Phenotypic and functional changes in cord blood stem cell progeny after cytokine activation

Author:
Ramirez, Carole

Publication Date:
2007

DOI:
https://doi.org/10.26190/unsworks/19584

Phenotypic and Functional Changes in Cord Blood Stem Cell Progeny after Cytokine Activation

A thesis submitted in fulfilment of the requirement for the degree of
DOCTOR OF PHILOSOPHY

By

CAROLE DANIELLA RAMIREZ

School of Women's and Children's Health
Faculty of Medicine
University of New South Wales
Sydney, Australia

August, 2007

ORIGINALITY STATEMENT

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

Signed …….....

Date …….....

Abstract

Human umbilical cord blood, an alternate source of haematopoietic stem cells (HSC), has been successfully used to reconstitute haematopoiesis in both related and unrelated transplant recipients. However, because CB has fewer total cells (and as a consequence fewer HSC and progenitor cells) CB transplant recipients often experience delayed engraftment as compared with that seen in bone marrow or mobilized peripheral blood transplant recipients. Delayed engraftment exposes patients to an increased risk of infection and bleeding. Cytokine-mediated expansion has been investigated to improve engraftment after CB HSC transplantation as a means to expand the total cell number and both the HSC and progenitors populations. However, its effect on HSC function remains controversial. We hypothesise that if cytokine-mediated expansion promotes divisional recruitment and multilineage differentiation it causes changes in phenotype and cell cycle related gene expression which may be detrimental to the engraftment capacity of haematopoietic cells. Therefore we investigated the relationship between cell division, phenotype and engraftment potential of CB $CD34^+$ cells following cytokine-mediated expansion. High resolution cell division tracking using the fluorescent dye CFSE was used to monitor changes as a consequence of cytokine-mediated expansion in phenotype and function in CB $CD34^+$ cells. Cytokine-mediated expansion caused upregulation of lineage and proliferation markers and adhesion molecules and downregulation of putative stem cell markers with concomitant cell division. However, these changes in phenotype as a consequence of cytokine-mediated expansion may not reflect or be predictive of a functional change in the expanded population. Cytokine-mediated expansion of CB $CD34^+$ also caused changes in cell

cycle related gene expression of G1 phase regulators. CB $CD34^+$ cells exhibited expression of all D cyclins, albeit at different levels and $p21^{WAF1}$ was differentially expressed across CB samples. The effect of cell division on the engraftment potential as a consequence of cytokine-mediated expansion was examined in CB $CD34^+$. Cytokine-mediated expansion of CB $CD34^+$ cells reduced, but did not completely eliminate engraftment potential, as a proportion of the expanded and divided cell populations retained their ability to engraft the NOD-SCID mouse. Overall, this study confirms reports in the literature that cytokine-mediated expansion induces changes in the phenotype of HSC and compromises their *in vivo* function.

Acknowledgements

First and foremost, I want to acknowledge my supervisor, Associate Professor Alison Rice. Thank you for introducing me to the world of stem cells. Your guidance, inspiration and friendship have kept me on track and supported me through all these years and did not falter even from a distance. I am truly grateful for all your help and critical input in preparing this thesis.

To my co-supervisor Dr Richard Lock, thanks for your help and advice with the molecular work over the years. I would also like to acknowledge your support when Alison relocated to Queensland. Thanks must also go to Dr Robert Nordon and his technical advice earlier in the study in particular with the cell tracking work.

Thank you to the Australian Cord Blood Bank for providing the cord blood samples and the Bone Marrow Transplant Unit of the Prince of Wales Hospital for providing adult bone marrow samples.

I want to thank past and present friends and colleagues at the Children's Cancer Institute for Medical Research Australia who in some way have helped me professionally or personally through the years. I would like to specially thank Rachael Papa, Jayne Murray and Janice Smith for their technical advice on the molecular studies and statistics. Rachael, thank you for your friendship and for your patience editing this thesis. Christine, thank you for all those cups of tea, your friendship and encouragement helped through very tough times in the last years.

To my friends Kathryn and Monica, even though our career paths have taken us to different cities and countries, you are still very close to my heart. To Tio Enrique, the other researcher in the family, thank you for our many long distance conversations and your infectious love for scientific research.

To my wonderful family, Mum, Dad, Romina and Michelle, thank you for being there through the years and for all the hours of babysitting so that I could focus once again and finalise my studies. Mum, thank you for always believing in me, even when I am stubborn and it is hard to be optimistic, without you I would not have made past my first year of studies. To the Ford family, thank you also for all your support.

Finally to Jason and Emma, my little burst of hope and love. Thank you for your love, patience and never-ending support through very hectic times. To you both I dedicate this thesis.

Publications and Presentations

Nordon RE, Nakamura M, **Ramirez C,** Odell R (1999) Analysis of growth kinetics by division tracking. *Immunology & Cell Biology* 77(6):523-529

Ramirez CD, Lock RB, MacKenzie K, Nordon RE and Rice AM. (2003) Phenotypic and functional changes in cord blood stem cell progeny after cytokine activation. *Haematology Society of Australia & New Zealand Congress,* Christchurch, New Zealand (Oral Presentation)

Ramirez CD Nordon RE Lock RB Rice AM (2002) Phenotypic And Gene Expression Changes In Cord Blood Stem Cell Progeny After Cytokine Activation. *44th Annual Scientific Meeting, American Society of Hematology*, *Blood.* 100 (11 suppl. 1):155b

Ramirez CD, Nordon RE, Rice AM (2000) Use of cell tracking to study the relationship between the ability to engraft the NOD/SCID mouse and the level of progenitor development. *Haematology Society of Australia & New Zealand Congress,* Perth (Oral Presentation)

Ramirez CD Nordon RE Rice AM (2000) Effect of cell division and progenitor development on the ability to engraft the NOD/SCID mouse. *Proceedings of the Australian Society for Medical Research (NSW Scientific Meeting),* Sydney (Oral Presentation)

Ramirez CD Nordon RE Rice AM (1999) Growth factor receptor and lineage marker expression in successive progeny of cord blood $CD34^+$ cells. *41st Annual Scientific Meeting, American Society of Hematology, Blood.* 94 (10 suppl. 1):132b

Ramirez CD Nordon RE Rice AM (1999) Growth factor receptor and lineage marker expression in successive progeny of cord blood $CD34^+$ cells. *Tow Poster Prize,* Prince of Wales Hospital, Sydney (Poster)

Other Publications

Castor A, Nilsson L, Åstrand-Grundström I, Buitenhuis M, **Ramirez C**, Anderson K, Strömbeck B, Garwicz S, Békássy AN, Schmiegelow K, Lausen B, Hokland P, Lehmann S, Juliusson G, Johansson B & Jacobsen SEW (2005) Distinct patterns of haematopoietic stem cell involvement in acute lymphoblastic leukaemia. *Nature Medicine***,** 11(6):630-637.

Liem NLM, Papa RA, Milross CG, Schmid MA, Tajbakhsh M, Choi S, **Ramirez CD**, Rice AM, Haber M, Norris MD, MacKenzie KL, and Lock R.B. (2004) Characterization of childhood acute lymphoblastic leukemia xenograft models for the pre-clinical evaluation of new therapies. *Blood* 103:3905-3914.

Ramirez CD, Catchpoole DR (2002) Etoposide-induced apoptosis in lymphoblastoid leukaemic MOLT-4 cells: Evidence that chromatin condensation, loss of phosphatidylserine asymmetry and apoptotic body formation can occur independently. *Apoptosis* 7 (1): 59-68

Ramirez CD, Catchpoole DR and Stewart BW (2000) Nuclear instability, typical of apoptosis, occurs during human lymphoblastoid cell differentiation following exposure to phorbol ester. *Cell Death and Differentiation* 7: 548-555

Awards

2003	Haematology Society of Australia & New Zealand Travel Grant
1999 - 2003	Lions Scholarship for Cord Blood Research
2000	Haematology Society of Australia & New Zealand Travel Grant
2000	Novartis Travel Grant
1998	UNSW Vacation Scholarship

List of Abbreviations

A	Absorbance
AGM	Aorta Gonads and Mesonephros
ALL	Acute lymphoblastic leukaemia
BM	Bone Marrow
BSA	Bovine serum albumin
CAFC	Cobblestone Area-forming cell
CB	Human Umbilical Cord Blood
CCIA	Children's Cancer Institute Australia for Medical Research
CD	Cluster of differentiation
CDK	Cyclin-dependent kinases
cDNA	Complementary DNA
CEM	CCRF CEM cell line
CFSE	Carboxyfluorescin Diacetate Succinimidyl ester
CFU-S	Colony Forming Unit Spleen
CKIs	Cyclin-dependent kinase inhibitors
CT	Cycle threshold
Cy3	Cyanine 3
Cy5	Cyanine 5
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide5'-triphosphate
DTT	Dithiothreitol
E	Embryonic Day
ECM	cell-extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
E-LTC-IC	extended long-term culture-initiating cell
FACS	Fluorescence-activated cell sorting
Fig.	Figure
FL	Flt3-Ligand
G-CSF	granulocyte Colony-stimulating factor
Glu	L-glutamine
H2O	Water

HEPES	N-2-hydroethylpiperazine-N'-2ethanesulfonic acid
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplantation
Ig	Immunoglobulin
IL	Interlukin
INK 4	Inhibitor of cyclin dependent kinase 4
LIF	Leukemia inhibitory Factor
LTC-IC	Long-term Culture-initiating cell
LT-HSC	Long-term haematopoietic stem cell
KCl	Potassium chloride
M	Mitotic phase
MACS	Magnetic Activated Cell Sorting
MgCl2	Magnesium chloride
MGDF	Megakaryocyte growth and Development Factor
ML-IC	Myeloid-lymphoid initiating cells
MNC	Mononuclear Cells
MPB	Mobilised Peripheral Blood
NOD-SCID	non-obese diabetic severe combined immunodeficient
p21	$p21^{WAFi/CIP1}$
p27	$p27^{KIP1}$
PECAM	Platelet Endothelial Cell adhesion Molecule
Rb	Retinoblastoma
Rho	Rhodamine
Rn	Magnitude of the signal generated by the PCR reaction
SCF	Stem Cell Factor
SCID	severe combined immunodeficient
TPO	Thrombopoietin
VCAM	Vascular cell adhesion molecule
VLA	Very late Antigen

Table of Contents

Chapter 1 Introduction

1.1 Clinical Problem

Allogeneic haematopoietic stem cell transplantation (HSCT) offers the chance of a cure for patients with relapsed or poor prognosis leukaemia. The primary aim of HSCT is to reconstitute a healthy haematopoietic system in patients that has been damaged by the disease and the high dose chemotherapy and radiation used to eradicate it. Human umbilical cord blood (CB) is an alternate source of HSC and it has been successfully used to reconstitute haematopoiesis in both related and unrelated transplant recipients (Cairo and Wagner, 1997, Rubinstein et al., 1998, Wagner et al., 1996, Eapen et al., 2007, Laughlin et al., 2004).

CB has a number of advantages as a stem cell source for allogeneic transplants including ease of collection with absence of risk to the donor, lower risk of transmitting infectious diseases and lower risk of both acute and chronic graft-versus-host disease (Kurtzberg et al., 1996, Wagner et al., 1996, Rocha et al., 2000). However, its main disadvantages are its reduced total cell number (and as a consequence reduced HSC and progenitor cells) per collection and delayed engraftment compared to other HSC sources such as bone marrow (BM) (Gluckman et al., 1997). Total nucleated cell number infused correlates strongly with engraftment. Hence, BM patients are routinely given 1-3 x 10^8 total cells to ensure engraftment. Significant effort has been directed towards developing therapies to facilitate engraftment including the use of cytokine-mediated expansion. Cytokine-mediated expansion aims to expand the total cell number and both

the HSC and progenitors populations to improve engraftment after CB HSCT (Bertolini et al., 1994, Moore and Hoskins, 1994). Hence, cytokines need to promote recruitment of HSC into proliferation (Srour et al., 1999, Metcalf, 1993, Ogawa, 1993) and as a result promote cells to progress through the cell cycle; but the resultant HSC progeny must retain their repopulation capacity. However, the effects of cytokine-mediated expansion on HSC function remain controversial.

This study focuses on the CB $CD34^+$ population and the cytokine combination of stem cell factor (SCF), thrombopoietin (TPO), Flt3-Ligand (FL) and interleukin (IL)-3 which we have previously confirmed as being able to promote divisional recruitment and multilineage development of CB $CD34^+$ cells (Case et al., 2001).

1.2 Aim and Scope

We hypothesised that if cytokine-mediated expansion promotes divisional recruitment and multilineage differentiation, it will cause changes in phenotype and cell cycle related gene expression which may be detrimental to the engraftment capacity of haematopoietic cells. That is, cytokine-mediated expansion may compromise HSC function. Therefore the aim of this thesis was to investigate the relationship between cell division, phenotype and engraftment potential of CB $CD34^+$ cells following cytokine-mediated expansion and to determine if cytokine-mediated expansion is detrimental to the long-term engraftment capacity of CB $CD34^+$ cells.

1.3 Overview of the study

To achieve this aim: Chapter 3 addresses optimisation of a high resolution cell division tracking technique to provide a tool to monitor changes in phenotype and function in CB $CD34^+$ cells as a consequence of cytokine-mediated expansion. Then using this technique, the effect of cell division of CB $CD34^+$ cells is correlated with the expression level of cytokine receptors, adhesion molecules and lineage markers following cytokine-mediated expansion. In chapter 4, the changes in cell cycle-related expression of candidate G1 phase genes are characterised prior to and following cytokine-mediated expansion of CB $CD34^+$ cells. Chapter 5 determines the engraftment potential of CB stem cell progeny generated *in vitro* to expand *in vivo* using the non-obese diabetic severe combined immunodeficient (NOD-SCID) mouse model of human engraftment.

Chapter 2 Literature Review

2.1 Haematopoiesis

Haematopoiesis is the process whereby the blood system is replenished throughout life. This process is complex and tightly regulated and leads to the production of approx 10^{12} blood cells. In order for haematopoiesis to be possible a unique and rare cell is required, the haematopoietic stem cell (HSC). HSC and its progenitors give rise to all the blood lineages in the body required for normal cell turnover and in response to stress, such as bleeding and infection (Smith, 2003, Weissman, 2000)

2.1.1 Differentiation and the hierarchy of haematopoiesis

Haematopoiesis is organized in a hierarchical manner (Fig. 2.1). At the top is the HSC and as it undergoes differentiation into progenitor cells, it loses the ability to self-renew. Through a series of intermediate progenitors, HSCs ultimately give rise to the multiple haematopoietic lineages. The first major branching point is thought to be with a cell becoming either a common lymphoid progenitor (giving rise to cells of the lymphoid lineage; CLP) or a common myeloid progenitor (giving rise to cells of the myeloid lineage; CMP). These progenitors in turn differentiate into precursors which are morphologically different. Immature precursors have a high proliferative potential and as they mature, they eventually become terminally differentiated resulting in the functional end cells seen in the

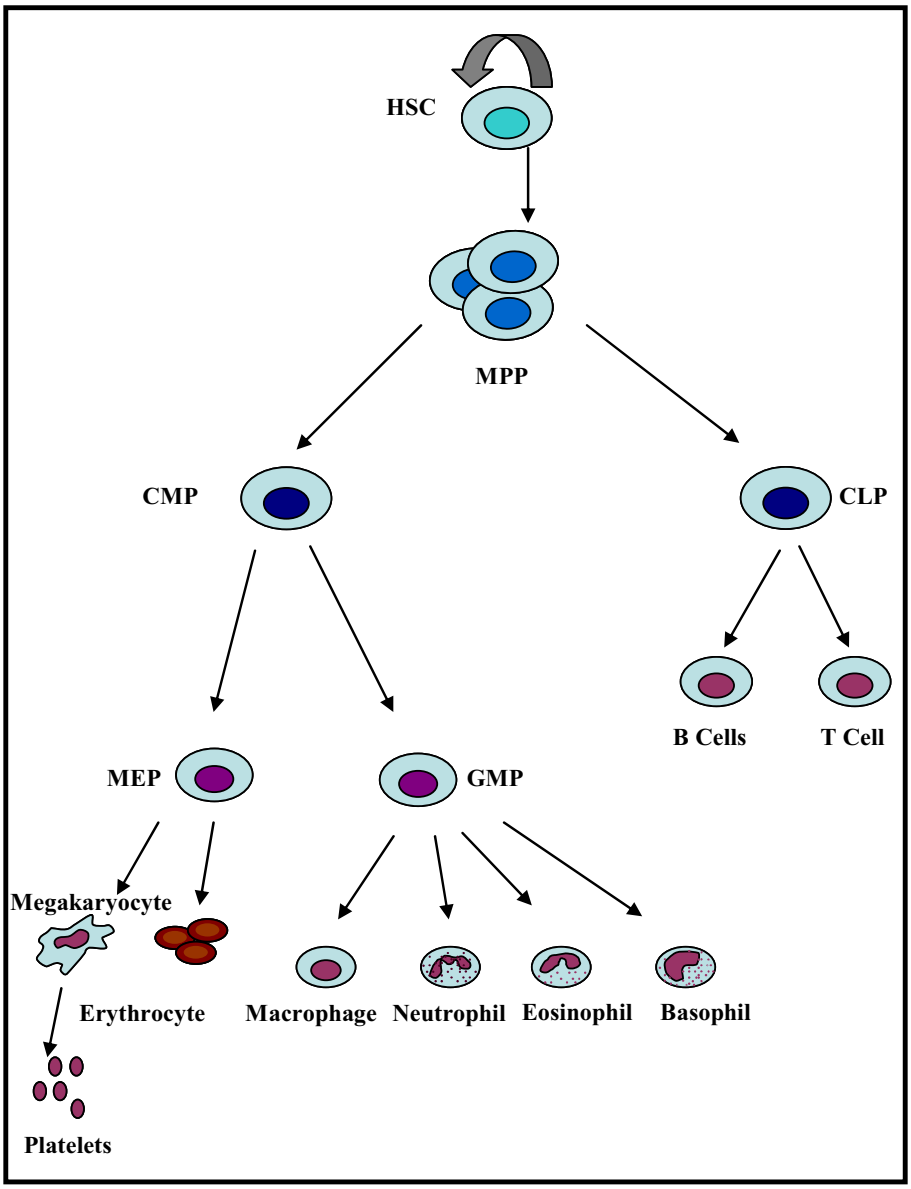

Figure 2.1 Hierarchy of hematopoiesis

Hematopoiesis is thought to be organised in a hierarchical manner. The hematopoietic stem cell (HSC) gives rise to the multipotent progenitor (MPP). This cell may then become a common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). The CLP gives rise to B and T cells. The CMP further differentiates to either megakaryocyte-erythrocyte (MEP- giving rise to megakaryocytes and erythrocytes) or granulocyte-monocyte progenitor (GMP – giving rise to macrophages, neutrophils, eosinophils and basophils).

blood such as erythrocytes, platelets, T and B lymphocytes, neutrophils, monocytes, macrophages, eosinophils and basophils (Akashi et al., 2000, Kondo et al., 1997, Smith, 2003).

2.1.2 Ontogeny of HSC

The development of haematopoiesis early in ontogeny provides proof that HSC are able to expand in the developing organism. However as ontogeny of HSC is beyond the scope of this thesis it will be only briefly summarised in this section. During development in vertebrates, haematopoiesis occurs sequentially in two distinct anatomical locations, reviewed by Burns and Zon, 2006 and Cumano and Godin, 2007. In the mouse, at embryonic day 7.5 (E7.5), the first stage termed primitive haematopoiesis starts and takes place within blood islands of the mammalian extra-embryonic yolk sac and it primarily produces primitive/embryonic nucleated red cells (Haar and Ackerman, 1971). Definitive haematopoiesis then occurs within the embryo proper in the aorta, gonads and mesonephros (AGM) region (Burns and Zon, 2006). Haematopoietic cells are generated between E9.5 and 12.5 days in the AGM in the absence of differentiation. These definitive HSC constitute the entire pool of HSCs that establishes adult haematopoiesis (Godin et al., 1999, de Bruijn et al., 2002, Godin et al., 1993) and after E11, they are the first cells capable of long term reconstitution of the haematopoietic compartment in lethally irradiated recipient mice (Muller et al., 1994, Medvinsky and Dzierzak, 1996, Medvinsky et al., 1993, Cumano and Godin, 2001). The definitive HSCs then colonize the foetal liver and sometime between E16 and E17, foetal liver HSCs migrate to the BM (Kondo et al., 2003). The BM then becomes the predominant site of haematopoietic cell development soon after birth and continues into

adult life. Subsequent haematopoietic organs (foetal liver, thymus, BM) are thought to be seeded by HSC migration through the circulation. Recently, the placenta has also been shown as a site for definitive haematopoiesis. However, it is not known if HSC are generated in situ or seeded by circulating HSC (Burns and Zon, 2006, Cumano and Godin, 2007).

2.2 The Haematopoietic Stem Cell (HSC)

A stem cell is described as a cell capable of self renewal and multilineage differentiation (Till and Mc, 1961, Weissman, 2000). The haematopoietic stem cell is an example of a multipotent stem cell (Jackson et al., 2002). This cell is sometimes loosely referred to as pluripotent, but in this study it will be referred as multipotent, as it is lineage specific and already confined to the haematopoietic system.

2.3 Identification of HSCs

The concept of a HSC came about through a number of observations nearly 60 years ago. In 1949, Jacobson noted that lead shielding of haematopoietic tissues prevented death from otherwise lethal doses of radiation. Another important observation was made by Lorenz in 1951, when intravenous infusion of syngeneic marrow after irradiation also prevented death. In 1956, three different groups, (Ford et al., 1956, Makinodan, 1956, Nowell et al., 1956), reported that lymphohaematopoeitic cells arising from infused BM repopulated haematopoietic tissues (Morrison et al., 1995). But it was the breakthrough assay developed by Till and McCulloch in 1961 that revolutionised our understanding of HSC by discovering the existence of a type of cell able to form

colonies consisting of blood cells of various lineages in the spleen of recipient mice. Their assay involved transplanting BM cells from non-irradiated donors intravenously into lethally irradiated mice. Their results showed that the BM contained highly proliferative progenitor cells (CFU-S), capable of giving rise to individual colonies of myeloid, erythroid, and megakaryocytic cells within the spleens of irradiated hosts (Till and Mc, 1961). In 1963, Siminovitch observed that these colonies were the progeny of single cells and some were also serial-transplantable (Siminovitch et al., 1963). It was then proposed that a population of radioprotective cells, HSC, must exist within the BM capable of multilineage differentiation as well as self-renewal. These studies provided functional criteria for defining HSC – self-renewal and differentiation. In 1968, Wu showed that the same cell type also gave rise to lymphocytes. Later in 1975, Lajtha and McCulloch demonstrated that CFU-S cells were more mature progenitors and not in fact HSC, as HSC are too primitive to form spleen colonies within 12 days (Lajtha, 1975). This was further examined through flow cytometric analysis of the CFU-S and this revealed that these cells are not a true HSC but a more mature multipotential population with only a relative limited capacity to generate haematopoietic cells (Spangrude et al., 1988).

