AN INVESTIGATION INTO THE ROLE OF CHEMOKINES IN HAEMOPOIETIC STEM CELL QUIESCENCE

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Abstract

Haemopoietic stem cells (HSC) maintain lifelong haemopoiesis through the monitoring and production of cells from multiple haemopoietic cell lineages. A key property of HSC is their ability to maintain quiescence. Quiescence refers to a state of inactivity in which the cell is not dividing and remains dormant. It is this property of the HSC that is thought to maintain genomic integrity and to allow the HSC to sustain haemopoiesis over the period of a lifetime. However, the regulation of quiescence in this context is not well understood. Numerous studies have aimed to understand the molecular mechanisms underlying HSC quiescence using high-throughput approaches. A previous microarray study by our group aimed to understand the transcriptional differences between quiescent and proliferating human HSC. Data from this microarray showed that the most up regulated group of genes in quiescent compared to proliferating human HSC were chemokine ligands, specifically within the CXC family. Although this was a novel finding at the time, the biological function of these chemokine genes was not studied until the current work presented here. In this thesis, we aimed to extend foregoing research and importantly, investigate the role of CXC chemokines in HSC properties, using both human and mouse systems.

First, we validated the results from the microarray study using gene expression analyses to show that chemokine ligands *CXCL1* and *CXCL2* were significantly up regulated in quiescent HSC ($CD34^+CD38^-$) in comparison to more proliferative progenitors ($CD34^+CD38^+$). Focusing on CXCL1, we showed positive expression of the ligand protein in human stem/progenitor cells using immunofluorescence and western blotting on human primary $CD34^+$ cells. In addition, we identified positive expression of receptor CXCR2 by gene and protein analyses on $CD34^+$ cells, indicating the presence of an autocrine chemokine signalling loop. To determine the biological function of CXCL1/CXCR2 signalling in human HSC, we used shRNA to reduce CXCL1 expression and a commercially available inhibitor (SB-225002) to block CXCR2 receptor signalling. Experiments on cell lines expressing CXCL1 and CXCR2 (HT 1080) showed that reduction of CXCL1 and over-expression reduced or increased cell viability and proliferation respectively. Experiments on human primary $CD34^+$ cells revealed that reduction of CXCL1 induced apoptosis and reduced colony formation. Similarly, inhibition of CXCR2 signalling in $CD34^+$ cells using SB-225002 induced apoptosis and reduced colony formation in a dose dependent manner. However, due to human sample availability and technical challenges, experiments need repeated in order for a valid conclusion to be

made and statistical analysis could not be carried out for some primary experiments. In addition, further experimental work is required to conclusively prove that human stem/progenitors express CXCL1 and CXCR2 as different techniques showed varying results. In summary, we provide some evidence that CXCL1 and CXCR2 is expressed by human HSC and may be an important survival pathway in normal human HSC which requires further experimental data to provide valid conclusions.

In order to gain a deeper understanding of the biological function of chemokine signalling in HSC biology, we used an *in vivo* murine system. First, we examined mRNA transcripts of CXC chemokines in mouse HSC populations. We screened a small selected group of CXC chemokines using primitive mouse HSC and single cell quantitative PCR using the Fluidigm™ platform. Gene expression analyses identified that *Cxcr2* and *Cxcl4* mRNA transcripts were detected including in the most rare, primitive HSC fraction. To elucidate the mechanism of action, we used a transgenic reporter and knock out mouse models for both genes of interest. Analysis of a *Cxcr2* null mice model (*Cxcr2*$^{-/-}$) validated previous research in which animals lacking *Cxcr2* show disrupted haemopoiesis with an expansion of myeloid cells in the haemopoietic organs. Interestingly, within the current work, analysis of steady state haemopoiesis revealed an expansion of the most primitive HSC in the BM of animals lacking *Cxcr2* and enhanced mobilisation demonstrated by an increase in the stem/progenitor activity in the spleen and PB. HSC functional analyses using BM reconstitution assays with wildtype (WT) or *Cxcr2*$^{-/-}$ HSC showed that there was a trend towards a reduction in engraftment in animals transplanted with HSC lacking *Cxcr2*. However, this result was not statistically significant due to high sample variability and due to time constraints and the length of this assay, this was not repeated. The data suggests that *Cxcr2* expressing HSC may be important for stem cell maintenance via a cell autonomous mechanism however experiments are required to be repeated to draw valid conclusions.

Cxcl4-Cre transgenic mice containing a RFP construct under the control of the Rosa26 promoter (*Cxcl4-Cre*) showed RFP expression in HSC and progeny. RFP expression in HSC populations was in accordance with *Cxcl4* mRNA transcripts therefore suggesting RFP expression was correlated with endogenous *Cxcl4* expression. Interestingly, flow cytometry analysis identified that not all (~50%) HSC showed positive expression for RFP. Flow cytometry sorting of positive and negative populations revealed that cells with enhanced colony formation potential reside within the RFP (*Cxcl4)* positive fraction. To extend this data, we aimed to knock out and reduce *Cxcl4* expression and examine the

phenotype. Targeted deletion of *Cxcl4 in vitro* using a *Cxcl4* shRNA vector demonstrated that *Cxcl4* reduction *in vitro* diminished colony formation in primary and secondary replating assays. Since data for human *CXCL4* mRNA were not conclusive from the original microarray, we reassessed the relevance of *CXCL4* in the human system. Gene expression analyses showed that *CXCL4* transcripts were indeed detected and furthermore, up regulated in primitive HSC ($CD34^{+}CD38^{-}CD90^{+}$) compared with proliferative progenitors ($CD34^{+}CD38^{+}$). Collectively, the data indicates that *CXCL4* may play an important role in mouse and human HSC biology, however further experimental work is required to address this.

In summary, the data presented in this thesis demonstrate that several chemokines including CXCL1, CXCL4 and receptor CXCR2 may have key roles in HSC survival and maintenance, both in the mouse and human systems. However, increased biological replicates and further experiments are required to draw valid conclusions. Enhanced understanding of the regulation of stem cell properties is critical for improving our ability to manipulate normal stem cells *in vitro* and *in vivo.* Furthermore, understanding normal stem cell regulation is fundamental for the research of diseases such as leukaemia in which leukaemic stem cells are less sensitive to drug treatment.

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Related Publications

S. D. J. Calaminus*, A. V. Guitart*, **A. Sinclair***, H. Schnachnter, S.P. Watson, T.L. Holyoake, K. Kranc* and L. Machesky*. 'Lineage tracing of Pf4-Cre marks hematopoietic stem cells and their progeny'. *PLoS ONE.* 7(12): e51361. doi: 10.1371/journal.poe.0051361.

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*Joint authorship.

Publications in Preparation

Under review

S. D. J. Calaminus, **A. Sinclair,** A. V. Guitart, K. Flegg, K. Anderson, G. Inman, S. Watson, O. Sansom, K. Kranc, T. L. Holyoake and L. Machesky. 'Alterations in hematopoietic wnt signalling in mice drive myelofibrosis and bone marrow failure'.

C. Hamilton, A. Fraser, C. Michels, M. Kurowska-Storarska, **A. Sinclair,** T. L Holyoake, M. Copland, P. Adu, R J. B. Nibbs and G. J. Graham. 'TLR stimulation induces CXCR4 down regulation which is associated with stem cell mobilisation'.

In preparation

A. Sinclair, S. D. J. Calaminus, F. Pellicano, S. M. Graham, R. Kinstrie, O. Sansom, G. J. Graham, K. Kranc, L. Machesky and T. L. Holyoake. 'CXC chemokines play a role in haemopoietic stem cell properties'.

F. Pellicano, L. Park, L. Hopcroft, **A. Sinclair**, M. Girolami, G. Leone, A. Whetton, K. Kranc and T. L. Holyoake. 'E2F1 is critical for survival of chronic myeloid leukaemia stem cells'.

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Author’s declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

List of Abbreviations

Full Name	Abbreviation
2-mercaptoethanol	2-ME
3-dimensional	3-D
4-(2-hydroxethyl)-1-piperazinethanesulfonic acid	HEPES
4-(2-hydroxethyl)-1-piperazinethanesulfonic acid buffered saline	HBS
4'6-diamidino-2-phenylindole dihydrochloride	DAPI
5-azacytidine	5-AZA
5-fluorouracil	5-FU
Acute myeloid leukaemia	AML
Adult stem cell	ASC
Allophycocyanin	APC
Ammonium chloride	NH_4Cl
Ammonium persulfate	APS
Aorta-gonad-mesonephros	AGM
Bacterial artificial chromosome	BAC
Base pair	BP
Bicinchoninic acid	BCA
Bone marrow	BM
Bone morphogenetic protein	BMP
Bovine serum albumin/insulin/transferrin	BIT
Bromodeoxyuridine	Brd-U
Calcium chloride	$CaCl_2$
Cell division cycle 6	CDC6
Chronic lymphocytic leukaemia	CLL
Chronic myeloid leukaemia	CML
Cluster of differentiation	CD
Cobblestone area forming cell	CAFC
Colony forming cell	CFC
Colony forming units-erythroid	CFU-E
Colony forming units-fibroblast	CFU-F
Colony forming units-granulocyte erythroid macrophage megakaryocyte	CFU-GEMM
Colony forming units-granulocyte macrophage	CFU-GM

Colony forming units-spleen	CFU-S
Common lymphoid progenitor	CLP
Common myeloid progenitor	CMP
Complimentary DNA	cDNA
Cord blood	CB
CXCL12-abundant reticular cells	CAR
Deoxyribonuclease	DNAse
Diacylglycerol	DAG
Dimethyl sulfoxide	DMSO
Duffy antigen receptor for chemokine	DARC
Dulbecco's modified eagle medium	DMEM
Embryonic day	E
Embryonic stem cell	ESC
Endothelial cells	EC
Enzyme-linked immunosorbent assays	ELISA
Epidermal growth factor	EGF
Erythropoietin	EPO
Ethylenediaminetetraacetic acid	EDTA
Fluorescein isothiocyanate	FITC
Fluorescence minus one	FMO
Fluorescent activated cell sorting	FACS
Foetal calf serum	FCS
Forward angle light scatter	FSC
G protein coupled receptor	GPCR
Germ stem cell	GSC
Glyceraldhehyde 3-phophate dehydrogenase	GAPDH
Glycosaminoglycan	GAG
Granulocyte macrophage-colony stimulating factor	GM-CSF
Granulocyte/macrophage progenitor	GMP
Granulocyte-colony stimulating factor	G-CSF
Gray	Gy
Green fluorescent protein	GFP
Growth factor	GF
Guanosine diphosphate	GDP

Guanosine nucleotide binding proteins	G proteins
Guanosine triphosphate	GTP
Haemopoietic stem cell	HSC
Hank's buffered salt solution	HBSS
Homeobox	HOX
Horseradish peroxidise	HRP
Human immunodeficiency virus	HIV-1
Human serum albumin	ALBA
Hydrochloric acid	HCl
Hypoxia-inducible factors	HIF
Individually ventilated cages	IVC
Induced pluripotent stem cells	IPS
Interleukin	IL
Iositol triphosphate	IP3
Isocove's modified dulbecco's medium	IMDM
Leukaemic stem cell	LSC
Long-term culture initiating cell	LT-CIC
Long-term repopulating HSC	LT-HSC
Low density lipoprotein	LDL
Luria's broth	LB
Lymphoid primed multipotent progenitor population	LPMPP
Magnesium chloride	$MgCl_2$
Megakaryocyte/erythroid progenitor	MEP
Mesenchymal stem cells	MSC
Metalloproteinases	MMP
MicroRNA	MiRNA
Minutes	Min
Mitogen activated protein kinase	MAPK
Mononuclear cells	MNC
Multidrug resistance	MDR
Multipotent progenitor population	MPP
Osteoblast	OB
Osteoclast	OC
Peridinin chlorophyll	PerCP
Peripheral blood	PB

Phosphate buffered saline	PBS
Phosphatidylinositide 3 kinase	PI3K
Phosphatidylinositol (4,5)-biphosphate	PIP2
Phospholipase C	PLC
Phycoerithrin	PE
Polycomb	PCG
Polymerase chain reaction	PCR
Polyvinylidene fluoride	PVDF
Potassium chloride	KCl_2
Puromycin	Puro
Quantitative-polymerase chain reaction	Q-PCR
Red blood cell	RBC
Restriction enzyme	RE
Reverse transcription	RT
Rho associated kinase	ROCK
Ribonucleases	RNAses
Room temperature	RT
Seconds	Sec
Severe combined immunodeficiency	SCID
Serum free medium	SFM
Short-term repopulating HSC	ST-HSC
Shrimp alkaline phosphatase	SAP
Side angle light scatter	SSC
Side population	SP
Sodium chloride	NaCl
Sodium dodecyl sulphate	SDS
Sodium dodecyl sulphate - polyacrylamide gel electrophoresis	SDS-PAGE
Stem cell factor	SCF
Super optimal broth with catabolite repression	SOC
RFP	RFP
Tetramethylethylenediamine	TEMED
Thrombopoietin	TPO
Tris ethylenediaminetetraacetic acid buffer	TE
Tyrosine kinase	TK
Vascular cell adhesion	VCAM-1

molecule-1	
Vascular endothelial growth factor	VEGF
Volts	V
White blood cell	WBC
Wildtype	WT
β-2-Microglobulin	β2M

1 Introduction

1.1 The history of stem cells

Homeostasis refers to the ability of a system to maintain a stable condition, even in response to perturbation. An excellent example of this is the human body, which is capable of continuously monitoring and regulating its conditions in order to maintain a constant of all variables. This is elegantly demonstrated when we consider the regulation of individual organs within an organism. For example, the haemopoietic system is responsible for producing cell types of all the blood lineages over the period of a lifetime. There is a constant turnover of large numbers of cell types under basal conditions, which is regulated in response to haemopoietic stress and injury. Importantly, it is now understood that stem cells are heavily involved in the production and regulation of all cell types in the biological system, including in haemopoiesis.

The identification of stem cells marked an important discovery in the field of biology and medicine. The haemopoietic system represents an important component in the stem cell time line, beginning with a variety of observations including the acceptance of donor bone marrow (BM) into irradiated hosts in the mid 1900's (Weissman and Shizuru, 2008). Subsequently, experiments by Till and McCulloch demonstrated that cells, when transplanted into irradiated mice, produced colonies in the spleen derived from a single cell (Wu et al., 1967, Becker et al., 1963, McCulloch and Till, 1960, Till and Mc, 1961, Siminovitch et al., 1963, Magli et al., 1982). These experiments implicated that there was an existence of cells with clonogenic activity within the BM which are now referred to as colony formation units-spleen (CFU-S) (Magli et al., 1982). These experiments led to translational research from 1968 onward in which the first human BM transplants were carried out. Collectively, these were the first studies that demonstrated that a specific type of cell had clonogenic activity and was capable of reconstituting a whole system. Stem cells from the haemopoietic system were subsequently identified and isolated, which provided a foundation for the understanding of stem cell biology (Weissman and Shizuru, 2008). Over the following years, stem cells have been discovered in a variety of other organs and these discoveries have revolutionised our understanding of how biological systems function.

Stem cells have been categorised into two main groups; embryonic stem cells (ESC) and adult stem cells (ASC) (Sylvester and Longaker, 2004). The main distinctions between

cells from these groups are in terms of residency and potency. An ESC is present at the beginning of development in the embryo and has the potential to produce cell types from all lineages of the embryo, which is described by the term ‘pluripotent’. In contrast, ASC are tissue specific and reside in particular adult organs. These cells are limited in their potential to produce cell types solely from a particular organ, which is described by the term ‘multipotent’. Although it is generally accepted that ASC have limited potential to particular lineages, recent studies have suggested there is an added ‘developmental plasticity’ of these cells (Korbling and Estrov, 2003, Wagers and Weissman, 2004). Indeed, there is evidence that purified HSC are capable of producing non-haemopoietic cell types, however this is thought to occur through fusion of haemopoietic and non-haemopoietic cells and is suggested to only occur during stress/injury and is a relatively rare event (Nygren et al., 2008).

The fusion of gametes results in the creation of a zygote which undergoes several rounds of cell division to generate a structure named the blastocyst, where the ESC reside (Donovan and Gearhart, 2001). ESC were identified and isolated from the inner cell mass of the blastocyst in mouse embryos in 1981 (Martin, 1981, Evans and Kaufman, 1981). This discovery was the beginning of a new area of research which would later prove to revolutionise the field of biology. ESC have been shown *in vitro* and *in vivo* to possess the ability to produce cell types of every lineage (Biswas and Hutchins, 2007). Over subsequent years, ESC have been used for several applications. To date (more than 30 years after their initial discovery), ESC have been used in the generation of chimeric mouse models and as a model to understand the mechanisms of lineage differentiation (Smith, 2001). Understanding the development of different lineages provides the potential for the production of large numbers of particular cell types for pharmacological screening and to create cells to be used in the treatment of disease. This has been termed regenerative medicine and research within this field has expanded exponentially over the past decade. Although proven to be a useful tool, there were issues associated with the use of ESC in research. An ethical debate surrounded the use of ESC experimentally as this involved the destruction of embryos. Furthermore, the transplantation of cells from one origin to patients of a different origin raised concerns of tissue rejection.

1.2 Regenerative medicine

The concept of generating patient specific cells became a reality with groundbreaking research by Yamanaka and his team. The research involved the integration of several

transcription factors associated with pluripotency into mature somatic cells to reprogramme them into a pluripotent state (induced pluripotent stem cells, IPS) (Takahashi and Yamanaka, 2006). This technique facilitated the idea of generating patient specific cells for therapy and overcame the ethical issues of using ESC and the issue of tissue rejection. However, the methodology in the original study was controversial as viruses were used to integrate transcription factors into mature cells. More recently, groups have developed methods to overcome this (Okita et al., 2008). Alternative strategies have been developed to reprogramme cell types, including nuclear transfer of somatic nuclei into oocytes, or the fusion of somatic cells with ESC (Yamanaka, 2007). In addition, the generation of IPS cells from diseased individuals has opened a new avenue of research as these studies provided insight into the mechanisms of disease. To date, researchers have successfully generated IPS cells from diseased individuals (Dimos et al., 2008, Soldner et al., 2009). If we focus on the haemopoietic system, IPS cells have been generated from haemopoietic cells derived from normal and malignant patient samples (Kumano et al., 2013). However, a key concern that still exists is the lack of understanding about the mechanisms of differentiation. ASC are studied to understand the pathways involved in differentiation. ASC, also referred to as tissue-specific stem cells are responsible for maintaining tissue homeostasis and producing the cell types required in particular organs.

Haemopoiesis has been well studied and to date serves as a model for a well characterised adult stem cell system (Weissman and Shizuru, 2008). Haemopoiesis is a well understood hierarchical model which is controlled and maintained from a stem cell population, the haemopoietic stem cell (HSC).

1.3 Haemopoiesis

Haemopoiesis is a hierarchical organisation in which the HSC are responsible for tissue homeostasis. Simply, the HSC reside at the top of the hierarchy and produce a cascade of more committed progenitor cells, which in turn produce terminally differentiated mature cells of all the blood lineages. Before we consider how haemopoiesis is regulated by the HSC population, the true characteristics of a stem cell must first be discussed.

1.3.1 Self renewal and differentiation

The definition of a true stem cell is the ability to elicit three main functions; self renewal, differentiation and the capacity to reconstitute a tissue *in vivo* (Roobrouck et al., 2008). To understand self renewal and differentiation, cell division must be discussed.

Mitosis is the process of cell division in which two daughter cells are generated. HSC can undergo symmetric and asymmetric cell divisions, which ultimately decides the daughter's cell fate (Morrison and Kimble, 2006, Domen and Weissman, 1999). Symmetric cell division involves the production of two identical daughter cells after mitosis. These identical cells can either be two HSC or two more differentiated daughter cells (Weissman and Shizuru, 2008). In this way, HSC can be produced which will either maintain the stem cell pool or more differentiated progeny can be produced which will ultimately provide mature cell types to replenish lost blood cells. In addition to symmetric cell division, HSC can also undergo asymmetric cell divisions in which progeny are produced that are not identical. Asymmetric division produces one stem cell and one differentiated cell. The combination of symmetric and asymmetric cell division is ultimately responsible for the maintenance of the haemopoietic system with the production of mature cell types when required while maintaining a functional stem cell pool (Figure 1-1) (Weissman and Shizuru, 2008).

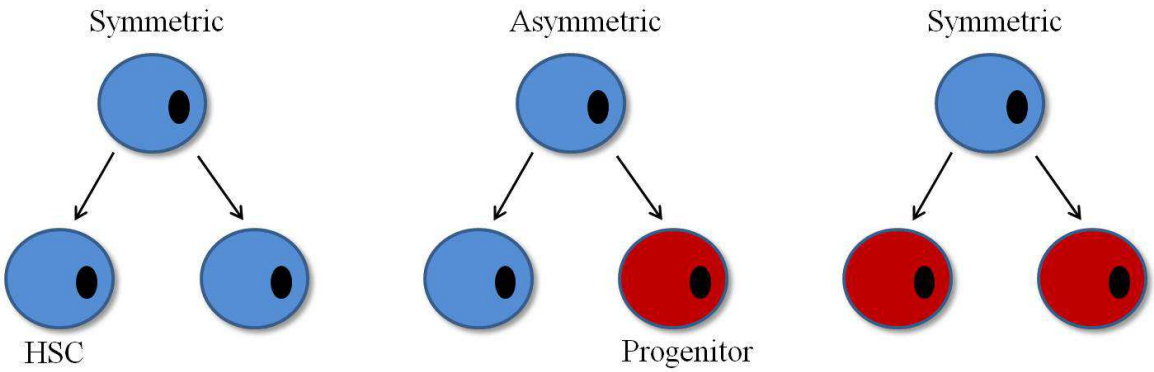

Figure 1-1 Symmetric versus asymmetric cell division.

HSC can undergo symmetric and asymmetric cell division in which identical progeny or two different progeny are generated respectively. Symmetric cell division can produce two identical HSC or more differentiated progenitors. Asymmetric cell division results in the production of one HSC and one progenitor. The combination of symmetric and asymmetric cell divisions ultimately controls haemopoiesis through the maintenance of a stem cell pool and the production of a cascade of more mature progenitors.

The majority of research on asymmetric cell division has been carried out using developmental model organisms including the *Drosophila melanogaster* (Morrison and Kimble, 2006, Gomez-Lopez et al., 2013). Studies have shown that both intrinsic and extrinsic mechanisms govern cell division. As an example, cell polarity and the distribution of cell components/proteins occurs prior to cell mitosis and governs subsequent cell fate. This has been shown in stem cells including in HSC, in which several proteins were found to segregate differentially after cell division (Gonczy, 2008, Beckmann et al., 2007). In addition, external mechanisms also control cell division with evidence that cell location alters the ultimate cell fate (Morrison and Kimble, 2006). The decision of symmetric versus asymmetric division is important not only for cell fate in normal development, but also in disease as previous studies have highlighted that asymmetric divisions are deregulated in cancer (Morrison and Kimble, 2006, Gomez-Lopez et al., 2013).

Self renewal is a fundamental property of HSC and it is thought that self renewal capacity is reduced as cell division occurs. Therefore more mature cells, including progenitor cells, show a reduced capacity to self renew. Differentiation is described as the production of a more mature, specialised cell down a particular lineage. HSC balance the processes of self renewal and multilineage differentiation to maintain a pluripotent HSC population poised to give rise to appropriate cell types when required including in response to blood loss,

infection or exposure to cytotoxic agents and oxidative stress (Seita and Weissman, 2010, Wilson et al., 2008).

Self renewal and differentiation are key to HSC function and can be measured experimentally. For simplicity, mouse and human experiments will be discussed separately.

1.3.1.1 Mouse

Both *in vitro* and *in vivo* assays can be used to experimentally to examine self renewal and differentiation. *In vitro* colony formation assays (colony formation cell, CFC assay) are widely used. This assay involves the culture of cell populations with the addition of particular cytokines designed to drive proliferation and differentiation. Cells are cultured for a period of time and the resulting colonies formed can be scored based on enumeration and classification of colonies. This gives an indication of proliferation and differentiation capacity. This assay is considered an assay for more mature progenitor cells, however, the colonies derived from a primary plating assay can be replated into a secondary plating assay in which the growth of colonies can act as an indicator for self renewal capacity. However, a CFC assay is relatively short term and is therefore more indicative of progenitor cell activity. The long-term culture initiating cell (LT-CIC) assay allows the detection and enumeration of the HSC population (Woehrer et al., 2013). In this assay, cells are plated on a layer of stromal cells (designed to mimic *in vivo* conditions) which support the survival, self renewal and differentiation of HSC. These cultures are maintained long term to identify true HSC populations in comparison to shorter experiments which identify progenitor populations only. In addition, limiting dilution of cell populations are used to give an indication of the frequency of LT-CIC per population. The cobblestone area forming cell (CAFC) assay involves the culture of a test population on a stromal layer and particular areas of HSC growth termed ‘cobblestones’ are scored based on stem/progenitor growth over a period of time (Breems et al., 1994, de Haan and Ploemacher, 2002).

However, *in vivo* experiments have provided unique insights into stem cell behaviour and are arguably more accurate in terms of quantifying HSC than *in vitro* assays (Domen and Weissman, 1999, Perry and Li, 2010). The CFU-S assays (as described previously) can provide an *in vivo* indication of stem/progenitor cell activity. The production of distinct colonies grown on the spleen of irradiated animals refers to the clonogenicity activity of

transplanted cells. Spleens are typically analysed on days 8 and 12 with the latter referring to a more primitive clonogenic cell than the former (Weissman and Shizuru, 2008). However, this assay is thought to involve more mature progenitor cells as opposed to stem cells due to the short time frame of the assay. The gold standard technique for assaying HSC function is the reconstitution of an ablated/diminished haemopoietic system (Harrison, 1980). A cell population can be transplanted into mouse models in which endogenous haemopoiesis has been ablated using irradiation or chemotherapy drug treatment. A true HSC will be capable of reconstituting the BM and providing progenitor and subsequent mature cells therefore rescuing haemopoiesis. Competitive repopulation involves the transplantation of donor test cells along with support BM cells which ensures host survival and markers can be used to elegantly track donor transplanted populations over time. Examples for host versus donor distinction are sex, expression of reporter genes or arguably the most commonly used, expression of cluster of differentiation (CD) cell surface markers including isoforms of CD45 (van Os et al., 2001, Domen and Weissman, 1999). CD45 was originally known as the leukocyte common antigen as is expressed on all leukocytes (Trowbridge and Thomas, 1994). Two alleles of CD45 are available and mouse strains have been developed with each allele on a C57/BL6 background (van Os et al., 2001). The generation of monoclonal antibodies against these alleles allowed for the distinction between donor versus host in competitive transplantation assays (Weissman and Shizuru, 2008). Limiting dilution experiments using a titration of the number of transplanted HSC are used and are more reliable in terms of quantifying the number of true stem cells in a population (Perry and Li, 2010). HSC transplantation assays not only give an indication of mutlilineage reconstitution, however self renewal can be experimentally examined through the ability of test populations to rescue haemopoiesis in secondary and tertiary recipient mice and these are the most stringent assays to report stem cell self renewal and therefore activity.

1.3.1.2 Human

Similar to *in vitro* assays described for mouse studies, the CFC, LT-CIC and CAFC assays can be used to assay human stem/progenitor behaviour (Domen and Weissman, 1999, Liu et al., 2013, Sarma et al., 2010, de Haan and Ploemacher, 2002). More recently, literature is emerging in which 3-dimensional structures are used to model the BM niche (Sharma et al., 2012). As discussed with the mouse *in vitro* assays, these assays are arguably not measuring true HSC activity.

The ability to transplant HSC into irradiated hosts and compare their differentiation and self renewal capacity has become a standard technique. However, studying human HSC is more complex. The use of immunocompromised mouse models as hosts for human HSC was a groundbreaking discovery in HSC research (Meyerrose et al., 2003). Original research showed that human haemopoietic tissue could engraft in immunocompromised (severe combined immunodeficient, SCID) mice which have a mutation resulting in defects in B and T cell development (Lapidot et al., 1992, Mosier et al., 1988). However, these mice still have natural killer cells which can attack foreign cells and therefore hinder the experiment. In order to study immune responses, there are other models available however further immune compromised mouse models have since been generated which work well as HSC xenograft models. Since this research, a variety of models have been described including non obese diabetic (NOD) SCID animals. These animals have additional defects in natural killer cell, macrophage and complement. Additional strains including, but not limited to, NOD/SCID/*β-2-Microglobulin (β2M),* NOD/SCID/IL-2R-$\gamma^{-/-}$ mice or RAG2$^{-/-}$ / IL-2R-$\gamma^{-/-}$ models are used which show more immunodeficiency than the NOD/SCID animals (Park et al., 2008, van der Loo et al., 1998, Wermann et al., 1996). Such models are commonly used in the literature and allow for the tracking of human HSC activity without the complications arising from rejection of the foreign transplanted cells by the host immune system (Domen and Weissman, 1999). However, disadvantages of using these mice include a shortened lifespan and their extreme sensistivity due to their compromised immune systems (Meyerrose et al., 2003). A diagram is displayed in Figure 1-2 for examples of the different assays available.

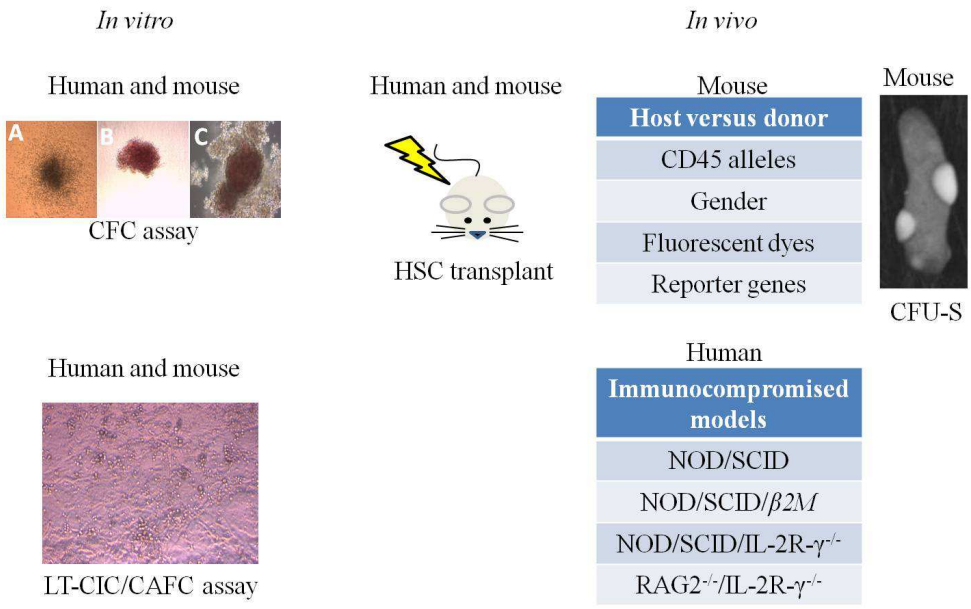

Figure 1-2 Commonly used methods for assaying HSC activity.

Schematic is adapted from published literature and based on the literature described above (Domen and Weissman, 1999). Various techniques have been developed to assay HSC activity. *In vitro* techniques include the culture of cell populations in particular growth conditions in which resulting colonies can be identified to give an indication of proliferation and differentiation status (CFC). LT-CIC and CAFC assays monitor cell growth over a longer time period in a co-culture with stromal cells to assess more primitive HSC activity. *In vivo* techniques involve the transplantation of cells into irradiated recipients where colonies grown on the spleen (CFU-S) or the capacity for multilineage reconstitution is evaluated. Donor cell activity can be tracked using various methods to distinguish host versus donor cells. Self renewal activity can be examined using the serial transplantation of cells into secondary and tertiary irradiated recipients. Mouse models for the transplantation of human HSC involve various immunocompromised mouse models of which the most common are stated. (Dr Francesca Pellicano and Dr Arunima Mukhopadhyay should be acknowledged for the CFU-S and LT-CIC images respectively).

1.3.2 The haemopoietic hierarchy

Irving Weissman's haemopoietic hierarchy hypothesis arose from work conducted in his laboratory beginning in 1998. This research was revolutionary and is now a generally accepted dogma (Weissman and Shizuru, 2008). The hypothesis was formed based on the stem cell activity of different mouse populations isolated using immunophenotypic cell surface markers. More specifically, cell sorting was used to isolate different cell types and

their activity was measured using BM reconstitution assays. These experiments identified that different populations of cells vary in terms of their ability to reconstitute the haemopoietic system. Furthermore, these experiments identified that certain cell types were capable of long term reconstitution (over a long period) while other cell types lost their potential for reconstitution over time. It was identified that a true HSC has a high capacity for multilineage differentiation and self renewal which allowed these cells to maintain haemopoiesis over a lifetime.

Based on these assays, the hypothesis states that the HSC population resides at the top of a haemopoietic hierarchy with a distinct population known as the LT-HSC (long term repopulating HSC). Experiments using transplantation of different populations into irradiated hosts identified that different populations showed differences in 1. the ability to reconstitute haemopoiesis and 2. the ability to results in multilineage reconstitution long-term (Weissman and Shizuru, 2008). The LT-HSC possess the ability to give multilineage reconstitution long term (at least 6 months) in a host with an ablated haemopoietic system (Weissman and Shizuru, 2008). Furthermore, these cells can be transferred to secondary hosts and will successfully maintain haemopoiesis. LT-HSC can give rise to a less primitive HSC population known as the ST-HSC (short term repopulating HSC) which has a reduced capacity for self renewal and repopulation i.e. cannot sustain haemopoiesis up to 6 months post transplantation and will not rescue haemopoiesis in a secondary host. HSC populations give rise to cells termed multipotent progenitor (MPP) populations which have limited self renewal and reconstitution potential. Similarly, experiments identified that populations can only give rise to particular cell lineages, providing evidence that more mature lineage restricted progenitor populations exist (Weissman and Shizuru, 2008). These are now referred to as a common myeloid progenitor (CMP) and common lymphoid progenitor which are capable of producing cell types of the myeloid and lymphoid lineages respectively (CLP) (Akashi et al., 2000, Kondo et al., 1997). Similarly, the CMP population gives rise to more mature progenitors in an intermediate stage named the granulocyte/macrophage progenitor (GMP) and megakaryocyte/erythroid progenitor (MEP) populations, which give rise to cell types of the myeloid lineage and which do not self renew. A simplified schematic of the haemopoietic hierarchy can be visualised in Figure 1-3.

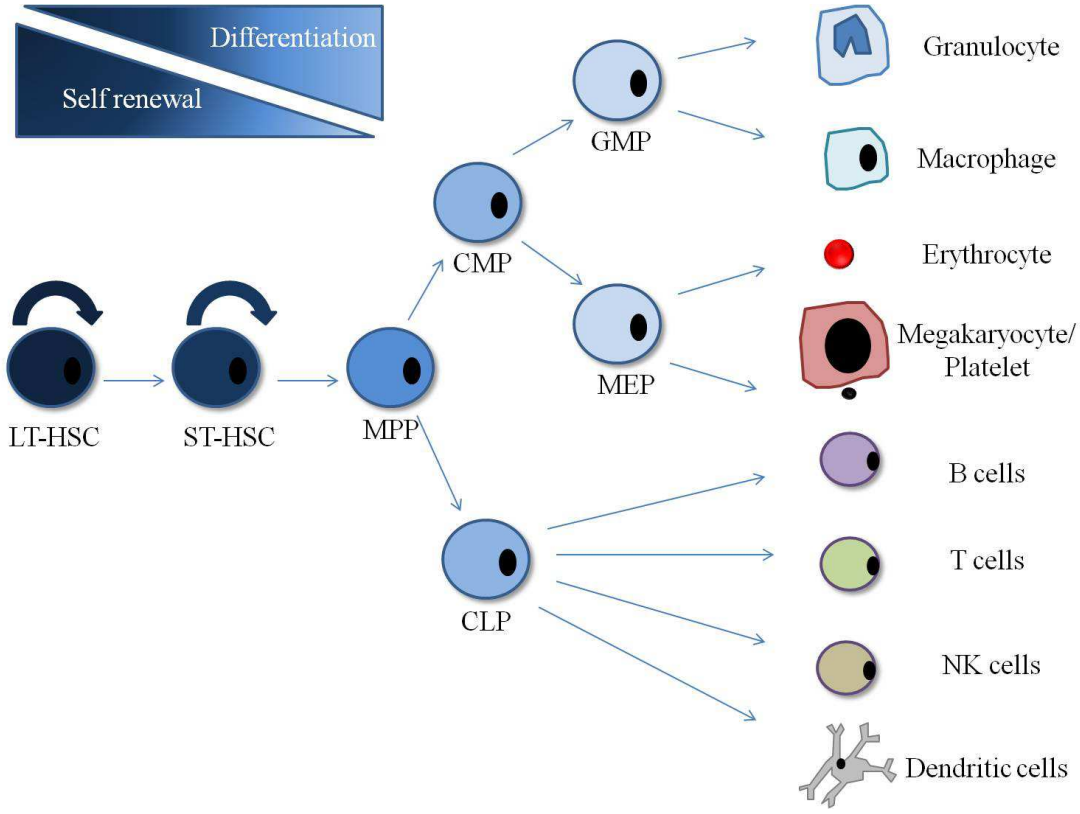

Figure 1-3 The haemopoietic hierarchy.

Schematic diagram adapted from published literature displaying the haemopoietic hierarchy as it is currently understood (Weissman and Shizuru, 2008). The haemopoietic system is a hierarchical system which is regulated and maintained by the HSC which reside at the top of a cell hierarchy. The LT-HSC are the most primitive cells which have the ability to reconstitute haemopoiesis in an ablated system and have a high capacity for self renewal. LT-HSC give rise to a more mature ST-HSC population which gives rise to a more mature progenitor population known as the MPP. HSC populations balance self renewal and differentiation to produce a cascade of more mature progenitor cells which are ultimately responsible for generating all of the diverse cell types of the myeloid and lymphoid lineages. The transition of HSC to mature cells results in a reduction in self renewal and long term repopulating capacity, which is associated with an increase differentiation.

It is generally understood that the sequence of differentiation stemming from the LT-HSC through to terminally differentiated cell types is irreversible with the generation of more lineage committed cells at each stage (Bryder et al., 2006). For example, it is proposed that cells after the MPP stage are either lineage restricted CMP or CLP which are subsequently destined for myeloid or lymphoid status respectively (Akashi et al., 2000, Kondo et al.,

1997). Some controversy over the potential of ST-HSC and MPP populations has arisen with the suggestion that priming for myeloid or lymphoid potential occurs at an earlier stage than previously proposed (Buza-Vidas et al., 2007). An alternative hypothesis has been put forward in which there is the existence of an additional lymphoid primed MPP (LPMPP) population. It is likely that the original Weissman hypothesis represents a simplified version of the haemopoietic system and there is additional complexity and plasticity involved. However, future research is required to address this in more detail.

The identification and isolation of cell populations has facilitated our understanding of HSC biology. Since the original experiments were carried out by Weissman *et al* on the identification and isolation of mouse HSC populations, an abundance of literature has extended this research. New markers have since been identified which have selected for a more enriched, primitive HSC populations.

1.3.3 HSC identification and isolation

The study of stem cells requires the ability to identify and isolate these cells for experimental research. HSC are generally identified due to their lack of expression of lineage positive cell markers and low staining of side population using DNA and RNA stains which will be discussed in detail in this section.

The discovery of HSC populations expressing particular cell surface markers has allowed for the identification and isolation of these populations (Wognum et al., 2003). There is some overlap between human and mouse HSC populations in terms of identification. For example, in both species HSC are identified through their lack of expression of lineage markers and low staining of DNA and RNA stains. HSC from both mouse and human are identified as existing in a population that are negative for staining of CD markers commonly expressed on mature lineage cells, including erythroid, granulocyte, B and T cells (defined from onwards as lineage negative). The addition of nucleotide stains, including RNA and DNA stains, has enhanced this population. As an example, Hoechst 33342 was identified as marking HSC populations in 1996 and was subsequently referred to as the side population (SP). Experiments showed that the most primitive HSC effluxed the dye which identified Hoechst 33342 negative cells as stem cells (Goodell et al., 1996, Goodell et al., 1997). ABC/G2 transporters are selectively expressed on stem cell populations are thought to result in the efflux of Hoechst 33342 solely in stem cell populations (Zhou et al., 2001, Kim et al., 2002).

However, the species will be discussed in more detail separately due to differences in expression of cell surface markers between species.

1.3.3.1 Mouse

Animal studies have provided vast advances in the identification and isolation of HSC and progenitor populations. In 1986, cells which were negative for a cocktail of lineage markers were identified as a population enriched with mouse cells that had reconstitution potential (Muller-Sieburg et al., 1986). In 1988, cells which were additionally negative for CD90 and positive for Sca-1(ly-6 A/E) were shown to reconstitute haemopoiesis in a proportion of recipients with ablated BM with only the cells positive for Sca-1 having the capacity for *in vivo* reconstitution (Spangrude et al., 1988). This research was extended to include c-Kit (CD117) as a positive marker for HSC, also known as the cell surface receptor which binds to stem cell factor (SCF) (Ogawa et al., 1991, Ikuta and Weissman, 1992). The combination of lineage negative with Sca-1 and c-Kit positive markers (lineage negative, Sca-1^{+}, c-Kit^{+}; LSK) was identified as the population containing all the HSC activity (Uchida et al., 1994). However, this population is now known to be heterogeneous and contains a mix of stem cell populations with progenitor cells (Bryder et al., 2006). Subsequently after these initial investigations, research by the Weissman, Jacobsen, Nakauchi and Morrison laboratories collectively identified markers which allowed for the isolation of purer HSC populations. Interestingly, one of these markers, CD34, was found to be a negative marker of LT-HSC in contrast to evidence from the human studies. The addition of LSK with the negative selection of cell surface markers CD34 and Flk-3 was reported to give rise to long term haemopoiesis with the acquisition of these markers selecting for ST-HSC and MPP populations (Osawa et al., 1996, Christensen and Weissman, 2001). More recently and arguably most commonly used in the literature are the cell surface markers CD150 (more commonly referred to as SLAM), CD244 and CD48 (Kiel et al., 2005). Currently $LSKCD150^{+}CD48^{-}$ and $LSKCD34^{-}Flk\text{-}3^{-}$ are the most commonly used sets of markers for LT-HSC identification in the mouse system. The former is arguably the most commonly used method for identification, due to the positive selection of marker CD150. Using this marker showed approximately 50% of the LT-HSC gave rise to BM reconstitution (Kiel et al., 2005). These studies bring us closer to identifying a HSC population capable of 100% BM reconstitution. This is the purest population currently available for mouse HSC to date. A figure with the most up to date mouse HSC hierarchy is displayed (Figure 1-4). Committed progenitor cell types are well

defined in the mouse system with a combination of cell surface markers including CD127, CD34 and CD16/32 (Doulatov et al., 2012).

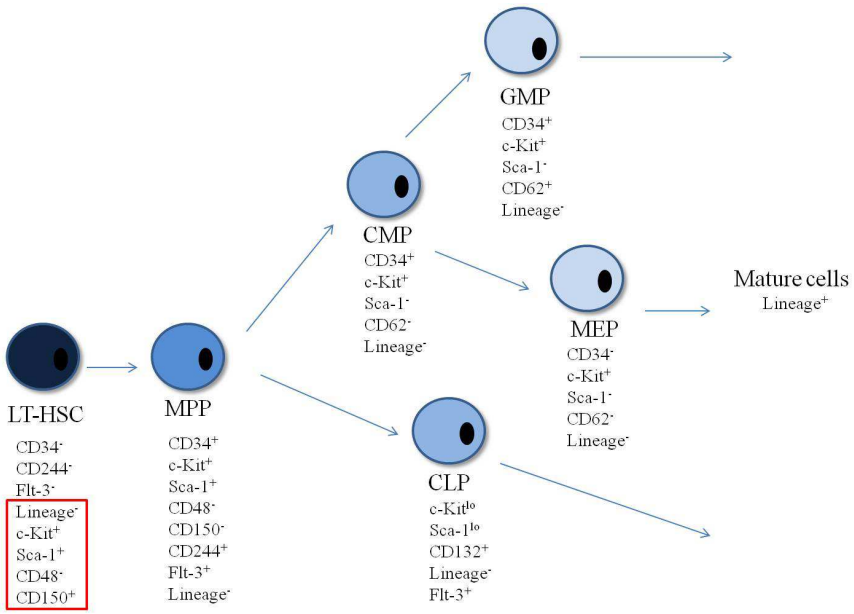

Figure 1-4 Mouse haemopoietic hierarchy.

Schematic demonstrates the most recent mouse haemopoietic hierarchy. The boxes denote the cells used to identify human stem/progenitors in this study. The box marked in red identifies the LT-HSC (lineage$^-$c-Kit$^+$Sca-1$^+$CD150$^+$CD48$^-$) used throughout in chapters 4 and 5 and used widely in the literature to separate identify LT-HSC. The ST-HSC and progenitor populations used in this study are described more detail in the materials and methods chapter 2 and were used widely in the literature at the time of doing the experiments in this thesis. Information in this schematic is based on the literature discussed in section 1.3.3.1.

1.3.3.2 Human

Focusing on human studies, early experiments identified that positive expression of CD34 marked a rare population of BM cells which were enriched for colony formation and capable of *in vivo* reconstitution of immunocompromised mice (Berenson et al., 1988, Sutherland and Keating, 1992, Civin et al., 1984, Andrews et al., 1989). Collectively, these studies suggested that CD34 marked a population of cells with stem/progenitor activity. In addition, the CD34 protein has also been shown to be expressed on endothelial cells (EC) and embryonic fibroblasts (Krause et al., 1996). To date, human HSC are now commonly identified in the literature as expressing CD34. It is a well known marker of a

heterogeneous stem/progenitor cell population (Stella et al., 1995). Experiments have detected stem cell activity in the $CD34^-$ fraction of human cells which indicated that CD34 is not a marker of all stem and progenitor populations (Bhatia et al., 1998, Goodell et al., 1997, Sonoda, 2008). Recently, a study has shown that this population in combination with additional marker CD93 does function as a HSC population and is more primitive than $CD34^+$ cells suggesting $CD34^-$ cells are at the pinnacle of the haemopoietic hierarchy (Anjos-Afonso et al., 2013, Danet et al., 2002). However, this research is novel and to date, CD34 is widely used in experimental haematology and clinical haematology in which $CD34^+$ cells are isolated and used for stem cell transplantation (Wognum et al., 2003). Interestingly, although CD34 is widely used as a human stem/progenitor marker, the function of the CD34 protein is not well understood. It is thought that this is due to a lack of data on functional assays on the protein as discussed in a detailed review (Nielsen and McNagny, 2008). As $CD34^+$ cells are known to represent a heterogeneous population containing stem and progenitor cells, additional surface markers have been sought after in order to further enrich the human stem cell population. Additional marker CD133 has been reported (Yin et al., 1997). However, more recent evidence suggest CD133 does not mark only stem cells but also more mature cell types (Meregalli et al., 2013). CD38 is a cell surface marker known to play roles in immunity, cell adhesion and calcium signalling (Mehta et al., 1996). Experiments have shown that a small proportion of $CD34^+$ cells express the CD38 protein (<10%) therefore representing a rare population of $CD34^+CD38^-$ cells (Bhatia et al., 1997). The combination of CD34 with CD38 showed that the $CD34^+CD38^-$ and $CD34^+CD38^+$ fractions differed in terms of cell cycle status and stem cell activity, including reconstitution into immunocompromised mice (Bhatia et al., 1997, Civin et al., 1996). These cell surface combinations are now widely used in studies with the $CD34^+CD38^-$ fraction representing a more primitive subset of cells. However, further purification of this population can enrich the stem cell population, for example by using the combination of CD34 and CD38 with CD45RA and CD90 (Thy-1). In addition, rhodamine123 has been used as a dye to mark stem cells which are negative for the dye. Briefly, rhodamine123 is a dye that labels mitochondria with increasing intensity proportional to cellular activation (Kim and Broxmeyer, 1998). CD45RA is a member of the CD45 family that is highly expressed on naive T lymphocytes; whereas CD90 is commonly used to identify thymocytes, but has also been implicated in a variety of different processes (Streuli et al., 1987, McKenzie et al., 2007, Mayani et al., 1993, Baum et al., 1992). Recently the combination of markers was used to identify a population with stem cell activity ($CD34^+CD38^-CD45RA^-CD90^+$) however the population containing $CD90^-$ cells also showed engraftment in serial transplantation assays (Notta et al., 2011,

Majeti et al., 2007). More recently, the addition of CD49f was conclusively shown to be a specific HSC marker and it was shown that the MPP population lost expression (Notta et al., 2011). CD49f is a member of the integrin family which associates with either integrin β1 or β4 to form receptors for laminin and Kalinin. It is expressed on a monocytes, T cells, platelets, endothelial and epithelial cells, and is involved in adhesion or co-stimulation for T cell activation/proliferation (Hughes, 2001). Although some controversy still exists, collectively $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}CD49f^{+}$ cells represent the highest reported purity of human HSC to date and a figure is displayed in Figure 1-5. Humans have well defined cell surface marker expression committed progenitor cell types using CD135, CD10 and CD7 (Doulatov et al., 2012). Although human HSC identification has progressed, human markers of the stem and progenitor populations in haemopoiesis are not as well identified as in the mouse system.

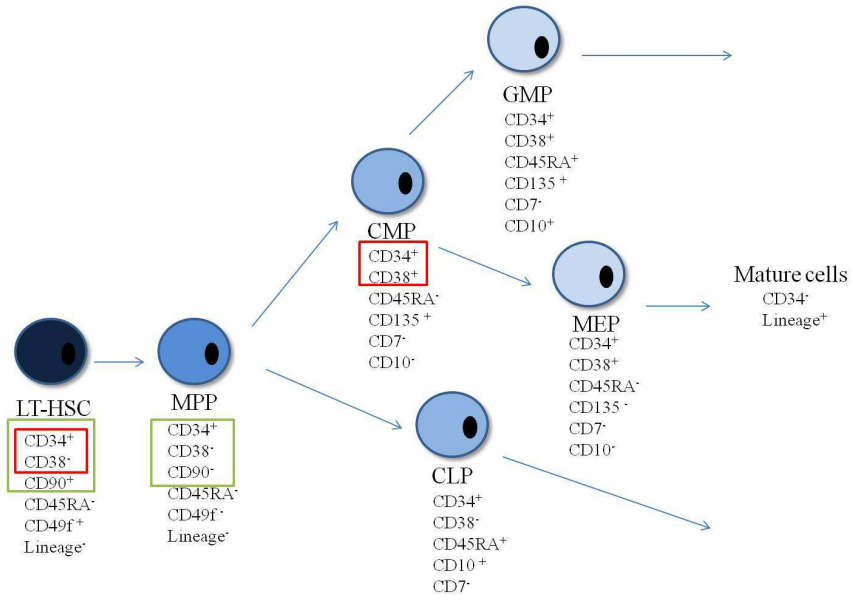

Figure 1-5 Human haemopoietic hierarchy.

Schematic demonstrates the most recent human haemopoietic hierarchy. The boxes denote the cells used to identify human stem/progenitors in this study. The boxes marked in red identify HSC ($CD34^+CD38^-$) with MPP ($CD34^+CD38^+$) used throughout in chapter 3 and used widely in the literature to separate HSC with more mature progenitor populations. The green boxes denote HSC ($CD34^+CD38^-CD90^+$) with MPP ($CD34^+CD38^-CD90^-$) and more committed progenitors ($CD34^+CD38^+$) used in chapter 5 which is used widely in the literature. Information in this diagram is based on the literature discussed in section 1.3.3.2.

Due to the identification of cell surface markers expressed by human and mouse HSC populations, flow cytometry cell sorting has emerged as the best technique for the isolation of HSC populations. Cell sorting using flow cytometry allows for the isolation of individual cells which is ideal for stem cell biology in which these cells are so rare. This also allows for the study of the stem cell behaviour at a single cell level. The ability to identify and isolate HSC from their environment allows for their study and this approach has enabled us to understand their behaviour.

1.3.4 HSC cellular fates

In addition to self renewal versus differentiation, HSC can undergo alternative cellular decisions (Domen and Weissman, 1999). It is understood that the numbers of HSC are fairly constant over the period of a lifetime and it is thought that this is a tightly regulated

process. One of the mechanisms to ensure that HSC numbers are regulated over the period of a lifetime is programmed cell death.

Apoptosis is understood to be a molecular mechanism which regulates numbers of cells within the haemopoietic system. Known regulators of apoptosis in the HSC population, include members of the BCL family (Domen and Weissman, 1999, Domen et al., 2000). The over expression of BCL-2 in the HSC compartment was shown to result in altered HSC numbers and activity in competitive transplantation assays (Domen et al., 2000). In addition to members of the BCL family, other candidates are involved including the anti-apoptotic protein MCL-1 (Opferman et al., 2005).

When we discuss cell death it is also important that we consider not only programmed cell death, but other mechanisms. Autophagy is a key survival process in which cellular components are degraded to maintain cell survival at basal levels and in response to stress (Murrow and Debnath, 2013). Recent research has identified that autophagy plays an essential role in HSC maintenance (Mortensen et al., 2011a, Mortensen et al., 2011b). Further research identified autophagy as a mechanism which protected HSC from metabolic stress with forkhead family transcription factor member FOXO3a proven to be a key mediator of this process (Warr et al., 2013).

The majority of HSC reside in the BM, however these cells can traffic into and out of circulation and home to sites of extramedullary haemopoiesis, which is termed as HSC mobilisation. Mobilisation plays important roles throughout development, with the migration of HSC to different sites of haemopoiesis and in the adult at basal levels. In the adult, the egress of HSC into the periphery is modulated in response to inflammation, stress or injury (Ratajczak and Kim, 2012). Furthermore, the administration of pharmacological agents has been found to increase HSC mobilisation. This process can be exploited with exogenous addition of particular cytokines which are shown to enhance mobilisation above basal levels. Mobilisation through cytokine treatment is also routinely used in clinical therapy to mobilise the donor stem cells from BM to the PB so they can be more easily harvested prior to transplantation. The majority of understanding of HSC mobilisation is with the use of cytokine stimulation granulocyte-cell stimulating factor (G-CSF) and chemokine receptor CXCR4 and ligand CXCL12 (Whetton and Graham, 1999). Although the mechanism of action is not clear, proposed mechanisms include the involvement of granulocytes, metalloproteinases (MMP) and proteolytic enzymes (Ratajczak and Kim, 2012).

The combination of the diverse cellular fates faced by the HSC allows the HSC population to be tightly regulated. As an example, when the HSC population becomes too large, apoptosis is induced and vice versa. Similarly, in response to haemopoietic injury or stress, differentiation is induced, ultimately leading to constant numbers of HSC and the stable production of a cascade of mature cell types. In addition to the cellular fates outlined above, HSC are known to exist in different stages of the cell cycle (Figure 1-6). As HSC face cellular decisions of self renewal versus differentiation it seems likely that the HSC is a dynamic, active population. However, surprisingly it is known that the majority of HSC exist in a quiescent state.

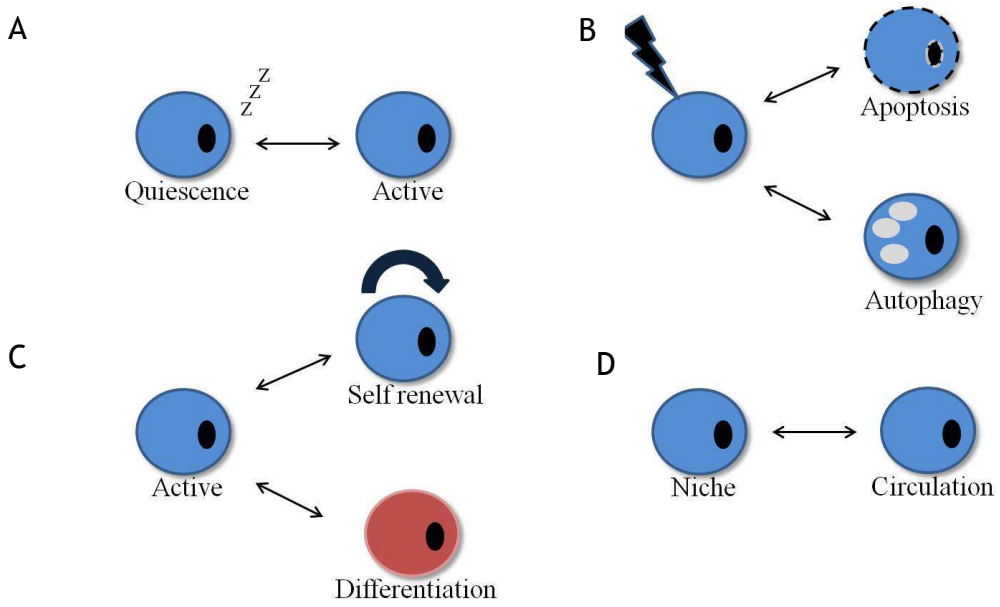

Figure 1-6 HSC cell fate decisions.

Schematic diagram is adapted from published literature (Domen and Weissman, 1999). HSC face several fate decisions that are thought to be controlled by both intrinsic and extrinsic mechanisms. The majority of steady state HSC are quiescent, however they can also reversibly move into an active stage of the cell cycle (A) in which they can either self renew through symmetric cell division or differentiate through asymmetric or symmetric cell division (C). HSC can reside within the BM niche or they can migrate into the circulation, which occurs at low levels during steady state haemopoiesis, but may be higher in response to stress or haematological injury (B). HSC respond to external signals and balance survival with cell death by programmed cell death (apoptosis) or autophagy in order to maintain a constant size of the HSC pool (D).

1.3.5 HSC kinetics

When HSC are in an active state of the cell cycle they have the option to follow paths of self renewal or differentiation via asymmetric and symmetric cell division. However, studies examining the cell cycle status of HSC populations have identified that the majority of HSC are inactive and only a small percentage of HSC are actively proliferating. Quiescence is now known to be a common property in stem cells. It is this function that is thought to be crucial for the ability of stem cells to maintain homeostasis and the capacity to respond to stress or injury with regeneration over the period of a lifetime. In particular, it's believed that the quiescent phenotype is to preserve genomic integrity and to 'escape' stress from external stimuli (Pietras et al., 2011, Li and Bhatia, 2011). it is generally

accepted that the majority of HSC are quiescent and are only induced to proliferate at low levels during basal haemopoiesis and at higher levels due to haematological stress (Li and Clevers, 2010).

Quiescence refers to a cell in a state of dormancy or inactivity in which the cell is not dividing, therefore is in G_0 phase of the cell cycle. Quiescence should not be misinterpreted with other processes in which cells are moved to $G_{0,}$ including senescence. Quiescence is viewed as a temporary state of inactivity in which cells are held in G_0 but can re-enter the cell cycle in response to particular stimuli. Instead, senescence has been defined as a form of replicative cell death in which proliferation is irreversibly arrested (Campisi, 2013). Senescence is known to be induced in response to stimuli including DNA damage, epigenetic changes or activation of tumour suppressor genes. Although there is some literature to suggest senescence may not be completely irreversible, there is a distinction between quiescent and senescent cells and it is generally accepted that senescent cells do not re-enter the cell cycle.

Experiments using the stem cell hair follicle system used dyes which were diluted upon cell division and identified that a proportion of cells were ‘label retaining’ (Cotsarelis et al., 1990). Similar observations were made in other systems containing stem cells, including the gut epithelium and epidermis (Cheung and Rando, 2013). Experiments in the haemopoietic system identified that adult HSC are mostly quiescent, with around 1-3% in cycle and 90% in G_0 (Harrison and Lerner, 1991, Goodell et al., 1996, Bradford et al., 1997, Cheshier et al., 1999). These cells were also shown to exhibit low RNA content and absence of markers involved in cell proliferation (Cheung and Rando, 2013). Haematological stress using chemotherapeutic reagent 5-fluorouracil (5-FU), results in the killing of mature, cycling cells and essentially depletes the BM system. Experiments found that the HSC population moved into an active cell cycle state in response to 5-FU treatment and replaced the haemopoietic system with the production of progenitors and subsequently mature cell types (Harrison and Lerner, 1991, Van Zant, 1984, Goodell et al., 1996, Dixon and Rosendaal, 1981). A global gene expression analysis demonstrated that 5-FU induced a proliferative signal in the HSC, which resulted in a preparative state in the HSC population. This was followed by active proliferation, before being re-introduced into quiescence depicted by distinct gene expression patterns at each stage (Venezia et al., 2004). Collectively, these studies suggest that the majority of HSC are in a state of quiescence, allowing cells to be ‘poised’ for entering the cell cycle to give rise to mature blood cells when required, while maintaining a dormant stem cell pool. The balance of

quiescence and proliferation is controlled for longevity and also for protection from genetic damage and stem cell exhaustion (Pietras et al., 2011).

What are the molecular mechanisms which underlie cell fate decisions? Evidence has shown that a combination of both intrinsic and extrinsic mechanisms are involved which will be discussed below.

1.3.6 Intrinsic regulation of HSC behaviour

Intrinsic factors which regulate HSC behaviour are cell autonomous which are already embedded within the cell. Factors include gene and miRNA expression, epigenetics, metabolism and protein expression including post translational modifications. A brief over view of the mechanisms involved in the intrinsic regulation of HSC behaviour are discussed.

The ability to identify and isolate rare HSC populations has allowed gene expression analyses. The identification of highly expressed genes in primitive HSC populations and studies using knock down or over expression has facilitated our understanding on their role in HSC maintenance. Genes previously identified include members of the Homeobox family (HOX) of genes, Polycomb (PCG) and Wnt families of genes (Eckfeldt et al., 2005). As quiescence is fundamental to HSC behaviour, genes involved in cell cycle regulation have been identified to be essential for HSC maintenance, including the Cyclin family members (Pietras et al., 2011, Zon, 2008). Furthermore, studies have shown that signalling pathways fundamental to development are also essential for HSC maintenance. These include the bone morphogenetic protein (BMP) family members, Notch and Hedgehog signalling (Eckfeldt et al., 2005). In addition, genetic mutations involved in malignancies of the haemopoietic system have been reported to regulate normal HSC including Scl and Lmo2. Finally, chemical treatment has identified particular genes/pathways fundamental for HSC maintenance. Examples include retinoic acid and prostaglandin E_2 which were found to alter signalling pathways involved in HSC maintenance including HOX and BMP pathways (Zon, 2008).

It is not solely gene expression which is fundamental for controlling HSC behaviour. In addition, there is the added complexity of protein expression and post translational modifications which can occur. Furthermore, transcription factors, epigenetics and microRNA (miRNA).are involved. Transcription factors including p53, Runx1, Cbp and

Gata2 have been identified to be essential for HSC maintenance (Pietras et al., 2011, Zon, 2008). MiRNA are a class of small noncoding RNA that are involved in the regulation of gene expression. More recently, miRNA were identified to be fundamental for intrinsic HSC regulation (Zhao et al., 2013, Alemdehy and Erkeland, 2012). Finally, modifications of chromatin are involved and it has been shown that epigenetic regulators including E2H2 and MLL are necessary for HSC self renewal (Zon, 2008).

Experiments have identified that HSC reside in a location which is of low oxygen levels (Zhang and Sadek, 2013). Over the past several years, studies have shown that HSC have lower rates of oxygen consumption and master regulators of metabolism including hypoxia-inducible factors (HIF) have been shown to be essential for HSC maintenance. It has now been identified that various aspects of metabolism including mitochondrial respiration and oxidative stress are fundamental for HSC activity.

It is clear that a complex network of gene, protein and other regulatory mechanisms are involved in the functioning of the HSC through intrinsic mechanisms. However, to add to this complexity, cell fate is not solely controlled by mechanisms intrinsic to the HSC. HSC behaviour is regulated by extrinsic factors which include growth factors (GF) and signalling pathways from within the BM niche where the adult HSC reside.

1.3.7 BM niche

There are distinct phases and locations of haemopoiesis which occur over the period of a lifetime. These can be divided into haemopoiesis during development in the early embryo and in the adult.

Studies have aimed to identify and understand where and when the first haemopoietic cells appear in the early embryo. Studies on haemopoiesis in early development have been facilitated using the embryos of model organisms, including the zebrafish, chick and mouse. In the developing embryo, the first haemopoietic cells have been identified to appear in the yolk sac and blood islands of the chick embryo (Dzierzak and Speck, 2008). In terms of the first HSC, elegant transplantation studies collectively identified that the first HSC appear at embryonic day 10.5 (E10.5) in the aorta-gonad-mesonephros (AGM) region of the developing embryo (Ivanovs et al., 2011). Experiments using explants of particular regions identified that these cells at this stage and region were capable of long term, multilineage reconstitution of irradiated hosts (Dzierzak and Speck, 2008). Some

controversy exists about whether the first HSC can be found in the AGM region or exist at an earlier timepoint of development in the yolk sac (Samokhvalov et al., 2007). During mid to late gestation, HSC migrate to the fetal liver, which is an important site of haemopoiesis in early development and they subsequently migrate to the predominant site of adult haemopoiesis, the BM.

Bone cavities are formed during early embryogenesis, the HSC migrate to the endosteum after birth and this remains the predominant site of haemopoiesis throughout adult life (Dzierzak and Speck, 2008). At the adult stage there are secondary sites of haemopoiesis, including the spleen, thymus and lymph nodes. In addition, HSC are capable of migrating to and from the BM into the periphery at low levels and is modulated in response to inflammation, stress or injury (Ratajczak and Kim, 2012). In this thesis, factors involved in adult haemopoiesis are investigated, therefore the BM and haemopoiesis in the adult system will be discussed onwards.

The BM has been termed the adult haemopoietic ‘niche’ due to the complex signalling and interactions that occur between HSC and other cells within the structure. The term niche was first introduced in 1978 by Schofield and is now accepted and commonly used (Schofield, 1978). The concept of a stem cell niche is not unique to the haemopoietic system. An excellent example of this is demonstrated in experiments on germ stem cells (GSC) in the model organism *Drosophila Melanogaster*. GSC were found in close contact with accessory cells and their interaction was found to be fundamental for stem cell properties, including proliferation and differentiation (Eckfeldt et al., 2005). A dynamic niche has been well described for other stem cell systems, including the intestinal epithelium and epidermal structures such as the hair follicle (Moore and Lemischka, 2006). Collectively, these studies have identified a tight interplay between stem/progenitor cells with other cell types, which cooperate to ultimately maintain tissue homeostasis and function. Since the original proposal of a BM niche, an abundance of literature has emerged to show how diverse cell types within the niche affect HSC behaviour and maintain a functioning haemopoietic system (Moore and Lemischka, 2006, Weissman, 2000).

The BM niche is composed of several cell types including osteoblasts (OB), osteoclasts (OC), stromal cells, EC, macrophages, sympathetic nervous system neurons, glial cells as described in a recent review (Smith and Calvi, 2013). The evidence of how these cells regulate HSC behaviour and haemopoiesis is discussed below. The majority of research on

the BM niche has been carried out on mouse models, however more recently some human research has emerged. Mouse and human research will be discussed separately to avoid confusion.

1.3.7.1 Extrinsic regulation of HSC behaviour

1.3.7.1.1 Mouse

MSC have been shown to play fundamental roles in haemopoiesis and have gained interest in the literature recently. Original experiments noted that individual cells derived from the BM could formed colonies of a fibroblast nature, which were colonies derived from single clonogenic cells (Friedenstein et al., 1968). These are now referred to as colony forming units fibroblast (CFU-F). In addition, these cells were known to exhibit self renewal and differentiation, indicating that these cells were of a stem cell origin (Friedenstein et al., 1982). These cells were termed MSC and were subsequently identified and isolated. Although there is controversy regarding the exact molecular signature of these cell types, a recent review describes the most up to date phenotype in human and mouse systems (Frenette et al., 2013). MSC reside in the BM niche where they can produce stromal cells, adipocytes, chondrocytes and OB which have been implicated to play a role in HSC behaviour. The interactions occur through a variety of cell surface receptors, secreted ligands and GF (Krause et al., 2013). Experiments have identified that these cells and their progeny express proteins, including CXCL12, SCF, Angiopoietin-1, various adhesion molecules (for example, VCAM-1), VEGF, Wnt and Notch ligands (Ding and Morrison, 2013). The receptors for these proteins are expressed by the HSC population and these interactions have been identified in the literature to play important roles in HSC properties, including quiescence, adhesion, maintenance and self renewal (Eckfeldt et al., 2005, Li et al., 2009, Yamashita et al., 2012, Reya et al., 2003, Kirito et al., 2005).

In addition, various experiments using gene depletion and over expression noted that other cell types within the niche, macrophages, adipocytes, sympathetic nerve neurons adipocytes, extracellular matrix and glial cells altered HSC numbers or mobilisation (Krause et al., 2013, Smith and Calvi, 2013). The ECM is composed of collagens, glycoproteins which can influence a variety of HSC functions including adhesion, proliferation, survival and differentiation (Muth et al., 2013).

Gene targeting studies have identified that a series of developmental signalling pathways are involved in HSC maintenance. Studies identified TGF-β, Wnt, Notch and Hedgehog

were essential for the extrinsic regulation of HSC behaviour (Pietras et al., 2011). A more in depth discussion is provided in the following paragraphs.

Cells which have gained a lot of interest in the BM niche are OB and OC (Mansour et al., 2012). OB and OC are involved in bone modelling and several studies have provided conclusive evidence that OB regulate HSC behaviour. It has been shown in mouse models that altering OB numbers alters the number of HSC (Visnjic et al., 2004, Calvi et al., 2003). In the study by Calvi *et al*, transgenic mice with activated parathoid hormone (PTH) were used which increase OB number and consequently HSC numbers. In another study, complimentary findings were reported. Visnjic *et al*, used transgenic mice expressing herpes thymidine kinase under the collagen type I promoter (Visnjic et al., 2004). In these mice a bone loss was reported which resulted in a decrease in BM cellularity including mature cell types and the HSC population. A recent study also implicated the role of BM macrophages. Winkler *et al*, used transgenic mice (Mafia) to deplete macrophages and found a reduction in OB numbers which resulted in an increase in HSC mobilisation (Winkler et al., 2010). Furthermore, experiments inhibiting or activating OC has shown alterations in HSC mobilisation although there are some discrepancies between studies (Miyamoto et al., 2011, Kollet et al., 2006). It has been shown that increasing OC number alters HSC mobilisation. The study by Miyamoto *et al* used three different transgenic mouse models which reduced osteoclast numbers and showed no effect on mobilisation. However, pharmacological inhibition of OC was shown to increase HSC mobilisation (Miyamoto et al., 2011). Recently, a mouse model (*Oc/Oc*) resulting in loss of osteoclast activity resulted in HSC effects but also dramatic effects on the BM niche (Mansour et al., 2012). Results showed increased MSC production and reduced osteoblast differentiation. Ultimately these niche changes resulted in a decrease in HSC homing. Further experiments identified a Notch signalling as a key signalling pathway in this process (Calvi et al., 2003). Collectively, there is conclusive evidence from mouse studies that both OB and OC play a fundamental role in the regulation of HSC properties. As these cells are located in a particular region of the niche in close proximity to the bone tissue, it was hypothesised that there may be distinct regions within the niche important for HSC behaviour.

Experiments have shown that cell types are located in particular areas of the BM niche which are essential for their development and maturation (Nilsson et al., 2001). To get an indication of the BM architecture and cell to cell interaction *in vivo*, studies on the BM niche have used histological analyses. These analyses have provided great insight into the location of particular cell types in the BM niche and indicated possible function. However,

histology provides only a two dimensional view of the niche, therefore more recently, intelligent imaging techniques, using intravital microscopy, have been employed to examine the niche with the intact three dimensional architecture *in vivo*. Studies have identified distinct areas within the BM niche in which HSC populations reside. More specifically, HSC have been identified to localise close to the endosteal surface of trabecular bone, endosteal niche which has been reported in HSC after transplantation (Nilsson et al., 2001, Lo Celso et al., 2009). The study by Nilsson *et al* used labelling of cells and transplantation into non ablated recipients. Stem cells were found to locate to the endosteal niche and more mature 'lineage restricted' cells were mostly located in the central marrow (Nilsson et al., 2001). Another study used an alternative technique of live imaging of transplanted, labelled cells in the calvarium and they noted that cells with varying maturity/differentiation located to different areas within the BM niche (Lo Celso et al., 2009). In addition, it was observed that HSC locate to the endosteal niche when required for expansion suggesting this area controls stem cell behaviour. In terms of a mechanism, a variety of studies have identified particular proteins involved, with calcium signalling as an example. HSC lacking a calcium sensing receptor were shown to have a defect in their localisation to the endosteal niche, which was shown to be disruptive to their function (Adams et al., 2006). Research has been conducted primarily on the calvarium which has been shown to be an ideal model for live cell imaging. However, differences in mouse models used, labelling of cells, exact cell populations studied and niche area studied will likely contribute to differences between studies. Indeed, a recent study has argued that different BM compartments are diverse and shown striking data in which heterogeneity in their structure and function is shown (Lassailly et al., 2013).

Although there is a great body of evidence to suggest that the endosteal niche is fundamental to HSC function, a vascular niche exists which is also important. The vascular niche is located in the centre of the BM close to the blood vessels. It contains sinusoids which contain a layer of EC (Kopp et al., 2005). It is proposed that different anatomical areas of the niche control different HSC behaviour with the general consensus that more mature progenitors reside in the vascular niche (Xie et al., 2009, Lo Celso et al., 2009). However, this is contradictory evidence in the literature and HSC have been reported to reside in the vascular niche with more mature progenitors located in the endosteal niche (Ding and Morrison, 2013). However, it has been suggested that experiments reporting that HSC reside close to the endosteum perhaps underestimate the vascular niche. There are also issues due to the close proximity of both niches and there are technical issues involved with the size of animal tissues (Doan and Chute, 2012). However, in spite of the

controversy in the literature regarding this issue, there is evidence that shows the vascular niche plays an important role in HSC behaviour (Eckfeldt et al., 2005). Studies have shown this involves blood vessels and the sympathetic nervous system. A study by Yamazaki *et al,* used TGF-β type II receptor null mice to confirm the role in HSC maintenance (Yamazaki et al., 2011). They found that non-myelinating Schwann cells were responsible for TGF-β expression through nerve cells. Furthermore, nerve denervation reduced Schwann cells and consequently decreased HSC cell number. Another study reported the function of EC in HSC function. Research by Winkler *et al,* used transgenic mice null for E-selectin and reported HSC proliferation and chemosensitivity was altered. In addition, pharmacological inhibition of E-selectin reduced HSC proliferation. E-selectin was found to be expressed in BM EC exclusively in the vascular niche (Winkler et al., 2012). This research supports the concept that HSC proliferation is important in the vascular niche and quiescence HSC reside in the endosteal niche (Kopp et al., 2005). CXCL12-abundant reticular cells (CAR) which express CXCL12 have been shown to play a role in maintaining the HSC quiescence pool through CXCR4. These cells have been reported in both the endosteal and the vascular areas within the BM niche in particular around the sinusoids in the vascular niche (Sugiyama et al., 2006).

However, collectively these studies must be interpreted with caution as mostly these experiments use *in vivo* analysis of the BM niche typically after transplantation. Transplantation stresses the haemopoietic system and may indeed alter cell interaction dynamics, in comparison to behaviour that occurs in a normal, physiological setting.

Studies examining oxygen sensing have identified that the BM niche is ‘hypoxic’, which refers to a state of low oxygen. Original experiments which used targeted deletion of essential hypoxic genes in the haemopoietic system found a reduction in HSC function and maintenance in response to deletion of these particular genes (Takubo et al., 2010). Furthermore, it has been proposed that particular HSC populations require distinct metabolic programs and require different oxygen levels according to their function (Warr et al., 2013). These signalling pathways are also related to DNA damage and oxidative stress and there is compelling evidence to show that these pathways are relevant to HSC and their niche (Moore and Lemischka, 2006).

