV (feline immunodeficiency virus)
Flt3L (Fms-related tyrosine kinase 3 ligand)
FOG-1 (Friend of GATA protein 1)
Fz (Frizzled)
GATA (GATA-binding factor)
G-CSF (granulocyte colony-stimulating factor)
GDFs (growth and differentiation factors)
GDP (guanosine diphosphate)
GlyA (glycophorin A)
GMP (granulocyte/macrophage progenitor)
GMP standards (Good Manufacturing Practices)
GM-CSF (granulocyte macrophage CSF)
GOI (gene of interest)
GRB2 (growth factor receptor-bound protein 2)
GSK3 (glycogen synthase kinase 3)
GTP (guanosine-5'-triphosphate)
GVHD (graft-versus-host disease)
GVL (graft-versus-leukemia)
GVT (graft-versus-tumour)
HDAC (histone deacetylases)
HDF (human dermal fibroblast)
HLA (human leukocyte antigen)
HPCs (haematopoietic progenitor cells)
HS (DNase I Super-hypersensitive)
HSCs (haematopoietic stem cells)
HSPC (haematopoietic stem and progenitor cells)
ICM (inner cell mass)
IGF (Insulin-like growth factor)

IGFBPs (IGF binding proteins)
iPS cells (induced pluripotent stem cells)
IL-3 (Interleukin-3)
Klf4 (Kruppel-like factor 4)
LCR (locus control region)
Lin28 (Lin-28 Homolog A)
LMPP (lymphoid-primed multipotent progenitor)
LoxP (locus of X-over P1)
LRP (low density lipoprotein receptor-related protein)
LTMR (long-term multilineage reconstitution)
LT-HSC (long-term self-renewal haematopoietic stem cells)
MAbs (monoclonal antibodies)
MACS (magnetic-activated cell sorting)
MAFK (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K)
MAPK (mitogen-activated protein kinases)
M-CSF (macrophage CSF)
MDS (myelodysplasia)
MECP (methyl-CpG-binding proteins)
mEpiSCs (mouse epiblast stem cells)
MEFs (mouse embryonic fibroblasts cells)
MEF-CM (MEF-Conditioned Medium)
MEK (Mitogen-activated protein kinase kinase)
MEP (megakaryocyte/erythroid progenitor)
MET (mesenchymal-epithelial-transition)
MNC (mononuclear cells)
mPB (mobilised peripheral blood)
mPBd (mobilised donor peripheral blood)
MSC (mesenchymal stem cells)
MTOR (mechanistic target of rapamycin)
MyRPs (myeloid-restricted repopulating progenitors)
Nanog (Homeobox Transcription Factor Nanog)
NK (natural killer)
NF-E2 (Nuclear Factor, Erythroid 2)
NOD/SCID (non-obese diabetic/severe combined immunodeficient)
NR2F2 (Nuclear Receptor Subfamily 2, Group F, Member 2)
nPB (normal peripheral blood)
Oct4 (octamer-binding transcription factor 4)
oriP (origin of viral replication)
OSKM (Oct4, Sox2, KLF4, and c-Myc)
PBS (phosphate buffered saline)
PBSC (peripheral blood stem cells)
PDGFR (Platelet-derived growth factor receptors)
PDK1 (3'-phosphoinositide-dependent kinase-1)

PDK (phosphoinositide-dependent kinase)
PE (phycoerythrin)
PerCP (peridin chlorophylla protein)
PI(3,4,5)P3 (phosphatidylinositol 3,4,5 triphosphates)
PI3K (phosphoinositide 3-kinase)
PKC (protein kinase C)
Poly I:C (Polyinosinic–polycytidylic acid potassium salt)
PP2A (protein phosphatase 2A)
P-Sp (para-aortic splanchnopleures)
qPCR (Quantitative polymerase chain reaction)
Ras (Rat sarcoma)
RAF kinase (proto-oncogene serine/threonine-protein kinase)
R-SMADs (receptor-regulated SMADs)
SCF (stem cell factor)
SCID (severe combined immunodeficiency)
SCNT (somatic cell nuclear transfer)
SCRM (Scottish Centre for Regenerative Medicine)
SF (SCF+Flt3L)
SF+IL3 (SCF+Flt3L+IL3)
SIV (simian immunodeficiency virus)
SNBTS (Scottish National Blood Transfusion Service)
SOS (Son of Sevenless)
Sox2 (SRY (sex determining region Y)-box 2) Sp (splanchnopleura)
SRCs (SCID-repopulating cells)
SSEA (stage-specific embryonic antigen)
SV40LT (Simian Vacuolating Virus 40 large T antigen)
TCF (T-cell factor)
TGF-β (transforming growth factor beta)
TPO (thrombopoietin)
TRA (tumor related antigen)
TSA (trichosatin A)
vCJD (new variant Creutzfeld–Jakob disease)
VPA (valproic acid)
Wnt (Int/Wingless)
YS (yolk sac)
ZBP-89 (BFCOL1, BERF-1, ZNF 148)

Chapter 1

Introduction

1.1 Introduction to stem cells

Stem cells are widely applied in regenerative medicine and have developed into a cutting edge topic in translational medicine. This might be attributed to two reasons: One reason is that the multipotential capacity of stem cells makes it possible to differentiate into various tissues, leading to promising treatments in degenerative diseases in clinic. An excellent case is the mesenchymal stem cells injected into joints to relieve degenerative arthritis (Centeno *et al.*, 2008). In addition, haematopoietic stem cell transplantation has become accepted effective therapy to treat haematopoietic disorders in clinic, for example, certain leukemias, myelodysplasia (MDS) and lymphoma. The successful application of haematopoietic stem cell transplantation in clinical treatments was a significant turning point with regard to the ultilisation of stem cells in translational medicine. Another reason for stem cells capturing so much focus is that stem cells potentially can provide a platform for scientists to study the mechanism of how they differentiate into a variety of cells and how the process can be controlled towards

a particular direction for translational research. In this part, I will present the definition of stem cells and summarise the fundamental knowledge of different stem cells. Moreover, I will give a general view of applications of stem cells in regenerative medicine.

1.1.1 Stem cells

Generally speaking, stem cells are defined by the characteristics of self-renewal as well as the capacity to differentiate into different types of cells. In other words, stem cells are capable of generating many daughter cells with the same characteristics as the mother cells without loss of their multipotent ability to form varieties of cells.

Self-renewal, the term means replicative or copy. The stem cells are capable of extensive proliferation *in vivo* or *in vitro* without oncogenic transformation under the appropriate conditions. However, there exists an essential issue: Can the stem cells still maintain their potency after several passages *in vitro*? Criteria to assess the quality and characteristics of stem cells have to be considered. In some cases, the self-renewal capacity of certain stem cells can be assessed rigorously by single cell or serial transfer into acceptable hosts. An excellent example are adult haematopoietic stem cells (HSCs) (Allsopp and Weissman, 2002; Iscove and Nawa, 1997). With regard to embryonic stem cells (ES cells), in animal species, the ES cells could be assessed by *in vivo* differentiation through the capacity of transferred ES cells to contribute to all somatic lineages and generate germ line chimerism. In current dogma, self-renewal is the special characteristic of stem cells, but not of commited progenitor cells. However, this view is challenged by the myeloid-restricted repopulating progenitors (MyRPs) with high self-renewal activity (Yamamoto *et al.*, 2013).

Potency is a pivotal and essential characteristic of stem cells. Potency can

be understood as the potential of stem cells to differentiate into multiple or limited cell lineages. Generally speaking, primitive cells with the capacity to differentiate into many cell lineages can be defined as stem cells. For example, ES cells are identified by their potential and ability to generate all somatic lineages in appropriate microenvironments or niches under well-defined conditions. The potency of ES cells to generate any cell types that make up the body is called pluripotency. Induced pluripotent stem cells are also considered to be pluripotent, the same as derived ES cells. Another potency which has to be distinguished from pluripotency is totipotency. Totipotent cells can differentiate into any cell types in a body including the extraembryonic and placental cells. However, progenitor cells can only produce one or two types of differentiated cells. Taking red cell progenitors as an example, in this case red cell progenitors have the potential to generate erythrocytes but not other blood lineages. Thus, the potency of cells only capable of producing one type of differentiated cell is best described as unipotency.

1.1.2 Adult stem cells

Different from ES/iPS cells, adult stem cells derive from the body after development and, although not pluripotent, have the multipotency to generate all cell types of the organ from which they originate, promisingly regenerating the damaged tissues or organs. In a word, the basic characteristics of adult stem cells are self-renewal as well as the capacity to generate differentiated cells. Though the most rigorous assessment of the two characteristics is to transplant the purified cell population into an acceptable host for observing self-renewal and reconstitution, it is not available for many types of adult stem cells. Thus it is necessary to develop an easily accessible method to assess cell potential *in vitro* rather than *in vivo*. An excellent case is haematopoietic stem cells, which have multipotency to

generate all blood lineage cells including erythrocytes, leukocytes and platelets. *In vitro* colony forming unit assay is the popular and routine method to assess haematopoietic stem/progenitor cells (HSPC). Part of my work focuses on culturing HSPC with different combinations of cytokines.

1.1.3 Applications

With the intriguing achievements and developments in stem cell research, stem cells have recently been applied in translational medicine especially regenerative medicine. Firstly, stem cells can be utilized in many diseases, for example, neurodegenerative disease, spinal injury, heart disease, diabetes, burned skin, and damaged tissues (Fisher *et al.*, 2013; Gutierrez-Fernandez *et al.*, 2013; Kim *et al.*, 2013; Lewis, 2013; Liu *et al.*, 2013). In autologous transplantation, when stem cells originating from patients are used in the same patients for treatments, there might be fewer immunogenic rejection issues. Secondly, tissues or cell lines deriving from stem cells provide a source for drug testing. They might be beneficial for patient-specific therapy, because every individual responds differently to the identical drug, and this would be useful for development of drug safety and effectivity.

1.2 Adult stem cells-haematopoietic stem cells

1.2.1 Haematopoiesis: development in the human

Overview

Haematopoiesis plays an essential role in human development and throughout life; recent research has focused on the embryonic developmental origin of the human haematopoietic system. Generally speaking, there are two independent anatomical sites as potential candidates for generating haematopoietic cells: yolk sac (YS) and aorta-gonad-mesonephros (AGM) region inside the embryo. During the third week of human development, haematopoiesis initiates outside the embryo, in the yolk sac, then proceeds and colonizes the liver, and actively develops in the thymus before lastly getting stabilization into adult life in the bone marrow. Alongside extra-embryonic haematopoiesis, the capacity to generate haematopoietic cells arises inside the embryo within splanchnopleura, but these only appear at 27 days of embryonic development. However, comparing characteristics of haematopoietic stem cells deriving from yolk sac with those from AGM region, yolk sac-derived stem cells show limited development capacity to differentiate toward myelo-erythroid lineages whereas stem cells derived from the AGM region inside the embryo exhibit lymphoid potentials (Tavian and Peault, 2005b).

Extra-embryonic generation of HSCs

Yolk sac In embryonic development of higher vertebrates, many mesoderm cells aggregate to form clusters at the origin of the vascular system in the extra-embryonic area. Within the homogeneous clusters, peripheral cells form endothelium while simultaneously the lumens of blood vessels are generated. The clusters attached to the inner wall of the lumen are named blood islands (Sabin, 1920). In Risau's study, cells at the periphery of these aggregates differentiate into endothelial cell precursors, while cells in the interior become primitive blood cells (Risau, 1991). However, mesoderm cells have the capacity to differentiate into both haematopoietic cells and endothelial cells, the simultaneous appearance of which led to the hypothesis that they might have a common ancestral precursor for endothelial and haematopoietic cells: the angioblast, later named as hemangioblast (Murray, 1932; Sabin, 1920; Wagner, 1980).
According to previous study, human yolk sac gives rise to mostly erythroid cells (Bloom and Bartelmez, 1940) and there is also the occasional presence of macrophages and primitive megakaryocytes (Fukuda, 1973). A functional study of haematopoiesis showed the presence of pluripotent stem cells, erythroid progenitors, and granulocyte, monocyte progenitors (GMP) at 4.5 weeks of human development. Subsequently in the fifth week, in the yolk sac early erythroid progenitors (BFU-E) drop dramatically and disappear in the sixth week, while BFU-E can be detected in the blood and their number undergoes a striking rise in the liver parenchyma (Migliaccio *et al.*, 1986). This is in accordance with the study by another group, who found that both erythroid progenitors and granulopoietic progenitors were identified in the yolk sac as early as 27 days, while they can be detected in the embryonic liver at 40 days (Tavian *et al.*, 1999).

The Liver The liver originates from a flat plate of endodermal cells within a five-somite embryo, lying ventrally to the endoderm of the foregut at the anterior intestinal portal. Due to the differentiation of the embryo, the plate of endoderm has to be folded. During the folding process of adjacent structures of the plate, there is a close spatial relationship between the cells of the endodermal plate and the caudal and ventral endothelial lining of the atrium and the sinus venosus. As a result, the folding leads to the formation of "T-shaped" diverticulum which projects ventrally and cephalically from the gut tract at the 20 somite-stage embryo (Severn, 1971).

Bone marrow In human haematopoietic ontogenesis, bone marrow is the last localized region and becomes the primary blood-forming tissues in adult mammals. Outside the embryo, human haematopoiesis starts in the yolk sac, then transiently proceeds in the liver and develops in the thymus, finally getting stabilized in bone marrow. An immunohistochemical study (Charbord *et al.*, 1996) of haematopoiesis demonstrated that haematopoiesis in fetal long bones appears during week 10.5, but is confined to primary logettes in the diaphyseal region until week 15, from which time onward the outer region of the diaphyseal area is replaced progressively by calcified trabeculae modelling adult-type bone. Primary logettes consist of an arteriole surrounded by a network of mesenchymal cells, actually the vascular sinuses. As the embryos develop, logettes increase in size and merge into larger ones, concomitant with the decreasing number, and finally disappear replaced by long bones with areas of fully calcified bone and areas of dense haematopoiesis. Surprisingly, $CD34^+$ haematopoietic cells are absent before the onset of haematopoiesis while the earliest blood cells present in diaphyseal area are $CD15^+$ myeloid cells from week 10 to 15, which means myeloid generation is the predominance of early haematopoiesis.

Intra-embryonic generation of HSCs

The extra-embryonic haematopoiesis system described above is the classic scheme for the development of human haematopoiesis. However, this was challenged in recent years. In mammals, early yolk sac is not the only origin of haematopoiesis, intra-embryonic para-aortic splanchnopleures (P-Sp) is an independent region that can generate HSC. Tavian and colleagues detected a small number of cells (2-3 cells) in rostral, duplicated section of aorta as early as 27 days, and these cells begin to proliferate to form clusters, which move towards the umbilical region of the aorta and the inside of the vitelline artery, disappearing after 40 days of gestation (Tavian *et al.*, 1996, 1999). The haematopoietic potential of these cells can be analysed through *in vitro* experiments using the corresponding isolated tissues or cells from different stages of development of gestation. Cells derived from aorta during day 27-40 of gestation have demonstrated haematopoietic potential. The point contradicting the classic view is that cells from day-19 splanchnopleura are able to establish long-term haematopoietic cell culture. However, at day-19 of gestation the aorta has not developed, which indicates the haematopoietic cells isolated from day-19 splanchnopleura (Sp) do not originate from aorta, further more they are not derived from yolk sac (Tavian and Peault, 2005a; Tavian *et al.*, 2001). With the development of embryos, the Sp eventually develops to the para-aortic splanchnopleura/aorta, while genital ridges and mesonephros originate from paraxial mesoderm. The aorta, genital ridges and mesonephros are comprised of AGM region, which are endowed with haematopoietic potential in mammalian embryos (Cumano, 2007; Dzierzak, 2008).

Cells from both early yolk sac and intraembryonic paraaortic splanchnopleures (P-Sp), cultured on MS-5 stromal cells, are capable of generating definitive HSCs with multilineage long-term reconstituting ability (Tavian *et al.*, 2001). The

haematopoietic differentiation potential of HSCs from these two regions are different: yolk sac derived haematopoietic cells are characterised by limited development potential, preferentially differentiating towards erythro-myeloid lineages; however, P-Sp or AGM derived haematopoietic cells exhibit myelo-lymphoid potentials (Tavian *et al.*, 2010).

As for the HSC origin in AGM region, there are currently two different models (Cumano, 2007; Tavian and Peault, 2005b). One is the haemogenic endothelium model, demonstrating that endothelial cells from the aortic floor transdifferentiate into haematopoietic cells. Another one is the subaortic generation model: in the human embryo, splanchnopleura derived angiogenic haematopoietic cells move through the periaortic mesenchyme and colonize the aortic floor during the fourth week of development (see Figure 1.1). Cortes *and colleagues* reported the phenotype of the cell population is Flk-1^{+}CD34^{-} in 1999 (Cortés *et al.*, 1999). In 2001, a monoclonal antibody BB9 was described to bind the antigen expressed in human bone marrow stromal cells (Ramshaw *et al.*, 2001). This novel marker can identify angiogenic haematopoietic cells or hemangioblasts, e.g. BB9^{+}CD34^{-}CD45^{-} angiogenic haematopoietic precursors from P-Sp migrates towards ventral aorta, concomitant with the generation of BB9^{+}CD34^{+}CD45^{+} haematopoietic stem cells and BB9^{+}CD34^{+}CD45^{-} endothelial cells.

During the commitment of the mesoderm towards a haematopoietic fate, bone morphogenetic proteins (BMP) signalling pathway plays a key role. The posterior fragment of the epiblasts in the early murine embryo can produce haematopoietic cells when cocultured with OP9 stromal cells (Nakano *et al.*, 1996), while anterior epiblasts lose the ability to generate haematopoietic cells in the same condition. However, the addition of BMP4 to the culture medium changes the situation, allowing the anterior epiblasts to retain the capacity to produce haematopoietic cells. There are also studies reporting that BMP4 promotes haematopietic cell differentiation (Chadwick *et al.*, 2003; Johansson and Wiles, 1995).

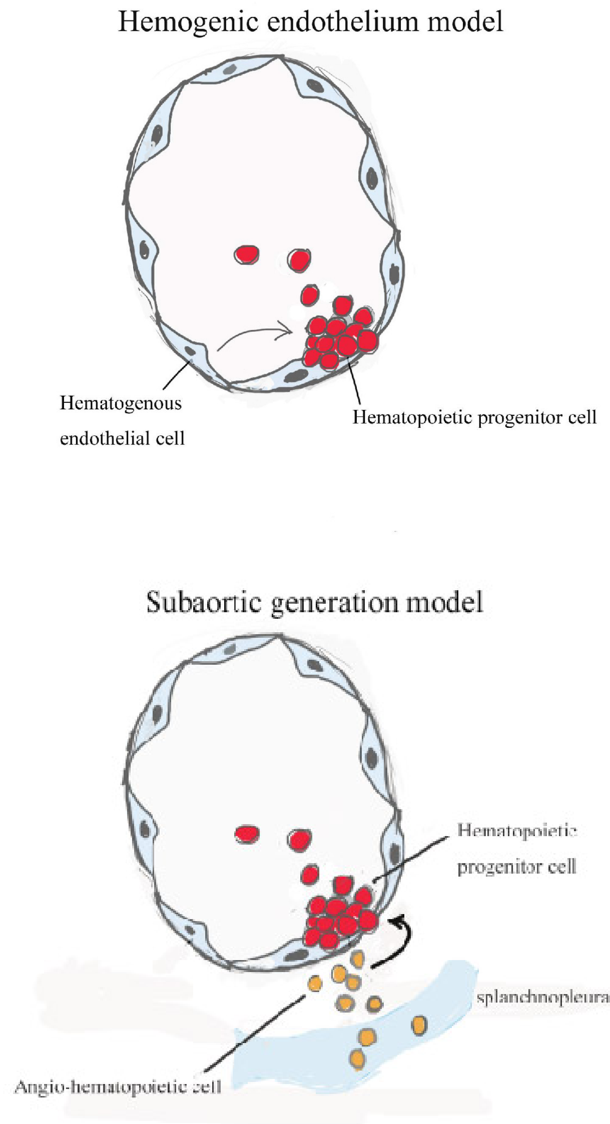


Figure 1.1: Two models of the HSC origin in AGM region

Haemogenic endothelium model: endothelial cells from the aortic floor transdifferentiate into haematopoietic cells. Subaortic generation model: in the human embryo, splanchnopleura derived angio-haematopoietic cells move through the peri-aortic mesenchyme and colonize the aortic floor during the fourth week of development.

Assessment of haematopoiesis through hemoglobin synthesis

The hemoglobin genes are differentially expressed during haematopoietic development. ε- and ζ-globin genes are expressed during the early embryonic period, while α- and γ-globin genes are expressed later in the fetal period followed by increased expression of β-globin gene and decreased γ-globin gene expression in adults. In addition, the δ-globin gene is also expressed at a low level in adults (Palis and Segel, 1998). The globin expression changes from embryonic globin to fetal globin (ζ-α, ε-γ) concomitant with the switch from primitive nucleated (megaloblasts) to definitive enucleated (macrocytes) erythrocytes, which indicates the transition from embryonic to liver haematopoiesis (Migliaccio *et al.*, 1986). The switch of fetal globin to adult globin (γ-β) is an important indication of maturation of erythroid differentiation, and a lack of adult globin can lead to thalassemia syndromes.

1.2.2 Definition and isolation of haematopoietic stem cells

Definition of haematopoietic stem cells and source of HSCs

HSCs are characterised by self-renewal and their potential to differentiate to generate all blood cell lineages throughout the life span of an individual, which is responsible for the long-term multilineage reconstitution (LTMR) in HSC transplant. Human haematopoietic stem and progenitor cells (HSPC) have been used since the end of 1960's (Bach *et al.*, 1968; De Koning *et al.*, 1969; Gatti *et al.*, 1968) for transplantation to reconstitute haematopoiesis following inherent deficiency (for example, severe immune deficiency) or deficiency induced by disease or by myeloablative chemotherapy. Initially HSPC were sourced from

bone marrow but are now commonly sourced from peripheral blood following their mobilisation from bone marrow by, for example, granulocyte colony stimulating factor (G-CSF). Another rich source of HSPC is umbilical cord blood (CB). HSPC are present in normal peripheral blood, but not at any clinically useful concentration. They were identified by their ability to form haematopoietic cell colonies in viscous methylcellulose-based culture medium supplemented with cytokines which stimulate growth of myeloid or erythroid colonies: these colonies are presumed to grow from a single originating cell referred to as a colony-forming unit (CFU), and so estimate the frequency of such cells. Haematopoietic colonies grow out over 12 to 14 days, and are subject to problems arising from any microbial contamination during this time. However, the frequencies of HSPC in different sources are different, which will be given in more detail in chapter 3.

Cell surface markers of HSPCs

In the 1990s flow cytometry began to be widely used to examine the expression of various surface markers on different cells, and it was recognised that the CD34 marker could be used to adequately define and quantify HSPC for clinical use (Sutherland and Keating, 1992; Sutherland *et al.*, 1996). This relatively instantaneous technique superseded the more laborious and slow CFU technique for HSPC characterisation. An alternative marker, AC133 (later named CD133), was also described which characterised HSPC (Yin *et al.*, 1997a) and has also been employed extensively for clinical and research use. Both markers can be used to enrich and isolate the cells which express them.

HSPC have most commonly been defined by their expression of CD34. CD34 is a monomeric cell surface antigen with a molecular mass of approximately 110 kD that is selectively expressed on human haematopoietic progenitor cells (Simmons *et al.*, 1992). Populations expressing CD34 are capable of long-term multilineage reconstitution, which is confirmed by the wide application of enriched

$CD34^+$ cells in HSPC transplantation in clinics. In Bhatia and colleagues' work, SCID-repopulating cells (SRCs) were only found in $CD34^+CD38^-$ fraction (Bhatia *et al.*, 1997). Actually, $CD34^-$ cells cells with reconstituting potential have also been confirmed by a series of successful engraftment experiments in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Bhatia *et al.*, 1998; Fujisaki *et al.*, 1999; Nakamura *et al.*, 1999). Some investigators demonstrated that the expression of CD34 is reversible on engrafting human haematopoietic stem/progenitor cells. The human haematopoietic stem/progenitor cells which lose the expression of CD34 in immunodeficient mice following the primary transplantation can retain the capacity to regenerate $CD34^+$ cells in secondary transplantation in sublethally irradiated secondary immunodeficient mouse recipients (Dao *et al.*, 2003; Hess *et al.*, 2003; Sato *et al.*, 1999; Tajima *et al.*, 2001; Zanjani *et al.*, 2003). The repopulating ability of $CD34^-$ cells indicates that further exploration of the inter-relationship between $CD34^+$ cells and $CD34^-$ cells is necessary. Recently, $Lin^-CD34^-CD38^-CD93^{hi}$ population has been reported to reside at the apex of HSPC hierarchy because of delayed primary and efficient secondary engraftment (Anjos-Afonso *et al.*, 2013) *in vivo*. In addition, CD34 can be found on endothelial cells (Shi *et al.*, 1998; Peichev *et al.*, 2000; Murohara *et al.*, 2000; Padfield *et al.*, 2010).

The next most commonly used definition of HSPC for clinical use has been the expression of CD133 (AC133), a unique 5-membrane-spanning cell surface molecule expressed on primitive human progenitor cells of haematopoietic (Miraglia *et al.*, 1997; Gallacher *et al.*, 2000), endothelial (Gill *et al.*, 2001a) and neural epithelial lineages (Corbeil *et al.*, 2000; Uchida *et al.*, 2000; Kosodo *et al.*, 2004; Weigmann *et al.*, 1997). Both CD34 and CD133 markers have been used for HSC enrichment and purification. While HSPC are generally recognised by CD34 expression for clinical assessment and less commonly by CD133 expression (said to identify "more primitive" HSPC – and popular in the Miltenyi method for HSPC enrichment), there is another much less commonly used marker, aldehyde dehydrogenase

activity expression, which may also define HSPC in a general sense (Storms *et al.*, 1999).

Isolation of HSCs

Magnetic enrichment can be employed to enrich cell populations robustly. MACS Microbeads (Miltenyi Biotech) coupled to specific monoclonal antibodies are used to label the specific cells. The labelled cells are then passed through the column nearby a strong magnetic field and retained in the column. When the magnet is removed, the retained cells are collected into a separate tube as the enriched fraction. The flow-through is collected as the depleted fraction. The magnetic method is appropriate for robustly separating large number of cells, but the enriched cells are not pure. Fluorescence activated cell sorting (FACS) is pivotal in separating cells accurately for further assays. FACS can use multiple antibodies, which are specific to combine cell surface markers of targeting cells, to sort pure cell population from large mixed populations.

1.2.3 Hierarchy of haematopoietic stem/progenitor cells

Human haematopoietic progenitors have been characterised by many investigators. Manz and colleagues isolated populations committed to granulocyte/macrophage (referred as granulocyte/macrophage progenitor GMP) lineages and megakaryocyte/erythroid lineages (referred as megakaryocyte/erythroid progenitor MEP) from the common myeloid progenitors (CMP) based on the expression of Interleukin 3 receptor (IL-3R) α and CD45RA from lineage negative $CD34^+CD38^+$ fraction ($Lin^-CD34^+CD38^+$), which exist in cord blood and bone marrow (Manz *et al.*, 2002). They also identified the phenotype of CMP as $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^{lo}CD45RA^-$, but Louise and colleagues found it difficult

to isolate CMP and MEP based on IL-3Rα expression, showing thrombopoietin (TPO) receptor as a good marker for a pure separation of MEP, the expression level of Flt3 within the CMP population signalling the pre-commitment towards GMP and MEP (Edvardsson *et al.*, 2006). As for the human lympho-haematopoietic system, Galy and colleagues (Galy *et al.*, 1995) isolated a subpopulation of Lin^{-}CD34^{+}CD45RA^{+}CD10^{+} cells co-expressing CD38^{+} and HLA-DR committed to lymphoid T, B, natural killer (NK), and dendritic cells. Recently there is a different perspective about HSCs and lymphoid progenitors, which occupy distinct bone marrow niches. HSCs prefer a perivascular niche created by endothelial cells and perivascular stromal cell, where some early lymphoid progenitors occupy an endosteal niche created by osteoblasts, and committed B-lineage progenitors depend on a different perivascular niche (Ding and Morrison, 2013). It was reported that conditional ablation of osteoblasts *in vivo* significantly depletes lymphoid progenitors but not HSCs (Visnjic *et al.*, 2004; Zhu *et al.*, 2007). This indicates that lymphoid progenitors might not be the descendants of HSCs and it is possible that development of lymphoid cells is different from other blood cells, which are differentiated from HSCs.

However, the most popular hypothesis proposes that long-term self-renewal haematopoietic stem cells (LT-HSC) give rise initially to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), and the CMP then produce GMP and MEP. CMP could generate all myeloid/erythroid lineages, while MEP committed to only red cells and platelets, and GMP have potential to generate granulocytes, monocytes, macrophage and all types of dendritic cells (Akashi *et al.*, 2000; Forsberg *et al.*, 2006; Karsunky *et al.*, 2003, 2008; Manz *et al.*, 2001; Nakorn *et al.*, 2003; Traver *et al.*, 2000) (figure 1.2). In 2005, the classical model with CLP and CMP was challenged by the proposing of LMPP (lymphoid-primed multipotent progenitor) Lin^{-}Sca-1^{+}CD34^{+}Flt3^{+} committing to GMP and CLP, accompanied by the upregulation of IL-7-receptor gene expression (in mouse) (Adolfsson *et al.*, 2005). (figure 1.3)

However, within IL-3R$^-$ cells, erythrocyte-committed progenitors were identified as Lin$^-$c-Kit$^+$Sca-1$^-$IL-7R$^-$IL-3R$^-$CD41$^-$CD71$^+$ cells (Terszowski *et al.*, 2005)(in mouse), which could generate CFU-E colonies at around 70% efficiency and produce reticulocytes *in vivo*. In Terszowski and colleagues' study, they isolated myeloid progenitors based on the expression of IL-3Rα, which plays an important role in identifying erythrocyte-committed progenitors. However, a contradiction came from another study indicating that mice lacking IL-3Rα still showed a normal steady haematopoiesis (Gillessen *et al.*, 2001; Nishinakamura *et al.*, 1996).
In the *in vitro* expansion of a human cord blood CD36$^+$ erythroid progenitor, the expression of glycophorin A (GlyA), transferrin receptor (CD71) and haemoglobin A proteins correlated with gene expression of key transcription factors of haematopoietic cells, indicating that the committed CD36$^+$ erythroid progenitor cells maintain, in part, many features of adult erythropoiesis at the cellular and molecular level (Scicchitano *et al.*, 2003). Chen and colleagues identified human bone marrow CD13$^+$CD36$^+$ cells as bipotential precursors of erythroid and myeloid commitment in normal haematopoiesis and found that CD13$^-$CD36$^+$ cells lose nearly all myeloid potential but still could differentiate into erythroid colonies (Chen *et al.*, 2007).

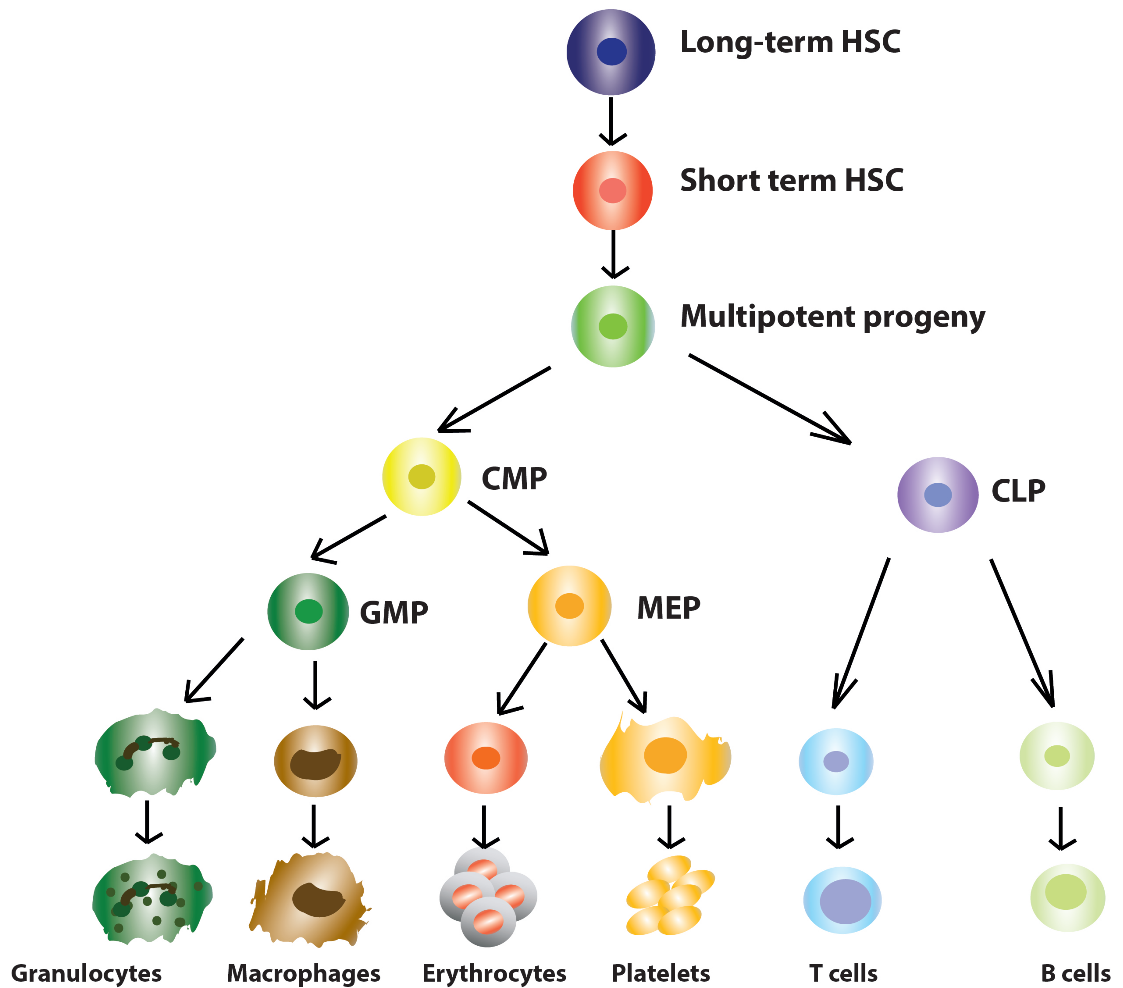


Figure 1.2: The classic model of hierarchy of haematopoiesis

In classic model, LT-HSC has the potential to self-renew and produce multipotent progenitors and, subsequently, multipotent progeny with no self-renewal capacity. Then multipotent progeny in turn give rise to CLP and CMP, which then produces GMP that can differentiates into granulocytes, monocytes, macrophage and all types of dendritic cells, and MEP that differentiates into platelets and erythrocytes.

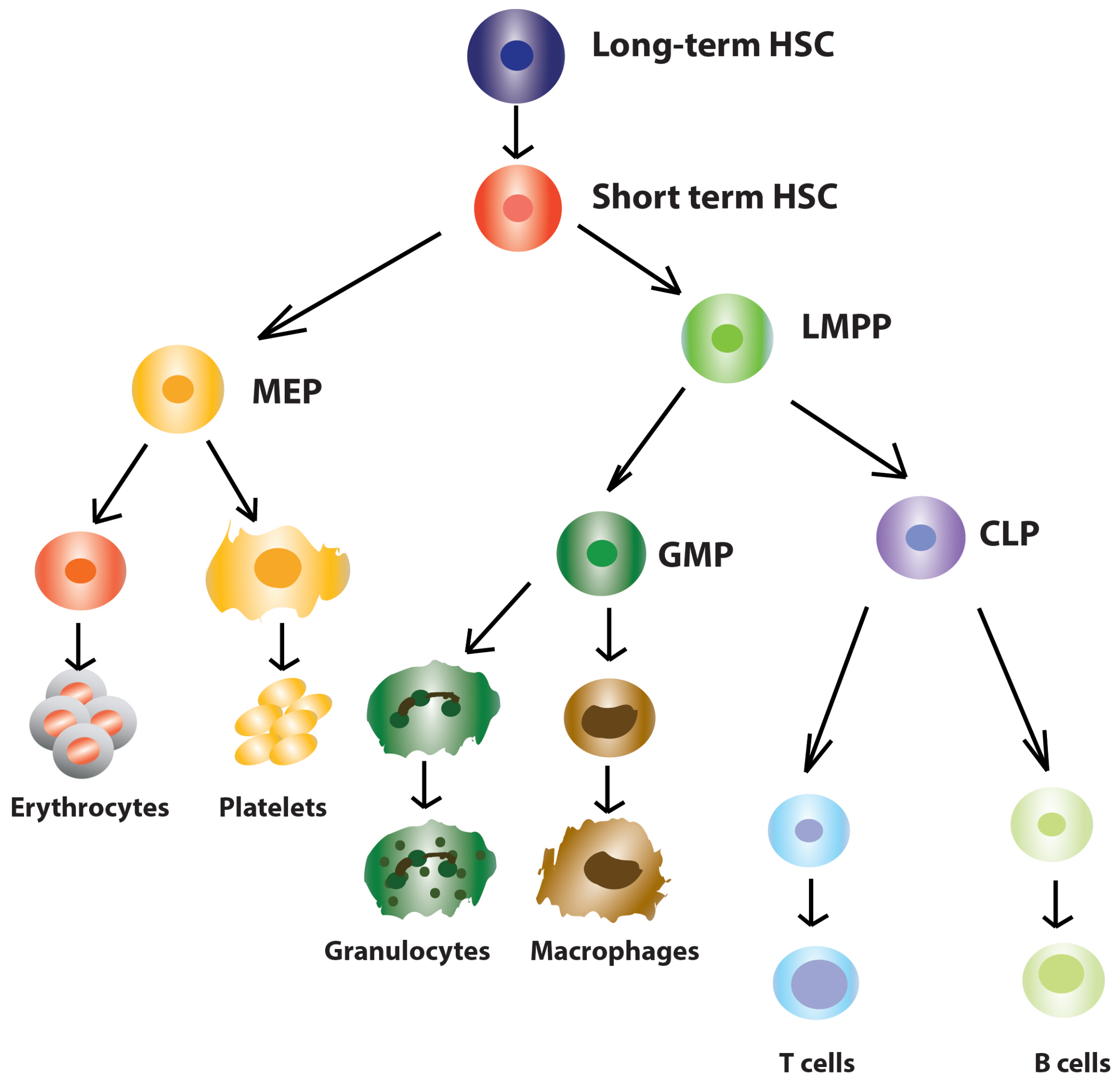


Figure 1.3: **The alternative model of hierarchy of haematopoiesis**

In the alternative model of hierarchy of haematopoiesis, LMPP was proposed to have the capacity to yield CLP and GMP, which differentiates into granulocytes, monocytes, macrophage and all types of dendritic cells. Short-term HSC generates MEP committing to platelets and erythrocytes.

1.2.4 Key transcription factors of haematopoietic stem cells

GATA family

The GATA-binding factor (GATA) gene family has six members and is a group of X-linked transcription factors discovered in the 1980s.
GATA1: GATA1, located on chromosome Xp11.23, is expressed in erythroid, megakaryocytic, eosinophilic, mast and multipotential precursor cells within the haematopoietic system (Cantor and Orkin, 2002). GATA-1 plays an essential role in erythropoiesis, including primitive erythroid precursors and terminal erythroid maturation. *In vitro* GATA-1$^-$ ES cells fail to produce primitive erythroid (EryP) precursors while still generating definitive erythroid (EryD) precursors, which are normal in number but undergo developmental arrest and death at the proerythroblast stage. Surprisingly, arrested GATA-1$^-$-definitive proerythroblasts express GATA target genes at normal levels. Simultaneously, transcripts of the related factor GATA-2 are elevated in GATA-1$^-$ proerythroblasts. These findings suggest the interchangeable relationships of GATA factors *in vivo* and imply that GATA-1 represses the expression of GATA-2 during erythropoiesis (Weiss *et al.*, 1994). Megakaryocytes lacking GATA-1 are delayed in their maturation, exhibit hyperproliferation, and generate fewer than normal, and enlarged platelets *in vivo*, revealing a function for GATA-1 in controlling cell cycle arrest.
GATA2: GATA2 is expressed in HSCs (Evans and Felsenfeld, 1989), multipotent haematopoietic progenitors, erythroid precursors, megakaryocytes, eosinophils, and mast cells (Minegishi *et al.*, 1999; Tsai *et al.*, 1994; Tsai and Orkin, 1997). GATA-2 functions in proliferation and maturation of stem and progenitor cells. A study showed that GATA-2 could rescue primitive erythropoiesis of GATA-1 knockdown mouse from embryonic lethality (Takahashi *et al.*, 2000). However, the mice which were rescued with GATA-2 transgene developed anemia and

abnormal hematopoiesis in adulthood. In GATA-2 overexpressing subclones of K562, the expression levels of erythroid-specific genes including α-, β-, γ-globin and transferrin receptor were increased, while the expression level of Gata-1 was unchanged (Harigae *et al.*, 2006). GATA-2 protein binding to the GATA site of the human α-globin locus control region (LCR) was increased in GATA-2 overexpressing K562 cells, which suggested that Gata-2 could induce erythroid-specific genes without competition with GATA-1 in K562 cells.

Other transcription factors belonging to GATA family: It has been reported that GATA3 induces luminal epithelial differentiation in the adult mammary gland (Kouros-Mehr *et al.*, 2006), and moreover GATA3 promotes the differentiation of Th0 cells towards Th2 cells while suppressing their differentiation to Th1 cells (Yagi *et al.*, 2011). GATA4, 5, 6 are thought to plays a critical role in mammalian cardiac development, maintenance of cardiac function and they are associated with may cardiovascular diseases (Perrino and Rockman, 2006; McCulley and Black, 2012; Zhou *et al.*, 2012a). In addition, they regulate the the differentiation of intestinal epithelial cells (Chang and Appasani, 2006) and are essential in regulation of hepatic function (Divine JK, 2004).

FOG-1

Friend of GATA protein 1 (FOG-1), which binds specifically to the amino zinc finger of GATA-1, contains nine predicted zinc fingers, four of which (zfs 1, 5, 6 and 9) individually mediate interactions with GATA-1 (Fox *et al.*, 1999). FOG-1 is not only expressed abundantly in erythroid and megakaryocytic cells, but also is co-expressed with GATA-1 during development and operates as the interacting partner for Gata-1 (Tsang *et al.*, 1997). Amigo and colleagues (Amigo *et al.*, 2009) used *in situ* hybridization, gain- and loss-of-function studies in zebrafish to explore the function of FOG-1 during myeloid and lymphoid development or

how FOG-1 expression is regulated in any tissue. They found that the suppression of FOG-1, in the presence of normal GATA-1 levels, induces severe anemia and thrombocytopenia and expands myeloid-progenitor cells, which indicates that FOG-1 is required during erythroid/myeloid commitment.

In Tsang and colleagues (Tsang *et al.*, 1997) study, FOG-1$^{-/-}$ mice died in mid-embryonic gestation due to severe anemia with arrest in erythroid maturation at a stage similar to that observed in the GATA-1^{-} mice, providing genetic evidence that these two factors act in a common pathway. However, FOG-1$^{-/-}$ mice exhibit complete failure of megakaryopoiesis in contrast to GATA-1$^{-/-}$ mice, indicating that FOG-1 also has a GATA-1 independent role in early megakaryopoiesis.

The direct physical interaction between GATA-1 and FOG-1 for normal erythropoiesis *in vitro* (Crispino *et al.*, 1999) was evidenced by the studies utilizing a point mutant of GATA-1 with markedly reduced affinity for FOG-1 (but normal DNA binding activity). The megakaryocytes from these patients resemble murine GATA-1 deficient megakaryocytes suggesting that a GATA-1: FOG-1 interaction is also critical for late stages of megakaryopoiesis. In addition, Chang's (Chang *et al.*, 2002) study showed that FOG-1 requires an interaction with either GATA-1 or GATA-2 as part of its essential role in early megakaryopoiesis.

Efforts have now been focused on understanding the mechanism by which FOG proteins influence GATA-mediated processes. Cantor's study showed that FOG-1 molecules containing extensive and overlapping deletions spanning the entire molecule (but retaining at least one GATA-binding zinc finger) are also able to rescue erythroid maturation of the FOG-1$^{-/-}$ cell line (Cantor and Orkin, 2002). However, this indicates that a simple interaction between FOG-1 and GATA-1 is sufficient to activate GATA-1.

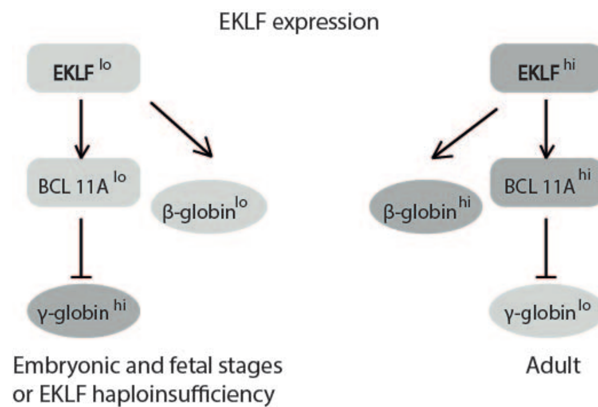


Figure 1.4: How EKLF expression affects the expression of fetal globin and adult globin

During embryonic and fetal development or in EKLF-haploinsufficient adults (left), EKLF levels are low, resulting in low levels of β-globin and BCL11A and high levels of γ-globin. In adults with two functional copies of EKLF (right), increased expression of EKLF in definitive red blood cells promotes high levels of β-globin and BCL11A expression, which in turn represses γ-globin expression.

EKLF and BCL11A

Erythroid Krüppel-like Factor (EKLF) is a zinc finger transcription factor that is necessary for the proper maturation of erythroid (red blood) cells. Mouse embryos deficient in EKLF by gene targeting died of anaemia during fetal liver erythropoiesis. And these embryos showed the molecular and haematological features of β-globin deficiency found in β-thalassaemia (Perkins *et al.*, 1995). EKLF knockout mice die from severe anemia at the fetal liver stage due to failure of adult β-globin gene activation. EKLF$^{-/-}$ mice containing a complete human β-globin locus transgene have reduced levels of β-globin, but elevated levels of γ-globin expression, compared to wild type mice containing the same transgene (Perkins *et al.*, 1995; Wijgerde *et al.*, 1996). Two other studies not only suggest a crucial role for EKLF in regulating the developmental switch between fetal and adult hemoglobin expression, but also uncover a regulatory relationship

between EKLF and a second transcription factor, B-cell lymphoma/leukemia 11A (BCL11A), which helps explain how EKLF affect the switch (Borg *et al.*, 2010; Zhou *et al.*, 2010a). The promoter region of BCL11A, the suppressor of HBG1/HBG2 gene, contains several EKLF binding sites, which means EKLF could activate the expression of BCL11A in order to further repress HBG1/HBG2. Another study indicated that EKLF gene dosage is essential for regulating the surface phenotype and molecular identity of maturing EryP (Isern *et al.*, 2010)(as illustrated in Figure 1.4).

DNase I Super-hypersensitive sites

DNase I Super-hypersensitive (HS) sites are normally located upstream of the human β-globin locus and are crucial in erythroid development (Forrester *et al.*, 1987, 1986; Tuan *et al.*, 1985). The sites required for the activation of the locus is evidenced by the studies of patients possessing deletions of HS sites but with an intact β-globin gene (Driscoll *et al.*, 1989). Subsequently, a series of studies demonstrated that HS sites play a pivotal role in high-level, erythroid-specific expression of human β-globin genes and suggest HS sites are required for the regulation of the entire human β-globin gene (Grosveld *et al.*, 1987; Ryan *et al.*, 1989a,b; Talbot *et al.*, 1989; Townes *et al.*, 1989). Further, competition of individual globin gene family members for interactions with the HS sequences are critical for human γ- to β-globin gene switching during erythroid development (Behringer *et al.*, 1990).

1.2.5 Application of haematopoietic stem cells in the laboratory

In vivo and *in vitro* standard assays for the assessment of haematopoietic stem cell activity are very important in haematopoietic stem cell studies. Today, the best *in vivo* assay that measures the long-term ability of HSC to self-renew and reconstitute method is to use NOD/SCID mouse model to define the repopulating capacity of human HSC (Pflumio *et al.*, 1996). Whereas the *in vitro* studies of HSCs has been limited in two-stage liquid culture and colony-forming unit assay in methylcellulose semisolid medium. Liquid culture is the fundamental approach to assess haematopoietic potential of HSPC and for further application such as differentiation. *In vitro* expansion of primary stem cell is difficult because it is easy to lose potential, there remains a similar problem for HSCs. Thus, much work has been done in amplification studies of HSPC. Usually, the addition of cytokines in medium also plays an essential role in HSC culture and distinct combinations of these cytokines exert different functions in cell fate decisions. This point will be discussed in chapter 4. However, the latter commonly employed *in vitro* assay for the quantification of committed haematopoietic stem/progenitors is easy and convenient. Cells to be tested are mixed with stimulatory cytokines in tissue culture medium made up in viscous methylcellulose which does not allow the cells to disperse – so that any cells proliferating in response to the cytokine stimulants remain localised and form a colony. Each colony is presumed to be derived from a single cell by clonal outgrowth, and different types of colonies represent different types of progenitor cell lineages. Initially the colony assays were relatively primitive and relied on use of various tumour cell supernatants, but were refined with better classification of the different cytokines and were eventually designed around defined mediums with known mixtures of recombinant cytokines (largely by Stem Cell Technologies, Inc).

Erythropoiesis is a multi-step process by which erythrocytes are produced. The

process involves proliferation and differentiation of HSCs into mature red cells. There is current interest here and elsewhere in development of erythropoiesis *in vitro* to try to produce a sustainable and infection-free supply of red blood cells for clinical blood transfusion as a substitute for donated blood cells. Blood transfusion, as an important therapy, is required crucially in surgery, leukemia patients and varieties of severe anemia patients. However, we have to confront the possibility of significant health consequences associated with blood transfusion, which might transmit diseases such as hepatitis, HIV, new variant Creutzfeld–Jakob disease (vCJD) and so on. Moreover, the generation of platelets and white cells are still the regions many scientists concentrate on.

1.2.6 Clinical application of haematopoietic stem cells in regenerative medicine

The origin of bone marrow and peripheral HSCs transplantation

HSCs are the only type of stem cells widely applied in clinical medicine. As early as the 1950s, scientists attempted to utilize the potential of bone marrow grafting in treating human diseases or animal models (Barnes *et al.*, 1956; Billingham RE, 1959; Kurnick *et al.*, 1958; Mannick *et al.*, 1959; Thomas *et al.*, 1959, 1957). In 1965, Mathé and colleagues attempted the first case of allogeneic marrow graft in a patient with leukemia, although finally the patient died from the complications due to chronic graft-versus-host disease (GVHD) (Mathe *et al.*, 1965). In 1968 and 1969, three infants with immune deficiency diseases were reported to receive successful haematopoietic cells transplants from human leukocyte antigen (HLA)-matched siblings (Bach *et al.*, 1968; De Koning *et al.*, 1969; Gatti *et al.*, 1968). Subsequently, the Seattle team successfully treated many patients using haematopoietic cells engraftment (Fefer *et al.*, 1977;

Storb *et al.*, 1977b,a,c; Thomas *et al.*, 1975, 1977a, 1979, 1977b,c, 1972). Since then, HSCs transplantation has been increasingly applied world-wide in routine treatment for haematopoietic cancers and genetic or acquired bone marrow failure, such as aplastic anemia, myelodysplastic syndrome, thalassemia sickle cell anemia, and so on (Thomas and Blume, 1999). In addition, the indications of HSCs transplantation also include some non-haematopoietic malignancies and autoimmune diseases.

Autologous and allogeneic grafts

Currently depending on HSCs sources, transplantation can be carried out in two different ways, autologous or allogeneic transplantation (patients themselves or donors).

Autologous transplantation utilizes bone marrow or mobilised peripheral blood of patients themselves and thus reduce the risk of rejection reaction by the recipients' immune system. Simultaneously, autologous grafts lack of graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) response, which is induced by donor-immune effector cells recognising specifically host tumor cells. In addition, the graft still keeps the genetic deficiency of the patient, so the prognosis for patients with genetic deficiency requires to be seriously considered.

Allogeneic transplantation occurs between two genetically dissimilar individuals of the same species. The major block for allogeneic transplantation is transplantation rejection, which is an immune response to the cell surface antigens that distinguish between "self" and "non-self". The specific tissue antigens are known as histocompatibility antigens. Thus it is essential to solve this problem in allogeneic transplantation. Currently, for successful allogeneic transplantations, the donor must match at least half of major HLA between host and donor. However, although allogeneic grafts are limited in availability of matched donors and

the possible GVHD, allogeneic grafts have the potential to induce GVL response unlike autologous grafts.

HSC banking

With the world-wide application of HSCs in the clinic, stem cell banks have been built to promote the utilization of umbilical cord blood or bone marrow transplantation. The storage of umbilical cord blood provides an opportunity for possible treatment in future when the donor suffers related diseases or for unrelated individuals. For those patients with blood cancers who require HSCs transplantation, when they cannot find immunologically matched relatives, they have to ask for help from the public. Then the establishment of human HSCs banks facilitates the procedure to find matched donors.

1.3 iPS introduction

1.3.1 Origins of iPS cells

ES cells are derived from the inner cell mass of preimplantation embryos upon the generation of a cystic blastocyst (Papaioannou, 2001). Following fertilization, a series of cleavage divisions occur as the embryo travels down into the uterus. The cells in the cleavage division stage are called blastomeres. Each cell from the blastomere has the potential to generate any particular cell type of the body. The first differentiation of the human embryo occurs around five days of development. Simultaneously, the outer layer cells separate from the inner cell mass (ICM) and finally will develop into part of the placenta. Implantation is an important time

point for ICM cells, once implanted, they quickly lose pluripotency and differentiate into other cell types with limited developmental potential. However, when ICM cells are removed from the implanted environment and cultured within the defined embryonic medium, the ICM cells can proliferate and maintain the capacity of pluripotency. These ICM derived cells with self-renewal and pluripotent ability are ES cells. In 1981, the first mouse ES cell line was reported (Evans MJ, 1981; Martin, 1981) but the derivation of a human ES cell line was in 1998 (Thomson *et al.*, 1998). With the development of ES medium, ES cells can be cultured long-term *in vitro*, providing a fundamental basis for regenerative medicine. Due to their pluripotent capacity, ES cells can be directed to differentiate into cell types which can be used for transplantation in the clinic, or drug research.

There are many advantages of ES cells, but for human ES cells it is difficult to scale up the pure stem cells due to the limit of obtaining embryonic tissues and isolating relatively rare cells. In addition, since we can only obtain limited embryonic resources at a specific time point at the embryonic stage, it is impossible to generate patient-specific ES cells for deriving differentiated cells or tissues. Thus many scientists worked diligently on the mechanism of embryonic development to explore alternative approaches to create pluripotent cells, these would be better than using embryos. Such cells can be derived from accessible tissues of the human body. In 1962, John B. Gurdon used somatic cell nuclear transfer (SCNT) technique to replace the nucleus in an egg cell of a frog with the nucleus from a mature intestinal cell, and then the modified egg cell developed into a fully functional, cloned normal tadpole. His work challenged the dogma that the fate of a mature specialised cell is irreversible. In his experiment, the nucleus from mature cells within the embryonic microenvironment can be reprogrammed to an immature, pluripotent state while it has lost its capacity to drive development to a fully functional organism in the specialized cells. In the following years many cell types have been tried, but failed until the birth of Dolly sheep at Roslin Institute in 1996 (Campbell *et al.*, 1996). However, the SCNT technique efficiency

is quite low. This leads to the question 'Would it be possible to reprogram an intact cell back into a pluripotent stem cell?'
Shinya Yamanaka perfectly answered this question in 2006, more than 40 years after John B. Gurdon's discovery. His group successfully reprogrammed fibroblasts from mice back to an embryonic stem cell-like state by introducing four genes that encode critical transcription factor proteins octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (Sox2), Kruppel-like factor 4 (Klf4), and V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (c-Myc), which regulate the function of other genes essential in embryonic early development (Takahashi and Yamanaka, 2006). Before that, his research was concerned with embryonic stem cells and development. Yamanaka identified several genes that kept embryonic stem cells immature, tried to introduce different combinations of these genes into mature fibroblast cells from mouse, and then examined the results under the microscope. Finally they found that the combination of four genes including Oct4, Sox2, Klf4, and c-Myc (OSKM) reprogrammed mouse somatic cells into immature stem cells with the capacity of pluripotency, which can be directed to differentiate into any cell types of the three germ layers. These induced pluripotent cells (iPS cells) are similar to ES cells and cell lines could be established after several weeks' culture. This is a milestone in science. Since then, we have learned that specialized cells have the possibility to turn back the developmental clock under certain micro-environmental conditions. In 2007, iPS cells were derived from human somatic cells using OSKM factors (Takahashi *et al.*, 2007) or a different gene combination of Oct4, Sox2, Homeobox Transcription Factor Nanog (Nanog) and Lin-28 Homolog A (Lin28) (Yu *et al.*, 2007).

1.3.2 Definition and characteristics of iPS cells

Induced pluripotent stem cells are characterized by two important capabilities: (1) the ability to differentiate into many tissues of three germ layers including ectoderm, mesoderm and endoderm, which means iPS cells have multiple potential to differentiate into cells or tissues of interest. (2) the capacity of renewal to generate many cells with the same characteristics as iPS cells. iPS cells can replicate and produce many copies of themselves, simultaneously maintaining their pluripotent and self-renewable capacity.

1.3.3 Current methods of producing iPS cells

Since the Yamanaka group invented iPS cells using OSKM factors to transduce mouse fibroblasts through retrovirus in 2006, reprogramming work has evoked promising future applications in regenerative medicine. A large number of scientists worked diligently on improving approaches of reprogramming. From the standpoint of the development of delivery tools, many types of viruses, microRNA, mRNA, protein, and plasmids are widely applied in the reprogramming process. These methods are summarised in table 1.1.

The summary of current methods to generate iPS cells

Delivery methods	Characteristics	Advantages	Disadvantages
Retrovirus vectors	A single-stranded, positive-sense RNA virus, derived from murine leukemia virus	The expression of transgene can be silenced as the withdrawal of pluripotent factors	Leaking problem; Limit in delivering the factors into non-dividing cells; The lower reprogramming efficiency than nonsilencing viral method; Unexpected activation of a cellular protooncogene as a result of retroviral integration.
Lentivirus vectors	A single-stranded, positive-sense virus, derived from HIV, SIV, EIAV, and FIV	Genes can be delivered into both dividing cells and non-dividing cells; Inducible system allows temporal control of factor expression.	Leaking problem; Insertional mutagenesis and malignant transformation as a result of the integration of genes into the genome.

The summary of current methods to generate iPS cells(*continued*)

Delivery methods	Characteristics	Advantages	Disadvantages
Adenoviral vectors	Double stranded DNA viruses	Replicate in the nucleus but outside chromosome; Can infect nucleus of cells but will lose its nature upon cell division.	Some human adenoviruses are potentially oncogenic.
Episomal plasmids		Avoid the problem of integration into genome; The established stable episomal vectors are lost at nearly 5% per cell cycle without drug selection.	Low reprogramming efficiency

The summary of current methods to generate iPS cells(*continued*)

Delivery methods	Characteristics	Advantages	Disadvantages
Sendai virus	A single-stranded, negative-sense and nonsegmented RNA virus	Replicates in cytoplasm; Can infect dividing and nondividing cells	Sustained replication of sendai virus vectors in cytoplasm even though viral vectors can be gradually diluted as the division of iPS cells (interference with viral RNA-dependent RNA polymerase using siRNA can erase completely the vector genome from targeting cells; Or temperature-sensitive sendai virus vectors).
mRNA		Easier to cease the action of mRNA of interesting genes in cell; Avoid the problem of integration into genome	Multiple rounds of transfection; Low transfection efficiency.

The summary of current methods to generate iPS cells(*continued*)

Delivery methods	Characteristics	Advantages	Disadvantages
Protein		Without any genetic manipulation; Circumvents the issue of gene insertion and mutagenesis	Very low efficiency 0.001% of input cells
microRNA		With less or without transcription factors	10% efficiency; Still need viral delivery system.

Table 1.1: A summary of iPS generation methods

Retroviral vectors

The first method applied in reprogramming was OSKM factors delivered into mouse fibroblasts through retrovirus. A retrovirus is an RNA virus which makes use of reverse transcriptase to replicate in host cells, subsequently it produces DNA, and this DNA can be incorporated into the host's genome, further allowing the virus DNA to replicate as part of host's genome. Most commonly used retroviral vectors derive from murine leukemia virus. Although the expression of transgenes can be silenced when pluripotent factors are withdrawn, there are reports of the expression of pluripotency-associated genes and transgene reactivation observed in germ line transmission chimeric mice. In addition, retrovirus is limited to delivering the factors into dividing cells, leading to a restricted range of cell types that can be reprogrammed. It has also been reported that the expression of retrovirus transgenes decreases gradually in accordance with the process of somatic cell reprogramming, thus the reprogramming efficiency is

lower than non-silencing viral method (Stadtfeld *et al.*, 2008a). Although cases of X-linked severe combined immunodeficiency (SCID) were cured by retrovirus-mediated *ex vivo* gene transfer (Hacein-Bey-Abina *et al.*, 2003; Kohn *et al.*, 2003), the follow-up showed four out of nine successfully treated patients later suffered leukemia, which were suspected to be triggered by unexpected activation of a cellular proto-oncogene as a result of retroviral integration within the LMO-2 locus (Howe *et al.*, 2008; Ste; Yi *et al.*, 2005).

Lentiviral vectors

Lentivirus vectors derive from HIV, simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), and feline immunodeficiency virus (FIV), and have been applied extensively in research (Knipe and Howley, 2007). Compared to retroviruses, lentiviruses can deliver genes into both dividing cells and non-dividing cells, and thus are more efficient in gene transduction. The integration of genes into the genome allows long-term gene expression. Once more, another advantage of lentivirus over retrovirus is inducible system, permitting temporal control of factor expression. On the other hand, inducible systems have the problem of leaking possibility, not complete insurance of ceasing expression of transduction factors.

Retrovirus and lentivirus transduction inevitably meet the problems of foreign DNA integration into host chromosome where they can cause insertional mutagenesis and malignant transformation (Nakagawa *et al.*, 2008; Okita *et al.*, 2007), and interfere with gene transcription (Kustikova *et al.*, 2005). In addition, with respect to integrated virus systems, there exists the difficulty of regulating the ectopic gene expression due to the possibility of integration near active regions in the chromatin.