The definition for a HSC is constantly evolving and it entails not a single feature but multiple criteria. When first characterised in the 1960s, HSC were defined as cells capable of both self renewal and multilineage differentiation. That is, a cell that was able to expand and give rise to a large number of lymphocytes, myeloid cells and erythrocytes. The current definition of a true HSC includes the property that the cell must be able to sustain the repopulation of a recipient's haematopoietic tissues (Ploemacher and Brons, 1989, Morrison and Weissman, 1994).

HSCs are rare and are slowly cycling in the steady state adult haematopoietic system. They can be isolated from haematopoietic tissues including BM, human umbilical cord blood (CB) and mobilised peripheral blood (MPB). HSCs in the mouse and human consist of up to 0.05% of cells in the BM (Morrison et al., 1995).

2.4 Characterisation of human haematopoietic stem cell

The knowledge of the biology of HSC and its progenitors comes mostly from murine studies as they are the best characterized HSC phenotypically and functionally (Uchida and Weissman, 1992, Uchida et al., 1996, Uchida et al., 1998, Domen and Weissman, 1999, Osawa et al., 1996, Eaves et al., 1997, Jones et al., 1996). Most of the information we currently know about human HSC progenitors comes from *in vitro* studies and xenotransplantation studies of human cells into immunodeficient animals, and large animal models (McCune, 1996, Bhatia et al., 1998, Zanjani et al., 1994, Berenson et al., 1988)

The phenotype of the human HSC is still evolving as currently the HSC population is isolated mostly by negative surface marker expression, unlike the murine HSC. Human haematopoietic cells expressing the surface glycoprotein CD34 (Andrews et al., 1986, Berenson et al., 1988, Civin et al., 1996), lacking expression of lineage commitment markers and having low levels of the lymphoid antigen Thy-1 (Craig et al., 1993), that is $CD34^{+}$ Lin^{-} $Thy^{+/lo}$, are enriched for primitive progenitors and HSC activity (Civin et al., 1996, Sutherland et al., 1989, Sutherland et al., 1990, Ema et al., 1990, Andrews et al., 1989, Andrews et al., 1990, Baum et al., 1992).

The CD34 positive population is heterogenous as it is enriched for stem cells and their progeny (Civin et al., 1984, Craig et al., 1993, Katz et al., 1985, Cardoso et al., 1995). CD34 is expressed on 1-4% of low density BM mononuclear (MNC) cells and < 1% of peripheral blood nucleated cells (Civin et al., 1984, Civin et al., 1987, Lu et al., 1987, Watt et al., 1987). Most $CD34^+$, that is 99% of cells are also positive for CD38. This population encompasses all those $CD34^+$ that express CD71, CD33 and CD10 (Terstappen et al., 1991, Lansdorp et al., 1992, McNiece, 2000, Miller et al., 1999). Thy-1 is co-expressed on 5 to 25% of $CD34^+$ cells (Morrison et al., 1995, Baum et al., 1992, Craig et al., 1993). Similar CD34 and Thy-1 expression patterns were observed in CB, BM and foetal liver (Craig et al., 1993) and Thy-1 decreased with increased expression of lineage markers like CD45RA, CD38 or CD71 (Lansdorp and Dragowska, 1992, Lansdorp et al., 1990, Terstappen et al., 1991). Another marker expressed in human haematopoietic progenitor cells and at high levels in the more primitive HSC is p-glycoprotein, a multidrug efflux pump (Chaudhary and Roninson, 1991). The low or negative expression of other markers is used to further enrich the HSC population. These include the transferring receptor, CD71 (Brandt et al., 1990, Lansdorp and Dragowska, 1992), the high molecular weight isoform of human leukocyte antigen, CD45RA (Lansdorp and Dragowska, 1992, Lansdorp et al., 1990), the myeloid marker CD33 (Andrews et al., 1989, Buhring et al., 1989, Watt and Visser, 1992, Andrews et al., 1990, Ema et al., 1990) and the HLA-DR antigen (Sutherland et al., 1989, Watt et al., 1987, Brandt et al., 1990). $CD34^+$ $CD38^{lo}$, $CD34^+$ $HLA\text{-}DR^{lo}$ and $CD34^+$ $Thy\text{-}1^+$ are all primitive populations and they have a high potential to proliferate and differentiate (Terstappen et al., 1991, Morrison et al., 1995, Baum et al., 1992) and reconstitute immunodeficient mice and irradiated humans (Baum et al., 1992, Cashman et al., 1997b, Young et al., 1999)

2.5 Purification of CD34 population

A number of techniques have been used to enrich the HSC population including density centrifugation, activation and/or cell cycle status and surface antigen expression. The most common method is isolation of the desired population based on the cell surface marker expression by staining with monoclonal antibodies to the respective antigens. Monoclonal antibodies has successfully permitted the development of cell sorting and are the main tool used for enrichment of mouse (Spangrude et al., 1988) and in particularly human HSC in techniques such as immunoadsorption (panning) procedures, magnetic separations, or in flow cytometry (Craig et al., 1993). These antibodies may be attached to immunomagnetic microbeads for separation though a column exposed to a magnetic field or fluorochromes for analysis through fluorescence-activated cell sorting (FACS). An example of immunomagnetic separation is the magnetic activated cell sorting (MACS) (Watt and Visser, 1992) system which relies on using directly or indirectly conjugated microbeads attaching to the desired cells (positive selection) or to the unwanted population with the unlabeled cells passing through the column (negative selection). The development of FACS allowed better characterisation and purification of subsets of haematopoietic populations (Weissman, 2002). In multiparameter cell sorting, a number of markers may be used simultaneously leading to purification of rare populations by FACS. Both methods may be used sequentially and may be used for positive or negative selection of the desired population depending on the selected antibodies and the phenotype of the population. For human HSC only CD34 expression is available for positive selection and any further enrichment is possible through negative depletion of lineage markers. Currently, as previously mentioned, studies have

ruled out the surface markers that are not expressed by HSC, but more candidate markers are needed to determine the positive phenotype of the human HSC.

Another approach to purify and sub-fractionate the HSC population is to use the supravital fluorescent dyes Rhodamine 123 (Rho) or Hoechst 33342. These techniques target the functional differences between stem and progenitor cells of different degree of maturation (Zinovyeva et al., 2000). The rhodamine 123 dye binds specifically to the mitochondria in viable cells (Johnson et al., 1980), its level of uptake increases with cell cycling and it is used to isolate cells that reside primitive HSC in G0 phase (Bertoncello et al., 1985) and that have long term repopulating ability (Visser and De Vries, 1990, Udomsakdi et al., 1991). Hoechst 33342 dye binds to the ABCG2 reporter (Zhou et al., 2001) at a lower level in stem cells compared to committed progenitors (Baines and Visser, 1983, Pallavicini et al., 1985, Neben et al., 1991).

2.6 Current assays to evaluate HSC function

As previously mentioned, the HSC is defined as a cell capable of self renewal, multilineage differentiation and able to sustain the reconstitution of haematopoiesis in a conditioned recipient following transplantation (Ploemacher and Brons, 1989, Morrison and Weissman, 1994). A number of stem cell assays have been developed to evaluate the functional characteristics in candidate HSC populations. However, they do not appear to completely assess the human HSC (Heike and Nakahata, 2002) and its long-term engraftment abilities (Coulombel, 2004, Hofmeister et al., 2007).

2.6.1 In vitro assays

There are a number of *in vitro* assays available that assess primitive human progenitors including the long-term culture-initiating cell (LTC-IC) assay (Sutherland et al., 1990, Bonnet, 2002); Cobblestone area-forming cell (CAFC) assay (Ploemacher et al., 1989, Breems et al., 1994) the myeloid-lymphoid initiating cells (ML-IC) assay (Punzel et al., 1999); and the extended-LTC-IC (E-LTC-IC) (Hao et al., 1996). However, these are stroma driven assays and they generate primitive myeloid progenitors, downstream of the HSC, with self-renewal or multilineage differentiation potential and may overestimate progenitor cell expansion (Hofmeister et al., 2007). As the focus of the thesis was to expand haematopoietic cells in a cytokine driven culture without stroma support, these systems were not considered suitable.

2.6.2 In vivo assays

The definitive assay for HSC function is repopulation capacity, that is, their ability to reconstitute the entire haematopoietic system of transplant recipients (Bonnet, 2002). The phenotype and function of the murine HSC was determined using *in vivo* competitive repopulation assays (Harrison, 1980). This quantitative competitive repopulating assay uses phenotypically distinguishable stem cell populations. However, as this assay cannot be used in the characterisation of human HSC, surrogate *in vivo* and *in vitro* assays were developed to evaluate human HSCs. A number of xenotransplantation assays have been developed to study human haematopoiesis including immune-deficient mice (McCune, 1996, Bhatia et al., 1998) primates and the foetal sheep (Zanjani et al., 1991, Berenson et al., 1988, Srour et al., 1992, Zanjani et

al., 1994). However for the purpose of this study, the non-obese diabetic severe combined immunodeficient (NOD/LtSz-*scid* or NOD-SCID) mouse model was the chosen animal model as it was established in the laboratory and it currently a reliable method for quantification of human HSC. Hence the other xenotransplantation models are outside the scope of this thesis.

2.6.2.1 The NOD/SCID mouse model

The NOD-SCID mouse strain (Shultz et al., 1995) was developed from the severe combined immunodeficient (SCID) strain. The SCID mouse model (McCune et al., 1988) allowed engraftment of human haematopoietic samples including foetal liver, BM and CB (Namikawa et al., 1990, Greiner et al., 1998, Lapidot et al., 1992, Vormoor et al., 1994, Cashman et al., 1997b, Pflumio et al., 1996, Hogan et al., 1997). However the engraftment levels were poor due to the residual immunity (normal NK cells, complement and myeloid cells) in the SCID mouse and to overcome this problem high cell doses were required (Dick et al., 1991).

The NOD-SCID mouse strain has a variety of immunological abnormalities including T- and B-cell deficiency, lower NK cell activity, defect in macrophage function, and absence of circulating complements (Shultz et al., 2000). The NOD-SCID is also highly radiosensitive and higher engraftment is achieved with lower cell doses than required for the SCID strain (Dick et al., 1997). The limitation of the NOD-SCID model is its inability to support human T-cell development, its short life span (Shultz et al., 2000) and like other animal models it does not represent the human stem cell niche. Dick and Lapidot developed a more efficient *in vivo* technique for engrafting human cells that is

based on conventional BM transplantation and it can be used with SCID and NOD-SCID as recipients and they are conditioned by sublethal total body irradiation of 375 to 400 cGy (Dick et al., 1991, Dick et al., 1997).

2.7 Regulation of Haematopoiesis

Regulation of haematopoiesis determines a number of possible fates for HSC and its progenitors including differentiation, self-renewal, apoptosis, quiescence and mobilization or migration (Weissman, 2000, Orkin and Zon, 2002, Domen and Weissman, 1999, Domen et al., 2000). Haematopoiesis and maintenance of the primitive HSC pool is tightly regulated (Sasaki et al., 2004), and involves cell division, differentiation commitment, maturation induction, cellular trafficking and mature cell activation. A number of models have been proposed to describe the nature of HSC regulation as either intrinsic, extrinsic or both (Bonnet, 2002, Krause, 2002). Extrinsic regulation is thought to occur through secreted factors such as cytokines (section 2.7.1 and 2.9.1) and the extracellular matrix (Whetton and Spooncer, 1998, Watt and Hogan, 2000, Metcalf, 1993) while intrinsic regulation refers mainly to stochastic models (Ogawa, 1999) and factors such as telomere shortening and regulation of transcription. Overall, regulation of haematopoiesis is likely to be the result of multiple processes including cell-cell interaction, cell-extracellular matrix interactions, cytokines, transcription factors (Zinovyeva et al., 2000) and other intrinsic factors of haematopoietic development (Bonnet, 2002).

2.7.1 Cytokine-mediated regulation of haematopoiesis

Cytokines are a large group of growth factors that mediate stimulatory or suppressive effects on essential cell functions such as quiescence, apoptosis, self renewal and differentiation (Verfaillie, 1993, Heinrich et al., 1993, Simmons et al., 1994). As a general rule, cytokines function by engaging a specific receptor and activating a variety of signalling pathways (Smith, 2003). Cytokines may regulate haematopoiesis in two ways, through secretion of multiples cytokines at low levels, regulating basal blood cell production; and in response to specific stresses where higher levels of particular cytokines are released for a short time to trigger increased cell production. Cytokines support differentiation of haematopoietic cells mainly through permissive rather than instructive means (Bryder and Jacobsen, 2000). The most primitive haematopoietic stem and progenitor cells require a combination of cytokines to activate multiple cytokine receptors for recruitment into active cell cycling (Ogawa, 1993, Metcalf, 1993, Brandt et al., 1990, McNiece et al., 1991) whereas single cytokines stimulate proliferation of committed progenitors (Moore, 1991).

2.7.2 Homing of the HSC

HSCs need to home to and lodge in the specialized niches of the BM microenvironment in order to engraft and reconstitute normal BM functions following transplantation. The 'stem cell niche' defined as a functional system composed of cells, matrix glycoprotein and three-dimensional spaces (Ballen, 2007) influences and regulates self renewal, survival, proliferation and differentiation of the HSC *in vivo* (Spradling et al., 2001, Krause, 2002, Bonnet, 2002). HSC within the niches are surrounded by the marrow

microenvironment through cell-cell interaction and cell-extracellular matrix (ECM) interactions and exposed to membrane bound, soluble or ECM associated cytokines and adhesion molecules (Krause, 2002, Whetton and Spooncer, 1998). HSC outside of their niche seem to have little function as they need the particular cues from these specific sites to control their fate (Scadden, 2006). Greater understanding of these complex systems may be advantageous for therapeutic purposes. However, current *in vitro* and *in vivo* assays cannot yet replicate the human stem cell niche; hence it is beyond the scope of this thesis.

During ontogeny, HSCs migrate from one anatomical site to another through the bloodstream. This migration is directed by interactions with adhesion molecules and their receptors expressed on HSC and endothelium of the microenvironments (Srour et al., 2001). Homing requires trafficking and migration of the transplanted cells from the injection site to the BM, mimicking the natural movement during development.

2.7.3 Adhesion Molecules

There is a close relationship between cytokines and adhesion molecules (Papayannopoulou et al., 1998). Cytokines that stimulate $CD34^+$ haematopoietic progenitor cells may also stimulate adhesion molecules, enhancing their function (Levesque et al., 1996, Levesque et al., 1995, Papayannopoulou et al., 1998). Adhesion molecules include selectins, integrins, CD44 and the immunoglobulin superfamily. The selectin family includes 3 proteins, E- (endothelial; CD62E), P- (platelet; CD62P) and L- (leucocyte; CD62L) selectins. However, it is the L-selectin that is constitutively expressed on leucocytes, including $CD34^+$ cells (Chan and Watt, 2001). β1 integrins are

adhesion molecules essential for the homing of haematopoietic cells during ontogeny and in adult tissues (Potocnik et al., 2000). HSC from chimeric mouse embryos lacking β1 integrins can differentiate into different lineages but cannot establish haematopoiesis in the foetal liver (Hirsch et al., 1996). This supports the hypothesis that similar mechanisms exist in migration during development and in response to cytokines in the adult system. β1 integrins include very late antigen (VLA)-4 (CD49d), VLA-5 (CD49e), LFA-1 (CD11a), MAC-1 (CD11b) and CD29. $CD34^+$ haematopoietic progenitors express VLA-4 and VLA-5 integrins (Chan and Watt, 2001, Morrison et al., 1997b, Williams et al., 1991). Myeloid and erythroid progenitors express LFA-1. MAC-1 is expressed in more mature haematopoietic progenitors than HSCs. A small $CD34^+$ population, including megakaryocyte progenitors express $\alpha_{IIb}\beta_3$ (CD41/CD61) integrin.

The VLA-4 integrin (α4β1) interaction with vascular cell adhesion molecule (VCAM)-1 may be an important regulator of HSC migration (Morrison et al., 1997a). VCAM-1 is expressed by BM stroma and sinusoidal endothelia (Williams et al., 1991, Miyake et al., 1991, Simmons et al., 1992, Jacobsen et al., 1996, Schweitzer et al., 1996). *In vivo* administration of antibodies directed against α4 integrin, were sufficient to cause mobilization of haematopoietic progenitors (Papayannopoulou and Nakamoto, 1993, Papayannopoulou et al., 1995). Deletion of α4 is embryonic lethal (Chan and Watt, 2001). VLA-4 plays a central role in homing and engraftment of transplanted human cells to the BM of sheep foetuses (Zanjani et al., 1999). It has been suggested that VLA-5 (α5β1) may be involved in the regulation of cell adhesion and matrix assembly whereas VLA-4 may be required for the speed of migration. The engraftment of CB $CD34^+$ was prevented by blocking function of VLA-4, VLA-5 or their shared subunit

β1 and inhibition of VLA-4 blocked homing and engraftment by murine HSCs (Peled et al., 2000, Papayannopoulou et al., 1995, Wagers et al., 2002). CD44 is widely expressed in haematopoietic and non-haematopoietic tissues and plays a role in adhesion between haematopoietic progenitors to stromal cells (Chan and Watt, 2001). A member of the immunoglobulin superfamily involved in homing of haematopoietic cells is the platelet endothelial cell adhesion molecule (PECAM)-1, also referred to as CD31. It may be involved in the formation of stem cell niches and transendothelial migration of haematopoietic progenitors (Chan and Watt, 2001).

The adhesion molecules of particular interest to this thesis are involved in transendothelial migration and migration within the marrow cavity (CD62L, CD44, and CD31) and lodgement within the marrow microenvironment (CD29, CD49d, CD49e, CD11a, and CD11b). The characterisation of the expression patterns of adhesion molecules following cytokine-mediated expansion in haematopoietic cells is essential to determine correlation between the changes to cell adhesion and homing and engraftment capacity.

2.7.4 Chemokines

Chemokines are important regulators of haematopoiesis that are involved in homing and engraftment. Chemokines can inhibit progenitor growth, regulate trafficking and homing of haematopoietic progenitors and mediate T-cell development in the thymus (Smith, 2003, Wright et al., 2002). The chemokine SDF-1 (receptor CXCR4) is essential for trafficking of haematopoietic cells in the developing embryo. It mediates homing of HSCs and progenitors to the BM following transplantation and also plays a

role in G-CSF mediated mobilization of blood derived stem cells for transplantation (Christopherson and Hromas, 2001). Murine and human HSCs express the CXCR4 receptor (Mohle et al., 1998, Wright et al., 2002). CXCR4 expression is induced by cytokine-mediated expansion and the engraftment of NOD-SCID mice by human HSCs can be blocked by an inhibitory antibody to CXCR4 (Peled et al., 1999). However, it has been demonstrated that CXCR4 expression on human haematopoietic cells is not required for their repopulation capacity (Rosu-Myles et al., 2000). SDF-1α induces the function of adhesion molecules, VLA-4 and LFA-1 on CB $CD34^+$ cells (Peled et al., 2000) and enhances their NOD-SCID repopulating ability following *in vitro* culture (Glimm et al., 2000). Due to the importance of chemokines in homing and engraftment, it is important to assess the effect that cytokine-mediate expansion may have on the expression of chemokines receptors like CXCR4 in haematopoietic cells.

2.8 Human umbilical cord blood a source of haematopoietic stem cells for transplantation

HSC transplantation (HSCT) is a therapeutic option for high risk or relapsed hematologic malignancies and non-malignant diseases. These therapies follow very large doses of chemotherapy that ablate the BM. The three sources of HSC for transplantation include BM, mobilised peripheral blood (MPB) and human umbilical CB. While BM is the classic source of HSC for transplantation (Kolb and Holler, 1997) and MPB is used extensively for both autologous and allogeneic HSC transplantation (Cutler and Antin, 2001), they are outside the scope of this thesis and will not be discussed further.

CB is an alternate source of stem and progenitor cells that has been successfully used to reconstitute haematopoiesis in both related and unrelated transplant recipients (Wagner et al., 1996, Rubinstein et al., 1998, Cairo and Wagner, 1997). Early studies showed that CB has a significantly higher number of early and committed progenitor cells than adult BM (Broxmeyer et al., 1990) and it is enriched with primitive stem cells producing long-term repopulating cells *in vivo* (Broxmeyer et al., 1992). CB has a number of advantages as a stem cell source for allogeneic transplants compared to BM and MPB. These include ease of collection with absence of risk to the donor, lower risk of transmitting infectious diseases, lower risk of both acute and chronic graft-versus-host disease (Kurtzberg et al., 1996, Wagner et al., 1996, Rocha et al., 2000). In addition, with the establishment of cord blood banking, better access and immediate availability of CB samples is on hand to transplantation centres (Cairo and Wagner, 1997).

Clinical studies have shown a strong correlation between engraftment and the cell dose infused (De Felice et al., 1999). However, CB samples are a one-off collection with a limited volume in the average CB unit collected. Moreover, CB has been shown to have fewer cells than BM in the log range. All these factors have initially restricted its use to paediatric patients (Gluckman et al., 1997).

To further complicate matters, engraftment after CB HSC transplant is significantly delayed as compared to haematopoietic reconstitution seen in patients receiving BM and MPB (Kurtzberg et al., 1996, Wagner et al., 1996). In the mid to late 1990s significant effort was directed towards developing therapies to facilitate engraftment and these therapies included cytokine-mediated expansion. The aim was to increase the total cell dose, including the number of stem and progenitor cells, to promote engraftment

(Bertolini et al., 1994, Moore and Hoskins, 1994). Currently, new approaches are being explored whereby patients are transplanted with 2 or more CB (Barker et al., 2005); the aim being to provide protection from neutropenia and thrombocytopenia. However further discussion of this work is beyond the scope of this thesis.

2.9 *Ex vivo* culture of haematopoietic cells with cytokines

HSC and progenitors expand in number during development and in HSC transplantation in order to repopulate the haematopoietic system. If the mechanisms behind these expansions could be better understood and manipulated then *ex vivo* expansion could be used to improve haematopoietic engraftment, in particular in CB transplantations. There are currently two main approaches that aim at expanding HSC *ex vivo*. These include culture systems supported by BM stroma and liquid cultures supported by different cytokines combinations. The focus of this thesis is on cytokine-mediated *ex vivo* expansion of HSC.