The studies outlined above clearly demonstrate that the BM niche is a complex interplay of several diverse cell types that work cooperatively to orchestrate haemopoiesis over a lifetime. Various studies have implicated the role of particular proteins in the regulation of

the haemopoietic niche, including BMP, Wnt and Notch signalling (Moore and Lemischka, 2006). These signalling pathways are also known to be involved in several other stem cell niche systems and therefore a conserved stem cell signature common among several tissues has been proposed (Eckfeldt et al., 2005). Understanding niche interactions is fundamental to understanding HSC behaviour. In addition, haematological diseases are known to exhibit BM abnormalities. Recent evidence showed that the niche becomes modified in response to disease, which ultimately resulted in the protection of malignant HSC from apoptosis (Krause et al., 2013).

1.3.7.1.2 Human

Perhaps not surprisingly human studies on the BM niche are rare. Recently, a study by Guezguez *et al.* examined HSC location in xenograft mouse models and using histology of BM trephines (Guezguez et al., 2013). In this study, the authors characterise the location of HSC within human BM biopsy specimens and use human-mouse xenografts. In accordance with the majority of literature on mouse studies, human HSC were located in the trabecular bone area in an endosteal niche. Furthermore, OB and Notch signalling were found to play a role in HSC function.

A collection of studies from both mouse and human models has shown that the BM niche is indeed complex and contains a variety of different cell types and signalling molecules which each play a fundamental role in regulating haemopoiesis. A summary of the main cell types with key molecules involved is presented in Figure 1-7.

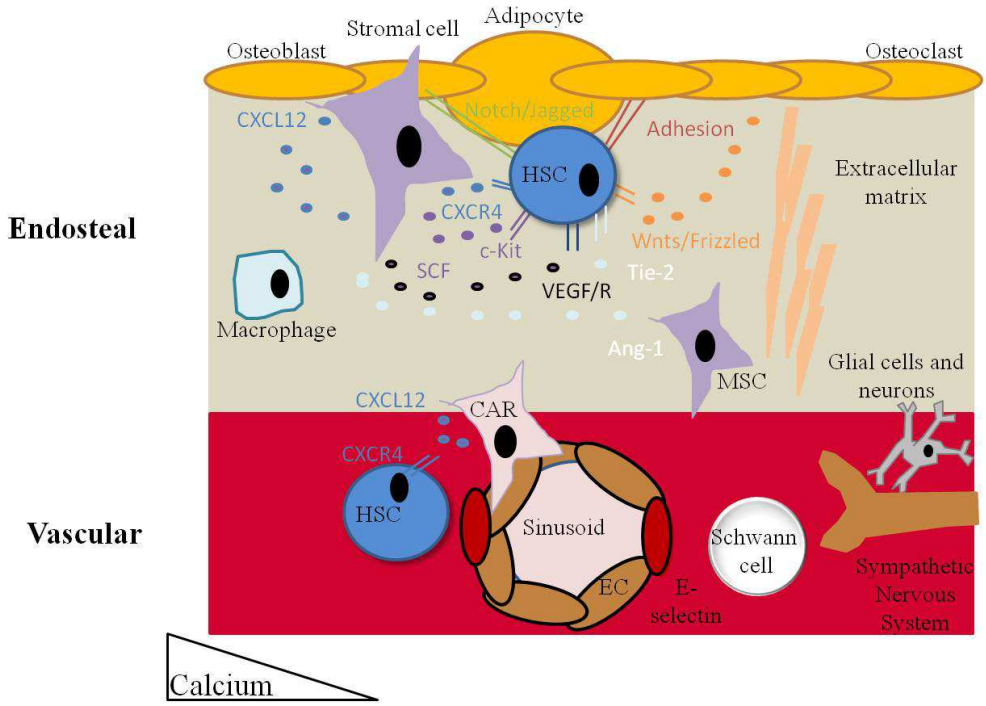

Figure 1-7 Schematic diagram of components of the BM niche.

The majority of research on the BM niche has been acquired from mouse studies. The BM niche is important for stem cell function and tissue homeostasis. The niche is a tight interplay of various components, cell types, GF, vessels, nerve fibres and matrices. Studies have highlighted that there are two distinct regions; the endosteal and vascular region which are both important for stem cell function. Although the majority of research has hypothesised that the endosteal region is where quiescent HSC are maintained and the vascular niche supports more mature, committed progenitor cells. HSC interact with the niche through adhesion molecules and gap junctions, cytokines/chemokines and GF and extracellular matrix. Schematic adapted from published literature (Ehninger and Trumpp, 2011, Geiger et al., 2013, Eckfeldt et al., 2005).

1.3.8 Methods for understanding HSC cellular fate decisions

Several methods can be used to identify novel candidate genes/pathways involved in regulating a particular biological process including hypothesis and data driven approaches. Data driven approaches apply a non biased approach to identify candidates. As an example, data driven approaches including gene expression analyses on a high-throughput scale can be used as a tool to identify candidates involved in biological processes (Bryder et al., 2006, Eckfeldt et al., 2005). High-throughput screening analyses are not limited to

gene expression and studies have shown that epigenetic, protein and phospho-protein screens can be carried out.

The experiments designed in this PhD thesis were based on a previous microarray study which aimed to identify transcriptional differences between quiescent and proliferative HSC populations. Although various previous studies have identified a variety of different factors which play a role in HSC quiescence, it is still not well understood (Li, 2011, Park et al., 2010, Liu et al., 2009). Several studies have aimed to identify novel transcriptional targets in quiescent HSC, however the literature is dominated by studies on mouse HSC (Cheung and Rando, 2013, Forsberg et al., 2010, Venezia et al., 2004, Passegue et al., 2005). Understanding the molecular mechanisms underlying quiescence in normal HSC can provide a foundation for when this becomes deregulated, for example in response to ageing and disease (Geiger et al., 2013, Li, 2011, Graham et al., 2002).

1.3.9 Study rationale

A previously published microarray study from our group aimed to use a high-throughput gene expression platform to identify candidates differentially regulated between quiescent and proliferative human HSC. This screen was carried out using human haemopoietic stem/progenitor populations sorted based on their cell cycle status with DNA and RNA flow cytometry markers ($CD34^+$) (Graham et al., 2007). Hoechst 33342 and Pyronin Y are widely used flow cytometry stains for the identification of populations with differential cell cycle status (Shapiro, 1981). In the previous study, viable $CD34^+$ cells were sorted for G_0 and dividing cells ($G_1/G_2/S/M$) using Hoechst and Pyronin Y. The subfractions were sorted using flow cytometry with G_0 defined as ($CD34^+$, $Hoechst^{low}$, $Pyronin\ Y^{low}$) and dividing defined as ($CD34^+$, $Hoechst^{low\text{-}high}$, $Pyronin\ Y^{high}$). Global gene expression analysis was conducted and the published results from this screen identified that the most up regulated group of genes in G_0 compared to dividing HSC were chemokine ligands including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL10, CXCL11 and CXCL13. The genes reported with the greatest differentiation expression between populations were CXCL1, CXCL2, CXCL6 and CXCL13. The majority of these ligands bind to a communal receptor CXCR2 (CXCL1, CXCL2 and CXCL6), suggesting this common signalling pathway may play a role in stem cell properties, in particular quiescence. We therefore focused on these ligands in the human system and the rationale of this PhD thesis was to investigate their biological function.

Although global expression studies are useful and have increased our understanding of particular biological processes, candidates must be validated and their individual role should be deciphered. The main aim of this study was to validate expression data from the original array and extend this research by executing an investigation into the biological function of chemokines in terms of HSC behaviour.

1.4 Chemokines

Chemokines are a family of small molecular weight cytokines which are secreted and bind specific chemokine receptors to elicit their effects (Murphy et al., 2000). Chemokines are named due to their ability to induce directed chemotaxis in nearby responsive cells and due to their presence within the cytokine family. The majority of the literature is based on the ability of chemokines to attract and activate leukocytes (Baggiolini, 1998). An abundance of literature is now available showing that chemokines play roles in a diverse range of biological functions. It would be impossible to cover all aspects of chemokine function in detail in this thesis, therefore a brief overview of chemokine function is described. The primary topic of this thesis is HSC biology therefore a detailed description of chemokines in haemopoiesis is provided.

1.4.1 Classification

Chemokines were first discovered with the identification of proteins that could induce directed cell migration. After the discovery of the first chemokines, the identification and isolation of others quickly followed (Murphy et al., 2000). The accumulation of literature with the identification of novel chemokines resulted in a random nomenclature, although some proteins had similar structures and functions. In addition, several groups often identified the same protein simultaneously, but with different nomenclature, making the literature confusing. To overcome this issue, a classification system was devised and proposed in a keystone chemokine symposium with the strategy for the classification of chemokines according to their structure and function. In 2000, a nomenclature system for chemokine receptors was devised (Murphy et al., 2000). This was extended to include ligands and receptors (Zlotnik and Yoshie, 2000). This novel classification system created a standardised nomenclature for the naming of chemokines and grouping according to their structure. The classification system is logical, generally accepted and widely used. Although a few studies still quote chemokines using their alias, for simplicity, the naming

of chemokines in this thesis will be according to the standardised classification which can be observed in Table 1-1 (Zlotnik and Yoshie, 2000).

Chemokines were named according to the presence and positioning of a cysteine residue in the N-terminus of the protein structure (Zlotnik and Yoshie, 2000) (Figure 1-8). Chemokines were classified into CXC, CC, XC and CX_3C groups according to the location of the cysteine peptide with other peptides. The CXC family members contain two cysteine peptides with an amino acid separating them, the CC group contain two cysteine peptides in juxtaposition, the XC family members contain a single cysteine peptide which follows an amino acid and finally the CX_3C group contain two cysteine peptides with the presence of three amino acids separating them. An identifying number was given for each individual chemokine. The standard nomenclature is used with the addition of L for ligand and R for receptor with numbering from 1 upwards for both (Table 1-1).

It can be observed from the list of chemokines that there are many different known chemokines which have different functions. To add to this complexity, the existence of chemokines varies between human and mouse species (Zlotnik and Yoshie, 2012). For simplicity, the table shows a list of human receptors and ligands from a recent review which is based on human chemokines. Mouse homologs are known to exist and there are mouse chemokines that have high homology to the human chemokines. However, as described by Zlotnik *et al* in their recent review of the classification system there is not always a homolog between species (Zlotnik and Yoshie, 2000). There are chemokines found in mouse which have no clear human homolog and in contrast there are human chemokines which have no clear mouse homolog. In addition, for several chemokines, there are mouse chemokines available which have similarity to sequences of human chemokines, however the exact homolog is not clear. For simplicity, mouse homologs available are discussed in more detail for specific chemokines throughout the text. Furthermore, variants of particular chemokines have been found (Zlotnik and Yoshie, 2012). These vary in terms of the protein structure, usually through the removal or addition of amino acids. These will be described in more detail for particular examples in the text.

Family	Receptor	Ligand
CXC	CXCR1	CXCL6, CXCL8
	CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
	CXCR3A	CXCL9, CXCL10, CXCL11, CXCL13
	CXCR3B	CXCL4, CXCL4L1
	CXCR4	CXCL12
	CXCR5	CXCL13
	CXCR6	CXCL16
	Unknown	CXCL14, CXCL17
CC	CCR1	CCL3, CCL3L1, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23, CCL26
	CCR2A/B	CCL2, CCL7, CCL8, CCL13, CCL16, CCL26
	CCR3	CCL3L1, CCL5, CCL7, CCL11, CCL13, CCL14, CCL15, CCL24, CCL26, CCL28
	CCR4	CCL17, CCL22,
	CCR5	CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL8, CCL11, CCL14, CCL16, CCL26
	CCR6	CCL20
	CCR7	CCL1, CCL19, CCL21
	CCR8	CCL1, CCL16
	CCR9	CCL25, CCL27, CCL28
	CCR10	CCL3L3, CCL4L2, CCL27, CCL28
XC	XCR1	XCL1, XCL2
CX_3C	CX_3CR1	CX_3CL1

Table 1-1 Chemokine classification system.

Chemokines are classified into subgroups based on the presence and positioning of a cysteine residue in the N-terminus of the protein structure. Chemokine ligands are given the letter L and a particular number with receptors given the letter R. The table shows all chemokines in each subfamily with the known binding partners. The chemokine data is in reference to the most recent review of the chemokine superfamily by Zlotnik and Yoshie (Zlotnik and Yoshie, 2012).

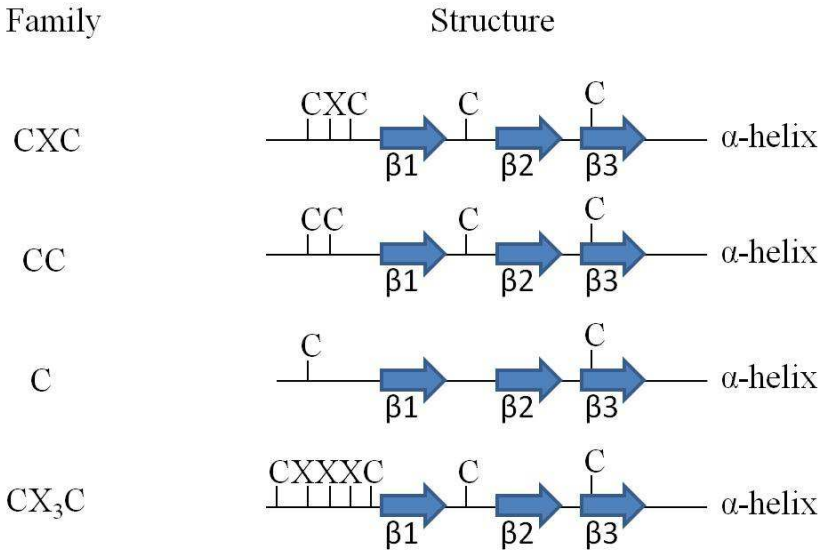

Figure 1-8 Protein structure of chemokine families.

Schematic is modified from published literature (Rollins, 1997). Schematic diagram demonstrates the protein structure of chemokine families. The chemokine protein structure contains an N-terminal loop connected with bonds between cysteine residues depicted by C. The tertiary protein structure of chemokines are very similar. Families are distinguished using the presence and positioning of amino acids (X) inbetween cysteine residues near to the N-terminus of the protein. Chemokines share a structured core consisting of three β-sheets (β1, β2 and β3) with an α-helix at the C-terminal of the protein structure with disulphide bonds which stabilise the structure.

1.4.2 Signalling

Chemokine ligands are expressed by the cell and the majority are secreted as soluble factors which interact with their cognate chemokine receptors. Chemokine receptors are expressed selectively on particular cell types which mediate the chemokine signalling response. An interesting aspect of chemokine biology that is highlighted by Table 1-1 is the observation that several ligands can bind the same receptor and similarly that several receptors can bind the same ligand. This adds a complexity to chemokine signalling as it is known that a high level of redundancy and compensatory mechanisms between chemokines exist. An example is the CXC family, which plays a predominant role in the induction of leukocyte migration. It is thought that several chemokines share roles in this function to ensure robustness in cell recruitment during inflammation (Mantovani, 1999,

Remick et al., 2001). Another example is the availability of viable knock-out mouse models of particular chemokines. This suggests that shared chemokine function has developed in order that fundamental biological processes are not affected by the absence of individual chemokine genes (Mantovani, 1999). However, the concept of redundancy has been questioned and there is evidence against this theory (Schall and Proudfoot, 2011). Studies have shown that different binding partners to the same receptor can result in different receptor behaviour or kinetics. An example is CXCL8 which binds both CXCR1 and CXCR2, however ligand binding shows different kinetics for each receptor (Holmes et al., 1991, Murphy and Tiffany, 1991). Furthermore, studies have shown that different ligands binding to the same receptor show different biological responses and there are key roles for specific chemokine interactions that are not shared between ligands. In addition, there are also specific ligand and receptor partners which are unique and have only one binding partner including CX_3CL1 and CX_3CR1.

The majority of chemokine ligands function by binding and signalling through chemokine receptors which are 7 transmembrane domain spanning guanosine nucleotide binding protein (G protein) coupled receptors (GPCR). Briefly, an extracellular domain at the N-terminus of the protein structure binds the target ligand and the intracellular domain at the C-terminus is responsible for signal transduction (Bockaert and Pin, 1999, Murdoch and Finn, 2000). The structure contains seven α helical transmembrane domains with three intracellular and extracellular loops. Receptor binding of the ligand results in receptor homodimerisation, internalisation, signal transduction and activation of downstream signalling pathways (Raman et al., 2011). The classical method of chemokine receptor signalling transduction is similar to that of alterative GPCR and involves signalling of heterotrimeric G-protein complexes as illustrated in Figure 1-9 (Bockaert and Pin, 1999, O'Hayre et al., 2008). Consequently to ligand/receptor binding, receptors are homodimerised and the G protein pathway is activated (Mellado et al., 2001). The G protein pathway includes activation of Gα, β and γ subunits which control the binding and hydrolysis of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). In the inactive state, the Gα, β and γ subunits are bound with GDP. Ligand binding results in the activation of the receptor and the G protein subunits. GDP then dissociates from the Gα subunit which is replaced by GTP. The Gα-GTP complex dissociates from the receptor and from the Gβ and γ subunits. The process is demonstrated in Figure 1-9. These separate complexes then activate downstream effectors, which ultimately leads to a cellular response. The Gα and Gβ/γ sub units activate distinct signalling pathways, of which some overlap. Following prolonged exposure to a ligand, the receptor can become desensitised

(Murdoch and Finn, 2000). Consequently, the internalised receptor can be ‘recycled’ to the cell surface or be targeted for degradation which is thought to be important for monitoring the signalling response (O'Hayre et al., 2008, Cotton and Claing, 2009).

To add to the signalling complexity, certain chemokine receptors have been shown to homodimerise or heterodimerise, a process which is thought to result in the activation of distinct signalling pathways and biological responses (Salanga et al., 2009, Mellado et al., 2001). This has been observed for several chemokine partners including CCL3/CCL4, CXCL4/CXCL8, and CCL2/CCL8. It has been proposed that receptor dimerization involves the JAK/STAT signalling pathways and downstream transcriptional targets which are important for chemokine regulation *in vivo.*

A series of downstream signalling pathways are activated in response to ligand binding which mainly involves the activation of protein kinases. The activation of signalling pathways is dependent upon the chemokine, the cell type which elicits the response and indeed the required biological response which is summarised in the comprehensive review by O’Hayre *et al* (O'Hayre et al., 2008). Briefly, signal transduction results in the activation of phospholipase C (PLC) which cleaves phosphatidylinositol (4,5)-biphosphate (PIP2) into iositol triphosphate (IP3) and diacylglycerol (DAG). This results in the activation of downstream signalling mediated by protein kinases. Migration and cell adhesion are mediated with phosphatidylinositide 3 kinase (PI3K) and tyrosine kinase (TK) activation as well as activation of Akt, Rac, Cdc42, Rho and Rho associated kinase (ROCK). Collectively, actomyosin is contracted and F-actin is polymerised which ultimately leads to cell motility. In addition, activation of Akt, PKC, TK, mitogen activated protein kinase (MAPK) and PI3K are thought to mediate the effects on survival and proliferation (O'Hayre et al., 2008).

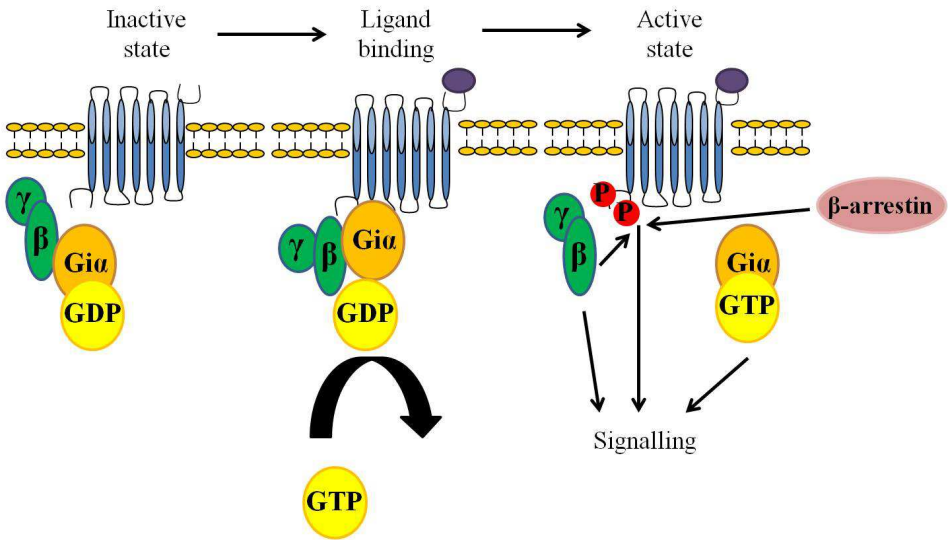

Figure 1-9 Chemokine activation using the G protein pathway.

In the inactive state, the receptor is free from ligand and the G subunits are bound. In response to ligand binding, G subunits are activated, recruited to the GPCR and facilitate the transfer of GDP to GTP. The Gα-GTP complex and the Gβ and γ subunit both dissociate and activate downstream signalling pathways to elicit the required response. Prolonged exposure to ligand binding to the GPCR results in phosphorylation of the C-terminus tail. As a consequence arrestins including β-arrestin are recruited to the GPCR which blocks further interaction of the GPCR with G subunits. Schematic adapted from published literature (O'Hayre et al., 2008).

An interesting aspect of chemokine biology is the presence of receptors which do not elicit a cellular response. These receptors are unique in terms of their function, are known as ‘atypical’ receptors, and are thought to bind chemokine ligands without resulting in signal transduction (Balkwill, 2012). It has been proposed that the lack of signal transduction is due to the lack of a particular amino acid sequence which is found in all other chemokine receptors (Graham, 2009, Ulvmar et al., 2011, Nibbs et al., 2003). Atypical receptors are well studied and include duffy antigen receptor for chemokine (DARC), CCXCKR, CXCR7, CCRL2 and D6 (Graham and Locati, 2013). The random nomenclature reflects the fact that these receptors do not transduce a signal and were not considered as classical chemokine receptors. It is suggested that these receptors are involved in regulating chemokine activity, which is commonly referred to as ‘scavenging’. Arguably the most well studied and understood of the atypical receptors is D6. Studies showed that binding of CC ligands to the receptor does not activation signalling pathways commonly activated in response to GPCR activation (Hansell et al., 2011, Graham, 2009). Indeed, experiments

using CCXCKR and D6 have showed these receptors can bind ligands which are subsequently internalised and degraded (Graham, 2009). Recent evidence has shown that binding of ligands to atypical receptor D6 does not result in the activation of a classical G protein signalling pathway, but involves a β-arrestin dependent signalling pathway (Borroni et al., 2013). This pathway ultimately increases D6 protein at the cell surface and is essential for the scavenging activity. As examples for the physiological role of this process, D6 plays a central role in the modulation of inflammatory responses and CXCR7 scavenging has been shown to regulate tumour cell survival (Lee et al., 2013, Graham and Locati, 2013, Hernandez et al., 2011).

The method of ligand and receptor binding is referred to as the classical method of signalling, however this is not the only method of chemokine signalling. In addition, chemokines can interact with adhesion molecules and glycosaminoglycans (GAG) (Allen et al., 2007). Chemokine ligands CXCL16 and CX_3CL1 are not secreted as soluble factors but are attached to the extracellular surface (Allen et al., 2007). However, there are cases in which these chemokines are cleaved and can also function as a soluble chemoattractants (Charo and Ransohoff, 2006, Laurence, 2006). More recently, extracellular ubiquitin has been implicated to bind chemokine receptors including CXCR4 (Saini et al., 2010)

1.4.3 Function

Although the classification system has grouped chemokines according to their structure and binding partner(s), these groups are not necessarily discrete in terms of function (Zlotnik and Yoshie, 2000). As an example, the CXC family contains members which contain a tri peptide motif with amino acids glutamate, leucine and arginine (ELR) between the N-terminus of the protein and the cysteine residue. The presence of this motif allows a distinction between members of the family with properties in leukocyte chemotaxis (Laurence, 2006). Members which contain this motif are produced in response to inflammatory factors and the presence of this motif allows the chemokines to attract neutrophils to inflammatory sites resulting in granule exocytosis and respiratory burst. As an example, CXCL4 does not contain the ELR motif and does not induce leukocyte migration. Experiments which have manipulated the structure of CXCL4 to include the ELR motif showed that this mutated protein structure was capable of leukocyte migration (Clark-Lewis et al., 1993). This ELR motif is also correlated with angiogenesis which is the process of blood vessel formation. ELR-CXC chemokines are proangiogenic and can stimulate EC chemotaxis. In contrast, those lacking the ELR motif are angiostatic (Strieter

et al., 2005). The pro and anti angiogenic chemokines separates the CXC family into discrete groups which signal through alternative receptors (Rossi and Zlotnik, 2000).

Chemokines are known to play a variety of diverse roles throughout the biological system (Rossi and Zlotnik, 2000). The most recognised biological function in chemokine biology is in the direction of inflammatory cell types and consequently their role in the immune response. However, it is now clear that chemokines are also involved in development, organogenesis and homeostasis (Mantovani, 1999, Rossi and Zlotnik, 2000, Zlotnik et al., 2011). For simplicity, chemokines are generally split into function according to their expression patterns and expression is thought to be either inflammatory or homeostatic. Inflammatory chemokines are induced in response to inflammatory conditions and are generally involved in leukocyte migration to sites of inflammation or injury. Homeostatic chemokines are constitutively active and direct trafficking of cell types under physiological conditions. Although this categorisation is useful when discussing function, chemokines are more complex and it is known that these functions overlap for some chemokines including CCL17, CCL20, CCL22, XCL1, XCL2 and CXC3CL1 (Zlotnik and Yoshie, 2012). As the topic in the thesis is of chemokines in haemopoiesis and chemokine biology is complex, only a brief overview of the role of inflammatory and homeostatic chemokines is discussed below with particular examples.

1.4.3.1 Inflammatory chemokines

Inflammation occurs in response to tissue damage or infection and a step by step process occurs involving the immune system to deal with the insult (Ortega-Gomez et al., 2013). These processes are mediated by inflammatory chemokines which act as ‘molecular cues’. Inflammation is complex therefore a brief overview is provided with a few examples.

Chemokines have a central role in leukocyte recruitment and leukocyte activation (Mackay, 2008). Early studies identified chemokines as proteins in cellular supernatants that were capable of mediating directed chemoattraction of leukocytes using modified Boyden chamber assays (Ransohoff, 2005). Inflammatory chemokines are up regulated in not only activated endothelial cells and smooth muscle cells, but also leukocytes, macrophages and monocytes (Balkwill, 2012). It has been shown that the inflammatory chemokine response varies on the type/cause of inflammation. The majority of inflammatory chemokines respond by an upregulation at the transcription level whereas other chemokines are stored in granules for immediate release when necessary. The

production of chemokines at the site of injury/infection is followed by the establishment of a chemoattractant gradient. Chemokines present themselves on the endothelium of post-capillary venules, resulting in the instantaneous activation of rolling leukocytes resulting in firm adhesion and diapedesis (Ransohoff, 2005).

Acute inflammation is characterised by a predominantly neutrophil influx resulting in adema, redness and further recruitment of a large number of neutrophils, whereas chronic inflammation involves the sustained recruitment and activation of T cells and monocyte/ macrophages. Generally speaking, the CXC chemokine family are primarily implicated in the recruitment of polymorphonuclear leukocytes (neutrophils) to sites of acute inflammation, whereas the CC family of chemokines are primarily involved in the recruitment of MNC to sites of chronic inflammation (Ransohoff, 2005). However, there is some overlap in the CC and CXC family and complexity involved. An example is helper T cells (Th1 versus Th2) which have been shown to respond to distinct chemokine signatures (Siveke and Hamann, 1998). The CXC members play a key role in wound repair as elegantly documented by studies using CXCR2 null mice (Charo and Ransohoff, 2006). As mentioned previously, the CXC family also play a pivotal role in the control of angiogenesis.

The CC sub family are referred to as monocyte chemoattractant proteins as they are generally involved with the recruitment of monocytes to sites of injury including (but not limited to) to trauma and infection (Charo and Ransohoff, 2006). Key chemokines involved in monocyte recruitment include CCL2, CCL7, CCL8 and CCL13. Studies on a CCR2 knock out mouse model have identified that this receptor and its binding partners is essential for this process in inflammation (Daly and Rollins, 2003). CCR7 and ligands including CCL19 and CCL21 are involved in T cells and dendritic cell recruitment. A knock out mouse model lacking CCR7 has demonstrated the role of this signalling as these mice are deficient in T cell dependent immunity and show disorganised lymph node T zones (Charo and Ransohoff, 2006).

Atypical receptors as described previously including D6 are thought to play key roles in inflammation, immune activation and anti-microbial resistance (Di Liberto et al., 2008). As an example, knocking out the D6 decoy chemokine receptor resulted in a higher susceptibility to tuberculosis infection compared to their wildtype counterparts regardless of bacterial load. These D6 deficient mice harboured elevated levels of systemic

chemokines and other inflammatory cytokines, collectively implying that D6 belongs to a network of chemokines that limit excessive inflammatory responses *in vivo*.

As an example of the role of chemokines in inflammation, chemokines have been demonstrated to be important in atherosclerosis, a chronic inflammatory disease process occurring in the arterial wall over a long period of time that results in angina, myocardial infarction and ischemic stroke (Weber et al., 2008). Formation of atherosclerotic plaques is a process of on-going inflammation that is characterised by endothelial damage, MNC recruitment and vascular smooth muscle cell proliferation processes that can be driven by adhesion molecules, chemokines and their receptors (Weber et al., 2008, Zernecke and Weber, 2014).

Chemokines have been implicated in some of these processes, such as development of atherosclerotic lesions (Weber et al., 2008). Briefly, subsequent to endothelial cell damage and activation, chemokines are expressed and presented on damaged endothelium by binding to GAG. A chemokine gradient is created which attracts monocytes to enter the sub-endothelial space of the vessel and to differentiate into macrophages. In particular CCL2 and CX3CL1 have important roles in recruitment of monocytes into the plaques. The role of these two chemokines, alongside their respective receptors CCR2 and CX3CR1 were investigated by Saederup *et al*., who demonstrated that these two chemokine/chemokine receptor pairs have both individual and additive roles in the recruitment of macrophages to atherosclerotic plaques (Saederup et al., 2008). In addition to macrophage recruitment, the retention of macrophages within the plaque aids in plaque progression. Indeed, Trogan *et al*., demonstrated that CCR7 expression and function are essential for emigration of macrophages from the atherosclerotic plaque (Trogan et al., 2006). Chemokines also play a role in retention of monocytes in established atherosclerotic plaques. As an example, a study by Baric *et al*., showed that when monocytes are exposed to oxidized lipids, pro-migratory chemokine receptor CCR2 levels decrease whilst pro-adhesive chemokine receptor CX3CR1 levels are increased, aiding in plaque formation. Atherosclerosis is just one example of how chemokines function in an inflammatory disease, chemokines are also known to be involved in non inflammatory settings.

1.4.3.2 Homeostatic chemokines

Homeostatic chemokines are named due to their expression in normal organs in the absence of inflammatory stimuli or alternatively are expressed by cells not involved in

inflammation response (Baggiolini and Loetscher, 2000, Rossi and Zlotnik, 2000). Homeostatic chemokines are important for lymphocyte trafficking, B and T cell production and during development including in organogenesis and more specifically secondary lymph node organogenesis (Zlotnik et al., 2011). Studies on knock out mice of particular chemokines has formed the basis of the research including CXCR4, CXCL12 and CXCR5 null mice (Locati and Murphy, 1999).

These chemokines are expressed at particular sites and bind to receptors that are expressed by lymphocytes (Baggiolini and Loetscher, 2000). Lymphoid cells including B and T cells are developed in secondary lymphoid organs, a process which is controlled by chemokines with main players CXCR4, CXCR5 and CCR7 involved (Baggiolini and Loetscher, 2000, Zlotnik and Yoshie, 2000). Most strikingly, CXCL12 null mice do not develop to adulthood and has been shown to have a defect in B cell lymphopoiesis (Nagasawa et al., 1996). The CXCR4 and CXCL12 signalling axis is now also known to be involved in stem cell migration in early development and adult hood, and in the development of the central nervous system (Balkwill, 2012). Chemokines are also involved in the development of other organs including the thymus in which chemokines coordinate T cell trafficking (Rossi and Zlotnik, 2000).

1.4.3.3 Chemokines in disease

Due to the fundamental roles of chemokines in various biological processes, it is perhaps not surprising that chemokines are connected with disease. Chemokines have been shown to be associated with autoimmune disorders, vascular disease and viral disease (Allen et al., 2007). Briefly, chemokines are involved in inflammatory diseases included arthritis, asthma, psoriasis and atherosclerosis as used as an example in section 1.4.3.1 (Charo and Ransohoff, 2006). In terms of viral disease, an abundance of evidence is on human immunodeficiency virus (HIV-1) with the involvement of CXCR4 and CCR5 (Charo and Ransohoff, 2006, Mellado et al., 2001). Experiments have shown that CXCR4 and CCR5 are required for mediating viral entry into host cells, with evidence that a polymorphism in CCR5 allows individuals to be resistant to viral infection, but more susceptible to West Nile virus (Glass et al., 2006, Mellado et al., 2001). Furthermore, HIV-1 replication is abrogated when CXCR4 and CCR5 are blocked (Moore et al., 2004).

Chemokines were first implicated in cancer with the observation that high levels of CXCL1 were secreted by melanoma cells (Richmond and Thomas, 1986). Chemokine

signalling is now known to be directly involved in cancer development through different mechanisms. An interesting observation was that chronic inflammation predisposes individuals to cancer (Hanahan and Weinberg, 2011). In addition, a heightened inflammatory state was found in response to cancers (Balkwill, 2012). The main players involved include receptors CXCR1, CXCR2, CXCR2, CXCR4, CCR2 and CCR5 and their ligands. Currently, it is understood that chemokine signalling is important in various aspects of cancer development. The up regulation of chemokines, particular inflammatory chemokines results in the recruitment and activation of immune cells to the site. The chemokines act to promote angiogenesis, metastasis, tumour cell proliferation/survival and provide a protective microenvironment (Borsig et al., 2013, Zlotnik et al., 2011). As an example, deregulation of CXCR4 is well recognised in several cancers including breast, ovarian and prostate cancer (Teicher and Fricker, 2010, Furusato et al., 2010). This up regulation can have diverse effects including tumour progression, trafficking, angiogenesis and the prevention of apoptosis. Chemokines have also been shown to be down regulated in response to disease. In this way, malignant cells exploit chemokine signalling for disease development.

Due to the abundance of literature on the role of chemokines in autoimmune disorders, viral disease and cancer development, chemokines have become popular as potential pharmacological targets. The best understood are CXCR4 and CCR5 in HIV-1, CXCR4 in cancers, CCR2 in atherosclerosis and CXCR1/CXCR2 in pulmonary disease (Charo and Ransohoff, 2006).

The data discussed above highlights the complexity of chemokine signalling and the diverse range of roles they play throughout the biological system. In this thesis, chemokines CXCR2/CXCR2 binding ligands and CXCL4 are focused on. Therefore, the main function of these chemokines is discussed in more detail below.

1.4.3.4 CXCR2 signalling

As can be seen in Table 1-1, human CXCR2 binds ligands CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8. These chemokines are expressed on a variety of cell types including neutrophils, basophils, monocytes, macrophages, dendritic cells, natural killer cells and mast cells (Koelink et al., 2012). CXCR1 and CXCR2 were originally shown to be expressed on granulocytes when CXCL8 was shown to bind to two receptors with different affinity (Murphy and Tiffany, 1991, Holmes et al., 1991). CXCR2

shares structural similarity to CXCR1 with a 77% homology which can also bind CXCL6 and CXCL8. There are differences between human and mouse CXCR2 signalling which is discussed for the later contents of this thesis. Mouse homologs have been identified against human CXCR1 and CXCR2 (Fan et al., 2007, Fu et al., 2005). CXCL1, CXCL2, CXCL3, CXCL5/6 and CXCL7 mouse homologs have been identified (Zlotnik and Yoshie, 2012). However, human CXCL1, CXCL2, and CXCL3 are structurally very similar and there is some controversy on the exact homolog for these ligands which can be confusing in the literature. Finally, to date a mouse homolog of human CXCL8 has not been identified. However, mouse cells can respond to human CXCL8 therefore it is suggested a chemokine with a similar sequence exists (Fan et al., 2007).

CXCR2 signalling is involved in a variety of processes including leukocyte migration, wound healing, survival, proliferation, senescence and disease. CXCR2 signalling is predominantly involved in leukocyte trafficking and signalling through CXCR2 is essential for the migration of neutrophils to sites of inflammation (Devalaraja et al., 2000, Romagnani et al., 2004, Eash et al., 2010). Recently, CXCR2 signalling has been found to be important in cell senescence. Research has shown that chemokine signalling via CXCR2 reinforces senescence (Acosta et al., 2008). The results showed that senescent cells activate CXCR2 and its ligands including CXCL1 and CXCL8 reinforcing growth arrest. CXCR2 signalling has also been implicated in several models of cancer. It has been proposed that senescence prevents progression to a more malignant state that is bypassed with the acquisition of additional mutations which suppress these effects (Acosta and Gil, 2009). In addition, studies have shown that CXCL8 is up regulated by tumour cells (Waugh and Wilson, 2008). More recently, CXCR2 ligands CXCL1 and CXCL2 were shown to induce inflammatory cell types to the tumour micronenvironment ultimately controlling cancer cell survival (Acharyya et al., 2012). CXCR2 is also involved in inflammatory disease and blocking agents are being tested (Busch-Petersen, 2006). In addition, CXCR2 is also known to be involved in cell proliferation and survival with ligand CXCL1 and CXCL8 in a variety of cell types (Tsai et al., 2002, Filipovic and Zecevic, 2008, Mockenhaupt et al., 2003).

1.4.3.5 CXCL4 signalling

CXCL4 is one of the oldest members of the chemokine family and was discovered in 1977 (Deuel et al., 1977). Dissimilar to other ligands within the CXC family, CXCL4 does not exhibit chemotactic activity for neutrophils and monocytes (Kasper and Petersen, 2011). Human and mouse CXCL4 share 64% identity (Watanabe et al., 1999). CXCL4 is predominantly produced in megakaryocytes and held in the α-granules of platelets where it released upon activation (Deutsch et al., 1955, Levine and Wohl, 1976). As seen in Table 1-1, CXCL4 functions through binding to a spliced variant of CXCR3 (CXCR3B) which has been shown in EC (Lasagni et al., 2003, Mueller et al., 2008). However, the binding of CXCL4 to CXCR3B is not simply how CXCL4 functions. Studies have reported that cells respond to the ligand which lack the receptor therefore other mechanisms of action are involved (Kasper and Petersen, 2011). CXCL4 have been shown to bind to GAG with a high affinity and there is evidence that proteoglycans serve as a functional receptor for CXCL4 (Kasper and Petersen, 2011). Additionally, CXCL4 has been shown to interact directly and bind integrins on EC surface (Aidoudi et al., 2008). CXCL4 is also able to interact with GF including VEGF and inhibit their interaction with receptors (Gengrinovitch et al., 1995, Bikfalvi, 2004). CXCL4 can also form complexes with other chemokines as a co-factor in other chemokine responses (Kasper and Petersen, 2011). A variant of human CXCL4 (CXCL4L1) has been discovered and isolated from platelets and differs in terms of localization, secretion and behaviour (Struyf et al., 2004, Dubrac et al., 2010, Lasagni et al., 2007).

CXCL4 was initially reported to have anti heparin activity and the main physiological role of the chemokine is in coagulation regulation (Eitzman et al., 1994). Currently it is known to play a role in wound healing, chemotaxis, inflammatory cell activation, proliferation, survival, angiogenesis, differentiation inhibition, cancer development and atherosclerosis (Kasper and Petersen, 2011, Vandercappellen et al., 2011). It has a well documented role as a physiological inhibitor of megakaryocytopoiesis and and angiogenesis (Lambert et al., 2007, Maurer et al., 2006). This thesis focuses on the role of chemokines in haemopoiesis, therefore previous published literature in this field is discussed in more detail below.

1.4.4 Chemokines in haemopoiesis

Chemokines play vital roles in haemopoiesis with their main roles including cell migration, proliferation, differentiation and survival. A summary of the role of chemokines in haemopoiesis is provided below with a focus on the literature involving the chemokines in this study.

Chemokines have been shown to positively and negatively regulate haemopoietic cell proliferation *in vitro* (Broxmeyer, 2001). In 1989, CCL3 and CCL4 were shown to increase myeloid colony formation, however under particular growth conditions they inhibited proliferation of early progenitors, both in culture and *in vivo* (Broxmeyer et al., 1989). A variety of studies have been carried out to show that CCL3 suppresses myelopoiesis, CFU-S, cell cycling and HSC/progenitor cells numbers (Broxmeyer, 2001). After the original pioneering research, several other chemokines were tested for myelosuppressive activity and it was identified that several chemokines displayed inhibitory activity against immature haemopoietic cells *in vitro* and *in vivo* including CC and CXC family members (Broxmeyer and Kim, 1999). Due to the high number of chemokines which were found to effect haemopoietic cell growth, targeted deletion of particular receptors was examined. Investigation of a *Cxcr2*$^{-/-}$ mouse model identified that signal transduction via this chemokine receptor is involved in negatively regulating myeloid growth (Broxmeyer et al., 1996). CCR2 has also been implicated in this process, with studies using a *Ccr2*$^{-/-}$ mouse model and there is evidence that signalling mediated by CCR2 is regulates apoptosis in these populations (Reid et al., 1999, Boring et al., 1997).

Chemokines have also been found to support HSC/progenitor survival. Specifically in the CXC group, CXCL4, CXCL8 and CXCL10 were shown to support the viability of myeloid progenitor cells in culture and to protect them from cytotoxic induced cell death (Han et al., 1997). Experiments have shown that HSC respond to CXCL8 and, in turn, CXCL8 can be detected in human HSC populations (Behringer et al., 1997).

Focusing on CXCL4 as this is a key player in this thesis, an abundance of research is available on the role of this chemokine on haemopoietic cells including megakaryocytopoiesis inhibition and cytoprotection, survival and adhesion of primitive stem/progenitor cells. CXCL4 has been shown to offer a protective effect on haemopoietic cells ($CD34^{+}$) treated with cytotoxic compound 5-FU in culture and *in vivo* (Aidoudi et al., 1996, Xi et al., 1996). It was reported that CXCL4 could increase the recovery of cells

following 5-FU treatment including the most primitive haemopoietic cells, the stem cells. In addition to viability, studies have shown that CXCL4 also promoted proliferation in addition to viability in stem/progenitor cells in response to 5-FU treatment. A similar protective effect on haemopoietic cells was found with other chemotherapeutic compounds also (Han et al., 1997). In addition, experiments have shown that culture of $CD34^+$ cells derived from human BM or CB with CXCL4 resulted in an increase in cell viability and numbers of the primitive sub population ($CD34^+CD38^-$ cells) (Huang et al., 2000, Han et al., 1997). In addition to the effects of CXCL4 on primitive haemopoietic cell survival, it has been shown to alter other properties. CXCL4 has also been shown to alter cell adhesion with an enhancement of adhesion of stem/progenitor cells to EC (Zhang et al., 2004, Dudek et al., 2003). It was also noted that CXCR4 expression was increased following culture with CXCL4, suggesting a possible mechanism of increased adhesion. Another report found that CXCL4 increased $CD34^+$ cell adhesion to EC and also stromal cells. The enhancement of adhesion by CXCL4 is thought to be mediated via CXCL8 and a link between CXCL4 and CXCL8 has been previously proposed (Pelus et al., 2002, Dudek et al., 2003).

Chemokines in haemopoiesis cannot be discussed without describing a well defined chemokine interaction, that of CXCL12 and CXCR4. Their interaction is responsible for a variety of diverse roles, including fundamental roles in haemopoiesis controlling the survival and migration of HSC. The first identification of a role for these genes in haemopoiesis came from studies on *Cxcr4*$^{-/-}$ and *Cxcl12*$^{-/-}$ embryos, which were shown to be embryonic lethal (Zou et al., 1998, Ma et al., 1998, Nagasawa et al., 1996). CXCL12 was shown to exert survival enhancing effects and to improve engraftment on human and mouse HSC and progenitor cells (Broxmeyer et al., 2003). CXCL12 was identified as a chemoattractant for $CD34^+$ positive cells and present in BM derived stromal cells (Aiuti et al., 1997) (Ponomaryov et al., 2000, Peled et al., 1999, Imai et al., 1999) and CXCR4 was shown to be expressed by $CD34^+$ HSC and progenitor cells (Viardot et al., 1998, Kim and Broxmeyer, 1998, Mohle et al., 1998). Although originally the CXCR4/CXCL12 axis was considered to be monogamous, CXCL12 has been shown to bind CXCR7. CXCR7 is expressed on HSC and possibly functions as a scavenging receptor (Pelus and Fukuda, 2008, Sun et al., 2010). In the adult BM, CXCR4/CXCL12 controls HSC homing/mobilisation and an inhibitor against CXCR4 (AMD3100) induces the release of HSC and progenitor cells from the BM into the periphery which synergises with G-CSF (Zlotnik and Yoshie, 2012, Broxmeyer et al., 2005). A study by Sugiyama *et al*, used targeted deletion of CXCR4 which severely reduced HSC numbers without altering

progenitor frequency suggesting that CXCR4-CXCL12 signalling also maintains the quiescent HSC pool (Sugiyama et al., 2006).

In addition to the CXCL12/CXCR4 axis, a role for other chemokines have been documented in haemopoietic cell mobilisation (Pelus et al., 2002). CXCL2 is a member of the CXC family ofchemokines which functions through binding to receptor CXCR2. A study published by reported that exogenous treatment of CXCL2 to mice resulted in the mobilisation of HSC from the BM into the circulation (King et al., 2001). An N-terminal truncated form of the protein (SB-251353) was also found to show the same effect on HSC mobilisation. This phenomenon was noted in mice in non-human primates (rhesus monkeys) (King et al., 2001). In addition, in comparison to mobilisation agent G-CSF, HSC mobilised into the periphery with CXCL2 and SB-225353 were reported to show increased homing and engraftment after transplantation of the cells in addition to a higher proportion of mobilised primitive cells (Pelus and Fukuda, 2006). Studies have aimed to examine the molecular mechanism, however this is not well understood. Research indicated the involvement of MMP including MMP-9 (King et al., 2001). An increase in MMP-9 in the plasma was noted prior to HSC mobilisation and MMP-9 blocking antibodies showed this was an essential component of the mobilisation. It is thought that MMP are involved in other mechanisms of mobilisation including G-CSF as a defect in HSC migration has been reported in MMP-9 null mice (Heissig et al., 2002). Furthermore, a study showed that mobilisation mediated by CXCL2, SB-251353 and G-CSF is dependent on proteases secreted from neutrophils (Pelus et al., 2004). Mobilisation by CXCL2 has been shown to require neutrophils from studies using antibodies against GR-1 (Pelus et al., 2004). Results for CXCL2 mediated mobilisation have also indicated the involvement of receptor CXCR2, however, the characterisation of cell types that are involved and the function of CXCR2 in HSC mobilisation signalling remains poorly understood (Pelus, 2008). It has been noted that the mobilising agent G-CSF up regulates expression of CXCL2 in BM neutrophils and it is proposed that granulocytes and monocytes are required to mediate mobilisation via CXCR2 and G-CSF (Nguyen-Jackson et al., 2010, Ratajczak and Kim, 2012, Semerad et al., 2002, Levesque et al., 2003, Pelus, 2008, Liu et al., 1997). In addition to CXCL2, another CXCR2 binding ligand, CXCL8 has been shown cause mobilisation of mouse HSC into the circulation (Laterveer et al., 1996). A study aimed to examine the molecular mechanism of CXCL8 mediated mobilisation and implicated neutrophils in the process by showing mobilisation did not occur in neutropenic mice (Pruijt et al., 1999). In addition, CXCL8 mobilisation was reported to be dependent on MMP as reported with CXCL2 mediated mobilisation. A summary of role of CXC

chemokines in stem cell properties is provided in Figure 1-10. Due to the complexity of chemokine signalling, the schematic focuses on CXCL2, CXCL4, CXCL8, CXCL12 and CXCR4.

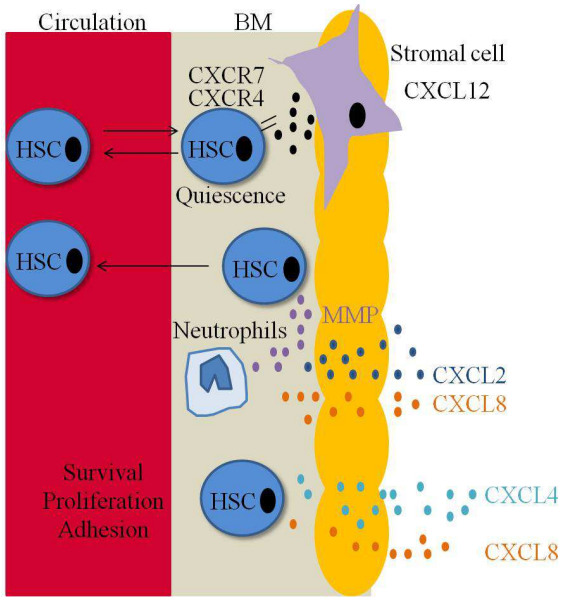

Figure 1-10 Chemokine regulation of HSC.

Schematic diagram shows a summary of the known role of CXC chemokines CXCL2, CXCL4, CXCL8, CXCL12 and their receptors in HSC properties. CXCR4 is expressed on HSC and responds to CXCL12 secreted by BM stromal cells and various other cells within the BM niche. This interaction regulates HSC migration to and from the niche and also quiescence. CXCR7 is also expressed on HSC and is thought to act as a scavenger receptor for CXCL12. CXCR2 ligands CXCL2 and CXCL8 can induce the mobilisation of HSC into the circulation which is thought to involve CXCR2 expressing neutrophils and MMP molecules. CXCL4 and CXCL8 have been shown to alter HSC survival, proliferation and adhesion however the molecular mechanism is not well understood.

Similarly to the evidence outlined above with chemokines in cancer, chemokines are known to be deregulated or to have roles in malignancies of the haematological system. CXCL8 and CCL25 have been identified as pro-survival or proliferative factors in various subtypes of chronic lymphocytic leukaemia (CLL) (Laurence, 2006). Due to their roles in normal haemopoiesis, it is perhaps not surprising that CXCL12 and CXCR4 are involved in haematological malignancies. CXCR4 is decreased and signalling impaired in response to oncogene BCR-ABL in chronic myeloid leukaemia (CML) and current therapy has been shown to alter CXCR4 signalling, leading to accumulation of CML cells in the niche and

potential protection through stromal cell interaction (Dillmann et al., 2009, Geay et al., 2005, Jin et al., 2008). Based on this research, inhibition of CXCR4 using small molecule inhibitor AMD3100 has shown promise in a leukaemic model *in vivo* (Weisberg et al., 2012). Multiple myeloma cells have been documented to express various chemokine ligands and receptors which play roles in cell homing, growth of tumours and disease progression (Aggarwal et al., 2006). Chemokines known to be expressed by acute myeloid leukaemia (AML) cells include CXCL1, CXCL6 and CXCL13 however the biological function is not clear (Bruserud et al., 2007).

Collectively, chemokines control fundamental process in haemopoiesis. Chemokines are continuing to be found to confer important properties in haemopoiesis. A recent study showed that CCL28 enhanced cell survival, cycling and long term repopulation in human HSC (Karlsson et al., 2013). Although there is a great body of work on the role of chemokines in haemopoiesis, the work in this thesis aims to build on results from a previously published microarray in which CXC chemokines were implicated in HSC biological properties with a particular focus on CXCL4 and CXCR2 signalling (Graham et al., 2007). Although there is some literature available on these chemokines, the data are not conclusive, in particular the data on their expression and functional roles.

1.5 Thesis aims

The following thesis is an investigation into the role of CXC chemokines in HSC properties, including survival, self renewal, differentiation and cell cycle status. The aims can be broken down as follows:

1. Examine CXC ligand and receptor expression on quiescent and proliferating human HSC populations.

It is fundamental that the results from the previously published microarray are validated. The expression of key CXC chemokine ligands from the original array (CXCL1, CXCL2 and CXCL6) were examined at the gene expression level between quiescent and proliferating human HSC populations. To ensure the protein was translated, protein expression analysis using multiple techniques was assessed. Furthermore, it was not clear from the previous research whether CXCR2 was expressed on HSC populations. The expression at the gene and protein level of CXCR2 was examined.

2. Investigate the effect of modulating expression of CXC ligands and receptor and the resulting biological effect on human HSC properties.

To elucidate the biological function, experiments were designed to modulate chemokine expression and examine the effect on human HSC properties. In this section of the thesis, chemokine ligand and receptor CXCL1 and CXCR2 were focused on due to the high expression in preliminary data and evidence in the literature. Experimental techniques included the use of over expression and knock down vectors against CXCL1. To compliment this, an inhibitor against CXCR2 signalling was used.

3. Examine the effect of CXCR2 signalling on haemopoiesis using a *Cxcr2*$^{-/-}$ mouse model.

Due to the presence of complex *in vivo* signalling involved in the haemopoietic system, it is possible that niche interactions of CXCR2 signalling may play a role in stem cell properties. Key experiments understanding chemokine function have used targeted deletion of the proteins, in particular the receptor. A mouse model in which *Cxcr2* has been deleted is viable and it was decided to use this model to examine if *Cxcr2* signalling is fundamental to stem cell properties. Haemopoiesis was examined in a *Cxcr2*$^{-/-}$ background. Experimental techniques involved the examination of steady state haemopoiesis, *in vitro*

stem cell functional assays and the effect of the haemopoietic system in response to stress, including ageing and BM reconstitution assays.

4. Examine which CXC ligands and receptors are expressed in mouse HSC populations and investigate their biological function.

There is limited literature of the role of chemokines in mouse haemopoiesis. Furthermore, the previous microarray study was carried out on human HSC which may not translate to the mouse system. A range of CXC chemokine ligands and receptors were examined for expression by single cell gene expression analysis on mouse HSC populations. Based on the expression, the biological function was investigated using a combination of transgenic mice, including a reporter model, examination of knock out mouse models and knock-down using shRNA *in vitro*.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

Cell line	Origin
HEK293	Human embryonic kidney
HT 1080	Human fibrosarcoma
PC-3	Human prostate cancer

Table 2-1 List of cell lines.

Details of the cell lines used in this study and their origin are listed in Table 2-1. HT 1080 and HEK293 cell lines were available 'in-house'. PC-3 cell lines were a kind gift from Dr Hing Leung. HT 1080 and PC-3 cells express high levels of CXCL1 and CXCR2 therefore were an ideal model for optimisation of techniques prior to the use of precious primary samples. HEK293 cells were used for lentiviral particle production.

2.1.2 Plasmids

Plasmids were used in this study to over express and knock down the expression of proteins of interest. Lentiviral transduction was designated as the appropriate technique as primary samples (human and mouse) are difficult to transduce due to their non-proliferative status. For knock down experiments, plasmids encoding a shRNA hairpin for human CXCL1 (Cat No. RHS4533-NM_001511) and mouse CXCL4 (Cat No. RMM4534-NM_019932) were purchased in a pLKO.1 vector containing a puromycin (puro) resistance cassette (pLKO.1-puro) (Thermo Fisher Scientific Inc., Hertfordshire, UK). Each shRNA construct was designed to contain a hairpin of 21 base paired (BP) stem separated by a 6 BP loop designed to target the gene of interest. ShRNA hairpins of interest were subcloned into the pLKO.1 plasmid containing a green fluorescent protein (GFP) tag (pLKO.1-GFP) as described in section 2.3.6.9.1. A plasmid encoding a shRNA hairpin with a sequence designed as non-targeting was used as a control (pLKO.1-Scr). *CXCL1* cDNA for over expression studies was cloned into a plasmid with a GFP tag (CXCL-PRRL) as described in section 2.3.6.9.2. Packaging plasmids were required to provide the accessory proteins required for the transcription and packaging of an RNA copy of the expression construct into recombinant pseudoviral particles. PLKO.1-GFP, pLKO.1-Scr and PRRL plasmids were kindly donated by Dr Kamil Kranc. Packaging

plasmids pCML-VSV-G and HIV-1 were kindly donated by Professor John Rossi and Professor Paolo Salomoni respectively.

2.1.3 Small molecule inhibitors

SB-225002 is a potent and selective antagonist of CXCR2 for the inhibition of ligands binding to the CXCR2 receptor (Figure 2-1) (Catusse et al., 2003, White et al., 1998). SB-225002 was purchased commercially (Cat No. 559405, Molecular Weight 352.1) (Merck Chemicals Ltd, Watford, UK). SB-225002 was solubilised in dimethyl sulfoxide (DMSO) at a stock solution of 1mg/mL, aliquoted and stored at -20ºC for long-term storage. A working concentration of SB-225002 was made fresh and diluted to the appropriate concentration as documented in the text, with a DMSO treated control as a vehicle control.

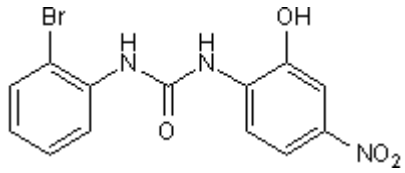

Figure 2-1 Chemical structure for SB-225002.

Image shows chemical structure for SB-225002 (chemical name N-(2-Bromophenyl)-N-(2-hydroxy-4-ni-trophenyl)urea).

2.1.4 Tissue culture supplies

Company	Product
Baxter Healthcare, Nottingham, UK	Sterile Water
Biolegend, London, UK	Mouse recombinant IL-3 Mouse recombinant IL-6 Mouse recombinant Stem Cell Factor (SCF)
Chugai Pharma, London, UK	Human recombinant G-CSF
Gilson, Bedfordshire, UK	Yellow and blue tips
Greiner, Bio-One, Gloucestershire, UK	Cryotubes Pipettes (5mL, 10mL and 25mL) Tissue culture flasks (25cm^2, 75cm^2 and 175cm^2) Tissue culture plates (6-well, 12-well, 24-well and 96-well)
Invitrogen, Paisley, UK	2-mercaptoethanol (2-ME) Dulbecco's modified eagle medium (DMEM) Dulbecco's phosphate buffered saline (PBS) Foetal calf serum (FCS)

	Isocove's modified Dulbecco's medium (IMDM) L-glutamine (200mM)
Miltenyi Biotec, Bisley, UK	c-Kit microBead™ kit mouse CD34 microBead™ kit human CliniMACS CD34 reagent CliniMACS microBead™ kit CliniMACS PBS/ ethylenediaminetetraacetic acid (EDTA) buffer
Nalgene Labware, Roskilde, Denmark	$25cm^2$ and $75cm^2$ non-adherent tissue culture flasks Cryo freezing container 'Mr Frosty' Vacubottles
Peprotech, London, UK	Human recombinant FLT-3L Human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF) Human recombinant CXCL1 Human recombinant IL-3 Human recombinant IL-6 Human recombinant SCF Human recombinant TPO
Sartorius, Hannover, Germany	Minisart 0.2µM sterile filters Minisart 0.45µM sterile filters
Scottish National Blood Transfusion, Glasgow, UK	20% Human serum albumin (ALBA) 4.5% Human albumin solution
Sigma-Aldrich, Dorset, UK	5-azacytidine (5-AZA) Bovine serum albumin (BSA) Carbonate-bicarbonate buffer DMSO Gelatin Hank's buffered salt solution (HBSS) Histopaque®-1077 Histopaque®-1119 Hydrochloric acid (HCl) Magnesium chloride ($MgCl_2$) Polybrene® Potassium chloride (KCl_2) Puro Sodium azide Trisodium citrate Trypan blue Trypsin-EDTA
StemCell™ Technologies, Grenoble, France	Ammonium chloride solution (NH_4Cl) Bovine pancreatic deoxyribonuclease (DNase) 1mg/mL BSA/insulin/transferrin (BIT) Low density lipoprotein (LDL) Methocult (human) $H4434^+$ Methocult (murine) $M3434^\times$ Serum substitute
Sterilin Ltd, Hounslow, UK	Disposable pipettes (5mL, 10mL and

	25mL) Pastettes Sterile plastic falcon tubes (15mL and 50mL)

[+] Human Methocult contains SCF, GM-CSF, IL-3 & erythropoietin (EPO)
[×] Mouse Methocult contains SCF, IL-3, IL-6 & EPO

Table 2-2 Tissue culture supplies.

2.1.5 Molecular biology supplies

Company	Product
Applied Biosystems, Foster City, CA, USA	96 and 384 well plates High capacity complimentary DNA (cDNA) reverse transcription (RT) kit SUPERase-In™ TaqMan® probes TaqMan® universal polymerase chain reaction (PCR) master mix
Bioline, London, UK	Crystal 5X DNA loading buffer blue HyperPAGE prestained protein marker ISOLATE II Genomic DNA kit MangoMix™ α-select competent cells (gold and silver efficiency)
Bio-Rad Laboratories Ltd, Sussex, UK	Combs Gel casting trays Immuno-Blot™ polyvinylidene fluoride (PVDF) membrane Immuno-Star™ WesternC™ Kit
Cambridge Bioscience, London, UK	Transdux™
Cell Signaling Technology®, New England Biolabs, Hitchin, UK	100 BP DNA ladder 1kiloBP DNA ladder Anti-goat IgG horseradish peroxidise (HRP) linked secondary antibody Anti-rabbit IgG HRP linked secondary antibody BamHI restriction enzyme (RE) EcorI RE HindIII RE NdeI RE Rabbit anti-human β-Tubulin antibody Shrimp alkaline phosphatase (SAP) SnabI RE SpeI RE T4 DNA ligase
Chemical Store, University of Glasgow, UK	Ethanol Isopropanol Methanol
Clontech, Saint-Germain-en-Laye, France	Retronectin™

Eurofins MWG Operon, Wolverhampton, UK	PCR primers
Invitrogen	CellsDirect™ One-Step PCR kit Miller's Luria Broth (LB) base® Super optimal broth with catabolite repression (SOC) medium SYBR®Safe Tris EDTA (TE) buffer
Qiagen, West Sussex, UK	HiSpeed® plasmid maxi kit QIAamp DNA blood mini kit QIAquick gel extraction kit QIAshredder kit RNeasy micro kit RNeasy mini kit
SG Wasseraufbereitung und Regenerierstation GmbH, Barsbuttel, Germany	Ultra pure water system
Sigma-Aldrich	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) Agarose Ammonium persulfate (APS) Boric acid Calcium chloride ($CaCl_2$) EDTA Formaldehyde solution (36.5%) Microagar Sodium chloride (NaCl) Sodium docedyl sulphate (SDS) Tetramethylethylenediamine (TEMED) Tris base TWEEN 20 for electrophoresis
Thermo Fisher Scientific Inc.	Bicinchoninic acid (BCA)™ protein assay kit

Table 2-3 Molecular biology supplies.

2.1.6 Flow cytometry supplies

Company	Product
BD Biosciences, Oxford, UK	Annexin-V Allophycocyanin (APC)/Fluorescein isothiocyanate (FITC) Anti-human CD34 APC monoclonal antibody Anti-human CD38 Peridinin chlorophyll (PerCP) monoclonal antibody Anti-human CD90 Phycoerithrin (PE)-Cy5 monoclonal antibody Anti-human Ki-67 FITC Ant-mouse CD4, CD5, CD8a, CD11b, GR1, TER119 & B220 biotinylated antibodies

	Fluorescent activated cell sorting (FACS) flow/ FACS clean IgG APC IgG FITC IgG PE-Cy5 IgG PerCP
Biolegend	Anti-mouse B220 PE-Cy5 Anti-mouse CD11b PE Anti-mouse CD11b PE-Cy7 Anti-mouse CD150 APC Anti-mouse CD16/CD32 APC-Cy7 Anti-mouse CD19 APC-Cy7 Anti-mouse CD34 FITC Anti-mouse CD34 PE Anti-mouse CD45.1 FITC Anti-mouse CD45.2 Pacific Blue™ Anti-mouse CD48 PE Anti-mouse CD4 PE Anti-mouse CD8a APC Anti-mouse CD8a PE Anti-mouse c-Kit APC Anti-mouse c-Kit APC-Cy7 Anti-mouse Gr-1 APC Anti-mouse Gr-1 APC-Cy7 Anti-mouse Sca-1 PE-Cy7 Anti-mouse TER119 FITC Streptavidin PerCP
R&D Systems, Abington, UK	Anti-human CXCL4 PE monoclonal antibody Anti-human CXCR2 FITC monoclonal antibody
Invitrogen	Streptavidin Pacific Blue™
Sigma-Aldrich	4’6-Diamidino-2-phenylindole dihydrochloride (dapi)

Table 2-4 Flow cytometry supplies.

2.1.7 Primers

2.1.7.1 PCR primer sequences

Primer	Sequence
Human *CXCL1* Forward	ATGGCCCGCGCTGCTCTCTCCGC
Human *CXCL1* Reverse	GCAGGGCCTCCTTCAGGAACAGCC
Mouse *Cxcl4* Forward	GGTACCACACCGGCAGATGATAG
Mouse *Cxcl4* Reverse	CACTATGTTGAGCCCCCTTCCTG
Neomycin Forward	TTTTGTCAAGACCGACCTGT
Neomycin Reverse	TGCGCTGCGAATCGGGAGCG
pLKO.1 Backbone Forward	GACTATCATATGCTTACCGT
pLKO.1 Backbone Reverse	AAACCCAGGGCTGCCTTGGAAAAG

Table 2-5 PCR primer sequences.

2.1.7.2 TaqMan® probes[+]

Probe	Catalog number	Spans exons
Human Cell Division Cycle 6 (CDC6)	Hs00153374_m1	Yes
Human CD34	Hs00990732_m1	Yes
Human CD38	Hs01120071_m1	Yes
Human CXCL1	Hs00236937_m1	Yes
Human CXCL2	Hs00601975_m1	Yes
Human CXCL4	Hs00427220_g1	Yes
Human CXCL6	Hs00605742_g1	Yes
Human CXCR2	Hs00174304_m1	Yes
Human Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)	Hs02758991_g1	Yes
Mouse CCL19	Mm00839967_g1	Yes
Mouse CCR7	Mm01301785_m1	Yes
Mouse CXCL1	Mm00433859_m1	Yes
Mouse CXCL13	Mm00444533_m1	Yes
Mouse CXCL2	Mm00436450_m1	Yes
Mouse CXCL3	Mm01701838_m1	Yes
Mouse CXCL4	Mm00451315_g1	Yes
Mouse CXCL5/6	Mm00436451_g1	Yes
Mouse CXCR2	Mm00438258_m1	Yes
Mouse CXCR5	Mm00432086_m1	Yes
β2M	Mm00437762_m1	Yes

[+] All Taqman® probes were purchased from Applied Biosystems.

Table 2-6 Taqman® probes.

2.1.8 Immunofluorescence supplies

Company	Product
Abcam, Cambridge, UK	Mouse IgG isotype control
BD Biosciences	Golgistop™
Carl Zeiss, Jena, Germany	AxioVision software
Fisher Scientific, Leicestershire, UK	Multi-spot microscope slides
Invitrogen	Alexa Fluor® 488 goat anti-mouse IgG Alexa Fluor® 594 donkey anti-goat IgG
R&D Systems	Goat IgG isotype control Mouse anti-human CXCR2 monoclonal antibody
Santa-Cruz Biotechnology, Inc, CA, USA	Goat anti-human CXCL1 polyclonal antibody
Sigma-Aldrich	Poly-L-lysine 0.1% (w/v) Triton-X-100
Vector Laboratories Ltd, Peterborough, UK	VECTASHIELD® hardest mounting medium with dapi

Table 2-7 Immunofluorescence supplies.

2.2 Medium and Solutions

2.2.1 Tissue culture

2.2.1.1 DMEM

DMEM	440mL
FCS	50mL
L-glutamine (200mM)	5mL
Penicillin/streptomycin solution ($10{,}000 UmL^{-1}/10{,}000 gmL^{-1}$)	5mL

2.2.1.2 DMEM+

+DMEM as prepared in section 2.2.1.1 with the addition of 20% FCS for viral production.

DMEM	390mL
FCS	100mL
L-glutamine (200mM)	5mL
Penicillin/streptomycin solution ($10{,}000 UmL^{-1}/10{,}000 gmL^{-1}$)	5mL

2.2.1.3 IMDM

IMDM	440mL
FCS	50mL
L-glutamine (200mM)	5mL

Penicillin/streptomycin solution ($10,000UmL^{-1}/10,000gmL^{-1}$)	5mL

2.2.1.4 IMDM+

+IMDM prepared as in section 2.2.1.3 with the addition of cytokines for mouse culture.

IMDM	50mL
Mouse SCF (10µg/mL)	200µl (40ng/mL)
Mouse IL-3 (10µg/mL)	100µl (20ng/mL)
Mouse IL-6 (10µg/mL)	100µl (20ng/mL)

2.2.1.5 PBS 2%FCS

PBS	490mL
FCS	10mL

2.2.1.6 DAMP solution

DNase I	2mL
$MgCl_2$	1.25mL
Trisodium citrate (0.155M)	53mL
ALBA	25mL
Dulbecco's PBS	418.75mL

2.2.1.7 Serum free medium (SFM)

BIT	25mL
L-glutamine (200mM)	1.25mL
Penicillin/streptomycin solution ($10,000UmL^{-1}/10,000gmL^{-1}$)	5mL
2-ME (50mM)	250µl
LDL (10mg/mL)	500µl
IMDM	97.25mL

2.2.1.8 SFM supplemented with a 5 GF cocktail[+]

SFM	50mL
Human IL-3 (50µg/mL)	20µl (20ng/mL)
Human IL-6 (50µg/mL)	20µl (20ng/mL)
Human G-CSF (20µg/mL)	50µl (20ng/mL)
Human FLT-3L (50µg/mL)	100µl (100ng/mL)
Human SCF (50µg/mL)	100µl (100ng/mL)

[+]The solution was filtered through a 0.2µM filter to sterilise the solution before use.

2.2.1.9 20% DMSO/4.5% ALBA

DMSO	20mL
4.5% ALBA	80mL

2.2.1.10 Freezing media 10% DMSO FCS

DMSO	5mL
FCS	45mL

2.2.1.11 PBS/0.1% BSA

PBS	50mL
BSA	0.5g

2.2.2 Western blotting

2.2.2.1 2X SDS sample buffer (Laemmli)

1.5M Tris-HCl, pH6.8	10mL
Glycerol	30mL
20% (w/v) SDS	6mL
Bromophenol blue	15mL
dsH_20	up to 100mL

2.2.2.2 10X TBS buffer[+]

NaCl	876.6g
Tris	121.1g
dH_20	10L

[+]A 1X solution was made using 100mL of the 10X solution with the addition of 900mL of dH_20 supplemented with 10mL of Tween-20.

2.2.2.3 Homemade gels

2.2.2.3.1 15% Resolving gel

dsH_20	2.3mL
30% Acrylamide	5.0mL
1.5M Tris (pH8.8)	2.5mL
10% SDS	0.1mL
10% APS	0.1mL
TEMED	0.01mL

2.2.2.3.2 Stacking gel

dsH_20	1.4mL
30% Acrylamide	0.33mL
1.0M Tris (pH6.8)	0.25mL
10% SDS	0.02mL
10% APS	0.02mL
TEMED	0.01mL

2.2.2.410X Running buffer[+]

Glycine	144.1g
Tris	30.3g
SDS	10g
dH_20	up to 2L
pH 8.3	

[+]A 1X solution was made using 100mL of the 10X solution with the addition of 900mL of dH_20.

2.2.2.510X Transfer buffer[+]

Glycine	144.1g
Tris	30.3g
dH_20	to 2L
pH 8.3	

[+]A 1X solution was made using 100mL of the 10X solution with the addition of 700mL of dH_20 and 200mL of methanol.

2.2.2.65% BSA/TBST blocking solution

1X TBST	100mL
BSA	5g

2.2.3 Flow cytometry

2.2.3.1Dapi[+]

Dapi	50mg
dsH_20	1mL

[+]The stock solution was diluted 1 in 50 in PBS to make a 1000X solution. Aliquots were stored at -20ºC and immediately prior to use a 1X solution was made using PBS result in a final concentration of 1µg/mL.

2.2.3.2 Annexin-V

Annexin-V APC/FITC	5µl
HBSS supplemented with dapi	95µl

2.2.4 Immunofluorescence

2.2.4.1 3.65% Formaldehyde

36.5% Formaldehyde solution	5mL
PBS	45mL

2.2.4.2 0.25% Triton-X-100

Triton-X-100	125µl
PBS	49.875mL

2.2.4.3 5% BSA blocking solution

BSA	5g
PBS	100mL

2.2.5 PCR

2.2.5.1 10X TBE[+]

Tris base		108g
Boric acid		55g
EDTA	9.3g	
dsH_20		up to 1L

[+]A 1X solution was made using 100mL of the 10X solution with the addition of 900mL of dH_20.

2.2.5.2 2% Agarose TBE[+]

1X TBE	125mL
Agarose	2.5g

[+]The solution was boiled using a microwave until the agarose powder was completely dissolved. After cooling, 12.5µl of SYBR®Safe DNA gel stain was added to the solution and gel was poured into casting trays and allowed to set before use.

2.2.5.3TaqMan® PCR reaction

10µl reaction	
Universal gene expression master mix	5µl
cDNA	1µl
TaqMan® assay	0.5µl
Nuclease free H_20	3.5µl

2.2.5.4 cDNA synthesis reaction

20µl reaction	
10X reverse transcriptase buffer	2µl
25X dNTP mix	0.8µl
10X RT random primers	2µl
Reverse transcriptase	1µl
RNAse inhibitor	1µl
RNA (1µg) and nuclease free H_20	13.2µl

2.2.5.5 PCR mix

50µl reaction	
MangoMix™	25µl
Template	2µl
Primers (10µM)	2µl
Nuclease free H_20	up to 50µl

2.2.5.6 RT and preamplification mix

5µl reaction	
0.2X TaqMan® probes mix	1.4µl
Cells direct 2X reaction	2.8µl
SUPERase-In	0.056µl
SuperscriptIII RT	0.112µl
TE buffer	0.672µl

2.2.6 Cloning

2.2.6.1 30% glycerol[+]

Glycerol	30mL
dsH_20	70mL

[+]The solution was autoclaved prior to use for sterilisation.

2.2.6.2 Bacterial glycerol stocks[+]

Bacteria in LB broth	500µl
30% glycerol	500µl

[+]The solution was prepared with a Bunsen burner and immediately stored on ice and transferred to -80ºC for storage.

2.2.6.3 RE digestion

DNA	1-3µg
RE	1µl
10X Buffer	1µl
10X BSA	1µl
Nuclease free H_20	up to 10µl

2.2.6.4 Ligation

DNA insert and backbone	appropriate volume[+]
T4 DNA ligase	1µl
DNA ligase buffer	1µl
Nuclease free H_20	up to 10µl

[+]A molar ratio of insert to vector as shown in Equation 1 was used to determine volume of DNA to use per reaction. Insert to vector ratios 3:1, 6:1 and 9:1 were used and a reaction minus DNA insert was used as a negative control with every reaction.

((ng vector) x (kb size of insert))/(kb size of vector)) x (molar ratio of (insert/vector)) = (ng insert)

Equation 1 Molar ratio of insert to backbone.