Currently there are two approaches to excise post-induction integrated DNA from iPS cells or progeny. In one approach, TTAA-specific transposon piggyBac system

which is a DNA entity encoding all required reprogramming factors (transgenes) flanked by transposon sequences. When piggyBac transposase is transfected into targeted cells, once colonies of reprogrammed cells are isolated, transient expression of the transposase enzyme results in excision of the insert (Woltjen *et al.*, 2009). Another way is to make use of Cre-LoxP recombination to remove the insert by transient expression of Cre enzyme (Kaji *et al.*, 2009; Soldner *et al.*, 2009).
However, to overcome the problems that arise from genomic integration by retroviral and lentiviral methods, scientists developed non-integrating virus vectors including adenovirus and sendai virus.

Adenoviral vectors

Adenoviruses (Ad) are medium-sized double stranded DNA viruses that are isolated from avian and mammalian species, and they are widely applied in *ex vivo* and *in vivo* gene delivery. The human adenovirus family consists of more than 50 serotypes, which can be classified into 6 subgroups (subgroups A-F). The fiber protein, one of three major protein components of adenoviruses, is responsible for the primary attachment of Ad virions to the cellular receptor, the coxsackie virus and adenovirus receptor (CAR). Due to low levels of expression or the lack of CAR in some target cells, e.g. skeletal and smooth muscle cells, endothelial cells, hematopoietic cells, and many tumor cells, these cells are refractory to Ad vector transduction, thus leading to the limited utility of Ad vectors. Given the problems, many strategies have been developed to redirect the cell tropism of the Ad vectors. In natural tropism, the commonly used adenovirus serotype 5 vectors have an interaction between its fiber knob and CAR. Although most gene delivery studies with adenoviral vectors focus on Ad5, many other serotypes can target relatively unique receptors. One good example is that $CD34^{+}$ haematopoietic stem cells can be efficiently transduced by Ad35 and Ad11 but

quite poor transduction results with Ad5 based vectors. Another strategy is to utilize bispecific molecules to block natural tropism for CAR-expressing cells and redirect the virus to a novel cell surface receptor, e.g. folate receptor, epidermal growth factor receptor, and so on. This strategy is called transductional targeting. In addition, genetic modification is another strategy, through which fiber knob is replaced by knob of an alternative Ad serotype or a receptor ligand (Knipe and Howley, 2007).

Following the initial attachment, adenoviruses are internalised through integrin engagement with the penton base of the virus. When adenovirus is transported into the nucleus, it replicates autonomously from chromosomes in the nucleus for the rest of the life cycle, so thus successfully reducing the risk of insertional mutagenesis to the host cell (Knipe and Howley, 2007). Therefore, the episomal nature allows adenovirus to infect the nucleus of cells but will reduce the quantity of adenovirus gradually upon cell division. Adenoviral vectors have been successfully applied in reprogramming human cells to iPS cells (Stadtfeld *et al.*, 2008b). However, some human adenoviruses are oncogenic, and they can transform cells in culture, resulting in tumours when inoculated into animals, though there is no evidence that adenoviruses cause cancer in humans (Knipe and Howley, 2007).

Episomal plasmids

Although episomal plasmids can enter the nucleus to carry out replication, they only replicate extra chromosomally. Thus they won't integrate into the genome, demonstrating more safety compared to integrated viruses. In Thomson's group, they produced human iPS cells using origin of viral replication/ Epstein-Barr nuclear antigen-1 (oriP/EBNA-1) episomal vectors to deliver reprogramming factors Oct4, Sox2, Nanog, Lin28, c-Myc, Klf4 and Simian Vacuolating Virus 40

large T antigen (SV40LT) (Yu *et al.*, 2009). General view of the reprogramming method, the oriP/EBNA-1 vectors do not need viral packaging, avoiding the problem of integration into genome. Multiple rounds of tranfections are not required, and a single transfection of episomal vectors is sufficient for the generation of iPS cells. Moreover, since the binding of Epstein-Barr virus replication protein EBNA1 enhances the retention of plasmid DNA (Middleton and Sugden, 1994), there might be higher transfection efficiency. In addition, the removal of episomal vectors from iPS cells can be achieved by simple cell culture in the absence of drug selection since the established stable episomes are lost at nearly 5% per cell cycle without drug selection owing to defects in plasmid synthesis and partioning (Nanbo *et al.*, 2007). However, the reprogramming efficiency of the episomal-based method is low, only three colonies from 10^6 human foreskin fibroblasts. In order to circumvent the low reprogramming problem, this group successfully improved reprogramming efficiency (nearly 220 colonies from 10^6 fibroblasts) through the combination use of a cocktail of episomal reprogramming vectors and small molecules containing MEK inhibitor PD0325901, GSK3-β inhibitor CHIR99021, TGF-β/Activin/Nodal receptor inhibitor A-83-01, HA-100 and human leukemia inhibitory factor within feeder-free chemically defined medium (Yu *et al.*, 2011). Yamanaka's group reported successful generation of iPS cells using p53 suppression and nontransforming L-Myc with episomal vectors without the assistance of small molecules (Okita *et al.*, 2011). In addition, Yamanaka's group successfully used human artificial chromosomes with Cre-LoxP (locus of X-over P1) system to produce iPS cells (Hiratsuka *et al.*, 2011).

Sendai virus

Sendai virus, also known as murine parainfluenza virus type1 or hemagglutinating virus of Japan, is a single-stranded, negative-sense and non-segmented RNA virus, belonging to the paramyxoviridae family. Sendai virus can infect various

animal cells except human cells, and they have been extensively applied as a recombinant viral vector capable of transient but strong expression (Griesenbach *et al.*, 2005). Sendai virus has notable advantages, allowing it to become an attractive and promising approach for gene therapy. Firstly, sendai viruses are replicated in the cytoplasm, circumventing the opportunity of integration into host genome; secondly, the entry of transduction factors into host cells does not require the status of cells in cell cycle. It has been reported that sendai virus vectors successfully reprogrammed human blood cells (Seki *et al.*, 2010). However, there still exists the problem of sustained replicated sendai virus vectors in the cytoplasm even though viral vectors can be gradually diluted with the division of iPS cells. To surmount the issue interference with viral RNA-dependent RNA polymerase using siRNA can completely erase the vector genome from targeted cells (Fusaki *et al.*, 2009). In addition, a more efficient way has been developed to shut down the viral replication by making use of temperature-sensitive sendai virus vectors (Ban *et al.*, 2011).

mRNA

mRNA, as a molecular tool, plays an important role in gene therapy with more advantages compared with the traditional virus delivery system. mRNA can exert its function in the cytoplasm rather than the nucleus, thus cells have no risks of the foreign genes being incorporated into the host genome. It is more promising from the standpoint of safety for future application in clinical and regenerative medicine.

Moreover, it is easier to stop the expression of genes in cells through withdrawal of transfection of mRNA. In contrast, more manipulations have to be applied to stop gene expression in virus transduction, such as the addition of special drugs for switching off the function of the virus plasmids with drug selection and the application of Cre-LoxP box, but the problem of leakage still has to be considered,

which means the function of virus plasmids might not be switched off. mRNA transfection could be repeated several times, but as a double-edged sword, which means multiple rounds of transfection have to be done for the work of targeting cells.
However, mRNA has its drawbacks. When mRNA without any modifications is transfected into cells, the cells will activate a strong innate immune response. To avoid the problem, modifications to mRNA are essential. The addition of cap structure and poly A tail makes cells no longer recognise the molecule as dangerous, significantly reducing the immune rejection. mRNA transfection of multiple genes including Oct4, Sox2, Nanog, Lin28, has been successfully applied in reprogramming fibroblast cells into induced pluripotency stem cells (iPS cells) (Mandal and Rossi, 2013).

Protein

Theoretically, the core principle of iPS cell generation is using varieties of delivery method to express enough quantities of reprogramming factors to help activate a reprogramming signalling pathway to redirect cell fates. In addition to delivery of DNA or mRNA reprogramming factors into cells to express reprogramming factors, direct delivery of protein reprogramming factors themselves can be achieved. The protein-based method provides a promising and safe approach to generate human iPS cells without any destruction of ex utero embryo and genetic manipulation. Obviously, protein-based method completely circumvents the issue of gene insertion and mutagenesis, resulting in human iPS cells suitable for drug discovery, disease modeling, and future clinical translational medicine. In 2009, Ding and colleagues successfully generated mouse iPS cells by combining the use of recombinant reprogramming proteins and the small molecule valproic acid (Zhou *et al.*, 2009). Another study showed the successful generation of human iPS cells with direct delivery of reprogramming proteins into somatic cells without any

chemical treatment (Kim *et al.*, 2009a). The difference between the two protein-based cases is that one group used refolded proteins after expression in E. coli while another group used reprogramming proteins expressed in mammalian cells. However, the efficiency of iPSC generation is lower using protein delivery system (about 0.001% of input cells), compared to virus methods. (about 0.01% of input cells) (Park *et al.*, 2008c; Takahashi *et al.*, 2007).

microRNA

A microRNA is a small non-coding RNA molecule, encoded by eukaryotic nuclear DNA, which functions in transcriptional and post-transcriptional regulation of gene expression (Chen and Rajewsky, 2007). miRNA can base pair with complementary sequences within mRNA molecules, leading to gene silencing through transcriptional repression or target degradation (Bartels and Tsongalis, 2009; Kusenda *et al.*, 2006). It has been demonstrated that miRNA cluster 302-367 improved mouse somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. The transient transfection of microRNAs-291-3p, -294, -295, and -302d promoted mouse iPS cell generation with three Yamanaka factors including Oct4, Klf4 and Sox2 (Judson *et al.*, 2009) not with four factors (the same plus c-Myc). MicroRNAs-291-3p, -294, and -295 are part of the microRNA-290 cluster, belonging to ES cell-specific cell cycle regulating microRNAs, which shorten the cell cycle though accelerating the G1/S transition (Wang *et al.*, 2008). However, the application of microRNA clusters 17-92, 106b-25, 106a-363 and 302-367 in reprogramming work improved efficiency, whether using three or four of Yamanaka factors (Li *et al.*, 2011a; Liao *et al.*, 2011). Human embryonic stem cell-specific cell cycle-regulating family of miRNA (hsa-miR-302b and hsa-miR-372) repress multiple target genes related to the cell cycle, epithelial-mesenchymal transition (EMT), epigenetic regulation and vesicular transport, all of which synergize to promote somatic cell reprogramming (Subramanyam *et al.*, 2011). The work

described above depends on interaction with transcription factors. To generate induced pluripotent cells with less or without transcription factors is a promising way forward to clinical application. In order to make the technique more relevant to clinical application, the combined use of mir-200c plus mir-302s and mir-369s family miRNAs successfully reprogrammed mouse and human somatic cells (Miyoshi *et al.*, 2011). Another group also generated iPS cells using microRNAs without addition of transcription factors (Anokye-Danso *et al.*, 2011). In this study, they demonstrated that microRNA-367 is required for the reprogramming. The former one employed the transient transfection of microRNAs-200c, -302s and -369s while the latter one used a lentivirus delivery system producing microRNA cluster 302-367. However, when scientists tried to reprogram mouse fibroblasts using microRNA cluster 302-367 delivered by piggyBac transposon (Lu *et al.*, 2012), they could not generate iPS cells as expected. Another group also failed using human adipose stem cells and a lentivirus producing microRNA-302s (Hu *et al.*, 2013).

This discrepancy might be attributable to the differences in approaches for delivering miRNA into cells (Bartel, 2009). In Xichen Bao's study (Bao *et al.*, 2013), they described that microRNA cluster 302-367 target transactivators, and the lack of the transactivators slowed the reprogramming process. Buganim and colleagues showed that the expression of the endogenous pluripotency regulator Sox2 can promote the transition of cells from intermediate states to pluripotent status (Buganim *et al.*, 2012). In another study, it is reported that microRNA-302s target Nuclear Receptor Subfamily 2, Group F, Member 2 (NR2F2) (Bao *et al.*, 2013), which binds to Oct4 promoter to further repress the expression of Oct4. In addition, epigenetic modification reagents are beneficial for improving reprogramming efficiency. One case is histone deacetylases (HDAC) 2 inhibitor valproic acid (Anokye-Danso *et al.*, 2011), another is mir-302-targeted co-suppression of four epigenetic regulators, AOF2 (Aliases for Lysine (K)-Specific Demethylase

1A), AOF1 (Aliases for Lysine (K)-Specific Demethylase 1B), MECP (methyl-CpG-binding proteins)1-p66 and MECP2 facilitating reprogramming (Lin *et al.*, 2011).

1.3.4 Significance of small molecules in signalling pathways in generating iPS cells

Since the first successful reprogramming was achieved by administration of OSKM factors, scientists have tried many approaches to reduce the types of reprogramming factors used for reprogramming. Oct4 and Sox2 are two most important transcription factors in the iPS reprogramming process, and a successful case of reprogramming murine fibroblasts using lentivirus Oct4 and small molecules was reported (Li *et al.*, 2011b). Therefore, one object of our experiments aims at using fewer factors in conjunction with small molecules.
Small molecules are a very promising approach to the generation of iPS cells, as substitutes of some genetic reprogramming factors in reprogramming work. Most small molecules inevitably are involved in signalling pathways. Thus, it is necessary to understand the mechanism of signalling pathways in the reprogramming process in order to improve reprogramming approaches to make it accessible to satisfy Good Manufacturing Practices (GMP) standards. Theoretically, signalling molecules that can regulate the expression of endogenous ES cells transcription factors are promising candidates for the substitutions of typical transcription factors. Therefore we investigated several signalling pathways or signalling molecules with respect to reprogramming process and the corresponding small molecules utilized in generating iPS cells.

Wnt pathway

The Int/Wingless (Wnt) signalling pathway is used to transmit signals into the inside of cells through cell surface receptors from outside of cells. Depending on whether β-catenin is involved in the pathway, there are two categories of Wnt signalling pathway: the canonical and the noncanonical pathway (Rao and Kühl, 2010). With respect to their relations to reprogramming procedures, the canonical pathway is widely reported, thus we will put emphasis on the canonical pathway.

The mechanism of the canonical Wnt pathway In the canonical Wnt pathway, β-catenin plays an important role in the working stream. Without Wnt signals, β-catenin would be degraded by a destruction complex, which consists of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α) (Komiya and Habas, 2008; MacDonald *et al.*, 2009). As the Figure 1.5 shows, Wnt binding to the N-terminal extra-cellular cysteine-rich domain of a Frizzled (Fz) and low density lipoprotein receptor-related protein (LRP)-5/6 leads to the translocation of Axin and the destruction complex to the plasma membrane, the cytoplasmic tail of LRP-5/6 (Komiya and Habas, 2008). In addition, phosphorylation of the destruction complex can result in Axin being recruited to cytoplasmic tail of LRP-5/6. Subsequently, Dishevelled (Dsh) also translocates to the membrane and binds to the complex including Fz, Axin and phosphorylated LRP5/6. However the disruption of the destruction complex in cytoplasm or the complex formed at the membrane of Fz/LRP5/6 induces the stabilization of β-catenin, resulting in the accumulation of β-catenin in the cytoplasm, which then translocates into the nucleus where it complexes with TCF/LEF to mediate transcriptional induction of target genes.

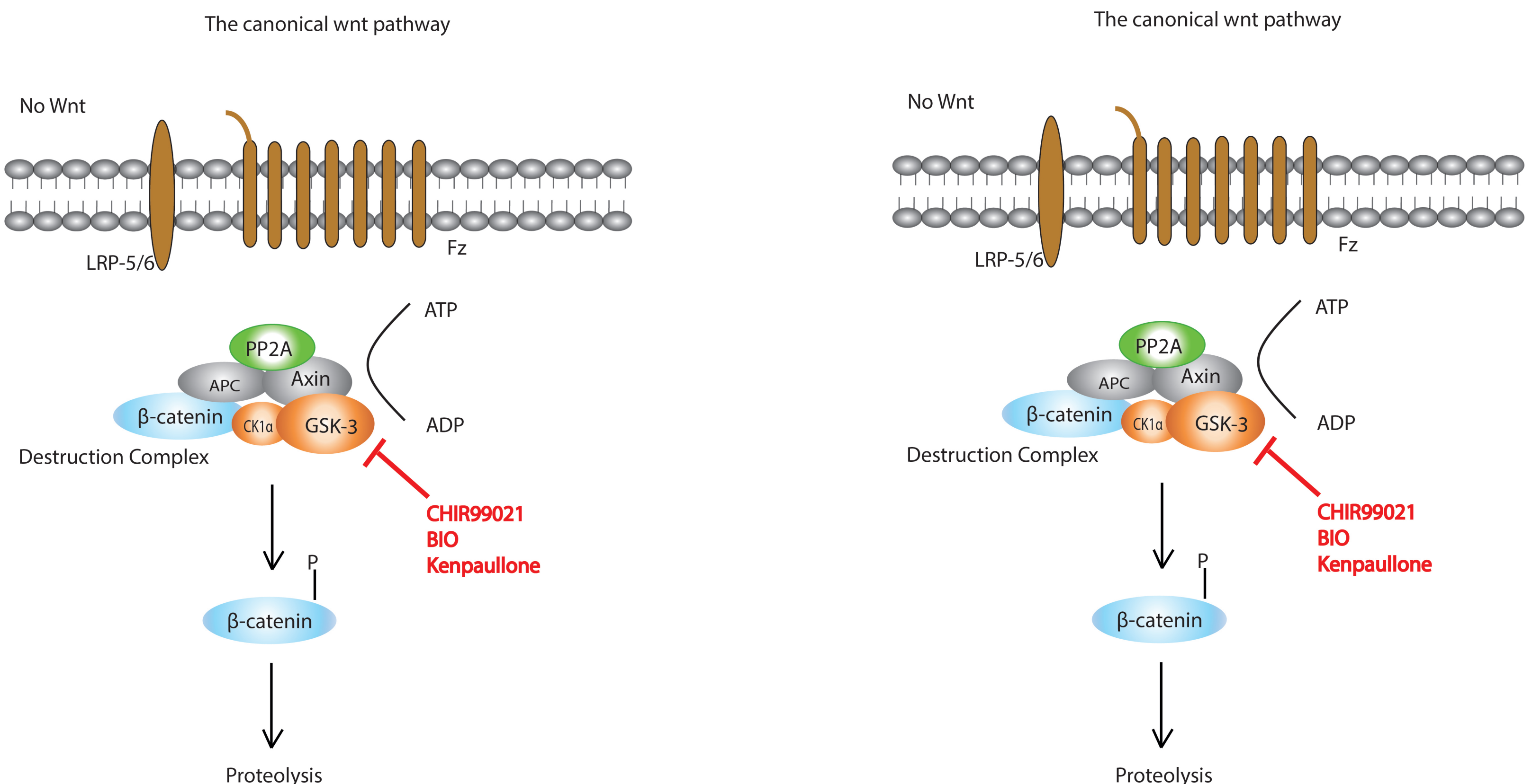


Figure 1.5: The canonical Wnt signalling pathway

The right figure shows that without Wnt signals, β-catenin can be degraded by a destruction complex consisting of Axin, APC, PP2A, GSK3 and CK1α. The left figure demonstrates the condition of the signalling pathway with Wnt. When Wnt binds to the N-terminal extra-cellular cysteine-rich domain of Fz and LRP-5/6, the destruction complex is disrupted and induces the stabilization of β-catenin, resulting in the accumulation of β-catenin in the cytoplasm, and then β-catenin translocates into the nucleus where it complexes with TCF/LEF to mediate transcriptional induction of target genes.

The function of Wnt signalling pathway in pluripotency The Wnt signalling pathway is essential for the maintenance of pluripotency and self-renewal of mouse and human ES cells, which has been reported by many scientists (Cai *et al.*, 2007; Ogawa *et al.*, 2006; Sato *et al.*, 2003; Singla *et al.*, 2006). In addition, GSK3-β inhibitor CHIR99021, which can activate Wnt signalling pathway, facilitates the conversion of mouse epiblast stem cells (mEpiSCs) and hES cells to naïve pluripotent state. Moreover, the Wnt signalling pathway contributes to the self-renewal of stem cells and cancer cells from many tissues (Reya and Clevers, 2005).

In the process of reprogramming, the role of the Wnt signalling pathway has to be emphasized. Marson and colleagues reported that Wnt3a improves the reprogramming efficiency in the absence of c-Myc retrovirus, indicating the coordinate function of signalling pathways and transcription factors (Marson *et al.*, 2008). The activation of β–catenin, the downstream factor of Wnt signalling pathway, promotes the generation of iPS cells from fibroblasts (Takahashi and Yamanaka, 2006). It has been reported that activation of the Wnt signalling pathway through inhibiting GSK3-β to alleviate the inhibitory effect of T-cell factor (TCF) on pluripotency (Niwa, 2011), is beneficial for promoting reprogramming. In addition, the application of GSK3-β inhibitor facilitates Oct4-Klf4 mediated reprogramming mouse embryonic fibroblast cells (MEFs) or human primary keratinocytes in combination with tranylcypromine, an inhibitor of lysine-specific demethylase1 (Hanna *et al.*, 2010; Li *et al.*, 2009a,b; Zhou *et al.*, 2010b). Recently there was another report demonstrating CHIR99021 plays a positive role in transdifferentiating fibroblasts into neural cells (Ladewig *et al.*, 2012). Kenpaullone, an inhibitor of GSK3-β and CDK, can replace KLF4 in OKM-mediated reprogramming of MEFs.

TGF-beta pathway

The transforming growth factor beta (TGF-β) signalling pathway is involved in regulating cell functions including cell proliferation, differentiation, apoptosis, migration and other functions, playing a key role in development and carcinogenesis. TGF-β is part of a complicated superfamily and its ligands include: Bone morphogenetic proteins (BMPs), Activin, Nodal and TGF-β's, Growth and differentiation factors (GDFs), and Anti-müllerian hormone (AMH). TGF-β ligands bind to the type II receptor (serine/threonine kinase receptors) dimer, which then catalyzes the transphosphorylation of type I receptor dimer (serine/threonine kinase receptors) to form a hetero-tetrameric complex with the ligand (Wrana *et al.*, 1992). Subsequently, the activated type I receptor then phosphorylates SSXS motif of receptor-regulated SMADs (R-SMADs) at C-terminal series, and R-SMADs can now bind the coSMAD SMAD4 to form a complex. The R-SMAD/coSMAD complexes accumulate in the nucleus, where they regulate the expression of target gene through cooperation with DNA-binding transcription factors and CREB-binding protein (CBP) or p300 coactivators (Derynck and Zhang, 2003).
In TGF-β signalling pathway, combinatorial interactions between tetrameric receptor complex and different signals allow versatility of TGF-β family responses. The combination of the same ligand to different compositions of receptor complex can induce different signalling pathways thus the receptor complex plays an important role in deciding the signalling pathway. For example, the BMP type II receptors (BMP-R II) can combine with three BMP type I receptors, ALK2 (ActR I), ALK3 (BMP-R I A), and ALK6 (BMP-R I B), which activates SMAD1/5/8. TGF-β type II receptors (TGF-β R II) can not only combine with ALK 5 (TGF-β R I), which interacts with SMAD2 and SMAD3, but also activates with ALK1 and ALK2, which activates SMAD1and SMAD5 (Valdimarsdottir and Mummery, 2005). Activin signalling occurs through ActRII, ActRII B combining with the type I receptor ALK4, the activation of which results in phosphorylation

of SMAD2 and SMAD3 (Oh *et al.*, 2000; Yamashita *et al.*, 1995). (see table 1.2)
However, the signalling pathways for the maintenance of pluripotency are dif-

Ligand	Type II receptors	Type I receptors	R-SMAD
BMP	BMP-R II	ALK2 (ActR I)	SMAD1, SMAD5, SMAD8
		ALK3 (BMP-R I A)	
		ALK6 (BMP-R I B)	
Activin/Nodal	ActR II, ActR II B	ALK4(ActR I B)	SMAD2
TGF-ß	TGF-ßR II	ALK5 (TGF-ßR I)	SMAD2, SMAD3
		ALK1	SMAD1, SMAD5
		ALK2 (ActR I)	

Table 1.2: Combinatorial interactions between receptors and signals in TGF-ßpathway.

ferent for murine and human ES/iPS cells. The blockage of BMP/SMAD1/5/8 signalling is necessary to maintain the undifferentiated state and pluripotency of murine ES cells whereas the activation of TGF-β activin/SMAD2/3 plays a necessary role in maintaining human ES/iPS cells pluripotency (Beattie *et al.*, 2005; James *et al.*, 2005; Valdimarsdottir and Mummery, 2005). TGF-β signalling plays an important role in inducing epithelial to mesenchymal transition (EMT) or transdifferentiation through regulating TGF β-activated SMAD signalling, Ras-MAPK-Erk kinases signalling, Rho GTPase signalling and PI3 kinase/AKT signalling pathways (Xu *et al.*, 2009). During development, EMT illustrates the differentiation plasticity, in which process the cells lose their epithelial characteristics including cell-cell contact and obtain mesenchymal cell characteristics with a migratory behaviour. Mesenchymal-epithelial-transition (MET), an early event in reprogramming, plays a crucial role in the generation of iPS cells (Esteban *et al.*, 2012). Thus factors beneficial for MET would help improve the reprogramming process. The hypothesis exactly explains the successful application of

inhibitor of TGF-β signalling in reprogramming. Inhibitors of TGF-β signalling, not only enhance the reprogramming efficiency, but also can replace the exogenous transcription factors c-Myc or Sox2 in induction of iPSCs from MEF cells (Ichida *et al.*, 2009; Maherali and Hochedlinger, 2009). In addition, the combination utilization of SB431542 (inhibitor of TGF-β receptor), PD0325901 (MEK inhibitor), and Tzy (Rho-ROCK inhibitor) in reprogramming human fibroblasts improves reprogramming efficiency and speed (Lin *et al.*, 2009a). Another report shows that the combination use of CHIR99021 (GSK3-β inhibitor), 616452 (an inhibitor of TGF-β type I receptor ALK5), tranylcypromine, VPA, vitamin C and bFGF with the lentivirus-Oct4 successfully reprogram murine fibroblasts into iPSCs (Rao and Kühl, 2010). A similar experiment using only Oct4 and chemical cocktails including sodium butyrate (HDAC inhibitor), A-83-01(an inhibitor of TGF-β type I receptor ALK4, ALK5, and ALK7), PD0325901, and PS48 (an activator of 3'-phosphoinositide-dependent kinase-1, PDK1) has been reported to induce human primary somatic cells to give rise to iPSCs (Zhu *et al.*, 2010).

MAPK/Erk pathway

The MAPK/ERK pathway is used to pass signals from a receptor on the surface of the cell to the DNA in the nucleus of the cell. Many proteins are involved in the pathway like Mitogen-activated protein kinases (MAPK), originally called ERK, Extracellular signal-regulated kinases.

When epidermal growth factor (EGF) binds to epidermal growth factor receptor (EGFR), a receptor-linked tyrosine kinase, the tyrosine kinase activity of the cytoplasmic domain of the receptor is activated. The phosphorylation of tyrosine residues of EGFR recruits growth factor receptor-bound protein 2 (GRB2)- Son of Sevenless (SOS) complex, leading to the activation of SOS, which allows Ras (a GTPase) to become activated through swapping its guanosine diphosphate (GDP) for guanosine-5'-triphosphate (GTP) (Schulze *et al.*, 2005; Zarich *et al.*,

2006). Apart from EGFR, other cell surface receptors can have similar effects including fibroblast growth factor receptor (FGFR) and Platelet-derived growth factor receptors (PDGFR). Then activated Rat sarcoma (Ras) initiates the kinase cascade through activating proto-oncogene serine/threonine-protein kinase (RAF kinase) (Avruch *et al.*, 2001), which then activates mitogen-activated protein kinase kinase (MEK) (MEK1 and MEK2), resulting in the activation of a mitogen-activated protein kinase (MAPK). Further, MAPK can regulate the expression of several transcription factors. The action of Ras-dependent MAPK/ERK pathway helps stabilize the c-Myc protein via phosphorylation of Ser 62, while GSK-3 activity is associated with the degradation of c-Myc protein (Sears *et al.*, 2000). In addition, Ras-MAPK-Erk kinases signalling, Rho GTPase signalling is beneficial for EMT process, the converse of which MET process is pivotal in early reprogramming. Moreover, TGF-β can also induce MAPK/ERK pathway apart from SMADs, both of which play a role in EMT transdifferentiation (Derynck and Zhang, 2003). The mechanism is illustrated in Figure 1.6. Although the inhibition of MAPK/ERK pathway reduces the stability of c-Myc, it facilitates the MET transdifferentiation, which is important in early reprogramming. However, the small molecule PD0325901, MEK inhibitor, is reported to block differentiation pathway of ES cells, supporting self-renewal (Ying *et al.*, 2008), and facilitates the reprogramming process (Hanna *et al.*, 2010; Li *et al.*, 2009a,b; Zhou *et al.*, 2010b; Lin *et al.*, 2009a; Zhou *et al.*, 2010b; Zhu *et al.*, 2010). Thus, according to this, it is more possible that MET process outweighs the effect of the decreased stability of c-Myc.

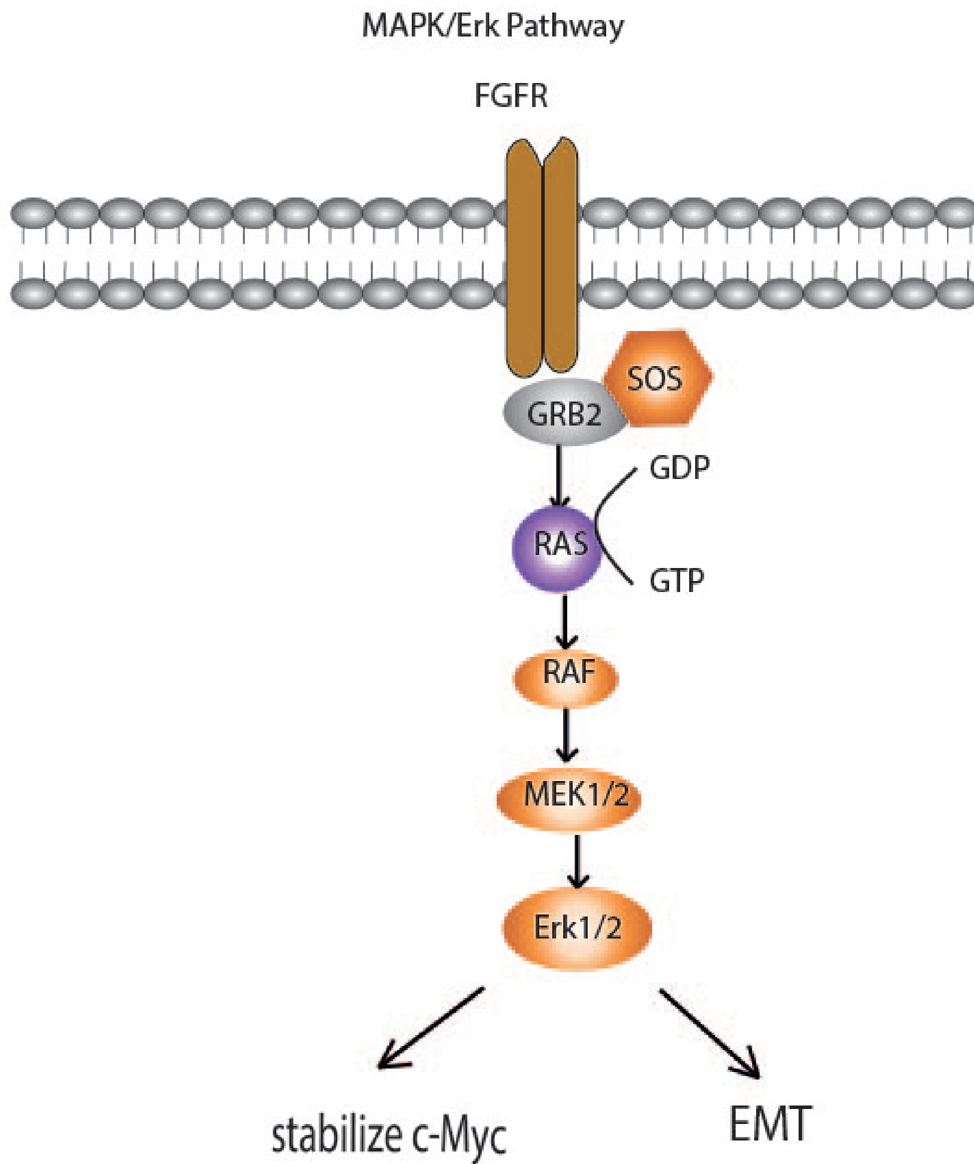


Figure 1.6: MAPK/Erk pathway *The combinding of EGF to its recetor EGFR activates the tyrosine kinase activity of the cytoplasmic domain of EGFR. The phosphorylation of tyrosine residues of EGFR recruits GRB2-SOS complex, leading to the activation of SOS, which allows Ras to become activated through swapping its GDP for GTP. Then activated Ras initiates the kinase cascade through activating RAF kinase which then activates MEK, resulting in the activation of MAPK. Further, MAPK can regulate the expression of several transcription factors, allowing to stabilize the c-Myc protein and facilitate EMT process.*

PI3-K/AKT pathway

The PI3K/AKT/MTOR signalling pathway is important in fundamental cellular functions such as cell proliferation, apoptosis, survival, cell growth and migration. The signalling pathway is activated by receptor tyrosine kinases, integrins, cytokine receptors, G-protein-coupled receptors and other stimuli that can induce phosphoinositide 3-kinase (PI3K) to generate phosphatidylinositol 3,4,5 trisphosphates (PI(3,4,5)P3), which is a second messenger responsible for the translocation of AKT to the plasma membrane where it can be activated by phosphoinositide-dependent kinase (PDK) 1 and PDK2 via phosphorylation (Yap *et al.*, 2008). The activation of protein kinase B (AKT) cascade can further activate mechanistic target of rapamycin (serine/threonine kinase) (MTOR). In many cancer cases, the PI3K-AKT signalling pathway is overactive, leading to the decreased apoptosis and enhanced proliferation (LoPiccolo *et al.*, 2008; Morgensztern and McLeod, 2005; Osaki *et al.*, 2004). Different forms of AKT (AKT1, AKT2, and AKT3) represent the major signalling arm of PI3K. However, prevention of cell apoptosis is the primary barrier to reprogramming. Therefore, the activation of PI3K/AKT/mTOR signalling pathway is beneficial for reprogramming through increasing cell proliferation and protection from cell apoptosis. IGF signalling can activate PI3-K/AKT pathway. Insulin-like growth factor (IGF) system plays an important role in the development of tissues or organs and postnatal growth, and maintenance of normal function of many types of cells (Jones and Clemmons, 1995; LeRoith *et al.*, 1995; Stewart and Rotwein, 1996). The IGF system is made up of three components: the IGF ligands (IGF-1 and IGF-2); cell surface receptors; and IGF binding proteins (IGFBPs). It is reported that IGF-1 receptor and ERBB2 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2) receptor signalling play an important role in long-term growth of multiple hES cell lines (Wang *et al.*, 2007). Telomere elongation can greatly

increase iPS generation efficiency (Marion *et al.*, 2009) while IGF-1 could modulate the activity of telomeres through PI3 kinase/AKT signalling pathway (Thum *et al.*, 2007; Werner *et al.*, 2008; Wetterau *et al.*, 2003). IGF-1 facilitates the histone H3 and H4 acetylation, which might be another possible way to improve iPS efficiency (Sun and D'Ercole, 2006). Moreover, the cooperation of IGF and FGF help maintain the stem cell niche of human pluripotent cells *in vitro* (Bendall *et al.*, 2007). In addition, hypoxia is reported to be beneficial for improving iPS efficiency (Yoshida *et al.*, 2009), which might contribute to the IGF-1 secreted by stem cells and progenitor cells under hypoxic conditions (Li and Geng, 2010a). IGF-1 was demonstrated to enhance expression of iPS reprogramming factors and antisenescence activity in human dermal fibroblast (Bhatia *et al.*, 1997). However, mesenchymal stem cells expessing IGF-1 promotes their differentiation into osteoblasts through an intact IRS1-PI3K signalling pathway (Granero-Moltó *et al.*, 2011).

1.3.5 Engineering the Stem Cell Niche and the related Micro- and Macro-environment

How to maintain 'stemness' of stem cells has a great relationship with the so called niche. Niche is a specific anatomic unit where stem cells are established and regulate their involvement in tissue regeneration, maintenance and repair. In the stem cell niche, stem cells have the greatest potential to self-renew or differentiate to form tissues. The niche provides a basic unit for stem cells to make responses to micro-environment and macro-environment through integrating signals.
In regenerative medicine, scientists are studying the components of the niche and trying to mimic the *in vivo* niche conditions for *ex vivo* culture. After all, during *in vitro* experiments, cell proliferation and differentiation are both occurring in flasks or plates, so that the reproduction of micro-environment is beneficial for

stem cells to mimic the expected growth conditions *in vivo*.

Adult stem cells have limited function when they lack a niche. A familiar case is haematopoietic stem cells. Many successful transplantation cases in clinic demonstrate that HSCs can self renew and regenerate various blood lineage cells when they are transplanted into patients. HSCs cannot generate such complete lineage cells *in vitro* even over a feeder layer and with defined culture medium. In addition, HSCs often undergo an ageing process in which their proliferation potential has decreased and they lose their multipotency. After all, the culture conditions *in vitro* cannot compare with the *in vivo* niche.

Another simple example is that human embryonic stem cells are often cultured in fetal bovine serum supplemented media containing basic fibroblast factor in the plate seeded by feeder layer, which is believed to be supportive in maintaining the pluripotent status of embryonic stem cells.

General introduction of micro- and macro-environmen

Both extrinsic and intrinsic factors influence the celluar function, and the two factors do not exert their influence independently, but work interdependently. The extrinsic environment plays a role in guiding cell function and fate. This extrinsic environment contains many aspects, biochemical microenvironment, physico-chemical environment and mechano-structural environment. The three classes of factors can represent the tripartite axes of cues which act on cells (Di Nardo *et al.*, 2011). All these factors work together for cell fate in the form of prestressed extracellular matrix, prestressed neighbouring cells, endocrine and paracrine signals, body movement, blood flow, forces and so on.

Biochemical microenvironment

In the human body, biochemical cues contain not only soluble ligands but also insoluble ligands, which are secreted by paracrine cells or signalling pathway network. Biochemical factors are of crucial importance in deciding cell differentiation, reprogramming status whether *in vivo* or *in vitro* environment. These biochemical factors include hormones, cytokines, peptides, growth factors and steroids. Take mesenchymal stem cells as an example. Although mesenchymal stem cells have the potential to differentiate into osteocytes, myocytes, chondrocytes, adipocytes, and so on, biochemical factors are essential for deciding which type of mature cells mesenchymal stem cells prefer to differentiating to. According to experience of our group, mesenchymal stem cells can differentiate into osteocytes with the addition of dexamethasone, ascorbate-2-phosphate, and βu-glycerophosphate in culture medium supplemented with glutamine and foetal calf serum. Similarly, embryonic stem cells can differentiate into neuron-like, pancreatic beta-cell-like, adipose-like, hepatocyte-like and osteoblast-like cells by the addition of biochemical factors such as dexamethasone, retinoic acid, and dimethyl sulfoxide (Trounson, 2006). Another example is the reduction use of bFGF in the medium of culturing hES cells resulted in rapid differentiation (Dvorak and Hampl, 2005). Different cell types and extracellular matrices play a significant role in the regulation of stem cells in a niche *in vivo*. For *in vitro* experiments, feeder cells, tissues or appropriate ligands in specific space and time dependent configurations try to recapitulate the *in vivo* microenvironment to supply a wide range of flexible culture systems in which multi-parametric factors can be defined *in vitro*. Therefore, the defined culture medium provides accessibility and possibility to study stem cell fate selection.

The macroenvironment

The biochemical microenvironment is not the only factor that exerts functions in stem cell culture, other non-biochemical cues also influence cell behaviour and fate. Nearly all these factors can be referred to two subgroups, physical-chemical factors and mechano-structural factors.

Physical-chemical factors are composed of pH, temperature, humidity, surface energy, oxygen, charge, and so on. In the incubator, pH and CO_2 concentration are ensured to maintain similar conditions to *in vivo* through strict regulation of acid-base balance. Generally, temperature can be adjusted in the range of 28 °C to 37 °C. In addition, some incubators have the special facility to control the concentration of oxygen levels although the actual oxygen tension at the cell surface is different from each other due to the low solubility of oxygen and the different height of the medium. In reference to the relationship of oxygen levels and cell types, the typical oxygen concentration 20% is appropriate for most cell lines, while lower oxygen concentration (5%) is reported to increase stem cell proliferation (Csete, 2005; Moussavi-Harami *et al.*, 2004; Studer *et al.*, 2000) and even much lower oxygen concentration increase MSC proliferation whilst maintaining an undifferentiated status (Grayson *et al.*, 2006). There are also reports indicating that low oxygen concentration improves the stemness of ES cells (Guo *et al.*, 2013; Szablowska-Gadomska *et al.*, 2011) and enhances reprogramming efficiency (Jee *et al.*, 2010). Above all, the control of oxygen concentration (hypoxia) in a stem cell culture system is related to cell amplification and cell fate. Reduced temperature is reported to facilitate MSC cells maintaining stemness and reduce apoptosis and proteasome activity (Stolzing and Scutt, 2006; Stolzing *et al.*, 2006). Surface tension and wettability also influence stem cell behaviour. For example, in MSC culture, low surface energy through adding CH_3 end groups over silanized hydrophobic surfaces is beneficial for maintaining the MSC cell phenotype, whilst higher surface energy promote osteogenesis (Curran *et al.*, 2006).

Electric, magnetic and ultrasound fields also play a role in myocyte differentiation (Abilez *et al.*, 2009; Genovese *et al.*, 2008; Radisic *et al.*, 2004; Sauer *et al.*, 1999; Serena *et al.*, 2008).

Mechano-structural microenvironment

Mechano-structural microenvironment refers to the static nature of the cell habitat including surface physical features, bulk properties and dimensional environment. Generally speaking, the innovation and changes of the mechano-structure could be presented in culture plate or flasks. Thus many companies produced scaffold or three dimentional (3D) culture plate to improve the Mechano-structural microenvironment. 3D culture plates have many advantages over 2D plates. There are reports demonstrating that 3D culture facilitates cell-cell interaction and maintenance of spherical morphologies. 3D scaffold provides a homogeneous, structureless 3D environment beneficial for stem cell proliferation, maintaining stemness and differentiation into other tissues like hepatic cells or neural cells (Baharvand *et al.*, 2006; Brännvall *et al.*, 2007). In addition, the rigid and porous scaffold not only supplies a framework for cells to adhere to and spread in three dimensions, but also provides space for cells responding (Di Nardo *et al.*, 2011). There are studies showing that a 3D scaffold promotes ES cells differentiating into haematopoietic cells (Liu *et al.*, 2006; Liu and Roy, 2005) and cardiac stem cells (Forte *et al.*, 2008).

1.3.6 The reduced use of transcription factors in reprogramming process

To reduce the number of transcription factors in reprogramming process irrespective of delivery methods is the aim of generating induced pluripotent cells. From the beginning, four to five transcription factors deliver by retrovirus were utilized in reprogramming, and the number has been reduced with the addition of small molecules. However, mouse somatic cells have been successfully reprogrammed into iPS cells by small molecule compounds without any addition of transcription factors (Hou *et al.*, 2013). Transcription factors used in reprogramming process were summarised in table 1.3, which gives a clue for reduction of transcription factors.

Transcription factors used in reprogramming process

Transcription factors	Cell type	Publications
OSKMNL	Human	$CD34^{+}CD38^{+}$ cells or $CD45^{+}CD34^{-}$ deriving from $CD34^{+}CD38^{+}$ cells, (+BMSC) electroporation (Park *et al.*, 2012).
OSKML	Human	$CD34^{+}$ cells from CB, PBMC, plasmids electroporation (Okita *et al.*, 2013). Mononuclear cells (MNC) or $CD34^{+}$ cells from CB and PB, a single episomal plasmids encoding OSKML, electroporation (Chou *et al.*, 2011). MNC or $CD34^{+}$ cells from BM, CB, electroporation (Hu *et al.*, 2011). Human neonatal foreskin fibroblasts, mRNA (Warren *et al.*, 2010).
OSKMN	Human	Neonatal human dermal fibroblasts, retrovirus (Lowry *et al.*, 2008).

Transcription factors used in reprogramming process (*continued*)

Transcription factors	cell type	Publications
OSKM	Human	Adult human dermal fibroblasts, retrovirus (Park *et al.*, 2008b; Takahashi *et al.*, 2007). Adult human dermal fibroblasts, dox-inducible lentivirus (Maherali *et al.*, 2008). Human, Excision of a retroviral cassette (Loh *et al.*, 2012). Neonatal human foreskin keratinocytes, a single polycistronic lentivirus (Carey *et al.*, 2009). Human keratinocytes, retrovirus (Aasen *et al.*, 2008). Human neonatal foreskin fibroblasts, OSKML mRNA (Warren *et al.*, 2010). $CD34^+$ cells from mPB, retrovirus (Loh *et al.*, 2009). $CD34^+$ cells from CB, BM, mPB, retrovirus (Ye *et al.*, 2009). PBMC, lentivirus (Gianotti-Sommer *et al.*, 2008). Peripheral MNC, a polycistronic lentiviral vector encoding OSKM (Staerk *et al.*, 2010). T cells from peripheral blood, lentiviruses (Loh *et al.*, 2010). $CD34^+CD38^+$ cells or $CD45^+CD34^-$ deriving from $CD34^+CD38^+$ cells, (+BMSC) electroporation (Park *et al.*, 2012).
	mouse	MEF, retrovirus (Maherali *et al.*, 2007; Okita *et al.*, 2007; Takahashi and Yamanaka, 2006) (c-Myc tumor). Fibroblasts, retrovirus (Wernig *et al.*, 2007). Fibroblasts, an inducible lentivirus (Stadtfeld *et al.*, 2008a). MEF, plasmids (Okita *et al.*, 2008). MEF, human artificial chromosome (Hiratsuka *et al.*, 2011).
	Monkey	Fibroblasts, retrovirus (Liu *et al.*, 2008).

Transcription factors used in reprogramming process (*continued*)

Transcription factors	cell type	Publications
OSNL	human	Somatic cells, lentivirus (Yu *et al.*, 2007). Endothelia cells from CB, lentivirus, efficiency low (Haase *et al.*, 2009). Human dermal fibroblasts, mES medium + hLIF + PD0325901 + A-83-01 + CHIR99021 (Li *et al.*, 2009a).
OSK	mouse	Fibroblast, retrovirus (Nakagawa *et al.*, 2008). Fibroblast, retrovirus (Wernig *et al.*, 2008).
OKM	mouse	MEF, retrovirus, 616452 + VPA (Ichida *et al.*, 2009). MEF, Pan-Src Family Kinase Inhibitors (Staerk *et al.*, 2011). Neural progenitor cells, lentivirus (Eminli *et al.*, 2008).
OSM	mouse	MEF, Kenpaullone, replacement of Klf4, retrovirus, lentivirus (Lyssiotis *et al.*, 2009).
OS	human	Human primary fibroblasts, retrovirus (Huangfu *et al.*, 2008). $CD133^{+}$ cells from CB, retrovirus (Giorgetti *et al.*, 2009).
OK	human	Human primary keratinocyte, lentivirus, tranylcypromine+CHIR99021 (Li *et al.*, 2009b).
	mouse	Neural progenitor cells, viral transduction Oct4, Klf4, BIX-01294 (functionally replace Sox2 and c-Myc) (Shi *et al.*, 2008b). MEF, retrovirus, BIX-01294 and BayK8644 (Shi *et al.*, 2008a). MEF, retrovirus, CHIR99021 (Li *et al.*, 2009b).

Transcription factors used in reprogramming process (*continued*)

Transcription factors	cell type	Publications
KSM	mouse	Neural progenitor cells, viral transduction Klf4, Sox2 and c-Myc, BIX-01294, PD0325901(Shi *et al.*, 2008b).
Oct4	human	Neonatal human epidermal keratinocytes, lentivirus, NaB (a histone deacetylase inhibitor), + PS48 (a small molecule activator of PDK1), + A-83-01. Adult human epidermal keratinocytes, lentivirus, NaB + PS48 + A-83-01+ Parnate + CHIR99021 (Zhu *et al.*, 2010).
	mouse	Fibroblasts, lentivirus, VPA + CHIR + 616452 + Tranyl + VitC (Li *et al.*, 2011b). Neural stem cells, retrovirus, BIX-01294 (Kim *et al.*, 2009b). MEF, retrovirus, a protein arginine methyltransferase (PRMT) inhibitor AMI-5 + A-83-01 (Yuan *et al.*, 2011).
none	mouse	Fibroblasts, FSK + VPA + CHIR + 616452 + Tranyl + DZNEP + TTNPB (Hou *et al.*, 2013).

Table 1.3: Transcription factors used in reprogramming process

1.3.7 Types of different cells which are reprogrammed into iPS cells

The first group of iPS cells were derived from mouse fibroblasts, since then scientists tried to reprogram fibroblasts from different tissues and animals. After mouse

fibroblasts, Yamanaka's group successfully reprogrammed human fibroblasts into iPS cells. Subsequently, human neonatal dermal fibroblasts, human primary fibroblasts, and human foreskin fibroblast cells, all of these cells are a reliable source of iPS cells. Regarding the reason for fibroblast cells as the first source of reprogramming, this might be because of the accessibility of fibroblast cells and their incredible capacity for regeneration. In addition, keratinocytes from skin can be reprogrammed 100 fold more efficient and 2 fold faster compared with reprogramming of human fibroblasts since keratinocytes express higher levels of endogenous Klf4 and c-Myc transcripts than fibroblasts (Aasen *et al.*, 2008). And other groups have successfully reprogrammed keratinocytes (Carey *et al.*, 2009; Li *et al.*, 2009b; Zhu *et al.*, 2010). Moreover, adipose stromal cells express levels of key pluripotent genes that may synergize with the suppression of cell senescence and induction of MET triggered by microRNAs-200c, -302s and -369s in order to initiate the reprogramming.

However, most reprogramming work up to now have been done on fibroblast cells, a kind of adherent cells with great regenerative potential. However, skin is not the only accessible cells, which makes us consider another type of accessible cells: blood cells. We can easily get blood cells from human or animals without serious surgery and are able to isolate cells according to our routine methods. But a big difference with fibroblast cells is that blood cells are suspension cells, which will increase difficulties during the reprogramming process. Although reprogramming blood cells without any treatments have not been reported, growth factor activated myeloid cells were reported to be successfully reprogrammed into iPS cells, demonstrating the synergistic action between hematopoietic growth factor, stromal activation signals, and episomal Yamanaka factor expression. In their study, growth factor activated myeloid progenitors were partially programmed to ES-like cells. The addition of Yamanaka factors and contact-dependent stromal cells resulted in the production of stable pluripotent cells (Park *et al.*, 2012). In addition, human $CD34^+$ cells (Chou *et al.*, 2011; Okita *et al.*, 2013), T cells

(Brown *et al.*, 2010), endothelial cells (Haase *et al.*, 2009), and mononuclear cells (Chou *et al.*, 2011; Hu *et al.*, 2011) were successfully reprogrammed using episomal plasmids in recent years.

Not only accessible cells like fibroblasts and blood cells, but also viscera cells can be a source of deriving iPS cells. Pancreatic β cells can be reprogrammed into iPS cells using inducible lentiviruses to ectopically express OSKM (Stadtfeld *et al.*, 2008a). Induced pluripotent stem cells can also be produced from hepatocytes (Imamura *et al.*, 2010). In addition, mouse liver and stomach cells can be the source of iPS cells (Aoi *et al.*, 2008). The reprogramming efficiency ranges between 0.1% and 0.2%, similar to reprogramming fibroblasts cells by ectopic expression of OSKM factors. Neural cells have specific advantage as the source for the generation of iPS cells. The endo-expression part of reprogramming factors, facilitates the reprogramming process (Kim *et al.*, 2009b). Duanqing Pei and colleagues reported the successful generation of iPS cells from cells of urine, which is the easiest accessible sample for human (Xue *et al.*, 2013; Zhou *et al.*, 2012b).

1.3.8 Application of iPS cells

iPS cells have many promising applications in clinical and biomedical research to study diseases, develop candidate drugs and support therapeutic treatments in regenerative medicine.

In biomedical research, cell culture-based assays are the basic approach to test drugs to learn about the functionality and side effects. Induced pluripotent cells are generated through changing fate of source cells and reprogramming their differentiation, thus iPS cell lines still enable genomic characteristics of source cells. Therefore, if cells from genetic disease patients can be utilised for reprogramming, these derived disease-specific iPS cell lines are characterized with the genetic defects of related diseases, thereby not only facilitating disease research but also

providing an opportunity for autologous cell therapies that would circumvent immune rejection and permit correction of gene defects prior to tissue reconstitution. Therefore, the patient disease-specific derived iPS cell lines lay a foundation for drug discovery and exploring fundamental principles and mechanisms.

Animal disease models play an essential role in studying human pathophysiology. Although murine models of human congenital and acquired diseases provide a useful platform for research, there still remains a limit. Take trisomy 21 for example, Down's syndrome critical region locates on distal human chromosome 21, while the corresponding region in mouse is on chromosome 16 and orthologous segments to human chromosome 21 are present on mouse chromosome 10, 17 (Nelson and Gibbs, 2004; Olson *et al.*, 2004). Thus the capacity of recapitulating human congenital diseases in murine models is limited due to their many differences. However, the iPS cell lines derived from normal and disease-specific patients can act as *in vitro* disease models to study human pathophysiology under defined *in vitro* conditions, or provide new insights for therapeutic interventions. Somatic cells have been successfully applied to generate iPS cell lines from patients with a wide range of diseases, including adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne muscular dystrophy, Becker muscular dystrophy, Parkinson disease, Huntingdon disease, juvenile-onset, type 1 diabetes mellitus, Down syndrome/trisomy 21, the carrier state of Lesch-Nyhan syndrome, and spinal muscular atrophy (Park *et al.*, 2008a). In 2012, iPSC-derived cardiomyocytes from dilated cardiomyopathy (DCM) patients recapitulate partially the morphological and functional phenotypes of DCM, despite limitations in the study such as cardiomyocyte immaturity and the lack of an *in vivo* environment (Sun *et al.*, 2012). In 2013, researchers in Stanford-Burnham Medical Research Institute used skin fibroblast cells from patients with arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) to recapitulate the adult-onset disease in a culture dish, generating the first maturation-based disease model for study (Kim

et al., 2013). The generation of iPS cells from these patients obviously provides a promising future for disease mechanism investigation research, and pharmacological interventions.

One appealing character of iPS cell lines is the capacity to de directed to differentiate into the interesting cells or tissues in the defined conditions, offering the potential to support treatment or tissue regeneration. In theory, easily accessible somatic cells biopsied from patients can be reprogrammed into iPS cells, and could then be directed to differentiate into cell types that are compromised or destroyed by the disease for therapeutic purpose, offering the promising potential for clinical application. In the clinic, allograft of tissue or organ can lead to graft-versus-host disease in recipients, resulting in damage to skin (rash), mucosa, liver, and the gastrointestinal tract. Thus immunosuppressive drugs are inevitably used to reduce immune rejection in recipients in clinic, but they have serious side-effects, including the possibility of developing cancer. However, due to iPS cells originating from patients, the immune rejection effects of autologous iPS derivatives might be minimized. Moreover, the study made by Boston University School of Medicine demonstrated that there was little evidence of immune rejection by mouse recipients when the embryoid bodies (EBs) or representative cell types spanning the three embryonic germ layers deriving from iPSCs were transplanted into the genetically identical recipients (Guha *et al.*, 2013). Therefore, the tremendous prospect of iPS derived tissues or organs in cell replacement therapy promotes the study of iPS cell development and has become the frontier in medical science. A large amount of work has been done in iPS cell differentiation into various types of cells. A group in Harvard University successfully directed iPS cells deriving from a patient with amyotrophic lateral sclerosis to differentiate into motor neurons (Dimos *et al.*, 2008). Moreover, there were reports demonstrating that iPS cells derived from human (Burridge and Zambidis, 2013; Gai *et al.*, 2009; Lieu *et al.*, 2013; Zhang *et al.*, 2009) and murine fibroblasts can produce functional cardiomyocytes (Kuzmenkin *et al.*, 2009; Mauritz *et al.*, 2008;

Pfannkuche *et al.*, 2009). As early as in 2007, iPS cells derived from fibroblasts are used to treat sickle cell anemia mouse model (Hanna *et al.*, 2007).

1.4 Hypothesis and Aims

The generation of iPS cells from somatic cells has promising future applications in clinical and biomedical research for study of diseases, development of candidate drugs and to support therapeutic treatments in regenerative medicine. However, the clinical applications have to meet GMP requirements without the risk of insertional mutagenesis associated with retrovirus. Chromatin modifying agents, facilitating gene expression through regulating epigenetics, are widely used in many protocols to generate iPS cells and culture of blood $CD34^+$ cells with chromatin-modifying agents can lead to an increase in marrow repopulating cells and in the case of valproic acid increased erythroid cell colony formation. This prompted us to see whether DNA modification reagents affect the functionality of pretreated HSPC and whether they are beneficial for increasing the expression of delivered genes. Is it possible to use the clinical accessible source e.g. peripheral blood to efficiently generate iPS cells through non-integration method? Is the reprogramming efficiency comparable from $CD34^+$ cells and MNC?

We undertook research to help understand what effects chromatin modifying agents have on mobilised peripheral blood (mPB) $CD34^+$ cells, and optimised the expansion medium protocol to facilitate reprogramming work. The final aim of the project is to utilize peripheral blood mononuclear cells (MNC), one of the most easily accessible tissues to generate iPS cells using an efficient non-viral, feeder cell free methodology, with the ultimate goal of moving this methodology towards clinical use.

Chapter 2

Materials and Methods

2.1 Cell source and sampling

All cells from human sources were obtained under ethical committee approval and institutional procedures including written informed consent from each patient. Ethical committee approval was granted for sampling haematopoietic stem/progenitor cells and mononuclear cells (MNC) from mobilised blood, umbilical cord blood and normal peripheral blood for studies in Scottish National Blood Transfusion Service (SNBTS) RD. Lipoaspirate samples and fat tissues were obtained from Doctor Chris West in Scottish Centre for Regenerative Medicine (SCRM) of University of Edinburgh.

Venous blood samples (10 ml) were collected into EDTA from donors or patients following mobilisation of haematopoietic stem cells by granulocyte colony stimulating factor (G-CSF) immediately following cell-separator leukapheresis collection of G-CSF mobilised peripheral blood stem cells (PBSC) for transplant. In most cases adult patients with different types of leukaemia donated for autologous PBSC transplant during disease remission; in some cases donors were

healthy adult volunteers donating for allogeneic transplant. Umbilical cord blood was aspirated from the umbilical placental veins following caesarean delivery and collected into heparinised 50 ml tubes. Simultaneously, small parts of umbilical cords were collected. Normal peripheral blood cells were recovered from blood bags containing the leukocyte-rich layer at the sedimented red-cell/plasma interface following routine preparation of platelet-rich plasma from fresh blood donations by the SNBTS.

2.2 Cell isolation and purification

2.2.1 MNC isolation

Mononuclear cells were isolated by buoyant density centrifugation of samples over Ficoll-Paque PLUS (GE Healthcare, cat. No. 17-1440-02). The different samples were diluted with relative ratios of 1:1 (mobilised blood/cord blood/fetal liver suspension cells) or 1:3 (buffy coat) in sterile Dulbecco's phosphate buffered saline (PBS, Life technologies, cat. No. 14190-094) with EDTA, and then layered over Ficoll-Paque PLUS (in sterile polypropylene centrifuge tubes) and centrifuged at 400×g for 25 minutes. Leucocytes were collected from the interface, washed twice with PBS and resuspended in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, cat. No.12-722F) with 2% fetal calf serum (FCS, GE Healthcare Life Sciences, Hyclone, cat. No. SH30070). Unless otherwise stated, separated mononuclear leukocytes were used for subsequent isolation of cell subpopulations or experiments directly. If required, isolated MNC were frozen using 10% DMSO in IMDM with 10 % FCS and stored at liquid nitrogen.

2.2.2 HSPC purification by Magnetic-activated cell sorting MACS

The $CD34^+$ subpopulation of mononuclear cells was enriched using Magnetic-activated cell sorting (MACS) positive selection (MACS CD34 isolation Kit, Miltenyi Biotec, cat. No. 130-046-702), according to the manufacturer's instructions. MACS Microbeads coupled to specific monoclonal antibodies were used to label the target cells. 10^8 mononuclear cells were incubated with 100 μl Fc-receptor blocking reagent at room temperature for 10 minutes to inhibit non-specific binding or Fc-receptor mediated binding of CD34-Microbeads to non-target cells. Subsequently, 100 μl CD34-Microbeads/10^8 cells were added, mixed well, and incubated for 30 minutes at 4 ℃. Then the $CD34^+$ haematopoietic stem/progenitor cells in mononuclear cells were labelled with MACS Microbeads coupled to anti-CD34 antibodies. The cells were washed and resuspended in an appropriate volume of PBS for magnetic separation. Using MS separation columns (Miltenyi Biotec, cat. No. 130-042-201) held in a MACS magnet, the cell suspension was loaded onto the column. $CD34^+$ cells were retained in the column, while the unlabeled cells passed through the column following elution with PBS and were collected as the CD34 depleted fraction (CD34d). $CD34^+$ cells were expelled using a plunger following removal of the column from the magnet by elution with PBS as CD34 enriched fraction (CD34e). The recovered cells from the column were used as the CD34 enriched fraction (CD34e). With respect to the enriched $CD34^+$ or $CD133^+$ population, CD34-Microbeads and CD133-Microbeads were added into the MNC together at the concentration of 100 μl CD34/133-Microbeads/10^8 cells. Other procedures using MACS are the same as the way of isolating $CD34^+$ population. If required, CD34e cells were frozen using 10% DMSO in IMDM with 10 % FCS and stored at liquid nitrogen.