2.9.1 Candidate cytokines for ex vivo expansion of human HSC

A number of groups have investigated different cytokines as candidates to promote *ex vivo* expansion of human HSC. The cytokines that may positively regulate the HSC include stem cell factor (SCF), Flt3 ligand (FL), interleukin (IL)-6, IL-11, IL-12, leukaemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and thrombopoietin (TPO) (Heike and Nakahata, 2002). Also IL-3 and IL-1 have been examined; however, their role in positively regulating HSC is controversial in the literature. Our lab has previously investigated using flow cytometry and factorial

analysis (Case et al., 2001) the cytokines SCF, TPO, FL and IL-3 (as single factors and in combination) as candidate cytokines to support and promote growth of primitive haematopoietic stem cells *in vitro*. This combination of cytokines is the focus of this thesis and effects of these cytokines on HSC and progenitors are described in the following sections.

2.9.1.1 Stem cell factor (SCF)

SCF is an early acting cytokine in human haematopoiesis and it functions by binding to its receptor *c-kit* (Broudy, 1997). SCF, also known as mast cell growth factor, steel factor and c-kit ligand, is constitutively expressed by BM stromal fibroblasts and endothelial cells and it is essential for survival, proliferation, adhesion/migration and differentiation of HSC (Broudy, 1997). SCF alone plays a protective role in apoptosis (Lyman and Jacobsen, 1998) but does not have a significant effect on growth in early myeloid and lymphoid cells (McNiece et al., 1991, Broxmeyer et al., 1991). However, in combination with other cytokines SCF stimulates growth in particular of primitive haematopoietic cells and their immediate progeny (McNiece et al., 1991, Broxmeyer et al., 1991). SCF acts synergistically with other cytokines including FL (Haylock et al., 1997), thrombopoietin (Ramsfjell et al., 1996) as described below.

2.9.1.2 Flt3 ligand (FL)

FL is a critical component in cytokine cocktails for optimal recruitment into the cell cycle of the most primitive human haematopoietic cells (Nordon et al., 1997, Zandstra et al., 1998, Shah et al., 1996, Haylock et al., 1997, Case et al., 2001). FL leads to short

term expansion (De Felice et al., 1999) and at high concentrations was also shown to promote human HSC self-renewal. FL alone does not efficiently induce proliferation of normal myeloid and lymphoid progenitors, but shows strong synergy with other haematopoietic cytokines and interleukins including SCF, G-CSF, GM-CSF IL-3, IL-6, IL-7, IL-11 and IL-12 (Lyman et al., 1993, Lyman et al., 1994, Lyman and Jacobsen, 1998). FL lacks an effect on erythroid progenitors but when combined with other growth factors, strongly promotes proliferation, myeloid differentiation and expansion of primitive and committed haematopoietic progenitors (Jacobsen et al., 1995, Rusten et al., 1996, Haylock et al., 1997). FL has a structural homology with SCF and overlapping functions (Zandstra et al., 1997). FL (Haylock et al., 1997, Lyman and Jacobsen, 1998), like SCF (Lyman and Jacobsen, 1998) protects cells from apoptosis, but is more efficient in supporting survival of human candidate HSC cells (Sitnicka et al., 2003).

2.9.1.3 Thrombopoietin (TPO)

TPO supports the survival of highly purified population of HSC, and in combination with other cytokines accelerates entry into the cell cycle of HSC (Sitnicka et al., 1996). Megakaryocyte Growth and Development Factor (MGDF), a truncated, polyethylene glycol-coated form of the TPO molecule, is used as a replacement in many studies. TPO acts synergistically with FL to maintain and expand human HSC (Piacibello et al., 1998b) and enhance the growth of candidate murine HSC (Ramsfjell et al., 1996). TPO, combined with SCF, stimulates multilineage growth *in vitro* on candidate murine and human stem cell populations (Ku et al., 1996, Ramsfjell et al., 1996, Sitnicka et al., 1996, Kobayashi et al., 1996) and plays a role in committed megakaryocyte and platelet

production (Kaushansky, 1995, Nocka et al., 1990, Broudy et al., 1995, Hunt et al., 1995, Banu et al., 1995, Gurney et al., 1994).

The cytokine combination TPO, SCF and FL has been shown to inhibit apoptosis, stimulate self-renewal division of primitive haematopoietic (Murray et al., 1999, Ramsfjell et al., 1999) and promote the development of multipotent progenitors as determined by *in vitro* and *in vivo* functional assays (Piacibello et al., 1999, Spence et al., 1998, Cashman et al., 1997a, Petzer et al., 1996, Zandstra et al., 1997, Zandstra et al., 1998, Ramsfjell et al., 1996, Conneally et al., 1997).

2.9.1.4 Interleukin-3 (IL-3)

The effect of IL-3 on expansion of haematopoietic cells and its contribution to self-renewal is controversial in the literature (Heike and Nakahata, 2002). Some studies suggest that IL-3 may have a negative effect on *in vitro* stem cell expansion in murine (Yonemura et al., 1996, Peters et al., 1996, Yonemura et al., 1997, Ogawa and Matsunaga, 1999, Bhatia et al., 1997) and human cells (Zandstra et al., 1997, Piacibello et al., 1998a). Another study has also shown a harmful effect on the repopulating ability of CB $CD34^+$ cells with addition of IL-3 to the cytokine combination of SCF, FL, TPO and IL-6/sIL-6R (Ueda et al., 2000). However, others studies have shown that IL-3 has a positive effect on the expansion of primitive cells, maintaining engraftment (Rossmanith et al., 2001) and in supporting SCF, FL and MGDF-induced expansion of multilineage, long-term reconstituting activity in primary and secondary recipients (Bryder and Jacobsen, 2000). Case et al., confirmed that SCF, FL, MGDF, and IL-3 are

able to promote divisional recruitment and multilineage development of CB $CD34^+$ cells (Case et al., 2001).

2.9.2 Clinical studies using cytokine-mediated expansion

The results of *in vitro* and *in vivo* studies on the use of cytokines as a candidate for *ex vivo* expansion have suggested a role for cytokine-mediated expansion in the clinic. The first reports of clinical studies focused on the safety of transplanting expanded haematopoietic cells in patients using MPB (Brugger et al., 1995, Alcorn et al., 1996). These studies found that cytokine expansion had little or no toxicity and could safely restore haematopoiesis in patients treated with high doses of chemotherapy, making the use of cytokine mediated expansion a possibility in the clinic. Further clinical studies showed that engraftment was possible by cultures initiated with small aliquots of PB, supported by cytokines (Brugger et al., 1995), supporting the use of cytokine-mediated expansion in samples where the volume was limited such as CB. A clinical study by Mc Niece et al found that reduction of neutropenia following transplantation could be achieved by expansion of PB progenitors and that the kinetics of engraftment was dose dependent (McNiece et al., 2000). However, another study showed that even though short-term haematopoietic recovery was seen in patients, long-term engraftment failed to occur in some of the patients. The early recovery may have been due to the progenitors present in the *ex vivo* expanded cells but the late recovery was provided by the unmanipulated PB progenitors given as a 'back up' in all cases (Holyoake et al., 1997). The loss of long-term reconstitution further highlights the issue of an engraftment defect following *ex vivo* expansion as observed in *in vivo* studies described above. This problem has also been the main hindrance with other clinical studies where

CB was used as the HSC source for *ex vivo* expansion (Hofmeister et al., 2007). Hence, an optimal cytokine combination is yet to be defined for *ex vivo* expansion of HSC and its progenitors to promote long-term engraftment and reduction of delayed engraftment in CB transplants.

2.9.3 Compromised engraftment following cytokine-mediated expansion

For cell expansion to occur, cytokines need to promote recruitment of HSC into proliferation (Srour et al., 1999, Metcalf, 1993, Ogawa, 1993), but if cytokines are to be used to stimulate HSC *in vitro*, the resultant HSC progeny must retain their repopulation capacity. Cytokines that are positive regulators have a role in counteracting apoptosis as they promote survival in HSC (Domen and Weissman, 1999, Cowling and Dexter, 1994). Hence, it is also important that the cytokines used *in vitro* promote survival and not apoptosis or cell death.

Numerous *in vitro* and *in vivo* studies have been performed to investigate the effects of cytokine exposure on haematopoietic cells (Zandstra et al., 1997, Bryder and Jacobsen, 2000, Heike and Nakahata, 2002) and on the engraftment capacity of these cells (Bhatia et al., 1997, Novelli et al., 1999, Sitnicka et al., 2003). However, the effects of cytokine-mediated expansion on HSC function remains controversial as some reports have shown that *ex vivo* expansion of haematopoietic cells with cytokines supports both short- and long term engraftment and is not detrimental to serial transplantation (Bhatia et al., 1997, Conneally et al., 1997). Alternatively, studies have also implicated that *ex vivo* culture of HSC, in the most favourable results, maintained long-term repopulating activity while in others studies expansion compromised it (Domen and Weissman, 1999,

Traycoff et al., 1996, Peters et al., 1996, Yonemura et al., 1997, Yonemura et al., 1996). These findings suggest the problem is an introduced engraftment defect in long-term repopulating cells by exposure to cytokines and highlights the importance of targeting and expanding the right population of haematopoietic cells.

2.10 Cell cycle and haematopoiesis

Haematopoiesis needs to be tightly regulated to maintain the stem cell pool, sustain the different blood cell lineages required in daily life and to quickly up-regulate their production in a stressful situation such as blood loss. Depending on the requirements of the haematopoietic system, cells are required to remain quiescent (G0 phase) or to enter the cell cycle for self-renewal, proliferation or differentiation. The cell cycle can be divided into 4 phases (Fig.2.2), S-phase where DNA synthesis occurs, the mitotic phase (M) and the two gaps that separate these processes, G1 (between M and S phase) and G2 (between S and M phase) (Myatt and Lam, 2007). The cell cycle and its regulation are central to the fate of haematopoietic cells, including expansion as cell proliferation requires entry into and successful progression through the cell division cycle (Sherr and Roberts, 1999, Sherr and Roberts, 2004). The role of cell cycle status in normal haematopoiesis and on the phenotype of the engrafting stem cell has been the subject of research for a number of years. However, to this day it still remains controversial.

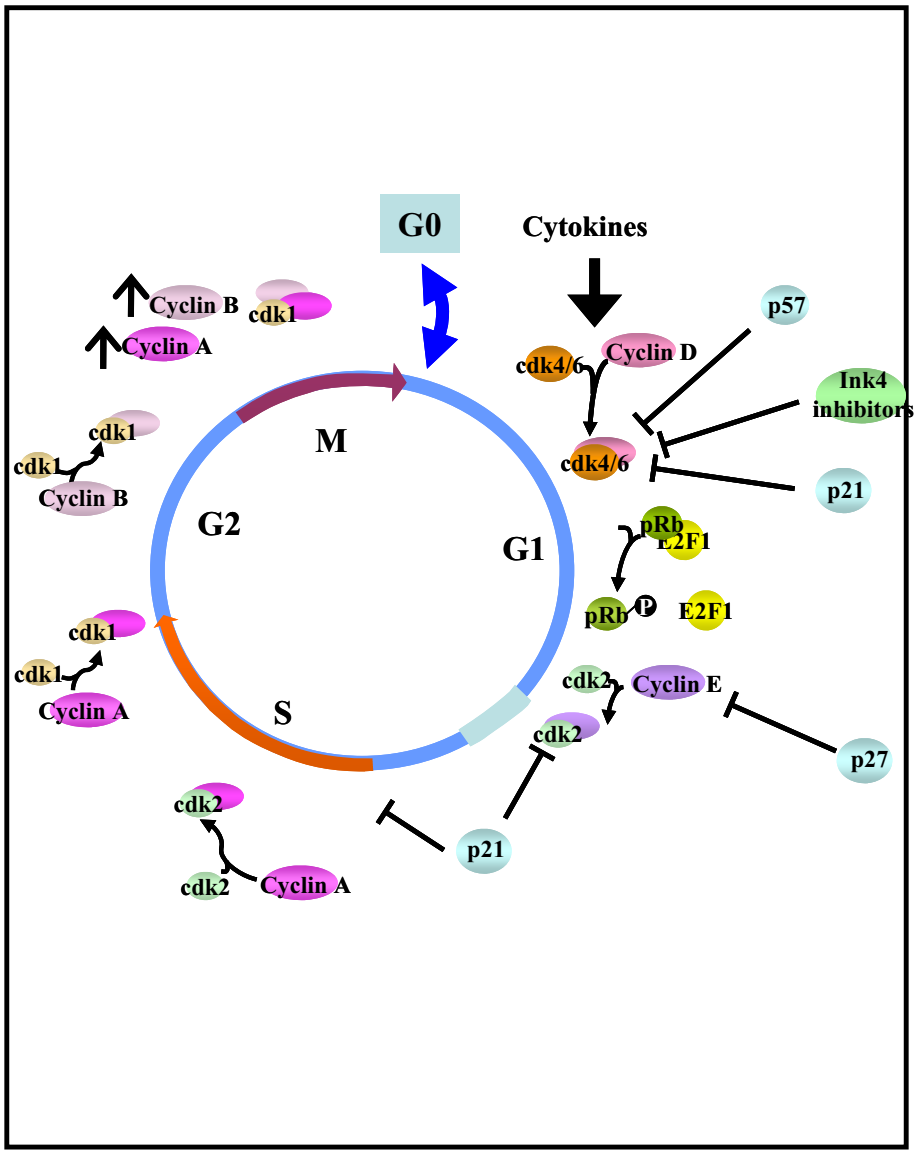

Figure 2.2 The Mammalian Cell cycle

Cyclins and cyclin-dependent kinases (CDK) tightly regulate progression through the cell cycle. During G1, D cyclins associate with CDK4 and CDK6 to form complexes. Activated cyclin-CDK complexes phosphorylate the retinoblastoma (Rb) protein. Rb is a transcriptional repressor that binds to E2F1 transcription factors in the hypophosphorylated state and blocks expression of S-phase genes. Phosphorylation of Rb by D cyclin/CDK4 or CDK6 early in G1 and cyclin E/CDK2 late in G1 phase lead to inactivation of Rb-E2F1 complex and allows transcription of S-phase genes allowing G1/S transition.

2.10.1 The relative quiescence of HSC

The model for haematopoiesis that current data mostly supports is a functional hierarchy of haematopoietic stem cells correlated to their quiescent state (Hao et al., 1996, Steinman, 2002). Hence, one of the main dogmas is that adult HSCs remain in a quiescent state, necessary to maintain long-term self-renewal and to prevent stem cell exhaustion (Ogawa, 1993). Primitive stem cells residing in the G0 phase of the cell cycle have the highest stem cell activity and only 3-4% of long-term self-renewing HSCs in adult mice are in S/G2/M phases of the cell cycle at one any time (Morrison and Weissman, 1994). The LTC-IC of primitive cells is highest in cells residing in the G0 phase, and those cells that have cycled and re-entered the G0 phase progressively lose their potential with cell cycling (Gothot et al., 1997, Gothot et al., 1998a). Hao et al. (1996) further supported this model by extending the LTC-IC assay (E-LTC-IC) and defining a more primitive progenitor with a higher level of quiescence. However, this dogma has been challenged by further studies (Quesenberry et al., 1999). Cheshier et al. (1999) through an *in vivo* assay showed that 75% of the long-term self renewing HSC (LT-HSC) were indeed quiescent but most LT-HSC entered the cell cycle by 30 days and 99% divided approximately every 57 days. Hence, while most HSCs are quiescent at anyone time, they do divide regularly and cycle slowly. This finding was confirmed by another study in a nonhuman primate model (Mahmud et al., 2001) where they showed that the nonhuman primate HSC cycled at a lower rate as compared with murine HSC. Hence the difficulty in stimulating the most primitive HSC populations to expand with cytokines may be due not only to the quiescent state of most of the population but also to the slow cycling rate of LT-HSC.

2.10.2 The role of cell cycle status on the engraftment capacity of HSC

A number of studies have demonstrated that a relationship exists between cell cycle status and the engraftment ability of haematopoietic stem cells. As mentioned above, the HSCs capable of repopulating the haematopoietic system are mainly quiescent cells (Ogawa, 1993, Fleming et al., 1993). However, cell cycle transit may change the functional phenotype of the HSC (Habibian et al., 1998, Quesenberry et al., 1999). This notion would support regulation of haematopoiesis to be cell cycle related and not hierarchical (Quesenberry et al., 1999). This is important for cytokine-mediated culture as this method aims at stimulating the cells to enter the cell cycle in order to expand. However, current reports differ on the cell cycle phenotype of the engrafting cell as described in the previous section.

LTC-IC cells from CB $CD34^+$ cells are mostly in G0/G1 but when stimulated with IL-3, SCF and G-CSF, these cells are driven to the S-phase of the cell cycle (Lucotti et al., 2000). MPB $CD34^+$ cells moving from G0 to the G1 phase of the cell cycle, following cytokine stimulation, lose their engraftment capacity (Gothot et al., 1998b). Other studies on CB $CD34^+$ cells showed similar repopulating capacity from G0 and G1 fractions (Wilpshaar et al., 2000). When foetal liver and foetal bone marrow were tested, cell cycle status of HSC was not detrimental to the repopulating capacity of the cells (Wilpshaar et al., 2002). Hence, the engrafting capacity of HSC cells is restricted to the quiescent fraction in the adult stem cell sources but not in the foetal or neonatal sources. Another report found that following cytokine culture, CB $CD34^+$ cells in S/G2/M phase lose their engraftment potential, whereas the cells in the G1 fraction were still able to engraft the NOD-SCID mice (Glimm et al., 2000). This finding further

confuses the field; therefore there is a need to unequivocally determine the engraftment capacity of defined CB $CD34^+$ cell populations that have undergone cytokine-mediated expansion.

The adverse effect of cell cycle activation during *ex vivo* expansion targets long-term stem cell function (Gothot et al., 1998b, Habibian et al., 1998, Fleming et al., 1993) but not short term engraftment kinetics (Szilvassy et al., 2000). The engraftment capacity seems to be reduced in cells expanded *in vitro* compared to unmanipulated stem cells, but the specific cell cycle status of the engrafting cell is still controversial. However, the detrimental effect on engraftment correlated to cell cycle transit does not seem to be irreversible (Habibian et al., 1998, Quesenberry et al., 1999) challenging the dogma that once cells enter the cell cycle they permanently lose their self-renewal and repopulating capability. This raises the issue of characterising the engrafting cell according to a phenotype that may fluctuate depending on cell cycle progression. Since *ex vivo* expansion induces cell cycle progression, the 'expanded' phenotypic markers may not accurately reflect functional potential (Habibian et al., 1998).

2.10.3 Regulation during G1 phase of the cell cycle

The focus on this study is on the regulation of the G1 phase of the cell cycle as it is the critical period when cells commit to proliferation or growth arrest following stimulation by mitogens such as cytokines. The processes involved in other parts of the cell cycle are outside the scope of this thesis.

A number of studies have shown a role for G1 cell cycle regulators not only in policing the progression of cells through the G1 phase but also in quiescence, proliferation, differentiation and apoptosis (Steinman, 2002). However, most of the studies have been performed on either cell lines or murine models and if using human samples mainly investigating specific lineages (eg. erythroid cells) rather than the effect of *ex vivo* expansion on cell cycle control of haematopoiesis. Candidate cell cycle related genes to be studied are p21, p27 and D cyclins as there are conflicting data on their involvement in haematopoiesis (Steinman, 2002, Yaroslavskiy et al., 1999, Taniguchi et al., 1999).

2.10.3.1 D cyclins are involved early in the cell cycle

Cyclins and cyclin-dependent kinases (CDK) tightly regulate progression through the cell cycle (Fig. 2.2). During G1, D cyclins associate with CDK4 and CDK6 to form complexes. Activated cyclin-CDK complexes phosphorylate the retinoblastoma (Rb) protein. Rb is a transcriptional repressor that binds to E2F1 transcription factors in the hypophosphorylated state and blocks expression of S-phase genes. Phosphorylation of Rb by D cyclin/CDK4 or CDK6 early in G1 and cyclin E/CDK2 late in G1 phase lead to inactivation of Rb-E2F1 complex and allows transcription of S-phase genes allowing G1/S transition (Sherr and Roberts, 1999).

D and E type cyclins govern rate of progression of mammalian cells through G1 phase of the cell cycle. However the focus of this study are the D cyclins as they are required after G0/G1 transition but not in the G1/S boundary (Sherr, 1993) and the target of cytokine stimulation. D type cyclins are differentially and combinatorially expressed in mammalian cells (Matsushime et al., 1994) and are functionally heterogenous (Ewen et

al., 1993). Some studies have shown cyclin D2 and cyclin D3 to be predominant in haematopoietic cells (Steinman, 2002) but their the pattern of expression differs between studies (Cheshier et al., 1999, Fink and LeBien, 2001, Furukawa et al., 2000, Ando et al., 1993, Gong et al., 1995, Della Ragione et al., 1997). Cyclin D1 expression is expressed at different levels in human adult (Furukawa et al., 2000) and foetal BM $CD34^+$ cells (Fink and LeBien, 2001) but not expressed in murine haematopoietic cell lines (Ando et al., 1993) and murine BM LT-HSCs (Cheshier et al., 1999). Hence, the exact role of expression of D cyclins in haematopoiesis is yet to be defined.

2.10.3.2 Cyclin-dependent kinases inhibitors

The activity of the cyclin-CDK complexes is negatively regulated by CDK inhibitors (CKIs). CKIs are classified into two groups, the INK4 family and the CIP/KIP family.

The INK 4 (inhibitors of cyclin dependent kinase 4) family consists of 4 proteins constitutively expressed, namely $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{ARF}$. They specifically inhibit CDK4 and CDK6 (Serrano et al., 1993). $p19^{ARF}$ binds to MDM2 and blocks destruction of p53 leading to an increase in p53. The CIP/KIP family ($p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$) are able to inhibit multiple cyclins and CDKs, including CDK4 and CDK6 but their greatest inhibitory activity is against CDK2.

2.10.3.3 $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ role in haematopoiesis

The focus of this thesis is on the CIP/KIP family members, $p21^{WAF1/CIP1}$ (p21) and $p27^{KIP1}$ (p27) as they are involved in the G1 phase of the cell cycle. p27 is expressed

both in proliferating and in differentiated cells (Zhu and Emerson, 2002) and inhibits a number of CDK complexes *in vitro* including D cyclins-CDK4 (or CDK-6) or cyclin A-CDK2 (Toyoshima and Hunter, 1994) and cyclin E-CDK-2, but *in vivo* it seems that cyclin E-CDK-2 may be the main target (Zhu and Emerson, 2002). p27 levels decrease following mitogenic stimulation of quiescent cells or increase following contact inhibition, mitogen withdrawal or other anti-proliferative signals (Vlach et al., 1997, Zhu and Emerson, 2002). p27 may also have a role in erythroid differentiation (Hsieh et al., 2000)

p21 inhibits G1 cyclin/CDK complexes, blocking G1/S transition (Steinman et al., 1998) and is involved in G1 arrest following DNA damage, in response to cytokines and loss of substrate adhesion and in maintenance of terminally differentiated cells in a nonproliferative state (Ju et al., 2007). p21 is positively regulated at the transcriptional level by p53 activation (Almasan et al., 1995). However, cytokines also have a role in regulating p21 independently of p53. p21 has been suggested to a have a dual role in haematopoiesis (LaBaer et al., 1997, Parry et al., 1999). At low levels, p21 is required to stabilise the cyclin D/CDK 4 complex whereas at higher levels of expression it inhibits these complexes. p21 has also been described as the switch governing entry of stem cells into the cell cycle. In the absence of p21, an increase in cell cycling leads to stem cell exhaustion (Cheng et al., 2000) making p21 an important candidate for the molecular phenotype of expanded cells.