The equation was used to calculate the quantity of insert to use in a ligation to achieve a particular molar ratio of insert to vector.

2.2.7 Transfection

2.2.7.1 2X HEPES-buffered saline (HBS)

NaCl	8g
KCl	0.37g
$Na_2HP0_42H_20$	106.5mg
Dextrose	1g
HEPES	5g
dsH_20	to 500mL
pH 7.05 to 7.1	

2.2.7.2 2M CaCl2

$CaCl_2$	147g
dsH_20	to 500mL

2.2.7.3 Transfection solution/T125cm^2 flask

dsH_20	440µl
2 X HBS	500µl
2M $CaCl_2$	60µl
pCML HIV-1 plasmid	6µg
pCML VSV-g plasmid	3.3µg
Plasmid for transfection	10µg

2.2.8 Microbiology

2.2.8.1Ampicillin (100mg/mL)$^+$

Ampicillin sodium salt	5g
dsH_20	50mL

$^+$The solution was filter sterilised through a 0.2µM filter, aliquoted and stored at -20ºC.

2.2.8.2 LB broth

Miller's LB base®	20g
dsH_20	up to 1L
Ampicillin (100mg/mL)$^+$	1mL

$^+$The solution was autoclaved immediately after prepared, allowed to cool and supplemented with ampicillin prior to use.

2.2.8.3 LB agar plates

Miller's LB base®	20g
Microagar	7g
Ampicillin (100mg/mL)$^+$	1mL

$^+$The solution was autoclaved and allowed to cool in a waterbath at 50ºC. Ampicillin was supplemented and 10mL of solution was added to individual sterile petri dishes. Dishes were allowed to solidify and stored at 4ºC for future use.

2.3 Methods

2.3.1 General tissue culture

A list of the materials used for tissue culture can be seen in Table 2-2.

2.3.1.1 Technique

Tissue culture was conducted using a laminar air flow hood. An aseptic technique was maintained with all materials sprayed in 70% alcohol prior to use.

2.3.1.2 Cryopreservation of cells

Primary cells and cell lines were stored in liquid nitrogen long term. For cryopreservation, a cell suspension was made in medium which was added to an equal volume of ALBA + 20% DMSO to give a final concentration of 10% DMSO (2.2.1.9). Cell lines were resuspended in a solution containing neat FCS supplemented with 10% DMSO (2.2.1.10). Cells were resuspended in the appropriate freezing solution at a concentration of 10^6 cells/mL and dispensed into cryotubes at a final volume of 2mL. The cryotubes were placed in a freezing container ('Mr Frosty') that contained neat isopropyl alcohol and were incubated in a -80ºC freezer overnight. This technique allowed a controlled reduction in temperature over time. Subsequently, cells were stored in -80ºC for short term or liquid nitrogen for long term storage.

2.3.1.3 Recovery of frozen samples

To recover the maximum number of cells from cryopreservation, it was necessary to exert extreme care with primary samples. On removal from liquid nitrogen, cells were immediately thawed in a waterbath at 37ºC. The cell solution was transferred to a 50mL falcon tube and thawing solution DAMP (2.2.1.6) was added dropwise to cells over approximately 20 minutes (min). Cells were centrifuged for 10 min at 200 x *g* and the step was repeated. After centrifugation, cells were washed in PBS/2% FCS (2.2.1.5) and resuspended in SFM supplemented with GF (2.2.1.8) overnight for recovery. Cell lines were thawed as described for primary cells and resuspended in appropriate culture medium for each cell line (2.2.1), washed in PBS and resuspended in medium overnight with a medium change the next day to remove dead cells/debris after thaw.

2.3.1.4 Assessment of viability

Cell viability and cell counting was carried out using the trypan blue exclusion method and performed using a counting chamber. Trypan blue is a coloured chemical that cannot pass the cell membrane barrier in live, viable cells with an intact membrane. Dead cells allow the absorption of the compound, therefore the cells display a blue colour under the microscope and can be discriminated from viable cells. Trypan blue stock solution (Cat No. T8154-100ML) was diluted in 1 in 10 in PBS to make a working solution. The cell suspension was diluted 1 in 2 in the trypan blue solution. 10µl of the solution was transferred to two sides of a haemocytometer counting chamber. Four squares within each counting chamber side were counted, averaged and repeated with the second side of the haemocytometer. To ensure accuracy, a minimum of 100 cells was counted across four squares. The cell count was multiplied by the dilution factor and 10^4 to get the cell count/mL.

2.3.1.5 CFC assay

The CFC/methycellulose is widely used to detect and quantify haemopoietic progenitor cells based on their ability to proliferate and differentiate to produce colonies in response to culture in a particular growth medium. Resulting colonies are scored based on number and type of colony which gives an indication of cell growth and differentiation. This assay can be of particular use for monitoring the activity of cells in response to treatment in culture or genetic manipulation. In addition, cells harvested from a primary CFC assay can be reseeded into replating assays which can be used to get an indication of the self renewal activity of cells. The particular cytokines used in this study were optimised to support growth of erythroid progenitors (blast forming units-erythroid and colony forming units-erythroid (CFU-E)), granulocyte-macrophage progenitors (colony forming units-granulocyte macrophage (CFU-GM)) and multi-potential granulocyte, erythroid, macrophage and megakaryocyte progenitors (colony forming units-granulocyte erythroid macrophage megakaryocyte (CFU-GEMM)).

Primary human (Cat No. 04444) or mouse (Cat No. 03434) cells were plated in Methocult™, vortexed to ensure homogenous mixing of cells/cytokines and plated in duplicate in 6 well plates using a sterile needle and syringe. DsH_20 was added to the surrounding wells in each plate to ensure humidity during the incubation period. Cells were incubated for a period between 10-14 days (based on growth) at 37ºC, 5% $C0_2$ and resulting colonies were counted and scored based on colony type (Figure 2-2) and images

taken using a standard light microscope. After counting, colonies were harvested resuspended in IMDM (2.2.1.3), counted and an equal number of cells was added to fresh aliquots of Methocult™ and plated as described above. It was noted that the resulting colonies formed in a secondary assay were all CFU-GM so colony numbers were counted and not scored based on type. Primary human CD34 enriched samples were seeded at 1,000 cells/mL/dish and replated at 10^4 cells/mL/dish. Primary mouse BM, spleen and PB were seeded at 10^4, 10^5 and 10^5 cells/mL/dish respectively and replated at the same density. Mouse c-Kit enriched cells after lentiviral transduction were seeded at 10^4 cells/mL/dish. A red blood cell (RBC) lysis step was carried out on mouse PB samples before plating as described in section 2.3.7.4 to remove RBC which would interfere with the analysis.

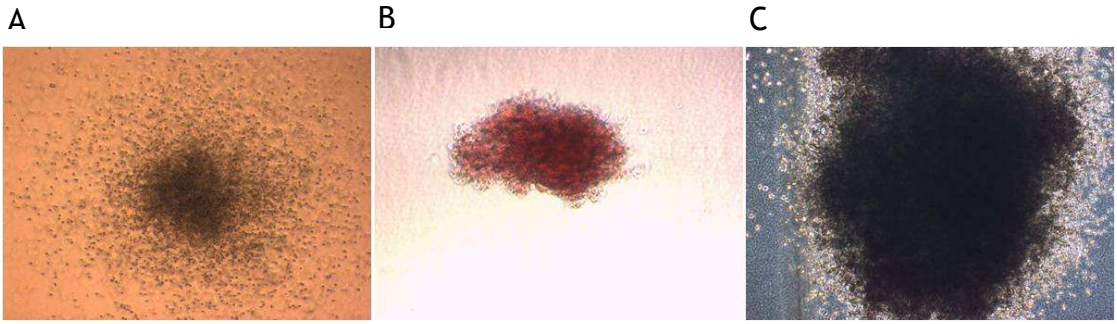

Figure 2-2 Representative images of colonies obtained in a CFC assay.

Images display representative examples of different types of colonies observed in this study. CFU-GM progenitor colonies consisted of colonies of varying sizes with a compact centre consisting of small white cells (A), CFU-E progenitor colonies were small, condensed and solely red in colour (B) and CFU-GEMM progenitor colonies were large in size and contained a mixture of white and red cells within the colony (C). This scoring system was used consistently to identify colony types throughout this study using both human and mouse samples.

2.3.1.6 Culture of cell lines and primary cells

HEK293, HT 1080 and PC-3 cell lines were maintained in DMEM (2.2.1.1) and HEK293 cell lines were maintained in a higher concentration of serum for viral production (2.2.1.2). Cell lines were maintained at a density of 10^5-10^6 cells/mL in tissue culture flasks ($T25cm^2$, $T75cm^2$ or $T175cm^2$). Cells were counted every two days and passaged with warm, fresh medium.

Primary human $CD34^+$ or mononuclear cells (MNC) were cultured in SFM supplemented with a high GF cocktail containing IL-3, IL-6, SCF, G-CSF and FLT3-L for cell survival (2.2.1.8). Cells were maintained at a density of 10^5-10^6 cells/mL in non-adherent tissue culture flasks ($T25cm^2$, $T75cm^2$ or $T175cm^2$). Primary mouse MNC or c-Kit enriched cells were cultured in IMDM supplemented with a GF cocktail containing IL-3, IL-6 and SCF for cell survival and to aid the integration of lentiviral particles (2.2.1.4).

2.3.1.7 Drug treatment

CXCR2 inhibitor SB-225002 was purchased commercially (Cat No. 559405-1mg). SB-225005 was solubilised in DMSO at a stock solution of 1mg/mL, aliquoted and stored at -20ºC. SB-225002 was made fresh and diluted to the appropriate concentration in medium prior to use with a DMSO treated control.

Recombinant human CXCL1 (Cat No. 300-11) was reconstituted in dsH_20 and further diluted in PBS/0.1% BSA (2.2.1.11). Recombinant protein was added to $CD34^+$ cells in culture in various concentrations with an appropriate vehicle treated control.

2.3.1.8 Granulocyte isolation

Histopaque® is a Ficoll gradient solution designed for cell separation techniques. Whole blood can be layered over the solution and after a centrifugation step allows cells of different densities to be isolated. Histopaque®-1077 is of a particular density that allows the isolation of MNC while removing erythrocytes, plasma and granulocytes. To allow isolation of granulocytes, Histopaque®-1077 can be combined with Histopaque®-1119. Human PB was collected from normal, healthy donors with informed consent. Blood was diluted in PBS and layered onto a double layer of Histopaque®-1077 and Histopaque®-1119. Solution was spun at 400 x *g* for 30 min and the layer containing the granulocytes was isolated, washed in PBS/2% FCS and used immediately for downstream applications.

2.3.2 Transfection

2.3.2.1 Lentiviral infection

Lentiviral transduction is a technique which was developed as an effective method for the introduction of stable expression of protein in any cell type, including primary haemopoietic cells which are largely non-dividing therefore do not integrate plasmid DNA. The method involves transiently transfecting HEK293 cell lines with vectors encoding the desired lentiviral vector and packaging plasmids. These cells then package the lentiviral expression construct into pseudoviral particles which are released into the cell supernatant which can be added to cells of interest to allow highly efficient transduction. HEK293 cells were plated at 80% confluence 24 hours prior to transfection in T125cm^2 tissue culture flasks. Immediately prior to transfection ($CaCl_2$ method), fresh medium was added. A solution containing plasmids was prepared as described in 2.2.7.3, mixed and incubated at 37ºC for 30 min. The mix was then added drop wise to the medium and cells were incubated overnight. The following day, the medium was removed and fresh medium supplemented with 20% FCS (2.2.1.2) was added to cells to remove the $CaCl_2$ mix precipitate and to allow viral production for subsequent collection. 48 hours after the addition of fresh medium, the medium containing viral particles was removed and filtered through a 0.44µm sterile filter. The cells of interest to be transduced were resuspended in the viral medium and the appropriate transduction reagent was added to the medium and the following protocols were carried out depending on the cell type. Viral particles were prepared fresh for every transduction and not used from frozen due to a decrease in viral transduction efficiency with frozen viral supernatant.

2.3.2.1.1 Cell lines

HT 1080 cells were resuspended in the viral medium with the addition of Polybrene® (Cat No. H9268) at a final concentration of 4µg/mL. Cells were cultured for 24 hours in the viral supernatant.

2.3.2.1.2 Primary human samples

Primary human samples are not actively dividing, difficult to transduce and required a different transduction reagent and a protocol of spin inoculation. Primary human cells were thawed and cultured over night as mentioned in sections 2.3.1.3 and 2.3.1.6. After overnight recovery, an appropriate number of cells were resuspended in viral medium with the addition of Transdux™ (Cat No. LV850A-1) at a 1 in 200 dilution, spun at 400 x *g* for 1.5 hours at 32ºC and subsequently cultured for several hours in the viral supernatant at 37ºC. Cells were infected with 2 rounds of infection with fresh supernatant containing viral particles at each round of infection.

2.3.2.1.3 Primary mouse samples

Primary mouse samples were enriched for stem/progenitor marker c-Kit as mentioned in section 2.3.3.4 to assess a more primitive cell population. For transduction of primary mouse samples, Retronectin™ (Cat No. T100A) (50µg/mL) was coated onto tissue culture 6 well plates and incubated overnight at 4ºC prior to transduction. Retronectin™ was removed, viral medium was added and centrifuged at 400 x *g* for 1.5 hours at 32ºC. Viral supernatant was removed, cells were seeded at $1x10^6$ cells cells/well, centrifuged at 400 x *g* for 1.5 hours at 32ºC and subsequently cultured for several hours in the viral supernatant at 37ºC. Cells were infected with 3 rounds of infection with fresh supernatant containing viral particles.

After 24 hours in final viral medium, cells were washed several times in PBS and resuspended in appropriate medium for 24 hours before downstream applications. Depending on the lentiviral vector of interest containing a puro resistance cassette or a sequence encoding GFP protein, cells were either cultured in puro (Cat No. P8833-10mg) (2µg/ml) for 7 days or sorted for GFP positive cells (GFP^+) using FACS as mentioned in section 2.3.3.13.

2.3.3 Stem cell selection

2.3.3.1 Collection of human primary cell samples

All samples were collected with the approval from the local research and ethics committee and with written informed patient consent from patients. Samples were obtained from patients undergoing autologous stem cell collection. Patients had been treated with G-CSF following chemotherapy and had excess $CD34^+$ cells remaining after those required for clinical use had been processed. The $CD34^+$ content is deemed 'normal' and used for these studies. A table listing the samples used in this study can be seen in (Table 2-8). A combination of male and female donors were used. BM samples used in this study were from normal healthy volunteers or purchased from commercial companies (AllCells and Lonza). Further information including age and gender was not available for the BM samples.

Sample ID	Age	Gender	Disease
121106	N/A	Male	Lymphoma
121121	N/A	Male	Lymphoma
130201	N/A	N/A	Lymphoma
121113	N/A	N/A	Germ Cell Tumour
Non CML 014	42	Male	N/A
Non CML 017	33	Male	Lymphoma
Non CML 019	64	Male	Mantle Cell Lymphoma
Non CML 021	31	Male	Myeloma
Non CML 022	50	Female	Relapsed Follicular Lymphoma
Non CML 023	61	Female	Hodgkin's Lymphoma
Non CML 024	59	Female	Relapsed Hodgkin's Lymphoma

Table 2-8 Patient sample information.

Table shows sample identification number, age, gender and disease status of the samples used in this study. N/A: Non applicable states that the information was not available for the particular sample.

2.3.3.2 Removal of RBC

To remove RBC from primary human material, a separation based on density gradients was used. Whole blood was diluted in PBS and layered onto Histopaque®-1077. The suspension was centrifuged at 400 x *g* for 30 min and the layer containing the MNC fraction was isolated and washed in PBS. This technique allows the isolation of MNC, leaving the plasma, granulocytes and RBC separated.

2.3.3.3 Stem cell enrichment

For enrichment of the human stem/progenitor population, the mononuclear fraction of PB and BM was enriched for cell surface marker CD34 using magnetic beads and a magnet separation method. The mononuclear layer of cells was counted and the appropriate number of cells was mixed with magnetic beads against human CD34 IgG (Cat No. 130-046-702). The cells were then loaded onto a column and washed to remove negative cells. The cell suspension was removed from the magnetic column and the positively selected cells were collected and washed. To ensure the enrichment worked correctly and to assess the efficiency, resulting cells were stained with an antibody against CD34 (Cat No. 555824) and FACS was performed. The samples used in this study were kindly processed by Dr Alan Hair.

2.3.3.4 c-Kit enrichment of mouse BM cells

For enrichment of the mouse stem/progenitor population, unmanipulated BM was enriched for cell surface marker c-Kit using magnetic beads (Cat No. 130-091-224) and a magnet separation method. BM was prepared as mentioned in section 2.3.7.4. The BM cell suspension was counted and the appropriate number of cells was mixed with magnetic beads against mouse c-Kit IgG. The cells were then loaded onto a column and washed to remove negative cells. The cell suspension was removed from the magnetic column and the positively selected cells were collected and washed. To ensure the enrichment worked correctly and to assess the efficiency, resulting cells were stained with an antibody against c-Kit (Cat No. 105825) and FACS was performed. Figure 2-3 demonstrates the enrichment of c-Kit positive cells after magnetic bead selection.

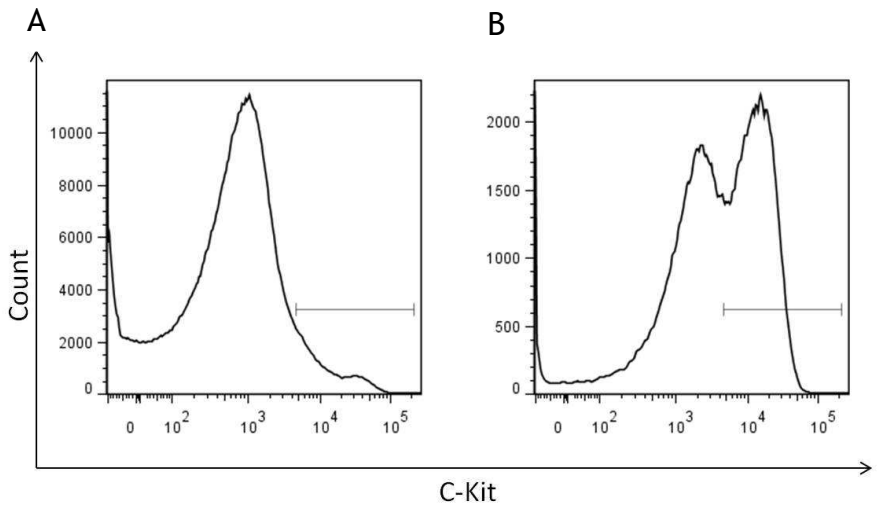

Figure 2-3 Representative plot of c-Kit staining in unmanipulated mouse BM and BM after c-Kit bead selection.

C-Kit staining can be visualised on the X axis. Viable cells were selected using forward angle light scatter (FSC) and side angle light scatter (SSC) and an unstained control was used to set the positive cell gate. A small percentage of positive cells for c-Kit staining can be seen in an unmanipulated BM sample (A) in contrast to cells stained after enrichment using c-Kit magnetic beads (B).

Flow cytometry and cell sorting

2.3.3.5 Flow cytometry

All reagents used for flow cytometry can be seen in Table 2-4. Flow cytometry permits the visualisation and sorting of cells according to the presence of antigens which are detected through fluorescently labelled antibodies, which when bound and excited with a laser can be detected. This allows a quantitative technique to compare protein expression between samples. A FACSCanto II flow cytometer (BD Biosciences) was used for flow cytometry analysis and a FACSAria (BD Biosciences) was used for sorting. With the exception of apoptosis assays, viable cells were gated on using FSC and SSC. Unstained cells, single colour controls and fluorescence minus one (FMO) controls were used for compensation analysis and to set appropriate gates for analysis. Data was acquired using BD FACSDiva (BD Biosciences) and analysis was performed using FlowJo (Tree Star Inc., Ashland, USA) software.

2.3.3.6 Antibody staining

All antibodies were titrated for optimal concentration before use. Antibodies used in this study are described in Table 2-4. Controls consisted of an appropriate isotype matched antibody at the same concentration. Isotype controls were used for all human antibodies used. Mouse primary cells were incubated in Fc block (CD16/CD32) (Cat No. 553141) and incubated on ice prior to antibody staining with the exception of mouse progenitor staining which uses a CD16/CD32 antibody.

2.3.3.7 Cell surface antibody staining

An appropriate number of cells were centrifuged at 300 x *g* for 5 min and washed several times. Cells were resuspended in PBS/2% FCS with antibodies at an appropriate concentration and incubated for the period and temperature as per the manufacturer's protocol. After staining, cells were washed, resuspended in 200µl PBS/2% FCS and analysed. Cells required for sorting were filtered using a 0.2µM filter prior to use.

2.3.3.8 Apoptosis assays

To examine apoptosis of cells in response to drug treatment or gene manipulation, Annexin-V (Cat No. 550475) and dapi (Cat No. D9542) were used. Briefly, protein phosphatidylserine is bound to the inner cell membrane, however during apoptosis this protein translocates to the outer cell membrane which can be detected using flow cytometry using stain Annexin-V. Annexin-V positive cells contain both early and late apoptotic cells which can be further discriminated with the addition of dapi. Dapi is used as a dead cell discriminator and is present only in late apoptotic cells due to the ability of the dye to penetrate the cell membrane when it becomes disrupted in late apoptosis. Approximately 10^5 cells were incubated with Annexin-V and a solution containing HBSS and dapi as mentioned in section 2.2.3.2 for 15 min at room temperature (RT) in the dark. Subsequently, cells were diluted in HBSS and analysed. Cells Annexin-V^-/$dapi^-$ were deemed as viable, with Annexin-V^+/$dapi^-$ early apoptotic and Annexin-V^+/$dapi^+$ late apoptotic (Figure 2-4).

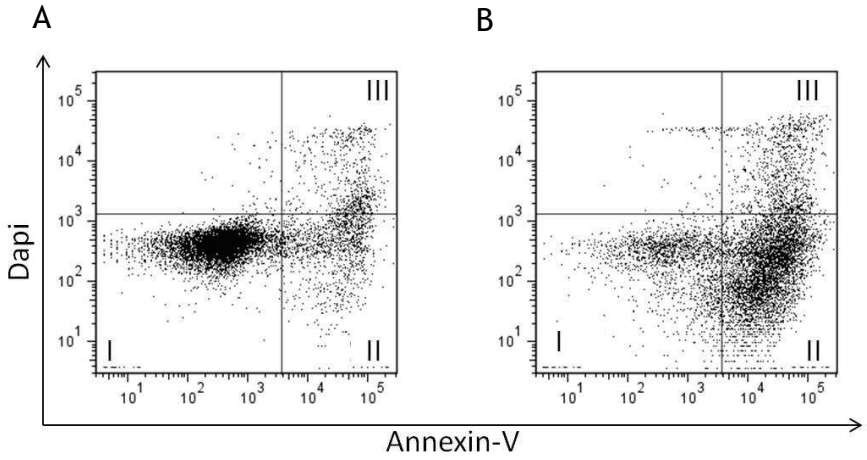

Figure 2-4 Representative plot of Annexin-V/dapi staining in viable and apoptotic cells.

Annexin-V and dapi are visualised on the X and Y axes respectively. Viable cells can be seen in quadrant I, early apoptotic II and late apoptotic III. The low percentage of cells positive for Annexin-V and dapi can be seen in unmanipulated cells (A) which increases in response to stimuli (B).

2.3.3.9 Cell cycle analysis

Ki-67 and dapi were used to examine the proportion of cells in different stages of the cell cycle. Ki-67 is a nuclear protein which is expressed during cellular proliferation (Gerdes et al., 1984). The protein is therefore only present in cells in active states of the cell cycle and is excluded from cells in G_0. Using this staining pattern, cells can be discriminated between G_0 and other phases using Ki-67 staining. Analysis of Ki-67 protein staining in combination with DNA dye dapi can be used to identify cells in all stages of the cell cycle (Jordan et al., 1996). Dapi stains intercalating DNA and distinguishes between cells in G_0/G_1 and $S/G_2/M$.

Cells were washed in PBS/2%FCS and resuspended in 1mL of PBS/3.65% formaldehyde (2.2.4.1) and incubated for 30 min on ice for fixation. 1mL of PBS/0.25% Triton-X-100 (2.2.4.2) was added without washing to permeabilise the cells which were incubated at 4ºC overnight. Cells were washed in PBS/2%FCS and resuspended in 1mL of PBS/2%FCS and the cell suspension was divided equally between two flow cytometry tubes for incubation with Ki-67-FITC (Cat no. 556026) or the appropriate isotype control. After incubation at RT for 40 min, the remaining unbound antibody was washed in PBS/2%FCS and the cells were resuspended in 100µl PBS/2% FCS with dapi. The cell solution was incubated for several hours at 4ºC, washed and analysed. In cases where all available channels of the flow cytometer were in use with other fluorochromes, Ki-67 staining was used in the absence of dapi. Figure 2-5 demonstrates a representative staining profile of cells stained with Ki-67 and dapi.

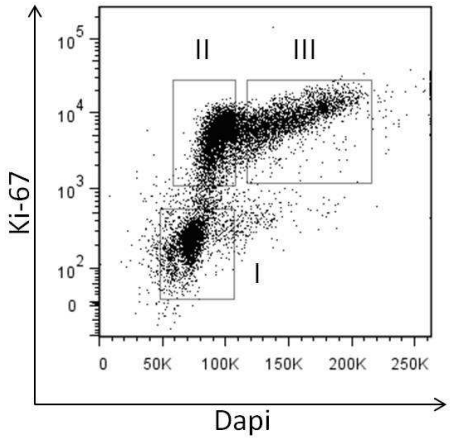

Figure 2-5 Representative plot of cell cycle staining using Ki-67 and dapi.

Dapi (linear) and Ki-67 (log) are visualised on the X and Y axes respectively. Viable cells were identified using FSC and SSC and gates were set up according to cells unstained with dapi and an isotype control for the Ki-67 antibody. Cells in G_0, G_1 and G_2/S/M phases can be identified in gates I, II and III respectively.

2.3.3.10 Selection of human stem cell fractions

The CD34 antigen represents a marker of stem/progenitor cells which can be further sub fractionated using additional cell surface markers. Primary CD34 enriched samples were centrifuged and washed in PBS/2% FCS. Cells were incubated with CD34, CD38 and CD90 (2.1.6) at optimal concentrations for 15 min at RT in the dark. Cells were washed in PBS/2% FCS to remove unbound antibody and resuspended in approximately $1x10^6$ cells/100µl PBS/2% FCS, filtered through a 0.7 µm mesh and analysed on the FACSAria. Appropriate gates were set up using single colour controls with appropriate isotypes and single cell sorting was carried out. Figure 2-6 demonstrates the gating strategy used for the identification of $CD34^+CD38^+$, $CD34^+CD38^-$, $CD34^+CD38^-CD90^+$ and $CD34^+CD38^-CD90^-$ populations.

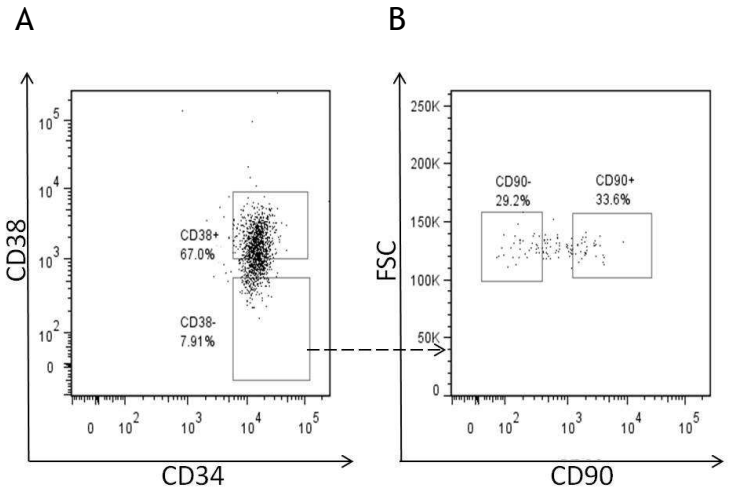

Figure 2-6 Representative plots of CD34, CD38 & CD90 staining.

Viable cells were gated using FSC and SSC and isotype controls were used to select positive populations. Within the viable cell gate, cells were analysed for $CD34^{+}CD38^{+}$ and $CD34^{+}CD38^{-}$ gates (A). For further purification within the $CD34^{+}CD38^{-}$ gate, cells were selected for $CD90^{-}$ or $CD90^{+}$ gates (B).

2.3.3.11 Selection of mouse stem and progenitor fractions

Mouse BM, spleen or PB cells were prepared as described in section 2.3.7.4. Cells were spun at 400 x *g* for 5 min and resuspended in PBS/2%FCS. Cells were stained with appropriate antibodies (2.1.6) at optimal concentrations for 30 min at 4ºC in the dark. Cells were washed in PBS/2% FCS to remove unbound antibody and resuspended in streptavidin and stained for a further 30 min at 4ºC. Cells were washed, filtered through a 0.7µm mesh and analysed or sorted. Appropriate gates were set up using single colour controls and FMO. Figure 2-7 demonstrates the gating strategy used to identify and isolate stem and progenitor populations from mouse cells.

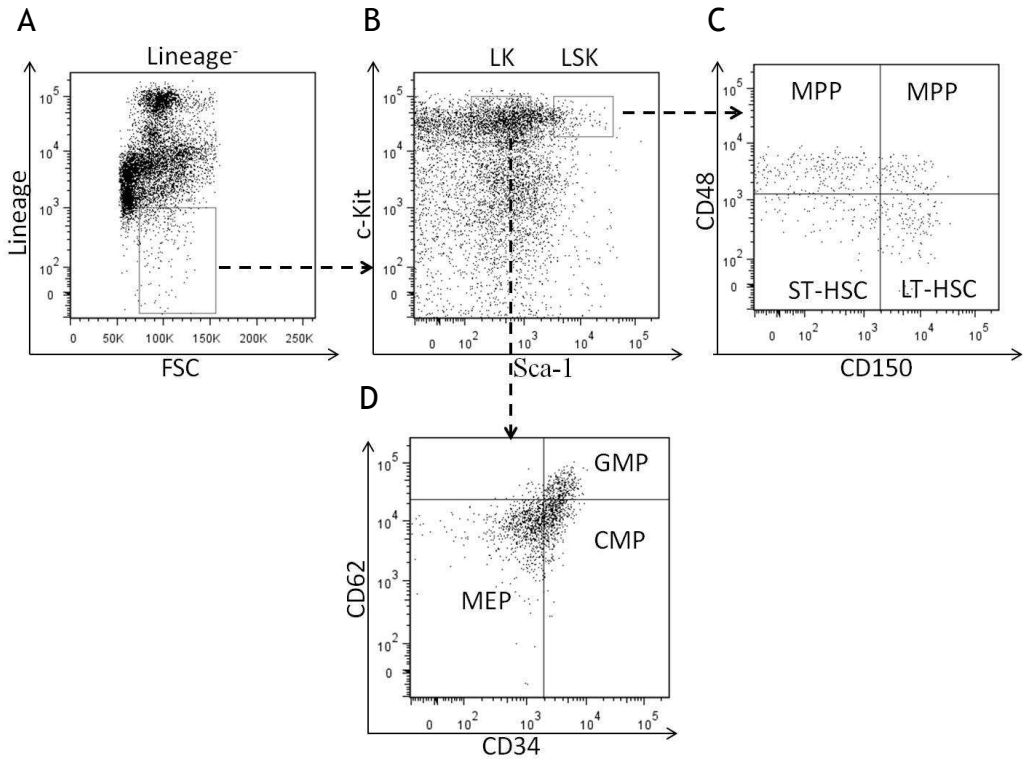

Figure 2-7 Representative plots for identification of mouse stem and progenitor cells.

Viable cells were identified using FSC and SSC. Gates were set up using single colour and FMO controls. Within the viable cells, lineage negative cells were selected (A) and lineage negative, c-Kit$^+$ (LK) or lineage negative, c-Kit$^+$, Sca-1$^+$ (LSK) gates were selected (B). Within LK population, progenitors were assayed using CD34 and CD62 staining (D). Within LSK cells, stem cell populations were examined using CD150 and CD48 staining. The stem/progenitor populations are marked according to literature available at the time of doing the experiments for this thesis.

2.3.3.12 Selection of mouse mature cell types

Mouse BM, spleen or PB cells were prepared as described in section 2.3.7.4. Cells were spun at 400 x *g* for 5 min and resuspended in PBS/2%FCS. Cells were stained with appropriate antibodies (2.1.6) at optimal concentrations for 30 min at 4ºC in the dark. Cells were washed in PBS/2% FCS to remove unbound antibody. Cells were washed, filtered through a 0.7µm mesh and analysed. Appropriate gates were set up using single colour controls and FMO. Figure 2-8 demonstrates the gating strategy used to identify and isolate various mature cell populations from mouse organs. Myeloid, B, erythroid and T cells were selected as GR1$^+$CD11b$^+$ (A), CD19$^+$B220$^+$ (B), TER119$^+$ (C) and CD4$^+$CD8$^+$, CD4$^-$CD8$^-$, CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ (D) respectively (Figure 2-8).

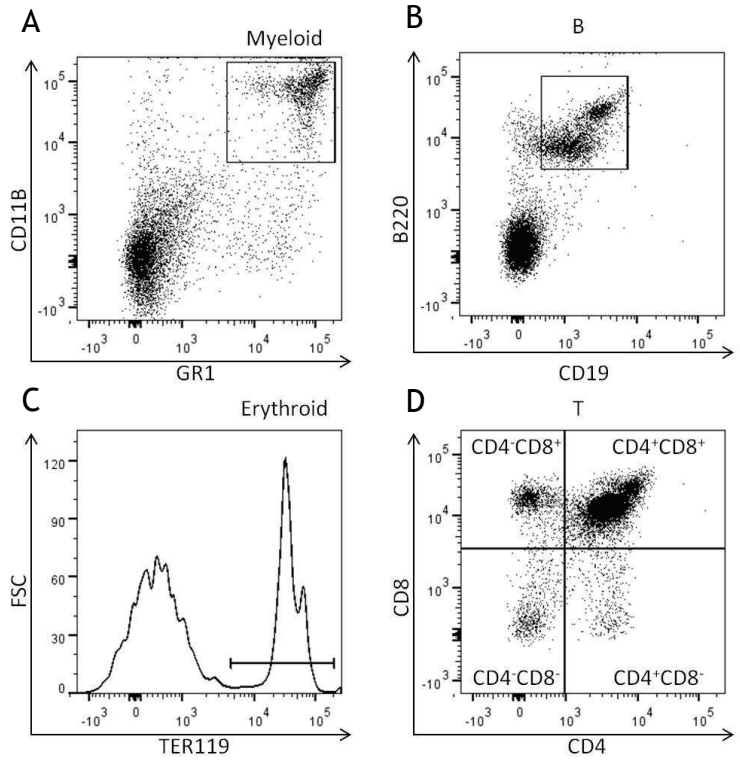

Figure 2-8 Representative plots for identification of mouse mature cell types.

Viable cells were identified using FSC and SSC. Gates were set up using single colour controls. Within the viable cells, particular cell types were selected using different antibodies. Panels A-C display representative images of myeloid ($GR1^+CD11B^+$), B ($B220^+CD19^+$) and erythroid ($TER119^+$) staining in a WT BM. Panel D displays different T cell populations identified in stained WT thymocytes.

2.3.3.13 Flow cytometry cell sorting

Cells were prepared in PBS/2% FCS at an appropriate concentration, approximately $1x10^6$ cells/300µl. Cells were filtered through a nylon sterile filter before use. Sorted cells were analysed for purity post-sort (Figure 2-9). A purity of ≥90% was achieved for all samples in this study.

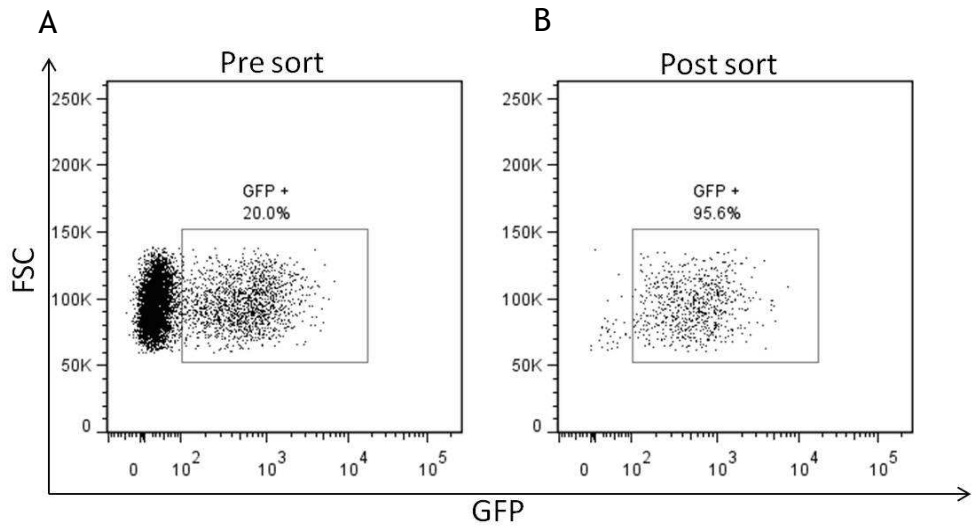

Figure 2-9 Representative plots demonstrating sorting efficiency.

Viable cells were gated using FSC and SSC and a GFP positive gate was identified using negative cells. Image shows small percentage of GFP positive cells prior to sort (A) and post sort (B).

2.3.4 Immunofluorescence and immunohistochemistry

2.3.4.1 Immunofluorescence

All reagents used for immunofluorescence can be found in Table 2-3. Cells at approximately $2x10^4$ cells/spot were added to a pre-coated microscope slide with Poly-L-lysine. Cells were allowed to attach to the slide for several hours during incubation at 37ºC. Cells were fixed with 3.75% formaldehyde solution (2.2.4.1) for 15 min at RT and washed several times in PBS. Cells were permeabilised to allow intracellular staining with 0.25% Triton-X-100 (2.2.4.2) for 15 min at RT and washed several times in PBS. Antigen sites were blocked using 5% BSA solution (2.2.4.3) for 30 min at RT before subsequent addition of appropriate primary antibody. After overnight incubation at 4ºC, the primary antibody was washed in PBS and the appropriate conjugated secondary antibody was added to the cells for 1 hour at RT. After washing, the cells were mounted with a coverslip using Vectashield with dapi (Cat No. H-1200). Fluorescence was analysed using a Zeiss Imager M1 microscope with equal exposure times between samples to ensure reliability and appropriate isotype controls. Data was acquired using AxioVision software and 3-dimensional (3-D) images were generated using Image J software.

2.3.5 Western blotting

Briefly, proteins were isolated, fractionated on a gel and transferred to a membrane for subsequent blotting against specific antibodies to analyse expression of protein of interest.

2.3.5.1 Preparation of protein lysates

RIPA lysis buffer (Cat No. PI-89901) was prepared immediately prior to use with the addition of protease and phosphatase inhibitors. Equal cell numbers were washed twice in ice cold PBS and transferred to an eppendorf. An appropriate volume of RIPA lysis buffer was added to cells and incubated for 15 min on ice. Cells were centrifuged for 10 min at 4ºC to remove the nucleic acid and debris leaving the supernatant containing protein which was used immediately or stored at -20ºC. In cases of small cell numbers, equal cell numbers were immediately resuspended in 2X SDS sample buffer (2.2.2.1) and stored at -20ºC for future use.

2.3.5.2 Protein quantification

In cases of small cell numbers, equal cell numbers were immediately resuspended in 2X SDS sample buffer and loaded neat onto a gel. Samples lysed using RIPA buffer were quantified using the BCA™ protein assay kit (Cat No. 23227) as per the manufacturer's instructions. The BCA assay is a biochemical assay used for determining the concentration of protein in a solution. Reagents are added to samples of proteins which results in colour change which is correlated to protein concentration which can be measured using colorimetric techniques such as absorbance (Smith et al., 1985). Briefly, BSA standards were prepared with varying concentrations of a standard protein in PBS. Standards were used to create an equation using a known concentration of protein standard against absorbance. Unknown lysates were then examined for protein concentration using the equation. The absorbance was read at 562nm using a plate reader. Based on the protein concentration of samples, equal amounts of protein were then added in each assay.

2.3.5.3 Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a denaturing separation method commonly used to analyse protein samples. Proteins migrate through a matrix at varying speeds according to their molecular weight in a gel solution containing SDS which is a negatively charged detergent. These can be applied with an electric current in which

negatively charged proteins migrate towards a positive electrode therefore allowing separation. Protein samples were diluted in 2X SDS buffer according to concentrations desired and boiled at 95ºC for 5 min. Samples were loaded onto a gel alongside a prestained protein ladder (Cat No. BIO-33065) to assess the molecular weight of proteins migrating through the gel. Samples were run in running buffer (2.2.2.4) for 80 volts (V) for 30 min and at 120V for the remaining 1 hour.

2.3.5.4 Membrane transfer

After protein separation, proteins were required to be transferred to a membrane which could be subsequently blotted with antibodies against the protein of interest. Briefly, an electrical current can be used to transfer proteins from a gel to a membrane. Proteins were transferred from the acrylamide gel to an Immun-Blot PVDF membrane. The PVDF membrane was activated in a solution of neat methanol. A solution of transfer buffer (2.2.2.5) was used to soak sponges, papers and PVDF membrane. The sponges and papers were layered onto a transfer system with the isolated gel layered below the PVDF membrane with the addition of sponges and gel blotting paper (1.0mm) paper on top. The transfer was run for 30V at 1 hour and successful transfer was noted due to the addition of the protein ladder from the gel to the membrane. The membrane was kept moist at all times to prevent drying out and damage to the proteins.

2.3.5.5 Immunolabelling

Immediately, post transfer, the PVDF membrane was carefully transferred to a solution to block antigen sites and prevent non specific binding (2.2.2.6). After 1 hour blocking at RT, the membrane was transferred to a fresh blocking solution with the addition of the appropriate primary antibody (2.1.5) with a gentle rotation at 4ºC overnight. After overnight incubation, the blot was washed several times with gentle shaking in a wash solution (2.2.2.2). The blot was then incubated in the appropriate secondary antibody conjugated to enzyme HRP for 1 hour at RT with gentle rotation. After incubation, the blot was washed several times and an ECL detection method was used to visualise the protein bands (ImmunoStar™Western C kit (Cat No. 170-5070) as per manufacturer's instructions. Protein bands were visualised using the Molecular Imager® ChemiDoc™ XRS machine (Bio-Rad Laboratories Ltd). After visualisation of bands showing protein of interest, the blot was blocked and probed with the appropriate primary antibody for housekeeping protein to ensure equal protein loading between samples. The blot was then probed with the appropriate secondary antibody, washed and visualised as detailed above.

Densitometry was used to quantify protein expression between samples using Quantity One® software.

2.3.6 Molecular biology

All reagents used can be found in Table 2-3.

2.3.6.1 Primer design

Primers were designed using NCBI software and the sequences can be seen in Table 2-5. Primers were synthesised commercially (Eurofins MWG Operon). Primers were reconstituted with the appropriate volume of nuclease free H_20 to achieve a stock concentration of 100µM. Dilutions were made to achieve the appropriate working concentration of 10µM and aliquots were prepared to ensure sterility and stored at -20ºC. PCR reagent concentrations and thermal cycling conditions were optimised for each primer set.

2.3.6.2 Generation of DNA

Approximately $5x10^6$ cells were centrifuged and washed in PBS. DNA was extracted using the Bioline DNA extraction kit (Cat No. BIO-52067) or QIAamp DNA blood mini kit (Cat No. 51104) as per the manufacturer's instructions. Animal ear/tail samples or MNC were incubated for several hours up to overnight in DNA lysis buffer plus Proteinase-k with intermittent vortexing to ensure complete digestion.

2.3.6.3 Generation of RNA

To ensure work was free from ribonucleases (RNAses), RNA extraction and downstream applications was carried out with the addition of RNAZap™ (Cat No. AM9780) on all surfaces and pipettes before use. Reagents were also treated with UV light before use. An appropriate number of cells were spun and washed in PBS before use. Depending on cells numbers, the RNA mini ($5x10^6\geq$) (Cat No. 74106) or micro kit ($5x10^6\leq$) (Cat No. 74004) was used as per manufacturer's instructions. The resulting RNA was quantified and examined for purity using a NanoDrop spectrophotometer ND-1000. RNA was kept on ice at all times and stored at -80ºC.

2.3.6.4 First strand synthesis

RNA was synthesised to cDNA using the High Capacity cDNA Archive kit according to the manufacturer's instructions. 1µg of RNA was converted to cDNA in a 20µl reaction (2.2.5.4). When low RNA yields were obtained, the maximum volume of RNA was added to a 20µl reaction. A non-template control with reagents minus RNA was included with every synthesis to ensure no contamination of reagents was present and cDNA was stored at 4ºC.

2.3.6.5 Standard PCR

PCR is a technique widely used in molecular biology. The technique is based on the principle of amplifying DNA using thermal cycling so DNA can be detected. The use of DNA primers which are sequences complementary to the DNA region of interest are used to selectively amplify particular regions of DNA. The process of DNA synthesis requires enzyme Taq polymerase. Positive control samples and a PCR reaction minus DNA was used to assess reagent contamination with every reaction (2.2.5.5). PCR reactions were run with appropriate PCR conditions according to primers used (2.1.7.1). PCR products were analysed using agarose gels with the addition of SybrSafe™ to visualise DNA with UV illumination. Molecular ladders at an appropriate size for analysis were run with samples to know exact size of PCR products. Agarose gels were made solubilising agarose powder in a solution of 1X TBE (2.2.5.2). The solution was heated in a microwave to dissolve the powder, the solution was cooled, SybrSafe™ was added and poured into a gel setting with combs. Once solidified, the gel was put in an electrophoresis tank in 1X TBE solution, samples were loaded into the wells and an electric current was used to allow the migration of DNA samples through the gel according to size. DNA was visualised using UV illumination using a molecular imager® ChemiDoc Chemidoc™ XRS visualisation system.

2.3.6.6 Quantitative-PCR

Real-time quantitative PCR (or Q-PCR) is based on the principles of standard PCR, however this technique allows gene expression differences to be analysed between different samples. In this thesis, Q-PCR was carried using the TaqMan® system and reagents. This technique is widely used and has a high sensitivity and specificity. Briefly, amplification is quantified using fluorescently labelled probes which are cleaved during amplification resulting in a fluorescent signal which allows detection and quantification

during the PCR reaction. To achieve this, each TaqMan® probe has been designed to contain a fluorophore and quencher. The quencher stops fluorescence emitted by the fluorophore when excited by the cyclers light source using fluorescence resonance energy transfer. The juxtaposition of the fluorophore and quencher inhibits fluorescence and therefore its detection. Amplification of the target sequence using Taq polymerase results in probe displacement which results in fluorescence emission. The fluorescence emitted correlates with the DNA template and is therefore quantified. RNA was converted into cDNA as mentioned in section 2.3.6.4 and was combined with TaqMan™ inventoried probes (Table 2-6), H_20 and 2X universal PCR mastermix in a 10µl reaction (2.2.5.3). Samples were prepared in triplicate with an appropriate housekeeping control for every sample. A non-template control was included in every reaction to exclude the possibility of contamination of reagents. Samples were loaded into a 384 well plate, centrifuged and loaded onto a 7900HT real-time PCR system (Applied Biosystems). The standard thermal cycling conditions were used as per the manufacturer's instructions: 50ºC for 2 min, 95ºC for 10 min and finally 40 cycles of 95ºC for 15 seconds (sec) and 60ºC for 1 min. Data was acquired using SDS software and analysed using RQ manager (Applied Biosystems). CT values were examined and compared between samples using fold change relative to calibrator using the ΔΔCT standard method for analysis (Schmittgen and Livak, 2008). To show the variation between biological replicates of calibrant samples, relative expression is shown using the $2^{-DeltaCT}$ as detailed in the text.

2.3.6.7 Single cell RT and preamplification

Due to small cell numbers of primary material, Q-PCR was carried out using small numbers of cells and the Fluidigm™ platform and TaqMan reagents®. A small number of cells (200) were sorted using a FACS into a 5µl mix containing a lysis buffer, Taqman® probe mix and 2X reaction mix (Cat No. 11753-100) (2.2.5.6). The cell suspension was vortexed and spun before added to a PCR machine for RT and preamplification of particular genes using Taqman® probes. The standard thermal cycling conditions were used as per the manufacturer's instructions: 50ºC for 15 min, 95ºC for 2 min and finally 18-22 cycles of 95ºC for 15 sec and 60ºC for 4 min. Subsequently, the cDNA sample was diluted with the addition of 20µl TE buffer (Cat No. 12090-015) and stored at -20ºC. The cDNA was used as described in sections 2.3.6.6 and 2.3.6.8.

2.3.6.8 Fluidigm

A high-throughput Q-PCR was carried out using the Fluidigm™ platform. Fluidigm™ has developed an approach to analyse large sets of gene expression analysis using very small cell numbers based on microfluidic technology. Q-PCR is carried out as described in section 2.3.6.6 with the addition of a system in which multiple samples and genes can be analysed simulateously. A 48 x 48 or 96 x 96 (sample x gene) chip format is available which dramatically increases the number of PCR reactions that can be achieved in one assay. After the preamplification of samples, the resulting cDNA was used with the Taqman™ probes of interest to examine gene expression of 48 genes across 48 samples. Briefly, cDNA and probes were mixed with appropriate sample or assay buffer and added to inlets of gene chips. The chip is primed before use and the loaded chip is run on the Fluidigm™ BioMark HD system. Data was acquired using the BioMark acquisition software and analysed using Fluidigm real-time PCR analysis software. Fold changes in gene expression were calculated according to methods described in the Q-PCR section (2.3.6.6).

2.3.6.9 Cloning of DNA fragments

2.3.6.9.1 CXCL1 and CXCL4 shRNA

Human CXCL1 and mouse CXCL4 shRNA hairpins were purchased in a pLKO.1 plasmid with an ampicillin resistance cassette and a puro resistance cassette as described in section 2.1.2. The plasmids purchased came with multiple plasmids each with a unique shRNA sequence, designed to ensure adequate coverage of the target gene. The sets purchased contained six and five plasmids for CXCL1 and CXCL4 respectively. It is predicted that some of the shRNA sequences will give at least 70% knock down of the gene of interest, however it is necessary to test which sequence from the set is best at gene reduction for future experiments. Each plasmid was transduced into positive control cells as mentioned in section 2.3.2.1 and selected in puro for seven days when an untransfected control had undergone apoptosis therefore all cells growing had stable integration of the expression vector. Resulting cells were tested for reduction in gene and protein levels using Q-PCR as mentioned in section 2.3.6.6 and western blotting as mentioned in section 2.3.5. The shRNA sequences found to result in the highest levels of gene reduction were subcloned into a plasmid with a reporter tag (GFP) for use in primary cell transduction. Two plasmids from human CXCL1 and one plasmid (due to time constraints) from mouse CXCL4 were

subcloned into a pLKO.1 plasmid containing a GFP insert. Briefly, 10µg of DNA from pLKO.1-CXCL1 or pLKO.1-CXCL4 and pLKO.1-Scr (GFP) were digested with NdeI and SpeI RE (New England Biolabs) (2.2.6.3). After 3 hours at 37ºC, digestion products were incubated with SAP at 37ºC for 45 min to dephosphorylate the ends of the cut fragments, reducing the probability of the cut fragments joining together. The products were then run on a 2% agarose gel and analysed using UV illumination. The inserts containing the shRNA sequences from pLKO.1-CXCL1 and pLKO.1-CXCL4 and the plasmid backbone from pLKO.1-Scr were cut using a sterile scalpel and the DNA was extracted from the gel (2.3.6.10). DNA was quantified and insert was ligated to the backbone using T4 DNA ligase and incubated overnight at 14ºC (2.2.6.4). Bacteria was transformed and grown as mentioned in 2.3.6.11 and individual clones were examined for the correct plasmid using RE digestion and sequencing analysis using primers spanning the shRNA sequence (2.1.7.1) (GATC Biotech, Konstanz, Germany). Bacterial stocks with the correct plasmid were made in glycerol as mentioned in 2.3.6.11, stored at -80ºC and cultured when required.

2.3.6.9.2 CXCL1 over expression

DNA was extracted from cell line PC-3 and PCR set-up with primers spanning the human CXCL1 coding sequence (2.1.7.1). The correct band was visualised using UV and extracted as mentioned in section 2.3.6.10. The insert was ligated into vector PCR® 2.1 TOPO® using the TOPO® TA cloning® kit according to the manufacturer's instructions. The ligation mix was incubated overnight at 14ºC and transformed into competent bacteria as mentioned in 2.3.6.11. Colonies were screened for the presence of a single insert in the correct orientation using RE digests with HindIII and the plasmid was sent for sequencing analysis. The CXCL1 insert was extracted from PCR® 2.1 TOPO® using BamHI and Ecor V RE digests and ligated into lentiviral plasmid PRRL backbone cut with BamHI and SnabI overnight at 14ºC and transformed into bacteria. Correct clones were sent for sequencing analysis and PRRL without the CXCL1 insert was used as an empty vector control.

2.3.6.10 Extraction of gel bands

DNA was extracted from agarose gel fragments using the Qiaquick gel extraction kit (Qiagen) as per manufacturer's instructions. The resulting DNA was quantified and examined for purity using a nanodrop spectrophotometer Nd-1000.

2.3.6.11 Growth of plasmids

All microbiology work was completed on a sterile bench with a bunsen burner. Competent bacteria were transformed with DNA plasmid according to the manufacturer's instructions. Briefly, bacteria were allowed to thaw on ice for 30 min and 1µl of plasmid DNA or 5µl of ligation product was incubated for 30 min. Bacteria were then incubated at 42ºC for 90 seconds (sec) resulting in the disruption of the cell membrane allowing the introduction of the DNA into the bacterial cells. Subsequently bacteria were incubated on ice for 2 min, SOC medium was added and incubated on a shaking platform for 1 hour at 37ºC to allow the production of the appropriate antibiotic resistance gene. Subsequently, approximately 50µl of bacterial suspension was added to agar plates grown in ampicillin and streaked or spread according to transformation reaction. Single colonies were selected and cultured for approximately 8-12 hours in LB medium plus ampicillin. The bacterial culture was then incubated approximately 8-12 hours in a large culture of LB medium plus ampicillin. Bacterial stocks containing correct the plasmid were made with the addition of 30% glycerol stocks and stored at -80°C for future use (2.2.6.1; 2.2.6.2).

2.3.6.12 Isolation of plasmid DNA

Plasmid DNA was isolated from bacteria using mini, midi or maxi kits according to volume of bacteria and manufacturer's instructions were followed. DNA was eluted in nuclease free H_20 and stored at -20ºC.

2.3.6.13 Verification of DNA sequence

The correct plasmid sequence was verified using RE digest and sequencing analysis. pLKO.1 plasmids were digested using NdeI and SpeI double digestion. PRRL-CXCL1 was digested using EcoRI single digestion. 1µg of DNA was incubated with enzymes with appropriate buffer and BSA solution for 3 hours at 37ºC as mentioned in section 2.2.6.3. Resulting DNA was visualised using UV for correct cuts. To ensure correct plasmid, DNA was sent for sequencing analysis using primers spanning the shRNA sequence with primers mentioned 2.1.7.1.

2.3.7 Animal work

2.3.7.1 Ethical issues

All animal work was carried out in accordance with regulations set by the Animals Scientific Procedures Act 1986 and UK Home Office regulations. Animals were housed at the Beatson Institute for Cancer Research or at the Veterinary Research Facility at the University of Glasgow. All experiments were carried out under my personal licence (60/12683) and Dr Kamil Kranc's project licence (60/4076). Stem cell analysis was carried out on animals between 6 and 12 weeks and animals were matched for sex where possible. Specifics of numbers of animals used and gender for each experiment is provided in detail in each figure legend.

2.3.7.2 Mouse models

2.3.7.2.1 Rosa26-RFP;Cxcl4-Cre

Rosa26-RFP;Cxcl4-Cre mice were a kind gift from Professor Laura Machesky and experiments were done in collaboration with Dr Simon Calaminus (Beatson Institute for Cancer Research, Glasgow, UK). Briefly, *Cxcl4-Cre* transgenic mice (C57/BL6 background) were crossed with mice containing a conditional tandem dimer red fluorescent protein (RFP) construct under the control of the Rosa26 promoter (C57/BL6 background). *Cxcl4-Cre* animals were constructed through a *Cre* recombinase cDNA insertion in bacteria into a bacterial artificial chromosome (BAC) clone containing *Cxcl4* (Tiedt et al., 2007). It should be noted that the BAC used to create the *Cxcl4-Cre* transgene contained the entire *Cxcl4* gene in addition to several others as described in Table 2-9. These genes have a variety of different functions including mouse neutrophil chemotaxis, megakaryocyte/platelet biology and HSC maintenance (Tiedt et al., 2007). The *Rosa26-RFP* animals were generated by targeting RFP into the ubiquitously expressed ROSA26 locus of C57/BL6 ES cells (Luche et al., 2007). A schematic of the *Rosa26-RFP;Cxcl4-Cre* mouse model is displayed in Figure 2-10. *Rosa26-RFP*$^{+}$*;Cxcl4-Cre*$^{+}$ mice were used with *Rosa26-RFP*$^{+}$*;Cxcl4-Cre*$^{-}$ mice as a control. Ear or tail samples were used to genotype the mice using Transnetyx at the Beatson Institute for Cancer Research.

Gene Name	Gene ID
Cxcl5	20311
Cxcl7	57349
Cxcl15	20309
Gm1960	330122

Table 2-9 List of genes contained on the BAC clone used in the construction of *Cxcl4-Cre* animals.

The BAC that was used for the transgenic animals contained *Cxcl4*, *Cxcl5*, *Cxcl7*, *Cxcl15* and *Gm1960* (Tiedt et al., 2007).

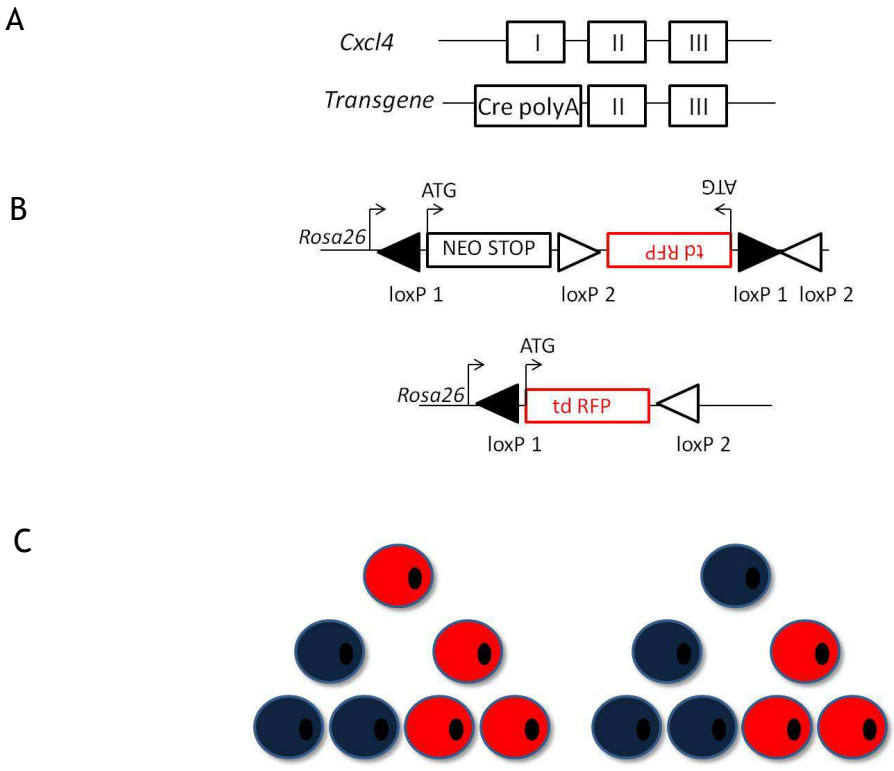

Figure 2-10 ***Rosa26-RFP;Cxcl4-Cre mouse model***

Panel A shows the transgene construct. Each box denotes the *Cxcl4* exons and the Cre denotes a codon-improved cDNA for Cre recombinase and polyA is the polyadenylation signal. Panel B displays the Rosa26-tdRFP construct. The tdRFP cassette is inserted in an anti-sense orientation. Two oppositely oriented WT loxP sites (loxP 1) flank the whole element. Two additional mutant loxP sites (loxP 2) surround the reversed tdRFP cassette with one of the loxP 1 sites. Cre mediates the inversion at the loxP sites and results in the removal of the NEO STOP cassette (1 and 2). The tdRFP is under full transcriptional control of the ROSA26 locus. Panel C demonstrates the activity of the RFP reporter. In cells in which *Cxcl4* is active, cells are all resulting progeny are RFP^{+} irrespective of the subsequent activity of *Cxcl4.* Images A and B are from the published literature on the constructs used (Luche et al., 2007, Tiedt et al., 2007). Image C is an original diagram.

2.3.7.2.2 Cxcl4 null animals

Cxcl4$^{-/-}$ mice were kindly donated by Professor Mortimer Poncz (Philadelphia children's hospital, Philadelphia, USA). Briefly, *Cxcl4*$^{-/-}$ mice were generated by replacing the entire coding region for *Cxcl4* with a neomycin resistance gene (C57/BL6 background). *Cxcl4*$^{-/-}$ mice were imported from Philadelphia children's hospital into the Veterinary research facility (University of Glasgow). *Cxcl4*$^{-/-}$ animals were crossed with WT C57/BL6 mice purchased in house (Harlan Laboratories, UK) to generate heterozygous animals. Unrelated heterozygous animals were then crossed to generate *Cxcl4*$^{+/+}$ (WT) and *Cxcl4*$^{-/-}$ (KO) animals which were used for the analysis detailed in chapter 5. Ear or tail samples were used to genotype animals as described in section 2.3.6.5 by using primers against endogenous *Cxcl4* or the *neomycin* cassette to ensure the sole use of WT or *Cxcl4*$^{-/-}$ animals in the analysis. The sequences of the primers were provided by Professor Mortimer Poncz.

2.3.7.2.3 CXCR2 null animals

Cxcr2$^{-/-}$ mice were kindly donated by Professor Owen Sansom (Beatson Institute for Cancer Research, Glasgow, UK). Briefly, *Cxcr2*$^{-/-}$ mice were generated by replacing the entire coding region for *Cxcr2* with a neomycin resistance gene (C57/BL6 background). Heterozygous with homozygous animals were crossed and *Cxcr2*$^{+/+}$ (WT) and *Cxcr2*$^{-/-}$ animals (KO) were used for analysis. Ear or tail samples were used to genotype animals using Transnetyx at the Beatson Institute for Cancer Research by using primers against endogenous *Cxcr2* or the *neomycin* cassette to ensure the sole use of WT or *Cxcr2*$^{-/-}$ animals in the analysis.

2.3.7.2.4 CD45.1^{+} animals

Animals containing the congenic marker CD45.1^{+} (Ly5.1) were kindly donated by Dr Kamil Kranc. This strain carriers the allele of the SJL mouse *Ptprc* gene locus. C57/BL6 animals are CD45.2^{+} (Ly5.2) therefore this strain can be used to discriminate between the animals used in this study as described in more detail in section 2.3.7.6.

2.3.7.3 Dissection

Animals were sacrificed using appropriate schedule 1 methods. Femur, tibia, hip bones, spleen and thymi were dissected and stored in PBS/2%FCS on ice. PB was taken by tail vein bleed prior to sacrifice or through collection from femoral vein post sacrifice and collected into tubes containing EDTA to ensure anti coagulation, stored at RT and analysed within several hours.

2.3.7.4 Cells

Bones from femur, tibia and hips were crushed in PBS/2% FCS using a mortar and pestle and made into a single cells suspension through filtering through a sterile 0.2µm filter. Spleen and thymi were mashed in PBS/2% FCS using a sterile plunger and filtered. Peripheral blood (PB) was analysed neat for cellularity analyses and RBC were lysed before downstream applications in NaCl solution according to manufacturer's instructions (Cat no. 07850).

2.3.7.5 Analysis

Single cell suspensions of organs were analysed using a haematology automated analyser (Hemovet). This was used to perform full blood counts. Bones, spleen and thymi were resuspended in a volume of 30mL, 12mL and 12mL respectively in PBS/2% FCS. Cells were spun and resuspended in appropriate antibodies and analysed by flow cytometry. White blood cell (WBC) counts were multiplied to percentage of cells to get cell count/mouse.

2.3.7.6 Transplantation assays

A BM reconstitution assay allows the detection of a primitive class of HSC that allow the survival of lethally irradiated mice transplanted with few numbers of cells due to their ability to repopulate haemopoeisis. This assay can be used to compare stem potential of different cell types or to compare stem cell activity in transgenic mice.

Lineage negative, Sca-1^+, c-Kit^+, $CD150^+$, $CD48^-$ (LT-HSC) cells from *Cxcr2*$^{-/-}$ or *Pf4*$^{-/-}$ with wildtype (WT) animals were sorted using flow cytometry into a cell suspension of $CD45.1^+$ BM MNC at a concentration of 10^2 LT-HSC donor cells plus $2x10^5$ BM $CD45.1^+$ cells per mouse. 200µl of the cell suspension was injected by intravenous transfer into

lethally irradiated mice at a maximum of 24 hours post irradiation. CD45.1^{+} animals were irradiated using a dosage of 7 gray (Gy) radiation using an x-ray irradiator at the Beatson Institute for Cancer Research and subsequently housed in individually ventilated cages (IVC) and treatment with Baytril. Every 4 weeks post transplant up to 16 weeks, PB was taken and prepared as described in section 2.3.7.4. Cells were stained for antibodies against CD45.1, CD45.2 and antibodies against mature cell types including mature B (CD19^{+}), T (CD4^{+}, CD8^{+}) and myeloid (GR-1^{+}, CD11B^{+}) cells (2.1.6). After 16 weeks, the BM, spleen and PB were taken from sacrificed mice and examined for CD45.1^{+} and CD45.2^{+} positive cells in mature cell types and within stem/progenitor cell populations (Figure 2-11).

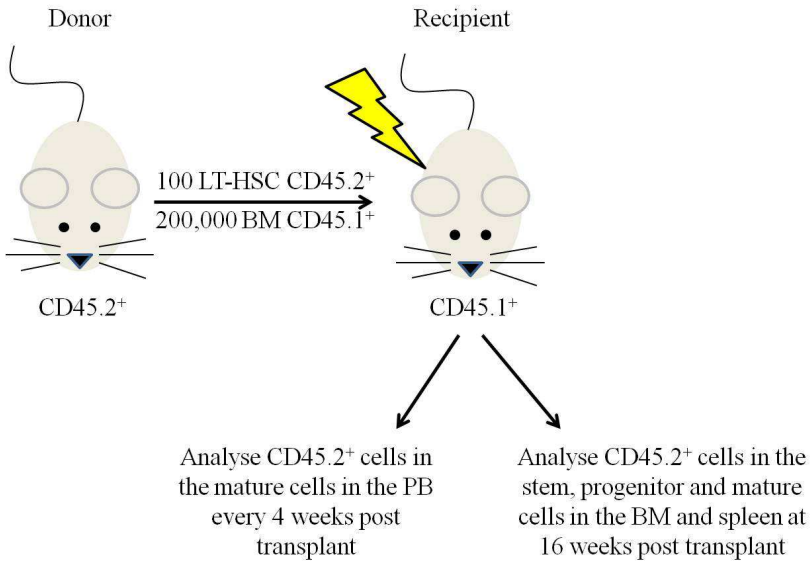

Figure 2-11 Schematic digram demonstrating BM transplantation assay.

CD45.2^{+} donor derived HSC were transplanted with CD45.1^{+} derived support BM in irradiated CD45.1^{+} recipients. PB was analysed every 4 weeks post transplant to examine CD45.2^{+} cells in the whole blood and mature cell types. At 16 weeks post transplant, BM and spleen was analysed for the CD45.2^{+} cells in the organs, stem, progenitor and mature cell types.

2.3.8 Statistics

The results are shown as the mean with standard deviation values unless otherwise stated. All statistical analyses were performed using the Graph Pad prism software package. To test if values came from a Gaussian distribution, normality was analysed using the Kolmogorov-Smirnov test. Depending on the results of the test, the appropriate parametric or non parametric tests were used. In cases of low sample numbers (i.e. $n \leq 3$), the normality test could not be used and therefore values were assumed to come from a Gaussian distribution. Specific details of the statistical test carried out for each data set are provided in the figure legends. A level of $P < 0.05$ was deemed significant and levels of $P < 0.01$ and $P < 0.001$ were deemed very significant. Values of $P > 0.05$ were deemed not significant.

3 Results I: The role of CXCL1/CXCR2 signalling in human HSC survival

3.1 Introduction

A key property of HSC is their ability to maintain quiescence, which is thought to important for their ability to sustain haemopoiesis over a long period of time (Pietras et al., 2011). A variety of methods have been used to identify novel genes in quiescence, including gene-targeted deletion/knock-in mouse models. In addition, high-throughput approaches including global gene expression studies have been used (Passegue et al., 2005, Venezia et al., 2004, Forsberg et al., 2010). Such screens have identified genes involved in quiescence including in metabolism and cell cycle regulation. Despite the identification of a variety of genes involved in quiescence, it is still not well understood. In addition, limitations in previous studies have been due to a lack of data on human HSC populations. A previously published microarray study by our group reported that the most up regulated group of genes in quiescent compared to proliferating human HSC were chemokine ligands, specifically within the CXC group (Graham et al., 2007). This was a novel finding, however their biological function in this context was unclear.

The previous microarray study reported that chemokine ligands *CXCL1*, *CXCL2*, *CXCL6* and *CXCL13* were the most up regulated genes in normal G_0 versus dividing HSC. As described in the introduction section, chemokines *CXCL1, CXCL2* and *CXCL6* share a common receptor, *CXCR2* and for simplicity we decided to focus on this signalling pathway. The literature available on these chemokines in terms of stem cell properties is scarce. Regarding CXCL1, a previous study reported that CXCL1 supported growth and regulated self renewal or adoption of a particular cellular fate in ESC (Krtolica et al., 2011). CXCL2 shares sequence similarity with CXCL1 and they share some functional activity, however CXCL2 has not been identified to play a role in ESC properties. CXCL2 has been shown to cause mobilisation of HSC from the BM into the periphery in animal studies (Pelus et al., 2002). The molecular mechanism is poorly understood with no data on expression of the chemokine on HSC, and it is not well defined which cell types are involved. CXCL6 was reported to be expressed in primitive ($CD34^+CD38^-$) BM derived HSC in another study, however there was no data to suggest its biological function in these cells (Lu et al., 2004). There is literature which showed CXCL6 was expressed on MSC, however in this context it was shown to play a role in angiogenesis (Kim et al., 2012).

Data obtained from a microarray study provides a global analysis of mRNA expression and alterations in different cell populations/treatments. However, it is important that the targets identified are validated before further study. The experiments in this chapter were designed to validate the microarray data and to extend the research through examining protein expression, in particular focusing on key chemokine ligand CXCL1. To examine the biological function *in vitro,* several approaches including an over expression and knock down approach of the protein were used. These techniques were first tested and optimised on cell lines before use on primary samples.

Chemokine ligands signal to their receptor and this can occur in an autocrine or paracrine fashion. To date, CXCR2 expression has not been detected on HSC. In terms of other stem cell systems, the receptor has been shown to be expressed on MSC and plays a role in cell migration (Ringe et al., 2007). A variety of cell types express CXCR2 including granulocytes and other inflammatory cell types which exist within the BM. Indeed an interaction between HSC and other cell types in the BM niche is already well known. Based on this, an aim of this chapter was to examine CXCR2 expression at the mRNA and protein level in HSC populations. Based on this research we would be able to identify whether chemokine ligands are potentially signalling through an autocrine loop or in a paracrine manner.

3.2 Aims and objectives

The specific aims of this chapter were:

I To validate gene expression data from a previous microarray study and investigate whether CXC ligands are up regulated in quiescent, primitive human HSC

It is fundamental that candidates identified from a microarray study are validated. In this objective we wanted to ensure we could validate microarray data which would merit further study of these genes.

II To examine protein expression of CXC ligands on HSC

It is well known that gene expression is not always indicative of protein expression or indeed function. In this objective we wanted to examine protein expression of CXC ligands to ensure protein was translated.

III To investigate whether HSC express receptor CXCR2

To date, the expression of receptor CXCR2 is not clear in the literature. We wanted to confirm whether human HSC express CXCR2 at the gene and protein level. This would give an indication of the mechanism of how chemokine ligands are eliciting their effects.

IV To determine the biological function of CXC signalling on HSC behaviour

We designed experiments to determine whether CXC signalling plays a biological role in terms of stem cell properties. To complete the objective the plan was to construct an over expression and knock down vector against chemokine ligands and use an inhibitor against receptor CXCR2. The resulting effect would be examined in terms of HSC properties including viability, self renewal/differentiation and cell cycle status.

3.3 Results

3.3.1 CXCL1, CXCL2 and CXCL6 are up regulated in primitive, BM derived HSC

Data from a published microarray reported differential expression of genes in human HSC populations that were sorted and isolated according to their cell cycle status (Graham et al., 2007). More specifically, $CD34^+$ cells were isolated using flow cytometry and a combination of DNA and RNA stains (Hoechst 33342 and Pyronin Y). Due to technicalities, including the lack of a UV laser to detect Hoechst staining, this approach could not be replicated. An alternative approach to sorting cells according to cell cycle status was required. Initially, alternative DNA stains were tested which can be used in viable cells and do not require a UV laser, however these were shown to be unreliable (data not shown). As an alternative approach, cell populations were sorted using cell surface markers CD34 and CD38. CD34 is a cell surface marker known to be expressed on a heterogeneous population of cells including primitive stem cells and progenitor cells and including CD38 allows a generally accepted discrimination between more primitive, and therefore more quiescent cells (Civin et al., 1996, Bhatia et al., 1997, Paz et al., 2007).

Cells used in the original microarray study were derived from normal controls in which the stem cells had been mobilised (Graham et al., 2007). It is possible that this treatment could alter the gene expression signature, in particular for chemokine expression. Indeed, CXCR2 binding ligands are modulated in response to G-CSF treatment (Richards et al., 2003, Eash et al., 2010). Therefore $CD34^+CD38^-$ and $CD34^+CD38^+$ cells were isolated from normal BM samples derived from the BM to examine whether high levels of chemokine expression is inherent to a primitive stem cell population regardless of cellular location and cytokine treatment.

Normal BM samples were enriched for cell surface marker CD34, stained with antibodies against CD34 and CD38 and sorted using flow cytometry into different cell populations according to their cell surface expression of CD34 and CD38. RNA was extracted, RT and examined for mRNA levels of CXC chemokines identified in the microarray; *CXCL1*, *CXCL2* and *CXCL6* using Q-PCR analysis. It can be seen in Figure 3-1 that all CXC ligands were down regulated in the $CD34^+CD38^+$ fraction in comparison to the $CD34^+CD38^-$ fraction at the mRNA level however *CXCL6* was not statistically significant. It is likely this has arisen from a smaller sample size and variation between individuals. From this result it can be inferred that CXC chemokines *CXCL1* and *CXCL2* are up

regulated in quiescent HSC derived from BM and mobilised PB samples as carried out in the original microarray study. It can also be inferred that sorting of $CD34^+$ cells into $CD34^+CD38^-$ and $CD34^+CD38^+$ fractions separates cells which are in different stages of the cell cycle as the results corroborate the findings from the original microarray. However, it should be noted that a huge variation between samples was noted in both populations as seen by relative expression. It is possible chemokine levels vary greatly between individuals and information regarding gender and age would have been useful, however this information was not available.

