



**SMALL MOLECULE TO ENHANCE THE HAEMATOPOIETIC
DIFFERENTIATION CAPABILITY IN MOUSE INDUCED
PLURIPOTENT STEM CELLS**

Amani Salem Alsayari

Presented to the Cardiff University in partial
Fulfilment of the requirements for the degree of PhD at

CARDIFF UNIVERSITY

School of Biosciences

January 2022

CONTENTS

LIST OF CONTENTS	ii
DECLARATION	v
ACKNOWLEDGEMENTS	vi
ABSTRACT	vii
LIST OF FIGURES	ix
LIST OF TABLES	xii
LIST OF ABBREVIATIONS	xiii
CHAPTER 1: Introduction	1
1.1 Development of the haematopoietic system in the embryo	2
1.2 Transcription factor networks in haematopoietic development	8
1.3 Haematopoiesis in adults	12
1.3.1 Regulation of pluripotency in iPSCs	14
1.4 Lineage Selection: Instructive vs. Selective Action of Cytokines	17
1.5 Clinical HSC Transplantation	19
1.6 Induced pluripotent stem (iPS) cells	21
1.6.1 Regulation of pluripotency in iPSCs	24
1.6.2 Differentiation of pluripotent stem cells to haematopoietic cells	25
1.6.3 Experimental and Clinical Uses of iPS in Haematology	26
1.7 Drug discovery	30
1.8 Aims and Objectives	35
CHAPTER 2: Material and Methods	40
2.1 Cell Culture	41
2.2.1 Maintenance of mouse iPSCs	41
2.2.2 Proliferation assay using Cell Titre-Blue® cell viability assay	41
2.2.3 Hanging drop culture to generate embryoid bodies	41
2.2.4 Differentiation of iPSCs (embryo-body formation) toward lymphoid and myeloid lineages	42
2.2.5 Maintenance of Human AML Cell lines	42
2.2 Colony forming cell assay (CFU).....	43
2.3 Flow cytometry Analysis	43
2.3.1 Pluripotent Stem Cell Marker Analysis	43

2.3.2 Apoptosis Analysis	43
2.3.3 Cell Cycle Analysis	43
2.3.4 iPSC Differentiation	45
2.3.5 Analysis of Transplantation	45
2.4 Transplantation Experiment	45
2.5 Gene Expression Analysis	46
2.5.1 Extraction of RNA	46
2.5.2 Conversion of RNA to cDNA	47
2.5.3 Q-PCR	47
2.6 Statistical Analysis	47
CHAPTER 3: Exploring the impact Phthalylsulfathiazole on haematopoietic differentiation from induced pluripotent stem cells and haematopoietic stem cells ..	52
3.1 Introduction	53
3.2 Aims and Objectives	56
3.3 Result	57
3.3.1 Morphology of undifferentiated IPS cells cultured in feeder free culture condition in mice	57
3.3.2 Flow Cytometry Evaluation of iPSCs to identify markers of stem cell pluripotency	58
3.3.3 iPSCs formed EBs in the HD culture condition	59
3.3.4 iPSCs formed embryoid bodies and expressed mesodermal germ layer markers	60
3.3.5 The effect of Phthalylsulfathiazole on iPS cells proliferation	61
3.3.6 Effects of Phthalylsulfathiazole in the differentiation of haematopoietic cells from iPSCs	62
3.3.7 Effects the Phthalylsulfathiazole in second transplantation	67
3.4 Discussion	71
CHAPTER 4: Examining the impact of Yohimbine on haematopoietic stem cell function and haematopoietic differentiation from induced pluripotent stem cells.....	74
4.1 Introduction	75
4.2 Aims and Objectives	80
4.3 Result	81
4.3.1 Evaluating Yohimbine's Impact on Functions of Haematopoietic Stem and	

Progenitor after Bone Marrow transplantation	81
4.3.2 Yohimbine treatment didn't impact survival of HSC and their progenitors	
After bone marrow transplant	88
4.3.3 Lack of impact of yohimbine on undifferentiated iPSC proliferation	89
4.3.4 Evaluating of small molecules in (improving) or (to enhance) haematopoietic differentiation potential of iPSCs	90
4.3.5 Gene Manifestation and qPCR of Differentiated iPSCs in Mice	94
4.3.6 iPSc Modification of Differentiation Capacities in Myeloid and Erythroid cells from iPSCs	100
4.4 Discussion	102
CHAPTER 5: Assessing the impact of Oxa-22 in normal stem cells and leukaemia cell line	103
5.1 Introduction	104
5.5.1 Leukaemogenesis and Leukaemia stem cells	104
5.5.2 Acute Myeloid Leukaemia (AML)	104
5.5.3 Cis-2-Methyl-5-termmethylammoniummethyl-1,3-oxathiolane iodide (Oxa- 22)	108
5.2 Aims and Objectives	112
5.3 Result	113
5.3.1 Impact of Oxa-22 on normal HSC function in <i>vivo</i>	113
5.3.2 Impact of small molecules on differentiation of AML cells	118
5.3.3 Impact of drugs on cell survival in leukaemia cell lines	122
5.3.4 Impact of small molecules on cell cycle activity of AML cells	125
5.4 Discussion	128
CHAPTER 6: General Discussion	131
6.1 General Discussion	131
References	135

DECLARATION

This thesis is a presentation of my original research work. No portion of the work has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my excellent supervisor Dr. Neil Rodrigues for the continuous support of my Ph.D study and related research, for his patience, motivation and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study. He has shown me, by his example, what a good scientist (and person) should be.

I sincerely thank **Dr.Badi Alotaibi** from the Rodrigues laboratory for his help in the lab and making the long work days enjoyable and unforgettable.

I thank Dr.Gui Jie Feng for her day-to-day support and supervision in the laboratory. Also, I thank Lubaid Saleh for sharing his knowledge and very useful discussions and for technical expertise with transplantation. experiments. Furthermore, I would like to thank my Saudi Arabia Government for their generous support in funding my academic pursuit.

Most importantly, none of this would have been possible without my family. I would like to thank my wonderful Parents: **my mother, Zahra**, who offered her encouragement through phone calls every day – despite my own limited devotion to correspondence. And **my beloved father, Salem**, who is giving birth to me at the first place and supporting me spiritually throughout my life. To my dear Father, I wish to have a good health to be proud of me when I get my Ph.D. Also, all my love and thanks go to my husband **Abdullah Alnahdi** for always being by my side and for understanding, support, assistance, patience and advice. My gratitude and appreciation goes to my little angels and joy, **Mohammed, Raed, Sarah and Hajar**, who have demonstrated so much patience and understanding during the period of my studies which sometimes was at the expense of spending time with them. Also, I would like to dedicate this work for my beloved grandfather **Eidah** and uncle **Salmin** who unfortunately passed away before seeing this accomplishment that they wished for me.

I would like to give my deepest appreciation to my aunt **Salha** (who is like a second mother to me) for her support throughout, and despite of the pain of losing her husband.

ABSTRACT

Haematopoiesis is the process of blood and immune cell production that, for life, is sustained by a rare population of bone marrow resident haematopoietic stem and progenitor cells (HSPCs). Over the last 50 years, the ability of HPSCs to rescue haematopoiesis has been used therapeutically in bone marrow transplantation following myeloablative therapy for haematological malignancies/disorders. Of further biological and clinical relevance, HSPC function must be strictly regulated in order to prevent the accumulation of genetic/epigenetic mutations that generate leukaemia stem cells (LSCs) which cause leukaemia. In this thesis, I have built on previous work in the laboratory where a multi-species stem cell based forward screen was conducted on a chemical library with the objective to (i) target HSPC function *in vitro* and *in vivo* to improve the practice of bone marrow transplantation and (ii) therapeutically target LSCs, which are the basis for therapy resistance and relapse in a sub-type of leukaemia called acute myeloid leukaemia (AML). Three lead small molecules were identified in this screen: Phthalylsulfathiazole, a sulphonamide, Yohimbine, an alpha-2 adrenergic receptor antagonist, and, Cis-2-Methyl-5-trimethylammonium methyl-1, 3-oxathiolane iodide (Oxa-22), M3 muscarinic acetylcholine receptor agonist. Here, I used induced pluripotent stem cells (iPSCs), a type of stem cell produced in the laboratory by genetic reprogramming of somatic cells, to assess the ability of Phthalylsulfathiazole and Yohimbine to potentiate the hematopoietic differentiation programme of iPSCs, which could eventually be used in bone marrow transplantation or transfusion medicine. As proof-of-principle for clinical application, we initially established a mouse iPSC culture and haematopoietic differentiation system in the laboratory. Gene expression analysis revealed that Phthalylsulfathiazole exposure appeared to enhance haematopoietic gene expression following haematopoietic commitment of iPSCs, while Yohimbine increased the haematopoietic gene expression programme during both haematopoietic specification from iPSCs and post-haematopoietic commitment. In functional experiments, iPSCs that were exposed to Yohimbine demonstrated improved granulocyte-macrophage potential in colony-forming cell assays. These data suggest that both Phthalylsulfathiazole and Yohimbine are promising agents to enhance haematopoietic differentiation from iPSCs *in vitro*. As Yohimbine and Oxa-22 has been shown to enhance HSPC function *in vivo* after transplantation, in the short-term, I also sought to evaluate whether these small molecules affected long-term functioning of HSCs, including haematopoietic differentiation potential. Yohimbine and Oxa-22 did not affect long-term HSC function or differentiation. Preliminary evidence, in contrast, suggests that Phthalylsulfathiazole administration *in vivo* may be detrimental to long-term HSC functioning. Finally, I assessed the impact of the small molecules to target human AML cell lines. In THP-1 cells, Yohimbine and Oxa-22, but not Phthalylsulfathiazole, alleviated the differentiation block normally

observed in AML cells, as assessed by immunophenotyping for myeloid markers, but without impacting either cell cycling status or cell survival. Collectively, the data presented in this thesis suggest that both Yohimbine and Oxa-22 have the conserved ability to potentiate haematopoietic differentiation from both iPSCs and AML cells *in vitro* and they do not adversely impact HSPC function *in vivo*. Further experimentation is required to elucidate the molecular mechanisms underscoring Yohimbine and Oxa-22 mediated normal and leukaemic function *in vitro* and *in vivo* with a view that these investigations could facilitate their application in clinical haematology settings.

LIST OF FIGURES

Figure 1.1 Simplified diagram of embryonic to adult haematopoiesis	3
Figure 1.2 A model of haematoendothelial differentiation include HSPC	5
Figure 1.3 A model of a core transcriptional regulatory networks of up-regulated and down-regulated genes in early development of murine haematopoietic stem cells....	9
Figure 1.4 Schematic diagram showing Classical Haematopoietic Hierarchy in the Adult mouse.....	16
Figure 1.5 Signaling network in mouse pluripotent stem cells	23
Figure 1.6 Standard workflow within Drug Development	31
Figure 1.7 Drug discovery library	37
Figure 3.1: Morphology of healthy mouse induced pluripotent stem cells (iPSCs) ...	57
Figure 3.2 Flow cytometric analysis of stem cell markers in undifferentiated iPSCs	58
Figure 3.3 iPSCs form EBs in hanging drop culture	59
Figure 3.4 Gene expression of stem cells markers and mesodermal genes detected by q-PCR in EBs derived mouse iPSCs	60
Figure 3.5 Phthalylsulfathiazole treatment did not affect the proliferation of undifferentiated	61
Figure 3.6 Temporal immunophenotypic analysis of iPS-derived cells exposed to Phthalylsulfathiazole during haematopoietic differentiation at day (0) and (2) of EB formation	64
Figure 3.7 Gene expression analysis in response to Phthalylsulfathiazole treatment in Day 0 in EBs.	65
Figure 3.8: Gene expression analysis in response to Phthalylsulfathiazole treatment at day 2 of EB formation Table 5.4 Role of muscarinic signalling (M3 in particular) in disease/cancer.	66
Figure 3.9: Assessing the impact of Phthalylsulfathiazole following secondary transplantation..	68
Figure 3.10: The impact of Phthalylsulfathiazole administration following secondary transplantation on the function of lymphoid haematopoietic lineages.....	69
Figure 3.11: The impact of Phthalylsulfathiazole administration following secondary transplantation on the function of myeloid haematopoietic lineages.	70
Figure 4.1: Flow cytometry gating strategy for haematopoietic stem cells at 16 weeks after transplantation..	82

Figure 4.2: Flow cytometry gating strategy for committed haematopoietic progenitors	83
Figure 4.3: The impact of Yohimbine administration on the function of haematopoietic stem and committed progenitor cells following transplantation.	84
Figure 4.4 (A): The impact of Yohimbine administration following transplantation on the function of haematopoietic lineages in Bone marrow	85
Figure 4.4 (B): The impact of Yohimbine administration following transplantation on the function of haematopoietic lineages in spleen	86
Figure 4.4 (C): The impact of Yohimbine administration following transplantation on the function of haematopoietic lineages in peripheral blood	87
Figure 4.5: The impact of Yohimbine administration following transplantation on HSC and committed myeloid progenitor cell survival	88
Figure 4.6: Yohimbine did not affect proliferation of iPSCs	89
Figure 4.7: Examining the effect of Yohimbine on haematopoietic differentiation in iPSC system	91
Figure 4.8: Temporal Immunophenotypic Analysis of iPSCs during Haematopoietic Differentiation in Response to Yohimbine exposure at Day 0 of EB formation	92
Figure 4.9: Temporal Immunophenotypic analysis of iPSCs during Haematopoietic Differentiation in Response to Yohimbine exposure at day 2 of EB formation	93
Figure 4.10: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 0 EBs	96
Figure 4.11: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 0 EBs	97
Figure 4.12: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 2 EBs	98
Figure 4.13: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 2 EBs	99
Figure 4.14: Representative morphology of haematopoietic colonies generated by different progenitor population from day12 EB-derived precursors	100
Figure 5.1: Experimental schematic of secondary transplantation of Oxa-22 treated recipients and analysis of peripheral blood engraftment	114
Figure 5.2. Effect of Oxa-22 treated HSPCs after secondary transplantation	115
Figure 5.3: Effect of Oxa-22 after transplantation on the function of haematopoietic lineage in bone marrow of secondary transplant recipients	116

Figure 5.4 Effect of Oxa-22 treatment on HSPC apoptosis from secondary transplantation	117
Figure 5.5: Flow cytometry gating strategy for assessing leukaemia cell line differentiation after exposure to Yohimbine, Phthalylsulfathiazole and Oxa-22	119
Figure 5.6. Oxa-22 and Yohimbine enhance THP1 cell line differentiation	120
Figure 5.7. No impact of small molecules on NOMO1 cell line differentiation	121
Figure 5.8: Flow cytometry gating strategy for assessing apoptosis in leukaemia cell lines	122
Figure 5.9: THP1 cell line apoptosis in response to Oxa-22 treatment <i>in vitro</i>	123
Figure 5.10. NOMO1 cell line apoptosis in response to Oxa-22 treatment <i>in vitro</i>	124
Figure 5.11: Flow cytometry gating strategy for Leukaemia cell line cell cycle with different drugs and different concentration	125
Figure 5.12. THP1 cell line cell cycle status in response to Oxa-22 treatment <i>in vitro</i>	126
Figure 5.13. NOMO1 cell line cell cycle status in response to Oxa-22 treatment <i>In vitro</i> ..	127

LIST OF TABLES

Table 1.1 Markers of development of HSPC in embryogenesis and adults	6
Table 1.2 Transcription factor functions in HSPC during embryogenesis	8
Table 1.3 Transcription factor important in HSC function and haematopoietic differentiation in adult	13
Table 1.4 Cytokines categorized by their differentiation function in homeostasis	18
Table 1.5: Pluripotency Networks in iPSCs	24
Table 1.6: Potential uses of iPSCs in disease modelling, pharmaceuticals, and	28
regenerative healthcare in haematology	28
Table 1.7: Prioritization of hit compounds from Fruit-fly and Zebrafish screens	38
Table 1.8: Summary of small molecules used in thesis	39
Table 2.1. List of antibodies used in thesis	48
Table 2.2. Primer pairs used for gene analysis of iPSCs clones using quantitative PCR	50
Table 3.1: Immunophenotypic markers of haematopoiesis in EBs	63
Table 4.1. Adrenergic receptor subtypes and physiological effects of catecholamine to the specific receptor	76
Table 5.1 Classification of AML mutations	106
Table 5.2 Different of FAB subtypes of leukaemia.	106
Table 5.3 Biological roles of M1-5 receptors.	110
Table 5.4 Role of muscarinic signalling (M3 in particular) in disease/cancer.	111

LIST OF ABBREVIATIONS

A		L	
AB	Antibody	LIF	Leukaemia Inhibitory Factor
AGM	Aorta Gonad	Lin ⁻	Lineage Negative
ALL	Acute Myeloid Leukaemia	LK	Lineage-, Sca-1-, c-kit+
ATP	Adenosine Triphosphate	LMP	Lymphoid Myeloid Precursor
AML	Acute Myeloid Leukaemia	LMPP	Lymphoid-Myeloid Multipotent Progenitor
		LSC	Leukaemic Stem Cell
		LSK	Lineage-, sca-1+, c-kit+
		LT-HSC	Long-term Haematopoietic Stem Cell
B		M	
BFGF	Basic Fibroblast Growth Factor	M	Molar(moles/l)
BMP4	Bone Morphogenic Protein 4	Mac-1	Macrophage -1 Antigen
BSA	Bovine Serum Albumin	MEF	Mouse Embryonic Fibroblast
C		MEP	Megakaryocytic-Erythroid Progenitor
Cebpa	ccat/Enhancer Binding protein α	MESC	Mouse Embryonic Stem Cell
cDNA	Complementary Deoxyribonucleic acid	MiPSC	Mouse Induce Pluripotent Stem Cell
CFU-G	Colony Forming Unit-Granulocyt	ml	Millilitre
CFU-M	Colony Forming Unit-Monocyte	MLP	Multi-Lymphoid Prognitor
CLP	Common Lymphoid Progenitor	Mm	Millimolar
CLL	Chronic Lymphocytic Leukaemia	mESC	Mouse Embryonic Stem Cells
CML	Chronic Myeloid Leukaemia	MPP	Multi-Potential Progenitor
D		MSC	Mesenchymal Stem Cell
Dapi	6-diamidino-2-phenylindole	MG	Milligram
DH2O	Distilled H2O		
DMEM	Dulbecco's modified eagle media		
DMSO	Dimethyl sulfoxide		
DNA	Deoxyribonucleic acid		
E		N	
EB	Embryoid Body	Ng	Nanogram
EC	Endothelial Cell	Nm	Nanomolar
EHT	Endothelial to Haematopoietic Transition		
EMT	Endothelial to Mesenchymal Transition		
EPCAM	Epithelial Cell Adhesion Molecule		
Erk	Extracellular signal -regulated Proteins		
F		O	
FAB	French- American- British	Oct4	Octamer-binding Transcription Factor
FBS	Fetal Bovine Serum		
FITC	Fluorescein Isothiocyanate		

<i>Flk1</i>	Fetal Liver Kinase 1		
FSC	Forward Scatter		
G		P	
<i>Gata-1</i>	Globin transcription factor 1	PBS	Phosphate Buffered Saline
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	PCR	Polymerase Chain Reaction
GMP	Granulocyte macrophage precursors	PerCP-Cy5.5	Peridinin-Chlorophyllprotein-cyanine5.5
		PS	Phosphatidylserine
		PSC	Pluripotent Stem Cell
H		Q	
HC	Haematopoietic Cell		
HE	Haemogenic Endothelium	QRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
HESC	Human Embryonic Stem Cell		
HP	Haematopoietic Progenitor		
HPC	Haematopoietic Progenitor Cells		
HSC	Haematopoietic Stem Cell		
I		R	
ICM	Inner Cell Mass	RBC	Red Blood Cell
<i>IL-3</i>	Interleukin-3	RPMI	Roswell Park Memorial Institute Medium
<i>IL-6</i>	Interleukin-6	RT	Room Temperature
IMDM	Iscove's Modified Dulbecco's	RT-PCR	Reverse Transcription-Polymerase Chain Reaction
		<i>Runx1</i>	Runt Related Transcription Factor 1
K		S	
KG	Kilogram	<i>SCF</i>	Stem Cell Factor
		<i>SCL</i>	Stem Cell Leukaemia
		SLAM	Signalling Lymphocytic Activation Molecule
		SOX2	Sry-box2
		SSEA	Stage Specific Embryonic Antigen
		ST-HSC	Short Term Haematopoietic Stem Cell
T		W	
TF	Transcription Factor	Who	World Health Organisation
		Wnt	Wingless
V		μ	
VEGF	Vascular Endothelial Growth Factor	μM	Micromolar
		μL	Microlitre

CHAPTER 1:
An Introduction

1.1 Development of the haematopoietic system in the embryo

There are specific anatomical locations for haematopoiesis – also known as blood cell production - during development in the embryo. Long-term haematopoietic stem cells (LT-HSC) – which produce all blood cells in the adult - are transported from the aorta site toward the aorta-gonads-mesonephros (AGM) at 8.5-10.5E, as well as placenta, and then to the foetal liver at 11-12.5 E (Sugiyama, Inoue-Yokoo et al. 2011). From E16, HSCs migrate toward the bone marrow (BM), which becomes residence for life for HSCs (Pietilä and Vainio 2005). As HSCs develop, researchers have identified two major stages of haematopoiesis during which blood progenitors are produced (Dzierzak and Bigas 2018) as illustrated in Figure 1.1.

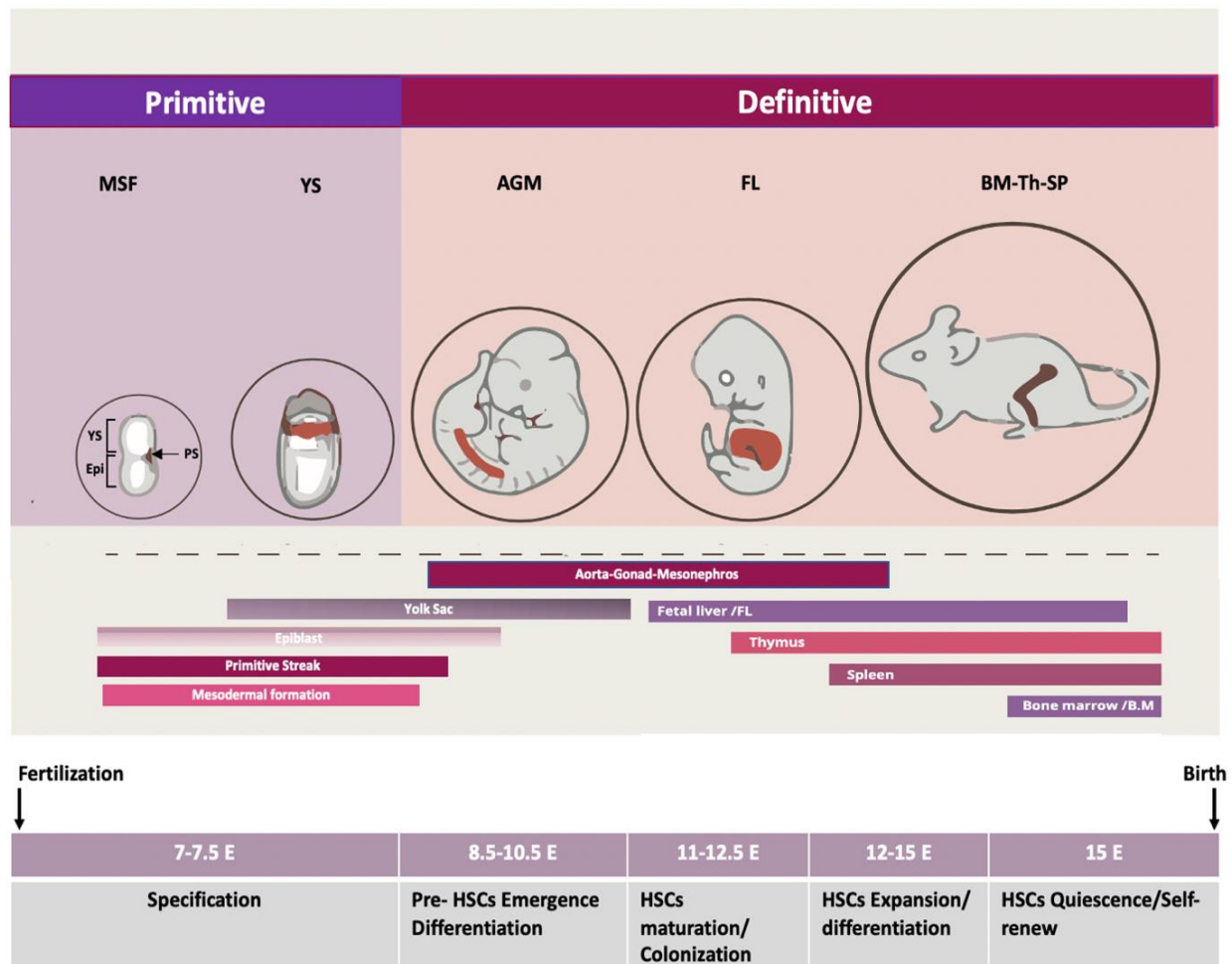


Figure 1.1: Simplified diagram of embryonic to adult haematopoiesis in a murine model (primitive and definitive haematopoiesis). Following fertilization, the embryo arises from a common mesodermal origin, but the process of haematopoiesis can be divided into primitive waves and definitive waves. The lower figure represents the timing and sites of specification, the emergence of maturation and the migration of HSCs for mesodermal formation. E= embryonic day, MS= Mesodermal formation, Epi=Epiblast, PS=Primitive Streak, YS=Yolk Sac, AGM= Aorta-Gonad- Mesonephros, FL: foetal liver, TH: Thymus, SP=Spleen, BM=Bone marrow. Adapted from (Dzierzak and Bigas 2018) (Baron, Isern et al. 2012).

The initial stages of haematopoietic cell development are termed primitive haematopoiesis (Jagannathan-Bogdan and Zon 2013). When gastrulation is completed, blood formation starts in two separate locations at day E6.5 in mice, inside the extraembryonic yolk sac (YS) via creation of blood structures known as “islands” and, also, at an intraembryonic location called the para-aortic splanchnopleure (P-Sp) (Baron, Isern et al. 2012). Afterwards, by E7-8.25, the myeloid lineage develops along with primitive erythroid cells (EryP) related to basic endothelial cells, basic megakaryocytes and some macrophages (Woolthuis and Park 2016). Primitive haematopoietic cells are relevant for transporting oxygen and vital nutrients, securing fast development of the foetus but, importantly, they do so without the need for full differentiation from a LT-HSC (Jagannathan-Bogdan and Zon 2013). Rather, primitive erythroid cells are usually generated alongside endothelial cells from a single precursor source known as haemangioblast (Hirschi 2012). This primitive phase of haematopoiesis eventually switches to a second phase, definitive haematopoiesis (Lange, Morgan et al. 2021). In definitive haematopoiesis, HSCs are elaborated from hemogenic endothelium (HE), as shown in Figure 1.2, which is related to the ventral wall of the dorsal (vDA) (Jagannathan-Bogdan and Zon 2013). At E8.25-10, initial HSCs are produced within the AGM; in the meantime, definitive HSCs develop inside the dorsal AGM location with elevated division frequency compared to the YS (Gao, Xu et al. 2018). In that way, development of myeloid and erythroid progenitors results in myeloid and, importantly, erythroid-myeloid progenitor (EMP) (Frame, McGrath et al. 2013). The AGM is viewed as a source of definitive HSCs and the natural origins of definitive haematopoiesis (Kondo 2010). The placenta is also now acknowledged as a major site of definitive HSC development (Gekas, Dieterlen-Lièvre et al. 2005). From E11- E12.5, HSCs migrate to the liver of a foetus (Lewis, Yoshimoto et al. 2021). In addition, it is the central location of HSC growth and stratification of the haematopoietic hierarchy that plays a considerable role in balanced division of self-renewing HSCs (Manesia, Xu et al. 2015). Hence, by creating a systematic multi-level hierarchical organization, haematopoietic progenitors effectively produce definitive erythrocytes, myeloid cells as well as lymphoid cells (Laurenti and Göttgens 2018). At E16-17, late phases of definitive haematopoiesis are occurring, taking place in the developing thymus and spleen; eventually, LT-HSCs migrate from the foetal liver and remain in the BM upon the formation of the skeletal system (Jagannathan-Bogdan and Zon 2013). Once in the BM, HSCs function and balance the processes of self-renewal, quiescence and differentiation of HSCs to support blood cell formation that is necessary throughout life (Huang, Cho et al. 2007). HSCs in adult BM have the functional capacity to newly reproduce the entire haematopoietic system upon transplantation to an individual exposed to radiation or high-dose chemotherapy (Sudo, Yokota et al. 2012). HSCs and their downstream precursors can also be defined immunophenotypically as they express distinct cell surface proteins as described in Table 1.1.

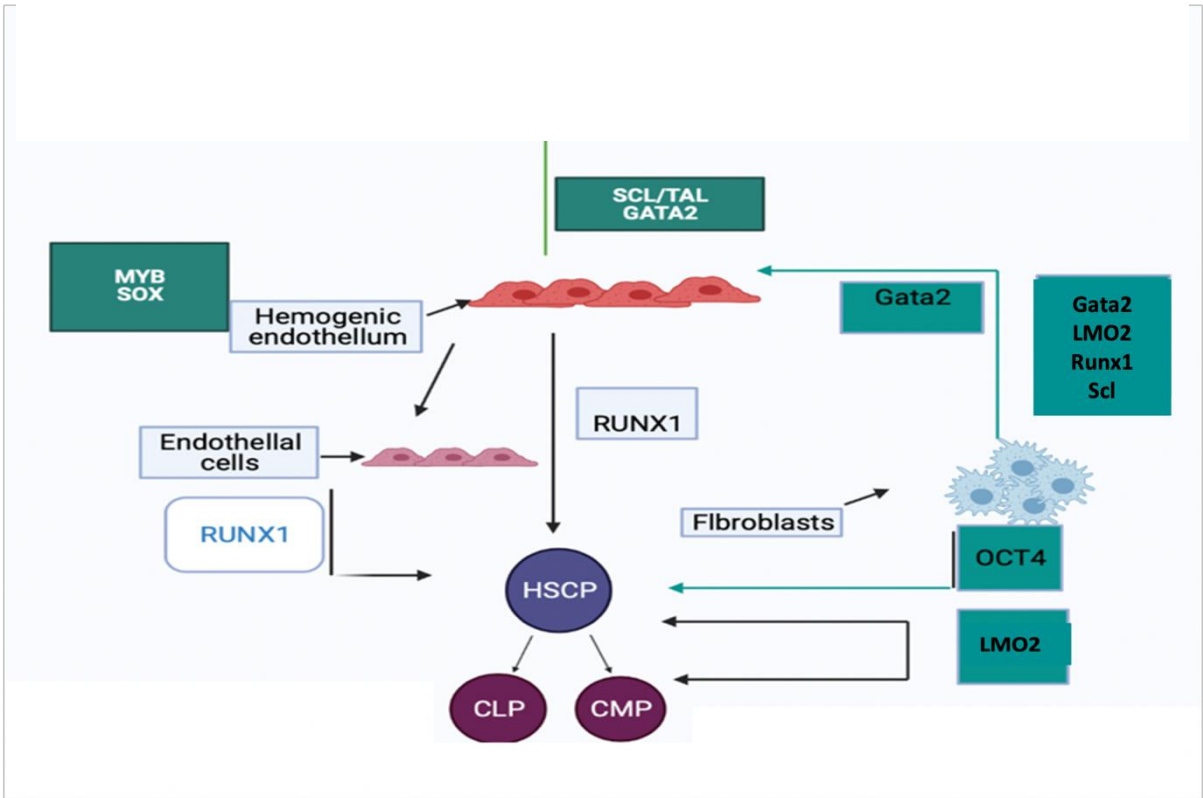


Figure 1.2: A model of haematoendothelial differentiation include HSPC. Haematopoietic stem cells. CLP: Common lymphoid progenitors CMP: Common Myeloid progenitors.

Table 1.1: Markers of development of HSPC in embryogenesis and adults

Marker	Function
c-KIT (stem cell encoded receptor with cluster of differentiation 117)	<ul style="list-style-type: none"> Has an association with the haematopoietic cells; moreover, it is expressed within the haemangioblast (Hirschi 2012). All HSCs incorporate this surface marker during the time of the whole embryonic development process of HPC within FL after 12.5 days and during the adulthood stages (Ogawa 1993).
Platelets and Megakaryocyte expressing CD41(GpIIb) (Integrin 2alpha)	<ul style="list-style-type: none"> Reduced expression of CD41 in YS, FL, as well as adult BM; nonetheless, elevated expression in haematopoietic progenitors within AGM (Rybtsov, Sobiesiak et al. 2011). CD41 precedes the expression of the pan-haematopoietic marker, CD45 (Mikkola, Fujiwara et al. 2003).
TER-119+	<ul style="list-style-type: none"> This specific marker widely refers to erythroid cells. Moreover, it represents 30-40% of d10 yolk sac cells, 80-90% of d14 foetal liver cells, in addition to 40-50% of the liver itself (Kina, Ikuta et al. 2000).
CD16-32+	<ul style="list-style-type: none"> EMP marker incorporates quite an analogous expression to the c-KIT gene (Kaimakis, de Pater et al. 2016).
Platelet endothelial cell adhesion molecule 1(CD31) (Pecam1)	<ul style="list-style-type: none"> Specialized agglutination protein expressed inside adult ECs reflecting 30% of cell population, a subdivision of leukocytes, as well as HSCs from (E8.5) during the adult stages; moreover, it is preserved in the adult mouse species. CD31 can trigger the production of endothelial cell structures.
CKIT+CD31+	<ul style="list-style-type: none"> Intra-aortic haematopoietic cluster cells (IAHCS) can be identified in the aorta site in extensive numbers. When Day 6 starts, the quantity of CD31CKIT is visibly increased, even though reduction trends become noticed by Day 12 (Baron, Kester et al. 2018).
cKIT+CD41+	<ul style="list-style-type: none"> Progenitors come from EHT in large quantities. The original source is similar to unformed progenitors as well as adult

	<p>HPCs that contain both primitive and specified HPCs. In addition, they contribute to determination of the novel pre-HSCs located in mesenchyme (Ottersbach 2019).</p>
Sca-1(Ly-6A)	<ul style="list-style-type: none"> Viewed as an integral marker of HSCs of adult BM, in addition to c-KIT. It can also be a valid marker for embryo associated HSCs in haematopoiesis later in development i.e. at the foetal liver stage (Ito, Li et al. 2003).
CD45	<ul style="list-style-type: none"> This is a marker reflecting pan-haematopoietic cells. It is observable in definitive and specific haematopoietic cells, even though low-level CD45+ might be visible or not in the AGM site. From biological perspective, CD45 manages and operates at late stages of erythroid formation in adult BM (Cortegano, Serrano et al. 2019).
Erythrocyte CD71+ (transfer-associated receptor)	<ul style="list-style-type: none"> This is a marker associated with primitive erythroblasts (EryPs) that can be distinguished from ES/iPSCs. Moreover, this is a relevant membrane protein accountable for the assimilation of the so-called iron-transferrin complex (Chao, Gong et al. 2015).

1.2 Transcription factor networks in haematopoietic development

A comprehensive and widespread system of transcription factors (TFs) responsible for managing HSC development is presented in Figure 1.3. Molecular activities within a cell, being closely interlinked, are dynamic in nature, leading to an organized series of gene expression changes that control cellular behaviour (Turner 2009). TFs master control of gene expression within a cell has been identified through mouse models and is vital for creation of definitive haematopoietic cells in the time of embryogenesis (Laurenti and Göttgens 2018). TFs have been associated with HSC specification and cell cycle regulation, which is why they are crucial in managing this entire coordinated activity (Laurenti and Göttgens 2018). One stereotypical haematopoietic TF is called GATA binding Protein 2 (*GATA2*) which represents a factor from the GATA group of zinc-finger TFs responsible for creation of HSCs, supporting dynamics of growth of haematopoietic progenitors in mature BM (Rodrigues, Tipping et al. 2012) and its proliferation in the AGM (Zhou, Zhang et al. 2019). Moreover, it regulates c-kit expression in the early stages of haematopoietic development (Oh, Lobry et al. 2013). *Gata2* expression is manifested initially in the aortic endothelium enriched with endothelial cells mainly regulated in EHT (Kang, Mesquitta et al. 2018). *Gata2* regulates HSC survival in utero, which is why deficiency of *Gata2* causes a depletion of HSCs in the developing embryo (de Pater, Kaimakis et al. 2013).

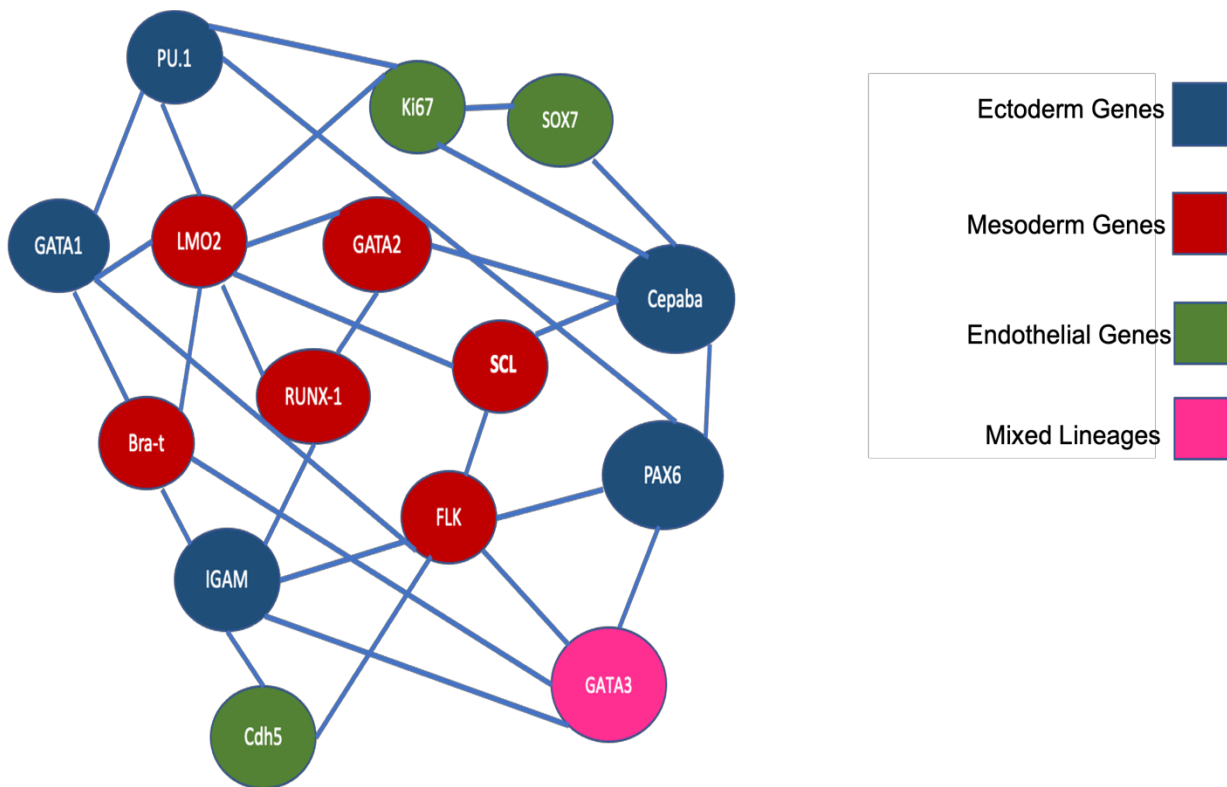


Figure 1.3: A model of a core transcriptional regulatory networks of up-regulated and down-regulated genes in early development of murine haematopoietic stem cells. The transcription factor regulatory network is highly interconnected. Red nodes indicate mesodermal genes, Blue nodes represent Endothelial genes, and Green nodes indicate Ectoderm genes (Goode, Obier et al. 2016, Gao, Chen et al. 2020).

Table 1.2: Transcription factor functions in HSPC during embryogenesis

Genes	Function
<p><i>GATA1</i></p>	<ul style="list-style-type: none"> Plays a role of an important TF in primitive haematopoiesis; moreover, it is responsible for operating activity of specified myeloid cells, along with managing the erythropoiesis process as well as cycles of macrophages-granulocytes (Scott, Simon et al. 1994). Technically, downward-regulation of <i>GATA1</i> expression is sufficient for enhancing expression of <i>C/EBP-α</i> along with <i>PU.1</i> in the myeloid lineage.
<p><i>GATA3</i></p>	<ul style="list-style-type: none"> Takes part in regulation of T cells and coordinates activity of endothelial cells (Ranganath and Murphy 2001).
<p><i>Purine box binding protein (PU.1)</i></p>	<ul style="list-style-type: none"> Viewed as the fundamental controller responsible for managing haematopoietic TFs. Considered important in forming the so-called CMPs (common myeloid progenitors) as well as production of common lymphoid progenitors (CLPs).
<p><i>The Stem Cell Leukaemia (SCL) or T-cell acute lymphocytic leukaemia 1(TAL1)</i></p>	<ul style="list-style-type: none"> Serves as an essential helix-loop-helix (bHLH) TF system that stimulates and operates basic haematopoiesis (especially by triggering haematopoiesis during the haemangioblast stage in the original mesoderm links (Göttgens, Barton et al. 2002). Important for developing haemogenic endothelium (D'Souza, Elefanty et al. 2005).
<p><i>Runt-related transcription factor 1 (RUNX1)</i></p>	<ul style="list-style-type: none"> It is a relevant TF for definitive haematopoiesis; it is engaged in defining HSCs as well as their transfer towards of adult phase of blood cells from HE (Yzaguirre, de Bruijn et al. 2017). <i>Runx1</i> inhibits the erythroid to stimulate the development of HSCs (Tober, Yzaguirre et al. 2013).
<p><i>FLK</i></p>	<ul style="list-style-type: none"> It is an essential marker for haemangioblast cells, playing a crucial haematopoietic role in the initial embryonic haematopoiesis stage (Hirschi 2012).

<p><i>T-brachyury Bra(T)</i></p>	<ul style="list-style-type: none"> • T-box is viewed as the TF associated with <i>brachyury (T)</i> that reflects on a certain protein technically encrypted by T gene. • It essentially contributes to developing mesoderm in time of early embryonic progress (Showell, Binder et al. 2004).
<p><i>LMO2</i></p>	<ul style="list-style-type: none"> • is considered a cysteine-filled LIM area protein 2. It has clear alpha and beta chains. Overall, it plays an important role in haematopoietic development, becoming highly relevant for erythropoiesis in yolk sac site.
<p><i>Integrin alpha M chin (IGAM) or cluster of differentiation molecule (CD11b):</i></p>	<ul style="list-style-type: none"> • A relevant protein sub-item containing as alpha as beta chains; it contributes to formation of a leukocyte sensitive integrin that is labelled as macrophage receptor 1 (Mac-1).

1.3 Haematopoiesis in adults

Haematopoiesis after birth is the coordinated process for creating 300 billion blood cells per day from the bone marrow (BM) in homeostasis or in response to the impact of damage or infection (Laurenti and Göttgens 2018). HSCs are the fundamental and continuous source of blood quality and immunity in the living organism. HSCs formally reside at the top of the haematopoietic hierarchy, responsible for generating all blood cell types (Seita and Weissman 2010). Despite being indispensable for blood cell production, the replicative dynamics of HSCs is highly limited, being both highly quiescent and representing a scarce pool of cells located in the BM; in the BM, HSC frequency is limited in BM to 0.05%, representing only 0.001% of all nucleated cells (associated with peripheral blood) (Lemos, Farias et al. 2018). Aside from BM, HSC can also be found in extramedullary sites in the spleen, liver and/or thymus (Oda, Tezuka et al. 2018).

Due to their scarcity, self-renewal is a principal function that sustains HSCs, either through symmetric or asymmetric division (Ting, Deneault et al. 2012). In turn, MPPs are able to reproduce blood lineages of a broad variability yet with limited ability for self-renewal over time (Laurenti and Göttgens 2018). Thanks to the blood lineage, oxygen is transported across the body and gas exchange is implemented. Moreover, other functions such as erythrocyte delivery, immunity operation/reactions, lymphocytes (T, B cells) functionality, recovery and haemostasis are also fulfilled. Just as described above during embryogenesis, nuclear TFs also tightly regulate HSC fate decisions in adults as exemplified in Table 1.3. Deregulated TF control of HSC fate can result in pathological impact – for example, BM might fail functionally, or leukaemia may develop (Greim, Kaden et al. 2014). In addition to intrinsic control of haematopoiesis, various haematopoietic cytokines (e.g., *SCF*, *interleukin 3-6*) are involved in the regulatory process (Metcalf 2008) (discussed in Section 1.4). The important haematopoietic TFs involved in haematopoietic development are outlined in Table 1.3.

Table 1.3: Transcription factor important in HSC function and haematopoietic differentiation in adult.

TF	Function
<i>Pax5</i>	<ul style="list-style-type: none"> Gradually ensures and identifies lymphoid predecessors of the B cell fate. Absence of <i>Pax5</i> leads to distinction at initial Pro-B stage.
<i>EBF</i>	<ul style="list-style-type: none"> <i>Ebf2</i> contributes to a transcription relating to the osteoblastic area, which supports the haematopoietic progenitors. <i>EBF</i>'s expression at the level of lymphoid progenitor cells has provoked disruption of B cell growth because other lymphoid-related lineages such as LDC, NK, and T have been reduced in cells manifesting <i>EBF</i> expression.
<i>E2A</i>	<ul style="list-style-type: none"> <i>E2A</i> serves as a HLH TF prerequisite for distinctions and specification of B cells.
<i>Cited2</i>	<ul style="list-style-type: none"> Crucial for progression (in mouse species) and contributes to adequate haematopoiesis in foetal liver as well as in mature bone marrow.
<i>Runx1</i>	<ul style="list-style-type: none"> Impact on dynamics of long-term recovery of HSCs (LT-HSCs) is quite minor. Expression in multiple myeloid cell types and at lower amounts in lymphoid cells. <i>RUNX1</i> inhibits erythroid differentiation by downregulation of the erythroid gene expression program. <i>RUNX1</i> can act as an activator and repressor during megakaryocytic differentiation (Kuvardina, Herglotz et al. 2015).
<i>Gata1</i>	<ul style="list-style-type: none"> implements relevant in-<i>vivo</i> functions for coordinating definitive/specified haematopoietic progenitors to distinguish as erythroid and megakaryocytic lineages. Is important for stable erythropoiesis and extension of erythroid as a reaction to anaemia.

1.3.1 HSCs – Immunophenotypic and Functional Definition

Identification of HSCs using multi-colour flow cytometry depends on different cell surface markers that can be identified by utilizing monoclonal antibody recognizing an antigen on the cell surface conjugated to a fluorochrome (Challen, Boles et al. 2009). In mice, a population enriched for HSCs has c-kit expression together with low Thy1 expression and markers of mature lineages (Lin⁻) are non-existent, while SCA-1 expression is also expressed (Ikuta and Weissman 1992). This population (termed LSK) defines LT-HSCs, ST-HSCs and MPPs in the BM. Thus, LSK is a heterogeneous pool enriched in HSC capacity. To functionally characterize HSC and progenitor capacity *in vivo*, long-term multi-lineage reconstitution in competitive transplantations is the gold-standard assay (Kwarteng and Heinonen 2016) and has led to the identification of multiple classes of HSC and progenitor cell. In the case of mice, after two weeks of transplantation, spleen units responsible for building colonies save the recipient from fatality (Till and McCulloch 1961) due to haematopoietic regeneration of multipotential progenitors. After three-six weeks, the active restoration of ST-HSC dominates to secure multi-lineage regeneration. LT-HSC reinforce haematopoiesis over the next 16 weeks for the remainder of life post-transplantation (Abbuehl, Tatarova et al. 2017). LT-HSC have a critical role in haematopoiesis in context of regeneration, particularly, throughout entire life of an organism (Pina and Enver 2007). LT-HSC are scarce, representing only 0.01% inside BM (Kiel, Yilmaz et al. 2005); (Yang, Bryder et al. 2005). LT-HSC are identified through Lin⁻ Sca1⁺ c-KIT⁺ CD150⁻ CD48⁺ and lack of CD34 and Flt3 expression (Kidoya, Muramatsu et al. 2019). In contrast, ST-HSC refer to a heterogeneous class of transitory reproductive cells that cultivate various MPPs and/or committed progenitors discovered (Morrison, Wandycz et al. 1997). ST-HSC tend to manifest CD34 expression (Qiu, Papatsenko et al. 2014). At first, MPPs are deprived of their erythroid-megakaryocytic capacities (Iwasaki and Akashi 2007). As a result, they transform into lymphoid-primed MPPs (LMPPs); alternatively, they can turn into early lymphoid progenitors (ELPs) (Challen, Boles et al. 2009) ST-HSCs secure short-term regeneration in case of transplantation intervention within four–six weeks and generally throughout three–four months. ST-HSC can be identified as Lin⁻ Sca1⁺ c-KIT⁺ CD150⁺ CD48⁺ (Challen, Boles et al. 2009). MPPs contribute to the growth of intermediary progenitors that transform into the CLPs that further develop into lymphoid progeny, as well as CMPs with erythromyeloid capacities (Cheng, Zheng et al. 2020). Granulocyte/macrophage progenitors (GMPs) are associated with the myelomonocytic lineage, and megakaryocyte/erythrocyte progenitors (MEPs), incorporating high expression of *GATA1*, are responsible for erythropoiesis that supports formation of precursors of red blood cells (RBCs) and platelets (Woolthuis and Park 2016).