In the literature, there is no consensus on the expression levels of p21 and p27 in untreated $CD34^+$ cells and following cytokine expansion. In CB $CD34^+$ stimulated to differentiate along the myeloid lineage, there was no expression of p21 in freshly

isolated $CD34^+$ cells but expression increased following cytokine exposure (Yaroslavskiy et al., 1999). Another study showed high levels of p21 that decreased shortly after incubation with cytokines (Taniguchi et al., 1999). For p27, one study showed that expression was present in both freshly harvested CB $CD34^+$ cells and differentiated cells but at low levels (Yaroslavskiy et al., 1999, Taniguchi et al., 1999) whereas another study showed that untreated CB $CD34^+$ cells had high p27 expression (Steinman, 2002). However the purpose of these studies was to examine the role of these CKI in differentiation of haematopoietic cells and not on their effect on cytokine-mediated expansion.

2.11 Self renewal and asymmetric cell division in haematopoietic cells

Numerous studies have demonstrated that primitive HSC undergo asymmetric division (Brummendorf et al., 1999, Huang et al., 1999) and reviewed by Ho and Wagner (2007). Asymmetric division results in two functionally different daughter cells. One cell is identical to the parent stem cell (self-renewal) and the other cell is capable of differentiating down specific lineages. However, as HSC proliferate, self renewal capacity gradually decreases with differentiation (Zhu and Emerson, 2002). In the absence of stress, the total pool of HSCs is constant, hence to maintain the HSC pool, self renewal needs to occur at close to half of all divisions of the HSC (Osawa et al., 1996, Kondo et al., 2003). Self renewal supports the hierarchical model of HSCs ability to proliferate to make differentiated cell types of a given tissue *in vivo* as well as maintaining the stem cell pool (van der Kooy and Weiss, 2000). Evidence of self renewal of HSC is shown through expansion *in vivo* during ontogeny and after transplantation (Domen and Weissman, 1999).

Serial transplantation of HSCs in mice is limited to around 5 to 7 rounds which indicates that HSC cannot self renew indefinitely (Kondo et al., 2003, Ogden and Mickliem, 1976). All normal somatic cells have an intrinsic limit to the number of times they can divide, a phenomenon referred to as the "Hayflick limit" (Shay and Wright, 2000) (Morrison et al., 1995). Telomerase activity appears to correlate with self-renewal capacity and is reduced as HSCs differentiate into multipotent progenitor populations (Morrison et al., 1996) and with donor age (Vaziri et al., 1994). Telomere shortening may be one factor that reduces HSC self-renewal potential (Allsopp and Weissman, 2002, Vaziri et al., 1994). Telomerase, a ribonucleoprotein complex responsible for

extending telomere sequence, is expressed by mouse foetal liver cells, CB and BM HSC (Morrison et al., 1996). However even though telomerase is induced in cycling HSCs following cytokine stimulation (Yui et al., 1998), it may not be sufficient to maintain telomere length, as telomeres still shorten with HSC division *in vivo* (Allsopp et al., 2001, Brummendorf et al., 2001). However, even though it is important to acknowledge their role in cell division and expansion, an extensive review of this issue are beyond the scope of this thesis.

A better understanding of self-renewal and the molecular factors involved in its regulation would impact on the maintenance of the HSC pool after *in vitro* cytokine-mediated expansion.

2.11.1 The effect of cell division on engraftment

The aim of cytokine-mediated expansion is to expand the total cell number and both the HSC and progenitors populations to facilitate engraftment after CB HSC transplantation. Hence, an important function of cytokine-mediated expansion is divisional recruitment. Moreover, the ideal cytokine combination needs to promote asymmetric cell division so as to maintain the primitive HSC pool in the expanded population.

As previously mentioned in section 2.9.1, studies have shown that cytokines such as FL (Nordon et al., 1997), SCF and TPO are able to promote proliferation of HSC populations and its progenitors (Ramsfjell et al., 1999, Case et al., 2001). However, the effect of cell division following cytokine expansion on the engraftment ability of HSC

is controversial. Some reports suggest that stimulation of cell division in HSC by cytokines may lead to differentiation and loss of engraftment potential *in vivo* (Young et al., 1999). While others show that engraftment potential is maintained in divided cells (Glimm and Eaves, 1999). Cell tracking technology is a useful tool to investigate the relationship between cell division and engraftment potential (Fig. 2.3).

2.11.2 Cell tracking technology

A number of fluorescent dyes have been used for over 20 years to track viable cells but the two most successful dyes to follow cell division tracking are PKH-26 and 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Lyons and Parish, 1994). These dyes are highly fluorescent, their stain is persistent and it is possible to monitor the proliferation history of viable cells by flow cytometry. CFSE has the advantage over PKH-26 due to its homogeneity in staining (Parish, 1999) and resolution of divisional clusters (Lansdorp and Dragowska, 1993) and cost. CFSE diffuses into cells and binds irreversibly to internal constituents of the cytoplasm and as the cell divides, the mean fluorescence is decreased by a factor of 2 as it is equally divided between the daughter cells (Fig.2.3). The divided cell populations or clusters are then distinguished from one another as the mean fluorescence is halved in each divisional cluster. CFSE has been used to track cell division in several cell types including lymphocytes and haematopoietic cells (Parish, 1999, Nordon et al., 1997). Modifications in staining and inclusion of cell

sorting allowed high resolution cell division tracking of subsets of human BM $CD34^+$ cells following cell culture (Nordon et al., 1997). Hence high resolution cell division tracking is a powerful technique that may be used to establish the relationship between cell division, phenotype and engraftment potential and to determine if cytokine-mediated expansion is detrimental to the long-term engraftment capacity of CB $CD34^+$ cells, the aim of this thesis.

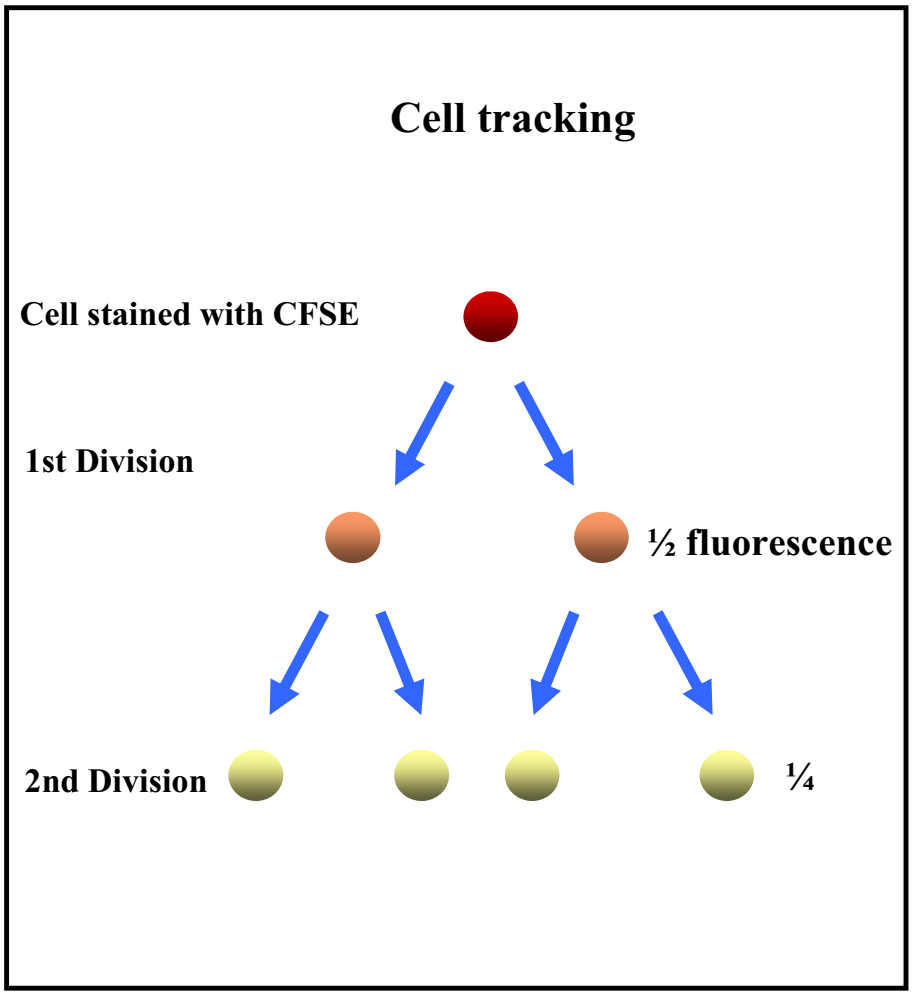

Figure 2.3 Cell tracking

CFSE fluorescent dye diffuses into cells and binds irreversibly to internal constituents of the cytoplasm. As the cell divides, the mean fluorescence is decreased by a factor of 2 as it is equally divided between the daughter cells. The divided cell clusters are then distinguished from one another as the mean fluorescence is halved in each divisional population.

2.12 Hypothesis and Aims of the study

Cytokine-mediated expansion of CB derived $CD34^+$ cells could be used to increase the total cell number by increasing HSC and progenitor cells necessary to facilitate engraftment after transplantation. We hypothesise that if cytokine-mediated expansion promotes divisional recruitment and multilineage differentiation it causes changes in phenotype and cell cycle related gene expression which may be detrimental to the engraftment capacity of haematopoietic cells. Hence the overall hypothesis is that cytokine-mediated expansion compromises haematopoietic stem cell function.

Aims:

(1) To optimise a flow cytometry based high resolution cell division tracking technique to provide a tool to monitor changes in phenotype and function in CB $CD34^+$ cells as a consequence of cytokine-mediated expansion.

(2) To correlate the effect of cell division of CB $CD34^+$ cells with the expression level of cytokine receptors, adhesion molecules and lineage markers following cytokine-mediated expansion.

(3) To characterise the changes in cell cycle-related expression of candidate G1 phase genes prior to and following cytokine-mediated expansion of CB $CD34^+$ cells.

(4) To determine the engraftment potential of CB stem cell progeny generated *in vitro* to expand *in vivo* using the NOD-SCID mouse model of human engraftment.

2.13 Significance of the study

Elucidation and prediction of the effects of cytokine stimulation on stem cell progeny will lead to the development of clinically relevant cytokine-mediated expansion protocols to further improve the safety of haematopoietic stem cell transplantation.

Chapter 3 Phenotypic analysis of CB CD34 positive cells and its progeny following cytokine-mediated expansion

3.1 Introduction

3.1.1 Changes in the phenotype of HSC following cytokine-mediated expansion

Successful cytokine expansion induces entry to the cell cycle and proliferation to increase the total number of cells in the sample. But it is important to ensure that that the right haematopoietic population is targeted and both the number of HSC and progenitor cells increases without differentiation occurring at the expense of self renewal. Cytokine-mediated expansion also has a role in homing and engraftment of haematopoietic cells as it induces chemokines such as CXCR4 (Peled et al., 1999).and stimulates adhesion molecules such as VLA-4 and VLA-5, enhancing their function (Levesque et al., 1996, Levesque et al., 1995, Papayannopoulou et al., 1998).

The specific phenotype of the engrafting human haematopoietic cell is yet to be defined and its characterisation is particularly problematic due to the fluctuating nature of phenotype during cell cycle progression (Habibian et al., 1998). This is further complicated by the dissociation of stem cell function and phenotype observed following cytokine-mediated expansion as seen in CB $CD34^{+}CD38^{-}$ cells (Dorrell et al., 2000).

In this study the cytokine combination SCF, MGDF, FL and IL-3 was chosen as previous studies in our laboratory (Case et al., 2001) confirmed that this cytokine cocktail is able to promote divisional recruitment and multilineage differentiation of CB $CD34^+$ cells. Since the effect of IL-3 on cytokine-mediated expansion of haematopoietic cells and its contribution to self- renewal is debatable as described in the literature review, closer examination of its role in expansion of CB samples in combination with SCF, MGDF and FL is of importance. Moreover, the effect of cell division on HSC function following cytokine expansion is controversial.

Hence, the examination of a number of cell surface markers including putative stem cell markers, lineage specific markers, growth factor receptors and adhesion molecules is necessary to assess any changes in phenotype following cytokine-mediated expansion of CB $CD34^+$ cells and the relationship of cell division on their expression.

3.1.2 Cell tracking technology

Cell tracking technology using the intracellular fluorescein-based dye CFSE has been used to study the proliferation history of viable cells including lymphocytes and haematopoietic cells by flow cytometry (Parish, 1999, Nordon et al., 1997). High resolution cell tracking achieved by sorting of the CFSE population enables cell tracking of cell divisions following culture of haematopoietic cells (Nordon et al., 1997). Hence, the high resolution cell tracking technique may be a useful tool to assess the relationship between phenotype and divisional history and hence characterise any changes in the cell surface phenotype of CB $CD34^+$ cells as a consequence of cytokine-mediated expansion.

3.1.3 Aims of the study

The hypothesis for this chapter is that cell tracking technology is a useful tool to establish the relationship between cell division, phenotype and engraftment potential and to determine if cytokine-mediated expansion is detrimental to the long-term engraftment capacity of CB $CD34^+$ cells.

The aim of this section of the study is to optimise a high resolution cell division tracking technique to provide a tool to monitor changes in phenotype and function in CB $CD34^+$ cells following cytokine-mediated expansion and to examine the effect of cell division of CB $CD34^+$ cells on the expression level of cytokine receptors, adhesion molecules and lineage markers following cytokine-mediated expansion.

3.2 Materials and Methods

3.2.1 Source of haematopoietic stem cells

After informed consent, human umbilical cord blood (CB) was collected from the umbilical vein following normal full term delivery of the baby, into 250 ml transfer bags containing 35 ml ACD (Paediatric collection bags, Baxter, Sydney). The samples were stored at room temperature and processed no longer than 24 hours after collection. The collection of CB for research purposes was approved by the Research and Ethics committees of the South Eastern Sydney Area Health Service and the University of New South Wales. Unless stated, all CB samples used in this study consisted of pooled CB collections. This was due to the large number of cells required per experiment.

3.2.2 $CD34^+$ cell isolation

CB contains relatively high levels of nucleated red cells compared to BM and MPB, which interfere with the $CD34^+$ isolation procedure and reduce the purity of the final product. Redout (Robbins Scientific, California, USA) was used to eliminate nucleated red cells from the mononuclear fraction. Redout induced aggregation of nucleated red cells by increasing their density allowing them to fall into the red cell layer of the density gradient when centrifuged (Rubinstein et al., 1995). Twenty ml aliquots of CB sample were placed into 50 ml centrifuge tubes (Falcon, Bedford, USA) and 50µL of Redout were added per tube. The aliquots were incubated for 20 minutes at 4°C.

3.2.2.1 Mononuclear Cell Preparation

Density gradient separation was used to isolate low-density mononuclear cells (MNC). CB samples were diluted with 15 ml of phosphate buffered saline (PBS, Gibco Invitrogen, Melbourne, Australia) and underlayed with 15 ml of Lymphoprep (density 1.077 g/ml, NycoMed, Oslo, Norway). Cells were centrifuged (Hettich Rotanta 96R, Tuttlingen, Germany) at 20°C, 800g for 30 minutes with acceleration at 5 and slow deceleration. The interphase, consisting of MNC, between the plasma and Lymphoprep was collected by aspirating with a syringe (Terumo, Sydney, Australia) and cannulae (Maersk Indoplast, Sydney, Australia) and placed in a fresh 50 ml centrifuge tube. PBS was added to the cells to a total volume of 40 ml and the sample were centrifuged at 20°C, 300g for 10 minutes with acceleration at 9 and deceleration at 5. A second wash was performed under the same conditions using the buffer required for Magnetic Activated Cell Sorting (MACS) described below (MACS buffer: PBS of pH 7.2 containing 2mM disodium ethylenediamine tetra-acetic acid [EDTA, Ajax Chemicals, Sydney, Australia], 0.5% bovine serum albumin [BSA, Sigma, St Louis, USA]). Cells were placed in a Neubauer chamber (Knittel Glaser, Germany) and counted using a light microscope (Zeiss, Germany). The viability of cells was measured by their ability to exclude trypan blue (Sigma, St Louis, USA) exclusion. The cell concentration was then adjusted to 10^8 cells per 300µl of MACS buffer. A small sample (pre-sample) was set aside for phenotyping by flow cytometry (see flow cytometry section below), which was used to calculate the proportion of $CD34^+$ cells in MNC and estimate the approximate yield of $CD34^+$ cells.

3.2.2.2 Magnetic Activated Cell Sorting of CB

$CD34^+$ cells were isolated from MNC using the Magnetic Activated Cell Sorting (MACS) system and the Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec, Germany) according to manufacturer's instructions and as previously described (Case et al, 1996). This technique enables enrichment of CD34 cells by labelling CD34 expressing cells within the MNC sample with magnetic microbeads and positively selecting them on separation columns in the magnetic field of a magnetic cell separator such as VarioMACS (Miltenyi, Germany).

In brief, 100µl of FcR blocking reagent per 10^8 MNC cells was added and gently mixed to inhibit non-specific binding of CD34 microbeads to non-target cells. 100µl of CD34 microbeads per 10^8 total cells were added to the MNC sample to label $CD34^+$ cells. After gently mixing and incubating at 4°C for 30 minutes, the sample was washed in MACS buffer and centrifuged at 4°C, 300g for 10 minutes. The cells were resuspended to $2x10^8$ cells/ml of MACS buffer.

Positive selection of $CD34^+$ cells was achieved in 2 steps. First, a LS^+ column (Miltenyi, Germany) was used as its loading capacity allowed positive selection of up to 10^8 labelled cells from up to $2x10^9$ total cells. To achieve between 90-95% purity in the $CD34^+$ population the sample was passed through a second column. Since the $CD34^+$ cell population in CB MNC ranged between 0.1-1 %, after the first column the number of cells collected was significantly less than the original sample. Hence, a MS^+ column (Miltenyi, Germany) was used for the second separation as it could select up to 10^7 positive cells. Prior to adding the sample, the LS^+ column was washed with 3 ml of

MACS buffer to remove the hydrophilic coating in the column and the effluent was discarded. The labelled sample was filtered through a 30μm MACS pre-separation filter (Miltenyi, Germany) and passed through the separation column placed on the VarioMACS magnet. The effluent was collected in a 14 ml sterile tube (Falcon, Bedford, USA). To maximise purity, the column was washed 3 times with 2 ml of MACS buffer. Once washed, the column was removed from the magnet and placed on top of a fresh sterile collection tube. The attached cells were removed by flushing the column 3 times with 3 ml of MACS buffer using the plunger supplied. The sample was centrifuged at 4°C, 300g for 10 minutes as above and resuspended in less than 500μL of MACS buffer. The sample was then passed through the second column.

The MS^+ column was washed with 500μl of MACS buffer before separation. The sample was filtered as above and passed through the MS^+ column, followed 2-3 times by 500μL of MACS buffer. The effluent (CD34- cells) was collected in a 4 ml sterile fluorescence activated cell sorter (FACS) glass tube (Falcon, Bedford, USA). After washing the column thoroughly with MACS buffer to remove unlabelled cells, the $CD34^+$ cells were eluted into a fresh sterile tube. $CD34^+$ cells were counted and cryopreserved for later usage. $CD34^-$ cells were also cryopreserved. A small sample (post-sample) was set aside for phenotyping by flow cytometry to test the purity of the $CD34^+$ cells and co-expression of CD38 (see flow cytometry section below).

3.2.3 Cryopreservation of CB cells

Both $CD34^+$ and $CD34^-$ fractions were resuspended in Foetal Calf Serum (FCS, Trace Biosciences, Sydney) at a concentration of $1x10^6$ cells/ml and transferred to 2 ml

internal thread cryovials (Iwaki, Montreal, Canada). Dimethyl sulfoxide (DMSO, Sigma, St Louis, USA) was added to each sample to a final concentration of 10%, prior to cryopreservation at -80°C in a Nalgene isopropanol bath (Nelge-Nunc International, Rochester, USA). After 24 hours cells were stored in the vapour phase of liquid nitrogen (-196°C) until use.

3.2.4 Thawing of cryopreserved CB $CD34^+$ cells

When required, vials of CB $CD34^+$ were removed from liquid nitrogen, rapidly thawed in a 37°C water bath and washed twice in a solution of phenol red free Iscove's Modification of Dulbecco's Medium (IMDM; Life Technologies, Sydney), 20% FCS and 0.2 μg/ml DNAse (Sigma, St Louis, USA). Trypan Blue exclusion was used to assess viability and it always exceeded 95%. Previous studies showed that usage of cryopreserved CB samples had no effect on expansion (Case et al. 1996).

3.2.5 Quantification of $CD34^+$ enrichment of CB samples

Flow cytometry was used to immunophenotype the CB samples to calculate $CD34^+$ content and purity. All antibodies used were mouse anti-human monoclonal antibodies. The samples collected (pre- and post-MACS separation) were stained with fluorescein isothiocyanate (FITC) conjugated CD45, phycoerythrin (PE) conjugated CD38 (B-D, Becton Dickinson, San Diego, CA) and CD34 phycoerythrin-cyanin 5 (PECy5) (Immunotech, France). Appropriate isotype-matched conjugated controls included mouse IgG1PE (Becton Dickinson, San Jose, USA) and IgG1PECy5 (Immunotech, France). To stain samples, 10μl of each antibody (Immunotech Abs, only 5μL) was

added to approximately $1x10^5$ cells in 4 ml FACS tubes. Samples were incubated at 4°C for 30 minutes and washed by adding 2 ml of PBS and centrifugation at 300g for 10 minutes. Cells were resuspended in 200μL of PBS with 0.2%BSA and 0.2% sodium azide and acquired by flow cytometry within 4 hours. Multiparameter flow cytometry was used to acquire 10,000 events using a FACSCalibur flow cytometer and flow cytometric data was analysed using CellQuest (both BD Immunocytometry Systems, San Jose, CA).