As a control to validate that the $CD34^+CD38^-$ fraction represents a more quiescent fraction than the $CD34^+CD38^+$ population, normal BM samples were examined for gene expression of genes associated with cell cycle status. *CDC6* is only transcribed during the G_1 phase of the cell cycle therefore it is predicated this will be at higher levels in $CD34^+CD38^+$ cells (Pelizon, 2003). In addition, gene expression analysis of cell surface marker CD38 was carried out to demonstrate the sorting efficiency. In one representative BM sample, *CDC6* and *CD38* mRNA levels were examined in $CD34^+CD38^-$ and $CD34^+CD38^+$ sorted populations. It can be seen from Figure 3-2 that both *CDC6* and *CD38* showed an increase in expression in the $CD34^+CD38^+$ fraction in comparison to the $CD34^+CD38^-$ fraction. The higher expression of CD38 in the $CD34^+CD38^+$ fraction confirms that the sorting was efficient. Differential expression of *CDC6* confirms that $CD34^+CD38^+$ are more proliferative than $CD34^+CD38^-$ cells, which justifies the use of these populations in this study. However due to one sample used, no significant differences are noted.

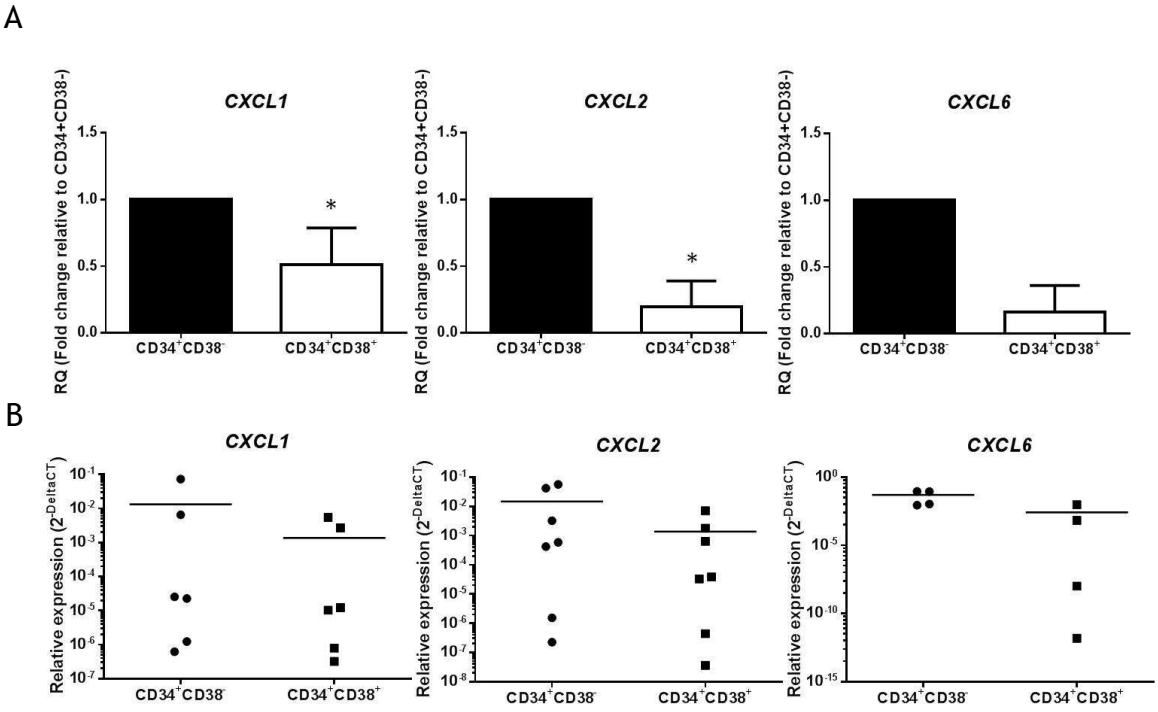

Figure 3-1 *CXCL1* and *CXCL2* are up regulated in CD34$^+$CD38$^-$ compared to CD34$^+$CD38$^+$ cells derived from normal BM samples.

Normal BM samples were freshly isolated or recovered from cryogenic storage. Cells were enriched for CD34 and recovered overnight in medium supplemented with GF for cell survival. Cells were stained for antibodies against CD34 and CD38 and sorted for CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ populations. RNA was extracted, RT and Q-PCR was carried out for *CXCL1*, *CXCL2* and *CXCL6* mRNA expression (A). Fold change was calculated relative to housekeeping control *GAPDH* according to the DeltaDeltaCT method. Data are presented as the mean fold change of expression in the CD34$^+$CD38$^+$ fraction using the CD34$^+$CD38$^-$ fraction as a calibrator which is set to the value of 1. The chemokines *CXCL1*¸*CXCL2* and *CXCL6* showed a mean 50 (P <0.05), 81 (P <0.05) and 84 (n.s) percent reduction in expression levels in the CD34$^+$CD38$^+$ fraction in comparison to the CD34$^+$CD38$^-$ fraction. The panels in B demonstrate relative expression ($2^{-DeltaCT}$) in both populations for each gene tested (B). Each dot displays an average of technical triplicates from independent samples. Statistical differences were analysed using the Wilcoxon matched paired test (n = 4-7) (* P <0.05). Details of age and gender from samples were not available.

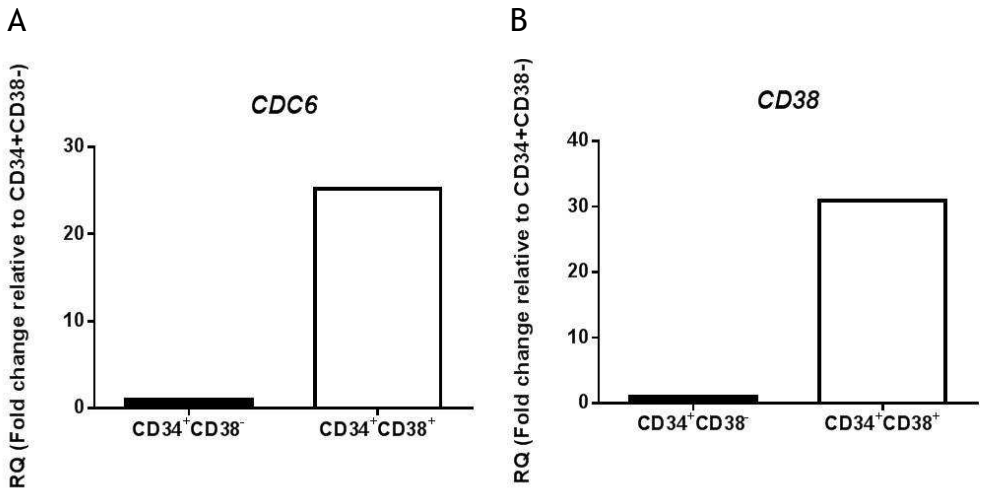

Figure 3-2 *CDC6* and *CD38* show up regulation in $CD34^+CD38^+$ compared to $CD34^+CD38^-$ cells derived from one normal, representative BM sample.

Normal BM samples were freshly isolated or recovered from cryogenic storage. Cells were enriched for CD34 and recovered overnight in medium supplemented with GF for cell survival. Cells were stained for antibodies against CD34 and CD38 and sorted for $CD34^+CD38^-$ and $CD34^+CD38^+$ populations. RNA was extracted, RT and Q-PCR was carried out for *CDC6* (A) and *CD38* (B) mRNA expression. Fold change was calculated relative to housekeeping control *GAPDH* according to the DeltaDeltaCT method. Data are presented as the mean fold change of expression in the $CD34^+CD38^+$ fraction using the $CD34^+CD38^-$ fraction as a calibrator which is set to the value of 1. Both *CDC6* and *CD38* showed an increase in expression in the $CD34^+CD38^+$ fraction with a 25.1 and 31.0 fold increase in expression levels in comparison to the $CD34^+CD38^-$ fraction. No statistical analysis was used due to the sample size of 1. Details of sample age and gender were not available.

3.3.2 CXCL1 is expressed in both $CD34^+CD38^-$ and $CD34^+CD38^+$ cells at the protein level

CXCL1 is up regulated in $CD34^+CD38^-$ cells in comparison to $CD34^+CD38^+$ cells at the mRNA level, however it is important to ensure gene expression is translated into protein expression. Examining protein expression for several ligands was thought to be potentially wasteful of primary material, time consuming and technically challenging due to the known quality issues associated with chemokine ligand antibodies. The experiments in the remaining study focus on chemokine ligand CXCL1 for several reasons. 1. CXCL1 was consistently found to be highly expressed in quiescent HSC in several microarrays from our group ((Graham et al., 2007); (Irvine *et al*., data unpublished)). 2. Previous research has shown that CXCL1 controls fundamental cellular processes including survival and proliferation (reviewed in the introduction section). Therefore this ligand seemed like an ideal candidate for further study.

CXCL1 was examined for protein expression on $CD34^+CD38^-$ and $CD34^+CD38^+$ cells. As previous results in this study found that *CXCL1* was up regulated in $CD34^+CD38^-$ cells regardless of cellular location, samples were derived from BM or mobilised PB samples depending on availability. As CXCL1 is a protein which is expressed intracellularly and secreted, intracellular flow cytometry analysis and enzyme-linked immunosorbent assays (ELISA) were thought to be ideal for examining CXCL1 protein expression. However, both techniques resulted in technical problems as antibody staining was found to be non specific and no signal was generated from the ELISA (data not shown). It was thought that this was due to a poor quality antibody against CXCL1 and due to the small cell numbers of primary cells available and used in the ELISA. As alternative approaches, immunofluorescence and western blotting were used to detect CXCL1 expression. Immunofluorescence allows the detection of intracellular protein expression and although not particularly quantitative, would show the presence or absence of signal. Western blotting allows validation of the immunofluorescence staining and provides a quantitative technique for examining different expression levels between populations. Antibodies against CXCL1 were optimised for both immunofluorescence and western blotting using positive control HT 1080 cell lines before use with precious primary samples.

It can be observed in Figure 3-3 and Figure 3-4 that CXCL1 was detected in HT 1080 cells by immunofluorescence staining and western blotting analysis. After optimisation of these techniques, human CD34 enriched samples were sorted for $CD34^+CD38^-$ and

$CD34^+CD38^+$ populations using CD34 and CD38 expression and examined for protein expression of CXCL1. Experiments showed that positive staining for CXCL1 was found in all populations tested. A representative figure of staining is seen for $CD34^+$ cells (Figure 3-3). As immunofluorescence is not quantitative, western blotting analysis showed that CXCL1 was detected in both $CD34^+CD38^-$ and $CD34^+CD38^+$ fractions in HSC derived from sources of mobilised PB and BM (Figure 3-4). Densitometry was used as a tool for quantification as the housekeeping protein expression showed unequal loading between samples. Densitometry analysis showed a 0.66 and 1.17 fold change for the $CD34^+CD38^+$ population in comparison to the $CD34^+CD38^-$ fraction for BM and PB respectively (n = 1). The small differences in expression likely represent no change and no conclusions can be drawn due to the sample size of n = 1.

Recombinant protein of human CXCL1 was tested against the antibody used for immunofluorescence and western blotting. Analysis showed multiple bands at ~8Kd, ~12Kd and ~16Kd (Figure 7-1). Primary samples showed multiple bands with strongest at 16Kd (Figure 7-2). Without sequencing methods it is difficult to confirm that the band found in cell extracts is CXCL1. It seems as though CXCL1 is expressed by human primary cells but perhaps a better antibody would clarify this. Using the recombinant antibody, the 8kD band is most likely the correct band for CXCL1 as it is the correct molecular weight for the CXCL1 protein. The higher band at 12kD is likely to be the unprocessed precursor protein which is larger and runs at a higher molecular weight. The band at 16kD could represent a dimer as chemokines frequently dimerise in SDS or the band could represent a non specific antibody band. Therefore the band visualised in cell extracts in this study could be CXCL1 protein which has dimerised or it could be a non specific band. Further experimental work is required to address this.

If the CXCL1 staining is correct, these results raise the question, why is an up regulation of CXCL1 observed in mRNA levels and not protein levels? The culture of HSC and progenitor cells with particular GF *in vitro* may play a role in modulating cell cycle status and therefore chemokine expression. If the addition of GF results in the activation of the quiescent fraction after a certain time period this would alter CXCL1 expression in the cells. A difference in expression would therefore not be observed in $CD34^+CD38^-$ cells in comparison to $CD34^+CD38^+$ cells. Alternatively, it is possible that CXCL1 is expressed on stem and progenitor cells and may play a biological role in both cell types. At this stage, this could not be concluded. However, collectively the combination of techniques provides

support that CXCL1 is expressed in HSC and progenitor populations and merited justification for further investigation into the role of CXCL1 in stem cell function.

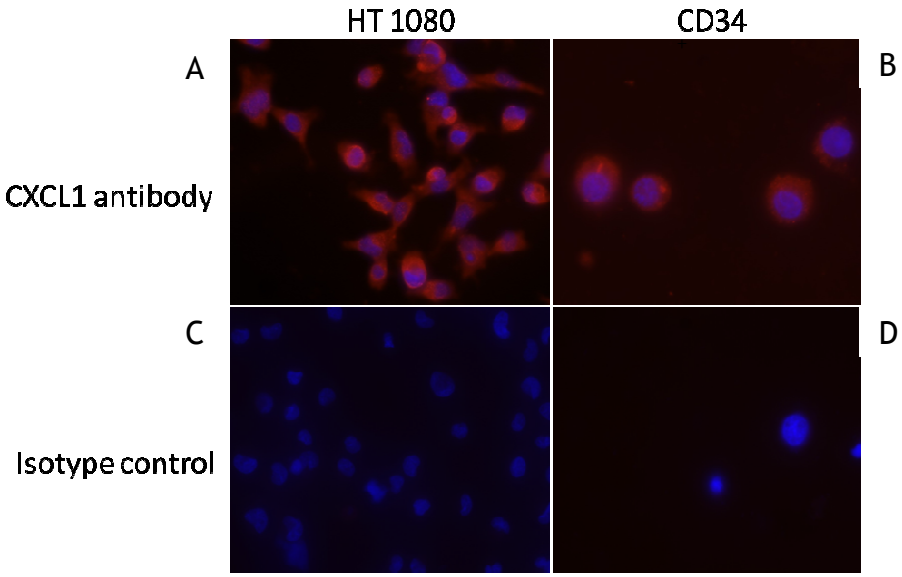

Figure 3-3 CXCL1 is expressed on HT 1080 cell lines and CD34$^+$ cells using immunofluorescence staining.

HT 1080 cell lines were cultured in medium, adhered to microscope slides and examined for CXCL1 expression using a fluorescently labelled antibody against CXCL1 and immunofluorescence analysis. Human CD34$^+$ cells were thawed, recovered overnight in medium supplemented with GF, sorted for CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ populations and subsequently cultured for 48 hours. Cells were then allowed to attach to slides and examined for CXCL1 expression. CXCL1 expression was visualised in red using an Alexa-Fluor-594 labelled antibody and the nuclei were visualised in blue using DNA stain dapi. Images display CXCL1 expression in HT1080 (A) and CD34$^+$ cells (B). Images were acquired using a Zeiss fluorescent microscope. Images shown are representative images from three independent experiments with appropriate isotype controls (C & D) used at the same concentration and exposure time as the primary antibody of interest. Patient samples used were of mixed age, gender and health status.

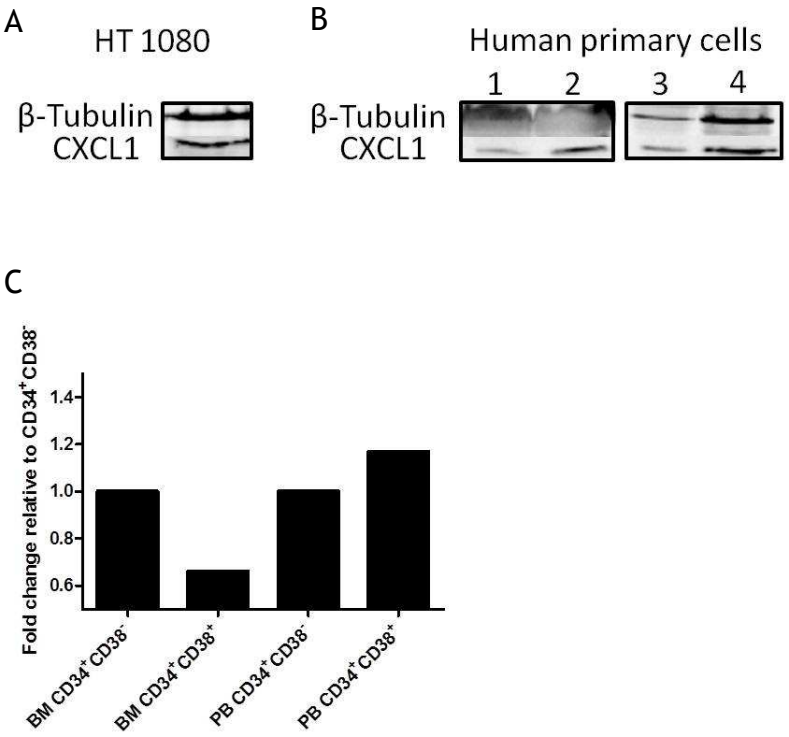

Figure 3-4 CXCL1 is expressed on HT 1080 and $CD34^+CD38^-$ and $CD34^+CD38^+$ cells using western blotting analysis.

HT 1080 cell lines were cultured in standard medium and protein lysates were prepared. Human $CD34^+$ cells were thawed, recovered overnight in medium and GF, sorted for $CD34^+CD38^-$ and $CD34^+CD38^+$ populations, cultured for 48 hours and protein lysates were prepared. Protein lysates were examined for CXCL1 protein expression by western blotting. A band was observed in HT 1080 cells (image taken from from Figure 3-7A), (*n* = 3) $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (A, B). Lanes 1-4 represent the following: 1. BM $CD34^+CD38^+$; 2. BM $CD34^+CD38^-$; 3. PB $CD34^+CD38^+$, 4. PB $CD34^+CD38^-$ (*n* = 1) (B). Housekeeping protein β-tubulin (~50kDa) was used as a loading control which was observed at the predicted molecular weight. Densitometry analysis was used to examine differential expression of CXCL1 between populations with each $CD34^+CD38^-$ population set to the value of 1 for comparison (C). A full image of the blot with molecular ladder can be seen in the supplementary Figure 7-2.

3.3.3 CXCR2 is expressed by human $CD34^+CD38^-$ and $CD34^+CD38^+$ cells

CXCL1 functions through binding to its receptor CXCR2 (reviewed in the introduction section). A lack of CXCR2 expression on HSC would suggest that CXCL1 is signalling in a paracrine fashion to other cell types, possibly in the BM niche where diverse cell types exist and can regulate HSC behaviour (reviewed in the introduction section). However, if the receptor is expressed by HSC, this would suggest that CXCL1 and potentially other ligands are signalling to the receptor in an autocrine loop. The expression of the CXCR2 receptor at the gene and protein level was therefore examined on human HSC and progenitor populations using analysis on $CD34^+CD38^-$ and $CD34^+CD38^+$ sorted populations.

Gene expression analysis showed that *CXCR2* is detected on human HSC and progenitor populations, however a marked variation in expression level was noted between samples. This was likely due to sample variability and an increased sample size was not possible in this study. Collectively the results showed a trend towards an up regulation in the $CD34^+CD38^+$ fraction in comparison to the $CD34^+CD38^-$ fraction with a 2.01 fold increase (n.s.) (n = 3) (Figure 3-6). This was not statistically significant and showed a high standard deviation therefore likely reflects no change in expression between populations. To examine protein expression of CXCR2, several techniques were used. Flow cytometry was initially tested with cell surface staining of the receptor which showed negative staining (data not shown). However, a previous study examining CXCR2 expression documented that flow cytometry with cell surface staining is not ideal for chemokine receptor expression. The receptor can become internalised and expressed in intracellular vesicles, therefore negative staining using flow cytometry cell surface analysis does not reflect a lack of expression (Acosta et al., 2008). Based on this research, immunofluorescence was used to examine CXCR2 staining on human HSC. Immunofluorescence staining using a CXCR2 antibody was optimised using human neutrophils as a positive control. It can be seen in Figure 3-6 that positive staining of CXCR2 can be clearly seen in human neutrophils with membranous and cytoplasmic staining as predicted.

Analysis using human HSC showed that $CD34^+CD38^-$ and $CD34^+CD38^+$ cells expressed the CXCR2 receptor at the protein level (Figure 3-6). The technique was not considered to be quantitative, but a more cell membranous staining pattern was observed in the $CD34^+CD38^-$ fraction and more intracellular staining was observed in the $CD34^+CD38^+$

fraction. 3-D images were generated to demonstrate CXCR2 staining patterns in the different cell populations. Studies using CXCR2 immunofluorescence expression have indicated that intracellular staining represents a more active protein (Acosta et al., 2008). However, this could reflect the difference in cell cycle status between the quiescent and the progenitor fraction. To validate this result, western blotting technique was used to examine CXCR2 expression in protein lysates, however preliminary experiments showed the antibody produced multiple strong bands and time constraints did not permit further study (data not shown).

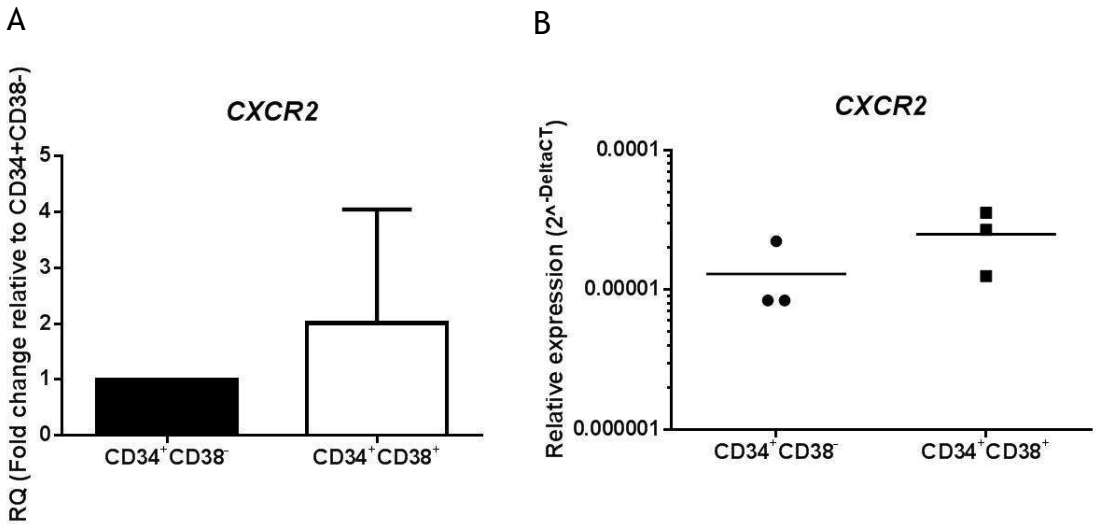

Figure 3-5 CXCR2 is expressed in human HSC $CD34^+CD38^-$ and progenitor $CD34^+CD38^+$ cells in BM samples at the mRNA level.

Normal BM samples were isolated fresh or thawed from cryogenically frozen. Cells were recovered overnight in medium supplemented with GF. Cells were sorted for $CD34^+CD38^-$ and $CD34^+CD38^+$ populations, RNA was extracted, RT and Q-PCR was carried out. Fold change in gene expression was calculated relative to housekeeping control *GAPDH* using the DeltaDeltaCT method. Data are presented as the mean fold change of expression in the $CD34^+CD38^+$ fraction using the $CD34^+CD38^-$ fraction as a calibrator which is set to the value of 1 (A). Panel B demonstrates the relative expression with each dot displaying the average from technical triplicates from three independent samples (B). Statistical analysis was performed using an unpaired student's *t* test with Welch's correction for unequal variance (n.s., $n = 3$). Details of sample age and gender were not available.

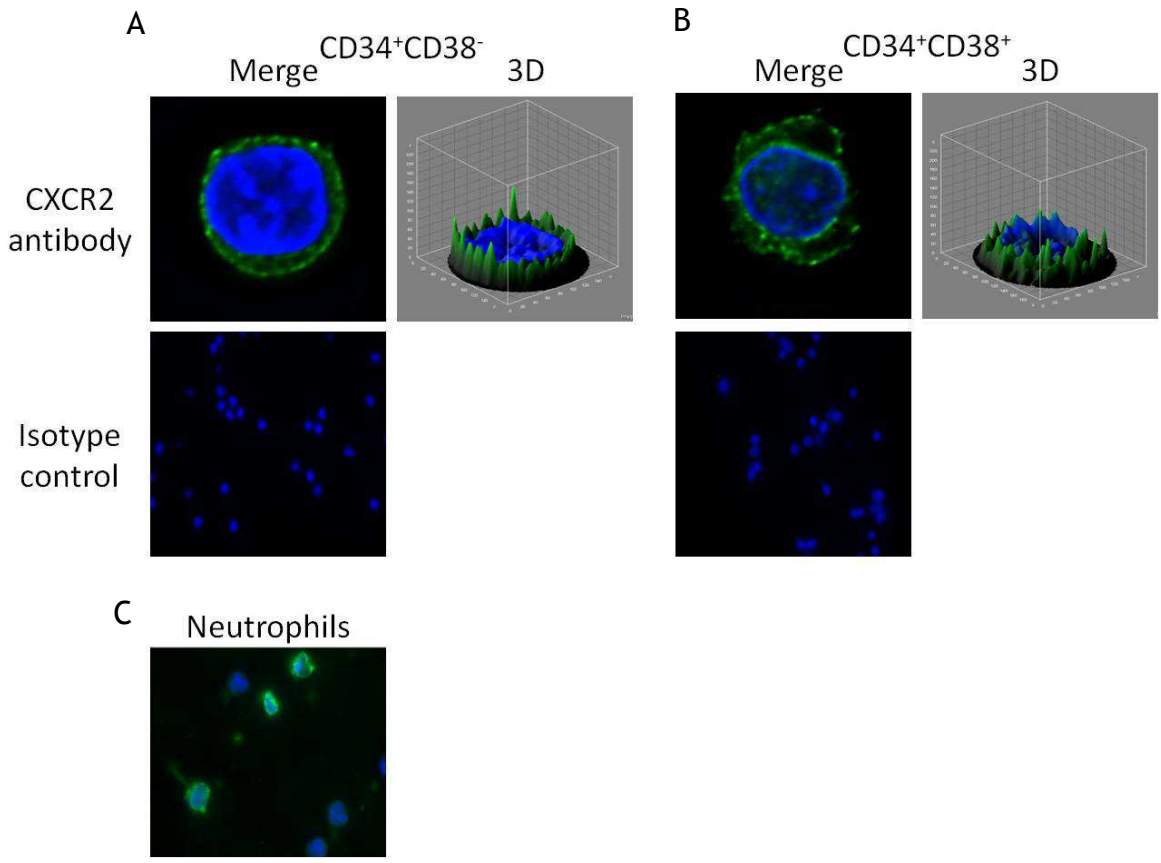

Figure 3-6 CXCR2 is expressed in human HSC $CD34^+CD38^-$ and $CD34^+CD38^+$ cells at the protein level using immunofluorescence staining.

Human PB neutrophils were isolated from a normal donor and used immediately after isolation. Human $CD34^+$ cells were thawed, recovered overnight in medium supplemented with GF, sorted for $CD34^+CD38^-$ and $CD34^+CD38^+$ populations and cultured for 48 hours. Cells were then adhered to slides and examined for CXCR2 expression. CXCR2 expression was visualised in green using an Alexa-Fluor-488 labelled antibody and the nuclei were visualised in blue using DNA stain dapi. Images display CXCR2 expression in $CD34^+CD38^-$ (A), $CD34^+CD38^+$ (B) and neutrophils (C). Images shown are representative images from three independent experiments with appropriate isotype controls used at the same concentration and exposure time as the primary antibody of interest. Patient samples used were of mixed age, gender and health status.

3.3.4 Modulation of CXCL1 in HT 1080 cell lines alters cell viability and proliferation

Experiments in sections 3.3.2 and 3.3.3 show both ligand CXCL1 and receptor CXCR2 are expressed on human $CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{+}$ cells. The next objective was to elucidate the biological significance of this signalling pathway. First, we aimed to modulate CXCL1 expression in cell lines to optimise the techniques and to examine the resulting phenotype. We designed experiments to knock down and over express CXCL1. Appropriate vectors were constructed and relevant techniques were optimised in HT 1080 cells.

To knock down expression of CXCL1, a lentiviral transduction approach with a shRNA vector was taken. A set of several vectors with different unique sequences against the CXCL1 protein was purchased and each vector was tested in HT 1080 cells. Several shRNA vectors against CXCL1 were shown to result in different levels of protein reduction using western blotting analysis which can be visualised in Figure 3-7. Densitometry showed the following fold changes of 1.0, 0.99, 0.75. 1.1, 0.84 and 0.86 for untransduced, sh1, sh2, sh3, sh4 and sh5 respectively in comparison to the Scr controlled which was set to the value of 1 (Figure 3-7). The two vectors corresponding to the greatest knock down of the CXCL1 protein were cloned into the same vector (pLKO.1), but with the presence of a GFP coding sequence. Both vectors were confirmed to significantly reduce gene expression levels of *CXCL1* in comparison to the control at the mRNA level (40% and 50% reduction in sh2 and sh5 in comparison to Scr control set to the value of 1, $P < 0.01$, $n = 3$) (Figure 3-7).

The effect of CXCL1 reduction in HT 1080 cells was examined by assessing proliferation and viability using cell counts. CXCL1 reduction (sh2 and sh5) in HT 1080 cells resulted in a decrease in cell counts in comparison to the control (Scr) after 48 hours in culture ($P < 0.05$, $n = 3$) (Figure 3-8). The cell counts obtained using sh1 and sh2 were below the density of the input cells ($10x10^{3}$) indicating a loss of cells in culture after CXCL1 reduction. This would infer cells were undergoing apoptosis in response to CXCL1 reduction. Apoptosis analysis using Annexin-V and dapi staining showed no differences in apoptosis ($n = 3$) (Figure 3-9). However a decrease in the percentage of GFP positive cells was observed with both hairpins in comparison to the control ($P < 0.01$ and $P < 0.001$ sh2 and sh5 respectively) ($n = 3$) (Figure 3-9).

Collectively, the data shows that CXCL1 reduction reduces cell proliferation in HT 1080 cells. As the reduction in cell counts was below that of the input, it was implicated that CXCL1 reduction also reduced cell survival. However, the apoptosis data suggested there was no/very little change in apoptosis in response to CXCL1 reduction. The apoptosis assay was carried out at a later time point than the cell counts and it is therefore possible that CXCL1 reduction did reduce cell viability and at the time of the apoptosis assay, these cells had disappeared from the culture. Indeed, analysis of the cell populations showed that there was a reduction in the percentage of GFP cells in response to CXCL1 reduction. Assuming that 100% of cells at the beginning of the assay are positive for GFP and a loss is found in response to CXCL1 reduction, this suggests positively transduced cells are being lost from the culture. It is predicted that this is due to apoptosis. To conclude this, apoptosis should be analysed at an earlier time point after transduction.

The data highlight that CXCL1 plays an important role in proliferation and survival in cell lines. To complement these experiments, an over expression vector (CXCL1-PRRL) was constructed to increase levels of CXCL1 and examine the resulting effect on HT 1080 cell properties. CXCL1-PRRL showed an increase in CXCL1 protein levels through mRNA (68.4 fold change, $P < 0.05$) ($n = 3$) and protein levels (1.4 fold change increase) ($n = 1$) (Figure 3-10). CXCL1-PRRL was found to increase cell counts after culture for 48 hours in comparison to the control ($P < 0.05$, $n = 3$) (Figure 3-11). Finally, apoptosis staining using Annexin-V and dapi staining showed an increase in the percentage of viable cells in the CXCL1-PRRL cells in comparison to the control ($P < 0.05$, $n = 3$) (Figure 3-12).

Taken together, the results show that over expression of CXCL1 in cell lines increases cell proliferation and cell viability. The results in combination with the knock down studies suggest that CXCL1 is a key factor involved in proliferation and survival in HT 1080 cells.

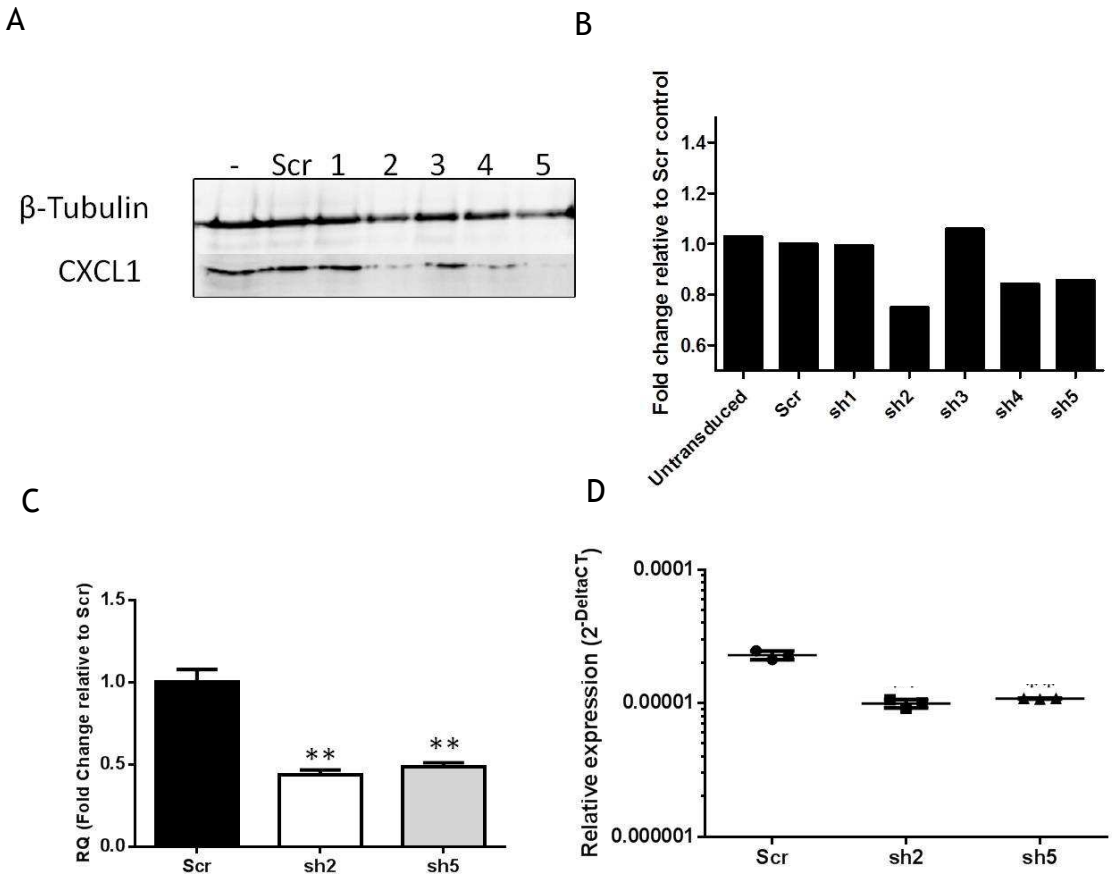

Figure 3-7 CXCL1 reduction using shRNA mediated lentiviral reduction reduces CXCL1 protein and mRNA levels in HT 1080 cell lines.

HT 1080 cells were lentivirally transduced with 5 plasmids (pLKO.1-CXCL1 or a pLKO.1-Scr). After selection in puro, protein lysates were examined for expression of CXCL1 or housekeeping β-tubulin (A). Blot represents representative image. Densitometry analysis shows the fold reduction of density intensity/mm^2 in response to CXCL1 reduction (B). A larger image of the blot with ladder can be seen in the supplementary Figure 7-3. The 2 vectors which corresponded to the best reduction in CXCL1 protein were cloned into a GFP plasmid and lentivirally transduced into HT 1080. GFP positive cells were sorted using flow cytometry, RNA was extracted, RT and analysed for *CXCL1* mRNA levels. Fold change in gene expression was calculated relative to housekeeping control *GAPDH* according to the DeltaDeltaCT method. Data are presented as the mean fold change of expression in the knock down cells using the control (Scr) as a calibrator which is set to the value of 1 (C). Data is also presented as relative expression ($2^{-DeltaCT}$) (D). A repeated measures one-way ANOVA was used with the Dunnetts's multiple comparison test to compare the control (Scr) with each hairpin (** P <0.01)

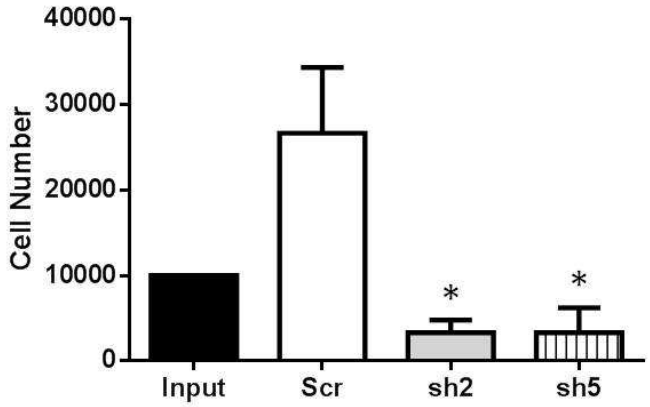

Figure 3-8 CXCL1 reduction decreases proliferation in HT 1080 cell lines.

HT 1080 cells were lentivirally transduced with 2 separate plasmids pLKO.1-CXCL1 (sh2 or sh5) or a pLKO.1-Scr (Scr). After lentiviral transduction, GFP positive cells were isolated and cultured for 48 hours and cells were counted using trypan blue exclusion method and total cell count per sample was calculated. Data are presented as the mean cell count obtained. The input represents the starting number of cells seeded for all conditions. Statistical analysis was performed using a repeated measures one-way ANOVA with Dunnett's multiple comparison test to measure differences between the control (Scr) and each hairpin (n = 3) (* P <0.05).

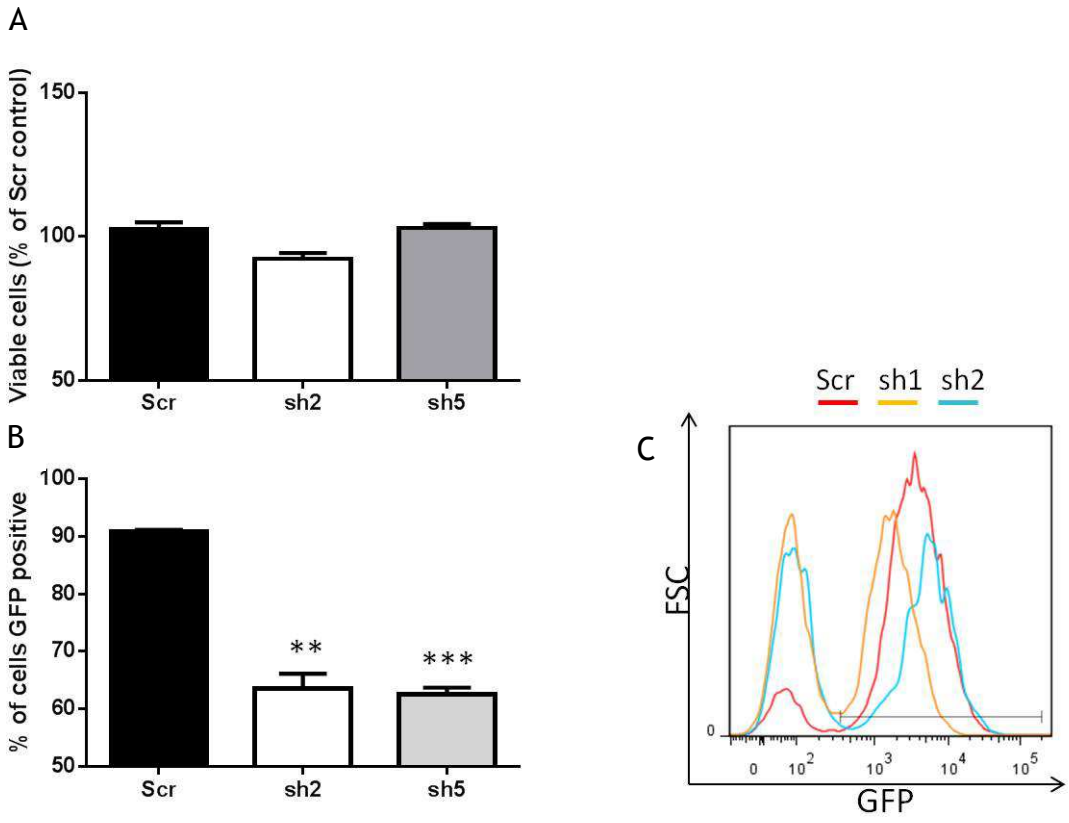

Figure 3-9 CXCL1 reduction reduces the percentage of GFP$^+$ cells in HT 1080 cell lines.

HT 1080 were lentivirally transduced with sh1, sh2 or a Scr control. After lentiviral transduction, GFP$^+$ cells were isolated and cultured for 96 hours and cells were examined for apoptosis using Annexin-V and dapi staining. Data are presented as the mean percentage of cells that were viable or dead (A). Cells were examined for percentage of GFP$^+$ cells using flow cytometry. Data are presented as the mean percentage of cells that were GFP$^+$ (B) after 96 hours in culture. Statistical analysis was performed using a repeated measures one-way ANOVA with Dunnett's multiple comparison test to assess differences between the control column with sh2 and sh5 (B). A statistically significant difference was found between the Scr group and both sh2 and sh5 (n = 3) (**P <0.01; *** P <0.001). A representative histogram of GFP$^+$ cells between conditions is shown (C).

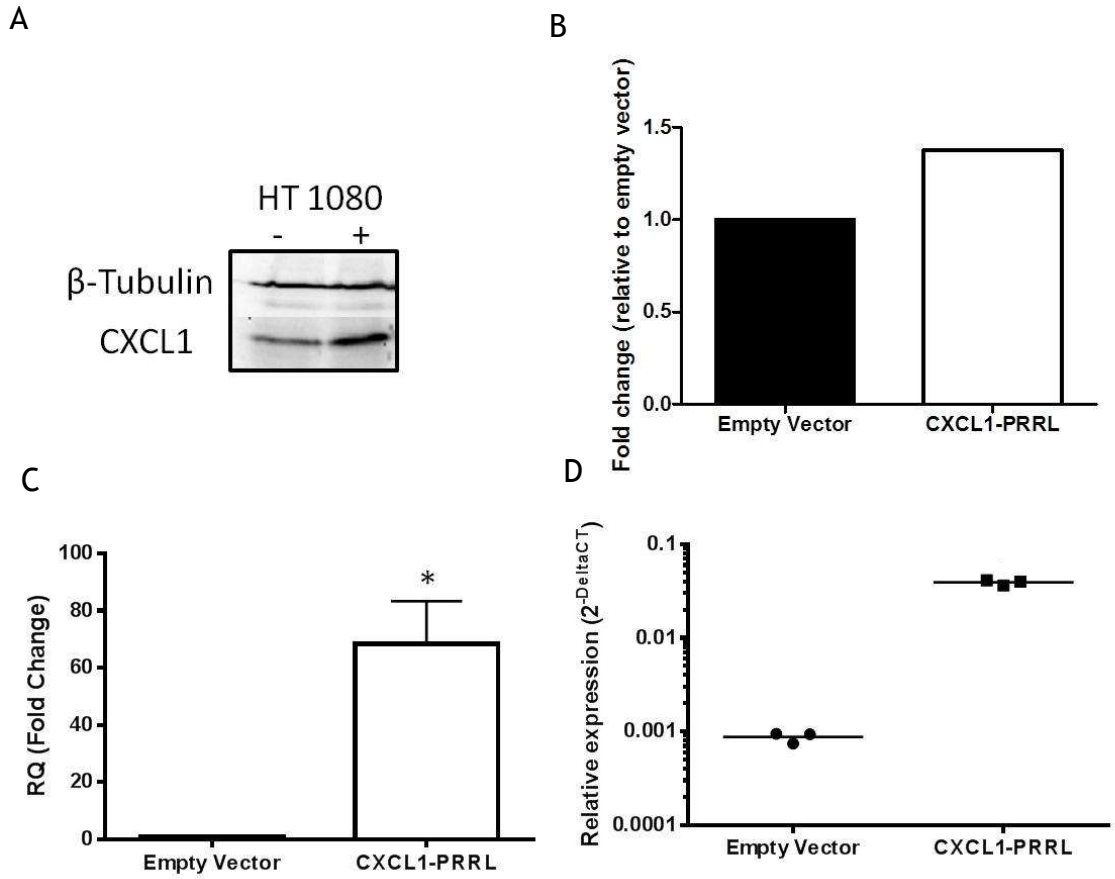

Figure 3-10 CXCL1 over expression vector CXCL1-PRRL increases CXCL1 expression by protein and mRNA analysis.

HT 1080 cells were lentivirally transduced with CXCL1-PRRL or a PRRL empty vector control. After transduction, cells were sorted for GFP^+ cells and protein lysates were made and RNA was extracted, RT and both were examined for expression of CXCL1 using western blotting or Q-PCR. Blot shows CXCL1 expression in empty vector (-) and CXCL1-PRRL transduced (+) cells using housekeeping protein β-tubulin as a loading control (A). Blot represents representative protein image and larger image of blot with molecular ladder is available in the supplementary Figure 7-4. Densitometry analysis demonstrates the fold change intensity/mm^2 in CXCL1-PRRL relative to the empty vector control (B). *CXCL1* gene expression was calculated relative to housekeeping control *GAPDH* using the DeltaDeltaCT method. Data are presented as mean fold change of expression in the CXCL1-PRRL cells using the empty vector cells as a calibrator which is set to the value of 1 (C). Relative expression ($2^{-DeltaCT}$) is shown with each dot displaying an average of technical triplicates in three independent experiments (D). Statistical analysis was performed using a two-tailed paired *t* test assuming equal variance ($n = 3$) (*$P < 0.05$).

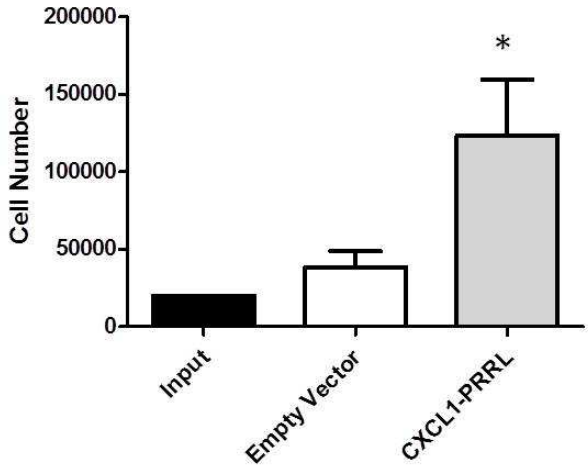

Figure 3-11 CXCL1 over expression increases proliferation in HT 1080 cell lines.

HT 1080 cells were lentivirally transduced with CXCL1-PRRL or a PRRL empty vector control. After transduction, cells were sorted for GFP^+ cells and cells were cultured for 48 hours in medium. Cells were counted using trypan blue exclusion method and total cell count per sample was calculated. Data are presented as the mean cell count. The input represents the starting number of cells seeded for both conditions. Statistical analysis was performed using a paired *t* test assuming equal variance comparing the empty vector control with the CXCL1-PRRL group ($n = 3$) ($^*P < 0.05$).

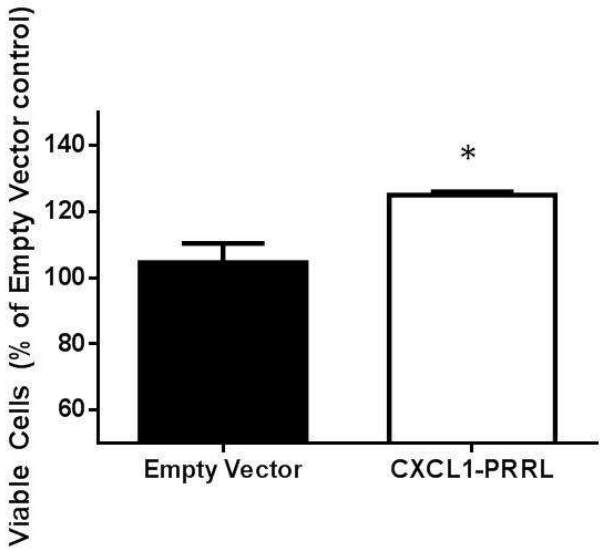

Figure 3-12 CXCL1 over expression increases cell viability in HT 1080 cell lines.

HT 1080 cells were lentivirally transduced with CXCL1-PRRL or a PRRL empty vector control. After transduction, cells were sorted for GFP positive cells and cells were cultured for 96 hours in medium. Cells were analysed for levels of apoptosis using Annexin-V and dapi staining. Data are presented as the mean percentage of viable cells. Statistical analysis was performed using a paired *t* test assuming equal variance comparing the empty vector control with the CXCL1-PRRL group ($n = 3$) ($^*P < 0.05$).

3.3.5 Reduction of CXCL1 in $CD34^+$ cells leads to a reduction in cell viability and colony formation capability

To determine if CXCL1 also affects survival and proliferation in human HSC and progenitor populations, a lentiviral transduction approach using shRNA was used. $CD34^+CD38^-$ and $CD34^+CD38^+$ cells were transduced with a vector encoding a CXCL1 shRNA (sh1) or a control (Scr).

To get an indication of differentiation and proliferation activity transduced cells were plated in a CFC assay. In this assay, CXCL1 reduction reduced colony numbers in both $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (Figure 3-13). The colonies grown in the CXCL1 knock down arm (~5% of the number obtained in the control arm) were smaller and less dense than those in the control arm (Figure 3-13). Due to limiting cell numbers after the lentiviral transduction, cells were available for flow cytometry analysis from the $CD34^+CD38^+$ fraction only. 72 hours post infection, $CD34^+CD38^+$ cells showed a reduction in viability in response to CXCL1 reduction as measured by Annexin-V and dapi staining (n = 1) (Figure 3-14). Due to the observed effects on $CD34^+CD38^-$ and $CD34^+CD38^+$ cells, the assay was repeated using unsorted $CD34^+$ cells. This was to increase the material available for assays as the sorting process loses a large amount of cells. The assays were repeated with the addition of a second shRNA vector with an alternative sequence against CXCL1 (sh5) to exclude the possibility of non-targeting effects of one single shRNA sequence. CXCL1 reduction using 2 vectors reduced the percentage of viable cells in comparison to the control in $CD34^+$ cells (Figure 3-15). In addition, CXCL1 reduction resulted in a reduction in the number of colonies obtained in comparison to the control (Figure 3-15).

Collectively, the data shows that CXCL1 reduction reduces colony formation and induces apoptosis in human stem/progenitor cells. Although this experiment has not been reproduced several times, it provides a good indication that CXCL1 plays a role in survival in human HSC. It is interesting that only a small percentage of cells underwent apoptosis in response to CXCL1 reduction, however a large reduction in colony numbers was observed in comparison to the control. The viability staining was carried out after 72 hours after transduction, whereas colony growth was examined approximately 10-14 days post transduction. A possible explanation is that apoptosis is induced over time and 72 hours is too early to detect dramatic changes in viability. Another possible explanation is that CXCL1 reduces cell viability and inhibits proliferation therefore a dramatic reduction in

colony formation is found. This is supported by the results in Figure 3-8 and Figure 3-11 in which proliferation is reduced and increased in HT 1080 cells in response to CXCL1 reduction and over expression respectively. It will be necessary to confirm that CXCL1 expression is reduced in $CD34^+$ cells in response to both shRNA vectors, however due to a lack of cells this was not possible in this study. It can be inferred from the experiments on HT 1080 cells that the vectors work to reduce CXCL1 expression. However, it will be important to confirm this in primary material.

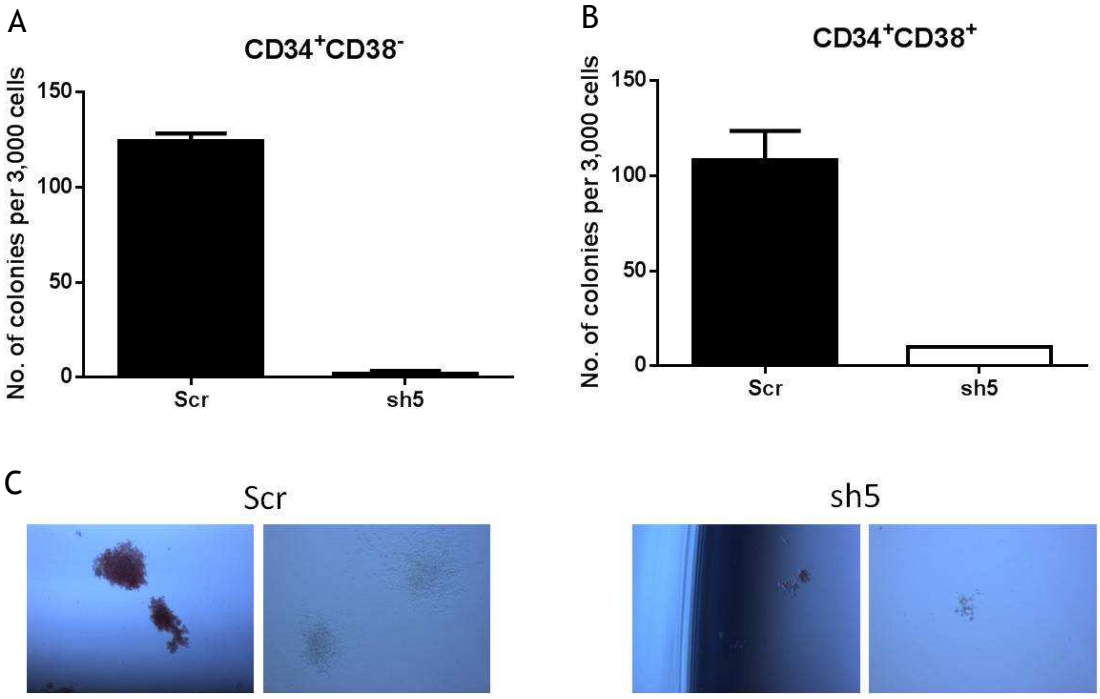

Figure 3-13 Reduction of CXCL1 reduces colony formation in human HSC $CD34^+CD38^+$ and $CD34^+CD38^-$ cells.

Human $CD34^+$ cells were recovered from frozen and cultured overnight in medium supplemented with GF. Cells were stained, sorted for $CD34^+CD38^-$ and $CD34^+CD38^+$ fractions and lentivirally transduced with viral supernatant for CXCL1 knock down (sh6) or with a control (Scr). 24 hours after the addition of fresh medium, cells were sorted for GFP^+ cells and 10^3 cells per mL of Methocult™ was plated in duplicate and cultured for 10-14 days and colonies were counted. Data are presented as the mean total number of colonies for Scr and sh6 arms in $CD34^+CD38^+$ (A) and $CD34^+CD38^-$ (B) fractions (n = 2). No statistical test was carried out due to the sample size of 2. Images are representative of colonies obtained in control or with CXCL1 knock down (C).

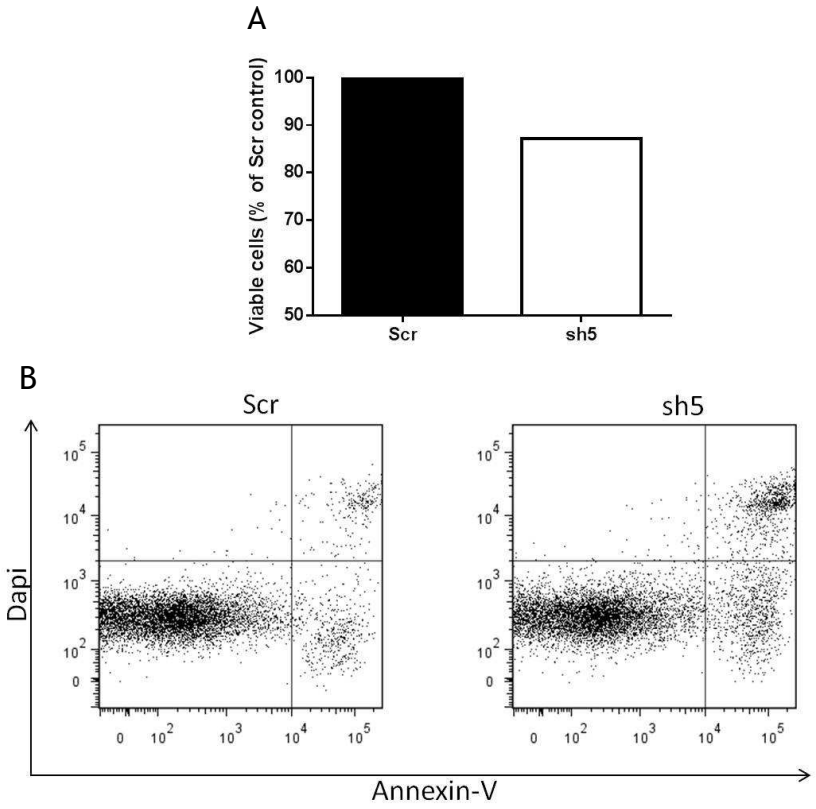

Figure 3-14 Cell viability of CD34^{+}CD38^{+} cells in response to CXCL1 reduction.

Human CD34^{+} cells were recovered from frozen and cultured overnight in medium supplemented with GF. Cells were stained and sorted for CD34^{+}CD38^{-} and CD34^{+}CD38^{+} fractions and lentivirally transduced with viral supernatant for CXCL1 knock down (sh5) or with a control (Scr). 24 hours after the addition of fresh medium, cells were sorted for GFP^{+} cells and cultured for 72 hours and analysed for Annexin-V and dapi staining. Data are presented as the percentage of cells that were viable or dead (with early and late apoptosis combined) for Scr and sh5 arms (A) (n = 1). Image shows Annexin-V and dapi staining in Scr and sh5 arms (B). No statistical test was carried out due to the sample size of 1.

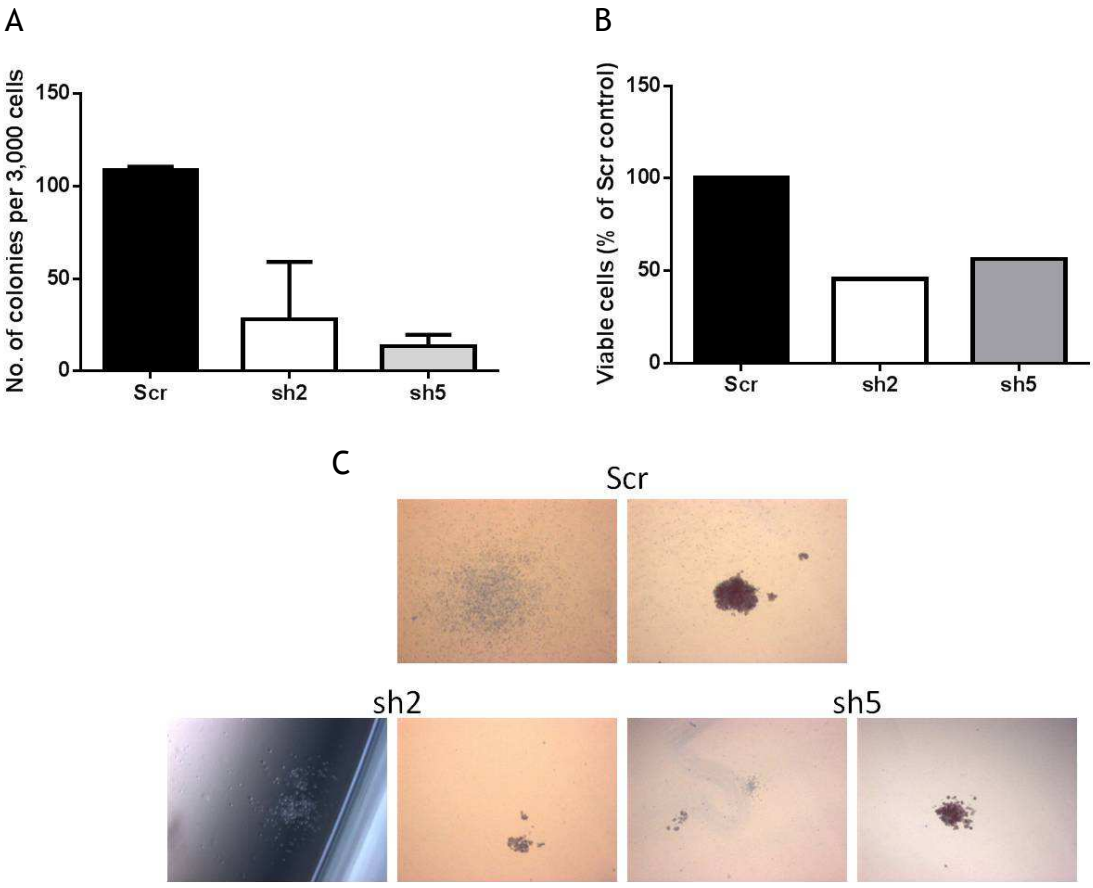

Figure 3-15 Cell viability and colony formation in response to reduction of CXCL1 in CD34$^+$ cells.

Human CD34$^+$ cells were recovered from frozen and cultured overnight in medium supplemented with GF. Cells were lentivirally transduced with viral supernatant for CXCL1 knock down using 2 separate vectors (sh2 and sh5) or with a control (Scr). 24 hours after the addition of fresh medium, cells were sorted for GFP$^+$ cells and 10^3 cells plated in duplicate in 1mL of Methocult™ and incubated for 10-14 days or cultured for 72 hours and analysed using Annexin-V and dapi for apoptosis staining. Data are presented as the total number of colonies obtained in Scr, sh2 and sh5 treatment arms (A) and as the percentage of cells that were viable or dead (with the combination of early and late apoptosis) for Scr, sh2 and sh5 arms (B). No statistical test was carried out due to the sample size of 1. Images are representative of colonies obtained in control or with CXCL1 knock down (C).

3.3.6 CXCL1 over expression in CD34$^+$ cells does not alter colony formation

To examine if over expression of CXCL1 enhances cell viability and colony formation in primary HSC and progenitor cells, cells were lentivirally transduced with a vector encoding the CXCL1 coding sequence (CXCL1-PRRL). Due to limitations in cell numbers, cells were lentivirally transduced and cultured in a colony formation assay only.

The results showed that there was no difference between CD34$^+$ cells transduced with an empty vector or with CXCL1-PRRL (n.s., $n = 3$) (Figure 3-16). There was no difference in the types of colonies between treatments therefore data are presented as the total number of colonies. To ensure that the vector was functioning correctly, gene expression analysis was examined and showed an increase in *CXCL1* levels in CD34$^+$ cells transduced with CXCL1-PRRL in comparison to the control ($P < 0.01$, $n = 3$).

It was hypothesised that an increase in colonies would be found in response to CXCL1 over expression. However the result found in Figure 3-16 does not support his hypothesis. Possible explanations include a saturation of CXCL1 levels in human primary cells or that a more in depth analysis including cell counts and apoptosis would show differences between conditions.

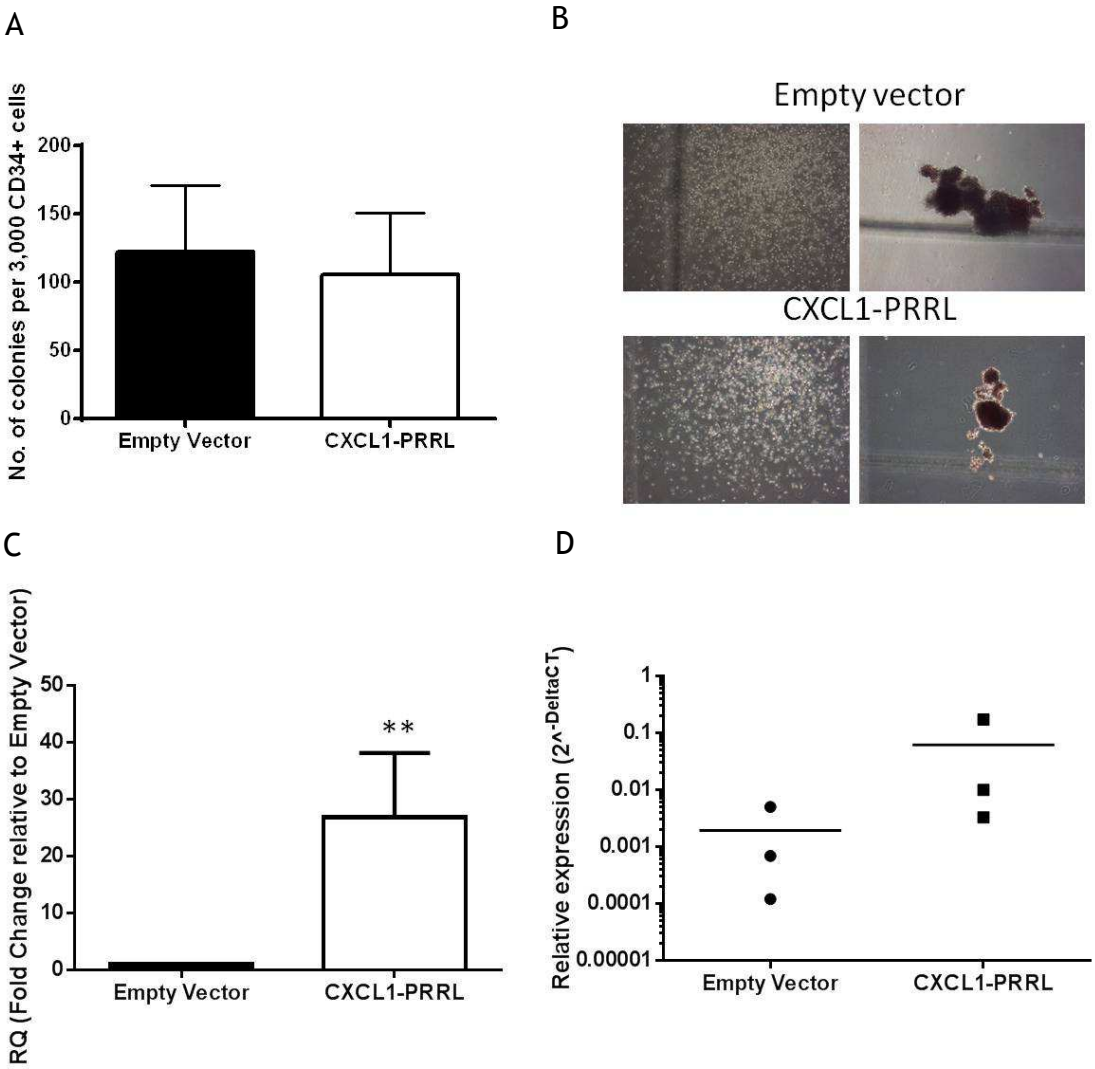

Figure 3-16 Over expression of CXCL1 does not affect colony numbers in CD34$^+$ cells.

Human CD34$^+$ cells were thawed and cultured overnight in medium and GF. Cells were lentivirally transduced with viral supernatant for CXCL1-PRRL or an empty vector control. 24 hours after the addition of fresh medium GFP positive cells were cultured and plated 1,000 cells per mL in Methocult™ and incubated for 10-14 days. Colonies were then counted. Data are presented as the total number of colonies with empty vector or CXCL1-PRRL arms (A). Images are representative of colonies obtained in control or with CXCL1 over expressionknock down (B). *CXCL1* gene expression was calculated relative to housekeeping control *GAPDH* using the DeltaDeltaCT method. Data are presented as mean fold change of expression in the CXCL1-PRRL cells using the empty vector cells as a calibrator which is set to the value of 1 in three independent samples in triplicate (C). Relative expression is displayed ($2^{-DeltaCT}$) with each dot displaying an average of technical triplicates from three independent experiments (D). Statistical analysis was performed using a paired *t* test ($n = 3$) (** $P < 0.01$). Patient samples used were of mixed age, gender and health status.

3.3.7 Recombinant CXCL1 treatment of CD34$^+$ cells does not alter cell viability or cell cycle status

To examine if human stem/progenitor cells can respond to CXCL1 signalling, CD34$^+$ cells were treated with recombinant CXCL1 (CXCL1) protein at a concentration typically used in the literature (100ng/mL). After 24 hours treatment with CXCL1 or an appropriate vehicle control, cells were examined for viability and cell cycle status. No difference was found in the viability (n.s., $n = 3$) or cell cycle status (n.s., $n = 3$) (Figure 3-17).

It is proposed that the exogenous treatment of CXCL1 would increase viability and proliferation of CD34$^+$ cells. A possible explanation is that the time point and concentration used are not optimal for this experiment. A more in depth analysis with longer time points and higher concentrations would allow a more definitive conclusion on whether human HSC or progenitor cells respond to CXCL1.

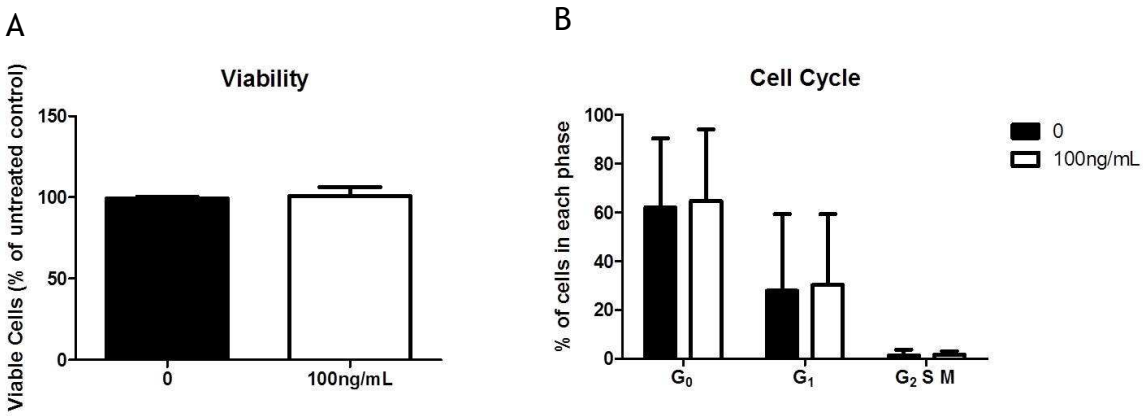

Figure 3-17 Treatment of CD34$^+$ cells with rCXCL1 does not alter cell viability or cell cycle status after 24 hours.

Human CD34$^+$ cells were thawed and cultured overnight in medium and GF. Cells were treated with 100ng/mL CXCL1 or with a vehicle control for 24 hours. Cells were then washed and analysed for viability using Annexin-V and dapi staining or fixed, permeabilised and stained for Ki-67 and dapi. Data are presented as percentage of viable cells (A) or percentage of cells in each phase of the cell cycle (B) for both conditions. A paired *t* test (A) and two-way repeated measures ANOVA with Sidak's multiple comparisons was used to examine significance between treatments (n.s., $n = 3$). Patient samples used were of mixed age, gender and health status.

3.3.8 CXCR2 inhibition on human $CD34^+$ cells using SB-225002 alters cell viability, cell cycle status and colony formation

Results in section 3.3.3 show CXCR2 is expressed on $CD34^+CD38^-$ and $CD34^+CD38^+$ cells by mRNA and protein analysis. We wanted to use an antagonist of CXCR2 signalling to complement the experiments using CXCL1 reduction. Compound SB-225002 was selected as it is an antagonist of CXCR2 signalling and has been shown to inhibit the binding of CXCL1 to CXCR2 (White et al., 1998). Due to the limiting availability of material and the observation that CXCR2 was expressed on both $CD34^+CD38^-$ and $CD34^+CD38^+$ fractions, the cells used in this study were unsorted $CD34^+$. Cells were treated for 72 hours with the compound at various concentrations ranging from 0.1µM up to 10µM and apoptosis, cell cycle and CFC assays were carried out.

With the use of inhibitors there is always concern that any effects observed may be due to off target non specific effects or general toxicity. SB-225002 is known to inhibit murine CXCR2 signalling (Bento et al., 2008). To examine the specificity of SB-225002 on CXCR2 inhibition, a stem/progenitor population (c-Kit^+) was isolated from WT or *Cxcr2*$^{-/-}$ mice and treated with various concentrations of SB-225002 *in vitro*. A decrease in the percentage of viable cells was found at 10µM in comparison to the untreated control in both WT and *Cxcr2*$^{-/-}$ cells (P <0.01) (n = 3) (Figure 3-18). It can be inferred from this result that 10µM is non specific as cells lacking *Cxcr2* respond to the compound. Alternatively, a possible explanation is that the compound does not function to inhibit *Cxcr2* in mouse cells and effects on viability are non specific which is not mediated through inhibition of CXCR2 signalling. Unfortunately the compound at lower concentrations does not affect the WT cells. This experiment cannot conclude whether *Cxcr2* inhibition using SB-225002 alters cell viability.

A reduction in the percentage of viable cells in comparison to a vehicle treated control was observed with 1µM (P <0.05) but not with 0.1µM (n.s.) (n = 3) (Figure 3-19). Cell cycle analysis was examined in concentrations from 0.1 to 1µM. An increase in the percentage of cells in G_0 phase of cell cycle (P <0.05) and a decrease in percentage of cells in G_1 phase of cell cycle was found (P <0.05) (n = 3) (Figure 3-20) in the 1µM treatment arm. There were no significant differences found with the 0.1µM treatment arm however a trend towards a decrease in G_0 and an increase in G_2, S and M phases was reported. However, the increase in G_0 cells with 1µM could also be due to the effects on viability and this should be further investigated.

Colony formation assays showed a trend towards a decrease in colony numbers in a primary plating assay with treatment with 1µM (*n* = 2) (Figure 3-21). However a sample size of 2 does not allow statistical analysis. Cells were harvested from the assay, counted and replated at equal numbers in Methocult™. The differentiation and proliferation potential in a secondary replating assay is thought to be indicative of self renewal activity. In a secondary colony formation assay, a trend towards a reduction in the number of colonies was found with both 0.1 and 1µM treatments (Figure 3-21) (*n* = 2). Again, sample size did not permit statistical analysis. Unfortunately, conclusions cannot be drawn, however some evidence is provided to suggest that CXCR2 signalling regulates survival in human HSC.

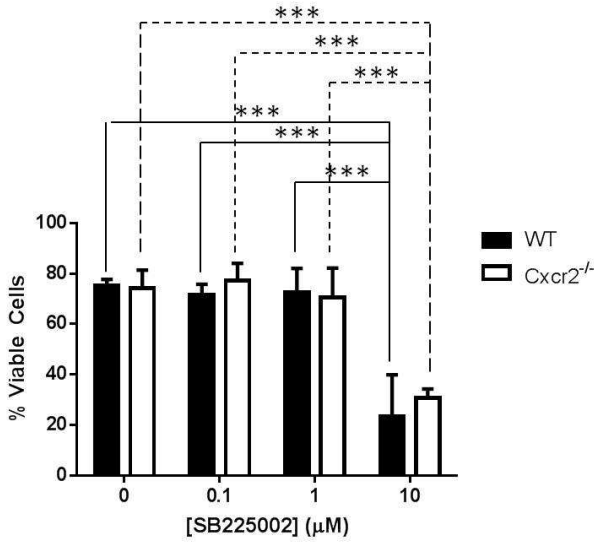

Figure 3-18 CXCR2 inhibition using SB-225002 decreases cell viability in c-Kit enriched cells derived from WT and *Cxcr2*$^{-/-}$ animals.