MPPs comprise two major classes of adult blood cells, yet they have shorter life capacities and limited division capacities compared to LT-HSCs and ST-HSCs (Kondo 2010). In the context of committed progenitors in the haematopoietic system, CLP regulates the adaptive immunity by differentiation between T, B and nature killer (NK) cells, and secures blood development for four–six weeks. In that way, Flt3 contributes to lymphoid progress and takes part in forming MEPs (Iwasaki and Akashi 2007). In contrast, CMP is found within the Lin⁻ Sca1⁻ c-KIT⁺ CD34⁺ CD16/32⁻ compartment to generate all possible myeloid progenitors, such as GMP, as depicted in Figure 1.4 (Challen, Boles et al. 2009). In a similar vein to mice, a human haematopoietic roadmap has been elucidated using flow cytometry and transplantation in xenogeneic mouse models (Doulatov, Notta et al. 2012), albeit in less detail than in mouse haematopoietic system (Kikushige, Yoshimoto et al. 2008). John Dick's laboratory has primarily been responsible for formulating this roadmap. Human HSCs are defined by CD34⁺ CD38⁻ Lin⁻ expression in cord blood, bone marrow, and CD90 (Majeti, Park et al. 2007). Classes of progenitors (MPPs), CMP and CLP have been identified in humans with a similar developmental potential to their mouse counterparts (Seita and Weissman 2010).

1.3.1.1 Human and Mouse haematopoiesis

Even though multiple common features are detected in basic cellular patterns, relevant distinctions between HSC of mice and humans are still evident, especially in certain functions of transcription factors and cellular mechanisms (Parekh and Crooks, 2012). Hence, HSC of mice have been noted to be dividing every 30–50 days for one time compared to more stationary reaction of human HSC with their division rates of 175–350 days (Doulatov, Notta, Laurenti and Dick, 2012). In case of human biology, HSC as well as haematopoietic progenitor cells (HPC) fail to manifest CD150, whereas CD48 has been manifested as in HSC as in HPC (Oguro, Ding and Morrison, 2013). The prolonged bioactivity of human HSC persists in the CD34⁺CD38⁻lin⁻ particle of cord blood as well as bone marrow. However, HSC of mice fails to manifest either CD34 or Flt-3, but manages to exhibit CD38. Such immunophenotypic distinctions might indicate of functional diversities in stem cell performance, as well as differentiation and microenvironmental interaction channels, stressing on the difference in haematopoiesis of mice and humans. CD150, which is an agent of the signalling lymphocyte activation molecule (SLAM) genus, is supposed to contribute to the performance of HSC of mice (Weksberg, Chambers, Boles and Goodell, 2008). In murine, Flt-3 signalling becomes significant for lymphopoiesis, except for survival or myelopoiesis of HSC. In turn, Kit Ligand (in *vitro*) was found to be a stronger survival and resilience factor unlike Flt3 ligand for bone marrow HSC of mice. Still, the opposite pattern is also valid for case of human HSC. The distinctions in species regarding the impact of cytokines on survival rates of HSC (in *vitro*)

provides a research value for projecting special systems aimed at expansion (*ex vivo*) as well as transduction of human-based HSC (Parekh and Crooks, 2012).

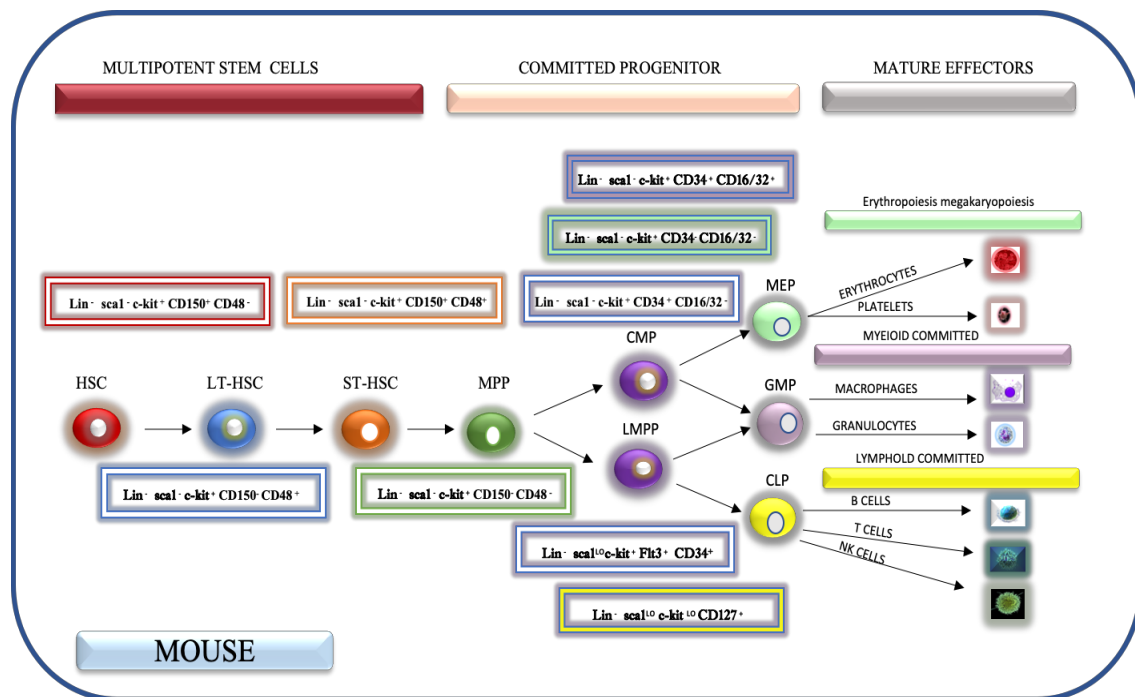


Figure 1.4: Schematic diagram showing Classical Haematopoietic Hierarchy in the adult mouse: This describes long-term (LT)HSCs and short-term (ST)HSCs, respectively at the apex of the differentiation hierarchy classes according to the extent of their repopulation capacity in competitive transplantation experiments. HSCs give rise to MPP cells that differentiate into multipotent progenitors (Lymphoid-primed multipotent progenitors LMPP and common myeloid progenitors (CMP), CMPs further differentiate into granulocyte-macrophage progenitor (GMP) and megakaryocyte/erythrocyte progenitors (MEP) lead to macrophages and granulocytes. The lymphoid lineage, initiated by Common lymphoid progenitors (CLP) which differentiate into mature white blood cells include T cells, B cells, natural killer NK cells.

1.4 Lineage Selection: Instructive vs. Selective Actions of Cytokines:

Cytokines are soluble proteins, that might be secreted or stay bound to membrane, and are biologically active molecules with various physiologic roles (Oppenheim 2001). For example, haematopoietic cytokines that have been discovered play a vital function in instructing haematopoietic lineage choice (LC), activating and managing the survival process, and the self-renewal of HPCs/HSC (Zhang and Lodish 2008, Pouzolles, Oburoglu et al. 2016). Cytokines render their roles in the BM setting mainly, but they can also operate inside the bloodstream system or lymphatic vessels, impacting immunity and inflammatory reactions (Zhang and An 2007). More than 50 cytokines are classified as chemokines – this includes but is not limited to interleukins (IL), interferons (IFN), Thrombopoietin (TPO), erythropoietin, and others (Endele, Etzrodt et al. 2014) and Table 1.4. The downstream activation of TFs is also regulated by cytokines. These proteins are also used as target inhibitors, which can be utilized in therapeutic setting (Hughes, Rees et al. 2011). Cytokines also launch the so-called mitogen activated protein kinase (MAPK) that transports signals across the channels (the p38 MAPK/NF- κ B signaling pathway) (Wang, Cui et al. 2016).

Table 1.4: Cytokines categorized by their differentiation function in homeostasis.

Marker	Function
<p><i>IL-3</i> (<i>Interleukun-3</i>)</p>	<ul style="list-style-type: none"> • Reproduction and/or survival factor for embryo's initial HSCs (Robin, Ottersbach et al. 2006). • Ensures HSC survival in the AGM and YS (Robin, Ottersbach et al. 2006). • Serves as <i>Runx1</i> target and impacts matured haematopoietic cells (Robin, Ottersbach et al. 2006). • Regulates embryonic HSCs at E9-E10 leading to formation of HSC fate (Gao, Xu et al. 2018).
<p><i>IL-6</i> (<i>Interleukun-6</i>)</p>	<ul style="list-style-type: none"> • Regulates cell distributions, differentiation, as well as survival in time embryonic progression (Heinrich, Behrmann et al. 1998). • Supports immunity reactions, homeostasis, metabolic processes, oncogenesis, inflammation and cell formation. • Improves pluripotent conditions of stem cell markers. • Ensures increase in expression within the human bone marrow aligned with the MSCs (mesenchymal stem cells) (Mi and Gong 2017).
<p><i>Stem Cells Factor</i> (<i>SCF</i>)</p>	<ul style="list-style-type: none"> • Serve as a ligand for tyrosine kinase receptor, c-kit. • Extrinsically relevant in coordinating HSC immobility (Williams et al. 1990) (Barker 1994). • Ensure erythropoiesis formation at the mesoderm phase of genesis. • interconnection with c-KIT and secure from HSC apoptosis (Zhang and Lodish 2008).
<p>Erythropoietin</p>	<ul style="list-style-type: none"> • Contributes to the growth of erythrocytes and development of neutrophil (Elliott and Sinclair 2012).
<p><i>Granulocyte-colony stimulating factor (G-CSF)</i></p>	<ul style="list-style-type: none"> • majorly extracellularly regulates the innate immune system and haemopoiesis (Roberts, 2005). • affects the differentiation, proliferation, and survival of all neutrophil lineage cells, from haemopoietic stem cells to mature neutrophils (Roberts, 2005).

<i>Granulocyte- macrophage colony- stimulating factor (GM- CSF)</i>	<ul style="list-style-type: none"> • a cytokine promoting the formation and maturation of a myeloid cell as well as the differentiation and sustenance of the dendritic cell <i>in vitro</i> (Shi et al., 2006)
---	--

1.5 Clinical HSC Transplantation

Transplantation related to HSCs has become an inspiration for establishing stem cell biology as a broad regenerative therapy for multiple ailments involving the blood and/or the immune system. Transplantation can be curative in the case of haematopoietic malignancies and/or non-malignant complications, including immunodeficiency disease and thalassemia (Copelan 2006). The basis for HSC transplantation in the clinic is that after receiving high-dose chemotherapy/radiation therapy, the blood system of patients with haematological cancers can be rescued by transplanting BM containing HSCs (Hatzimichael and Tuthill 2010) (Copelan 2006). The efficiency of transplantation of HSCs from the original donor to a recipient relies on their ability to automatically home to recipient haematopoietic tissues after infusion. The mechanisms involved in homing after transplanting include migrating haematopoietic stem cells via the blood through endothelial vasculature into various organs and their bone marrow niche mediated by chemokines such as CXCL12 and adhesion molecules (Perlin, Sporrij et al. 2017). Firm adhesion and rolling of progenitors to the endothelial cells into tiny marrow sinusoids under flow of blood, followed by trans-endothelial migration across the somatic extracellular/endothelium matrix barrier (Lapidot, Dar et al. 2005). While bone marrow derived, including mobilized peripheral blood, HSCs are the principal HSC source used for transplantation, umbilical cord blood is rich in HSCs and routinely used in transplantation of paediatric patients, but HSC numbers are too low for full reconstitution of adults (Copelan 2006). Active investigations are currently being undertaken to explore whether cord blood HSCs may be expanded in culture for use in adult patients (Fan, Zhang et al. 2011).

Clinical HSC transplantation can occur in two different settings. First, there is autologous transplantation that is based on the reinfusion of a patient's native HSCs and is used in treating multiple myeloma and lymphoma (Khaddour, Hana et al. 2019). This approach is challenged by a risk that malignant cells presented in the original pool of transplanted cells might provoke a relapse (Khaddour, Hana et al. 2019). Second, there is the allogeneic transplantation that requires populations of myeloid, B-lymphoid and T-lymphoid cells to be regenerated throughout three or six months after transplantation from a related, immunologically matched donor. The types of donor are divided into three categories: transplant from an identical twin,

related matched tissue donor, or matched unrelated donor (Khaddour, Hana et al. 2021). Donors should be physically related or have similar HLA and defining HLA (human leukocyte antigen) system complexes has helped in selecting well-matched sibling donors. However, recent studies have introduced choosing unrelated donors. Patients having fairly common HLA genotype, a matched mature donor may be identified for most cases (Little, Green et al. 2016). The evaluation of the patient's search is a complex process with multiple variables, ultimately affecting the choice of the donor selection options and intervention, if needed. In particular, the process includes the sources of donors to be used, the total number of donors needed for the successful match, and the adherence to an established timeline for the transplantation (Rogers, Robertson et al. 2019).

In treating leukaemia, the Graft-versus-leukaemia (GVL) represents a crucial element of the general beneficial impact of allogeneic bone marrow transplantation (BMT). Numerous clinical studies have indicated a direct link between GVL impact, to chronic and acute graft-versus-host disease (GVHD) (Slavin,1990). Besides, several murine models investigations concerning human leukaemia show that GVL can be separated from GVHD partially. Irrespective of the GVHD allogeneic BMT can be beneficial relative to syngeneic BMT. Similarly, the depletion of T lymphocytes is linkable to the increased relapse incidence (Slavin,1990). BMT with GVHD, through recombinant cytokines like human IL2 that can invigorate effector cells by anti-leukaemic reaction. Combined, the present data from experimental men and animals indicate that GVL is partially separable from GVHD. Thus, further comprehension of target and effector GVL's cells and our abilities to trigger anti-tumour effector cells, particularly the MHC non-restricted ones, can result in new ways of intensifying anti-tumour effector mechanisms with no induction of severe, clinically hidden GVHD (Slavin,1990). Most successful trials in the direction may also result in enhanced results after autologous BMT. The median variation between period to graft-versus-host (GVH) and GV receptiveness in the whole cohort was a fortnight. No association was established between the T-cell dosage and the comparative start of GVL compared to GVH reactivity, showing that temporal separation of GVH and GVL reactions is not an expression of the overall infused donor T cells (Hari, Logan and Drobyski, 2004).The result showing that GVH and GVL responses are non-temporally synchronous occurrences and increase the potential of targeted alloreactive donor T cells elimination following bone marrow transplant can be an efficient way of separating GVL/GVH reactivity (Hari, Logan and Drobyski, 2004).

1.6 Embryonic stem cells, Induced pluripotent stem cells and Nuclear Transfer Stem Cells

In 1981, Evans and Kaufman and peers managed to isolate embryonic stem cells (ES) cells from the blastocyst of a mouse. In turn, in 1998, Thomson and colleagues were the first to isolate human ES from an *in-vitro* egg that was fertilized. In initial stages of embryo growth, the inner cell mass (ICM) is created via the blastocyst at day 3.5 *in vivo* in the case of mice. Moreover, trophectoderm (TE) cultivates an additional extra-embryonic stratum, which serves as a coat that maintains the ICM and further cultivates the placenta (Hackett and Surani 2014). This ICM results in formation of the primitive/elementary endoderm, which further forms both the visceral and parietal YSs as well as epiblast cells (Hadjantonakis, Siggia et al. 2020). At the late stages of embryogenesis, the epiblast may transform into certain tissues (Muhr and Ackerman 2020). ES cells are generated from this ICM, which can continuously self-renew under specific stimulation, or differentiate into the embryo's diverse repertoire of somatic cell lineages (Warmflash, Arduini et al. 2012). Qualities of this sort made the ES cell highly useful in examining developmental biology principles and mechanisms maintaining tissue development and potential therapies in regenerative medicine (Doğan 2018). Still, there are impediments hindering further successful clinical research including, importantly, an ethical problem associated with isolation of ICM i.e. destruction of human embryos to derived human ES cells (Volk and Theoret 2013).

In the context of stem cell science, biology, genetic engineering and regenerative medicine, the capacity to reprogram somatic cells into pluripotent stem cells was considered as a seminal scientific, medical and ethical breakthrough (Morris and Daley 2013). Shinya Yamanaka offered a theory that factors supporting identity of ES cells might biologically stimulate multi-potency of somatic cells. Yamanaka, a winner of the Nobel Prize for ultimately proving this theory correct, discovered in 2006 that introducing an admixture of TFs (particularly Oct3/4, Sox2, c-Myc, and Klf4) into a somatic cell might activate multi-potency in these cells. These reprogrammed cells were coined induced pluripotent stem cells (iPSCs). It was discovered that iPSCs share many traits in common with ES cells such as common morphology, qualities of growth and development, and expression of cell surface marker proteins. Figure 1.5 shows a depiction of common signalling in iPSCs and ES cells include (FGF4, Wnt, BMP4, and LIF) (Mossahebi-Mohammadi, Quan et al. 2020).

Importantly, use of iPSCs could solve the ethical dilemma of using ES cells in the lab, which requires the destruction of potentially viable embryos (Takahashi and Yamanaka 2006). Ultimately, the initial reprogramming approach using retroviruses to deliver OCT3-4/Sox2/c-Myc and Klf4 to somatic cells stimulated multi-potency among cells with reduced differentiation

potential (0.02%) and allowed for the possible reactivation of c-Myc, which creates a risk of oncogenic potential and is thus problematic for clinical applications of iPSCs. However, further research showed that c-Myc is expendable for mouse fibroblast direct reprogramming (Wernig, Meissner et al. 2008). As predicted, mice obtained from Myc (-) iPSCs never developed tumours during the research period (Rand, Sutou et al. 2018). Human iPSCs have now been made from the adult dermal fibroblasts in the absence of MYC (Nakagawa, Koyanagi et al. 2008).

One vector known as stem cell cassette (STEMCCA) simultaneously transfers four reprogramming genes (Oct3/4, Sox2, c-Myc, and Klf4) to somatic cells to obtain iPSCs (Sommer, Stadtfeld et al. 2009). While this vector contains c-myc, it has several advantages that include enhancing the efficiency of reprogramming. It also uses one vector containing the four transcription factors as opposed to co-transducing four distinct transcription factors in four vectors. This reduces viral integrations in that the single vector minimizes viral reactivation and insertional mutagenesis risks (Medvedev, Shevchenko et al. 2010). Furthermore, it is an inducible construct which facilitates improved viral transgene expression control (Sommer, Stadtfeld et al. 2009). Additionally, STEMCCA, with loxP sites flanking the reprogramming genes, can create integration-free iPSCs by use of the Cre/loxP system that allows removal of STEMCCA following full reprogramming, which would allow iPSC cell derived products to be used, in principle, for clinical applications such as transplantation.

Other ethical sources of pluripotent stem cells can also be engineered in the laboratory. For instance, somatic cells might be reprogrammed using a method that transports their nuclear contents into oocytes, termed nuclear transfer (NT). Nuclear Transfer of somatic cells at a certain phase of growth to selected oocytes (enucleated and unfertilized) that induce reprogramming was a technique pioneered by John Gurdon, a co-winner of 2012 Nobel Prize with Yamanaka. Specifically, in the 1950s, he used the nuclear content of epithelium cells of tadpoles' intestines and transferred nuclear material from them into oocytes, which ended up with the successful cloning a frog (Gurdon 1962). Based on this technology, in 1996, Prof. Ian Wilmut successfully cloned Dolly the Sheep, which was based on the transplantation of the nucleus content from mammary gland cells into an enucleated sheep egg (Gurdon and Uehlinger 1966).

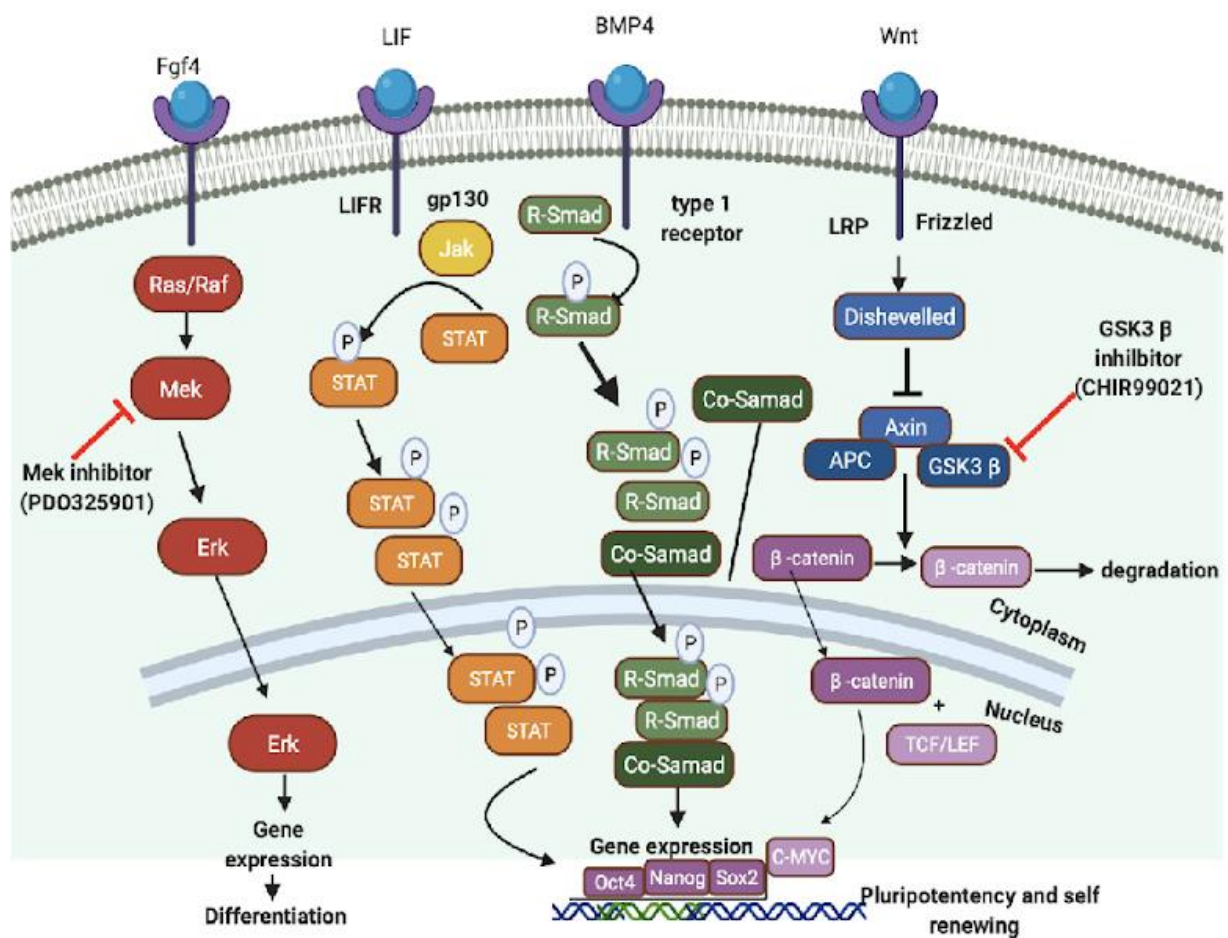


Figure 1.5: Signalling network in mouse pluripotent stem cells. Maintaining a balanced network of signalling input is important for the maintenance of pluripotent stem cells. signalling pathways achieve by maintaining a long term, proliferative state with differentiation. Adapted from (Mossahebi-Mohammadi, Quan et al. 2020).

1.6.1 Regulation of pluripotency in iPSCs

iPSCs manifest a pluripotent transcriptional program akin to ES cells (Ayob and Ramasamy 2018). As such, they share common morphological and development qualities as ES cells. As alluded to earlier, in the context of DNA binding, TFs are able to activate (or in some case) restrain gene expression (Fietze and Farnham 2011). TFs are crucial for direct gene activation, growth options, and for securing antibody effector functions. The TFs involved in maintaining the pluripotency network in iPSCs are highlighted in Table 1.5.

Table 1.5: Pluripotency Networks in iPSCs

Pluripotency Networks	Function
Oct3-4(Pou5F1)	<ul style="list-style-type: none"> • The reduction of Oct3-4 expression accelerates the identification and distinction of ESCs from both trophectoderm and placental cell structures. • On the other hand, expression of Oct3-4 elevates distinction capacities resulting in primitive formation of both endoderm and mesoderm layers (Matoba, Niwa et al. 2006).
Sox2	<ul style="list-style-type: none"> • Is associated with the SOX gene supercluster, thus representing visible HMG box transcript. It reacts to proteins originating from the POU site. In case of mice, it is demonstrated in the initial phases of an embryo (Pan and Schultz 2011).
SSEA-1(CD15/Lewis x)	<ul style="list-style-type: none"> • Its expression increases in terms of distinction of human cell structures; nonetheless, it reduces upon distinction patterns in mouse cell structures, which is why SSEA-1 might be defined on the surface of murine embryo, as well as EC cell structures (Xu, Hardin et al. 2016).
Nanog	<ul style="list-style-type: none"> • A homeodomain transcription-related protein plays a role in developing multi-efficient system. • Nanog directs the process of ESC self-renewal. Active expression of the protein leads to a broad proliferation of ESCs (Pan and Thomson 2007).

C-MYC	<ul style="list-style-type: none"> Serves as an essential HLH/leucine zipper TF. Moreover, it represents the so-called proto-oncogene used for recoding and reprogramming objectives (Chappell and Dalton 2013).
KLF	<ul style="list-style-type: none"> Refers to 17 components from the Kruppel-like zinc-finger cluster of TFs (Kenchegowda, Swamynathan et al. 2010). It ensures an observable influence on all reprogramming phases, as it suppresses somatic gene expression in the initial phases and advances multi-efficient genes during the sequential stages, respectively.

1.6.2 Differentiation of pluripotent stem cells to haematopoietic cells

Two systems of culture to differentiate into haematopoietic cells are in use. Comprehensive research has resulted into the cultivation of a feeder-free (FF) culture system for multi-potent stem cells taken from mice. The FF system implies that cells can adhere to the extracellular matrix surface for extensive development. The central aim of FF methodology is to reach a proper balance between maintenance of exponential development of multi-potent stem cells and preventing random specification of cell-related colonies through chemical identification of all elements utilized for cultivating a culture (Zakrzewski, Dobrzyński et al. 2019).

The feeder layer (FL) system incorporates adherent growth-centred cells with highly bioactive and viability potential. The cells identified can be utilized as a subunit to determine other medium cells developed at relatively low or clonal density. This feeder system was first published in 1955 by Puck and Marcus (Puck and Marcus 1955, Llames, García-Pérez et al. 2015).

iPSCs may eventually be differentiated into types of cells usable in treating haematopoietic illnesses. For example, as a model system for examining normal developmental processes and a source of cells/tissues, mouse iPSCs cells offers the starting point for human iPSC differentiation into transplantable haematopoietic descendants, which may be employed therapeutically (Nakamura, Okamoto et al. 2016). Two distinct investigational systems are utilized in most experiments for generating blood precursors mainly from iPSCs cells: the induction of haematopoietic differentiation using stromal cells or synthesis of embryoid bodies (EBs). EB disruption through collagenase and trypsin treatment generates single-cell suspensions. It then forms suspensions that contain haematopoietic progenitors which may be enumerated and analysed using colony forming cell (CFC) or flow cytometry assays. Cells within maturing EBs manufacture different cytokines and eventually the initial phases of

haematopoiesis happen in serum and with haematopoietic specific growth factors (Lim, Inoue-Yokoo et al. 2013).

1.6.3 Experimental and Clinical Uses of iPSCs in Haematology

Cultivation of iPSCs is not only viewed as an option to bypass ethical problems and solve the allogeneic issues of molecular rejection of ES cells (Medvedev, Shevchenko et al. 2010). From a scientific perspective, it provides a new unique opportunity for potentially regulating diseases and genetic manipulation of patient's biology for treatment of disease (Shi, Inoue et al. 2017). Yet, issues remain for clinical translation of iPSCs – for example, producing enough therapeutically valuable cells, like HSCs, remains a problem. Moreover, incompletely differentiated iPSCs might result in teratomas (Doss and Sachinidis 2019). Of concern also, expression during *in-vitro* differentiation of HLA molecules may cause immune rejection of transplanted iPSC cellular products (Taylor, Peacock et al. 2012). Generation of homozygous HLA-typed iPSCs that can fit several therapeutic and clinical cases is a priority (Drukker 2008). As alluded to above, transplantation of HSCs via the allogeneic method involving immune-compatible donors has been effectively practiced over more than six decades to treat different malignant as well as non-malignant disease (Leonard, Tisdale et al. 2020). However, risks persist. For example, host-dependent incompatibility and variation; low presence of HLAs; and restricted reproduction and spreading of compatible and adjustable HSCs (Guilcher, Truong et al. 2018). Thus, in the case of sickle cell disease, HLA-Matched sibling for Allogeneic haematopoietic cell transplantation (AHSCT) has shown relatively high survival rates of cells, but the problem is that more than 85% of recipients cannot find an appropriate HLA-matched donor or some other additional sources of HSCs (Leonard, Tisdale et al. 2020). As a procedure, AHSCT is evidently challenged by factors of donor matching, transplant rejection, and risks of developing graft-vs-host disease (GVHD). However, if risks are managed, AHSCT can be a potential curative solution for patients (Leonard, Tisdale et al. 2020). To produce HSCs directly from iPSCs a lot of scientific efforts have been made as HSCs cannot be easily generated from iPSCs (Demirci, Leonard et al. 2020). An approach to this challenge is to use differentiated cell types and enforce the expression of several master transcription factors that facilitate HSC behaviour (Rieger and Schroeder 2012). A total of seven transcription factors, namely *Runx.1*, *LCOR*, *PU.1*, *HOX*, *HOXA9*, and *HOXA5*, confer HSC-like engraftment or self-renewal with multi-lineage capacity (Sugimura, Jha et al. 2017). Even though molecular and functional differences are still noted for these engineered cells and authentic HSCs with respect to the durability of engraftment and full recapitulation of the terminally differentiated cells, the findings of the experiments indicated the potential for modelling of HSC based disease and developing therapeutic interventions in genetic blood disorders (Demirci, Leonard

et al. 2020). In fact, the genetic manipulation approach used to produce HSCs may provide reasonably promising results in a clinical or therapeutic perspective (Morgan, Gray et al. 2017).

iPSCs can be used for the purposes of disease modelling with a potential to correct monogenic disorders for potential, autologous therapeutic application in a wide range of fields including haematology. In a proof of principle mouse study, a model of Faconi's Anaemia (FA) using iPSCs has been developed (Müller, Tarasov et al. 2012). FA is the most common bone marrow failure syndrome which is caused by X-linked chromosomal instability due to mutations one of 13 genes (Wu 2013). Corrected Fanconi-anaemia-specific iPSCs were engineered in order to provide haematopoietic progenitors of myeloid and erythroid lineages that were disease-free (Raya, Rodríguez-Pizà et al. 2009). It may therefore be possible to reprogram non-haematopoietic somatic cells to generate genetically corrected FA-specific iPSCs which we could produce large number of autologous, genetically stable HSCs that could be used in blood stem cell transplantation to treat FA patients (Raya, Rodríguez-Pizà et al. 2009). Another disease that could benefit from iPSC technology in therapeutic terms is Sickle Cell Anaemia. In further proof of principle studies, by gene-specific targeting, human sickle haemoglobin allele has been successfully corrected in iPSCs (Hanna, Wernig et al. 2007). Further potential uses of iPSCs in disease modelling, pharmaceuticals and regenerative healthcare in haematology are shown in Table 1.6.

Table 1.6: Potential uses of iPSCs in disease modelling, pharmaceuticals, and regenerative healthcare in haematology

Use of iPSCs in Therapy	Regenerative healthcare
Model of Faconi's Anaemia (FA)	<ul style="list-style-type: none"> • FA is viewed a common BM dysfunction syndrome triggered by disorder of X-related chromosome impacted by mutation and deviation of one gene out of 13 other genes (Raya, Rodríguez-Pizà et al. 2009). • In the future, these updated HSCs can be implemented in terms of SCs surgical transplantation to assist in recovery of FA patients (Li, Cascino et al. 2017).
Thalasseмииas	<ul style="list-style-type: none"> • Refers to a group of diseases characterized by radical production and release of haemoglobin (meaning single-point deviations or nucleotide elimination inside b-golbin gene) or null generation of B-globin (Rieger and Schroeder 2012). • Commonly speaking, thalassemia is regarded as one of the most broadly distributed hereditary illnesses (Marengo-Rowe 2007).
Sickle Cell Disease (SCD)	<ul style="list-style-type: none"> • To secure valid evidence for future researches on targeted genes, a successful manipulation on amplifying the sickle haemoglobin allele inside human iPSCs has been completed (Hanna, Wernig et al. 2007). • Thus, the cellular connections responsible for reproduction of iPSCs have been a source of huge interest among scientists. iPSCs as well as their artificially made populations incorporate morphology patterns like ES cells. The central treatment approach to work with SCD is a regular blood transfusion along with iron chelation to control haemochromatosis effects.
Haemophilia A	<ul style="list-style-type: none"> • Is more widespread compared to congenital dyserythropoietic anemia (CDA). This is a hereditary blood disorder that forms as an X-linked recessive pattern. • The therapeutic approach is based mostly on genetic management, cell coordination, synthetic production of tissues, and sophisticated production of iPSCs. • For instance, a mouse species with newly transplanted iPSCs demonstrated a prolonging (more than 3 months) interval of survival,

	revealing a visible enhancement of novel endothelial progenitor cells within the specific liver parenchyma environment (Mannucci 2020).
Myeloproliferative Neoplasms (MPNs)	<ul style="list-style-type: none"> • Tests on generating iPSC with reference to CD34+ secondary blood cells derived from two patients incorporating JAK2-V617F actualized the availability of myeloproliferative disorder (MPD) of various kinds. The first patient was associated with polycythemia vera (PV); the second one had primary myelofibrosis (PMF) (Mesa, Verstovsek et al. 2007).
Myelodysplastic syndrome (MDS)	<ul style="list-style-type: none"> • Kotini and colleagues successfully derived del(7q) in addition to typical isogenic karyotypically iPSCs from patients diagnosed with MDS and who were showing measurable symptoms of negatively affected haematopoietic specification (Kotini, Chang et al. 2015).

1.7 Drug discovery and the use of model systems to aid drug discovery

Chemical libraries entail the collection of various real-kept chemicals or/and HTS real chemical compounds (R Liu, 2017). The compound library began with compounds produced from combinatorial manufacture. Besides, compound libraries are obtainable from conventional synthesis as well as phytochemicals or e-replicated natural products. The focal point for developing the compound library has moved to chemical relevance, functional relevance, and biological relevance from library size (Wassermann, Camargo and Auld, 2014). The meritorious designs for library kinds bear similarity, and the binary methods - computational (for virtual compound libraries) and experimental (for actual compound libraries). These methods are complementary in the development process and drug discovery. The two kinds of libraries are run simultaneously in drug discovery projects and the outcomes of one relative to the other for finding new promising drug leads. Such libraries include GSK Published Kinase Inhibitor Set (367 inhibitors of 20 chemotypes) and chemical libraries Joint European Compound Library (300,000 compounds) (Dranchak et al., 2013). Drug Discovery Oxford: Davies intended to form a similar firm capable of bestriding the borderline between biology and chemistry. The effect is two components, one offering pharmaceutical services and the other employed on the firm study. The aim was to make the traditional approaches of discovering drugs on their heads. Pharmaceutical firms utilize a 'gene-to-screen' methodology, allowing the use of genomic technologies for identifying biological enzymes or receptors. Enzymes or receptors are utilised as targets where drugs act. VASTox begins with huge digital libraries for available chemical data. The digital libraries are subject to probation for simultaneously identifying possible proteins and receptors, which will respond to drugs and

suitable molecules capable of targeting them. This approach is a more cost-effective approach of finding new drug targets and models, receiving an enormous industrial interest.

Pharmaceutical and chemical companies across the world have chemical compound libraries (Hughes, Rees et al. 2011). Screening chemical compounds to determine potential pharmacological effects plays a vital role in the drug discovery and development process (Hughes, Rees et al. 2011). Along with cell-based therapy and developmental biology, pluripotent stem cells, including iPSCs, have widespread applications in areas associated with drug discovery and development (McNeish 2004).

Drug discovery projects commence with initial research, usually in academia, in which data is generated and a hypothesis developed. The hypothesis states that the activation or inhibition of an identified pathway or protein will lead to a therapeutic effect to address a particular disease state (Hughes, Rees et al. 2011). The outcome from this initiation activity helps in selecting a target which usually needs further validation before progressing into the next phase of where the drug discovery effort is justified in the lead discovery phase. In the next lead discovery phase, an intensive search commences to identify a drug-like biological therapeutic or small molecule, typically referred to as development candidate, that will proceed into preclinical phase, and if approved, into clinical development (Steinmetz and Spack 2009).

Upon successfully passing through all these phases, the drug becomes a marketed medicine which can be prescribed by all authorised healthcare providers (Hughes, Rees et al. 2011). Evidence-based findings consider the early stages of selecting promising drug candidates critical for successful drug. Generally, the process of drug discovery and development has five major stages including target selection, drug discovery, drug development, drug approval and clinical use (Deore, Dhumane et al. 2019) (Figure 1.6).

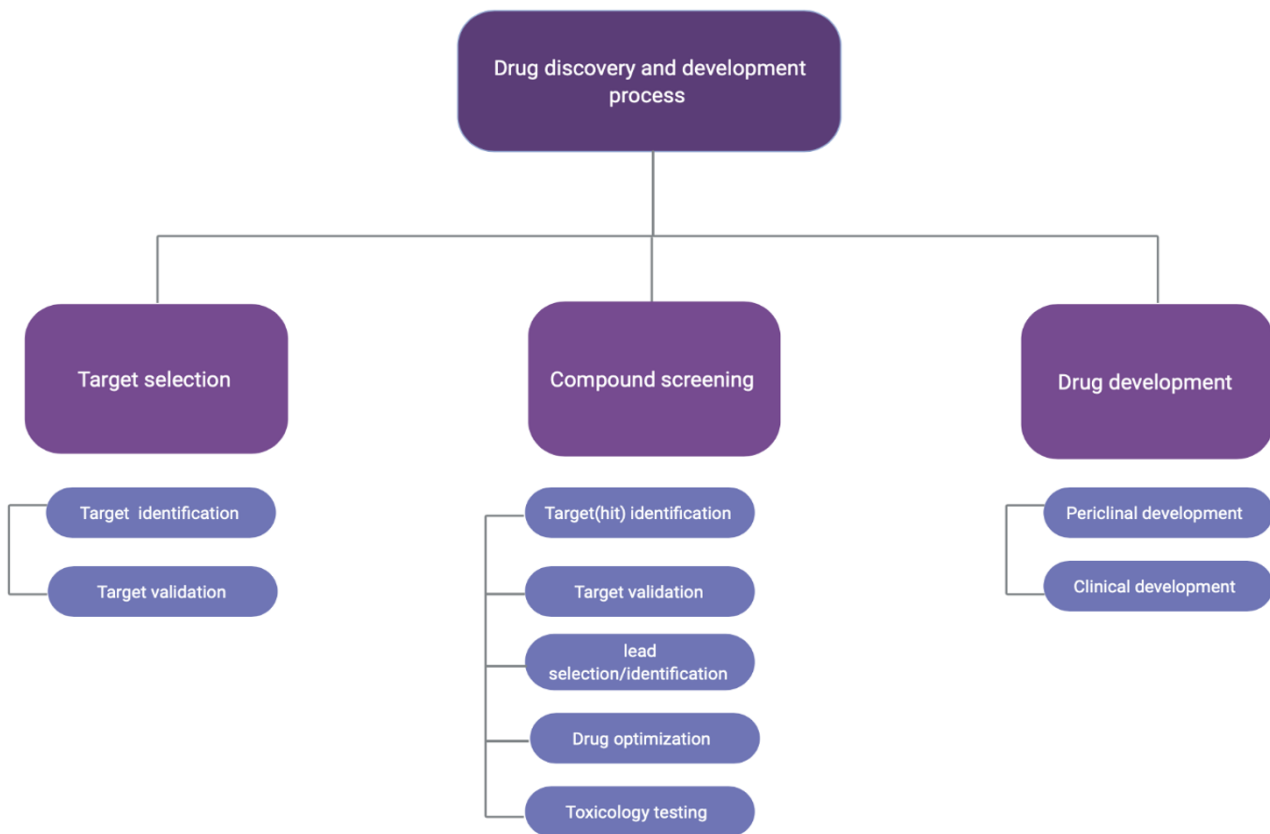


Figure 1.6: Standard workflow within Drug Development. A typical drug discovery and development process consists of three stages: 1-Target selection, 2-Compound screening, and Drug Development Adapt from (Hughes, Rees et al. 2011).

There are multiple of process of drug discovery: First, target selection that practice of picking up a single object from a pool of other numerous objects. With picking up a proposed target of the research, a set of limitations and issues requires assessment. In terms of drug discovery, target selection is an attempt to identify an agent with a special biological structure and mechanism of action that is expected to cause the positive therapeutic effect. Its study and application is considered by checks and balances in scientific, pharmaceutical and strategic disciplines. Second, compound Screening means the detection and selection of specific compounds that can be potentially used for drug manufacturing before moving to the next time-consuming and expensive phases of pre-clinical and clinical development and trials(JG Lombardion, 2004) Screening also aims at identifying the possible adverse effects from using such compound(s). Third, pre-Clinical development which production of drugs traditionally starts with work before clinical trials that involves collections of safety data, in addition to other routine pre-development and preparation steps (R Gaspar,2009). Finally, clinical development that stage of research and evaluation of a drug substance designed to define to what extent the drug will be useful and/or detrimental (toxic) to human recipients (R Gaspar,2009).

The complete process involving drug development must involve all the phases of in the drug discovery and subsequent developments for the drug to be able in the market (Hughes, Rees et al. 2011). Discovery may entail screening of appropriate chemical libraries, selection of active ingredient from a natural design or remedy obtained from an understanding of a given target (Hughes, Rees et al. 2011). Introduction of new and improved medicines to the market enhances health and safety in the society (Taylor 2015). Despite much improving from the novel stages of pharmaceutical sciences, a drug lifespan continues to be lengthy, a challenge compounded by a costly process (Taylor 2015). On average, many drug candidates last over ten years to get through the approval stages (Takebe, Imai et al. 2018). As such, less than 1% of all synthesized drug compounds enter the developmental phases involving trials. For example, less than 1 percent of the over 5000 drugs that were in development were approved by the Food and Drug Administration (FDA) (Mignani, Huber et al. 2016). In many cases, the approval of any new drug takes more than 10 years making the process of development of drug very high risk, costly, and lengthy (Samardzhieva and Khan 2018). A lot of new candidate compounds do not reach the clinical trials and with cost implications: the pivotal clinical trial median from 2015 to 2016 was about \$19 million (Moore, Heyward et al. 2020). For a drug to be approved, it must enter and be successful clinical trials which comprises of three phases. In Phase I trials, most drugs fail to pass the toxicity tests. In Phase II and Phase III trials, efficacy problems cause many drugs to fail, although toxicity issues still significantly contribute

to failure of most of them (Van Norman 2019). Because toxicity and efficacy form the two most common reasons for drug development failure (Van Norman 2019).

Failure of drugs in the clinical trial stages is both very costly on the basis money, labour and time, but it poses great danger to patients involved in the trials. Failures in clinical trials could either be prevented by through various approaches of scientific curiosity, discipline, or rigor, or be unavoidable because of inadequate advances in sciences (Van Norman 2019). Unavoidable drug development failures usually lack well-performing models resulting from an insufficient knowledge base associated with underlying biology and/or chemistry (Mohs and Greig 2017). Preventable drug development failures are explained by a lack of optimal study designs, safety data and dosages (Fogel 2018). Overall, measures to minimize cases of avoidable failures include learning from earlier mistakes, particularly, data collection, sharing, and analysis in both failed and successful drug tests. Moreover, the development of new models based on these data can be helpful in improving the conditions of the existing unavoidable failures (Fogel 2018). Typical drug development involves in *vitro* screening although many chemicals fail clinically, either due to disruption of the target failed yield the expected effect in animals or due to causing unexpected side effects (Hughes, Rees et al. 2011). However, the use of in *vivo* animal models for drug screening at the initial stages could improve drug success rate. Furthermore, information about distribution, absorption, metabolism, toxicity, and excretion (ADMET), pharmacodynamics and pharmacokinetics can be obtained (Hughes, Rees et al. 2011).

Cell replacement therapy can be applied in the treatment of many debilitating diseases (Lovell-Badge 2001). The cells provide a wide range of strategies for development of drugs that are candidates for regenerative medicine, simply put, the therapeutic repair and/or regrowth of damaged cells (Mao and Mooney 2015). The pluripotent stem cell system has a unique strength which is its ability to genetically manipulate them, providing a tractable and high-throughput model to measure the impact of drugs and in drug development (Keller 2005, Lou and Liang 2011). Modern drug discovery often involves the use of other high-throughput organisms. For example, zebrafish models are a relatively high-throughput vertebrate model with relevance to mammalian biology and they are relatively inexpensive experimental tool when compared to larger vertebrate models (Patton, Zon et al. 2021). Optical clarity, visualisation and miniature size make it preferable to other animal models. Using zebrafish as a drug discovery tool, new stem cells modulators have been identified. A case in point is the identification of prostaglandin PGE2 synthesis that increases the formation of HSC (North, Goessling et al. 2007). A group of biologically active compounds was analysed for their impact on stem cell induction in zebrafish AGM region for the purpose of discovering new modulators involved in HSC formation as well as homeostasis. The screening revealed that the PGE2 is

the key effector prostanoid that the zebrafish produces, and it is inhibited affect by Cox1 (Ptgs1) as well as Cox2. Furthermore, the research findings suggested that a stable derivative of PGE2 contributed to the more efficient recovery of the kidney marrow in adult zebrafish in the aftermath of irradiation injury. The inclusion of PGE2 in the treatment of zebrafish embryos helped elevate the expression of important haematopoietic transcription factors *Runx1/c-myb*. The application of the 50 μ M dmPGE2 followed by the progenitor recovery and recovery of the myeloid and lymphoid populations exhibited the strong increase in the kidney marrow repopulation. The dmPGE2 treatment also resulted in the increase in the number of receptors of target cells, including stem, progenitor, and endothelial. The overall survival and the reduced kidney marrow recovery were noted with along inhibiting Cox activity. To explore the function of PGE2 in mammalian HSCs, transplantation experiments were conducted in mice. During the experiment the WBM (CD45.1) exposed to dmPGE2 *ex vivo* was admixed with diverse doses with a predetermined amount of untreated competitor cells (CD45.1/CD45.2) followed by the injection of the mixture into congenic recipient mice (CD45.2), which improved reconstitution capability.

Drosophila (fruit-fly) has conventionally been regarded as another tractable genetic model to understand biology at the organismal level (Pandey and Nichols 2011). *Drosophila* has improved the identification of therapeutics and provided insights into a variety of other illnesses (Lu and Vogel 2009). The studies on *Drosophila* have contributed to the betterment of the therapeutic treatments and empowered clinicians to learn more about diverse diseases (Kasai and Cagan 2010). *Drosophila* may be further useful in the research efforts targeting new drug discovery (Kasai and Cagan 2010). This strategy might help to define compounds with improved whole-animal efficiency and eventually minimize whole-animal toxicity levels. As a result, early attempts to organize whole-animal testing of fruit-flies are regarded as promising preliminary data for further testing in mammals (Pandey and Nichols 2011).

1.8 Aims and Objectives:

- Several screening methods were initially conducted by Dr. Neil Rodrigues in collaboration with VASTox/Summit (Oxford). Compounds considered a positive hit from either of the 2 fruit fly screens were then tested on the embryos of the zebrafish for blood stem cell activity as judged by *Runx1* expression, an essential TF for blood cell development (Kulkeaw and Sugiyama 2012). Zebrafish embryos were dipped in a medium that contained the test small molecule. The appearance of HSC in the zebrafish can be evaluated easily by expression of *Runx1*, at a time-point (22-26 sp) instantaneously after the embryonic blood cell enters circulation (Growney, Shigematsu et al. 2005). Two compounds, Fluvastatin and Fluphenazine, that were identified in our initial screen (Dr. Neil Rodrigues, unpublished observations) have been reported by independent laboratories to be potential inhibitors of leukaemia stem cell function (Sassano, Katsoulidis et al. 2007, Sachlos, Risueño et al. 2012).
- The objective of the Value-added screening technologies Oxford (VASTox) project, a screen containing 12000 compounds was carried out: Therefore, in this thesis will achieve this by following aims:

1- Identify stem cell active compounds which population that account for most of the replenishment of corresponding tissue in both cancer and normal stem cell setting.