The percentage of $CD34^+$ cells before MACS isolation (pre-sample), acquired by flow cytometry, and the MNC cell count were used to calculate the expected yield of $CD34^+$ cells in the CB sample. This value was used as quality control for the MACS procedure. The percentage of $CD34^+$ cells after MACS isolation, tested by analysing the post-sample by flow cytometry, was used to calculate the purity of the enriched sample (Figure 3.1). The mean purity of MACS $CD34^+$ cell separations used in this study was 91.3 ± 0.75% (n=62). A minimum of 80% purity was the cut-off for samples used in all experiments.

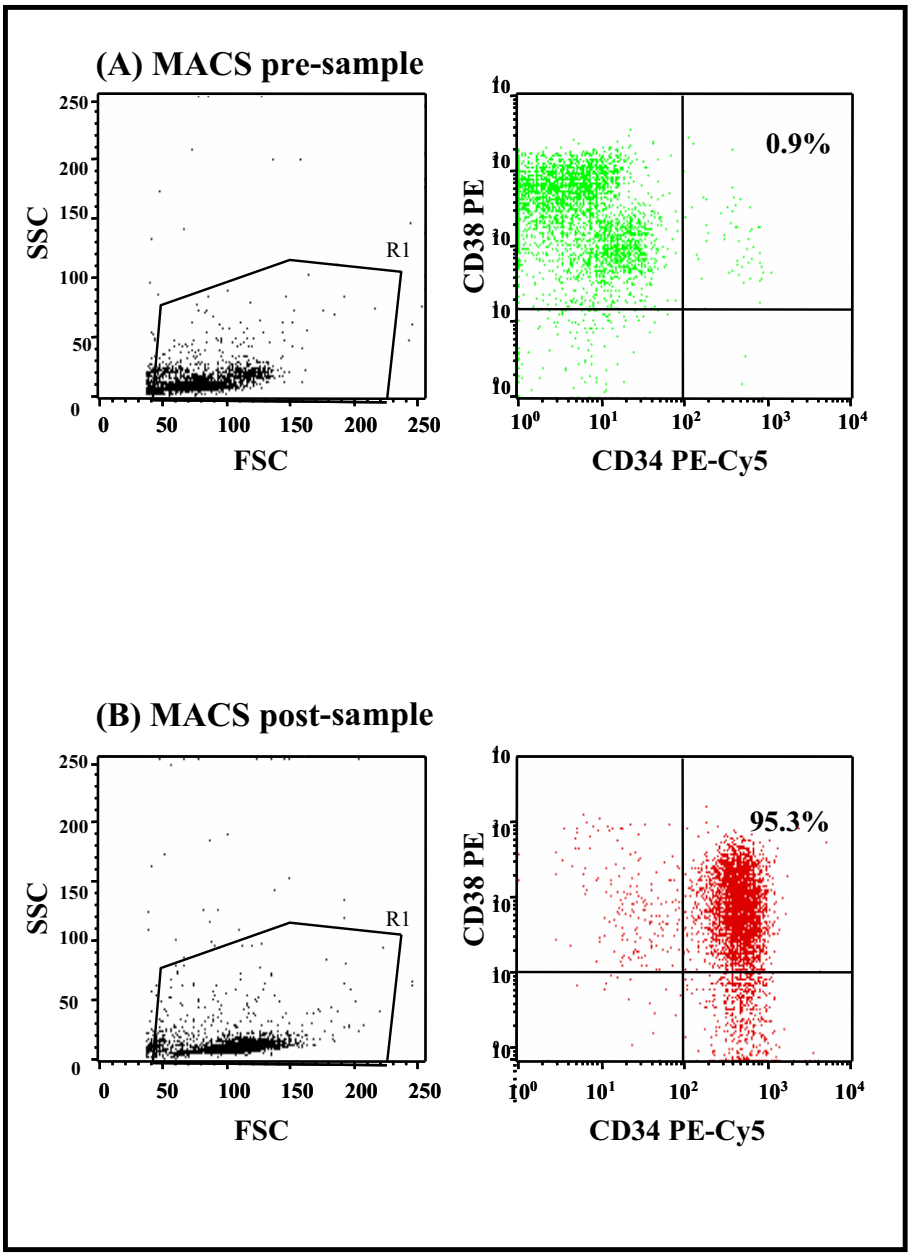

Figure 3.1 MACS separation of CB CD34+ cells

Analysis by flow cytometry of a CB sample prior to and following MACS separation. The expected yield of CB samples was estimated by us ing the percentage of $CD34^+$ cells in the pre -sample (A). The proportion of $CD34^+$ cells after MACS separation (B) was used to calculate the purity of the CB samples (Mean purity of post sample 91.3±0.75, n=62).

3.2.6 Cell tracking by flow cytometry

3.2.6.1 Cell tracking dye CFSE

The cell tracking fluorescent dye 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) was used in this study to investigate the division history of CB $CD34^+$ cells following cytokine-mediated expansion. Twenty five mg anhydrous CFSE (Molecular probes, Eugene, OR) was dissolved in DMSO to a final concentration of 10mM. This stock was stored at -20°C and thawed as required. For every experiment, a fresh working solution was made up to 10μM by diluting 5μL of 10mM CFSE in 5 ml PBS. The main stock lasted approximately nine months at -20°C before a decrease in fluorescence was noted by flow cytometry. It was observed that when reconstituting CFSE, the first working stocks were toxic to the cells. This was overcome by a number of freeze/thaw cycles of the main stock.

3.2.6.2 CFSE staining of CB $CD34^+$ cells

A number of dose finding experiments were undertaken to determine the optimal concentration of CFSE required for staining of CB $CD34^+$ cells. Staining with CFSE was performed as previously described by Nordon et al. (Nordon et al., 1997) with some modifications. In brief, $CD34^+$ cells were washed and resuspended in PBS. Cells were counted and the concentration of cells adjusted to $5x10^6$ cells/ml. Cells were incubated at 37°C for 5 minutes, stained with CFSE concentrations varying from 0.5μM to 2μM, then incubated at 37°C for 10 minutes in the dark. After incubation the cell suspension was diluted 1:10 with ice cold IMDM and 20% FCS and washed twice. Cells were

incubated overnight at 37°C in IMDM and 10%FCS to allow any residual unbound dye to diffuse out of the cells. The CFSE stained samples ($CFSE^+CD34^+$ cells) were examined by a FACSCalibur flow cytometer with excitation at 488 nm and using the FL1 detection channel as emission of CFSE was at 522 nm. Flow cytometric data was analysed using CellQuest program.

3.2.6.3Flow cytometric sorting of $CFSE^+CD34^+$ cells

$CFSE^+CD34^+$ cells were sorted to achieve high resolution cell tracking after cell culture (Nordon et al. 1997). Prior to sorting, CFSE stained cells were washed in PBS and labelled with 5µl of anti-CD34 PECy5 antibody per 10^6 cells. In some experiments, samples were also stained with 10µl per 10^6 cells of either CD38 PE or CD33 PE (Becton Dickinson, San Jose) and CD71 PE (Caltag, Burlingame, USA). Isotype controls, mouse IgG1PE and IgG1PECy5 were used to establish sorting regions. The samples were incubated at 4°C for 30 minutes, washed by adding 2 ml of PBS and centrifugation at 300g for 10 minutes. Three sort regions (R) were established to collect the desired cell population ($CD34^+CFSE^+$ cells) as shown in Figure 3.4 and Figure 3.6. The cells were sorted with a $FACStar^{plus}$ flow cytometer (a two-way sorter, BD Immunocytometry Systems, San Jose, USA). Sorts achieved >95% purity. $CFSE^+CD34^+$ cells were analysed using the FACSCalibur flow cytometer and CellQuest software.

3.2.7 Cytokines

All cytokines in this study were purified recombinant human (rh) cytokines and used at a final concentration of 20 ng/ml. rh SCF and rh MGDF were generous gifts from Amgen (Thousand Oaks, CA). rh FL was kindly provided by Immunex (Seattle, WA). rh IL-3 was a generous gift from Novartis (Basel, Switzerland).

3.2.8 Cytokine-mediated expansion of CB $CD34^+$ cells

Sorted $CFSE^+CD34^+$ cells at a concentration of $5x10^4$ - $1x10^5$ per ml were cultured for up to 7 days in serum free medium consisting of phenol red free IMDM, StemPro™-34 Nutrient supplement (Life Technologies), 2 mercaptoethanol (0.1 M), sodium pyruvate (100 mM), penicillin (100 U/ml), kanamycin (50 μg/ml) (both from Sigma, St Louis, USA) and SCF+MGDF+FL. For some experiments, IL-3 was added to the cytokine combination to examine its effect on phenotype and cell division. All cell cultures were maintained in a Sanyo MCO-17A1 CO2 incubator at 37°C in a humidified atmosphere in the presence of 5% CO2.

3.2.9 Immunophenotyping of sorted $CFSE^+CD34^+$ cells

The immunophenotype of CB $CD34^+$ cells was monitored by flow cytometry to asses any changes due to cell division following cytokine-mediated expansion. The following mouse anti-human monoclonal phycoerythrin (PE) conjugated antibodies were used: CD38, CD19, CD33 (Becton Dickinson, San Jose); CD71 (Caltag, Burlingame, USA); CD41 (Dako, Denmark); CD90 (Immunotech, Marseille, France) and CD61, CD117,

CD130, CXCR4, CD62L, CD44, CD31, CD29, CD49d, CD49e, CD11a, CD11b (Pharmigen, Becton Dickinson, San Diego, CA). To compare CD34 co-expression with other markers, CD34 phycoerythrin-cyanin 5 (PECy5) (Immunotech, France) was used in all combinations. Appropriate isotype-matched conjugated controls included mouse IgG1PE (Becton Dickinson, San Diego, CA) and IgG1PECy5 (Immunotech, France). Samples were adjusted to a concentration of 10^6 cells/ml and 10 μl of each antibody (for CD34PECy5 and CD71PE only 5μL) was added to approximately 10^5 cells in 4 ml FACS tubes. Samples were incubated at 4°C for 30 minutes and washed by adding 2 ml of PBS and centrifugation at 300 *g* for 10 minutes. Cells were resuspended in 200 μl of PBS with 0.2%BSA and 0.2% sodium azide and acquired by flow cytometry within 4 hours. Multiparameter flow cytometry was used to acquire 10,000 events using the FACSCalibur flow cytometer and analysed using CellQuest software. The cell surface marker expression was analysed by calculating the percentage of positive expression of the antigen (*x*) in question. The mean expression of antigen *x* was divided by the mean expression of the appropriate isotype control.

3.2.10 Statistical Analysis

Results were expressed as mean ± SEM. Where applicable one-way ANOVA was used to assess statistical significance, otherwise student *t*-test was used to evaluate statistical significance. Statistical analysis was performed using Graph Prism software. A p-value ≤ 0.05 was considered statistically significant.

3.2.10.1 Analysis of division tracking data

In order to analyse the cell tracking data, the following values were calculated as described by Nordon et al. (1999) from histogram data acquired in experiments.

Progenitor number As a cell (a progenitor) divides, it gives rise to two daughter cells, its progeny. Hence, progenitors are the precursors that generate progeny during a cell tracking experiment. The number of progenitors can be calculated by dividing the number of cells in generation ***i*** by 2^{i-1}, where undivided cells are the first generation. The progenitor number producing cells in the *i*th generation at time t, P*i*(t) is defined as:

$$\boldsymbol{P}i\,(\mathrm{t}) = \frac{\boldsymbol{N}i\,(\mathrm{t})}{\boldsymbol{2}^{i-1}}$$

where $\boldsymbol{N}i(\mathrm{t})$ is the number of cells in generation ***i*** at time *t*.

Proliferation Index (*PI*) is defined as a measure of the proliferative process independent of cell death and is calculated by dividing the sum of the number of cells in all generations by the sum of all progenitors $\boldsymbol{P}_i$, at time point *t*.

$$PI = \frac{\sum \boldsymbol{N}_j(\mathrm{t})}{\sum \boldsymbol{P}_j(\mathrm{t})} = \frac{\text{progeny }_j}{\text{progenitors }_j}$$

Frequency distribution of progenitors across consecutive generations is calculated by dividing the number of progenitors in each generation by the total number of progenitors at time *t*.

$$f_i\,(\mathrm{t}) = \frac{\boldsymbol{P}_i\,(\mathrm{t})}{\sum \boldsymbol{P}_i(\mathrm{t})_{\,i}}$$

The *mean generation number* or average number of divisions at time point t is calculated by multiplying each generation *i* by its frequency f_i *(t)* and adding all the generations at time point *t*.

$$m\,(\mathrm{t}) = \sum_i f_i\,(\mathrm{t})$$

3.3 RESULTS

3.3.1 Optimisation of high resolution cell tracking technique

3.3.1.1 Titration of CFSE staining of CB CD34$^+$ cells

A number of CFSE concentrations were tested on CB CD34$^+$ cells to achieve high fluorescence intensity and low toxicity. The viability of the cells was determined by their ability to exclude trypan blue. Cells were stained with CFSE concentrations varying from 0.5μM to 2μM (Figure 3.2). When lower concentrations (0.5 to 1μM) were used, CFSE fluorescence decreased close to background fluorescence within the timeline of the experiment (up to 7 days) and resolution of divisions by flow cytometry was difficult.

The optimal concentration for CFSE cell tracking was 1.5μM. In later experiments when another stock of CFSE was used the dye required titration again and the new optimal concentration was 2μM. The optimal concentrations of CFSE were not toxic to the cells when compared to unstained controls as examined by trypan blue exclusion.

3.3.1.2 Loss of CFSE fluorescence with culture

CFSE fluorescence decreases with culture due to dye leaching and it can be noticed as a shift to the left on the CFSE histogram. Hence to determine the new shifted position of

the undivided cells, $CFSE^{+}CD34^{+}$ cells were cultured up to 3 days with and without cytokines (SCF, MGDF, FL and IL-3) and analysed every day by FACS. Since $CFSE^{+}CD34^{+}$ cells cultured in the absence of cytokines did not divide, they were used as an experimental control. The decrease in fluorescence due to leaching was readily determined and the position of the undivided population after cytokine stimulation was confirmed by overlaying on the CFSE histogram the control and the cytokine cultured sample (Figure 3.3).

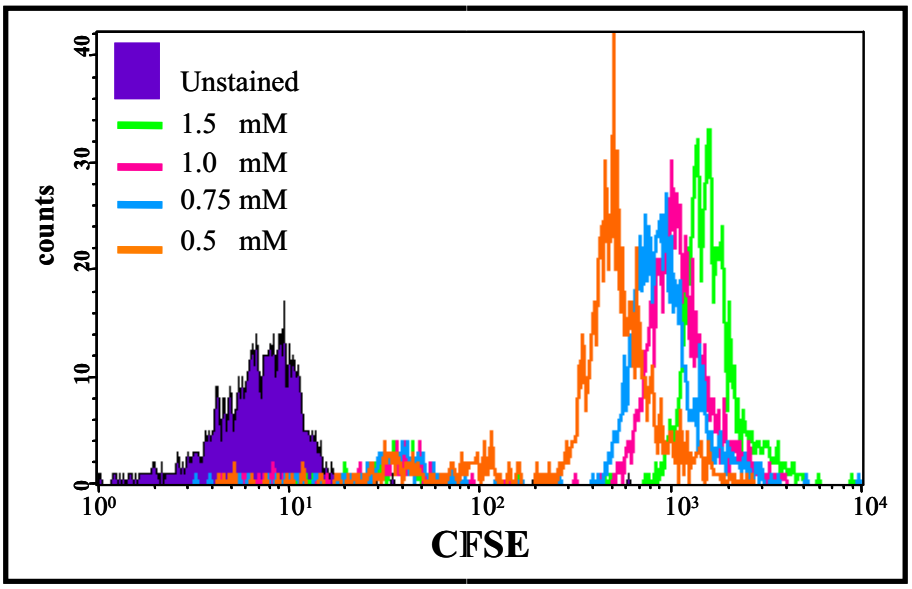

Figure 3.2 Titration of CFSE staining. MACS isolated $CD34^{+}$ cells were stained with 0.5 to 1.5μM CFSE (overlays) to examine optimal staining conditions. Solid histogram depicts unstained $CD34^{+}$ cells.

3.3.1.3 Optimisation of high resolution cell tracking by sorting of CFSE$^+$CD34$^+$ cells

As cells undergo numerous cell divisions, CFSE fluorescence approaches background fluorescence and there is a decrease of resolution between divisional clusters. To achieve a clear separation of each cell division after cytokine expansion, cell sorting was performed on CB CFSE$^+$CD34$^+$ cells. Based on previous studies (Nordon et al., 1997) the sorting window used was 36-40 channels wide in the centre of the CFSE fluorescence peak using a 1024 channel log amplifier (Figure 3.4 B). This sorting strategy selected a more homogenous CFSE stained population compared to unsorted cells and increased the cell tracking resolution of CB CD34$^+$ cells expanded in culture as divisional clusters were clearly visible.

3.3.1.4 Limit of high resolution cell tracking by the length of cytokine culture

In order to accurately determine the changes in phenotype and function as a consequence of cytokine-mediated expansion then it is essential to show high resolution cell division. Hence we designed an experiment whereby sorted CFSE$^+$CD34$^+$ cells were incubated with SCF+MGDF+FL+IL-3 and cultured for up to 7 days, samples were taken daily for flow cytometric analysis. Results indicated that high resolution of divided cell populations could be accurately identified after up to 3 days culture. By day 5 of culture, fluorescence in the later divisions was reduced to background, making it difficult to resolve the populations of divided cells. For this reason, cells were cultured for no longer than 3 days whenever they were stained with CFSE (Figure 3.5).

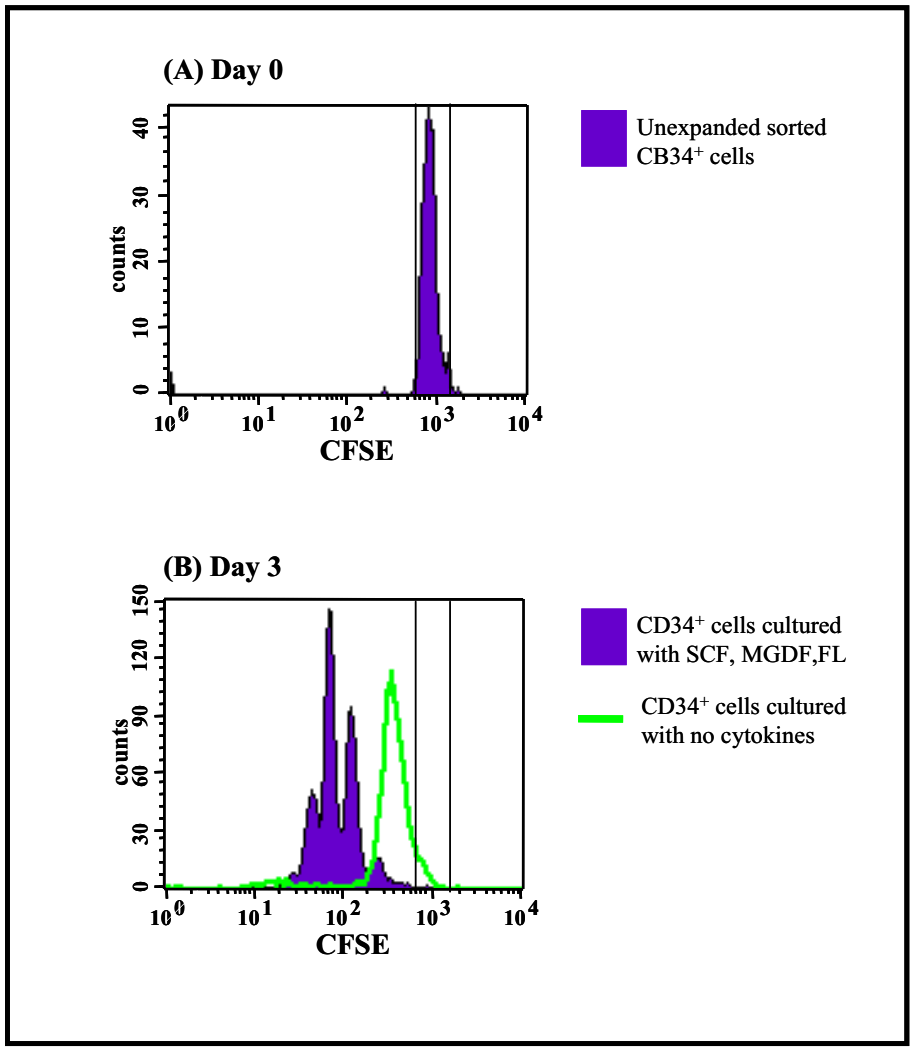

Figure 3.3 CFSE expression of CB CD34+ cells cultured with no cytokines overlaps CFSE expression of undivided cells from cultured samples

CFSE stained CB $CD34^+$ were sorted on day 0 for a narrow gate centre of the CFSE peak (a) and cultured for 3 days with and without cytokines (b). The region between the black lines represents CFSE expression of CB CD34+ cells prior to culture. The shift in CFSE expression of undivided cells due to loss in fluorescence (green line) overlaps the cell population cultured without cytokines.

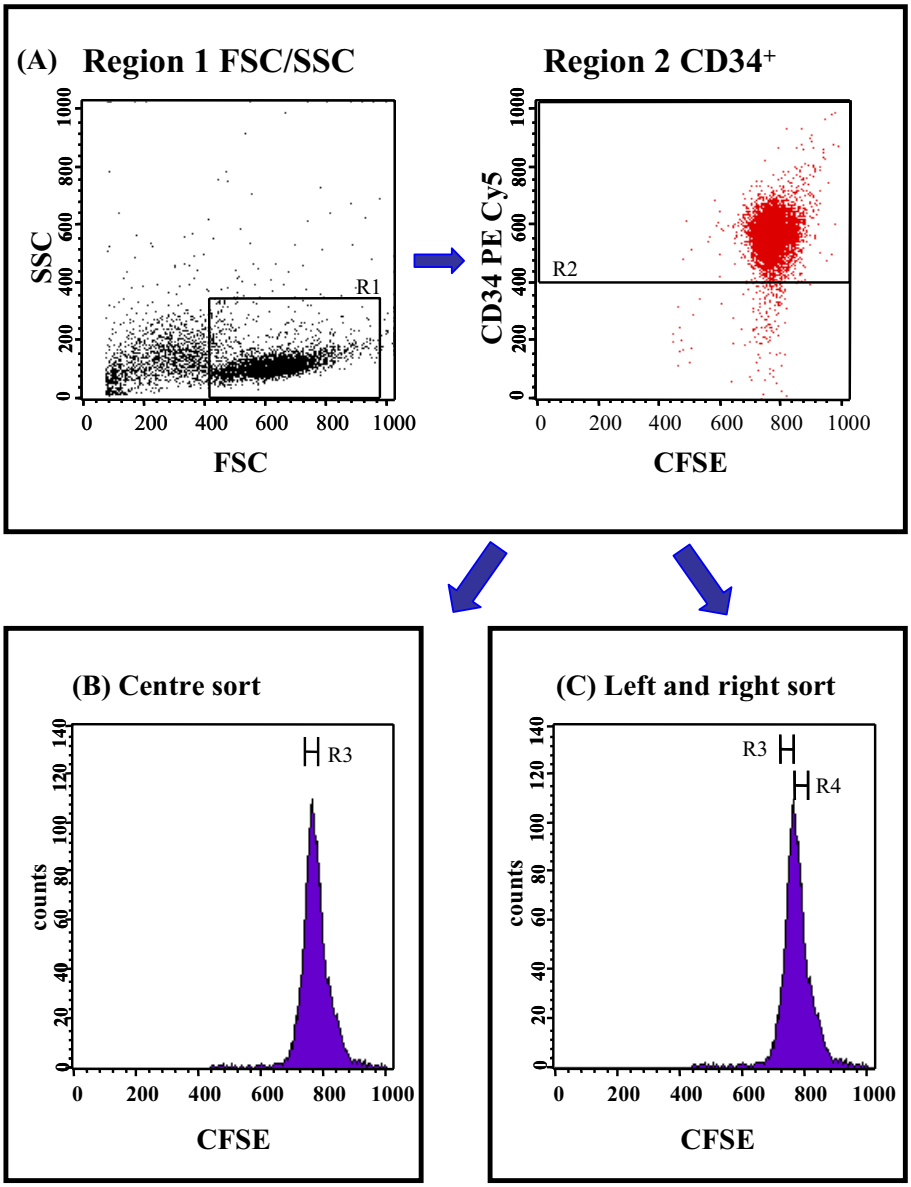

Figure 3.4 Sorting subpopulations of CFSE stained cells

CB CD34+ CFSE stained cells were sorted to increase the resolution of divisions after culture. R1 included cells with low light forward scatter and side scatter ; R2 included CD34+ cells positive and R3, cells within a narrow gate, centre of the CFSE peak. The width of the CFSE gate was 36-40 channels on a 1024 channel log amplifier.