2.2.3 Mesenchymal stem cells (MSC) isolation

MSC isolation from umbilical cord

After washing several times using PBS, the small fragment of umbilical cord was diced into 1 mm^3 pieces, and then collect all small pieces into a conical tube. Add collagenase A (Roche Applied Science, cat. No. 10103578001) at the final concentration of 1 mg/ml, and incubate at 37 °C, 5% CO_2 for 2 hours, followed by centrifugation at 300×g for 5 minutes. Repeat washing remnants until no more rubbish washed out. Finally resuspend remnants in EGM-2 (Lonza, cat. No. CC-3162) in T25 flask coated with collagen I (Life technologies, cat. No. A10644-01) or gelatine, and incubate the flask at 37 °C, 5% CO_2 overnight. Check whether cells have stuck down onto the flask under the microscope. Change the medium every other day. When the cells reach 80% confluency, passage cells, and culture in fresh MSC medium containing Dulbecco's Modified Eagle Medium, high glucose (DMEM, high glucose, Life Technologies, cat. No. 10566-016) + 10% FCS + 1% MEM Non-essential Amino Acid Solution (NEAA, Sigma-Aldrich, cat. No. M-7145) + 1% pyruvate (Sigma-Aldrich, cat. No. S8636) + 1% penicillin/streptomycin (P/S, Sigma-Aldrich, cat. No. P4333) with or without 4 ng/ml basic fibroblast factor (bFGF, Peprotech, cat. No. 100-18B).

MSC isolation from fat tissues or lipoaspirate

After collecting fat tissues from patients, mince the tissue into very fine pieces using cheese grater and transfer all small pieces into a conical tube. If lipoaspirate, the step is not necessary. Centrifuge the tube at 300×g for 5 minutes. After spinning, the tissue of interest will be between blood at the bottom and some liquid fat at the top of the tube. Transfer the middle layer to a new 50 ml tube

with a 1 ml tip and resuspend the mince to a final volume of 30 ml. Add the same volume of digestion solution made up of DMEM with 1/10 35% BSA and 1/10 10 mg/ml collagenase II (Life technologies, cat. No. 17101-015) into the fat mince mixture or lipoaspirate mixture, and shake well. Transfer the tube to the shaking water bath at 37 °C for 30 minutes. After incubation, centrifuge the tube at 300×g for 5 minutes. After aspirating the supernatant, resuspend the pellet in 25 ml fresh MSC medium containing DMEM, high glucose + 10% FCS + 1% NEAA + 1% pyruvate + 1% P/S within a T75 flask at 37 °C, 5% CO_2 overnight. After 48 hours, check whether cells have stuck down onto the flask under the microscope. Change half of the medium every other day. When the cells reach 80%-90% confluency, passage cells at 1: 3 ratio.

2.2.4 FACS sorting of different populations

MACS enriched $CD34^+$ or $CD133^+$ cells were suspended in 100 μl PBS and were directly stained using 5 μl anti-human monoclonal antibodies (MAbs) conjugated to fluorescein isothiocynate (FITC), phycoerythrin (PE), peridin chlorophylla protein (PerCP) or allophycocyanin (APC) for 30 minutes at 4 °C in the dark. Samples were centrifuged at 300×g for 5 minutes and washed once with PBS. Appropriate antibody combination controls using $CD34^-$ cells were used to set selection gates for FACS sorting. Cells were sorted using a FACSAria 2 (Becton Dickinson, UK) equipped with with 448 nm, 633 nm and 405 nm lasers. Dead cells and debris were excluded using FSC/SSC gating before setting sorting gates for further collection of target cell populations. In chapter 3, we used mouse anti-human MAbs conjugated to CD34 PE and CD133 PerCP to sort $CD34^+CD133^-$, $CD34^+CD133^+$, $CD34^-CD133^+$ subpopulations.

2.3 Cell culture

2.3.1 HSC cells *ex vivo* culture

In chapter 4, human mobilised blood CD34e cells (10^5 cells/ml) were cultured in basic medium consisting of IMDM supplemented with 10% FCS and 1% P/S with cytokines. Recombinant human (rhu) stem cell factor (SCF, 100 ng/ml; Peprotech, cat. No. 300-07), Fms-related tyrosine kinase 3 ligand (Flt3L, 10 ng/ml; Peprotech, cat. No. 300-19), and Interleukin-3 (IL-3, 20 ng/ml; Peprotech, cat. No. 200-03) were employed in the following combinations: SCF+Flt3L (SF) or SCF+Flt3L+IL3 (SF+IL3). Valproic acid (VPA, 1 mM; Sigma-Aldrich, cat. No. P4543) and 5-Aza-2-deoxycytidine (5-Aza, 1 μM; Sigma-Aldrich, cat. No. 2353-33-5) were added into the culture medium (see table 2.1). Cells in the media were incubated at 37 °C in humidified atmosphere containing 5% CO_2 for eight days. After eight days, the nucleated cell number, immunophenotype, and colony-forming unit assay were carried out. Cell number was counted by hemocytometer and expressed as fold increase.
In chapter 6, CD34e cells and mononuclear cells have been cultured in Stempro® CD34+ medium (Life technologies, cat. No. A14059) supplemented with 100 ng/ml SCF, 10 ng/ml Flt3L, and 20 ng/ml IL3 for several days before reprogramming. The culture time period depends on the proliferation status of cells.

2.3.2 Haematopoietic cell line culture

K562 (ATCC, cat. No. CCL-243™) and U937 (ATCC, cat. No. CRL-1593.2™) cell lines were cultured at a density of 5×10^5 cells per ml in IMDM + 10% FCS

SCF+Flt3L	SCF+Flt3L+IL3
SCF+Flt3L+VPA	SCF+Flt3L+IL3+VPA
SCF+Flt3L+5-Aza	SCF+Flt3L+IL3+5-Aza

Table 2.1: The different combinations of drugs supplemented in basic medium, which is made up of IMDM + 10% FCS + 1% P/S.

+ 1% P/S at 37 °C in humidified atmosphere containing 5% CO_2. A stock solution of 5 mmol/L hemin was made as the following steps: 32.5 mg hemin bovine (SIGMA, H9039-1G) was dissolved in 0.5mL of 1 M NaOH (Riedel-deHaen, cat. No. 1310-73-2) for around 30 minutes; and then 10 ml of Glasgow Minimum Essential Medium (GMEM, Sigma-Aldrich, cat. No. G6148) with 10% bovine serum albumin (BSA, Sigma-Aldrich, cat. No. 9048-46-8) was added following the addition of 0.5 ml of 0.5 M Tris base (Pharmacia Biotech, code No. 17-1321-01). Finally the mixture was neutralized with 0.5 ml of 1 M HCl (Sigma, cat. No. 32-0331), filtered through a 0.22 μm filter and stored a 4 °C for more than a week. If required, hemin was applied in the culturing medium for four days at the final concentration 30 μM. After transfection, cells were culture for 3 days and then harvested for assessments.

2.3.3 MSC culture

MSC basic culture

Fifty to eighty thousand MSC cells derived from human umbilical cord were seeded into 12-well plates coated with matrigel and incubated in MSC medium made up of DMEM (high glucose) + 10% FCS + 1% NEAA + 1% pyruvate + 1% P/S,

at 37 °C, 5% CO_2. When cells reached 80-90% confluency, cells were passaged using TrypLE™ Express Enzyme (Life technologies, cat. No. 12604-021) at 37 °C until cells cells have detached. Subsequently, add 1 ml of pre-warmed MSC medium, transfer cells in conical tube for centrifugation. After determine viable cell density, subculture cells in appropriate format.

MSC bone differentiation

MSC bone differentiation method is two-step process. Cells were induced for 7 days followed by 21 days' culture in bone differentiation medium. When MSC cells reached 70-80% confluency, culture cells in osteocytes induction medium consisting of IMDM + 1% Glutamine + 5% FCS + 1% P/S supplemented with 10 nM Dexamethasone (Sigma-Aldrich, cat. No. D4902) and 50 μM Ascorbic acid-2-phosphate magnesium salt (Acros Organics, cat. No. 84309-23-9). After 7 days' induction culture, the induction medium was replaced with bone differentiation medium consisting of IMDM + 1% Glutamine + 5% FCS + 1% P/S with addition of 10 nM Dexamethasone, 50 μM Ascorbic acid-2-phosphate magnesium salt and 3.5 mM ß-glycerophosphate (Sigma-Aldrich, cat. No. G-9891). Keep replacing half of medium every second day. The cells were stained with Alizarin Red S (Sigma-Aldrich, cat. No. A5533) separately on day 0, day 7, day 14, day 21 after culturing in bone differentiation medium.

MSC fat differentiation

When MSC cells reached 70-80% confluency, cells were cultured in adipocytes differentiation medium (MesenCult® MSC Basal Medium, Stemcell technologies,

cat. No. 05401; MesenCult® Adipogenic Stimulatory Supplements, Stemcell technologies, cat. No.05403) for 14 days. Change fresh medium every three days. The cells were stained with Sudan Black B staining system (Sigma-Aldrich, cat. No. 021K6086) separately on day 0, day 7, and day 14.

2.3.4 iPS culture

Established iPS cell lines were maintained in 6-well plates in 2 ml of Essential 8™ Medium (E8 medium, Life technologies, cat. No. A1517001) and the medium was replaced every day. At 70-90 % confluency, iPS cells were passaged. Spent medium was discarded, and cells were rinsed twice with PBS before adding of 1 ml of 0.5 mM EDTA in PBS. After incubation at 37 °C for 3-5 minutes, EDTA was replaced with 2 ml of pre-warmed E8 medium and colonies were broke up into small pieces using a 5 ml glass pipette. Cells were replated at a 1:3 ratio in matrigel-coated plates.

2.4 Reprogramming procedures

2.4.1 Generation of iPS cells from MSC cells by mRNA transfection and small molecules

50, 000-80, 000 MSC cells were seeded into one well from 12-well plates coated with matrigel and incubated in MSC medium at 37 ℃, 5% CO_2. When cells grew to 70-80% confluency, cells were transfected with GFP mRNA, Oct4 mRNA, (Oct4+ Sox2) mRNA or OKSML mRNA mix (Stemgent; Oct4 mRNA cat. No. 05-0014, Klf4 mRNA cat. No. 05-0015, Sox2 mRNA cat. No. 05-0016, Lin28

mRNA cat. No. 05-0017, c-Myc mRNA cat. No. 05-0018, and nGFP mRNA cat. No. 05-001) using TransIT®-mRNA Transfection Kit (Mirus, cat. No. MIR 2250) respectively. The mRNA mix was bought from Stemgent company and prepared as a molar stoichiometry of 3:1:1:1:1:1 for the OKSML and nGFP mRNAs. Alongside the mRNA mixture, our lab synthesized Oct4 and Sox2 mRNA by ourselves (see more details in 2.15). After 4-6 hours post transfection, the medium was replaced with fresh MSC medium with addition of small molecules, CHIR99021 (3 μM; Selleck, cat. No. S1263), tranylcypromine (5 μM; Sigma-Aldrich, cat. No. P8511), and ascorbic acid (vitamin C, 10 μg/ml; Sigma-Aldrich, cat. No. A4403), bFGF (10-100 ng/ml), VPA (1 mM) with or without 616452 (Repsox2, 1 μM; Sigma-Aldrich, cat. No. 446859-33-2). During the eight days' transfection, MSC medium was replaced by ES medium gradually before every transfection (Day1-2, 1/4 ES medium; day 3-4, 1/2 ES medium; day 6-7, 3/4 ES medium; day8, ES medium). After eight rounds of transfections, the medium with small molecules was replaced every four days. The iPS-like colonies were picked on day 20 to day 30 after mRNA transfection and maintained in the culture condition for hES cells. The medium for culturing iPS-like colonies was changed every day.

2.4.2 Generation of iPS cells from fibroblast cells by episomal reprogramming vectors

Plate 200, 000 human dermal fibroblast cells (Lonza, cat. No. CC-2511) in 2 ml MSC medium per well in a 6-well plate 18-24 hours before transfection. When cell confluency reached 40-80%, cells were harvested and nucleofected with episomal iPS reprogramming vectors containing Oct4, Sox2, Nanog, Lin28, Klf4, and L-Myc (Invitrogen, cat. No. A14703) or Epi5^{TM} episomal vectors (Life technologies, cat. No. A15960) with a Nucleofector 4D device (lonza, USA) using an Amaxa

P2 Primary Cell 4D Nucleofector® X Kit, program DT-130. Subsequently, 10^5 transfected cells were immediately seeded in one well of BD Matrigel Matrix basement membrane (matrigel, BD Bioscience, cat. No. 356237) coated 6-well plates in MSC medium without antibiotics. On the second day post transfection, replace the medium with N2B27 medium consisting of DMEM/F-12 (Life technologies, cat. No. 11330-032) + 1% N-2 Supplement (Life technologies, cat. No. 17502-048) + 2% B-27 Supplement (Life technologies, cat. No. 17504-044) + 1%NEAA + 1% Glutamine + 0.1% ß-Mercaptoethanol (Life technologies, cat. No. 21985-023) with addition of 100 ng/ml bFGF or CHALP including CHIR99021 (3 μM), PD0325901 (0.5 μM; Sigma-Aldrich, cat. No. PZ0162-5MG), hLIF (Human leukemia inhibitor factor, 10ng/ml; Peprotech, cat. No. 300-05), 616452 (Repsox2, 1μM), HA-100 (10 μM; Santa Cruz Biotechnology, cat. No. 84468-24-6), and bFGF (100 ng/ml). Replace the medium every other day until day 15. After that, replace with E8 Medium every other day until iPS colonies appear and grow to an appropriate size for transfer. The iPS-like colonies were picked using 200 μl pipette at around day 15 to day 20 after transfection and maintained in E8 medium. Replace E8 medium every day until passage.

2.4.3 Generation of iPS cells from blood CD34 enriched cells or mononuclear cells by episomal reprogramming vectors

CD34 enriched cells from human cord blood or mobilised blood were isolated as described in 2.2.2 by MACS and cultured at the density of 10^5-3×10^5 in HSPC expansion medium comprising StemPro® 34 SFM (Life technologies; cat. No. 10639-011) + recombinant human (rhu) SCF (100 ng/ml) + Flt3L (10 ng/ml) + IL-3 (20 ng/ml) for several days before transfection. 10^6 actively proliferating,

expanded $CD34^+$ cells from CB or mPB were nucleofected with 3-4 μg episomal vectors using an Amaxa P3 Primary Cell 4D Nucleofector® X Kit and a Nucleofector 4D device using, program E0-100. Following addition of pre-warmed expansion medium cells were immediately replated and cultured under three conditions. (1) 10^5-2×10^5 electroporated cells were immediately seeded onto matrigel-coated 6-well plate directly in 1.5 ml expansion medium without antibiotics on day 0 followed by adding 0.5ml of fresh N2B27 medium with addition of 100 ng/ml bFGF on day 1. From day 2 to day 6, 0.5-1 ml of spent medium was replaced with N2B27 medium with addition of 100 ng/ml bFGF without disturbing the attaching cells every other day until day 7. From day 7 to day 9, all medium was replaced with N2B27 medium supplemented with 100 ng/ml bFGF every day. After day 9, all spent medium was replaced with E8 medium. (2) 3×10^5 electroporated cells were cultured in expansion medium in 24 well plate in plastic without any feeder for three days. (3) 3×10^5 electroporated cells were immediately replated in 0.8 ml expansion medium onto irradiated human bone marrow stromal cell feeders (BMSC, a gift from Orthopedics group in Center for Regenerative Medicine of University of Edinburgh) in a 12-well plate. For the second and third treatment, after three days, transfected cells cultured in 24-well plates with/without BMSC feeder (the second and the third treatment) were harvested and every 10^5 cells were replated onto matrigel-coated 6-well plates in 1.5 ml expansion medium without antibiotics. Then the following steps were the same as the first treatment. Colonies appeared day 8 after seeding onto matrigel-coated plates and grew large enough for picking after another 3-5 days. The reprogramming procedure used for MNC was similar to that for CD34 enriched cells, but slightly different. The electroporation program is EN-138. 10^6 thawed or fresh MNC were expanded in 0.5 ml HSPC expansion medium for nearly 10 days until cells were in active proliferation status. After nucleofection, the time to replace N2B27 medium with E8 medium was delayed to day 11-12.

2.5 Preparation of MEF- Conditioned Medium (MEF-CM)

4×10^6 irradiated MEF were plated in a T75 Flask coated with 0.5% gelatin, in complete MEF medium consisting of DMDM (4.5 g/L glucose) + 10%FCS + 2 mM L-glutamine + 1%NEAA + 1%pyruvate + 1%P/S. On the following day, the MEF medium was replaced with 37.5 ml KnockOut medium with addition of 4 ng/ml bFGF, and incubated for 24 hours at 37 ℃, 5% CO_2. After 24 hours MEF-CM from the flasks were collected and sterilized by 0.22 μM filter. Considering depletion of L-Glutamine and bFGF from the MEF-CM, L-Glutamine (to 2 mM final), and bFGF (to 4 ng/ml final concentration) were added back prior to using. MEF-CM medium was aliquoted and frozen at -20 ℃.

2.6 *In vitro* differentiation of iPS cells into three germ layer cells

Around 2×10^5 iPS cells were cultured in E8 medium until 50% confluency followed by replacing medium with ectoderm, endoderm, and mesoderm differentiation medium respectively (R&D systems, cat. No. SC027). The time for differentiation to each of the three germ layers is different. For ectoderm differentiation, ectoderm medium was changed every day until day 4, when cells were ready for staining with ectoderm marker Otx2. For mesoderm differentiation, medium was replaced every 12-16 hours in first 24 hours, then in the following 12 hours, cells were fixed and staining with brachyury antibody. For endoderm differentiation, endoderm media 1 was used on day 1, replaced with endoderm media 2 from day 2 to day 4, and characterized on day 4 by staining with Sox17 antibody.

2.7 Plasmids construction

GFP fragment was cut from pcDNA 3.1 EGFP-poly(A83) and ligated Oct4 or Sox2 gene into the digested vector to produce pcDNA 3.1 Oct4-poly(A83) or pcDNA 3.1 Sox2-poly(A83) plasmids. Sox2 insert gene was generated using PCR technique from the template pOBT7-Sox2 (clone 2823424) and Oct4 gene was cut from pCR4TOPO-Oct4 (clone 40125986).

Sox2 gene was produced using 5'-AAGCTTCAGCGCCCGCATGTACAACATGA-3' forward primer and 5'-GAATTCAGTTCGCTGTCCGGCCCTCA-3' reverse primer, applying AmpliTaq Gold® Fast PCR Master Mix (Life technologies, cat. No. 4390939) according to the manufacturer's protocol. PCR was run on Unocycler (VWRc1732-1200) in the condition of 95 °C 15 minutes followed by 40 cycles of 94 °C 30 seconds, 58 °C 30 seconds and 72 °C 40 seconds. The vector pcDNA 3.1 EGFP-poly(A83) was digested using restriction enzyme HindIII and EcoRI at 37 °C for 1-2 hour. Subsequently, the digested vectors were examined on 1% agarose gel, and the desired DNA fragments were cut out followed by the purification using peqGold Gel Extraction Kit (Peqlab, cat. No. 12-2501-02). Then the plasmid and inserted gene digested with restriction enzymes HindIII and EcoRI were ligated at 16 °C for 15 min - 24 hours. DNA from ligation reactions was diluted by 5 fold in 10 mM Tris-HCl (pH7.5) and 1 mM EDTA, before adding 1 μl of the diluted ligation mix (1-10 ng DNA) to the competent cells. After leaving the cells on ice for 30 minutes, cells were heat-shock at 42 °C for 45 seconds without shaking followed by incubation on ice for 2 minutes. Then 0.9 ml of room temperature S. O. C. medium (Invitrogen, cat. No. 15544-034) without antibiotics was added into the mix followed by shaking at 225 rpm for 45 min-1 hour at 37 °C for transformation to develop antibiotic resistance. Simultaneously, 100 μl of the diluted the control plasmid DNA 1:100 with S. O. C. medium was spread on LB agar plate with antibiotics and incubated at 37 °C overnight to allow plasmid replication. On the second day, colonies were picked

from the transformation plate with autoclaved pipette tips and inoculated in the corresponding media followed by shaking at 37 °C overnight. On the next day, spin down 3 ml of culture and proceed with plasmid miniprep kit (Quiagen, cat. No. 27106). After plasmid isolation, clone was examined by restriction digestion and electrophoresis. Finally sequence was verified by addgene company.

2.8 Isolation of total RNA

Total RNA was isolated from targeted cells using Rneasy mini RNA Kit (Qiagen, cat. No. 74104). Cells of interest were harvested, centrifuged, and lysed by 350 μl RLT buffer. After adding the same volume of 70% ethanol, the mixture was immediately transferred to Rneasy mini spin column followed by centrifugation at the speed of $> 8000\times$g for 15 sec. Subsequently, spin column was washed using 700 μl RW1 buffer, followed by twice wash with RPE buffer. Then centrifuge the column in a new microcentrifuge to allow ethanol to evaporate. Finally centrifuge the column at the speed of $> 8000\times$g for 1 min following the addition of 33 μl nuclease-free water to elute RNA into a nuclease-free tube. Measure RNA concentration using Nanophotometer (Implen, UK).

2.9 Synthesis of cDNA

cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystem, cat. No. 4368814). In a sterile RNase-free microcentrifuge tube, dilute 5 μg high quality RNA in a total volume of 50 μl nuclease-free water (1:10) and then add 10 μl of diluted RNA to 10 μl of master mix. The master mix was

prepared on ice as following:

10× Reaction Buffer	2 μl
dNTP Mix	0.8 μl
Random primers	2 μl
Reverse transcriptase	1 μl
Nuclease-free water	up to 10 μl

The total reaction system volume is 20 μl.

The cDNA synthesis program we used is as following on Applied Biosystem® thermal cyclers:

	Stage1	Stage2	Stage3	Stage4
Temperature(°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

After reaction, measure the concentration of cDNA for downstream application.

2.10 Quantitative polymerase chain reaction (qPCR)

The High Capacity cDNA Reverse Transcription kit (Applied Biosystem, cat. No. 4368814) was used to convert 1μg of RNA to cDNA. The quantitative validation of the expression of selected genes was performed by QRT-PCR (Applied Biosystems

StepOne Real-Time PCR Systems) using custom PrimerDesign primers and applying the Sybre Green PCR master mix (Applied Biosystem, cat. No. 4368577), following the manufacturer's protocol. The geNormPLUS 12 gene kit (Primerdesign, cat. No. gePLUS-SY-12) is used for normalisation. Reactions were run in triplicate on a StepOne Plus instrument (Applied Biosystems, UK). Running conditions were 95 °C 10 minutes followed by 40 cycles of 95 °C 15 seconds and 60 °C 60 seconds. Amplification was performed for each cDNA (20 ng) sample in triplicate. qPCR data were presented as ΔCt value in gene expression normalized to ACTB and TOP1 or fold change ($2^{-\Delta\Delta Ct}$) compared to somatic cells controls (fibroblasts and $CD34^{+}$).

Master mix	10 μl
Reconstitued primer/probe	1 μl
20 ng cDNA	1.5 μl
Nuclease-free water	7.5 μl

The total reaction system volume is 20 μl.

The primers used in qPCR were listed in the following table.

Oct4	Sense Primer	CACTAAGGAAGGAATTGGGAACA
	Anti-sense Primer	GGGATTAAAATCAAGAGCATCATTG
Sox2	Sense Primer	GGAGAGTAAGAAACAGCATGGA
	Anti-sense Primer	TTTGCGTGAGTGTGGATGG
Nanog	Sense Primer	GCTGTGTGTACTCAATGATAGATTT
	Anti-sense Primer	GAGGTTCAGGATGTTGGAGAG

2.11 Digestion of pcDNA3.1 EGFP-poly(A83), pcDN3.1 Oct4-poly(A83) and pcDN3.1 Sox2-poly(A83)

pcDNA3.1 EGFP-poly(A83), pcDNA3.1 Oct4-poly(A83) and pcDNA3.1 Sox2-poly(A83) were digested using Xba I enzyme (Promega, cat. No. C-R6181-X) to obtain the linear cDNA. The reaction system is as follows

RE 10× Buffer	10 μl
XbaI enzyme	10 μl
pcDNA3.1 EGFP/ Oct4/ Sox2 -poly(A83)	15 ng
Acetylated BSA, 10 $\mu g/\mu l$	1 μl
DEPC water	to100 μl

In a sterile PCR tube, add RE 10× buffer, pcDNA, Acetylated BSA and DEPC water and mix by pipetting. Then add 10 μl XbaI enzyme into the mixture. Mix gently and microcentrifuge for several seconds to keep all mixture at the bottom of the tube. Finally keep the reaction system at 37 °C for two hours.

2.12 Purification of plasmid DNA

The plasmid DNA was purified using peqGold Gel Extraction Kit (Peqlab, cat. No. 12-2501-02). Transfer the reaction to a clean 1.5 ml microcentrifuge tube, and add another three volumes binding buffer, followed by 1 volume DEPC water. Mix gently and microcentrifuge the sample at 10000×g for 1 minute. After

discarding the liquid, add 750 μl of CG wash buffer diluted with ethanol followed by centrifugation at 10000×g for 1 minute. Repeat wash and remove the flow-through liquid. Subsequently, dry the column matrix by centrifuge at 10000×g for 1 minute. At last, 33 μl elution buffer was applied to elute DNA. Then the DNA concentration was measured by Nanophotometer.

2.13 Agarose gel electrophoresis

2.13.1 Pouring a standard 1% agarose gel

0.5 g agarose (Sigma-Aldrich, cat. No. 15510-027) was dissolved into 50 ml of TBE buffer in a conical beaker followed by the addition of 4 μl SYBR® Safe DNA gel stain (Invitrogen, cat. No. S33102) into the cool agarose. Then the agarose liquid was poured into a gel tray with the well comb in place, leaving at room temperature for at least half an hour. Finally the comb was removed until the agarose gel has completely solidified.

2.13.2 Loading samples and running an agarose gel

The agarose gel was placed into electrophoresis unit filled with TBE buffer.
For RNA agarose gel analysis,1 μg RNA was diluted into 10 μl of RNase free water with addition of 5 μl of RNA loading buffer. Then the mixture was incubated at 70 °C for 5 minutes followed by chill on ice for two minutes before loading on the gel. For DNA agarose gel analysis, 0.5-1 μg of purified DNA was diluted into 10 μl of DEPC water with 2 μl of loading dye. Then a molecular weight ladder

and the sample were loaded into the wells of agarose gel, running at 75-100 v until the dye front is approximately 70-80% of the way down the gel. Finally, DNA or RNA fragments were visualized under any device with UV light.

2.14 mRNA synthesis using mMESSAGE mMACHINE® T7 kit-high yield capped RNA transcription kit

When the frozen reagents were thawed, all reagents were microcentrifuged briefly before opening to prevent loss and/or contamination of material present around the rim of the tube. Then transcription reaction was assembled at room temperature as following

Nuclease-free Water	to 20 μl
T7 2× NTP/ARCA	10 μl
10× T7 Reaction Buffer	2 μl
linearised template DNA	1 μg

The reaction was mixed completely and incubated at 37 °C for one hour and then TURBO Dnase (Ambion, cat. No. AM2238) was added to remove the remaining DNA in the reaction. Following this, mRNA was purified by lithium chloride precipitation and measured using Nanophotometer.

2.15 Transfection of mRNA and plasmids

2.15.1 mRNA transfection using Mirus TransIT®-mRNA Transfection Kit

At 70-80% confluency, MSCs were begun to be transfected with mRNA using Mirus TransIT®-mRNA Transfection Kit (Mirus, cat. No. MIR 2255). 1 μg mRNA (about 1 $\mu g/\mu l$) was diluted into 100 μl of Opti-MEM® I Reduced Serum medium (OptiMEM, Life technologies, cat. No. 11058-021) followed by the addition of 2 μl mRNA boost reagent. Then 2 μl of TransIT-mRNA Transfection was added into the mixture and incubated at room temperature for 2-5 minutes to form complex. Subsequently the complexes was added into wells by drop-wise and the plate was rocked to distribute evenly complexes over the cells, followed by incubation at 37 ℃, 5% CO_2 overnight.

2.15.2 Transfection of plasmids using TransIT®-2020 reagent

When MSCs reached 40-80% confluency, cells were ready for transfection with plasmid using TransIT®-2020 reagent (Mirus, cat. No. MIR 5400). 2.5 μl of plasmid DNA (about 1 $\mu g/\mu l$) was diluted into 250 μl of OptiMEM I Reduced-Serum medium followed by the addition of 7.5 μl TransIT®-2020 reagent, and incubated at room temperature for 15-30 minutes to form complex. Then the complexes was added into wells by drop-wise and the plate was rocked to distribute evenly complexes over the cells, followed by incubation at 37 ℃, 5% CO_2 overnight.

2.15.3 Transfection of plasmids using nucleofector 4D

P2 primary cell kit (Lonza, cat. No. V4XP-2032) and P3 primary cell kit (Lonza, cat. No. V4XP-3012) were used for nucleofecting plasmids into cells. An appropriate number of cells were resuspended in 20 μl or 100 μl of transfection solution with supplement in a sterile tube, followed by adding 1 μg or 4 μg plasmid DNA (about 1 $\mu g/\mu l$).Then cells were transferred into the strips or cuvettes from the kit and electroporated using the recommended program for human dermal fibroblasts, CD34 enriched cells or mononuclear cells. After electroporation, cells were resuspended in warm appropriate medium and incubated in appropriate wells at 37 ℃, 5% CO_2 overnight.

2.16 Flow cytometry and analysis

Flow cytometry was used to measure the phenotype and frequency of cell populations of interest. Six-parameter four-colour analysis using 488nm and 633nm lasers for excitation employed fluorescein isothiocynate (FITC); phycoerythrin (PE); peridin chlorophylla protein (PerCP) and allophycocyanin (APC) fluorochrome antibody conjugates, with forward-scatter (FSC) and side-scatter (SSC) for acquisition of data. Cells were simultaneously stained with up to four MAbs, each conjugated with one of the four fluorochromes- FITC, PE, PerCP and APC (See table 2.2 for antibodies used).

Unstained samples were used in comparison to stained samples to set boundaries (regions, gates) for determining positive staining (see more details in 3.1). In general, for surface marker staining, 100 μl of sample in PBS (supplemented

with 0.1% BSA and 0.1% azide) was incubated with an optimal concentration of appropriate antibodies for 20-30 minutes at 4 °C in the dark. Cells were washed once with 2 ml supplemented PBS and finally resuspended in 300 μl PBS. Data for all samples was acquired on the same day of staining using a FACS Calibur flow cytometer linked to an Apple Macintosh computer equipped with CellQuest software (Becton Dickinson), pre-calibrated and compensated with Becton Dickson calibration beads and software.

For cytoplasmic staining, cells have to be fixed using 2% paraformaldehyde (PFA) on ice for 20 minutes and permeabilised in 0.1% saponin (Sigma-Aldrich, cat. No. S7900) at room temperature for 20 minutes, followed by incubation with antibodies in PBS supplemented with 0.1% saponin and 0.1% BSA. All incubation steps are required to be manipulated in 0.1% saponin in cytoplasmic staining. The following steps are the same as surface staining. The number of events acquired for each sample was MNC 50000, CD34 depleted cells 50000, CD34 enriched cells 5000-10000, CFU 20000-50000, iPS cells 20000. Data was transferred to a PC (Microsoft Windows) computer for analysis using FCS Express software (De Novo Software, www.denovosoftware.com). The antibodies used and combinations used are shown in table 2.2.

2.17 *In vitro* colony assay

Methylcellulose based culture media supplemented with cytokines for colony-forming unit assay is the standard method for enumeration and assessment of blood stem and progenitor cells. An appropriate number of the cells to be tested were mixed in tissue culture medium made up in viscous methylcellulose which does not allow the cells to disperse – so that any cells proliferating in response

anti-human Ab	isotype	fluorochrome	supplier	cat. No.
CD36	IgM	FITC	eBioscience	11-0369-41
CD38	IgG1, κ	FITC	BD Biosciences	560982
Oct4	IgG2a, κ	AF488	eBioscience	53-5841
SSEA4	IgG3	AF488	eBioscience	53-8843
CD235a	IgG2b, κ	PE	eBioscience	12-9987
CD133	IgG1	PE	Miltenyi Biotec	130-098-826
TRA-1-81	IgM, κ	PE	BD Bioscience	560885
CD34	IgG1, κ	PerCP	BD Biosciences	345803
CD34	IgG1, κ	PerCP-eFluor® 710	eBioscience	9046-0349
CD133	IgG1	APC	Miltenyi Biotec	130-098-829
CD45	IgG1, κ	APC	BD Biosciences	555485
TRA-1-60R	IgM, κ	AF647	Biolegend	330605
SSEA3	IgM, κ	AF647	Biolegend	330307

Table 2.2: Antibodies used in flow cytometry

to the cytokine stimulants remain localised and form a colony. StemMACS HSC-CFU complete with Epo (Miltenyi Biotec, cat. No. 130-091-280) containing IMDM, 2 mM L-Glutamine, 1% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M β-mercaptoethanol, 50 ng/ml SCF, 20 ng/ml granulocyte macrophage CSF (GM-CSF), 20 ng/ml G-CSF, 20 ng/ml IL-3, 20 ng/ml IL-6, 3 U/ml erythropoietin (EPO) was used to evaluate clonogenic growth of BFU-E, CFU-GEMM, CFU-G, CFU-M, and CFU-GM. An appropriate number of cells were suspended in 0.1 ml IMDM with 2% FCS, mixed thoroughly with 1 ml HSC-CFU-Complete medium and then plated in 6-well plates using a 1 ml syringe with a blunt-end needle. The number of plated cells depended on the

fraction of different cells. We obtained appropriate colony frequencies by plating 1000 $CD34^+$ HSCs following MACS separation. After 12-14 days, colonies (>50 cells) were scored according to their appearance under ×4 lens, inverted microscope (Wilovert S, Helmut Hund GmbH, Germany).

2.18 iPS colonies live staining

Remove the iPS colonies medium from 24-well plate and add 250 μl fresh hES medium. Add 3 μl anti-human SSEA-4 Alexa Fluor® 488 Ab (eBioscience, cat. No. MC-813-70) into the colonies medium and incubate at 37 ℃, 5% CO_2 for 0.5 hour. Remove medium and wash twice with PBS. Finally replace PBS with 500 μl OptiMEM for further observation under the fluorescent microscope. After taking photographs, replace with ES culture medium immediately and return the plate into incubator.

2.19 *In situ* immunofluorescence staining

Cells were cultured in chamber slides or 24 well plates as required. After washing twice in PBS, cells were fixed using 4% PFA for 15 minutes followed by washing three times in PBS for 5 minutes. Subsequently, permeabilise cells with 100 μl of 0.5% Triton X-100 for 3 minutes and then wash twice with PBS. After blocking cells for 1 hour in blocking buffer which is made up of PBS supplemented with 0.3% Triton X-100 and 5-10% normal serum from the same species as the secondary antibodies, incubate with primary antibodies in PBS in addition with 1% BSA and 0.3% Triton X-100 overnight at 4 °C. Then cells were washed three

times in PBS for 5 minutes and incubated for 1-2 hours at room temperature, in the dark , with fluorochrome conjugated secondary antibodies diluted in the same buffer as primary antibodies. After washing three times in PBS for 5 minutes, cells were stained with DAPI for 10 minutes. Finally, mount the slide using an anti-fading agent and coverslips, allowing to dry overnight on the bench. On the second day, slides were ready for microscopy after sealing the edges of the cover slips with nail polish. If 24-well plate, add 0.5 ml PBS for microscopy. All antibodies used are shown in table 2.3.

Ab types	species	supplier	cat. No.
anti-human Oct4 primary Ab	rat	eBioscience	14-5841-82
anti-human Nanog primary Ab	rabbit	Santa Cruz	sc-33759
anti-human SSEA4 primary Ab	mouse	Santa Cruz	sc-21704
anti-human SSEA3 primary Ab	rat	Santa Cruz	sc-73066
anti-human tra-1-81primary Ab	mouse	Santa Cruz	sc-21706
anti-human tra-1-60 primary Ab	mouse	Millipore	MAB4360
anti-human Otx2 primary Ab	goat	R&D systems	SC027
anti-human Brachyury primary Ab	goat	R&D systems	SC027
anti-human Sox17 primary Ab	goat	R&D systems	SC027
anti-rat AF555 secondary Ab	goat	Invitrogen	A21434
anti-rabbit AF594 secondary Ab	goat	Invitrogen	A11037
anti-mouse AF488 secondary Ab	goat	Invitrogen	A11017
anti-goat AF594 secondary Ab	donkey	Invitrogen	A11058

Table 2.3: Antibodies used in in situ immunofluorescence staining.

2.20 Statistical analysis

The results were reported as mean ± SD unless otherwise stated. Student's paired t test or analysis of variance (ANOVA) was used to test the probability of significant differences between samples when their variances were identical, otherwise nonparametric test was utilized. P value < 0.05 were considered significant. All statistics was analysed using GraphPad Prism 6.

Chapter 3

The selection of HSPC based on CD34 and CD133 markers affects haematopoietic functional heterogeneity

3.1 Introduction

HSCs have self-renewal ability and multiple potential to differentiate to generate all blood cell lineages throughout the lifespan of an individual, and they are responsible for the long-term multilineage reconstitution. However, haematopoietic progenitor cells (HPCs), unlike HSCs, can produce some blood cell lineages but not all within the defined culture conditions.

With the wide application of flow cytometry in examining the expression of various surface markers on different cells, people realised that anti-CD34 Ab is an excellent

marker to adequately define and quantify HSPC for clinical use (Sutherland and Keating, 1992). In addition, CD133, as an alternative marker, has been used extensively for clinical and research use. However, the expression of CD34 and CD133 on HSPCs is not permanent, which has been mentioned in chapter1. In addition, according to the recent publication, $Lin^{-}CD34^{-}CD38^{-}CD93^{hi}$ population might be at the top of the HSPC hierarchy. Moreover, CD34 or CD133 are also expressed on other types of cells (Corbeil *et al.*, 2000; Gill *et al.*, 2001b; Hilbe *et al.*, 2004; Murohara *et al.*, 2000; Padfield *et al.*, 2010; Peichev *et al.*, 2000; **?**; Uchida *et al.*, 2000).

The functional relevance of CD34 or CD133 is not understood, and their use is largely empirical. While there may be little practical difference in which of these is used to characterise HSPC for haematopoiesis, we are aware that they are not contiguously or homogeneously expressed by all HSPC (Tura *et al.*, 2007a). In this chapter we will explore how the selection of HSPC based on markers CD34 and CD133 affects haematopoietic functional heterogeneity. While either CD34 or CD133 may be employed successfully for clinical characterisation of HSPC for transplantation to restore haematopoiesis, it is not known if that holds true for other HSPC functions. For example, we have recently examined their different expression in relation to where endothelial cell progenitors (EPC) originate, and find these EPC are exclusively within the $CD34^{+}CD133^{-}$ subpopulation (Tura *et al.*, 2007a) in agreement with other reports (Case *et al.*, 2007; Timmermans *et al.*, 2007). It would be useful to determine whether other functional heterogeneity reflects heterogeneity of expression of these almost “fundamental” HSPC markers. Thereby it may be possible to define and isolate more homogeneous subpopulations based on these markers for examination in the context of *in vitro* HSPC expansion, differentiation and the factors that modify these.

Using heterogeneous HSPC, it is not, for example, feasible to determine which cells may be responders, which bystanders, and which characteristics (phenotype, gene

expression) change directly in response to factors and which are changed indirectly by, for example, secondary response to paracrine stimuli from the activated direct responders. While there is a wide range of other immunophenotype markers, many with recognised functions, which further define subcategories of HSC, however none are synonymous with HSC and most are more discretely expressed on subsets of HSC and other cells. It is necessary to resort to utilising co-expression of such markers with the more fundamental CD34 and CD133 markers to define functionally or lineage-related useful homogeneous subsets of HSPC for manipulation studies. However, the scope of CD34/CD133 heterogeneity needs to be defined firstly, and that is the intent of this study.

In this chapter, we studied the relationship between HSC subpopulations based on immunophenotype, myelopoiesis and erythropoiesis in a standard CFU assay. In the process of selecting HSPC, various methods can be used for separating prospective cell populations depending on the required purpose. This study used magnetic bead selection to enrich and deplete CD34 and/or CD133 cells from mononuclear cells (MNC) from different sources. And then FACS sorting was used to sort pure subpopulations from MACS based primary selection. Subsequently, colony forming units assay in methylcellulose semisolid medium was employed as the *in vitro* assay for the quantification of committed HSPC. Cells to be tested are mixed with stimulatory cytokines in tissue culture medium made up in viscous methylcellulose which did not allow the cells to disperse – so that any cells proliferating in response to the cytokine stimulants remained localised and formed a colony. Each colony is presumed to be derived from a single cell by clonal outgrowth, and different types of colonies represent different types of progenitor cell lineages. It is intended to examine whether haematopoietic colony formation is homogeneously distributed over all HSPC subpopulations defined by CD34 and CD133, or whether any subpopulation defined by these markers shows preference for myeloid or erythroid colony types.

3.2 Experimental strategy

In our laboratory, HSPC are sourced from mobilised blood, cord blood and bone marrow based on the CD34$^+$ selection by magnetic-activated cell sorting (MACS). However, the CD34$^+$ population after MACS selection is not pure, though much more concentrated. The CD34 enriched fraction is named as CD34e population, and the CD34 depleted fraction is CD34d population. When the two populations are cultured in StemMACS HSC-CFU complete with Epo, the CD34d cells could generate much higher erythroid colonies proportion than CD34e cells. This suggests that there are different subpopulations from CD34e cells and CD34d cells capable of forming colonies. Considering most cells of CD34e population are CD34$^+$/CD133$^+$, therefore we decided to sort pure subpopulations using fluorescence-activated cell sorting depending on markers CD34 and CD133 for further colony forming units assay to verify the functionality of various populations.

3.3 Results

3.3.1 The expression of HSPC markers (CD34 or CD133) on MNC from different sources

Manual Method of estimating the frequencies of subpopulations in MNC from different sources using flow cytometry

Flow cytometry is a simple way to estimate the frequencies of different populations. Manual gating analysis is the traditional strategy, convenient and enabling

cytometrists to capture the biological meaning, although easily influenced by personal experience. Automated gating identification using computational analysis is a recent developed strategy with the potential to find rare and hidden populations, offering a highly functional alternative to the traditional way in multiple color staining. But computational analysis might lead to overlap and insignificant population, which requires cytometrists to review and validate the results of computational analysis. In addition, it requires much professional knowledge in bioinformatics and not as easy as the traditional way to handle, thus the manual strategy is still the priority in the simple staining.

In this section, I took the frequencies of $CD34^{+}/CD133^{+}$, $CD34^{+}/CD133^{-}$ or $CD34^{-}/CD133^{+}$ as an example to explain the manual method process. Mononuclear cells were stained with anti-CD34 PercP and anti-CD133 APC antibodies, and sample data was collected on the flow cytometer for analysis, using appropriate predetermined flow cytometry instrumental settings for fluorescence amplification and channel compensation specific for blood cells. The estimation of CD34 and CD133 expression was analyzed as shown in Figure 3.1. All of the HSPC (either CD34 or CD133) can be defined as CD34 OR CD133; the dual cells expressing both markers ($CD34^{+}/CD133^{+}$) can be defined as CD34 AND CD133; while the single-positive subpopulations can be defined by NOT CD133 AND CD34 ($CD34^{+}/CD133^{-}$) and by NOT CD34 AND CD133 ($CD34^{-}/CD133^{+}$). Figure 3.2 shows an example of gated “all HSPC” (CD34 or CD133) of MNC from each of mobilised blood (mPB), bone marrow (BM), normal peripheral blood (nPB) and cord blood (CB) which were used to determine the frequencies of $CD34^{+}/CD133^{+}$, $CD34^{+}/CD133^{-}$ or $CD34^{-}/CD133^{+}$ in mononuclear cells (MNC) employing the logical gating method.

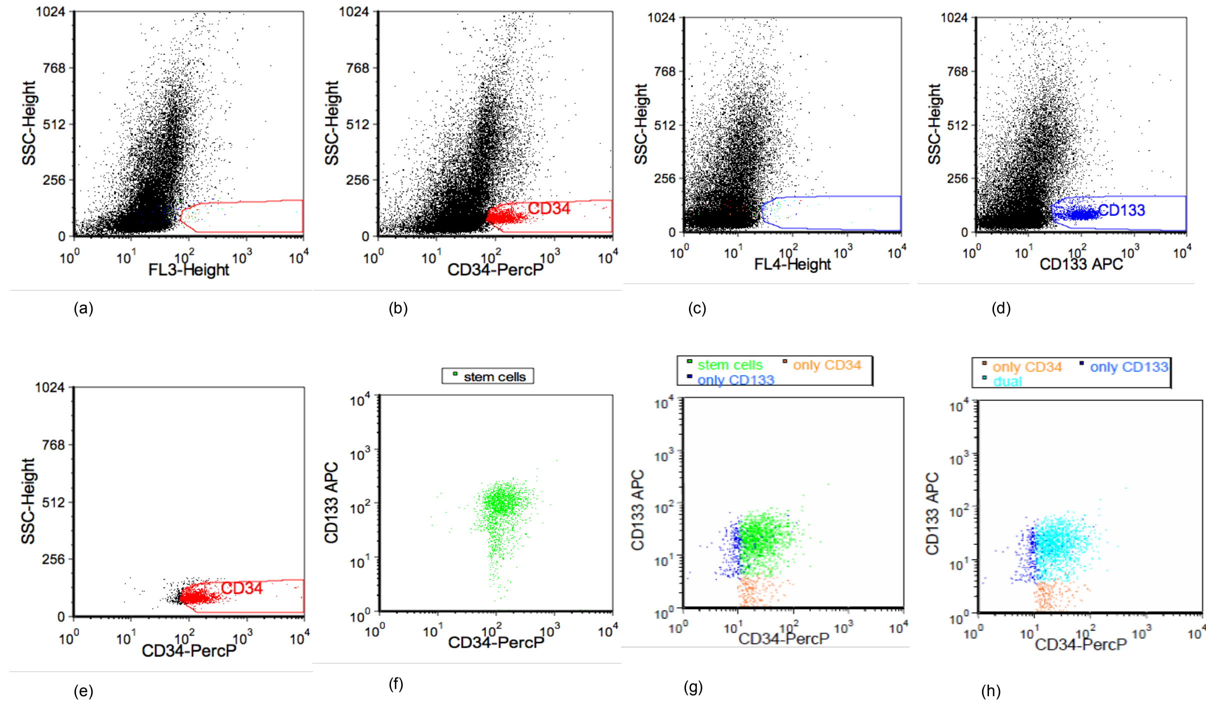


Figure 3.1: Determining CD34 and CD133 subpopulations in flow cytometry by regional and logical gating methods. *Using FCS Express flow cytometry analysis software, the fluorescence resulting from each antibody was plotted on the x-axis of a dotplot scattergram versus the side (orthogonal) scatter on the y-axis, and a polygon region was drawn demarcating the low side-scatter CD34^{+} cells (b) and the low side-scatter CD133^{+} cells (d), with reference to the equivalent unstained sample (a,c). Then the total "stem cell" (HSPC) gate was identified as CD34 or CD133, that is stem cells = CD34 OR CD133 (in Boolean terms . When we select (gate) on "stem cells"only, as (e) shows, all cells expressing CD34 or CD133 are in the dot plot of FL3 v. SSC without showing other cells. The "stem cells" (CD34 OR CD133) can be assigned a colour (green) on a plot of FL3 v. FL4. Single-positive cells (only CD34) (CD34^{+}CD133^{-}) = NOT CD133 AND CD34 (in Boolean terms) are assigned orange color, (only CD133) (CD34^{-}CD133^{+}) = NOT CD34 AND CD133 (in Boolean terms) are assigned blue color, showing, where the residual cells remain green. However if the double-positive cells (dual) (CD34^{+}CD133^{+}) are defined by CD34 AND CD133 (in Boolean terms) and assigned cyan colour they can be shown as in (h) by choosing which gates to illustrate (i.e. which colours).*

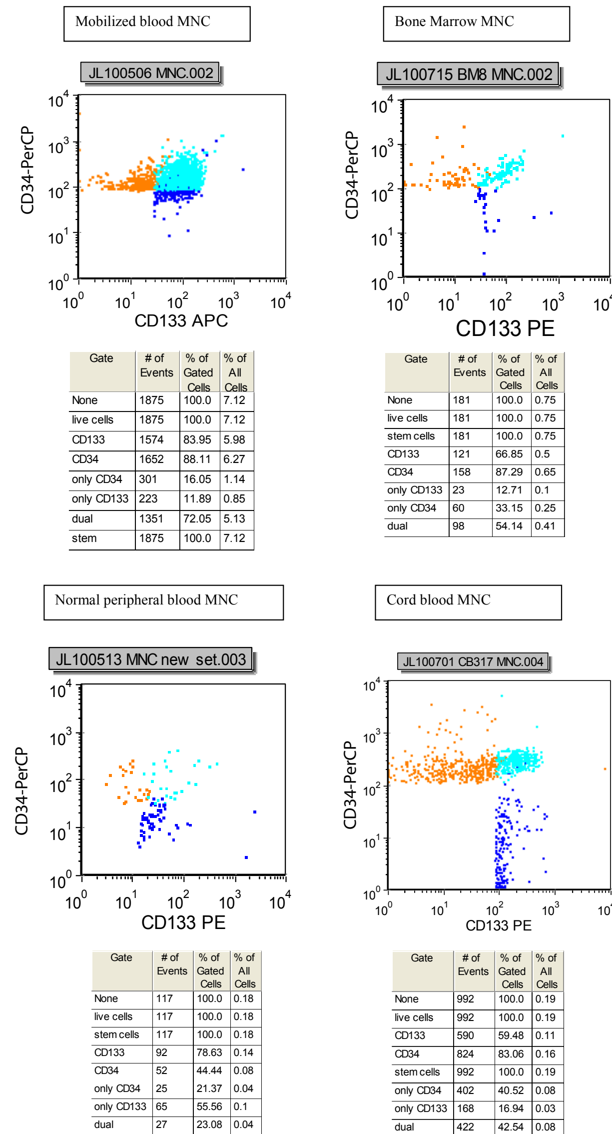
The frequencies of CD34$^+$/CD133$^+$, CD34$^+$/CD133$^-$ or CD34$^-$/CD133$^+$ fractions from different sources

As Figure 3.3 demonstrates, in mobilised peripheral blood (including patients and donors) the dual CD34$^+$/CD133$^+$ cells are the predominant subpopulation while in bone marrow and normal peripheral blood the percentages of the single-positive CD34$^+$CD133$^-$ cells and CD34$^-$CD133$^+$ cells outnumber CD34$^+$CD133$^+$ cells. Cord blood gave an intermediate distribution of subpopulations. The data for subpopulations based on CD34 and CD133 provides a basis for the exploration of functional assay in relation to these different subpopulations.

3.3.2 Assessment of Cell isolation method by MACS

Cell enrichment, as the first step to provide homogeneous known cell populations for experiments, is very important for cell functional assays. MACS selection provides an efficient way to select cells of interest for further experiments. Figure 3.4 shows the flow chart of an example (mPB392) of isolating CD34 enriched cells from mononuclear cells and we find in this case that CD34$^+$ cells were concentrated from 0.1 % to 21.5% - 83% (43.0%$\pm$ 15.0%): though not pure, still more concentrated than in mononuclear cells. Comparing the expression of CD34 between CD34-enriched fraction (CD34e) and CD34-depleted fraction (CD34d), most CD34$^+$ cells were removed from CD34d fraction, though still 0.48% CD34$^+$ cells were left.

Generally speaking, the CD34e cells were collected from the column and the cells from flow through were named as the CD34d cells. All samples were labelled and separated within 24 hours. However, the frequency of CD34$^+$ cells in CD34e fractions ranged from 21.5% to 83% depending on different sources, in which cord blood usually gave a higher purity (64% - 83%).



Mobilized blood MNC

Gate	# of Events	% of Gated Cells	% of All Cells
None	1875	100.0	7.12
live cells	1875	100.0	7.12
CD133	1574	83.95	5.98
CD34	1652	88.11	6.27
only CD34	301	16.05	1.14
only CD133	223	11.89	0.85
dual	1351	72.05	5.13
stem	1875	100.0	7.12

Bone Marrow MNC

Gate	# of Events	% of Gated Cells	% of All Cells
None	181	100.0	0.75
live cells	181	100.0	0.75
stem cells	181	100.0	0.75
CD133	121	66.85	0.5
CD34	158	87.29	0.65
only CD133	23	12.71	0.1
only CD34	60	33.15	0.25
dual	98	54.14	0.41

Normal peripheral blood MNC

Gate	# of Events	% of Gated Cells	% of All Cells
None	117	100.0	0.18
live cells	117	100.0	0.18
stem cells	117	100.0	0.18
CD133	92	78.63	0.14
CD34	52	44.44	0.08
only CD34	25	21.37	0.04
only CD133	65	55.56	0.1
dual	27	23.08	0.04

Cord blood MNC

Gate	# of Events	% of Gated Cells	% of All Cells
None	992	100.0	0.19
live cells	992	100.0	0.19
CD133	590	59.48	0.11
CD34	824	83.06	0.16
stem cells	992	100.0	0.19
only CD34	402	40.52	0.08
only CD133	168	16.94	0.03
dual	422	42.54	0.08

Figure 3.2: **Estimation of the frequencies of CD34$^+$/CD133$^+$, CD34$^+$/CD133$^-$ or CD34$^-$/CD133$^+$ in mononuclear cells (MNC) from different sources: examples.**

An example from each of mobilised blood, bone marrow, normal peripheral blood (buffy coat) and cord blood were used to measure the frequencies of CD34$^+$/CD133$^+$, CD34$^+$/CD133$^-$ or CD34$^-$/CD133$^+$ in mononuclear cells (MNC) by the gating method shown in Figure 3.1. The software generates statistics for the gates, expressed as % of Gated Cells (where the "stem cell" [HSPC] gate is used for selection.

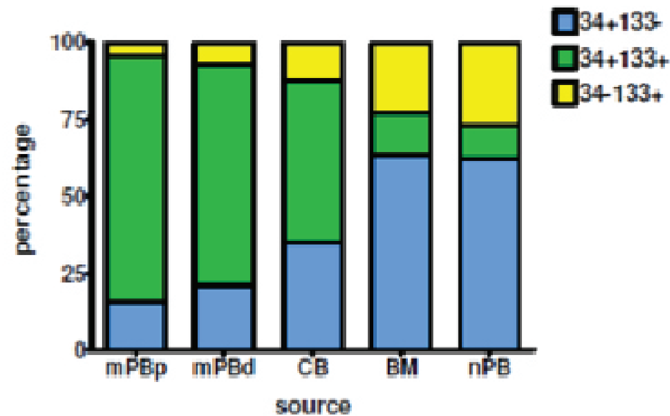


Figure 3.3: The proportions of CD34^{+}CD133^{+}, CD34^{+}CD133^{-} or CD34^{-}CD133^{+} from different sources.

The comparison of percentages of CD34^{+}CD133^{+}, CD34^{+}CD133^{-} or CD34^{-}CD133^{+} fractions in different HSPC sources including normal (non-mobilised) peripheral blood (nPB), bone marrow (BM), umbilical cord blood (CB) and G-CSF-mobilised peripheral blood samples (mPBp, autologous patients; or mPBd, allogeneic donors). Data is from Olga Tura and our colleagues (Tura et al.*, 2007a) in the group. In mobilised sample, the dual CD34^{+}/CD133^{+} cells comprise the major subpopulation whereas the single-positive (CD34^{+}CD133^{-} or CD34^{-}CD133^{+} cells) together outnumber the CD34^{+}CD133^{+} cells in bone marrow and normal peripheral blood. The CD34^{+}CD133^{-} cells are the major population in bone marrow and in the quite rare HSPC in peripheral blood. The CD34^{-}CD133^{+} cells are minimal in mobilised blood.*

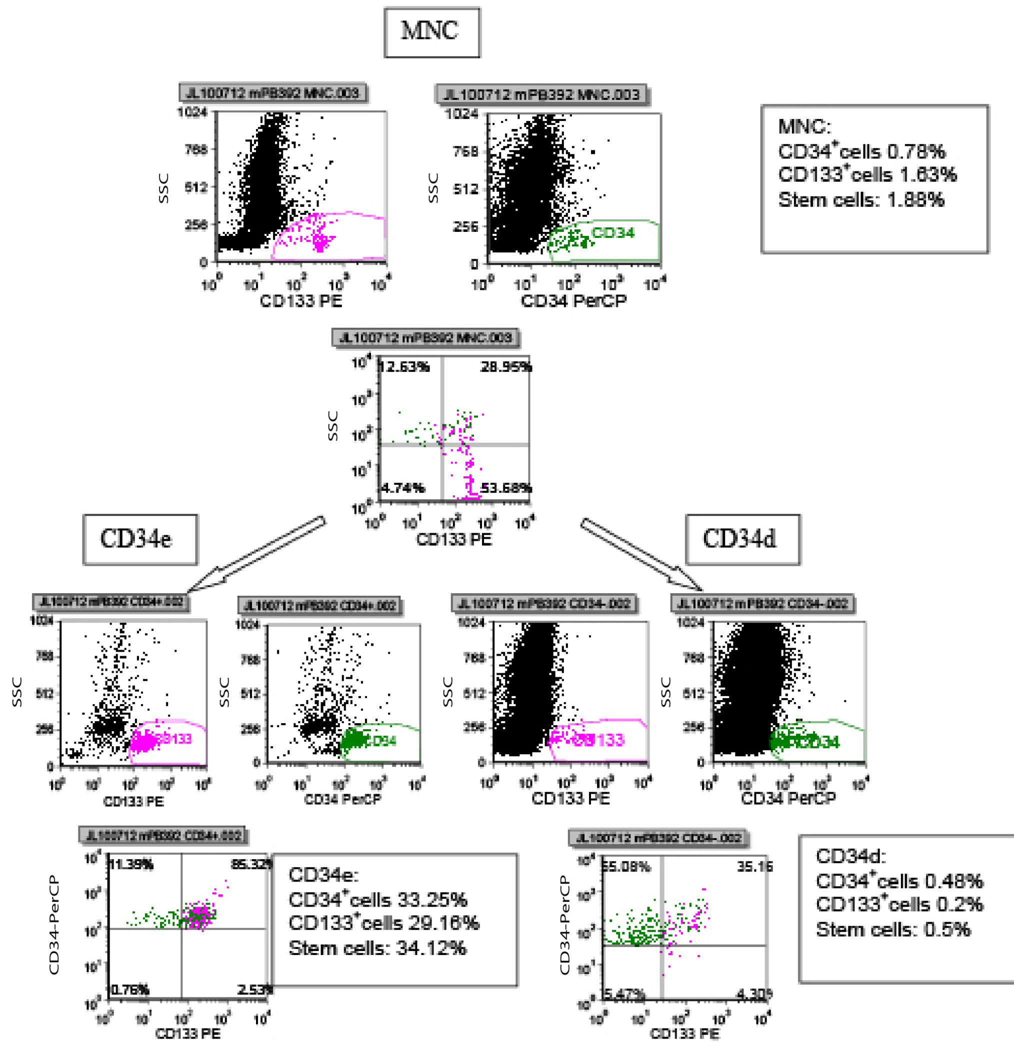
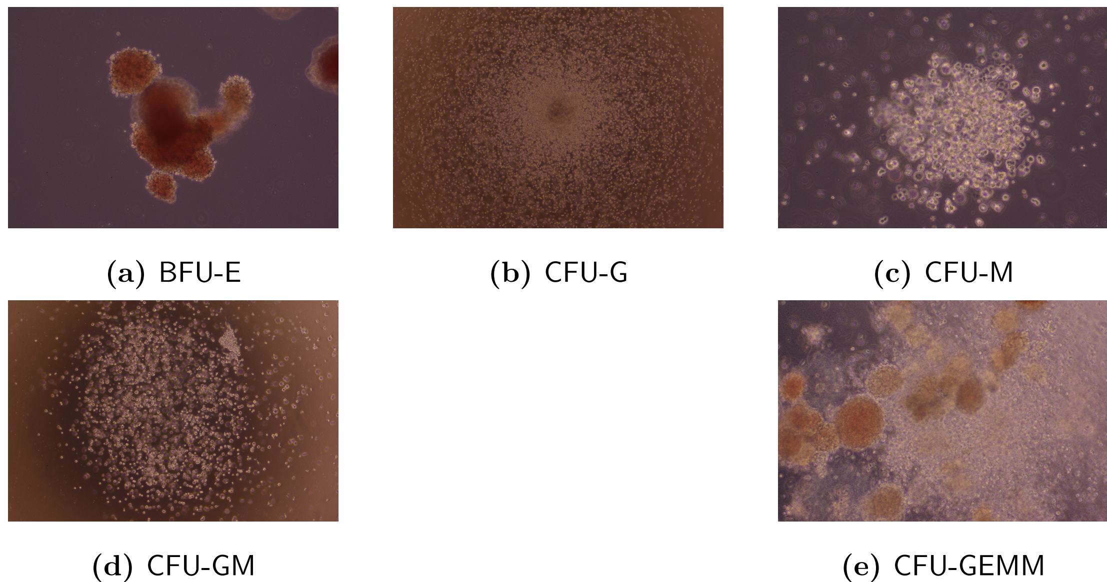


Figure 3.4: **An example showing a CD34$^+$ cell enrichment and depletion from mononuclear cells using anti-CD34-labelled magnetic bead selection.** *CD34 enriched cells (CD34e) were "positively" selected from mononuclear cells using the MACS method. After isolation, CD34$^+$ cells were concentrated in the CD34e fraction, to 34% in this case, but there were still CD34$^+$ cells left in CD34d fraction (0.78% in MNC reduced to 0.48% in CD34-depleted MNC).*

3.3.3 Assessment of myeloid and erythroid CFU from $CD34^+$ enriched cells population (CD34e) and $CD34^+$-depleted MNC (CD34d)

500-1000 CD34 enriched cells or 20, 000- 30, 000 CD34d cells from mobilised patient peripheral blood (mPB), mobilised donor peripheral blood (mPBd), and normal peripheral blood (nPB) were plated in "complete" methylcellulose medium supplemented with required cytokines. Colonies were scored on day 12-14 after initiation.



(a) BFU-E (b) CFU-G (c) CFU-M

(d) CFU-GM (e) CFU-GEMM

Figure 3.5: Examples of the typical BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM.

Figure 3.6 shows that in most samples (with some exceptions) CD34e cells produced considerably more myeloid colonies than erythroid colonies, comparing the erythroid colonies and myeloid colonies generated by the CD34d cells from the same sample. According to the comparison of erythroid colonies and myeloid colonies generated by CD34d cells, it was found that the erythroid colony forming

ability of CD34d cells prevailed compared to myeloid colony forming ability (see Figure 3.7).