To examine the specificity of SB-225002 on CXCR2 inhibition, c-Kit^{+} cells were enriched from WT or *Cxcr2*$^{-/-}$ animals. Cells were cultured with GF for 72 hours in the presence of various concentrations of SB-225002 or a vehicle control. Cells were then analysed for apoptosis using Annexin-V and dapi staining. Data are presented as the mean percentage of viable cells in response to treatment. Animals were between 8 and 12 weeks and male. Statistical analysis was performed using a repeated measure's two-way ANOVA with the Sidak's multiple comparison test to compare the treatments within each group and for each treatment between groups (*** P <0.001, n = 3).

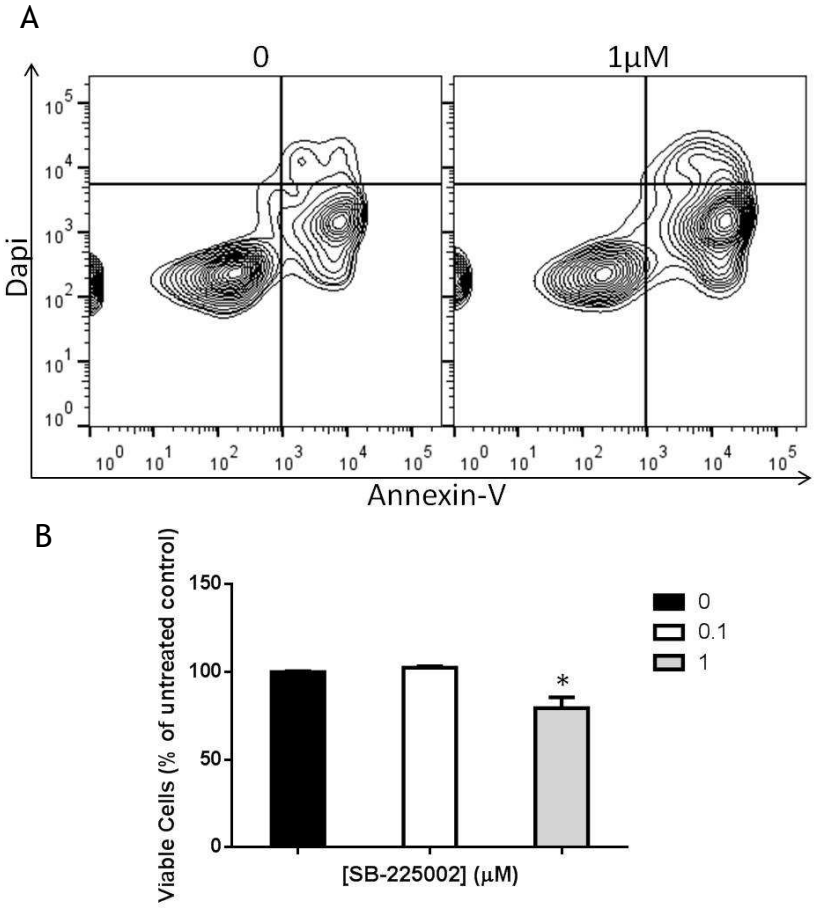

Figure 3-19 CXCR2 inhibition using SB-225002 reduces cell viability in CD34$^+$ cells *in vitro.*

CD34$^+$ cells derived from mobilised PB were thawed and recovered overnight in medium supplemented with GF. Cells were cultured for 72 hours with medium and GF in the presence of the desired concentration of inhibitor or the appropriate vehicle control. Cells were washed and stained for Annexin-V and dapi and analysed. Data are presented as the mean percentage of viable cells relative to the vehicle treated control (set to 100%) (B) with representative dot plots for Annexin-V and dapi staining with vehicle treated and 1μM treatment arms (A). Statistical analysis was performed using a one-way repeated measure ANOVA with Tukey's multiple comparisons test to compare differences between untreated and each treatment (n = 3) (n.s., * P <0.05). Patient samples used were of mixed age, gender and health status.

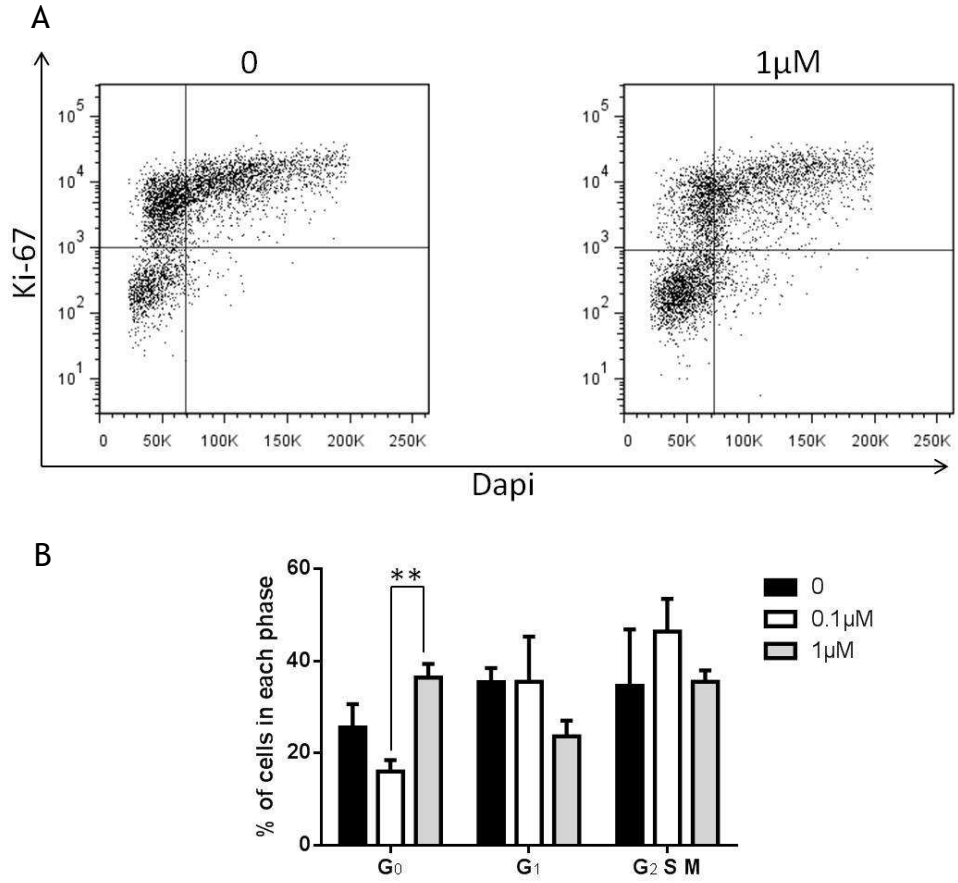

Figure 3-20 CXCR2 inhibition using SB-225002 on CD34$^+$ cells alters cell cycle status.

CD34$^+$ cells derived from mobilised PB were thawed and recovered overnight in medium supplemented with GF. Cells were cultured for 72 hours in medium and GF in the presence of the desired concentration of inhibitor or appropriate vehicle control. Cells were washed, fixed, permeabilised and stained for Ki-67 and dapi and analysed. Data are presented as the mean percentage of cells in each phase of the cell cycle; G_0, G_1 or G_2, S and M (B) with representative dot plots for Ki-67 and dapi staining (A). Statistical analysis was performed using a repeated measures two-way ANOVA with Tukey's multiple comparison test to assess differences between treatments (n = 3) (**P <0.01). Patient samples used were of mixed age, gender and health status.

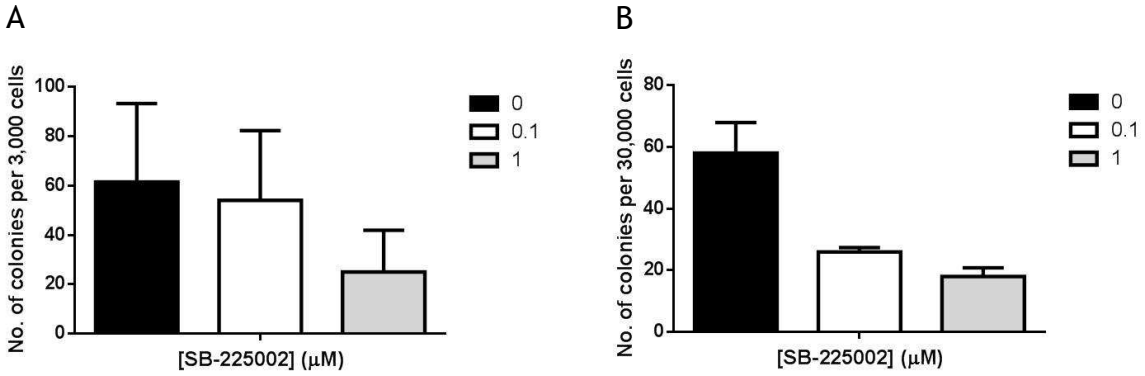

Figure 3-21 CXCR2 inhibition using SB-225002 decreases colony formation in primary and secondary colony assays in CD34$^+$ cells *in vitro.*

CD34$^+$ cells derived from mobilised PB were thawed and recovered overnight in medium supplemented with GF. 1,000 CD34+ cells were plated per mL of methylcellulose™ with the addition of the desired concentration of inhibitor or appropriate vehicle control. The mix was vortexed and plated in duplicate and incubated for 10-14 days. After this time period, colonies were counted, cells were resuspended, counted and replated at 10,000 cells per mL of methycellulose™ with the addition of fresh inhibitor. All cells were replated and no differences were noted in colony type between conditions therefore data is presented as the mean total number of colonies after culture in primary (A) and secondary colony formation assay (B). No statistical significance tests were carried out due to the sample size of 2. Patient samples used were of mixed age, gender and health status.

3.4 Discussion

Published microarray data from our laboratory reported than *CXCL1*, *CXCL2* and *CXCL6* were up regulated in quiescent, human HSC populations although *CXCL6* did not reach significance (Graham et al., 2007). Significantly differentially expressed candidates from microarray studies offer important discoveries. However, it is understood that these candidates should be experimentally validated as in particular, microarray expression data can contain a high level of noise. In addition, although gene expression differences can be validated, there are cases where the protein is not translated or indeed not functional. Considering these points, the first objectives in this study was to validate this gene expression data from the previous microarray study and extend the research to investigate the biological function.

In this study, CD34 and CD38 cell surface markers were used to isolate populations taking the assumption that cells in these populations are in different states of the cell cycle with the $CD34^{+}CD38^{-}$ population existing as more quiescent than the $CD34^{+}CD38^{+}$ population. The results obtained in this chapter showed that chemokines were up regulated in the quiescent fraction using CD34 and CD38 cell surface markers. In addition, the differential expression of *CD38* and *CDC6* in sorted samples provided conclusive evidence that the sorted populations showed differential expression of *CD38* therefore the sort was efficient and *CDC6* therefore the cells were in differential status of the cell cycle. It can be argued that the $CD34^{+}CD38^{+}$ fraction is more of a progenitor population, therefore experiments should compare chemokine gene expression of chemokines in different populations within the stem $CD34^{+}CD38^{-}$ fraction to get a more in depth analysis of a stem cell population. Ideally, a more enriched HSC population should have been used in this study, however this would have reduced the material obtained and therefore the ability to perform techniques accurately.

The microarray study did not implicate the receptor CXCR2 as being differentially expressed between quiescent and proliferating human HSC populations and it remains unclear from the available literature whether this particular receptor is expressed on any subsets of HSC. One study stated that CXCR2 was not expressed by human stem or progenitor populations ($CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{+}$), including cells isolated from adult BM or mobilised PB (Rosu-Myles et al., 2000). However, the technique used in this study to analyse protein expression was flow cytometry for cell surface staining only. In

the results in this chapter, it can be clearly seen that CXCR2 is expressed on human HSC and progenitor populations, both at the mRNA and protein level by immunofluorescence. One previous study showed positive staining of CXCR2 on $CD34^+$ cells isolated from PB from normal controls, however this was variable between samples (Emadi et al., 2005). The results here are completely novel with the only chemokine receptor known to be expressed by HSC or progenitor cells is CXCR4 which is involved in cell survival and mobilisation (reviewed in the introduction section). However, to fortify the results in this chapter another method can be used to examine CXCR2 expression.

Experiments in this chapter show that CXCR2 inhibition using SB-225002 on $CD34^+$ cells resulted in a decrease in cell viability, cell cycle status and colony formation. This implicates that CXCR2 may be controlling these stem cell properties. In the literature, the compound has been tested *in vitro* and *in vivo* to show it can be used to prevent CXCR2 ligand CXCL1 and CXCL8 induced neutrophil chemotaxis at similar concentrations (Catusse et al., 2003, Lane et al., 2001, White et al., 1998). However, due to the nature of compounds it could not be conclusively concluded that SB-225002 mediated CXCR2 inhibition is responsible for the cellular effects. The literature also suggests that higher concentrations of the compound can inhibit CXCR1 signalling. It would therefore be useful to examine whether human HSC express CXCR1 as this is unclear in the literature. One study showed that human $CD34^+$ cells derived from the cord blood (CB) of normal donors expressed high levels of CXCR1, however this should be validated (Rosu-Myles et al., 2000). However, as both CXCR2 inhibition and CXCL1 reduction showed a reduction in cell viability and colony formation, collectively this provided evidence that CXCL1-CXCR2 signallin may play a role in HSC survival and maintenance but these experiments should be repeated to allow significance to be assessed.

There is little literature available on the role of CXCR1 or CXCR2 on stem cell properties. A study on CB cells showed that inhibition against CXCR1 and CXCR2 decreased the percentage of stem cells, defined by CD133 positive staining, suggesting these receptors are fundamental to cell survival (Khalaf et al., 2010). A study on myeloid metaplasia with myelofibrosis showed that inhibition of CXCR1 and CXCR2 on $CD34^+$ cells resulted in an increase in proliferation and skewed differentiation, however this was in a disease setting and not in normal haemopoiesis (Emadi et al., 2005). The predominant role of CXCR2 signalling is in directed cell movement. CXCR2 expressing cells including granulocytes are directed towards different sites due to ligand expression. The studies in this chapter did

not examine cell migration, however it will be interesting to examine whether CXCR2 expressing human HSC migrate in response to ligand stimulation.

The results show that *CXCL1* is expressed on $CD34^{+}CD38^{-}$ cells at the mRNA level. Protein analysis through several techniques provided opposite results. Flow cytometry and ELISA showed negative expression, while immunofluorescence and western blotting showed positive expression. It is possible that technical reasons are responsible for a lack of signal with flow cytometry and ELISA. However, this can not be concluded. Further experiments are required to firmly conclude the expression of CXCL1 on human HSC.

Results showed CXCL1 reduction in both cell lines (HT 1080) and primary $CD34^{+}$ cells reduced cell survival and proliferation. Previous studies have shown that CXCL1 knock down decreases cell viability in melanoma cell lines (Botton et al., 2011). Furthermore, CXCL1 knock down and over expression have been shown to decrease and increase cell proliferation in epithelial ovarian cancer cell lines (Bolitho et al., 2010). CXCL1 reduction has also been shown to reduce cell proliferation in other cell types, including oligodendrocyte progenitors (Zhou et al., 2005). Collectively, the role of CXCL1-CXCR2 signalling in survival and proliferation is not novel, however this has not been shown on primary HSC. To conclude this chapter, the results provide some evidence that CXCL1-CXCR2 is a novel signalling pathway that may play a role in stem cell survival and proliferation however repetition of experiments are required to conclude this hypothesis.

4 Results II: Analysis of haemopoieisis and stem cell activity in *Cxcr2*$^{-/-}$ mice

4.1 Introduction

Results from chapter 3 provide evidence that the *CXCR2* signalling pathway may support human HSC and progenitor survival *in vitro.* However, the haemopoietic structure is incredibly complex with HSC interacting with various other cell types in the BM niche. Consequently, HSC are influenced by external cues as well as internal cues (reviewed in the introduction section). With a particular focus on *Cxcr2* signalling, there is evidence in the literature that *Cxcr2* binding ligands play a role in HSC mobilisation (Pelus et al., 2002). However the role of *Cxcr2* signalling in HSC behaviour is largely unstudied and not well understood. Gene-targeting studies have been used to identify key roles of signalling pathways in biological processes and a mouse model in which mouse *Cxcr2* is excised, is available for study. In addition, the mouse system provides the advantages of more material for in depth experiments including the use of *in vivo* assays.

In this chapter, we wanted to extend the research in the first part of this study and examine how *Cxcr2* signalling controls stem cell properties *in vivo* using a mouse model which lacks *Cxcr2* (*Cxcr2*$^{-/-}$). This model has been generated and animals are viable and live to adulthood. The mice have previously been characterised and animals lacking *Cxcr2* display an increase in the numbers of mature myeloid cells and progenitors in the haemopoietic organs including the BM, spleen and PB. This phenotype has been shown to be environment dependent (Cacalano et al., 1994, Broxmeyer et al., 1996). It is proposed from previous literature that *Cxcr2* negatively regulates myeloid cell production and this regulation is mediated through *Cxcr2* binding ligands. However, whether mouse HSC express *Cxcr2* and whether this signalling plays a role in the function of the stem cells has not been assessed to date.

The results in this chapter were designed to investigate whether HSC and progenitor populations derived from *Cxcr2*$^{-/-}$ animals differ in terms of their frequency and function in comparison to WT controls. Data from the human experiments suggests that CXCR2 is controlling cell survival. Therefore the prediction is that in a *Cxcr2*$^{-/-}$ mouse model, the HSC will show loss of function in terms of stem cell activity.

4.2 Aims and Objectives

The specific aims of this chapter were:

I To examine *Cxcr2* expression on mouse HSC

The results in chapter 3 show that human HSC show positive expression of CXCR2. However whether mouse HSC express *Cxcr2* is not known to date.

II To investigate how a lack of *Cxcr2* alters the frequency of mature cells in the haemopoietic organs

This objective was to examine how *Cxcr2* signalling affects the haemopoietic system. Previously published data shows differences in the frequency of mature haemopoietic cells in *Cxcr2*$^{-/-}$ animals. However this has been shown to be dependent on the environment the animals are housed in. We wanted to examine the phenotype of the mice used in this study.

III To investigate whether a lack of *Cxcr2* alters HSC and progenitor populations in terms of their frequency, properties and function

This objective was to understand whether a lack of *Cxcr2* signalling affects the number and function of HSC/progenitor populations.

4.3 Results

4.3.1 CXCR2 is expressed on mouse HSC

Data from chapter 3 showed human HSC populations express CXCR2 and an autocrine signalling loop with ligand CXCL1 is involved in stem cell viability and proliferation. It is unclear in the literature whether mouse HSC populations express the receptor. One study identified positive expression in lineage negative, $CD45^+$ cells derived from mouse BM (Yoon et al., 2012). However, this population represents a mix of stem and progenitor cells. A similar study examined a more primitive population (LSK) for *Cxcr2* expression. Variation was found between samples with both positive and negative expression reported (Wright et al., 2002). It is therefore unclear in the literature whether *Cxcr2* is expressed on mouse HSC. Furthermore, recent research has identified cell surface markers expressed by mouse HSC and more primitive populations can be isolated and studied. The first objective in this chapter was to examine *Cxcr2* expression on mouse HSC. Due to the small numbers of stem cells available from mouse HSC populations (including the most primitive subset), single cell Q-PCR was used to assess expression of *Cxcr2* on sorted populations. Mouse BM was harvested, stained for a cocktail of antibodies against stem cell markers and sorted for LSK in addition to $CD150^+CD48^-$ (LT-HSC), $CD150^-CD48^-$ (ST-HSC), $CD150^+CD48^+$ and $CD150^-CD48^+$ (MPP) populations and examined for *Cxcr2* expression. The terminology used in this thesis follows previous research (Khandanpour et al., 2010, Kiel et al., 2005). The experiments in section 4.3.1 were carried out in collaboration with Dr Amelie Guitart.

Results showed *Cxcr2* was expressed at the mRNA level on all HSC populations examined with no trend towards differences in expression between populations (Figure 4-1). Relative expression was used to compare expression with a stem cell gene *p21* (Cheng et al., 2000). This is the first study to show that *Cxcr2* is expressed on mouse HSC populations including the most primitive LT-HSC. The lack of difference in expression between stem and progenitor populations suggests that signalling is important for stem and progenitor function. Alternatively, it is possible that CXCR2 is differentially expressed at the protein level in these populations. However, protein expression of CXCR2 was not examined due to time constraints in this study and the low cell numbers that are available from mouse HSC and progenitor populations. Protein expression analyses will be useful to confirm the protein is translated.

To determine whether an autocrine loop of *Cxcr2* signalling exists on mouse HSC, mouse homologs of human *Cxcr2* binding ligands *Cxcl1, Cxcl2* and *Cxcl5/6* (as highlighted from the original microarray) were examined for gene expression using the methodology described above. Results showed that the ligands tested were not detected on any of the populations using single cell Q-PCR (data not shown). It can be interpreted from these results that either a *Cxcr2* autocrine signalling loop is not present on mouse HSC, or alternatively, other CXC ligands are expressed which were not assessed in this particular experiment. Alternatively, it is possible that *Cxcr2* expressing HSC signal to other cell types in the BM niche in a paracrine manner. To support this, previous studies have shown that CXCR2 expression is detected on BM derived lineage negative cells which respond to *Cxcl5* expressed by EC (Yoon et al., 2012). Therefore, there is literature to support the result that CXCR2 is expressed on stem/progenitor populations and they can respond to ligand expression from other cell types present in the BM niche.

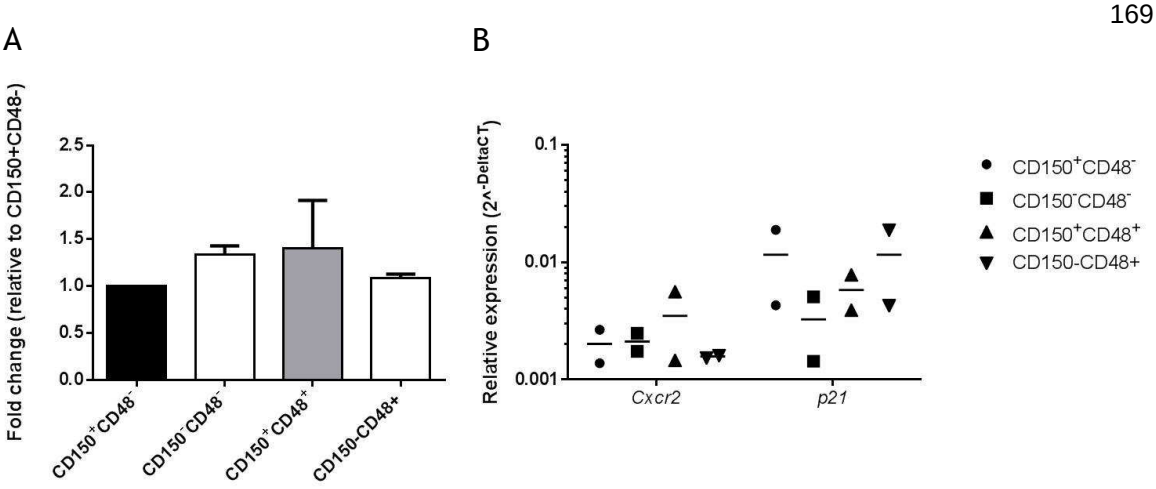

Figure 4-1 Mouse HSC populations express *Cxcr2* at the mRNA level.

Normal BM was freshly isolated and stained for antibodies against LSK, CD150 and CD48. 500 cells were sorted, RT and Q-PCR was carried out for *Cxcr2* mRNA expression. Fold change was calculated relative to housekeeping control *β2M* (A). Data is also displayed as the relative expression ($2^{-DeltaCT}$) (B). Results show the mean of technical triplicates from two independent experiments using two separate WT animals, both male at age 6-12 weeks (n = 2) (A). Panel B shows relative expression in comparison to a stem cell gene *p21*. No statistical analysis was carried out due to the sample size of 2.

4.3.2 *Cxcr2*$^{-/-}$ animals display differential numbers of mature haemopoietic cells

Preliminary experiments were carried out on *Cxcr2*$^{-/-}$ animals on a Balb/c background. However, analysis of the stem cell populations in these animals showed little/no staining of stem cell marker Sca-1 which was confirmed in the literature (data not shown) (Spangrude and Brooks, 1993). A lack of Sca-1 staining made the stem cell analysis difficult to interpret without the ability to examine populations which were Sca-1^{+}. In addition, animals on a Balb/c background are not commonly used in BM reconstitution assays with no clear way to discriminate between donor and host cells. Consequently, experiments were carried out on animals on a C57/BL6 background in which normal Sca-1 staining was observed (Figure 4-7). C57/BL6 animals are commonly used for BM reconstitution assays due to the existence of two strains with different cell surface markers (Weissman, 2000). This allows the discrimination of donor versus host cells in transplantation assays.

Previous literature has documented that the observed phenotype in *Cxcr2*$^{-/-}$ animals is dependent upon the environment in which the animals are housed in (Broxmeyer et al., 1996). In this section, immunophenotypic analysis and cell counts were used to assess the cellularity and frequency of mature haemopoietic cells (myeloid (GR1, CD11B), lymphoid (CD19 and B220) and erythroid cells (TER119)) in the haemopoietic organs (BM, spleen, PB and thymus). *Cxcr2*$^{-/-}$ animals were examined with age and sex matched WT littermates as controls.

4.3.2.1 BM

Analysis of the BM showed a trend towards an increase in the cellularity in the absence of *Cxcr2*, which was not statistically significant (n.s., $n = 12$) (Figure 4-2). Immunophenotypic analysis showed a significant decrease in erythroid cells in the absence of *Cxcr2* ($P < 0.05$, $n = 12$) (Figure 4-2). A significant increase was found in the granulocyte cells in the *Cxcr2*$^{-/-}$ mice ($P < 0.001$, $n = 12$) (Figure 4-2). Finally a trend towards a decrease was found in the B cells in the *Cxcr2*$^{-/-}$ mice which was not significant (n.s., $n = 12$) (Figure 4-2).

4.3.2.2 Spleen

Analysis of the spleen showed a trend towards an increase in the cellularity in the *Cxcr2*$^{-/-}$ animals, which was not statistically significant (n.s., $n = 6$) (Figure 4-3). This was most

likely due sample variation as an increase in spleen size in the *Cxcr2*$^{-/-}$ animals was clearly noted after dissection. There was an increase in the numbers of erythroid cells in the *Cxcr2*$^{-/-}$ mice (P <0.01. n = 6) and in the granulocyte cells (P <0.05, n = 6) (Figure 4-3) (Figure 4-4). There was no statistically significant differences in the B and T cell numbers between strains (n.s., n = 6) (Figure 4-3).

4.3.2.3 PB

PB analysis showed an increase in cellularity in the *Cxcr2*$^{-/-}$ animals (P <0.05, n = 12) (Figure 4-5). There was no difference in the numbers of erythroid cells in the *Cxcr2*$^{-/-}$ animals (*n.s.*, n = 6) (Figure 4-5). A trend towards an increase was found in the granulocytes (n.s., n = 6) (Figure 4-5) which was not significant due to small sample size and inter sample variability. Finally no difference in the numbers of B cells was found between strains (n.s., n = 6) (Figure 4-5). T cells information is not available due to technical problems during the staining.

4.3.2.4 Thymi

Analysis of the thymi showed no difference in cellularity (n.s., n = 6) with no difference in the number of T cells between strains (n.s., n = 6) (Figure 4-6).

Collectively, the data in this section is in accordance with results from previous reports (Broxmeyer et al., 1996, Cacalano et al., 1994). Previously it has been shown that the number of myeloid cells are increased in *Cxcr2*$^{-/-}$ animals which is only displayed in animals housed in a normal environment and not under germ-free conditions (Broxmeyer et al., 1996). Although the use of animals in this chapter were housed in a ‘clean’ facility, it is likely this was not germ-free and therefore the reason why a difference in myeloid cells was observed. As reported previously, an increase in myeloid cells was observed in the BM, spleen and PB with an overall increase in WBC cellularity. It has been proposed that *Cxcr2* is a negative regulator of myeloid cells therefore a lack of *Cxcr2* in the organism results in an expansion of the myeloid compartment. In addition to differences in the number of myeloid cells between strains, differences were found in numbers of erythroid cells in the BM and spleen between strains. It is possible that a reduction in the BM is due to the increase of myeloid cells which reduces the overall number of erythroid cells. However, an increase of erythroid cells was found in the spleen of *Cxcr2*$^{-/-}$ animals. It is possible that *Cxcr2* plays a role in both myeloid and erythroid regulation. The lack of

difference in the cellularity of thymus and numbers of T cells suggests that this is not altered by *Cxcr2* signalling.

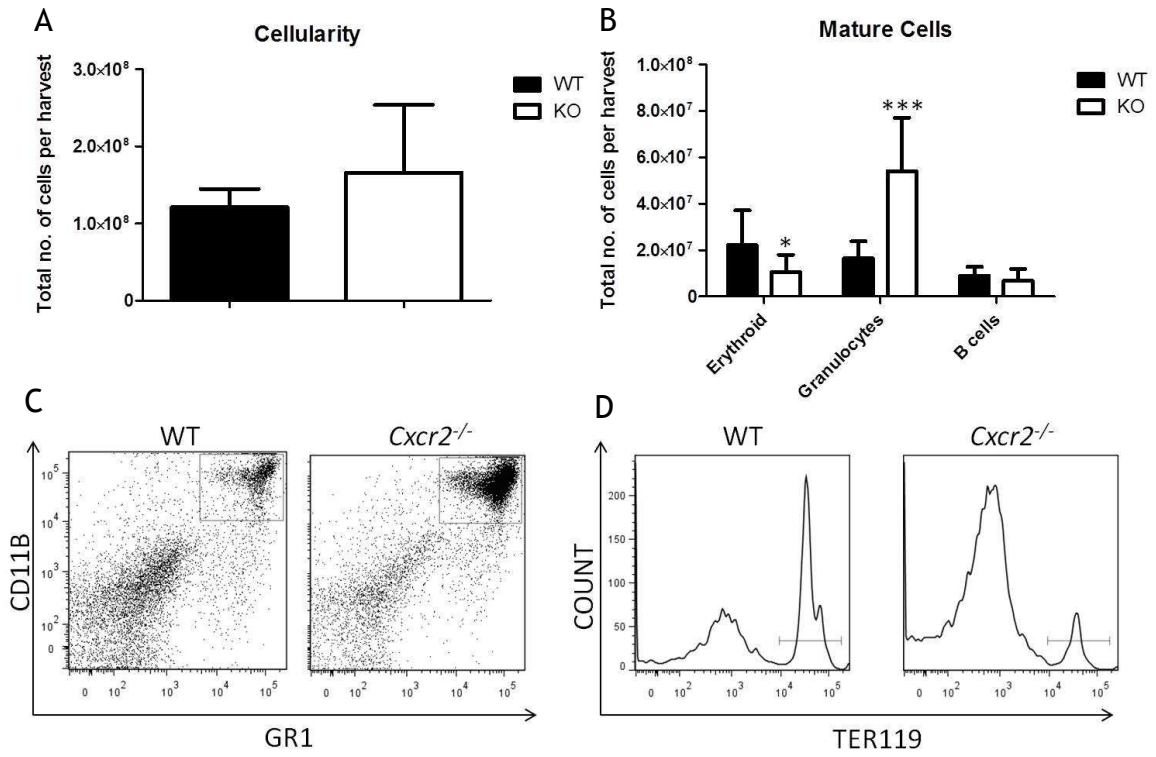

Figure 4-2 Cellularity and absolute numbers of mature cells in the BM between WT and *Cxcr2*$^{-/-}$ animals.

Whole BM was assessed for cellularity and the absolute number of mature cells was assessed using flow cytometry and WT and *Cxcr2*$^{-/-}$ (KO). Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of mature cells; erythroid (TER119^{+}), granulocyte (GR1^{+}CD11B^{+}) or B cells (CD19^{+}B220^{+}) (B). Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (* P <0.05; *** P <0.001, n = 12). Plots display a representative image of staining observed with GR1^{+}CD11B^{+} dotplots (C) or TER119^{+} histogram (D). Animals were between 6 to 12 weeks and mixed gender (WT 7 male, 5 female; *Cxcr2*$^{-/-}$ 6 male, 6 female).

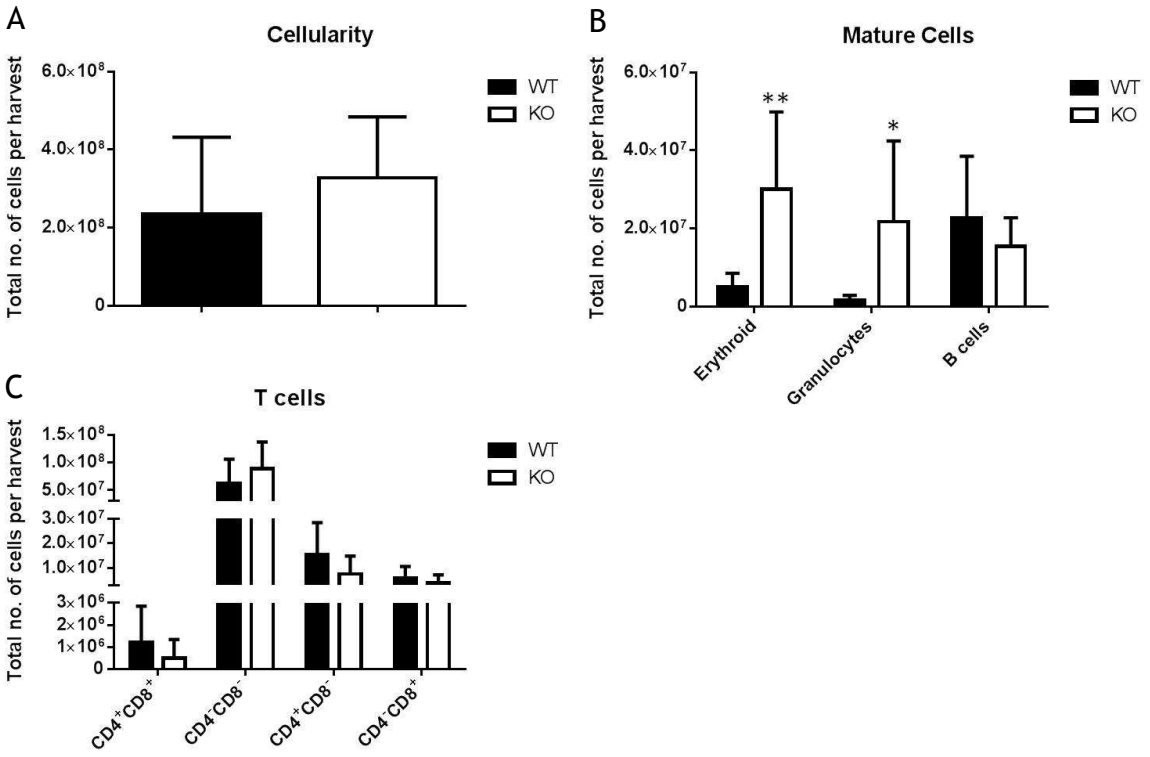

Figure 4-3 Cellularity and absolute numbers of mature cells in the spleen between WT and *Cxcr2*$^{-/-}$ animals.

Spleen was assessed for cellularity and the absolute number of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of mature cells; erythroid (TER119^{+}), granulocyte (GR1^{+}CD11B^{+}), B cells (CD19^{+}B220^{+}) (B) or T cell subsets (CD4^{+}CD8^{+} , CD4^{-}CD8^{-}, CD4^{+}CD8^{-} and CD4^{-}CD8^{+}) (C). Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (* P <0.05; ** P <0.01, n = 6). Animals were between 6 to 12 weeks and mixed gender (WT 3 male, 3 female; *Cxcr2*$^{-/-}$ 4 male, 2 female).

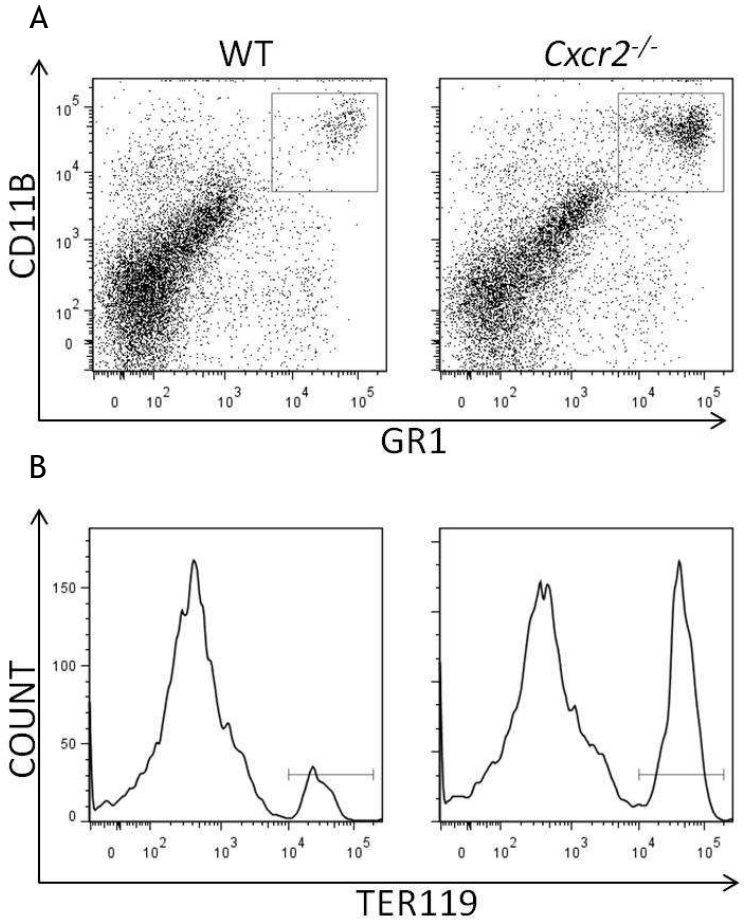

Figure 4-4 Flow cytometry plots of mature cells in the spleen between WT and *Cxcr2*$^{-/-}$ animals.

Plots display a representative image of staining observed in GR1^{+}CD11B^{+} dotplots (A) or TER119^{+} histogram (B) in *Cxcr2*$^{-/-}$ or WT spleens.

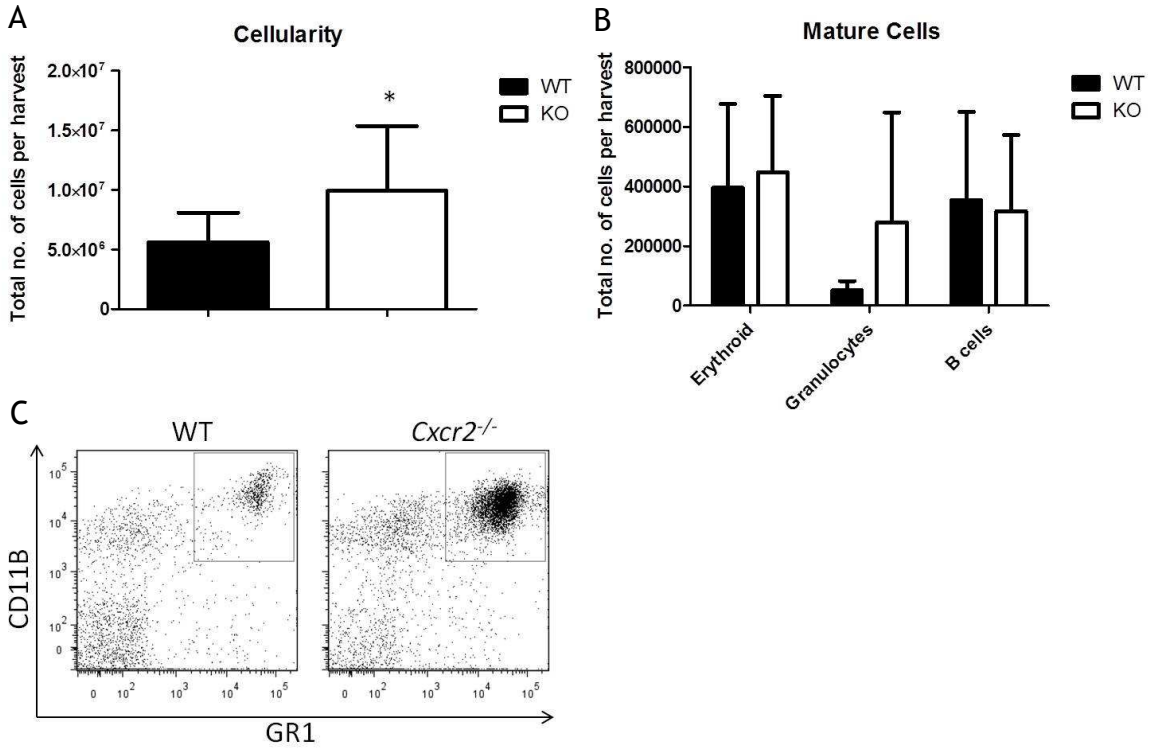

Figure 4-5 Cellularity and absolute numbers of mature cells in the PB between WT and *Cxcr2*$^{-/-}$ animals.

PB was assessed for cellularity and the percentage of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of mature cells; erythroid (TER119^{+}), granulocyte (GR1^{+}CD11B^{+}) or B cells (CD19^{+}B220^{+}) (B). Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (* P <0.05, n = 6). Plots display a representative image of staining observed in GR1^{+}CD11B^{+} dotplots (C). Animals were between 6 to 12 weeks and mixed gender (WT 3 male, 3 female; *Cxcr2*$^{-/-}$ 4 male, 2 female).

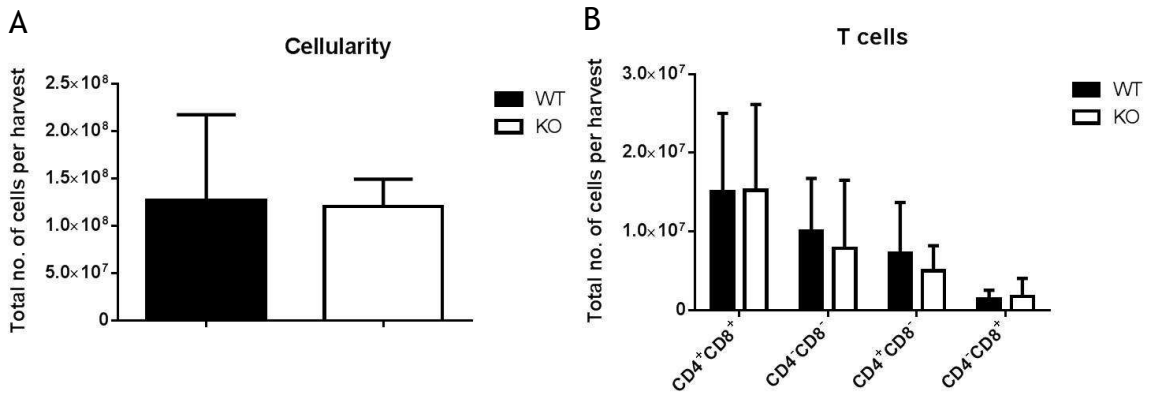

Figure 4-6 Cellularity and absolute numbers of mature cells in the thymi between WT and *Cxcr2*$^{-/-}$ animals.

Thymi were assessed for cellularity and the percentage of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of T cell subsets ($CD4^+CD8^+$, $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$) (B). Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (n.s., $n = 6$). Animals were between 6 to 12 weeks and mixed gender (WT 3 male, 3 female; *Cxcr2*$^{-/-}$ 4 male, 2 female).

4.3.3 Cxcr2$^{-/-}$ animals show differences in the frequencies of stem and progenitor cells in the BM and spleen

Analysis of the stem and progenitor compartment in terms of frequency and functionality has not been assessed in *Cxcr2*$^{-/-}$ animals to date.

A method for assessing whether a gene plays a key role in stem or progenitor function is to examine the frequencies of stem and progenitor cells in animals lacking the gene. As an example, a reduction or expansion of the stem cell compartment can give an indication of genes involved in stem cell maintenance or cell cycle. Analysis of stem and progenitor populations can be examined using flow cytometry with a variety of cell surface markers to distinguish different cell types (as described in methods section). Stem cell populations can be identified as described in section (4.3.1). Progenitor populations can be analysed using the lineage negative fraction, with Sca-1 negative and c-Kit positive cells (LK) which can be further sorted into lineage restricted progenitor populations using CD16/CD32 and CD34 staining for the identification of GMP, CMP and MEP populations.

4.3.3.1 BM

4.3.3.1.1 Stem cell frequency

There was a decrease in the number of lineage negative cells in the *Cxcr2*$^{-/-}$ animals (P <0.05, n = 12) (Figure 4-7). Within the lineage negative fraction, the LSK and further enriched stem cell populations were examined. There was a trend towards an increase in the number of LSK cells which was not significant most likely due to high variability between *Cxcr2*$^{-/-}$ samples (n.s., n = 12) (Figure 4-7). The CD150^{+}CD48^{-} fraction (LT-HSC) showed an increase in the *Cxcr2*$^{-/-}$ animals (P <0.05, n = 12) (Figure 4-7). Finally, there were no differences between CD150^{-}CD48^{-} (ST-HSC), CD150^{+}CD48^{+} (MPP) or CD150^{-} CD48^{+} (MPP) fractions but a trend towards an increase in all populations was noted (n.s., n = 12) (Figure 4-7). Representative flow cytometry plots are shown (Figure 4-8).

As these animals show an expansion of myeloid cells within the BM, the reduction in the percentage of lineage negative cells and subsequently cell number is possibly due to this disruption of mature cells within the organ. The data collectively suggests that *Cxcr2*$^{-/-}$ animals have an expansion of the stem cell populations, including the most primitive (LT-HSC) fraction in the BM. This suggests that *Cxcr2* is important for steady state haemopoiesis. This could suggest that CXCR2 signalling is negatively regulating stem cell production. Stem cell frequency gives no conclusive indication of stem cell function. However, often ‘loss of function’ in HSC is initially associated with transient expansion in stem cells numbers due to increased proliferation but eventual exhaustion and depletion of repopulating stem cells. As an example, HSC with no cell cycle inhibitors show a transient expansion which results in exhaustion and reduced HSC activity in BM reconstitution assays (Cheng et al., 2000). To conclude this, *in vivo* cell cycle analysis and BM reconstitution assays are required.

4.3.3.1.2 Progenitor frequency

In terms of the progenitor populations, the LK fraction contains progenitor cells and shows a trend towards a decrease in the *Cxcr2*$^{-/-}$ condition (n.s., n = 6) (Figure 4-9). There were no statistically significant differences between GMP, CMP and MEP populations in terms of absolute numbers however there was a trend towards an increase in the GMP population and decrease in the CMP and MEP populations (n.s., n = 6) (Figure 4-9). It is likely that differences might be found with an increase in sample size as a high volume of variation was noted between animals and a sample size of n = 6 was used in this assay. Observation

of representative flow cytometry plots of GMP, CMP and MEP staining demonstrates that the distribution of cell types between strains is different with an increase in GMP and CMP populations and a decrease in MEP populations in *Cxcr2*$^{-/-}$ animals (Figure 4-9).

It is not surprising that the flow cytometry plots show a differential distribution of GMP, CMP and MEP populations due to the differences in mature cell types (granulocytes and erythroid) that we see in the mature cells in the BM in section 4.3.2. This does however suggest that the deregulation of myeloid cells that occurs in the *Cxcr2*$^{-/-}$ mice occurs at the primitive, progenitor level. However, this cannot be concluded due to a lack of significance in absolute progenitor numbers between strains.

4.3.3.2 Spleen

4.3.3.2.1 Stem cell frequency

There was a trend towards an increase in the number of lineage negative cells in the *Cxcr2*$^{-/-}$ animals in the spleen (n.s., $n = 6$) (Figure 4-10). There was a significant increase in the number of LSK cells in the *Cxcr2*$^{-/-}$ animals ($P < 0.05$, $n = 6$) (Figure 4-10). Finally, All HSC fractions showed a trend towards an increase in the *Cxcr2*$^{-/-}$ animals which was not statistically significant, most likely due to the low sample size for this result (n.s., $n = 3$) (Figure 4-10).

The data suggests that *Cxcr2*$^{-/-}$ animals have an expansion of the stem cell populations both in the BM and spleen and show extramedullary (spleen) haemopoiesis. This suggests that the HSC are both expanded and there is enhanced mobilisation in the *Cxcr2*$^{-/-}$ animals. This is in accordance with previous literature which shows an increase in the numbers of CFU in BM and spleen derived cells in *Cxcr2*$^{-/-}$ conditions in comparison to WT controls (Broxmeyer et al., 1996, Cacalano et al., 1994). However, this study extends this previous research and shows detailed analysis of the stem cell populations.

4.3.3.2.2 Progenitor frequency

In terms of progenitor cells in the spleen, the LK fraction showed an increase in the *Cxcr2*$^{-/-}$ animals ($P < 0.05$, $n = 6$) (Figure 4-11). There were trends towards increases in all progenitor populations including the GMP, CMP and MEP populations (n.s. for GMP and CMP respectively with $P < 0.001$ for MEP, $n = 3$) (Figure 4-11). It can be seen from representative flow cytometry plots that the distribution of progenitor populations differ between strains with a greater population of GMP and CMP populations in the *Cxcr2*$^{-/-}$ animals (Figure 4-11). The increase in progenitor populations is in accordance with results in the previous sections which showed an increase in mature myeloid and erythroid cells in the spleen derived from *Cxcr2*$^{-/-}$ animals.

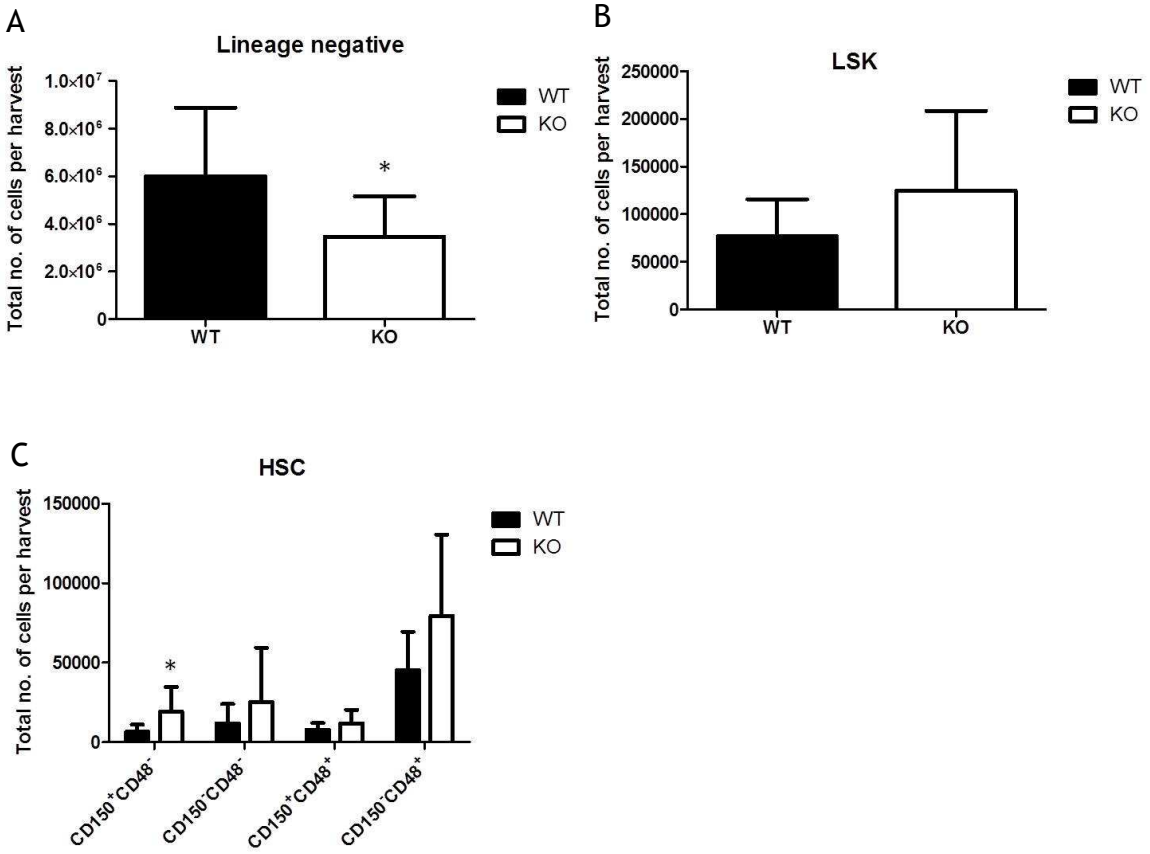

Figure 4-7 Absolute numbers of stem cell populations between WT and *Cxcr2*$^{-/-}$ animals in the BM.

Whole BM was made into a single cell suspension and stained for antibodies to examine the stem cell populations and analysed using flow cytometry. Data are presented as the mean absolute cell numbers for lineage negative (A), LSK (B) or HSC (C) populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (* $P < 0.05$, $n = 12$). Animals were between 6 to 12 weeks and mixed gender (WT 7 male, 5 female; *Cxcr2*$^{-/-}$ 6 male, 6 female).

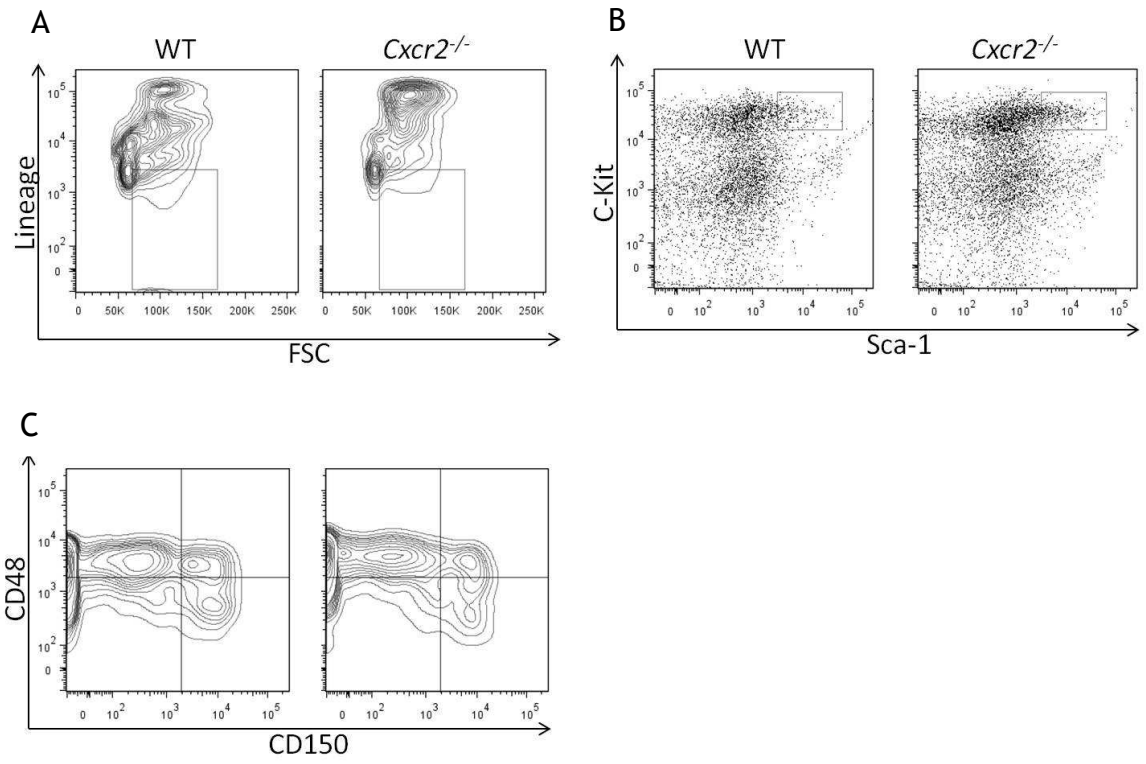

Figure 4-8 Representative flow cytometry plots of lineage negative, LSK and HSC populations between WT and *Cxcr2*$^{-/-}$ animals.

Plots display a representative image of lineage negative (A), LSK (B) and HSC populations (C) between WT and *Cxcr2*$^{-/-}$ animals.

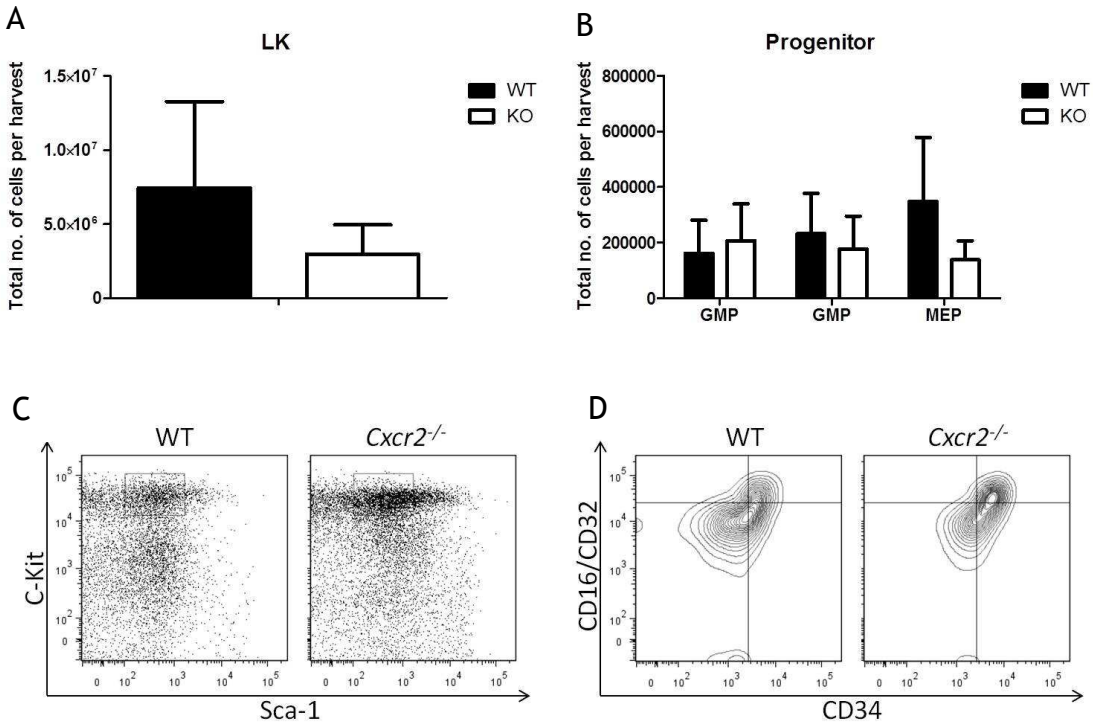

Figure 4-9 Absolute numbers of progenitor populations between WT and *Cxcr2*$^{-/-}$ animals in the BM.

The BM was made into a single cell suspension and stained with antibodies to examine the progenitor populations and analysed using flow cytometry. Data are presented as the mean absolute cell numbers for LK (A) or progenitor populations (B) between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (n.s., *n* = 6). Images demonstrate representative flow cytometry plots for LSK (C) and progenitor (D) staining between WT and *Cxcr2*$^{-/-}$ animals. Animals were between 6 to 12 weeks and mixed gender (WT 3 male, 3 female; *Cxcr2*$^{-/-}$ 1 male, 5 female).

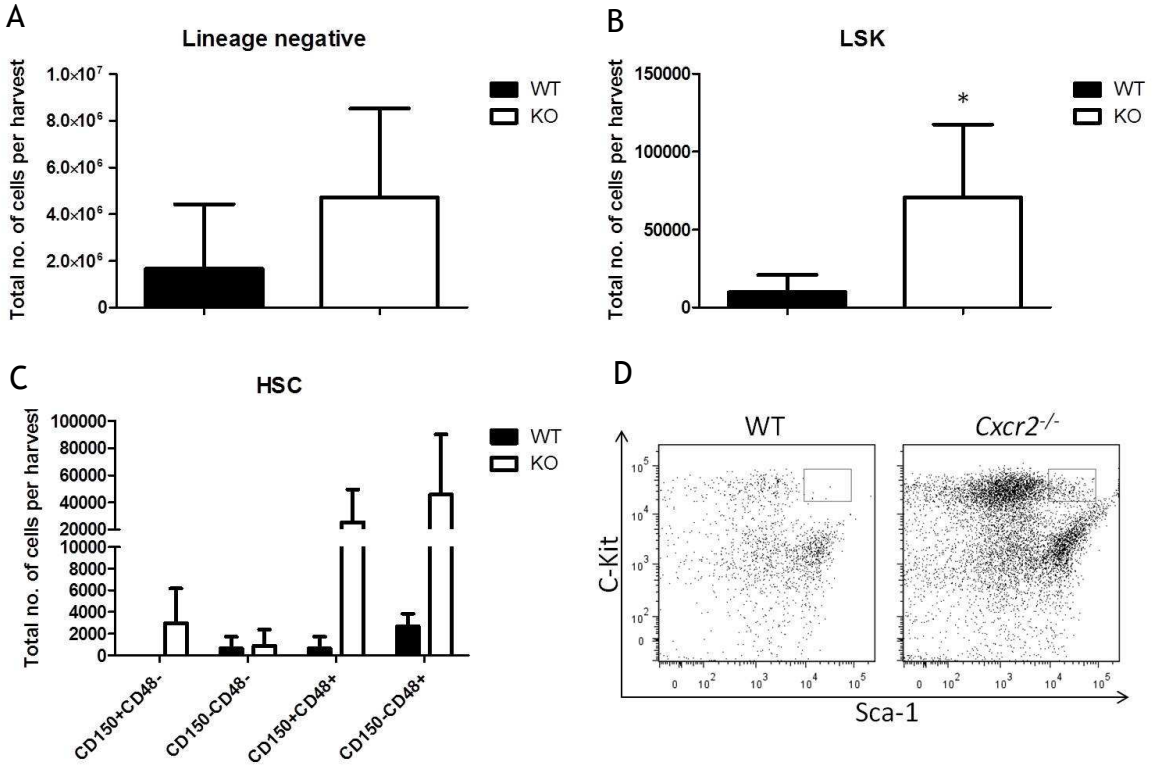

Figure 4-10 Absolute numbers of stem cell populations between WT and *Cxcr2*$^{-/-}$ animals in the spleen.

Spleen was made into a single cell suspension and stained for antibodies to examine the stem cell populations and analysed using flow cytometry. Data is presented as the mean absolute cell numbers for lineage negative (A), LSK (B) or HSC (C) populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (* P <0.05, n = 6). Dotplot shows representative flow cytometry staining profile for LSK staining between WT and *Cxcr2*$^{-/-}$ animals (D). Animals were between 6 to 12 weeks and mixed gender (WT 3 male, 3 female; *Cxcr2*$^{-/-}$ 1 male, 5 female).

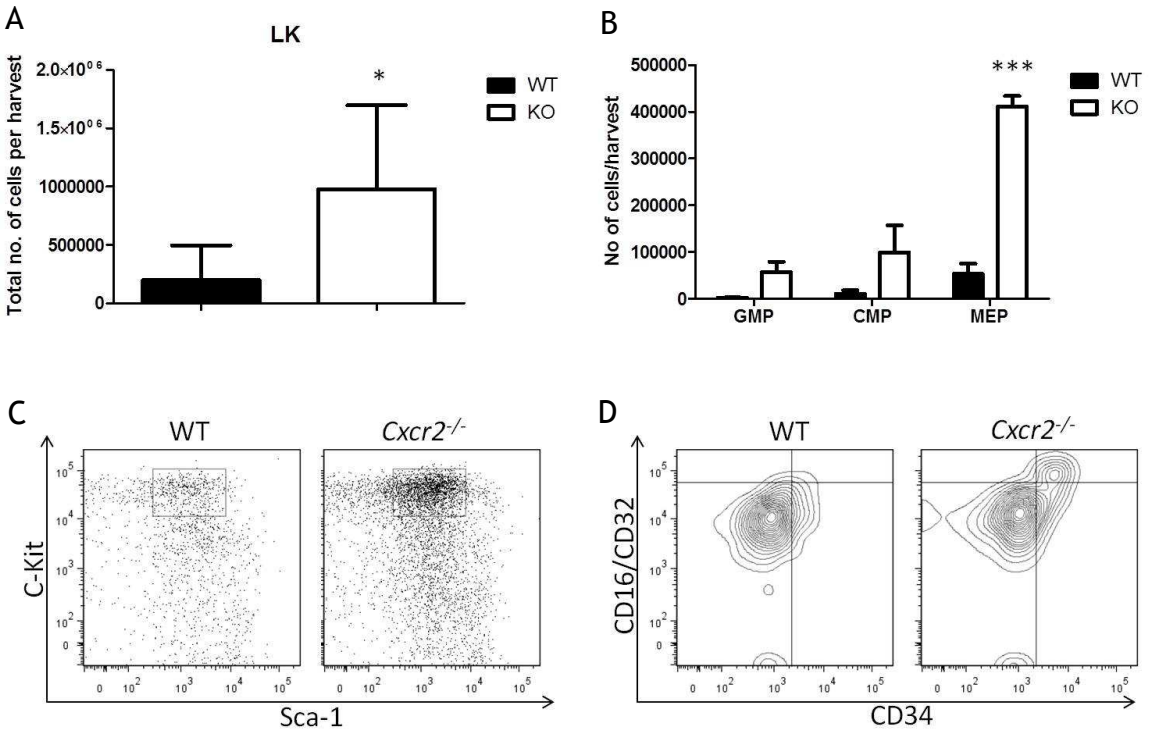

Figure 4-11 Absolute numbers of progenitor populations between WT and *Cxcr2*$^{-/-}$ animals in the spleen.

The spleen was made into a single cell suspension and stained with antibodies to examine the progenitor populations and analysed using flow cytometry. Data are presented as the mean absolute cell numbers for LK (A) and progenitor (B) populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a two-tailed unpaired student's *t* test with Welch's correction for unequal variance (* P <0.05; *** P <0.001, n = 3). Dotplots display representative flow cytometry staining observed for LK (C) and progenitor populations (D) between WT and *Cxcr2*$^{-/-}$ animals. Animals were between 6 to 12 weeks and same gender (WT 3 female; *Cxcr2*$^{-/-}$ 3 female).

4.3.4 *Cxcr2*$^{-/-}$ animals show an increase in colony numbers derived from the spleen and PB

HSC and progenitor cells are contained within the BM, spleen and PB and have the capacity to differentiate into cells of myeloid and lymphoid lineages. The CFC assay measures the differentiation and proliferation capacity using their ability to form colonies in culture as described in the previous chapter. This assay was used to examine the differences in proliferation and differentiation capacity of cells derived from the BM, spleen and PB from WT and *Cxcr2*$^{-/-}$ animals. To examine the self renewal capacity of BM derived cells, colonies grown in a primary plating assay were harvested and replated into a secondary assay. Using this assay, the self renewal capacity of BM stem/progenitor cells was compared between strains.

4.3.4.1 BM

BM derived from *Cxcr2*$^{-/-}$ and WT animals showed both strains generated colonies in methylcellulose and had comparable lineage differentiation potential. No difference in CFU was found in primary plating assays derived from BM cells between strains (n.s., n = 8) (Figure 4-12). No difference in colony types were observed between strains therefore colonies were counted as total number of colonies (data not shown). After colony counts, colonies were harvested from plates, pooled and reseeded into a secondary colony formation assay to get an indication of the self renewal activity of the stem/progenitor cells. No difference in colonies in a secondary colony formation assay was found in cells derived from the BM however huge variation was noted between samples in the *Cxcr2*$^{-/-}$ condition (n.s., n = 3) (Figure 4-12).

4.3.4.2 Spleen

An increase in CFU-GM colonies was found in cells derived from the *Cxcr2*$^{-/-}$ spleen (P <0.001, n = 3) (Figure 4-13). This was not statistically significant which likely reflects a small sample size and sample variability. Similarly, a trend towards an increase in CFU-E colonies was found (n.s., n = 3). Finally, no CFU-GEMM colonies were found in either condition. The increase in CFU-GM and CFU-E colonies resulted in an overall trend towards increase in the total number of colonies in the *Cxcr2*$^{-/-}$ animals in comparison to the WT controls (n.s., n = 3).

4.3.4.3 PB

An increase in CFU-GM colonies was found in the PB of *Cxcr2*$^{-/-}$ animals (P <0.01., n = 6) (Figure 4-14). No difference in CFU-E or CFU-GEMM colonies was found between strains (n.s., n = 6). However the increase in CFU-GM colonies collectively resulted in a trend towards an increase in the total number of colonies in the *Cxcr2*$^{-/-}$ animals which failed to reach significance (n.s., n = 6)

Data from previous literature which showed an increase in CFU in the spleen and PB in cells derived from *Cxcr2*$^{-/-}$ animals in comparison to controls (Broxmeyer et al., 1996). This indicates that stem/progenitor activity exists in the circulation and extramedullary sites of haemopoiesis including the spleen and PB. The results in section 4.3.3.2 with immunophenotypic analyses support this. The lack of significance with colony assays in the spleen and PB likely represents inter sample variation. However, in the study by Broxmeyer *et al.*, an increase in CFU in BM derived cells was reported in BM cells which lack *Cxcr2*. The lack of difference in CFU found in the BM between *Cxcr2*$^{-/-}$ and WT cells in this chapter could, and most likely reflects technical issues. Observation of the CFU numbers obtained from BM samples in the Broxmeyer *et al.*, study showed a much higher number of CFU obtained in the WT condition than in the results in this thesis (>80 CFU per sample in comparison to ~20 per sample). Therefore it is likely that technical issues resulted in small CFU numbers and may not be as accurate. To support this idea, an increase in myeloid, stem and progenitor populations is noted in *Cxcr2*$^{-/-}$ animals using flow cytometry analysis as described in sections 4.3.2.1 and 4.3.3.1. Therefore it was predicted that BM derived *Cxcr2*$^{-/-}$ cells would produce an increase in CFU in comparison to WT controls.

The results from the replating assay suggests that there is no difference in self renewal activity between strains however low numbers of CFU were noted, small sample sizes were used and high variation was noted between samples. Therefore this result is not conclusive to whether cells lacking *Cxcr2* in the BM show altered proliferation/differentiation or self renewal activity.

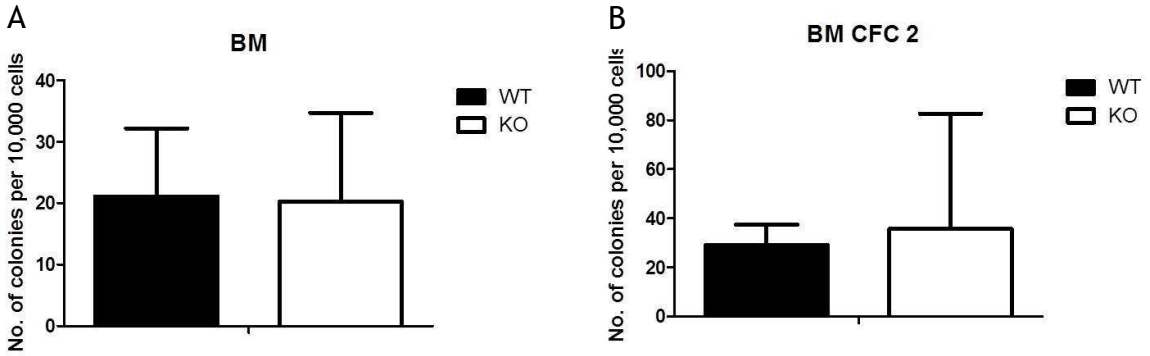

Figure 4-12 WT and *Cxcr2*$^{-/-}$ CFC analysis in BM derived cells showed no difference between strains in primary or secondary plates.

Whole BM from either *Cxcr2*$^{-/-}$ or WT animals was made into a cell suspension, WBC counted and 10^4 cells plated per mL in Methocult™ and incubated for 10-14 days. Colonies were subsequently scored, counted and replated into a secondary assay. Data are presented as the mean total number of colonies in a primary (A) (n = 8) and secondary replating assay (B) (n = 3). Statistical analysis was carried out using a two-tailed unpaired student's t test with Welch's correction for unequal variance (n.s.). Animals were between 6 to 12 weeks and mixed gender (CFC1 WT 5 male, 3 female; *Cxcr2*$^{-/-}$ 5 male, 3 female; CFC2 WT 1 male, 2 female; *Cxcr2*$^{-/-}$ 2 male, 1 female).

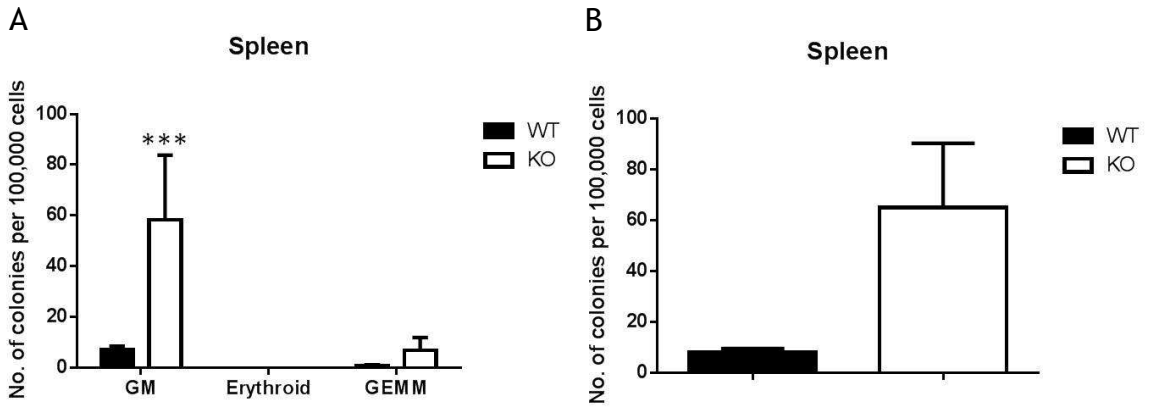

Figure 4-13 WT and *Cxcr2*$^{-/-}$ CFC analysis in spleen derived cells.

Spleen was isolated from *Cxcr2*$^{-/-}$ or WT animals, WBC counted and 10^5cells plated per mL in Methocult™ and incubated for 10-14 days. Colonies were subsequently scored and counted. Data are presented as the mean total number and type of colonies (A) and total number of colonies (B) (n = 3). Statistical analysis was carried out using a two-way ANOVA with Sidak's multiple comparisons to assess differences between groups (A) (*** P <0.001) and a two-tailed unpaired student's t test with Welch's correction for unequal variance (B) (n.s., P = 0.058). Animals were between 6 to 12 weeks and same gender (WT 3 female; *Cxcr2*$^{-/-}$ 3 female).

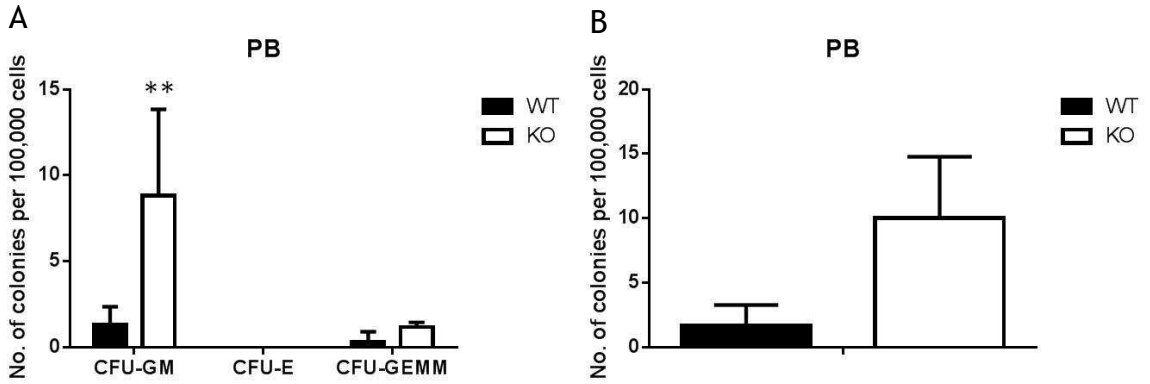

Figure 4-14 WT and *Cxcr2*$^{-/-}$ CFC analysis in PB derived cells.