3.3.1.5 Enrichment of primitive HSC from CD34$^+$ cells to increase the homogeneity of the unexpanded population

CB samples enriched for CD34$^+$ cells by MACS separation were used as the starting population for the experiments described above. However this population has been described in the literature as heterogenous and other populations such as CD34$^+$CD38$^-$ and CD34$^+$Lin$^-$ have been shown to be enriched for a more primitive population (section 2.4). However, two major limitations in this study were the cell numbers available per CB sample and the availability of CB samples. Hence, it was important to calculate the incurred loss of yield if the sorting strategy involved sorting a subpopulation of CD34$^+$ cells to enrich the CB sample and then sorting a subpopulation of CFSE stained cells to improve resolution of cell tracking (section 3.3.1.3). Hence experiments were designed to sort CB CD34$^+$ cells for a narrow gate of CFSE and either the CD34$^+$CD38$^-$ population (sort #1) or the lineage negative (CD34$^+$CD71$^-$CD33$^-$) population (sort #2; for sorting gates see Fig. 3.6). The sort described in section 3.3.1.3, that is CB CD34$^+$ sorted for a narrow region centre of CFSE peak was also performed (sort# 3) to compare the difference in yield from all 3 sorts.

The results were discouraging as sorting to further enrich the CD34$^+$ population greatly decreased the number of cells in the sorted population. When comparing only the enrichment of the samples, the CD34$^+$CD38$^-$ fraction was only 17.5% of the original CD34$^+$ sample and the lineage negative fraction represented 23.2% of the total CD34$^+$ population. The yield of sort #1 was less than 1/10 of the control sort (#3). The yield of sort #2 was only 15.6% of the yield acquired from population in sort #3. Hence, the loss of sample due to enrichment of the CD34$^+$ population was too high to pursue in the

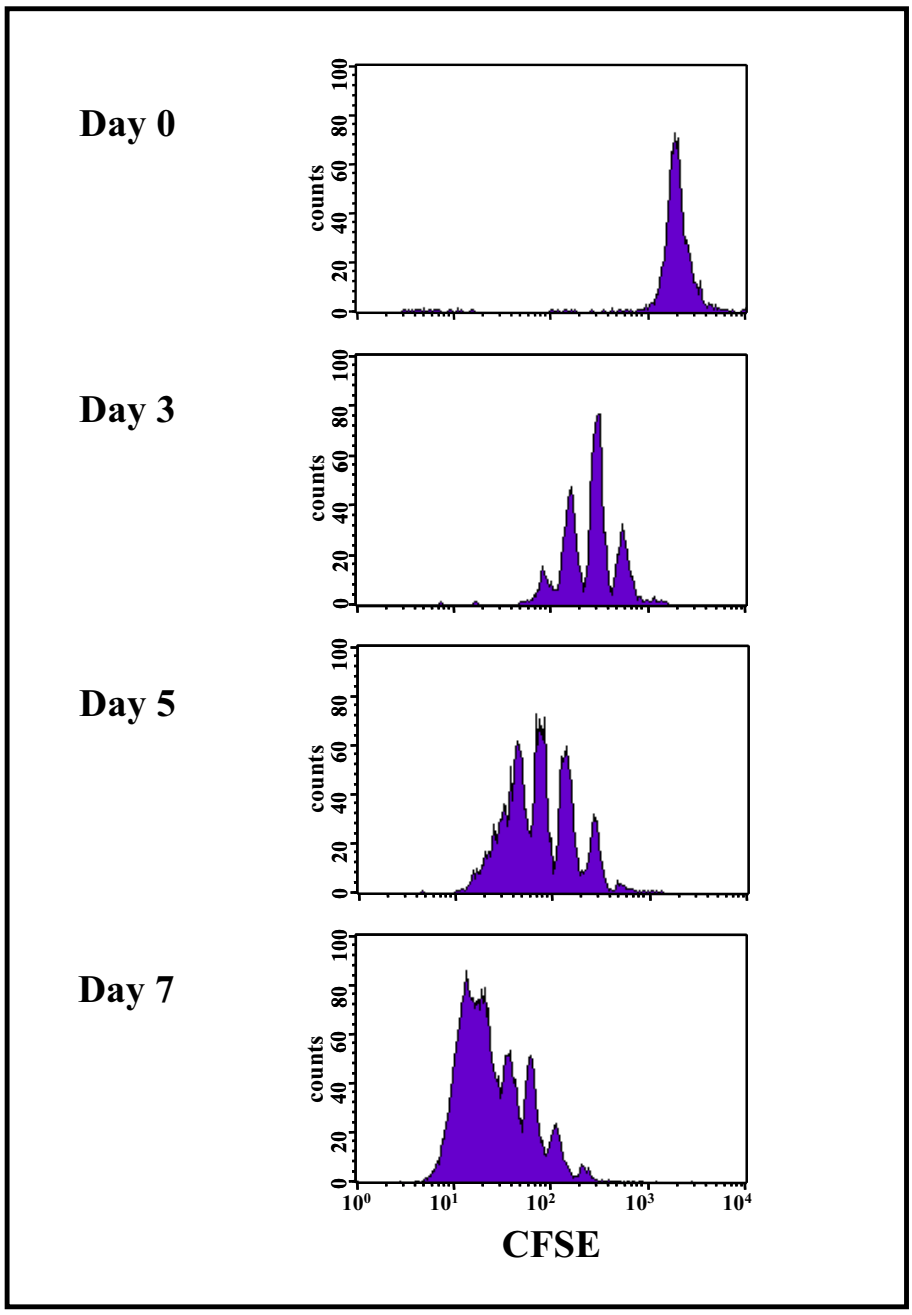

Figure 3.5 The resolution of cell divisions diminishes with prolonged culture
CB $CD34^+$ cells stained with 1.5mM CFSE were sorted for 36 channels centre of the CFSE peak and tracked by flow cytometry up to 7 days in culture with SCF, MGDF, FL and IL3. Optimal resolution of divisions decreases by 5 days of culture.

current study. Hence for all experiments the starting population or unexpanded population of the CB samples was $CD34^+$ cells.

3.3.2 Characterisation of the $CFSE^+$ sorted $CD34^+$ population

Once it was determined that the optimal duration for high resolution cell tracking using CFSE of CB $CD34^+$ cultured with cytokines (SCF, MGDF, FL and IL-3) was 3 days, the sorting strategy was examined further in subsequent experiments. The sorting strategy was tested to ensure that the sorted population was representative of the original heterogenous CB $CD34^+$ sample. Hence, the populations either side of the sorted population described in section 3.3.1.3 were tested. $CFSE^+CD34^+$ cells were sorted 36 channels wide (in a 1024 channel log amplifier) to the left and right of the centre of the CFSE fluorescence peak (Figure 3.4 B) and the respective populations cultured for 3 days with SCF, MGDF, FL and IL-3. In later experiments, the sorted $CFSE^+CD34^+$ populations were cultured with and without IL-3 in combination with the cytokine cocktail (SCF, MGDF and FL) to investigate its effect on proliferation.

Following cytokine-mediated expansion with all four cytokines, the two sorted populations differed as cells sorted to the right of the peak (R) underwent more cell divisions. The mean generation number (the average number of divisions) after 3 days of cytokine exposure was 3.80±0.25 (n=3) for R sorted cells and 3.22±0.26 (n=2) for L sorted cells as shown on Table 3.1. The mean proliferation index (PI) was 9.61±1.94 (n=3) for R sorted cells and 5.68±0.84 (n=2) for L sorted cells (Table 3.1).

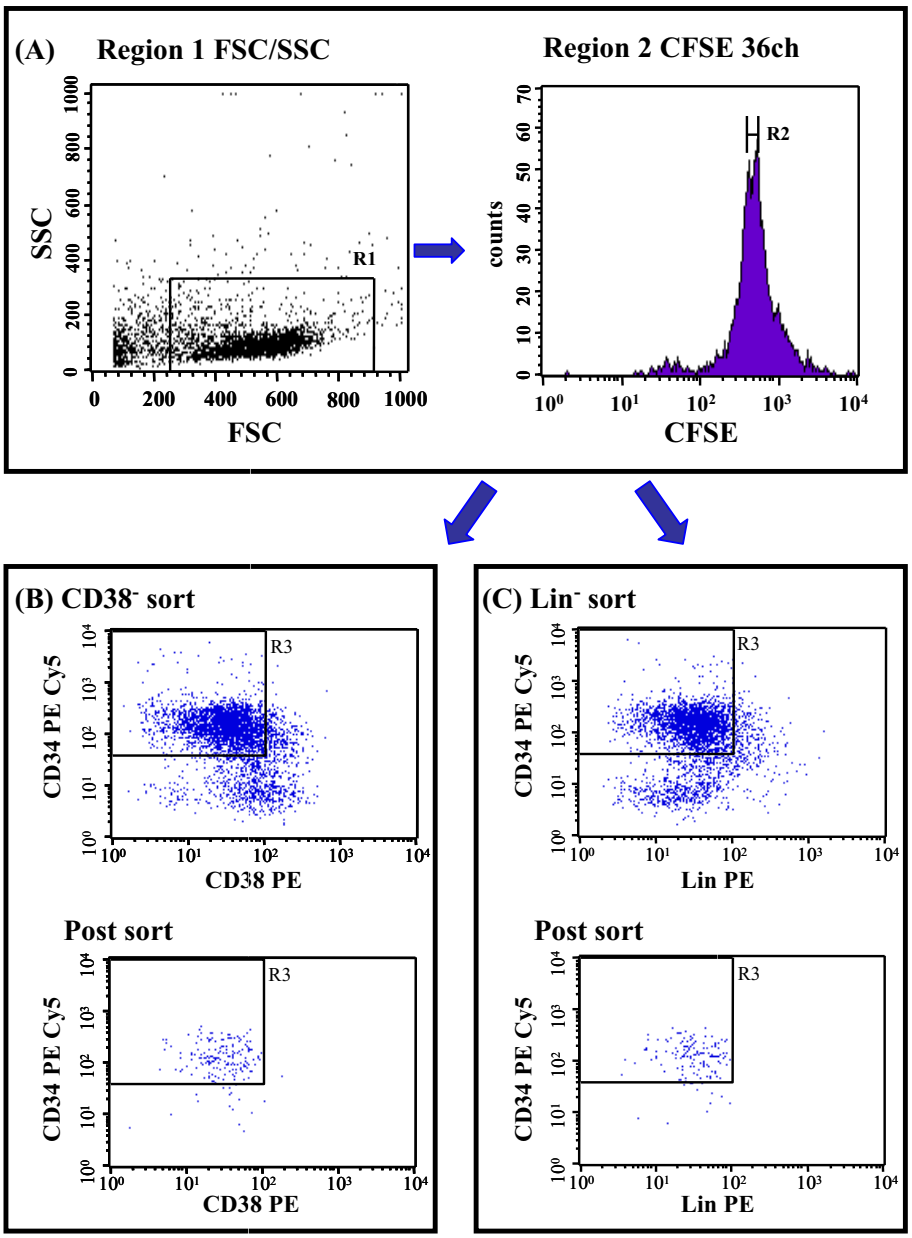

Figure 3.6 Sorting of subpopulations to enrich CD34$^+$ population

The feasibility of using enriched subpopulations of CFSE$^+$CD34$^+$ cells as a starting population for later experiments was tested. MACS isolated CFSE$^+$CD34$^+$ cells were sorted for (A) viable cells (region 1), CFSE 36 channels in the centre of the peak (region 2) and either (B) CD34$^+$ and CD38$^-$ or (C) CD34$^+$ and CD33$^-$/CD71$^-$ (Lin$^-$). Region 3 was set according to IgG1 PE and IgG PE Cy5 isotype controls

3.3.3 Effect of IL-3 on the proliferation of $CFSE^{+}CD34^{+}$ cells

When IL-3 was removed from the cytokine combination, the mean generation number decreased in both L and R sorted population as seen in Figure 3.7. R was 3.32±0.25 (n=2) and L was 3.00±0.15 (n=2). The PI was also affected with the lowest value in the L sorted population, 4.76±0.71 (n=2). The R population had a PI value of 6.32±1.30 (n=2). Hence, the proliferation of CB $CD34^{+}$ cells decreased when IL-3 was removed from the cytokine cocktail (Table 3.1; Fig. 3.7).

The changes in proliferation between the L and R sorted populations and by the addition of IL-3 were not statistically significant as analysed using one-way ANOVA (PI p = 0.2251; mean generation number p=0.2038). However, these populations were further examined as it was still important to characterise whether the differences in proliferation may have an impact on the phenotype of the sorted populations following cytokine culture with and without IL-3.

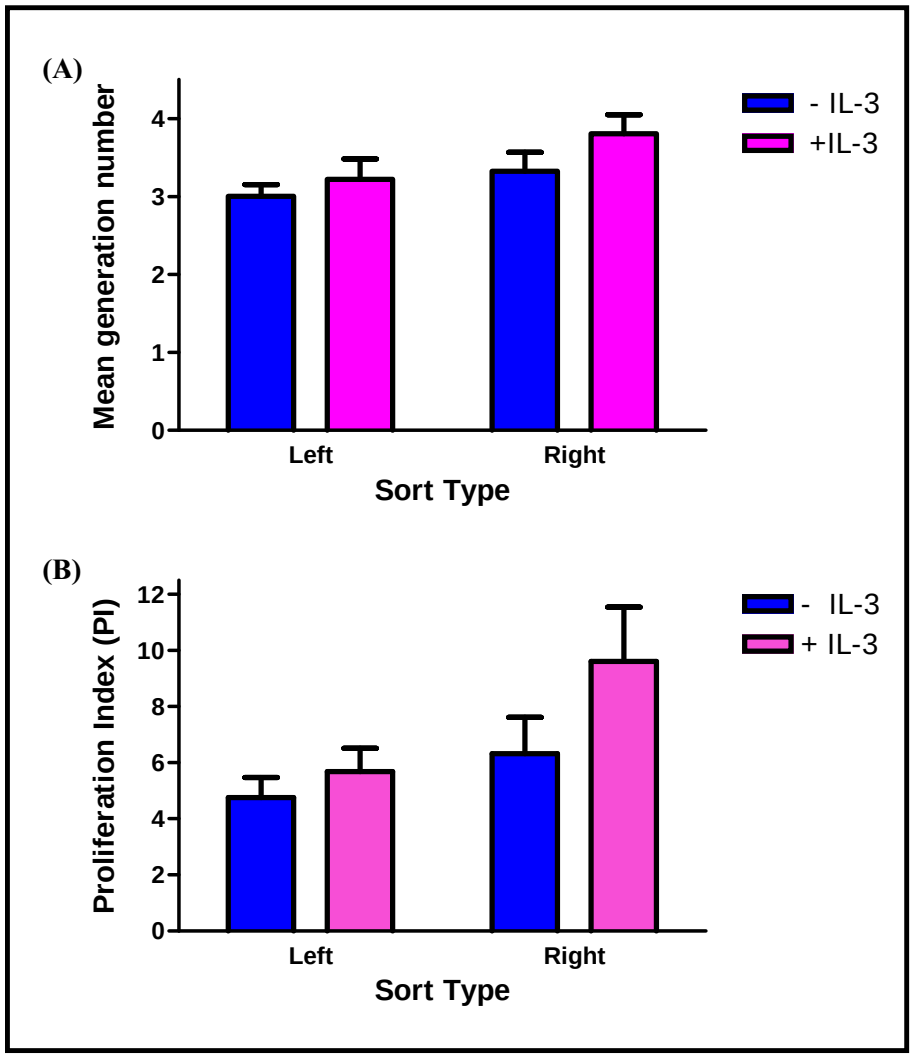

Figure 3.7 Kinetics analysis of divisional tracking

(A) Mean Generation Number and (B) proliferation index calculated for CFSE+CD34+ cells sorted left (L) and right (R) of the centre of CFSE peak and cultured for 3 days with SCF, MGDF, FL ± IL-3 (Means ± SEM).

Table 3.1 Mean generation number and Proliferation Index of $CFSE^+CD34^+$ subpopulations sorted to the left (L) or to the right (R) of the CFSE peak and cultured for 3 days with cytokines SCF, MGDF and FL with (+) and without (-) IL-3.

Sorted population	Mean generation number	Proliferation Index (PI)
L+ (n=2)	3.22±0.26	5.68±0.84
R+ (n=3)	3.80±0.25	9.61±1.94
L- (n=2)	3.00±0.15	4.76±0.71
R- (n=2)	3.32±0.25	6.32±1.30

Values shown are mean ± SEM

3.3.4 Characterisation of the immunophenotype of sorted $CFSE^+CD34^+$ following cytokine-mediated expansion

In order to examine the changes in phenotype following cytokine-mediated expansion of CB $CD34^+$ cells, cell surface marker expression was investigated as a function of cell division history.

Unexpanded $CFSE^+CD34^+$ cells were sorted 36 channels wide (in a 1024 channel log amplifier) to the left and right of the centre of the CFSE fluorescence peak (Figure 3.4 C) and the populations were cultured for 3 days in the presence of SCF, MGDF, FL ± IL-3. This experiment was performed on 3 independent pooled CB samples. The cell surface markers studied involved putative stem cell markers (CD34/Thy-1), lineage

specific markers (CD38/CD19/CD33/CD41/CD71/CD61) and growth factor receptors (CD117- c-kit receptor and CD130-IL-6 receptor associated). Expression of these markers was characterized with respect to divisional history (Fig. 3.8 and 3.9).

3.3.4.1 Comparison of phenotype between R and L sorted populations

The phenotype of L and R sorted populations (described in 3.3.2) was examined after 3 days of culture with SCF, MGDF, FL ± IL-3 (Fig. 3.8 and 3.9). In Table 3.2, the peak fluorescence intensity (PF) of each marker was compared between the 4 populations on day 3: (i) L sort +IL-3; (ii) R sort +IL-3; (iii) L sort –IL-3 and (iv) R sort –IL-3 . The L sorted population without IL-3 (iii) in its cytokine cocktail had the highest PF for CD34 (253.0) and the lowest PF for CD33 (78.7), CD117 (23.9), and megakaryocyte markers CD41 (47.1) and CD61 (42.5) out of the four groups. In the L sorted population with IL-3 (i), the PF of c-kit receptor (CD117) was highest and the PF of CD38 and CD71 was lowest. The R sorted population with IL-3 included (ii) had the highest PF for lineage markers CD38, CD33, CD71 and CD61 and second highest PF for CD41 (highest in R sort – IL-3).

Table 3.2 Peak fluorescence intensity (PF) of cell surface markers in $CFSE^{+}CD34^{+}$ subpopulations sorted to the left (L) or to the right (R) of the CFSE peak and expanded in culture for 3 days with cytokines SCF, MGDF and FL with (+) and without (-) IL-3.

	Sorted $CFSE^{+}CD34^{+}$ population			
	L +	R+	L-	R-
CD34	184.5	226.2	253.0	170.3
CD38	159.1	227.6	173.9	174.5
CD33	92.3	120.3	78.7	87.8
CD71	216.4	437.2	254.0	381.6
CD117	38.9	34.3	23.9	35.0
CD41	49.2	110.8	47.1	135.8
CD61	116.0	132.3	42.5	88.1

Next the changes in PF of surface markers were examined in relation to cell division in the 4 sorted populations (Fig.3.8). CD34 PF decreased in all 4 sorted populations (i-iv) with increased cell division whereas CD33 and CD71 gradually increased in all 4 populations with cell division (Fig. 3.8). Figures 3.8 A and B show that in the L and R sorted populations cultured in the presence of IL-3 (i-ii), CD41 and CD61 PF increased between undivided and divided populations. The PF of CD38 fluctuated as cells underwent cell division. CD117 PF in all populations (i-iv) and CD61 (Fig 3.9 C-D) in the populations cultured without IL-3 (iii-iv) did not change greatly with cell division.

The phenotype data was also analysed as percentage of cells expressing each marker to examine more closely the proportion of the population expressing each marker and its

relationship to divisional history. CD34 expression (Fig. 3.9) was down-regulated in all sorted population (i-iv) between cells that have divided once (83.6±2.30%) and cells that had undergone a total of 4 divisions [41.37%; population (iii)] or a total of 5 divisions [16.5±3.28%; populations (i-ii) and (iv)]. Interestingly, CD34 expression was lower in undivided cells compared to divided cells in all four populations (i-iv).

The expression of all lineage specific markers (CD38, CD33, CD71, CD41 and CD61) and of the c-kit receptor was upregulated with increased cell division, with the exception of CD19 and CD130 which were below the sensitivity of detection (Fig. 3.9). CD38 expression was upregulated as cells underwent cell divisions in all investigated populations (i-iv), and its maximum expression occurred after cells had undergone 3 divisions (90.09±1.21%) in all populations except for R sorted cells cultured without IL-3 where CD38 peaked after 2 divisions (89.97%).