But there is still an important problem that we absolutely can not ignore: the frequency of different populations. CD34e cell populations were not pure and the frequency ranged from 21.5% to 83% depending on sample source and isolation method. The CD34e fraction would always have a higher frequency of CD34$^+$ cells than the CD34d fraction. Similarly, there were usually some progenitor cells (identified as CD34$^+$ or CD133$^+$ cells) left in CD34d population, so that the CD34d cell population should really be considered only as mononuclear cells with fewer CD34$^+$ cells compared to CD34e populations. To this point, colony cell number/1000 plated cells were used to assess the colony forming ability of the whole population of cells plated.

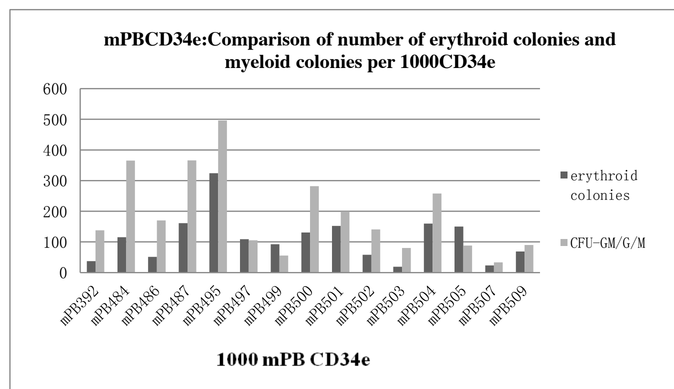


Figure 3.6: Comparison of colony forming ability of CD34e cells from mobilised blood.

In most samples, CD34e cells produced more myeloid colonies than erythroid colonies.

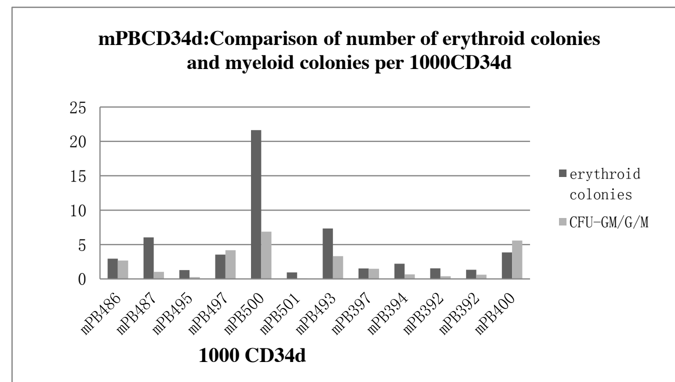


Figure 3.7: Comparison of colony forming ability of CD34d cells from mobilised blood.

From the figure, we can find that 1000 CD34d cells produced more erythroid colonies than myeloid colonies

3.3.4 CD34e cells have reduced potential to generate CFU whose cells express glycophorin A, while CD34d cells retain capacity to generate colonies whose cells express glycophorin A

When we depleted CD34^{+} cells from MNC, the residual CD34d population still retained some range of potency to form BFU-E, CFU-GEMM and CFU-G/GM/M colonies. Figure 3.8 demonstrates the differences in CFU generated by CD34e, and CD34d cells. Dramatically, the CD34e population did not produce more erythroid colony proportions than the CD34d population, and even more remarkable, the CD34e population generated around half of erythroid colony proportion produced by CD34d population. However, the CD34e population had more potential to produce CFU-G/GM/M compared to CD34d population and mononuclear cells.

Nevertheless, we harvested the colonies produced by the CD34e population and CD34d population, and stained the cells with anti-GlyA Ab and anti-CD45 Ab. Then flow cytometry analysis was used to calculate the mean of expression of GlyA and CD45 on colony cells respectively. As shown in Figure 3.9, nearly 65% of colonies generated by the CD34d population had GlyA expression while the colonies formed by CD34e populations expressed only 36% GlyA. Generally speaking, the staining of GlyA was consistant with the percentages of erythroid colonies shown in Figure 3.8. Thus, colony-forming units assay is a reasonable approach to measure the multilineage differentiation potential of HSPC cells.

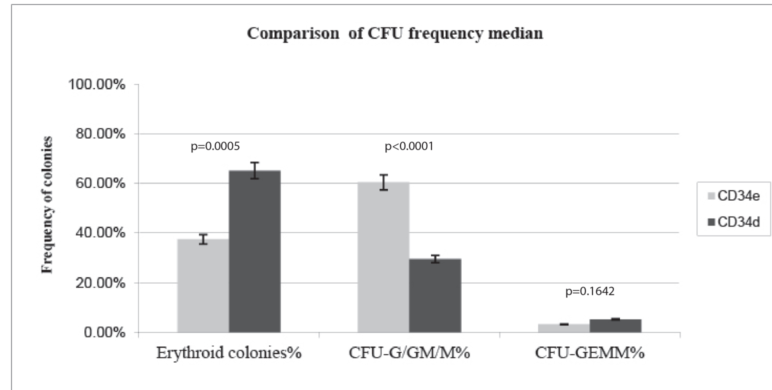


Figure 3.8: Comparison of percentages of erythroid colonies, myeloid colonies (CFU-G/GM/M) and CFU-GEMM generated by CD34e and CD34d. *n=15*

It shows the comparison of the mean proportions of CFU generated by CD34e cell populations, CD34d cell populations and mononuclear cells. The CD34e population produced 29% erythroid CFU, nearly 30% lower than CD34d cells, while CD34e cells had more potential to generate myeloid CFU compared to CD34d cells.

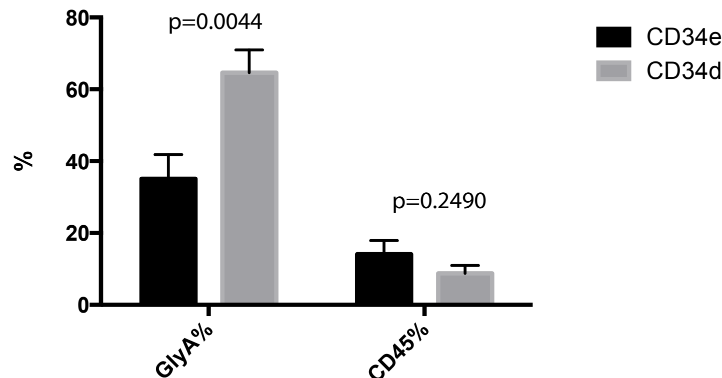


Figure 3.9: Comparison of expression of GlyA and CD45 on colony cells generated by CD34e and CD34d.

It indicates the mean of GlyA and CD45 expression on harvested colony cells generated from CD34e and CD34d cells. Clearly the expression of GlyA on recovered CD34d was higher than on the recovered CD34e cells population, consistent with the CFU result.

3.3.5 Isolation of HSPC ($CD34^+$ or $CD133^+$) subpopulations and corresponding functional heterogeneity

$CD34^+CD133^+$ subpopulation has specific potential to produce CFU-G/GM/M rather than BFU-E

$CD34^+CD133^+$ subpopulation of CB358 In order to produce further data from more purified cells, cord blood (CB) MNC (8 samples) were selected using $CD34^+$ and $CD133^+$ Microbeads firstly, and then all $CD34^+$ or $CD133^+$ enriched cells from cord blood were further sorted to isolate the $CD34^+CD133^+$ fraction by fluorescence activated cell sorting (FACS). 250 cells of $CD34^+CD133^+$ fraction were plated in methylcellulose medium supplemented with the required cytokines. After 12-14 days, when we scored colonies and found every 1000 cells of the $CD34^+CD133^+$ subpopulation from CB358 generated 237 CFU-G/GM/M (average of three repeated wells) compared to 72 erythroid colonies (average of three repeated wells), indicating their specific potential to produce white cells rather than red cells. With respect to proportions of different types of colonies in each well, $CD34^+CD133^+$ fraction generated 74% CFU-G/GM/M outnumbering 6% CFU-GEMM and 18% erythroid colonies (see Figure 3.10). This was different from colony percentages for the $CD34^+CD133^-$ subpopulation.

$CD34^+CD133^+$ subpopulation from other samples 250-500 cells from FACS sorting $CD34^+CD133^+$ cells were plated for the colony-forming unit assay. After 12-14 days, we scored colonies and found that every 1000 $CD34^+CD133^+$ cells could produce 387 CFU-G/GM/M and 73 erythroid colonies on the average. According to the colony count from our experiment, the $CD34^+CD133^+$ population generated CFU-G/GM/M predominately (79%), with a mean of 12% erythroid colonies.

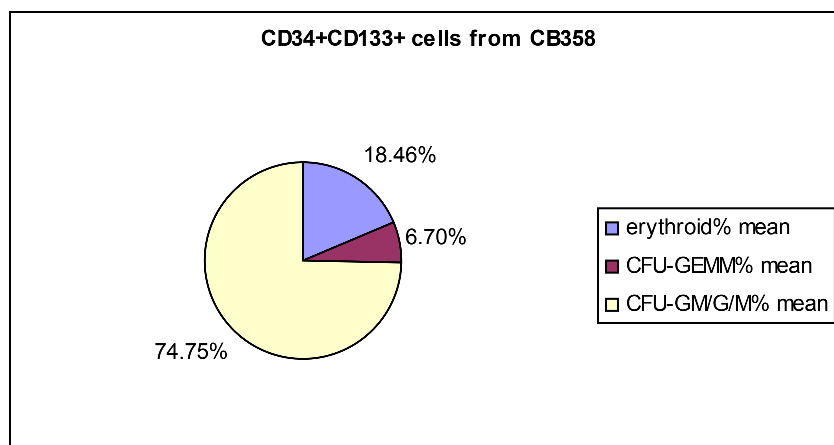


Figure 3.10: **Comparison of percentages of BFU-E, CFU-GEMM and myeloid colonies (CFU-G/GM/M) generated by $CD34^+CD133^+$ cells.** *It shows the proportions of different colony-forming units generated by $CD34^+CD133^+$ cells. 74 % myeloid colonies were predominantly produced by $CD34^+CD133^+$ cells compared to 18% BFU-E.*

The $CD34^+CD133^-$ subpopulation generates higher proportions of erythroid colonies and myeloid colonies

$CD34^+CD133^-$ subpopulation of CB358 Figure 3.11 shows 500 cells of $CD34^+CD133^-$ subpopulation sorted by FACS were plated in methylcellulose medium containing appropriate growth factors. Subsequently, after 12 days growth, we obtained on average 154 erythroid colonies outnumbering the average 63 CFU-G/GM/M produced by every 1000 cells from $CD34^+CD133^-$ fraction, demonstrating very different proportions of colonies, with 68% BFU-E compared to 28% CFU-G/GM/M.

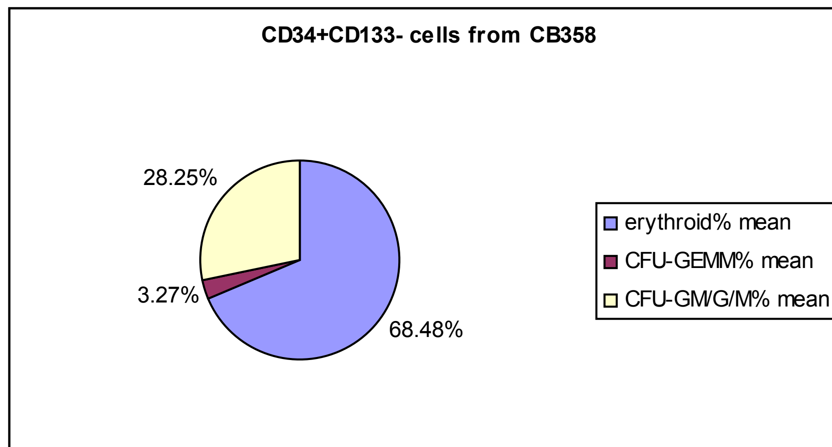


Figure 3.11: **Comparison of proportions of BFU-E, CFU-GEMM and myeloid colonies (CFU-G/GM/M) generated by $CD34^+$ $CD133^-$ cells.**
Compared to $CD34^+CD133^+$ cells, $CD34^+CD133^-$ cells generated only 28% myeloid colonies but much higher BFU-E (68%), nearly the opposite case of $CD34^+CD133^+$ subpopulation.

$CD34^{+}CD133^{-}$ subpopulation from other samples When we studied the $CD34^{+}CD133^{-}$ fraction from MNC cells of other cord blood (7 repeated samples), the colony forming units assay showed a similar result. Generally speaking, average 199 erythroid colonies and 54 CFU-G/GM/M could be scored from colony-forming unity assay deriving from every 1000 cells from $CD34^{+}CD133^{-}$ fraction. From the stand point of the frequency of various colonies, that is 74% erythroid colonies compared to 20% myeloid colonies, significantly different from the colony-forming unit assay of $CD34^{+}CD133^{+}$ cells.

The poor colony formation efficiency of CD34$^-$CD133$^+$ subpopulation

Compared to other subpopulations, the CD34$^-$CD133$^+$ population is a small fraction of HSPC cells and we usually sorted less than 1000 cells. With respect to the cells of CB358 CD34$^-$CD133$^+$ population, we only obtained 470 cells after FACS sorting. After 12-14 days, we observed 3 CFU-G in the whole well. Due to the limited potential of producing colonies, it's difficult to draw a definitive conclusion.

In addition, we sorted other seven samples, and four of them generated less than 10 colonies totally deriving from every 1000 CD34$^-$CD133$^+$ cells. In other three cases, the number of CFU we obtained is as following 27, 12, 10. Generally speaking, the plating efficiency of cells from CD34$^-$CD133$^+$ population is much lower than cells from CD34$^+$CD133$^+$ and CD34$^+$CD133$^-$ fraction. From the result listed in table 3.1, we found that for the three samples, every 1000 CD34$^-$CD133$^+$ cells can form more eythroid colonies than myeloid colonies.

No. of colonies/1000 cells	CB120502	CB351	CB350
Erythroid colonies	17	12	9
CFU-GEMM	1	0	0
CFU-G/GM/M	9	0	1
Total	27	12	10

Table 3.1: The colony forming units generated by CD34$^-$CD133$^+$ cells. *We sorted 8 samples, in which of five produced less than 10 colonies per 1000 cells, and we only listed samples that generated more than 10 colonies.*

The comparison of percentages of erythroid colonies and myeloid colonies generated by different subpopulations

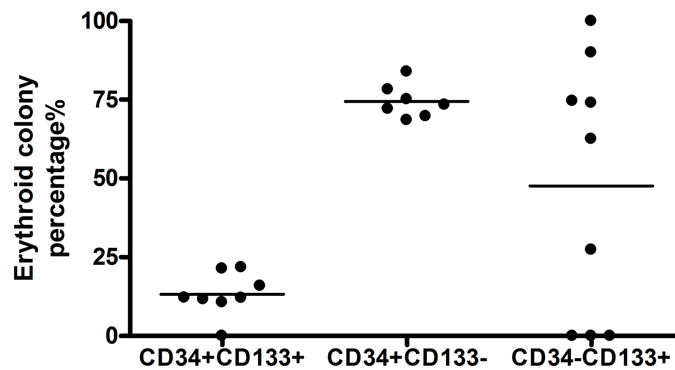


Figure 3.12: Comparison of proportions of erythroid colonies by different subpopulations.

The cells of CD34$^+$CD133$^-$ subpopulation produced 74% erythroid colonies on the average, predominantly higher compared to other subpopulations. It has to be mention that we doubt claim definitive results with regard to the potential of CD34$^-$CD133$^+$ cells due to the limited colony count produced by CD34$^-$CD133$^+$ cells. Statistics showed significantly difference between proportions of erythroid colonies generated by CD34$^+$CD133$^+$ subpopulation and CD34$^+$CD133$^-$ subpopulation ($p<0.0001$).

As the colony-forming unit assay (Figure 3.12) shows, CD34$^+$CD133$^-$ subpopulation could generate 74% erythroid colonies and 12% myeloid colonies compared to nearly 13% erythroid colonies and 79% myeloid colonies deriving from CD34$^+$CD133$^+$ subpopulation. The noticeable contrast demonstrated CD34$^+$CD133$^-$ cells with more potential to produce erythroid cells rather than myeloid cells.

The comparison of colony count from different subpopulations

With respect to actual number of erythroid colonies produced by 1000 cells, every 1000 cells from $CD34^{+}CD133^{-}$ fraction produced 199 erythroid colonies outnumbering 73 colonies generated by $CD34^{+}CD133^{+}$ population, while the opposite was the case for the generation of myeloid colonies. Cells from $CD34^{+}CD133^{+}$ fraction gave a predominant production of myeloid colonies, that is 399 CFU-G/GM/M per 1000 $CD34^{+}CD133^{+}$ cells compared to only 54 CFU-G/GM/M by $CD34^{+}CD133^{-}$ cells (Figure 3.3.5). Therefore, $CD34^{+}CD133^{-}$ subpopulation could be considered to harbor prospective erythroid progenitors.

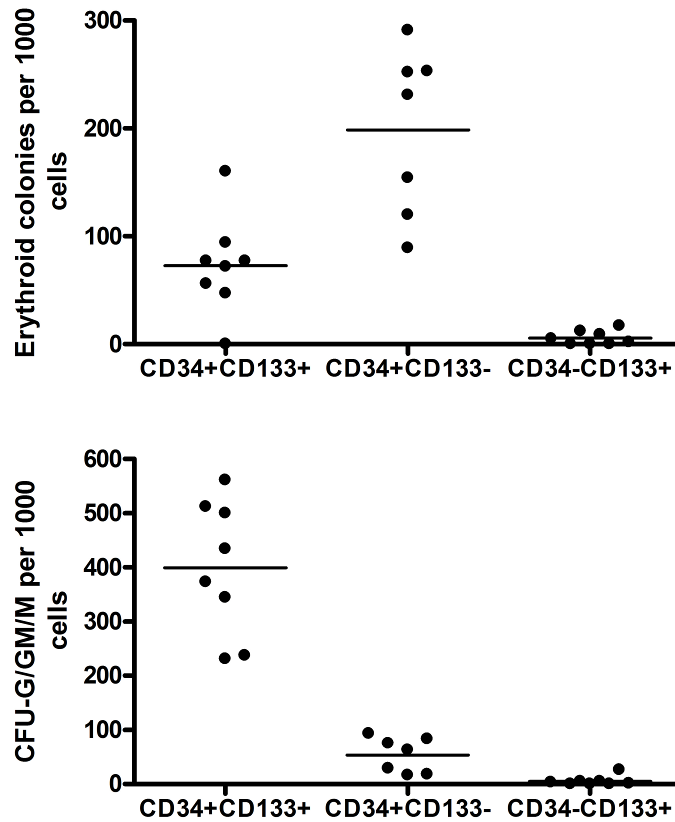


Figure 3.13: **Comparison the count of erythroid colonies and myeloid colonies generated by different subpopulations.**

Every 1000 cells of CD34^{+}CD133^{+} subpopulation generated average 73 erythroid colonies and 387 myeloid colonies while every 1000 CD34^{+}CD133^{-} cells could produce 199 erythroid colonies and 54 myeloid colonies on the average. Statistics showed significantly difference between the colony forming ability of CD34^{+}CD133^{+} subpopulation and CD34^{+}CD133^{-} subpopulation (or CD34^{-}CD133^{+} subpopulation) ($p<0.0001$).

3.4 Discussion

3.4.1 Colony forming units assay is the basic method to measure the functional reconstituting potential of cells from different sources

Cell isolation is an important step to give enriched or purified cell populations for further experiments. The positive selection with immunomagnetic beads (MACS), the general isolation procedure used in our experiment, could achieve enrichment of $CD34^+$ cells to 83%, but the lowest frequency was 21.5%. The highest and the lowest frequency were from mobilised blood, which might be attributable to mobilised blood samples from patients with different diseases and their various responses to G-CSF. However, most positive isolations succeeded to enrich cells expressing CD34 and/or CD133 depending on the combination use of isolation kits.

In our experiments, we proposed to use the colony forming units assay as the basic method to measure the functional reconstituting potential of cells from different sources. Today, the best *in vivo* assay that measures the long-term potential of HSC to self-renew and reconstitute is to make use of the NOD/SCID mouse model to define the repopulating capacity of human HSC (Pflumio *et al.*, 1996). However this is a complex assay requiring *in vivo* work, and the more amenable *in vitro* CFU assay for the quantification of committed haematopoietic progenitors is widely used and accepted. Colony formation is assessed after 12-14 days, and the number of BFU-E, CFU-GEMM, CFU-G, CFU-GM and CFU-M is counted.

In order to generate optimal numbers of colonies from plated cells, it is necessary to initiate the CFU assay with different numbers of cells. The CD34 enriched population, which was expected to have a frequency of above 50% (though this

was not always achieved), was plated at 1000-2000 cells/well. And when the CD34 enriched cells had a frequency above 70%, 500 cells/well would be beneficial to achieve an appropriate colony number. For haematopoietic stem/progenitor cells isolated by FACS sorting with 100% frequency, the optimal cell density for plating CD34$^+$CD133$^+$ cells is 250 cells/well and 500 cells/well for CD34$^+$CD133$^-$ cells according to our experience. Since colony-forming unit ability from different subpopulations are different, for each new population from various sources we often employed different (say 2-fold) concentration of cells for plating in methylcellulose medium, then selected the most optimal for scoring, since too few or too many colonies are not optimal.

3.4.2 CD34d cells show potential to generate a much higher proportion of erythroid colonies than CD34e cells.

The comparison of the average number of different types of colonies generated by CD34e cells and CD34d cells showed that the CD34d cells generated a much higher proportion of erythroid colonies (60%) than the corresponding CD34e cells (29%), whereas the CD34e cells showed higher proportion of myeloid CFU-G/GM/M colonies (71%) in contrast to CD34d cells (40%). In order to get further information about colonies, we harvested colonies and recovered total cells to obtain the cell count and immunophenotype which agreed well with the colony numbers and type frequency.

These results have unexpectedly required us to consider the unexpected but evident erythroid colony formation potential of the CD34-depleted (supposedly HSPC depleted) MNC population. Our data is consistent with the results published by van den Akker and colleagues (van den Akker *et al.*, 2010), who found

that the expansion of erythroblasts from total PBMC or CD34-depleted PBMNC cells generated significantly more erythroblasts compared to the corresponding CD34 enriched cells purified from the PBMC. They concluded that *in vitro* erythroid cell expansion potential predominantly resides in CD34-depleted cells, outweighing the contribution of the CD34$^+$ cells. In 2005, Leberbauer and colleagues (Leberbauer *et al.*, 2005) had described a culture method achieving a 109 fold expansion of erythroblasts from mononuclear cells isolated from cord blood without prior CD34 enrichment, on which van den Akker and colleagues made the hypothesis and designed further experiments to demonstrate the CD34-depleted cells contribution to erythroid expansion potential. None of these studies proposed the nature of the specific stem cells which made the substantial contribution to erythroid expansion, which requires to be further explored.

3.4.3 The candidate progenitor cells existing in CD34d cells responsible for producing a much higher erythroid forming proportion

Unquestionably, there were some CD34$^+$ cells left in CD34d populations. If it were hypothesized that the colony formation capacity of CD34d is mainly attributed to the residual CD34$^+$ cells, then the proportion of erythroid colonies generated by CD34d population should be comparable to that of the CD34e cells. In fact the result contradicted this hypothesis, indicating that the CD34d cells preferentially produced myeloid colonies. In order to facilitate comparison of colony formation potential from different cells, we had calculated the colony number/1000 plated cells. But to take the frequency of stem cells from different populations into account, colony number/1000 plated cells was at most the assessment of the colony forming ability of the entire population but not the stem cells in that population. Therefore we assessed the colony count per equal stem cells (normalised) (more

details can be found in my Msc thesis (Liu, 2010)). From our calculated result, equivalent numbers of stem cells (refers to all CD34$^+$ OR CD133$^+$ cells) from the CD34-depleted population appeared to yield many more colonies per stem cell than the CD34-selected population. According to this, we predict that the greater colony formation potential of the equivalent stem/progenitor cell number from CD34-depleted population must be contributed to by other (non-CD34$^+$) HSPC existing in CD34-depleted cells.

As data from our group has previously shown (Tura *et al.*, 2007a), HSPC exist which can be CD34$^+$CD133$^+$, CD34$^+$CD133$^-$ or CD34$^-$CD133$^+$, and that since CD34$^-$CD133$^+$ cells exist in different samples, including bone marrow, mobilised blood, cord blood and normal peripheral blood, we had to consider the possible role of CD34$^-$CD133$^+$ cells in the CD34$^-$depleted cell populations. CD34$^-$CD133$^+$ cells are the least frequent among defined HSPC [CD34$^+$ OR CD133$^+$] in any source, and are especially low in mobilised blood or cord blood. We concentrated HSPC through MACS method, and used FACS sorting to obtain pure CD34$^-$CD133$^+$ cells. But the number of CD34$^-$CD133$^+$ subpopulation cells is so small that we could only get 470 to 1000 cells total, not enough for colony assay triplicates. It is difficult to draw a definitive conclusion.

An extremely rare CD133$^+$CD7$^-$ population from CD34$^-$CD38$^-$Lin$^-$ cells has been reported at a frequency of 0.2%, and the enriched cells had a high progenitor activity at a frequency equivalent to purified fractions of CD34$^+$ stem cells (Gallacher *et al.*, 2000). Also it has been possible to detect human CD133$^+$CD7$^-$CD34$^-$CD38$^-$Lin$^-$ cells in bone marrow 8 weeks after transplantation of human CD133$^+$CD7$^-$CD34$^-$CD38$^-$Lin$^-$ cells in non-obese/severe combined immunodeficiency (NOD/SCID) mice. However, CD34$^-$CD133$^+$ might not be the first choice for the prospective HSPC functioning in the CD34d cells population, and cannot numerically account for all colonies in CD34d fractions, more purified populations are required to do further functional analysis. In a paper

published in August 2013 (Anjos-Afonso *et al.*, 2013), Lin$^-$CD34$^-$CD38$^-$CD93^{hi} population was reported to be above the CD34$^+$ population in haematopoietic hierarchy and show delayed primary and efficient secondary engraftment in engrafting nonobese diabetic (NOD)/SCID-$\beta 2^{-/-}$ recipient mice. Thus, the classic hierarchy is challenged, and Lin$^-$CD34$^-$CD38$^-$CD93^{hi} population is at the apex of the human haematopoietic stem cell hierarchy.

Besides CD34 and CD133 there is another “primary” marker of HSPC. In 1999, Storms and colleagues (Storms *et al.*, 1999) isolated human hematopoietic stem/progenitor cells using the increased aldehyde dehydrogenase (ALDH) activity of a fluorescent substrate of ALDH, by fluorescence activated cell sorting. ALDH is an enzyme implicated in the metabolism of aldehydes to their corresponding carboxylic acids such as the retinoid metabolism and the resistance of HSCs to alkylating agents (Sahovic *et al.*, 1988; Takebe *et al.*, 2001). The high expression of ALDH has been detected on human and murine derived hematopoietic stem cells and neural stem cells (Armstrong *et al.*, 2004; Hess *et al.*, 2004; Matsui *et al.*, 2004). In a study by David and colleagues (Hess *et al.*, 2006), an enriched ALDH^{hi}CD133$^-$Lin$^-$ population showed enhanced colony forming ability/1000 cells due to increased BFU-E production, compared to ALDH^{hi}CD133$^+$Lin$^-$ population. The co-expression of CD34 on the ALDH^{hi}CD133$^-$Lin$^-$ population was 75%compared to 95% on ALDH^{hi}CD133$^+$Lin$^-$ population, which offered another clue for erythroid progenitors. An ALDH^{hi}SSC^{low}CD34$^-$ population has been detected in a study examining the co-expression of CD34, CD90, CD117 and CD133 in ALDH^{hi}SSC^{low} cells from human apheresis samples (Sharma *et al.*, 2010). Thus, an ALDH^{hi}CD34$^-$ population could be considered as another prospective population which might be responsible for the enhanced erythroid expansion and erythroid colony forming potential of CD34$^-$ cells compared to CD34$^+$ cells. The potential of this ALDH^{hi}CD34$^-$ population requires investigation of their erythroid expansion and colony forming ability.

3.4.4 The relationship of functional heterogeneity to haematopoietic stem/progenitor cell phenotype heterogeneity

The CD133 gene encodes a pentaspan transmembrane glycoprotein and has been described as a marker for human hematopoietic stem and progenitor cells. Successful cases have been reported about the application of CD133 selection in clinics. Koehl and colleagues reported the first successful autologous transplantation of CD133 selected hematopoietic progenitor cells (Koehl *et al.*, 2002). Following this, many transplantations of CD133 selected HSPC grafts were used clinically (Barfield *et al.*, 2007; Burt *et al.*, 2010), which clearly demonstrated the multipotency of CD133^{+} cells *in vivo* to differentiate into all blood elements including erythroid cells and leucocytes. The co-expression of CD34 and CD133 was found on primitive progenitors and on some leukemias (Miraglia *et al.*, 1997), and in human HSPC sources from different origins the CD34^{+}CD133^{+} double-positive are the majority HSPC proportion (compared to single-positive populations), which was verified by our group's data. It is of interest to find that highly purified CD34^{+}CD133^{+} population (using FACS sorting) have the potential to generate predominantly (nearly 80%) CFU-G/GM/M, and 12% erythroid colonies, consistent with the result of Yin and colleagues (Yin *et al.*, 1997b). When we isolated CD34^{+}CD133^{-} fraction of other cord blood (7 repeated samples) through FACS sorting, 74% erythroid colonies derived from CD34^{+}CD133^{-} cells compared to 20% myeloid colonies, significantly different from the colony-forming unit assay of CD34^{+}CD133^{+} cells. With regard to authentic colony count, every 1000 cells of CD34^{+}CD133^{-} subpopulation produced 199 erythroid colonies outnumbering 73 erythroid colonies generated by 1000 CD34^{+}CD133^{+} cells on the average, while the opposite was the case for the generation of myeloid colonies, that is 387 CFU-G/GM/M per 1000 CD34^{+}CD133^{+} cells compared to only 54 CFU-G/GM/M by CD34^{+}CD133^{-} cells.

This preferential myeloid colony formation appears to indicate that the cells co-expressing CD34 and CD133 are the predominant myeloid progenitor. Cells of $CD34^+CD133^-$ subpopulation might be the promising erythroid progenitors. In addition, from the colony assay results of sorted $CD34^-CD133^+$ cells, three of them generated more than ten colonies, most of which are erythroid colonies, while others produced less than ten colonies, most of which were myeloid colonies. This indicates CD133 might not be a good marker of haematopoietic stem/progenitor cells, since CD133 single positive cells could not give a definitive result as well as $CD34^+CD133^-$, and sometimes even produced no colonies.

3.5 Concluding remarks

The mononuclear cells enriched for $CD34^+/CD133^+$ double-positive marker expression are essentially committed to myeloid colony production, but retain minor multipotency (CFU-GEMM potential and erythroid potential). The mononuclear cells remaining after $CD34^+$ cell depletion (CD34d cells) retain erythroid colony potential: while these are contaminated with $CD34^+$ or $CD133^+$ cells they cannot account for all CFU potential, mainly erythroid, and some must come from cells expressing neither CD34 nor CD133. Such cells are probably more mature erythroid precursors which have lost these stem/progenitor marker expression. The $CD34^+CD133^-$ fraction has greater capacity to generate erythroid colonies compared to the $CD34^+CD133^+$ fraction, and $CD34^+CD133^-$ cells could be considered to harbor prospective erythroid progenitors. The results are consistent with a model where cells expressing CD34 and CD133 are predominantly myeloid progenitor, but retain some multipotency, while cells expressing CD34 but not CD133 have predominantly erythroid potential and may progress to lose CD34 but still retain erythroid colony potential which is no longer associated with any cell defined by CD34 or CD133 as an HSPC.

Chapter 4

The relationship of cytokines and Chromatin modification reagents in *ex vivo* proliferation of haematopoietic stem/progenitor cells

4.1 Introduction

A long term aim in the field of treating blood and other disorders is to be able to successfully culture and expand HSPC, without a concomitant loss in their ability to self-renew and reconstitute. Currently, HSPC *in vitro* expansion is not an effective approach to increase the number of haematopoietic stem cells for further application in transplantation, gene and stem cell therapies. Many scientists have

tried to increase the number of cord blood (CB) stem/progenitor cells by culturing CD34$^+$ cells *in vitro* with various culture conditions. A variety of cytokines and other factors have been employed for the *ex vivo* culture, expansion and differentiation of CD34$^+$ haematopoietic stem/progenitor cells. Among the most commonly used are SCF, Flt3L, TPO and EPO. The important roles of Flt3L and TPO in the *ex vivo* expansion of HSPCs has been demonstrated (Dao *et al.*, 1997; De Felice *et al.*, 1999; Petzer *et al.*, 1996; Piacibello *et al.*, 1997; Shah *et al.*, 1996). From our group's experience in culturing HSPCs, the essential cytokines and their concentrations are 100 ng/ml SCF and 10 ng/ml Flt3L. This is the optimal dosage employed in *ex vivo* expansion of HSPCs (Tura *et al.*, 2007b). Flt3L is structurally homologous to SCF and colony stimulating factor 1, both of them binding to class III tyrosine kinase receptor expressed on primitive human and murine haematopoietic progenitor cells. In synergy with other cytokines, Flt3L supports the proliferation and differentiation of HSPCs into multilineage progenitors (Slanicka Krieger *et al.*, 1998). SCF is a transmembrane growth factor and can activate the KIT (CD117) receptor which is a type III transmembrane receptor tyrosine kinase (Kent *et al.*, 2008). The activation of KIT receptor results in downstream effectors to regulate HSPC proliferative and self-renewal behaviours (Audet *et al.*, 2002; Kent *et al.*, 2008; Zandstra *et al.*, 1997). IL-3 binding to IL-3 receptor alpha subunit (IL3RA), forms a complex with combination of a common beta chain, which is shared by IL-5 and GM-CSF. The complex comprising of IL3, IL3RA and beta chain, activates the JAK/STAT, MAPK and PI3-kinase signaling modules (Cooney, 2002), which are implicated in regulation of neutrophil, eosinophil and basophil cell apoptosis (Didichenko *et al.*, 2008; Epling-Burnette *et al.*, 2001; Weinmann *et al.*, 1999; Yousefi *et al.*, 1996). Associated with IL3RA was JAK2, which was reported to play a role in embryonic erythropoiesis in mice (Cooney, 2002; Parganas *et al.*, 1998). Keller and colleagues' study (Keller *et al.*, 1995) shows that SCF and IL-3 directly improved the survival of HSPCs derived from murine bone marrow, while G-CSF, IL-6, leukemia inhibitory factor, IL-11,

IL-l, GM-CSF, and macrophage CSF (M-CSF) had no such effect. In Chaurasia and colleagues' study (Chaurasia *et al.*, 2011) showing valproic acid (VPA) is beneficial for cord blood CD34$^+$ cells differentiating into erythroid cells, cytokines including IL3, SCF, Flt3L and TPO were applied in *ex vivo* expansion of CD34$^+$ cells. In our experiments, we employed either two (SCF+Flt3L) or three (SCF+Flt3L+IL3) cytokines in the culture medium when expanding mobilized blood CD34 enriched cells.

Epigenetic altering reagents have gained widespread applications in clinical settings and basic science, since modifications of chromatin structures such as histone acetylation and DNA methylation were shown to regulate the expression of genes. Acetylation of lysine at N terminal tails of nucleosomal core histone H3 and H4 will relax chromosomes and facilitate the expression of genes. Histone deacetylase (HDAC) inhibitors, such as VPA, have been used in the clinic to treat neural disease (Chiu *et al.*, 2013). With respect to basic science, it has been reported to improve the efficiency of reprogramming somatic cells to induced pluripotent stem cells. Also, VPA enhances the *in vitro* expansion of human CD34$^+$ cells isolated from cord blood, bone marrow and mobilized peripheral blood in the presence of cytokine cocktails (De Felice *et al.*, 2005). In addition, its application in expanding CD34$^+$ cells from cord blood is beneficial for the differentiation of cord blood CD34$^+$ cells to erythroid progenitor and precursor cells (Chaurasia *et al.*, 2011). In contrast, using microarray data and functional profiling, other scientists concluded that VPA inhibits erythroid differentiation and activates the myelo-monocytic pathway (Chateauvieux *et al.*, 2011). DNA methyltransferase (DNMT) can silence the expression of genes through DNA methylation of a gene's promotor. 5-aza-2'-deoxycytidine (5-Aza, Decitabine), as a nucleoside analogue, can inhibit DNMT1 which can lead to a significant loss of methyltransferase activity and demethylation of DNA (Patel *et al.*, 2010) and had

been used in clinic (Leone *et al.*, 2002) as well. It was reported that human bone marrow CD34$^+$ cells exposed to a cytokine cocktail with the addition of HDAC inhibitor trichosatin A (TSA) and 5-Aza resulted in a significant amplification of CD34$^+$CD90$^+$ cells that possessed the phenotypic properties and the proliferative potential of HSPCs (Milhem *et al.*, 2004). In an effort to isolate the actual role played by these factors in erythroid priming and differentiation, cells were not exposed to EPO until they went into colony forming units assay. We also looked into the role played by IL3 in the system by either including, or excluding it from the basic two cytokine (SCF+Flt3L) CD34$^+$ cell culture medium when chromatin modifying reagents were being used.

4.2 Experimental strategy

HSPC culture is a basic and important method to expand HSPC without losing the capacity to reconstitute and self-renew. Based on our group's experience in culturing HSPC, 100ng/ml SCF and 10ng/ml Flt3L are the necessary cytokines in maintaining HSPC expansion. The addition of 20ng/ml IL3 in the cytokine cocktails increased HSPC proliferation. To test the characteristics of expanded HSPCs, we made colony forming units assay by plating 1000 expanded HSPC cells into HSC-CFU-Complete medium (with Epo) (Miltenyi Biotec, Surrey, United Kingdom). In addition, to test whether modifications of chromatin structures affect HSPC expansion and erythroid priming, we use HDAC inhibitor and DNA methyltransferase inhibitor in the expansion medium. Subsequently, after eight days culture, HSPC were used for colony forming units assay and flow cytometry analysis. However, we designed a reasonable 'matrix' to culture cells (see table 4.1).

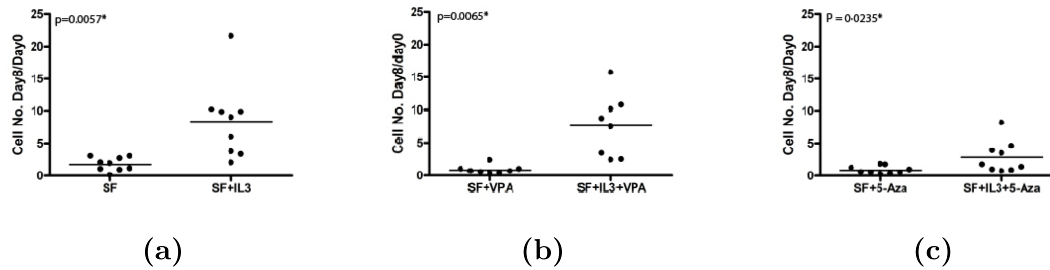
SCF+Flt3L	SCF+Flt3L+IL3
SCF+Flt3L+VPA	SCF+Flt3L+IL3+VPA
SCF+Flt3L+5-Aza	SCF+Flt3L+IL3+5-Aza

Table 4.1: **The different combinations of cytokines and drugs supplemented in basic medium, which is made up of IMDM + 10% FCS + 1% P/S.**

4.3 Results

4.3.1 The effect of different cytokine combinations in cell proliferation

When CD34e cells are concentrated from mobilized blood, expanding them in culture has been a pivotal problem for further application. In our experiments, their basic medium is made up of IMDM + 10% FCS + 1% P/S supplemented with cytokine cocktails: SCF+Flt3L (SF) or SCF+Flt3L+IL3 (SF+IL3). After CD34 enriched cells were cultured in the medium with different cytokines at 37℃, 5% CO_2 for eight days, cells were collected for immunophenotypic analysis and colony forming units assay. The media with the cytokine combination of SF+IL3 significantly increased the amplification of CD34 enriched cells compared to the cytokine combination of SF alone regardless of the addition of other drugs, such as VPA or 5-Aza (see table 4.1). It can be seen from Figure 4.1 that the media with the cytokine combination of SF+IL3 improved cell number by 8.4 fold, much higher than SF (1.8 fold). The number of cells increased 6.9 fold after adding VPA into the medium with SF+IL3 in contrast to the addition of VPA in the medium with SF (0.8 fold). The addition of 5-aza in the medium with SF+IL3 increased cell number by 2.7 fold compared to 0.8 fold increased by the medium consisting of SF and 5-Aza.



(a) (b) (c)

Figure 4.1: (a–c) Proliferation of CD34 enriched cells cultured in basic medium supplemented with SF or SF+IL3 (a), SF+VPA or SF+IL3+VPA (b), SF+5-Aza or SF+IL3+5-Aza (c) for eight days.

*CD34e cells cultured in the presence of SF or SF+IL3 showed an expansion of 1.8 fold and 8.4 fold respectively as compared to day 0 (a). The addition of VPA into SF gave an expansion of 0.8 fold, much lower than 6.9 fold in the presence of SF+IL3+VPA (b). Similarly, in the presence of SF+IL3 the addition of 5-Aza resulted in expansion (2.7 fold), however, this was lower compared to that seen in the presence or absence of VPA. *Statistically significant.*

Generally speaking, in the same conditions, the addition of IL3 plays a significant role in cell proliferation. However, according to Figure 4.1, the drugs VPA or 5-Aza has not played any positive role in total cell amplification in the medium supplemented with SF. While in the medium with SF+IL3, the addition of VPA increased cell number by 4.2 fold more than 5-Aza. This might be attributed to the toxic effect of 5-Aza to cells, which could be seen by light microscopy.

4.3.2 The immunophenotype variance of CD34$^+$ cells after different treatments

The CD34$^+$ cell frequency was (27±4) % using MACS. After culturing the same quantity of CD34 enriched cells in different medium for eight days, the frequency of CD34$^+$ cells changed. Comparing the SCF+Flt3L group (including

SF, SF+VPA, SF+5-Aza) with SCF+Flt3L+IL3 group (SF+IL3, SF+IL3+VPA, SF+IL3+5-Aza), we find that the combination of SF or SF+5-Aza could help maintain and increase the frequency of CD34$^+$ cells slightly more than SF+IL3 group, but they were generally comparable. (see table 4.2)

	%D0	%control D8	%VPA D8	%5-Aza D8
SCF+Flt3L	27±4	40±3	61±4	54±6
SCF+Flt3L+IL3	27±4	26±3	59±4	38±7

Table 4.2: The frequency of CD34$^+$ cells prior to and following drug treatment

Flow cytometry results show that without IL3 there is little effect on CD36 expression on CD34$^+$ cells regardless of whether VPA, 5-Aza, or a combination of both is used. However, when IL3 is added to the culture medium there is a profound effect on the numbers of CD34$^+$/CD36$^+$ double positive cells, with this population increasing.

Although we found an increase in the number of glycophorin A (GPA/CD235a) positive cells in cultured mobilised peripheral blood in our experiments, the number of CD235a cells increased in four of the eight mobilised bloods, whereas the other four showed no increase, mPB498 (Figure 4.2) is an example where no increase occurred and mPB505 did show an increase of CD235a (Figure 4.2). Interestingly, all three myeloma patient samples increased in CD235a, the other increase was observed in a lymphoma patient sample, indicating that this could be a patient-specific effect.

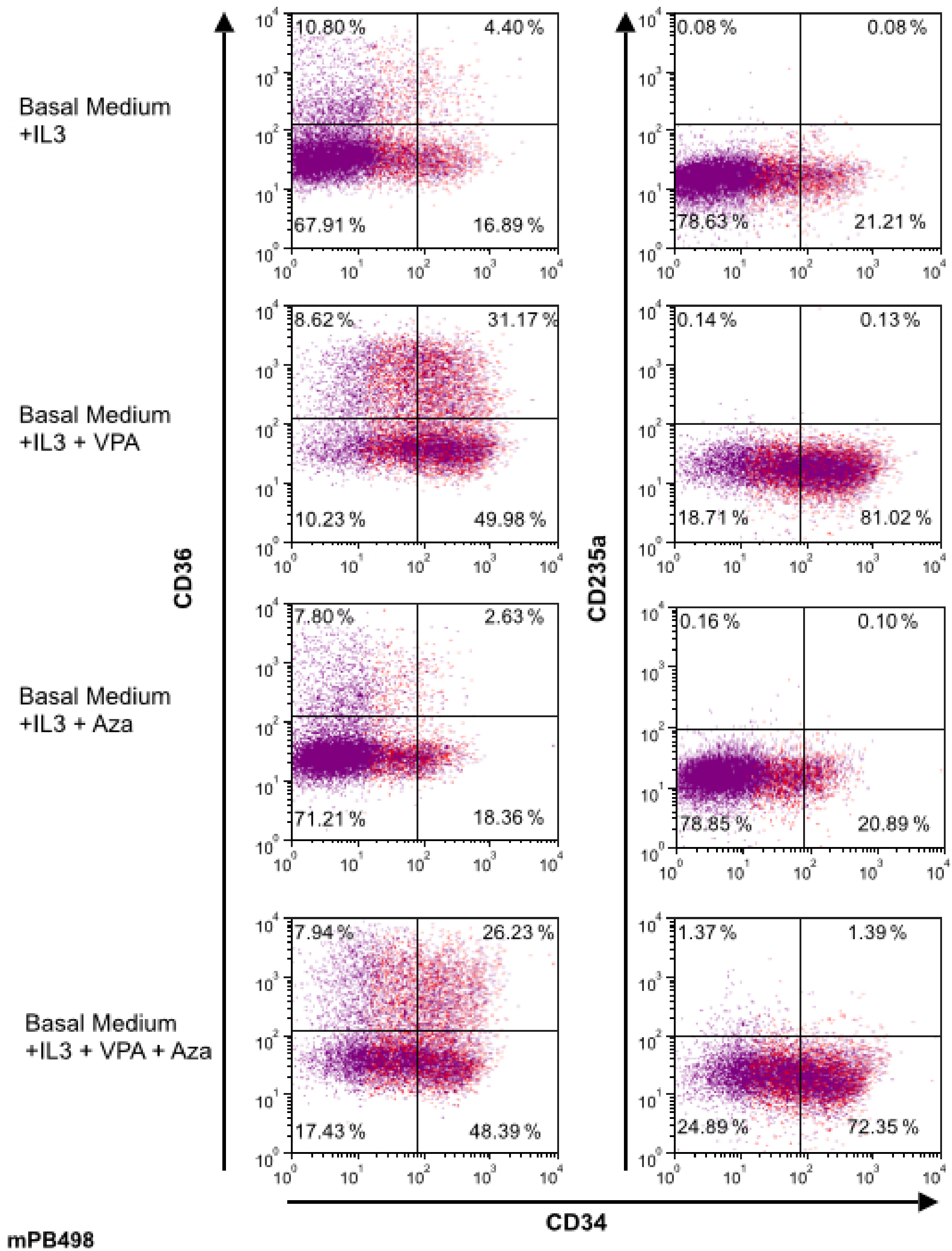
When cells from CD34 enriched fraction were cultured in the medium supplemented with different cytokine combinations and chromatin modification

reagents for eight days, an appropriate quantity of cells were collected for immunophenotypic analysis. According to Figure 4.3, a small population of cells deriving from SCF+Flt3L groups (including SF, SF+VPA, SF+5-Aza) was CD235a$^+$CD36$^+$CD34$^-$ which is a red cell population, while this population cannot be found in SCF+Flt3L+IL3 groups. And the cytokine combination of SF+VPA could help CD34 enriched cells generate a relatively larger population of CD235a$^+$CD36$^+$CD34$^-$ in contrast to SF and SF+5-Aza.

Flow cytometry results show that without IL3 there is little effect on CD36 expression on CD34$^+$ cells regardless of whether VPA, 5-Aza, or a combination of both is used. However, when IL3 is added to the culture medium there is a profound effect on the numbers of CD34$^+$/CD36$^+$ double positive cells, with this population increasing (Figure 4.4). This phenomenon is restricted to cells treated with VPA, when 5-Aza is added there is no observed effect, while using a combination of VPA and 5-Aza returns the numbers to near the value of the VPA-only treated cells (Figure 4.2). Statistically, the hypothesis that there is no difference with VPA in the culture when IL3 is present can be rejected at $P<0.01$ using a paired t test. A graphical representation of the effect of the various treatments on CD34$^+$/CD36$^+$ cell totals is given in the box plot, Figure 4.4.

4.3.3 The number of CD34$^+$ cells in the medium supplemented with different cytokine combinations and chromatin modification reagents

The CD34 enriched fraction was not pure, so we assessed the number of CD34$^+$ cells in the CD34 enriched fraction after eight days culture, which could give a general view of self-renewal capacity of stem cells. We used flow cytometry to determine the phenotype of CD34 enriched cells and then gated the CD34$^+$ cells


Basal Medium
+IL3
Basal Medium
+IL3 + VPA
Basal Medium
+IL3 + Aza
Basal Medium
+IL3 + VPA + Aza
CD36
CD235a
CD34
mPB498
10.80 %
4.40 %
67.91 %
16.89 %
0.08 %
0.08 %
78.63 %
21.21 %
8.62 %
31.17 %
10.23 %
49.98 %
0.14 %
0.13 %
18.71 %
81.02 %
7.80 %
2.63 %
71.21 %
18.36 %
0.16 %
0.10 %
78.85 %
20.89 %
7.94 %
26.23 %
17.43 %
48.39 %
1.37 %
1.39 %
24.89 %
72.35 %

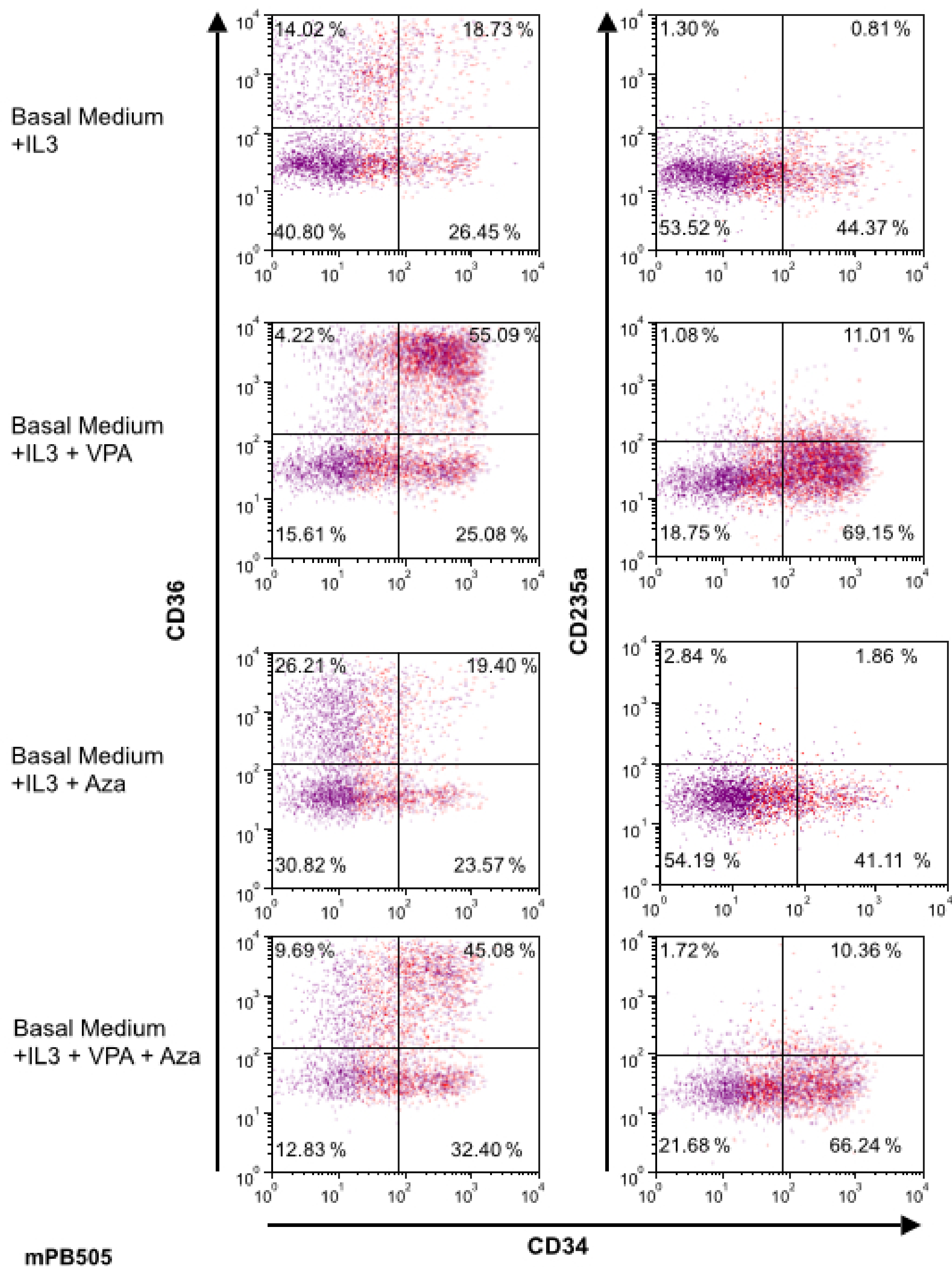
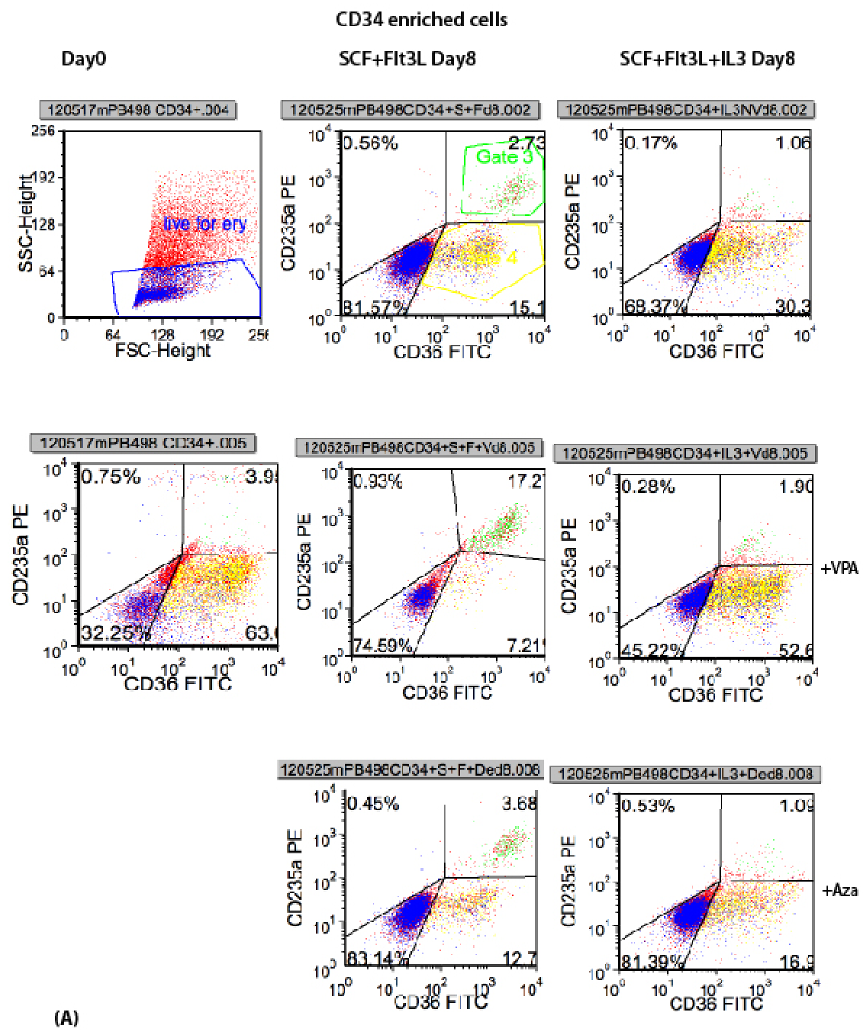


Figure 4.2: **Two example of the immunophenotype of CD34 enriched cells after culturing for eight days.**


CD34 enriched cells
Day0
SCF+Flt3L Day8
SCF+Flt3L+IL3 Day8
SSC-Height
FSC-Height
live for ery
CD235a PE
CD36 FITC
Gate 3
0.56%
2.73
81.57%
15.1
0.17%
1.06
68.37%
30.3
0.75%
3.9
32.25%
63.
0.93%
17.2
74.59%
7.21
0.28%
1.90
45.22%
52.6
+VPA
0.45%
3.68
83.14%
12.7
0.53%
1.09
81.39%
16.9
+Aza
(A)

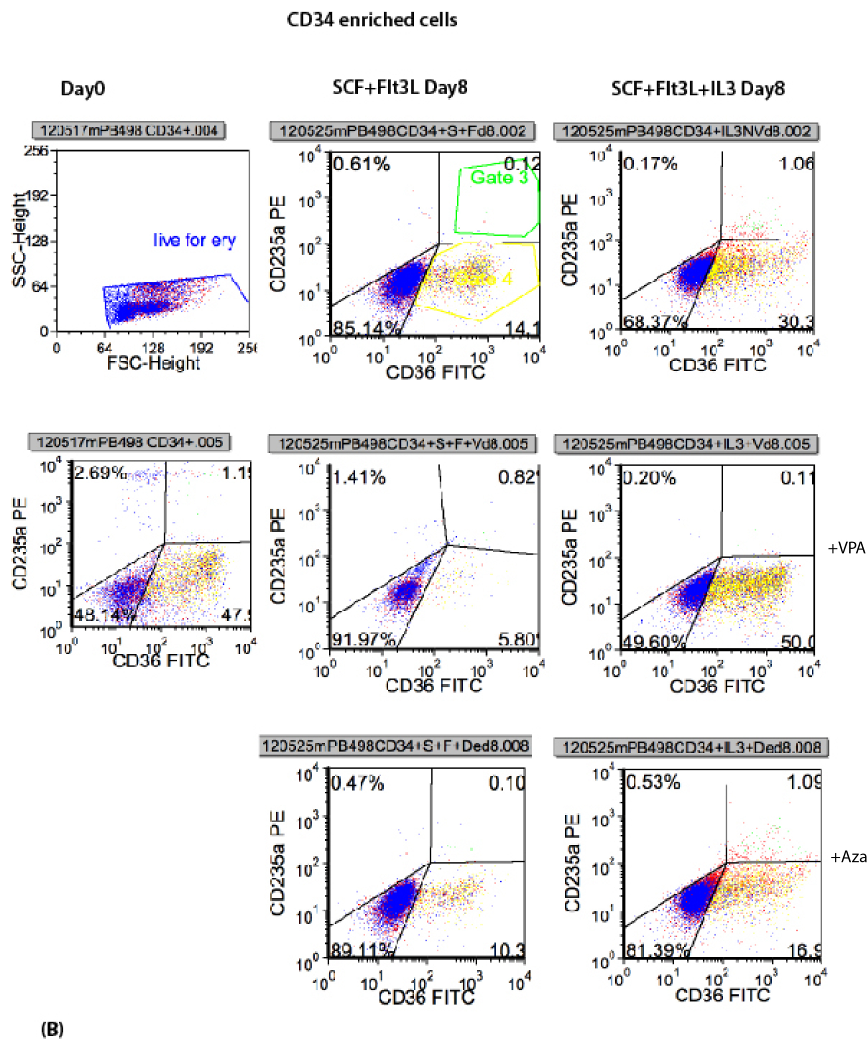


Figure 4.3: **Immunophenotype of CD34 enriched cells prior and after culturing for eight days.**

When CD34 enriched cells were cultured in serum containing medium supplemented with cytokine combinations SCF+Flt3L (SF) or SCF+Flt3L+IL3 (SFL3) with the addition of chromatin modification reagents (VPA or 5-za-dC) for eight days, an appropriate quantity of cells were collected and stained. (A) showed immunophenotype of CD34 enriched cells when cells distributed in most levels of side scatter were gated. A small population of CD235a^{+}CD36^{+} cells (green color cells) only appeared in SCF+Flt3L groups (including SCF+Flt3L, SCF+Flt3L+VPA and SCF+Flt3L+5-Aza-dC) but not in SCF+Flt3L+IL3 groups. (B) demonstrated CD34 enriched cells distributed in low side scatter. Compared to (A), we cannot find CD235a^{+}CD36^{+} population (green cells) when we set the gate in low side scatter. Since CD34^{+} cells are localizing in low side scatter areas, CD235a^{+}CD36^{+} population (green cells) is CD34^{-}.

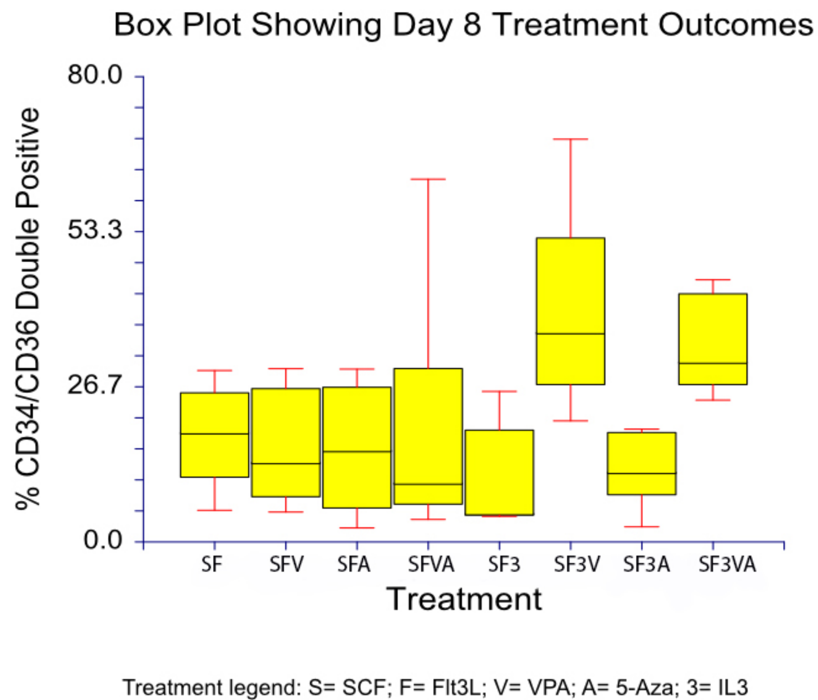


Figure 4.4: The frequency of CD34$^+$/CD36$^+$ population from CD34 enriched cells after eight days treatment.