PB was isolated from WT and *Cxcr2*$^{-/-}$ animals, WBC counted, RBC lysed and 100,000 cells plated per mL in Methocult™ and incubated for 10-14 days. Colonies were subsequently scored and counted. Data are presented as the mean total number and type of colonies (A) and total number of colonies (B) (n = 3). Statistical analysis was carried out using a two-way ANOVA with Sidak's multiple comparison test to assess differences between groups (A) (** P <0.01) and a two-tailed unpaired student's t test with Welch's correction for unequal variance (B). Animals were between 6 to 12 weeks and same gender (WT 3 female; *Cxcr2*$^{-/-}$ 3 female).

4.3.5 Analysis of viability in *Cxcr2*$^{-/-}$ HSC populations

The results from section 4.3.3.1.1 showed an expansion of LT-HSC in the BM of *Cxcr2*$^{-/-}$ animals indicating expansion of stem cells with a lack of *Cxcr2*. To test whether HSC derived from *Cxcr2*$^{-/-}$ or WT animals differed in terms of their stem cell properties, it was aimed to examine viability and cell cycle status of HSC derived from both strains. BM was harvested and stained for antibodies to identify HSC populations with the addition of Annexin-V and Ki-67 to measure cell death and proliferation respectively. The results are representative of one sample therefore no statistical analysis has been carried out and only trends are discussed.

Results showed a trend towards a decrease in the percentage of Annexin-V^{+} cells in cell populations in the *Cxcr2*$^{-/-}$ animals in the whole BM, lineage negative, LSK, CD150^{+}CD48^{-}, CD150^{-}CD48^{-}, CD150^{+}CD48^{+} and CD150^{-}CD48^{+} HSC populations (Figure 4-15). A sample size of n = 1 was used therefore no standard deviation or statistical significance is recorded.

Although it cannot be concluded, the results suggest that the stem/progenitor populations are more viable (less Annexin-V^{+}) in the *Cxcr2*$^{-/-}$ animals in comparison to the WT littermates (n = 1). Analysis of viability of the WT cells, showed a percentage of Annexin-V^{+} cells in whole BM which is likely basal levels of apoptosis (16.4%). This is not surprising as the BM contains a mixture of diverse cell types including terminally differentiated mature cell types which have a short life span and are regulated by apoptosis. In addition, the cells were examined *ex vivo* which means they are taken from their niche environment where their survival and maintenance are regulated by extrinsic signals. The percentage of apoptotic cells was decreased as a more primitive cell population was analysed from lineage negative (9.77%) to LSK and LT-HSC population (5.0% and 5.5% respectively). This indicates that the Annexin-V staining is correct as the expression pattern of staining correlates to the pattern expected. Interestingly, an increase in the percentage of Annexin-V^{+} cells was found in the CD150^{+}CD48^{+} population which suggests this particular MPP population exhibits a higher level of basal apoptosis than the other HSC populations. It is unclear why cells lacking *Cxcr2* show increased viability in comparison to controls. It is possible that *Cxcr2* inhibition results in a compensation of other chemokines including *Cxcr1* which results in enhanced cell viability. However, this cannot be concluded without analysis of *Cxcr1* and other chemokines in the absence of *Cxcr2*.

Ki-67 staining was used to examine proliferation in HSC populations. Although this stain used alone cannot discriminate between cells in G_0 versus G_1, the percentage of Ki-67^+ cells shows cells in G_2, S and M phases and therefore can indicate cells that are actively proliferating. Data showed a decrease in the percentage of Ki-67^+ cell populations suggesting a decrease in cell proliferation in the *Cxcr2*$^{-/-}$ animals in the whole BM, lineage negative and LK populations (Figure 4-15). No difference was found between WT and *Cxcr2*$^{-/-}$ animals in the LSK or other HSC populations including the $CD150^+CD48^-$ and $CD150^+CD48^+$ populations. A trend towards an increase was found in the $CD150^+CD48^+$ and $CD150^-CD48^+$ populations (n = 1) (Figure 4-15). No statistical tests were carried out due to the sample size of 1.

In the WT cells, it can be seen that the BM and LK fractions show the highest levels of proliferation which is decreased in the HSC populations, with the lowest levels noted in the most primitive $CD150^+CD48^-$ fraction. This staining pattern is as would be predicted as the most primitive HSC are known to be less proliferative. No difference in Ki-67^+ cells is found between HSC populations between strains with the exception of the MPP population ($CD150^-CD48^+$) and this trend is found in the less primitive LK and lineage negative fraction also. Although with a sample size of 1, conclusions cannot be drawn, it can be inferred that the MPP population becomes more proliferative in the absence of *Cxcr2*. In contrast, the whole BM, lineage negative and LK fraction seem to be less proliferative in the absence of *Cxcr2*. Higher proliferation in the MPP population could result in cell exhaustion and *Cxcr2* controls cell cycle differently in MPP in comparison to more mature cell types. A more in depth analysis of cell cycle analysis with the addition of DNA stains to distinguish between cells in G_0 and G_1 can be used. Alternatively, the cycling status of cells can be examined *in vivo* with the use of label retaining dyes including bromodeoxyuridine (Brd-U).

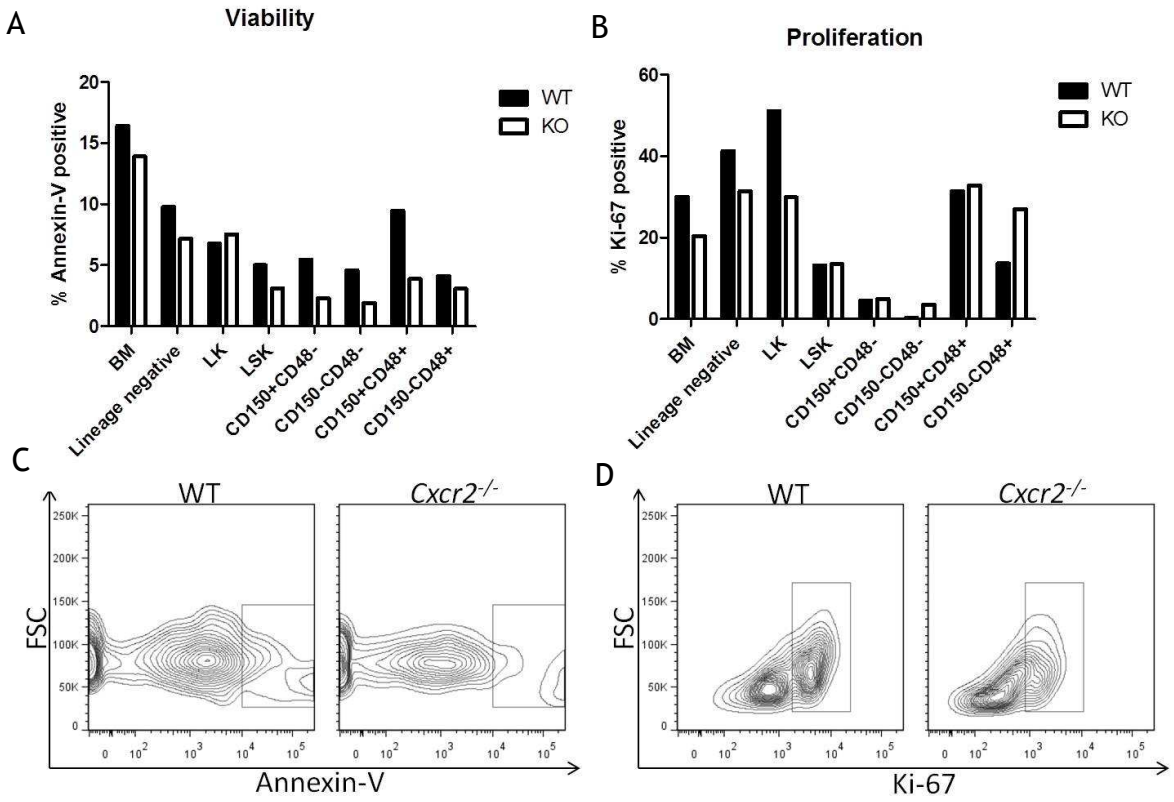

Figure 4-15 *Cxcr2*$^{-/-}$ HSC viability and proliferation.

WT or *Cxcr2*$^{-/-}$ stem and progenitor populations were stained for Annexin-V and analysed for viability or fixed, permeabilised and stained for Ki-67. Data is presented as the mean percentage of Annexin-V^{+} cells (A) or Ki-67^{+} cells (B) in various cell populations in BM derived cells in the WT and *Cxcr2*$^{-/-}$ conditions. The sample size is one, therefore no standard deviation or statistical analysis is shown (n = 1). Dotplots of representative samples show flow cytometry staining of Annexin-V^{+} cells in the CD150^{+}CD48^{+} fraction (C) or Ki-67 staining in LK fraction (D). Animals were between 6 to 12 weeks and same gender (WT 1 female; *Cxcr2*$^{-/-}$ 1 female).

4.3.6 Analysis of engraftment in a BM reconstitution assay with WT or *Cxcr2*$^{-/-}$ HSC

The gold standard technique for assaying HSC functional activity is the BM reconstitution assay. More specifically, this technique involves the isolation of HSC and transplantation into a host in which the BM has been ablated with lethal irradiation. After transplantation, the HSC will home and engraft into the BM niche, where they will balance self renewal and multilineage differentiation to produce progeny including the mature cell types of haemopoiesis. A decrease in engraftment and multilineage differentiation infers that the HSC population is not functional. The use of donor cells (CD45.2^{+}) from a different genetic background than the host (CD45.1^{+}) allows the contribution of donor cells to be tracked using flow cytometry.

WT or *Cxcr2*$^{-/-}$ HSC were isolated from animals on a CD45.2^{+} background and were transplanted into several host CD45.1^{+} animals which had been previously irradiated. The HSC were transplanted with unmanipulated BM from a CD45.1^{+} animal to act as a 'support' for the survival of the hosts with ablated haemopoietic systems. Monoclonal antibodies against CD45.1 and CD45.2 were used with flow cytometry analysis to examine donor engraftment in different cell populations over time. It was hypothesised that *Cxcr2* null HSC would show a decrease in engraftment potential in comparison to the control WT HSC.

4.3.6.1 Primary BM reconstitution assays

To examine whether HSC derived from WT or *Cxcr2*$^{-/-}$ animals contained differences in engraftment potential, PB from recipient animals was examined for the percentages of CD45.1^{+} versus CD45.2^{+} cells every 4 weeks up to 16 weeks post transplant. This time frame allows the donor HSC to engraft into the BM niche and result in multilineage BM reconstitution. The engraftment of donor cells was tracked in the PB over 16 weeks using CD45.1 and CD45.2 staining. Furthermore, within the CD45.2^{+} fraction, the percentage of myeloid (GR1^{+} and CD11B^{+}), B (CD19^{+}) and T (CD4^{+} and CD8^{+}) cells were examined. This was to examine whether HSC from *Cxcr2*$^{-/-}$ animals had the potential for multilineage differentiation. Mice were sacrificed at 16 weeks post transplant and haemopoietic organs were examined for the presence of donor derived cells.

From 4 weeks up to 16 weeks post transplant, a trend towards a decrease in the percentage of CD45.2^{+} cells was found in the PB of animals transplanted with *Cxcr2*$^{-/-}$ donor HSC in comparison to the control (n.s., *n* =7, 6) (Figure 4-16). In the WT control, a trend towards increase in CD45.2^{+} cells was found over the 16 week period indicative that the transplant was successful. No significant differences were noted between conditions, and it was observed that a great deal of variation was found within both conditions. It can be seen from Figure 4-16 that several samples showed no/little engraftment up until the 16 week timepoint in the recipients transplanted with WT HSC. This is likely due to a technical problem during the injection as WT HSC should home and engraft in irradiated recipients. Similarly, some samples in the *Cxcr2*$^{-/-}$ condition showed no/little engraftment and the majority of samples showed a small percentage of engraftment. However these were not excluded from the analysis as this would introduce bias. It can be seen from the WT donor cells that percentage of engraftment increases over time which is as expected in a BM reconstitution assay. Similarly, in the *Cxcr2*$^{-/-}$ condition, some HSC do engraft and the percentage engraftment increases over time. This indicates that the HSC that do engraft are capable of self renewal, however the overall percentage engraftment is lower than in comparison to the control. A possible explanation is a defect in cell homing in HSC lacking *Cxcr2.*

In addition to examining engraftment of donor cells, the cells that did engraft (CD45.2^{+}) were examined for their potential to produce cell types of different lineages. Within the CD45.2^{+} cells, the percentage of cells positive for mature cells of all the myeloid, lymphoid and T cell lineages was examined. Data showed no difference in the ability of

any of the recipient animals to produce all the mature cell types over a period of 16 weeks post transplant (n.s., n = 7, 6) for WT and *Cxcr2*$^{-/-}$ conditions respectively at 16 weeks post transplant (Figure 4-16). These results indicate that although the HSC derived from *Cxcr2*$^{-/-}$ show a trend towards decreased engraftment, the HSC that engraft are capable of multilineage differentiation. This would support the hypothesis that HSC lacking *Cxcr2* show a defect in homing, as it is clear from the result in Figure 4-16 that some HSC can engraft and produce multilineage differentiation. Interestingly, there was no difference in the percentage of myeloid cells in the PB in animals transplanted with WT or *Cxcr2*$^{-/-}$ HSC. As the *Cxcr2*$^{-/-}$ animals show increased number of myeloid cells in the haemopoietic organs at steady state, this suggests that it is not a *Cxcr2*$^{-/-}$ cell autonomous HSC that is responsible for this expansion. As the host animals are WT with *Cxcr2* not deleted, it is possible that a lack of this receptor is required in all tissues to result in the observed phenotype. However, this is speculation.

At 16 weeks post transplant, the animals were sacrificed and haemopoietic organs were analysed for donor derived cells in mature cell types and stem/progenitor cells. To achieve this, CD45.1 and CD45.2 staining was examined in myeloid (GR1 and CD11B), lymphoid (CD19), progenitors (LK, GMP, CMP and MEP) and stem cells (LT-HSC, ST-HSC, MPP) in the BM and spleen.

The results showed a similar pattern obtained in the PB analysis (Figure 4-16). A trend towards a decrease in engraftment was found in the BM and spleen (n.s., n = 7, 6). A trend towards a reduction in engraftment was noted in the mature cells in both in the BM (n.s.) and spleen (P<0.05 for myeloid and n.s. for T cells respectively, n = 7, 6) (Figure 4-17). In terms of progenitors, there was a trend towards a reduction in the LK, GMP, CMP and MEP populations in the WT and *Cxcr2*$^{-/-}$ conditions respectively (n.s., n = 7, 6) (Figure 4-18). Similarly, the LK population in spleen derived cells showed trend towards a decrease in engraftment which was not statistically significant (n.s., n = 7, 6) (Figure 4-18).

Analysis of the stem cell populations within the BM showed a decrease in the engraftment of lineage negative (P <0.05), LSK (n.s.), CD150^{+}CD48^{-} (n.s.), CD150^{-}CD48^{-} (n.s.), CD150^{+}CD48^{+} (n.s.) and CD150^{-}CD48^{+} (n.s.) populations in the WT and *Cxcr2*$^{-/-}$ conditions respectively (Figure 4-18). Similarly, the lineage negative and LSK populations in the spleen showed a trend towards decrease in donor cell engraftment in recipients transplanted with *Cxcr2*$^{-/-}$ HSC (n.s.) (Figure 4-18).

Collectively, the data shows that *Cxcr2* cell autonomous signalling is important for stem cell functional activity. The lack of significance across the analysis represents heterogeneity in engraftment observed in both WT and *Cxcr2*$^{-/-}$ donor derived cells and is likely technical. The evidence that *Cxcr2*$^{-/-}$ HSC can still contribute to mature cell types, stem and progenitor cells in the BM and spleen infers that *Cxcr2*$^{-/-}$ HSC can still function, however this is to a lesser extent than the WT. If the *Cxcr2*$^{-/-}$ HSC can produce multilineage reconstitution over time then they are still functional and it is possible that their defect is not in stem cell function but in cell homing after transplantation. It is unclear from these experiments whether the reduction in engraftment found with *Cxcr2*$^{-/-}$ donor cells is due to a decrease in stem cell function or a decrease in cell homing. If *Cxcr2*$^{-/-}$ could not engraft in the BM but engrafted in alternative sites of haemopoeisis, an increase of engraftment would be found in the spleen or PB from *Cxcr2*$^{-/-}$ transplanted animals. However, this was not the case. It may be possible that the cells cannot home and undergo apoptosis and therefore show a reduction in engraftment, however this cannot be concluded at this stage.

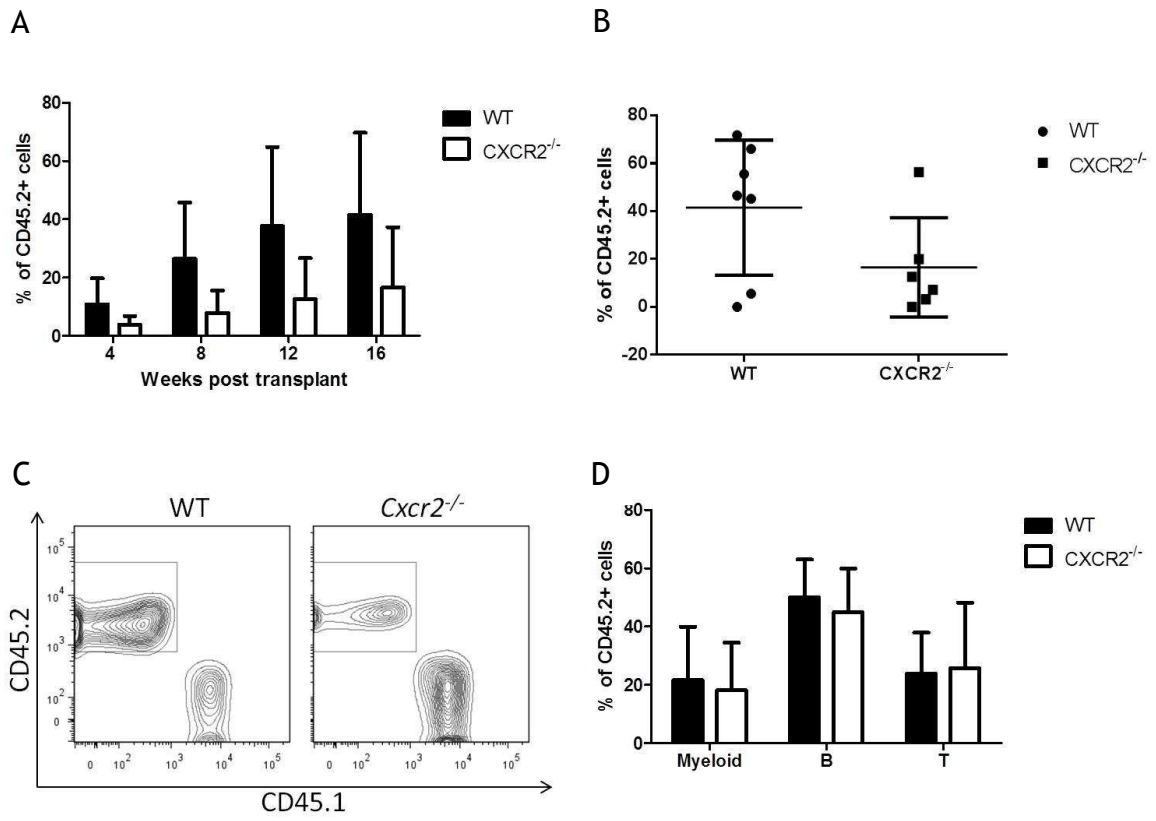

Figure 4-16 WT and *Cxcr2*$^{-/-}$ HSC show no significant differential engraftment in a primary BM transplantation assay.

Data are presented as the mean percentage of CD45.2^{+} cells in recipient mice transplanted with either WT or *Cxcr2*$^{-/-}$ HSC (LT-HSC, 10^2 cells per mouse) along with CD45.1 support marrow ($2x10^5$ cells per mouse) over a 16 week period (A) (n.s. n = 7, 6). Each recipient animal is displayed as a single symbol on the graph at 16 weeks post transplant (B) (n.s., n = 7, 6). Representative flow cytometry plots of CD45.1 and CD45.2 staining in recipients with WT or *Cxcr2*$^{-/-}$ HSC are shown (C). The percentage of myeloid (GR1^{+}, CD11B^{+}), B (CD19^{+}) and T (CD4^{+}, CD8^{+}) cells found in the PB within CD45.2^{+} donor cells are shown (D). Antibodies against CD4 and CD8 were used in the same fluorophore therefore T cells are labelled as double positive cells (CD4^{+}, CD8^{+}) only. Statistical analysis was carried out using two-way repeated measures ANOVA with Sidak's multiple comparison test to compare conditions at each timepoint (A) and a student's unpaired t test with Welch's correction for unequal variance (D) (n.s.) (n = 7, 6). Animals were between 6 to 12 weeks and mixed gender (donor WT 2 female; *Cxcr2*$^{-/-}$ 2 female; recipients WT 4 male, 3 female; *Cxcr2*$^{-/-}$ 4 male, 2 female).

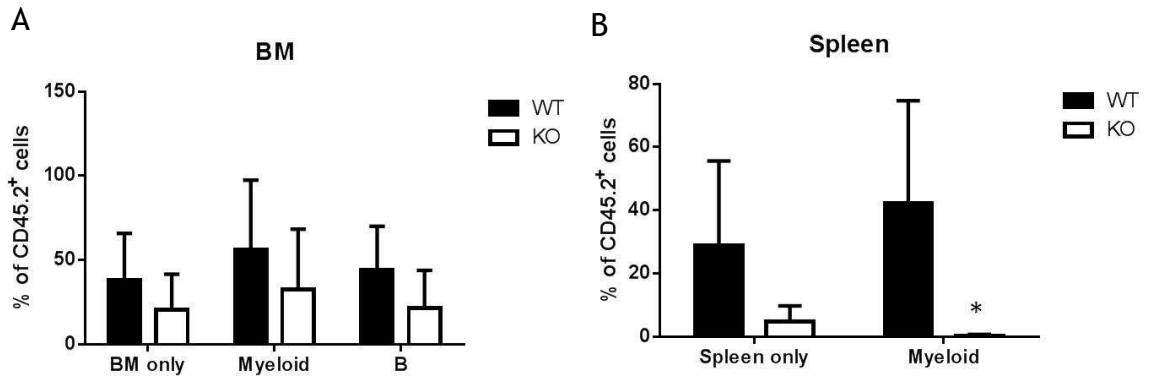

Figure 4-17 WT and *Cxcr2*$^{-/-}$ HSC show no differential engraftment in BM but show a decrease in myeloid cells in the spleen.

Data are presented as the mean percentage of CD45.2^{+} cells within whole BM (A) or spleen (B) myeloid and B cells in recipient mice transplanted with either WT or *Cxcr2*$^{-/-}$ HSC after 16 weeks post transplant. Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (* P <0.05, n = 7, 6). Spleen B cell data was not available (B). Animals were between 6 to 12 weeks and mixed gender (donor WT 2 female; *Cxcr2*$^{-/-}$ 2 female; recipients WT 4 male, 3 female; *Cxcr2*$^{-/-}$ 4 male, 2 female).

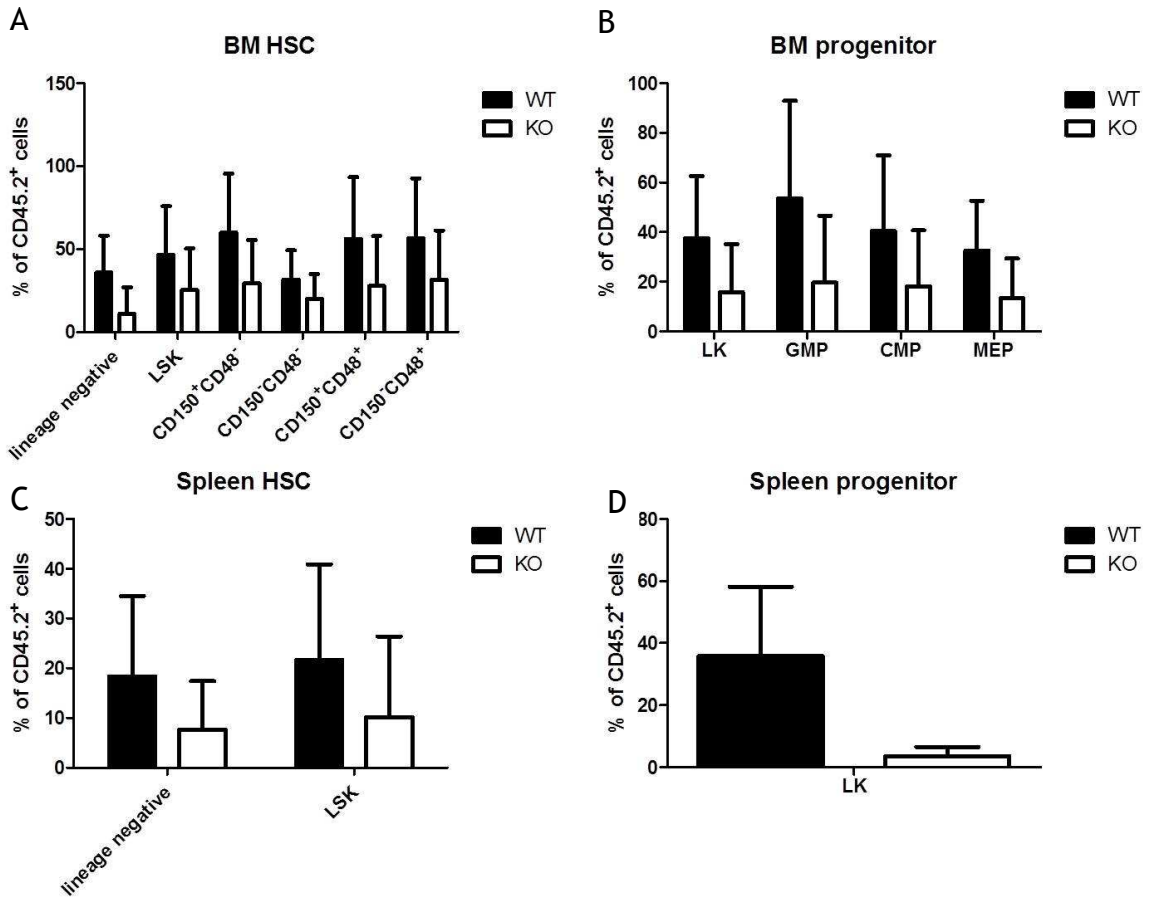

Figure 4-18 WT and *Cxcr2*$^{-/-}$ HSC show no differential engraftment in BM and spleen derived stem and progenitor cells.

Data are presented as the mean percentage of CD45.2^{+} cells within BM and spleen HSC and progenitor cell types. The percentage of CD45.2^{+} cells within BM HSC (A), BM progenitor (B), spleen HSC (C) and spleen progenitor (D) was assessed in recipient mice transplanted with either WT or *Cxcr2*$^{-/-}$ HSC after a 16 week period (A). Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n.s. $n = 7, 6$). Animals were between 6 to 12 weeks and mixed gender (donor WT 2 female; *Cxcr2*$^{-/-}$ 2 female; recipients WT 4 male, 3 female; *Cxcr2*$^{-/-}$ 4 male, 2 female).

4.3.7 Survival curve of WT and *Cxcr2*$^{-/-}$ animals over a year period

A BM reconstitution assays stresses the haemopoietic system so HSC will be forced to reconstitute the BM and functional activity can be assessed. As an alternative approach to stress the haemopoietic system, *Cxcr2*$^{-/-}$ and WT animals were aged for 52 weeks and the haemopoietic system was examined.

4.3.7.1 Survival curve

Firstly, it was observed that the *Cxcr2*$^{-/-}$ animals showed a decrease in survival in aged animals with 2 animals sacrificed out of 5 (67% survival) in comparison to WT animals with no deaths up to 52 weeks old (100% survival) (Figure 4-19). Limited assays were carried out on *Cxcr2*$^{-/-}$ animals post-mortem and it therefore cannot be concluded the reason for death of the animals. However, BM was analysed in one of the sacrificed mice and compared with a WT control at a similar age. The results showed that phenotype of the young mice is exaggerated with such an increase in the granulocyte population that the erythroid cells within the BM are at a very low level (Figure 4-19). Flow cytometry analysis of these populations on a WT aged mice was used as a comparison and it can be observed that an increase in myeloid cells and decrease in erythrocytes is strikingly observed in aged *Cxcr2*$^{-/-}$ animals. Based on this analysis, it is hypothesised that the BM became depleted of erythrocytes and this is likely to be the cause of premature death. However, this is purely speculative as more in depth assays were not carried out at this stage. Based on the survival animals, the experiments described in the following section used 5 WT and 3 *Cxcr2*$^{-/-}$ animals for analysis.

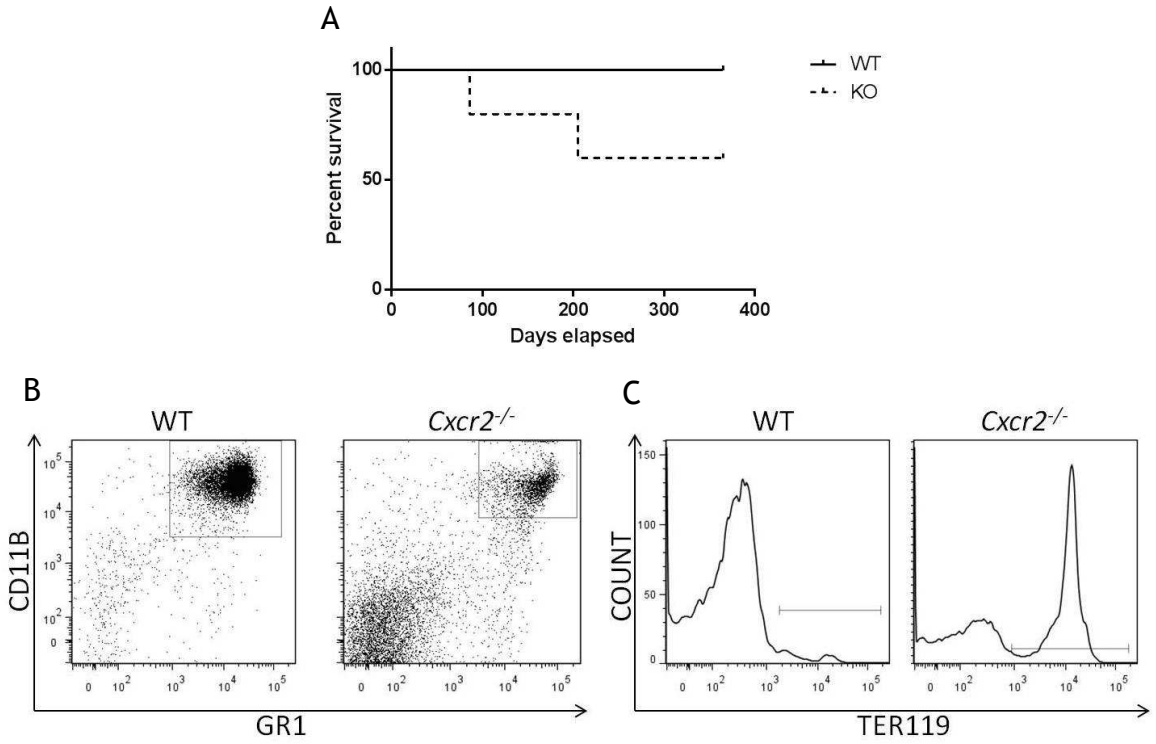

Figure 4-19 Survival curve for aged WT and *Cxcr2*$^{-/-}$ animals.

The data are presented as a survival curve for the percentage survival of WT or *Cxcr2*$^{-/-}$ animals up to a 52 week period where the surviving animals for sacrificed for analysis (n = 5) (A). Flow cytometry plots show granulocyte (B) and erythroid staining in the BM of a *Cxcr2*$^{-/-}$ animal that was sacrificed before the year period. Analysis of a WT control at a similar age was used as comparison (B, C). Animals were all F due to availability and between 48 and 52 weeks of age. A log-rank (Mantel-Cox) test was used to compare survival curves using percentage of survival over time and showed no statistically significant difference between the two survival curves (n = 5).

4.3.7.2 Cellularity and frequency of mature cell types

BM, spleen, PB and thymus were analysed for cellularity and absolute numbers of mature cell types including granulocytes, erythroid, B, and T cells in aged WT and *Cxcr2*$^{-/-}$ animals as described in section 4.3.2.

4.3.7.2.1 BM

The BM showed no difference in cellularity between WT and *Cxcr2*$^{-/-}$ conditions (n.s., n = 5, 3) (Figure 4-20). There was a significant decrease in erythroid cells in the *Cxcr2*$^{-/-}$ condition (P <0.01, n = 5, 3) (Figure 4-20). A trend towards an increase was found in the granulocytes in the *Cxcr2*$^{-/-}$ conditions (n.s., n = 5, 3) (Figure 4-20). Finally a trend towards a decrease was found in the B cells in the *Cxcr2*$^{-/-}$ condition which was not significant (n.s., n = 5, 3) (Figure 4-20).

4.3.7.2.2 Spleen

The spleen showed no difference in cellularity between WT and *Cxcr2*$^{-/-}$ conditions (n.s.) which likely reflects sample variation as an increase was noted in spleen size in *Cxcr2*$^{-/-}$ animals upon dissection (Figure 4-21). There was no difference in erythroid cells between strains (n.s.) (Figure 4-21). There was a trend towards an increase in the granulocytic population in the *Cxcr2*$^{-/-}$ condition (n.s.) (Figure 4-21). There was a trend towards a decrease in the B and T cell populations in the *Cxcr2*$^{-/-}$ condition (n.s., n = 5, 3) (Figure 4-21).

4.3.7.2.3 PB

The PB showed a trend towards increase in cellularity in the *Cxcr2*$^{-/-}$ conditions (n.s., n = 5, 3) (Figure 4-22). There was a trend towards an increase in erythroid cells in the *Cxcr2*$^{-/-}$ condition (n.s.) (Figure 4-22). A trend towards an increase was found in the granulocytes in the *Cxcr2*$^{-/-}$ condition (n.s.) (Figure 4-22). Finally a trend towards a decrease was found in the B cells in the *Cxcr2*$^{-/-}$ condition (n.s., n = 2, 3) (Figure 4-22). The lack of samples in these assays was due to technical problems with staining. T cells subsets were not analysed in the PB due to technical problems.

4.3.7.2.4 Thymi

The thymi showed no difference in cellularity between WT and *Cxcr2*$^{-/-}$ conditions (n.s., n = 5, 3) (Figure 4-23). There was no difference in T cells in the thyme however only double positive cells ($CD4^+CD8^+$) were analysed (n.s., n = 5, 3) (Figure 4-23).

Collectively, the data shows the same trend in the frequency of mature cell types between the conditions as in the young mice. The lack of significance for particular cell types including granulocytes in the BM, spleen and PB reflects a small sample size and variation between samples.

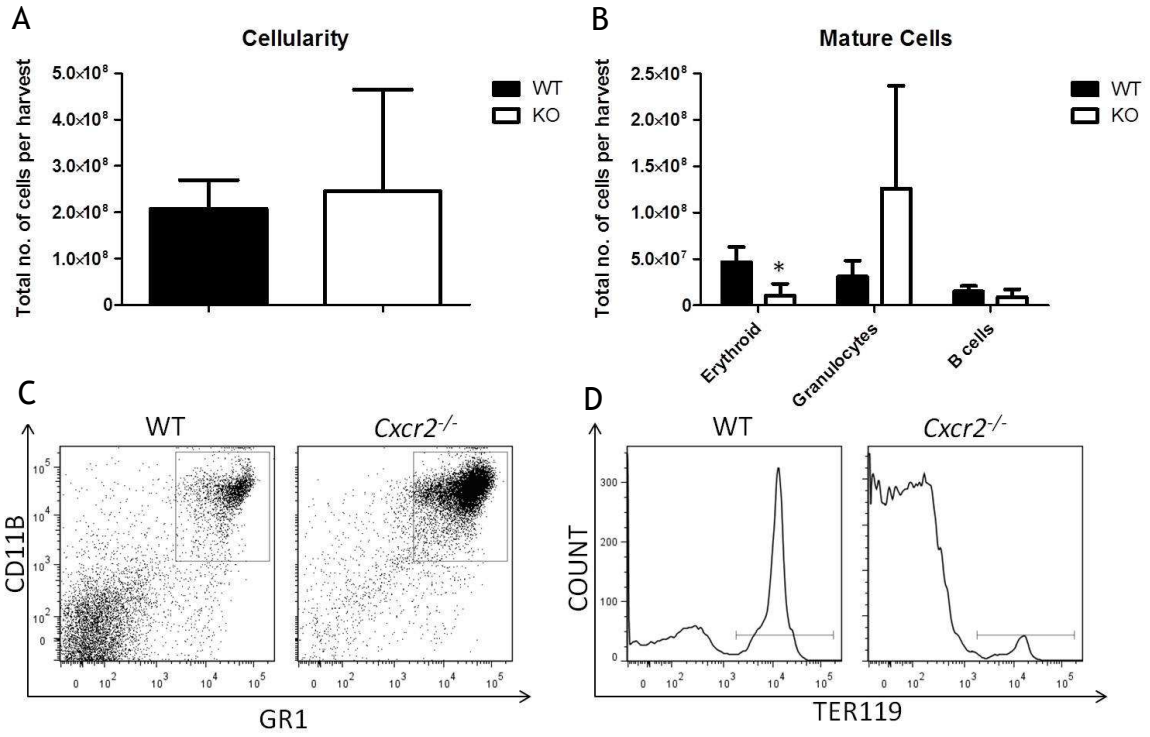

Figure 4-20 Cellularity and absolute numbers of mature cell types in BM of WT and *Cxcr2*$^{-/-}$ aged animals.

Whole BM was assessed for cellularity and the absolute number of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of mature cells; erythroid (TER119^{+}), granulocyte (GR1^{+}CD11B^{+}) or B cells (CD19^{+}B220^{+}) (B). Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (* P <0.05; n = 5, 3). Plots display a representative image of staining observed with GR1^{+}CD11B^{+} dotplots (C) or TER119^{+} histogram (D). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

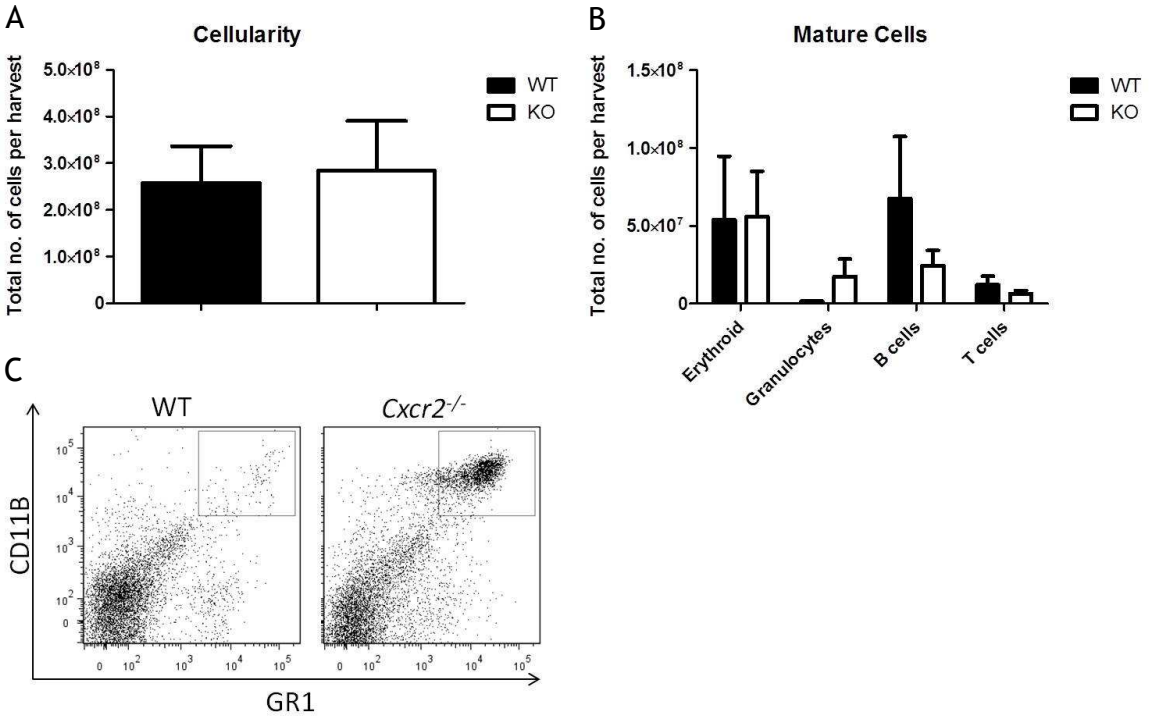

Figure 4-21 Cellularity and absolute numbers of mature cell types in spleen of WT and *Cxcr2*$^{-/-}$ aged animals.

Spleen was assessed for cellularity and the absolute number of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of mature cells; erythroid (TER119^{+}), granulocyte (GR1^{+}CD11B^{+}), B cells (CD19^{+}B220^{+}) or T cells (CD4^{+}CD8^{+}) (B). Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n = 5, 3). Plots display a representative image of staining observed with GR1^{+}CD11B^{+} dotplots (C). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

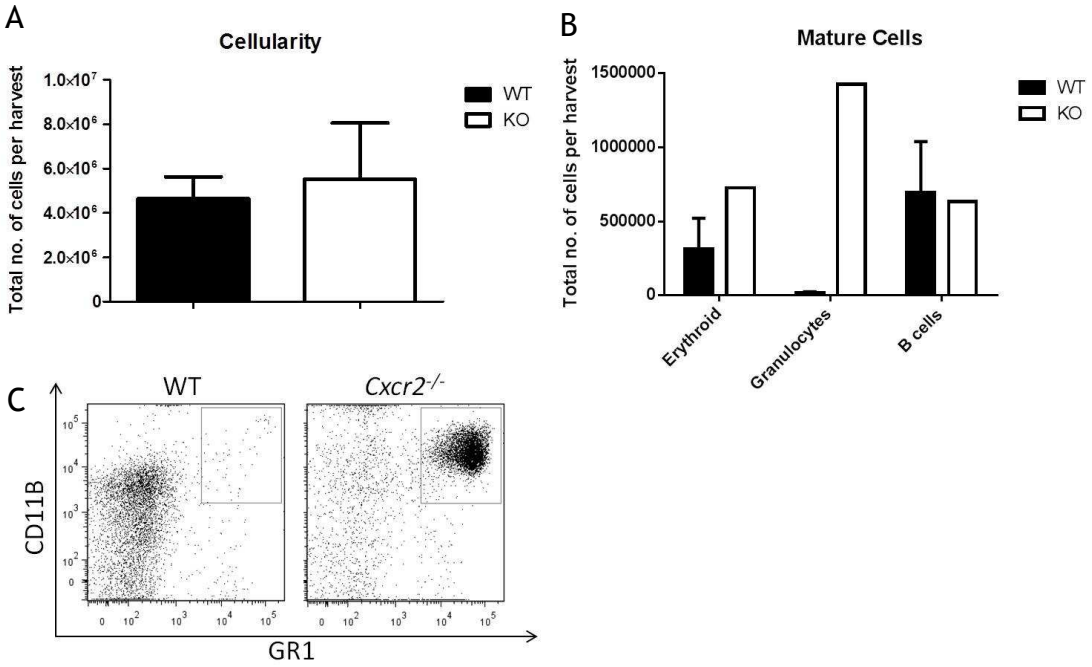

Figure 4-22 Cellularity and absolute numbers of mature cell types in PB of WT and *Cxcr2*$^{-/-}$ aged animals.

PB was assessed for cellularity and the absolute number of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) (n = 5, 3) or absolute numbers of mature cells; erythroid (TER119^{+}), granulocyte (GR1^{+}CD11B^{+}) or B cells (CD19^{+}B220^{+}) (B) (n = 2, 1). Statistical analysis was carried out using a student's unpaired t test with Welch's correction for unequal variance (A) and no statistical test was carried out in panel B. Plots display a representative image of staining observed with GR1^{+}CD11B^{+} dotplots (C). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

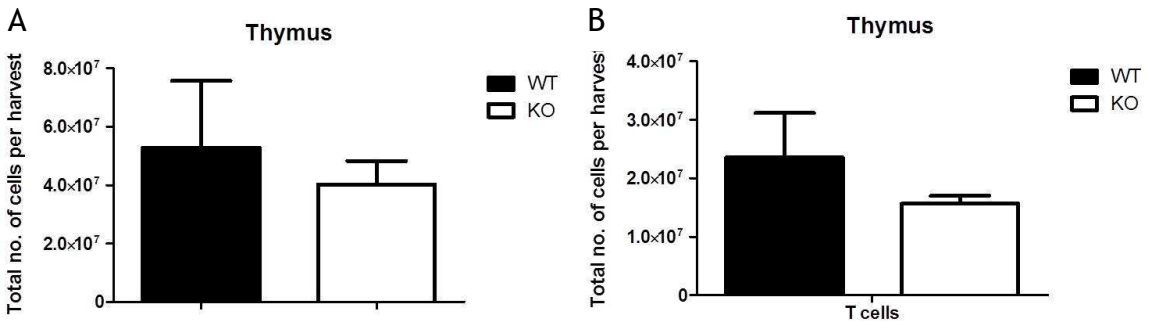

Figure 4-23 Cellularity and absolute numbers of mature cell types in thymi of WT and *Cxcr2*$^{-/-}$ aged animals.

Thymi was assessed for cellularity and the absolute number of mature cells was assessed using flow cytometry. Data are presented as the mean total number of cells (WBC) between strains (A) or absolute numbers of T cells ($CD4^+CD8^+$) (B). For this particular experiment, CD4 and CD8 antibodies were used with the same fluorochrome therefore only double positive population data is available ($CD4^+CD8^+$). Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n = 5, 3). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

4.3.7.3 Frequency of stem and progenitor cells in aged WT and *Cxcr2*$^{-/-}$ mice

The frequency of stem and progenitor populations was examined in aged WT and *Cxcr2*$^{-/-}$ animals as described in section 4.3.3.

4.3.7.3.1 BM

4.3.7.3.1.1 Stem cell frequency

There was a trend towards a decrease in the number of lineage negative cells in the *Cxcr2*$^{-/-}$ animals which can be seen with representative flow cytometry plots (n.s., n = 5, 3) (Figure 4-24; Figure 4-25). There was a trend towards an increase in the number of LSK cells in the *Cxcr2*$^{-/-}$ animals (n.s., n = 5, 3) (Figure 4-24; Figure 4-25). Finally there were no significant differences between the various HSC populations (n.s., n = 3, 5) (Figure 4-24; Figure 4-25).

4.3.7.3.2 Progenitor populations

In terms of progenitors, the LK fraction showed no difference between strains (n.s., n = 5, 3) (Figure 4-26). There were no statistically significant differences between GMP, CMP and MEP populations between strains however representative flow cytometry plots show the differential distribution of populations between strains (n.s., n = 3, 5) (Figure 4-26). However the trends showed the same as found in the young animals. The lack of significance likely reflects the small sample size.

The lineage negative and LSK frequency in the BM show a similar trend to those obtained in young animals (Figure 4-7). However, no difference was noted between strains for the HSC populations. This could be due to smaller sample sizes in the ageing analysis or it is possible that there are no differences in the frequency of HSC populations in aged animals. Interestingly, an increase (9.7 fold) is noted in the absolute number of LT-HSC in WT BM derived from young and aged animals. This is in accordance with previous literature to show that LT-HSC are increased in frequency in response to ageing, but display decreased functionality (Snoeck, 2013). Although an increase is still noted between young and aged LT-HSC in the *Cxcr2*$^{-/-}$ animals, the fold increase is lower than that observed with the WT (2.8 fold). It is possible that an expansion of LT-HSC occurs at an earlier stage in the *Cxcr2*$^{-/-}$ animals therefore no differences are seen in aged mice as an expansion is also

noted in the WT. This would infer that the HSC have reduced self renewal potential, however this cannot be concluded at this stage.

4.3.7.3.3 Spleen

4.3.7.3.3.1 Stem cell populations

There was a trend towards an increase in the number of lineage negative cells in the *Cxcr2*$^{-/-}$ animals in the spleen (n.s., n = 5, 3) (Figure 4-27). There was no differences in the number of LSK cells between strains (n.s., n = 5, 3) (Figure 4-27). Finally there were no significant differences between the various HSC populations (n.s., n = 3, 5) (Figure 4-27).

4.3.7.3.3.2 Progenitor populations

In terms of progenitors, the LK fraction showed a trend towards an increase in the *Cxcr2*$^{-/-}$ condition (n.s., n = 5, 3) (Figure 4-28). There were no statistically significant differences between GMP, CMP and MEP populations between strains (n.s., n = 3, 5) (Figure 4-28). However the lack of significance likely reflects the small sample size, the differential distribution of cell types can be seen with representative flow cytometry plots between strains and the data showed the same trends as in the young mice.

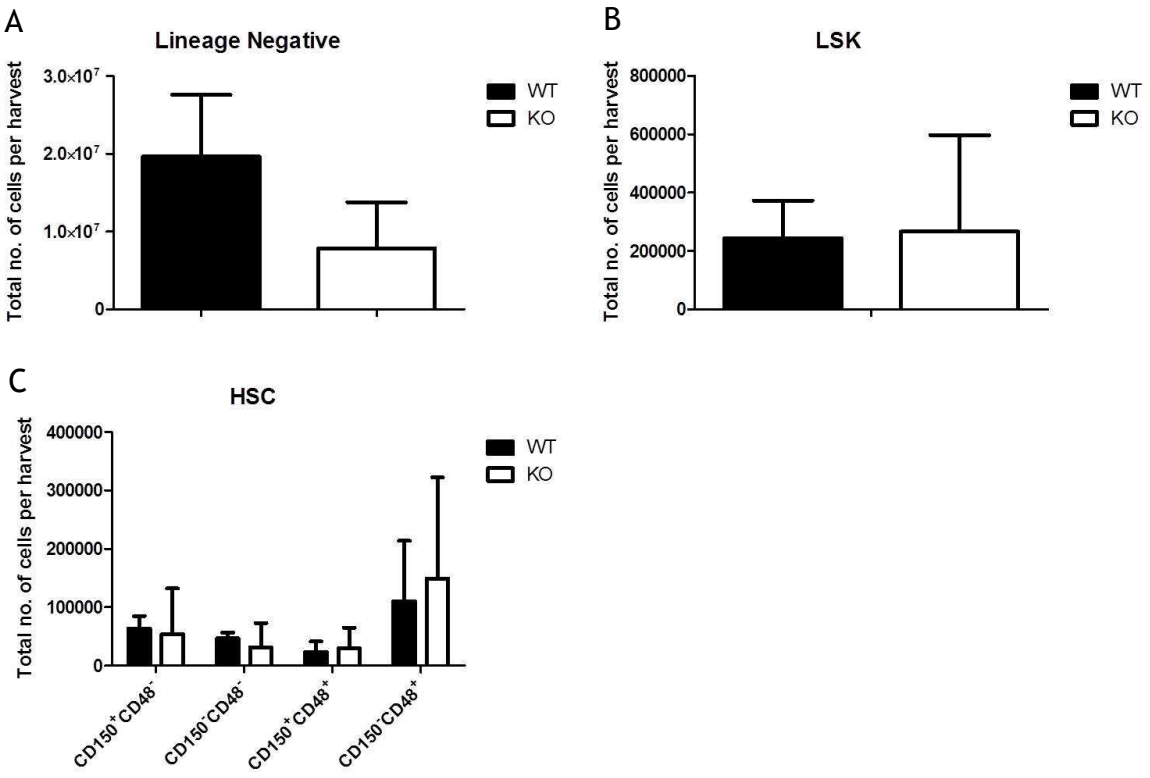

Figure 4-24 Absolute numbers of stem cell populations in the BM between WT and *Cxcr2*$^{-/-}$ aged animals.

Whole BM was made into a single cell suspension and stained for antibodies to examine the stem cell populations and analysed using flow cytometry. Data are presented as the mean±standard deviation absolute cell numbers for lineage negative, LSK or HSC populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., n = 5, 3). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

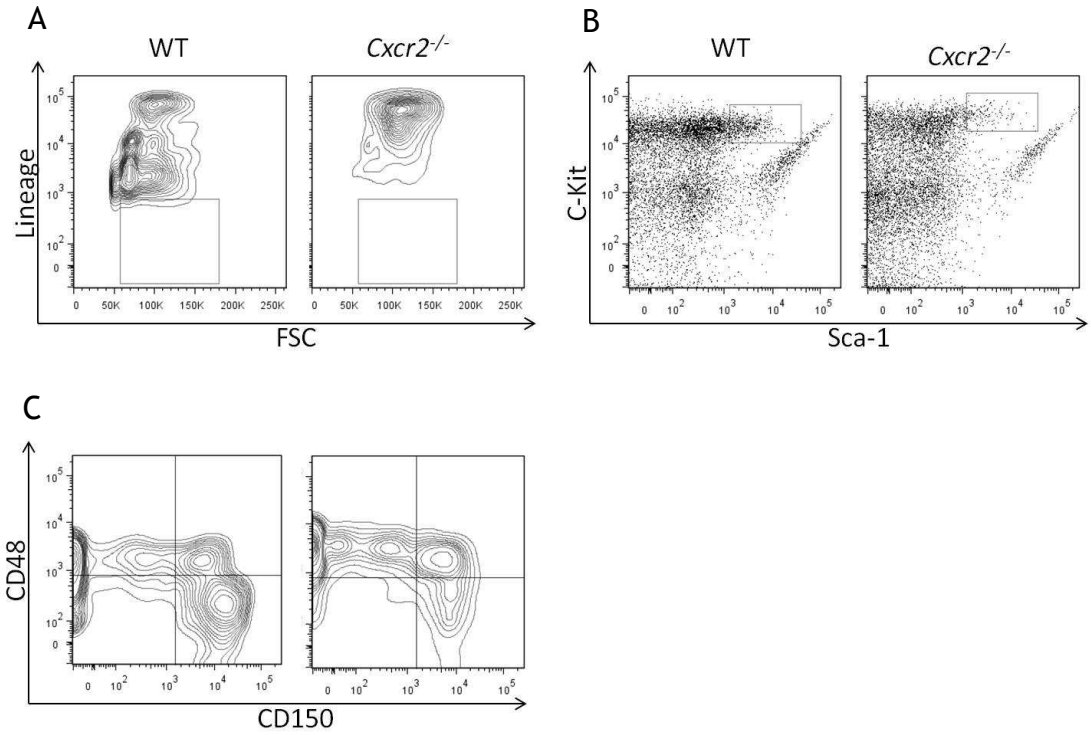

Figure 4-25 Representative flow cytometry plots of lineage negative, LSK and HSC populations between WT and *Cxcr2*$^{-/-}$ animals in aged animals.

Images show representative flow cytometry plots of lineage negative (A), LSK (B) and HSC staining (C) in WT and *Cxcr2*$^{-/-}$ animals. Animals were all F due to availability and between 48 and 52 weeks of age.

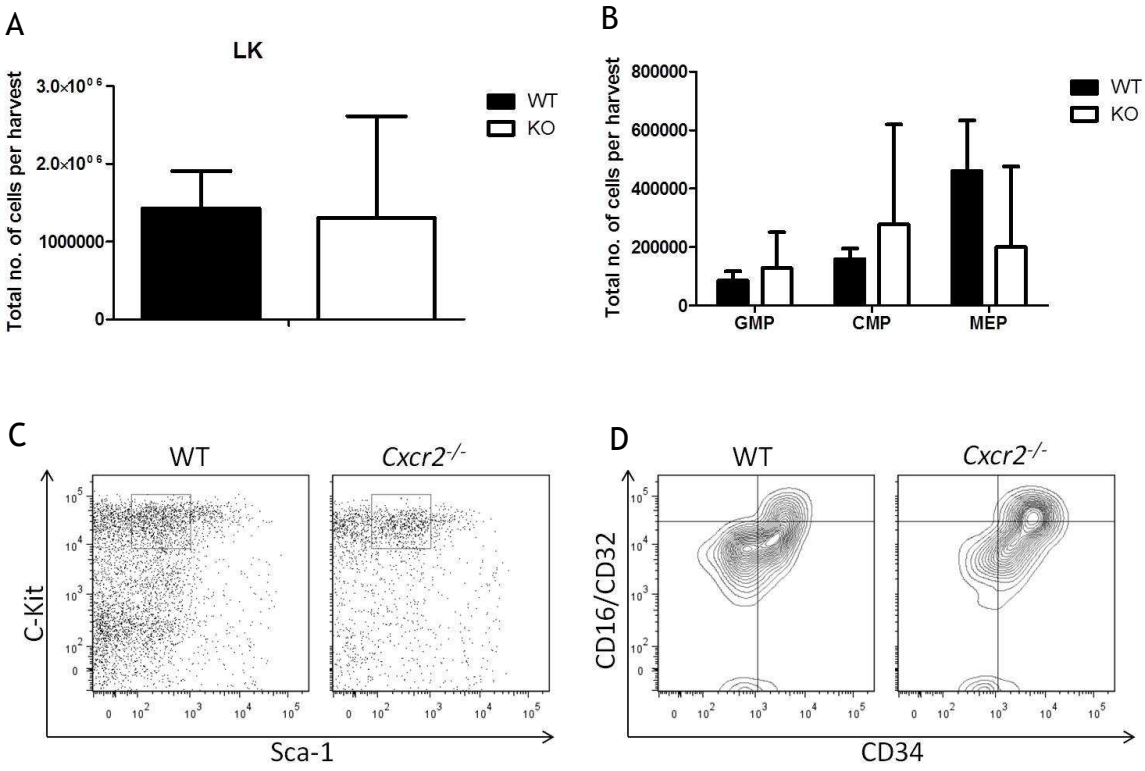

Figure 4-26 Absolute numbers of progenitor populations between WT and *Cxcr2*$^{-/-}$ animals.

Whole BM was made into a single cell suspension and stained for antibodies to examine the progenitor populations and analysed using flow cytometry. Percentages of cells within whole BM was multiplied by the WBC cellularity of the BM and absolute numbers compared. Data are presented as the mean absolute cell numbers for LK, CMP, GMP and MEP populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., $n = 5$, 3). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

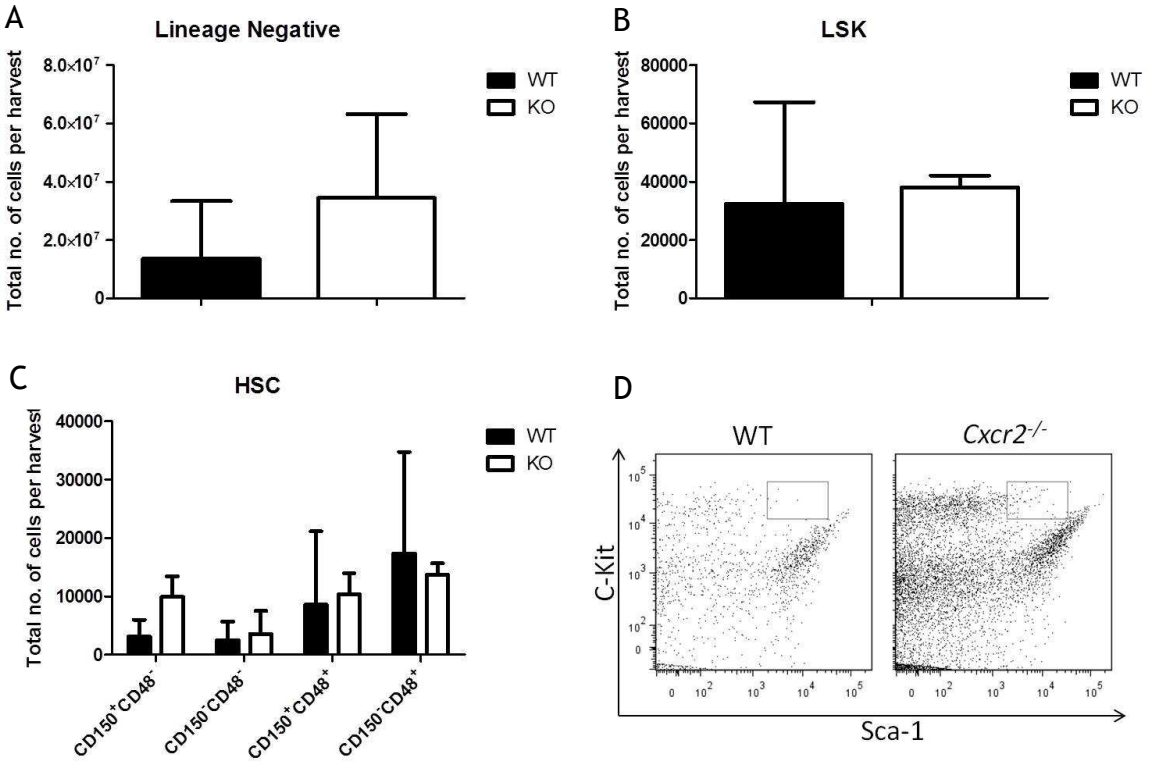

Figure 4-27 Absolute numbers of stem cell populations between WT and *Cxcr2*$^{-/-}$ aged animals in spleen.

Spleen was made into a single cell suspension and stained with antibodies to examine the stem cell populations and analysed using flow cytometry. Percentages of cells within whole spleen was multiplied by the WBC cellularity of the spleen and absolute numbers compared. Data is presented as the mean absolute cell numbers for lineage negative, LSK or HSC populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., $n = 5$, 3). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

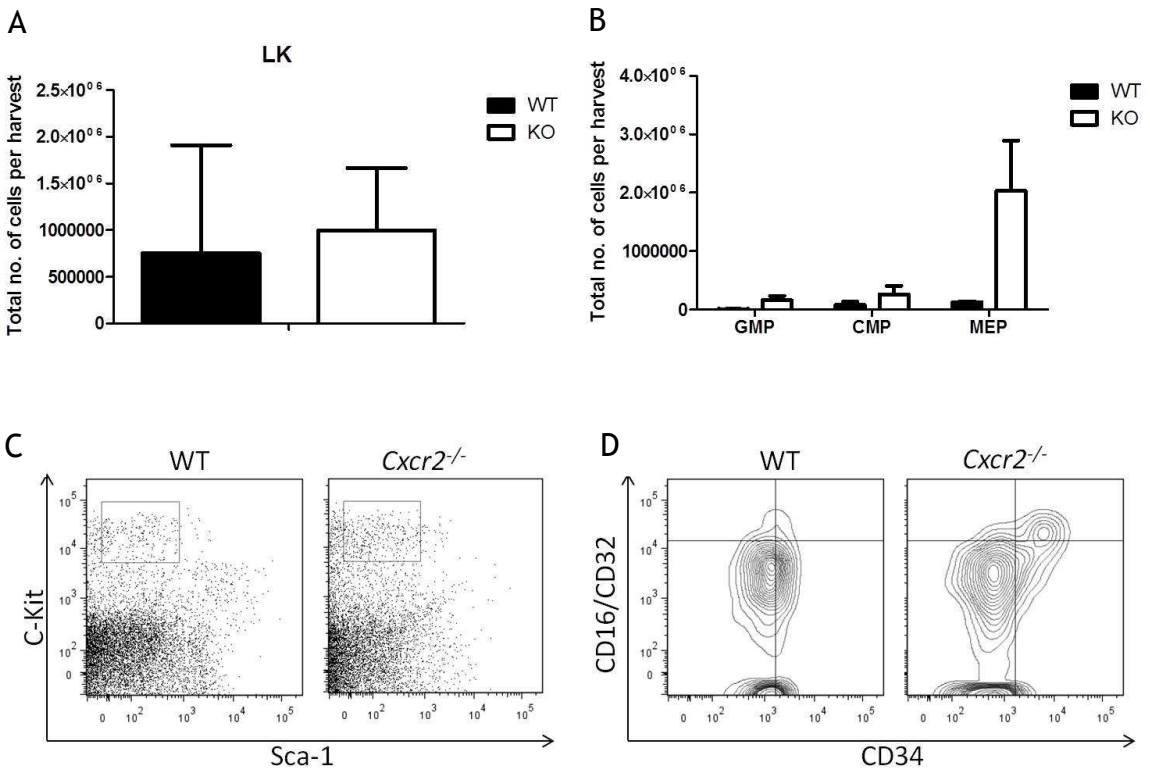

Figure 4-28 Absolute numbers of progenitor populations between WT and *Cxcr2*$^{-/-}$ animals in the spleen.

Spleen was made into a single cell suspension and stained with antibodies to examine the progenitor populations and analysed using flow cytometry. Percentages of cells within whole spleen was multiplied by the WBC cellularity of the spleen and absolute numbers compared. Data is presented as the mean absolute cell numbers for LK, CMP, GMP and MEP populations between WT and *Cxcr2*$^{-/-}$ animals. Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., $n = 5$, 3). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

4.3.7.4 CFC assays

As an *in vitro* measure of the stem/progenitor activity in a population, cells from aged animals were plated in Methocult™ and examined for colony growth as described in section 4.3.4.

4.3.7.5 BM

No difference in colonies was found in the BM between strains. Colonies were pooled and counted as total number of colonies as no difference in different types were found (n.s., n = 3, 1) (Figure 4-29). No statistical analysis was carried out due to the sample size of 1 in the *Cxcr2*$^{-/-}$ population. This was due to contamination in several samples. Colonies were reseeded into a secondary colony formation assay to get an indication of the self renewal activity of the stem/progenitor cells. A trend towards an increase was noted in the *Cxcr2*$^{-/-}$ condition, however small sample size does not allow for statistical analysis (n.s., n = 3, 1) (Figure 4-29).

4.3.7.6 Spleen

There is a trend towards an increase in CFU-GM colonies was found in the spleen. No difference in CFU-E or CFU-GEMM colonies were found. The increase in CFU-GM colonies causes an overall trend towards increase in the total number of colonies in the *Cxcr2*$^{-/-}$ animals (n.s., n = 5, 3) (Figure 4-30). The lack of significance is likely due to variation between samples in this assay and a small sample size in one of the conditions.

Not enough material from PB in aged animals was available for colony formation assays. It is predicted an increase would be found in the *Cxcr2*$^{-/-}$ condition as reported in the young animals.

Taken collectively, the data shows the same trend as in young animals. The lack of difference in CFU in the BM of *Cxcr2*$^{-/-}$ animals is likely technical due to small numbers of CFU obtained.

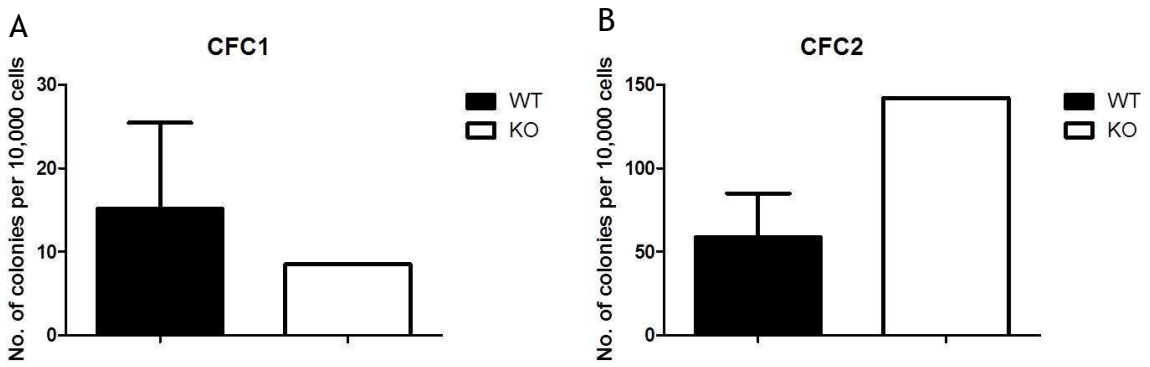

Figure 4-29 WT and *Cxcr2*$^{-/-}$ CFC analysis in BM derived cells shows no difference between strains in primary or secondary plates in aged animals.

Whole BM was made into a cell suspension, WBC counted and 10^5 cells plated per mL in Methocult™ and incubated for 10-14 days. Data are presented as the mean total number of colonies in a primary (A) and secondary replate (B) (n = 3, 1). No statistical test was carried out due to the sample size of 1 in one of the conditions. Animals were between 6 to 12 weeks and the same gender (WT 3 female; *Cxcr2*$^{-/-}$ 1 female).

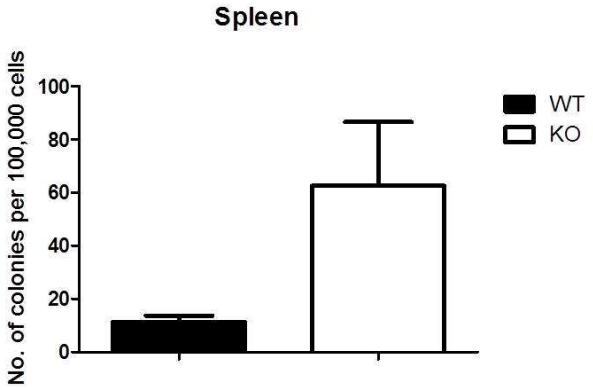

Figure 4-30 WT and *Cxcr2*$^{-/-}$ CFC analysis in spleen derived cells shows no difference between strains in aged animals.

The spleen was made into a cell suspension, WBC counted and 10^5 cells plated per mL in Methocult™ and incubated for 10-14 days. Data are presented as the mean total number of colonies in a primary plating assay (n = 5, 3). Statistical analysis was carried out using a student's unpaired *t* test with Welch's correction for unequal variance (n.s.). Animals were between 6 to 12 weeks and the same gender (WT 5 female; *Cxcr2*$^{-/-}$ 3 female).

4.3.7.7 Stem cell viability and cell cycle status

The stem and progenitor populations were examined for viability and cell cycle status in aged mice as described in section 4.3.5.

Data shows a trend towards a decrease in the percentage of Annexin-V^+ cells in cell populations in the *Cxcr2*$^{-/-}$ animals in the LSK, $CD150^+CD48^-$, $CD150^-CD48^-$ (P <0.05), $CD150^+CD48^+$ (P <0.01) and $CD150^-CD48^+$ populations (P <0.05). A total of 5 and 3 animals for WT and *Cxcr2*$^{-/-}$ conditions were analysed respectivel (Figure 4-31). A trend towards an increase in Annexin-V^+ cells was found in the *Cxcr2*$^{-/-}$ animals in the whole BM, lineage negative and LK fractions but this is not statistically significant and likely represents no differences between populations (n.s., n = 5, 3) (Figure 4-31).

The data suggests that the stem/progenitor populations are more viable in the *Cxcr2*$^{-/-}$ animals in comparison to the WT littermates. This is in accordance with data obtained on HSC populations in young mice. Furthermore, the increase in sample sized in aged animals allowed statistical analysis. It can seen from data on the WT cells that there is a basal level of apoptosis in BM cells which decreases in the LSK and more primitive HSC populations which suggests the staining is correct. Interestingly, in the WT mice in both young and aged mice show a high percentage of Annexin-V^+ cells in the $CD150^+CD48^+$ (MPP) population in comparison to the other HSC fractions suggesting this is a more apoptotic population. In addition, the $CD150^+CD48^+$ population in the *Cxcr2*$^{-/-}$ mice shows a lower percentage of Annexin-V^+ cells in comparison to the WT.

Ki-67 staining was used to examine proliferation, although this stain used alone cannot discriminate between cells in G_0 versus G_1, percentage of positive cells can indicate proliferation. Data shows a trend towards decrease in the percentage of Ki-67^+ cell populations *Cxcr2*$^{-/-}$ animals in the whole BM, lineage negative and LK populations (Figure 4-31) (n.s., n = 4, 2).

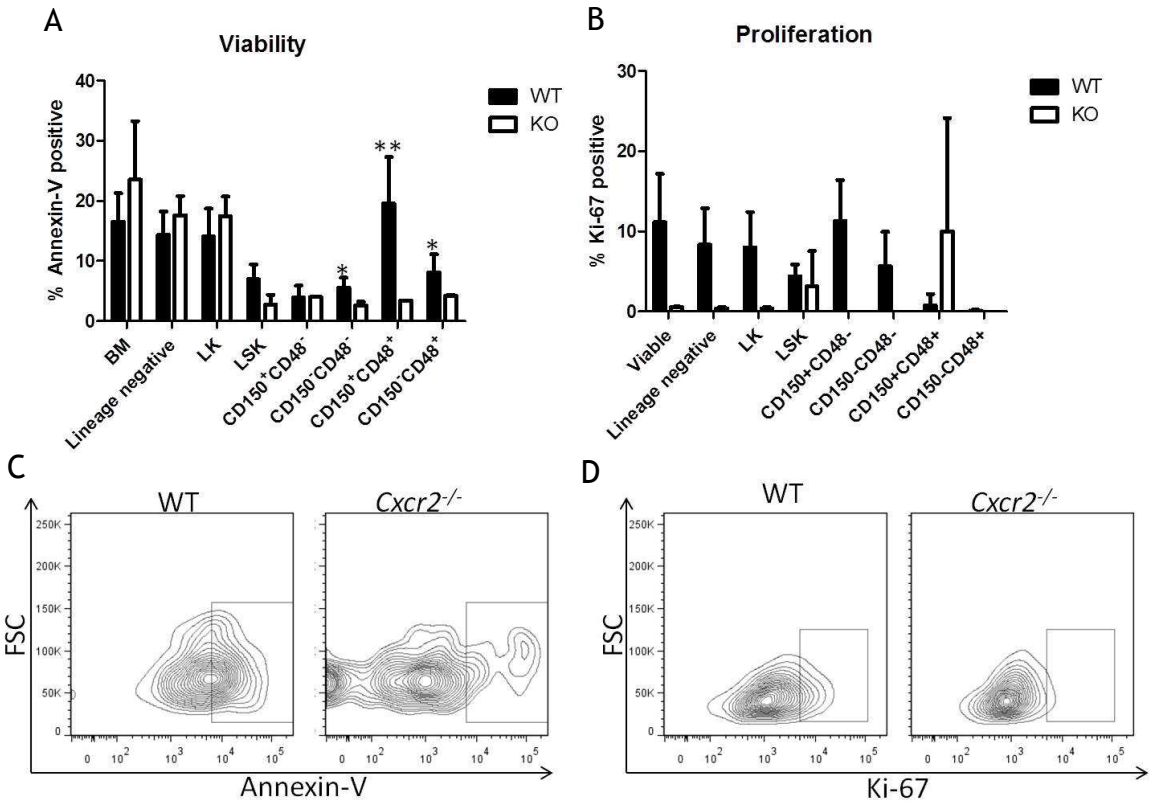

Figure 4-31 *Cxcr2*$^{-/-}$ HSC show an increase in viability in aged HSC.

WT or *Cxcr2*$^{-/-}$ stem and progenitor populations were stained for Annexin-V and analysed for viability or fixed, permeabilised and stained for Ki-67. Data are presented as the mean percentage of Annexin-V^{+} cells (n = 5, 3) (A) or Ki-67^{+} cells (B) (n = 4, 2). Statistical analysis was carried out using a student's unpaired t test with Welch's correction for unequal variance. Sample size is too low for the *Cxcr2*$^{-/-}$ conditions in the Ki-67 experiment, therefore no statistical analysis is shown. Representative plots of Annexin-V staining in the CD150^{+}CD48^{+} fraction (C) or Ki-67 staining in the LK fraction (D) are displayed. Animals were between 6 to 12 weeks and the same gender (A WT 5 female; *Cxcr2*$^{-/-}$ 3 female; B WT 4 female; *Cxcr2*$^{-/-}$ 2 female).