2-Determine the capacity of Phthalylsulfathiazole and Yohimbine to induce iPSCs to produce haematopoietic cell subtypes that could eventually be used for therapeutic applications (e.g. mature blood cells for transfusion, blood stem cells and progenitors for transplantation) using flow cytometry and QPCR.

3-To tested three of our compounds including Yohimbine, Oxa-22, and Phthalylsulfathiazole for their effectiveness in targeting AML cell lines *in vitro* as a first pass, surrogate for leukaemia stem cell activity *in vivo* by apoptosis and cell cycle by flow cytometry.

4-To test the *in vivo* impact of Yohimbine, Oxa-22, and Phthalylsulfathiazole on haematopoietic stem cells (HSC), select transplantation experiments were conducted as the gold-standard assay for HSC function using flow cytometry.

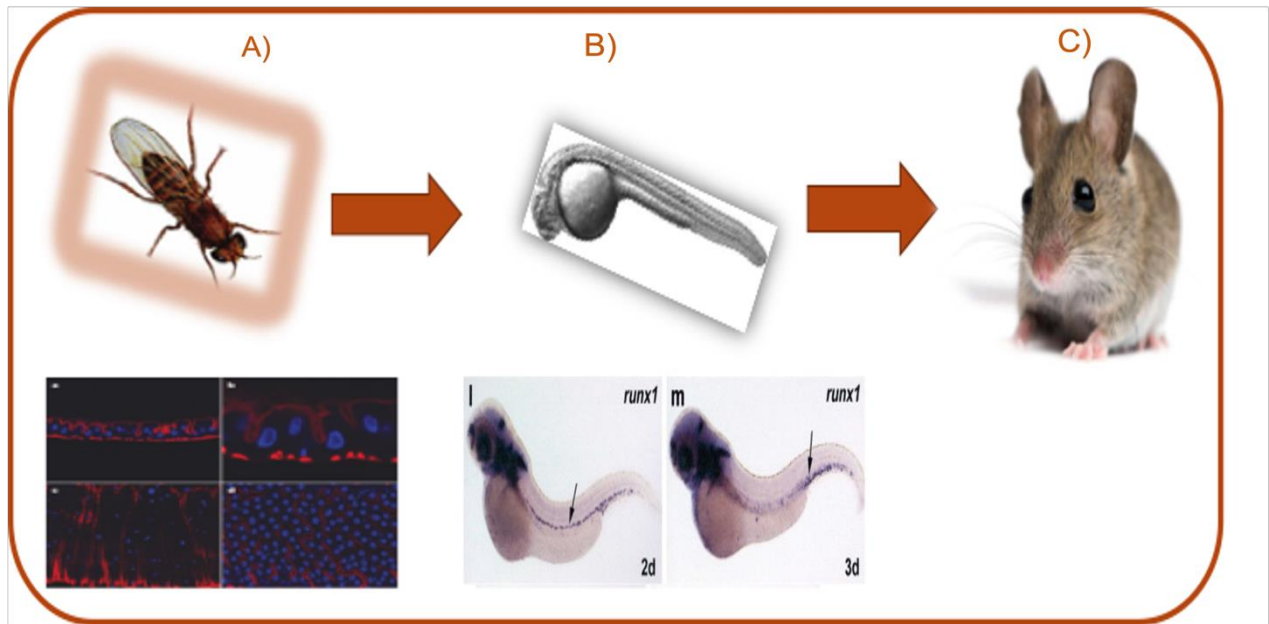
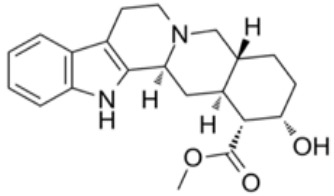
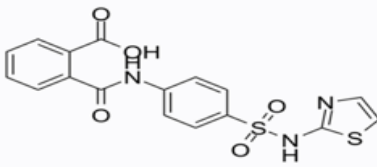
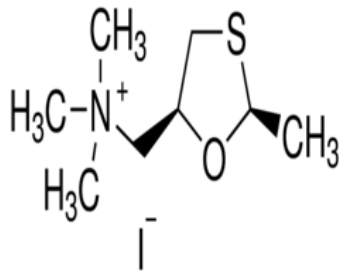


Figure 1.7: Drug screen library to identify stem cell active compounds in normal and cancer cells: A) Fruit-fly brain or asymmetry screen: For the digestive stem cell screen, DAPI staining assists in revealing the cell nuclei. The nuclei size help in distinguishing their source. Observing large nuclei pointed to the presence of a somatic cell and a small nucleus suggested a stem cell from the digestive system or the gut. B) Zebrafish: Emergence of HSCs tested on the embryos of the zebra fish for blood stem cell activity as evaluated by the *Runx1* expression from the ventral wall of the dorsal aorta.

Table 1.7: Prioritization of hit compounds from Fruit-fly and Zebrafish screens.

Screen	Hit (+) or No Hit (-)						
Fruity-fly-Brain /Asymmetry	+	+	+	-	-	+	-
Fruity-fly-Gut	+	-	-	+	-	+	+
Zebra-fish Blood	+	+	-	+	+	-	-
Priority for mouse screen	1	2	3	4	5	6	7

Table 1.8: Summary of small molecules used in thesis.

Small molecules	Yohimbine	Phthalylsulfathiazole	Oxa-22
Biological/clinical Function	<ul style="list-style-type: none"> Utilized for managing erectile dysfunction it may augment long manifestation therapy for post-traumatic stress disorders. 	<ul style="list-style-type: none"> Suppress reproduction of cancer cells Acts through inhibiting folic acid's bacterial biosynthesis that is required for cell development. 	<ul style="list-style-type: none"> Glandular secretions induction and smooth muscle contractions.
Concentrations	100µM, 10µM, 1µM, 0.1µM	10µM, 1µM, 0.1µM, 0.01µM,	100µM, 10µM, 1µM, 0.1µM
Vendor Empirical Formula Molecular Weight	Sigma-Aldrich C ₂₁ H ₂₆ N ₂ O ₃ HCL 390.91	Sigma-Aldrich C ₁₇ H ₁₃ N ₃ O ₅ S ₂ 403.43	Sigma-Aldrich C ₈ H ₁₈ INOS 303.20
Chemical structure			

CHAPTER 2:
Material and Methods

2.1 Cell Culture

2.1.1 Maintenance of mouse iPSCs

Mouse iPSCs were generated by Dr. Gui Jie Feng in the laboratory of Dr. Neil Rodrigues as described by (Sommer, C. A., et al. (2009)). Mouse iPSCs were cultured in gelatine coated plates with ES-2i medium consisting of DMEM knockout medium (Invitrogen, UK) supplemented with 10% Foetal Bovine Serum (FBS), 2mM Glutamine, 100U/ml pen/ strep, 1x non-essential amino acid, 1mM sodium pyruvate, 55mM of 0.1 mM β -mercaptoethanol, 1:100 Leukaemia inhibitor factor (LIF) conditioned medium, 0.5 μ M PD0325901 and 3 μ M CHIP99021. 6 well plates were coated with 0.1% of gelatine in PBS for 30 min at 37°C prior to use. iPSCs were passaged every 2-3 days or when the cells reached 70% confluence. Cells were incubated in a 37°C incubator with 5% CO₂. Medium was changed daily.

2.1.2 Proliferation assay using Cell Titre-Blue® cell viability assay

Assessment of cell proliferation was carried out using cell Titre-Blue ® reagent (Promega). Undifferentiated iPSCs were placed on a gelatine coated 96 plate one day before the addition of drugs (Yohimbine, Phthalylsulfathiazole and Oxa-22) at the concentration between 1nM to 10mM in 100ul/well and incubated at 37°C in 5% CO₂. After two days, the proliferation assay was performed by adding 20 μ l/well of Cell Titre-Blue single reagent incubating at 37°C between 1-4 hours. Fluorescence generated by the samples was measured using a fluorescent plate reader (Clario Star) at the wavelength of Excitation 560 nm /Emission 590 nm.

2.1.3 Hanging drop culture to generate embryoid bodies

iPSCs were cultured to approximately 70% confluency before being trypsinized and harvested by centrifugation. Cells were then resuspended in Hanging drop (HD) medium (ES medium without LIF and 2i) at a density of 2.4×10^5 /ml. Hanging drops were produced by blotting 25 μ l/drop of cell suspension onto the inside lid of a 10cm petri dish (40-50 drops/plate). The lid was inverted and put on top of a dish in which 5-10ml dH₂O were added to maintain the humidity. The plates were then placed in a 37 °C incubator with 5% CO₂. Two days later, embryoid bodies (EBs) were harvested and transferred into a Petri dish with 10ml of fresh HD medium, and fresh HD medium were replaced every two days until the EBs were harvested. EBs were harvested by centrifugation at 300xg for 5min at room temperature (RT), after washing the cell pellets with PBS. Cell pellets were frozen at -80°C before RNA extraction were conducted.

2.1.4 Differentiation of iPSCs toward haematopoietic cells

iPSCs were maintained in a feeder-free system using ES medium (knockout DMEM supplemented with 10% FBS, 2mM glutamine, 1mM sodium pyruvate, 100µM non-essential amino acids, 0.1mM β-mercaptoethanol and LIF condition medium) in the presence of MAPK inhibitor PD0325901 and GSK3b inhibitor CHIR99021. After trypsinization, the single cells were resuspended in HD medium (ES medium except that PD, CHIR and LIF were removed). These single cell suspensions (2.4×10^5 /ml) were then used to generate hang drops (25ul/drop) on the lid of the Petri dishes with different drugs and incubated upside down for 2 days in a 37°C incubator. During this time EBs were gradually formed in each drop and these EBs were then transferred into the liquid culture in the Petri dishes containing HD medium supplemented with a cocktail of cytokines (3U/ml) Erythropoietin (rhEpo), 30ng/ml of *IL-3*, 30ng/ml of *IL-6*, 150ng/ml of *SCF*) for an additional 4 and 8 days in the presence/absence of drugs. On day 6 or day 12, the EBs were harvested by centrifugation and single cell suspension were obtained for fluorescence labelling of the cell surface markers with corresponding antibodies, these samples were then subjected to flow cytometry analysis for detecting possible myeloid and lymphoid cell populations using BD LRS Fortesa™. The medium was changed weekly and was supplemented with fresh small molecules and cytokines. All cytokines were purchased from PeproTech.

2.1.5 Maintenance of Human AML cell lines

THP1 and NOMO1 cells were grown in Roswell Park Memorial Institute medium (RPMI)1640 (Gibco,UK) containing 10% heat inactivated FBS and 2 mM L-glutamine with different small molecules (Yohimbine, Phthalylsulfathiazole or Oxa-22). The cells were cultured at 37°C in a humidified 5% CO₂ incubator and were passaged every 2-3 days. Cells were harvested at 72 hours after treatment with drugs and non-viable cells excluded from the analysis. THP1 is a non-adherent human monocytic leukaemia cell line (Bosshart and Heinzlmann 2016). NOMO1 is derived from the bone marrow of 31 -year-old woman with acute myeloid leukaemia (AML FAB M5a) (Drexler, Quentmeier et al. 2004).

2.2 Colony forming cell assay (CFU)

Using ES-Cult™M3120 (Stem Cell Technologies) Methylcellulose Medium the colony formation cell assay was performed to determine haematopoietic progenitor function *in vitro*. EBs cells were harvested and 2.4×10^5 plated in ES-Cult™M3120. There were two steps to generate haematopoietic progenitors:

A. Primary plating

EBs from HD cultures were added to primary media which contained approximately 1% of MethoCult (ES-Cult™M3120) (Stem Cell Technologies) with the following recombinant cytokines: 30 ng/ml of *mIL-3*, 20 ng/ml of *hIL-6*, 160 ng/ml of *mSCF*, 3u/ml of *rhEpo*, 2mM L-Glutamine, 1:100 penicillin streptomycin amphotericin B, 15 % of FBS, and different small molecules and IMDM (Sigma, UK, catalog 36150).

B. CFC plating

EBs were harvested from primary plating and then disaggregated into single cells using a 1 mL syringe and 19G blunt end needle. Single cells were transferred to methylcellulose medium supplemented with approximately 1% of MethoCult (ES-Cult™M3120) (Stem Cell Technologies) with recombinant cytokines (30 ng/ml of *mIL-3*, 20 ng /ml of *hIL6*, 160 ng/ml of *mSCF*, and 3u/ml of *rhEpo*), 2mM L-Glutamine, 1:100 penicillin streptomycin amphotericin B, 15 % of FBS, BIT9500 serum substitute which includes [1% BSA, 10 ug/ml insulin, 200ug/ml transferrin and IMDM]. The plate was incubated at 37°C with 5% CO₂. The plate was gently tilted to allow to distribute evenly. After 14 days, colonies in each dish were counted under a light microscope and were identified as colonies of CFU-G (granulocytic) CFU-GM (granulocyte/monocyte) CFU-M (macrophage), CFU-E (erythroid).

2.3 Flow cytometry Analysis

Flow cytometry were gated according to forward and side scatter characteristics to exclude cell debris. A minimum of 1,000 events in the population of interest was collected for each sample analysis. A total of 1000 ungated events were acquired for each sample. Cells at specific time points were stained with antibodies as listed in Table 2.2 and 2.3 were analysed using a multiple laser BD LSR Fortessa™ (BD Biosciences, UK). Flow cytometer was used to detected and measure characteristics of population.

2.3.1 Pluripotent Stem Cell Marker analysis

iPSCs were cultured on gelatin coated in a T25 flask. Then, cells were harvested by Trypsin/EDTA (0.25%) and washed with PBS and pelleted in 2 ml-Eppendorf tubes to contain 500,000 cells. Cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT) followed by incubation in permeabilization buffer (0.1% TritonX100/2%BSA (ThermoFisher)/PBS) for 15 minutes at room temperature (RT) before being washed with wash buffer (0.1% Triton X-(Sigma, UK)100 in PBS). Cells were incubated with primary antibody Oct3/4 (purified mouse anti Oct3-4), SSEA-1 (purified mouse anti -SSEA-1) and Nanog (Alex Flour @488 mouse anti-mouse Nanog) for 30 minutes at RT before washing with wash buffer. Cells were incubated with a secondary antibody that was conjugated with FITC-fluorescent for 30 minutes at RT in the dark. Cells were then washed twice with Flow cytometry buffer (2%BSA in PBS) and re-suspended in 500µl Flow cytometry buffer followed by acquisition on a BD Fortessa.

2.3.2 Apoptosis Analysis

After staining with cell surface markers, cells were incubated with Annexin V in binding buffer for 30 minutes in the dark. The apoptotic cell populations were analysed by flow cytometry after addition of DAPI. For human cell lines, cells were treated with the three small molecules (Yohimbine, Phthalylsulfathiazole and Oxa-22) and apoptosis was measured by staining cells with Annexin V-PE (Biolegend, UK).

2.3.3 Cell Cycle Analysis

To investigate the cell cycle status of bone marrow cells, *Ki67* a cell proliferation marker, was used for intracellular staining. LSK cells from bone marrow were fixed in 0.2% PFA before the cell membrane was permeabilised using 0.1%TritonX100. The samples were incubated with APC-conjugated antibody to *Ki67* for 30 minutes in the dark. DAPI was added just before the analysis by flow cytometry. For leukaemia human cell lines, stained cells were fixed by 4% of PFA and incubate for 10 min at RT. Then, following permeabilization, cells were fixed with 0.2% PFA and permeabilized by 0.1% Triton, stained with *Ki67* antibody and incubated at room temperature for at least one hour or overnight in the dark.

2.3.4 Analysis of haematopoietic cells from iPSCs

On day 6 and day 12 of liquid culture, EBs were harvested by centrifugation and single cell suspension were obtained for fluorescence labelling of the cell surface marker with corresponding antibodies, these samples were then subjected to flow cytometry analysis for detecting possible myeloid and lymphoid cell populations. After staining, cells were washed with wash buffer (PBS in 0.1% FBS) prior analysis. Then, cells were washed twice and re-suspended in 200 ml in Flow cytometry buffer (PBS 2%FBS) and stored in dark on the ice prior to acquisition. Cells at specific time points were stained with antibodies as listed in Table 2.1. Subsequent data analysis was performed using Flow Jo, LLC (10.2). Human AML cell lines were harvested at 72 hours after treatment with drugs and non-viable cells excluded from the analysis. Data acquisition and manual colour compensations for the fluorescent panels were performed using the Flow cytometry Diva Software.

2.3.5 Analysis of transplantation

For HSPCs, bone marrow cells labelled with a cocktail of biotinylated antibodies to lineage markers (CD3, CD4, CD8, B220, GR-1, MAC-1 and Ter119) followed by PB-streptavidin (SA-PB) to allow for the exclusion mature haematopoietic cells such as lymphocytes, monocyte, and erythroid cells. For myeloid progenitor, bone marrow cells were labelled with the antibody for lineage markers as above. They were also stained with sca-1/PE, c-kit-APC, then, CD16-32/PE-CY7 and CD34-FITC. LK cells were gated for lineages negative, Sca-1 positive. Within the LK population, it was further divided into 3 sub-populations: the granulocyte-macrophage progenitor (GMP) is CD16/32+/CD34+, the common myeloid progenitor (CMP) is CD 16-32lo/CD34+ and megakaryocyte-erythroid (MEP) is CD16-32lo/CD34lo. Peripheral blood samples containing mature cells were stained with fluorescence labelled antibodies to granulocytes (Gr-1/PE-CY7), monocytes (Mac-1/PB), erythrocytes (TER119/APC-CY7), T-cells (CD4-8/PE), and B-cells (B220/FITC). 10µl of blood was collected and lysed using NH₄Cl.

2.4 Transplantation

All mice used in this study were C57/BL6 mice, starting from age 8 to 12-week-old. The animal experiments were performed following UK Home Office regulations. Cells were stained at a concentration of $1-5 \times 10^5$. There are two types of transplantation:

Post-transplant administration of drug: Whole bone marrow cells (5×10^5) from CD45.2 (donor) were transplanted with supporting bone marrow cells into irradiated recipients (CD45.1) via

tail vein injection. After the injections were completed, the recipient mice were treated twice daily with Yohimbine at different concentrations (1mg/kg, 10mg/kg, 100mg/kg, PBS) for 10 days. Bone marrow cells were harvested from the recipient mice at week 16. Briefly, both femurs and tibias were collected from each mouse, then crushed by pestle and mortar to release bone marrow cells. After washing with PBS containing 2% FBS. Cell numbers were estimated by trypan blue method under the microscope. For the peripheral blood samples, red blood cells were lysed using ammonium chloride (NH_4Cl) by incubating for 5 minutes. Whole bone marrow cells or peripheral blood cells were then stained with fluorochrome-conjugated antibodies dependent on the individual experimental designs following analysis by flow cytometry analysis as described above in section 2.3.5.

Competitive transplant: Donor mice underwent pulsing with drug as mentioned above. Recipient mice received two doses of total body irradiation (TBI) at 400cGy separated by 4-6hours. 24 hours later, recipients were transplanted with a total of 7.5×10^5 cells composed of donor cells from drug treated mice as well as competitive cells (of the same recipient strain). The ratios of donor: competitor cells were dependent on the changes in cellularity in response to the compound.

2.5 Gene expression analysis

2.5.2 Extraction of RNA

RNA extraction was carried out by using 500 μl of Trizol (Ambion, UK) to obtain the cell lysis in accordance with the manufacturer's instructions. Briefly, it was necessary to put cell lysate into Special Phase Lock Gel Heavy Phasing (PLGHP) which has three layers: the RNA-containing aqueous phase, the Phase lock Gel Heavy phase, phenol chloroform phase. Then cell lysate was incubated for 5 minutes at 15-30 $^{\circ}\text{C}$. For 15 seconds, 100 μl of chloroform was then added. Later, cell lysate was centrifuged at speed 12,000x g for 10 minutes at temperature 2-8 $^{\circ}\text{C}$. Furthermore, 250 μl of isopropyl alcohol and the sample incubated for 10 minutes at between 15 and 30 $^{\circ}\text{C}$. After that, the sample was cleaned using 1000 μl of ethanol (75%), with further centrifuging at 7,500 x g for 5 minutes at temperature 2-8 $^{\circ}\text{C}$. Then, the RNA pellet was dissolved in 30 μl RNeasy sterilized water, with further incubation for 10 minutes at temperature interval 55-60 $^{\circ}\text{C}$. RNA content and quality were measured and estimated utilizing a Nano Drop 2000 spectrophotometer (Thermo-scientific) and was later kept at sub-temperature -80 $^{\circ}\text{C}$.

2.5.3 Conversion of RNA to cDNA:

First stand cDNA was synthesized using QuantiTect ®reverse transcription kit (QIAGEN) in a 100 °C Thermal cycle. Briefly, 1ug RNA with RNase-free H₂O was mixed with a mastermix of 1µL reverse-transcription master mix-4ul Quantscript RT buffer (5x) RT primer Mix-2ul of genomic DNA elimination reaction. Samples were incubated at 42°C for 15 minute and 95 °C at 3 minutes to amplify cDNA using PCR. Reactions were carried out in a total volume of 20 ul. cDNA was stored at -20°C.

2.5.4 Q-PCR:

Relative gene expression was analysed by conducting real time-time quantitative polymerase chain reaction (qPCR). Reactions were carried out in a total volume of 20 ul. Q-PCR was performed with SYBR green (2X Qpcr Bio Sybrgreen MIX LO-ROX) based on manufacturers guideline: cDNA-primer, SYBER green, and RNeasy free water. Analysis was performed using Quantstudio 7 Flex Real-Time system (Applied Biosystem, UK) at 95 °C for 5 seconds, then 60 °C for 30 seconds, for 40 cycles. The threshold cycle (CT) Values were measured in triplicate and expression level were calculated against an internal control gene Hypoxanthine phosphoribosyl transferase (HPRT). The sequences of primers used for qPCR listed in the Table 2.2.

2.6 Statistical analysis:

Results obtained from multiple experiments are expressed as the mean ± one-way ANOVA-Ordinary-one data sets. The data were analysed using standard error of mean (SEM).

Table 2.1. List of antibodies used in thesis.

Antigen	Fluorochrome	Dilution	Species specificity	Clone
Pluripotency Antibodies				
OCT3-4	Primary antibody: Purified mouse anti oct3-4 /Sox2/SSE-1 Secondary antibody: FITC Alex Flour ®488 mouse anti-mouse nango	1:200	Mouse	MiPS(C8)
Sox2				
SSEA-1				
Nanog				
Differentiation Antibodies				
CD41	FITC	1:200	Mouse	MiPS(C8)
CD31	PE-CY7			
CD16-32	PE			
CD45	PE			
CD71	PE			
Ter119	APC-CY7			
cKIT	APC			
CD14	PE	Human	Thp1/Nomo1	
CD11B	APC	1:200	Human	Thp1/Nomo1
Haematopoietic stem cells				
Lin-	Pacific Blue (PB)	1:500	Mouse	Bone marrow
Sca-1	PE			
C-kit	APC			
CD150	PE-CY7			

CD48	FITC			
Progenitor cells (HSPC)				
Lin-	Pacific Blue (PB)	1:500	Mouse	Bone marrow
Sca-1	PE			
C-kit	APC			
CD16-32	PE-CY7			
CD34	FITC			
Lineage marker				
Ter119	APC-CY7	1:500	Mouse	Peripheral blood
GR-1	FITC			
MAC-1	Pacific Blue (PB)			
B-220	APC			
CD4-CD8	PE			

Table 2.2. Primer pairs used for gene analysis of iPSCs clones using quantitative PCR

Primer name	DNA sequence 5' to 3'	PCR size (bp)
Endoderm Markers		
<i>Gata1</i> F2	CTCTCATCCGGCCCAAGAAG	165
<i>Gata1</i> -R2	G/TGGGCGGTTACCTGATG	
<i>GATA2</i> F3	CTCATCAAGCCCAAGCGGA	165
<i>GATA2</i> R3	CAGTGGCCTGTTAACATTGTGC	
<i>GATA3</i> Fw1	AAGCTCAGTATCCGCTGACG	114
<i>GATA3</i> Re1	GATACCTCTGCACCGTAGCC	
<i>Sox7</i> Fw1	TCAGGGGACAAGAGTTCGGA	148
<i>Sox7</i> Re1	CCTTCCATGACTTTCCAGCA	
<i>Cdh5</i> Fw1	GCCTGGTCACTCACTCTTCC	107
<i>Cdh5</i> Re1	CTCTGTGGTGCAATTACGGT	
<i>PU.1</i> Fw1	AAGCAGGGGATCTGACCAAC	94
<i>PU.1</i> Re1	AAGTCATCCGATGGAGGGG	
<i>Itgam</i> Fw1	TGGCCTATAACAAGCTTGGCTTT	93
<i>Itgam</i> Re1	AAAGGCCGTTACTGAGGTGG	
Mesodermal markers		
<i>Bra</i> Fw1	GGCTGGGAGCTCAGTTCTTT	179
<i>Bra</i> Re1	TGTCCACGAGGCTATGAGGA	
<i>Runx1</i> F2	CCTTCAGGAGAGGTGCGTTT	104
<i>Runx1</i> R2	CTCGTGCTGGCATCTCTCAT	
<i>Ki67</i> Fw1	CCATCATTGACCGCTCCTTTAG	154
<i>Ki67</i> Re1	GTATCTTGACCTTCCCCATCAG	
<i>FLK</i> Fw1	GATGTGTGGTCTTTCGGTGTG	

<i>FLK</i> Re1	CATGCCAGCAGTCCAGCATG	178
<i>Cebpa</i> Fw1	AGACCGAGAGACTTTTCCGC	123
<i>Cebpa</i> Re1	TTTTTGCTCCCCCTACTCGG	
<i>Lmo2</i> F2	GTGTGGTCTTCACTCTTAG	187
<i>Lmo2</i> R2	GTTACTATGGCTAGCCTGTG	
<i>SCL</i> Fw4	G TTCACCAACAACAACCGGG	122
<i>SCL</i> Re4	TGAGGACCTGAGGGTAGAAGG	
Ectodermal marker		
<i>Pax6</i> Fw1	CACCTGGAGTGTCAGTTCCC	105
<i>Pax6</i> Re1	CTCGATCACACGCTCTCTCC	
Pluripotent Gene		
OCT4-F2	TG TTCAGCCAGACCACCATC	100
OCT4-R2	GCTTCCTCCACCCACTTCTC	
Sox2-F2	CAGCTCGCAGACCTACATGA	110
Sox2-R2	CCTCGGACTTGACCACAGAG	
Housekeeping Gene		
HPRT Fw1	CAGTCCCAGCGTCGTGATTA	168
HPRT Re1	TGGCCTCCCATCTCCTTCAT	

CHAPTER 3:

**Exploring the impact of Phthalylsulfathiazole on
haematopoietic differentiation from induced pluripotent
stem cells and in haematopoietic stem cells**

3.1 Introduction

The sulphonamide group is the key foundation for diverse bio-active compounds used in the drug manufacturing industry (Yadav, Manoli et al. 2016). Sulphonamide group derived drugs have typical biological properties such as being anti-bacterial, anti-thyroid, anti-tumor, and anti-neuropathic pain (Supuran 2017). Sulphonamide based drugs are quickly absorbed from the gastrointestinal tract, which makes them effective (Levison and Levison 2009). Gerhard Domagk discovered the anti-bacterial characteristics of sulphonamides in 1939 (van Miert 1994). Sulphonamides tend to suppress enzymes that take part in the process of folic acid production, which is relevant in synthesis of purine, thymidine, methionine, glycine as well as formyl methionyl-transfer RNA (Zheng and Cantley 2019). As a result, sulphonamides suppress protein production, proliferation, and metabolic activity (McDonald, Winum et al. 2012). Although the anti-tumour value of sulphonamides has been poorly characterised, they represent an important class of drug for fighting cancer (Scozzafava, Owa et al. 2003). Sulphonamides tend to suppress cell reproduction during mitosis by disturbing spindle microtubules assembly dynamics (Wan, Fang et al. 2021). Such perturbations might potentially destroy cancer cell structures in terms of apoptosis using the bcl-2-dependent channel (Campbell and Tait 2018).

Chloroquinoxaline sulphonamide (CQS) represents a sulphonamide anti-tumour agent that has passed several clinical tests after showing promising effects in Human Tumour Colony Forming Assay. CQS suppresses colony formation, affecting the growth rates of melanoma as well as ovarian carcinomas (Fisherman, Osborn et al. 1993).

Several sulphonamides have become an anticancer agent by engaging in mechanisms of impacting carbonic anhydrase (CA) (Singh, Lomelino et al. 2018). CA is a group of enzymes that work as catalysts in reinforcing molecular reactions between CO₂ and H₂O and they are engaged to generate bicarbonate and hydrogen ions within the pH environment (Mboge, Mahon et al. 2018). Bicarbonate is critically important for cells, as this substrate and anion is applied in metabolic and physiological processes such as the biosynthesis of arginine, uracil, or fatty acids. In the case of tumour development, two CA isomers (namely CA IX and CA XII) play a substantial role, as cancer cells tend to reproduce quickly in contrast to healthy cells, and thus need increased amounts of bicarbonate for cell cycling (Mboge, McKenna et al. 2015). In the intestine, H⁺ ions are generated and transported to the cells of intestine, leading

to a reduced pH level in the intestinal environment, causing a hostile setting where normal cell development and growth is heavily restrained (Aoi and Marunaka 2014).

Acyl sulphonamides, such as LY573636-sodium, has also been categorized as a beneficial drug in fighting tumour, inhibiting growth of malignant cells and initiating apoptosis in relation to many severe tumours, including those of haematopoietic origin such as AML, B-ALL, large B-cell and mantle cell lymphoma (Haritunians, Gueller et al. 2008). This anti-reproductive process in cancer cells which is mediated by LY573636-sodium has been associated with apoptosis, deprivation of mitochondrial membrane, and stimulation of ROS and re-activation of normal differentiation potential (Haritunians, Gueller et al. 2008). This specific impact makes LY573636 a viable candidate for mixed strategies of treatment for patients with excessive or drug-resistant forms of cancer (Haritunians, Gueller et al. 2008).

The bacterial microbiome located in the intestines is highly relevant in managing and coordinating haematopoiesis (Yan, Baldrige et al. 2018). There is a link between negative haematologic effects and instability of the microbiome environment in intestines (simply titled as dysbiosis). Microbiome deregulation refers to several micro-organisms, including viruses, fungi, archaea, and bacteria, which colonize the human body and form the ecological system necessary for human health. Besides, antibiotics have depleting effects on intestinal bacteria microbiome as well as a suppressive impact on haematopoiesis. In an experimental setting germ-free (GF) mouse do not have microbiota and they tend to have a significantly smaller haematopoietic stem/progenitor cell (HSPC) population (Yan, Baldrige et al. 2018). In addition, GF mice have the impaired T-cell functions and abnormal splenic myeloid counts. In a comparable way, oral antibiotics eliminate the intestinal bacteria and may suppress haematopoiesis. Studies have shown that adult specific-pathogen-free (SPF) mice that were treated with oral antibiotics over the course of 1 week or longer developed BM suppression. These results indicate that antibiotics could affect the engraftment of HSPCs in the aftermath of transplantation, implying that microbiome is crucial in posttransplant setting (Yan, Baldrige et al. 2018). Investigations conducted on murine models confirmed that antibiotic-based depletion of microbiota as well as BM suppression are caused by the lack of microbial products stable to heat in bloodstream that could trigger haematopoiesis due to basal inflammatory signalling (Yan, Baldrige et al. 2018). Such processes can be common in many patients, especially those in need of prolonged antibiotic-based therapy like those patients receiving treatment from HSCT (Yan, Baldrige et al. 2018).

In haematopoiesis specifically, as sulphonamides act as inhibitors of folic acid synthesis, it will be important to ascertain their impact on other haematopoietic lineages, haematopoietic differentiation and long-term HSC functioning (Fernández-Villa, Aguilar et al. 2019). For

example, folic acid participates in the regulation of erythropoiesis. (Koury and Ponka 2004). Folic acid shortage in this process leads to an onset of megaloblastic anaemia (Koury and Ponka 2004). Megaloblastic anaemia reduces the number of red blood cells associated with collapse of DNA synthesis and a block in erythrocytic specification and apoptosis (Shahzad, Qadir et al. 2020). Thus, folic acid deficiency results in anaemia caused by perturbed erythropoiesis. Studies revealed that patients affected by folic acid shortage and diagnosed with anaemia have erythroblasts which accumulate in the S-stage, related to an imbalance and impairment of cell cycling (Shahzad, Qadir et al. 2020). In patients suffering from anaemia, similar negative patterns can also be observed in BM cells and megaloblasts (Yadav, Manoli et al. 2016).

3.2 Aims and objectives

The aims of this chapter seeks to explore the effects of Phthalylsulfathiazole on haematopoiesis derived from induced pluripotent stem cell and haematopoietic stem cells by:

1- Identify the ability of Phthalylsulfathiazole, along with a combination of cytokines, to augment differentiation of haematopoietic cells in iPSCs. The mouse iPSC culture system was firstly established and characterized, and haematopoietic differentiation culture conditions was established prior to testing the impact of Phthalylsulfathiazole.

2- Determine the ability of Phthalylsulfathiazole, along with a combination of cytokines, to augment differentiation of haematopoietic cells in iPSCs.

3- Investigate the haematopoietic function, we performed transplantation experiments on HSCs that were exposed to Phthalylsulfathiazole *in vivo* by apoptosis and cell cycle in flow cytometry.

3.3 Results

3.3.1 Morphology of undifferentiated iPSCs cultured in feeder free culture condition in mice.

Firstly, iPSCs were established in the laboratory. TTFs (tail-tip fibroblasts) were isolated from B6 mice and transduced with lentivirus containing STEMCCA as described in (Sommer et al., 2009). Reprogramming transcription factors Oct4, Sox2, c-Myc, and Klf4 are encoded in this lentiviral construct. This led to the generation of various clones of iPSCs that were developed in the laboratory by Dr. Gui Jie Feng. iPSCs clones were maintained in the undifferentiated state using ES cell culture medium (knockout DMEM supplemented with 10% FBS, 2mM glutamine, 1mM sodium pyruvate, 100 μ M non-essential amino acids, 0.1mM β -mercaptoethanol and LIF condition medium) in the presence of MAPK inhibitor PD0325901 and GSK3b inhibitor CHIR99021, termed 2i medium (Qi-Long Ying, 2008) (Figure 3.1).

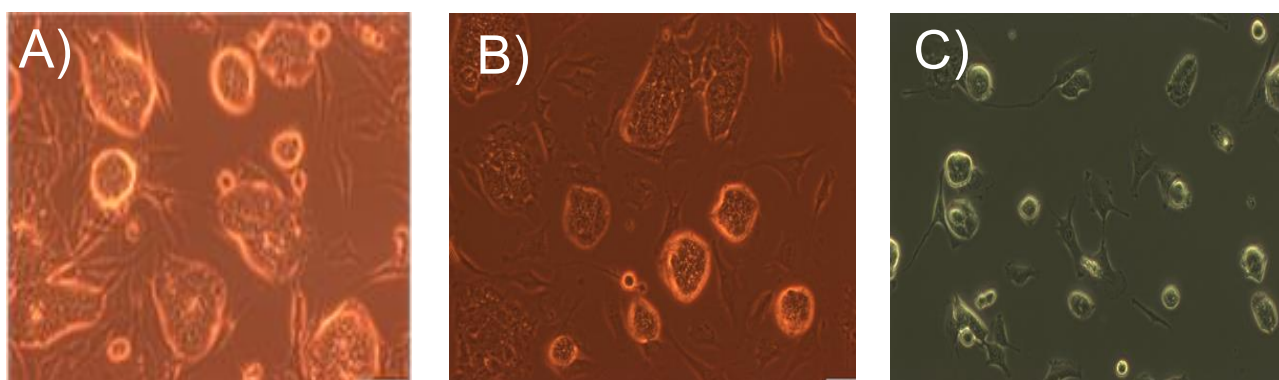


Figure 3.1: Morphology of healthy mouse induced pluripotent stem cells (iPSCs). Clone 19 was maintained in cell culture by feeder free system in the presence of 2i (iPS19) and was used for all experiments in the thesis. iPS19, passage 19; B) iPS19, passage 12; C) iPS19, passage 17. Different magnification on a microscope include (4X,10X).

3.3.2 Flow cytometry evaluation of iPSCs to identify markers of stem cell pluripotency

To confirm iPSCs express genes for pluripotent stem cells in the generated iPSC lines, flow cytometry analysis was carried out to identify the common stem cell markers such as Oct3/4, SSEA1 and Nanog (Zhao, Ji et al. 2012). Undifferentiated iPSCs were harvested and labelled with antibodies directed against Oct3-4, SSEA-1, Nanog and analysed by flow cytometry. Flow cytometry analysis showed abundant expression of Oct3/4 (83.5%), nearly half population of iPSCs showed positive for SSEA-1 (51.9%) and relative low percentage of iPSCs showed positive for Nanog (17.6%) in undifferentiated iPSCs (Figure 3.2). These data demonstrate the pluripotent nature of iPSCs lines generated.

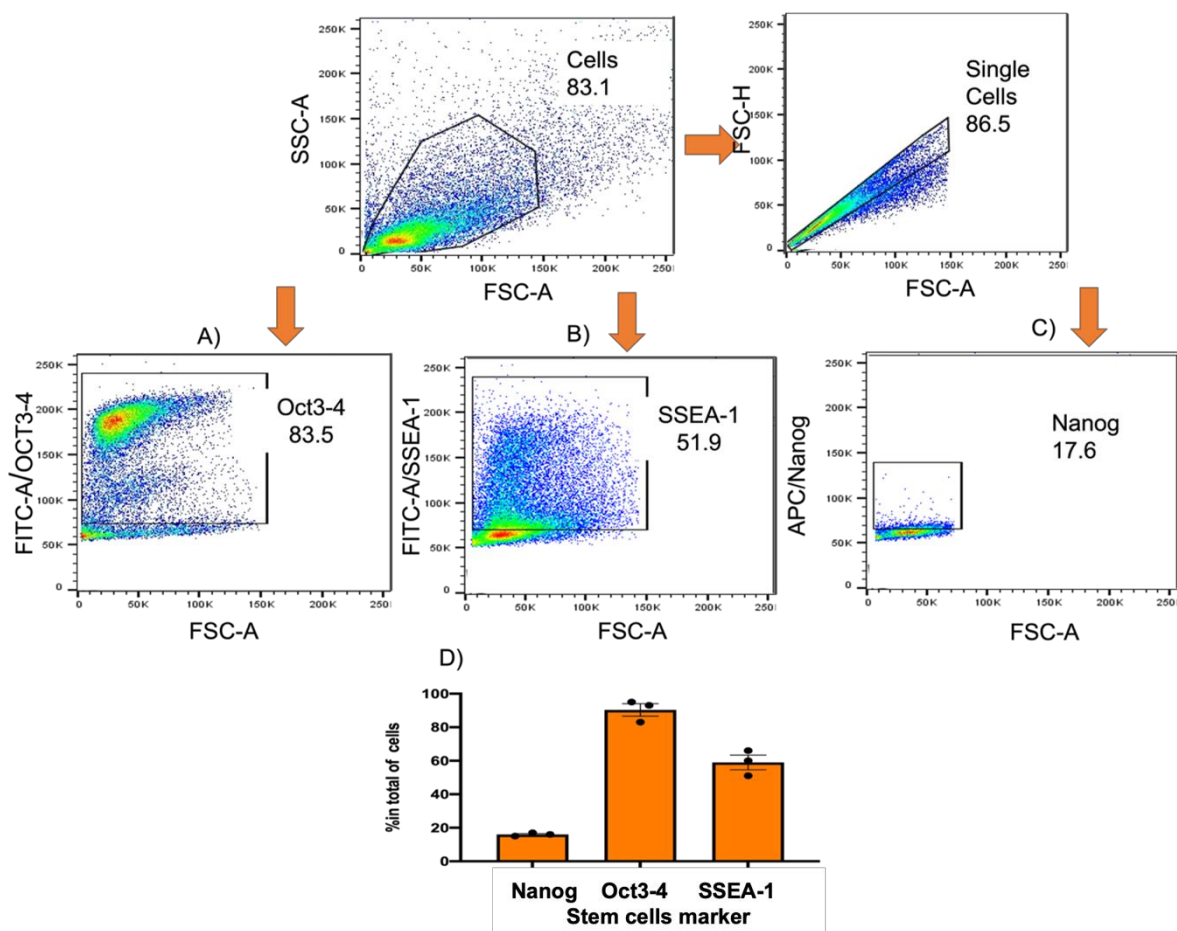


Figure 3.2: Flow cytometric analysis of stem cell markers in undifferentiated iPSCs.

iPSCs were stained using fluorochrome-conjugated antibodies directed against Nanog, Oct3-4 and SSEA-1. A) Oct3/4 using Purified mouse anti-Oct3-4 primary antibody and secondary antibody (Goat Anti mouse Ig FITC) B) SSEA-1 using primary antibody (Purified mouse anti-SSEA-1) and secondary antibody (Goat Anti mouse Ig FITC). C) Nanog using Alex Flour® 488 mouse anti-mouse Nanog antibody. D) Graph showed the stem cells marker. The data were analysed using standard error of mean (SEM).

3.3.3 iPSCs formed EBs in HD culture condition

iPSCs can form EBs and spontaneously differentiate to all the germ layers (mesoderm, endoderm and ectoderm) observed in an embryo (Rungarunlert, Techakumphu et al. 2009). Hanging drop (HD) culture is one method to generate EBs (Rungarunlert, Techakumphu et al. 2009). To test the ability of the generated iPSCs to induce EB formation iPSCs, undifferentiated iPSCs were resuspended in HD medium (ES medium without LIF and 2i) and cultured in a format of droplets (HDs) as described in Chapter 2. Consequently, iPSCs aggregated and formed uniform EBs in each droplet by day 2. After a further 3 to 7 days of culture, EBs further developed and expanded in size; the size was like that formed from a mouse ES cell line KH2 (Hou, Jin et al. 2016) (Figure 3.3A). Thus, these data suggest that iPSCs have multipotent differentiation potential.

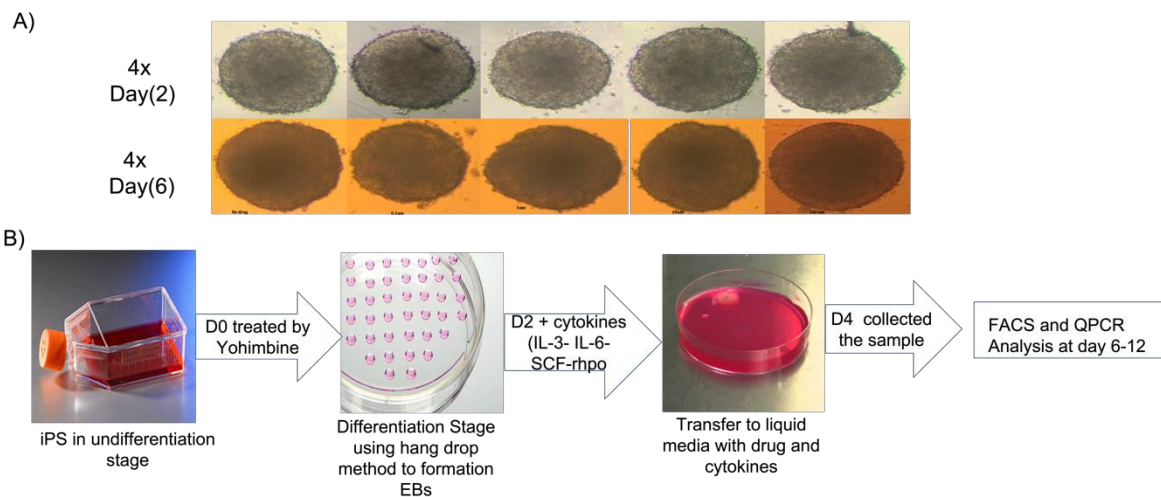


Figure 3.3: iPSCs form EBs in hanging drop culture. iPSCs were cultured in HD medium in hanging drops and panel A and B are shown from two iPSC cultures. A) Morphology EBs in different days. B) A schematic showing EB culture and hanging drop with haematopoietic cytokines (*IL-3- IL-6-SCF-rhpo*).

3.3.4 iPSCs formed embryoid bodies and expressed mesodermal germ layer markers

To confirm the partially differentiated status of EBs derived from our iPSCs, we assessed the differentiation status of EBs over time using q-PCR for pluripotent stem cell markers and germ layer marker gene expression, particularly focussing on the mesoderm, as this is where blood cells are derived from (Tani, Chung et al. 2020). As shown in Figure 3.4 gene expression of pluripotent stem cell markers (OCT3/4 and Sox2) gradually disappeared, beginning at day 4 (Figure 3.4A and 3.4B). Conversely, gene expression of the mesoderm markers, *Itgam*, *Cdh5*, *SCL*, and *Bra (T)*, were demonstrated upregulation by day 7, and 8 days (Figure 3.4C - 3F). Thus, during differentiation of iPSCs mesoderm markers are activated and pluripotent stem cell markers are repressed.

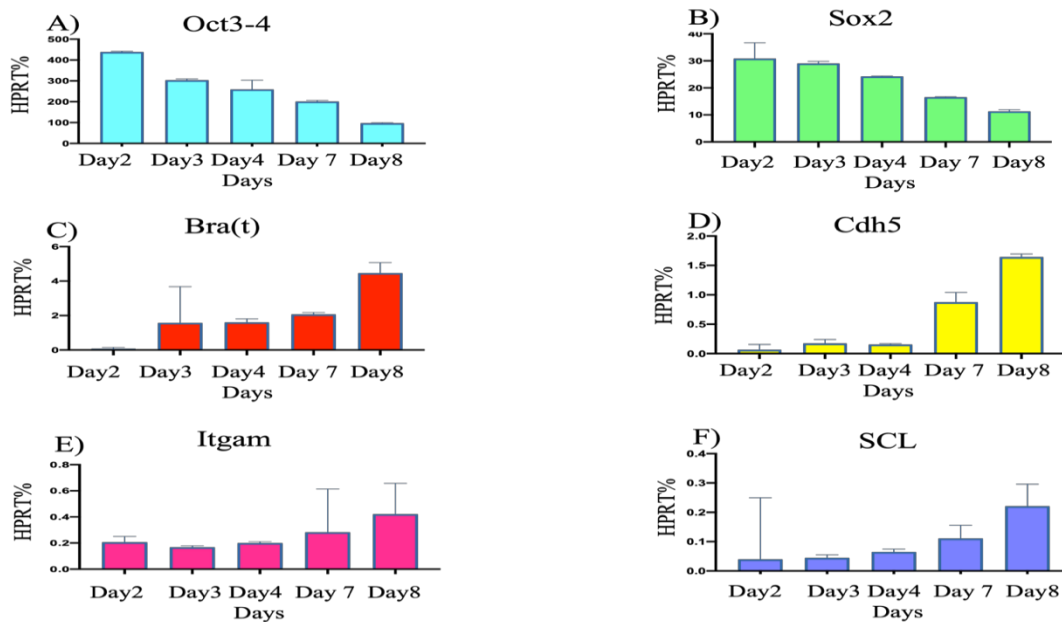


Figure 3.4: Gene expression of stem cells markers and mesodermal genes detected by q-PCR in EBs derived mouse iPSCs. A) Gene expression of Oct3-4 B) Gene expression of Sox2 in undifferentiated iPSCs. Quantitative RT-PCR analysis of mesodermal marker genes include C) Gene expression of brachyury (T), D) Gene expression of *cdh5*, E) Gene expression of *Itgam*, F) Gene expression of *Scl*. Expression of genes is in EBs formed over period up to 8 days. Hypoxanthine phosphoribosyltransferase (HPRT) was used as housekeeping gene control. Quantitative PCRs for gene expression shown are the mean and standard error of three independent experiments. Statistical significance for different days was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

3.3.5 The effect of Phthalylsulfathiazole on iPSC proliferation

As pluripotent stem cells share molecular features of cancer cells (Liu, Yu et al. 2013) and sulphomanide drugs have been shown to have anti-neoplastic ability (Liu, Tian et al. 2012), we first tested the effect of Phthalylsulfathiazole on the proliferation of undifferentiated iPSCs by carrying out proliferation assays. iPSCs were cultured in the presence of various concentrations of Phthalylsulfathiazole for 3-day period and Cell titre blue reagents were added to cells to measure the metabolic activity of the cells. According to the findings described in Figure 3.5, no significant distinction in proliferation was found between Phthalylsulfathiazole-treated and Phthalylsulfathiazole-free cells were identified. This indicates that Phthalylsulfathiazole, at the concentration up to 1mM, does not affect the proliferation of iPSCs (Figure 3.5).