When CD34 enriched cells were cultured in basic medium (IMDM+10%FCS+P/S) supplemented with SF without IL3 (SF, SFV, SFA, SFVA group), there is little effect on CD36 expression on CD34$^+$ cells regardless of whether VPA, 5-Aza or a combination of both is used. But when IL3 was added, the use of VPA evidently increased the frequency of CD34$^+$/CD36$^+$ double positive population (SF3V, SF3VA). N = 8, For SF3V P =0.000547; for SF3VA P = 0.001458.

in the histogram followed by calculation of the exact number. The CD34$^+$ cell number is shown in table 4.3. When VPA was employed in the medium supplemented with SCF, Flt3L and IL3, the number of CD34$^+$ cells increased the largest degree (13.4±2.3 fold) compared to other combinations. The CD34$^+$ cell number deriving from SF+IL3 group with different drugs (SF+IL3, SF+IL3+VPA, SF+IL3+5-Aza) was higher than SCF+Flt3L group with the same drugs (SF, SF+VPA, SF+5-Aza)(table 4.3).

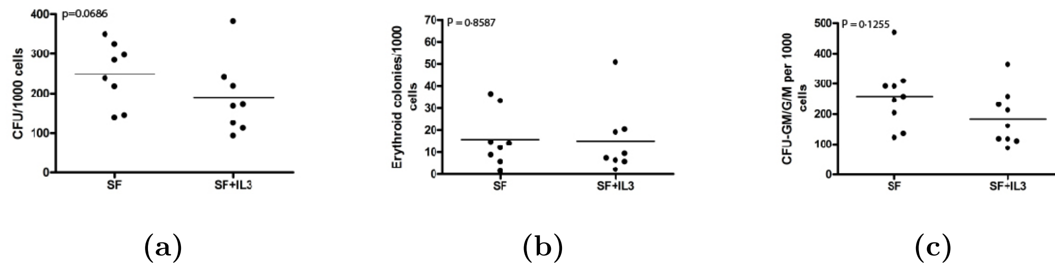
Increasing fold of CD34$^+$	control D8/D0	VPA D8/D0	5-Aza D8/D0
SCF+Flt3L	2.6±0.6	2.0±0.8	1.8±0.6
SCF+Flt3L+IL3	7.2±1.3	13.4±2.3	2.9±0.5

Table 4.3: Increasing fold of CD34$^+$ cells following drug treatment
Given the different number of cells at the beginning of culture, cells number is expressed by their increasing fold, which is cell number on Day8/Day0.

4.3.4 Comparison of haematopoietic stem cell functionality of CD34 enriched cells after treating with SCF+Flt3L or SCF+Flt3L+IL3

According to Figure 4.5a, the mean number of colonies generated by CD34 enriched cells after culturing with SF or SF+IL3 is different but does not demonstrate a significant difference. As Figure 4.5b and 4.5c indicate, the ability to generate erythroid colonies and myeloid cell colonies (CFU-GM/G/M) by CD34 enriched cells after culturing in basic medium supplemented with SF or SF+IL3 are not significantly different.
Generally speaking, the addition of IL3 efficiently improves cell proliferation, while haematopoietic functionality of CD34 enriched cells after culturing with SF+IL3

does not change compared to SF according to the data we collected from mobilized blood. The capacity of colony-forming unit of CD34e cells after treating with SF or SF+IL3 is equivalent.



(a) (b) (c)

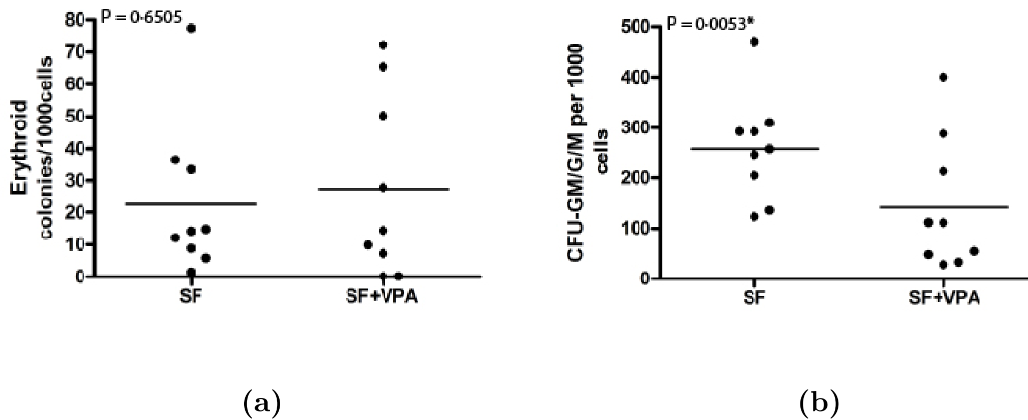
Figure 4.5: (a–c)CFU, erythroid colonies and white cell colonies generated by CD34 enriched cells after culturing in basic medium with SF or SF+IL3 for eight days.

The mean number of total colonies generated by CD34 enriched cells after culturing with SF or SF+IL3 does not demonstrate a significant difference (a), nor did erythroid colony (b) or white cell colony numbers (c).

4.3.5 Does the addition of drugs including VPA and 5-Aza exert an effect on cultured cells?

The effect of VPA in haematopoietic stem cell functionality of CD34 enriched cells when in combined use with SF

From Figure 4.6, when cultured in basic medium with SF, the addition of VPA has not increased the potential of CD34 enriched cells differentiating into erythroid cells, whereas it leads to a statistically significant reduction in myeloid cell colonies. However, there is not a significant change from the stand point of percentages of erythroid colonies and myeloid cell colonies.



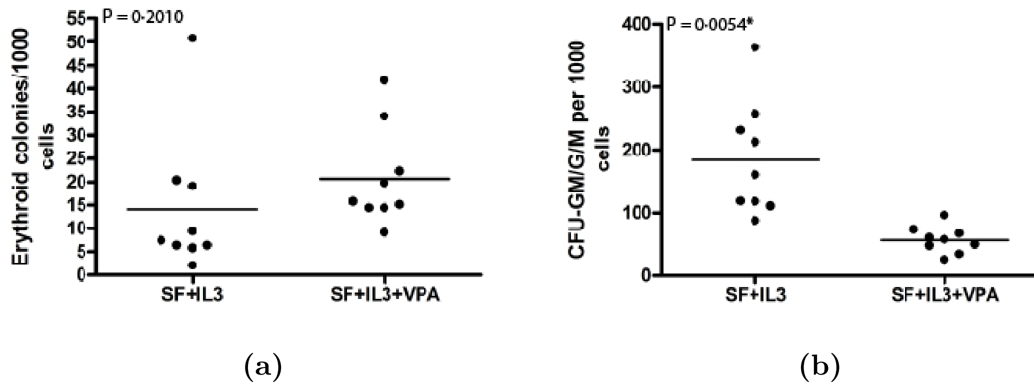
(a) (b)

Figure 4.6: The generation of erythroid colonies and myeloid colonies deriving from CD34 enriched fraction after treatment with SF or SF+VPA **Statistically significant.*

The effect of VPA in haematopoietic stem cells functionality of CD34$^+$ cells when in combined use with SF+IL3

As Figure 4.7 shows, the addition of VPA in the medium with SF+IL3 has not changed the erythroid colonies forming ability of CD34 enriched fraction, but significantly reduced the potential of the CD34 enriched fraction to differentiate into myeloid cell colonies.

Though the actual number of erythroid colonies has not changed, the number of myeloid colonies was reduced. This resulted in the relative proportion of erythroid colonies produced by CD34 enriched cells after treatment with SF+IL3+VPA to be higher than the treatment of SF+IL3. (27% vs 7%)



(a) (b)

Figure 4.7: The generation of erythroid colonies and white cell colonies deriving from CD34 enriched faction after treatment of SF+IL3 or SF+IL3+VPA.

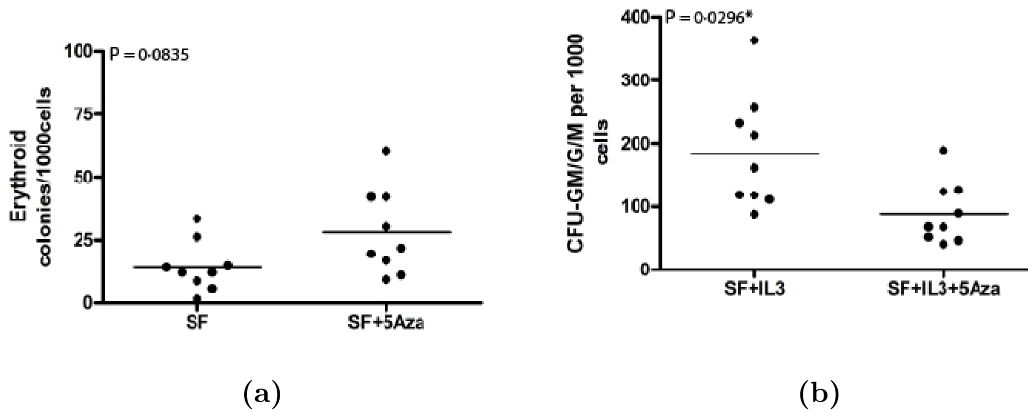
**Statistically significant.*

The effect of 5-Aza in haematopoietic stem cells functionality of CD34$^+$ cells when in combined use with SF

Figure 4.8 demonstrates that the treatment of SF+5-Aza has not resulted in significant changes in the potential of CD34 enriched cells differentiating into erythroid cell colonies or myeloid cells colonies. Although the percentage of erythroid colonies has increased 9% by the combination of SF+5-Aza, this is not statistically significant.

The effect of 5-Aza in haematopoietic stem cells functionality of CD34 enriched cells when in combined use with SF+IL3

As Figure 4.9 shows, the supplement of SF+IL3+5-Aza in the basic medium has not changed the erythroid colony forming ability of CD34 enriched cells, but has significantly reduced the potential of CD34 enriched cells to differentiate into myeloid cell colonies.



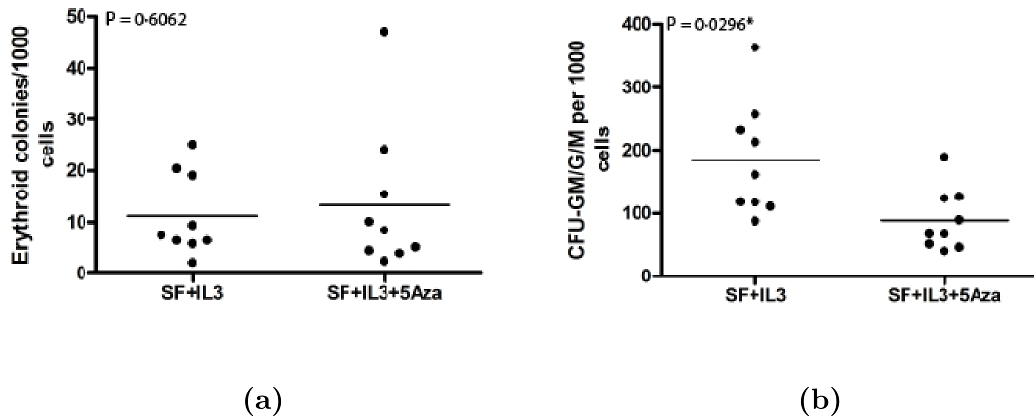
(a) (b)

Figure 4.8: The production of erythroid colonies and white cell colonies by CD34 enriched fraction after treatment of SF or SF+5-Aza.
**Statistically significant.*

4.4 Discussion

4.4.1 The effect of IL3 and chromatin modification reagents (VPA, 5-Aza) in cell proliferation

An intractable problem for *ex vivo* expansion of HSPC for further application in haematopoietic cell transplantation and gene therapy is a concomitant loss of self-renewal and reconstitution potential in expanding HSPC. Thus it has been an important strategy to find a way of maintaining HSPC self-renewal capacity. When $CD34^{+}$ cells are purified through MACS, a limited number of $CD34^{+}$ cells are obtained for expansion. From our experience in expanding $CD34^{+}$ cells, 100 ng/ml SCF and10 ng/ml Flt3L are the optimal dosages of cytokines in the culture medium (Tura *et al.*, 2007b). Since IL3 was reported to promote stem cell survival (Didichenko *et al.*, 2008; Epling-Burnette *et al.*, 2001; Weinmann *et al.*, 1999; Yousefi *et al.*, 1996) and has been in widespread use, we designed comparison experiments to check the effects of IL3 in synergy with other factors in culture



(a) (b)

Figure 4.9: The generation of erythroid colonies and white cell colonies deriving from CD34 enriched cells after treatment of SF+IL3 or SF+IL3+5-Aza.

**Statistically significant.*

medium. In addition, chromatin modification reagents are pivotal in regulating gene expression and have been reported to be involved in haematopoietic cells proliferation and differentiation. In order to further explore the functions of chromatin modification reagents in $CD34^+$ cells proliferation and differentiation, the HDAC inhibitor valporic acid and methylation inhibitor 5-Aza were utilized in our experiments.

Within the basic medium supplemented with SCF+Flt3L, the addition of IL3 played an active role in cell proliferation. Without the utilization of chromatin modification reagents, the cytokine combination of SCF, Flt3L and IL3 increased cell number by 8.4 fold on average, much higher than the other combination. In our experiments, the enrichment of $CD34^+$ cells using MACS microbeads does not yield 100% frequency. Therefore, an appropriate measurement is required to be developed to assess the number and quality of $CD34^+$ cells after eight days culture. In our work, we made use of flow cytometry to help calculate the $CD34^+$ cells in the CD34 enriched fraction. The number of $CD34^+$ cells in the CD34

enriched fraction has been increased with the addition of IL3 compared to the control. These demonstrate that the addition of IL3 to the *in vitro* cell growth medium improved the expansion of $CD34^+$ cells.

The addition of chromatin modification reagents VPA or 5-Aza to the medium did not increase the number of CD34 enriched cells in the medium supplemented with SCF and Flt3L alone. In contrast, when VPA or 5-Aza were added to the basic medium with the addition of SF+IL3, VPA improved cell amplification in synergy with IL3 whereas the effect of 5-Aza in proliferation of $CD34^+$ cells is not obvious, in accordance with De Felice and colleagues' work (De Felice *et al.*, 2005). Generally speaking, VPA enhances the proliferation of HSPC in combination use of cytokine SF+IL3.

When CD34 enriched cells were treated with different cytokine combinations and chromation modification reagents, the immunophenotype of cells concomitantly changed. The appearance of the small population $CD235a^+CD36^+CD34^-$ were from CD34 enriched fraction cultured with SCF+Flt3L groups (including SF, SF+VPA, SF+5-Aza), indicating the cytokine combination of SF is beneficial for erythroid cell differentiation since CD235a is a maker of erythroid cells. However, this does not contradict the colony assay result, since erythroid cells except the primitive ones do not have the potential to form erythroid colonies. In addition, our work demonstrated an increase in the number of $CD34^+/CD36^+$ double positive erythroid progenitors when CD34 enriched cells were cultured with the addition of IL3 and VPA, but this phenomenon is restricted to cells treated with VPA whereas the addition of 5-Aza did not, indicating the synergistical effect of IL3 and VPA in enhancing erythroid development *in vitro* culture.

4.4.2 The functionality of HSPCs after expansion with SF or SF+IL3

According to our results, the frequency of CD34$^+$ cells after culturing in cytokine combination of SF is higher than treating with SF+IL3 (40% ± 3% vs 26% ± 3%), but their colony-forming unit potential are equal. With regard to the erythroid lineage and myeloid lineage forming ability, there is not a significant change between CD34 enriched cells cultured in the medium supplemented with SF+IL3 and SF. Therefore, the haematopoietic functionality of CD34 enriched cells after culturing with SF+IL3 remains similar to the medium with SF. Above all, the addition of IL3 in the culture medium supplemented with SCF and Flt3L not only improves the expansion of HSPC in a large degree, but also maintains the haematopoietic functionality of HSPCs.

4.4.3 The effect of epigenetic altering reagents in functionality of expanded HSPC

Regardless of the cytokine combination of SF or SF+IL3, VPA reduced the potential of CD34$^+$ cells differentiating into myeloid cells lineages *in vitro* semisolid culture, but has not produced a significant difference in erythroid cell colony-forming unit. Due to the decreased number of myeloid cell colonies, the proportion of erythroid cell colonies increased relatively after adding VPA to the media supplemented with SF+IL3. This gives an appearance of an increase in numbers of red cell colonies, but this is a ratio effect rather than a total red cell colony number effect due to a decrease in CFU-G/GM/M colony formation. Chaurasia and colleagues' study (Chaurasia *et al.*, 2011) showed the role of VPA in promoting the preferential differentiation of cord blood CD34$^+$ cells to endothelial cell progenitors (EPC) *in vitro*, partly in accordance with our result such as the

proportion of erythroid colonies and the increasing number of $CD34^+/CD36^+$ double positive erythroid progenitors. But from the actual number of erythroid colonies, we have not observed a general increase after adding VPA although this has occurred on two samples out of nine occasionally. Considering the differences of the two studies, their $CD34^+$ cells were isolated from cord blood and expanded in the cytokine combination of SCF, Flt3L, TPO and IL3 for seven days, while the cytokines in our media included SCF, Flt3L and IL3. In addition, the specific dose for every cytokine is different depending on the experience of various groups.

As mobilised peripheral blood is capable of repopulating human bone marrow, it seems unlikely that there is any inherent deficit in the stem and progenitor cells used in these assays. We speculate that what is happening is that the use of GCSF predisposes those stem and progenitor cells already committed to being granulocytes and macrophages to be refractory to further *in vitro* expansion. In contrast, the erythroid and uncommitted stem and progenitor populations are affected by HDACIs and hence expand in number and move down the erythroid and megakaryocyte lineage pathway. This would explain why Chaurasia and colleagues found no difference in the ratios of white cell colonies when comparing those cells that had been expanded with and without VPA, i.e. cord blood stem and progenitor cells have no pre-exposure to recombinant cytokines. Therefore, in *ex vivo* assays of cytokine naïve stem and progenitor cells there is a predisposition to make erythroid and megakaryocytic lineages, however G,M, and GM progenitors are still able to expand. It is also possible that the ability of VPA to enhance erythroid and megakaryocytic development is purely an artefact of *in vitro* tissue culture: side effects in patients who are treated with sodium valproate include anaemia, leucopenia and pancytopenia, these being the antithesis of blood cell expansion. However, there has been a report that fetal hemoglobin synthesis increased in epileptic children receiving VPA, but analysis of this data showed that overall hemoglobin levels were actually lower in the VPA study group compared

to the control group (Kieslich *et al.*, 2003). Therefore, although VPA could find utility in *ex vivo* expansion of erythroid and megakaryocytic cells and progenitors, it does not have this effect *in vivo*.

According to our experiments, the frequency of CD34$^+$ cells in CD34 enriched fraction after culture in the medium supplemented with 5-Aza increased but their colony-forming unit capacity did not increase as expected. In addition, 5-Aza is quite toxic to cells, which could be observed under the light microscope when cells were treated with 5-Aza for eight days. In the UK and the US the drug decitabine (5-Aza) is used for the treatment of myelodysplastic syndromes, chronic myelomonocytic leukaemia and acute myeloid leukaemia (NICE, March 2011). Therefore, it is unsurprising that the *in vitro* effect of this drug is to lower the numbers of white cell colonies in CFU assays. However, it is of interest that this effect is only apparent when IL3 is also part of the culture medium.

The utility of VPA and 5-Aza for *in vitro* expansion of HSC and HPC suggests that these molecules are, at least in part, taking the place of an *in vivo* pathway that allows blood stem and progenitor cell division and expansion without the epigenetic changes associated with differentiation. One such pathway involved in globin gene synthesis involves the transcription factor ZBP-89 which interacts with GATA-1 and NF-E2 (Nuclear Factor, Erythroid 2)/MAFK (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K). Reduction in ZBP-89 (BFCOL1, BERF-1, ZNF 148) levels leads to decreased histone acetylation and a consequent reduction in globin gene syntheses, which can be partially restored by addition of VPA (Woo *et al.*, 2011). Future research may elucidate how to manipulate such pathways using biological methods and small molecules inter alia. These could be utilized in the search for an *ex-vivo* source of clinical-grade erythrocytes, e.g. derived from iPS cells.

4.5 Concluding remarks

IL3 is an important cytokine in improving $CD34^+$ cell amplification and maintaining the functionality of $CD34^+$ cells. VPA and IL3 also work synergistically to increase the numbers of $CD34^+/CD36^+$ double positive erythroid progenitors. In the cytokine (SF+IL3) induced culture, VPA increased $CD34^+$ cell number and reduced the potential of CD34 enriched cells differentiating into myeloid cells lineages.

Chapter 5

Assessment of methods for delivering mRNAs and plasmid DNA into cells

5.1 Introduction

The successful delivery of mRNAs or plasmids into cells is a very important step in genetic manipulation. Most viral methods are widely applied in regulating gene control but there is the risk of insertional mutagenesis and difficulty in controlling expression. Non-viral methods are a different way to deliver mRNAs and plasmids. The delivery methods are required to be effective, so the transfection efficiency should be high enough to allow most cells to express a delivered gene of interest in order for the gene product to function downstream. Simultaneously the highly efficient method should not cause serious damage to cells and needs to maintain cell viability. However, generally speaking, with regards to most non-viral methods, high transfection efficiency usually leads to a relatively higher cell

death rate so we have to seek out a balance between transfection efficiency and cell viability. In this chapter, I will study different non-viral methods for delivering mRNAs or plasmids into cells, looking at efficiency and viability.

5.2 Experimental strategy

Non-viral methods of transfection include electroporation and using different chemical transfection reagents. To decide which method to use for delivery, many factors have to be considered such as cells that are to be targeted, delivery material, size, how long the expression of targeted genes is required, and the downstream work. For mRNA, the essential modifications including the addition of cap structure and polyA tail are necessary to help significantly reduce their toxicity to transfected cells and also increase the half-life. Considering the short half-life of mRNA, we designed a simple experiment to measure the expression of GFP mRNA indirectly using flow cytometry to check the expression of GFP protein. With the knowledge of the robust expression of mRNA, we tried electroporation and chemical methods to deliver mRNA of targeted genes into cells. Since duration of the expression of mRNA is limited, repeated transfections have to be operated if the expression of mRNA of interest is required in downstream work. Electroporation caused more damage to cells so it is impossible to electroporate cells repeatedly. Thus we tried chemical methods to transfect cells repeatedly to observe the cell viability and efficiency. DNA modification reagents VPA and 5-Aza facilitate gene expression through regulating epigenetics, which enable us to see whether DNA modification reagents are beneficial for increasing the expression of delivered genes. So we tried to pre-culture cells with DNA modification reagents for four days followed by electroporating cells with GFP mRNA. As for plasmid delivery without drug selection, the transfection is

not permanent but still can maintain expression longer than mRNA. We tried electroporation and chemical methods to deliver GFP plasmids into cells to compare the expression period and efficiency.

5.3 Results

5.3.1 The persistence of *in vitro* synthesized mRNA measured indirectly by the expression of corresponding protein

As we know, the half-life of mRNA is very short. For example, the mRNA of bacteria and yeast only have 2 minutes and 1-60 minutes life respectively, and for animal cells their mRNA can last 4-24 hours. Thus, we have to consider how long the *in vitro* synthesized mRNA using mMESSAGE mMACHINE kit (see section 2.15.1) could exist in cells. To simplify the process and optimise economy, we indirectly learned about the maintenance of mRNA in cells *in vitro* through measuring the expressed GFP protein using flow cytometry translated by GFP mRNA, which have been transfected into cells using chemical methods. In our lab, we chose pcDNA3.1 EGFP-poly (A83) as the template to synthesize GFP mRNA *in vitro* through mMESSAGE mMACHINE kit. After verifying the purity of GFP mRNA, they were electroporated into U937 cells (non-adherent cells) followed by culturing in IMDM+10% FCS overnight. After 24, 48, 72, 96, 144 hours, transfected cells were collected to assess GFP expression using flow cytometry. The successful translation of GFP mRNA in U937 cells could be observed by being fluorescence positive in flow cytometry. As shown in Figure 5.1, GFP was expressed until 72 hours post electroporation, but the expression had decreased by more than 50%.

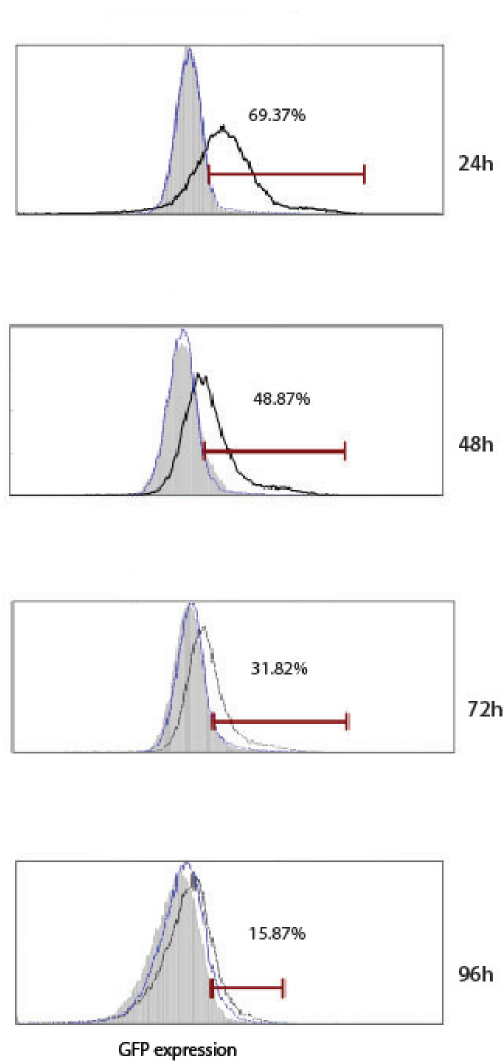


Figure 5.1: The GFP expression on different time points after *in vitro* synthesized GFP mRNA electroporation into U937 cells.

U937 cells were electroporated with GFP mRNA synthesized in vitro through mMESSAGE mMACHINE kit and then cells were cultured in IMDM+10% FCS at 37 °C, 5% CO2. After 24, 48, 72, 96, 144 hours, transfected cells were collected and detected using flow cytometry. Here we show the GFP positive cells at different time points post electroporation.

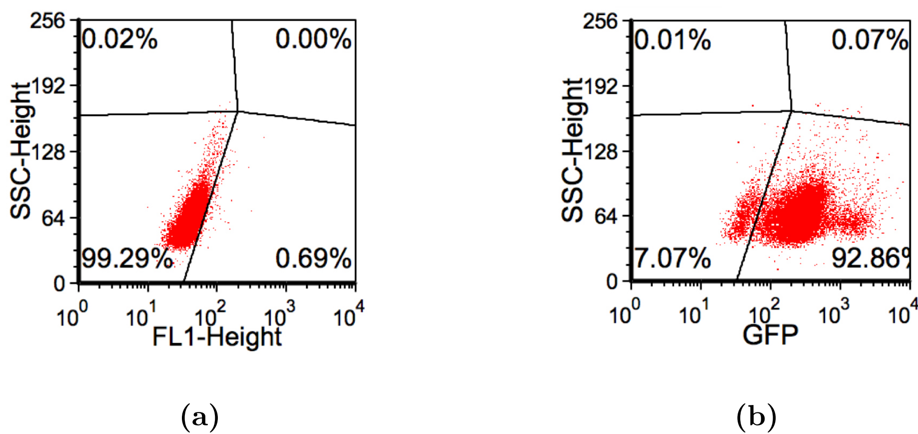
5.3.2 mRNA delivery

GFP mRNA can be successfully transfected into suspension cells by electroporation

In our experiment, we chose GFP mRNA due to its convenience as it can be detected using flow cytometry and fluorescence microscopy. 2 μg GFP mRNA was electroporated into 1.25×10^6 U937 cells using Bio-Rad electroporator in OptiMEM. After incubating at 37 °C, 5% CO_2 for 24 hours, we use flow cytometry to detect the expression of GFP. Figure 5.2 shows one example with around 93% transfection efficiency. I repeated electroporation several times and found that the efficiency of electroporating GFP mRNA into U937 cells can range from 40% to 95%.

The influence of chromatin modification reagents in electroporation efficiency of mRNA into suspension cells

To examine the effect of adding VPA or 5-Aza into the culture medium could improve transfection efficiency, we treated U937 cells with VPA or 5-Aza for four days and then electroporated cells with GFP mRNA. In three samples, the absence of VPA or 5-Aza in culture medium produced a higher transfection efficiency than the addition of VPA or 5-Aza. The addition of VPA changed transfection efficiency by 4%-12% (two samples decreased efficiency by 12%, one sample increased by 4%) compared to the untreated cells, and the treatment with 5-Aza decreased the transfection efficiency by 40%-50% compared to untreated cells. Generally speaking, the pretreatment with DNA modification reagents did not significantly increase the efficiency of electroporating GFP mRNA into U937 cells. (see Figure 5.3)



(a) **(b)**

Figure 5.2: The GFP expression of U937 cells after electroporation 24 hours

1.25×10^6 U937 cells were collected and washed twice using OptiMEM. 2 μg GFP mRNA was electroporated into 1.25×10^6 U937 cells in 300 μl OptiMEM using Bio-rad electroporator at the parameter of 300v, 150uF. Then transfected cells were cultured in IMDM + 10% FCS overnight. After 24hours, transfected cells were collected for flow cytometry to check the expression of GFP. Figure 5.2a shows the control for U937 cells electroporated in the same condition except the GFP mRNA was omitted. Figure 5.2b showed the GFP expression of U937 cells after electroporating with GFP mRNA. Compared to the control, the percentage of GFP positive cells was around 93%.

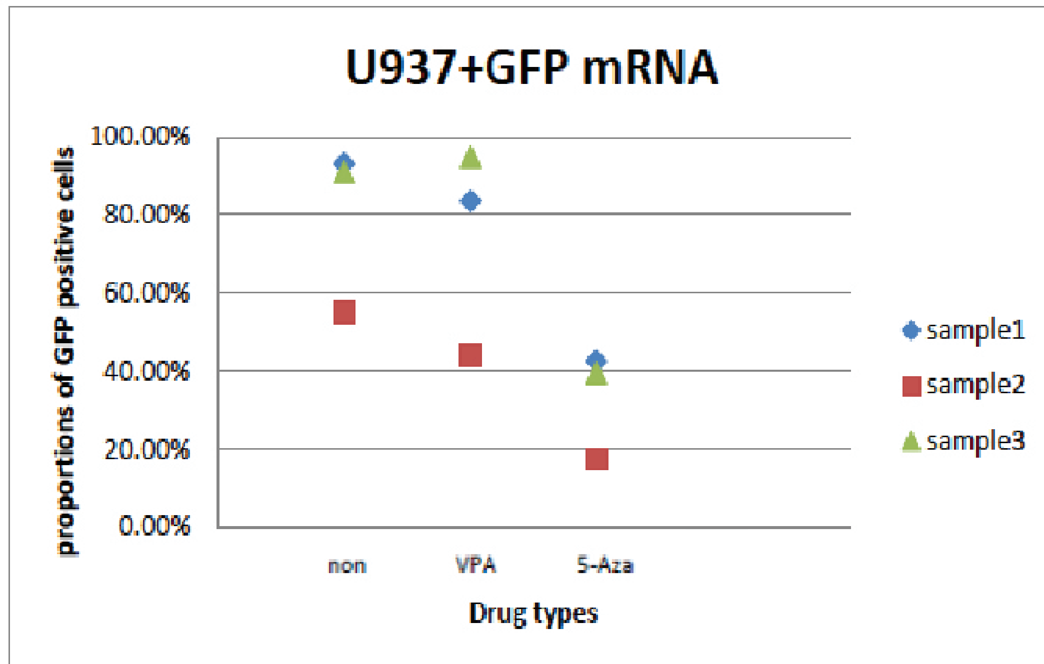
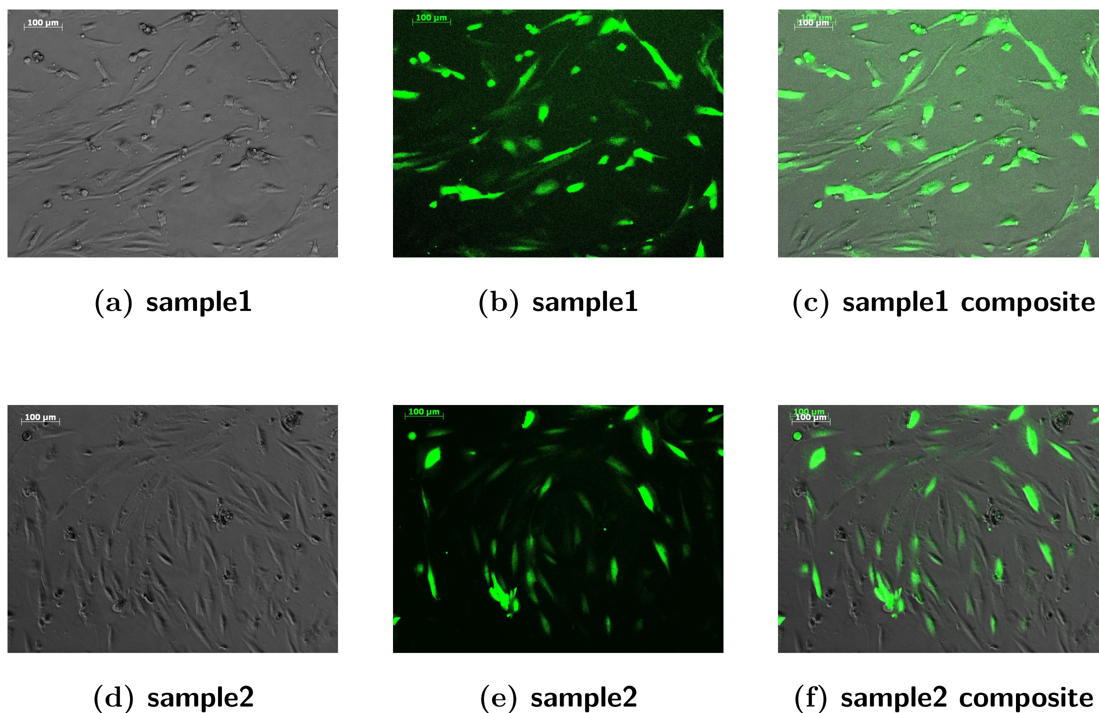


Figure 5.3: The GFP expression of U937 cells after electroporation when U937 cells were pretreated with DNA modification reagents.

U937 cells were cultured in IMDM+10% FCS with or without VPA, 5-Aza for four days. Then a quantity number of U937 cells after different treatments were electroporated with GFP mRNA. When observing the GFP expression after 24 hours using flow cytometry, Figure 5.3 shows that U937 cells without any DNA modification reagents (VPA or 5-Aza) gave a higher transfection efficiency. The treatment with 5-Aza led to a large reduction of transfection efficiency than without any DNA modification reagents. As for VPA, sample 2 and 3 showed 7%-12% decreased efficiency, while for sample3 the addition of VPA increased efficiency about 4%.

GFP mRNA was transfected into fibroblasts by chemical method

When cells grow to around 60-90% confluency, fibroblast cells from adult skin were transfected with mRNA using Mirus TransIT-mRNA transfection kit. After 24 hours, GFP expression can be observed using fluorescence microscopy. When cells proliferate in a good condition, the transfection efficiency can be optimised around 65-75%, as Figure 5.4 shows. However, when cells have to be repeatedly transfected, as many as eight times of transfection would result in 90% of cell death if just a small quantity of bFGF (4-10 ng/ml) was used in the culture medium. When the dose of bFGF was increased to 40 ng/ml, cells viability after repeated transfections improved much.



(a) **sample1** (b) **sample1** (c) **sample1 composite**

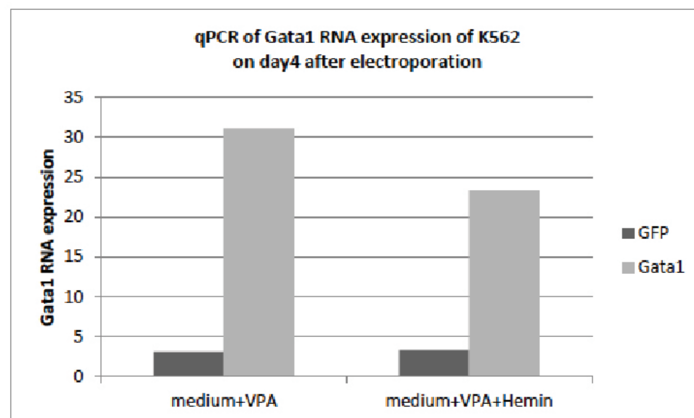
(d) **sample2** (e) **sample2** (f) **sample2 composite**

Figure 5.4: **GFP expression of adult skin fibroblasts 24 hours after transfecting GFP mRNA using Mirus transfection kit.**

Gata1 mRNA was transfected into suspension cells by electroporation

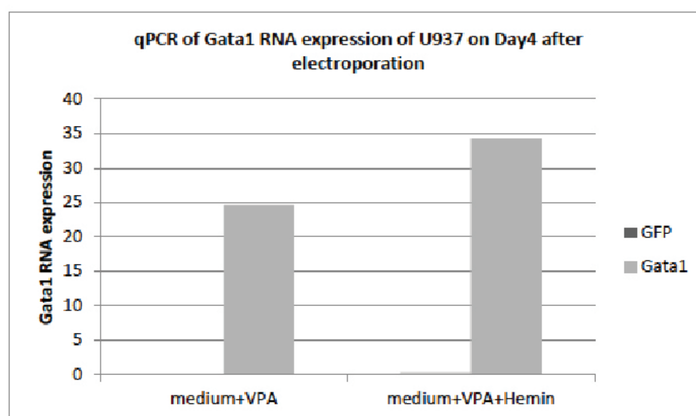
When K562 cells were transfected with GFP/Gata1 mRNA using electroporation method, transfected cells were cultured in IMDM supplemented with 10% FCS and VPA for four days followed by total cell RNA isolation and qPCR to measure the expression of transfected mRNA. Compared with GFP mRNA control, K562 cells transfected with Gata1 mRNA expressed much higher fold (31.12 vs 3.07) of Gata1 RNA (Figure 5.5a). Hemin (an iron-containing porphyrin) was reported to increase the rate of embryonic globin synthesis (Rutherford *et al.*, 1981) in K562 cell lines so hemin was tried to facilitate the expression of Gata1. When 0.5 mM hemin was added into the culture medium, K562 cells expressed 23 fold of Gata1 RNA compared with K562 cells transfected with GFP mRNA(Figure 5.5a).

Repeating this experiment in U937 cells, regardless of the addition of hemin in culture medium, the transfection of Gata1 mRNA into U937 cells increased the expression of Gata1 RNA compared to U937 cells transfected with GFP mRNA (Figure 5.5b).



Gata1 RNA expression of K562 cells	Medium+VPA	Medium+VPA+hemin
GFP transfection	3.07	3.29
Gata1 transfection	31.12	23.26

(a)



Gata1 RNA expression of U937 cells	Medium+VPA	Medium+VPA+hemin
GFP transfection	0.12	0.19
Gata1 transfection	24.59	34.3

(b)

Figure 5.5: **qPCR of Gata1 RNA expression of K562 or U937 on day 4 after electroporation with GFP or Gata1 mRNA.**

Compared with GFP mRNA control, K562 cells and U937 cells transfected with Gata1 mRNA expressed much higher fold (31.12 vs 3.07, 25 fold vs 0 fold) of Gata1 mRNA. The addition of hemin in culture did not bring about stable changes.

5.3.3 Plasmid DNA delivery

Comparison of the effect of different reagents used for transfecting pcDNA3.1 EGFP-poly (A83) into MSC cells

In our lab, there are two ways to transfect plasmid DNA into MSC cells: electroporation and chemical transfection reagents. We used chemical transfection reagents to transfect episomal reprogramming vectors into MSCs derived from fat tissues.

MSC cells were seeded at the density of 50, 000 cells per well in a 12-well plate, and transfected with 1 μg pcDNA3.1 EGFP-poly (A83) using TransIT®-LT1 Transfection Reagent (cat. No. MIR 2300), TransIT®-2020 Transfection Reagent (cat. No. MIR 5400), or jetPEI® transfection reagent (101-01N*). After 24 hours, GFP expression was observed using fluorescence microscopy (see Figure 5.6) and we found that the cells transfected using jetPEI® transfection reagent express more GFP protein than the other two transfection reagents, but it caused a higher death rate of cells after transfection and nearly half of cells were detached off from the plate. After 6 days, GFP expression could still be observed using fluorescence microscopy in all groups. But after 8 days, only the cells transfected using TransIT®-2020 Transfection Reagent expressed GFP protein. To verify the result, cells were collected from 12-well plate and used for flow cytometry, demonstrating that only cells transfected using TransIT®-2020 Transfection Reagent can express GFP after 8 days of transfection although the expression was not high (Figure 5.7).

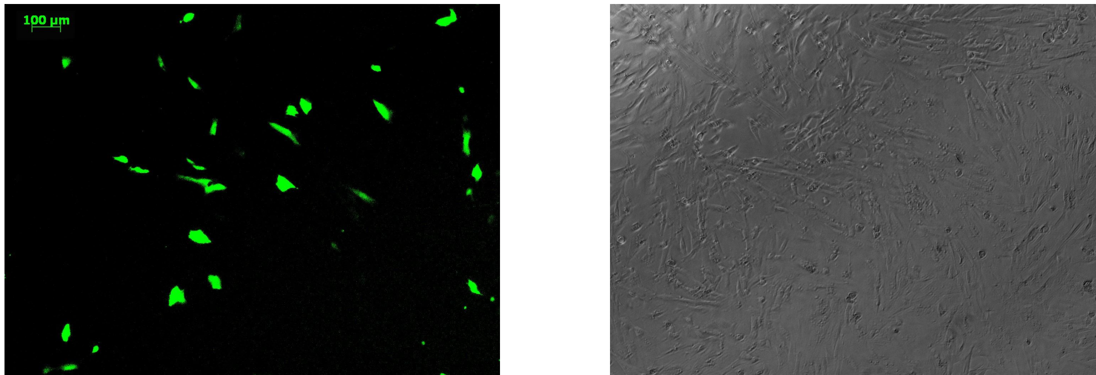
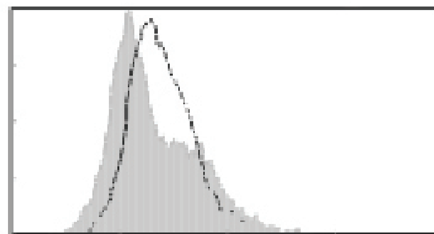
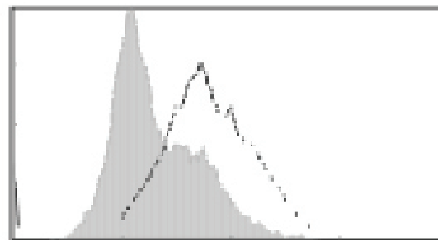


Figure 5.6: The cells 24h hours post transfection of GFP plasmid DNA using TransIT®-2020 Transfection Reagent using fluorescence microscopy.

Electroporation: How long can the expression of GFP be sustained?

When human dermal fibroblast (HDF) cells reached nearly 80-90% confluency, cells were collected and washed with serum free medium OptiMEM. 10^5 cells were electroporated with GFP plasmid using P2 primary cell 4D-Nucleofector® X kit followed by incubating in 6-well plate within fibroblast medium at 37 °C, 5% CO_2. On day 4, as many as 80-90% cells expressed GFP. With the proliferation of cells, the GFP expression was reduced gradually, and 50-60% of cells expressed GFP on day 9. Untill day 20, there were still 40-45% of cells expressed GFP. (see Figure 5.8)





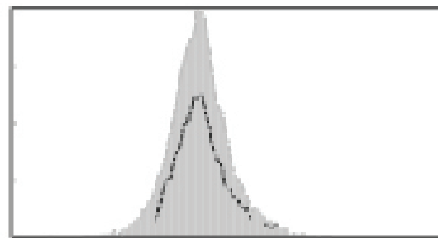
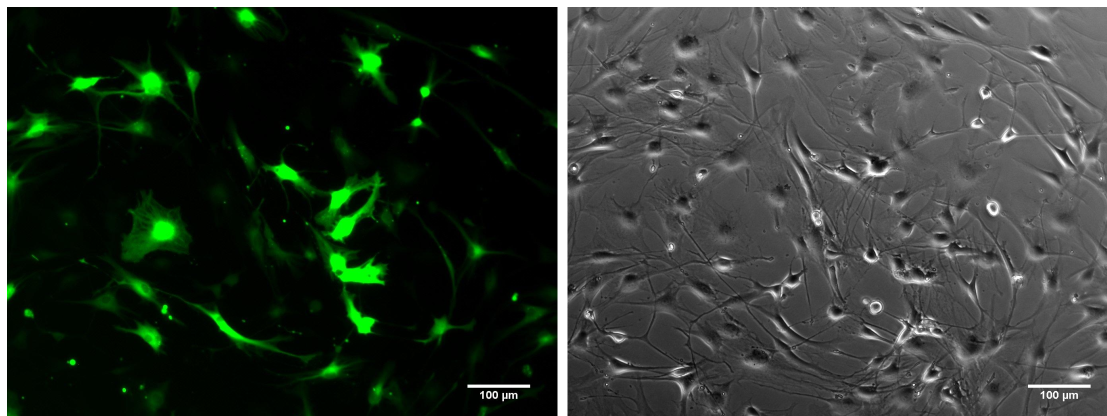
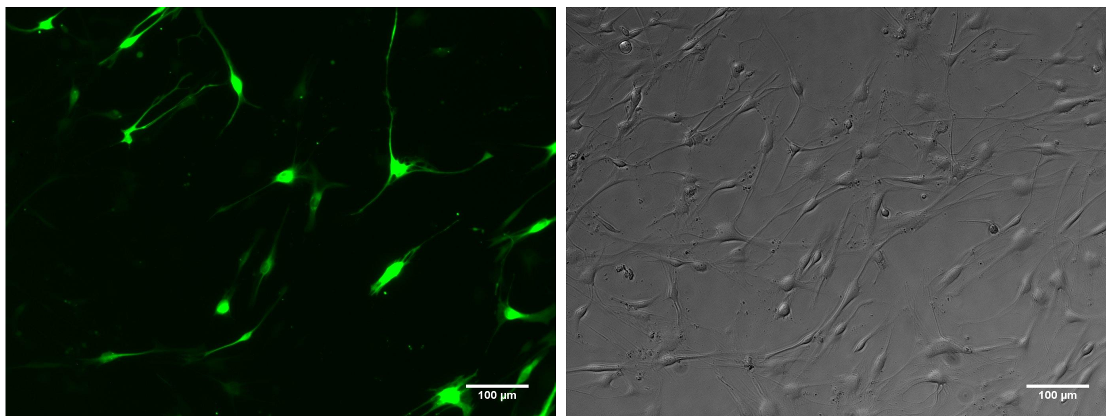


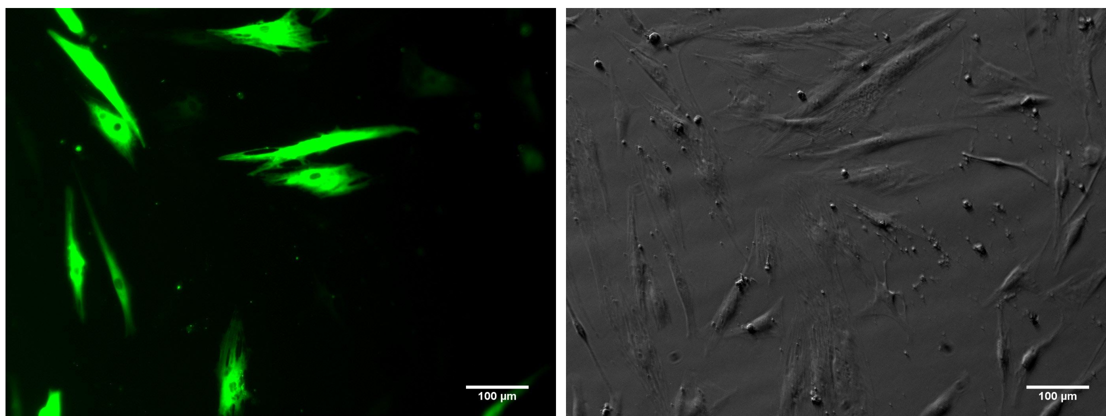
Figure 5.7: **The GFP expression on 8th day after transfection using various transfection reagents.**



(a) *Day4, 80-90%*



(b) *Day9 Plate27, 50-60%*



(c) *Day20, 40-45%*

Figure 5.8: **The GFP expression after transfecting GFP plasmids into HDF cells using fluorescence microscopy at different time points.**

5.4 Discussion

The aim of my project is to reprogram blood cells to iPS cells using non-integration methods. So we focused our delivery methods on mRNA and plasmids. It was also important that expression of delivered material was sufficient to bring about reprogramming and that viability of target cells was maintained.

5.4.1 Synthesized mRNAs can work *in vitro*, but not the priority tool for hierarchy regulation process

We successfully synthesized mRNAs *in vitro* in the lab, and they can be transfected into suspension cells and adherent cells both by electroporation and chemical methods. After a single round of transfection, their expression decreased more than 50% after 72 hours and disappeared after 96 hours through indirectly detection GFP using flow cytometry. The evidence from fluorescence microscopy and flow cytometry demonstrated that synthesized mRNAs can successfully work as a delivery tool. According to the basis, we considered using mRNAs to reprogram cells to generate iPS cell lines. Due to the limited life span of mRNA, successful reprogramming requires repeated transfections of mRNAs. This brought a serious problem to the viability of cells. In our work, eight rounds of transfections has led to 70%-80% cell death. We therefore changed to repeat transfection every other day or every two days to allow cells recover. But finally we did not successfully reprogram cells using mRNAs, only obtained partially reprogrammed cells. This might because the expressed reprogramming factors were not sufficient enough to reprogram cells due to intermittent transfections. After all, reprogramming is a complicated process and easy to be affected by many reasons. It is difficult to sort out an appropriate balance between transfection

efficiency and cell viability. mRNA is not a suitable tool for hierarchy regulation process, but can be applied in a simple process or the end of the process chain. For example, it can be used as an enzyme to function in a short period time without other requirement to remove it.

5.4.2 The delivery of Plasmid DNA into cells through electroporation can be more efficient than using chemical methods

Plasmid DNA is another useful tool for delivering transcription factors into cells. We tried to transfect plasmids into cells using both chemical methods and electroporation. Using chemical methods to deliver GFP plasmid into cells, GFP expression is not high and disappeared after eight days. However, electroporation can maintain the expression of GFP as high as 80-90% after 24 hours and on day 20, there were still 20-45% of cells expressing GFP (Figure 5.8). In addition, the special solutions from the nucleofector 4D kit helped maintain cell viability after electroporation. The powerful electroporation not only improves transfection efficiency but also maintain cell viability. Thus we finally chose electroporation using nucleofector 4D to deliver episomal vectors into cells for reprogramming. In next chapter, we presented the successful generation of iPS cell lines from blood cells and fibroblasts.

5.5 Concluding remarks

Synthesized mRNAs can work *in vitro*, and can be applied in a simple process, where the outcome is to express the product of the transfected mRNA. However, it is probably not optimal for the introduction of material to alter the hierarchical

processes involved in reprogramming as repeated rounds of transfections are required resulting in poor survival. The electroporation method to deliver plasmids using Nucleofector 4D device (lonza, USA) with the corresponding kits for different types of cells provides an efficient approach to deliver factors into cells and maintain their expression long enough for further application.

Chapter 6

iPS production

6.1 Introduction

In chapter 1, we have reviewed the used methods to reprogram somatic cells to generate iPS cells. There is the risk of insertional mutagenesis and malignant tumours when using viral methods. Therefore in our protocol we were trying to utilize non-viral methods including mRNA-mediated gene delivery or episomal plasmid DNA-based reprogramming methods, along with/without a combination of small molecules.

mRNA-mediated reprogramming is safe but requires multiple rounds of transfections. Moreover, unmodified mRNA results in damage to cells since unmodified mRNA easily induces innate immune response (Andries *et al.*, 2013), so a series of modifications, capping and polyadenylation are necessary to improve functions of mRNA deriving from genes of interest. In our lab mRNA was synthesized using ambion mMESSAGE mMACHINE kit, a 7-methyl guanosine cap at the 5' end mimics most eukaryotic mRNAs *in vivo*. The poly A tail at the 3' end of the

mRNA was provided as part of the plasmid itself. The efficiencies of different mRNA transfection methods are distinct. Electroporation could achieve greater than 90% efficiency in our experiment, and similar results were also reported by other researchers (Van Tendeloo *et al.*, 2001). But electroporation easily leads to cell death and requires a large quantity of cells in serum free system, thus it is not a feasible way to repeat electroporation. Other delivery methods have been developed such as lipofection, which employs mRNA encapsulation in cationic lipids comprising hydrophilic and hydrophobic domains, facilitating endocytosis-mediated mRNA uptake. Transfection of modified mRNA encoding GFP into six human cell types showed 50%–90% highly expression, indicating evidence of effective mRNA transfection into cells (Warren *et al.*, 2010). Therefore, mRNA transfection using lipofection makes multiple rounds of transfection feasible and allows cells to survive well for downstream applications. Similar methods are used for delivering plasmids into cells. mRNA transfection of multiple genes including Oct4, Sox2, Nanog, lin28, has been successfully applied in reprogramming fibroblast cells into iPS cells but only one paper was published. It is still worth noting that many repeated transfections have to be done in the protocol. Oct4 and Sox2 are two of the most important transcription factors in the iPS reprogramming process, and a successful case of reprogramming murine fibroblasts using lentivirus Oct4 and small molecules was reported (Li *et al.*, 2011a). In our project, we tried to use mRNA to reprogram cells in conjunction with small molecules.

Episomal vectors are popular due to their relatively longer expression and safer replication. This type of plasmid can replicate once per cell cycle extra-chromosomally and be partitioned to daughter cells stochastically. The introduction of oriP/EBNA-1 (Epstein-Barr nuclear antigen-1) in episomal vectors help increase expression of targeted genes. EBNA-1 can act in trans to increase the expression of cloned genes and does not induce a cellular immune response. In

addition, the transfected cells can lose the oriP-based plasmid gradually through cell cycle without selection. Thus established iPS cell lines will lose episomal vectors concomitant with the passage of iPS cells without any requirement to remove vectors (Leight and Sugden, 2000; Van Craenenbroeck *et al.*, 2000). In addition, it has been reported that knockdown of p53 helped improve reprogramming efficiencies and prevent differentiation (Hong *et al.*, 2009; Spike and Wahl, 2011). The introduction of mp53DD in episomal system, a dominant negative mutation of the p53 protein, allows for transient expression of the dominant negative mutant over an extended period of time (Kawamura *et al.*, 2009).

Moreover, the use of small molecules is a very promising approach to the generation of iPS cells, as substitutes for some genetic reprogramming factors in reprogramming work (Figure 6.1). The use of CHIR99021, a GSK3-β inhibitor,

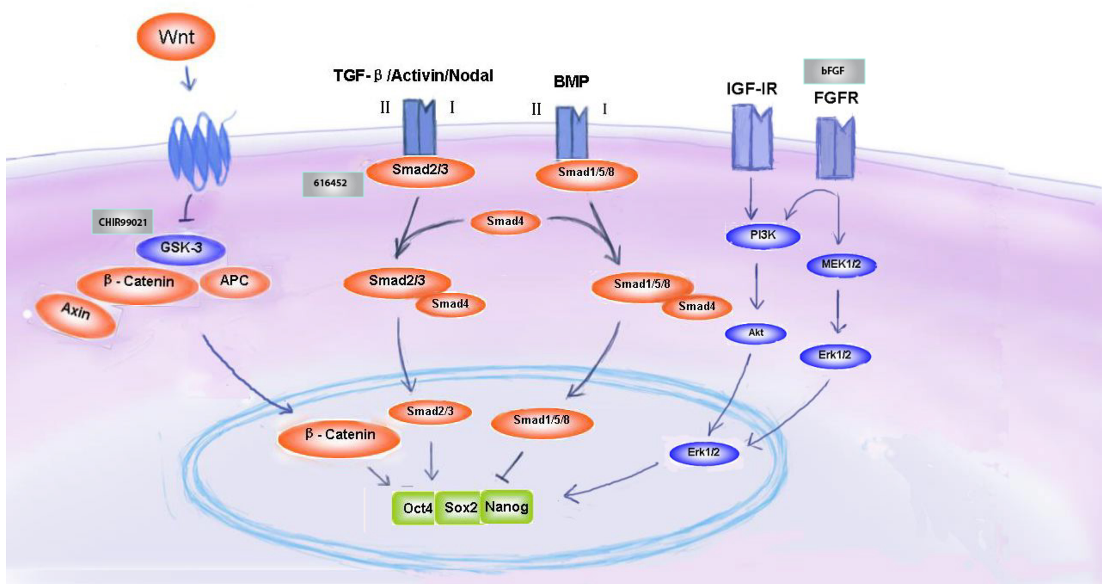


Figure 6.1: The important signalling pathway in reprogramming process

leads to the accumulation of β-catenin in the nucleus, which further collaborates

with T-cell-specific factors (TCF) to regulate the target gene expression, improving the completion and efficiency of the reprogramming process. 616452, a TGF-β inhibitor, not only plays a cooperative role in reprogramming murine fibroblasts but also is reported to successfully replace Sox2 or cMyc in reprogramming murine fibroblasts (Maherali and Hochedlinger, 2009). TGF-β is a part of a complicated superfamily. Its members could be classified into two subgroups, the first subgroup involving bone morphogenetic proteins (BMPs) which activates Smad1/5/8 signalling through ALK 1/2/3/6, and the second subgroup containing TGF-β activin and nodal which activates Smad2/3 via ALK4/5/7(Valdimarsdottir and Mummery, 2005). It is worth noting that the effects of activating the two subgroups are not the same for murine and human ES/iPS cells. But mesenchymal-epithelial-transition (MET), an early event in reprogramming, plays a crucial role in the generation of iPS cells (Esteban *et al.*, 2012), while TGF-β signalling actively promotes the opposite process epithelial to mesenchymal transition (EMT) or transdifferentiation through regulating TGF β-activated Smad signalling, Ras-MAPK-Erk kinases signalling, Rho GTPase signalling and PI3 kinase/Akt signalling pathways (Xu *et al.*, 2009). Therefore, considering the contradiction, we designed a control experiment to check the role of 616452 in reprogramming human iPS work. Tranylcypromine (also named Parnate), lysine-specific demethylase 1 inhibitor, is reported to increase reprogramming efficiency effectively in generating human iPS cells in the absence of exogenous Sox2 (Li *et al.*, 2009c). Valproric acid (VPA), HDAC inhibitor, could relax chromosome structure and make it accessible for transcription factors, enhancing downstream gene expression to further exert its positive role in improving reprogramming efficiency. In addition, it was reported that the addition of VPA allows for reprogramming of human fibroblasts to iPS cells without addition of genetic factors Klf4 and c-myc (Huangfu *et al.*, 2008). Ascorbic acid (Vitamin C) has been shown to improve the speed and efficiency of iPSC generation from both mouse and human somatic cells and promotes the transition of pre-iPSC colonies to a fully reprogrammed

state (Esteban *et al.*, 2010).

Basic fibroblast growth factor (bFGF), also named FGF2, can bind to different FGF receptors, FGFR Ib, FGFR Ic, FGFR 2c, FGFR 3c, FGFR 4. The reduction of bFGF in the medium of culturing hES cells resulted in rapid differentiation, which indicated the bFGF signalling pathway is important for maintenance of embryonic stem pluripotency (Dvorak and Hampl, 2005). The complex of FGF and FGFR could lead to the activation of two signalling pathways (1) MAPK/ extracellular signal-regulated kinase (ERK), PI3-K/AKT and (2) phospholipase C gamma/protein kinase C (PKC) pathways (Valdimarsdottir and Mummery, 2005). MAPK activation by FGFR resulted in phosphorylation of downstream transcription factors such as c-Myc, c-Jun, and c-Fos (Sears *et al.*, 2000), but Ras-MAPK-Erk kinases signalling is beneficial for EMT process, which does not facilitate reprogramming process. The inhibition of MAPK using U0126 resulted in the loss of Oct3/4 and Nanog (Li *et al.*, 2007). However, another study showed that inhibition of MAPK did not affect expression of Oct3/4 and Nanog levels (Armstrong *et al.*, 2006). In section 1.3.4, we have tried to explain the pathway. However, the possibility exists that the specific drug may act in more than one pathways, therefore, we have looked at the direct effect of the the drug in the whole reprogramming process. For example, FGF can also affect the PI3-K/AKT pathway, which is important in cell proliferation, differentiation, survival, and cellular transformation. The activation of PI3K/AKT/mTOR signalling pathway is beneficial for reprogramming through increasing cell proliferation and protection from cell apoptosis. The inhibition of PI3-K results in significantly downregulation of Oct3/4, Nanog, and SOX2 (Armstrong *et al.*, 2006). In addition, a study showed that activated PI3-K, rather than MAPK, can mediate pluripotent marker expression. In the mean time, bFGF mediated PI3-K signalling may have a direct role in modulating the downstream of Wnt signalling pathway to maintain long

term culture of undifferentiated hES cells (Ding *et al.*, 2010).

The reprogramming process is carried out with feeder cells or in feeder-free culture systems. The feeder-free system is considered to be beneficial for enabling greater accessibility to chemicals since the low density in which cells were seeded in a monolayer facilitates optimal interaction with the transfection regents (Braam *et al.*, 2008). Given the chemical transfection applied in our reprogramming work, a feeder-free system is our priority. In addition, feeder-free system is easier to manipulate than feeder system.