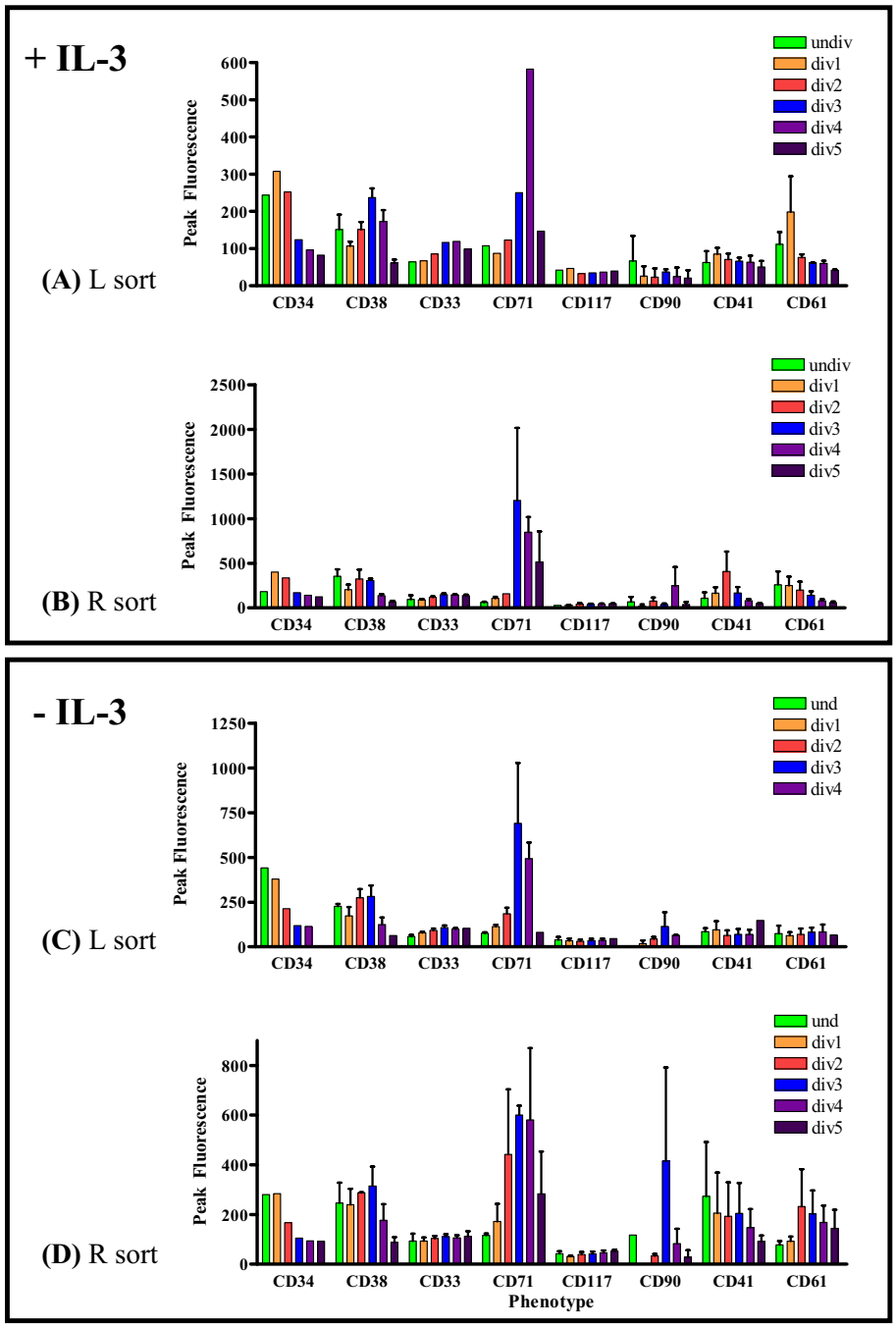

Figure 3.8 Expression of cell surface markers for CB CD34+ cells
Graphs showing peak fluorescence per marker within each division for CFSE+CD34+ cells sorted left (L) and right (R) of the centre of CFSE peak. The samples were cultured for 3 days with SCF, MGDF, FL ± IL-3

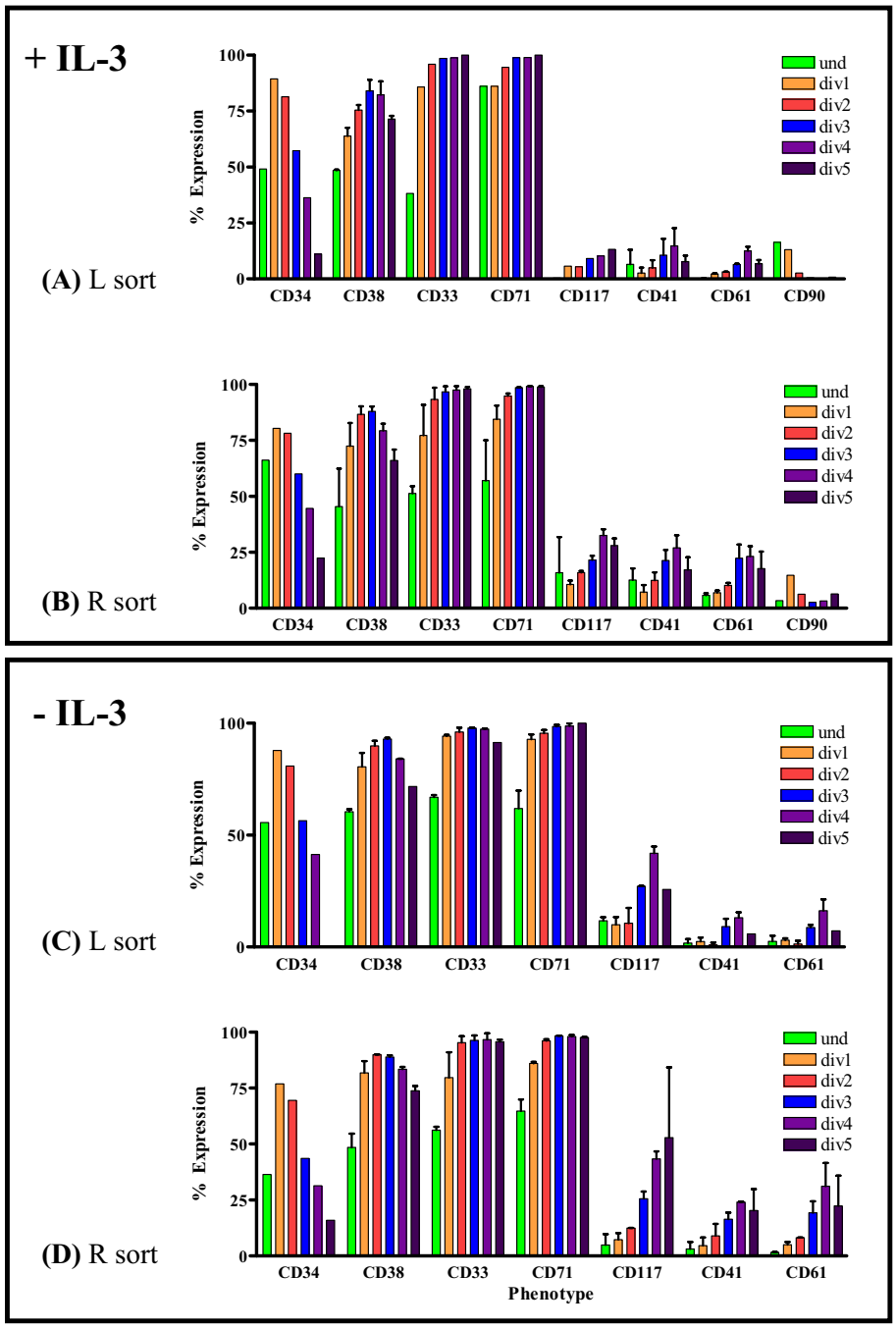

Figure 3.9

Graphs showing percentage of expression per marker within each division for CFSE+CD34+ cells sorted left (L) and right (R) of the centre of CFSE peak. The samples were cultured for 3 days with SCF, MGDF, FL ± IL-3

3.3.4.2 Phenotype of centre sorted populations without IL-3 and cultured up to 7 days

In a later experiment, the $CFSE^{+}CD34^{+}$ population was sorted for 36-40 channels in the centre of the CFSE peak and cultured for 7 days with SCF, MGDF and FL. As previously shown, extension of culture to 7 days with cytokines causes the resolution of CFSE divisions to decrease (Fig. 3.10) but the length of culture was necessary to correlate the phenotypic results to studies described in Chapter 4. Hence, after 7 days of expansion the phenotype was analysed for the population as a whole without investigating divisional history.

Expression of CD34 (Fig. 3.10 A and 3.11) and Thy 1 expression (Fig.3.11) were down-regulated in successive divisions by day 3 and further downregulated in the whole population after 7 days of culture. CD33, an early myeloid marker increased throughout expansion at a minimal level. CD38 expression fluctuated over time.

At day 3 of culture, lineage markers such as erythroid marker CD71 and megakaryocytes markers CD61/CD41 (αIIbβ3 integrin; Fig 3.10 A.) were upregulated with increasing division number. C-kit expression increased in the first division, but was down-regulated thereafter. By day 7 of culture, CD71, CD61, CD41 and c-kit receptor were all upregulated following cytokine expansion with SCF, MGDF and FL (Fig. 3.11).

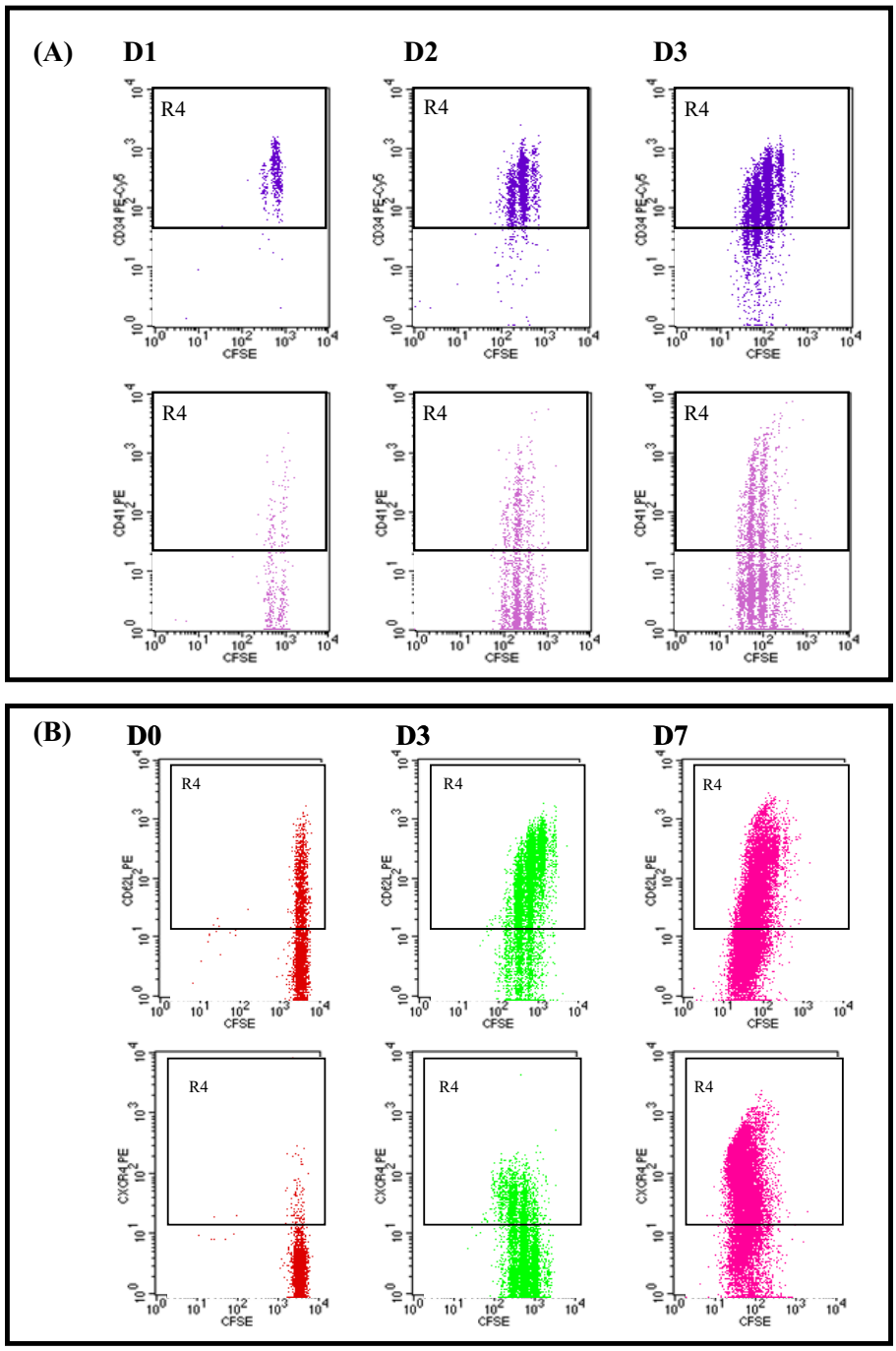

Figure 3.10

Representative plots showing comparison of expression changes concomitant to CFSE between markers over time (A) CD34, CD41 expression (up to 3 days in culture) and (B) CD62L and CXCR4 (up to 7 days in culture)

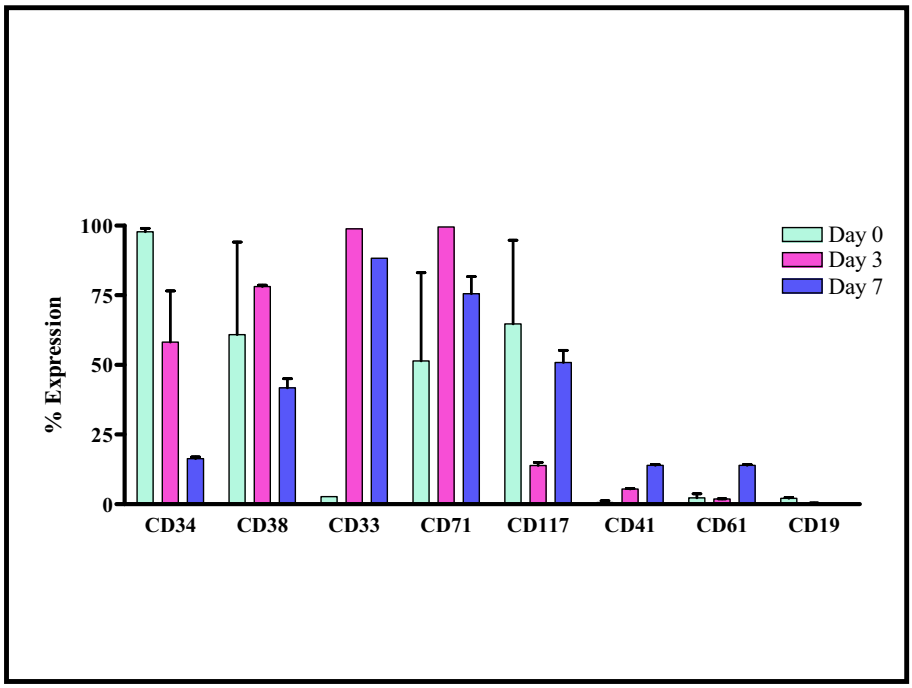

Figure 3. 11 Surface marker expression in CB CD34+ cells
Graph showing percentage of expression per marker for CFSE+CD34+ cells sorted for the centre of CFSE peak. The samples were cultured for 7 days with SCF, MGDF, FL ± IL-3

3.3.4.3 Adhesion molecule expression in centre sorted populations and cultured up to 7 days with SCF, MGDF and FL

The expression of chemokine receptor CXCR4 and adhesion molecules involved in transendothelial migration and migration within the marrow cavity (CD62L, CD44, CD31) and lodgement within the marrow microenvironment (CD29, CD49d, CD49e, CD11a, CD11b) were also characterized with respect to divisional history on day 3 and the expression of the whole population on day 0, 3 and 7 (Table 3.3; Fig. 3.12) following cytokine-mediated expansion with SCF, MGDF and FL. $CFSE^{+}CD34^{+}$ cells were sorted on day 0 for a narrow gate (36-40 channels) on the centre on the CFSE peak.

3.3.4.3.1 Adhesion molecule expression of $CFSE^{+}CD34^{+}$ cells correlated to cell division after 3 days culture

Figure 3.12 A shows expression of adhesion molecules concomitant with cell division following 3 days of culture with SCF, MGDF and FL. L selectin (Fig. 3.10 B) overall expression was upregulated with 3 days expansion, but its expression decreased in later divisions. The expression of β1 intergrins VLA-4 (CD49d), VLA-5 (CD49e) and LFA-1 (CD11a) expression was upregulated in all undivided and divided cells after 3 days expansion. The expression of CD44 and PECAM-1 (CD31) was upregulated in all undivided and divided cells after 3 days expansion. β1 intergrin (CD29) and MAC-1 (CD11b) expression was not upregulated by day 3. The expression of the chemokine receptor CXCR4 increased with cell division and all cells were positive in divisions 4 and 5, but its expression was not upregulated in undivided cells (Fig. 3.10 B).

3.3.4.3.2 Adhesion molecule expression of $CFSE^{+}CD34^{+}$ cells after 7 days culture

L selectin (CD62L) overall expression was upregulated from 34.3±1.08 (n=2) on day 0 to 82.8±1.44 (n=2) following 3 days of cytokine-mediated expansion (Table 3.3; Fig. 3.12 B). However, at 7 days of culture, CD62L was down-regulated (45.4±1.93, n=2) but its level of expression remained higher than seen on unexpanded cells. Expression in unexpanded cells of CD49d (80.7±7.95%, n=3) and CD11a (74.59±2.1, n=2) was increased with expansion by 7 days (99.5±0.5% and 98.7±0.63%, n=2 respectively). CD49e expression was also high in unexpanded cells (80.4±2.97%, n=3) and further up-regulated in expanded cells (97.99±0.81%, n=2). CD44 and CD31 expression was very high in both unexpanded (99.43±0.2%, n=2 and 98.93±0.17%, n=2) and expanded cells

(99.5±0.5%, n=2). β1 intergrin and MAC-1 expression was induced after 7 days expansion (10.39±1.89%, n=2 and 23.02±1.38%, n=2 respectively). CXCR4 expression was only 4.35±1.81% (n=3) day 0 but was induced with cytokine culture and upregulated to 66.27±1.75% (n=2) after 7 days.

Table 3.3 Expression (%) of adhesion molecules in centre sorted $CFSE^{+}CD34^{+}$ cells and expanded in culture up to 7 days with cytokines SCF, MGDF and FL.

	Day 0	Day 3	Day 7
CD62L	34.31±1.08	82.80±1.44	45.40±1.92
CD29	0.09±0.09	0.04±0.04	10.39±1.89
CD31	98.93±0.17	99.26±0.44	97.25±0.85
CXCR4	4.35±1.81	22.53±1.57	66.27±1.75
CD11a	74.59±2.10	99.01±0.41	98.70±0.63
CD11b	0.46±0.11	0.27±0.17	23.02±1.38
CD49d	80.67±7.95	99.39±0.28	99.46±0.50
CD49e	80.40±2.96	99.11±0.38	97.99±0.81
CD44	99.43±0.19	99.44±0.49	99.50±0.48

Values shown are mean ± SEM

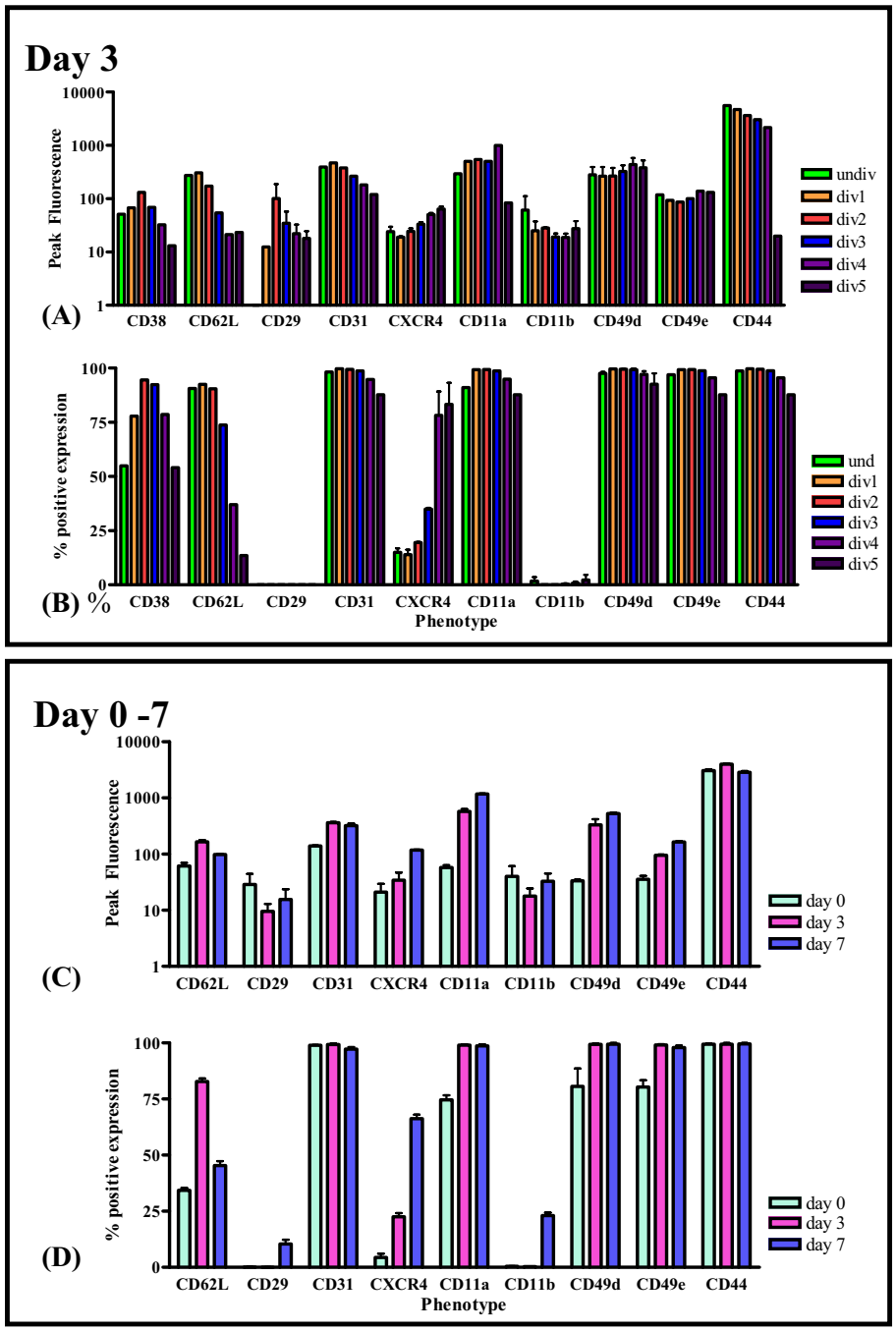

Figure 3. 12 Adhesion molecules expression in CB CD34+ cells

Expression (%) per marker within each division (A-B) and as a whole population (C-D) for CFSE+CD34+ cells for a narrow peak on the centre of CFSE peak. The samples were cultured for 7 days with SCF, MGDF, FL ± IL-3

3.4 DISCUSSION

3.4.1 High resolution cell tracking of CB CD34$^+$ cells following cytokine-mediated expansion

In this part of the study, a high resolution cell tracking technique was optimised that allows accurate separation and clear identification of cell division of CB CD34$^+$ cells over 3 days of cytokine culture. The technique using the intracellular CFSE dye enabled us to investigate the changes in cell proliferation and phenotype in CB CD34$^+$ cells following cytokine-mediated expansion and can be used combined with other functional assays as described in Chapter 5.