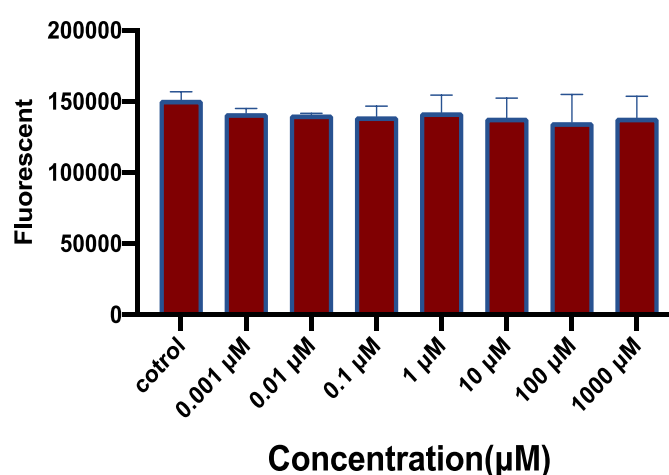


Figure 3.5: Phthalylsulfathiazole treatment did not affect the proliferation of undifferentiated iPSCs. iPSCs were cultured in the presence of various concentration of Phthalylsulfathiazole for 3 days and metabolic products were measured by the Cell Titer blue methods. The graph shown are the mean and standard error of three independent experiments. Statistical significance for different concentration of drug was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

3.3.6 Effects of Phthalylsulfathiazole in the differentiation of haematopoietic cells from iPSCs

I next tested whether Phthalylsulfathiazole could impact haematopoietic differentiation of iPSCs. Diverse doses (0.1 μ M, 1 μ M, 10 μ M and 100 μ M) of Phthalylsulfathiazole were used in these experiments. In this series of experiments, exposure to Phthalylsulfathiazole was started at day 0 (designated D0) of EB to assess the effect of enhancing blood lineage commitment to mesoderm, focussing on blood formation. In contrast, in parallel experiments, exposure to Phthalylsulfathiazole commenced on day 2 (D2) of EB formation, to assess whether the Phthalylsulfathiazole-influenced expansion following blood cell differentiation. In either setting, EBs were removed and added to a liquid culture incorporating Phthalylsulfathiazole (or vehicle for controls) as well as haematopoietic cytokines Interleukin 3 (*IL-3*), Interleukin 6 (*IL-6*), Stem Cell Factor (*SCF*) and Erythropoietin (*rhpo*) to stimulate haematopoietic cells to differentiate into types of blood cells (Fig 3.3A). At day 12 of liquid culture, EBs were harvested and subjected to Q-PCR assessment of haematopoietic transcription factors and immunophenotyping to assess formation of haematopoietic lineages as described in Table 3.1.

For immunophenotyping, no changes were noted in D0 or D2 experiments in haematopoietic lineages assessed (Fig. 3.6), indicating Phthalylsulfathiazole does not affect the abundance of haematopoietic populations during blood commitment or expansion post-commitment during haematopoietic differentiation of iPSC. In terms of gene expression profiling, at D0, only *GATA2* expression was significantly increased at 1 μ M of Phthalylsulfathiazole, but multiple other haematopoietic genes, *Runx*, *Flk* and *SCL* were reduced with increasing concentration of Phthalylsulfathiazole, albeit without reaching statistical significance (Fig 3.7). In striking contrast at D2, *Sox7* expression was increased at doses of 0.01 μ M and 10 μ M and both *CEBP α* and *Pax6* were increased at 10 μ M. In addition, *PU1* and *Runx1*, *Gata1* and *Flk* each showed non-significant trends of upregulated expression in response to increasing concentrations of Phthalylsulfathiazole (Fig 3.8). Thus, the transcriptional profiling data provided here suggests that Phthalylsulfathiazole may have opposing effects on haematopoietic commitment (D0) versus expansion of haematopoietic lineages following haematopoietic commitment (D2), with detrimental effects on the former and beneficial effects on the latter. To examine if a transcriptional signature indicative of an expansion of haematopoietic lineages in response to Phthalylsulfathiazole treatment translated to increased functional potential, a CFC assay was performed, which did not work due to technical difficulties with the assay and was not repeated due to Covid-19 restrictions.

Table 3.1: Immunophenotypic markers of haematopoiesis in EBs.

Combination of Markers	Functions
CKIT+Ter119+ CKIT+CD71+	Makes CFU-E centered on the survival and further growth of erythroid cells in context of reproduction and specification (Dulmovits et al., 2017).
CKIT+CD41+	These cells contain committed progenitors as well as adult HPCs that contain both primitive and specified HPCs. They contribute to determination of the novel pre-HSCs located in mesenchyme (Ottersbach 2019).
C-KIT+CD31+	By scRNA-seq, Intra-aortic haematopoietic cluster cells (IAHCS) can be identified in the aorta site in extensive numbers. When Day 6 starts, the quantity of CD31+CKIT+ is visibly increased, even though reduction in expression become noticed by Day 12 in vertebrate embryos that increase HSC production. The expression of IAHCS include Runx.1 (Baron, Kester et al. 2018).
CKIT+CD45	Through the ESC differentiation, using <i>Gata2Venus</i> (G2V) and Ly6a(SCA1)-GFP(LG), from mouse model of HP/SC emergence. reduction in C-KIT+CD45+ by 0.4%–2.2% during the derivation can be observed by the twelfth day; by day 6, it reduces to 1.3% (Cortegano et al., 2018).

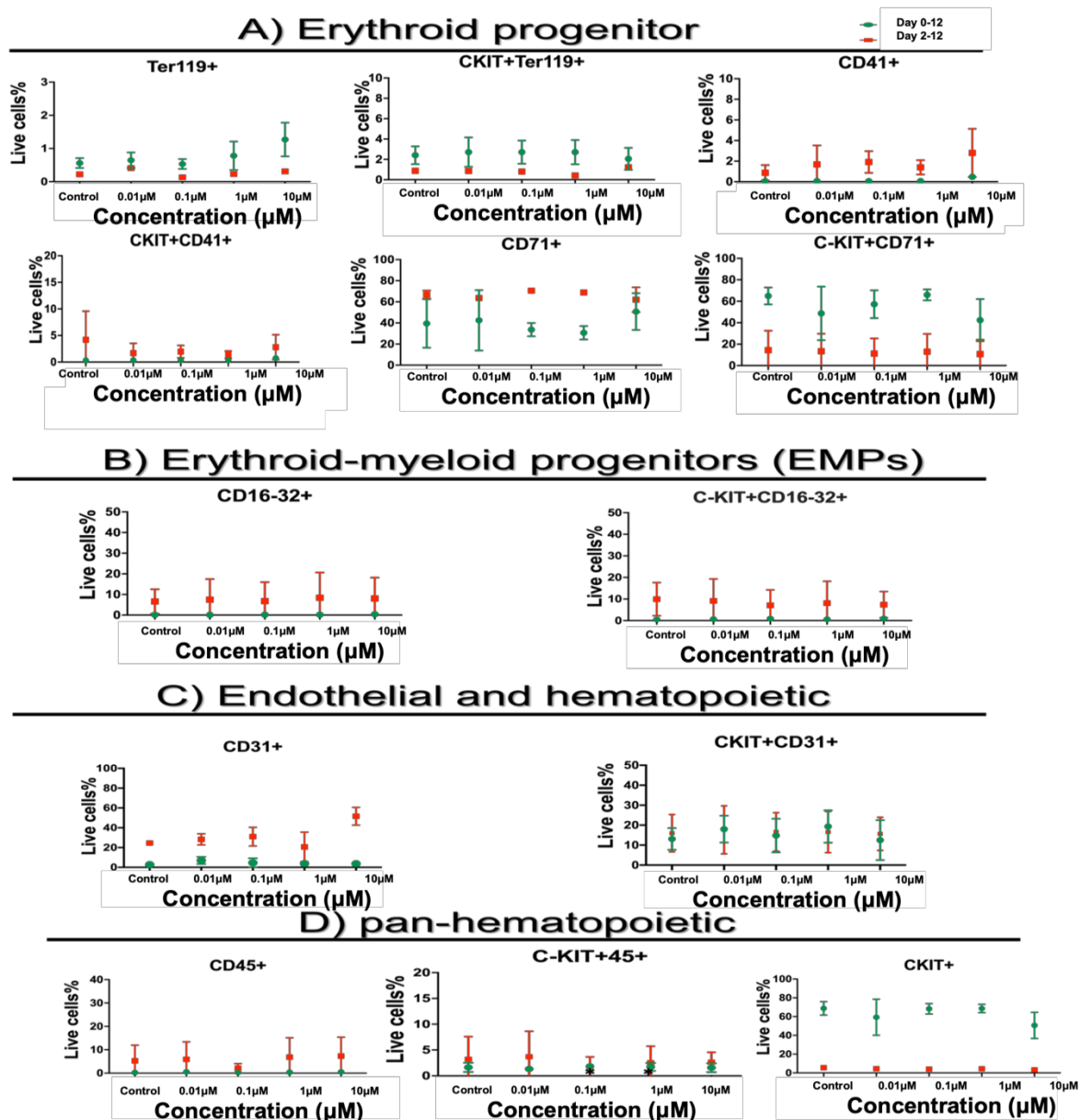


Figure 3.6: Temporal immunophenotypic analysis of iPS-derived cells exposed to Phthalylsulfathiazole during haematopoietic differentiation at day (0) and (2) of EB formation. EBs were cultured for 12 days in liquid culture with haematopoietic cytokines and was sulphonamide in the medium. EBs were stained with conjugated specific antibody for haematopoietic differentiation and analysed by flow cytometry. Graphs showing the percentage from differentiated of A) Erythroid progenitor B) Erythroid-myeloid C) Endothelial and haematopoietic D) pan-haematopoietic at days 12. (Green data points=D0/12, Red data points= D2/12). Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

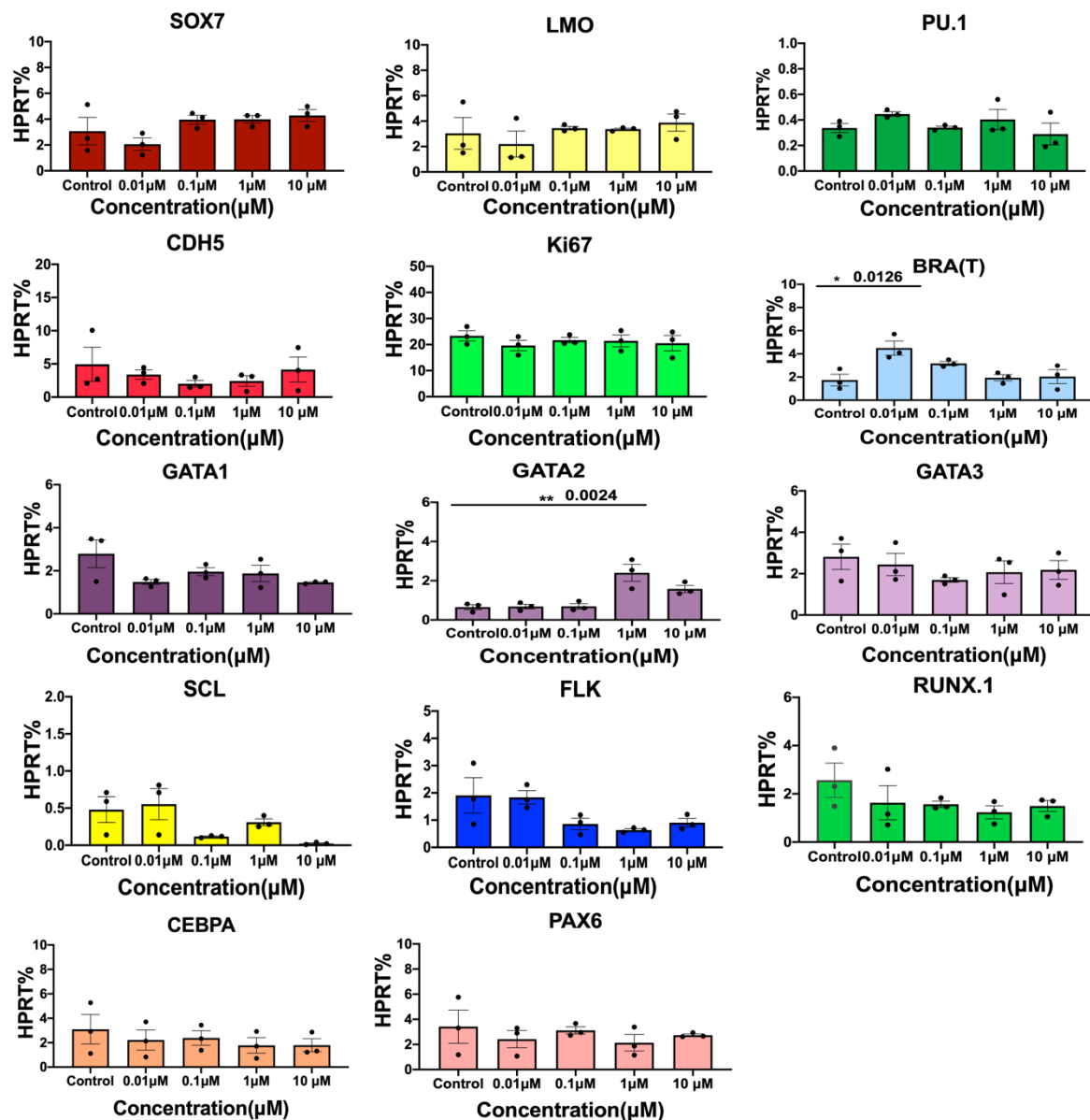


Figure 3.7: Gene expression analysis in response to Phthalylsulfathiazole treatment in Day 0 in EBs. D0 EBs treated with Phthalylsulfathiazole compared with untreated EBs at day 12. EBs were cultured for 12 days in liquid culture with haematopoietic cytokines with Phthalylsulfathiazole in the medium. Data represent means \pm standard error 3 independent experiment analysing. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

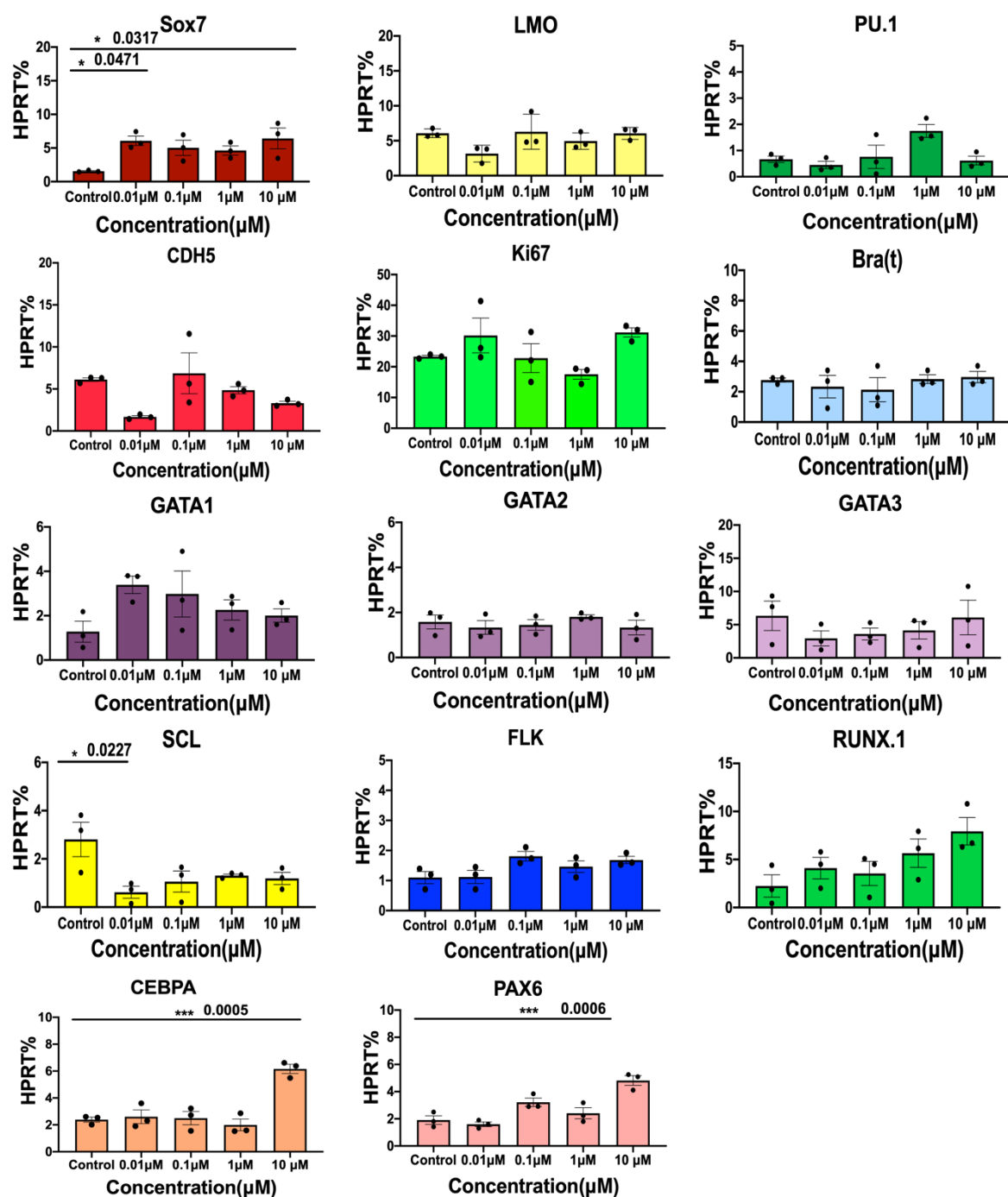


Figure 3.8: Gene expression analysis in response to Phthalylsulfathiazole treatment at day 2 of EB formation. D2 EBs treated with Phthalylsulfathiazole compared with untreated EBs at day 12. EBs were cultured for 12 days in liquid culture with haematopoietic cytokines and Phthalylsulfathiazole in the medium. Data represent means \pm standard error 3 independent experiment. Statistical significance for different concentration was determined by one way ANOVA test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

3.3.5 Assessing the impact of Phthalylsulfathiazole on long-term HSC function *in vivo*

Having shown a potential positive effect of Phthalylsulfathiazole on *in vitro* haematopoietic differentiation, I next turned to examine the impact of Phthalylsulfathiazole on haematopoiesis *in vivo* using transplantation assays. Previous data from the Rodrigues laboratory (Lubaid Saleh thesis, Cardiff University 2017) has shown that *in vivo* administration of Phthalylsulfathiazole to wild-type, non-conditioned mice cause a decrease in myeloid progenitors that is counterbalanced by an increase in Gr-1+ neutrophils in peripheral blood. In experiments where bone marrow cells from these Phthalylsulfathiazole treated mice were extracted and transplanted into lethally irradiated hosts to test their HSC functionality *in vivo*, it was found that *in vivo* Phthalylsulfathiazole-treated HSCs performed similarly to vehicle treated HSCs. In separate experiments, where Phthalylsulfathiazole was administered after bone marrow transplantation, Phthalylsulfathiazole was found to have no impact on haematopoiesis. Here, we explored the impact of Phthalylsulfathiazole on long-term functioning of HSCs in the context of their differentiation capacity. Mouse bone marrow from CD45.2 were transplanted into CD45.1 recipient mice that underwent lethal irradiation. Twice a day, Phthalylsulfathiazole at various doses (1mg/kg, 10mg/kg, 100mg/kg or PBS control) was administered to recipients by IP injection throughout an overall period of 10 days after 2 weeks after transplantation (Figure 3.9A). We then conducted secondary transplantation at 16 weeks after primary transplantation where primary recipients were treated with Phthalylsulfathiazole after transplantation. 2000 HSC enriched LSK cells were isolated and transplanted to secondary recipients for a further 16 weeks. Phthalylsulfathiazole treatment in secondary transplants was associated with a general non-significant decrease in myeloid lineages in BM and PB and CD4+ and CD8+ in BM and spleen respectively (Figure 3.10 - 3.11). Further experimentation will be needed to confirm these observations as a limited number of recipients were analysed. These preliminary results suggest however that Phthalylsulfathiazole treatment may have detrimental effects on long-term HSC functioning, including haematopoietic differentiation.

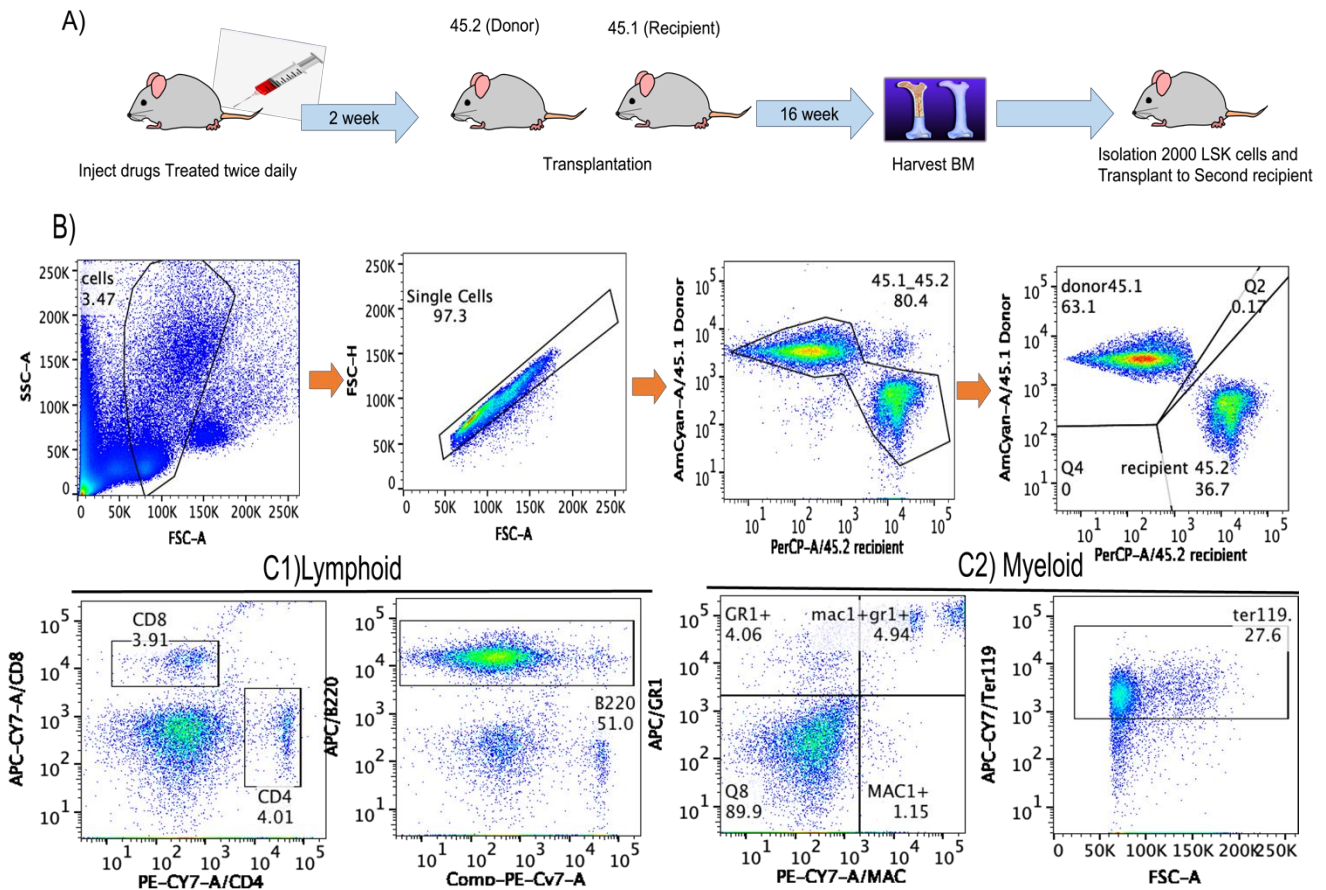


Figure 3.9: Assessing the impact of Phthalysulfathiazole following secondary transplantation. A) Method for secondary transplantation. B) Flow cytometry analyses showed the second transplantation C) Flow cytometry Analyses showed lymphoid and myeloid.

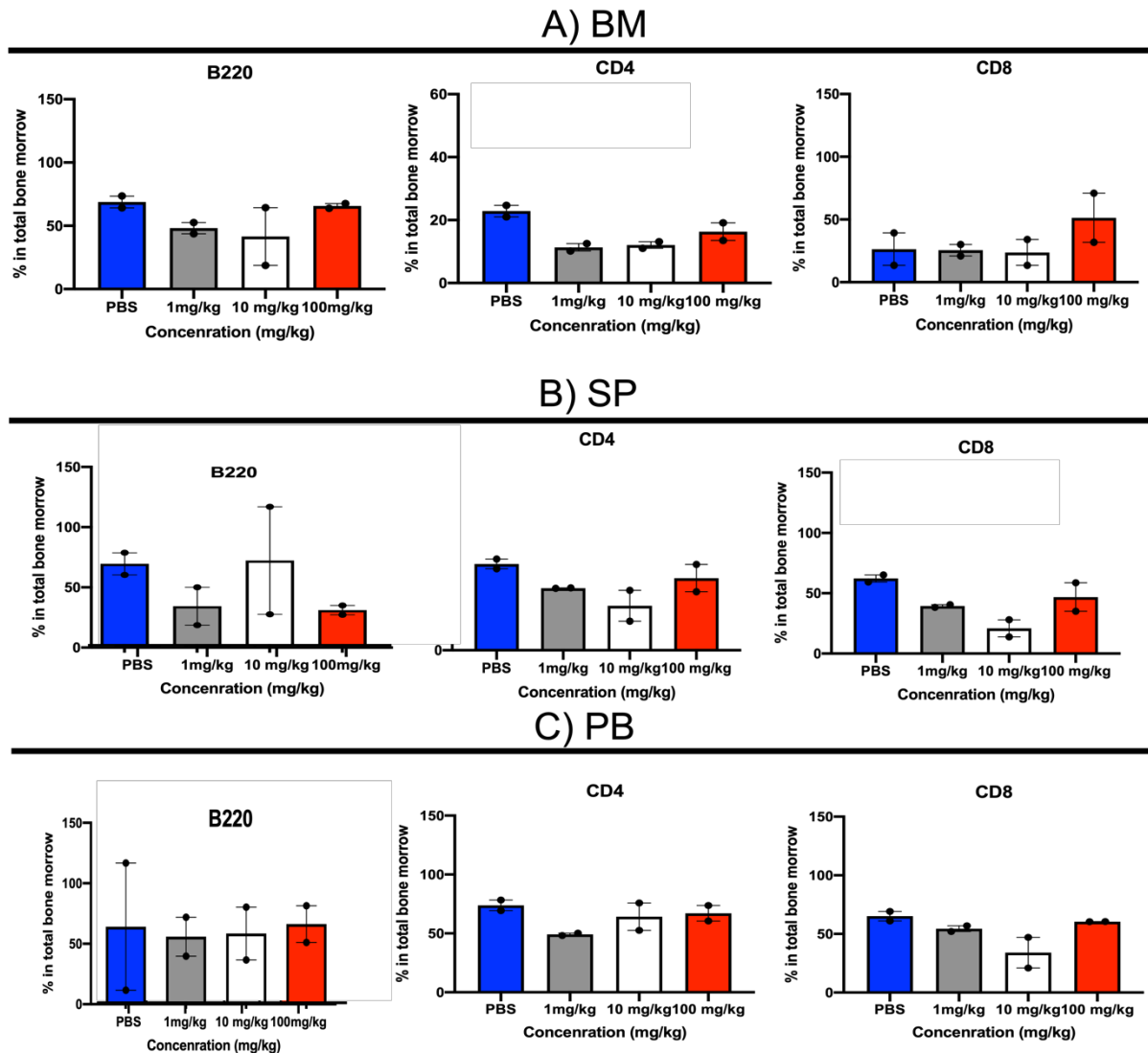


Figure 3.10: The impact of Phthalysulfathiazole administration following secondary transplantation on the function of lymphoid haematopoietic lineages. Mice were treated with different concentrations of PBS or 1mg/kg, 10 mg/kg, or 100 mg/kg Phthalysulfathiazole twice daily for 10 days following transplantation. After 16-week, bone marrow harvest and isolation 2000 LSK cells and transplant to second recipient A) Bone marrow B) Spleen C) Blood. Error bars represent mean + SEM from 2 recipients. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

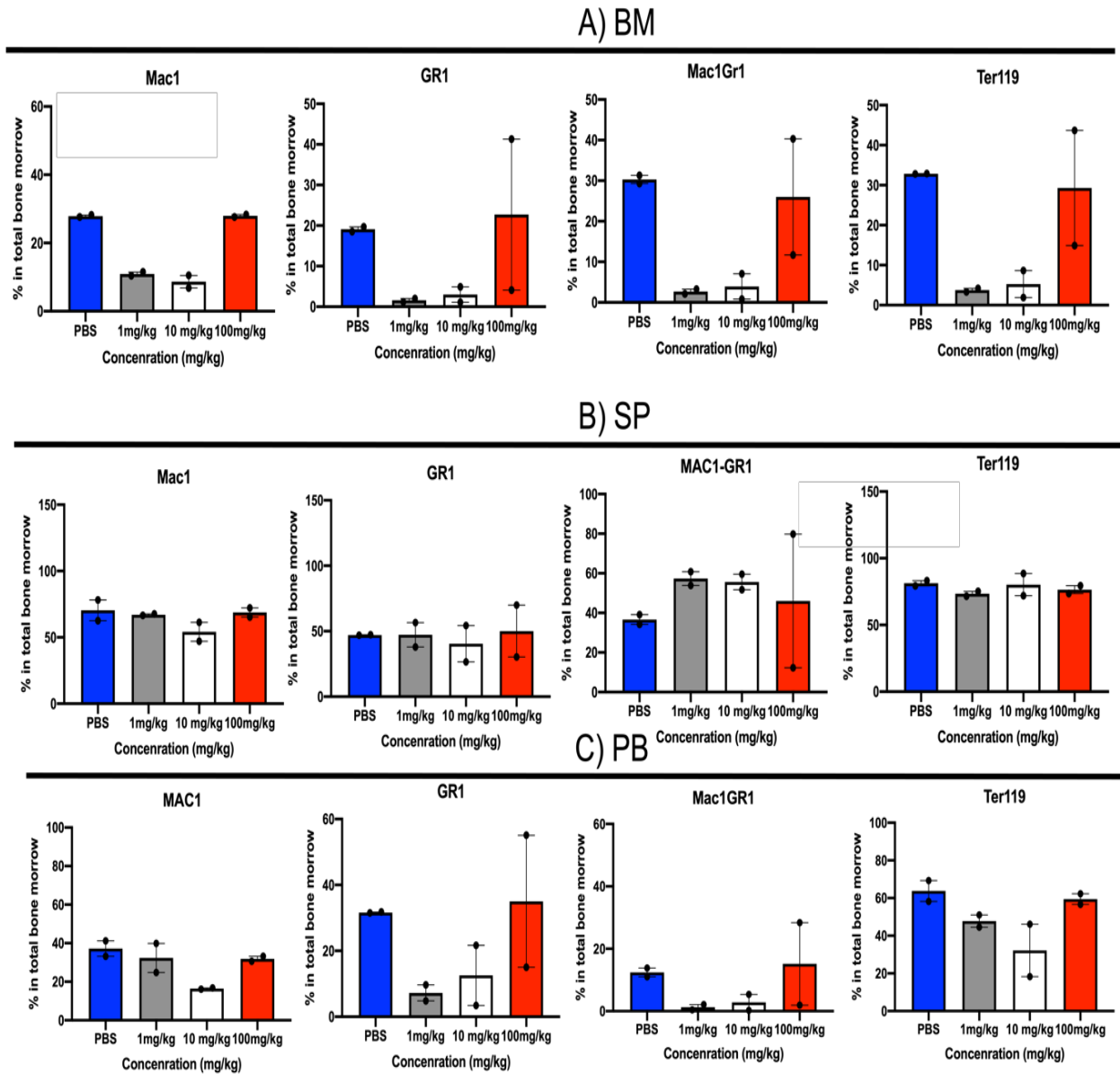


Figure 3.11: The impact of Phthalysulfathiazole administration following secondary transplantation on the function of myeloid haematopoietic lineages. Mice were treated with different concentrations of PBS or 1mg/kg, 10 mg/kg, or 100mg/kg Phthalysulfathiazole twice daily for 10 days following transplantation. After 16-week, bone marrow harvest and isolation 2000 LSK cells and transplant to second recipient A) Bone marrow B) Spleen C) Blood. Error bars represent mean + SEM from 2 recipients. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4 Discussion

iPSCs have potential therapeutic application in transplantation medicine and, also, in drug discovery to improve transplantation and the targeting of cancer cells. In this chapter, we successfully established the iPSC model in the laboratory, cultured iPSCs to maintain their pluripotent stem cell status and characterized their stem cell potential through flow cytometry. As expected, undifferentiated iPSCs, cultured in the presence of LIF and other inhibitors of differentiation, express markers of pluripotency such as Oct3/4, Nanog and SSEA-1 (Ben-Shushan E et al, 1998). Interestingly, Nanog expression was significantly lower than either Oct3/4 or SSEA-1 in undifferentiated iPSCs; this is consistent with Nanog playing an essential role in establishing the pluripotent gene network in iPSCs – yet it is dispensable for maintenance of this network after establishment (Cambers I et al, 2007).

By removal of LIF, as pluripotency factor and anti-differentiation factor, we also partially differentiated iPSCs to embryoid bodies (EBs) that contain the three germ layers constituting the body (mesoderm, endoderm and ectoderm). We focused our analysis on examining gene expression of the pluripotency markers and mesoderm, which is the germ layer through which blood cells originate. Our study did not examine the baseline expression of pluripotency and mesoderm genes, as a positive control for the entire time-period of the experiment, nor did it formally assess formation of the other germ layers, ectoderm and endoderm, to prove pluripotency. However, as predicted, we saw a general trend of reduction in pluripotency markers during EB differentiation and an associated upregulation of *SCL/TAL1*, *bra (t)*, *cdh5* and *itgam* (Zambidis, Peault et al. 2005), (Mao, Xue et al. 2013), (Fridley, Nair et al. 2014), indicating mesoderm lineage commitment. Overall, this analysis provided proof of principle of a tractable iPSC model to assess the impact of Phthalylsulfathiazole on properties of iPSCs and on haematopoietic differentiation *in vitro*.

Undifferentiated iPSCs share a common molecular signature with neoplastic cells. For example, high expression of Oct3/4 has implicated as a marker of prognosis and therapy resistance in cervical cancer (Kim, Cho et al. 2015). Given that past research has found that sulphonamides can act as antineoplastic drugs, we first used Cell Titre blue assay to study the impact of Phthalylsulfathiazole on cellular proliferation. However, my research found no impact of Phthalylsulfathiazole on proliferation of iPSCs within a range of 0.001µM to 1000µM. Nevertheless, Phthalylsulfathiazole could be potentially utilized to selectively attack other aspects of leukaemic cell biology (e.g. cell survival) and this will be explored in Chapter 5. While the possibility of Phthalylsulfathiazole mediated targeting of leukaemia cell proliferation has not been directly tested using the pluripotent stem cell model described here – it could, in

principle, target either proliferation, cell survival or the differentiation capabilities of leukaemia cells, which are largely incapable of progressing through a normal differentiation programme in leukaemia (Van Etten 2007). This notion could be probed experimentally where pluripotent stem cells express a leukaemia related oncogene and leukaemic/haematopoietic differentiation is induced in iPSCs (Zakrzewski, Dobrzyński et al. 2019), and the cellular and molecular responses to Phthalylsulfathiazole treatment could be examined. During directed differentiation to haematopoietic lineages, Phthalylsulfathiazole appeared to have opposing actions, as evidenced by transcriptional profiling of haematopoietic related genes –some inhibition of haematopoietic commitment was noted yet addition of Phthalylsulfathiazole appeared to enhance haematopoietic gene expression overall following haematopoietic commitment. The functional consequences of this will need to be confirmed in CFC assay. However, if proven to be true, this data would be consistent with an expansion of haematopoietic progenitors observed in folate deficient mice, where folate deficiency impacts these populations (Chuncharunee, Carter et al. 1993).

In preliminary experiments to assess the impact of Phthalylsulfathiazole on long-term haematopoiesis *in vivo*, a possible detrimental lineage-specific trend was observed on secondary transplantation of HSPCs that were previously exposed to Phthalylsulfathiazole during primary transplantation, which is consistent with suggestions that sulphonamides suppress haematopoiesis *in vivo* (Weinzierl and Arber 2013). For example, negative drug impact from sulphonamide antimicrobial drug combinations have been recorded for several species, including immune-mediated biological effects (Cudmore, Jalim et al. 2015). This may occur in its role as a folic acid inhibitor (Shahzad, Qadir et al. 2020), but also through the formation of antibodies, which involves cross-reactions of the drugs and haematopoietic cells (Brackett, Singh et al. 2004). A similar effect may be obtained through drug antigen-antibody complexes that fix complement, passively holding haematopoietic cells together (Haritunians, Gueller et al. 2008, Wang, Kai et al. 2012) and this phenomenon is frequently associated with sulphonamides (Haritunians, Gueller et al. 2008, Wang, Kai et al. 2012).

Therefore, if all data in this chapter is taken together, it appears that Phthalylsulfathiazole may have diametrically opposing effects on iPSCs/HSCs which is contextually dependent on whether cells are exposed to Phthalylsulfathiazole *in vitro* or *in vivo*. Gene expression data showing enhanced expression of haematopoietic genes after *in vitro* exposure of differentiated EBs is consistent with the initial screen in functional experiments, which showed an increase in multipotent progenitors, as judged by the abundance of CFC-mix in CFC assays after *in vitro* exposure of bone marrow cells to Phthalylsulfathiazole (data not shown). This bodes well for the potential use of Phthalylsulfathiazole in *ex vivo/in vitro* settings - for example, using iPSCs to produce potential blood/immune cellular products for use in a variety of clinical

haematology settings or for *ex vivo* cord blood transplantation. The apparent detrimental impact of Phthalylsulfathiazole on long-term functioning of HSCs requires further investigation, but if proven to be correct and relevant in humans, it may preclude use clinical haematology settings, like transplantation. Taken together with previous data from the laboratory that has shown that treatment Phthalylsulfathiazole has minimal beneficial effects anyway, this means that future *in vivo* applications of Phthalylsulfathiazole in clinical haematology are likely limited. Further experiments that clarify the molecular and functional distinction between *in vitro* and *in vivo* impacts of Phthalylsulfathiazole on haematopoietic cells are needed to clarify this.

CHAPTER 4:

Examining the impact of Yohimbine on haematopoietic stem cell function and haematopoietic differentiation from induced pluripotent stem cells

4.1 Introduction

The sympathetic nervous system (SNS) is a key component of the nervous system, responsible for down- and up-regulating several homeostatic mechanisms in living organisms. Post- and pre-ganglionic neurons transmit signals via the sympathetic system (Waxenbaum, Reddy et al. 2021). At the synapses in the ganglia, the preganglionic neurons emit acetylcholine (LeBouef, Yaker et al. 2021). This neurotransmitter activates nicotinic acetylcholine receptors in postganglionic neurons. In response to stimulation, catecholamines, like norepinephrine and epinephrine, emanate from postganglionic neurons activating adrenergic receptors, which are G-protein coupled receptors (Gordan, Gwathmey et al. 2015). When adrenergic receptors are activated in target tissues, it leads to physiologic effects linked to the sympathetic nervous system (Grisanti, Perez et al. 2011) such as cognition, emotion, motor control, endocrine regulation, and memory processing (Kobayashi 2001). There are two main classes of adrenergic receptors, α and β , with multiple subtypes of receptors for both α and β receptors (Molinoff 1984). Of these, α 2-adrenergic receptors mediate various biological impact associated by norepinephrine and epinephrine (Table 4.1). α 2-adrenergic signaling acts as a critical regulators of intraocular pressure, blood pressure and neurotransmitter release (Philipp, Brede et al. 2002).

As illustrated by Parayato et al. (2020), adrenergic receptors signal via the Gi/Gs-coupled GPCRs. After attaining agonistic occupancy of the Gs' coupling, stimulation happens by adenylate cyclase, which manufactures the cAMP through the ATP as shown in Figure 1.

Table 4.1. Adrenergic receptor subtypes and physiological effects of catecholamine binding to the specific receptor

Adrenergic receptors	Alpha (α -) adrenergic receptors		Beta (β -) adrenergic receptors	
	Alpha-1(α 1)	Alpha-2 (α 2)	Beta-1(β 1)	Beta-2(β 2)
Distribution	Vascular-liver-smooth muscle	Presynaptic-Autonomic nerve terminals-platelet	Heart-Kidneys-placenta	Vascular smooth muscle-uterus-GI tract-lung-placenta
Functions	Increase in blood flow and blood pressure and closure of bladder sphincters	Inhibits (Norepinephrine-Acetylcholine-insulin) release	Increase Automaticity, Contractility Renin release	Relaxation, decrease in Motility
Receptor-Transduction-Messenger-Effector	G_q linked to activation of PLC-DAG- IP_3	G_i linked to inhibition of adenylyl cyclase-c.AMP	Increase cAMP G_s - adenylyl cyclase	Increase c.AMP G_s - Adrenaline
Selective Antagonists	Prazosin	Yohimbine	Atenolol	Butaxamine

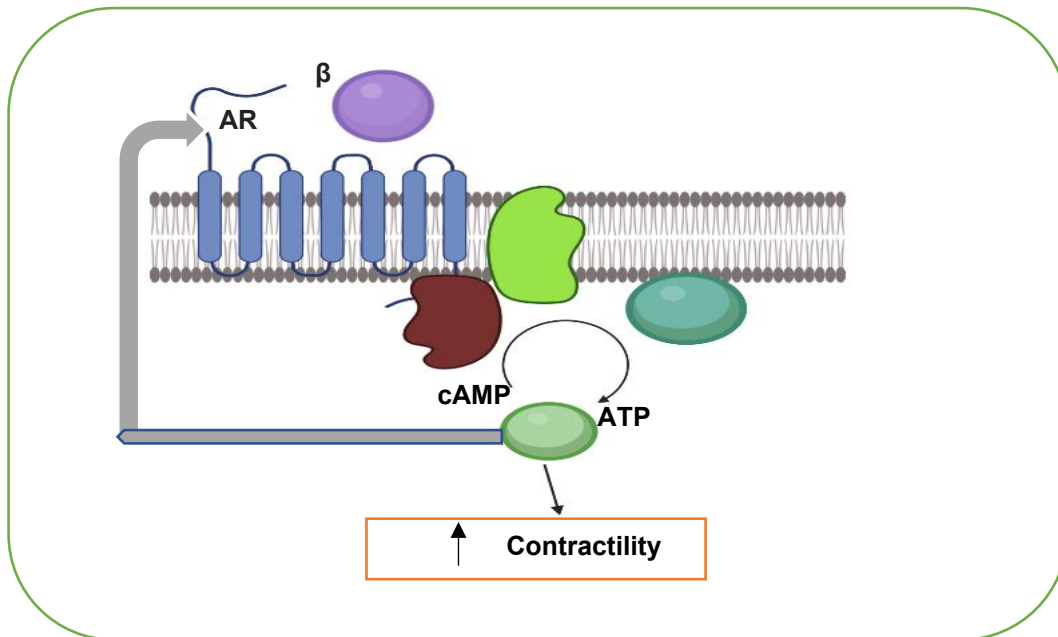


Figure 4.1: Adrenergic receptor signaling. The G_{α} activation both adenylyl cyclase (AC), which increases intracellular cAMP levels and L-type calcium channels, that allowed Ca^{2+} to enter into cardiomyocytes. The cAMP activates PKA which phosphorylates (P).

Compounds activating or blocking adrenergic receptors have been widely utilized clinically, particularly in cardiovascular diseases and congestive heart failure. α -adrenergic antagonists lower high blood pressure, which is a risk factor for heart attack and stroke (Temprano 2016). Additionally, α -selective agents can be used for the treatment of some cardiac arrhythmias as well as in ameliorating retention of urine for patients with prostatic hypertrophy (Ho, Yan et al. 2010). Compounds selectively activating β -adrenergic receptors, are also utilized for cardiac stimulation in the treatment of certain congestive heart failure types, while β 2-selective agonists remain potent bronchodilators in treating asthma (Rambacher and Moniri 2020). Additionally, they are utilized in the arrest of premature labour because they relax the uterine smooth muscles (Barnes 1993). Adrenergic blockers are also crucial when treating vasospastic disorders like acrocyanosis and in Raynaud's syndrome, to improve blood flow (Temprano 2016).

In haematopoiesis, sympathetic neural control is directly exerted on the bone marrow niche, which is marked by sympathetic innervations (Hanoun, Maryanovich et al. 2015). β and α are expressed in haematopoietic progenitor cells in the bone marrow compartment at different stages of lineage commitment (Muthu, Iyer et al. 2007). In terms of blood/immune cells, migration of human neutrophils, oxidative metabolism, and CD11b/CD18 expression are inhibited through β -AR signalling. Inhibitory β -AR can also occur on NK cells that express α -AR for undefined functional roles (Scanzano and Cosentino 2015). Monocytes express β -AR that is normally anti-inflammatory. Even in particular conditions, proinflammatory effects may occur (Scanzano and Cosentino 2015). Murine DC is utilized in expressing β -AR that modulates interactions of DC-T cells. In humans, DC β 2-AR can impact CD4+ T cells' Th1/2 differentiation (Scanzano and Cosentino 2015). β 2-AR dysregulation in astrocytes and microglia may result in neuroinflammation in neurodegenerative and autoimmune diseases. Adrenergic stimulation has a crucial function in innate immune cell development (Sharma and Farrar 2020). The critical AR expressed in the innate immune cells include β and possibly β 2-AR, but α -AR can occur in certain cell kinds and under certain conditions (Sharma and Farrar 2020). The adaptive immune system responds to adrenergic stimulation (Scanzano and Cosentino 2015). Most available information concerning adrenergic pathways for DC has been collected in murine DC. AR has the effect of mediating influences of the sympathetic nervous system on interactions of DC-T cells, leading to the right adaptive immunity response (Scanzano and Cosentino 2015).

In principle, therefore, stimulating adrenergic signalling may have a beneficial impact in clinical haematology applications such as mobilization, transplantation and transfusion medicine. Proof of principle studies targeting adrenergic receptors have been demonstrated in mice by use of the α -adrenergic antagonist, prazosin, which intensifies the growth of platelets, and the

development of granulocyte/macrophage cell types as evidenced by spleen-colony assays *in vivo* and CFC assays *in vitro* (Maestroni, Conti et al. 1992). For example, there was an increase in the number of CFU-GM from transplanted bone marrow exposed to prazosin (Maestroni, Conti et al. 1992). Yohimbine, an alkaloid compound that is medically applied to antagonize the α -2 adrenoreceptor (Tam, Worcel et al. 2001) and is associated with management of erectile dysfunction (ED) in male patients (Tam, Worcel et al. 2001), appears to have similar, but less potent, effects to prazosin in enhancing haematopoietic reconstitution *in vivo* (Maestroni, Conti et al. 1992).

4.2 Aims and objectives

In previous data obtained from the Rodrigues laboratory (Lubaid Saleh thesis, 2017), it was shown that Yohimbine improves the early *in vivo* reconstitution capacity of haematopoietic and immune cell consistent with enhancement of progenitor function rather than HSCs, which is promising for potential utilization in clinical bone marrow transplantation, where patients often die due to infections in the early stages after transplantation (Morales 2000). The aims in this chapter using flow cytometry and QPCR:

- 1- Determine the long-term effect of Yohimbine on *in vivo* HSC function after transplantation
- 2- To confirm the pattern of activity of Yohimbine, whether Yohimbine can enhance the ability of iPSCs to differentiate to haematopoietic cells *in vitro*.
- 3- Evaluate the development of haematopoietic cells from iPSCs after exposure to Yohimbine.

Results

4.3.1 Evaluating the impact of Yohimbine on Functions of

Haematopoietic Stem and Progenitor after Bone Marrow transplantation

As previously shown, the use of Yohimbine improved early haematopoietic reconstitution after transplantation (Saleh, 2017). Nevertheless, the effects and mechanisms of Yohimbine on the functions of haematopoietic stem and progenitor *in vivo* remain unclear. In an attempt to understand whether Yohimbine regulates the regenerative abilities of haematopoietic stem and progenitor cells (HSC, HPC1, HPC2, and MPP), committed progenitors (CMP, GMP, MEP) and mature haematopoietic populations (Gr1+, Mac1+, Ter119, B cells, T cells) transplantation assays were conducted to measure HSC functionality (Till and Mc 1961, Becker, Mc et al. 1963) using the CD45.1/CD45.2 congenic mouse system (Micklem, Ford et al. 1972, Harrison and Roderick 1997). Mouse BM cell populations from CD45.2 were transplanted into CD45.1 recipient mice that were exposed to lethal irradiation. Twice a day, Yohimbine was administered at various doses (1mg/kg, 10mg/kg, 100mg/kg or PBS control) to recipients via IP injection throughout an overall period of 10 days for 2 weeks after transplantation. At 16 weeks after transplantation, a time-point where HSC function is normally assessed (Kwarteng and Heinonen 2016), recipients were sacrificed and BM samples were obtained, and flow cytometry was used to analyse engraftment (Figure 4.1-4.2). Our analysis showed that Yohimbine did not impact haematopoietic stem cell and progenitor engraftment, total engraftment of bone marrow or the proportion of mature haematopoietic lineages from spleen, peripheral blood, or bone marrow (Figure 4.3- 4.4). Thus, while Yohimbine improves early haematopoietic engraftment following myeloablative conditioning, it does not affect the functionality, including differentiation capacity, of the long-term HSC pool.