6.2 Experimental strategy

To generate iPS cells from accessible tissues without the risk of insertional mutagenesis and malignant transformation is our major objective, since one aim is to eventually provide a promising and accessible road to clinical use. Thus, we chose mRNA and episomal vectors combined with small molecules to reprogram MSC cells, fibroblast cells or blood cells. Firstly, we transfected Oct4/Sox2 mRNA into MSC cells with the addition of small molecules including CHIR99021, 616542, ascorbic acid, VPA, tranylcypromine (C6VVT) and observed a few iPS-like colonies. Although the colonies did not expand, this promising colony formation encouraged us to further improve our protocol to pursue large enough colonies for further analysis. However, we observed very few colonies and these were difficult to expand even if we tried to adjust the types of small molecules used in the reprogramming medium. We therefore analysed the disadvantages of the mRNA transfection method for reprogramming. Multiple rounds of transfecting Oct4/Sox2 mRNA into cells resulted in cell death, and when we finished nearly

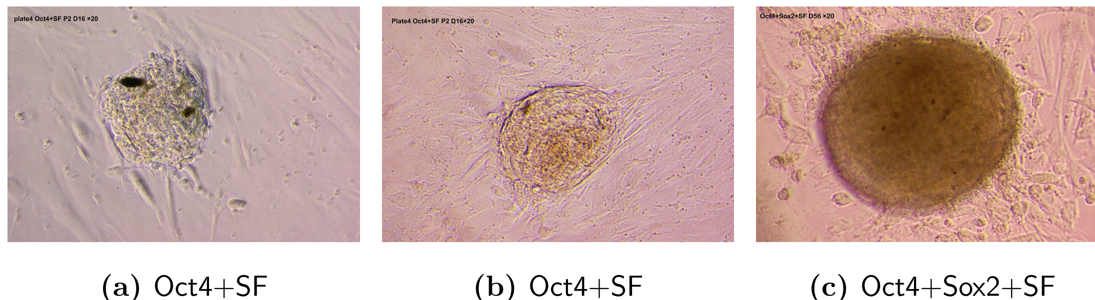
8-10 rounds of transfection, cells had to rest for several days to recover for the subsequent transfections. The intermittent transfections make it difficult for cells to receive enough reprogramming signals for generating iPS cells. Therefore, we considered using episomal vectors to replace mRNA to reprogram cells. Two problems have to be seriously considered in successful reprogramming process. One is to optimize the transfection efficiency to maintain sufficient expression of reprogramming genes for generating iPS cells. In chapter 5, we compared the transfection efficiency of different methods to find that electroporation using the nucleofector 4D machine gives the highest transfection efficiency. The other aim is to avoid the integration problem as far as possible. From this point of view, we chose episomal vectors which do not require integration for expression. In our protocol, we used episomal reprogramming vectors with an oriP/EBNA-1 backbone for delivering the reprogramming genes, Oct4, Sox2, Lin28, L-Myc, and Klf4. Reprogramming cells from a peripheral blood source is the final aim of my project since it is the easiest accessible source of cells for potential use in clinics. Therefore, I attempted to reprogram cells from peripheral blood, and used fibroblasts as a control cell type.

6.3 Results

6.3.1 Partially reprogrammed iPS-like colonies generated by Oct3/4 mRNA transfected cells cultured in hES medium with small molecules

We attempted to reprogram MSC cells using synthesized Oct3/4 mRNA in combination use with small molecules by chemical transfection reagents. When cells reached about 70-80% confluency, we started the first round of transfection.

MSC cells morphology began to change to be round or with obvious potential to clump together after several transfections and partially iPS-like colonies could be observed after 20-30 days post transfections with Oct3/4/Sox2 mRNA in hES medium with the addition of CHIR99021, tranylcypromine, ascorbic acid, bFGF and 616452. Figure 6.2c shows a compact nice colony from the well after transfecting with Oct4 mRNA and Sox2 mRNA into MSC cells in the ES medium supplemented with CHIR99021, tranylcypromine, ascorbic acid, VPA, bFGF, 616452, SCF and Flt3L, but the colony grew very slowly without obvious proliferation. After culturing for three months, the colony detached from the matrigel-coated plate and did not stick down onto the well again. In addition, the reprogramming efficiency was low, there were only a few (one to three) partial reprogramming colonies in each well.



(a) Oct4+SF (b) Oct4+SF (c) Oct4+Sox2+SF

Figure 6.2: Partially reprogrammed iPS-like colonies derived from transfected MSC cells.

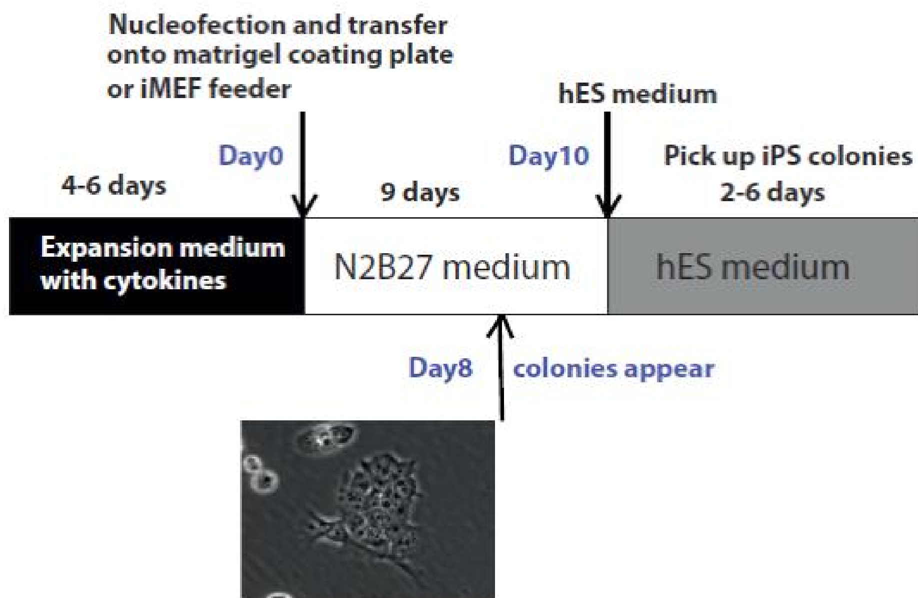
Figure 6.2a and 6.2b were colonies from MSC cells post transfection of Oct4 mRNA with SCF+ Flt3L+ CHIR99021+ 616452+ tranylcypromine+ ascorbic acid+ VPA+ bFGF. Figure 6.2c showed a colony from MSC cells post transfection of Oct4/Sox2 mRNA with SCF+ Flt3L+ CHIR99021+ 616452+ tranylcypromine+ ascorbic acid+ VPA+ bFGF. All photos were taken under 20× inverted microscope. SF= SCF+Flt3L

In our transfection process, MSC cells transfected with Oct4 mRNA had greater proliferative potential than did those transfection with GFP mRNA, though they

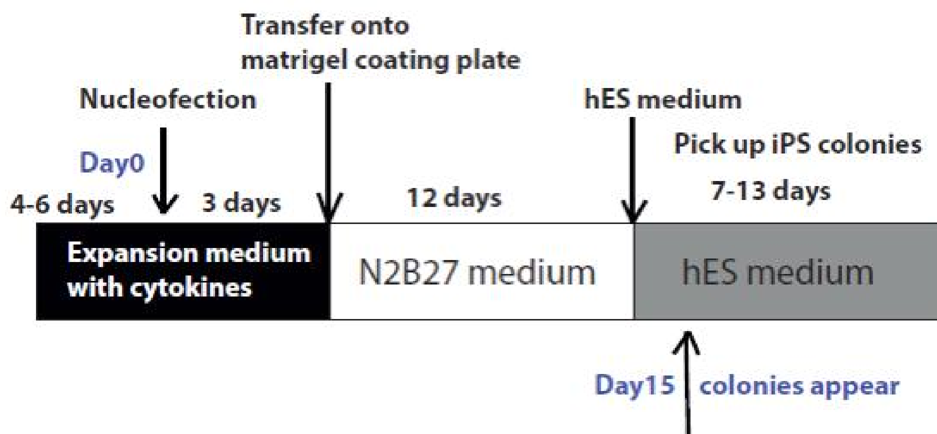
were cultured in the same hES medium with addition of small molecules. Actually, more than 50% cells were dead after eight transfections with GFP mRNA.

6.3.2 The generation of iPS colonies from CD34$^+$ cells from cord blood and fetal liver using episomal vectors

In our episomal vector system, we delivered five reprogramming genes, Oct4, Sox2, Lin28, L-Myc nd Klf4 via episomal vectors with an oriP/EBNA-1 backbone, together with Epi5^{TM} p53 & EBNA-1 vectors. CD34$^+$ cells were isolated from frozen samples or fresh fetal liver suspension cells. Every 0.5×10^4-10^5 cells were cultured in 0.5 ml Stempro medium supplemented with 100 ng/ml SCF, 10 ng/ml Flt3L and 20 ng/ml IL3 within 24 well plate for 4-6 days. If the number of cells was less than 50000, all cells were suspended in 0.5 ml media. When cells were in evident proliferative state, 10^6 cells were collected for each electroporation using nucleofector 4D. After transfection, cells were seeded in different conditions. (1) 10^5-2×10^5 electroporated cells were immediately seeded onto matrigel-coated 6-well plate directly in expansion medium without the addition of antibiotics (plate33.1). The expansion medium was gradually replaced with fresh N2B27 medium with addition of 100 ng/ml bFGF until day10, and from then medium was changed with E8 medium every other day as described in Figure 6.3a. (2) Moreover, 3×10^5 electroporated cells were cultured in expansion medium in 24 well plate in plastic without any feeder for three days and then reseeding onto matrigel-coated 6-well plate at the density of 10^5 cells/well (plate33.2). (3) In addition, 3×10^5 electroporated cells were plated in 6-well plate with irradiated bone marrow stromal cells (BMSC) as the feeder layer for three days followed by reseeding cells onto matrigel-coated 6-well plate (see Figure 6.3b).



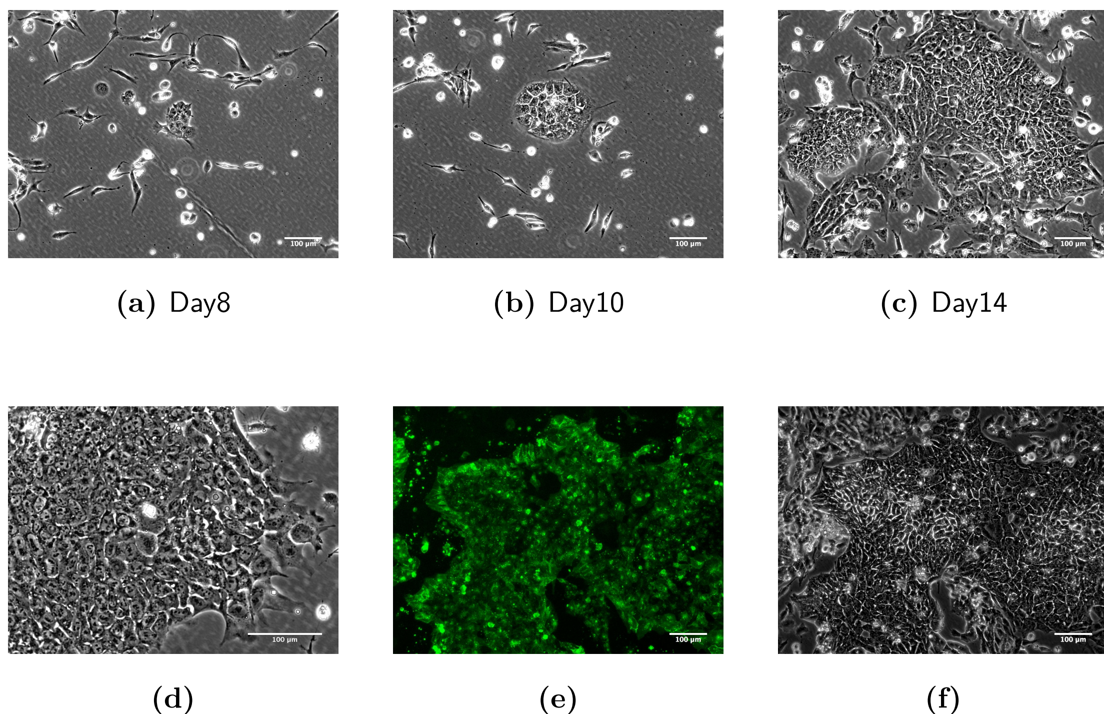
(a) The iPS induction protocol (CD34$^+$ cells, immediate seeding)



(b) The iPS induction protocol (CD34$^+$ cells, 3days culture)

Figure 6.3: The iPS induction protocol from CD34$^+$ cells

For $CD34^+$ cells which were seeded onto matrigel-coated plates immediately after transfection, iPS colonies appeared as early as day 8 after transfection (plate33.1). After several days' culture, colonies were large enough to pick for further passage. Figure 6.4 shows the iPS colony growth and live staining. Colonies were performed live staining with surface marker stage-specific embryonic antigen (SSEA)-4 AF488 for fluorescent microscope observation to recognise undifferentiated iPS colonies (Figure 6.4e). These SSEA-4^+ colonies were picked and passaged onto 24 well matrigel-coated plates.



(a) Day8 **(b)** Day10 **(c)** Day14

(d) **(e)** **(f)**

Figure 6.4: *(a)-(c) An example of the morphology change of iPS colony derived from cord blood $CD34^+$ cells on day8, 10, 14. (d) iPS colony morphology when the colony grow large enough for picking up. (e) The live staining of SSEA4 AF488 in iPS colonies. (f) The light image of iPS colony with SSEA4 staining.*

For cells which were cultured in 24 well plate for another three days (plate33.2

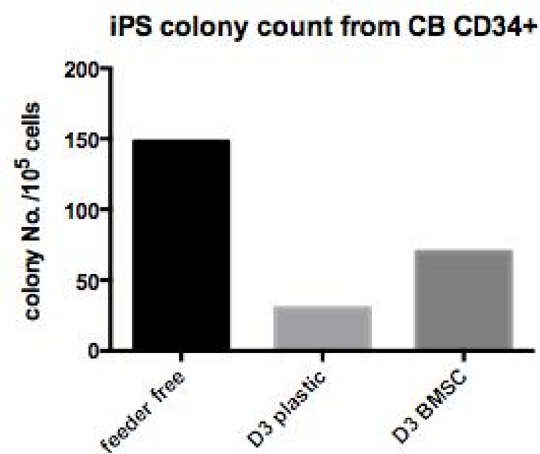
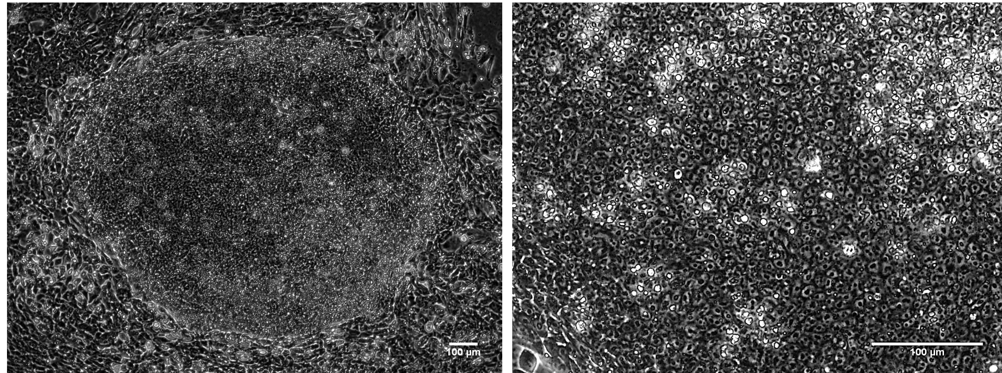


Figure 6.5: **Comparison of iPS colony count from cord blood CD34$^+$ cells after different treatments.**

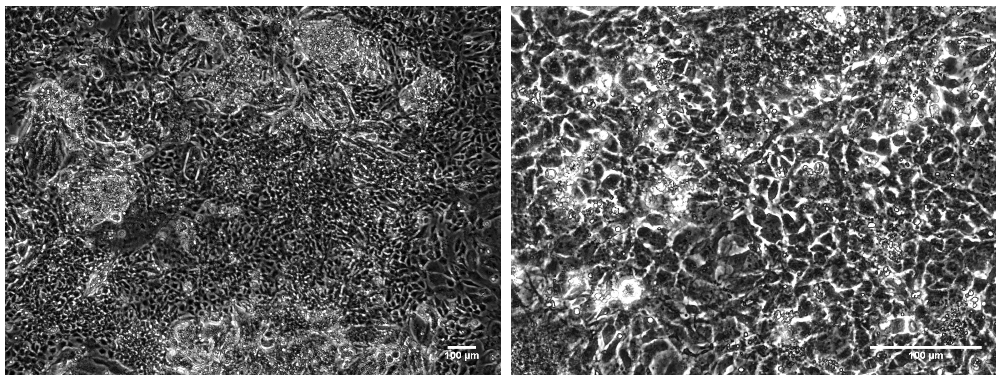
feeder-free: Transfected cells were seeded onto matrigel-coated plate immediately after electroporation. D3 plastic: Transfected cells were seeded onto matrigel-coated plate immediately after 3 days' culture in 24 well plate without any coating or feeder. D3 BMSC: Transfected cells were seeded onto matrigel-coated plate immediately after 3 days' culture on irradiated BMSC feeder in 24 well plate.

and plate33.3), colonies appeared around day 14 -15 after transfection. As table 6.1 shows, electroporated cells undergoing 3 days culture on BMSC produced more colonies than plastic (70 colonies/10^5 cells vs 30 colonies/10^5 cells). From the time points of colony appearance and colony count, the immediate seeding of electroporated cord blood $CD34^+$ cells onto matrigel-coated plates was optimal (148 per 10^5 cells) compared to other treatments (Figure 6.5, table 6.1).

Based on the result from cord blood $CD34^+$ cells, we tried to seed $CD34^+$ cells from fetal liver suspension cells after electroporation with episomal vectors immediately onto matrigel-coated plates. The appearance of iPS colonies from fetal liver $CD34^+$ cells was three days later than cord blood $CD34^+$ cells. It took around 11-12 days to generate iPS colonies. In a 6-well plate, 10^5 seeded cells produced 110-143 iPS colonies. The addition of CHIR33021, PD0325901, A83-01, HA100, LIF (CHALP) did not improve the reprogramming process, in fact, their addition not only delayed the appearance of iPS colonies, but also impacted the quality of iPS colonies. The iPS-like colonies from CHALP well looked small without compact edges and took a longer time to grow larger although the cells in iPS-like colonies were clearly demarcated from each other with a cobblestone-like appearance. Figure 6.6 compares the morphology of colonies from N2B27 system and N2B27+CHALP system (see table 6.1).



(a) *N2B27*



(b) *N2B27+ CHALP*

Figure 6.6: **The morphology of colonies from N2B27 system and N2B27+ CHALP system.** *(a) showed iPS colonies from N2B27 system demarcated from each other with cobblestone-like appearance and compact edge. (b) showed that the addition of CHIR33021, PD0325901, A83-01, HA100, LIF (CHALP) did not improve the reprogramming process, in fact, impacted the quality of iPS colonies.*

Cell type	plate	Seed density	Colony count
CB CD34^{+} cells	plate33.1, feeder-free, seed immediately after transfection	10^5 cells/well	148
		3×10^5 cells/well	430
	plate33.2, 3 days' culture in plastic	10^5 cells/well	30
	plate33.3, 3 days' culture on feeder	10^5 cells/well	70
FL CD34^{+} Seed immediately after transfection	feeder-free, N2B27	10^5 cells/well	143
			110
	feeder-free, N2B27+ CHALP	10^5 cells/well	5

Table 6.1: **The iPS-like colony count deriving from CD34^{+} cells from cord blood and fetal liver.**

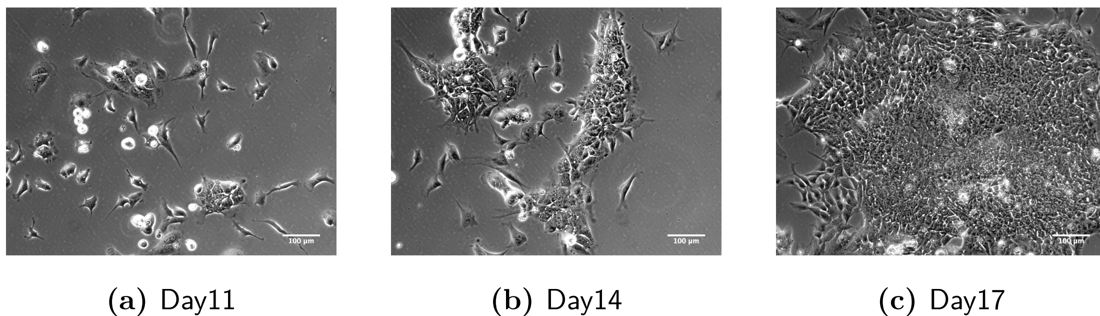
The colony number was counted at maximum.

6.3.3 The generation of iPS colonies from mononuclear cells using episomal vectors

We next attempted to reprogram mononuclear cells using Epi5^{TM} episomal vectors. Mononuclear cells were isolated from fresh or frozen buffy coat and mobilised blood. Once MNC cells were thawed, they were cultured in expansion medium made up of Stempro supplemented with 100 ng/ml SCF, 10 ng/ml Flt3L, and 20 ng/ml IL3 at 37°C in humidified atmosphere containing 5% CO_2 for 9-10 days. When there was an obvious increase in cell number, cells were ready for transfection with episomal vectors. The transfected cells were treated in three ways. (1) The transfected cells were immediately plated onto matrigel-coated 6-well plates at the density of 10^5-2×10^5 cells per well; (2) 3×10^5 cells were cultured for another 3 days in 24 well plate with irradiated human placenta fibroblasts as feeder cells, followed by reseeding onto matrigel-coated 6-well plates at the density of 10^5 cells per well; (3) 3×10^5 cells were cultured in 24-well plate in plastic for 3 days and then reseeding onto matrigel-coated 6-well plates.

In the first treatment, initially 10^5 of transfected cells were plated onto a well of 6-well plate, but in the culture process, some of cells died, therefore in subsequent experiments the seeding density was increased to 2×10^5 cells in order to get a more appropriate seeding density. It took around 9-10 days to observe a few tiny colonies, cells of which showed a round morphology and clear cell-to-cell boundaries. In the following week, these cells gradually adhered to each other and proliferated to form compact iPS-like colonies (Figure 6.7). In the third treatment, colonies appeared on day 12. However, in the second treatment, colonies appeared on day 19 in the well in which transfected cells were cultured for another 3 days in 24 well plate with feeder for further reseeding onto matrigel-coated 6-well plate, much later than the other two treatments. Figure 6.8 shows the colony count after the three different treatments. For the cells which were plated onto matrigel-coated plate immediately after transfection (the first treatment), around 19-24

colonies/10^5 cells were obtained from buffy coat MNC, and 27-33 colonies/10^5 cells from mobilised blood. For the cells which were plated onto matrigel-coated plate after 3 days culture in plastic (the third treatment), we observed around 17-19 colonies/10^5 cells from buffy coat, 33 colonies/10^5 cells from mobilised blood. For the cells which were plated onto matrigel-coated plate after 3 days culture on irradiated human placenta fibroblast feeder (the second treatment), we observed around 6-8 colonies/10^5 cells (see table 6.2). A further culture of three days did not facilitate the generation of iPS colonies, and even the three days culture with feeder decreased and delayed iPS generation.



(a) Day11 **(b)** Day14 **(c)** Day17

Figure 6.7: The development of iPS-like colonies derived from MNC

6.3.4 The generation of iPS colonies from fibroblasts using episomal vectors

We used fibroblasts as the control to carry out the reprogramming experiments using episomal vectors. The protocol to reprogram fibroblasts is simpler than $CD34^+$ cells or peripheral blood MNC, and Figure 6.9 describes the process. 10^5 fibroblasts were washed using OptiMem and resuspended in nucleofector transfection reagent. After electroporation, fibroblasts were cultured at room temperature for 10 minutes and then cultured in N2B27 medium onto matrigel-coated plate After 15 days, iPS-like colonies appeared and the medium was

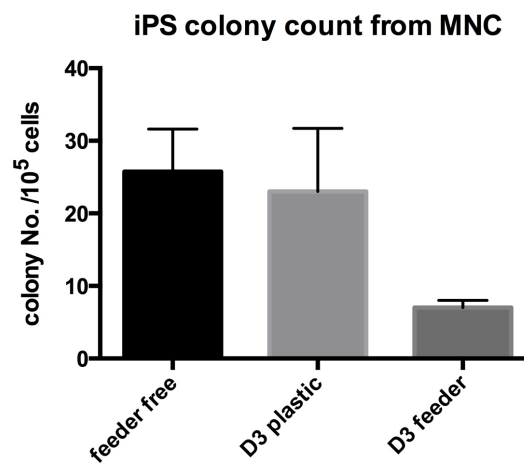


Figure 6.8: Comparison of iPS colony count from mononuclear cells after different treatments.

feeder-free: Transfected cells were seeded onto matrigel-coated plate immediately after electroporation. D3 plastic: Transfected cells were seeded onto matrigel-coated plate immediately after 3 days' culture in 24 well plate without any coating or feeder. D3 feeder: Transfected cells were seeded onto matrigel-coated plate immediately after 3 days' culture on feeder in 24 well plate. The differences among means are statistically significant. N=3-4, P=0.0166

Cell type	plate	Seed density	Colony count
Buffy coat MNC	feeder-free, seed immediately after transfection	10^5 cells/well	19
			24
	3 days' culture in plastic	10^5 cells/well	17
			19
	3 days' culture on feeder	10^5 cells/well	7
			8
mPB MNC	feeder-free, seed immediately after transfection	2×10^5 cells/well	65
		10^5 cells/well	27
	3 days' culture in plastic	6×10^4 cells/well	20
	3 days' culture on feeder	10^5 cells/well	6

Table 6.2: **The number of iPS-like colony deriving from mononuclear cells from buffy coat and mobilised blood.**

The colony number was counted at maximum.

Cell source	Number of iPS cell lines
Cord blood CD34$^+$ cells	50
Fetal liver suspension CD34$^+$ cells	22
Buffy coat MNC	22
mPB MNC	12
Fetal liver adherent cells	5
Fibroblasts	5

Table 6.3: The number of established iPS cell lines from different sources

replaced with human ES medium. From day 20, colonies were large enough to pick. We also attempted to reprogram fetal liver adherent cells, and found that the process was longer than blood cells and fibroblasts. For fibroblasts, every 10^5 fibroblast cells can generate 108 iPS colonies but for fetal liver adherent cells, only 7 iPS colonies were observed.

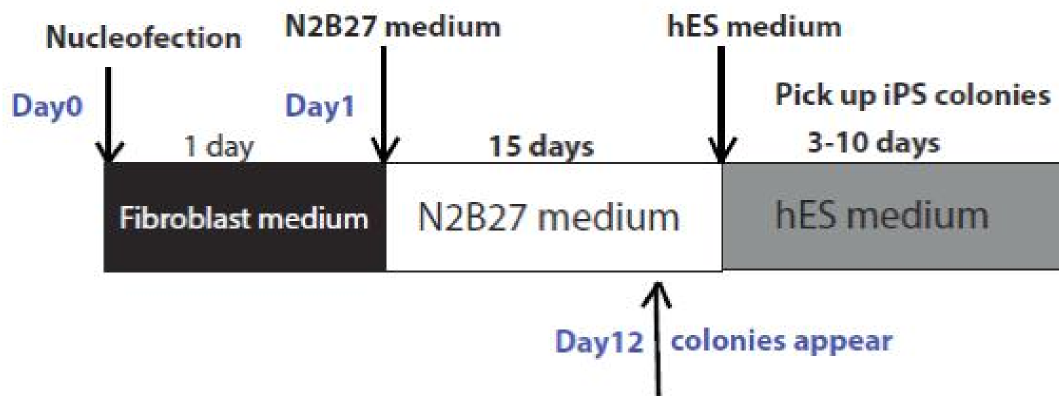


Figure 6.9: iPS induction protocol from fibroblasts

Generally speaking, the episomal method is very successful and we can generate as many iPS cell lines as we want from blood sources. The iPS cell lines we generated from different sources are listed as below (Table 6.3):

6.4 Discussion

6.4.1 The successful establishment of an efficient non-integration method to reprogram mononuclear cells in a feeder-free system

In our lab, we have developed an efficient and easy way to reprogram $CD34^+$ cells, and mononuclear cells from fresh or frozen samples using episomal vectors in a feeder-free system without assistance of small molecules. For $CD34^+$ cells, every input 10^5 cells onto a matrigel-coated plate can produce as many as 148 colonies (0.148% reprogramming efficiency). The optimized methodology can produce iPS colonies as early as the eighth day and grew large enough for picking on day 11-day 13 and onward. In comparison with published non-integration methods, the efficiency is much higher than 0.016% reported by Bin-Kuan Chou and colleagues (Chou *et al.*, 2011) and 0.03% reported by Mack and colleagues (Mack *et al.*, 2011) to generate iPS colonies from $CD34^+$ cells. In the research by Keisuke Okita and colleagues (Okita *et al.*, 2013), the maximum reprogramming efficiency of $CD34^+$ cells was up to 0.1%. Using our optimised protocol to reprogram MNC, every input 10^5 cells in a feeder-free system can generate around 33 colonies. The cell sources except buffy coat for reprogramming were frozen samples. And the feeder-free system facilitates downstream work - picking colonies. The successful development of an efficient way to reprogram frozen mononuclear cells using an integration-free method in a feeder-free system provides a promising future for potential clinical application. Mononuclear cells are isolated from peripheral blood which is one of the most easily accessible tissues from patients. Thus it is possible to reprogram mononuclear cells from any patient to generate patient-specific iPS cells for future personalised therapies, drug testing and other applications.

6.4.2 The significant points in our episomal vectors reprogramming system

The advantage of Epi5^{TM} reprogramming vectors

In our reprogramming protocol, we used three episomal reprogramming vectors with an oriP/EBNA-1 (Epstein-Barr nuclear antigen-1) backbone to deliver the reprogramming genes, Oct4, Sox2, Lin28, L-Myc, and Klf4. This combination did not require any assistance of small molecules to successfully produce iPS cells. We attempted to reprogram fibroblast cells and CD34^{+} cells using episomal iPSC reprogramming vectors (Cat. No. A14703, invitrogen) containing six reprogramming factors (Oct4, Sox2, Nanog, Lin28, Klf4, and L-Myc, OSNLKM) in conjugation with small molecules, but did not develop iPS cell lines. Comparing the two system, there are two significant differences: Epi5^{TM} reprogramming vectors deliver five genes without Nanog, and the system also contains mp53DD (a dominant negative mutation of the p53 protein) allowing for transient expression of the dominant negative mutant over an extended period of time which can help increase cell proliferation and reduce apoptosis. In addition, EBNA1 was also reported to enhance iPS formation (Okita *et al.*, 2013) and had a correlation with the p53 (Li *et al.*, 2002; Saridakis *et al.*, 2005). Additional inhibition of the p53/p21 pathway increased cell proliferation and further accelerated iPS cell formation (Hanna *et al.*, 2009).

The important role of cell proliferative state in reprogramming

Another important reason for successful reprogramming is to begin manipulating the transfection when cells are in active proliferating state. When samples are thawed, the appropriate cytokine cocktail plays an essential role in maintaining

cell proliferation state. In section 4.3.1, we presented the optimised cytokine cocktail containing 100 ng/ml SCF, 10 ng/ml Flt3L, and 20 ng/ml IL3, which improved cell proliferation to a large extent in eight days compared to the combination of 100 ng/ml SCF, 10 ng/ml Flt3L. Before transfection, cells were cultured in Stempro medium supplemented with the optimised cytokine cocktail for several days to achieve the evident proliferation, when it is easier for cells to receive exotic plasmids. In addition, cell proliferation has been reported to accelerate the stochastic course of reprogramming (Hanna *et al.*, 2009). In another reported case (Park *et al.*, 2012), growth factors including SCF, TPO and Flt3L-activated myeloid progenitors with episomal reprogramming have more potential to generate hiPS cells at an optimised efficiency of around 50%. And they provided evidence to indicate that the efficient reprogramming of cord blood myeloid progenitors was attributed to the high expression levels of common epigenetic regulatory circuits (e.g., ESC, MYC, PRC1, PRC2 modules) instead of key reprogramming factors. In another transdifferentiation case, the addition of SCF and Flt3L in the reprogramming medium improves the speed and efficiency of reprogramming (Szabo *et al.*, 2010). There might suggest other possible roles for cytokine cocktails in the reprogramming process.

The feeder-free system facilitates the operation of the reprogramming process

In addition, feeder-free system is especially appropriate for blood cells. Once cells adhered to the matrigel-coated plate, most of cells developed to form iPS-like colonies. Without doubt, this helps reduce difficulties to pick colonies and facilitates colonies attaching to the matrigel-coated plate. In many protocols, transfected cells are cultured in 24 well plate for 2-3 days followed by seeding onto iMEF feeder, but what the real significance of another 2-3 days culture in 24 well plate? In the process of reprogramming cord blood $CD34^+$ cells, we

made controls to seed transfected cells onto matrigel-coated plates after three days culture in plastic or BMSC feeders. As a result, we found that three days culture in plastic or BMSC feeder before seeding cut down the reprogramming efficiency from 0.148% to 0.03% or 0.07% and delayed the appearance of iPS colonies. But for the process of reprogramming MNC, the conditions are different. Compared to seeding transfected cells immediately after electroporation, the reprogramming efficiency of three days' culture in plastic before seeding was similar (nearly 0.02%). However, three days culture in irradiated human placenta fibroblast feeder reduced the reprogramming efficiency to 0.005%. It is interesting to find that the presence of feeder cells is not a necessary condition for reprogramming and the presence of feeders reduced the reprogramming efficiency. Seeding onto matrigel-coated plates is an excellent and easy way for improving the reprogramming process. It is possible that some unknown agents that the feeder cells produced suppressed the reprogramming process for blood cells.

6.4.3 An analysis for the failure of the mRNA-reprogramming process

Problems of cell viability and insufficient expression of reprogramming factors in the mRNA-reprogramming process

In this part, we will focus our discussion on mRNA transfection methods. From six repeated experiments to reprogram cells using Oct4 and Sox2 mRNA and small molecules, we found only three wells produced one tight colony. Two of them derived from MSC cells transfected with Oct4 and Sox2 mRNA with the combination of small molecules including CHIR99021, 616452, tranylcypromine, valproric acid, vitamin C with or without SCF and Flt3L in ES medium. The third colony was generated by MSC cells transfected with Oct4 mRNA in ES

conditioned medium. The cells in the three wells received 10-20 transfections intermittently. This is because when cells receive eight rounds ofxx mRNA transfections, nearly half of cells were dead so transfection has to be stopped so as to allow cells to recover. When cells grow to 70-90% confluency, transfection was repeated until colonies appear. However, the colonies we picked from wells transfected with mRNA and small molecules did not increase in size, indicating that they have not the potential to proliferate. We suspect it is possible that MSCs are partially reprogrammed, but have not yet arrived at the final destination. This might be attributable to the expression levels of reprogramming factors being not sufficient enough to fulfil the reprogramming process and multiple transfections are detrimental to cell survival.

The different role of TGF-β receptor signaling pathways in maintaining human and murine stem cell pluripotency.

Comparing the successful generation of iPSCs from mouse fibroblasts with the transduction of Oct4 and the above small molecules, it is worth noting the discrepencies between our experiments, such as the source cells and the nonviral delivery system. We considered the different roles of TGF-β receptor signalling pathways in maintaining human and murine stem cell pluripotency. The blockage of BMP/Smad1/5/8 signalling through ALK 1/2/3/6 is necessary to maintain murine stem cell pluripotency whereas the activation of TGF-β activin/Smad2/3 through ALK 4/5/7 plays a necessary role in maintaining human ES/iPS cell pluripotency. ALK5 Inhibitor II, 616452, plays a negative role from the standpoint of TGF-β activin pathway in maintaining human ES/iPS cells. But TGF-β signalling plays an important role in inducing epithelial to mesenchymal transition or transdifferentiation through regulating TGF-β-activated Smad signalling, Ras-MAPK-Erk kinases signalling, Rho GTPase signalling and PI3 kinase/Akt signalling pathways (Xu *et al.*, 2009). Mesenchymal-epithelial-transition (MET),

an early event in reprogramming, plays a crucial role in the generation of iPS cells (Esteban *et al.*, 2012). Surprisingly, removing 616452 from the combination of small molecules in reprogramming human MSC cells, we did not find any essential improvements. Given the role of 616452 in reprogramming human cells, we continued to use it in our small molecule combinations.

The candidate small molecules in future reprogramming process

Insulin-like growth factor (IGF) system plays an important role in the development of tissues or organs and postnatal growth, and maintenance of normal function of many types of cells (Jones and Clemmons, 1995; LeRoith *et al.*, 1995; Stewart and Rotwein, 1996). The IGF system is made up of three components: the IGF ligands (IGF-1 and IGF-2); cell surface receptors; and IGF binding proteins (IGFBPs). It is reported that IGF-1 receptor and ERBB2 receptor signalling play an important role in long-term growth of multiple hES cell lines (Wang *et al.*, 2007). Telomere elongation can greatly increase iPS generation efficiency (Marion *et al.*, 2009) while IGF-1 could modulate the activity of telomerase through PI3 kinase/Akt signalling pathway (Thum *et al.*, 2007; Werner *et al.*, 2008; Wetterau *et al.*, 2003). IGF-1 facilitates histone H3 and H4 acetylation, which might be another possible way to improve iPS reprogramming efficiency (Sun and D'Ercole, 2006). Moreover, the cooperation of IGF and FGF help maintain the stem cell niche of human pluripotent cells *in vitro* (Bendall *et al.*, 2007). In addition, hypoxia is reported to be beneficial for improving iPS cell reprogramming efficiency (Yoshida *et al.*, 2009), which might be due to the IGF-1 secreted by stem cells and progenitor cells under hypoxic conditions (Li and Geng, 2010b). IGF-1 was demonstrated to enhance expression of iPS reprogramming factors and antisenescence activity in human dermal fibroblasts (Li and Geng, 2010b). However,

mesenchymal stem cells expressing IGF-1 promotes their differentiation into osteoblasts through an intact IRS1-PI3K signalling pathway (Granero-Moltó *et al.*, 2011).

A new report demonstrated that in the reprogramming process the virus works not only as a delivery tool, but also activates the toll-like receptor 3 (TLR3) pathway enabling efficient induction of pluripotency by viral or mRNA approaches (Lee *et al.*, 2012). Polyinosinic–polycytidylic acid potassium salt (Poly I:C) is a double-stranded homopolymer used to mimic double-stranded RNA to study cell signalling at the level of TLR3, which recognises double-stranded RNA and affects the immune response against viral pathogens. Poly I:C is a TRIF-dependent toll-like receptor-3 (TLR3) ligand involved in the TLR3-mediated MyD88-independent pathway (Takeda and Akira, 2004). The inhibition of MyD88-independent pathway reduced the reprogramming efficiency of human or mouse fibroblasts. It is possible to improve reprogramming efficiency by other non-viral methods through activating the TLR3-mediated MyD88-independent pathway. This can be an option to enhance reprogramming efficiency if necessary.

6.4.4 The combination of small molecules CHALP did not improve reprogramming efficiency

In Junying Yu's group, they produced human iPS cells using oriP/EBNA-1(Epstein-Barr nuclear antigen-1) episomal vectors to deliver reprogramming factors with the combination of small molecules including CHIR33021, PD0325901, A83-01, HA100, LIF (CHALP)(Yu *et al.*, 2009). The small molecule PD0325901, MEK inhibitor, can block the differentiation pathway of ES cells, support self-renewal (Ying *et al.*, 2008), and facilitate the reprogramming process (Hanna *et al.*, 2010; Li *et al.*, 2009a; Lin *et al.*, 2009b; Zhou *et al.*, 2010b; Zhu *et al.*, 2010). Given the role played by small molecules combination CHALP, we added

CHALP from day 1 to day 15 in reprogramming medium to look into whether they can increase reprogramming efficiency. However, we found a contradictory result: With the addition of CHALP into reprogramming medium, the reprogramming efficiency fell from 143 colonies/10^5 cells to 5 colonies/10^5 cells. In addition, the colonies did not have defined edge and are difficult to grow up to large compact colonies as Figure 6.6 showed. The CHALP combination of small molecules appears to be optimal for fibroblast cells but may not be optimal for blood cells.

In addition, it has been reported that c-KIT$^+$ human first trimester amniotic fluid stem cells (AFSCs) have been fully reprogrammed to pluripotent cells just by culturing on matrigel in hES cell medium supplemented with VPA without any other foreign factors (Moschidou *et al.*, 2012). We tried some CD34e cells from 1st trimester fetal liver in reprogramming medium or in combination with small molecules, but failed to get any colonies. Compared to Moschidou's work, we selected CD34 using MACS selection instead of CD117 due to its extreme low expression in cells.

6.5 Concluding remarks

We successfully developed an efficient and easy way to reprogram CD34$^+$ cells, and mononuclear cells from frozen samples using episomal vectors in a feeder-free system without any assistance of small molecules. The application of episomal vectors with an oriP/EBNA-1 backbone for delivering five reprogramming genes, Oct4, Sox2, Lin28, L-Myc, and Klf4, and the efficient electroporation play the most important role in the reprogramming process. In addition, it is pivotal to begin manipulating the transfection when cells are in active proliferating status.

Problems of cell viability and insufficient expression of reprogramming factors in the mRNA transfection process resulted in the unsuccessful reprogramming.

Chapter 7

The identity of pluripotency of derived iPS cell lines

7.1 Introduction

Induced pluripotent stem cells have similar characteristics to ES cells. Generally speaking, the methods for identifying ES cells can be applied in inspecting the pluripotency of iPS cells. When iPS cells were first produced by Yamanaka's lab, iPS cells derived from human somatic cells were reported to express hES-specific surface antigens including SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and TRA-2-49/6E (alkaline phosphatase) (Takahashi *et al.*, 2007). In addition, from the perspective of RNA, human iPS cell lines expressed many undifferentiated ES cell marker genes including OCT3/4, SOX2, and NANOG.

According to the paper published by the international stem cell initiative (International Stem Cell *et al.*, 2007) , all ES cell lines they tested expressed several markers of human embryonic stem cells including glycolipid antigens SSEA-3, SSEA-4, the keratan sulfate antigens TRA-1-60, TRA-1-81, GCTM2 and GCT343, and

the protein antigens CD9, Thy1, tissue-nonspecific alkaline phosphatase, class 1 HLA, and developmentally regulated genes NANOG, OCT4, TDGF1, DNMT3B, GABRB3 and GDF3. Although the different expressions of several lineage markers existed, there were not any significant qualitative differences.

SSEA3 (Shevinsky *et al.*, 1982) and SSEA4 (Kannagi *et al.*, 1983) are stage-specific embryonic antigens, which are expressed on 4- to 8-cell stage mouse embryos and a human teratocarcinoma cell line. Tumor related antigen (TRA)-1-60 and TRA-1-81 are antigens expressed on the cell surface of human embryonal carcinoma (EC) cells, but their expressions are weak or absent on *in vitro* differentiating cells or in xenograft tumors, and in other germ cell tumor cell lines without the typical features of human EC cells (Andrews *et al.*, 1984). Oct4, a POU transcription factor expressed by early mammalian embryo cells and germ cells, was reported to play a pivotal role in identifying the pluripotential founder cell population in the mammalian embryo (Nichols *et al.*, 1998). Sox2 synergizes with Oct4 to activate transcription of the Fgf4, Utf1, Sox2, and Fbx15 genes (Nishimoto *et al.*, 1999; Tomioka *et al.*, 2002; Yuan *et al.*, 1995). Nanog is a divergent homeodomain protein expressed in pluripotent mouse and human cell lines, and is essential in propagation of undifferentiated ES cells (Chambers *et al.*, 2003). These pluripotent markers are specific for undifferentiated ES cells, and have been widely used in checking iPS cells since their discovery (Takahashi and Yamanaka, 2006).

However, it is still essential to verify that iPS cells have the multiple potential to differentiate into three germ layers through functionality testing. For *in vivo* functionality tests, the current standard way to verify the pluripotency of iPS cells is to inject iPS cells subcutaneously to dorsal flank of a SCID mouse to do a teratoma formation test. After nine weeks, the histological examination of teratoma showed different cell types from ectoderm, endoderm and mesoderm layers. However, it is time-consuming and expensive. The convenient alternative is to use *in vitro* functional test, where the iPS cells are differentiating into cell types

of three germ layers *in vitro*.

7.2 Experimental strategy

After derivation of iPS cell lines, the culture method is identical to ES cells. The passaged cells exhibited hES morphology characterised by a cobblestone-like appearance with individual cells clearly demarcated from each other and high nuclear-to-cytoplasm ratio in the colonies. However, more details are required to identify iPS cells and verify their pluripotency. Firstly, we looked into the pluripotent markers including SSEA4, SSEA3, TRA-1-60, TRA-1-81, Oct4, and Nanog expressed by iPS cells using flow cytometry and immunofluorescence. The expression levels of pluripotent markers primarily identifies iPS cells. To verify that the iPS cells we generated have the capability of differentiating into three germ layers, iPS cells were cultured *in vitro* for differentiating into ectoderm, mesoderm, endoderm layer cells. The functionality experiment provides sufficient evidence to verify the multiple pluripotency of the iPS cells derived in this work.

7.3 Results

7.3.1 The pluripotent markers expressed by iPS cells

Flow cytometry

We chose at least three cell lines from each source to detect pluripotent markers using flow cytometry. When iPS cells in 6-well plate were 90%-100% confluent,

cells were collected and fixed using 2% PFA, followed by permeabilisation with 0.1% saponin. After incubating with appropriate antibodies, cells were washed using PBS supplemented with saponin and FCS. The cells were then assessed by flow cytometry. In data analysis, a gate was set for excluding differentiated iPS cells and then the percentage of iPS cells positive for markers on undifferentiated cells were calculated by histogram analysis. All iPS cell lines we generated expressed high levels of pluripotent markers including SSEA4, SSEA3, TRA-1-60, TRA-1-81, Oct4, and Nanog (Figure 7.1, 7.2). No significant discrepancies were found for each marker of iPS cells from different sources.

Immunofluorescence

In order to directly observe the expression of pluripotent markers, we randomly selected three iPS cell lines derived from each source including cord blood CD34$^+$ cells, fetal liver suspended CD34$^+$ cells, fetal liver adherent cells, fibroblast cells and mononuclear cells from peripheral blood. Cells from each iPS cell line were cultured in matrigel-coated 24 well plate or chamber slides, followed by fixation, permeabilization, blocking and staining with Oct4, Nanog, SSEA4, SSEA3, TRA-1-60 and TRA-1-81 primary antibodies and and detected using fluorochrome conjugated secondary antibodies. After staining with DAPI, slides were mounted and dried in dark overnight. Finally, slides were examined using fluorescent microscopy. As seen in Figure 7.3, iPS cells expressed Oct4, Nanog, SSEA4, SSEA3, TRA-1-60 and TRA-1-81. Compared with the immunostaining of surface markers including SSEA4, SSEA3, tra-1-60 and tra-1-81, the intracellular staining with Oct4 and Nanog showed well demarcated cellular nucleus and membrane. The presence of DAPI in the nuclei provides sufficient evidence of the authenticity of staining.

7.3.2 Pluripotent gene expression of established iPS cell lines

Genorm analysis

Before carrying out the quantitive PCR of gene of interest (GOI), we have to validate the stability of expression of reference genes for normalisation in oder to remove any nonspecific variations. According to Vandesompele and colleagues' work (Vandesompele *et al.*, 2002), we evaluated 12 commonly used housekeeping genes including 18s, GAPDH, YWHZ, EIF4A2, ACTB, TOP1, UBC, B2W, SDHA, CYC1, ATP5B, and RPL13A for gene-stability measurement. The method presumes the expression ratios of two control genes are identical in all samples regardless of cell type or experimental conditions. Thus, the higher variation of the expression ratios of the two genes, the less stable expression. Using qBaseplus software developed by their group, they define the expression stability value of the reference gene (M) as the average pairwise variation of one particular gene with other housekeeping genes. High reference target stability (average geNorm $M \leq 0.5$) is accepted (see Figure 7.4). In addition, the software supplies another indicator for measurement—the coefficient of variation of the normalized reference gene relative quantities (CV) to determine how many control genes are required for reliable normalisation. In our genorm analysis, M of three reference genes (18s, TOP1, ACTB) meet the requirement and they are considered as control genes. When geNorm $V < 0.15$, below which additional control gene is not significant. According to this, the optimal number of reference targets in this experimental situation is 2 or 3. (see Figure 7.5)

The successful expression of pluripotent genes

Based on the selected reference genes, we measured the mRNA expression of Oct4, Nanog and Sox2 from 24 established iPS cel lines deriving from cord blood $CD34^+$ cells, fetal liver $CD34^+$ cells, fetal liver fibroblast cells, adult fibroblast cells, and mononuclear cells. For each gene, there was no significant difference between lines derived from the same or different sources.(see Figure 7.6)

Compared to genes for normalisation, ΔCt of Oct4, Nanog, and Sox2 are significant suggesting the pluripotent gene expression, although their mRNA expressions are different. Average ΔCt of Oct4, Nanog, and Sox2 is respectively 0.9, 5.4, and 2.6, which indicate the mRNA expression of Oct4 is highest, Nanog is lower than the other two genes.(see Figure 7.7)

Using unmanipulated fibroblasts and cord blood $CD34^+$ cells as the negative control, we found that mRNA expression of Oc4 was 1682 fold higher than negative control, Sox2 expression was 1182 fold compared to negative control, and Nanog expression was only 143 fold higher than negative control. (see Figure 7.8)

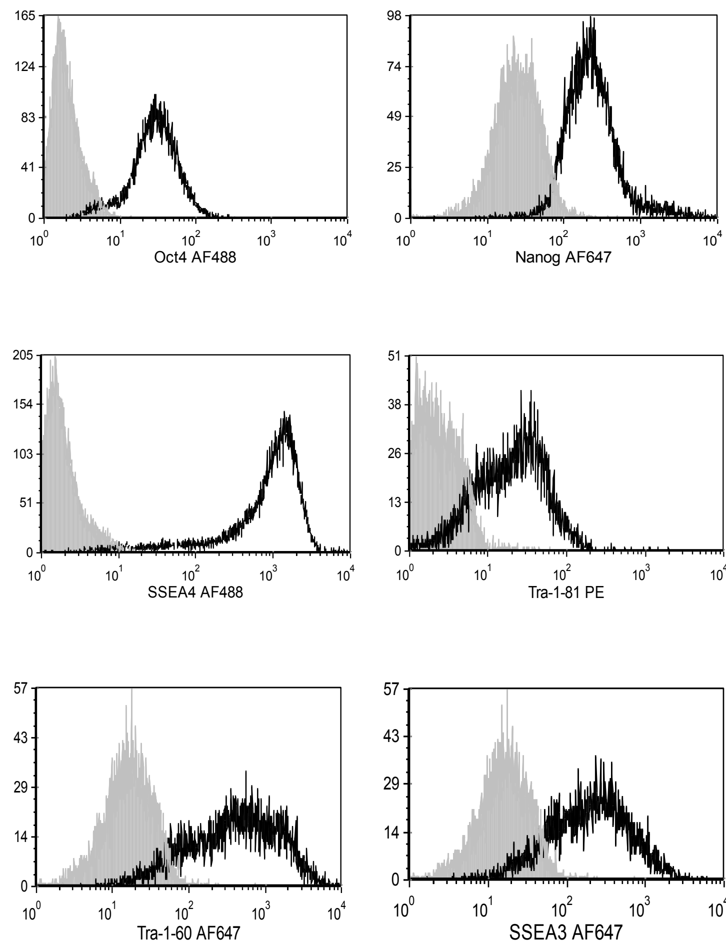


Figure 7.1: The immunophenotype of iPS cells by flow cytometry

Harvested iPS cells were fixed using 2% PFA, permeabilised with 0.1% saponin, followed by incubating with appropriate antibodies for flow cytometry. In histogram plot, iPS cells we produced were stained positive with Oct4 AF488, Nanog AF647, SSEA4 AF488, TRA-1-81 PE, TRA-1-60 AF646 and SSEA3 AF646.

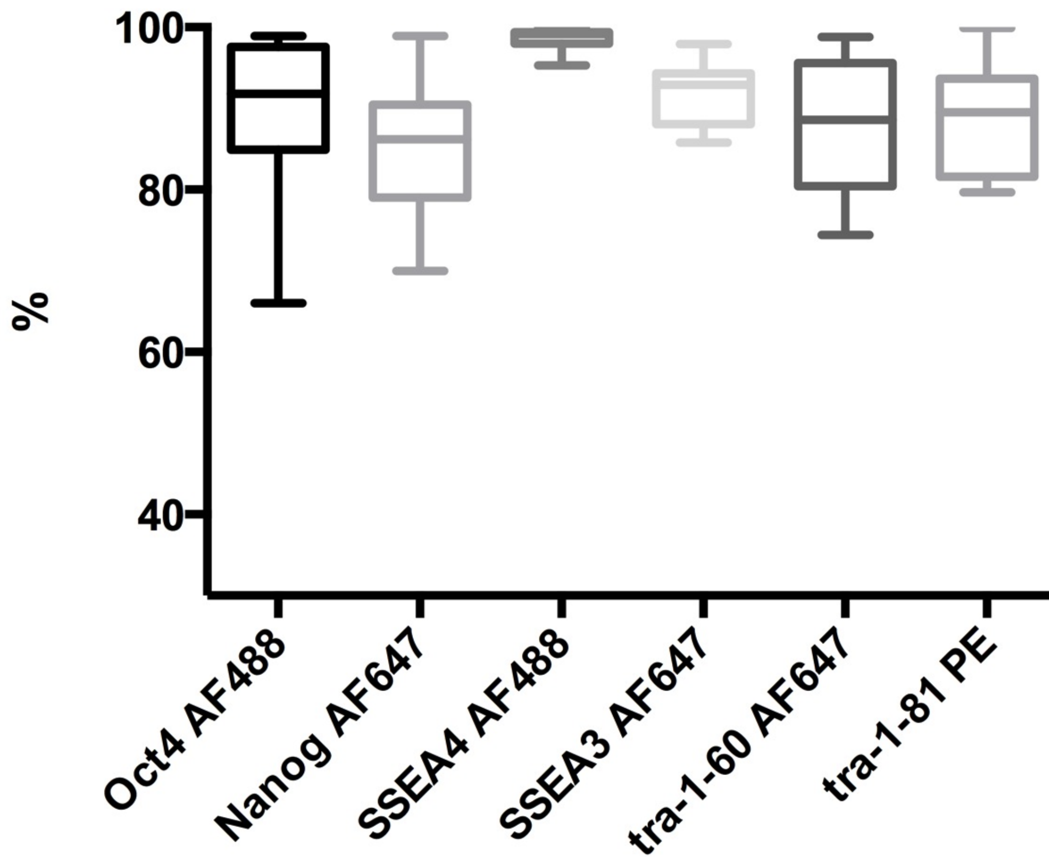


Figure 7.2: The frequency of hES/iPS markers detected by flow cytometry
The pluripotent markers are expressed by box whiskers. For Oct4 AF488, N=18; for Nanog AF647 and SSEA4 AF488, N=17; for SSEA3 AF647, tra-1-60 AF647, tra-1-81 PE, N=16.

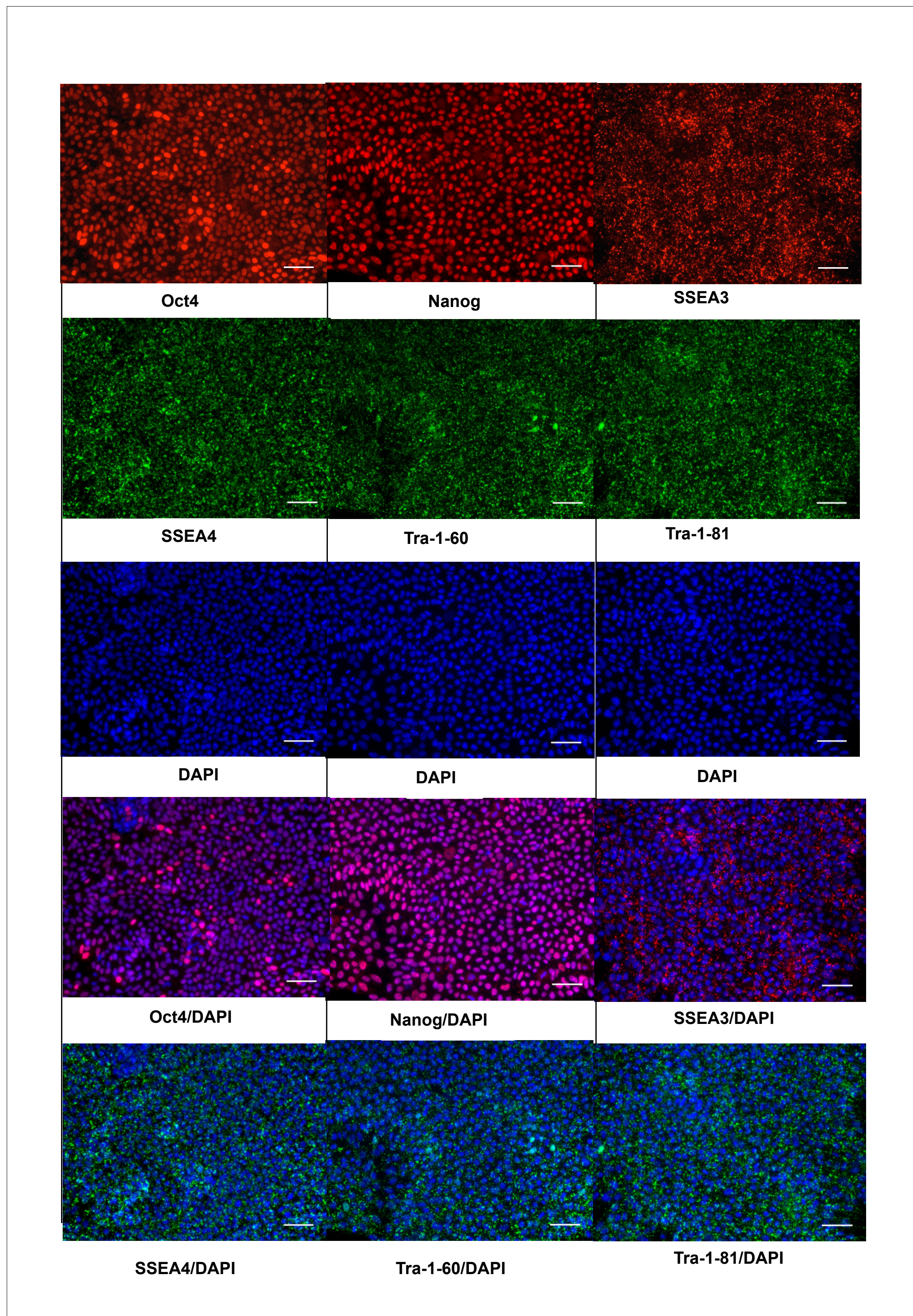


Figure 7.3: One example of the immunofluorescence result of iPS cell lines. *iPS cells were stained with anti-human Oct4, Nanog, SSEA4, SSEA3, TRA-1-60 and TRA-1-81 primary antibodies respectively and then incubated with fluorochrome conjugated secondary antibodies. The photos in the first and second row showed the expression of Oct4, Nanog, SSEA4, SSEA3, TRA-1-60 and TRA-1-81 markers. The photos in the third row suggested the presence of DAPI staining. The photos in the last row are the composite ones. Scale bar 50um*

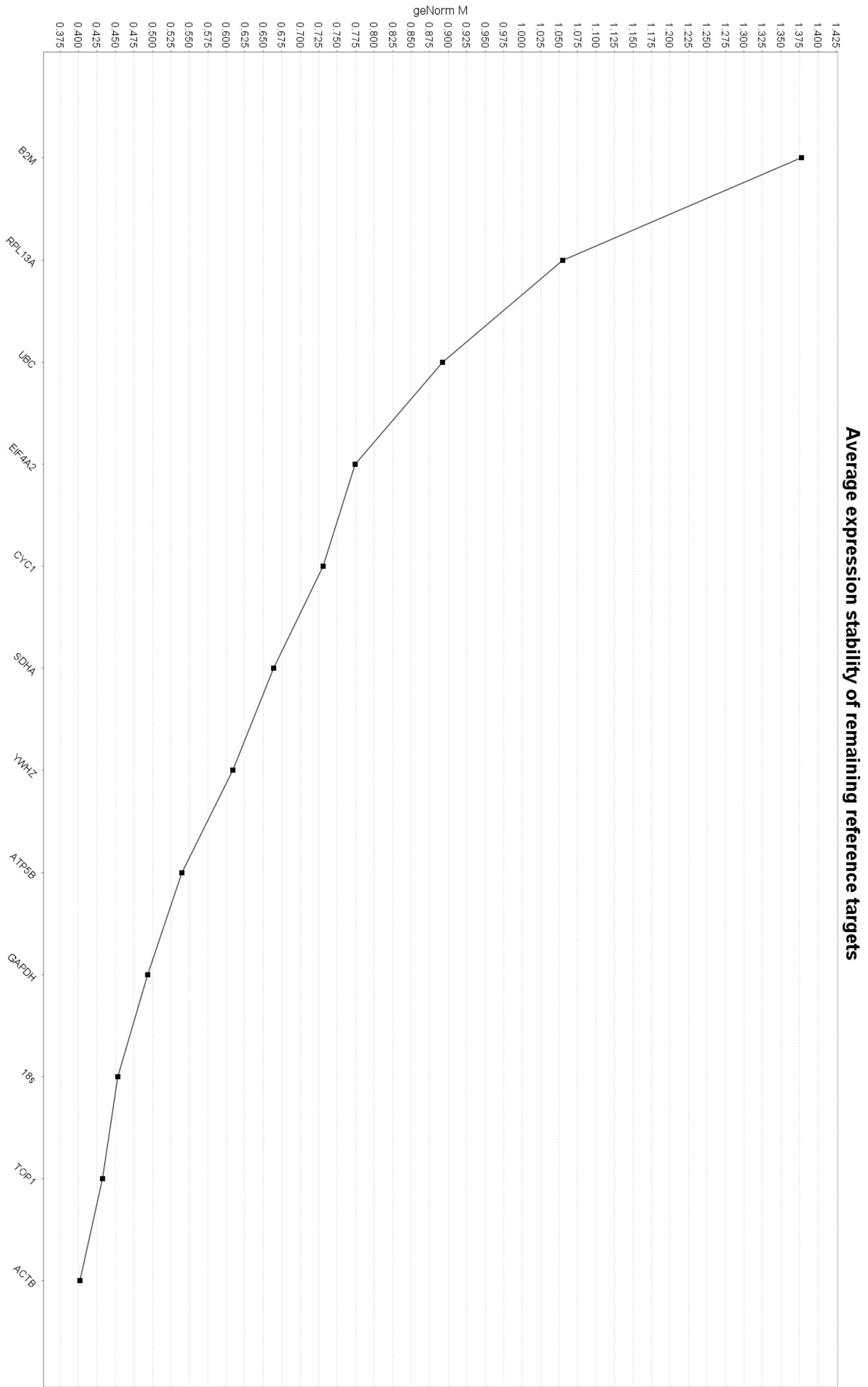


Figure 7.4: Average expression stability of reference genes

The graph shows the average expression stability value (M) of reference genes at each stepwise exclusion of the least stably expressed reference genes. The higher M of the gene mean the least stable expression, and the the genes are ranked according to increasing expression stability. Based on the ranked M value of commonly used 12 housekeeping genes, the three genes with the lowest M value at the right (average $M \leq 0.5$) are 18s, TOP1, and ACTB.