4.4 Discussion

The results in this chapter focus on the analysis of a mouse model which lacks *Cxcr2*. The results are found to be in accordance with published literature which shows an increase in the myeloid cells in the haemopoietic organs, and an increase in CFU in the spleen and PB in the *Cxcr2*$^{-/-}$ animals (Broxmeyer et al., 1996, Cacalano et al., 1994). However, it has not been examined to date whether this signalling pathway may play a role in HSC function.

Previous literature which first documented the phenotype of the *Cxcr2*$^{-/-}$ mice showed data to suggest that *Cxcr2* signalling controlled myeloid regulation (Broxmeyer et al., 1996). Therefore it was proposed this signalling pathway is controlling negative regulation of myeloid cells. However, if *Cxcr2* signalling was solely controlling myeloid regulation, there would be no additional phenotype in *Cxcr2*$^{-/-}$ animals particularly involving the HSC population. More specifically, this does not explain the expansion of the LT-HSC in the BM and enhanced mobilised with extramedullary haemopoiesis in the spleen and PB. In addition, this does not explain why *Cxcr2*$^{-/-}$ HSC show a defect in BM engraftment.

The expansion of the myeloid cells is due to a lack of *Cxcr2* signalling. However it was interesting to observe that the progenitor populations (GMP, CMP and MEP) show a trend towards differences in the *Cxcr2*$^{-/-}$ animals in comparison to the WT controls. If this was to be repeated and gain statistical significance, this could suggest that *Cxcr2* regulates myeloid cell production at a more primitive level than at the mature cells. In addition to an increase in myeloid cells, the *Cxcr2*$^{-/-}$ animals show an increase in the HSC in comparison to the controls in the BM. It is possible that *Cxcr2* signalling contributes to establishing numbers of HSC in addition to mature cells. A previous study has noted that *Cxcr2* signalling is involved in the regulation of oligodendrocyte numbers as *Cxcr2*$^{-/-}$ animals show differences in comparison to the controls (Padovani-Claudio et al., 2006).

In addition to the expansion of LT-HSC in the BM of *Cxcr2*$^{-/-}$ animals, the HSC were shown to be more viable than the WT counterparts. Although a sample size of 1 in the young animals does not allow a valid conclusion to be drawn. However, from the human data in the previous chapter it is predicted that a lack of *Cxcr2* would decrease cell viability. It is possible that mechanisms of signalling differ between species and this should be further examined. It could be inferred that *Cxcr2* signalling negative controls stem cell viability, or alternatively the lack of the receptor is causing a compensatory effect resulting in an increase in viability. As an example, it could be that *Cxcr2* reduction results in an

increase in *Cxcr1* expression. In support of this, *Cxcr1* and *Cxcr2* have been shown to show compensatory effects with each other. Furthermore, mRNA transcripts of CXCR1 have been shown to be expressed in the BM and spleen. In a previous study, *Cxcr2*$^{-/-}$ animals showed an over expression of *Cxcr1* in endothelium in comparison to levels in WT mice (Sanchez et al., 2007). Studies inhibiting *Cxcr1* have shown a decrease in cell viability, it is therefore likely that an increase in expression could support viability however this has not been tested in this study (Ginestier et al., 2010).

The true functional assay of a stem cell is the ability to reconstitute a host with a lethally ablated BM. In this experiment, it was found that there was a trend towards a reduction in the ability of the HSC derived from the *Cxcr2*$^{-/-}$ donor to engraft to the same level as the WT donor HSC. This suggests that *Cxcr2* signalling may have an effect on stem cell function in a cell autonomous manner. However, inter experimental variation resulted in the difference between conditions to be not significant. Therefore, it cannot be concluded that *Cxcr2* null HSC show a defect in functional activity with the results in this thesis. However, the data indicates there may be difference between samples. If this is the case, it is unclear from this experiment whether the HSC derived from the *Cxcr2*$^{-/-}$ donor show a trend towards a decrease in engraftment due to a defect in homing or due to a problem within the BM niche. Indeed, CD45.2^{+} cells derived from the *Cxcr2*$^{-/-}$ donors were found to produce multilineage reconstitution and a percentage of CD45.2^{+} cells from the *Cxcr2*$^{-/-}$ recipients were found in all mature cells, stem and progenitor cells. Furthermore, the percentage engraftment from donor *Cxcr2*$^{-/-}$ HSC increased over time which suggests the HSC were capable of self renewal. It is known that *Cxcr2* signalling plays a role in neutrophil mobilisation and it is possible that this receptor may play a role in HSC mobilisation (Eash et al., 2010). Indeed, a variety of studies have shown that administration of *Cxcr2* ligands results in the immediate mobilisation of HSC from the BM into the periphery which is *Cxcr2* dependent (Pelus and Fukuda, 2006). Furthermore, previous research reported that *Cxcr2* signalling in combination with *Cxcr4* (a key HSC mobilisation gene) regulated neutrophil mobilisation (Eash et al., 2010). Whether these genes may play a role together in HSC mobilisation has not been assessed. To support this idea, the evidence that CFU are found in extramedullary sites of haemopoiesis in *Cxcr2*$^{-/-}$ animals does indicate that there may be a defect in HSC mobilisation. The results show that *Cxcr2*$^{-/-}$ HSC do not locate to the PB or spleen instead of the BM as a reduction in engraftment was found in all haemopoietic sites. Therefore if the HSC cannot home to the BM it is likely that they undergo apoptosis.

Data from this chapter showed that mouse HSC populations, including the most primitive LSKCD150^{+}CD48^{-} fraction express the receptor, *Cxcr2*. However due to limited cell numbers and time constraints in this study, although mRNA data is available on the receptor. To strengthen this result, protein expression should be assayed. To date, very few chemokine receptors have been reported on HSC. The majority of research has been on CXCR4 which responds to CXCL12 resulting in HSC migration to and from the BM. Gene expression analysis for CXCR2 binding ligands on HSC showed no positive expression for the ligands tested. This could be technical, or that they are expressed on a less primitive stem cell population. It cannot be concluded from the experiments conducted in this study what is the mechanism of action of CXCR2 signalling. This presents a future avenue of research.

5 Results III: Human and mouse HSC express CXCL4 which regulates HSC self renewal

5.1 Introduction

The results shown in chapters 4 and 5 provide evidence to support that CXCR2 signalling plays a role in stem cell properties including survival and proliferation in both human and mouse systems. However, a discrepancy between the human and mouse experiments exists. In chapter 4, it was shown that CXCR2 binding ligands (*Cxcl1, Cxcl2* and *Cxcl5/6)* mRNA transcripts were found in human HSC. However, in chapter 5 it was shown that the mouse homologs of these specific genes were not expressed by the mouse HSC populations tested. Explanations for this result were previously discussed. In addition to *Cxcr2* binding ligands, another member within the CXC family of chemokines (*Cxcl4*) was examined for expression on mouse HSC populations. We investigated *Cxcl4* expression on mouse HSC for several reasons; 1. *Cxcl4* is within the CXC family of chemokine ligands and is structurally similar to *Cxcr2* binding ligands; 2. Examination of previously unpublished microarray data from our lab identified *Cxcl4* as being highly expressed in mouse HSC populations at levels comparable to stem cell gene *Cxcr4* (data not shown, unpublished data); 3. Literature searching identified that *Cxcl4* supports human HSC survival and adhesion (reviewed in the introduction section). This chemokine therefore seemed to be an ideal candidate to further pursuit. The experiments in this chapter were designed to elucidate the biological role of *Cxcl4* in mouse HSC behaviour. Furthermore, we wanted to examine *CXCL4* expression in human HSC to examine whether this is a novel stem cell factor highly expressed in both mouse and human HSC populations.

Cxcl4 is a chemokine belonging to the CXC group, which is predominantly expressed by activated platelets and promotes blood coagulation (reviewed in the introduction section). There is some literature available on the role of *Cxcl4* signalling in human stem/progenitor cells (reviewed in introduction section) with evidence that *Cxcl4* inhibits haemopoeisis and promotes stem/progenitor survival, however this is not well understood. Furthermore, as far as we have understood, there are no data on mouse *Cxcl4* in terms of HSC behaviour. In this chapter, expression of the chemokine was examined in both mouse and human HSC systems. In addition, the functional role was investigated in the mouse system using transgenic *Cxcl4-Cre* driven reporter mice, *Cxcl4* null mice ($Cxcl4^{-/-}$) and reduction of *Cxcl4* expression *in vitro* using shRNA mediated lentiviral transduction.

5.2 Aims and Objectives

The specific aims of this chapter were:

I To examine *Cxcl4* expression on mouse HSC populations

It was hypothesised that this chemokine may play a role in HSC properties therefore mRNA expression was analysed in mouse HSC populations using Q-PCR.

II To examine whether a *Cxcl4-Cre* driven reporter mouse model activity correlates with gene expression in HSC populations

A reporter mouse model in which *Cxcl4* drives RFPexpression is available. We wanted to use this mouse model to validate the gene expression data. Furthermore, it was aimed to use this mouse model to elucidate the biological function of *Cxcl4.*

III To investigate how a lack of *Cxcl4* alters stem/progenitor cell behaviour

We wanted to use different approaches to modulate *Cxcl4* expression in an aim to understand the role of this chemokine in HSC behaviour. We used animals lacking *Cxcl4* (*Cxcl4*$^{-/-}$*)* and examined whether these animals displayed differences in stem/progenitor cell frequency, properties and function. As an alternative approach we aimed to reduce *Cxcl4* expression *in vitro* using shRNA and to examine the resulting phenotype.

V To examine the expression of *CXCL4* on human HSC

The microarray study used for this thesis was not conclusive for *CXCL4* expression on human HSC, therefore this objective was to assess whether this gene is also expressed in human HSC and may play a role in human HSC properties.

5.3 Results

5.3.1 CXCL4 is expressed on mouse HSC

Data in chapter 3 showed that *Cxcr2* is expressed on mouse HSC populations, however *Cxcr2* binding ligands were not detected by Q-PCR analysis. Other chemokines in the same functional group were examined for expression, in particular *Cxcl4* (also known as platelet factor 4). This chemokine is not a known ligand for *Cxcr2,* but is part of the CXC functional group and is structurally related to other CXC chemokine ligands. Furthermore there is evidence that *Cxcl4* can bind Cxcl8 *in vitro* (Dudek et al., 2003). In addition, *Cxcl4* was reported to be highly expressed in mouse HSC from a search in unpublished microarrays from our group (data not shown). The experiment in this section was carried out with Dr Amelie Guitart.

Q-PCR using the Fluidigm™ platform was used to examine *Cxcl4* expression in mouse HSC populations. Gene expression was examined in the LSK fraction sorted using CD150 and CD48 using the terminology described in chapter 4. Data showed detectable expression of *Cxcl4* in the LT-HSC ($CD150^{+}CD48^{-}$), ST-HSC ($CD150^{-}CD48^{-}$) and MPP ($CD150^{+}CD48^{+}$ and $CD150^{-}CD48^{+}$) populations. Examination of differences between populations revealed a trend towards decrease in the $CD150^{-}CD48^{-}$ fraction (n.s.), an increase in the $CD150^{+}CD48^{+}$ population (P <0.01) and a decrease in the $CD150^{-}CD48^{+}$ fraction (P <0.01) with the fold change calculated using the $CD150^{+}CD48^{-}$ fraction as the calibrator and set to the value of 1 (n = 3) (Figure 5-1). Gene expression of *Pf4* is compared to a gene highly expressed in HSC populations (*P21*) to demonstrate expression at relatively high levels (Cheng et al., 2000).

In summary, results show that *Cxcl4* is highly expressed on mouse HSC populations with the highest expression found in the MPP population. The observation that *Cxcl4* is highly expressed in mouse HSC populations including the LT-HSC population merited further investigation into the role of this chemokine in stem cell activity. As *Cxcl4* is well known to be expressed in cell types of megakaryocyte/platelet lineage, it is possible that *Cxcl4* is actively transcribed in the MPP population and marks cells destined for a particular lineage at an early stage in lineage differentiation. However, this is speculation as was not investigated further.

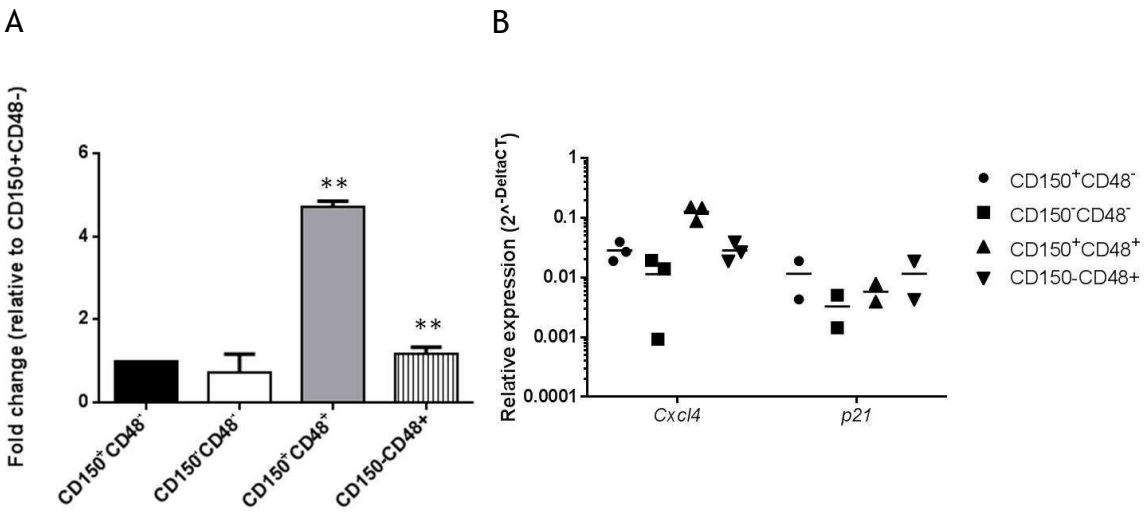

Figure 5-1 *Cxcl4* is expressed on mouse HSC at the mRNA level.

Normal BM was freshly isolated and stained for antibodies against LSK, CD150 and CD48. 500 cells were sorted, RT, preamplified and Q-PCR was carried out to assess *Cxcl4* mRNA expression. Results are shown relative to housekeeping control *β2M* fold change using the $CD150^{+}CD48^{-}$ population as a calibrator set to 1 (A) and displayed as relative expression levels ($2^{-DeltaCT}$) (B). Results displays the mean expression level from three independent experiments performed in triplicate (A). Panel B shows relative expression levels ($2^{-DeltaCT}$) with a stem cell gene to compare expression levels. Each dot represents average expression from three (*Cxcl4*) and two (*p21*) independent experiments (B). Animals were 6-12 weeks of age and males. Statistical test carried out was a repeated measures one-way ANOVA using Dunnett's multiple comparison test to compare differences between the LT-HSC fraction and each population for panel A only as *P21* expression was examined in two samples only ($n = 3$, ** $P < 0.01$).

5.3.2 Lineage tracing of Cxcl4 marks a proportion of HSC with enhanced colony formation activity

The gene expression data identifies that *Cxcl4* is highly expressed in HSC populations including the LT-HSC fraction. *Cxcl4-Cre (*commonly referred to as *Pf4-Cre)* is a widely used transgenic model which has been studied extensively previously (Tiedt et al., 2007). In this study, we used this reporter model to demonstrate that *Cxcl4-Cre* recombines in a proportion of BM derived HSC. This transgenic mouse model expresses Cre-recombinase in cells in which endogenous *Cxcl4* is expressed. The cross of *Cxcl4-Cre* with a reporter strain allows cells in which Cre-recombinase activity is active, to be identified and isolated. Briefly, *Cxcl4-Cre* mice were crossed with a tandem repeat *RFP* under the *Rosa26* promoter which should show RFP expression in cells expressing *Cxcl4* and their progeny. The *Rosa26-RFP*$^{+}$*;Cxcl4-Cre*$^{+}$ transgenic mouse model was used with *Rosa26-RFP*$^{+}$*;Cxcl4-Cre*$^{-}$ and *Rosa26-RFP*$^{-}$*;Cxcl4-Cre*$^{-}$ models used as controls which showed no difference in phenotype and were used interchangeably. The experiments in this section were carried out in collaboration with Dr Simon Calaminus.

5.3.2.1 *Cxcl4-Cre* is expressed in HSC and subsequent progeny

The transgenic model has been used previously to show RFP expression in cells positive for *Cxcl4* expression including megakaryocytes and platelets (Tiedt et al., 2007). However, an in depth analysis of the reporter model in HSC populations has not been examined to date. Results from section 5.3.1 demonstrate that endogenous *Cxcl4* is expressed in mouse HSC. We wanted to use the reporter mouse model to 1. Provide validation endogenous *Cxcl4* is expressed in HSC and 2. Elucidate the biological function of *Cxcl4*. Mice were analysed for expression of *Cxcl4* using RFP as a marker with flow cytometry analysis in combination with antibodies to identify HSC populations.

Firstly, it was noted that positive RFP expression was found within the haemopoietic organs tested (BM, spleen and thymi) with the highest expression in the BM followed by the spleen and thymi (n = 3, Figure 5-2). The controls were negative for RFP expression as expected (data not shown). Representative plots can be visualised in (Figure 5-3).

Examination of the stem cell compartment showed that RFP^{+} cells were found in HSC populations. Results showed a proportion of RFP^{+} cells in the BM, LSK and HSC compartments including the most primitive CD150^{+}CD48^{-} fraction (n = 9) (Figure 5-2).

As a positive control, BM derived megakaryocytes and PB derived platelets were isolated and examined for RFP expression within $CD41^+$ cells. Megakaryocyte and platelets are $CD41^+$ and are known to express *Cxcl4* therefore should show positive expression for RFP. Results showed RFP^+ cells in platelets (94.3%) and the majority of megakaryocytes (50.2%) (n = 3) (Figure 5-4). This result is as expected and has been shown previously suggesting that the transgenic model functions correctly. Although all of the platelets are positive for RFP expression, only 50% of megakaryocytes were found to be positive for RFP. However, previous results have shown that immature megakaryocytes may have incomplete recombination and therefore RFP expression which reflects the cell type and not the model (Tiedt et al., 2007). As a technical control, RFP^+ and RFP^- cells were sorted from *Rosa26-RFP*$^+$*;Cxcl4-Cre*$^+$ mice and examined for the presence of genomic *Cre*. Results showed the presence of *Cre* in both RFP^+ and RFP^- fractions using standard PCR (Figure 5-2).

The *Cxcl4-Cre* model has been used previously for megakaryocyte and platelet biology but *Cxcl4* expression in the HSC population has not been identified. This is likely due to differences in the techniques with previous research using histology which is not as sensitive as flow cytometry for RFP expression. The combination of detection of endogenous *Cxcl4* mRNA in HSC (Figure 5-1) and the positive RFP expression in HSC (Figure 5-2) suggests that *Cxcl4-Cre* reflects transcriptional activity of *Cxcl4* promoter in these populations.

As described in section 2.3.7.2.1, the BAC used to create the transgenic mice in this study also contained other genes, one of which is known to play a role in stem cell maintenance. Therefore, the results should be concluded with caution.

Gender was not noted for the animals used in this section. Without knowing whether the gender was similar or different between groups does not allow a valid conclusion to be drawn. Experiments should be repeated with the gender variable controlled.

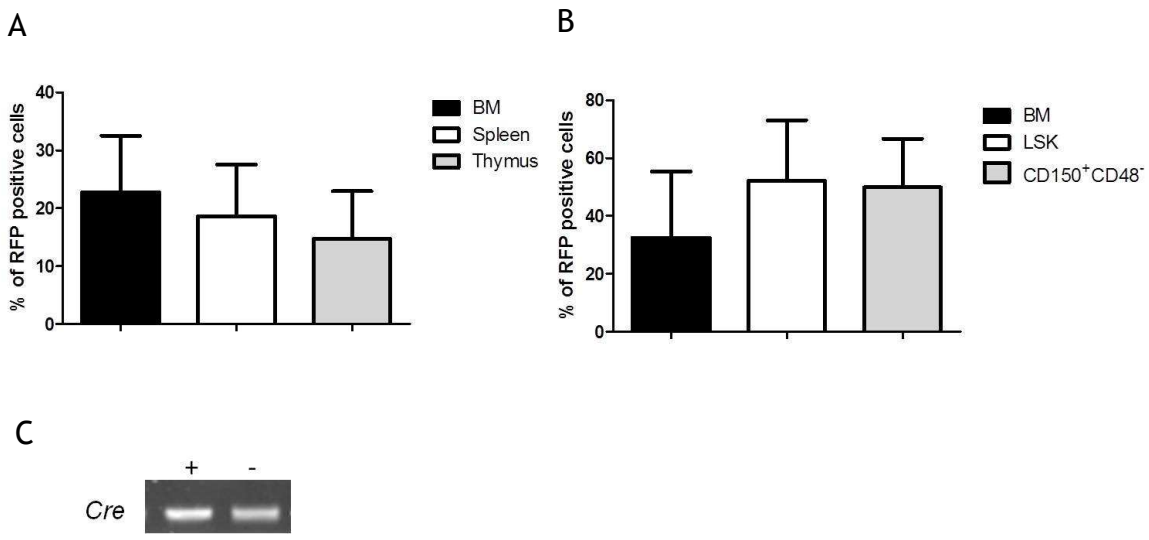

Figure 5-2 Mature haemopoietic organs and HSC populations express RFP which is under the control of the *Cxcl4* promoter.

BM, spleen and thymi were isolated, stained for HSC populations and examined for RFP expression using flow cytometry. Data are presented as the mean percentage of RFP^+ cells in mature haemopoietic organs BM, spleen and thymi (A) in BM and HSC populations LSK and LSK,$CD150^+CD48^-$ from three independent experiments (n = 3) (B). Image represents the presence of *Cre* in DNA isolated from LSK sorted cells which are RFP^+ (+) and RFP^- (-). Animals were 6-12 weeks of age. Animals were given by Dr Simon Calaminus (Beatson Institute for Cancer Research) and gender was not noted.

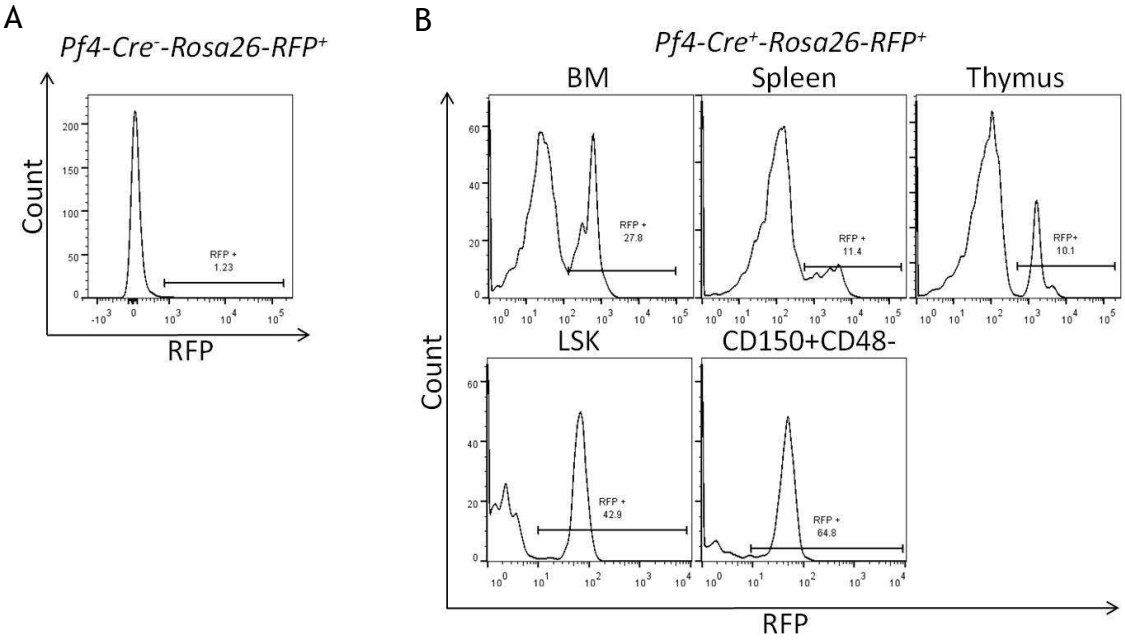

Figure 5-3 Representative plots of RFP expression in organs in *Pf4-Cre*$^{+}$*-Rosa26-RFP*$^{+}$ mice.

BM, spleen and thymi were isolated, stained for HSC populations and examined for RFP expression using flow cytometry. Data shows representative image of plot for RFP expression of cells in mature haemopoietic organs BM, spleen and thymi in BM and HSC populations LSK and LSK,CD150^{+}CD48^{-} from three independent experiments (B). Panel A demonstrates staining profile for all controls used in this study (A). Animals were given by Dr Simon Calaminus (Beatson Institute for Cancer Research) and gender was not noted.

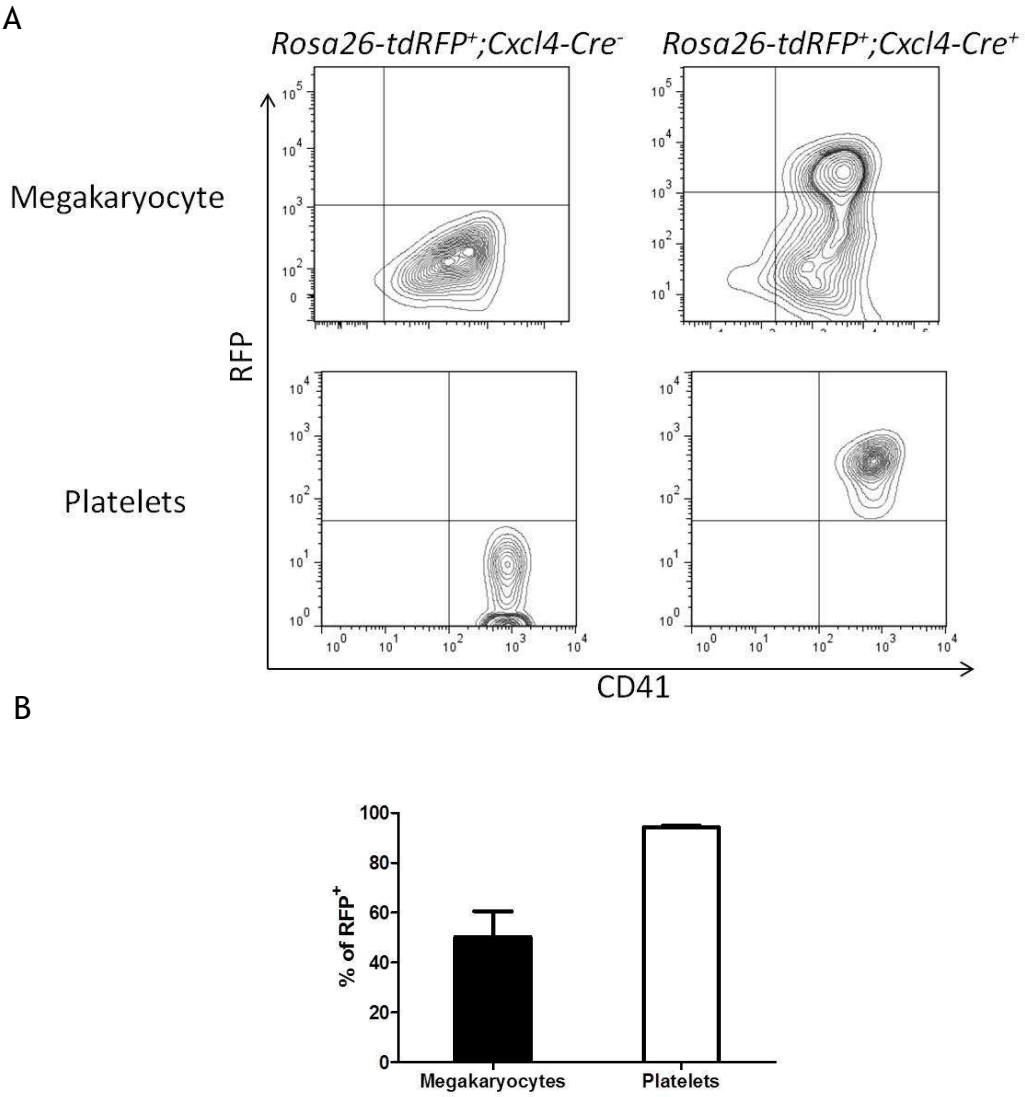

Figure 5-4 Positive control cells megakaryocytes and platelets express RFP which is under the control of the *Cxcl4* promoter.

BM derived megakaryocytes and PB derived platelets were isolated, stained for CD41 and analysed for RFP expression using flow cytometry. Data are presented as the mean percentage of RFP^+ cells within the $CD41^+$ fraction from three independent experiments (*n* = 3) (B). Representative flow cytometry plots show CD41 and RFP expression in cell populations between strains (A). Animals were given by Dr Simon Calaminus (Beatson Institute for Cancer Research) and gender was not noted.

5.3.2.2 Lineage tracing of *Cxcl4* marks a stem/progenitor population with increased colony formation activity

Interestingly, experiments in section 5.3.2.1 showed that only a proportion of HSC expressed RFP. Technical controls were carried out to suggest that this was a true biological result (Figure 5-2). It can be inferred from the data that *Cxcl4* is only transcriptionally active in a subset of cells. Therefore, the question arose, why are only a proportion of cells positive for *Cxcl4* expression in the HSC? RFP^+ and RFP^- negative cells were isolated from the BM from the *Cxcl4-Cre* and seeded into a CFC assay to get an indication of how these populations differ in terms of differentiation and proliferation capacity.

Results showed an increase in the number of colonies obtained in RFP^+ sorted cells in comparison to cells lacking RFP expression in a primary CFC assay ($P < 0.05$, $n = 3$) (Figure 5-5). To get an indication of self renewal activity, cells were replated into a secondary colony formation assay and results showed a trend towards a decrease in colony numbers between RFP^+ versus RFP^- cells, which was not statistically significant and likely represents sample variation (n.s., $n = 3$) (Figure 5-5).

The results indicate that $Cxcl4^+$ cells have enhanced colony formation capability in comparison to negative cells. This infers that $Cxcl4^+$ cells exhibit an increase in viability, differentiation or proliferation in comparison to the negative counterparts. Previous literature has shown that human haemopoietic cells respond to exogenous *Cxcl4* which results in an enhancement in cell viability, therefore this result would be in accordance with literature available (Han et al., 1997). It might be expected that an increase in primary colonies would result in an increase in colonies in a secondary plating, however this was not found. One explanation is that $Cxcl4^+$ cells are more proliferative and exhaust therefore produce less colonies in a replating assay. However, this cannot be concluded. Furthermore, the transgenic model used does not mark active transcription. Therefore, it is possible that *Cxcl4* is modulated in response to culture conditions which could skew the results in a secondary plating assay. It is also possible that RFP expression is not solely dependent on *Cxcl4* transcriptional activity due to the random integration of the *Cxcl4-Cre* transgene.

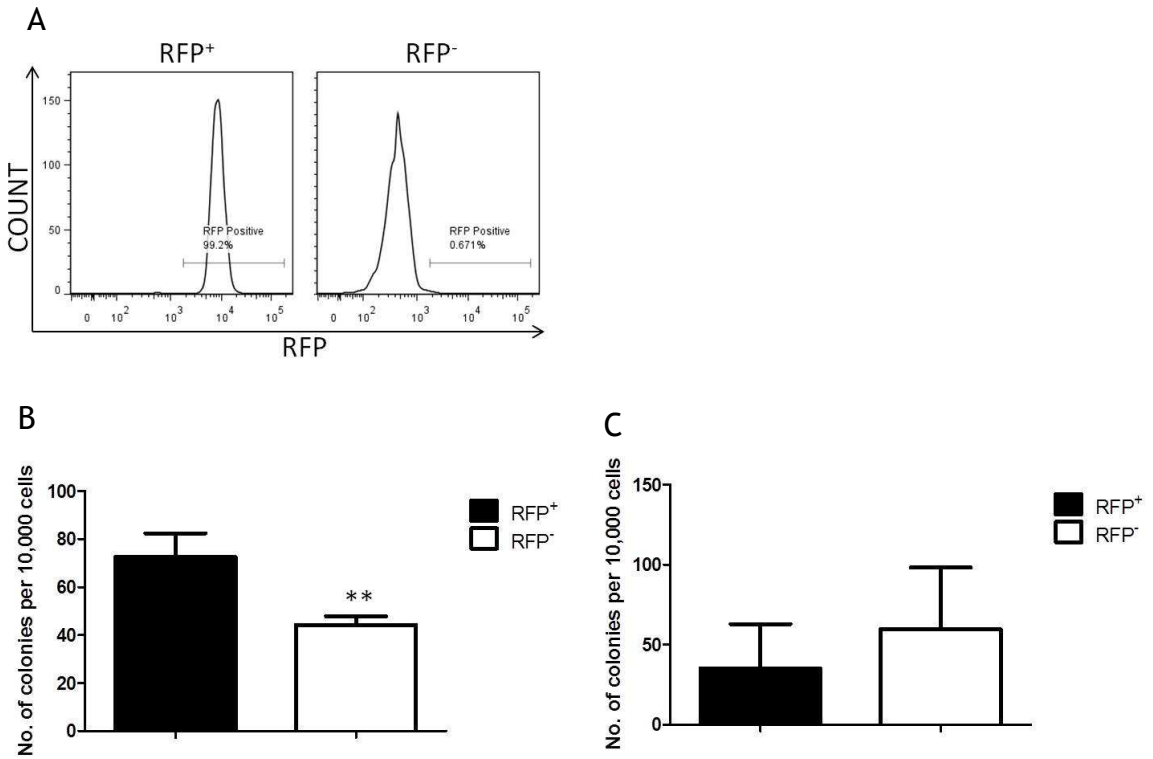

Figure 5-5 *Cxcl4*$^{+}$ BM cells show enhanced colony capability in a primary plating assay over *Cxcl4*$^{-}$ counterparts.

Representative flow cytometry plots display the RFP status of cells prior to plating into a CFC assay (A). Data are presented as the mean total colony number from cells sorted for RFP expression. Results show primary (B) and secondary (B) plating assays (n = 3). A ratio paired t test was carried out to assess statistical significance (** P <0.01). Animals were given by Dr Simon Calaminus (Beatson Institute for Cancer Research) and gender was not noted.

5.3.3 *Cxcl4* reduction *in vitro* reduces colony formation activity in mouse stem/progenitor cells

The results from section 5.3.2.2 implicate that *Cxcl4* may mark a stem/progenitor population with enhanced colony formation activity. To validate this, *Cxcl4* expression was reduced using a vector with a shRNA hairpin targeting the mouse *Cxcl4* coding sequence. Positively transduced cells were plated in a colony formation assay and colony growth was compared with a non-targeting shRNA control.

5.3.3.1 Optimisation of construct for *Cxcl4* reduction *in vitro*

As described in chapter 3, different shRNA sequences against a target gene can result in varying levels of gene modification. A set of 5 pLKO.1 vectors each with a different shRNA sequence were tested for their knock down efficiency in *Cxcl4* expressing mouse cell lines. Cell lines used were c-Kit positive mouse BM cells which were immortalised for long term culture using integration of oncogenes (Gil Smith, data not shown). These cells were chosen for optimisation experiments for two reasons: 1. These cells were shown to be positive for *Cxcl4* expression and 2. Their immortalisation using oncogenes transformed them into cell lines allowing an abundance of cells to carry out the required experiments. Cells were transduced using lentivirus particles and subsequently cultured in puro for several days before RNA was extracted and Q-PCR was used to test for *Cxcl4* expression. Each vector resulted in the following fold change decrease in comparison to the control which was set to the value of 1; sh1 (0.70, n.s.), sh2 (0.60, P <0.01), sh3 (0.80, n.s.), sh4 (0.33, P <0.01) and sh5 (0.14, P <0.001) (n = 3) (Figure 5-6). An interesting observation was that all vectors resulted in some amount of *Cxcl4* reduction and fewer cells were obtained in comparison to the control cells (data not shown). The construct which resulted in the best reduction in *Cxcl4* expression (sh5) was sub cloned into a pLKO.1 vector with a GFP reporter tag. The *Cxcl4*-pLKO.1-GFP vector will be described as sh1 for simplicity. This vector was tested in cell lines and confirmed to show a reduction in gene expression of *Cxcl4* (n = 3, P <0.001) in comparison to a control which was set to the value of 1 (Figure 5-6). Ideally, protein expression would be examined for the effect of the shRNA on *Cxcl4* protein reduction. However, due to difficulties with antibodies against mouse *Cxcl4* and time constraints, this experiment was not carried out.

5.3.3.2 Reduction of *Cxcl4* in c-Kit$^+$ cells reduces colony formation capability

A stem/progenitor population (c-Kit enriched mouse BM) was transduced with the shRNA vector (sh1) described in section 5.3.3.1. C-Kit enriched mouse BM cells were deemed appropriate for this study as a more primitive fraction would have provided fewer cells for the assay. Furthermore, primitive HSC are known to be difficult to transduce due to their non proliferative status. After transduction, the GFP$^+$ cells were sorted and subsequently cultured in a colony formation assay. Results showed a significant decrease in colonies in a primary assay with *Cxcl4* reduction in comparison to the number of colonies obtained in the control arm (P <0.05, n = 3) (Figure 5-7). No differences in the different colony types between conditions were observed therefore data was acquired as the total colony number obtained. This data suggests that *Cxcl4* reduction reduces colony formation therefore *Cxcl4* plays a role in stem/progenitor survival and proliferation. To examine self renewal potential, cells from a primary plating assay were plated into a secondary assay. In a secondary replating assay, *Cxcl4* reduction showed a significant reduction in colony numbers in comparison to the control (P <0.01, n = 3) (Figure 5-7). To ensure that the shRNA vector reduced *Cxcl4* expression in primary mouse cells, cells were harvested from the primary plating assay and examined for gene expression levels of *Cxcl4* using Q-PCR. Results showed a mean 0.50 reduction in *Cxcl4* gene expression levels in comparison to the control cells which was set to the value of 1. The decrease in gene expression was not statistically significant due to variation that was present in technical triplicates derived from 3 independent samples (n.s., n = 3) (Figure 5-7). This result indicates that *Cxcl4* controls stem/progenitor cell survival, proliferation and self renewal. A more in depth analysis of apoptosis and cell cycle status in response to *Cxcl4* reduction is needed to confirm these results, however was not completed due to time constraints.

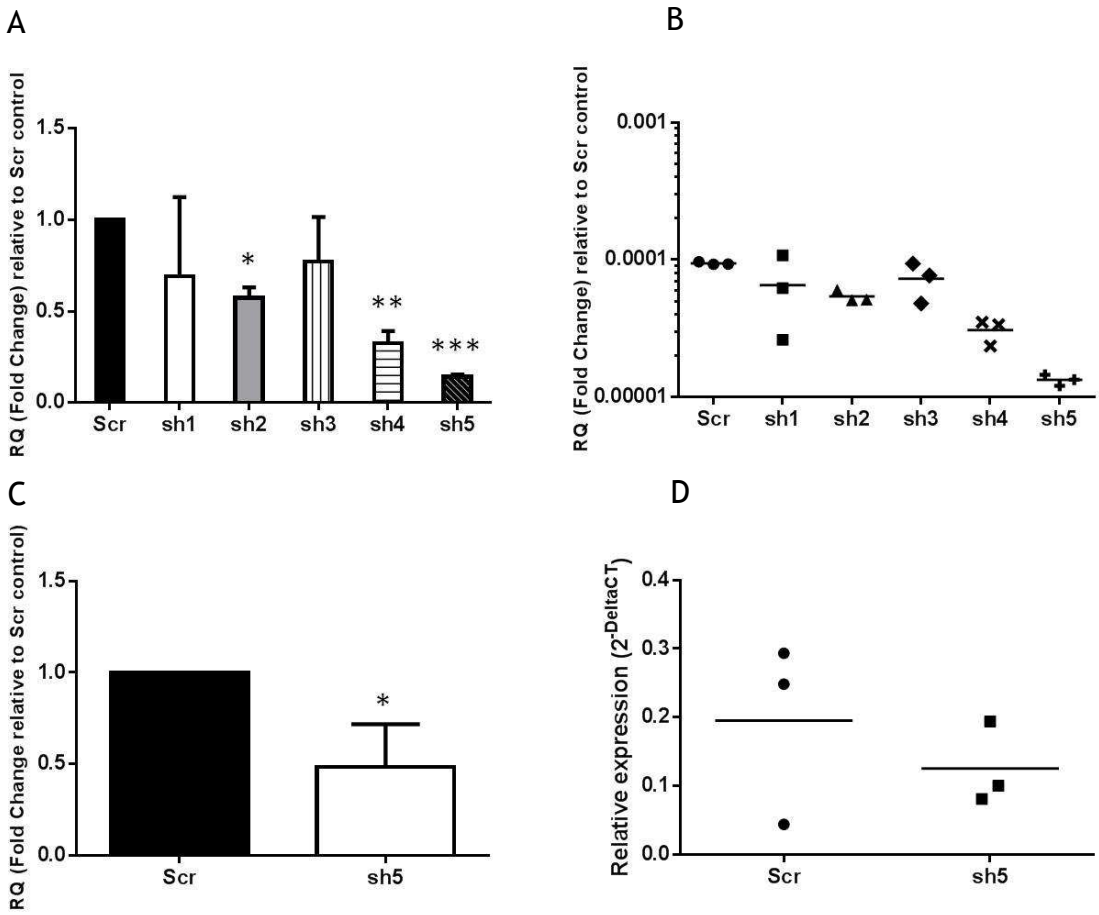

Figure 5-6 Reduction of *Cxcl4* using shRNA results in a reduction in *Cxcl4* expression in mouse cell lines.

Cell lines were transduced using lentivirus for *Cxcl4* shRNA or a control hairpin. Subsequently RNA was extracted, RT and Q-PCR carried out for *Cxcl4* expression. Data are presented as the mean fold change in *Cxcl4* expression in transduced cell lines with 5 vectors in a pLKO.1 puro background (A) and 1 vector in a pLKO.1-GFP background (C) compared to the control which is set to the value of 1. Data was calculated using housekeeping gene *Gapdh* and using the DeltaDeltaCT method. Relative expression is displayed in panels B and D with each individual dot as an average of technical triplicates in three independent experiments. A one-way repeated measures ANOVA with Dunnett's multiple comparisons test was used to assess statistical significance between each shRNA vector and the Scr control (A) and a ratio paired *t* test was used for panel C (* P <0.05; ** P <0.01; *** P <0.001, n = 3). Animals were 6-12 weeks of age and males were used.

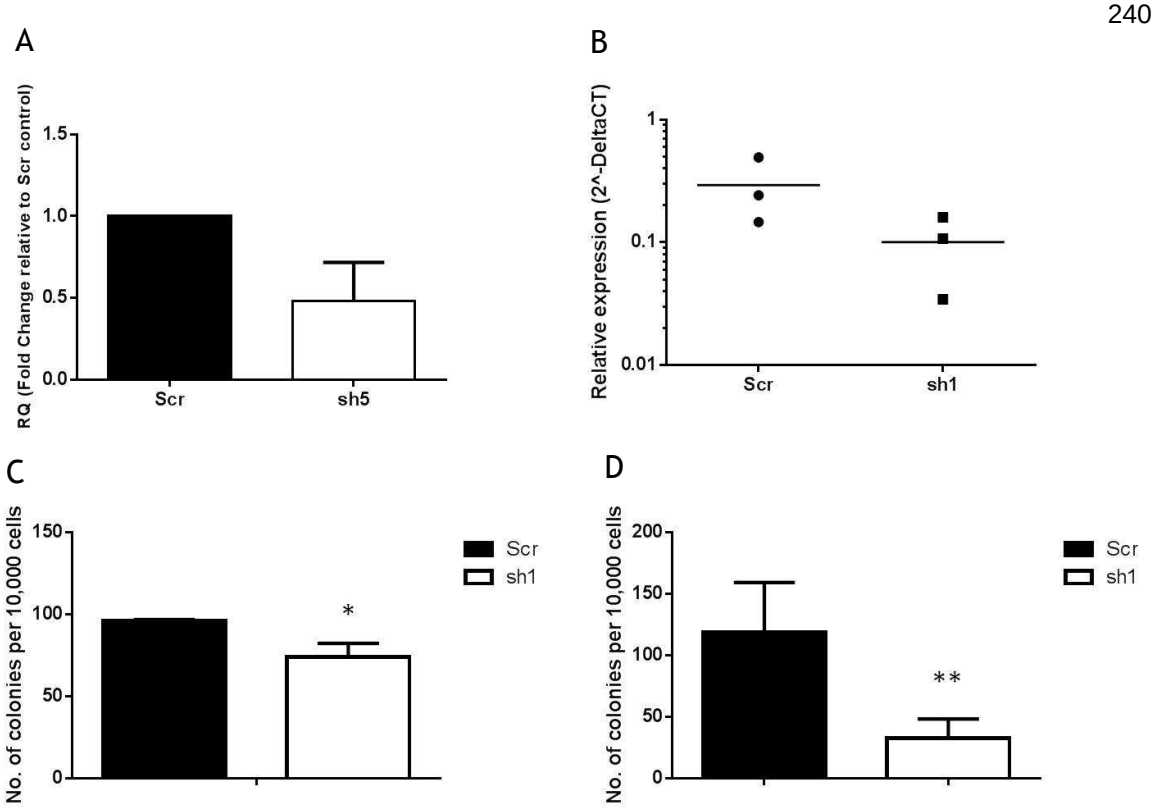

Figure 5-7 *Cxcl4* reduction in c-Kit$^+$ mouse BM cells reduces colony formation in primary and secondary plating assays.

C-Kit$^+$ BM cells were transduced with shRNA against *Cxcl4* or a control hairpin. GFP$^+$ cells were sorted into Methocult™ and cultured for 10-14 days and colonies were counted. Subsequently, cells were put into a replating assay, cultured and colonies were counted. Data are presented as the mean fold change in *Cxcl4* expression in the *Cxcl4* shRNA condition in comparison to the control, which is set to the value of 1 (A). Standard deviation represents 3 independent experiments, each with 3 technical replicates. Data were calculated using the DeltaDeltaCT method and housekeeping gene *Gapdh.* Data is also presented as relative expression with each dot representing an average of three technical replicates from three independent experiments (B). Data are presented as mean colony numbers in shRNA condition and control in primary (C) and secondary (D) colony formation assays. Statistical analysis carried out was a ratio paired two-tailed *t* test (A, B & C) and a paired two-tailed *t* test (B) (* P <0.05; ** P <0.01, n = 3). Animals were 6-12 weeks of age and males were used.

5.3.4 Analysis of haemopoiesis in *Cxcl4*$^{-/-}$ animals

The results in sections 5.3.2.2 and 5.3.3.2 provide evidence that *Cxcl4* controls colony formation and self renewal in mouse stem/progenitor populations. To compliment these experiments, analysis of animals lacking the *Cxcl4* gene were analysed (*Cxcl4*$^{-/-}$). *Cxcl4*$^{-/-}$ mice have been generated previously and shown to exhibit an increase in platelet counts and defects in blood coagulation in comparison to WT controls (Zhang et al., 2001) (Lambert et al., 2007). However, to date the HSC compartment and functional activity of these populations has not been assessed. In this study, the *Cxcl4*$^{-/-}$ mouse model was used to examine the frequency of stem and progenitor populations and to examine their functional activity in *in vitro* and *in vivo* assays. As the previous results show *Cxcl4* supports the survival and self renewal of HSC, experiments were designed to test whether similar results could be obtained in cells lacking *Cxcl4.*

Cxcl4$^{-/-}$ animals and age/sex matched WT controls were analysed (C57/BL6 background). Animals were genotyped prior to use using optimised PCR conditions for primers to detect endogenous *Cxcl4* or *Neomycin* (Figure 5-8). The haemopoietic system was assessed by measuring the frequency of mature cells/HSC/progenitor cells in the haemopoietic organs (BM, spleen, PB and thymus). Functional activity of the HSC populations was experimentally tested using *in vitro* and *in vivo* assays including colony formation assays with replates and BM reconstitution assays. These methods were described previously in chapter 4.

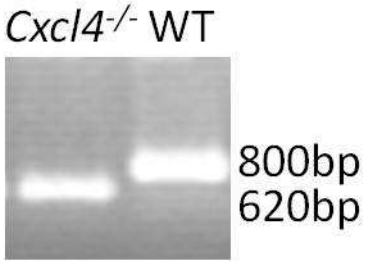

Figure 5-8 Genotyping analysis of WT and *Cxcl4*$^{-/-}$ animals.

Genomic DNA was extracted from tail tips/ear notch samples from animals and PCR was carried out using two sets of primers against mouse endogenous *Cxcl4* and *Neomycin*. Image shows a representative image of PCR products in WT and *Cxcl4*$^{-/-}$ DNA. DNA derived from WT cells shows the presence of endogenous *Cxcl4* (800bp), while DNA derived from cells lacking *Cxcl4* show the presence of a band corresponding to *Neomycin* expression (620bp). Genotyping was performed to identify whether animals were WT, *Cxcl4*$^{-/-}$ or *Cxcl4*$^{+/-}$. Positive (known WT and *Cxcl4*$^{-/-}$ DNA) and negative controls (no DNA template) were run to ensure the PCR reaction worked correctly and no contamination of reagents was present. Animals were only used in this study if genotyping gave a clear result of a single band for either *Cxcl4* or *Neomycin* to ensure that heterozygotes were not included in any experiments.

5.3.4.1 Mature cell types in haemopoietic organs

The frequency of mature cell types was examined in the BM, spleen, PB and thymi as described previously.

5.3.4.1.1 BM

BM analysis showed no difference in the cellularity in the *Cxcl4*$^{-/-}$ animals in comparison to the controls (n.s., *n* = 6, 7) (Figure 5-9). There were no differences in the numbers of erythroid cells, granulocytes or B cells in the BM between the strains (n.s., *n* = 6, 7) (Figure 5-9).

5.3.4.1.2 Spleen

Spleen analysis showed no difference in cellularity between the strains (n.s., *n* = 6, 7) (Figure 5-10). No differences in erythroid, granulocytes, B or T cells was found between strains (n.s., *n* = 6, 7) (Figure 5-10).

5.3.4.1.3 PB

PB analysis showed no difference in the cellularity in the *Cxcl4*$^{-/-}$ animals in comparison to the WT animals (n.s., *n* = 6, 8) (Figure 5-11). A trend towards an increase was noted in platelet counts in the *Cxcl4*$^{-/-}$ animals (n.s., *n* = 6, 8). Finally, no differences were noted in the numbers of mature cells in the PB between strains (n.s., *n* = 6, 7) (Figure 5-11).

5.3.4.1.4 Thymi

Analysis of the thymi showed no difference in the cellularity between strains (n.s., *n* = 6, 7) with no difference in T cells between strains (n.s., *n* = 6, 7) (Figure 5-12).

In summary, results showed no differences in the cellularity or numbers of mature cell types in the haemopoietic organs between *Cxcl4*$^{-/-}$ animals and WT controls. This infers that *Cxcl4* is not involved in the regulation of mature haemopoietic cells. An increase in platelet count found in the *Cxcl4*$^{-/-}$ animals is in accordance with previous literature and the lack of significance likely reflects small sample size with previous research examining a much larger sample size

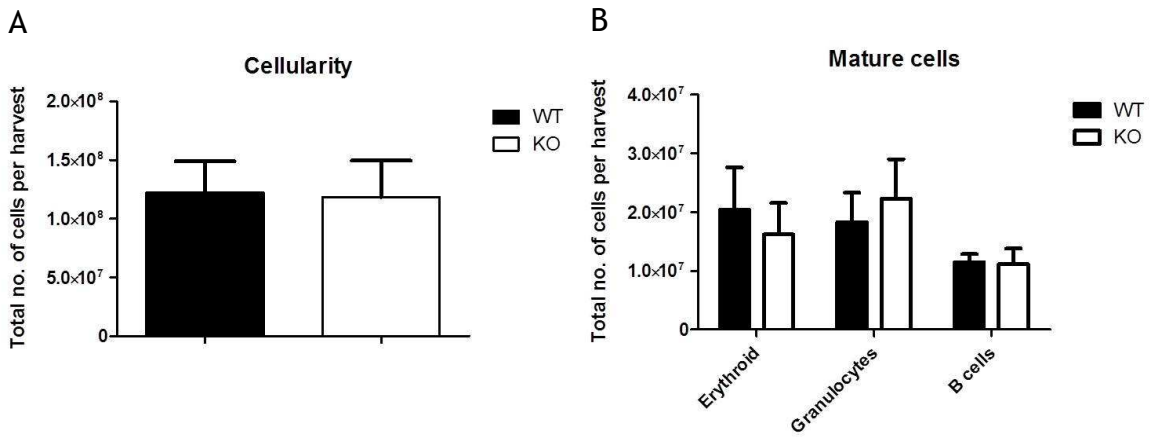

Figure 5-9 Cellularity and absolute numbers of mature cells in the BM between WT and *Cxcl4*$^{-/-}$ animals.

BM was isolated, assessed for cellularity, stained against mature markers and examined using flow cytometry. Data are presented as the mean cellularity (A) and absolute numbers of erythroid cells (TER119^{+}), granulocytes (GR1^{+}CD11B^{+}) and B (CD19^{+}B220^{+}) cells (B) in the BM between WT and *Cxcl4*$^{-/-}$ animals. A Mann Whitney U test showed no statistical significance between conditions (n.s., n = 6, 7). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

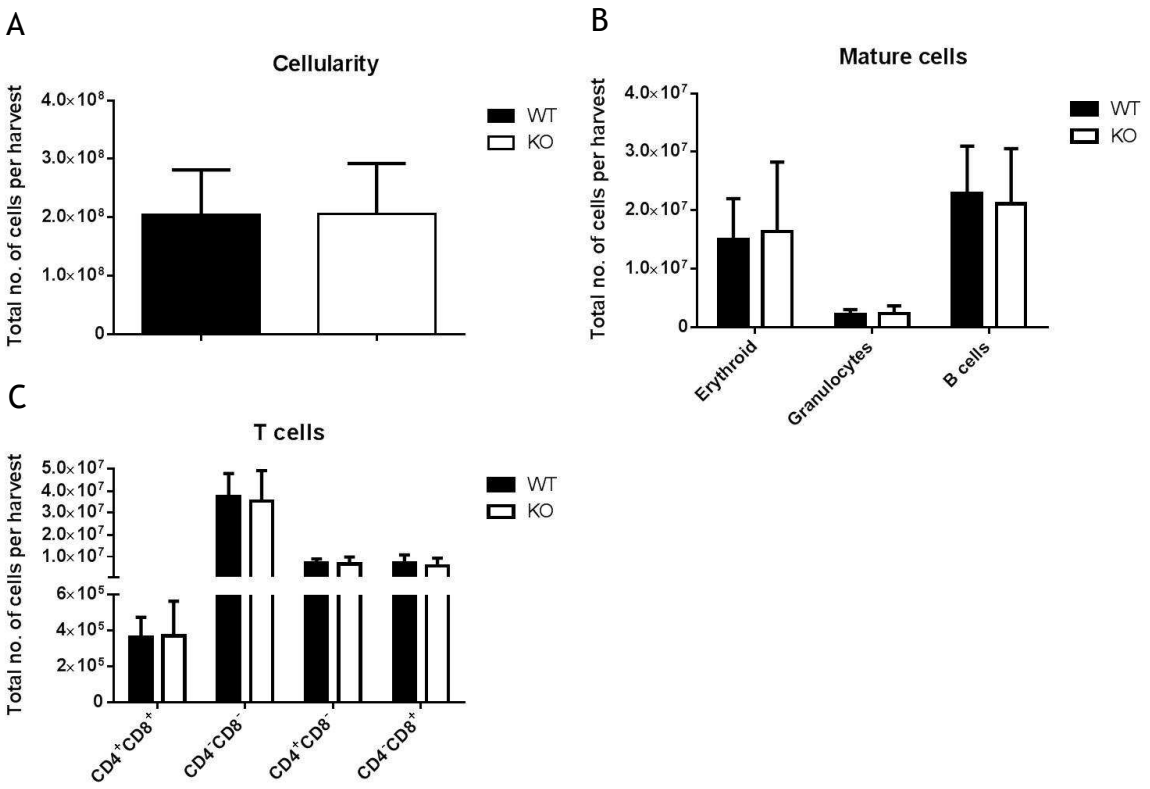

Figure 5-10 Cellularity and absolute numbers of mature cells in the spleen between WT and *Cxcl4*$^{-/-}$ animals.

Spleen cells were isolated, assessed for cellularity and stained with antibodies against mature cell types and analysed using flow cytometry. Data are presented as the mean cellularity (A) and absolute numbers of erythroid cells (TER119^{+}), granulocytes (GR1^{+}CD11B^{+}), B (CD19^{+}B220^{+}) (B) and T cell subsets (CD4^{+}CD8^{+}, CD4^{-}CD8^{-}, CD4^{+}CD8^{-} and CD4^{-}CD8^{+}) (C) in the spleen between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a two-tailed unpaired *t* test (A) and two-tailed unpaired *t* test with Welch's correction for unequal variance (B & C) (n.s., n = 6, 7). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

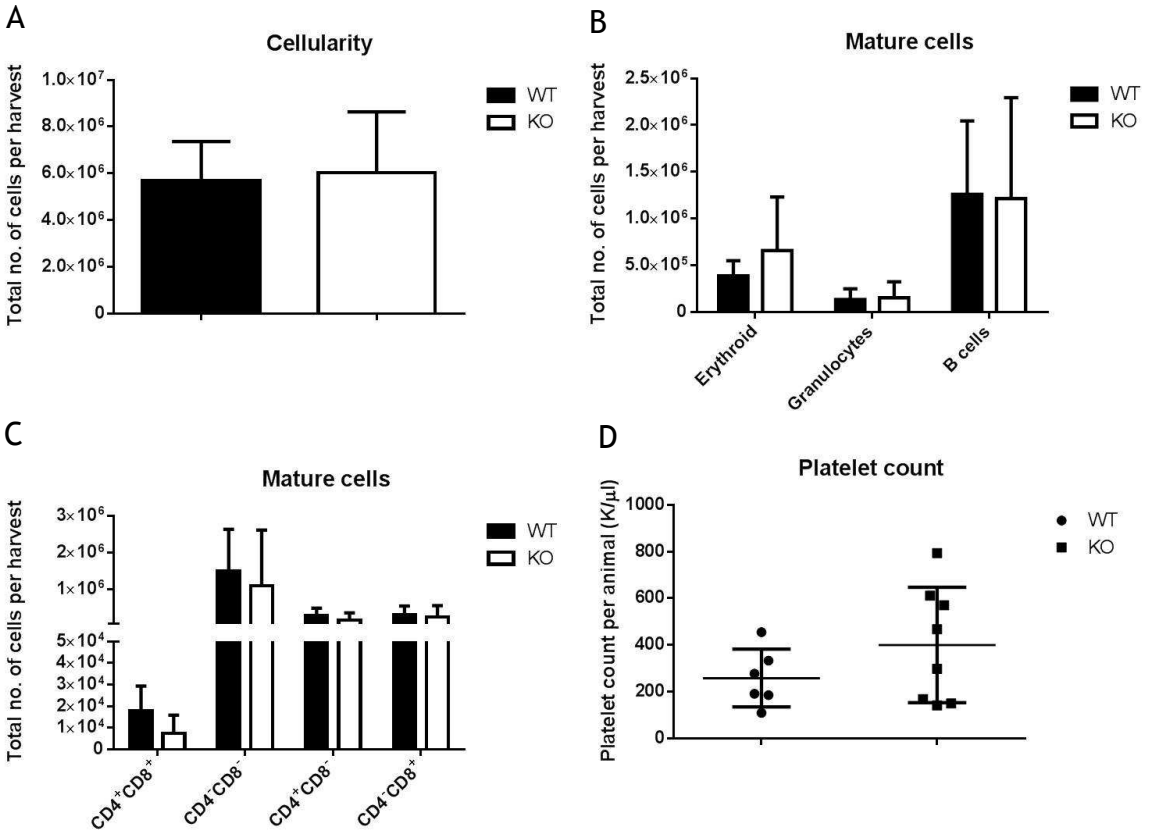

Figure 5-11 Cellularity and absolute numbers of mature cells in the PB between WT and *Cxcl4*$^{-/-}$ animals.

PB was harvested, assessed for cellularity, RBC were lysed and stained with antibodies to identify mature cell populations. extracted with an anticoagulant and assessed for cellularity neat and platelet count. Data are presented as the mean cellularity (A), absolute numbers of erythroid cells (TER119^{+}), granulocytes (GR1^{+}CD11B^{+}), B (CD19^{+}B220^{+}) (B), T cell subsets (CD4^{+}CD8^{+}, CD4^{-}CD8^{-}, CD4^{+}CD8^{-} and CD4^{-}CD8^{+}) (C) and platelet counts (D) in the PB between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., n = 6, 7 (A-C), n = 6, 8 (D)). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

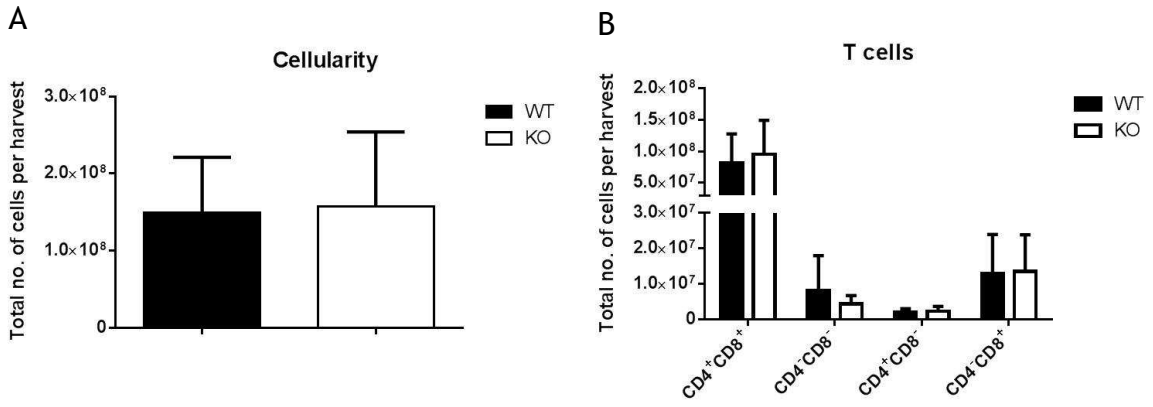

Figure 5-12 Cellularity and absolute numbers of mature cells in the thymi between WT and *Cxcl4*$^{-/-}$ animals.

Thymi were isolated, assessed for cellularity and stained with antibodies against mature cell types and analysed using flow cytometry. Data are presented as the mean cellularity (A) and absolute numbers of T cell subsets ($CD4^+CD8^+$, $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$) (B) in the thymi between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., $n = 6, 7$). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

5.3.4.2 The numbers of stem and progenitor populations in mice lacking *Cxcl4*

Assessment of the frequency of stem and progenitor populations in *Cxcl4*$^{-/-}$ mice has not previously been carried out. The frequency of stem and progenitor populations was examined using antibody staining against a variety of cell surface markers and analysed using flow cytometry as mentioned previously.

5.3.4.2.1 BM

5.3.4.2.1.1 HSC populations

No difference was found in the number of lineage negative cells in the *Cxcl4*$^{-/-}$animals in comparison to controls (n.s., *n* = 6, 7) (Figure 5-13).Within the lineage negative fraction, the LSK and further enriched stem cell populations were examined. There was no difference in the LSK cells between strains (n.s., *n* = 6, 7) (Figure 5-13). There were no differences noted between HSC populations between strains (n.s., *n* = 6, 7) (Figure 5-13).

5.3.4.2.1.2 Progenitor populations

In terms of progenitors, the LK fraction contains progenitor cells and showed no differences between strains (n.s., *n* = 6, 7) (Figure 5-14). There were no statistically significant differences between GMP, CMP and MEP populations in terms of absolute numbers (n.s., *n* = 6, 7) (Figure 5-14).

5.3.4.2.2 Spleen

5.3.4.2.2.1 HSC populations

A trend towards an increase in the number of lineage negative cells in the *Cxcl4*$^{-/-}$animals was reported which was not statistically significant (n.s., *n* = 6) (Figure 5-15). There was a trend towards increase in the number of LSK cells in the *Cxcl4*$^{-/-}$condition which was not statistically significant (n.s., *n* = 6) (Figure 5-15). All HSC fractions showed no differences in the absolute number of HSC populations between conditions (n.s., *n* = 6) (Figure 5-15).

5.3.4.2.2.2 Progenitor populations

In terms of progenitor cells in the spleen, the LK fraction showed a trend towards an increase in the *Cxcl4*$^{-/-}$animals which was not statistically significant (n.s., *n* = 6). There were no differences in the progenitor populations including the GMP, CMP and MEP populations (n.s., *n* = 3) (Figure 5-16).

In summary, the data collectively shows that a lack of *Cxcl4* in animals does not alter stem or progenitor frequency in either the BM or spleen. As only female animals were examined, future experiments should compare stem cell function in male animals in case the effects are gender specific. Frequency does not always confer to stem cell function, therefore experiments were designed to test functionality of stem/progenitor cells in *Cxcl4*$^{-/-}$ animals.

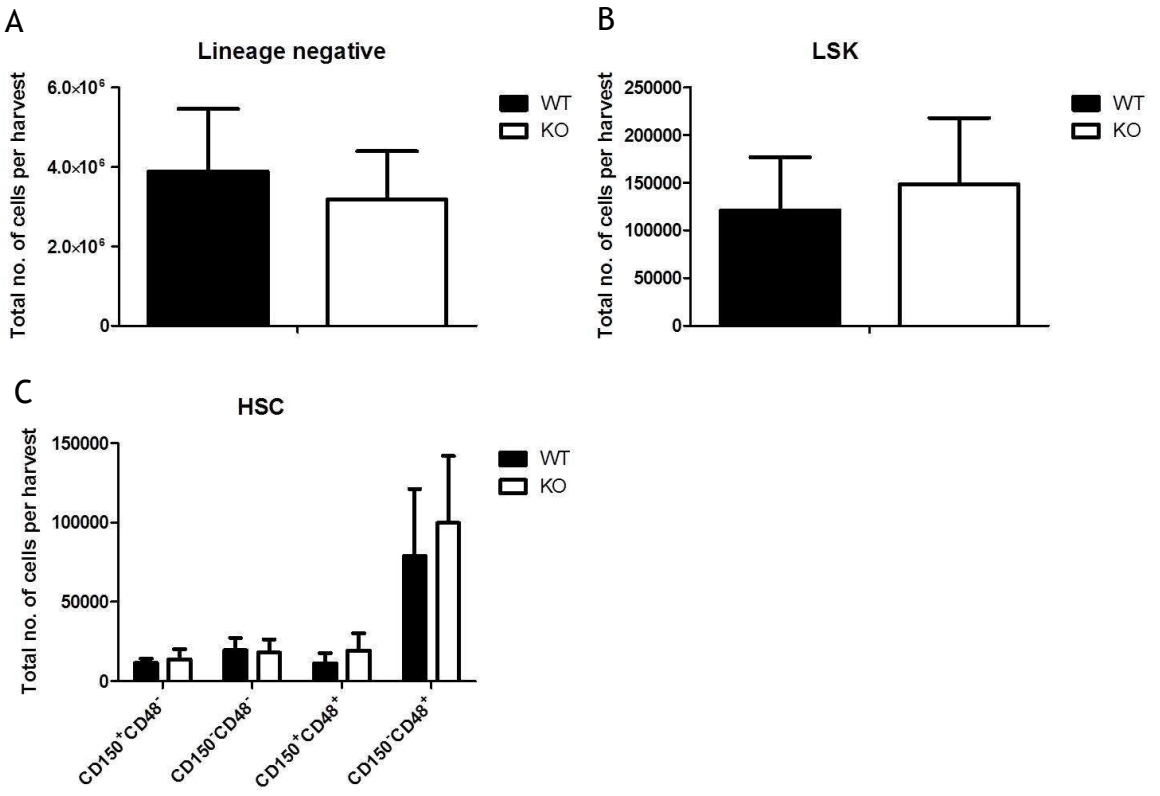

Figure 5-13 The numbers of HSC in the BM of WT and *Cxcl4*$^{-/-}$ animals.

BM was isolated and stained for antibodies against HSC markers and examined using flow cytometry. Data are presented as the mean absolute numbers of lineage negative (A), LSK (B) and HSC populations (C) in the BM between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., n = 6, 7). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

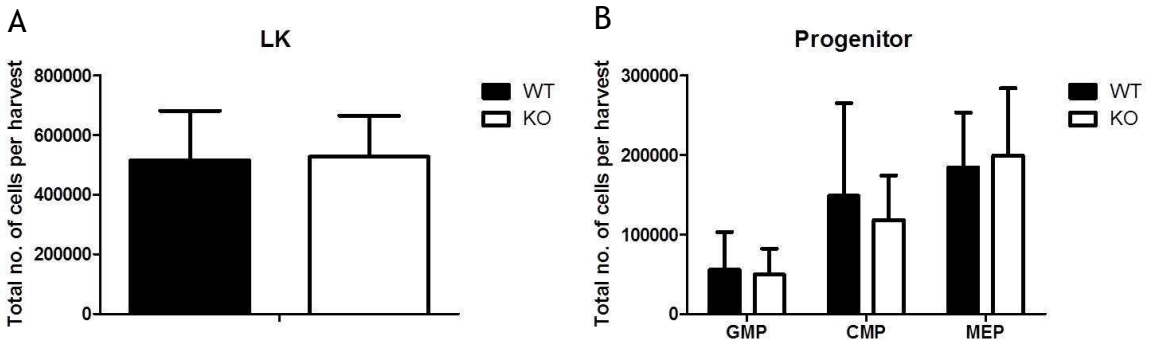

Figure 5-14 The numbers of progenitor cells in the BM of WT and *Cxcl4*$^{-/-}$ animals.

BM was isolated and stained against progenitor markers and examined using flow cytometry. Data are presented as the mean absolute numbers of LK (A) and progenitor populations (B) in the BM between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a student's unpaired *t* test with Welch's correction for unequal variance (n.s., $n = 6, 7$). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

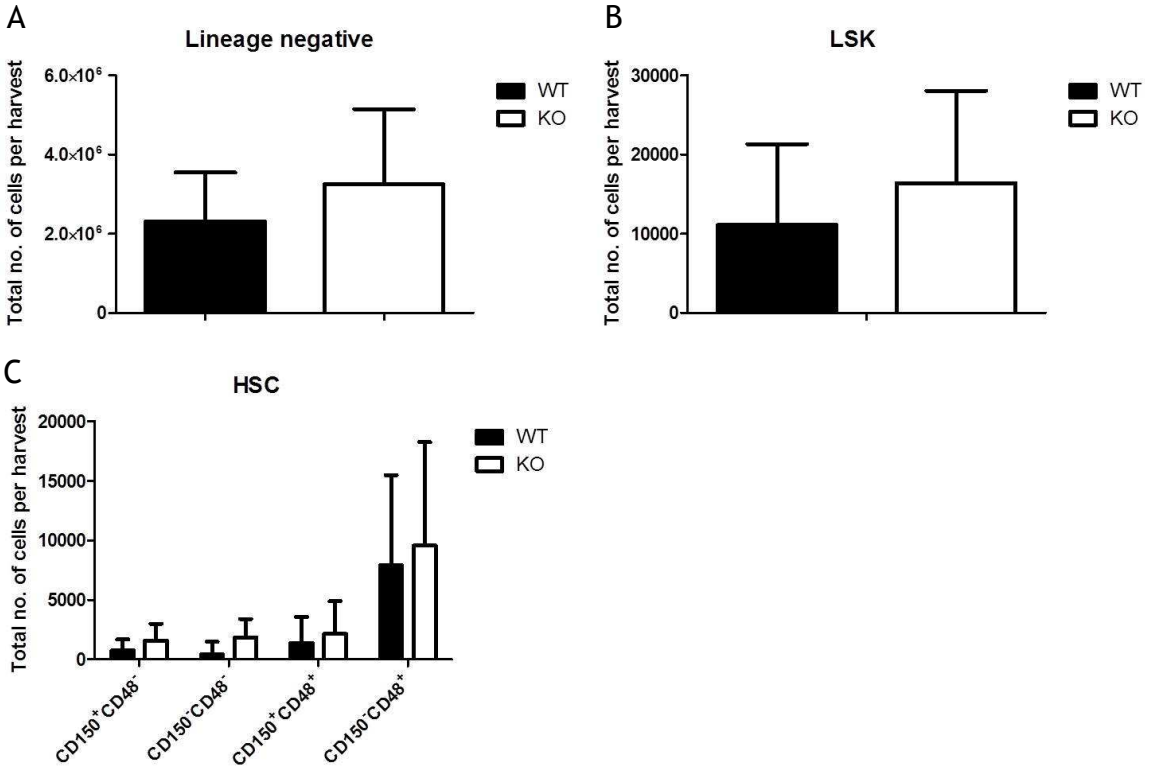

Figure 5-15 The numbers of HSC in the spleen of WT and *Cxcl4*$^{-/-}$ animals.

The spleen was isolated and stained with antibodies against HSC markers and examined using flow cytometry. Data are presented as the mean absolute numbers of lineage negative (A), LSK (B) and HSC populations (C) in the spleen between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a student's unpaired *t* test assuming equal variance between conditions (n.s., $n = 6, 7$). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

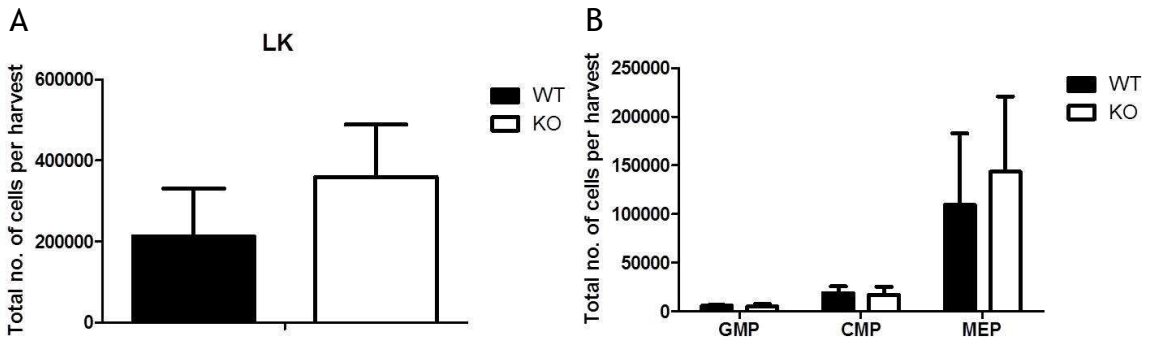

Figure 5-16 The numbers of progenitors in the spleen of WT and *Cxcl4*$^{-/-}$ animals.

The spleen was isolated and stained with antibodies against HSC markers and examined using flow cytometry. Data are presented as the mean absolute numbers of LK (A) and progenitor populations (B) in the spleen between WT and *Cxcl4*$^{-/-}$ animals. No statistical differences were reported using a student's unpaired *t* test assuming equal variance (n.s., *n* = 6, 7 (A), *n* = 3 (B)). Animals were 6-12 weeks of age and gender was the same (WT 6 females; *Cxcl4*$^{-/-}$ 7 females).