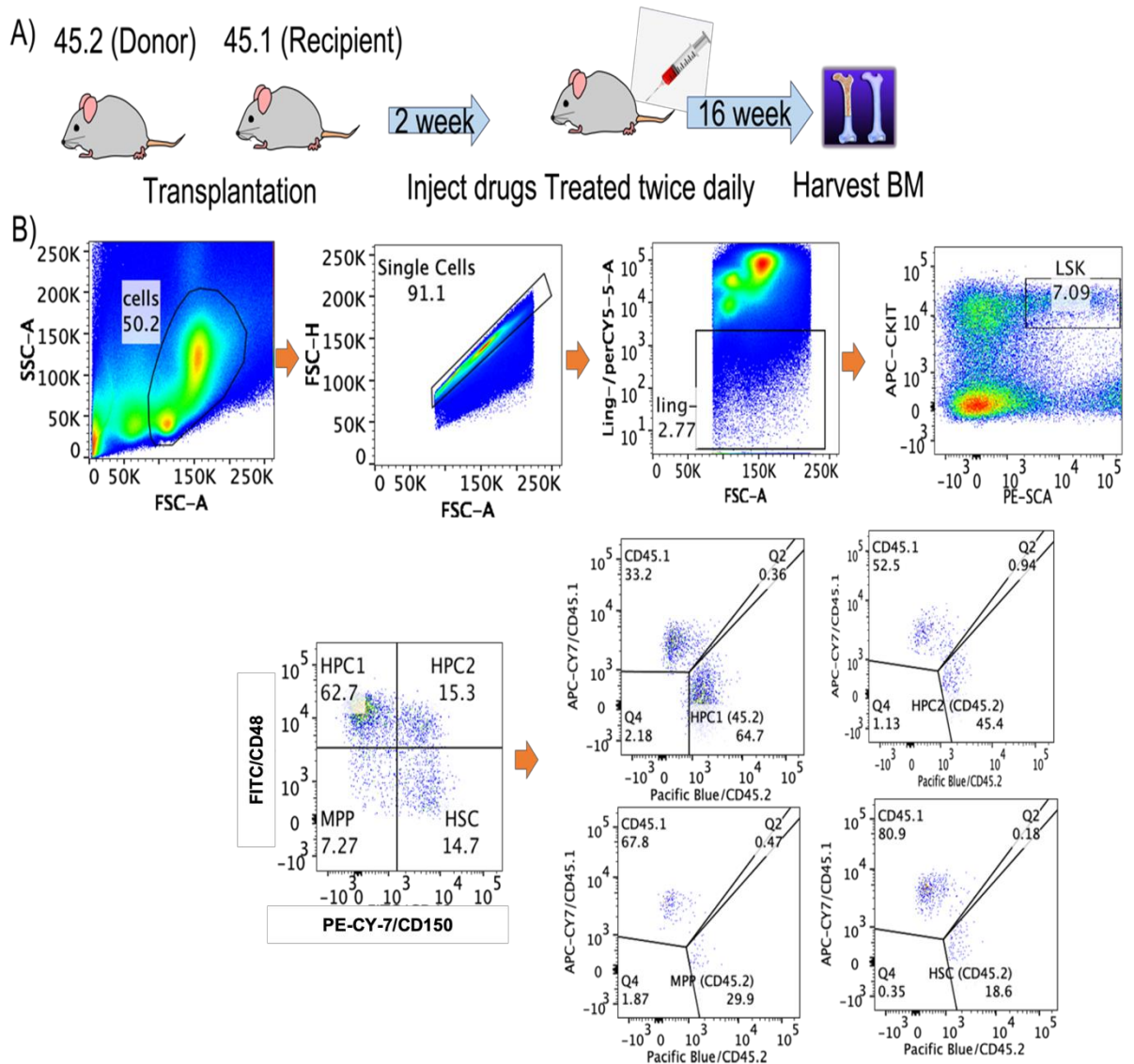


Figure 4.1: Flow cytometry gating strategy for haematopoietic stem cells at 16 weeks after transplantation. A) Scheme of experiment: transplanted cells from donor (CD45.2) to recipient cells (CD45.1). B) To analyse haematopoietic stem and progenitor populations, the Lineage- population gated from donor 45.2 cells and Lineage-/lowSca-1+c-kit+ (LSK) cells LSK were gated on CD48 and CD150 to determine HSCs (CD150+CD48-) and Primitive progenitors include HPC1 (CD150-CD48+), HPC2 (CD150+CD48+), and MPPs (CD150-CD48-).

C)

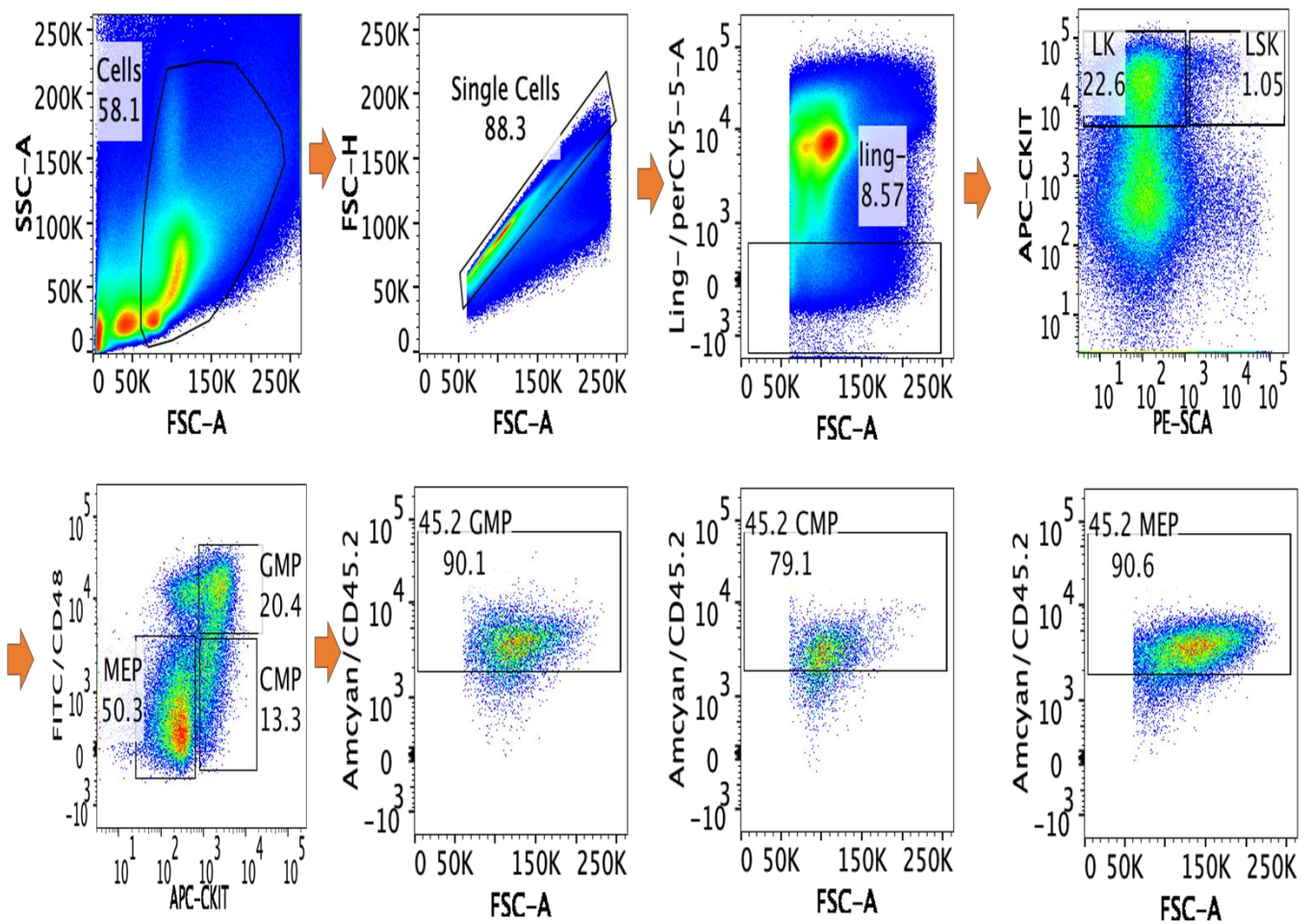
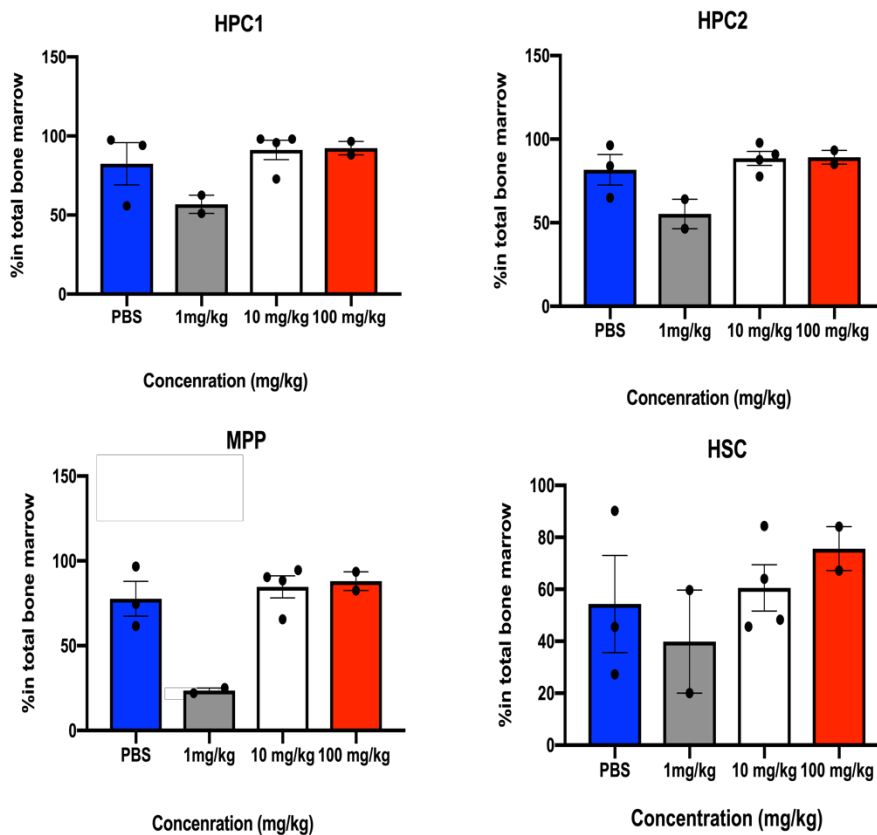


Figure 4.2: Flow cytometry gating strategy for committed haematopoietic progenitors. To analyse committed progenitor populations, the Lineage- population was gated from donor CD45.2 cells (A+B) and Lineage-/lowSca-1-c-kit+ were gated onto CD16/32 and CD34 to determine to identify CD16/32+ CD34+ GMP (granulocyte-macrophage progenitor), Cd16/32loCD34+ CMP (common myeloid progenitor), and CD16/32lo CD34lo MEP (megakaryocyte-erythroid) population (B).

A)



B)

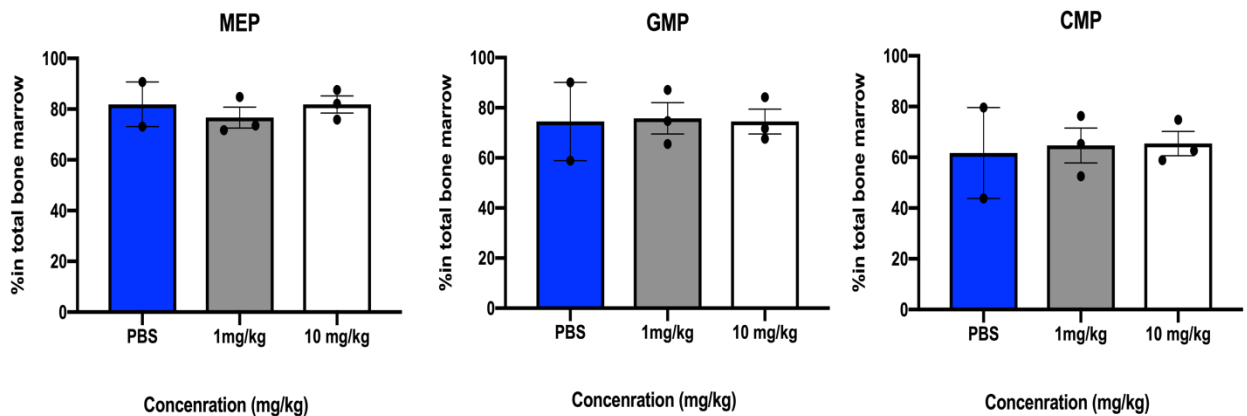


Figure 4.3: The impact of Yohimbine administration on the function of haematopoietic stem and committed progenitor cells following transplantation. Mice were treated with different concentrations of PBS or 1mg/kg, 10 mg/kg, or 100 mg/kg Yohimbine twice daily for 10 days following transplantation. After 16-week, bone marrow harvest and cell surface marker were stained for HSCs (A) and committed myeloid progenitors (B) and analysis by flow cytometry. Error bars represent mean + SEM from 3 recipients. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

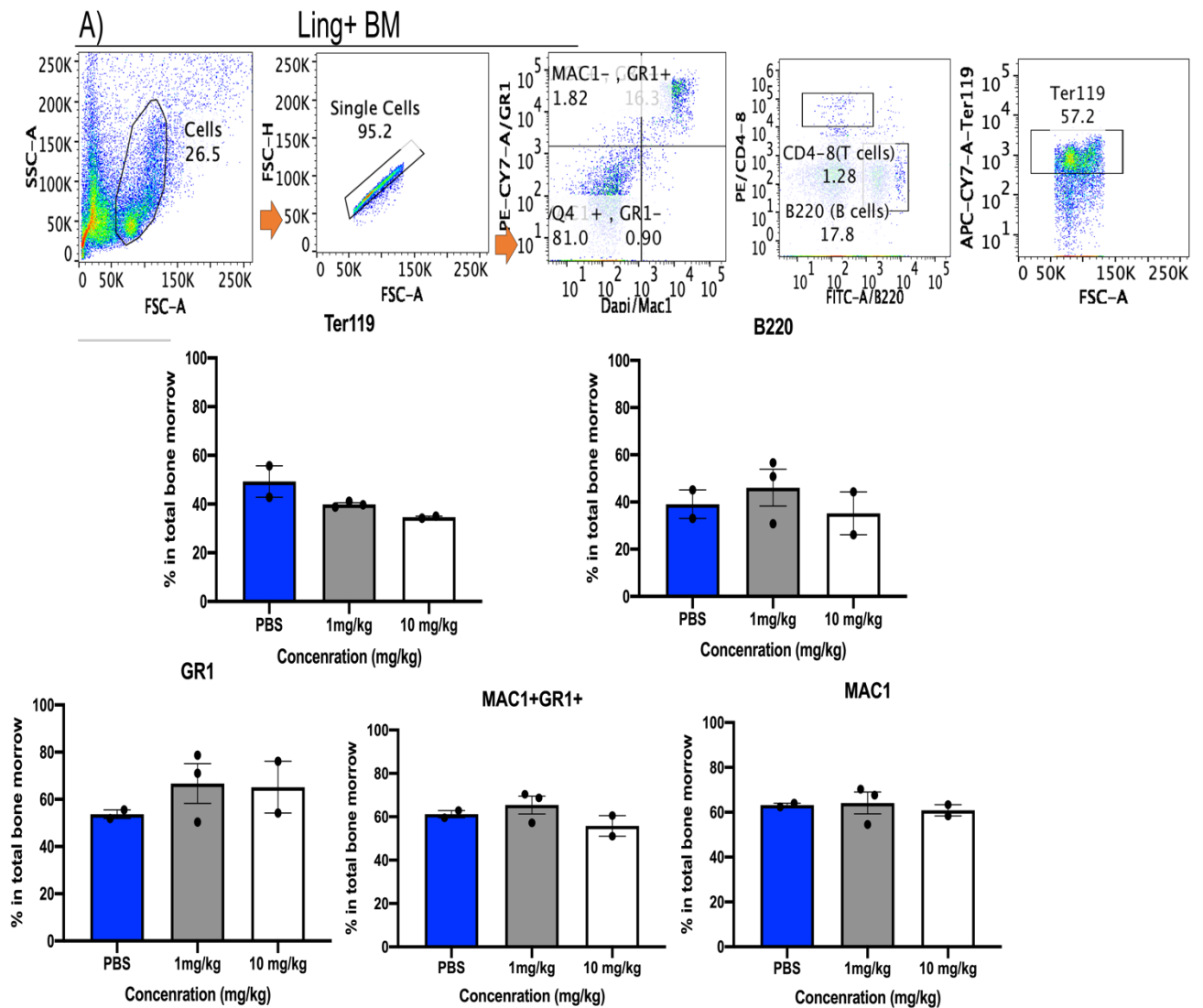


Figure 4.4 (A): The impact of Yohimbine administration following transplantation on the function of haematopoietic lineages in Bone marrow. The gating strategy of flow cytometry analysis of T-cells (CD4/CD8), B-cells (B220), myeloid cells (Mac1/Gr1), and erythroid cells (Ter119) using flow cytometry analysis. Mice were treated with different concentrations of PBS or 1mg/kg, 10 mg/kg, or 100 mg/kg Yohimbine twice daily for 10 days following transplantation. After 16 week, bone marrow were harvested and were stained for myeloid and lymphoid markers and analysed by flow cytometry. Error bars represent mean + SEM from 2 or 3 recipients. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

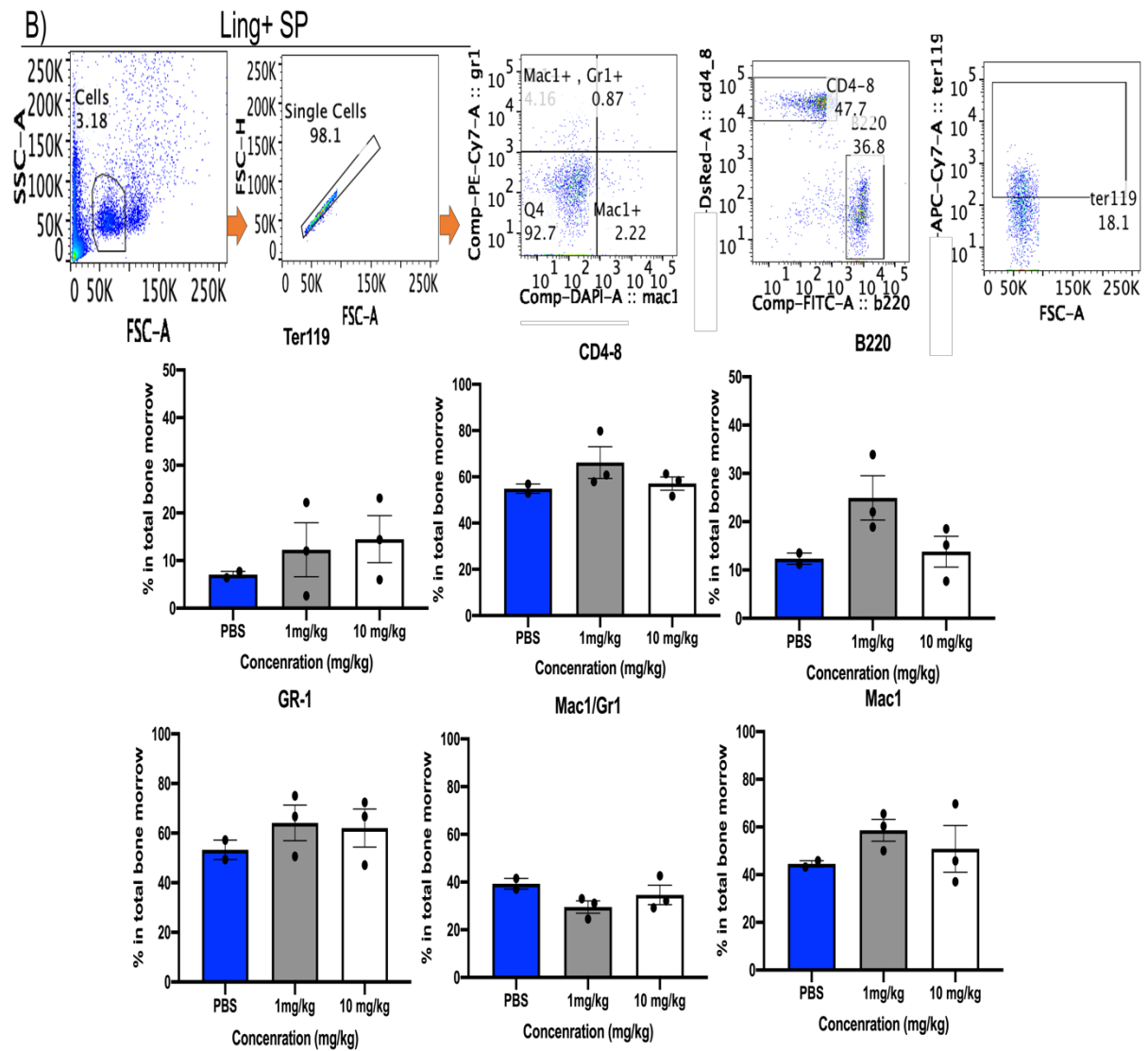


Figure 4.4 (B): The impact of Yohimbine administration following transplantation on the function of haematopoietic lineages in spleen. The gating strategy of flow cytometry analysis of T-cells (CD4/CD8), B-cells (B220), myeloid cells (Mac1/Gr1), and erythroid cells (Ter119) using flow cytometry analysis. Mice were treated with different concentrations of PBS or 1mg/kg, 10 mg/kg, or 100 mg/kg Yohimbine twice daily for 10 days following transplantation. After 16 weeks, bone marrow was harvested and were stained for myeloid and lymphoid markers and analysed by flow cytometry. Error bars represent mean + SEM from 2 or 3 recipients. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

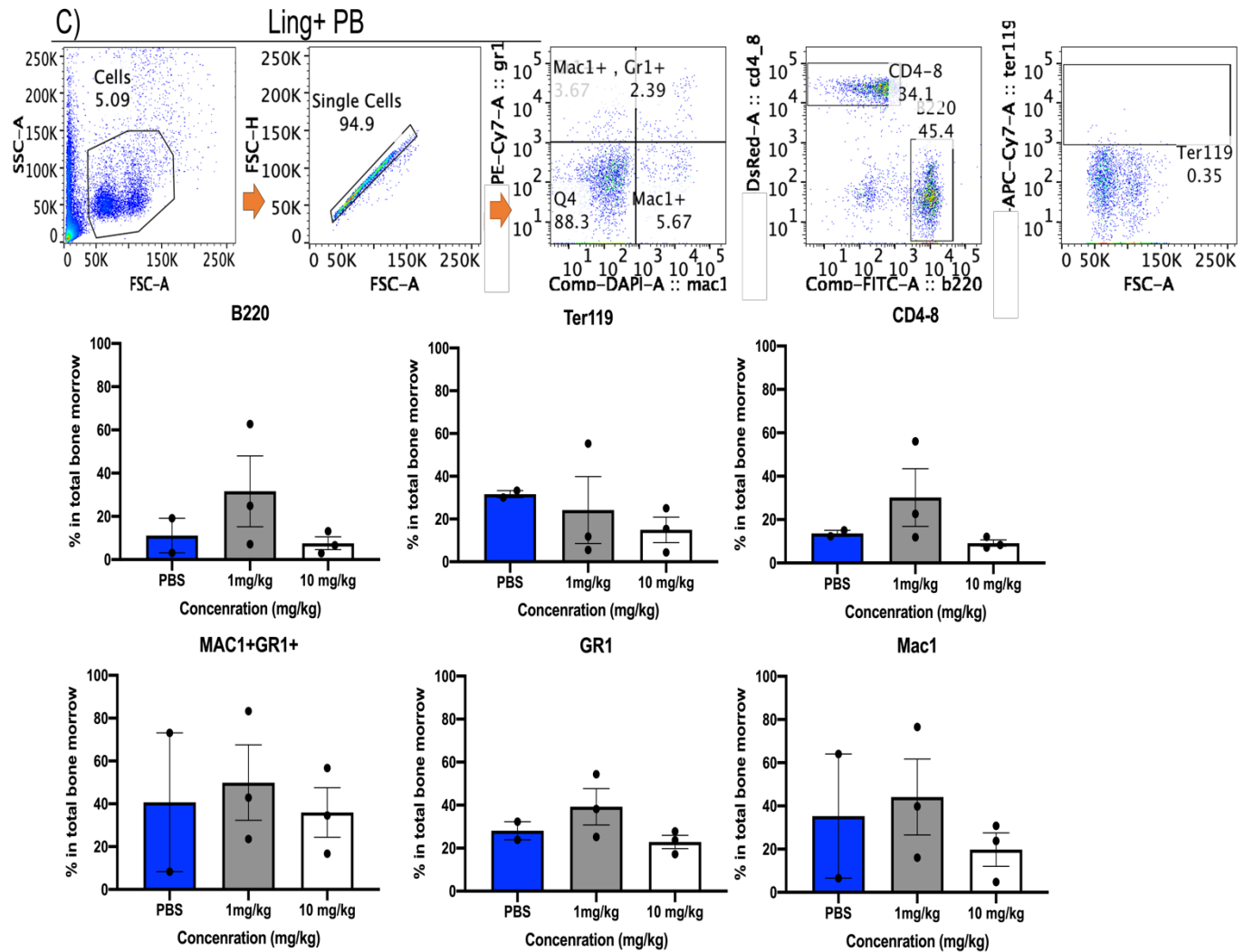


Figure 4.4 (C): The impact of Yohimbine administration following transplantation on the function of haematopoietic lineages in peripheral blood. The gating strategy of flow cytometry analysis of T-cells (CD4/CD8), B-cells (B220), myeloid cells (Mac1/Gr1), and erythroid cells (Ter119) using flow cytometry analysis. Mice were treated with different concentrations of PBS or 1mg/kg, 10 mg/kg, or 100 mg/kg Yohimbine twice daily for 10 days following transplantation. After 16 weeks, bone marrows were harvested and were stained for myeloid and lymphoid markers and analysed by flow cytometry. Error bars represent mean + SEM from 3 recipients. Statistical significance for different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.2 Yohimbine treatment does not impact survival of HSC and their progenitors after bone marrow transplantation

To assess whether Yohimbine treatment in the post-transplantation period influenced HSC or survival of committed progenitors, BM samples from recipients treated by Yohimbine were obtained and were further evaluated by Annexin V assay to track cell death within the LSK (Lin-Sca-1+c-kit+ HSC enriched fraction) or LK (Lin-Sca-1-ckit+ committed myeloid progenitor fraction). This analysis showed that Yohimbine treatment did not affect early or late apoptosis processes of HSPCs (Figure 4.5). The summary data confirm that yohimbine doesn't play role in survival of HSC.

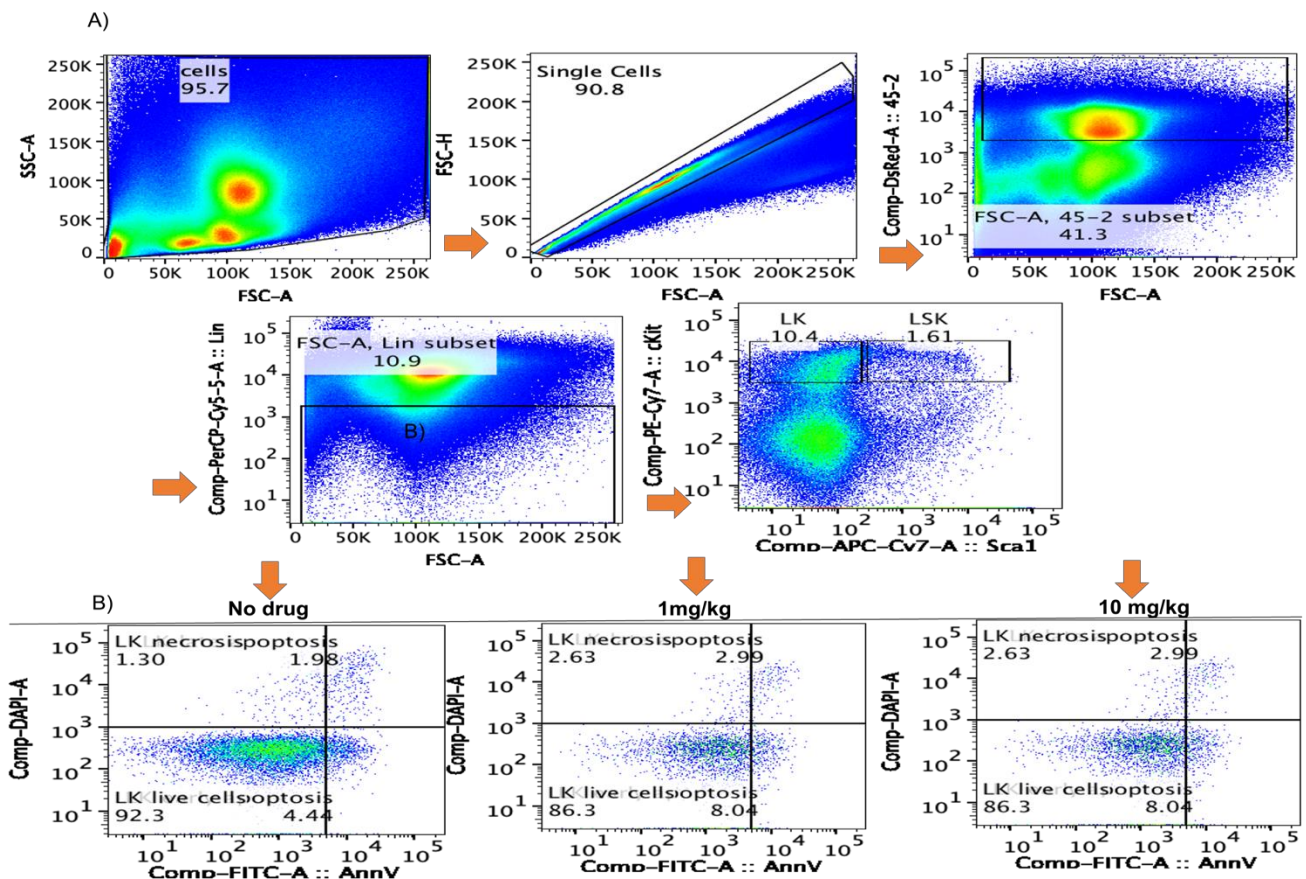


Figure 4.5: The impact of Yohimbine administration following transplantation on HSC and committed myeloid progenitor cell survival. A) The gating strategy of flow cytometry analysis for the Annexin V assay. Different stages of apoptosis were determined by Annexin V /DAPI: early apoptosis (AnnV+ DAPI-) and late apoptosis (AnnV+ DAPI+) B) Flow cytometry analysis with different concentration of drug. Error bars represent mean + SEM from 3 recipients.

4.3.6 Lack of impact of Yohimbine on undifferentiated iPSC proliferation

Previous findings by Lubaid Saleh in Dr. Rodrigues's laboratory and other investigators have indicated that Yohimbine can impact embryonic growth of blood cells in zebrafish (unpublished data). Moreover, as described above, previous data suggests that Yohimbine enhances early engraftment, suggesting an impact on haematopoietic differentiation. We therefore elected to assess whether Yohimbine augments haematopoietic differentiation of iPSCs. Firstly, to test the effect of Yohimbine on undifferentiated iPSCs, a proliferation assay was carried out. iPSCs were cultivated with various concentrations of Yohimbine over a 3-day period. The Cell Titre Blue assay was employed to estimate metabolic activity following exposure to Yohimbine (Riss, Moravec et al. 2016). Findings displayed in Figure 4.6 demonstrate that exposure of Yohimbine at the doses from 0.01 μ M to 1mM does not affect the proliferation of undifferentiated iPSCs (Figure 4.6).

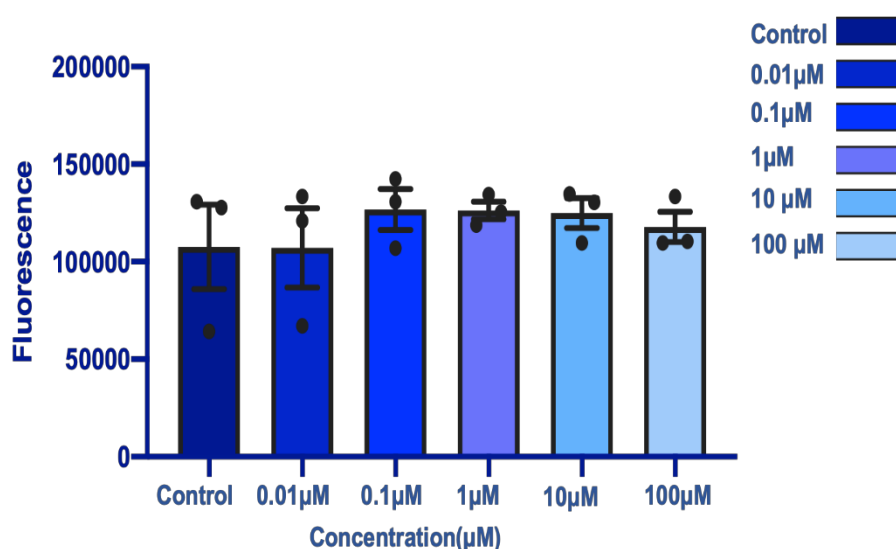


Figure 4.6: Yohimbine did not affect proliferation of iPSCs. iPSC cells were cultured in the presence of various concentrations of Yohimbine for 3 days and metabolic products were measured by Cell Titre blue method. The mean and standard error are presented for three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

4.3.6 Evaluating the effects of Yohimbine to enhance haematopoietic differentiation potential of iPSCs as assessed by immunophenotypic analysis

Next, Yohimbine capacities to promote differentiation from iPSCs to haematopoietic cells were tested directly. Initially, iPSCs were differentiated to EBs by withdrawal of LIF and by means of the hanging drop (HD) technique described in Chapter 3 (Dang, Kyba et al. 2002). To test the capacity of Yohimbine to enhance of the formation of EBs and subsequently on haematopoietic cells, during the formation of EBs in hanging drop culture, diverse doses (0.1µM, 1µM, 10µM and 100 µM) of Yohimbine were used. After 2 days passed, EBs were removed and added to liquid culture incorporating Yohimbine as well as haematopoietic cytokines Interleukin 3 (*IL-3*), Interleukin 6 (*IL-6*), Stem Cell Factor (*SCF*) and Erythropoietin (*rhpo*). These elements were integrated to stimulate and direct specification of haematopoietic lineage during and after EB formation at d0 of hanging drop culture and were termed d0 experiments (Lim, Inoue-Yokoo et al. 2013). In contrast, to test the impact of Yohimbine after mesoderm specification in EBs, separate cultures were established where Yohimbine and haematopoietic cytokines were added after EB formation at day 2 – these experiments were termed d2 experiments (Figure 4.7A). Yohimbine added during EB formation did not affect gross morphology of EBs (Figure 4.7B). EBs harvested at day 6 and 12 after liquid culture in d0 or d2 experiments were stained using antibodies for haematopoietic differentiation and analysed by flow cytometry (Figure 4.7C). Cell surface markers for erythroid progenitor, erythroid-myeloid progenitors, endothelial and haematopoietic cells or pan-haematopoietic were largely unaffected by the addition of Yohimbine before or after EB formation and during expansion in liquid culture for 6 or 12 days (Figure 4.8 – 4.9).

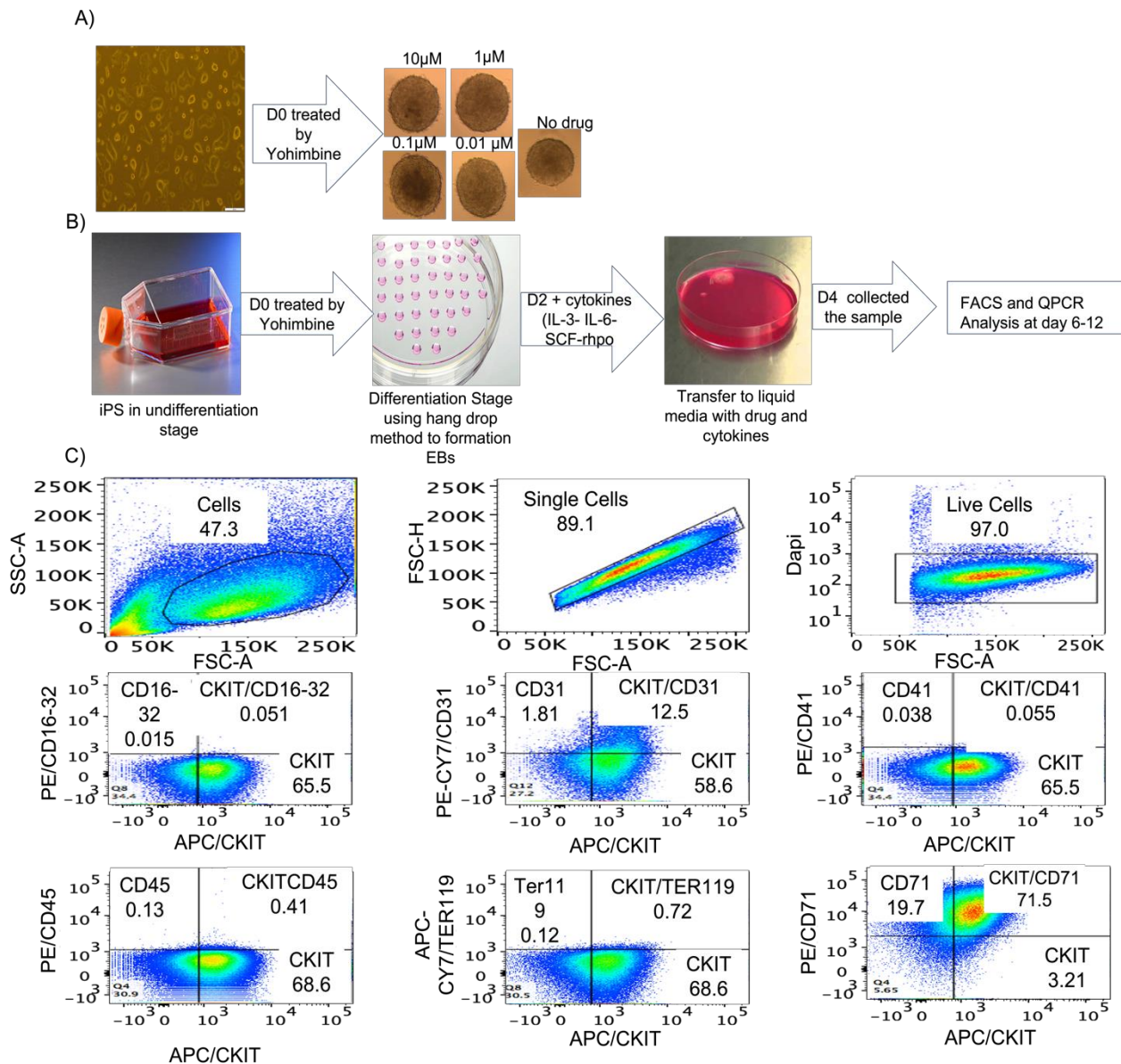


Figure 4.7: Examining the effect of Yohimbine on haematopoietic differentiation in iPSC system. A) Schematic of experimental design to examine the impact of Yohimbine on haematopoietic differentiation of iPSCs using the feeder free system. In some experiments, Yohimbine was added at d0 during EB formation and throughout liquid culture supplemented with haematopoietic cytokines (termed d0 experiments). In other experiments, Yohimbine was added in liquid culture after EB were formed by hanging drop method (termed d2 experiments). B) Yohimbine did not induce gross abnormalities in the formation of EBs from iPS cells at day 6 after induction. In the left image shows undifferentiated iPSCs before addition of drug. C) Dot plot of Flow Cytometry Profiles of haematopoietic cells derived from murine iPSCs stained for CD31, CD16-32, Ter119, CD45, CD71 and CD41. Gates were determined using isotype control for each marker. n=3.

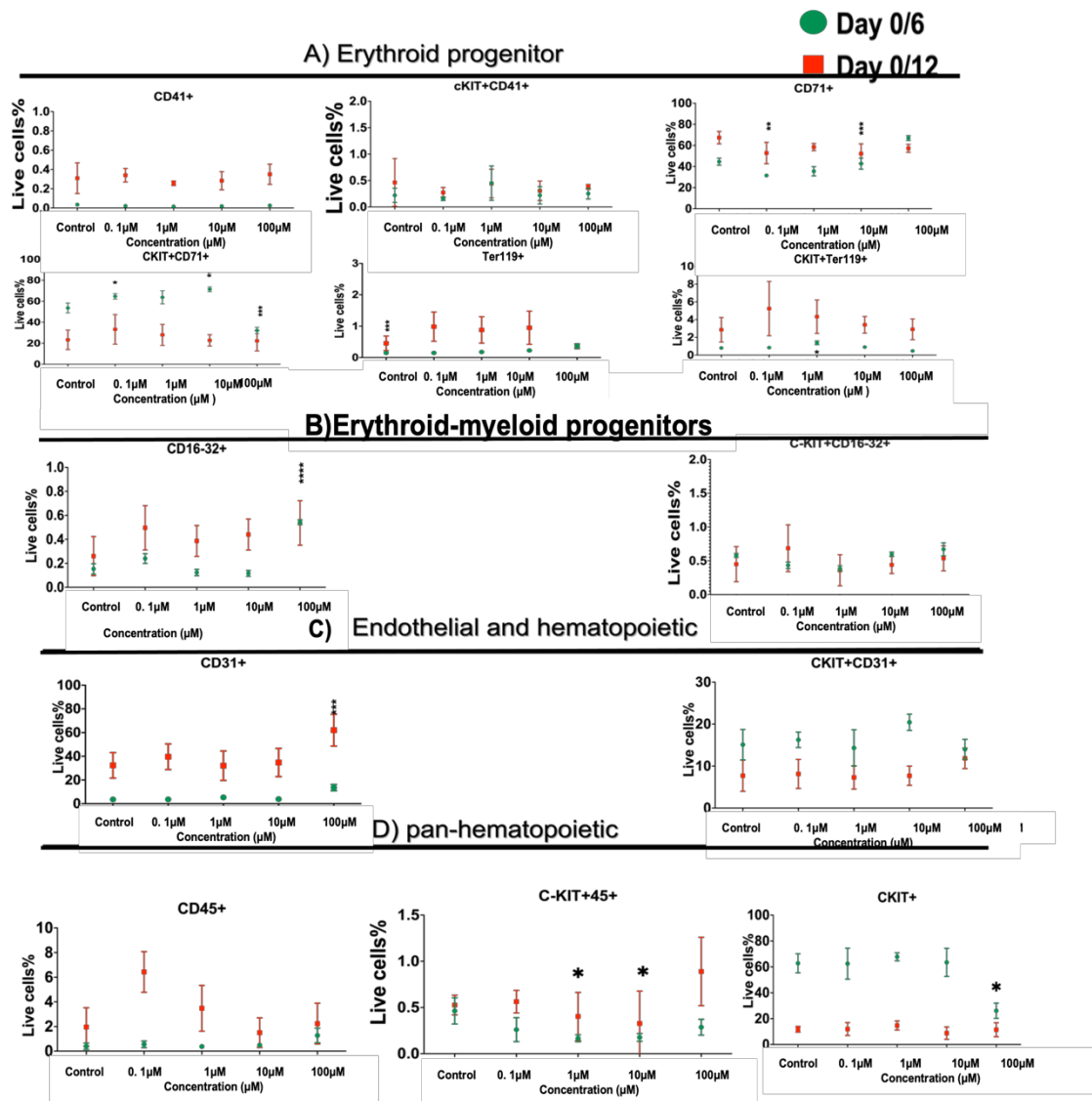


Figure 4.8: Temporal Immunophenotypic Analysis of iPSCs during Haematopoietic Differentiation in Response to Yohimbine exposure at Day 0 of EB formation. Graphs showing the percentage from differentiated of A) marker erythroid progenitor at days 6,12. (D6, Green-D12, Red; n=3). B) marker of erythroid-myeloid progenitors. C) Marker of endothelial and haematopoietic. D) Marker of Pan-haematopoietic (D6, Green-D12, Red; n=3). Statistical significance was of different concentration determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

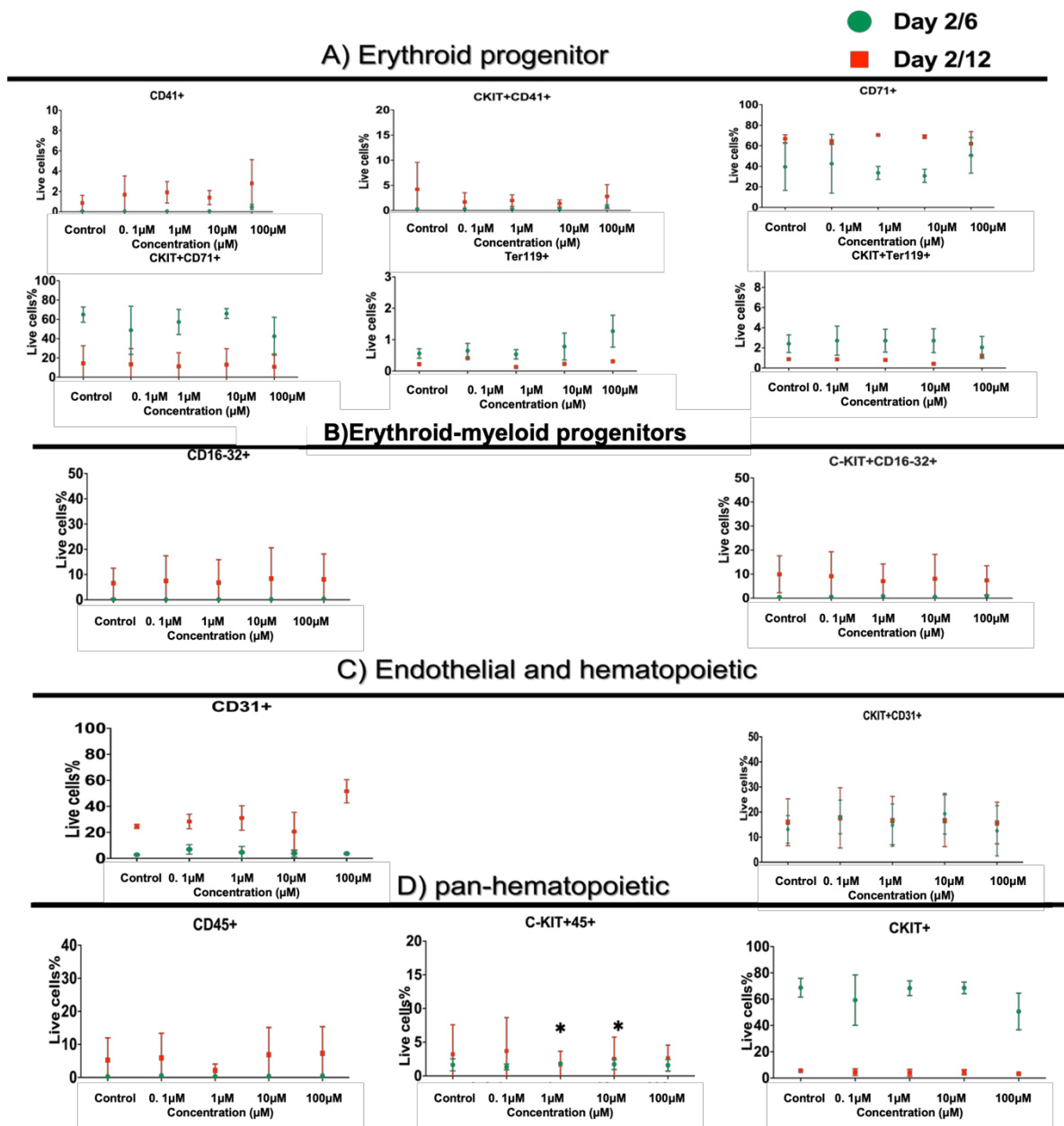


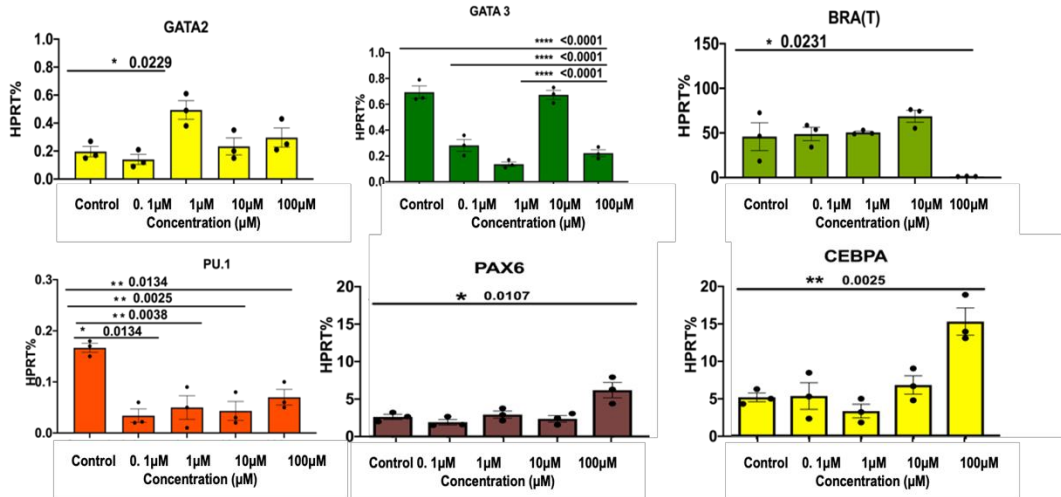
Figure 4.9: Temporal Immunophenotypic analysis of iPSCs during Haematopoietic Differentiation in Response to Yohimbine exposure at day 2 of EB formation. Graphs showing the percentage from differentiated of A) marker erythroid progenitor at days 6,12. (D6, Green-D12, Red; n=3). B) marker of erythroid-myeloid progenitors. C) Marker of endothelial and haematopoietic cells. D) Marker of Pan-haematopoietic cells (D6, Green-D12, Red; n=3). Statistical significance was of different concentration determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

4.3.6 Gene Expression Analysis of EBs following stimulation with Yohimbine

While cell surface markers indicative of haematopoietic differentiation appeared to be unaltered by exposure to Yohimbine during iPSC differentiation, I next performed comprehensive analysis of haematopoietic gene expression by Q-PCR to assess if Yohimbine-treated EBs demonstrated altered haematopoietic affiliated transcriptional programming. Gene expression analysis for d0 or d2 exposure to Yohimbine was performed. In d0 experiments, where EBs were exposed to Yohimbine during formation and were collected after a total of 6 days of liquid culture in haematopoietic cytokines and Yohimbine, upregulation of *GATA2* expression was observed at 1µM of Yohimbine, while *CEBPA* and *PAX6* were elevated at 100µM of Yohimbine (Figure 4.10 – 4.11). In d0 experiments where EBs were collected after 12 days of liquid culture in haematopoietic cytokines and Yohimbine, Yohimbine-treatment increased *GATA1* expression at 10µM, and 1µM; as well as *Cdh5* at 100µM of Yohimbine. An accompanying growth of *GATA2* and *SCL* expression was observed at 10µM of Yohimbine; similarly, *Sox7* and *Bra(T)* increased at 100µM; *RUNX1* expression increased at 0.1µM; *CEBPA* expression increased at 10µM and 100µM; and *PAX6* increased at 100µM of Yohimbine (Figure 4.10 – 4.11). With some exceptions (e.g., *PU.1* expression), these data generally indicate Yohimbine enhances expression of haematopoietic linked transcription factors during the initial formation of EBs and specification of haematopoietic lineages from iPSCs. Notably, these Yohimbine mediated changes in gene expression during the haematopoietic differentiation of iPSCs largely occurred at concentrations of between 10µM and 100µM Yohimbine.