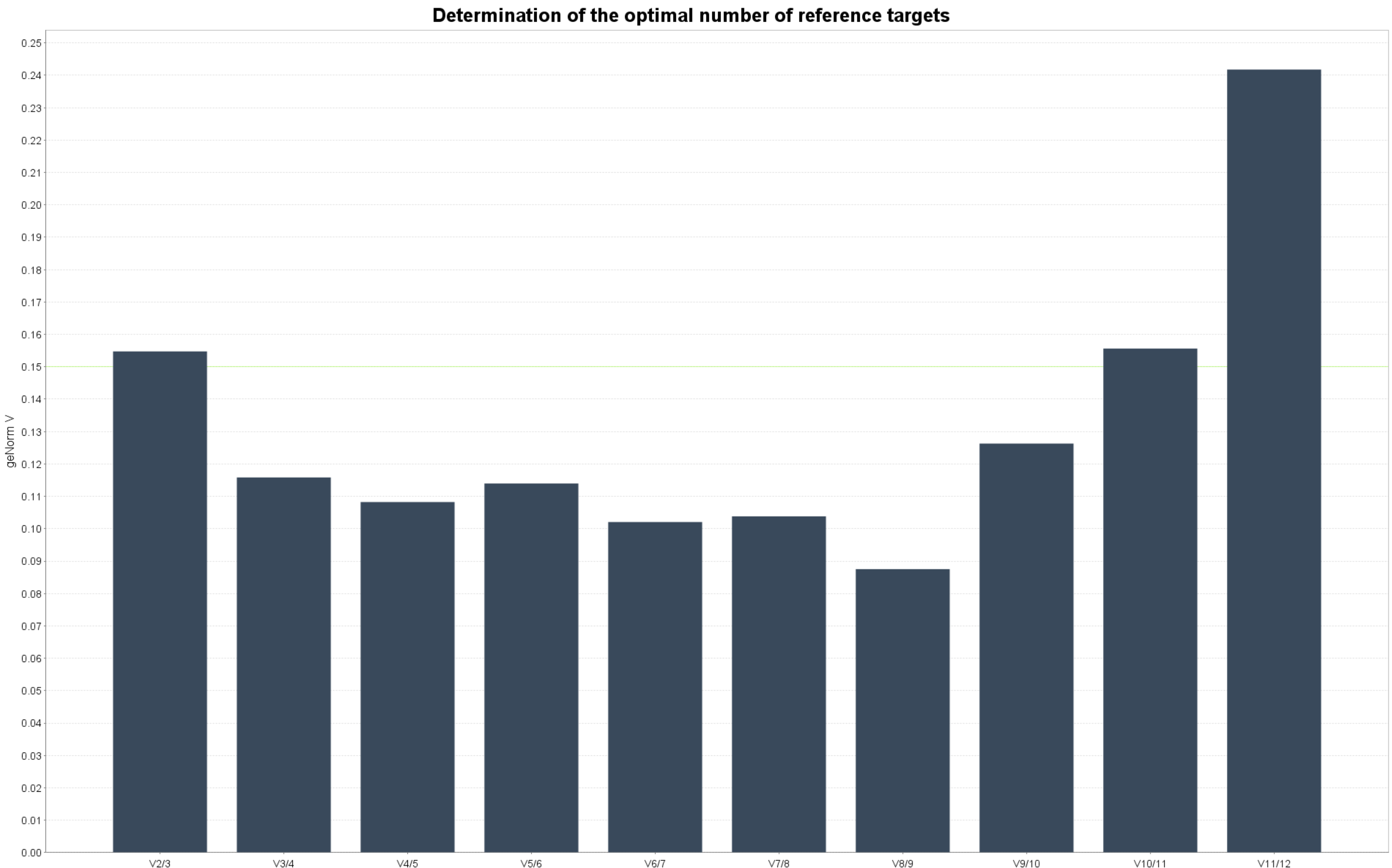


Figure 7.5: Determination of the optimal number of reference genes

The optimal number of reference genes for normalisation is determined through pairwise variation analysis between normalisation factors NF_n and NF_{n+1}. Starting with the two most stably expressed genes on the left, the chart indicates sequential addition of each reference gene in the two genes with the inclusion of a 3rd, 4th, 5th gene etc. moving to the right. The measure is named as pairwise variation, V(n/n+1). When geNorm $V < 0.15$, below which inclusion of additional control gene is not necessary. In our result, V2/3 is at the edge of 0.15, and V3/4 is between 0.11 and 0.12.

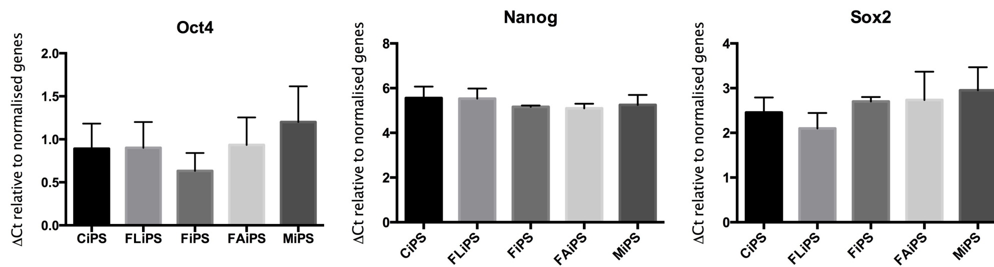


Figure 7.6: The $\triangle$Ct of Oct4, Nanog and Sox2

For the mRNA expression of Oct4, Nanog or Sox2 genes, there was no significant difference between lines derived from the same or different sources. N=24, $p > 0.05$

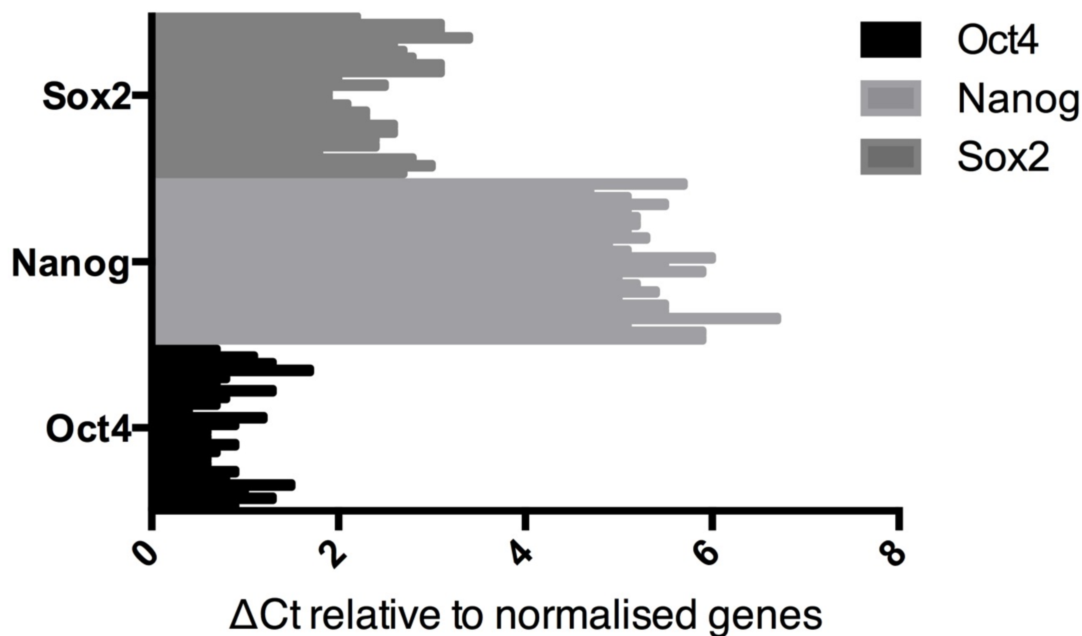


Figure 7.7: The $\triangle$Ct comparison of pluripotent genes of iPS cells

Compared to genes for normalisation, average $\triangle$Ct of Oct4, Nanog, and Sox2 is respectively 0.9, 5.4, and 2.6. N=24

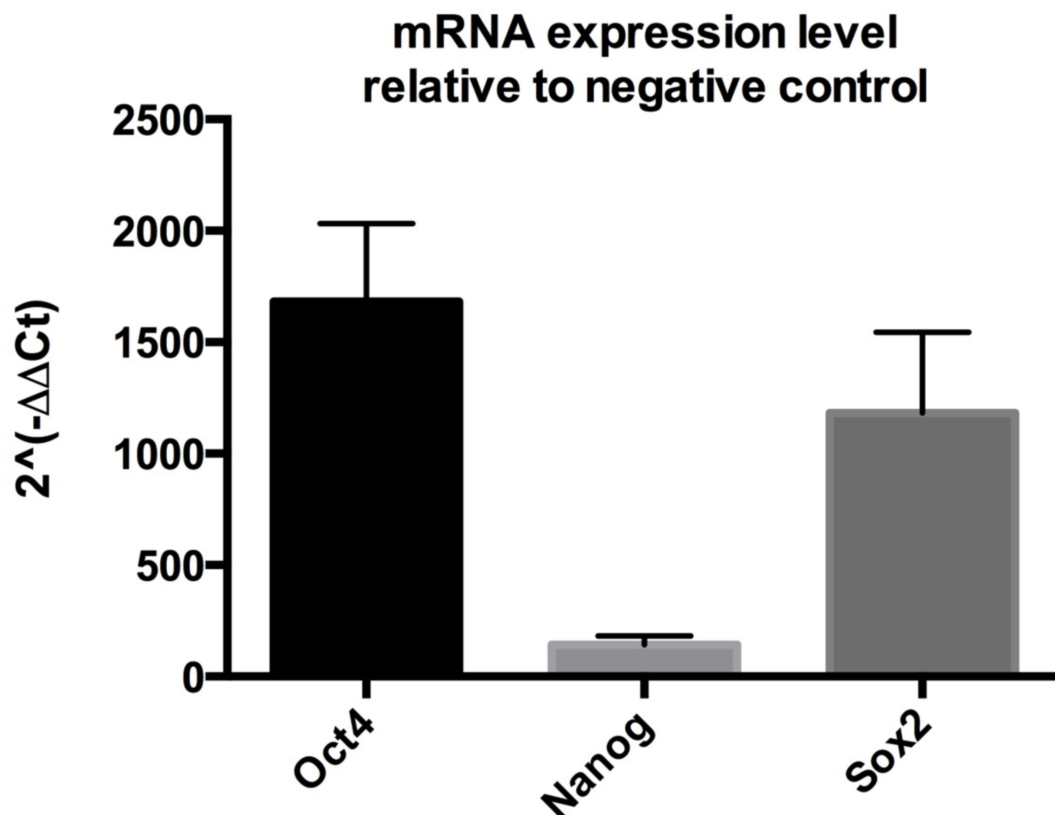


Figure 7.8: The ΔΔCt comparison of pluripotent genes of iPS cells compared to negative control *Based on* $2^{-\Delta\Delta Ct}$ *calculation method, mRNA expression of Oc4 was 1682 fold higher than negative control, Sox2 expression was 1182 fold compared to negative control, and Nanog expression was only 143 fold higher than negative control. N=24*

7.3.3 *In vitro* differentiation into three germ layer cells

In order to determine whether a cell is a real pluripotent stem cell, it is pivotal to assess its ability to differentiate into three germ layers. Human pluripotent stem cell functional identification kit was used to differentiate iPS cells we produced from different sources into ectoderm, endoderm, and mesoderm layers. iPS cells were cultured in human ES cell medium until 50% confluency, and then the spent medium was replaced with differentiation medium for ectoderm, mesoderm, and endoderm layers respectively. The differentiation time period for the three germ layers is different. Figure 7.9 shows the flowchart of the procedure. At the end of differentiation cells were characterised by immunofluorescence using goat anti-human Otx2 Ab for ectoderm cell types, goat anti-human brachyury Ab for mesoderm cell types, and goat anti-human Sox17 Ab for endoderm cell types (see Figure 7.10). In our experiments, we randomly selected three of iPS cell lines derived from mononuclear cells, cord blood $CD34^+$ cells, fetal liver suspended $CD34^+$ cells, fibroblast cells, and fetal liver adherent cells, and they have the capacity to differentiate into cell types of ectoderm, mesoderm, endoderm layers. (Figure 7.11)

7.4 Discussion

7.4.1 Pluripotent markers can identify iPS cells

In our reprogramming experiments, when colonies appeared, it is necessary to distinguish the real iPS colonies from non-iPS colonies through observing the cell morphology of the cell under a high magnification microscope. Cells from real iPS colonies are close to each other with prominent nucleoli and high nuclear-cytoplasm ratio, and this type of cells forms compact colonies with dense colony

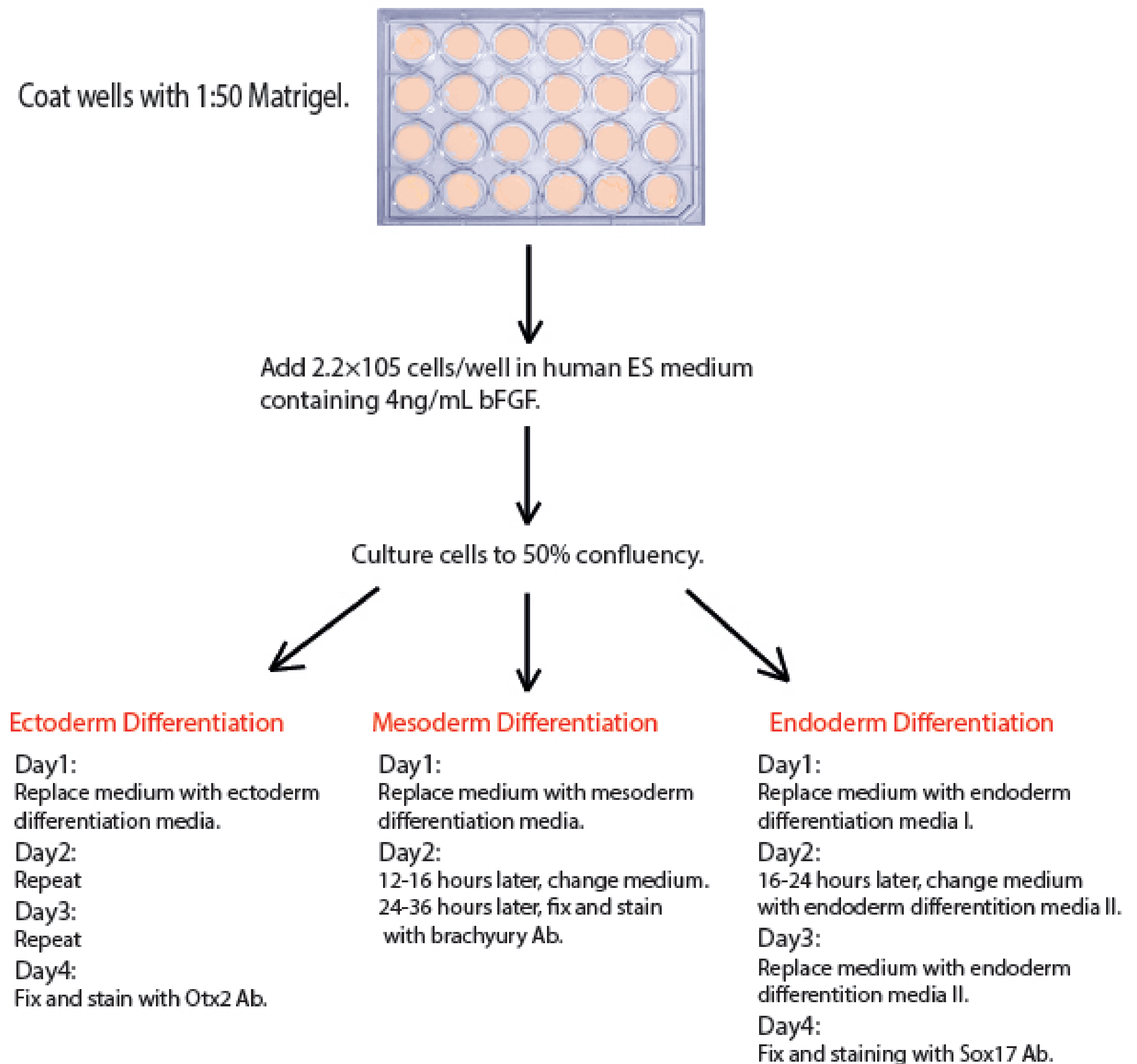
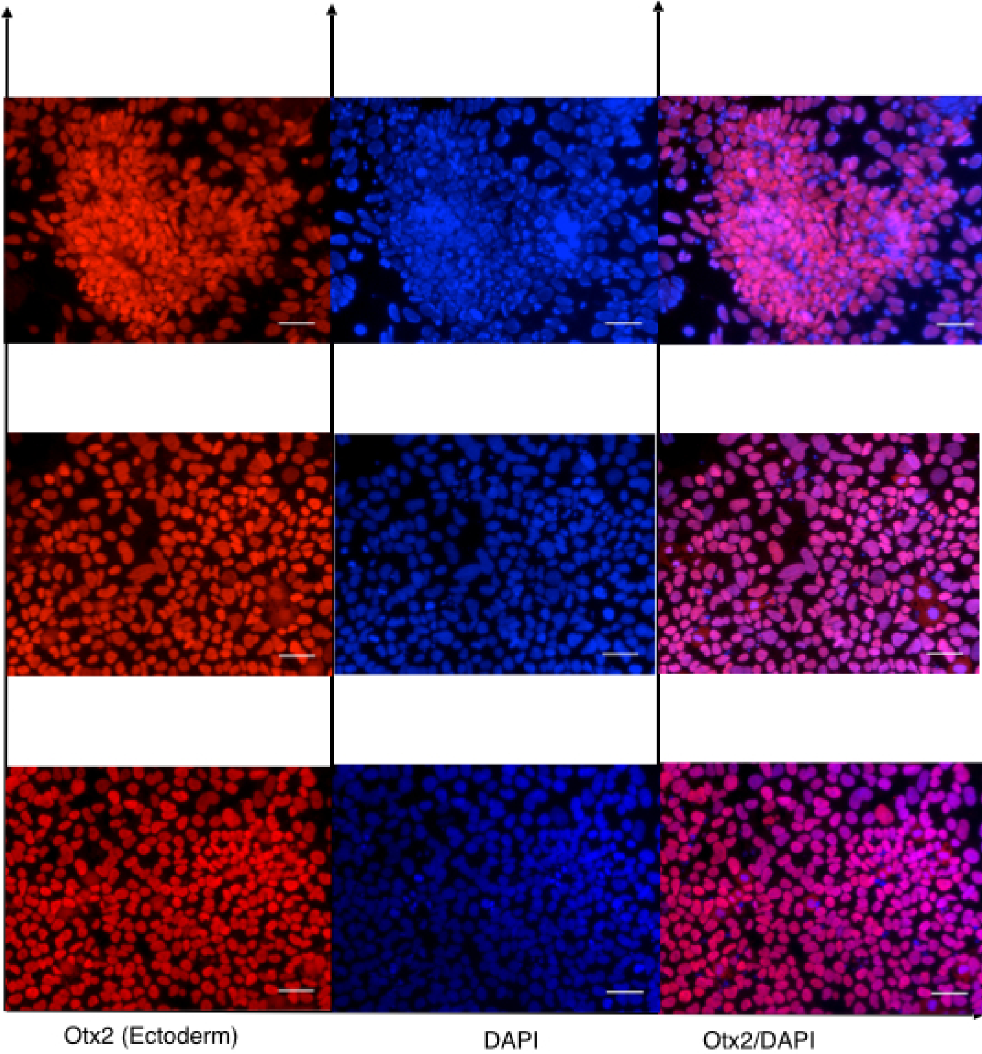
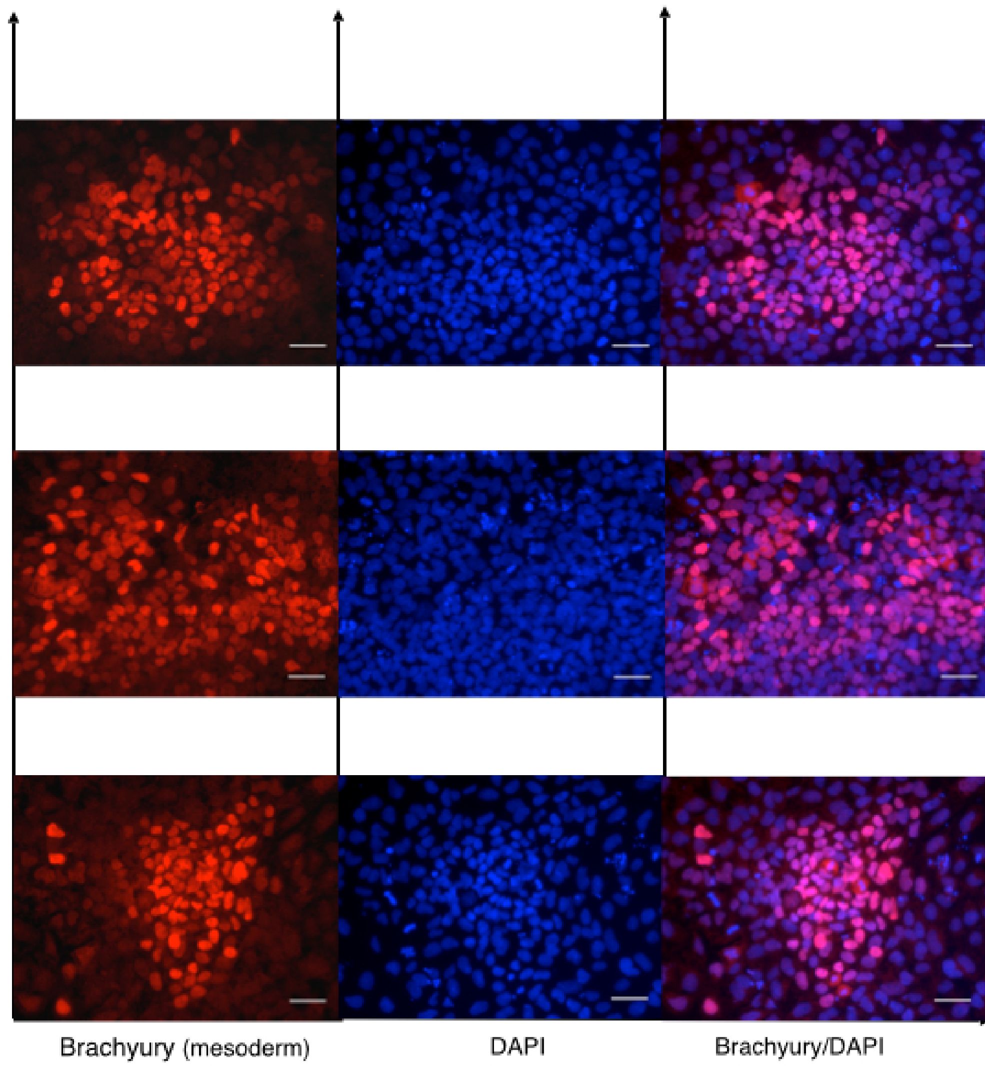


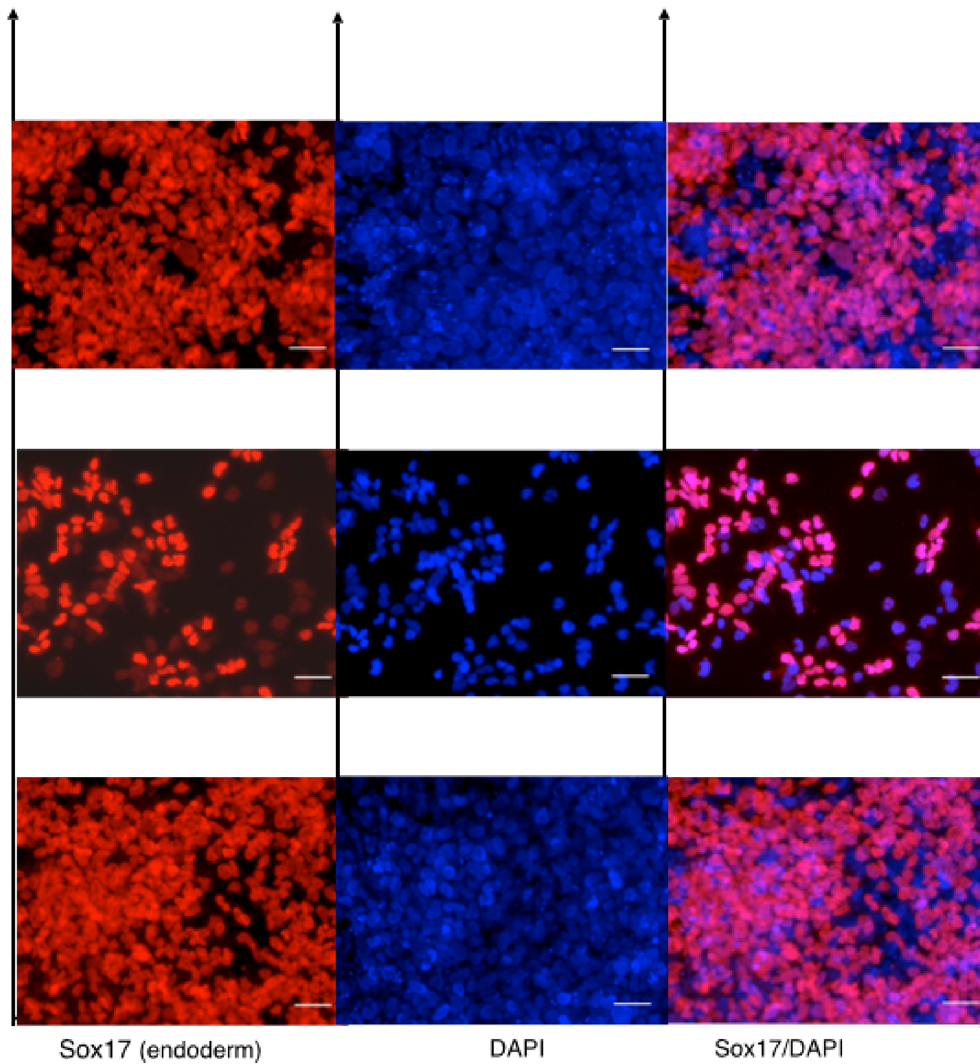
Figure 7.9: iPS cells differentiation procedure



(a) *Ectoderm cell types differentiated from iPS cells. scale bar 50µm*



(b) Mesoderm cell types differentiated from iPS cells. scale bar 50µm



(c) *Endoderm cell types differentiated from iPS cells. scale bar 50µm*

Figure 7.10: **An example of the positive staining of markers for three germ layers**

Goat anti-human Otx2 Ab for ectoderm cell types, goat anti-human brachyury Ab for mesoderm cell types, and goat anti-human Sox17 Ab for endoderm cell types were used in immunofluoresence.

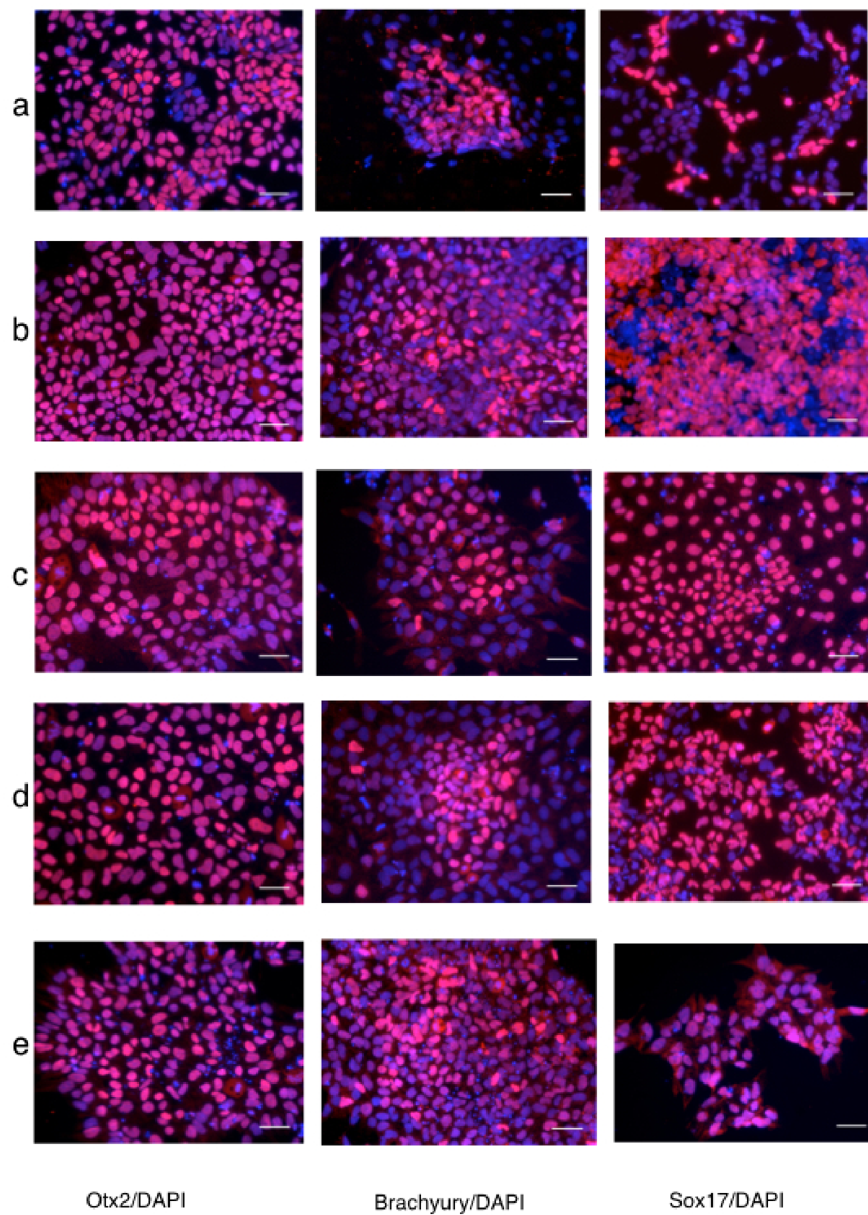


Figure 7.11: **iPS cell lines derived from different sources have the capacity to differentiate into cell types of ectoderm (Otx2), mesoderm (Brachyury), endoderm (Sox17) layers.**

iPS cell lines from mononuclear cells (a), cord blood CD34$^+$ cells (b), fetal liver suspension CD34$^+$ cells (c), fetal liver adherent cells (d), fibroblast cells (e). scale bar 50 μm

centres and defined borders. This is a simple but robust way to exclude non-iPS colonies. However, it is necessary to provide more reliable evidence to verify iPS cells. The flow cytometry and immunofluorescence studies make use of pluripotent markers to identify iPS cells. We chose surface markers (SSEA-4, SSEA-3, TRA-1-60, TRA-1-81) and intracellular markers (Oct4, Nanog). Although small differences were present, these markers confirmed that the derived iPS cells expressed markers consistent with pluripotency.

7.4.2 Pluripotent gene expression of established iPS cell lines

The appropriate selection of reference genes for normalisation is the essential prerequisite for obtaining reliable results to compare differences of gene expressions from various tissues. The expressions of commonly used reference genes from different types of tissues are different so it is necessary to select stable expressed reference genes for each tissue in each experimental system. Vandesompele and colleagues (Vandesompele *et al.*, 2002) developed a gene-stability measure M as the average variation between a particular gene and other control genes using qBaseplus software. According to the ranked M value of commonly used reference genes belonging to different functional classes, the lower M value demonstrates the more stable expression. For established iPS cell lines from different sources in our case, the three genes with the lowest M value (average $M \leq 0.5$) are 18s, TOP1, and ACTB. After validation of stability of reference genes, it is necessary to determine how many reference genes should be used for normalisation? They applied normalisation factor calculation based on the geometric mean of multiple reference genes. The optimal number of reference genes for normalisation is determined through pairwise variation analysis between normalisation factors NF_n

and NF_{n+1}. When geNorm V < 0.15, below which inclusion of additional control gene is not necessary. In our result, V2/3 is at the edge of 0.15, and V3/4 is between 0.11 and 0.12. Considering stability of genes and normalisation factors, we determined three reference genes including 18s, TOP1, and ACTB. However, in analysis we found that the Ct value of 18s is 10 fold lower than the other two reference genes, which might decrease the accuracy of comparison of gene of interest. Thus in the final normalisation analysis, we used TOP1 and ACTB as normalisation genes.

Compared to negative controls, the mRNA expressions of Oct4, Nanog, and Sox2 were significant. Although the iPS cells derived from different sources, there were no significant differences of the transcription of pluripotent genes among various sources. This indicates the protocol of reprogramming using episomal vectors is reliable and reproducible. However, the mRNA expression levels of the three genes are different and expression levels are as following Oct4 >Sox2 >Nanog.

7.4.3 *In vitro* differentiation test verifies the pluripotency of iPS cells

However gene expression alone is not sufficient to verify the pluripotency of iPS cells unless these cells are differentiated into cell types of three germ layers. As mentioned in section 7.1, the literature has examples of both *in vitro* and *in vivo* tests which have been used to verify the pluripotency of ES and iPS cells. Although the *in vivo* teratoma formation test is frequently used as gold standard, it is costly in both time and animal welfare and time-consuming. In addition, teratoma is a type of tumour which has to be removed from humans in clinical practice. Therefore it is preferable to find an efficient and cheap approach to verify the pluripotency of iPS cells. The *in vitro* tests described in this chapter

are cheaper than the *in vivo* teratoma formation tests and only require three days to complete. In the *ex vivo* differentiation test, cell density at the beginning of the experiment would affect the differentiation result. In the *in vitro* tests 50% confluency is optimised for differentiation into the three germ layers and too low confluency would result in low differentiation efficiency.

7.5 Concluding remarks

The iPS cell lines established in this work have pluripotent capacity as demonstrated by their differentiation into cell types of three germ layers and they are characterised by the expression of Oct4, Nanog, SSEA4, SSEA3, TRA-1-60 and TRA-1-81.

Chapter 8

Conclusion

Blood is one of the most easily accessible tissues from which to obtain cells for the generation of iPS cells but there are many other types of cells which can be utilised including, for example, HSPC, MNC and endothelial cells. It is necessary to look at different populations amenable for use as reprogramming sources and how chromatin modifying factors affect the cells, as chromatin modifying factors are useful for producing iPS cells. The largest fraction of $CD34^+$ cells, the $CD34^+CD133^+$ population, is essentially committed to myeloid colony production while the $CD34^+CD133^-$ population has a greater capacity to generate erythroid colonies. The addition of IL3 to the *in vitro* cell growth medium improved the expansion and maintained the functionality of $CD34^+$ cells. The optimised cytokine cocktail for expanding $CD34^+$ cells was 100 ng/ml SCF, 10 ng/ml Flt3L, and 20 ng/ml IL3, which maintained the cells in an active proliferating state. The cytokine cocktail also works well with MNC.

The proliferating cells were amenable to electroporation for the effective delivery of transcription factors (Oct4, sox2, klf4, L-myc, and lin28) within a backbone

of oriP/EBNA-1. This work has successfully developed an efficient and easy way to reprogram $CD34^+$ cells, mononuclear cells from frozen samples and other cell types in order to generate induced pluripotent cells using episomal vectors in a feeder-free system without any assistance of small molecules. The iPS cell lines we generated not only maintained the successful expression of ES markers including Oct4, nanog, SSEA3. SSEA4, tra-1-60 and tra-1-81, but also have the capacity to successfully differentiate into cell types of ectoderm, mesoderm and endoderm layers *in vitro*. The highest reprogramming $CD34^+$ cell efficiency is 0.148%. Compared with published non-integration methods, the efficiency is much higher than 0.016% reported by Bin-Kuan Chou and colleagues (Chou *et al.*, 2011) and 0.03% reported by Mack and colleagues (Mack *et al.*, 2011) to generate iPS colonies from $CD34^+$ cells. In the research by Keisuke Okita and colleagues (Okita *et al.*, 2013), the maximum reprogramming efficiency of $CD34^+$ cells was up to 0.1%. Using our optimised protocol to reprogram frozen MNC from adult peripheral blood, every input 10^5 cells in a feeder-free system can generate around 33 colonies (0.033%), much higher than the reported non-T cell stimulation method to reprogram MNC from peripheral blood regardless of viral or non-viral delivery tool (0.0007% by Bin-Kuan Chou and colleagues (Chou *et al.*, 2011), 0.001% by Keisuke Okita and colleagues (Okita *et al.*, 2013)). However, our reprogramming efficiency is lower than 0.06% efficiency of reprogramming MNC using T cell stimulation medium and EBNA episomal vectors reported by Keisuke Okita and colleagues (Okita *et al.*, 2013).

The optimized methodology can produce iPS colonies from $CD34^+$ cells as early as the eighth day and grew large enough for picking on day 11-day 13 and onward. During the process of reprogramming MNC, iPS colonies appeared on day11, later than $CD34^+$ cells. In addition, the feeder-free system facilitates downstream work - picking colonies. The successful development of an efficient way to reprogram frozen mononuclear cells using an integration-free method in a feeder-free system

provides a promising future for potential clinical application. Mononuclear cells are isolated from peripheral blood which is one of the most easily accessible tissues from patients. Thus it is possible to reprogram mononuclear cells from any patient to generate patient-specific iPS cells for future personalised therapies, the establishment of disease models for research, drug testing, the potential therapeutic treatments in regenerative medicine and other applications.

Bibliography

Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilić, J., Pekarik, V., Tiscornia, G., Edel, M., Boué, S. and Belmonte, J.C.I. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nature biotechnology*, **26**, 1276–1284.

Abilez, O., Benharash, P., Miyamoto, E., Gale, A., Xu, C. and Zarins, C.K. (2009). P19 progenitor cells progress to organized contracting myocytes after chemical and electrical stimulation: implications for vascular tissue engineering.

Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A.M., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M. and Jacobsen, S.E.W. (2005). Identification of flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell*, **121**, 295–306.

Akashi, K., Traver, D., Miyamoto, T. and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*, **404**, 193–197.

Allsopp, R.C. and Weissman, I.L. (2002). Replicative senescence of hematopoietic stem cells during serial transplantation: does telomere shortening play a role? *Oncogene*, **21**, 3270–3.

Amigo, J.D., Ackermann, G.E., Cope, J.J., Yu, M., Cooney, J.D., Ma, D., Langer, N.B., Shafizadeh, E., Shaw, G.C., Horsely, W. *et al.* (2009). The role and regulation of friend of gata-1 (fog-1) during blood development in the zebrafish. *Blood*, **114**, 4654–4663.

Andrews, P.W., Banting, G., Damjanov, I., Arnaud, D. and Avner, P. (1984). Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma*, **3**, 347–61.

Andries, O., Filette, M.D., De Smedt, S.C., Demeester, J., Poucke, M.V., Peelman, L. and Sanders, N.N. (2013). Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mrna to respiratory cells. *Journal of Controlled Release*, **167**, 157–166.

Anjos-Afonso, F., Currie, E., Palmer, H.G., Foster, K.E., Taussig, D.C. and Bonnet, D. (2013). Cd34(-) cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. *Cell Stem Cell*, **13**, 161–74.

Anokye-Danso, F., Trivedi, C.M., Juhr, D., Gupta, M., Cui, Z., Tian, Y., Zhang, Y., Yang, W., Gruber, P.J., Epstein, J.A. and Morrisey, E.E. (2011). Highly efficient mirna-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell stem cell*, **8**, 376–388.

Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T. and Yamanaka, S. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science*, **321**, 699–702.

Armstrong, L., Stojkovic, M., Dimmick, I., Ahmad, S., Stojkovic, P., Hole, N. and Lako, M. (2004). Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. *Stem Cells*, **22**, 1142–51.

Armstrong, L., Hughes, O., Yung, S., Hyslop, L., Stewart, R., Wappler, I., Peters, H., Walter, T., Stojkovic, P., Evans, J., Stojkovic, M. and Lako, M. (2006). The role of pi3k/akt, mapk/erk and nf-kappa beta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. *Human molecular genetics*, **15**, 1894–1913.

Audet, J., Miller, C.L., Eaves, C.J. and Piret, J.M. (2002). Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. *Biotechnol Bioeng*, **80**, 393–404.

Avruch, J., Khokhlatchev, A., Kyriakis, J.M., Luo, Z., Tzivion, G., Vavvas, D. and Zhang, X.F. (2001). Ras activation of the raf kinase tyrosine kinase recruitment of the map kinase cascade. *Recent Progress in Hormone Research*, **56**, 127–156.

Bach, F., Albertini, R., Joo, P., Anderson, J. and Bortin, M. (1968). Bone-marrow transplantation in a patient with the wiskott-aldrich syndrome. *The Lancet*, **292**, 1364–1366.

Baharvand, H., Hashemi, S.M., Ashtiani, S.K. and Farrokhi, A. (2006). Differentiation of human embryonic stem cells into hepatocytes in 2d and 3d culture systems in vitro. *International Journal of Developmental Biology*, **50**, 645.

Ban, H., Nishishita, N., Fusaki, N., Tabata, T., Saeki, K., Shikamura, M., Takada, N., Inoue, M., Hasegawa, M., Kawamata, S. and Nishikawa, S.I. (2011). Efficient generation of transgene-free human induced pluripotent stem cells (ipscs) by temperature-sensitive sendai virus vectors. *Proceedings of the National Academy of Sciences*, **108**, 14234–14239.

Bao, X., Zhu, X., Liao, B., Benda, C., Zhuang, Q., Pei, D., Qin, B. and Esteban, M.A. (2013). Micrornas in somatic cell reprogramming. *Current opinion in cell biology*.

Barfield, R.C., Hale, G.A., Burnette, K., Behm, F.G., Knapp, K., Eldridge, P. and Handgretinger, R. (2007). Autologous transplantation of cd133 selected hematopoietic progenitor cells for treatment of relapsed acute lymphoblastic leukemia. *Pediatr Blood Cancer*, **48**, 349–53.

Barnes, D., Corp, M., Loutit, J. and Neal, F. (1956). Treatment of murine leukaemia with x rays and homologous bone marrow. *British Medical Journal*, **2**, 626.

Bartel, D.P. (2009). Micrornas: target recognition and regulatory functions. *Cell*, **136**, 215–233.

Bartels, C.L. and Tsongalis, G.J. (2009). Micrornas: novel biomarkers for human cancer. *Clinical chemistry*, **55**, 623–631.

Beattie, G.M., Lopez, A.D., Bucay, N., Hinton, A., Firpo, M.T., King, C.C. and Hayek, A. (2005). Activin a maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem cells*, **23**, 489–495.

Behringer, R., Ryan, T., Palmiter, R., Brinster, R. and Townes, T. (1990). Human gamma-to beta-globin gene switching in transgenic mice. *Genes & development*, **4**, 380–389.

Bendall, S.C., Stewart, M.H., Menendez, P., George, D., Vijayaragavan, K., Werbowetski-Ogilvie, T., Ramos-Mejia, V., Rouleau, A., Yang, J., Bossé, M., Lajoie, G. and Bhatia, M. (2007). Igf and fgf cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature*, **448**, 1015–1021.

Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D. and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proceedings of the National Academy of Sciences*, **94**, 5320–5325.

Bhatia, M., Bonnet, D., Murdoch, B., Gan, O.I. and Dick, J.E. (1998). A newly discovered class of human hematopoietic cells with scid-repopulating activity. *Nature medicine*, **4**, 1038–1045.

Billingham RE, B.L. (1959). Quantitative studies on tissue transplantation immunity. iv. induction of tolerance in newborn mice and studies on the phenomenon of runt disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **242**, 439–77.

Bloom, W. and Bartelmez, G. (1940). Hematopoiesis in young human embryos. *Am. J. Anat.*, **67**, 21–53.

Borg, J., Papadopoulos, P., Georgitsi, M., Gutiérrez, L., Grech, G., Fanis, P., Phylactides, M., Verkerk, A.J., van der Spek, P.J., Scerri, C.A., Cassar, W., Galdies, R., van IJcken, W., Ozgur, Z., Gillemans, N., Hou, J., Bugeja, M., Grosveld, F.G., von Lindern, M., Felice, A.E., Patrinos, G.P. and Philipsen, S. (2010). Haploinsufficiency for the erythroid transcription factor klf1 causes hereditary persistence of fetal hemoglobin. *Nature genetics*, **42**, 801–805.

Braam, S.R., Denning, C., Van Den Brink, S., Kats, P., Hochstenbach, R., Passier, R. and Mummery, C.L. (2008). Improved genetic manipulation of human embryonic stem cells. *Nature methods*, **5**, 389–392.

Brännvall, K., Bergman, K., Wallenquist, U., Svahn, S., Bowden, T., Hilborn, J. and Forsberg-Nilsson, K. (2007). Enhanced neuronal differentiation in a three-dimensional collagen-hyaluronan matrix. *Journal of neuroscience research*, **85**, 2138–2146.

Brown, M.E., Rondon, E., Rajesh, D., Mack, A., Lewis, R., Feng, X., Zitur, L.J., Learish, R.D. and Nuwaysir, E.F. (2010). Derivation of induced pluripotent stem cells from human peripheral blood t lymphocytes. *PLoS One*, **5**, e11373.

Buganim, Y., Faddah, D.A., Cheng, A.W., Itskovich, E., Markoulaki, S., Ganz, K., Klemm, S.L., van Oudenaarden, A. and Jaenisch, R. (2012). Single-cell expression analyses during cellular reprogramming reveal an early stochastic and a late hierarchic phase. *Cell*, **150**, 1209–22.

Burridge, P.W. and Zambidis, E.T. (2013). Highly efficient directed differentiation of human induced pluripotent stem cells into cardiomyocytes. *Methods Mol Biol*, **997**, 149–61.

Burt, R.K., Testori, A., Oyama, Y., Rodriguez, H.E., Yaung, K., Villa, M., Bucha, J.M., Milanetti, F., Sheehan, J., Rajamannan, N. and Pearce, W.H. (2010). Autologous peripheral blood cd133+ cell implantation for limb salvage in patients with critical limb ischemia. *Bone Marrow Transplant*, **45**, 111–6.

Cai, L., Ye, Z., Zhou, B.Y., Mali, P., Zhou, C. and Cheng, L. (2007). Promoting human embryonic stem cell renewal or differentiation by modulating wnt signal and culture conditions. *Cell research*, **17**, 62–72.

Campbell, K.H., McWhir, J., Ritchie, W. and Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, **380**, 64–66.

Cantor, A.B. and Orkin, S.H. (2002). Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene*, **21**, 3368–3376.

Carey, B.W., Markoulaki, S., Hanna, J., Saha, K., Gao, Q., Mitalipova, M. and Jaenisch, R. (2009). Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc Natl Acad Sci U S A*, **106**, 157–62.

Case, J., Mead, L.E., Bessler, W.K., Prater, D., White, H.A., Saadatzadeh, M.R., Bhavsar, J.R., Yoder, M.C., Haneline, L.S. and Ingram, D.A. (2007). Human cd34+ac133+vegfr-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp Hematol*, **35**, 1109–18.

Centeno, C.J., Busse, D., Kisiday, J., Keohan, C., Freeman, M. and Karli, D. (2008). Regeneration of meniscus cartilage in a knee treated with percutaneously implanted autologous mesenchymal stem cells. *Medical Hypotheses*, **71**, 900 – 908.

Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A. and Bhatia, M. (2003). Cytokines and bmp-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood*, **102**, 906–915.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003). Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, **113**, 643–55.

Chang, A.I. and Appasani, K. (2006). Stem cells & regenerative medicine: from molecular embryology to tissue engineering and therapeutics.

Chang, A.N., Cantor, A.B., Fujiwara, Y., Lodish, M.B., Droho, S., Crispino, J.D. and Orkin, S.H. (2002). Gata-factor dependence of the multitype zinc-finger protein fog-1 for its essential role in megakaryopoiesis. *Proceedings of the National Academy of Sciences*, **99**, 9237–9242.

Charbord, P., Tavian, M., Humeau, L. and Peault, B. (1996). Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood*, **87**, 4109–4119.

Chateauvieux, S., Eifes, S., Morceau, F., Grigorakaki, C., Schnekenburger, M., Henry, E., Dicato, M. and Diederich, M. (2011). Valproic acid perturbs hematopoietic homeostasis by inhibition of erythroid differentiation and activation of the myelo-monocytic pathway. *Biochem Pharmacol*, **81**, 498–509.

Chaurasia, P., Berenzon, D. and Hoffman, R. (2011). Chromatin-modifying agents promote the ex vivo production of functional human erythroid progenitor cells. *Blood*, **117**, 4632–41.

Chen, K. and Rajewsky, N. (2007). The evolution of gene regulation by transcription factors and micrornas. *Nature Reviews Genetics*, **8**, 93–103.

Chen, L., Gao, Z., Zhu, J. and Rodgers, G.P. (2007). Identification of cd13$^+$cd36$^+$ cells as a common progenitor for erythroid and myeloid lineages in human bone marrow. *Experimental hematology*, **35**, 1047–1055.

Chiu, C.T., Wang, Z., Hunsberger, J.G. and Chuang, D.M. (2013). Therapeutic potential of mood stabilizers lithium and valproic acid: beyond bipolar disorder. *Pharmacol Rev*, **65**, 105–42.

Chou, B.K., Mali, P., Huang, X., Ye, Z., Dowey, S.N., Resar, L.M., Zou, C., Zhang, Y.A., Tong, J. and Cheng, L. (2011). Efficient human ips cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res*, **21**, 518–29.

Cooney, R.N. (2002). Suppressors of cytokine signaling (socs): inhibitors of the jak/stat pathway. *Shock*, **17**, 83–90.

Corbeil, D., Roper, K., Hellwig, A., Tavian, M., Miraglia, S., Watt, S.M., Simmons, P.J., Peault, B., Buck, D.W. and Huttner, W.B. (2000). The human ac133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J Biol Chem*, **275**, 5512–20.

Cortés, F., Debacker, C., Péault, B. and Labastie, M.C. (1999). Differential expression of kdr/vegfr-2 and cd34 during mesoderm development of the early human embryo. *Mechanisms of development*, **83**, 161–164.

Crispino, J.D., Lodish, M.B., MacKay, J.P. and Orkin, S.H. (1999). Use of altered specificity mutants to probe a specific protein–protein interaction in differentiation: the gata-1: Fog complex. *Molecular cell*, **3**, 219–228.

Csete, M. (2005). Oxygen in the cultivation of stem cells. *Annals of the New York Academy of Sciences*, **1049**, 1–8.

Cumano, .G.I., A. (2007). Ontogeny of the hematopoietic system. *Annu Rev Immunol*, **25**, 745–785.

Curran, J.M., Chen, R. and Hunt, J.A. (2006). The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. *Biomaterials*, **27**, 4783–4793.

Dao, M.A., Hannum, C.H., Kohn, D.B. and Nolta, J.A. (1997). Flt3 ligand preserves the ability of human cd34+ progenitors to sustain long-term hematopoiesis in immune-deficient mice after ex vivo retroviral-mediated transduction. *Blood*, **89**, 446–56.

Dao, M.A., Arevalo, J. and Nolta, J.A. (2003). Reversibility of cd34 expression on human hematopoietic stem cells that retain the capacity for secondary reconstitution. *Blood*, **101**, 112–118.

De Felice, L., Di Pucchio, T., Mascolo, M.G., Agostini, F., Breccia, M., Guglielmi, C., Ricciardi, M.R., Tafuri, A., Screnci, M., Mandelli, F. and Arcese, W. (1999). Flt3lp3nduces the ex-vivo amplification of umbilical cord blood committed progenitors and early stem cells in short-term cultures. *Br J Haematol*, **106**, 133–41.

De Felice, L., Tatarelli, C., Mascolo, M.G., Gregorj, C., Agostini, F., Fiorini, R., Gelmetti, V., Pascale, S., Padula, F., Petrucci, M.T., Arcese, W. and Nervi, C. (2005). Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. *Cancer Res*, **65**, 1505–13.

De Koning, J., Van Bekkum, D., Dicke, K., Dooren, L., Van Rood, J. and Radl, J. (1969). Transplantation of bone-marrow cells and fetal thymus in an infant with lymphopenic immunological deficiency. *The Lancet*, **293**, 1223–1227.

Derynck, R. and Zhang, Y.E. (2003). Smad-dependent and smad-independent pathways in tgf-β family signalling. *Nature*, **425**, 577–584.

Di Nardo, P., Minieri, M. and Ahluwalia, A. (2011). Engineering the stem cell niche and the differentiative micro-and macroenvironment: Technologies and tools for applying biochemical, physical and structural stimuli and their effects on stem cells. In *Stem Cell Engineering*, 41–59, Springer.

Didichenko, S.A., Spiegl, N., Brunner, T. and Dahinden, C.A. (2008). Il-3 induces a pim1-dependent antiapoptotic pathway in primary human basophils. *Blood*, **112**, 3949–58.

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R. *et al.* (2008). Induced pluripotent stem cells generated from patients with als can be differentiated into motor neurons. *science*, **321**, 1218–1221.

Ding, L. and Morrison, S.J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, **495**, 231–235.

Ding, V.M., Ling, L., Natarajan, S., Yap, M.G., Cool, S.M. and Choo, A.B. (2010). Fgf-2 modulates wnt signaling in undifferentiated hesc and ips cells through activated pi3-k/gsk3 signaling. *Journal of Cellular Physiology*, **225**, 417–428.

Divine JK, H.H.J.C.W.D.H.M.S.T., Staloch LJ (2004). Gata-4, gata-5, and gata-6 activate the rat liver fatty acid binding protein gene in concert with hnf-1alpha. *Am J Physiol Gastrointest Liver Physiol.*, **287**, 1086–1099.

Driscoll, M.C., Dobkin, C.S. and Alter, B.P. (1989). Gamma delta beta-thalassemia due to a de novo mutation deleting the 5'beta-globin gene activation-region hypersensitive sites. *Proceedings of the National Academy of Sciences*, **86**, 7470–7474.

Dvorak, P. and Hampl, A. (2005). Basic fibroblast growth factor and its receptors in human embryonic stem cells. *Folia Histochem Cytobiol*, **43**, 203–8.

Dzierzak, .S.N.A., E. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol*, **9**, 129–136.

Edvardsson, L., Dykes, J. and Olofsson, T. (2006). Isolation and characterization of human myeloid progenitor populations—tpor as discriminator between common myeloid and megakaryocyte/erythroid progenitors. *Experimental hematology*, **34**, 599–609.

Eminli, S., Utikal, J., Arnold, K., Jaenisch, R. and Hochedlinger, K. (2008). Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous sox2 expression. *Stem Cells*, **26**, 2467–74.

Epling-Burnette, P.K., Zhong, B., Bai, F., Jiang, K., Bailey, R.D., Garcia, R., Jove, R., Djeu, J.Y., Loughran, J., T. P. and Wei, S. (2001). Cooperative regulation of mcl-1 by janus kinase/stat and phosphatidylinositol 3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. *J Immunol*, **166**, 7486–95.

Esteban, M.A., Wang, T., Qin, B., Yang, J., Qin, D., Cai, J., Li, W., Weng, Z., Chen, J., Ni, S., Chen, K., Li, Y., Liu, X., Xu, J., Zhang, S., Li, F., He, W., Labuda, K., Song, Y., Peterbauer, A., Wolbank, S., Redl, H., Zhong, M., Cai, D., Zeng, L. and Pei, D. (2010). Vitamin c enhances the generation of mouse and human induced pluripotent stem cells. *Cell stem cell*, **6**, 71–79.

Esteban, M.A., Bao, X., Zhuang, Q., Zhou, T., Qin, B. and Pei, D. (2012). The mesenchymal-to-epithelial transition in somatic cell reprogramming. *Current opinion in genetics & development*.

Evans, T. and Felsenfeld, G. (1989). The erythroid-specific transcription factor eryf1: a new finger protein. *Cell*, **58**, 877–885.

Evans MJ, K.M. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature*, **292**, 154–156.

Fefer, A., Buckner, C.D., Thomas, E.D., Cheever, M.A., Clift, R.A., Glucksberg, H., Neiman, P.E. and Storb, R. (1977). Cure of hematologic neoplasia with transplantation of marrow from identical twins. *The New England journal of medicine*, **297**, 146.

Fisher, S.A., Doree, C., Brunskill, S.J., Mathur, A. and Martin-Rendon, E. (2013). Bone marrow stem cell treatment for ischemic heart disease in patients with no option of revascularization: A systematic review and meta-analysis. *PLoS One*, **8**, e64669.

Forrester, W.C., Thompson, C., Elder, J. and Groudine, M. (1986). A developmentally stable chromatin structure in the human beta-globin gene cluster. *Proceedings of the National Academy of Sciences*, **83**, 1359–1363.

Forrester, W.C., Takegawa, S., Papayannopoulou, T., Stamatoyannopoulos, G. and Groudine, M. (1987). Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. *Nucleic acids research*, **15**, 10159–10177.

Forsberg, E.C., Serwold, T., Kogan, S., Weissman, I.L. and Passegué, E. (2006). New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. *Cell*, **126**, 415–26.

Forte, G., Carotenuto, F., Pagliari, F., Pagliari, S., Cossa, P., Fiaccavento, R., Ahluwalia, A., Vozzi, G., Vinci, B., Serafino, A., Rinaldi, A., Traversa, E., Carosella, L., Minieri, M. and Di Nardo, P. (2008). Criticality of the biological and physical stimuli array inducing resident cardiac stem cell determination. *Stem Cells*, **26**, 2093–2103.

Fox, A.H., Liew, C., Holmes, M., Kowalski, K., Mackay, J. and Crossley, M. (1999). Transcriptional cofactors of the fog family interact with gata proteins by means of multiple zinc fingers. *The EMBO journal*, **18**, 2812–2822.

Fujisaki, T., Berger, M.G., Rose-John, S. and Eaves, C.J. (1999). Rapid differentiation of a rare subset of adult human lin- cd34- cd38- cells stimulated by multiple growth factors in vitro. *Blood*, **94**, 1926–1932.

Fukuda, T. (1973). Fetal hemopoiesis. i. electron microscopic studies on human yolk sac hemopoiesis. *Virchows Arch B Cell Pathol*, **14**, 197–213.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K. and Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on sendai virus, an rna virus that does not integrate into the host genome. *Proceedings of the Japan Academy, Series B*, **85**, 348–362.

Gai, H., Leung, E.L.H., Costantino, P.D., Aguila, J.R., Nguyen, D.M., Fink, L.M., Ward, D.C. and Ma, Y. (2009). Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts. *Cell biology international*, **33**, 1184–1193.

Gallacher, L., Murdoch, B., Wu, D., Karanu, F., Fellows, F. and Bhatia, M. (2000). Identification of novel circulating human embryonic blood stem cells. *Blood*, **96**, 1740–7.

Galy, A., Travis, M., Cen, D. and Chen, B. (1995). Human t, b, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity*, **3**, 459–473.

Gatti, R., Meuwissen, H., Allen, H., Hong, R. and Good, R. (1968). Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *The Lancet*, **292**, 1366–1369.

Genovese, J.A., Spadaccio, C., Langer, J., Habe, J., Jackson, J. and Patel, A.N. (2008). Electrostimulation induces cardiomyocyte predifferentiation of fibroblasts. *Biochemical and biophysical research communications*, **370**, 450–455.

Gianotti-Sommer, A., Rozelle, S.S., Sullivan, S., Mills, J.A., Park, S.M., Smith, B.W., Iyer, A.M., French, D.L., Kotton, D.N., Gadue, P., Murphy, G.J. and Mostoslavsky, G. (2008). *Generation of human induced pluripotent stem cells from peripheral blood using the STEMCCA lentiviral vector*. Cambridge (MA).

Gill, M., Dias, S., Hattori, K., Rivera, M.L., Hicklin, D., Witte, L., Girardi, L., Yurt, R., Himel, H. and Rafii, S. (2001a). Vascular trauma induces rapid but transient mobilization of vegfr2(+)ac133(+) endothelial precursor cells. *Circ Res*, **88**, 167–74.

Gill, M., Dias, S., Hattori, K., Rivera, M.L., Hicklin, D., Witte, L., Girardi, L., Yurt, R., Himel, H. and Rafii, S. (2001b). Vascular trauma induces rapid but transient mobilization of vegfr2(+)ac133(+) endothelial precursor cells. *Circ Res*, **88**, 167–74.

Gillessen, S., Mach, N., Small, C., Mihm, M. and Dranoff, G. (2001). Overlapping roles for granulocyte-macrophage colony-stimulating factor and interleukin-3 in eosinophil homeostasis and contact hypersensitivity. *Blood*, **97**, 922–928.

Giorgetti, A., Montserrat, N., Aasen, T., Gonzalez, F., Rodriguez-Piza, I., Vassena, R., Raya, A., Boue, S., Barrero, M.J., Corbella, B.A., Torrabadella, M., Veiga, A. and Izpisua Belmonte, J.C. (2009). Generation of induced pluripotent stem cells from human cord blood using oct4 and sox2. *Cell Stem Cell*, **5**, 353–7.

Granero-Moltó, F., Myers, T.J., Weis, J.A., Longobardi, L., Li, T., Yan, Y., Case, N., Rubin, J. and Spagnoli, A. (2011). Mesenchymal stem cells expressing insulin-like growth factor-i (mscigf) promote fracture healing and restore new bone formation in irs1 knockout mice: Analyses of mscigf autocrine and paracrine regenerative effects. *Stem Cells*, **29**, 1537–1548.

Grayson, W.L., Zhao, F., Izadpanah, R., Bunnell, B. and Ma, T. (2006). Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3d constructs. *Journal of cellular physiology*, **207**, 331–339.

Griesenbach, U., Inoue, M., Hasegawa, M. and Alton, E. (2005). Sendai virus for gene therapy and vaccination. *Current opinion in molecular therapeutics*, **7**, 346.