5.3.4.3 WT and *Cxcl4*$^{-/-}$ stem/progenitors show no difference in viability or cell cycle status

Although results in section 5.3.4.2 show animals lacking *Cxcl4* do not show different frequencies of stem or progenitor cells, we wanted to examine stem cell functionality. We examined viability and cell cycle status in HSC as described in chapter 4.

BM cells harvested from WT and *Cxcl4*$^{-/-}$ animals were stained for LSK and Annexin-V. Results showed no difference in Annexin-V^{+} cells between different populations between strains (n.s., $n = 3$) (Figure 5-17).

Ki-67 staining showed no differences in Ki-67^{+} cells between strains (n.s., $n = 3$) (Figure 5-17). However, a trend towards an increase is noted in all populations.

The staining pattern obtained suggests that the assay was technically sound with the highest percentage of viable and quiescent cells in the HSC compartments. Therefore, it can be inferred from the data that there are no differences in cell viability or proliferation in HSC populations between WT and *Cxcl4*$^{-/-}$ conditions. As there is trend towards an increase in Ki-67^{+} cells in the *Cxcl4*$^{-/-}$ cells, it suggests that this experiment should be repeated with more animals and perhaps examining male animals.

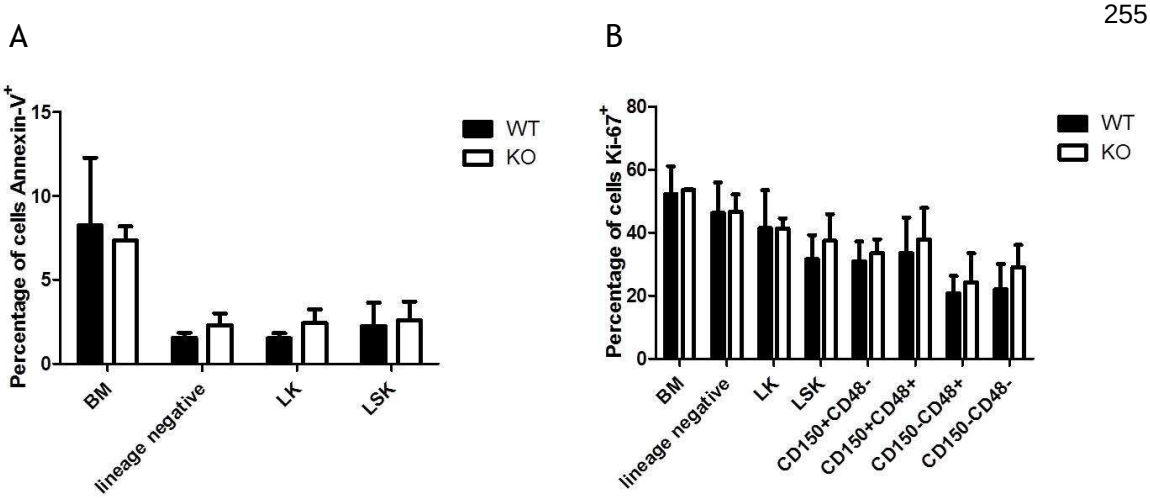

Figure 5-17 Viability and cell cycle status in HSC derived from WT and *Cxcl4*$^{-/-}$ animals.

BM cells were stained with antibodies against lineage, Sca-1, c-Kit, CD150 and CD48 and Annexin-V or Ki-67 and analysed using flow cytometry (LSK only for panel A). Data are presented as the mean percentage of Annexin-V^{+} (A) cells in BM, lineage negative, LK and LSK cells (A) or the percentage of Ki-67^{+} cells in BM, lineage negative, LK, LSK and HSC populations (B) between strains. Statistical test carried out was a student's unpaired *t* test assuming equal variances (B) and with Welch's correction (A) (n.s., $n = 3$). Animals were 6-12 weeks of age and gender was the same (WT 3 females; *Cxcl4*$^{-/-}$ 3 females).

5.3.4.4 *Cxcl4*$^{-/-}$ BM cells show no difference in colony numbers compared to controls

Colony formation primary and secondary replating assays were used to get an indication of whether stem/progenitor cell function is impaired in cells lacking *Cxcl4.*

5.3.4.4.1 BM

Results showed no difference in colony numbers in cells derived from the BM in a primary plating assay (n.s., $n = 6$) (Figure 5-18). In a secondary replating assay, results showed a trend towards a decrease in the *Cxcl4*$^{-/-}$ condition which was not statistically significant due to high variation between samples (n.s, $n = 5$) (Figure 5-18).

High variation found between individual samples resulted in a lack of statistical significance. However, this result in combination with data obtained in section 5.3.3.2 provides evidence to support that *Cxcl4* reduction reduces colony formation capability. This could be due to a role of *Cxcl4* in differentiation, survival or proliferation. This could also infer *Cxcl4* plays a role in stem cell self renewal. However, the CFC assay is a more progenitor assay and more in depth experiments are required to confirm the role of *Cxcl4* in stem cell properties.

5.3.4.4.2 Spleen

Results showed no difference in colony numbers in cells derived from the spleen in a primary plating assay, however a trend towards an increase in the CFU-GM colony types was noted (n.s., $n = 4$) Figure 5-19). No difference in the CFU-E or CFU-GEMM colonies was found (n.s., $n = 4$) (Figure 5-19). When the colony types were counted collectively, a trend towards an increase in the number in the *Cxcl4*$^{-/-}$condition was noted which was not statistically significant (n.s., $n = 4$) (Figure 5-19).

The trend towards increase in the number of colonies in the spleen cells in the *Cxcl4*$^{-/-}$ condition suggests there is increased stem/progenitor activity in the absence of *Cxcl4.* This is in accordance with the trend towards an increase reported in the frequency of LK and LSK populations in the *Cxcl4*$^{-/-}$condition (Figure 5-15; Figure 5-16). However, the results were not statistically significant.

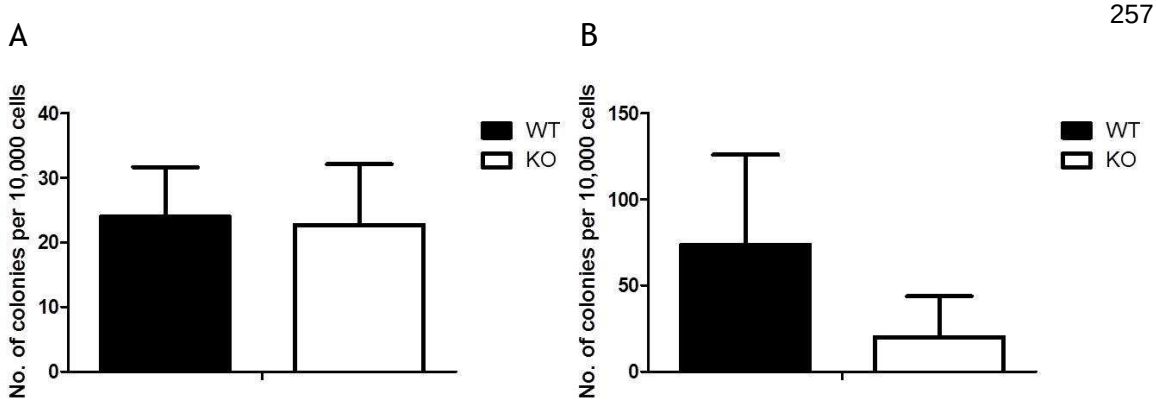

Figure 5-18 *Cxcl4*$^{-/-}$ BM cells show no difference in colony numbers in primary or secondary replating assays in comparison to WT cells.

BM cells harvested from WT and *Cxcl4*$^{-/-}$ animals were cultured in Methocult™ for 10-14 days and colonies were counted, cells harvested and plated in a secondary plating assay. Data are presented as the mean number of total colonies between strains in a primary (A) (n.s., $n = 6$) and secondary replating assay (B) (n.s., $n = 5$). Statistical test carried out was a student's unpaired *t* test with the assumption of equal variance (A) and with Welch's correction (B). Animals were 6-12 weeks of age and gender was the same (CFC1 WT 6 females; *Cxcl4*$^{-/-}$ 6 females; CFC2 WT 5 females; *Cxcl4*$^{-/-}$ 5 females).

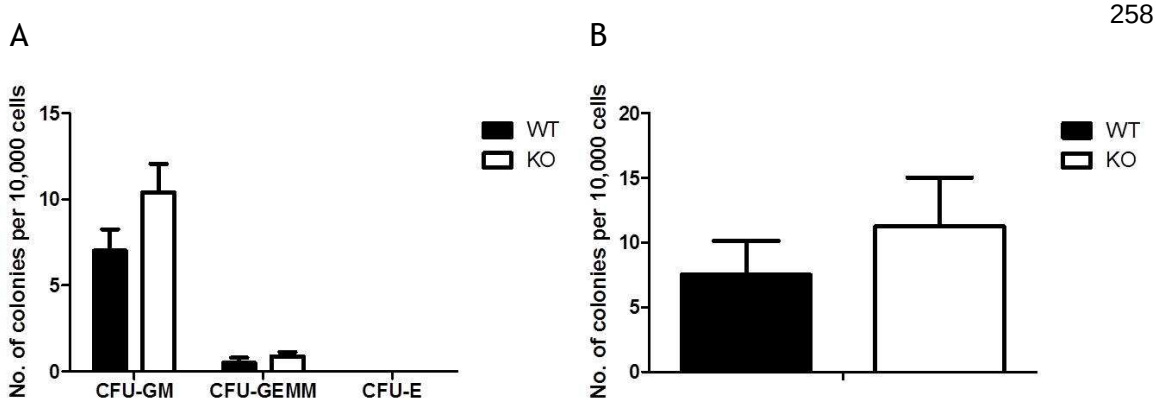

Figure 5-19 *Cxcl4*$^{-/-}$ spleen cells show no difference in colony numbers in comparison to WT cells.

Spleen cells harvested from WT and *Cxcl4*$^{-/-}$ animals were cultured in Methocult™ for 10-14 days and colonies were counted. Data are presented as the mean number of different colony types (A) and total colonies between strains (B) in a primary assay. Statistical test carried out was a student's two-tailed unpaired *t* test assuming equal variance (n.s., $n = 4$). Animals were 6-12 weeks of age and gender was the same (WT 4 females; *Cxcl4*$^{-/-}$ 4 females).

5.3.4.5 WT and *Cxcl4*$^{-/-}$ show no difference in BM reconstitution potential

Collectively, the results show that cells in which *Cxcl4* is reduced show a reduction in colony formation. The gold standard technique for assaying HSC activity is the BM reconstitution assay. LT-HSC were isolated from WT or *Cxcl4*$^{-/-}$ BM and examined for the ability to engraft in a recipient with a lethally ablated BM as described previously in chapter 4.

PB from irradiated recipient animals were examined for the percentages of CD45.1^{+} versus CD45.2^{+} cells to track engraftment as described previously in chapter 4. Furthermore, within the CD45.2^{+} fraction, the percentage of myeloid (GR1^{+} and CD11B^{+}), B (CD19^{+}) and T (CD4^{+} and CD8^{+}) cells were examined to assess the donor contribution to multilineage differentiation. Due to availability of mice, different sexes were used in this assay. This should be noted as same sex would make the experiment more reliable.

At up to 16 weeks post transplant, no differences in the percentage of donor cells in the PB was found between WT and *Cxcl4*$^{-/-}$ groups (n.s., n = 7, 6) (Figure 5-20). Panel B in Figure 5-20 shows engraftment levels obtained in individual animals from each group and it can be seen that although some inter group variation exists, the means are similar.

Within the CD45.2^{+} cells, the percentage of cells positive for mature cells was examined at 16 weeks post transplant. Data showed no difference in the ability of the recipient animals to produce mature cell types at 16 weeks post transplant (n.s., n = 7, 6) for WT and *Cxcl4*$^{-/-}$ conditions (Figure 5-20). The data trended towards an increase in myeloid cells and decrease in T cells in *Cxcl4*$^{-/-}$ derived cells, however this was not statistically significant and likely represents heterogeneity between samples.

Primary recipients were sacrificed at 16 weeks post transplant and the engraftment of donor cells was examined in the mature cell types (myeloid, erythroid and B cells) and stem cells within the BM and spleen. No differences were found in the engraftment of any cell types in the BM or spleen between WT and *Cxcl4*$^{-/-}$conditions (n.s., n = 7, 6).

In terms of the stem cell populations, no differences were found in $CD45.2^+$ cells between groups in the BM (n.s., n = 7, 6). Similar results were obtained with the spleen between conditions (n.s., n = 7, 6).

If *Cxcl4* was regulating self renewal, the assay to conclude this is a secondary BM transplantation assay. In this assay, donor derived stem cells are harvested from primary recipients and transplanted into secondary recipients and examined for engraftment. Due to time constraints in this study, this experiment could not be completed. It can be seen in Figure 5-20 that an increase in $CD45.2^+$ cells over 16 weeks is found in the recipients transplanted with WT HSC. This is as expected as it indicates that the HSC are self renewing over time and able to contribute to haemopoiesis. A defect in self renewal activity would reduce the ability of the donor cells to contribute to haemopoiesis. In the recipients transplanted with *Cxcl4*$^{-/-}$ HSC, an increase in $CD45.2^+$ cells are found up to 12 weeks. However, at the 16 week time-point, a reduction/no change in engraftment is found in comparison to the 12 week time-point (Figure 5-20). This could potentially represent a defect in self renewal which would complement the previous results, however this is not conclusive. To assess defects in self-renewal conclusively, engraftment in secondary transplantation assays needs to be assessed.

Donor cells from animals were aged matched (6-12 weeks) but differed in sex due to availability (WT F, KO M). Recipient animals were age matched (6-12 weeks) and were gender matched but due to toxicity related deaths final analysed animals were skewed (WT 3M, 4F; and *Cxcl4*$^{-/-}$ 1M, 5 F). This experiment should be repeated with cells derived from donors matched for gender. This is important in stem cell biology and there is evidence to support that HSC behave differently in response to gender (Nakada et al., 2014).

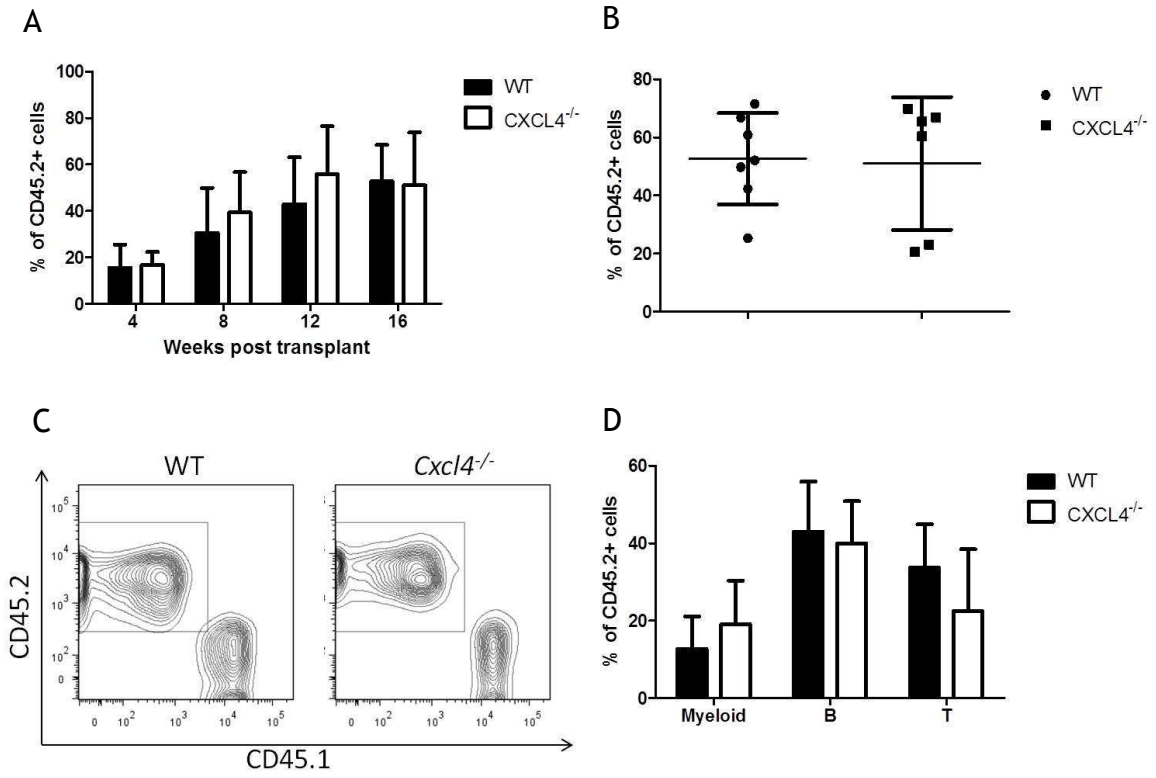

Figure 5-20 WT and *Cxcl4*$^{-/}$ HSC^{-} show no difference in the engraftment or multilineage differentiation capacity after BM transplantation.

LT-HSC (10^2) were sorted from BM from WT or *Cxcl4*$^{-/-}$animals and mixed with $2.0x10^5$ BM cells from mice on a CD45.1 background. Data are presented as the mean percentage of CD45.2$^+$ cells in recipient mice transplanted with either WT or *Cxcl4*$^{-/-}$HSC in the PB across 16 weeks (A). Each recipient animal is displayed as a single symbol on the graph at 16 weeks post transplant to demonstrate heterogeneity in engraftment between samples (B). A representative flow cytometry plot is used to demonstrate engraftment between conditions (C). Within the CD45.2$^+$ cells, the percentage of mature cell types was examined in the PB including myeloid (GR1$^+$CD11B$^+$), B (CD19$^+$) and T (CD4$^+$CD8$^+$) cells (D). Antibodies against CD4 and CD8 were used in the same fluorophore therefore T cells are labelled as double positive cells (CD4$^+$, CD8$^+$) only. Data are presented as the mean percentage of mature cell types within the CD45.2 $^+$ fraction (n = 7, 6) (B). A two-way ANOVA was used with Sidak's multiple comparisons to compare differences between WT and *Cxcl4*$^{-/-}$ null animals at each time point (A). A two tailed unpaired *t* test was used to compare differences in percentage of mature cells within CD45.2$^+$ cells between WT and *Cxcl4*$^{-/-}$ conditions (D). Animals were 6-12 weeks of age and mixed gender (donor WT 2 females; *Cxcl4*$^{-/-}$ 2 males; recipient WT 3 males, 4 females; *Cxcl4*$^{-/-}$ 1 male and 5 females).

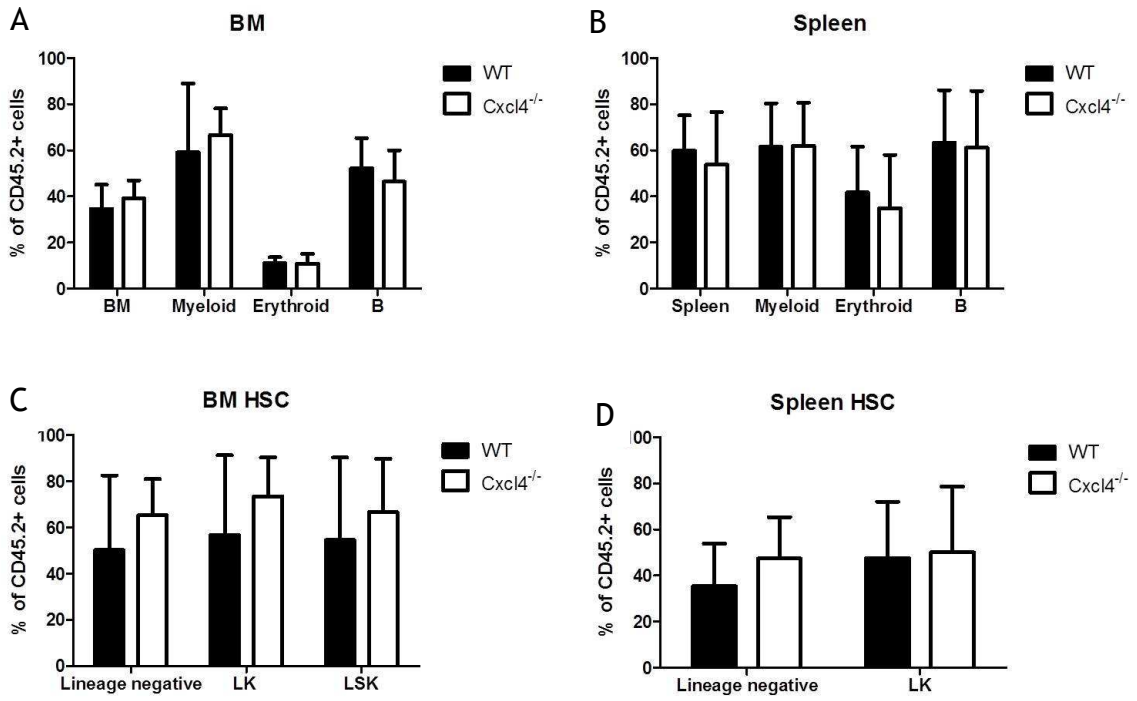

Figure 5-21 WT and *Cxcl4*$^{-/-}$ show no differences in the contribution to mature, stem and progenitors in a BM reconstitution assay.

LT-HSC (10^2) were sorted from BM from WT or *Cxcl4*$^{-/-}$animals and mixed with $2.0x10^5$ BM cells from mice on a CD45.1 background. Animals were sacrificed at 16 weeks post transplant and examined for the percentage of CD45.2$^+$ cells within the BM and spleen HSC and progenitor cell types (A-D). Data are presented as the mean percentage of CD45.2$^+$ cells within each cell fraction stated (n = 7). No statistically significant differences were reported using a student's unpaired t test with Welch's correction for unequal variance (n.s.). Animals were 6-12 weeks of age and mixed gender (donor WT 2 females; *Cxcl4*$^{-/-}$ 2 males; recipient WT 3 males, 4 females; *Cxcl4*$^{-/-}$ 1 male and 5 females).

5.3.5 CXCL4 is highly expressed on human HSC and up regulated on the most primitive, quiescent fraction

To relate the mouse work to the human system, *CXCL4* expression was examined on human HSC populations. Data from a published microarray on human HSC populations was shown to be inconclusive for *CXCL4* expression ((Graham et al., 2007); data not shown). Furthermore, there was no literature to suggest *CXCL4* is expressed by human HSC. To examine this, human HSC populations were sorted and examined for *CXCL4* expression at the mRNA and protein level.

5.3.5.1 *CXCL4* gene expression

Primitive HSC ($CD34^+CD38^-$) and more proliferative progenitor ($CD34^+CD38^+$) fractions in BM and PB derived human samples were stained, sorted and examined for *CXCL4* expression. Results showed high levels of expression in the HSC populations from both sources, with an up regulation in the most primitive fraction ($CD34^+CD38^-$). The results showed a 0.03 and 0.12 fold change in *CXCL4* levels in the BM and PB respectively in the $CD34^+CD38^+$ fraction using the $CD34^+CD38^-$ fraction which was set to the value of 1 (Figure 5-22). This suggests that *CXCL4* is highly expressed in human HSC in comparison to progenitor populations. The $CD34^+CD38^-$ HSC fraction can be further enriched for a purer, more primitive population. Gene expression was examined in $CD34^+CD38^+$ versus $CD34^+CD38^-CD90^-$ and $CD34^+CD38^-CD90^+$ fractions. PB derived samples were used due to the availability of material. Results showed an up regulation in the $CD34^+CD38^-CD90^-$ and the $CD34^+CD38^+$ fraction in comparison to the $CD34^+CD38^-CD90^+$ fraction which was set to the value of 1 (n = 3) (Figure 5-22). Collectively, the results show that *CXCL4* is expressed in human HSC populations with highest expression in the most primitive, quiescent HSC fraction ($CD34^+CD38^-CD90^+$).

5.3.5.2 CXCL4 protein expression

To ensure that the CXCL4 protein was translated, protein expression was examined using intracellular flow cytometry. PB derived HSC were stained to assess HSC ($CD34^+CD38^-CD90^+$) and progenitor ($CD34^+CD38^+$) fractions with the addition of a monoclonal antibody against CXCL4.

Results showed that all HSC populations stained positive for CXCL4 in comparison to isotype control stained cells ($CD34^+$; $CD34^+CD38^-$ and $CD34^+CD38^+$). This was not in

accordance with the gene expression data and the flow cytometry plots suggest this is non specific staining due to a shift in the whole population (Figure 5-23). It is understood that there can be technical problems associated with chemokine antibodies using intracellular staining and this was not further pursued due to time constraints. Ideally western blotting or immunofluorescence should be used for conclusive evidence of CXCL4 protein expression in human HSC.

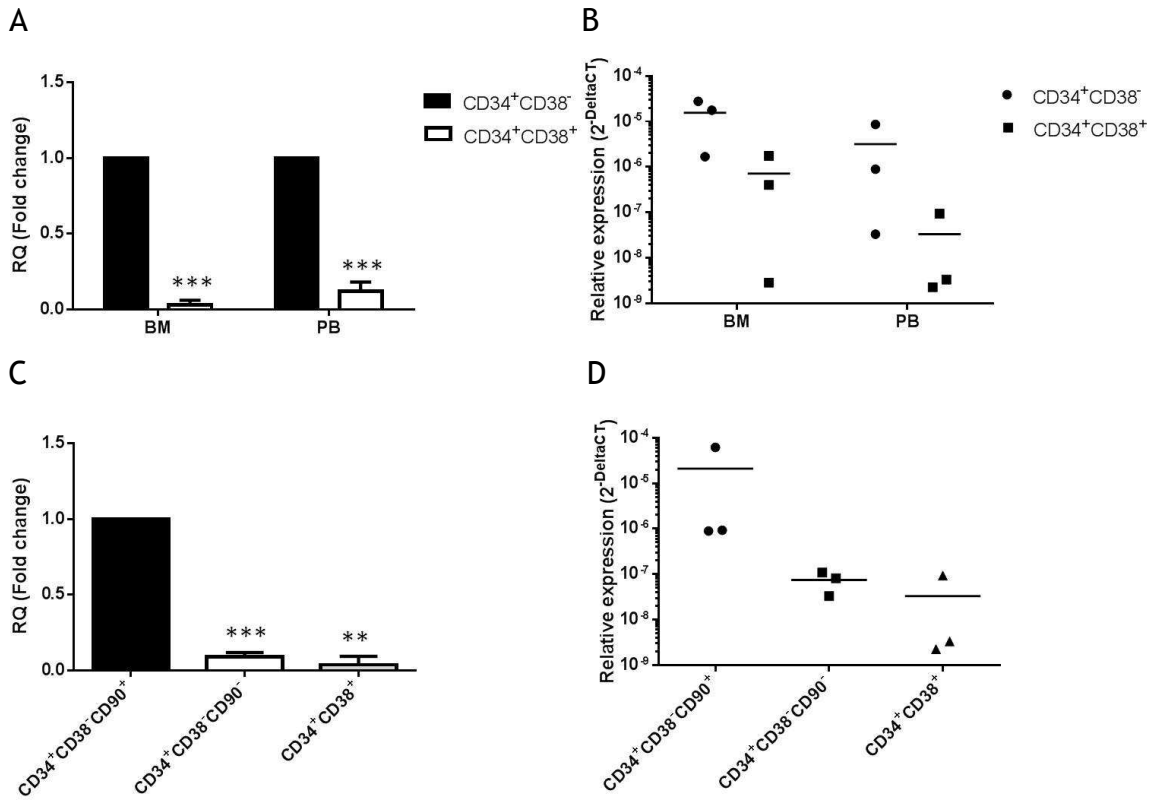

Figure 5-22 *CXCL4* is highly expressed in human HSC with an up regulation in the more primitive fraction.

BM or PB derived cells were sorted for CD34, CD38 and CD90 cell populations (500 cells), RT and Q-PCR was carried out. Gene expression was calculated using expression levels of housekeeping control *GAPDH* and using the DeltaDeltaCT method. Data are presented as the mean fold change in *CXCL4* expression in BM and PB derived $CD34^+CD38^+$ fraction using the $CD34^+CD38^-$ as a calibrator set to the value of 1 (A) (*** P <0.001) (n = 3). Panel C displays the fold change in *CXCL4* expression in the $CD34^+CD38^-CD90^-$ and $CD34^+CD38^+$ fraction using the $CD34^+CD38^-CD90^+$ as a calibrator set to the value of 1 (C) (** P <0.01; *** P <0.001) (n = 3). Panels B and D show relative expression using $2^{-DeltaCT}$. Each dot represents the average of technical triplicates from three independent experiments. A paired t test (A) and a repeated measures one-way ANOVA with Dunnett's test for multiple comparisons (C) was used to analyse statistical differences between the $CD34^+CD38^-CD90^+$ population against the other populations. Patient samples used were of mixed age, gender and health status.

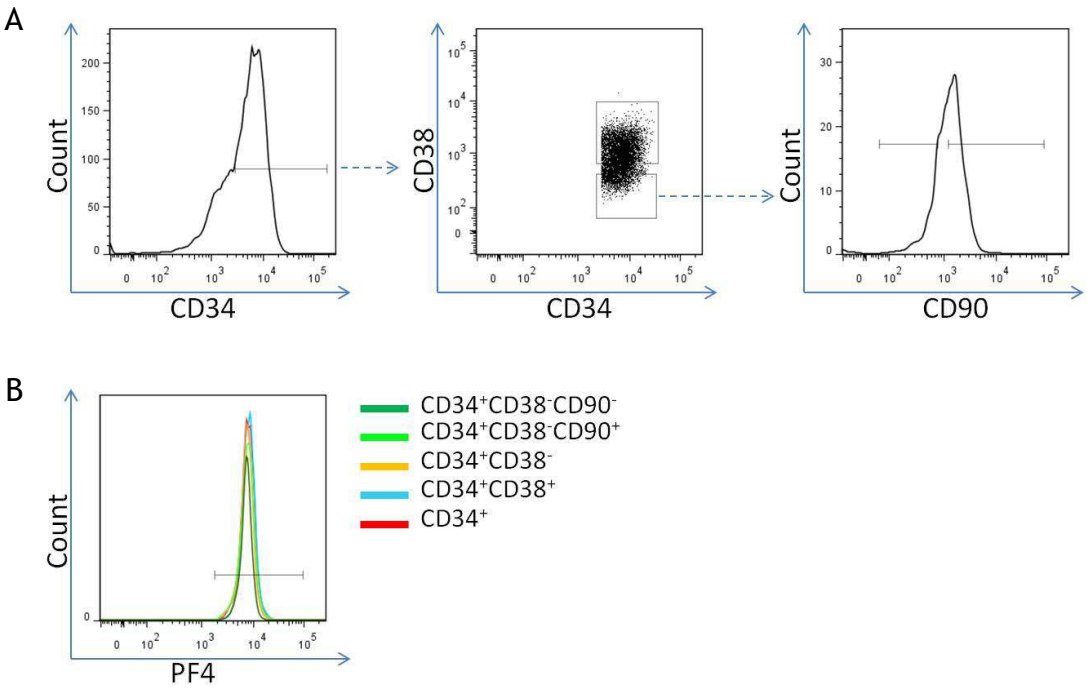

Figure 5-23 *CXCL4* flow cytometry monoclonal antibody is not appropriate to detect CXCL4 in human HSC.

Human PB samples were stained with antibodies for CD34, CD38, CD90, fixed, permeabilised and analysed for CXCL4 expression. Data are presented as the flow cytometry plots for the gating strategy used for different human HSC populations (A) and the observed histogram for $CXCL4^{+}$ expression in each population (B) (n = 1). Gates are set according to isotype control which correlated with unstained cells.

5.4 Discussion

Collectively, the data from this chapter show that human and mouse HSC express *CXCL4* and the chemokine may play a role in stem cell properties, such as stem cell self renewal.

The *Cxcl4-Cre* reporter model was previously used to study platelet biology as it was originally thought that *Cxcl4* was expressed solely on platelets and megakaryocytes. However, data from this study identifies that *Cxcl4* is also expressed on HSC populations and subsequent mature cells in haemopoiesis. The question arises, why was this not identified in previous studies? Previously this model has been shown to be specific for platelet and megakaryocyte cells (Bertozzi et al., 2010) (Tiedt et al., 2007). However, other studies have shown that *Cxcl4* driven reporter models show expression outside these cells (Chagraoui et al., 2011). It is possible that technical reasons and differences in data analysis are responsible for discrepancies between studies. More specifically, previous studies have used immunohistochemistry, while flow cytometry is thought to be more sensitive. Secondly, varying reporter genes have been used, such as *lacZ*, which possibly show variations in expression. As data in this chapter showed endogenous *Cxcl4* expression in HSC populations at the mRNA level, this provides further evidence that *Cxcl4* is expressed in HSC populations. The identification that *Cxcl4-Cre* is expressed in HSC is important, not only for the study of *Cxcl4* in HSC biology, but also for those who use the *Cxcl4-Cre* models. Although these models have provided invaluable data regarding platelet biology, any HSC phenotype, for example using these mice, may be misinterpreted as involving platelet and megakaryocyte biology, which may not be the case and should be interpreted with care. As a positive control using the *Cxcl4-Cre* model, platelets and megakaryocytes were examined for RFP expression and it was predicted these cell types should show maximum RFP expression. The results showed that approximately 100% of platelets showed RFP^+ expression and approximately 50% of the megakaryocytes were RFP^+. This is perhaps due to the presence of immature megakaryocytes which have yet to produce *Cxcl4* and subsequently RFP expression, which has been noted in another study with integrin β1. In contrast, the *Cxcl4-Cre* transgene, although useful, has its pitfalls. Integration can be random therefore an active model for lineage tracing would be better.

In addition, a proportion of RFP^+ cells are noted in thymocytes which cannot be explained by megakaryocytes and platelets. This suggests that *Cxcl4* becomes transcriptionally active in stem cells which then results in a proportion of positive cells in all lineages including thymocytes. The reason only a proportion of mature cells are RFP^+ suggests that only a

subset of HSC switch on *Cxcl4* therefore mature cell types contain a mix of both positive and negative cells. Indeed, HSC analysis shows not all HSC are RFP^{+}. Again, cells which are marked with *Cxcl4* transcription with a dynamic reporter would help address these questions.

As *Cxcl4* is thought to be a 'platelet specific' gene, it is surprising that expression was found in a stem cell population. In support of this, genes thought to be associated solely with platelet biology or other lineage specific paths have been identified to be expressed in stem cell populations (Pina et al., 2012). Indeed, platelets are a major source of CXCL12 and CXCR4 (Chatterjee and Gawaz, 2013). One example to support this has been reported in a previous study in which another 'platelet associated' gene (*Vwf*) was noted to be expressed in HSC in the mouse system (Kent et al., 2009). Furthermore, this particular gene was noted to be highest and consistently expressed in the self renewing HSC fraction which supports the results and conclusions proposed in this chapter. In addition, recent research published in Nature by Sten Jacobsen and his group used a *Vwf* driven GFP reporter mouse model (Sanjuan-Pla et al., 2013). Their research showed only a subset of HSC were positive for *Vwf* transcription. They have data to show that this population marks a mouse HSC subset primed for platelet-specific gene expression. They suggest the HSC hierarchy starts with platelet primed HSC at the apex. Possibly *Cxcl4* is playing a similar role in HSC biology, however experiments will need to address this.

Results using shRNA to knock down *Cxcl4 in vitro* showed a decrease in colony formation in cells with reduced *Cxcl4* in primary plating assays, which was more exaggerated in secondary replating assays. This result indicates that *Cxcl4* is controlling colony formation, but to a greater extent self-renewal. This could be supported by the experiments using $Cxcl4^{-/-}$ BM which showed a reduction of colonies in a secondary replating assay in the cells lacking *Cxcl4* however it should be noted that this result was not statistically significant. It is possible that $Cxcl4^{-/-}$mice are less sensitive than WT cells with *Cxcl4* reduction *in vitro*. Furthermore, a transgenic reporter mouse model under the promoter of *Cxcl4-Cre* showed that *Cxcl4* negative cells showed decreased colony formation in a primary plating assay. In the secondary replate, no difference was found between conditions. One possibility for the discrepancy in these results is that the transgenic reporter model does not mark active *Cxcl4* expression and only reports expression in which a *Cxcl4* transcription was active at some point. It is possible that the cells respond to culture conditions and switch on or switch off *Cxcl4* transcription and show skewed results. Therefore more conclusive evidence was obtained using *Cxcl4* reduction *in vitro*

and from *Cxcl4*$^{-/-}$ cells. Possible future work will be to study a transgenic model which marks active *Cxcl4* transcription with a reporter gene. In this way, the expression pattern of *Cxcl4* and its activity can be traced over time. In addition, the *Cxcl4*$^{-/-}$mice were all female and the transplantation assays had mixed genders. It is possible that the *Cxcl4* is gender specific and further experiments should address this. This could be explained by the experiment in which *Cxcl4* is reduced *in vitro* as these animals were male.

The key experiment to investigate self renewal is to examine the ability of a HSC population to reconstitute a lethally ablated BM in a secondary transplantation assay. This experiment is currently under way and it is hypothesised that a decrease in engraftment will be noted in recipients engrafted with *Cxcl4*$^{-/-}$ HSC populations, however this was outside the timeframe of this study. An experiment to compliment this could use WT HSC transduced with *Cxcl4* shRNA and the engraftment in secondary recipients should be examined. However, this experiment was outside the time frame in this study.

The data in this chapter examines the role of *Cxcl4* in adult haemopoiesis. It is possible that the chemokine may play a role in early haemopoiesis in addition to adult haemopoiesis, however this should be further investigated.

The results in this chapter showed that *CXCL4* is highly expressed in a primitive human HSC population, however the role is unclear. Data from mouse studies suggested that it was playing a role in self renewal and this should be examined in human studies. There is little literature on the expression of *CXCL4* on human HSC populations. One study examined expression between BM, CB and G-CSF mobilised PB and noted a much higher expression in BM derived cells (Ng et al., 2004). Results in this chapter showed both BM and PB derived samples had an up regulation in expression in the $CD34^+CD38^-$ fraction, however expression between samples was not compared. It is possible that BM derived cells express higher levels due to the increase in quiescence in these cells in comparison to PB sources. The data in this chapter investigated a more in depth analysis of *Cxcl4* expression in the stem cell compartment. Although there is little literature currently to suggest human HSC express *CXCL4*, there is evidence to support that the cells can respond to the chemokine. Studies have shown that recombinant *CXCL4 (rCXCL4)* supports the viability of BM cells, including the $CD34^+$ fraction, and provides a protective effect from cytotoxic drugs (Han et al., 1997). Furthermore, *rCXCL4* was found to increase quiescence in $CD34^+$ cells in comparison to the control (Huang et al., 2000). Another study noted that *CXCL4* enhanced the adhesion of $CD34^+$ cells to intact stroma (Dudek et al., 2003).

However, self renewal has not been assessed. It is possible that cells respond to the chemokine and plays several functions in these cells, including promoting cell viability and self renewal. This also suggested that *CXCL4* may be acting in an autocrine fashion, with evidence that human HSC express and respond to the chemokine. This is in accordance with data from the mouse system, in which the mouse stem/progenitor cells expressed *CXCL4* and also responded to modulation.

To extend this research in a possible future avenue, the mechanism of action can be examined. CXCL4 is a curious chemokine and in the literature it is not well understood which receptor it binds to or how it elicits its effects. Although there are various proposed mechanisms of action, currently it is unclear in the literature therefore future experiments are required to address this, particularly in the context of HSC properties.

6 Conclusion

6.1 Concluding remarks and future work

The haemopoietic system is an elegant hierarchical organisation in which a stem cell population is responsible for producing and maintaining all the mature haemopoietic cell types over the period of a lifetime (Weissman, 2000). The biological decisions of the HSC population are tightly controlled for the maintenance of the stem cell pool and the production of multi-lineage differentiated cells for basal haemopoiesis and when required in response to haemopoietic stress or injury (Passegue et al., 2005). HSC respond to intrinsic and extrinsic factors in the BM niche (Zon, 2008, Pietras et al., 2011, Blank et al., 2008). The deregulation of cellular fate decisions consequently disrupts stem cell maintenance and subsequently results in defects in the haemopoietic system. There is evidence to suggest that stem cell fate decisions are deregulated in response to ageing and in malignant transformation, including diseases of the haemopoietic system, such as leukaemia (Geiger et al., 2013) (Warr et al., 2011). However to begin to understand cancer and cancer stem cell properties in particular, it is essential to first understand the regulation of normal stem cells. It is therefore fundamental that the molecular mechanisms underlying cellular fate decisions are well understood.

A variety of studies have aimed to understand the molecular mechanisms of HSC cellular fate regulation. Studies have used a combination of data driven approaches using high-throughput expression studies and hypothesis driven research for the identification of novel candidates in the regulation of HSC fate. One of these studies compared the global gene expression profile between quiescent and proliferating HSC populations through a microarray that aimed to identify novel transcriptional targets that may be key to HSC regulation (Graham et al., 2007). However, the nature of a microarray alone does not allow for interpretation of the biological role of these chemokine ligands within the haemopoietic hierarchy. Therefore, it was aimed to expand current knowledge that is critical to the current understanding of HSC fate regulation. The aim of this present study was to validate findings from the study conducted by Graham *et al*. as well as focus on the biological role of CXC chemokines within haemopoiesis, including cell cycle regulation. In this conclusion chapter, the results obtained from this study are discussed in accordance with previous literature and possible future avenues for research are proposed.

6.1.1 High-throughput screening as a tool to identify novel candidates in biological processes

The genes examined in this study were identified from a microarray screen comparing gene expression in quiescent (G_0) and dividing (G_1, G_2, S and M) normal HSC populations (Graham et al., 2007). This highlights the advantages of using a high-throughput approach to identify novel candidates in biological processes. Systems biology is a field of study which combines biology with informatics in an aim to understand complex interactions within biological systems. Systems biology has emerged over the past several years as a novel high-throughput approach to compare expression patterns between different cell populations which has aided biological and biomedical research (Soon et al., 2013). Systems biology approaches allow global expression patterns to be compared between different cell populations at the epigenetic, genetic, protein and phospho-protein levels. This approach allows a data driven method which has the ability to identify several differentially regulated components in a variety of cellular processes. This can be applied to studying genes involved in biological processes, such as HSC cellular fates or disease populations. Furthermore, analysis can identify candidates for further study that can prove useful for drug discovery research. In terms of stem cell biology, these techniques have allowed us to increase our understanding of the biological system.

Various studies have employed this approach to examine the differential expression between HSC populations. However, only one previous study to date has identified CXCR2 signalling in this context. This is likely due to differences in samples used, method of cell isolation, culture conditions, data acquisition and the informatics analysis. This highlights an important issue in which although high-throughput screening has increased our understanding, there are disadvantages involved. The main limiting factors are expense and the need for large amounts of material for screens which is sometimes not possible when studying rare populations, including stem cell populations. Importantly, the expression data obtained is combined with bioinformatics analysis and there are several different approaches in the analysis step (Slonim, 2002). Consequently, it is possible that significant and biologically relevant candidates are neglected due to the particular threshold values used in the analysis step or even due to technical problems with the assay. As an example, in this study the chemokine *CXCL4* was investigated due to its structural similarity to the candidates identified from the screen and from unpublished data and evidence in the literature. However, *CXCL4* was not originally identified as differentially expressed between quiescent and proliferating normal human HSC in the original

microarray. On the contrary, it is understood that there is a high level of 'noise' obtained from global expression studies, leading to the identification of candidates which are not biologically significant. Therefore, it is crucial for candidates to be validated after the array step. Collectively these points highlight that there is the need for data driven expression data in combination with hypothesis driven research approaches. This includes the use of appropriate bioinformatics analysis, a comprehensive review of the literature and most importantly experimental validation.

6.1.2 The role of CXCR2 signalling in HSC properties

The aim of this study was to extend previous research with the main question addressing what is the biological role of particular CXC chemokines in terms of HSC properties? Importantly, the current study corroborated the microarray data from the Graham *et al,* study to show that CXCL1 and its receptor CXCR2 are expressed by human HSC populations. Furthermore through inhibition experiments, the current study demonstrated that CXCL1 and CXCR2 may play a pro survival role in human HSC. However, it is important that these experiments are repeated to obtain an appropriate number of biological replicates so that proper conclusions can be made. In addition, as discussed in section 3.3.2 further experiments are required to conclude that human HSC express CXCL1 due to technical issues with antibodies.

CXCR2 is a promiscuous receptor, which is capable of binding several ligands, which are structurally very similar, thus suggesting redundancy between them (Rossi and Zlotnik, 2000). It was therefore surprising that one key ligand for the receptor showed such a dramatic effect on the viability of HSC, considering several other ligands for the receptor were also found to be expressed on HSC. However, the results obtained in this study were supported by evidence in the literature which shows that although CXCR2 ligands share similar roles in some processes, in other processes, such as autocrine driven viability and proliferation, CXCL1 and CXCR2 pathways play clearly distinct roles. This has been reported in various cell types, including oligodendrocyte precursor cells and epithelial ovarian carcinoma cells (Bolitho et al., 2010, Filipovic and Zecevic, 2008, Botton et al., 2011, Tsai et al., 2002). To extend this research, future experiments should validate this research. In addition, experiments to extend this research might examine the mechanism of action with examination of signalling pathways involved. Experiments from other cell types implicate the involvement of ERK1/2 and epidermal growth factor (EGF) in CXCR2 signalling (Miyake et al., 2013). These have been shown to influence cell proliferation in

human ES cells and could therefore be potential candidate pathways to explore (Schuldiner et al., 2000). It is not surprising that a pro survival signalling pathway is expressed in normal HSC and up regulated in the quiescent sub population as stem cells are designed to be robust and viable in order to protect the haemopoietic system. Indeed, this is supported by the evidence that a variety of pro survival genes and pathways are up regulated in the most primitive HSC population. Survival is an important feature of HSC as the balance of survival and programmed cell death in the HSC population is tightly regulated to control the numbers of the stem cell pool (Wagers et al., 2002).

For experimental research, it should be considered that HSC do not exist in isolation *in vivo*, they reside in the BM where complex signalling occurs, with both intrinsic and extrinsic factors involving several diverse cell types. Examining human signalling pathways *in vitro* gives a good indication of human function. However, *in vivo* assays can add the advantage of examining the HSC population in the presence of complex signalling in the niche. This is important to this particular study as there is evidence that other cell types in the niche can contribute to determining HSC cell fate (Yin and Li, 2006). To extend the human research in this study, possible future experiments could examine the CXCL1 and CXCR2 interaction on human HSC in the context of the niche. Several approaches to modelling the niche interaction have been developed which could be used. Furthermore, human HSC could be transduced with plasmids to inhibit CXCL1 or CXCR2 expression and the effect *in vivo* observed using BM transplantation assays.

The majority of experiments examining stem cell function have used mouse models. The advantages are the availability of more material and more elegant *in vivo* models of HSC function. The availability of a *Cxcr2*$^{-/-}$ mouse model allowed the human work to be strengthened and to give a more in depth analysis of the role of this signalling pathway in HSC biological properties *in vivo*.

Briefly, the results in this study show that *Cxcr2*$^{-/-}$ animals exhibit extramedullary haemopoiesis amd an expansion of viable LT-HSC in the BM. The data indicates *Cxcr2*$^{-/-}$ HSC may display a reduced ability of LT-HSC to engraft the BM of an irradiated host. However, this result needs to be repeated due to variation between replicates.

To strengthen these results, in addition to repetition of the experiments in this thesis, future work could examine which cell types *Cxcr2* expressing HSC signal to. From the results in this thesis it is therefore currently unclear in the animal system which ligands are involved

in *Cxcr2* signalling and in addition, which cell types are expressing these ligands. One possibility is that CXCR2 binding ligands are expressed by the HSC which were not examined in this study. A well-studied chemokine signalling pathway in haemopoiesis is CXCR4 and ligand CXCL12, which involves signalling between the HSC and stromal cells (Sugiyama et al., 2006). There is literature to show that CXCR2 ligands are expressed by cell types residing in the BM niche, including EC and the possibility of a HSC/stromal cell interaction is also possible (Miyake et al., 2013). Alternatively, it is possible that CXCR2 ligands are expressed by a more proliferative progenitor population which signals to LT-HSC. Figure 6-1 highlights possibilities of how CXCR2 signalling is mediated in the mouse system which future experiments can assess.

For future research, it will be essential to repeat the transplantation experiments with the adidition of serial transplantation assays. If repetition shows that *Cxcr2* expressing HSC show a disadvantage in transplantation ability in comparison to the WT controls, experiments should aim to test why. Two hypotheses are proposed from this result in which either the *Cxcr2*$^{-/-}$ HSC exhaust faster than the WT cells, subsequently losing reconstitution potential, or alternatively the *Cxcr2*$^{-/-}$ HSC have a defect in migration and cannot home to the BM for engraftment. If we first consider the first hypothesis that the *Cxcr2*$^{-/-}$ HSC are exhausting faster than the control counterparts. The tight regulation of self renewal and multilineage differentiation is responsible for the maintenance of haemopoiesis and deregulation in these properties can result in stem cell exhaustion. Cell cycle blocking can inhibit self renewal and in contrast, cell cycle activation can lead to stem cell exhaustion which has been elegantly shown using manipulation of these properties (Yoshida et al., 2008). Deregulation of key self renewal genes results in the activation of cell cycle and consequently results in stem cell exhaustion and therefore reconstitution potential. Future work to further examine this should analyse the cell cycle status of WT and *Cxcr2*$^{-/-}$ HSC populations. Analysis *ex vivo* shows that there is no difference in proliferation as measured using Ki-67 staining in HSC populations between *Cxcr2*$^{-/-}$ cohorts and their control counterparts. However, possible future experiments to conclude this involve *in vivo* cell cycle assays, including the treatment and subsequent analysis of Brd-U to track proliferation of the cells *in vivo* or administrating 5-FU that will introduce stress to the system, which can be analysed between strains. In addition, to compare the self renewal capacity between *Cxcr2*$^{-/-}$ HSC and their WT counterparts, secondary transplantations can be examined and these experiments are currently underway.

In contrast, *Cxcr2* is well-studied for its effects on cellular migration and there is evidence to support this hypothesis in the HSC system. The *Cxcr2*$^{-/-}$ mouse model shows the presence of circulating HSC in the periphery and enhanced numbers of HSC in the spleen. Furthermore, CXCR2 binding ligands have been identified as factors which, when added exogenously to mice, results in mobilisation (Pelus and Fukuda, 2006). In addition, G-CSF treatment, which also leads to HSC mobilisation, has been shown to modulate the expression of *Cxcl1* and *Cxcl2* in the BM niche (Pelus, 2008). Future work should examine the ability of HSC from WT and *Cxcr2*$^{-/-}$ HSC to home to the BM immediately post injection into irradiated hosts which will address this hypothesis.

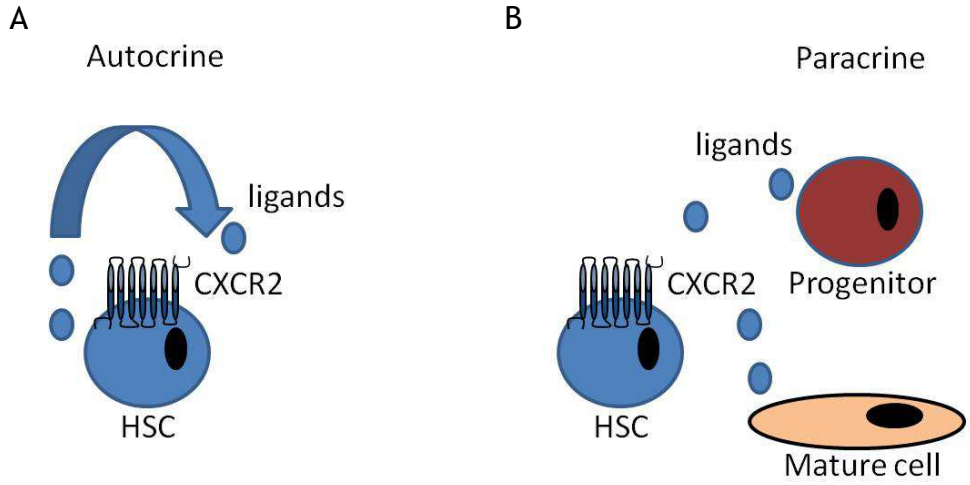

Figure 6-1 Potential mechanisms of CXCR2 signalling within mouse BM.

The schematic diagram illustrates potential mechanisms mediating CXCR2 signalling in mouse HSC. It is unknown from experiments in this thesis how CXCR2 ligands are binding to the receptor and which cell types are involved are in the mouse system. It is possible that an autocrine loop exists with ligands (panel A). Alternatively, paracrine signalling could be involved with ligands expressed by more mature progenitor cells or mature cells within the BM niche including EC (panel B).

6.1.3 The role of CXCL4 signalling in HSC properties

A key gene identified in this study as important in HSC regulation is CXCL4. The current study shows that CXCL4 is expressed by both human and mouse primitive HSC populations. Furthermore, experiments using transgenic mouse models in combination with knock down, collectively identifies CXCL4 alters stem cell colony formation ability and indicate CXCL4 may play a role in stem cell properties. The observation that CXCL4

is up regulated in the most primitive fraction of human HSC supports this hypothesis as it is predicted a key stem cell gene would be highly expressed in this HSC population. The results are fortified by evidence in the literature in which human $CD34^+$ cells have been shown to respond to exogenous CXCL4 with effects on cell viability, adhesion and stem cell expansion (Dudek et al., 2003, Lu et al., 2003, Li et al., 2006, Han et al., 1997). Importantly, the expression of CXCL4 on human and mouse HSC and its role in stem cell properties are novel. One possibility is that CXCL4 is involved in self renewal. HSC self renewal is a fundamental process of HSC and to date a variety of self renewal genes have been identified (Zon, 2008). However, there is still much left to be discovered in this field and the results in this study identify a novel gene involved in the process of self renewal. However, as discussed in chapter 5, serial BM transplantations are required to confirm this result and cannot be concluded with the results in this thesis.

CXCL4 was originally thought of as a lineage specific gene. A variety of studies have identified this gene as expressed solely on megakaryocytes and platelets, however the current study identifies that this gene is expressed by HSC populations. This finding is corroborated by emerging literature that suggests that genes associated with lineage commitment, in particular megakaryocyte/platelet expression, are expressed by HSC and involved in biological roles. An example of this is *Vwf* which shows expression in self renewing mouse HSC populations (Kent et al., 2009). Alternatively, it is possible that CXCL4 is active in a population of HSC which are destined for a particular lineage, however this would require future investigation.

As a possible avenue for future research, the mechanism of action of CXCL4 activity is currently not well understood in the literature and could be investigated (Kasper and Petersen, 2011). As the results show HSC express CXCL4 and modulation of the protein demonstrates a phenotype *in vitro*, this suggests the signalling is occurring in an autocrine manner. However, CXCL4 signalling is complex and not standard as exhibited by the other structurally similar chemokine ligands with 'classical' GPCR signalling (Rossi and Zlotnik, 2000). As detailed in the introduction, CXCL4 signalling is complicated. Expression of CXCR3B to date is not known on human/mouse HSC and could be a possible avenue of future work. Similarly, it is possible CXCL4 is functioning through binding to integrin receptors which have indeed been shown to be important for HSC behaviour (Yin and Li, 2006). Additionally, CXCL4 can heterodimerise with other chemokine ligands (Slungaard, 2005). In the context of HSC, an interesting study has observed the ability of CXCL4 to adhere and bind to $CD34^+$ cells through binding of

CXCL8 and with a $CD34^+$ chondroitin sulphate-containing moiety (Dudek et al., 2003). This would suggest a link between CXCL4 and CXCR2 in HSC biology. Furthermore, CXCL4 can bind GAG which may also transducer the signal. I think that understanding the mechanism of action of CXCL4 signalling in stem cells is particularly important and requires further examination. Figure 6-2 demonstrates possible mechanisms of CXCL4 signalling which can be studied further in future research. Understanding the mechanism of action will not only be important for furthering our understanding of haemopoiesis, but also for the other roles of the protein, including in megakaryocytic/platelet research.

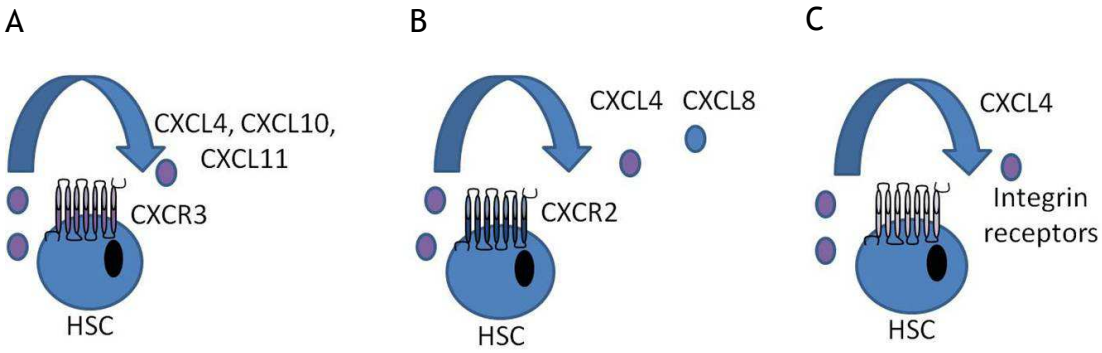

Figure 6-2 Potential signalling mechanisms for CXCL4.

The schematic diagram illustrates potential mechanisms mediating CXCL4 signalling in human and mouse HSC. It is unknown from experiments in this thesis how CXCL4 mediates its effects. It is possible that an autocrine loop exists with ligands including CXCR3B, integrin receptors or GAG (panels A and C). Alternatively, CXCL4 could elicit its effects through binding other ligands such as CXCL8 (panel B).

Finally, in this thesis we have identified a role for CXCL4 in mouse HSC biology. Gene expression analyses have shown that this ligand is expressed by human HSC and indeed is up regulated in the most primitive subsets. This would infer that CXCL4 is important for stem cell behaviour in human HSC. However, due to time constraints, an investigation into the function in human HSC could not be carried out. Future experiments could use inhibitors or shRNA plasmids to block/reduce CXCL4 signalling and examine the phenotype in human HSC.

6.1.4 Understanding normal HSC regulation can be applied to studying disease models

Currently, HSC cellular fates are not well understood and understanding this process in normal HSC is essential before understanding how the process is deregulated in response to disease. Global gene expression studies have been used as an approach for investigating the differences between normal and cancer cells. This approach has provided a starting point for many important discoveries for potential future therapies. A well understood chemokine signalling pathway in HSC biology is CXCR4 and CXCL12, which is shown to exhibit roles in normal HSC, but also is deregulated in leukaemia. Based on experimental data, research is examining the therapeutic advantage of CXCR4 inhibitors in cancer (Burger and Burkle, 2007). The modulation of chemokine signalling can therefore represent a novel therapy in haematological malignancies.

The role of chemokines in malignancies was described in the introduction section. Although the role of CXC chemokines in haematological malignancies was outside the scope of this particular study, the previously published microarray was used to compare normal dividing and proliferating HSC, but also to examine transcriptional differences between normal and leukaemic HSC populations (Graham et al., 2007). This was carried out using CML patient samples which were analysed in comparison to normal HSC populations. Briefly, CML is a disease in which the HSC compartment is transformed with a fusion oncogene (BCR-ABL) which allows the HSC to show deregulated cell fate, including increased survival and proliferation, which are responsible for the disease pathogenesis (Calabretta and Perrotti, 2004, Sawyers, 1999, Rowley, 1973) (panel A, Figure 6-3). CML therefore represents an ideal model in which the leukemic HSC are responsible for the pathogenesis of the disease and therefore should be targeted for the eradication of the disease. Indeed, studies have shown that it is the HSC fraction that is less sensitive to standard therapy both *in vitro* and *in vivo* and which consequently prevents the eradication of the disease (Jiang et al., 2007, Holtz et al., 2002, Bhatia et al., 2003, Graham et al., 2002). It is therefore fundamental that we understand how these leukaemic stem cells (LSC) are deregulated for future novel candidates for therapy to be identified. Informatic analyses were used to show that chemokine ligands were down regulated in quiescent leukaemic HSC in comparison to normal counterparts (Graham et al., 2007) (panel B, Figure 6-3). The data from this study suggests CXCL1/CXCR2 and CXCL4 signalling is a pro survival pathway in human HSC, therefore it is surprising that this would be down regulated in leukaemia. LSC are known to up regulate survival pathways in comparison to

their normal counterparts. However, this was not further examined in this study. Results from an unpublished study indicate that CML LSC up regulate chemokine ligands after treatment with standard therapy drugs, therefore it can be speculated that these genes may indeed play a role in the survival of LSC (unpublished data) (panel C, Figure 6-3). In addition, the data from the microarray identified that chemokine ligands were expressed at higher levels in quiescent CML cells in comparison to proliferating cells. The identification of genes up regulated in quiescent CML LSC is important as this population is less sensitive to current therapy (panel D, Figure 6-3).

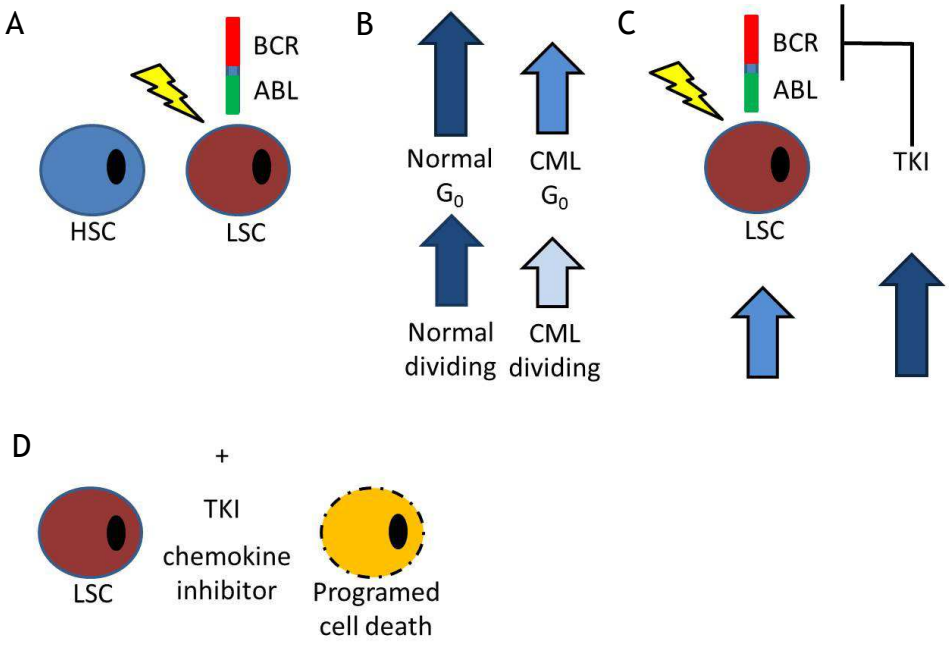

Figure 6-3 CXC chemokines are deregulated in CML and may provide a novel therapy.

CML occurs due to mutation resulting in the juxtaposition of BCR and ABL genes in a novel chromosome. The result of this mutation in a stem cell population results in the generation of a LSC and the pathogenesis of the disease (A). A previously published microarray identified that chemokines (*CXCL1*, *CXCL2* and *CXCL6*) are up regulated in CML G_0 versus dividing, however expression is down regulated in CML G_0 in comparison to normal G_0 (B). Interestingly, treatment of LSC with standard therapy has been shown to increase chemokine expression (C). It is possible that this is due to up regulation of this chemokine as a survival pathway. Consequently, inhibition of chemokine signalling may represent a novel future therapy (D).

Interestingly, both CXCL1 and CXCR2 have been implicated in playing a biological role in diseases, including solid tumours. Tumours in a preclinical lung cancer model have been

noted to be decreased on a *Cxcr2*$^{-/-}$ background (Keane et al., 2004). Similar results have been obtained for CXCR2 in prostate and breast cancer models (Waugh and Wilson, 2008, Snoussi et al., 2010). In terms of a possible mechanism, previous research suggests primary MSC express CXCR2 and facilitate the metastasis of mammary cancer cells to the BM (Halpern et al., 2011). Furthermore, CXCL1 has also been identified to play a role in promoting tumourigenesis, tumour migration and angiogenesis (Dhawan and Richmond, 2002, Halpern et al., 2011). Based on the abundance of the literature, CXCR2 inhibitors have emerged as a useful pharmaceutical target. In contrast, there is also evidence that CXCR2 signalling is involved in tumour prevention with evidence to show that CXCR2 ligands, including CXCL1, are involved in the recruitment of immune cells to clear tumour cells (Acosta and Gil, 2009). Future research could examine whether CXCL1 and CXCR2 is a survival pathway in CML HSC.

Focusing on CXCL4, a previous study shows that CXCL4 can inhibit tumour growth (Vandercappellen et al., 2011). Although there is little literature available on CXCL4 in leukaemia, a recent study notes that the gene is expressed in murine HSC populations in a CML model (Zhang et al., 2012). Furthermore, *CXCL4* expression is reported to be modulated in the absence of hypoxia gene HIF1a, which has previously been shown to mediate cellular responses to hypoxia within the BM niche and is essential for HSC maintenance (Miyamoto et al., 2007, Kranc et al., 2009, Takubo et al., 2010, Zhang et al., 2012). Hypoxia related genes have also been implicated in haemopoietic malignancies therefore are an important avenue for leukaemia research. As an example, in human AML, targeting HIF genes compromises AML functions, implicating these genes in future therapies and HIF genes have been shown to be essential in CML (Zhang et al., 2012, Wang et al., 2011). In addition, CXCL4 has been shown to regulate adhesion of both normal and leukaemic HSC to EC (Zhang et al., 2004). Therefore, CXCL4 in normal HSC biology and leukaemia may be interesting for further pursuit. Furthermore, experiments in this thesis show that CXCL4 is expressed in mouse HSC which was validated using *Cxcl4-Cre*. As discussed in chapter 5, this therefore raises concerns in studies using this model for megakaryocyte/platelet biology. This observation also opens up new possibilities for the use of this mouse model in haemopoietic studies including disease. *Cxcl4-Cre* can therefore be used in combination with other mouse strains to examine disease. As an example, experiments using *Cxcl4-Cre* coupled to inhibition/activation of β-catenin have shown this drives a myelofibrosis phenotype (data not shown).

A possible future avenue to extend the research in this study would be to explore the biological roles of CXCL1/CXCR2 and CXCL4 signalling in disease models, including leukaemia, however this was not within the scope of this study. Possible future experiments should examine expression and function of these genes and pathways in leukaemic HSC in comparison to normal HSC. Several mouse models of CML are currently available and could provide insight into whether these chemokines play a role in leukaemia initiation and maintenance and could provide a novel therapy (Koschmieder and Schemionek, 2011). Future experiments could examine disease initiation and maintenance in animals lacking *Cxcr2* or *Cxcl4.* Animals on a *Cxcr2*$^{-/-}$ or *Cxcl4*$^{-/-}$ background could be crossed with animal models of CML or alternatively, BCR-ABL^{+} cells can be generated using retroviral transduction which can be transplanted into *Cxcr2*$^{-/-}$ or *Cxcl4*$^{-/-}$ hosts.

7 Supplementary

7.1 Western blotting images

Original western blot images from the following figures are shown below (Figure 3-4, Figure 3-7 and Figure 3-10). Images can be found in Figure 7-2, Figure 7-4 and Figure 7-4 respectively. In addition, a western blot image of human rCXCL1 is displayed (Figure 7-1). Original, full blots are provided with an overlay of the molecular weight marker with sizes of bands.

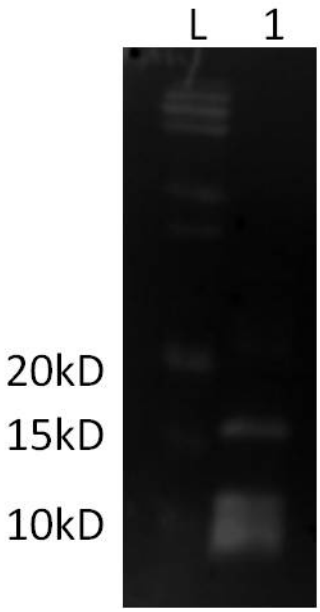

Figure 7-1 Raw western blot image of human rCXCL1.

Raw image of western blot with human rCXCL1 protein which has been run on a 15% gel. L = molecular marker ladder. 1 = rCXCL1. Approximate sizes of the marker are shown on the left of the image in kD. Three bands were found with the protein at ~8kD, ~12kD and ~16kD.

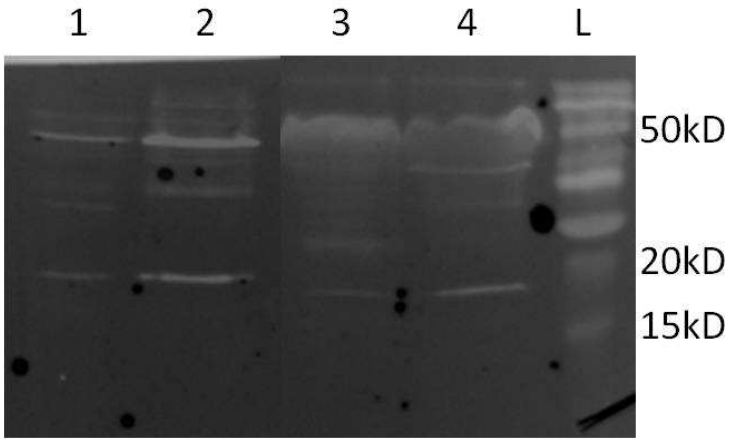

Figure 7-2 Raw western blot image for primary human cells sorted for $CD34^+CD38^-$ and $CD34^+CD38^+$ populations.

Raw image of western blot with human primary $CD34^+$ cells which has been run on a 15% gel. L = molecular marker ladder, 1 = PB $CD34^+CD38^+$, 2 = PB $CD34^+CD38^-$, 3 = BM $CD34^+CD38^+$, 4 = BM $CD34^+CD38^-$ with both CXCL1 and β-Tubulin antibodies. Approximate sizes of the marker are shown on the right of the image (kD). Several bands were found with the CXCL1 antibody with the most prominent at ~16kD. A single band was found in the housekeeping control at the estimated size of 50kD.

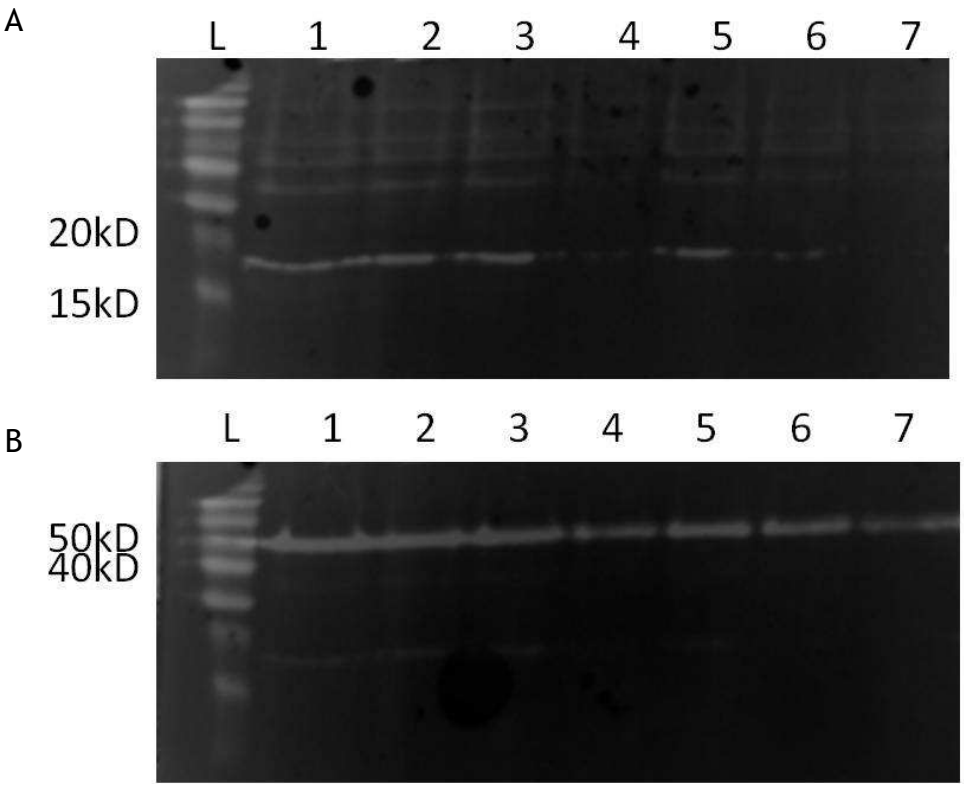

Figure 7-3 Raw western blot image for HT1080 cells transduced with plasmids to reduce CXCL1 or control.

Raw image of western blot with HT1080 cells which have been transduced with plamids to knock down CXCL1 (sh) or the control (Scr). The gel has been run on a 15% gel. L = molecular marker ladder, 1 = unstransduced, 2 = Scr, 3 = sh1, 4 = sh2, 5 = sh3, 6 = sh4 and 7 = sh5 with CXCL1 antibody (A) and β-Tubulin (B) antibodies. Approximate sizes of the marker are shown on the left of each panel (kD). Several bands were found with the CXCL1 antibody with the most prominent at ~16kD. A single band was found in the housekeeping control at the estimated size of 50kD.

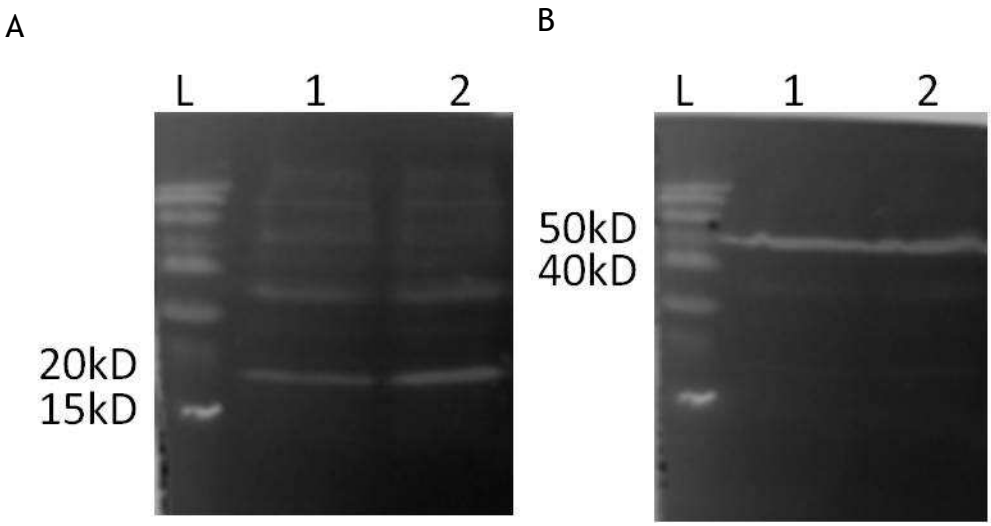

Figure 7-4 Raw western blot image for HT1080 cells transduced with empty vector or CXCL1 over expression vector.

Raw image of western blot with human HT1080 cell lines which has been run on a 15% gel. L = molecular marker ladder, 1 = Empty Vector, 2 = CXCL1-PRRL with CXCL1 antibody (A) or β-Tubulin antibody (B). Approximate sizes of the marker are shown on the left of each panel (kD). Several bands were found with the CXCL1 antibody with the most prominent at ~16kD (A). A single band was found in the housekeeping control at the estimated size of 50kD (B).

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