A distinct dose response and gene expression signature was observed in d2 experiments, where EBs were exposed to Yohimbine after formation and were collected at day 6 or 12 of liquid culture in haematopoietic cytokines. In these experiments at either day 6 or 12 of liquid culture, expression of *CEBPA* increased at 100µM of Yohimbine (Figure 4.12 – 4.13). There was considerable growth in *GATA3* and *PU1* expression at 1µM, *Bra(T)* increased at both 1µM and 10µM of Yohimbine and *RUNX1*, *CEBPA*, and *PAX6* expression at 1µM, and *CDH5* increased at 10µM in comparison to Yohimbine-free EBs (Figure 4.12 – 4.13). Interestingly, addition of Yohimbine following EB formation elicited these changes in gene expression generally at a lower dose (1µM and 10µM), implying that once EBs are formed they display greater sensitivity to Yohimbine compared to EBs during formation. These data support the idea that, overall, haematopoietic promoting genes are upregulated after exposure to Yohimbine either during or after EB formation, though the precise pattern of gene expression is dependent on the timing of exposure to Yohimbine and/or the specific dose of Yohimbine.

A)



B)

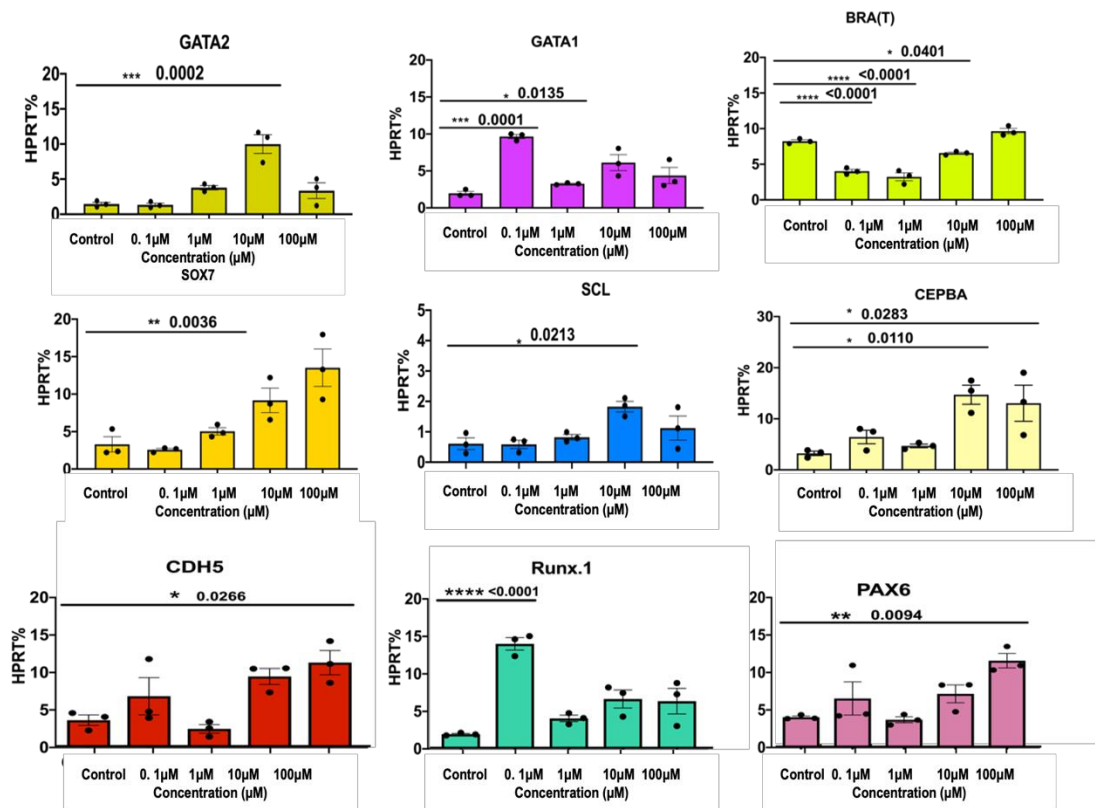


Figure 4.10: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 0 EBs. Gene expression was analysed at Day 6 (A) and day 12 (B) of liquid culture with haematopoietic promoting cytokines. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

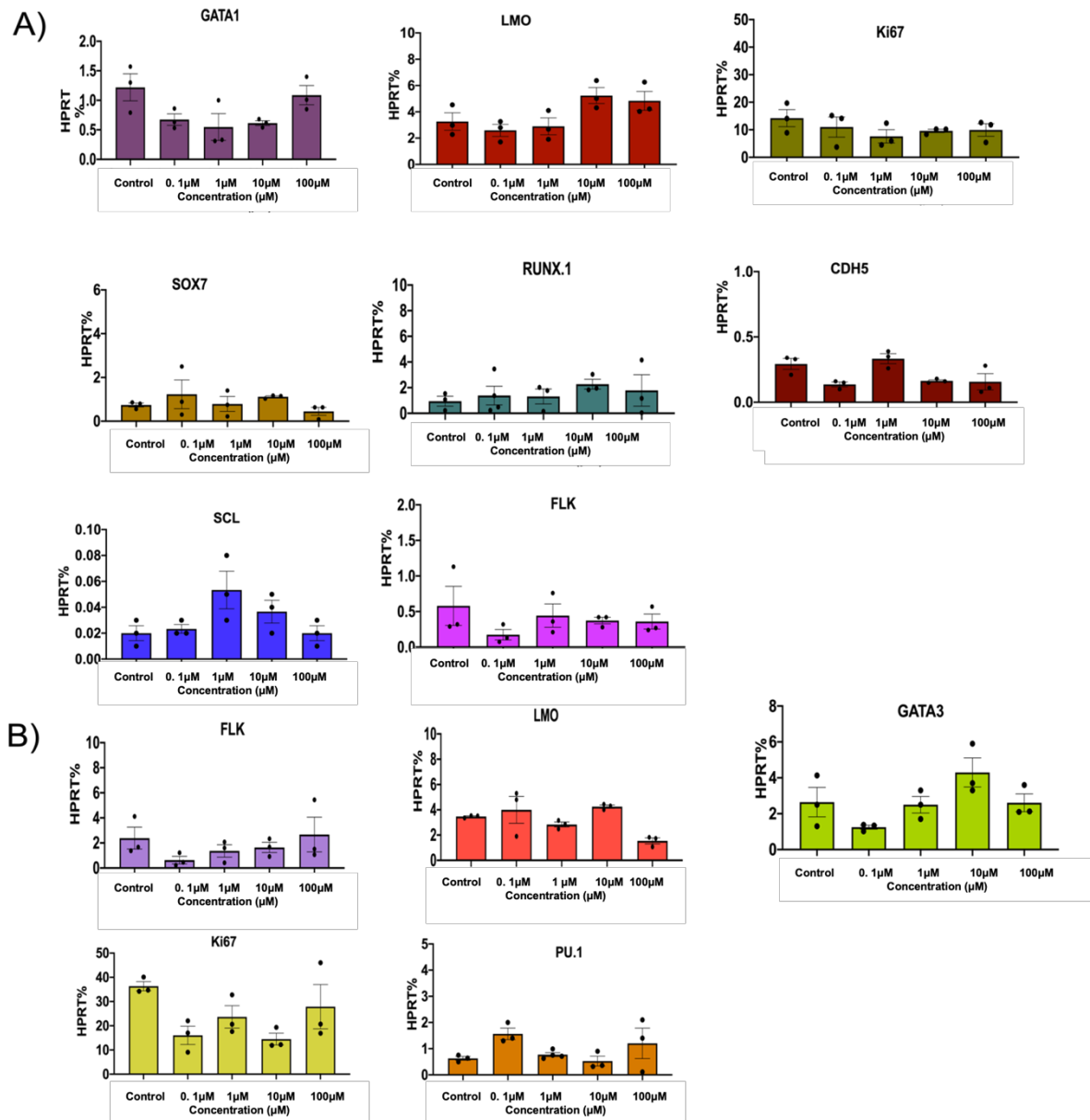


Figure 4.11: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 0 EBs. EBs treated with yohimbine at d0 and EBs were collected at day 6 (A) and day 12 (B). Data represent means \pm standard error 3 independent experiment. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

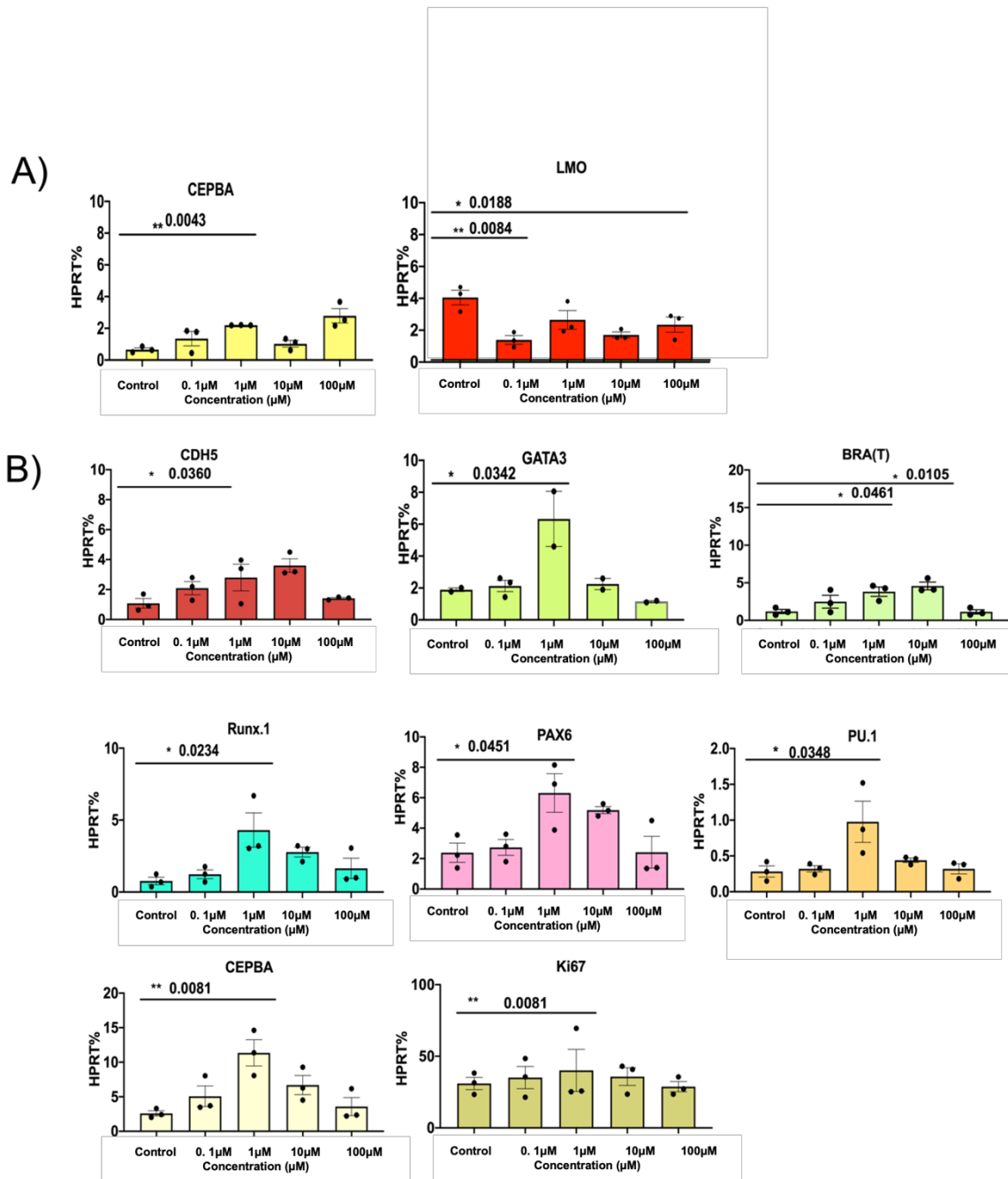


Figure 4.12: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 2 EBs. EBs treated with Yohimbine starting at d2 were collected at day 6(A) and day 12(B). Data represent means \pm standard error 3 independent experiment. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

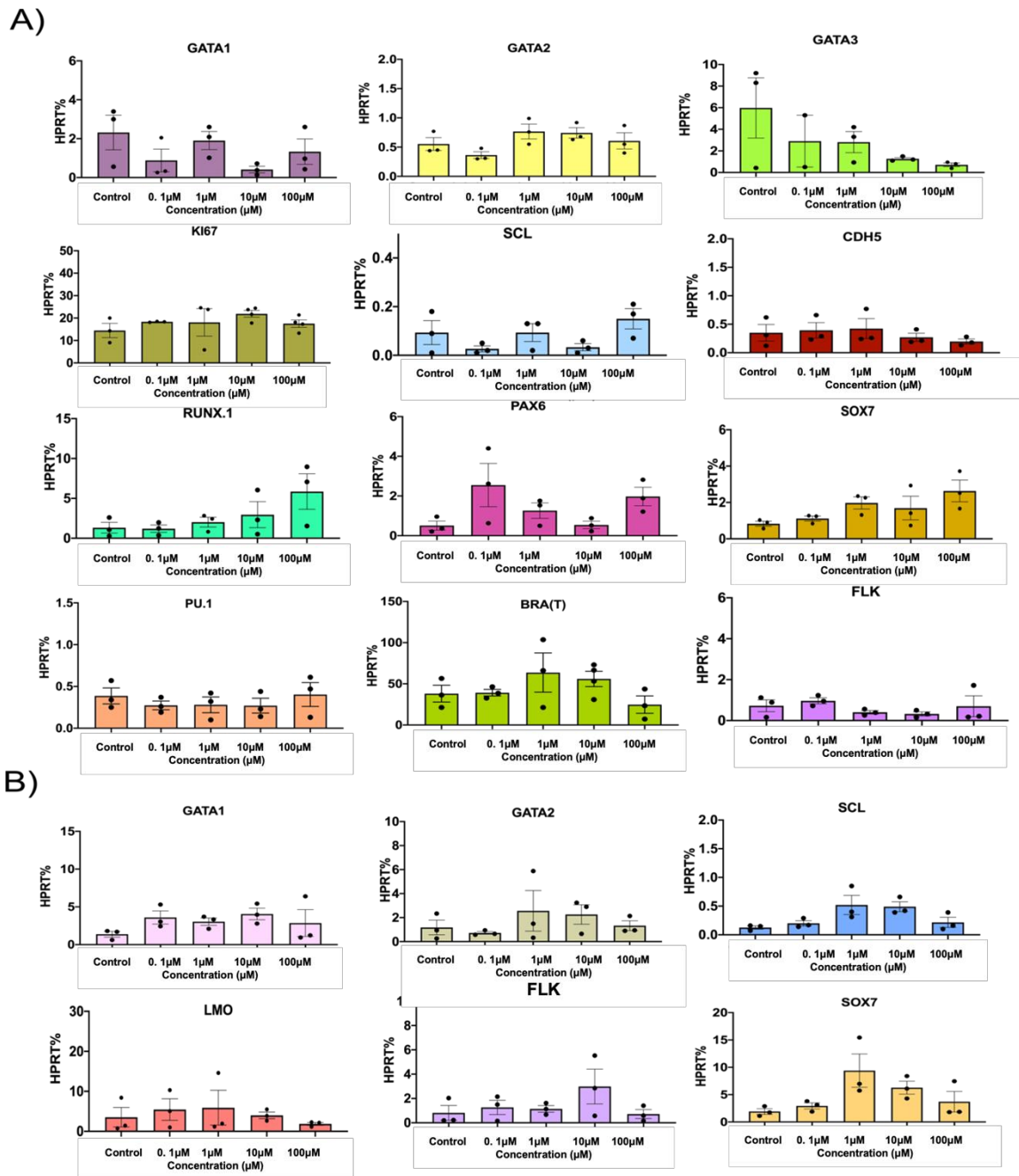


Figure 4.13: Gene expression analysis in differentiated iPSCs in response to Yohimbine treatment in day 2 EBs. EBs treated with yohimbine at day 2 after EB formation were collected at day 6 (A) and day 12 (B). Data represent means \pm standard error 3 independent experiment. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

4.3.6 Yohimbine enhances myeloid functional potential of iPSCs during haematopoietic differentiation

To functionally the impact Yohimbine-stimulated haematopoietic differentiation of iPSCs following exposure to Yohimbine, colony forming assays were conducted as described in Chapter 2 and Figure 4.14A. After exposure to Yohimbine during the two-step CFC differentiation assay, considerable, statistically significant growth was observed in CFU-GM progenitors, but not in other CFU subtypes, derived from iPSCs (Figure 4.14) indicating that Yohimbine enhances the myeloid functional potential of iPSCs during haematopoietic differentiation.

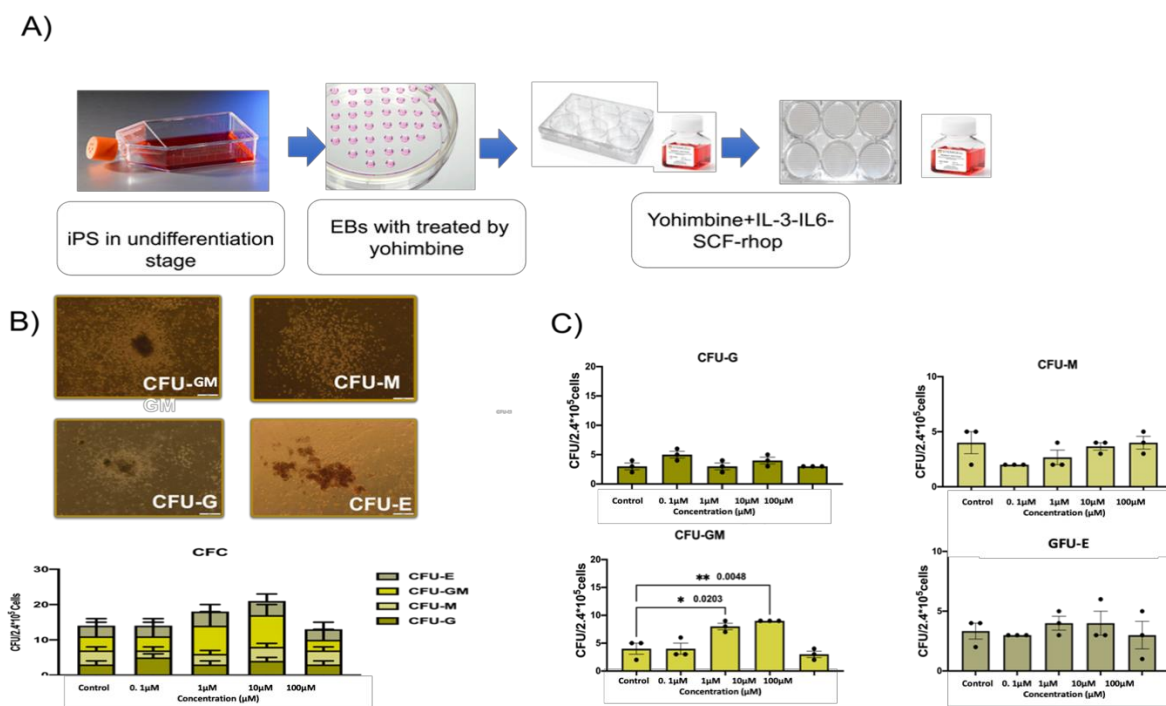


Figure 4.14: Representative morphology of haematopoietic colonies generated by different progenitor population from day12 EB-derived precursors. A) a schematic showing how CFC assay is conducted original colony magnification are (200 μ M), respectively. EBs collected after d12 in HD culture were assayed for colony formation in methylcellulose. B) Colonies were imaged after 12 days in culture. Colonies were categorized. C) Graphs showed methylcellulose CFC assay for erythroid (CFU-E), granulocyte/macrophages progenitors (CFU-GM), macrophages (CFU-M), granulocyte (CFU-G). Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The data were analysed using standard error of mean (SEM).

4- Discussion

There are major challenges in optimising treatment regimens in clinical haematology. For example, there is a shortage of blood cell components required for transfusion medicine (particularly red blood cells (RBCs) and platelets) that could theoretically be supplemented by enhancing or directing patient specifically iPSCs toward these haematopoietic lineages (Li, Cascino et al. 2017). Second, in transplantation medicine, reconstitution or rescue of the blood system in patients with BM containing HSPCs following high-dose chemotherapy is marked by slow rates of haematopoietic reconstitution (Hatzimichael and Tuthill 2010). As a result, patients often deal with risks of succumbing to infections, which would require administration of novel drugs to accelerate early haematopoietic reconstitution capacities, but without a long-lasting impact on the durability or functional capacities of transplanted HSCs (Hatzimichael and Tuthill 2010). In this chapter, I firstly examined the impact of Yohimbine on long-term HSC function. Our previous data demonstrated accelerated haematopoietic reconstitution following administration of Yohimbine after transplantation (Lubaid Saleh thesis, 2017). Here, my data show, promisingly, the minimal impact of Yohimbine on long-term functioning of HSCs, including differentiation capabilities. Thus, Yohimbine acts in a short-term manner to accelerate early haematopoietic reconstitution without long-term detrimental impacts on HSCs (Seita and Weissman 2010). While the precise roles of adrenergic regulation of haematopoiesis during BMT requires further experimentation using loss and gain of function approaches, this data is largely consistent with the notion that the regenerative capacities are under adrenergic control, mainly during regeneration phase following BMT (Maestroni, Conti et al. 1992). Also, of relevance to transplantation medicine in haematology, it is possible that Yohimbine, in its capacity as a vasomodulator, may also be considered as a novel HSPC mobilizing agent. Further experiments are needed to explore this possibility. While HSPC mobilization using G-CSF and/or CXCR4 antagonist AMD3100 is now the standard procedure for harvesting HSPCs for transplantation rather than harvesting directly from the BM, there are multiple issues that exist in current mobilisation regimens including cost, timeframe, side-effects, and problems mobilizing some donors (Smith-Berdan, Bercasio et al. 2019). Evidence for the use of vasomodulators has been demonstrated by Sildenafil citrate, also known as Viagra, which when taken by mouth in combination with AMD3100 leads to highly effective HSC mobilization that fares well in comparison to the standard 5-day regimen of G-CSF/Filgrastim/Neupogen (Smith-Berdan, Bercasio et al. 2019). In my studies examining the effect of Yohimbine in relation to haematopoietic differentiation of iPSCs, a tendency toward improvement of myeloid lineage was revealed. This was evidenced by the myeloid gene expression programme during differentiation of iPSCs following exposure to Yohimbine correlating with Yohimbine mediated enhancement of myeloid functionality in CFCs. Importantly, the gene expression programs activated by specific concentrations of Yohimbine

correlated specifically with concentrations of Yohimbine that enhanced myeloid function in the CFC assay. For example, myeloid related genes *GATA2*, *SCL*, *C/EBP α* were all activated at the same concentrations of Yohimbine that were effective for enhanced CFC-GM output in the CFC assay (1 μ M-10 μ M). Furthermore, these gene expression changes were elicited when Yohimbine was added at the initial stages of iPSCs differentiation i.e. prior to EB formation, suggesting that Yohimbine is an important driver of early haematopoietic specification. In support of this notion, it is known that *Gata2* plays pivotal roles in haematopoietic fate establishment (Eich, Arlt et al. 2018) and that it is also required in haemogenic endothelium for the emerging HSCs (Kang, Mesquitta et al. 2018). Similarly, *SCL* is required for early haematoendothelial specification (Lange, Hoffmann et al. 2020). However, Yohimbine also appears to impact the expansion of haematopoietic cells later following EB formation. After EB formation, Yohimbine stimulates a distinct, but nonetheless complementary myeloid gene expression, including *LMO*, and *PU.1*, that presumably coordinates enhanced myeloid differentiation. For example, *GATA2* and *Lmo2* coordinately feature in the regulation of haematopoietic differentiation (Coma, Allard-Ratick et al. 2013).

In closing, Yohimbine may be an important, promising therapeutic agent both in haematology and more widely in medicine, with applications ranging from transplantation to engineering myeloid cells for the treatment of solid cancer, and in drug screening applications. On the latter point, the ability to enhance myeloid cell output via Yohimbine stimulation during iPSC differentiation, may allow for large scale production of blood cell products for drug screening and further biological experimentation, where numbers are normally moderate under standard differentiation conditions and insufficient for these purposes (Tam, Worcel et al. 2001). Further work will be needed to explore whether pharmacologic targeting of adrenergic mediated haematopoietic reconstitution in the clinical setting will alleviate cytopenia, reduce the risk of patients experiencing opportunistic infections and, therefore, reduce the necessity for supportive care post-transplant. As Fig 4.3 at 100mg/kg, toxicology, as a scientific discipline, stands on the fundamental tenet of analysing a correlation between a toxic reaction and the quantity of poison used, i.e. a connection between the response and the dose. The toxic influences on a living organism refer to the quantity of exposure. Toxicity status emerges when exposure level prevails over a specific dose within a certain timeframe. The adequate therapeutic amount of drug dosage is limited enough. This means that the broader range of severe toxic influences may be examined only in contexts of evident overdose. Toxicity effect starts because of certain chemicals, while the body's response to these substances is directly associated with the dose. Furthermore, chemicals regarded as toxic can be eventually less detrimental and even useful at controlled lower doses. Safe and controlled doses for a diversity of chemicals have been legally established for treating humans.

CHAPTER 5:

Assessing the impact of Cis-2-Methyl-5-trimethylammonium methyl-1, 3-oxathiolane iodide (Oxa-22) on normal haematopoietic stem cells and on acute myeloid leukaemia cells

5.1 Introduction

5.1.1 Leukaemogenesis and Leukaemia stem cells

In the United Kingdom, leukaemia is the 15th most prevalent cancer type, representing 2% of all cancers (Cancer Research UK (2014)). Leukaemia represents the pathological process of hoarding undifferentiated WBCs (the so-called blasts (blasts) within the BM (Percival, Lai et al. 2017)). Leukaemia is divided into several sub-types with chronic and acute forms of the disease affecting both the myeloid and lymphoid lineages. Among chronic types, there is chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia (CML) while the two acute types are referred to as acute lymphocytic leukaemia (ALL) and acute myeloid leukaemia (AML) (Cancer Research UK (2014)).

Promoting and driving disease in all forms of leukaemia are relatively rare leukaemia stem cells (LSCs) (Horton and Huntly 2012) (Vu and Kharas 2018). These LSCs incorporate various biological properties that enable disease propagation. Thus, cells are found to be quiescent, and therefore highly resistant to many standard chemotherapeutic drugs, which require cells to be in actively cycling status (Saito, Kitamura et al. 2010). For this reason, many AML patients experience relapse after chemotherapy (Forman and Rowe 2013). Like healthy cells, LSCs display identifiable immunophenotypic surface markers (for instance, TIM3 (Akashi 2015)); additionally, they are able to self-renew, and incorporate differentiation into new populations of leukaemia cells (Horton and Huntly 2012). Finally, LSCs and leukaemia cells in general are dangerous for influencing other anatomical tissues in the organism, for example, causing conditions of hepatomegaly, splenomegaly and/or lymphadenopathy (Khwaja, Bjorkholm et al. 2016).

5.1.2 Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia (AML) is known as a collection of clonal haematopoietic stem cell diseases characterized by an inability to differentiate and by excessive proliferation patterns in the stem cell compartment, leading to concentration of non-functional cells titled as myeloblasts (Thomas and Majeti, 2017). The pathogenesis of AML includes a variety of molecular shifts disrupting approximately each aspect of the cell transformation process. These mechanisms involve the management of cell proliferation and dissemination, differentiation, self-renewal and recovery, survival rate, cell cycle checkpoint control, as well as DNA repair and chromatin stability. Adequate regulatory mechanisms get disrupted or misappropriated by these leukaemogenic episodes. Nevertheless, getting insight over these shifts is key for developing novel therapeutic approaches (Licht and Sternberg, 2005). The AML's molecular pathogenesis is structurally complicated, yet the numerous genetic flaws elaborated before can be practically understood to approach the improved meaning of the leukaemic cell's biological characteristics. In several examples, a vivid correlation between the molecular flaws and the biological patterns becomes evident; alternatively, an indirect or not fully covered interpretation for the disorder's molecular biological basis can be present (Licht and Sternberg, 2005). Upon having a finished and fuller understanding of such correlations, it is expected that more accurate and targeted therapies for curing AML might be designed. As current therapeutic strategies focus on managing the differentiation block as well as dissemination dynamics of AML, approaches on recovering genomic stability, inducing apoptosis of leukaemia cells, as well as repairing cell cycle checkpoint control must be thoroughly considered in the discourse of conducting future experimental therapies (DiNardo and Cortes, 2016). AML is viewed as the bone marrow disorder, a disease associated with of haematopoietic stem cells involving genetic defects in blood cell precursors. It leads to excessive production and release of neoplastic clonal myeloid stem cells. Technically, extramedullary expressions in forms of myeloid sarcomas or leukaemia cutis might take place; however, the disorder happens because of anomaly regarding haematologic cellular development (H Dohner, 2017). A minor number of case studies managed to define the cause-and-effect variables, including previous chemotherapy or other chemical experiences, but the most number of examples confirm that origins related to genetic mutations, via chromosomal anomalies or isolated gene changes, and with no evident causative factor. Distinguishing between these genetic anomalies is relevant in case of risk stratifying patients, as it predicts further adequate treatment strategy (Kantarjian et al., 2021). AML patients will represent a big variety of diagnostic cases. Some histories of disorder will be analysed and proven using a standard blood work; others are to be present with patients having symptomatic problems, including infection, disseminated intravascular coagulation or bleeding issues. Technically, bone marrow analysis is of most importance for

making the diagnosis and taking tissue biopsy for examination to properly categorize the AML subtype and expected severity level (Kantarjian et al., 2021). AML can be further categorized into three major risk groups: positive, moderate, and negative. The categories presented are designed by referring to cytogenetics and recent identification of molecular disorder subgroups distinguished from those contributing to cytogenetic risk factors. The newly identified molecular subgroups demonstrate alternative reactions to the routine therapeutic approaches. The risk groups managed to define the reaction to the routine therapy and survival rates in a single big retrospective study examining patients below the age of 55. The analysis revealed that the general survival dynamics at 5 years reached 44%; nonetheless, it reached other values upon enabling the risk categories. Hence, survival rates for positive, moderate, and negative risk groups were 64%, 41%, and 11%, accordingly. Generally, survival rates reduced among older adults, even though the differentiation of survival parameters was systematic and constant (Buccisano et al., 2010). AML management includes induction therapy measures along with post-remission treatment procedures. Induction therapy is designed to reach complete remission (CR) with, hopefully, having zero measurable residual disease (MRD). Previous research works revealed increased survival rates in patients who reached CR regardless of induction therapy typology. Selection of the original induction therapy depends on a patient's functionality (measured by comorbidities and general performance level), the disorder's biological profile (measured by predicting risk groups and recently identified molecular profiles of leukaemia cells) and the patient's overall health objectives. There are two widely applied induction therapies in case of AML, such as cytotoxic chemotherapy (targeted and non-targeted) and hypomethylating agents therapy (targeted and non-targeted) (Schlenk, 2014). Post-remission therapy is designed to prevent relapse of the disorder. There are two standard approaches: 1) complementary post-remission cytotoxic chemotherapy (for instance, moderate or high dose of cytarabine; targeted and non-targeted), or 2) allogeneic haematopoietic stem cell transplantation (titled as Allo SCT). Therapy selection is defined by the specific risk level and potential benefits from using a particular treatment plan. The threat of non-relapse mortality (NRM) gets increased in case of Allo SCT; nevertheless, the threat of relapse is lower. Additionally, Allo SCT is characterized by elevated morbidity level (associated with chronic graft vs host disorder, secondary complications and malignancies, or infection provoked by chronic suppression of immunity) (Schlenk, 2014). The ultimate reduction risk in terms of disorder relapse should be higher than the risk of NRM to rationalize the use of Allo SCT as a post-remission solution. Patients with negative profiles and the majority with moderate risk. In turn, patients with positive risk profiles might not worry about relapse scenario at higher probability if essential chemotherapeutic efforts are done, which means that health threats of Allo SCT will not be rationalized in this case. Thus, for a positive risk group of patients, induction therapy is supported by the consolidation therapy that presumes high dose cytarabine (HiDAC)

(Schlenk, 2014). Among all forms of leukaemia, AML remains to be the most prevalent type, developing in different life periods, from early childhood to the elderly, with the largest incidence in the elderly (Almeida and Ramos 2016). AML represents a quickly developing malignant white blood cell disorder provoked by somatic cell mutations within the haematopoietic stem or progenitor cells (Grove and Vassiliou 2014) and associated with a blockade of normal differentiation potential in the myeloid lineage (Table 5.1). If AML is not treated quickly, in matter of weeks or few months, disease-related symptoms will be lethal for a patient (Khwaja, Bjorkholm et al. 2016). Patients with AML demonstrate non-specific clinical signs – for instance, increased fatigue, breath shortness, high intentionality, and fever (Khwaja, Bjorkholm et al. 2016). The FAB classification in its system of disease classification of 4th edition, categorized AML by eight sub-types, from M0 to M7 (Alharbi, Pettengell et al. 2013) based on the level of progression, and differentiation intensity based on the leukaemia's source (Table 5.2). In the United Kingdom, statistics of remission among patients below 60 represents 80%; meanwhile, total survival rates are somewhere between 40 and 45% (Kantarjian, Kadia et al. 2021). For patients above 60, remission statistics raised toward 65% in a period between 1980 and 2000, although total survival rates remained at 12% after five years (Kantarjian, Kadia et al. 2021). Thus, AML remains a largely fatal disease in the elderly population, where the disease is most prevalent.

Table 5.1 Classification of AML mutations. The classification has been upgraded with inclusion of genetic mutations that have the positive prognostic potential. Also stimulated European Leukaemia Net (ELN) to be refined and upgraded with adding new risks for AML progression, such as favourable type, intermediate type, and adverse type (Herold, Rothenberg-Thurley et al. 2020).

Types of mutations	Genetic mutations
Mutations in nucleophosmin	NPM1 with FLT3-internal tandem duplications (ITD) (DiNardo and Cortes 2016).
Mutations in epigenetic modifiers: regulation of DNA methylation and chromatin modification	DNMT3, TET2, ASXL1, and IDH1/IDH2.
Mutations in transcription factors and master regulators	<i>CEBPA</i> , <i>RUNX1</i> , and <i>GATA2</i> .
Tumor suppressors	TP53.
Spliceosome complex	SRSF2, SF3B1, and ZRSR2.
Choesin complex	STAG1, STAG2, and SMC3.

Table 5.2 Different of FAB subtypes of leukaemia

FAB Subtype	
M0	Minimally Differentiated AML
M1	Myeloid Leukaemia without Maturation
M2	Myeloid Leukaemia with Maturation
M3	Acute Pro-granulocytic Leukaemia
M4	Myelomonocytic Leukaemia
M5	Monocytic Leukaemia
M6	Erythroleukaemia
M7	Megakaryocytic Leukaemia

5.1.2 Cis-2-Methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide (Oxa-22)

Cis-2-Methyl-5-trimethylammonium methyl-1, 3-oxathiolane iodide, Oxa-22, is an M3 muscarinic acetylcholine receptor agonist. Acetylcholine exerts several of its physiological processes through activation of a G-protein-coupled receptor (GPCR) family referred to as muscarinic acetylcholine receptors (mAChRs) (Kruse, Hu et al. 2012). The mAChRs have an important function in physiology because they control the heart rate, ensure contraction of the smooth muscles, regular the glandular secretion, and monitor other critical functions of the central nervous system (CNS) (Kruse, Kobilka et al. 2014). Consequently, acetylcholine, in its capacity as a neuromodulator, alters the conditions of neuronal networks and changes their reaction to both external and internal stimuli.

Muscarinic ACh receptors (mAChRs) are types of membrane receptor that initiate a number of metabolic steps to modulate cell activity (Broadley and Kelly 2001). Although all the five subtypes of mAChR (M1-M5) share high sequence homology levels, they have pronounced variation in the physiological response they mediate (Kruse, Hu et al. 2012) (Malbon 2005). Somatic and autonomic nervous systems are the primary areas where the cholinergic receptors operate with regard to signalling transduction. Ligand acetylcholine activates these receptors, which further subdivide into nicotinic and muscarinic. Nicotinic and muscarinic receptors are secondary to separate activating ligands (Carlson and Kraus 2022). Nicotinic receptors respond to the agonist nicotine and muscarinic receptors respond to muscarine. However, nicotinic receptors are ionotropic ligand-gated receptors and muscarinic receptors are G-protein coupled receptors (Carlson and Kraus 2022). In addition, nicotinic receptors operate within the central nervous system and at the neuromuscular junction, whereas muscarinic ones operate in peripheral and central nervous systems and mediate the stimulation to visceral organs (Carlson and Kraus 2022). There is a distinction in the way the two receptors perform signal transduction. In addition, the distinctions among receptor subtypes foster different biological outcomes both for the pharmacologic targets and the development of diseases (Carlson and Kraus 2022).

Clinical targeting muscarinic receptors could treat diverse pathophysiological conditions such as chronic obstructive pulmonary disease and overactive bladder through its action on smooth muscle contraction, gland secretion, indirect relaxation of vascular smooth muscle, and miosis (Broadley and Kelly 2001). Even though muscarinic receptor agonists and antagonists are characterised by many physiologic actions, the development of therapeutics is adversely affected by the absence of the small-molecule ligands capable of inhibiting or activating the specific mAChRs with high selectivity. Consequently, the specific physiological as well as

pathophysiological functions of the mAChR subtypes are still unclear. Nonetheless, the studies on the generation and phenotypic analysis of M1- to M5-knockout mice have been vital in expanding the current knowledge on biology of the individual mAChRs (Kruse, Kobilka et al. 2014).

Table 5.3: Biological roles of M1-5 receptors.

Muscarinic receptor subtype	Role in biology
M1- M3	Dilation and constriction in the vasculature (Gericke, Sniatecki et al. 2011).
M2	One type of functional subtype in the heart. along with Gi/o-coupled M2 receptor, makes it possible to design mAChR subtype-selective ligands (Kruse, Hu et al. 2012).
M3	Gq/11-coupled M3 mAChR ('M3 receptor', from rat) bound to the bronchodilator drug tiotropium and determine the binding mode for this medicament. The M3 receptor structure makes it possible to conduct a structural comparison between mammalian GPCR subfamily members with varied G-protein coupling selectivity (Kruse, Hu et al. 2012).
M4	The muscarinic acetylcholine receptor, cholinergic receptor, muscarinic 4 (CHRM4), pathway governs the self-renewal of the BFU-E. The pharmacological inhibition of CHRM4 ameliorates anemias in myelodysplastic syndrome (MDS), aging, and haemolysis. Genetic down-regulation of CHRM4 and the pharmacologic slowdown of CHRM4 by PD102807 enhanced the BFU-E self-renewal. Muscarinic acetylcholine receptor controls the self-renewal of early erythroid progenitors (Trivedi, Inoue et al. 2019).

Table 5.4: Role of muscarinic signalling (M3 in particular) in disease/cancer.

Type of cancer	Role the M3 in cancer
Brain Cancer	<ul style="list-style-type: none"> • Activation of M3 by cholinergic agonists increase the dissemination of a primary astrocytoma cell line. • M3 is expressed in astrocytoma.
Breast cancer	<ul style="list-style-type: none"> • Breast cancer cell line signify muscarinic receptors cell proliferation and angiogenesis. • The proliferation of the breast cancer cells is controlled by post-muscarinic receptor activating the signalling of ERK.
Colon cancer	<ul style="list-style-type: none"> • Colon epithelial cells express M1R and M3R • The receptors' activation fosters the cell proliferation that atropine suppresses.
Leukaemia	<ul style="list-style-type: none"> • M3 mAChR subtype receptor (M3-mAChR) has a role in enhancing cancer progression and dissemination (termed metastasis) and inducing the growth of cancer cells across a range of cancer subtypes and blockade of M3 has been suggested as a potential therapeutic strategy. An example of this is in gastric cancer where in mouse models gastric antral Lgr5+ gastric cancer stem cells robustly express M3-mACh and a coordinated denervation procedure based on botulinum toxin A as well as a blockade of muscarinic acetylcholine receptor 3 (M3R) successfully suppressed tumorigenesis (Hayakawa, Sakitani et al. 2017). It is also possible that M3-mAChR may play a role in leukaemia, where M3-mAChR expression has been differentially expressed across different subtypes with the highest expression observed in CML, and lowest expression in T-ALL. Expression of M3-mAChR in AML was found at high levels in the M1 and M2 subtypes (Suriyo, Chotirat et al. 2019). The functional significance of this expression of M3-mAChR in AML is unclear.

5.2 Aims and objectives

- As a prelude to understanding the impact of Oxa-22 on leukaemic cells, it is necessary to have a knowledge of its modulation of normal haematopoiesis. Previous work in the laboratory (Lubaid Saleh, Cardiff University 2017) demonstrated that Oxa-22 treatment increased the immunophenotypic abundance of HSCs and when Oxa-22 treated HSCs were transplanted *in vivo*, they enhanced both lymphoid and erythrocytic reconstitution. When Oxa-22 was administered to recipients of untreated HSCs to assess whether engraftment could be accelerated, minimal impacts were observed apart from accelerated erythrocytic reconstitution at two weeks after transplant.

The aims of this chapter:

1-To test the long-term impact of Oxa-22 in post-transplant haematopoiesis, serial transplantation was performed in this chapter to assess the long-term impact of *in vivo* treatment on HSPC behaviour.

2-To assessed whether treatment altered AML cell behaviour – in particular, apoptosis which is important to eliminate leukaemic cells by reactivating cell death pathways and is a promising therapeutic approach in (Cassier, Castets et al. 2017).

3- Determine the role of Oxa-22 could reactivate differentiation of myeloid markers CD11b and CD14. In AML normal differentiation potential is blocked; (Miraki-Moud, Anjos-Afonso et al. 2013).

4-To produce high-capacity *in-vitro* models of analysis with biologically important therapeutic end-point, namely cell death to screen and monitor the effect of on the Phthalylsulfathiazole on cancer cells.

5.3 Results:

5.3.1 Impact of Oxa-22 on normal HSC function *in vivo*

Transplanted mice that were treated post-transplant with Oxa-22 every other day for five days underwent secondary transplantation at 16 weeks after primary transplantation to measure long-term HSC functionality including differentiation capacity. Mice transplanted 5×10^5 from BM (expressing CD45.2) to 5×10^5 wild-type competitor (expressing CD45.1) BM cells (in a 1:1 ratio) following transplantation into lethally irradiated CD45.1 expressing syngeneic recipients. Oxa-22 was administered at different doses (10ng/kg, 100ng/kg or PBS control) to recipients by IP injection. At 16 weeks after transplantation, recipients were sacrificed and BM samples were obtained, and flow cytometry was used to analyse engraftment. Every 4 weeks during the study period following transplantation, peripheral blood was collected (Figure 5.1A). There was no impact on peripheral blood engraftment for the duration of the experiment (Figure 5.1B). At 16 weeks after secondary transplantation, mice were sacrificed, and the bone marrow of the mice was analysed. Analysis of primary recipients suggested an augmented HPC1, HPC2 compartments at 1 and 100 ng/kg, trend 2-fold and 1.5 increases in HSC and MPP compartments, respectively, while committed progenitor compartments were unchanged (Figure 5.2). Oxa-22 treated recipients also showed no alteration in differentiated haematopoietic cell engraftment except for TER119, which was reduced (Figure 5.3). Taken together with previous data from the laboratory, these findings showed that Oxa-22 may play a role in modulating HSC function without a direct impact on differentiation ability or apoptosis (Figure 5.4).