Grosveld, F., van Assendelft, G.B., Greaves, D.R. and Kollias, G. (1987). Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell*, **51**, 975–85.

Guha, P., Morgan, J.W., Mostoslavsky, G., Rodrigues, N.P. and Boyd, A.S. (2013). Lack of immune response to differentiated cells derived from syngeneic induced pluripotent stem cells. *Cell stem cell*.

Guo, C.W., Kawakatsu, M., Idemitsu, M., Urata, Y., Goto, S., Ono, Y., Hamano, K. and Li, T.S. (2013). Culture under low physiological oxygen conditions improves the stemness and quality of induced pluripotent stem cells. *Journal of cellular physiology*.

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Teresa Vallejo-Cremades, M., Fuentes, B., Cerdan, S. and Diez-Tejedor, E. (2013). Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. *Stem Cell Res Ther*, **4**, 11.

Haase, A., Olmer, R., Schwanke, K., Wunderlich, S., Merkert, S., Hess, C., Zweigerdt, R., Gruh, I., Meyer, J., Wagner, S., Maier, L.S., Han, D.W., Glage, S., Miller, K., Fischer, P., Scholer, H.R. and Martin, U. (2009). Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell*, **5**, 434–41.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C. and Cavazzana-Calvo, M. (2003). A serious adverse event after successful gene therapy for x-linked severe combined immunodeficiency. *New England Journal of Medicine*, **348**, 255–256.

Hanna, J., Wernig, M., Markoulaki, S., Sun, C.W., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M. and Jaenisch, R. (2007). Treatment of sickle cell anemia mouse model with ips cells generated from autologous skin. *Science*, **318**, 1920–3.

Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C.J., Creyghton, M.P., van Oudenaarden, A. and Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature*, **462**, 595–601.

Hanna, J., Cheng, A.W., Saha, K., Kim, J., Lengner, C.J., Soldner, F., Cassady, J.P., Muffat, J., Carey, B.W. and Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse escs. *Proceedings of the National Academy of Sciences*, **107**, 9222–9227.

Harigae, H., Okitsu, Y., Yokoyama, H., Fujiwara, T., Inomata, M., Takahashi, S., Minegishi, N., Kaku, M. and Sasaki, T. (2006). Induction of erythroid-specific genes by overexpression of gata-2 in k562 cells. *International journal of hematology*, **84**, 38–42.

Hess, D., Karanu, F., Levac, K., Gallacher, L. and Bhatia, M. (2003). Coculture and transplant of purified cd34+ lin- and cd34- lin- cells reveals functional interaction between repopulating hematopoietic stem cells. *Leukemia*, **17**, 1613–1625.

Hess, D.A., Meyerrose, T.E., Wirthlin, L., Craft, T.P., Herrbrich, P.E., Creer, M.H. and Nolta, J.A. (2004). Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. *Blood*, **104**, 1648–55.

Hess, D.A., Wirthlin, L., Craft, T.P., Herrbrich, P.E., Hohm, S.A., Lahey, R., Eades, W.C., Creer, M.H. and Nolta, J.A. (2006). Selection based on cd133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood*, **107**, 2162–9.

Hilbe, W., Dirnhofer, S., Greil, R. and Woll, E. (2004). Biomarkers in non-small cell lung cancer prevention. *Eur J Cancer Prev*, **13**, 425–36.

Hiratsuka, M., Uno, N., Ueda, K., Kurosaki, H., Imaoka, N., Kazuki, K., Ueno, E., Akakura, Y., Katoh, M., Osaki, M., Kazuki, Y., Nakagawa, M., Yamanaka, S. and Oshimura, M. (2011). Integration-free ips cells engineered using human artificial chromosome vectors. *PLoS One*, **6**, e25961.

Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K. and Yamanaka, S. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature*, **460**, 1132–5.

Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., Liu, K., Ge, J., Xu, J., Zhang, Q., Zhao, Y. and Deng, H. (2013). Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. *Science*, **341**, 651–4.

Howe, S.J., Mansour, M.R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M.H., Pike-Overzet, K., Chatters, S.J., de Ridder, D., Gilmour, K.C., Adams, S., Thornhill, S.I., Parsley, K.L., Staal, F.J., Gale, R.E., Linch, D.C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D.K., Schmidt, M., von Kalle, C., Gaspar, H.B. and Thrasher, A.J. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of scid-x1 patients. *The Journal of clinical investigation*, **118**, 3143.

Hu, K., Yu, J., Suknuntha, K., Tian, S., Montgomery, K., Choi, K.D., Stewart, R., Thomson, J.A. and Slukvin, I. (2011). Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. *Blood*, **117**, e109–19.

Hu, S., Wilson, K.D., Ghosh, Z., Han, L., Wang, Y., Lan, F., Ransohoff, K.J., Burridge, P. and Wu, J.C. (2013). Microrna-302 increases reprogramming efficiency via repression of nr2f2. *Stem Cells*, **31**, 259–268.

Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W. and Melton, D.A. (2008). Induction of pluripotent stem cells from primary human fibroblasts with only oct4 and sox2. *Nat Biotechnol*, **26**, 1269–75.

Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K., Huangfu, D., Akutsu, H., Liu, D.R., Rubin, L.L. and Eggan, K. (2009). A small-molecule inhibitor of tgf-beta signaling replaces sox2 in reprogramming by inducing nanog. *Cell Stem Cell*, **5**, 491–503.

Imamura, M., Aoi, T., Tokumasu, A., Mise, N., Abe, K., Yamanaka, S. and Noce, T. (2010). Induction of primordial germ cells from mouse induced pluripotent stem cells derived from adult hepatocytes. *Mol Reprod Dev*, **77**, 802–11.

International Stem Cell, I., 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., 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., 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., Olsson, C., Otonkoski, T., Park, K.Y., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M.F., Piekarczyk, M.S., Pera, R.A., 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. and Zhang, W. (2007). Characterization of human embryonic stem cell lines by the international stem cell initiative. *Nat Biotechnol*, **25**, 803–16.

Iscove, N.N. and Nawa, K. (1997). Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. *Curr Biol*, **7**, 805–8.

Isern, J., Fraser, S.T., He, Z., Zhang, H. and Baron, M.H. (2010). Dose-dependent regulation of primitive erythroid maturation and identity by the transcription factor eklf. *Blood*, **116**, 3972–3980.

James, D., Levine, A.J., Besser, D. and Hemmati-Brivanlou, A. (2005). Tgfβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development*, **132**, 1273–1282.

Jee, M.K., Kim, J.H., Han, Y.M., Jung, S.J., Kang, K.S., Kim, D.W. and Kang, S.K. (2010). Dhp-derivative and low oxygen tension effectively induces human adipose stromal cell reprogramming. *PloS one*, **5**, e9026.

Johansson, B.M. and Wiles, M.V. (1995). Evidence for involvement of activin a and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. *Molecular and Cellular Biology*, **15**, 141–151.

Jones, J.I. and Clemmons, D.R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev*, **16**, 3–34.

Judson, R.L., Babiarz, J.E., Venere, M. and Blelloch, R. (2009). Embryonic stem cell–specific micrornas promote induced pluripotency. *Nature biotechnology*, **27**, 459–461.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P. and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature*, **458**, 771–775.

Kannagi, R., Cochran, N.A., Ishigami, F., Hakomori, S., Andrews, P.W., Knowles, B.B. and Solter, D. (1983). Stage-specific embryonic antigens (ssea-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J*, **2**, 2355–61.

Karsunky, H., Merad, M., Cozzio, A., Weissman, I.L. and Manz, M.G. (2003). Flt3 ligand regulates dendritic cell development from flt3+ lymphoid and myeloid-committed progenitors to flt3+ dendritic cells in vivo. *The Journal of experimental medicine*, **198**, 305–313.

Karsunky, H., Inlay, M.A., Serwold, T., Bhattacharya, D. and Weissman, I.L. (2008). Flk2+ common lymphoid progenitors possess equivalent differentiation potential for the b and t lineages. *Blood*, **111**, 5562–5570.

Kawamura, T., Suzuki, J., Wang, Y.V., Menendez, S., Morera, L.B., Raya, A., Wahl, G.M. and Belmonte, J.C.I. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature*, **460**, 1140–1144.

Keller, J.R., Ortiz, M. and Ruscetti, F.W. (1995). Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. *Blood*, **86**, 1757–64.

Kent, D., Copley, M., Benz, C., Dykstra, B., Bowie, M. and Eaves, C. (2008). Regulation of hematopoietic stem cells by the steel factor/kit signaling pathway. *Clin Cancer Res*, **14**, 1926–30.

Kieslich, M., Schwabe, D., Cinatl, J., J. and Driever, P.H. (2003). Increase of fetal hemoglobin synthesis indicating differentiation induction in children receiving valproic acid. *Pediatr Hematol Oncol*, **20**, 15–22.

Kim, C., Wong, J., Wen, J., Wang, S., Wang, C., Spiering, S., Kan, N.G., Forcales, S., Puri, P.L., Leone, T.C., Marine, J.E., Calkins, H., Kelly, D.P., Judge, D.P. and Chen, H.S. (2013). Studying arrhythmogenic right ventricular dysplasia with patient-specific ipscs. *Nature*, **494**, 105–10.

Kim, D., Kim, C.H., Moon, J.I., Chung, Y.G., Chang, M.Y., Han, B.S., Ko, S., Yang, E., Cha, K.Y., Lanza, R. *et al.* (2009a). Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell stem cell*, **4**, 472.

Kim, J.B., Sebastiano, V., Wu, G., Arauzo-Bravo, M.J., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrich, M., van den Boom, D., Meyer, J., Hubner, K., Bernemann, C., Ortmeier, C., Zenke, M., Fleischmann, B.K., Zaehres, H. and Scholer, H.R. (2009b). Oct4-induced pluripotency in adult neural stem cells. *Cell*, **136**, 411–9.

Knipe, D.M. and Howley, P.M. (2007). *Fields Virology 5th*. Lippincott Williams Wilkins, Philadelphia.

Koehl, U., Zimmermann, S., Esser, R., Sorensen, J., Gruttner, H.P., Duchscherer, M., Seifried, E., Klingebiel, T. and Schwabe, D. (2002). Autologous transplantation of cd133 selected hematopoietic progenitor cells in a pediatric patient with relapsed leukemia. *Bone Marrow Transplant*, **29**, 927–30.

Kohn, D.B., Sadelain, M. and Glorioso, J.C. (2003). Occurrence of leukaemia following gene therapy of x-linked scid. *Nature Reviews Cancer*, **3**, 477–488.

Komiya, Y. and Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, **4**, 68–75.

Kosodo, Y., Roper, K., Haubensak, W., Marzesco, A.M., Corbeil, D. and Huttner, W.B. (2004). Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. *EMBO J*, **23**, 2314–24.

Kouros-Mehr, H., Slorach, E.M., Sternlicht, M.D. and Werb, Z. (2006). Gata-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell*, **127**, 1041–1055.

Kurnick, N., Montano, A., Gerdes, J.C. and Feder, B.H. (1958). Preliminary observations on the treatment of postirradiation hematopoietic depression in man by the infusion of stored autogenous bone marrow. *Ann Intern Med*, **49**, 973–986.

Kusenda, B., Mraz, M., Mayer, J. and Pospisilova, S. (2006). Microrna biogenesis, functionality and cancer relevance. *Biomedical Papers*, **150**, 205–215.

Kustikova, O., Fehse, B., Modlich, U., Yang, M., Düllmann, J., Kamino, K., von Neuhoff, N., Schlegelberger, B., Li, Z. and Baum, C. (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. *Science*, **308**, 1171–1174.

Kuzmenkin, A., Liang, H., Xu, G., Pfannkuche, K., Eichhorn, H., Fatima, A., Luo, H., Saric, T., Wernig, M., Jaenisch, R. and Hescheler, J. (2009). Functional characterization of cardiomyocytes derived from murine induced pluripotent stem cells in vitro. *FASEB J*, **23**, 4168–80.

Ladewig, J., Mertens, J., Kesavan, J., Doerr, J., Poppe, D., Glaue, F., Herms, S., Wernet, P., Kögler, G., Müller, F.J., Koch, P. and Brustle, O. (2012). Small molecules enable highly efficient neuronal conversion of human fibroblasts. *Nature Methods*, **9**, 575–578.

Leberbauer, C., Boulme, F., Unfried, G., Huber, J., Beug, H. and Mullner, E.W. (2005). Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. *Blood*, **105**, 85–94.

Lee, J., Sayed, N., Hunter, A., Au, K.F., Wong, W.H., Mocarski, E.S., Pera, R.R., Yakubov, E. and Cooke, J.P. (2012). Activation of innate immunity is required for efficient nuclear reprogramming. *Cell*, **151**, 547–558.

Leight, E.R. and Sugden, B. (2000). Ebna-1: a protein pivotal to latent infection by epstein-barr virus. *Rev Med Virol*, **10**, 83–100.

Leone, G., Teofili, L., Voso, M.T. and Lubbert, M. (2002). Dna methylation and demethylating drugs in myelodysplastic syndromes and secondary leukemias. *Haematologica*, **87**, 1324–41.

LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, J., C. T. (1995). Molecular and cellular aspects of the insulin-like growth factor i receptor. *Endocr Rev*, **16**, 143–63.

Lewis, C.J. (2013). Stem cell application in acute burn care and reconstruction. *J Wound Care*, **22**, 7–8, 10, 12–6.

Li, J., Wang, G., Wang, C., Zhao, Y., Zhang, H., Tan, Z., Song, Z., Ding, M. and Deng, H. (2007). Mek/erk signaling contributes to the maintenance of human embryonic stem cell self-renewal. *Differentiation*, **75**, 299–307.

Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A.Y., Qin, J. and Gu, W. (2002). Deubiquitination of p53 by hausp is an important pathway for p53 stabilization. *Nature*, **416**, 648–653.

Li, W., Wei, W., Zhu, S., Zhu, J., Shi, Y., Lin, T., Hao, E., Hayek, A., Deng, H. and Ding, S. (2009a). Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell*, **4**, 16–9.

Li, W., Zhou, H., Abujarour, R., Zhu, S., Young Joo, J., Lin, T., Hao, E., Scholer, H.R., Hayek, A. and Ding, S. (2009b). Generation of human-induced pluripotent stem cells in the absence of exogenous sox2. *Stem Cells*, **27**, 2992–3000.

Li, W., Zhou, H., Abujarour, R., Zhu, S., Young Joo, J., Lin, T., Hao, E., Scholer, H.R., Hayek, A. and Ding, S. (2009c). Generation of human-induced pluripotent stem cells in the absence of exogenous sox2. *Stem Cells*, **27**, 2992–3000.

Li, Y. and Geng, Y.J. (2010a). A potential role for insulin-like growth factor signaling in induction of pluripotent stem cell formation. *Growth Hormone & IGF Research*, **20**, 391–398.

Li, Y. and Geng, Y.J. (2010b). A potential role for insulin-like growth factor signaling in induction of pluripotent stem cell formation. *Growth Hormone & IGF Research*, **20**, 391–398.

Li, Y., Zhang, Q., Yin, X., Yang, W., Du, Y., Hou, P., Ge, J., Liu, C., Zhang, W., Zhang, X., Wu, Y., Li, H., Liu, K., Wu, C., Song, Z., Zhao, Y., Shi, Y. and Deng, H. (2011a). Generation of ipscs from mouse fibroblasts with a single gene, oct4, and small molecules. *Cell Res*, **21**, 196–204.

Li, Y., Zhang, Q., Yin, X., Yang, W., Du, Y., Hou, P., Ge, J., Liu, C., Zhang, W., Zhang, X., Wu, Y., Li, H., Liu, K., Wu, C., Song, Z., Zhao, Y., Shi, Y. and Deng, H. (2011b). Generation of ipscs from mouse fibroblasts with a single gene, oct4, and small molecules. *Cell Res*, **21**, 196–204.

Liao, B., Bao, X., Liu, L., Feng, S., Zovoilis, A., Liu, W., Xue, Y., Cai, J., Guo, X., Qin, B. *et al.* (2011). Microrna cluster 302–367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *Journal of Biological Chemistry*, **286**, 17359–17364.

Lieu, D.K., Fu, J.D., Chiamvimonvat, N., Tung, K.C., McNerney, G.P., Huser, T., Keller, G., Kong, C.W. and Li, R.A. (2013). Mechanism-based facilitated maturation of human pluripotent stem cell–derived cardiomyocytes. *Circulation: Arrhythmia and Electrophysiology*, **6**, 191–201.

Lin, S.L., Chang, D.C., Lin, C.H., Ying, S.Y., Leu, D. and Wu, D.T. (2011). Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic acids research*, **39**, 1054–1065.

Lin, T., Ambasudhan, R., Yuan, X., Li, W., Hilcove, S., Abujarour, R., Lin, X., Hahm, H.S., Hao, E., Hayek, A. and Ding, S. (2009a). A chemical platform for improved induction of human ipscs. *Nat Methods*, **6**, 805–8.

Lin, T., Ambasudhan, R., Yuan, X., Li, W., Hilcove, S., Abujarour, R., Lin, X., Hahm, H.S., Hao, E., Hayek, A. and Ding, S. (2009b). A chemical platform for improved induction of human ipscs. *Nat Methods*, **6**, 805–8.

Liu, H. and Roy, K. (2005). Biomimetic three-dimensional cultures significantly increase hematopoietic differentiation efficacy of embryonic stem cells. *Tissue engineering*, **11**, 319–330.

Liu, H., Lin, J. and Roy, K. (2006). Effect of 3d scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells. *Biomaterials*, **27**, 5978–5989.

Liu, H., Zhu, F., Yong, J., Zhang, P., Hou, P., Li, H., Jiang, W., Cai, J., Liu, M., Cui, K., Qu, X., Xiang, T., Lu, D., Chi, X., Gao, G., Ji, W., Ding, M. and Deng, H. (2008). Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell*, **3**, 587–90.

Liu, J. (2010). *Investigation of the relationship of haematopoietic stem and progenitor cell phenotype heterogeneity to functional heterogeneity*. Master's thesis, College of Medicine and Veterinary Medicine, The University of Edinburgh.

Liu, Y., Cao, D.L., Guo, L.B., Guo, S.N., Xu, J.K. and Zhuang, H.F. (2013). Amniotic stem cell transplantation therapy for type 1 diabetes: A case report. *J Int Med Res*, **41**, 1370–7.

Loh, Y.H., Agarwal, S., Park, I.H., Urbach, A., Huo, H., Heffner, G.C., Kim, K., Miller, J.D., Ng, K. and Daley, G.Q. (2009). Generation of induced pluripotent stem cells from human blood. *Blood*, **113**, 5476–9.

Loh, Y.H., Hartung, O., Li, H., Guo, C., Sahalie, J.M., Manos, P.D., Urbach, A., Heffner, G.C., Grskovic, M., Vigneault, F., Lensch, M.W., Park, I.H., Agarwal, S., Church, G.M., Collins, J.J., Irion, S. and Daley, G.Q. (2010). Reprogramming of t cells from human peripheral blood. *Cell Stem Cell*, **7**, 15–9.

Loh, Y.H., Yang, J.C., De Los Angeles, A., Guo, C., Cherry, A., Rossi, D.J., Park, I.H. and Daley, G.Q. (2012). Excision of a viral reprogramming cassette by delivery of synthetic cre mrna. *Curr Protoc Stem Cell Biol*, **Chapter 4**, Unit4A 5.

LoPiccolo, J., Blumenthal, G.M., Bernstein, W.B. and Dennis, P.A. (2008). Targeting the pi3k/akt/mtor pathway: effective combinations and clinical considerations. *Drug Resistance Updates*, **11**, 32–50.

Lowry, W.E., Richter, L., Yachechko, R., Pyle, A.D., Tchieu, J., Sridharan, R., Clark, A.T. and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A*, **105**, 2883–8.

Lu, D., Davis, M.P., Abreu-Goodger, C., Wang, W., Campos, L.S., Siede, J., Vigorito, E., Skarnes, W.C., Dunham, I., Enright, A.J. and Liu, P. (2012). Mir-25 regulates wwp2 and fbxw7 and promotes reprogramming of mouse fibroblast cells to ipscs. *PloS one*, **7**, e40938.

Lyssiotis, C.A., Foreman, R.K., Staerk, J., Garcia, M., Mathur, D., Markoulaki, S., Hanna, J., Lairson, L.L., Charette, B.D., Bouchez, L.C., Bollong, M., Kunick, C., Brinker, A., Cho, C.Y., Schultz, P.G. and Jaenisch, R. (2009). Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of klf4. *Proc Natl Acad Sci U S A*, **106**, 8912–7.

MacDonald, B.T., Tamai, K. and He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Developmental cell*, **17**, 9–26.

Mack, A.A., Kroboth, S., Rajesh, D. and Wang, W.B. (2011). Generation of induced pluripotent stem cells from cd34+ cells across blood drawn from multiple donors with non-integrating episomal vectors. *PLoS One*, **6**, e27956.

Maherali, N. and Hochedlinger, K. (2009). Tgfbeta signal inhibition cooperates in the induction of ipscs and replaces sox2 and cmyc. *Curr Biol*, **19**, 1718–23.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K. and Hochedlinger, K. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell*, **1**, 55–70.

Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C. and Hochedlinger, K. (2008). A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell*, **3**, 340–5.

Mandal, P.K. and Rossi, D.J. (2013). Reprogramming human fibroblasts to pluripotency using modified mrna. *Nature protocols*, **8**, 568–582.

Mannick, J., Lochte Jr, H., Ashley, C.A., Thomas, E.D. and Ferrebee, J. (1959). A functioning kidney homotransplant in the dog. *Surgery (US)*, **46**.

Manz, M.G., Traver, D., Miyamoto, T., Weissman, I.L. and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood*, **97**, 3333–3341.

Manz, M.G., Miyamoto, T., Akashi, K. and Weissman, I.L. (2002). Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci U S A*, **99**, 11872–7.

Marion, R.M., Strati, K., Li, H., Tejera, A., Schoeftner, S., Ortega, S., Serrano, M. and Blasco, M.A. (2009). Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. *Cell stem cell*, **4**, 141–154.

Marson, A., Foreman, R., Chevalier, B., Bilodeau, S., Kahn, M., Young, R.A. and Jaenisch, R. (2008). Wnt signaling promotes reprogramming of somatic cells to pluripotency. *Cell stem cell*, **3**, 132.

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 USA.*, **78**, 7634–7638.

Mathe, G., Amiel, J.L., Schwarzenberg, L., Cattan, A. and Schneider, M. (1965). Adoptive immunotherapy of acute leukemia: experimental and clinical results. *Cancer Res*, **25**, 1525–31.

Matsui, W., Huff, C.A., Wang, Q., Malehorn, M.T., Barber, J., Tanhehco, Y., Smith, B.D., Civin, C.I. and Jones, R.J. (2004). Characterization of clonogenic multiple myeloma cells. *Blood*, **103**, 2332–6.

Mauritz, C., Schwanke, K., Reppel, M., Neef, S., Katsirntaki, K., Maier, L.S., Nguemo, F., Menke, S., Haustein, M., Hescheler, J. *et al.* (2008). Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation*, **118**, 507–517.

McCulley, D.J. and Black, B.L. (2012). Transcription factor pathways and congenital heart disease. *Current topics in developmental biology*, **100**, 253.

Middleton, T. and Sugden, B. (1994). Retention of plasmid dna in mammalian cells is enhanced by binding of the epstein-barr virus replication protein ebna1. *Journal of virology*, **68**, 4067–4071.

Migliaccio, G., Migliaccio, A., Petti, S., Mavilio, F., Russo, G., Lazzaro, D., Testa, U., Marinucci, M. and Peschle, C. (1986). Human embryonic hemopoiesis. kinetics of progenitors and precursors underlying the yolk sac—-liver transition. *Journal of Clinical Investigation*, **78**, 51.

Milhem, M., Mahmud, N., Lavelle, D., Araki, H., DeSimone, J., Saunthararajah, Y. and Hoffman, R. (2004). Modification of hematopoietic stem cell fate by 5aza 2'deoxycytidine and trichostatin a. *Blood*, **103**, 4102–10.

Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J.D. and Yamamoto, M. (1999). The mouse

gata-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood*, **93**, 4196–4207.

Miraglia, S., Godfrey, W., Yin, A.H., Atkins, K., Warnke, R., Holden, J.T., Bray, R.A., Waller, E.K. and Buck, D.W. (1997). A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood*, **90**, 5013–21.

Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., Saito, T., Nishimura, J., Takemasa, I., Mizushima, T., Ikeda, M., Yamamoto, H., Sekimoto, M., Doki, Y. and Mori, M. (2011). Reprogramming of mouse and human cells to pluripotency using mature micrornas. *Cell stem cell*, **8**, 633–638.

Morgensztern, D. and McLeod, H.L. (2005). Pi3k/akt/mtor pathway as a target for cancer therapy. *Anti-cancer drugs*, **16**, 797–803.

Moschidou, D., Mukherjee, S., Blundell, M.P., Drews, K., Jones, G.N., Abdulrazzak, H., Nowakowska, B., Phoolchund, A., Lay, K., Ramasamy, T.S. *et al.* (2012). Valproic acid confers functional pluripotency to human amniotic fluid stem cells in a transgene-free approach. *Molecular Therapy*, **20**, 1953–1967.

Moussavi-Harami, F., Duwayri, Y., Martin, J.A., Moussavi-Harami, F. and Buckwalter, J.A. (2004). Oxygen effects on senescence in chondrocytes and mesenchymal stem cells: consequences for tissue engineering. *The Iowa orthopaedic journal*, **24**, 15.

Murohara, T., Ikeda, H., Duan, J., Shintani, S., Sasaki, K., Eguchi, H., Onitsuka, I., Matsui, K. and Imaizumi, T. (2000). Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest*, **105**, 1527–36.

Murray, P.D.F. (1932). The development in vitro of the blood of the early chick embryo. *Proceedings Of the Royal Society Of London Series B-Containing Papers Of a Biological Character*, **111**, 497–521.

Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N. and Yamanaka, S. (2008). Generation of induced pluripotent stem cells without myc from mouse and human fibroblasts. *Nat Biotechnol*, **26**, 101–6.

Nakamura, Y., Ando, K., Chargui, J., Kawada, H., Sato, T., Tsuji, T., Hotta, T. and Kato, S. (1999). Ex vivo generation of cd34+ cells from cd34- hematopoietic cells. *Blood*, **94**, 4053–4059.

Nakano, T., Kodama, H. and Honjo, T. (1996). In vitro development of primitive and definitive erythrocytes from different precursors. *Science*, **272**, 722–724.

Nakorn, T.N., Miyamoto, T. and Weissman, I.L. (2003). Characterization of mouse clonogenic megakaryocyte progenitors. *Proc Natl Acad Sci U S A*, **100**, 205–10.

Nanbo, A., Sugden, A. and Sugden, B. (2007). The coupling of synthesis and partitioning of ebv's plasmid replicon is revealed in live cells. *The EMBO journal*, **26**, 4252–4262.

Nelson, D.L. and Gibbs, R.A. (2004). The critical region in trisomy 21. *Science*, **306**, 619–621.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the pou transcription factor oct4. *Cell*, **95**, 379–91.

Nishimoto, M., Fukushima, A., Okuda, A. and Muramatsu, M. (1999). The gene for the embryonic stem cell coactivator utf1 carries a regulatory element which selectively interacts with a complex composed of oct-3/4 and sox-2. *Mol Cell Biol*, **19**, 5453–65.

Nishinakamura, R., Miyajima, A., Mee, P.J., Tybulewicz, V. and Murray, R. (1996). Hematopoiesis in mice lacking the entire granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 functions. *Blood*, **88**, 2458–2464.

Niwa, H. (2011). Wnt: what's needed to maintain pluripotency? *Nature Cell Biology*, **13**, 1024–1026.

Ogawa, K., Nishinakamura, R., Iwamatsu, Y., Shimosato, D. and Niwa, H. (2006). Synergistic action of wnt and lif in maintaining pluripotency of mouse es cells. *Biochem Biophys Res Commun*, **343**, 159–66.

Oh, S.P., Seki, T., Goss, K.A., Imamura, T., Yi, Y., Donahoe, P.K., Li, L., Miyazono, K., ten Dijke, P., Kim, S. and Li, E. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A*, **97**, 2626–31.

Okita, K., Ichisaka, T. and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature*, **448**, 313–7.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T. and Yamanaka, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. *Science*, **322**, 949–53.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K.i., Shibata, T., Kunisada,

T., Takahashi, M., Takahashi, J., Saji, H. and Yamanaka, S. (2011). A more efficient method to generate integration-free human ips cells. *Nature methods*, **8**, 409–412.

Okita, K., Yamakawa, T., Matsumura, Y., Sato, Y., Amano, N., Watanabe, A., Goshima, N. and 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**, 458–66.

Olson, L., Richtsmeier, J., Leszl, J. and Reeves, R. (2004). A chromosome 21 critical region does not cause specific down syndrome phenotypes. *Science*, **306**, 687–690.

Osaki, M., Oshimura, M., and Ito, H. (2004). Pi3k-akt pathway: its functions and alterations in human cancer. *Apoptosis*, **9**, 667–676.

Padfield, G.J., Tura, O., Haeck, M.L., Short, A., Freyer, E., Barclay, G.R., Newby, D.E. and Mills, N.L. (2010). Circulating endothelial progenitor cells are not affected by acute systemic inflammation. *Am J Physiol Heart Circ Physiol*, **298**, H2054–61.

Palis, J. and Segel, G. (1998). Developmental biology of erythropoiesis. *Blood Reviews*, **12**, 106 – 114.

Papaioannou, V. (2001). Stem cells and differentiation. *Differentiation*, **68**, 153–154.

Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M., Grosveld, G. and Ihle, J.N. (1998). Jak2 is essential for signaling through a variety of cytokine receptors. *Cell*, **93**, 385–95.

Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K. and Daley, G.Q. (2008a). Disease-specific induced pluripotent stem cells. *Cell*, **134**, 877–86.

Park, I.H., Lerou, P.H., Zhao, R., Huo, H. and Daley, G.Q. (2008b). Generation of human-induced pluripotent stem cells. *Nat Protoc*, **3**, 1180–6.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W. and Daley, G.Q. (2008c). Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*, **451**, 141–6.

Park, T.S., Huo, J.S., Peters, A., Talbot, J., C. C., Verma, K., Zimmerlin, L., Kaplan, I.M. and Zambidis, E.T. (2012). Growth factor-activated stem cell circuits and stromal signals cooperatively accelerate non-integrated ipsc reprogramming of human myeloid progenitors. *PLoS One*, **7**, e42838.

Patel, K., Dickson, J., Din, S., Macleod, K., Jodrell, D. and Ramsahoye, B. (2010). Targeting of 5-aza-2'-deoxycytidine residues by chromatin-associated dnmt1 induces proteasomal degradation of the free enzyme. *Nucleic Acids Res*, **38**, 4313–24.

Peichev, M., Naiyer, A.J., Pereira, D., Zhu, Z., Lane, W.J., Williams, M., Oz, M.C., Hicklin, D.J., Witte, L., Moore, M.A. and Rafii, S. (2000). Expression of vegfr-2 and ac133 by circulating human cd34(+) cells identifies a population of functional endothelial precursors. *Blood*, **95**, 952–8.

Perkins, A.C., Sharpe, A.H. and Orkin, S.H. (1995). Lethal beta-thalassaemia in mice lacking the erythroid caccc-transcription factor eklf. *Nature*, **375**, 318–22.

Perrino, C. and Rockman, H.A. (2006). Gata4 and the two sides of gene expression reprogramming. *Circulation research*, **98**, 715–716.

Petzer, A.L., Zandstra, P.W., Piret, J.M. and Eaves, C.J. (1996). Differential cytokine effects on primitive (cd34+cd38-) human hematopoietic cells: novel responses to flt3-ligand and thrombopoietin. *J Exp Med*, **183**, 2551–8.

Pfannkuche, K., Liang, H., Hannes, T., Xi, J., Fatima, A., Nguemo, F., Matzkies, M., Wernig, M., Jaenisch, R., Pillekamp, F., Halbach, M., Schunkert, H., Šaric, T., Hescheler, J. and Reppel, M. (2009). Cardiac myocytes derived from murine reprogrammed fibroblasts: intact hormonal regulation, cardiac ion channel expression and development of contractility. *Cellular Physiology and Biochemistry*, **24**, 73–86.

Pflumio, F., Izac, B., Katz, A., Shultz, L.D., Vainchenker, W. and Coulombel, L. (1996). Phenotype and function of human hematopoietic cells engrafting immune-deficient cb17-severe combined immunodeficiency mice and nonobese diabetic-severe combined immunodeficiency mice after transplantation of human cord blood mononuclear cells. *Blood*, **88**, 3731–40.

Piacibello, W., Sanavio, F., Garetto, L., Severino, A., Bergandi, D., Ferrario, J., Fagioli, F., Berger, M. and Aglietta, M. (1997). Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. *Blood*, **89**, 2644–53.

Radisic, M., Park, H., Shing, H., Consi, T., Schoen, F.J., Langer, R., Freed, L.E. and Vunjak-Novakovic, G. (2004). Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proceedings of the National Academy of Sciences*, **101**, 18129–18134.

Ramshaw, H.S., Haylock, D., Swart, B., Gronthos, S., Horsfall, M.J., Niutta, S. and Simmons, P.J. (2001). Monoclonal antibody bb9 raised against bone marrow stromal cells identifies a cell-surface glycoprotein expressed by primitive human hemopoietic progenitors. *Experimental hematology*, **29**, 981–992.

Rao, T.P. and Kühl, M. (2010). An updated overview on wnt signaling pathways a prelude for more. *Circulation research*, **106**, 1798–1806.

Reya, T. and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature*, **434**, 843–850.

Risau, W. (1991). Embryonic angiogenesis factors. *Pharmacol Ther*, **51**, 371–6.

Rutherford, T., Clegg, J., Higgs, D., Jones, R., Thompson, J. and Weatherall, D. (1981). Embryonic erythroid differentiation in the human leukemic cell line k562. *Proceedings of the National Academy of Sciences*, **78**, 348–352.

Ryan, T., Behringer, R., Martin, N., Townes, T., Palmiter, R. and Brinster, R. (1989a). A single erythroid-specific dnase i super-hypersensitive site activates high levels of human beta-globin gene expression in transgenic mice. *Genes & development*, **3**, 314–323.

Ryan, T.M., Behringer, R.R., Townes, T.M., Palmiter, R.D. and Brinster, R.L. (1989b). High-level erythroid expression of human alpha-globin genes in transgenic mice. *Proceedings of the National Academy of Sciences*, **86**, 37–41.

Sabin, F.R. (1920). Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contributions To Embryology*, **9**, 215–U56.

Sahovic, E.A., Colvin, M., Hilton, J. and Ogawa, M. (1988). Role for aldehyde dehydrogenase in survival of progenitors for murine blast cell colonies after treatment with 4-hydroperoxycyclophosphamide in vitro. *Cancer Res*, **48**, 1223–6.

Saridakis, V., Sheng, Y., Sarkari, F., Holowaty, M.N., Shire, K., Nguyen, T., Zhang, R.G., Liao, J., Lee, W., Edwards, A.M., Arrowsmith, C.H. and Frappier, L. (2005). Structure of the p53 binding domain of hausp/usp7 bound to epstein-barr nuclear antigen 1: implications for ebv-mediated immortalization. *Molecular cell*, **18**, 25–36.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. and Brivanlou, A.H. (2003). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of wnt signaling by a pharmacological gsk-3-specific inhibitor. *Nature medicine*, **10**, 55–63.

Sato, T., Laver, J.H. and Ogawa, M. (1999). Reversible expression of cd34 by murine hematopoietic stem cells. *Blood*, **94**, 2548–2554.

Sauer, H., Rahimi, G., Hescheler, J. and Wartenberg, M. (1999). Effects of electrical fields on cardiomyocyte differentiation of embryonic stem cells. *Journal of cellular biochemistry*, **75**, 710–723.

Schulze, W.X., Deng, L. and Mann, M. (2005). Phosphotyrosine interactome of the erbb-receptor kinase family. *Molecular systems biology*, **1**.

Scicchitano, M.S., McFarland, D.C., Tierney, L.A., Narayanan, P.K. and Schwartz, L.W. (2003). In vitro expansion of human cord blood cd36¡ sup¿+¡/sup¿ erythroid progenitors: Temporal changes in gene and protein expression. *Experimental hematology*, **31**, 760–769.

Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K. and Nevins, J.R. (2000). Multiple ras-dependent phosphorylation pathways regulate myc protein stability. *Genes & development*, **14**, 2501–2514.

Seki, T., Yuasa, S., Oda, M., Egashira, T., Yae, K., Kusumoto, D., Nakata, H., Tohyama, S., Hashimoto, H., Kodaira, M., Okada, Y., Seimiya, H., Fusaki, N., Hasegawa, M. and Fukuda, K. (2010). Generation of induced pluripotent stem cells from human terminally differentiated circulating t cells. *Cell stem cell*, **7**, 11–14.

Serena, E., Flaibani, M., Carnio, S., Boldrin, L., Vitiello, L., De Coppi, P. and Elvassore, N. (2008). Electrophysiologic stimulation improves myogenic potential of muscle precursor cells grown in a 3d collagen scaffold. *Neurological research*, **30**, 207–214.

Severn, C.B. (1971). A morphological study of the development of the human liver. i. development of the hepatic diverticulum. *Am J Anat*, **131**, 133–158.

Shah, A.J., Smogorzewska, E.M., Hannum, C. and Crooks, G.M. (1996). Flt3 ligand induces proliferation of quiescent human bone marrow cd34+cd38- cells and maintains progenitor cells in vitro. *Blood*, **87**, 3563–70.

Sharma, S., Shariatmadar, S. and Krishan, A. (2010). Electronic volume, aldehyde dehydrogenase, and stem cell marker expression in cells from human peripheral blood apheresis samples. *Cytometry B Clin Cytom*, **78**, 123–9.

Shevinsky, L.H., Knowles, B.B., Damjanov, I. and Solter, D. (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell*, **30**, 697–705.

Shi, Q., Rafii, S., Wu, M.H., Wijelath, E.S., Yu, C., Ishida, A., Fujita, Y., Kothari, S., Mohle, R., Sauvage, L.R., Moore, M.A., Storb, R.F. and Hammond, W.P. (1998). Evidence for circulating bone marrow-derived endothelial cells. *Blood*, **92**, 362–7.

Shi, Y., Desponts, C., Do, J.T., Hahm, H.S., Scholer, H.R. and Ding, S. (2008a). Induction of pluripotent stem cells from mouse embryonic fibroblasts by oct4 and klf4 with small-molecule compounds. *Cell Stem Cell*, **3**, 568–74.

Shi, Y., Do, J.T., Desponts, C., Hahm, H.S., Scholer, H.R. and Ding, S. (2008b). A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell*, **2**, 525–8.

Simmons, D., Satterthwaite, A., Tenen, D. and Seed, B. (1992). Molecular cloning of a cdna encoding cd34, a sialomucin of human hematopoietic stem cells. *The Journal of Immunology*, **148**, 267–271.

Singla, D.K., Schneider, D.J., LeWinter, M.M. and Sobel, B.E. (2006). wnt3a but not wnt11 supports self-renewal of embryonic stem cells. *Biochemical and biophysical research communications*, **345**, 789–795.

Slanicka Krieger, M., Nissen, C., Manz, C.Y., Toksoz, D., Lyman, S.D. and Wodnar-Filipowicz, A. (1998). The membrane-bound isoform of stem cell factor synergizes with soluble flt3 ligand in supporting early hematopoietic cells in long-term cultures of normal and aplastic anemia bone marrow. *Exp Hematol*, **26**, 365–73.

Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G.W., Cook, E.G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., Isacson, O. and Jaenisch, R. (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell*, **136**, 964–977.

Spike, B.T. and Wahl, G.M. (2011). p53, stem cells, and reprogramming: Tumor suppression beyond guarding the genome. *Genes Cancer*, **2**, 404–419.

Stadtfeld, M., Maherali, N., Breault, D.T. and Hochedlinger, K. (2008a). Defining molecular cornerstones during fibroblast to ips cell reprogramming in mouse. *Cell Stem Cell*, **2**, 230–240.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G. and Hochedlinger, K. (2008b). Induced pluripotent stem cells generated without viral integration. *Science*, **322**, 945–949.

Staerk, J., Dawlaty, M.M., Gao, Q., Maetzel, D., Hanna, J., Sommer, C.A., Mostoslavsky, G. and Jaenisch, R. (2010). Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell*, **7**, 20–4.

Staerk, J., Lyssiotis, C.A., Medeiro, L.A., Bollong, M., Foreman, R.K., Zhu, S., Garcia, M., Gao, Q., Bouchez, L.C., Lairson, L.L., Charette, B.D., Supekova, L., Janes, J., Brinker, A., Cho, C.Y., Jaenisch, R. and Schultz, P.G. (2011). Pan-src family kinase inhibitors replace sox2 during the direct reprogramming of somatic cells. *Angew Chem Int Ed Engl*, **50**, 5734–6.

Stewart, C.E. and Rotwein, P. (1996). Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev*, **76**, 1005–26.

Stolzing, A. and Scutt, A. (2006). Effect of reduced culture temperature on antioxidant defences of mesenchymal stem cells. *Free Radical Biology and Medicine*, **41**, 326–338.

Stolzing, A., Sethe, S. and Scutt, A.M. (2006). Stressed stem cells: temperature response in aged mesenchymal stem cells. *Stem cells and development*, **15**, 478–487.

Storb, R., Prentice, R. and Thomas, E. (1977a). Treatment of aplastic anemia by marrow transplantation from hla identical siblings. prognostic factors associated with graft versus host disease and survival. *Journal of Clinical Investigation*, **59**, 625.

Storb, R., Prentice, R.L. and Thomas, E.D. (1977b). Marrow transplantation for treatment of aplastic anemia: an analysis of factors associated with graft rejection. *New England Journal of Medicine*, **296**, 61–66.

Storb, R., Weiden, P.L., Prentice, R., Buckner, C.D., Clift, R.A., Einstein, A.B., Fefer, A., Johnson, F.L., Lerner, K.G., Neiman, P.E., Sanders, J.E. and Thomas, E.D. (1977c). Aplastic anemia (aa) treated by allogeneic marrow transplantation: the seattle experience. *Transplant Proc*, **9**, 181–5.

Storms, R.W., Trujillo, A.P., Springer, J.B., Shah, L., Colvin, O.M., Ludeman, S.M. and Smith, C. (1999). Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci U S A*, **96**, 9118–23.

Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B. and McKay, R. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of cns precursors in lowered oxygen. *The Journal of Neuroscience*, **20**, 7377–7383.

Subramanyam, D., Lamouille, S., Judson, R.L., Liu, J.Y., Bucay, N., Derynck, R. and Blelloch, R. (2011). Multiple targets of mir-302 and mir-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nature biotechnology*, **29**, 443–448.

Sun, L.Y. and D'Ercole, A.J. (2006). Insulin-like growth factor-i stimulates histone h3 and h4 acetylation in the brain in vivo. *Endocrinology*, **147**, 5480–90.

Sun, N., Yazawa, M., Liu, J., Han, L., Sanchez-Freire, V., Abilez, O.J., Navarrete, E.G., Hu, S., Wang, L., Lee, A. *et al.* (2012). Patient-specific induced pluripotent stem cell as a model for familial dilated cardiomyopathy. *Science translational medicine*, **4**, 130ra47.

Sutherland, D.R. and Keating, A. (1992). The cd34 antigen: structure, biology, and potential clinical applications. *J Hematother*, **1**, 115–29.

Sutherland, D.R., Anderson, L., Keeney, M., Nayar, R. and Chin-Yee, I. (1996). The ishage guidelines for cd34+ cell determination by flow cytometry. *Journal of hematotherapy*, **5**, 213–226.

Szablowska-Gadomska, I., Zayat, V. and Buzanska, L. (2011). Influence of low oxygen tensions on expression of pluripotency genes in stem cells. *Acta Neurobiologiae Experimentalis*, **71**, 86–93.

Szabo, E., Rampalli, S., Risueno, R.M., Schnerch, A., Mitchell, R., Fiebig-Comyn, A., Levadoux-Martin, M. and Bhatia, M. (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*, **468**, 521–526.

Tajima, F., Deguchi, T., Laver, J.H., Zeng, H. and Ogawa, M. (2001). Reciprocal expression of cd38 and cd34 by adult murine hematopoietic stem cells. *Blood*, **97**, 2618–2624.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, **126**, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, **131**, 861–72.

Takahashi, S., Shimizu, R., Suwabe, N., Kuroha, T., Yoh, K., Ohta, J., Nishimura, S., Lim, K.C., Engel, J.D. and Yamamoto, M. (2000). Gata factor transgenes under gata-1 locus control rescue germline gata-1 mutant deficiencies. *Blood*, **96**, 910–916.

Takebe, N., Zhao, S.C., Adhikari, D., Mineishi, S., Sadelain, M., Hilton, J., Colvin, M., Banerjee, D. and Bertino, J.R. (2001). Generation of dual resistance to 4-hydroperoxycyclophosphamide and methotrexate by retroviral transfer of the human aldehyde dehydrogenase class 1 gene and a mutated dihydrofolate reductase gene. *Mol Ther*, **3**, 88–96.

Takeda, K. and Akira, S. (2004). Tlr signaling pathways. *Semin Immunol*, **16**, 3–9.

Talbot, D., Collis, P., Antoniou, M., Vidal, M., Grosveld, F. and Greaves, D.R. (1989). A dominant control region from the human beta-globin locus conferring integration site-independent gene expression. *Nature*, **338**, 352–355.

Tavian, M. and Peault, B. (2005a). Analysis of hematopoietic development during human embryonic ontogenesis. *Methods Mol Medl*, **105**, 413–424.

Tavian, M. and Peault, B. (2005b). Embryonic development of the human hematopoietic system. *Int J Dev Biol*, **49**, 243–50.

Tavian, M., Coulombel, L., Luton, D., Clemente, H., Dieterlen-Lievre, F. and Peault, B. (1996). Aorta-associated cd34+ hematopoietic cells in the early human embryo. *Blood*, **87**, 67–72.

Tavian, M., Hallais, M.F. and Péault, B. (1999). Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development*, **126**, 793–803.

Tavian, M., Robin, C., Coulombel, L. and Péault, B. (2001). The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm. *Immunity*, **15**, 487–495.

Tavian, M., Biasch, K., Sinka, L., Vallet, J. and Péault, B. (2010). Embryonic origin of human hematopoiesis. *International Journal of Developmental Biology*, **54**, 1061.

Terszowski, G., Waskow, C., Conradt, P., Lenze, D., Koenigsmann, J., Carstanjen, D., Horak, I. and Rodewald, H.R. (2005). Prospective isolation and global gene expression analysis of the erythrocyte colony-forming unit (cfu-e). *Blood*, **105**, 1937–1945.

Thomas, E., Storb, R., Clift, R.A., Fefer, A., Johnson, F.L., Neiman, P.E., Lerner, K.G., Glucksberg, H. and Buckner, C.D. (1975). Bone-marrow transplantation (first of two parts). *N Engl J Med*, **292**, 832–43.

Thomas, E., Buckner, C., Banaji, M., Clift, R., Fefer, A., Flournoy, N., Goodell, B., Hickman, R., Lerner, K., Neiman, P., Sale, G., Sanders, J., Singer, J., Stevens, M., Storb, R. and Weiden, P. (1977a). One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. *Blood*, **49**, 511–533.

Thomas, E.D. and Blume, K.G. (1999). Historical markers in the development of allogeneic hematopoietic cell transplantation. *Biology of Blood and Marrow Transplantation*, **5**, 341–346.

Thomas, E.D., Lochte Jr, H.L., Lu, W.C. and Ferrebee, J.W. (1957). Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *New England Journal of Medicine*, **257**, 491–496.

Thomas, E.D., Lochte Jr, H.L., Cannon, J.H., Sahler, O.D. and Ferrebee, J.W. (1959). Supralethal whole body irradiation and isologous marrow transplantation in man. *Journal of Clinical Investigation*, **38**, 1709.

Thomas, E.D., Storb, R., Fefer, A., Slichter, S., Bryant, J., Buckner, C.D., Neiman, P., Clift, R., Funk, D. and Lerner, K. (1972). Aplastic anaemia treated by marrow transplantation. *The Lancet*, **299**, 284–289.

Thomas, E.D., Fefer, A., Buckner, C.D. and Storb, R. (1977b). Current status of bone marrow transplantation for aplastic anemia and acute leukemia. *Blood*, **49**, 671–681.

Thomas, E.D., Flournoy, N., Buckner, C.D., Clift, R.A., Fefer, A., Neiman, P.E. and Storb, R. (1977c). Cure of leukemia by marrow transplantation. *Leukemia Research*, **1**, 67–70.

Thomas, E.D., Buckner, C.D., Clift, R.A., Fefer, A., Johnson, F.L., Neiman, P.E., Sale, G.E., Sanders, J.E., Singer, J.W., Shulman, H., Storb, R. and Weiden, P.L. (1979). Marrow transplantation for acute nonlymphoblastic leukemia in first remission. *The New England journal of medicine*, **301**, 597.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. *science*, **282**, 1145–1147.

Thum, T., Hoeber, S., Froese, S., Klink, I., Stichtenoth, D.O., Galuppo, P., Jakob, M., Tsikas, D., Anker, S.D., Poole-Wilson, P.A., Borlak, J., Ertl, G. and Bauersachs, J. (2007). Age-dependent impairment of endothelial progenitor cells is corrected by growth-hormone-mediated increase of insulin-like growth-factor-1. *Circ Res*, **100**, 434–43.

Timmermans, F., Van Hauwermeiren, F., De Smedt, M., Raedt, R., Plasschaert, F., De Buyzere, M.L., Gillebert, T.C., Plum, J. and Vandekerckhove, B. (2007). Endothelial outgrowth cells are not derived from cd133+ cells or cd45+ hematopoietic precursors. *Arterioscler Thromb Vasc Biol*, **27**, 1572–9.

Tomioka, M., Nishimoto, M., Miyagi, S., Katayanagi, T., Fukui, N., Niwa, H., Muramatsu, M. and Okuda, A. (2002). Identification of sox-2 regulatory region which is under the control of oct-3/4-sox-2 complex. *Nucleic Acids Res*, **30**, 3202–13.

Townes, T., Ryan, T., Behringer, R., Palmiter, R. and Brinster, R. (1989). Dnase i super-hypersensitive sites direct high level erythroid expression of human alpha-, beta-and beta s-globin genes in transgenic mice. *Progress in clinical and biological research*, **316**, 47.

Traver, D., Akashi, K., Manz, M., Merad, M., Miyamoto, T., Engleman, E.G. and Weissman, I.L. (2000). Development of cd8-positive dendritic cells from a common myeloid progenitor. *Science*, **290**, 2152–2154.

Trounson, A. (2006). The production and directed differentiation of human embryonic stem cells. *Endocrine reviews*, **27**, 208–219.

Tsai, F.Y. and Orkin, S.H. (1997). Transcription factor gata-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood*, **89**, 3636–3643.

Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W. and Orkin, S.H. (1994). An early haematopoietic defect in mice lacking the transcription factor gata-2. *Nature*, **371**, 221–226.

Tsang, A.P., Visvader, J.E., Turner, C.A., Fujiwara, Y., Yu, C., Weiss, M.J., Crossley, M. and Orkin, S.H. (1997). Fog, a multitype zinc finger protein, acts as a cofactor for transcription factor gata-1 in erythroid and megakaryocytic differentiation. *Cell*, **90**, 109–119.

Tuan, D., Solomon, W., Li, Q. and London, I.M. (1985). The" beta-like-globin" gene domain in human erythroid cells. *Proceedings of the National Academy of Sciences*, **82**, 6384–6388.

Tura, O., Barclay, G.R., Roddie, H., Davies, J. and Turner, M.L. (2007a). Absence of a relationship between immunophenotypic and colony enumeration analysis of endothelial progenitor cells in clinical haematopoietic cell sources. *J Transl Med*, **5**, 37.

Tura, O., Barclay, G.R., Roddie, H., Davies, J. and Turner, M.L. (2007b). Optimal ex vivo expansion of neutrophils from pbsc cd34+ cells by a combination of scf, flt3-l and g-csf and its inhibition by further addition of tpo. *J Transl Med*, **5**, 53.

Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H. and Weissman, I.L. (2000). Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A*, **97**, 14720–5.

Valdimarsdottir, G. and Mummery, C. (2005). Functions of the tgfbeta superfamily in human embryonic stem cells. *APMIS*, **113**, 773–89.

Van Craenenbroeck, K., Vanhoenacker, P. and Haegeman, G. (2000). Episomal vectors for gene expression in mammalian cells. *Eur J Biochem*, **267**, 5665–78.

van den Akker, E., Satchwell, T.J., Pellegrin, S., Daniels, G. and Toye, A.M. (2010). The majority of the in vitro erythroid expansion potential resides in cd34(-) cells, outweighing the contribution of cd34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. *Haematologica*, **95**, 1594–8.

Van Tendeloo, V.F., Ponsaerts, P., Lardon, F., Nijs, G., Lenjou, M., Van Broeckhoven, C., Van Bockstaele, D.R. and Berneman, Z.N. (2001). Highly efficient gene delivery by mrna electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mrna and to electroporation of plasmid cdna for tumor antigen loading of dendritic cells. *Blood*, **98**, 49–56.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative rt-pcr data by geometric averaging of multiple internal control genes. *Genome biology*, **3**, research0034.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J. and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood*, **103**, 3258–3264.

Wagner, R. (1980). Endothelial cell embryology and growth. *Adv. Microcirc.*, **9**, 45–75.

Wang, L., Schulz, T.C., Sherrer, E.S., Dauphin, D.S., Shin, S., Nelson, A.M., Ware, C.B., Zhan, M., Song, C.Z., Chen, X., Brimble, S.N., McLean, A., Galeano, M.J., Uhl, E.W., D'Amour, K.A., Chesnut, J.D., Rao, M.S., Blau, C.A. and Robins, A.J. (2007). Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and erbb2 receptor signaling. *Blood*, **110**, 4111–4119.

Wang, Y., Baskerville, S., Shenoy, A., Babiarz, J.E., Baehner, L. and Blelloch, R. (2008). Embryonic stem cell–specific micrornas regulate the g1-s transition and promote rapid proliferation. *Nature genetics*, **40**, 1478–1483.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, G.Q., Brack, A.S., Collins, J.J., Cowan, C., Schlaeger, T.M. and Rossi, D.J. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mrna. *Cell Stem Cell*, **7**, 618–30.

Weigmann, A., Corbeil, D., Hellwig, A. and Huttner, W.B. (1997). Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. *Proc Natl Acad Sci U S A*, **94**, 12425–30.

Weinmann, P., Gaehtgens, P. and Walzog, B. (1999). Bcl-xl- and bax-alpha-mediated regulation of apoptosis of human neutrophils via caspase-3. *Blood*, **93**, 3106–15.

Weiss, M.J., Keller, G. and Orkin, S.H. (1994). Novel insights into erythroid development revealed through in vitro differentiation of gata-1 embryonic stem cells. *Genes & development*, **8**, 1184–1197.

Werner, C., Hanhoun, M., Widmann, T., Kazakov, A., Semenov, A., Poss, J., Bauersachs, J., Thum, T., Pfreundschuh, M., Muller, P., Haendeler, J., Bohm, M. and Laufs, U. (2008). Effects of physical exercise on myocardial telomere-regulating proteins, survival pathways, and apoptosis. *J Am Coll Cardiol*, **52**, 470–82.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M.C., Hochedlinger, K., Bernstein, B.E. and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent es-cell-like state. *Nature*, **448**, 318–U2.

Wernig, M., Meissner, A., Cassady, J.P. and Jaenisch, R. (2008). c-myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell*, **2**, 10–2.

Wetterau, L.A., Francis, M.J., Ma, L. and Cohen, P. (2003). Insulin-like growth factor i stimulates telomerase activity in prostate cancer cells. *J Clin Endocrinol Metab*, **88**, 3354–9.

Wijgerde, M., Gribnau, J., Trimborn, T., Nuez, B., Philipsen, S., Grosveld, F. and Fraser, P. (1996). The role of eklf in human beta-globin gene competition. *Genes & Development*, **10**, 2894–2902.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hämäläinen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M. *et al.* (2009). piggybac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*, **458**, 766–770.

Woo, A.J., Kim, J., Xu, J., Huang, H. and Cantor, A.B. (2011). Role of zbp-89 in human globin gene regulation and erythroid differentiation. *Blood*, **118**, 3684–93.

Wrana, J.L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.F. and Massague, J. (1992). Tgfβ signals through a heteromeric protein kinase receptor complex. *Cell*, **71**, 1003–1014.

Xu, J., Lamouille, S. and Derynck, R. (2009). Tgf-beta-induced epithelial to mesenchymal transition. *Cell Res*, **19**, 156–72.

Xue, Y., Cai, X., Wang, L., Liao, B., Zhang, H., Shan, Y., Chen, Q., Zhou, T., Li, X., Hou, J., Chen, S., Luo, R., Qin, D., Pei, D. and Pan, G. (2013). Generating a non-integrating human induced pluripotent stem cell bank from urine-derived cells. *PLoS One*, **8**, e70573.

Yagi, R., Zhu, J. and Paul, W.E. (2011). An updated view on transcription factor gata3-mediated regulation of th1 and th2 cell differentiation. *International immunology*, **23**, 415–420.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H. and Nakauchi, H. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell*, **154**, 1112–1126.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T.K., Andries, M., Smith, J.C., Heldin, C.H. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type ii receptors and induces certain activin-like effects. *The Journal of cell biology*, **130**, 217–226.

Yap, T.A., Garrett, M.D., Walton, M.I., Raynaud, F., de Bono, J.S. and Workman, P. (2008). Targeting the pi3k–akt–mtor pathway: progress, pitfalls, and promises. *Current opinion in pharmacology*, **8**, 393–412.

Ye, Z., Zhan, H., Mali, P., Dowey, S., Williams, D.M., Jang, Y.Y., Dang, C.V., Spivak, J.L., Moliterno, A.R. and Cheng, L. (2009). Human-induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. *Blood*, **114**, 5473–80.

Yi, Y., Hahm, S.H. and Lee, K.H. (2005). Retroviral gene therapy: safety issues and possible solutions. *Current gene therapy*, **5**, 25–35.

Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J. and Buck, D.W. (1997a). Ac133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*, **90**, 5002–5012.

Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J. and Buck, D.W. (1997b). Ac133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*, **90**, 5002–12.

Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature*, **453**, 519–523.

Yoshida, Y., Takahashi, K., Okita, K., Ichisaka, T. and Yamanaka, S. (2009). Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell*, **5**, 237–41.

Yousefi, S., Hoessli, D.C., Blaser, K., Mills, G.B. and Simon, H.U. (1996). Requirement of lyn and syk tyrosine kinases for the prevention of apoptosis by cytokines in human eosinophils. *J Exp Med*, **183**, 1407–14.

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, I. and Thomson, J.A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science*, **318**, 1917–20.

Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I. and Thomson, J.A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. *Science*, **324**, 797–801.

Yu, J., Chau, K.F., Vodyanik, M.A., Jiang, J. and Jiang, Y. (2011). Efficient feeder-free episomal reprogramming with small molecules. *Plos one*, **6**, e17557.

Yuan, H., Corbi, N., Basilico, C. and Dailey, L. (1995). Developmental-specific activity of the fgf-4 enhancer requires the synergistic action of sox2 and oct-3. *Genes Dev*, **9**, 2635–45.

Yuan, X., Wan, H., Zhao, X., Zhu, S., Zhou, Q. and Ding, S. (2011). Combined chemical treatment enables oct4-induced reprogramming from mouse embryonic fibroblasts. *Stem Cells*.

Zandstra, P.W., Conneally, E., Petzer, A.L., Piret, J.M. and Eaves, C.J. (1997). Cytokine manipulation of primitive human hematopoietic cell self-renewal. *Proc Natl Acad Sci U S A*, **94**, 4698–703.

Zanjani, E.D., Almeida-Porada, G., Livingston, A.G., Zeng, H. and Ogawa, M. (2003). Reversible expression of cd34 by adult human bone marrow long-term engrafting hematopoietic stem cells. *Experimental hematology*, **31**, 406–412.

Zarich, N., Oliva, J.L., Martínez, N., Jorge, R., Ballester, A., Gutiérrez-Eisman, S., García-Vargas, S. and Rojas, J.M. (2006). Grb2 is a negative modulator of the intrinsic ras-gef activity of hsos1. *Molecular biology of the cell*, **17**, 3591–3597.

Zhang, J., Wilson, G.F., Soerens, A.G., Koonce, C.H., Yu, J., Palecek, S.P., Thomson, J.A. and Kamp, T.J. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circulation research*, **104**, e30–e41.

Zhou, D., Liu, K., Sun, C.W., Pawlik, K.M. and Townes, T.M. (2010a). Klf1 regulates bcl11a expression and [gamma]-to [beta]-globin gene switching. *Nature genetics*, **42**, 742–744.

Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., Siuzdak, G., Schöler, H.R., Duan, L. and Ding, S. (2009). Generation of induced pluripotent stem cells using recombinant proteins. *Cell stem cell*, **4**, 381–384.

Zhou, H., Li, W., Zhu, S., Joo, J.Y., Do, J.T., Xiong, W., Kim, J.B., Zhang, K., Scholer, H.R. and Ding, S. (2010b). Conversion of mouse epiblast stem cells to an earlier pluripotency state by small molecules. *J Biol Chem*, **285**, 29676–80.

Zhou, P., He, A. and Pu, W.T. (2012a). Regulation of gata4 transcriptional activity in cardiovascular development and disease. *Current topics in developmental biology*, **100**, 143.

Zhou, T., Benda, C., Dunzinger, S., Huang, Y., Ho, J.C., Yang, J., Wang, Y., Zhang, Y., Zhuang, Q., Li, Y., Bao, X., Tse, H.F., Grillari, J., Grillari-Voglauer, R., Pei, D. and Esteban, M.A. (2012b). Generation of human induced pluripotent stem cells from urine samples. *nature protocols*, **7**, 2080–2089.

Zhu, J., Garrett, R., Jung, Y., Zhang, Y., Kim, N., Wang, J., Joe, G.J., Hexner, E., Choi, Y., Taichman, R.S. *et al.* (2007). Osteoblasts support b-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood*, **109**, 3706–3712.

Zhu, S., Li, W., Zhou, H., Wei, W., Ambasudhan, R., Lin, T., Kim, J., Zhang, K. and Ding, S. (2010). Reprogramming of human primary somatic cells by oct4 and chemical compounds. *Cell Stem Cell*, **7**, 651–5.