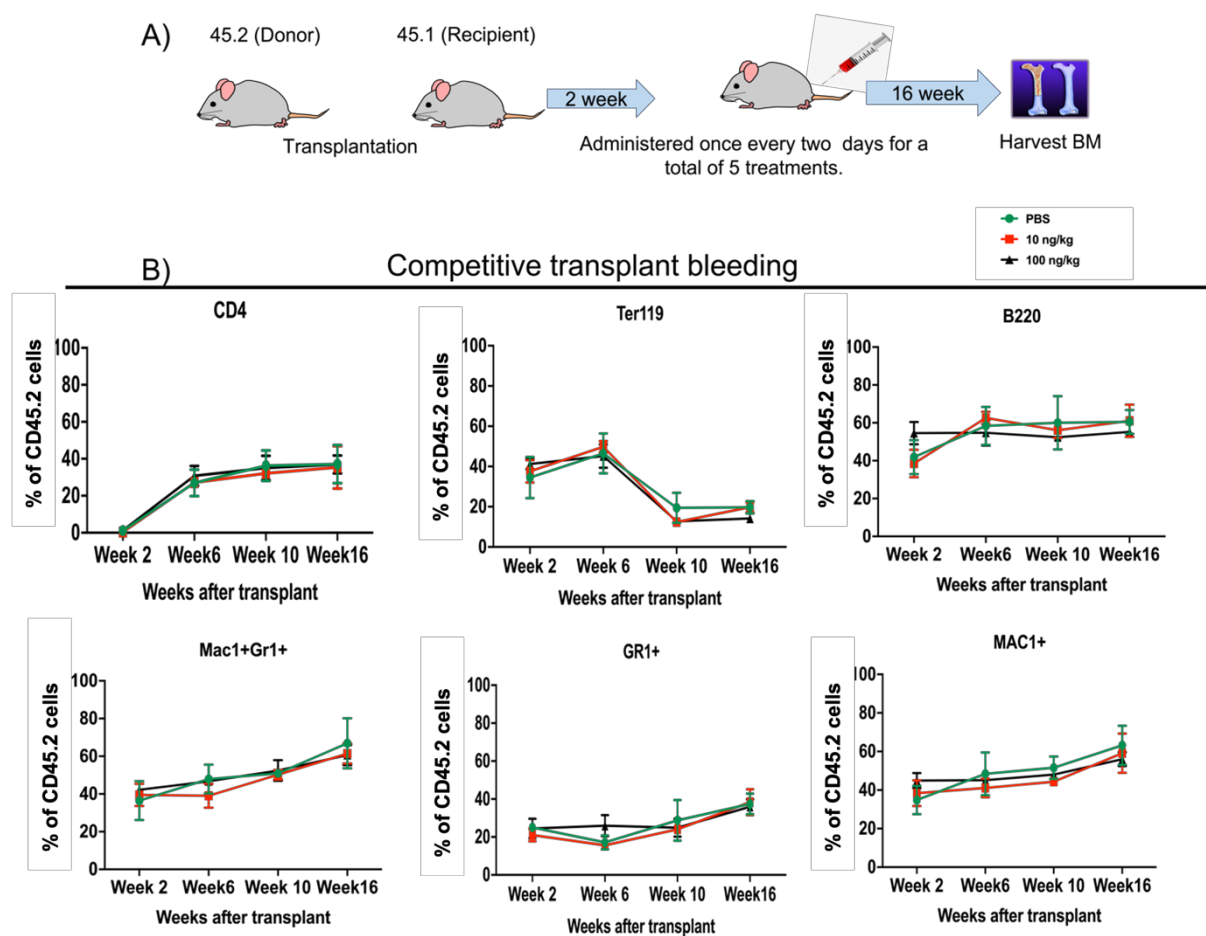


Figure 5.1: Experimental schematic of secondary transplantation of Oxa-22 treated recipients and analysis of peripheral blood engraftment. A) Scheme of experiment: transplanted cells from donor (CD45.2) to recipient cells (CD45.1) inject mice by yohimbine after two week from transplantation. **B)** Effect of Oxa-22 on lineage specific markers in peripheral blood. Error bars represent mean +SEM for 2-3 recipients. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

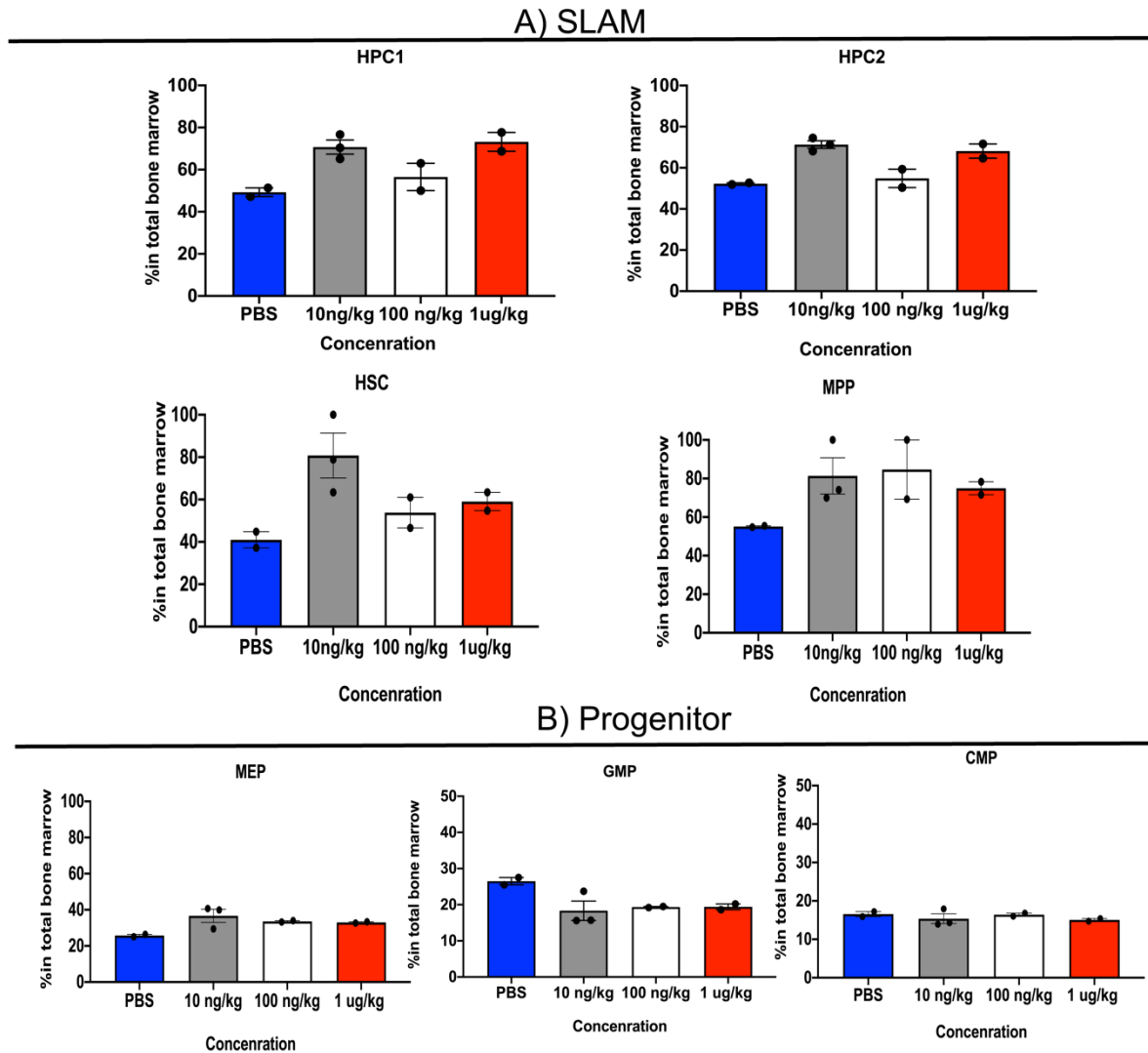


Figure 5.2. Effect of Oxa-22 treated HSPCs after secondary transplantation. At 16-week after secondary transplant, BM were harvested and stained for haematopoietic lineage. Error bars represent mean +SEM for 2-3 recipients. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$.**

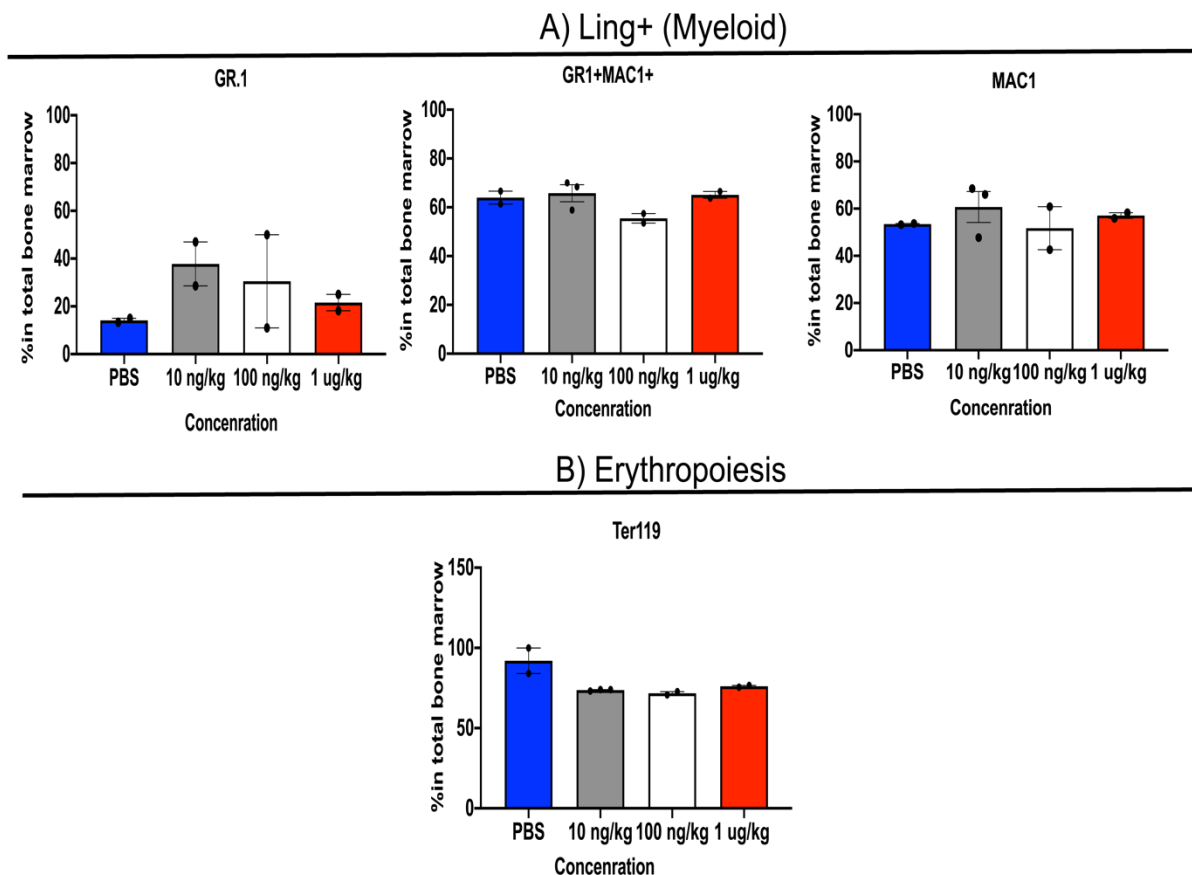


Figure 5.3: Effect of Oxa-22 after transplantation on the function of haematopoietic lineage in bone marrow of secondary transplant recipients. Mice treated with different concentration, after 16-week after secondary transplant, BM were harvested and stained for haematopoietic lineage. Myeloid (A) Lymphoid (B), and erythropoietic (C) engraftment are displayed. Error bars represent mean +SEM for 2-3 recipients. Statistical significance of different concentration was determined by one way ANOVA test. *p<0.05, **p<0.01, ***p<0.001.

LSK Annexin

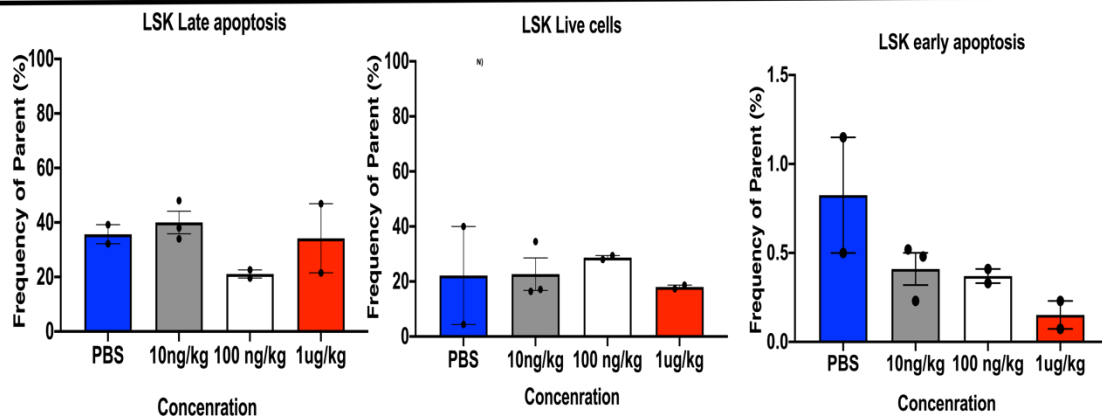


Figure 5.4 Effect of Oxa-22 treatment on HSPC apoptosis from secondary transplantation. Different stages of apoptosis were determined by Annexin V including LSK live cells, LSK early apoptosis, and LSK late apoptosis. Error bars represent mean +SEM for 2-3 secondary recipients. Statistical significance of different concentration was determined by one way ANOVA test. *p<0.05, **p<0.01, ***p<0.001.

5.3.2 Impact of Oxa-22 on differentiation of AML cells

Having assessed the impact of Oxa-22 on normal haematopoiesis, we next assessed the ability of Oxa-22 to impact leukaemic haematopoiesis. We used the AML cell lines, THP1 and NOMO-1, to assess whether Oxa-22 affected the cell fate of leukaemia cell line. We also examined the impact of Yohimbine and Phthalylsulfathiazole on AML cell lines as a comparison. We first asked whether Oxa-22 could relieve the differentiation block observed in AML, as judged by the myeloid markers CD11b and CD14 (Manzotti, Parenti et al. 2015) (Figure 5.5). The percentage of the differentiated cells in both the untreated and treated conditions within 72 hours were measured. A notable increase in the differentiation of marker CD11b was seen in THP-1 cells that were treated using Yohimbine at concentrations of 0.1 μ M and 100 μ M and increased in CD14 at 0.1 μ M and 1 μ M. Similarly, THP-1 cells treated with Oxa-22 showed a significant increased for CD14 at 0.1, 1 μ M and 1 μ M in THP-1 cells, while Phthalylsulfathiazole had no impact on differentiation status of THP-1 cells. In striking contrast no alterations in differentiation marker expression were noted in response to exposure of any of the small molecules in NOMO-1 AML cells. These findings demonstrate that Oxa-22 and Yohimbine, may play a significant role in enhancing the differentiation of THP-1 AML cells as shown in Figure 5.6.

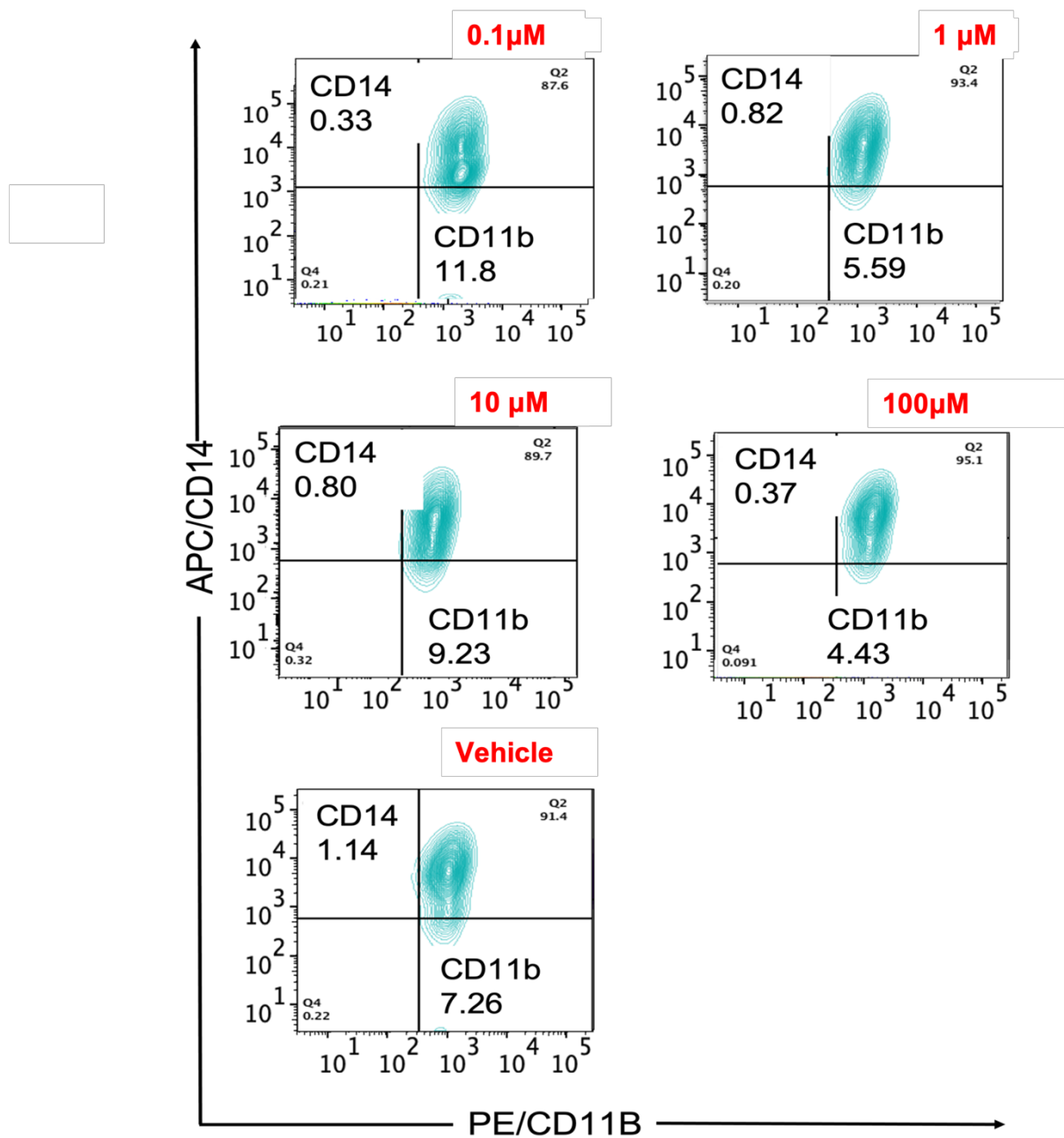
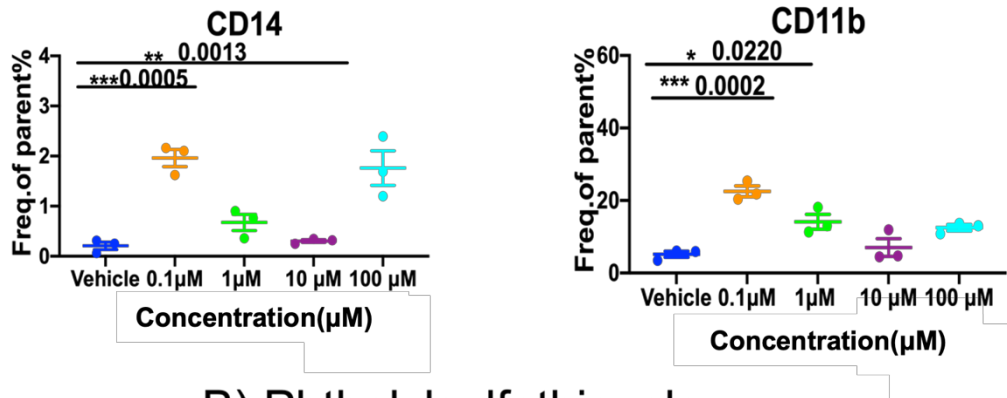
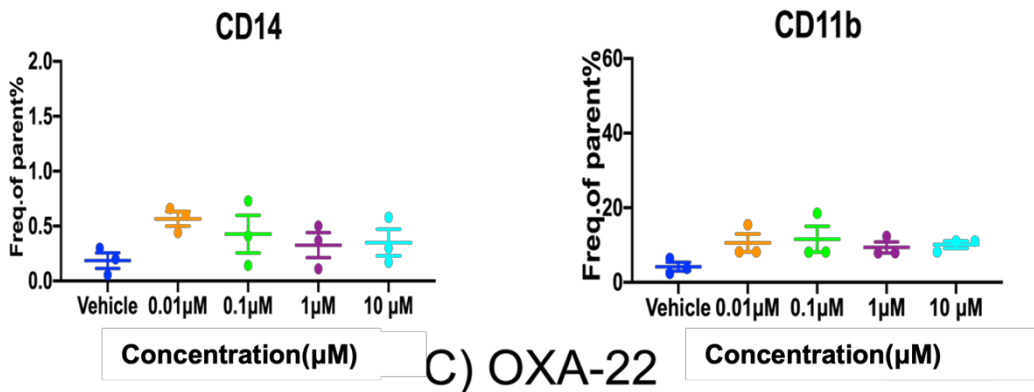


Figure 5.5: Flow cytometry gating strategy for assessing leukaemia cell line differentiation after exposure to Yohimbine, Phthalylsulfathiazole and Oxa-22. Gating scheme of quantification of CD14, CD11b in NOMO1 and THP1.

A) Yohimbine



B) Phthalylsulfathiazole



C) OXA-22

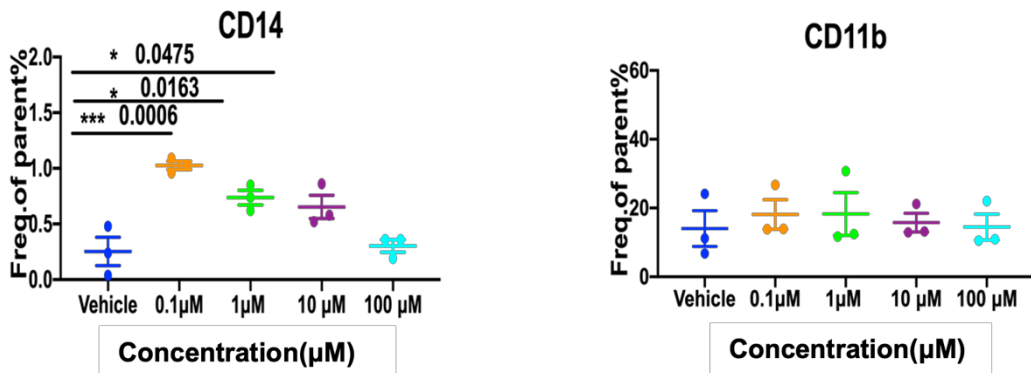
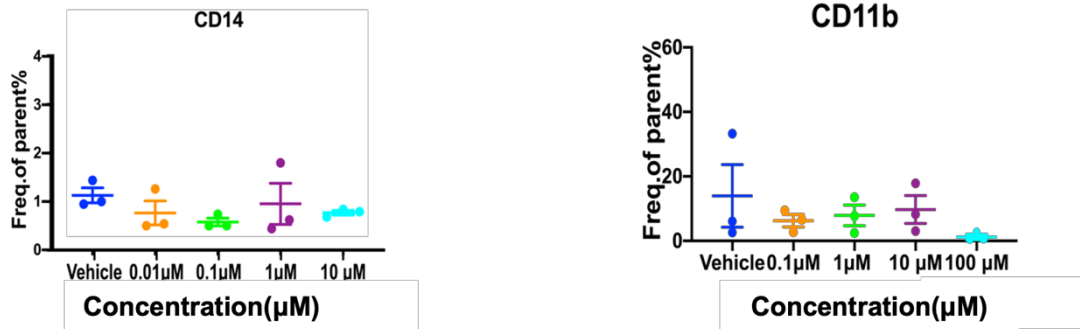
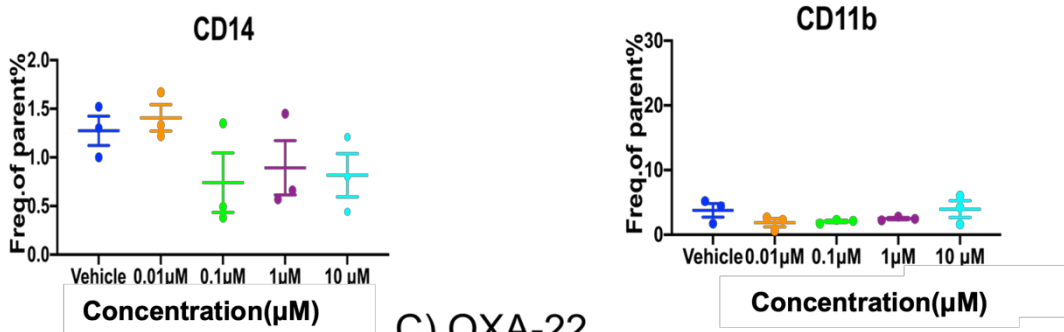


Figure 5.6. Oxa-22 and Yohimbine enhance THP1 cell line differentiation. Quantification data of CD14, CD11b in THP1 with different drugs. Error bars represent mean +SEM for 3 independent experiments. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A) Yohimbine



B) Phthalylsulfathiazole



C) OXA-22

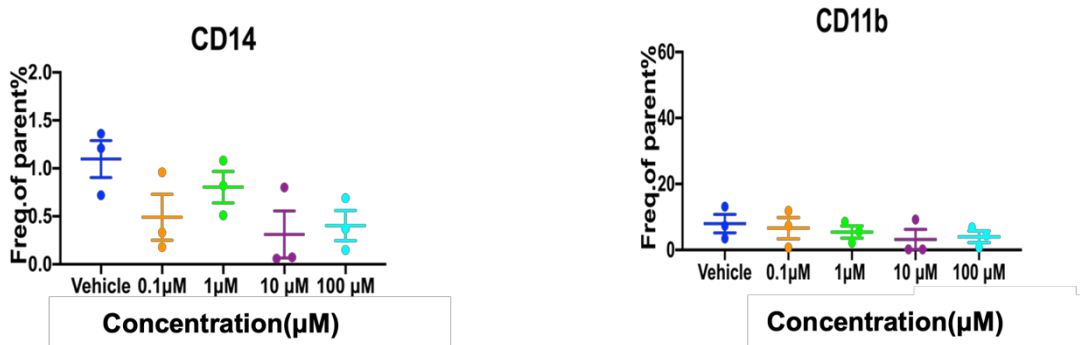


Figure 5.7. No impact of small molecules on NOMO1 cell line differentiation. Quantification data of CD14, CD11b in NOMO1 with different drugs. Error bars represent mean +SEM for 3 replicate. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.3 Impact of Oxa-22 on cell survival in leukaemia cell lines

Next, leukaemia cell lines were used to determine the change is associated with cell survival in NOMO1 and in THP1 treated with Oxa-22. Figure 5.8 shows representative gating for early apoptosis, necrosis, and Late-apoptosis, and live cells. The results shown in Figures 5.9 and 5.10 demonstrate that Oxa-22 and the other small molecules have no impact on cell survival in NOMO1 and THP1 leukaemia cell lines.

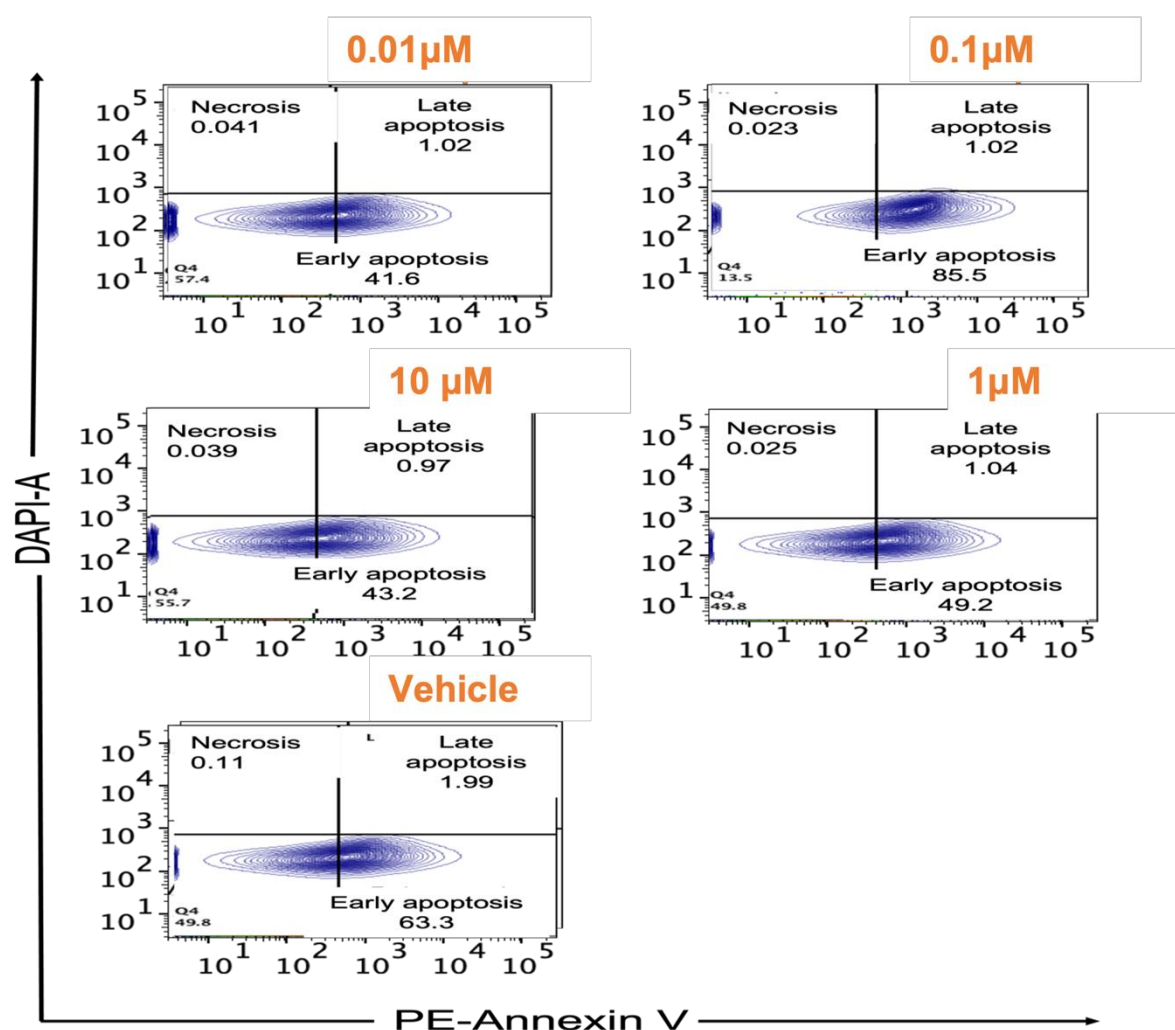


Figure 5.8: Flow cytometry gating strategy for assessing apoptosis in leukaemia cell lines. Quantification data of early apoptosis, necrosis, and Late- apoptosis, and live cell in Nomo1 and THP1 cells as judged by the Annexin V assay.

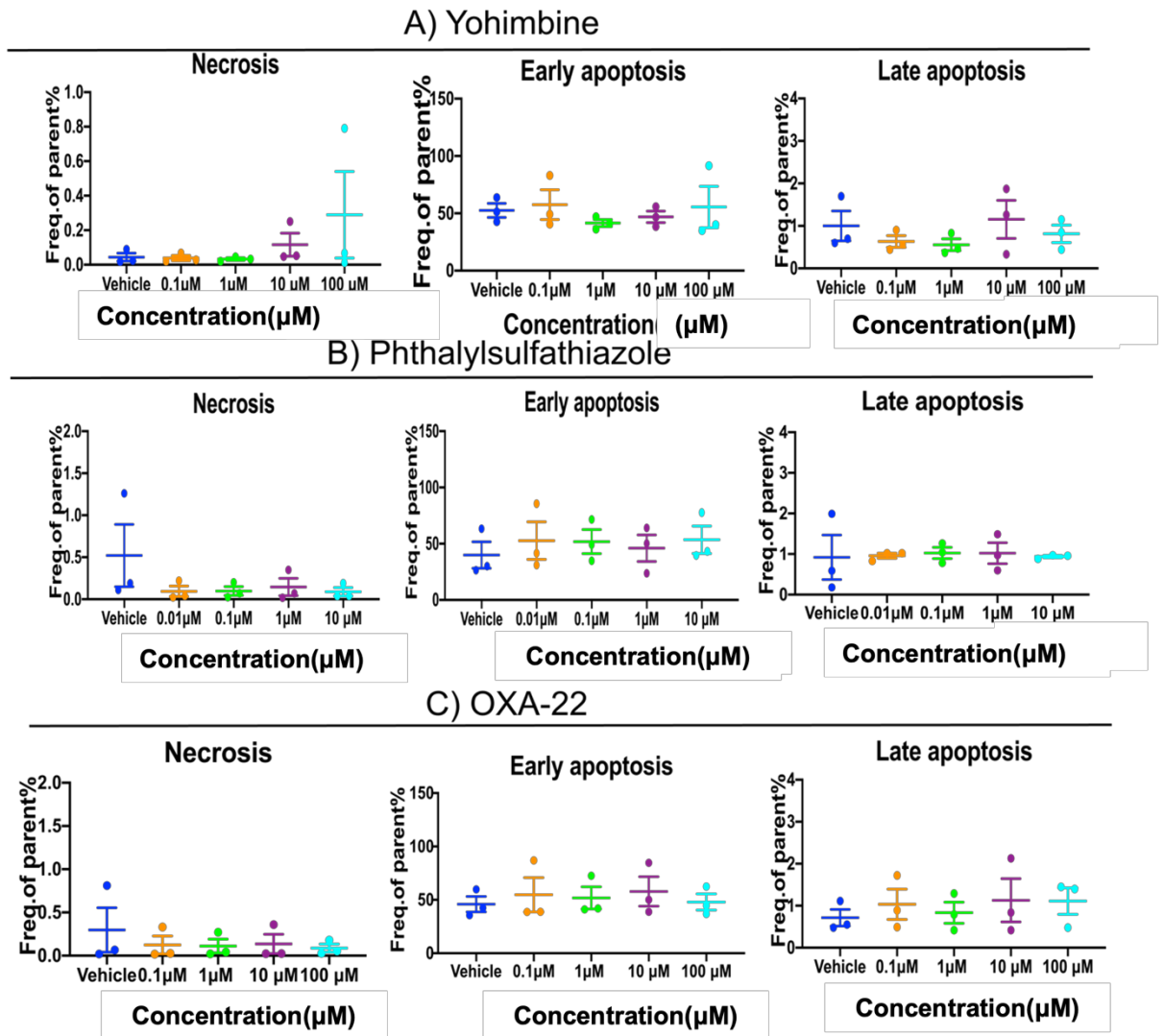
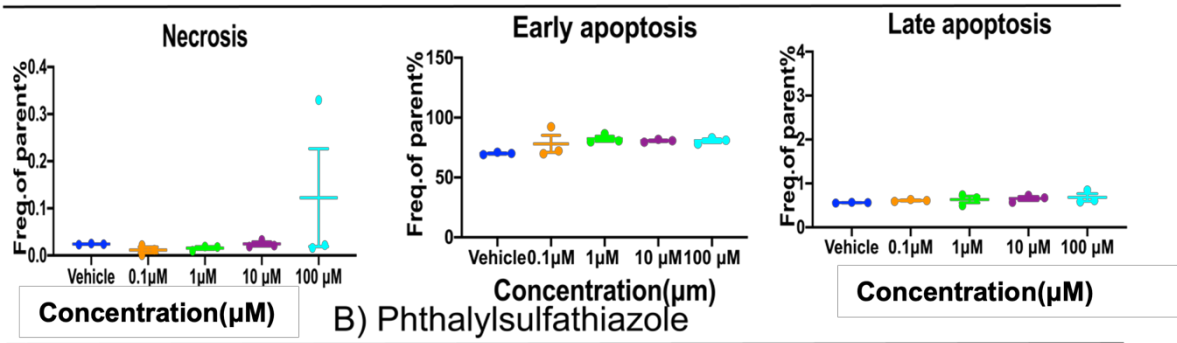
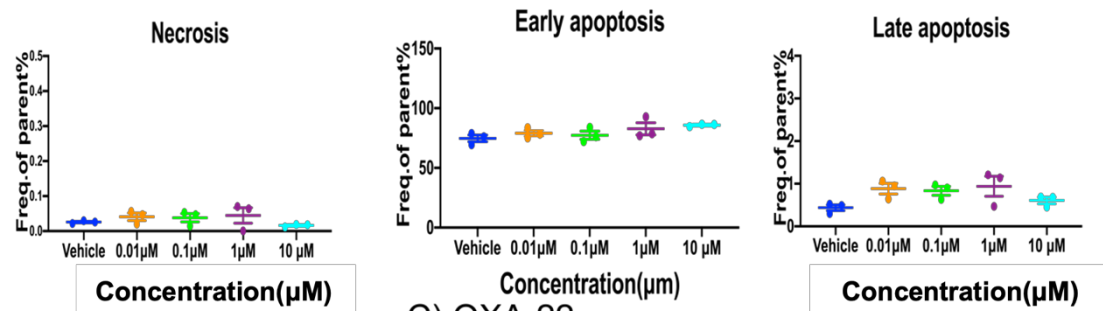


Figure 5.9: THP1 cell line apoptosis in response to Oxa-22 treatment *in vitro*. Quantification data of early apoptosis, necrosis, and Late- apoptosis, and live cell in THP1 with different drugs. Error bars represent mean +SEM for 3 replicate. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A) Yohimbine



B) Phthalylsulfathiazole



C) OXA-22

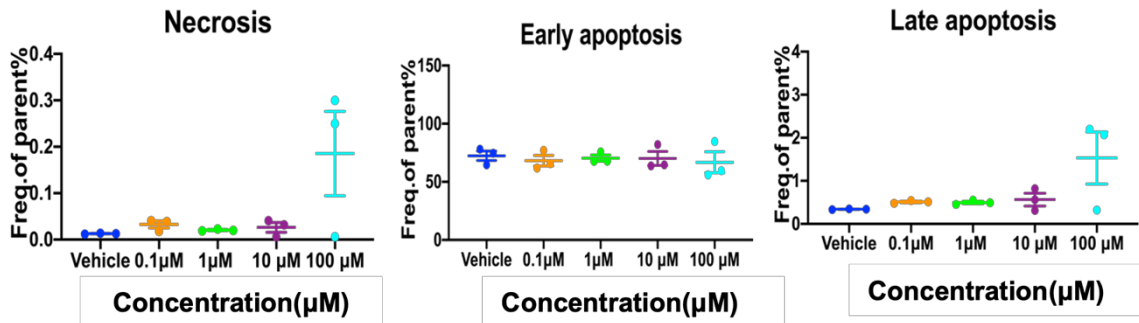


Figure 5.10. NOMO1 cell line apoptosis in response to Oxa-22 treatment *in vitro*. Quantification data of early apoptosis, necrosis, and Late- apoptosis, and live cell in Nomo1 with different drugs. Error bars represent mean +SEM for 3 replicates. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.4 Impact of small molecules on cell cycle activity of AML cells

In the absence of an impact of cell survival in response to Oxa-22, we next examined whether Oxa-22, and Yohimbine caused cell cycle alterations as assessed by *Ki67*, a marker of cell proliferation. However, at three days after the cells are treated with each different drug, there was no significant difference in G0, G1, and SG2 fractions compared to untreated control in each AML cell line (Figure 5.11 —5.13).

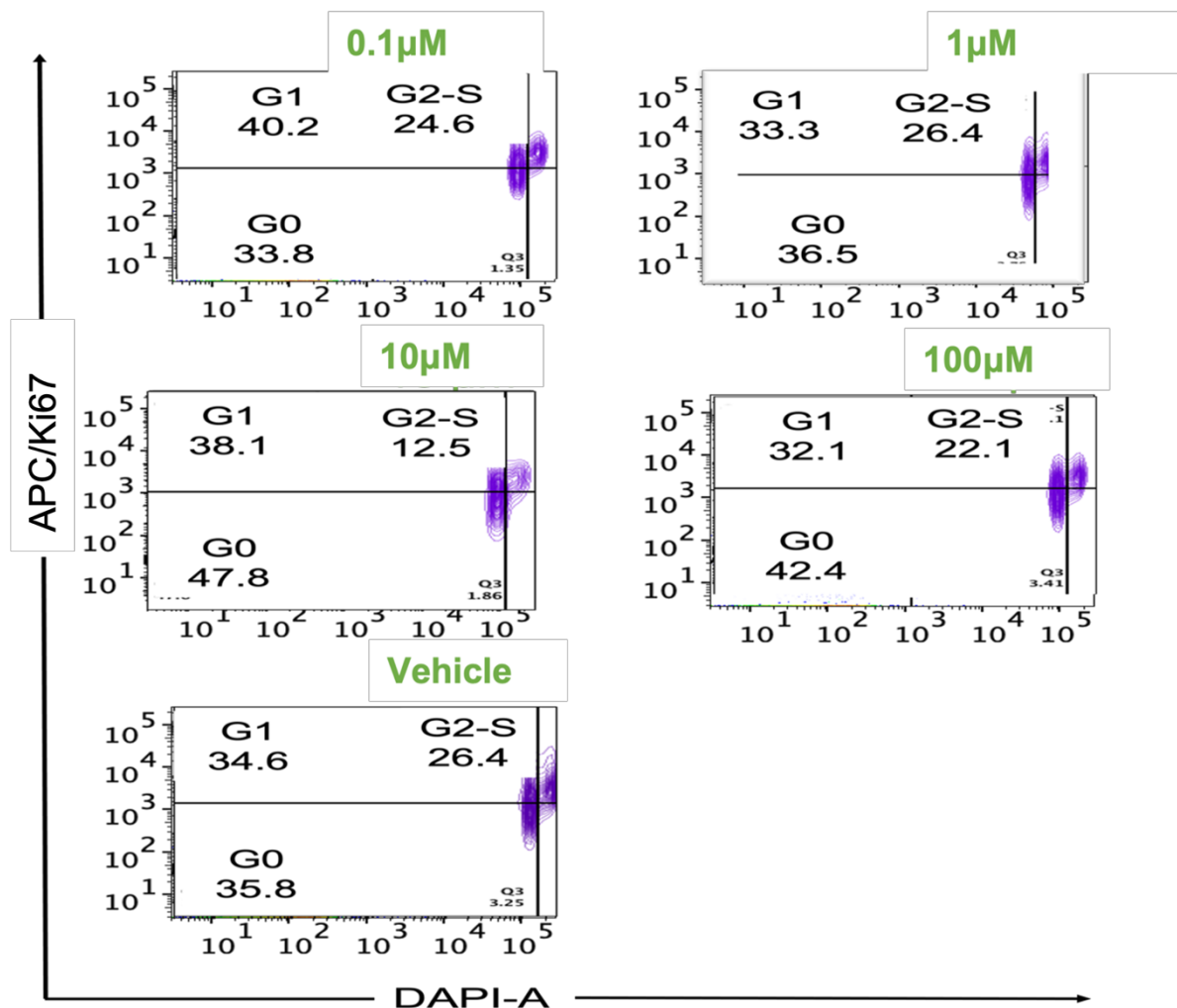


Figure 5.11: Flow cytometry gating strategy for Leukaemia cell line cell cycle with different drugs and different concentration. Quantification data of G0, G1, and G2S in THP1. Statistical significance was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

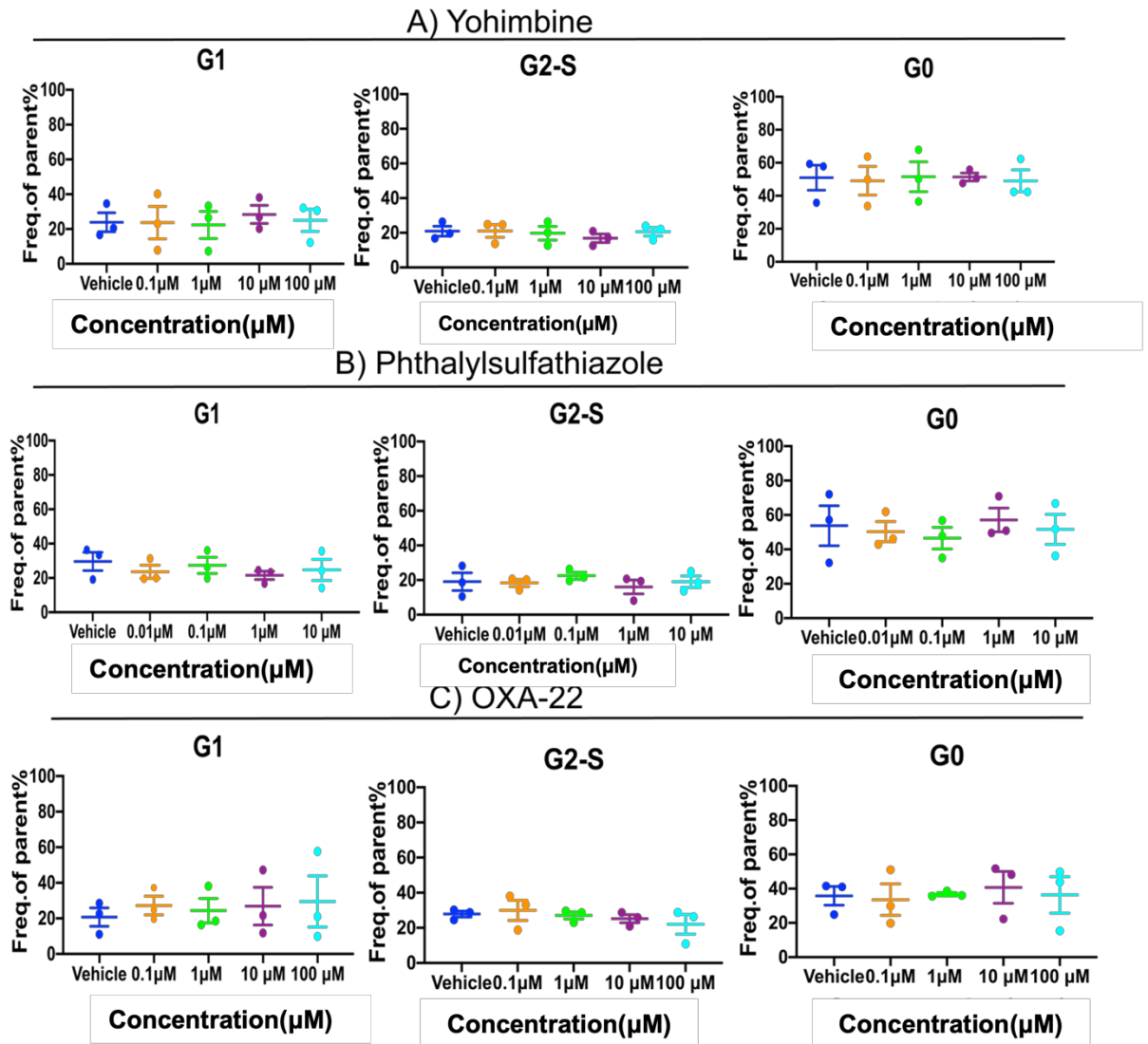


Figure 5.12. THP1 cell line cell cycle status in response to Oxa-22 treatment *in vitro*. Quantification data of G0, G2S, and G1 in THP1. Error bars represent mean +_SEM for 3 replicate. Statistical significance of different concentration-S was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

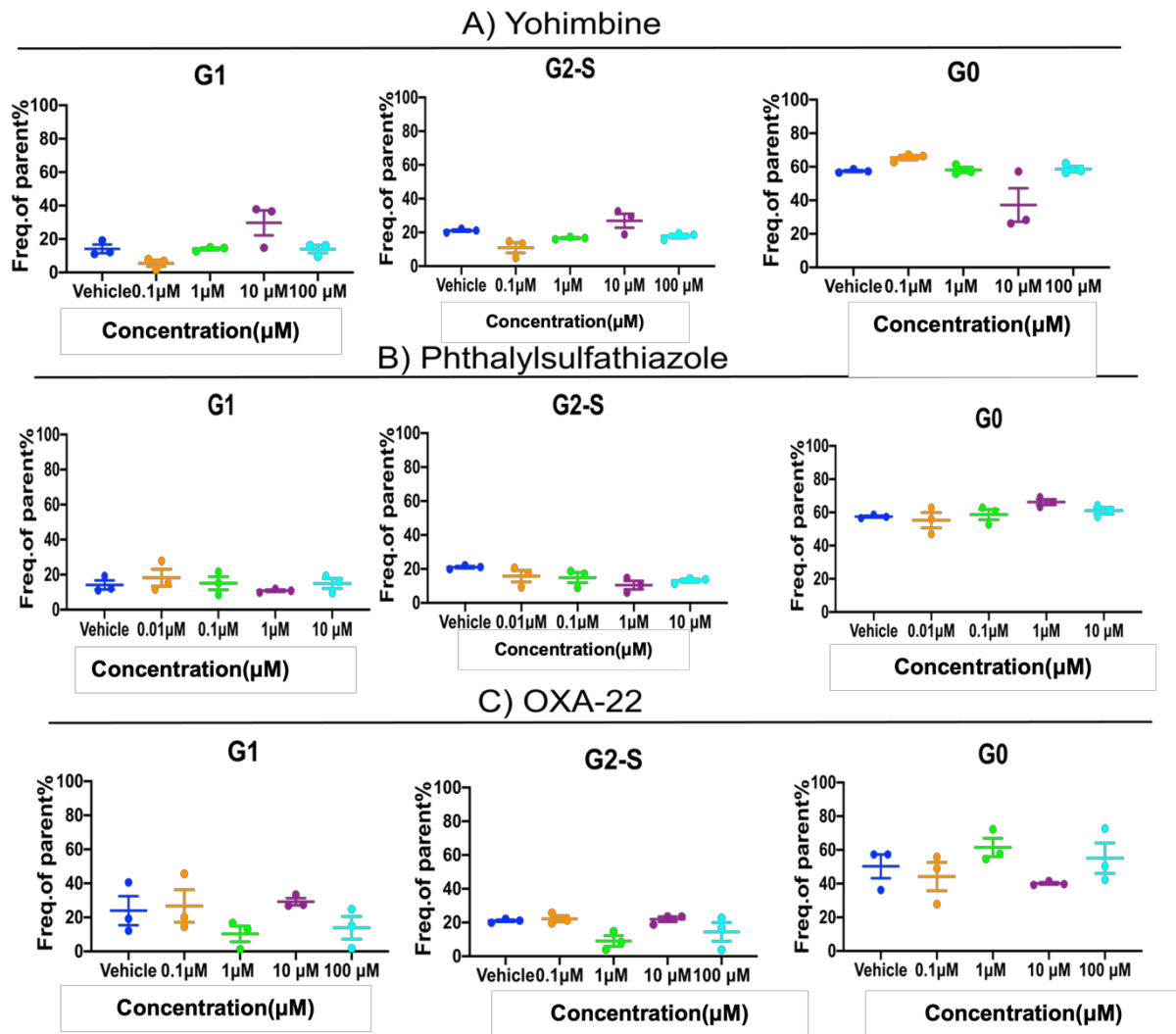


Figure 5.13. NOMO1 cell line cell cycle status in response to Oxa-22 treatment *in vitro*. Quantification data of G0, G2S, and G1 in NOMO1. Error bars represent mean +SEM for 3 replicate. Statistical significance of different concentration was determined by one way ANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.4 Discussion

In this chapter, building on previous experiments that demonstrated that administration of Oxa-22 after bone marrow transplantation leads to improved early erythrocytic regeneration without an impact on other lineages or HSPCs, I sought to evaluate the impact of this treatment on long-term HSC functioning in secondary transplantation. No significant change in lineage-specific behaviour was noted in these experiments, but, promisingly, preliminary analysis demonstrated augmentation of HSPC pools (specifically, HSC, MPP, HPC1 and HPC2 cells) after secondary transplantation of these *in vivo* Oxa-22 treated HSCs. Further experimentation is required to confirm these observations to statistical significance, but if proven to be correct, suggests that together with its impact on early haematopoietic reconstitution in transplantation settings, Oxa-22 has a positive impact on HSC self-renewal. There is evidence to suggest that muscarinic receptor signalling may have an impact on the self-renewal of haematopoietic cells. For example, M4-mAChR appears to be a critical regulator of BFU-E self-renewal. Pharmacological inhibition of this receptor expands both mouse and human erythroid progenitors and increases production of erythroid cells in CD34+ cells in patients with myelodysplastic syndrome and, in mouse models of aging and haemolysis, M4-mAChR targeting corrected anaemia *in vivo* (Trivedi, Inoue et al. 2019). The molecular mechanisms underlying this impact on self-renewal remain unknown and require elucidation through mouse genetic approaches to knockdown M4-mAChR and RNA-sequencing. Whether similar such mechanisms operate with Oxa-22 (i.e. M3-mAChR) signalling in HSCs remains possible. This would have two direct benefits in terms of clinical translation of Oxa-22. Firstly, if Oxa-22 was to be used to aid reconstitution after bone marrow transplantation, short-term gains in haematopoietic reconstitution, as observed in our previous experiments in the laboratory, could lead to durable engraftment in patients and lessen the likelihood of graft failure that can occur in the clinical setting (Hutt 2018). Second, if Oxa-22 was to be used as an anti-leukaemia therapeutic, as is envisaged in the experiments conducted in the second part of this chapter, targeting leukaemia cells together with either preservation of, or, preferably, enhancement of normal HSPC functioning would alleviate the broad suppression of haematopoiesis that is observed in leukaemia patients (Cheng, Sun et al. 2018). As a differentiation-inducing agent, all-trans retinoic acid (ATRA) has been used in clinics for treating patients with a sub-type of AML, acute promyelocytic leukaemia (APL) where a fusion protein (PML-RARA) is generated through chromosomal translocation. At (15; 17) (q22; q12) (Petrie, Zelent et al. 2009), where it has dramatically improved the prognosis for these types of patients. Differentiation therapy has been proposed as an attractive approach to treating different subtypes of AML, which are more refractory to treatment (DiNardo and Cortes 2016), as it alleviates the block observed in myeloid cell maturation observed across sub-types of AML and restores normal haematopoiesis (Petrie, Zelent et al. 2009). In this chapter, we directly assessed whether Oxa-

22 and, also Yohimbine and Phthalylsulfathiazole, could enhance the differentiation of non-APL AML cells and found in one AML cell line out of two examined, an evident growth in CD11b differentiation was observed after addition of Oxa-22 and, in addition, CD11b and CD14 differentiation was enhanced after *in vitro* exposure to Yohimbine. These data suggest that both adrenergic and cholinergic signalling regulates AML differentiation cell fate decisions. This data would be consistent with data showing autonomic sympathetic noradrenergic signals have been shown to regulate HSPC and leukocyte trafficking by reducing BM vascular adhesion and homing (García-García, Korn et al. 2019) and the suggestion that muscarinic signalling participates in leukaemia (Cabadak, Aydin et al. 2011). Of interest to targeting AML LSCs directly, targeting M3-mChAR may be feasible as it seems like a key regulator of cancers stem cells in gastrointestinal (GI) cancers. Accumulating evidence shows that acetylcholine is produced from nerves cells and stimulates and transforms GI epithelial stem cells, as a result, it causes cancer progression (Konishi, Hayakawa et al. 2019). While our findings are provocative, many unanswered questions remain as to the therapeutic potential of Oxa-22 and Yohimbine in AML. Our observations were made on a single AML cell line, with no effects on the other AML cell line tested. This lack of impact of the small molecules could represent (i) the technical heterogeneity of the cell lines tested or (ii) the biological heterogeneity of the AML cell lines tested. On the latter point, expression of adrenergic and muscarinic receptor subtypes was not examined in this chapter and the responsiveness of the small molecules observed here may simply reflect the presence/absence of the respective receptors. Our studies merit further investigations into how the responsiveness of the small molecules relate to the (i) AML sub-type and (ii) adrenergic and muscarinic receptor expression subtype. It is possible for example that these small molecules will only target certain AML subtypes. How the *in vitro* results here relate to *in vivo* targeting of AML cells, and in particular the biologically and clinically relevant LSCs, (Al-Hussaini and DiPersio 2014), also remains unresolved. Previous data from our laboratory has shown that Oxa-22 and sulphonamide drive *in vivo* proliferation of AML LSCs (Lubaid Saleh thesis 2017), which suggests that if this potential was harnessed appropriately, it could also drive differentiation or, indeed, enhance other cell fates (e.g. self-renewal and apoptosis) that would eliminate AML LSCs by exhausting their malignant stem cell potential *in vivo*. In this respect, it has been shown in xenografting experiments with patient samples that G-CSF is capable of driving LSCs into cell cycle and when combined with cell-cycle sensitive chemotherapy destroy LSC potential (Saito, Uchida et al. 2010). Given that G-CSF is also haematopoietic differentiation factor (Greenbaum and Link 2011), combining Oxa-22 or Yohimbine with G-CSF treatment might be an attractive therapeutic strategy to target AML LSCs.

CHAPTER 6:

General Discussion:

Using high-throughput screening of small molecules in stem cell assays or surrogate stem cell assays, previous data from Dr. Rodrigues's laboratory has identified 3 lead small molecules: Phthalylsulfathiazole, Yohimbine and Cis-2-Methyl-5-termethyammoniummethyl-1,3-oxathiolane iodide (Oxa-22). Each of these molecules has showed promise in targeting stem cell activity, both in the initial screening studies, and in further, specific investigations of normal and malignant stem cells within the haematopoietic system.

We have previously found that both Yohimbine and Oxa-22 can enhance the frequency of HSCs *in vivo* and that HSCs exposed to both these small molecules have enhanced functional capabilities to enhance early lymphoid reconstitution after transplantation (Lubaid Saleh thesis, Cardiff University 2017). This finding has potential application in clinical bone marrow transplantation that has several challenging limitations in practice (Al Hamed, Bazarbachi et al. 2019). For example, recipients might need considerable transfusion support because of cytopenias caused by chemotherapeutic toxicity (Gajewski, Johnson et al. 2008) (Brudno and Kochenderfer 2016). Similarly, patients may experience issues with reduced marrow engraftment and high risks of infection due to slow engraftment after transplantation (Cho, Lee et al. 2018) (Gajewski, Johnson et al. 2008). The latter issue can be circumvented by identification of novel or repurposed drugs, such as Yohimbine and Oxa-22, which can augment early haematopoietic reconstitution, yet they must not impinge on long-term HSC functionality *in vivo* following transplantation. Promisingly, in my thesis I found that Yohimbine and Oxa-22 had minimal impact on long-term HSC function, as assessed by transplantation experiments.

Separately, there are supply shortages in blood elements required for transfusion medicine (e.g. lymphocytes, red blood cells and platelets) both for transplantation and in other settings (García-Roa, Del Carmen Vicente-Ayuso et al. 2017) that could theoretically be supplemented by enhancing or directing pluripotent stem cells (including iPSCs) to these particular haematopoietic lineages. In this thesis, to test whether our small molecule candidates could enhance haematopoietic differentiation of iPSCs, I established and characterised a mouse iPSC system using the STEMCCA lentiviral reprogramming cassette containing pluripotent stem cell factors (Sommer, Stadtfeld et al. 2009), and I established haematopoietic differentiation protocols using the hanging drop EB method. From these studies, it was found that both Phthalylsulfathiazole and Yohimbine are promising agents to enhance haematopoietic differentiation from iPSCs *in vitro*, as evidenced by gene expression analysis and functional CFC assays. Although the ability of Oxa-22 to alter iPSC fate was not directly tested here, these data support and validate our overall stem cell screening strategy and assays conducted in lower organisms, then in mammals, which has led to identification of

small molecules active conserved across a range of stem cell settings – tissue-specific: haematopoietic and gut, embryonic: iPSCs and neoplastic: LSCs.

In regard to compounds that act on LSCs, our laboratory identified two other compounds in our screen, thioridazine, an anti-psychotic drug, and fluvastatin, a cholesterol lowering drug, which have now been characterised as potent inhibitors of LSC activity by other independent laboratories (Sachlos, Risueño et al. 2012) (Hartwell, Miller et al. 2013). Furthermore, previous data from our laboratory has shown that Phthalylsulfathiazole and Oxa-22 causes increased proliferative activity of LSCs *in vivo* models. This data suggests that if Phthalylsulfathiazole and Oxa-22 were used in combination with standard chemotherapies one may be able to deplete proliferating leukaemia cells and cause exhaustion of LSCs, which are being driven into excessive cycling from a state of relative quiescence. Given the similarities between pluripotent stem cells and neoplastic stem cells (Kasai, Chen et al. 2014) (Yu, Pestell et al. 2012), it will be of interest to characterize and investigate those compounds from the screen which drive, or indeed inhibit, pluripotency in our iPSCs (i.e. enhance stem self-renewal or drive differentiation) as these are the compounds that are likely to be active on LSCs (Sachlos, Risueño et al. 2012), either by driving their propagation to increase their susceptibility to chemotherapy, or propelling normal differentiation – a hallmark that is forestalled in cancer. As LSC exhibit conserved behaviour with other types of neoplastic stem cells (Valent, Sadovnik et al. 2019) (McCracken, George et al. 2016), it will be of interest to test such candidate small molecules across a range of cancers where cancer stem cells are deemed to be biologically and clinically relevant.

Autologous bone marrow transplantation, which is routinely used to treat myeloma and lymphoma (Al Hamed, Bazarbachi et al. 2019), brings an obvious risk of malignancy relapse due to the presence of contaminating cancer cells from the donor/patient (Dasari and Tchounwou 2014). Various methods have explored the possibility of purging cancer cells from stem cell preparations including using pulsed electric fields (Riezzo, Pascale et al. 2017). In addition, donor shortages are an issue in allogeneic bone marrow transplantation (Riezzo, Pascale et al. 2017) (Peffault de Latour 2016). Both these problems could potentially be obviated by use of Yohimbine and Oxa-22 in iPSCs to produce generate HSCs, as both increase HSC frequency *in vivo*. Making iPSCs autologous (i.e. patient specific) in both settings is important in reducing the immunogenicity and therefore associated complications (e.g. GVHD) (Ortuño-Costela, Cerrada et al. 2019)(Sackett, Brown et al. 2016). Making bona fide, engraftable HSCs from iPSCs, or least a mixture of HSPCs containing engraftable HSCs, is not trivial, with only limited success using highly engineered PSCs expressing transcription factors *ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1* and *SPI1*, and a complex, an expensive array of growth factors (Sugimura, Jha et al. 2017). This lack of ease in producing

HSPCs from iPSCs (and pluripotent stem cells in general) may be due to the inability to recapitulate the complexity of the bone marrow niche during *in vitro* differentiation, which is now an area of intense investigation (Sugimura, Jha et al. 2017)(Jung, Dunbar et al. 2015). In addition to using Yohimbine and Oxa-22 to induce iPSC derived HSCs in a cell autonomous manner, as both adrenergic and cholinergic signalling regulate the components of the bone marrow niche such as and non-myelinating Schwann cells cells (Fielding and Méndez-Ferrer 2020) and osteoblasts, osteoclasts, and mesenchymal stem cells (Fielding and Méndez-Ferrer 2020), respectively, it may be possible to use Yohimbine and Oxa-22 to engineer or manipulate an artificial niche that will support iPSC differentiation to HSCs *in vitro*. Thus, much of this potential *in vitro* application will rely on the ability to understand, through next generation sequencing, cell autonomous and non-cell autonomous molecular mechanisms that Yohimbine and Oxa-22 regulate in potentiating HSC number *in vivo*. Furthermore, Yohimbine and Oxa-22 may be useful experimental tools to specifically dissect adrenergic or cholinergic signalling in the bone marrow niche *in vivo*.

Finally, I present data in in this thesis suggest that both Yohimbine and Oxa-22 can reinstate differentiation of AML cells *in vitro*, though not through an impact on cell cycling or apoptosis. Though this data has only been demonstrated in one AML cell line, the data presented in this thesis suggest a conserved action of for these molecules, across species, this time on the biological process of differentiation from both mouse iPSCs and human AML cells *in vitro*. Future studies should be focussed on understanding the mechanisms underlying the impact of these molecules on differentiation in both settings. As previous data from the laboratory has shown that Oxa-22 increases the proliferation of AML cells *in vivo*, it will be of interest to determine whether this proliferation is also linked to increased differentiation *in vivo* when combined with standard chemotherapeutics or other agents, as when added alone Oxa-22 (or Phthalylsulfathiazole) appear to accelerate disease.

Reference:

Abbuehl, J.-P., et al. (2017). "Long-term engraftment of primary bone marrow stromal cells repairs niche damage and improves haematopoietic stem cell transplantation." *Cell stem cell* 21(2): 241-255. e246.

Al Hamed, R., et al. (2019). "Current status of autologous stem cell transplantation for multiple myeloma." *Blood Cancer J* 9(4): 44.

Alharbi, R. A., et al. (2013). "The role of HOX genes in normal haematopoiesis and acute leukaemia." *Leukaemia* 27(5): 1000-1008.

Al-Hussaini, M. and J. F. DiPersio (2014). "Small molecule inhibitors in acute myeloid leukaemia: from the bench to the clinic." *Expert review of haematology* 7(4): 439-464.

Almeida, A. M. and F. Ramos (2016). "Acute myeloid leukaemia in the older adults." *Leuk Res Rep* 6: 1-7.

Aly, R. M. (2020). "Current state of stem cell-based therapies: an overview." *Stem Cell Investig* 7: 8.

Aoi, W. and Y. Marunaka (2014). "Importance of pH homeostasis in metabolic health and diseases: crucial role of membrane proton transport." *Biomed Res Int* 2014: 598986.

Barker, J. (1994). "SI/SId haematopoietic progenitors are deficient in situ." *Experimental haematology* 22(2): 174-177.

Bennett, J. M., et al. (1976). "Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group." *Br J Haematol* 33(4): 451-458.

Bosshart, H. and M. Heinzelmann (2016). "THP-1 cells as a model for human monocytes.

Brackett, C. C., et al. (2004). "Likelihood and mechanisms of cross-allergenicity between sulfonamide antibiotics and other drugs containing a sulfonamide functional group." *Pharmacotherapy* 24(7): 856-870.

Broadley, K. J. and D. R. Kelly (2001). "Muscarinic receptor agonists and antagonists." *Molecules* 6(3): 142-193.

Brudno, J. N. and J. N. Kochenderfer (2016). "Toxicities of chimeric antigen receptor T cells: recognition and management." *Blood* 127(26): 3321-3330.

Buccisano, F., Maurillo, L., Spagnoli, A., Del Principe, M., Fraboni, D., Panetta, P., Ottone, T., Consalvo, M., Lavorgna, S., Bulian, P., Ammatuna, E., Angelini, D., Diamantini, A., Campagna, S., Ottaviani, L., Sarlo, C., Gattei, V., Del Poeta, G., Arcese, W., Amadori, S., Lo Coco, F. and Venditti, A., 2010. Cytogenetic and molecular diagnostic characterization combined to postconsolidation minimal residual disease assessment by flow cytometry improves risk stratification in adult acute myeloid leukaemia. *Blood*, 116(13), pp.2295-2303.

Cabadak, H., Küçükbrahimoğlu, E., Aydın, B., Kan, B., & Zafer Gören, M. (2009). Muscarinic receptor-mediated nitric oxide release in a K562 erythroleukaemia cell line. *Autonomic And Autacoid Pharmacology*, 29(3), 109-115. doi: 10.1111/j.1474-8673.2009.00431.

Campbell, K. J. and S. W. G. Tait (2018). "Targeting BCL-2 regulated apoptosis in cancer." *Open Biol* 8(5).

Carlson, A. B. and G. P. Kraus (2022). *Physiology, Cholinergic Receptors*. StatPearls. Treasure Island (FL), StatPearls Publishing.

Cassier, P. A., et al. (2017). "Targeting apoptosis in acute myeloid leukaemia." *Br J Cancer* 117(8): 1089-1098.

Challen, G. A., et al. (2009). "Mouse haematopoietic stem cell identification and analysis." *Cytometry Part A: The Journal of the International Society for Advancement of Cytometry* 75(1): 14-24.

Chanput, W., et al. (2014). "THP-1 cell line: an *in vitro* cell model for immune modulation approach." *International immunopharmacology* 23(1): 37-45.

Cheng, H., et al. (2018). "Haematopoiesis and microenvironment in haematological malignancies." *Cell Regeneration* 7(1): 22-26.

Cho, S. Y., et al. (2018). "Infectious complications after haematopoietic stem cell transplantation: current status and future perspectives in Korea." *Korean J Intern Med* 33(2): 256-276.

Chuncharunee, S., et al. (1993). "Chronic administration of transforming growth factor-beta suppresses erythropoietin-dependent erythropoiesis and induces tumour necrosis factor *in vivo*." *Br J Haematol* 84(3): 374-380.

Coma, S., et al. (2013). "GATA2 and *Lmo2* control angiogenesis and lymphangiogenesis via direct transcriptional regulation of neuropilin-2." *Angiogenesis* 16(4): 939-952.

Copelan, E. A. (2006). "Haematopoietic stem-cell transplantation." *New England Journal of Medicine* 354(17): 1813-1826.

Cudmore, W., et al. (2015). "Pancytopenia in two horses following administration of potentiated sulfonamide antimicrobials." *Equine Veterinary Education* 27(10): 519-523.

Dang, S. M., et al. (2002). "Efficiency of embryoid body formation and haematopoietic development from embryonic stem cells in different culture systems." *Biotechnol Bioeng* 78(4): 442-453.

Dasari, S. and P. B. Tchounwou (2014). "Cisplatin in cancer therapy: molecular mechanisms of action." *Eur J Pharmacol* 740: 364-378.

Dehn, J., et al. (2019). "Selection of unrelated donors and cord blood units for haematopoietic cell transplantation: guidelines from the NMDP/CIBMTR." *Blood, The Journal of the American Society of Haematology* 134(12): 924-934.

DiNardo, C. D. and J. E. Cortes (2016). "Mutations in AML: prognostic and therapeutic implications." *Haematology 2014, the American Society of Haematology Education Program Book 2016(1)*: 348-355.

Doss, M. X. and A. Sachinidis (2019). "Current challenges of iPSC-based disease modeling and therapeutic implications." *Cells* 8(5): 403.

Drukker, M. (2008). "Immunological considerations for cell therapy using human embryonic stem cell derivatives." *StemBook* [Internet].

D'Souza, S. L., et al. (2005). "*SCL/Tal-1* is essential for haematopoietic commitment of the haemangioblast but not for its development." *Blood* 105(10): 3862-3870.

Dual cholinergic signals regulate daily migration of haematopoietic stem cells and leukocytes." *Blood, The Journal of the American Society of Haematology* 133(3): 224-236.

Doulatov, S., Notta, F., Laurenti, E. and Dick, J., 2012. Hematopoiesis: A Human Perspective. *Cell Stem Cell*, 10(2), pp.120-136.

Döhner, H., Estey, E., Grimwade, D., Amadori, S., Appelbaum, F., Büchner, T., Dombret, H., Ebert, B., Fenaux, P., Larson, R., Levine, R., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G., Sanz, M., Sierra, J., Tallman, M., Tien, H., Wei, A., Löwenberg, B. and Bloomfield, C., 2017. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*, 129(4), pp.424-447.

Elliott, S. and A. M. Sinclair (2012). "The effect of erythropoietin on normal and neoplastic cells." *Biologics: targets & therapy* 6: 163.

Endele, M., et al. (2014). "Instruction of haematopoietic lineage choice by cytokine signaling." *Experimental cell research* 329(2): 207-213.

Fernández-Villa, D., et al. (2019). "Folic Acid Antagonists: Antimicrobial and Immunomodulating Mechanisms and Applications." *Int J Mol Sci* 20(20).

Fielding, C. and S. Méndez-Ferrer (2020). "Neuronal regulation of bone marrow stem cell niches." *F1000Res* 9.

Fisch, C. and H. J. Sacks (1950). "High phthalylsulfathiazole (sulfathalidine) level associated with granulocytopenia; case report." *Gastroenterology* 16(4): 782-784.

Fisherman, J. S., et al. (1993). "Chloroquinoxaline sulfonamide: a sulfanilamide antitumor agent entering clinical trials." *Invest New Drugs* 11(1): 1-9.

Forman, S. J. and J. M. Rowe (2013). "The myth of the second remission of acute leukaemia in the adult." *Blood, The Journal of the American Society of Haematology* 121(7): 1077-1082.

Fridley, K. M., et al. (2014). "Differential expression of extracellular matrix and growth factors by embryoid bodies in hydrodynamic and static cultures." *Tissue Eng Part C Methods* 20(12): 931-940.

Frietze, S. and P. J. Farnham (2011). *Transcription factor effector domains. A handbook of transcription factors*, Springer: 261-277.

Gajewski, J. L., et al. (2008). "A review of transfusion practice before, during, and after haematopoietic progenitor cell transplantation." *Blood* 112(8): 3036-3047.

Gao, X., et al. (2018). "The haematopoietic stem cell niche: from embryo to adult." *Development* 145(2): dev139691.

García-Roa, M., et al. (2017). "Red blood cell storage time and transfusion: current practice, concerns and future perspectives." *Blood Transfus* 15(3): 222-231.

Gautam, D., et al. (2006). "A critical role for beta cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in *vivo*." *Cell Metab* 3(6): 449-461.

Gautam, D., et al. (2010). "Beneficial metabolic effects caused by persistent activation of beta-cell M3 muscarinic acetylcholine receptors in transgenic mice." *Endocrinology* 151(11): 5185-5194.

Gekas, C., et al. (2005). "The placenta is a niche for haematopoietic stem cells." *Dev Cell* 8(3): 365-375.

Gericke, A., et al. (2011). "Role of M1, M3, and M5 muscarinic acetylcholine receptors in cholinergic dilation of small arteries studied with gene-targeted mice." *Am J Physiol Heart Circ Physiol* 300(5): H1602-1608.

Gordan, R., et al. (2015). "Autonomic and endocrine control of cardiovascular function." *World J Cardiol* 7(4): 204-214.

Göttgens, B., et al. (2002). "Transcriptional regulation of the stem cell leukaemia gene (*SCL*)—comparative analysis of five vertebrate *SCL* loci." *Genome research* 12(5): 749-759.

Greim, H., et al. (2014). "The bone marrow niche, stem cells, and leukaemia: impact of drugs, chemicals, and the environment." *Annals of the New York Academy of Sciences* 1310(1): 7.

Grove, C. S. and G. S. Vassiliou (2014). "Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer?" *Disease models & mechanisms* 7(8): 941-951.

Growney, J. D., et al. (2005). "Loss of *Runx1* perturbs adult haematopoiesis and is associated with a myeloproliferative phenotype." *Blood* 106(2): 494-504.

Guilcher, G. M., et al. (2018). Curative therapies: allogeneic haematopoietic cell transplantation from matched related donors using myeloablative, reduced intensity, and nonmyeloablative conditioning in sickle cell disease. *Seminars in haematology*, Elsevier.

Gurdon, J. B. (1962). "Adult frogs derived from the nuclei of single somatic cells." *Developmental biology* 4(2): 256-273. García-García, A., et al. (2019).

Hackett, J. A. and M. A. Surani (2014). "Regulatory principles of pluripotency: from the ground state up." *Cell stem cell* 15(4): 416-430.

Hamaguchi, M., et al. (1991). "FDP D-dimer induces the secretion of interleukin-1, urokinase-type plasminogen activator, and plasminogen activator inhibitor-2 in a human promonocytic leukaemia cell line."

Hanna, J., et al. (2007). "Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin." *Science* 318(5858): 1920-1923.

Hanoun, M., et al. (2015). "Neural regulation of haematopoiesis, inflammation, and cancer." *Neuron* 86(2): 360-373.

Hareison, D. E. and T. H. Roderick (1997). "Selection for maximum longevity in mice." *Exp Gerontol* 32(1-2): 65-78.

Haritunians, T., et al. (2008). "Novel acyl sulfonamide LY573636-sodium: effect on haematopoietic malignant cells." *Oncol Rep* 20(5): 1237-1242.

Harrison, D. E. and T. H. Roderick (1997). "Selection for maximum longevity in mice." *Exp Gerontol* 32(1-2): 65-78.

Hartwell, K. A., et al. (2013). "Niche-based screening identifies small-molecule inhibitors of leukaemia stem cells." *Nat Chem Biol* 9(12): 840-848.

Hatzimichael, E. and M. Tuthill (2010). "Haematopoietic stem cell transplantation." *Stem cells and cloning: advances and applications* 3: 105.

Hayakawa, Y., et al. (2017). "Nerve growth factor promotes gastric tumorigenesis through aberrant cholinergic signaling." *Cancer cell* 31(1): 21-34.

Heinrich, P. C., et al. (1998). "Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway." *Biochemical journal* 334(2): 297-314.

Herold, T., et al. (2020). "Validation and refinement of the revised 2017 European LeukaemiaNet genetic risk stratification of acute myeloid leukaemia." *Leukaemia* 34(12): 3161-3172.

Hillestad, L. K. (1957). "Acute promyelocytic leukaemia." *Acta Med Scand* 159(3): 189-194.

Hirschi, K. K. (2012). "Haemogenic endothelium during development and beyond." *Blood* 119(21): 4823-4827.

Ho, D., et al. (2010). "Modulation of beta-adrenergic receptor signaling in heart failure and longevity: targeting adenylyl cyclase type 5." *Heart Fail Rev* 15(5): 495-512.

Hoover, D. B. (2017). "Cholinergic modulation of the immune system presents new approaches for treating inflammation." *Pharmacology & therapeutics* 179: 1-16.

Horton, S. J. and B. J. Huntly (2012). "Recent advances in acute myeloid leukaemia stem cell biology." *haematologica* 97(7): 966.

Hou, D.-R., et al. (2016). "Derivation of Porcine Embryonic Stem-Like Cells from In *Vitro*-Produced Blastocyst-Stage Embryos." *Scientific Reports* 6(1): 25838.

Hughes, J. P., et al. (2011). "Principles of early drug discovery." *British journal of pharmacology* 162(6): 1239-1249.

Hutt, D. (2018). "Engraftment, graft failure, and rejection." *The European Blood and Marrow Transplantation Textbook for Nurses*: 259-270.

Ikuta, K. and I. L. Weissman (1992). "Evidence that haematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation." *Proceedings of the National Academy of Sciences* 89(4): 1502-1506.

Iwasaki, H. and K. Akashi (2007). "Myeloid lineage commitment from the haematopoietic stem cell." *Immunity* 26(6): 726-740.

Licht, J. and Sternberg, D., 2005. The Molecular Pathology of Acute Myeloid Leukaemia. *Hematology*, 2005(1), pp.137-142.

Jung, M., et al. (2015). "Modeling Human Bone Marrow Failure Syndromes Using Pluripotent Stem Cells and Genome Engineering." *Mol Ther* 23(12): 1832-1842.

Kantarjian, H., et al. (2021). "Acute myeloid leukaemia: current progress and future directions." *Blood cancer journal* 11(2): 1-25.

Kasai, T., et al. (2014). "Cancer stem cells converted from pluripotent stem cells and the cancerous niche." *J Stem Cells Regen Med* 10(1): 2-7.

Kean, B. H., et al. (1962). "The Diarrhea of Travelers: V. Prophylaxis with Phthalylsulfathiazole and Neomycin Sulphate." *JAMA* 180(5): 367-371.

Kenchegowda, D., et al. (2010). "Regulation of Gastrokine-1, Uroplakin-1B and Uroplakin-3B Promoter Activities by KLF4, KLF5 and Oct1." *Investigative Ophthalmology & Visual Science* 51(13): 706-706.

Khaddour, K., et al. (2021). *Haematopoietic Stem Cell Transplantation*. StatPearls. Treasure Island (FL), StatPearls Publishing

Khwaja, A., et al. (2016). "Acute myeloid leukaemia." *Nature reviews Disease primers* 2(1): 1-22.

Kidoya, H., et al. (2019). "Regnase-1-mediated post-transcriptional regulation is essential for haematopoietic stem and progenitor cell homeostasis." *Nature communications* 10(1): 1-16.

Kiel, M. J., et al. (2005). "SLAM family receptors distinguish haematopoietic stem and progenitor cells and reveal endothelial niches for stem cells." *Cell* 121(7): 1109-1121.

Kim, B. W., et al. (2015). "Clinical significance of OCT4 and SOX2 protein expression in cervical cancer." *BMC Cancer* 15: 1015.

Kikushige, Y., & Miyamoto, T. (2013). TIM-3 as a novel therapeutic target for eradicating acute myelogenous leukemia stem cells. *International Journal Of Hematology*, 98(6), 627-633. doi: 10.1007/s12185-013-1433-6

Kondo, M. (2010). "Lymphoid and myeloid lineage commitment in multipotent haematopoietic progenitors." *Immunological reviews* 238(1): 37-46.

Konishi, M., et al. (2019). "Role of muscarinic acetylcholine signaling in gastrointestinal cancers." *Biomedicines* 7(3): 58.

Koyuncu, I., et al. (2019). "Evaluation of the anticancer potential of a sulphonamide carbonic anhydrase IX inhibitor on cervical cancer cells." *J Enzyme Inhib Med Chem* 34(1): 703-711.

Kruse, A. C., et al. (2012). "Structure and dynamics of the M3 muscarinic acetylcholine receptor." *Nature* 482(7386): 552-556.

Kruse, A. C., et al. (2014). "Muscarinic acetylcholine receptors: novel opportunities for drug development." *Nat Rev Drug Discov* 13(7): 549-560.

Kulkeaw, K. and D. Sugiyama (2012). "Zebrafish erythropoiesis and the utility of fish as models of anemia." *Stem Cell Research & Therapy* 3(6): 1-11.

Kwarteng, E. O. and K. M. Heinonen (2016). "Competitive Transplants to Evaluate Haematopoietic Stem Cell Fitness." *J Vis Exp*(114).

Laurenti, E. and B. Göttgens (2018). "From haematopoietic stem cells to complex differentiation landscapes." *Nature* 553(7689): 418-426.

Lemos, N., Farias, M., Kubaski, F., Scotti, L., Onsten, T., & Brondani, L. et al. (2018). Quantification of peripheral blood CD34+ cells prior to stem cell harvesting by leukapheresis: a single center experience. *Hematology, Transfusion And Cell Therapy*, 40(3), 213-218. doi: 10.1016/j.htct.2018.01.002

Leonard, A., et al. (2020). "Curative options for sickle cell disease: haploidentical stem cell transplantation or gene therapy?" *British journal of haematology* 189(3): 408-423.

Lepor, H. (2016). "Alpha-blockers for the Treatment of Benign Prostatic Hyperplasia." *Urol Clin North Am* 43(3): 311-323.

Levison, M. E. and J. H. Levison (2009). "Pharmacokinetics and pharmacodynamics of antibacterial agents." *Infect Dis Clin North Am* 23(4): 791-815, vii.

Li, M., et al. (2017). "Application of induced pluripotent stem cell technology to the study of haematological diseases." *Cells* 6(1): 7.

Lim, W. F., et al. (2013). "Haematopoietic cell differentiation from embryonic and induced pluripotent stem cells." *Stem cell research & therapy* 4(3): 1-11.

Little, A. M., et al. (2016). "BSHI Guideline: HLA matching and donor selection for haematopoietic progenitor cell transplantation." *International journal of immunogenetics* 43(5): 263-286.

Liu, A., et al. (2013). "Pluripotency transcription factors and cancer stem cells: small genes make a big difference." *Chin J Cancer* 32(9): 483-487.

Liu, Z.-I., et al. (2012). "A novel sulfonamide agent, MPSP-001, exhibits potent activity against human cancer cells *in vitro* through disruption of microtubule." *Acta Pharmacologica Sinica* 33(2): 261-270.

Llames, S., et al. (2015). "Feeder layer cell actions and applications." *Tissue Engineering Part B: Reviews* 21(4): 345-353.

Lu, B. and H. Vogel (2009). "Drosophila models of neurodegenerative diseases." *Annu Rev Pathol* 4: 315-342.

Maestroni, G. J., et al. (1992). "Effect of adrenergic agents on haematopoiesis after syngeneic bone marrow transplantation in mice." *Blood* 80(5): 1178-1182.

Mannucci, P. M. (2020). "Haemophilia therapy: the future has begun." *haematologica* 105(3): 545.

Manzotti, G., et al. (2015). "Monocyte-macrophage differentiation of acute myeloid leukaemia cell lines by small molecules identified through interrogation of the Connectivity Map database." *Cell Cycle* 14(16): 2578-2589.

Mao, X. G., et al. (2013). "*CDH5* is specifically activated in glioblastoma stemlike cells and contributes to vasculogenic mimicry induced by hypoxia." *Neuro Oncol* 15(7): 865-879.

Marengo-Rowe, A. (2007). *The Thalassemias and Related Disorders*. Baylor University Medical Center Proceedings, 20(1), 27-31. doi: 10.1080/08998280.2007.11928230.

Matoba, R., et al. (2006). "Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling." *PLoS ONE* 1(1): e26.

Mboge, M. Y., et al. (2015). "Advances in Anti-Cancer Drug Development Targeting Carbonic Anhydrase IX and XII." *Top Anticancer Res* 5: 3-42.

Mboge, M. Y., et al. (2018). "Carbonic Anhydrases: Role in pH Control and Cancer." *Metabolites* 8(1).

McCracken, M. N., et al. (2016). "Normal and Neoplastic Stem Cells." *Cold Spring Harb Symp Quant Biol* 81: 1-9.

McDonald, P. C., et al. (2012). "Recent developments in targeting carbonic anhydrase IX for cancer therapeutics." *Oncotarget* 3(1): 84.

Metcalf, D. (2008). "Haematopoietic cytokines." *Blood, The Journal of the American Society of Haematology* 111(2): 485-491.

Mi, F. and L. Gong (2017). "Secretion of interleukin-6 by bone marrow mesenchymal stem cells promotes metastasis in hepatocellular carcinoma." *Bioscience reports* 37(4): BSR20170181.

Mignani, S., et al. (2016). "Why and how have drug discovery strategies in pharma changed? What are the new mindsets?" *Drug discovery today* 21(2): 239-249.

Miraki-Moud, F., et al. (2013). "Acute myeloid leukaemia does not deplete normal haematopoietic stem cells but induces cytopenias by impeding their differentiation." *Proceedings of the National Academy of Sciences of the United States of America* 110(33): 13576-13581

Molinoff, P. B. (1984). " α - and β -Adrenergic Receptor Subtypes." *Drugs* 28(2): 1-15.

Morales, A. (2000). "Yohimbine in erectile dysfunction: the facts." *Int J Impot Res* 12 Suppl 1: S70-74.

Morgan, R. A., et al. (2017). "Haematopoietic stem cell gene therapy: progress and lessons learned." *Cell stem cell* 21(5): 574-590.

Morrison, S. J., et al. (1997). "Identification of a lineage of multipotent haematopoietic progenitors." *Development* 124(10): 1929-1939.

Mossahebi-Mohammadi, M., et al. (2020). "FGF signaling pathway: a key regulator of stem cell pluripotency." *Frontiers in cell and developmental biology* 8: 79.

Malbon, C. C. (2005). "G proteins in development." *Nature reviews Molecular cell biology* 6(9): 689-701.

Muthu, K., et al. (2007). "Murine haematopoietic stem cells and progenitors express adrenergic receptors." *J Neuroimmunol* 186(1-2): 27-36.

Oda, A., et al. (2018). "Niche-induced extramedullary haematopoiesis in the spleen is regulated by the transcription factor Tlx1." *Scientific Reports* 8(1): 1-16.

Oppenheim, J. J. (2001). "Cytokines: past, present, and future." *International journal of haematology* 74(1): 3-8.

Ortuño-Costela, M. D. C., et al. (2019). "The Challenge of Bringing iPSCs to the Patient." *Int J Mol Sci* 20(24).

Oguro, H., Ding, L. and Morrison, S., 2013. SLAM Family Markers Resolve Functionally Distinct Subpopulations of Hematopoietic Stem Cells and Multipotent Progenitors. *Cell Stem Cell*, 13(1), pp.102-116.

- Pan, G. and J. A. Thomson (2007). "Nanog and transcriptional networks in embryonic stem cell pluripotency." *Cell research* 17(1): 42-49.
- Pan, H. and R. M. Schultz (2011). "Sox2 modulates reprogramming of gene expression in two-cell mouse embryos." *Biology of reproduction* 85(2): 409-416.
- Petrie, K., et al. (2009). "Differentiation therapy of acute myeloid leukaemia: past, present and future." *Current opinion in haematology* 16(2): 84-91.
- Peffault de Latour, R. (2016). "Transplantation for bone marrow failure: current issues." *Haematology Am Soc Haematol Educ Program* 2016(1): 90-98.
- Percival, M.-E., et al. (2017). "Bone marrow evaluation for diagnosis and monitoring of acute myeloid leukaemia." *Blood reviews* 31(4): 185-192.
- Perlin, J. R., et al. (2017). "Blood on the tracks: haematopoietic stem cell-endothelial cell interactions in homing and engraftment." *Journal of Molecular Medicine* 95(8): 809-819.
- Pouzolles, M., et al. (2016). "Haematopoietic stem cell lineage specification." *Current opinion in haematology* 23(4): 311-317.
- Puck, T. T. and P. I. Marcus (1955). "A rapid method for viable cell titration and clone production with HeLa cells in tissue culture: the use of X-irradiated cells to supply conditioning factors." *Proceedings of the National Academy of Sciences of the United States of America* 41(7): 432.
- Parekh, C. and Crooks, G., 2012. Critical Differences in Hematopoiesis and Lymphoid Development between Humans and Mice. *Journal of Clinical Immunology*, 33(4), pp.711-715.
- Rambacher, K. M. and N. H. Moniri (2020). "The β 2-adrenergic receptor-ROS signaling axis: An overlooked component of β 2AR function?" *Biochemical pharmacology* 171: 113690.
- Ranganath, S. and K. M. Murphy (2001). "Structure and specificity of GATA proteins in Th2 development." *Molecular and cellular biology* 21(8): 2716-2725.
- Raya, Á., et al. (2009). "Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells." *Nature* 460(7251): 53-59.
- Resende, R. R. and A. Adhikari (2009). "Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation." *Cell Communication and Signaling* 7(1): 1-20.
- Riezzo, I., et al. (2017). "Donor Selection for Allogenic Haemopoietic Stem Cell Transplantation: Clinical and Ethical Considerations." *Stem Cells Int* 2017: 5250790.

Rinner, I., et al. (1999). "A possible role for acetylcholine in the dialogue between thymocytes and thymic stroma." *Neuroimmunomodulation* 6(1-2): 51-55.

Riss, T. L., et al. (2016). "Cell viability assays." *Assay Guidance Manual* [Internet].

Robin, C., et al. (2006). "An unexpected role for *IL-3* in the embryonic development of haematopoietic stem cells." *Developmental cell* 11(2): 171-180.

Rungarunlert, S., et al. (2009). "Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors." *World J Stem Cells* 1(1): 11-21.

Sachlos, E., et al. (2012). "Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells." *Cell* 149(6): 1284-1297.

Sackett, S. D., et al. (2016). "Modulation of human allogeneic and syngeneic pluripotent stem cells and immunological implications for transplantation." *Transplant Rev (Orlando)* 30(2): 61-70.

Saito, Y., et al. (2010). "Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukaemia stem cells." *Sci Transl Med* 2(17): 17ra19.

Samardzhieva, I. and A. Khan (2018). "Necessity of bio-imaging hybrid approaches accelerating drug discovery process (mini-review)." *Int J Comput Appl* 182(6): 1-10.

Sanchez, P., et al. (2014). "Induced differentiation of acute myeloid leukaemia cells by activation of retinoid X and liver X receptors." *Leukaemia* 28(4): 749-760.

Sassano, A., et al. (2007). "Suppressive effects of statins on acute promyelocytic leukaemia cells." *Cancer research* 67(9): 4524-4532.

Scanzano, A. and M. Cosentino (2015). "Adrenergic regulation of innate immunity: a review." *Front Pharmacol* 6: 171.

Schmidt, R. and K. Plath (2012). "The roles of the reprogramming factors Oct4, Sox2 and Klf4 in resetting the somatic cell epigenome during induced pluripotent stem cell generation." *Genome biology* 13(10): 1-11.

Schnerch, D., et al. (2012). "Cell cycle control in acute myeloid leukaemia." *American journal of cancer research* 2(5): 508.

Schlenk, R., 2014. Post-remission therapy for acute myeloid leukaemia. *Haematologica*, 99(11), pp.1663-1670.

Scozzafava, A., et al. (2003). "Anticancer and antiviral sulfonamides." *Curr Med Chem* 10(11): 925-953.

Saito, Y., et al. (2010). "Induction of cell cycle entry eliminates human leukaemia stem cells in a mouse model of AML." *Nat Biotechnol* 28(3): 275-280.

Seita, J. and I. L. Weissman (2010). "Haematopoietic stem cell: self-renewal versus differentiation." *Wiley Interdiscip Rev Syst Biol Med* 2(6): 640-653.

Shahzad, S., et al. (2020). "Folic acid-sulfonamide conjugates as antibacterial agents: design, synthesis and molecular docking studies." *RSC Advances* 10(70): 42983-42992.

Sharma, D. and J. D. Farrar (2020). "Adrenergic regulation of immune cell function and inflammation." *Semin Immunopathol* 42(6): 709-717.

Shi, Y., et al. (2017). "Induced pluripotent stem cell technology: a decade of progress." *Nature reviews Drug discovery* 16(2): 115-130.

Showell, C., et al. (2004). "T-box genes in early embryogenesis." *Developmental dynamics: an official publication of the American Association of Anatomists* 229(1): 201-218.

Smith-Berdan, S., et al. (2019). "Viagra enables efficient, single-day haematopoietic stem cell mobilization." *Stem Cell Reports* 13(5): 787-792.

Sommer, C. A., et al. (2009). "Induced pluripotent stem cell generation using a single lentiviral stem cell cassette." *Stem cells* 27(3): 543-549.

Sugimura, R., et al. (2017). "Haematopoietic stem and progenitor cells from human pluripotent stem cells." *Nature* 545(7655): 432-438.

Supuran, C. T. (2003). "Indisulam: an anticancer sulfonamide in clinical development." *Expert Opin Investig Drugs* 12(2): 283-287.

Supuran, C. T. (2017). "Advances in structure-based drug discovery of carbonic anhydrase inhibitors." *Expert opinion on drug discovery* 12(1): 61-88.

Suriyo, T., et al. (2019). "Variation of nicotinic subtype $\alpha 7$ and muscarinic subtype M3 acetylcholine receptor expression in three main types of leukaemia." *Oncol Lett* 17(1): 1357-1362.

Swenson, E. S., et al. (2007). "Limitations of green fluorescent protein as a cell lineage marker." *Stem cells* 25(10): 2593-2600.

Tam, S. W., et al. (2001). "Yohimbine: a clinical review." *Pharmacol Ther* 91(3): 215-243.

Tani, S., et al. (2020). "Understanding paraxial mesoderm development and sclerotome specification for skeletal repair." *Exp Mol Med* 52(8): 1166-1177.

Temprano, K. K. (2016). "A Review of Raynaud's Disease." *Mo Med* 113(2): 123-126.

Till, J. E. and E. A. McCulloch (1961). "A direct measurement of the radiation sensitivity of normal mouse bone marrow cells." *Radiation research* 14(2): 213-222.

Ting, S. B., et al. (2012). "Asymmetric segregation and self-renewal of haematopoietic stem and progenitor cells with endocytic Ap2a2." *Blood, The Journal of the American Society of Haematology* 119(11): 2510-2522.

Tober, J., et al. (2013). "Distinct temporal requirements for *Runx1* in haematopoietic progenitors and stem cells." *Development* 140(18): 3765-3776.

Trivedi, G., et al. (2019). "Muscarinic acetylcholine receptor regulates self-renewal of early erythroid progenitors." *Science translational medicine* 11(511).

Jung, M., et al. (2015). "Modeling Human Bone Marrow Failure Syndromes Using Pluripotent Stem Cells and Genome Engineering." *Mol Ther* 23(12): 1832-1842.

Valent, P., et al. (2019). "Immunotherapy-Based Targeting and Elimination of Leukaemic Stem Cells in AML and CML." *Int J Mol Sci* 20(17).

Van Etten, R. A. (2007). "Aberrant cytokine signaling in leukaemia." *Oncogene* 26(47): 6738-6749.

Van Kesteren, C., et al. (2002). "E7070: a novel synthetic sulfonamide targeting the cell cycle progression for the treatment of cancer." *Anticancer Drugs* 13(10): 989-997.

van Miert, A. S. (1994). "The sulfonamide-diaminopyrimidine story." *J Vet Pharmacol Ther* 17(4): 309-316.

Volk, S. W. and C. Theoret (2013). "Translating stem cell therapies: the role of companion animals in regenerative medicine." *Wound Repair and Regeneration* 21(3): 382-394.

Vu, L. P. and M. G. Kharas (2018). "Targeting the residual leukaemia cells after chemotherapy." *Cancer cell* 34(3): 353-355.

Wan, Y., et al. (2021). "Sulfonamide derivatives as potential anti-cancer agents and their SARs elucidation." *Eur J Med Chem* 226: 113837.

Wang, J., et al. (2016). "Isoalantolactone inhibits the migration and invasion of human breast cancer MDA-MB-231 cells via suppression of the p38 MAPK/NF- κ B signaling pathway." *Oncology reports* 36(3): 1269-1276.

Wang, Z., et al. (2012). "Investigation of antigen-antibody interactions of sulfonamides with a monoclonal antibody in a fluorescence polarization immunoassay using 3D-QSAR models." *Int J Mol Sci* 13(5): 6334-6351.

Weinzierl, E. P. and D. A. Arber (2013). "The Differential Diagnosis and Bone Marrow Evaluation of New-Onset Pancytopenia." *American Journal of Clinical Pathology* 139(1): 9-29.

Weksberg, D., Chambers, S., Boles, N. and Goodell, M., 2008. CD150⁺ side population cells represent a functionally distinct population of long-term hematopoietic stem cells. *Blood*, 111(4), pp.2444-2451.

Woolthuis, C. M. and C. Y. Park (2016). "Haematopoietic stem/progenitor cell commitment to the megakaryocyte lineage." *Blood, The Journal of the American Society of Haematology* 127(10): 1242-1248.

Xu, J., et al. (2016). "Stage-specific embryonic antigen-1 (SSEA-1) expression in thyroid tissues." *Endocrine pathology* 27(4): 271-275.

Yadav, M. K., et al. (2016). "Comparative Assessment of Vitamin-B12, Folic Acid and Homocysteine Levels in Relation to p53 Expression in Megaloblastic Anemia." *PLoS ONE* 11(10): e0164559.

Yan, H., et al. (2018). "Haematopoiesis and the bacterial microbiome." *Blood* 132(6): 559-564.

Yang, L., et al. (2005). "Identification of Lin⁻Sca1⁺ kit⁺ CD34⁺ Flt3⁻ short-term haematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients." *Blood* 105(7): 2717-2723.

Yu, Z., et al. (2012). "Cancer stem cells." *Int J Biochem Cell Biol* 44(12): 2144-2151.

Yzaguirre, A. D., et al. (2017). "The role of *Runx1* in embryonic blood cell formation." *RUNX proteins in development and cancer*: 47-64.

Zahalka, A. H. and P. S. Frenette (2020). "Nerves in cancer." *Nat Rev Cancer* 20(3): 143-157.

Zakrzewski, W., et al. (2019). "Stem cells: past, present, and future." *Stem Cell Res Ther* 10(1): 68.

Zambidis, E. T., et al. (2005). "Haematopoietic differentiation of human embryonic stem cells progresses through sequential haematoendothelial, primitive, and definitive stages resembling human yolk sac development." *Blood* 106(3): 860-870.

Zhang, C. C. and H. F. Lodish (2008). "Cytokines regulating haematopoietic stem cell function." *Current opinion in haematology* 15(4): 307.

Zhang, J.-M. and J. An (2007). "Cytokines, inflammation and pain." *International anesthesiology clinics* 45(2): 27.

Zhao, W., et al. (2012). "Embryonic stem cell markers." *Molecules* 17(6): 6196-6236.

Zheng, Y. and L. C. Cantley (2019). "Toward a better understanding of folate metabolism in health and disease." *Journal of Experimental Medicine* 216(2): 253-266.