High resolution of divisional clusters of CB CD34$^+$ cells after cytokine expansion was achieved through cell sorting. However, the CFSE sorting strategy was examined to test the bias of the sort and investigate whether the CFSE sorted subpopulation (narrow centre sort) was representative of the initial population (MACS enriched CD34$^+$ cells) and if it was discriminating against any cell type. Hence two subpopulations either side of the centre of the CFSE peak were examined as described earlier. Differences were observed between the two populations (L and R sorted) where the R sorted cells had a higher proliferation index and a higher mean generation number. This finding pointed to a difference in proliferation related to the intensity of CFSE staining and possibly a more quiescent phenotype for the L sorted population compared to the R sorted population. Hence, the phenotype was investigated to further characterise the two sorted populations.

Even though the starting population was the same in both L and R sorted fractions in terms of $CD34^+$ expression, CFSE staining intensity separated distinct populations with different phenotype and proliferation abilities. The phenotype of L and R sorted populations differed with R sorted cells displaying a more mature phenotype. Both the MFI and the percentage of cells expressing lineage markers (CD41, CD61 and CD71) were higher in R sorted cells with concomitant lower expression of CD34. The higher CFSE fluorescence in the R sorted population may be due to higher amount of esterease present in mature cells, enabling the cell to break down the CFDASE dye to CFSE and hence produce a higher fluorescence (Nordon et al., 1999). This further supports the concept that the R sorted population has a more mature phenotype when compared to the L sorted population. Flow cytometric analysis of the size and internal complexity of the L and R sorted cells showed that the R population had a slightly higher FSC and SSC population compared to the L population and this is more pronounced after 3 days cytokine expansion. This further supports the finding that the L sorted population is more primitive than the R sorted population and is in accordance with previously published data showing that HSCs and progenitors have a low FSC and SSC on FACS analysis (Udomsakdi et al., 1992).

This finding highlights the heterogenous nature of the MACS enriched $CD34^+$ population and the limitations of using CD34 to isolate human haematopoietic cells. It also showed that by sorting the centre narrow gate of the CFSE peak the subpopulation is still representative of the original sample even though it is a proportion (less that 30%) of $CFSE^+CD34^+$ cells. Hence, the centre sorted $CFSE^+CD34^+$ cells, comprising both L and R populations, is still heterogenous in phenotype even though it is more homogenous in CFSE staining as required for high resolution tracking.

3.4.2 Effect of IL-3 on proliferation and phenotype of cytokine-mediated expansion of CB CFSE$^+$CD34$^+$ cells

The effect of IL-3 on expansion of haematopoietic cells and its contribution to self-renewal is controversial as described in Chapter 2. In this study, addition of IL-3 to SCF+MGDF+FL cytokine cocktail increased the number of cell divisions in CFSE$^+$CD34$^+$ cells after 3 days of culture but it did not affect the distribution of phenotype.

CD117 (c-kit receptor) has been used as a marker to distinguish between undifferentiated and mature cells (Mirabelli et al., 2008). Recently, c-kit was also shown to regulate the maintenance of quiescent HSC (Thoren et al., 2008). In the current study, c-kit expression did not vary greatly within the sorted populations (Table 3.2). However, when c-kit expression was determined within specific cell divisions in the L and R sorted populations with and without IL-3 added to the cytokine combination, c-kit expression increased with cell division in the all the populations cultured without IL-3. The lower CD117 expression in the populations with IL-3 may be due to this cytokine contributing to an increase in cell number at the expense of primitive cells. This would correlate with the lower mean generation number and proliferation index seen in the sorted populations cultured without IL-3.

Previous studies in our lab found no significant difference in engraftment levels when IL-3 was added to the cytokine cocktail to expand CB CD34$^+$ cells prior to transplanting them into NOD/SCID mice (Rice AM, unpublished data). Others studies have shown that IL-3 has a positive effect on the expansion of primitive cells, maintaining

engraftment (Rossmanith et al., 2001) and in supporting SCF, FL and MGDF induced expansion of multilineage, long-term reconstituting activity in primary and secondary recipients (Bryder and Jacobsen, 2000). Hence, depending on the cytokine cocktail, IL-3 does not seem to be detrimental for engraftment and may be advantageous as it increases the total number of cells following cytokine culture.

In this study, the increase in proliferation with the addition of IL-3 led to a decrease in the number of cells available in the undivided fraction and early divisions following cytokine culture. This was not advantageous for later experiments where sorting was required of these populations; hence it was decided for practical reasons that IL-3 would not be included in the cytokine combination SCF+MGDF+FL in future experiments.

3.4.3 Effect of cell division on the expression level of cytokine receptors, adhesion molecules and lineage markers following cytokine-mediated expansion of CB CD34$^+$ cells

The phenotype of the human HSC is still not accurately defined and currently a more primitive population is isolated mostly by negative surface marker expression. The CD34$^+$ population in this study was heterogenous as uncultured CD34$^+$ cells had a high expression of CD38, the tranferrin receptor (CD71) and the myeloid marker CD33. This phenotype has also been shown by other groups in human CD34$^+$ cells (Terstappen et al., 1991, Lansdorp et al., 1992, McNiece, 2000, Miller et al., 1999). The phenotype of CB CD34$^+$ cells changed following cytokine-mediated expansion. CD34 expression decreased with culture and as cells divided whereas lineage specific markers and adhesion molecules were mostly upregulated.

Interestingly, CD34 expression was lower in undivided cells compared to cells that had undergone cell divisions. This change in CD34 expression, which was originally high in the unexpanded population, is of interest. There are number of reports in the literature showing a fluctuating phenotype and dissociation of phenotype following cell cycle progression and cytokine-mediated expansion. That is, the change in phenotype following cytokine culture may not accurately reflect the functional potential of the manipulated cells (Habibian et al., 1998, Danet et al., 2001, Dorrell et al., 2000, Ogawa, 2002).

Cytokines also play a role in regulation of homing and adhesion molecule expression. Hence their expression following cytokine-mediated expansion was of interest in this study. Expression of adhesion molecules VLA-4, VLA-5 and LFA-1 was high in $CD34^+$ cells and further upregulated with cytokine-mediated expansion. These may be of functional significance as experiments where VLA-4 or VLA-5 was blocked had a detrimental effect on engraftment both in CB $CD34^+$ cells and murine HSCs (Peled et al., 2000, Papayannopoulou et al., 1995, Wagers et al., 2002). SDF-1α induces the function of adhesion molecules, VLA-4 and LFA-1 on CB $CD34^+$ cells (Peled et al., 2000) and enhances their NOD-SCID repopulating ability following *in vitro* culture (Glimm et al., 2000). Hence, the high and maintained expression of these adhesion molecules may be favourable in augmenting engraftment capacity in the NOD-SCID model for the expanded CB $CD34^+$ population.

MAC-1 was only expressed after 7 days of expansion which correlates with its reported expression in the literature in more mature haematopoietic progenitors than HSCs (Chan

and Watt, 2001). β1 integrin was also only expressed late in expansion. The late expression of these markers suggests that the expanded population is still immature.

The adhesion molecules CD62L, CD44 and CD31 are involved in transendothelial migration and migration within the marrow cavity. CD44 plays a role in adhesion between haematopoietic progenitors to stromal cells and CD31 may be involved in the formation of stem cell niches and transendothelial migration of haematopoietic progenitors (Chan and Watt, 2001). CD44 and CD31 were highly expressed on unexpanded cells and maintained on $CD34^+$ cells after cytokine-mediated expansion, suggesting maintenance of migration function in these cells.

L-selectin (CD62L) expression on $CD34^+$ cells which was upregulated by 3 days of culture but downregulated thereafter may be of interest as together with the chemokine receptor CXCR4 were the molecules most affected by cytokine-mediated expansion. A previous murine study has shown altered adhesion molecule expression and reduced functional binding to the ECM following expansion with cytokines, which may affect engraftment (Berrios et al., 2001). There are also conflicting reports on the expression of adhesion molecules following cytokine mediated expansion with some finding that expression remains unchanged (Dravid and Rao 2002, Reems, et al 1997) and others showing a significant increase in adhesion molecules and CXCR4 expression (Herrera, et al 2004, Chute et al., 1999, Denning-Kendall et al., 1999). CXCR4 was induced by cytokine-mediated expansion in CB $CD34^+$ in this study, as seen in previous reports of CB cells stimulated with SCF and IL-6 (Peled et al., 1999). However, it has also been demonstrated that CXCR4 expression on human haematopoietic cells is not required for their repopulation capacity (Rosu-Myles et al., 2000). Expression of adhesion molecules

has also been suggested as a predictor of time to haematopoietic recovery in BM and MPB (Watanabe et al., 1998), but since expression of markers such as CD62L and CXCR4 in CB is significantly lower it has been suggested that increasing expression of some adhesion molecules may improve engraftment (Liu et al., 2003). In a study using CB haematopoietic cells, neutrophil recovery correlated with CXCR4 expression and the time to platelet recovery correlated with both CD62L and CXCR4 expression (Liu et al., 2003). Interestingly, a relationship between L-selectin and CXCR4 has previously been shown in lymphocytes, whereby CD62L modulates CXCR4 expression and function during adhesion and migration (Ding et al., 2003). This group also showed that L-selectin stimulation increases expression of CXCR4 in murine lymphocyte cell lines. L-selectin knockout experiments have shown impairment in leukocyte migration to CXC chemokines (Hickey et al., 2000). Therefore, these two findings are of importance with regard to adhesion and homing, as cytokine-mediated expansion of CB $CD34^+$ cells upregulated adhesion molecule expression and it specifically upregulated both CD62L and the chemokine receptor CXCR4. Comparing these results to the literature, these findings would suggest that cytokine-mediated expansion with the cytokine combination SCF, MGDF and FL may improve the engraftment capacity of CB $CD34^+$ cells.

Overall, cytokine-mediated expansion induced up-regulation of lineage and proliferation markers and adhesion molecules and down-regulation of putative stem cell markers with concomitant cell division. The changes and in some cases maintenance of adhesion molecule expression would suggest that cytokine-mediated expansion may be beneficial to the engraftment capacity of CB $CD34^+$ cells. However, the changes in phenotype of putative stem cell and lineage markers with cell division as a consequence

of cytokine-mediated expansion shown in this study may not reflect or be predictive of a functional change in the expanded population and this will be investigated in Chapter 5.

3.4.4 Limitations of the study

The overall hypothesis of this thesis was that cytokine-mediated expansion compromises haematopoietic stem cell function. The aim of this section was to optimise a high resolution cell division tracking technique to provide a tool to examine the effect of cell division on the phenotype and function of CB $CD34^+$ cells following cytokine-mediated expansion. As discussed in section 3.3.1.5 it would have been preferable to further purify the starting population used in all experiments ($CD34^+$ cells). However further purification of the $CD34^+$ population was limited by three main factors: cell number, machine limitations and the unreliability of the "stem cell phenotype". These issues will be discussed below.

CB was used as the $CD34^+$ cell source for this study but two of its main limitations are the cell numbers available per sample and the availability of CB samples. As discussed in section 3.3.1.5 other sorting strategies to further purify the $CD34^+$ compartment were investigated but the loss of cell number due to enrichment was too high. Hence it was not feasible to pursue further.

Other approaches to further purify the $CD34^+$ populations such as Rhodamine 123 and Hoechst 33342 were considered but were not suitable to our approach for a number of reasons. As described in section 2.5, Rhodamine 123 binds specifically to the mitochondria in viable cells, its level of uptake increases with cell cycling and HSC

have a low Rhodamine 123 fluorescence. One main disadvantage of this staining is that it does not stain a specific separate population and it is a continuum. Side population (SP) studies by Hoechst staining also have limitations as discussed in a recent review on the SP phenotype (Challen and Little 2006). This strategy has been mainly used to enrich murine BM and not human CB cells. Importantly, SP populations have a low or negative expression of CD34 or CD133, making this technique non-compatible with this project where the starting population was $CD34^+$ cells. One of the main concerns of using the Rhodamine 123 and Hoechst dyes is that the population isolated may be heterogenous due to the mechanics behind the staining.

Another important point regarding further $CD34^+$ purification was that even if the above issues were not present, this study was also limited by the use of FACS Calibur flow cytometer for acquisition and analysis. Rhodamine 123 dye can be detected on FL2 and FL3, limiting the remaining channels available to 2 (FL1 needed for CFSE) to use for phenotype characterisation of the generated populations following expansion. In addition, Hoescht 33342 requires a UV laser for detection, which is not present on the FACS Calibur. Overall, after researching a number of approaches to pursue this study, the best experimental design at the time was used in full awareness of its limitations.

3.4.5 Future studies and Alternative approaches to the current study

The purpose of this section was to first optimise the cell tracking methodology and later characterise the cell populations generated following cytokine-mediated expansion with SCF, MGDF and FL. The changes caused by this particular combination of cytokines and specifically the early changes, were of interest to this study. Functional *in vitro*

assays such as CFU and LTC-IC assays have previously been used to assess the functional capabilities of HSC and their progenitors. However, some of these assays are stroma driven and they generate primitive myeloid progenitors, downstream of the HSC, with self-renewal or multilineage differentiation potential and may overestimate progenitor cell expansion (Hofmeister et al., 2007). As the focus of the thesis was to expand haematopoietic cells in a cytokine-driven culture without stroma support, these assays were not considered suitable as a test for HSC function. Hence, the functional test used later in this study (Chapter 5) to assess the functional ability of the generated populations was to assess their engraftment capacity by transplanting the different populations into the NOD/SCID mouse model.

Nevertheless, in order to enhance the phenotypic findings from this chapter and to compare them to current literature, *in vitro* assays could be performed and these alternative strategies will be discussed further. The available *in vitro* assays for the study of human HSC function may not be optimal; however they provide a baseline for further *in vivo* studies. Hence, a possible extension to this section would have been to compare the HSC activity *in vitro* in the generated cell divisions, following cytokine-mediated expansion. Briefly, an experimental design for this proposed study would be to culture CB $CD34^+$ cells as described in sections 3.2.6 to 3.2.8. After 3 days expansion with SCF, MGDF and FL, the divided population could then be sorted as described in section 5.2.6 and figure 5.2. These sorted populations could then be compared to the unexpanded population using for example the LTC-IC assay following expansion and cell division. The results of these assays would then be compared to the results generated through *in vivo* studies.

Another possible extension to this section would be to examine changes in adhesion and migration in the expanded populations. Of particular interest would be to perform chemotaxis assays with SDF-1 as the chemoattractant as an increase in CXCR4 was shown after cytokine expansion. Hence, as for the experimental design for the LTC-IC assay, different sorted populations would then be compared to unexpanded cells and their ability to migrate towards a SDF-1 gradient assessed by a chemotaxis assay. To complement this assay, it would also be of interest to determine the levels of SDF-1 in untreated and treated cells by an assay such as ELISA.

Chapter 4

Cell cycle-related expression of haematopoietic cells following cytokine-mediated expansion

4.1 Introduction

4.1.1 Background

The cell cycle and its regulators play an important role in haematopoiesis and in the expansion of haematopoietic stem cells. The role of cell cycle status in normal haematopoiesis and in the phenotype of the engrafting stem cell has been the subject of research for a number of years. However, to this day it still remains controversial.

Haematopoiesis needs to be tightly regulated in order to maintain the stem cell pool to sustain the different blood cell lineages required in daily life and to quickly up-regulate their production in a stressful situation such as blood loss. The model that current data mostly supports is a hierarchical arrangement of haematopoietic stem cells on the basis of their quiescent state (Steinman, 2002). That is, the primitive stem cells in G0 phase of the cell cycle have the highest stem cell activity (Morrison and Weissman, 1994). *In vitro* evidence to support this model include work by Gothot et al., (1997; 1998) where he demonstrated that the LTC-IC potential of primitive cells is highest in G0 cells and

those cells that have cycled and re-entered G0 phase progressively lose their potentiality with cycling.

There is also strong evidence that a relationship exists between cell cycle status and the engraftment ability of haematopoietic stem cells, but current reports differ on the phenotype of the engrafting cell. The cell cycle phenotype of the $CD34^+$ cells that are able to reconstitute haematopoiesis in the mice differs from G0 in MPB (Gothot et al., 1998), to either G0 or G1 in CB (Wilpshaar et al., 2000). Glimm et al. also found that cells in S/G2/M phase lose their engraftment potential, as do G0 cells after 5 days in culture (Glimm et al., 2000). Cell cycle activation has a detrimental effect on long-term stem cell function (Gothot et al., 1998, Habibian et al., 1998, Fleming et al., 1993) but not on short term engraftment kinetics (Szilvassy et al., 2000). Hence, engraftment capacity seems to be reduced in cells expanded in vitro compared to unmanipulated stem cells, but the specific cell cycle status of the engrafting cell is still controversial. A fluctuating phenotype in haematopoietic cells has also been shown to be linked to cell cycle progression (Habibian et al., 1998). Upon analysis of the phenotypic changes induced by ex vivo expansion, the 'expanded' phenotypic markers may not accurately reflect functional potential.

The role of G1 phase cell cycle regulators in haematopoiesis is of particular interest as they control the fate of cells following mitogen exposure. Even though the understanding of the machinery of the mammalian cell cycle has advanced greatly in the last decades, to date there have been no comprehensive studies focusing on the G1 cell cycle regulators and their function in human haematopoietic cells, particularly in CB stem cells, in steady state and following culture with cytokines. A number of studies

have shown a role for G1 cell cycle regulators not only in policing the progression of cells through the G1 phase but also in quiescence, proliferation, differentiation and apoptosis (Steinman, 2002). However, most of the studies have been performed on either cell lines or murine models and if using human samples mainly investigating specific lineages (eg. erythroid cells) rather than the effect of ex vivo expansion on cell cycle control of haematopoiesis. Candidate cell cycle related genes to be studied are p21, p27 and D cyclins as there are conflicting data on their involvement in the haematopoiesis (Steinman, 2002, Yaroslavskiy et al., 1999, Taniguchi et al., 1999).

Research in this area is particularly relevant if we are to continue to pursue cytokine-mediated expansion of haematopoietic samples as a means to increase the cell number in small samples (such as CB) required for transplantation. The relationship between cytokine mediated expansion and its effect on cell cycle regulation was the main focus in this chapter.

4.1.2 Aim

The hypothesis for this section was that cytokine-mediated expansion of haematopoietic cells causes changes in expression of cell cycle related genes, in particular G1 phase related genes. Since reports in the literature differ on the expression of some cell cycle related genes in different haematopoietic cell types, it was important to investigate the specific changes that occur in CB $CD34^+$ and also using the cytokine combination specified for this study. Also identification of candidate molecular markers, in particular those involved in cell cycle control, could overcome the problem of phenotype dissociation and provide a means to predict engraftment potential.

The aim of this section was to characterise the changes in cell cycle-related expression of candidate G1 phase genes prior to and following cytokine-mediated expansion of CB $CD34^+$ cells with SCF, MGDF and FL.

4.2 Materials and Methods

4.2.1 Cells

4.2.1.1CB CD34$^+$ cell preparation

CB CD34$^+$ cells were processed as previously described in Chapter 3 sections 3.1 -3.4. CD34$^+$ cells used for gene expression included 5 CB samples (pooled CB# 1-4 and single CB# 5) and CD34$^+$ cells from 1 BM sample.

4.2.1.2 BM CD34$^+$ cell preparation

A cryopreserved BM sample was obtained from the Bone marrow Transplant (BMT) Unit, Prince of Wales Hospital (Randwick, NSW, Australia) The usage of BM samples for research purposes was approved by the Research and Ethics committees of the South Eastern Sydney Area Health Service and the University of New South Wales.

The frozen BM sample (50 ml) was processed according to the standard operating procedures used at the above mentioned BMT Unit. Briefly, the frozen bag was thawed at 37°C using a water bath. The blood was transferred into two 50 ml tubes and 12.5 ml of 10% Dextran (Baxter Healthcare, Toongabbie, Australia) was slowly introduced per tube while mixing, followed by 12.5 ml of 5% Human Serum Albumin in PBS (HSA; Australian Red Cross, Sydney, Australia). The sample was mixed gently for 5 minutes at room temperature, and a small sample was collected to determine the cell

concentration. The tubes were centrifuged at 300g for 15 min at 10°C. Half of the supernatant was removed and 10 ml of 10% Dextran was added to the pellets while mixing, followed by 10 ml of HSA. As small clots appeared, 10 ml of 20% Acid Citrate Dextrose (ACD) was also added to the tubes. The sample was then diluted in PBS to a concentration of 10^7 cells/ml. MNC were acquired by density gradient separation and $CD34^+$ cells were isolated by MACS as described in Chapter 3, section 3.2.2 and 3.2.3, respectively.

Table 4.1 CB samples used in RT-PCR and RQ-PCR assays

Sample ID	No. of samples	Cell No. (pre-freeze)	Cell No. (post-thaw)	Fold expansion at day 7 (Δ) *
CB# 1	8	4.1×10^6	3.8×10^6	na[#]
CB# 2	8	9.7×10^6	4.9×10^6	14.2
CB# 3	5	5.7×10^6	5.2×10^6	11.1
CB# 4	5	5.3×10^6	5.1×10^6	5.43
CB# 5	1	2.7×10^6	2.2×10^6	6

* See chapter 6 section 6.2.12. # not available

4.2.1.3 Cell lines

Cell lines (Table 4.2) were used to optimise RNA isolation techniques and as positive controls for the genes studied. CCRF-CEM (CEM), Molt-4, Jurkat and Nalm-6 cell lines were cultured in Roswell Park Memorial Institute 1640 media (RPMI; Invitrogen,

Melbourne, Australia) and 10% FCS. MCF7 cell line was cultured in Dulbecco's modified essential medium (DMEM; Invitrogen) and 10% FCS. Both RPMI and DMEM were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (both from Sigma, St Louis, USA) and 2 mM L-glutamine (Invitrogen). All cell lines were maintained in a Sanyo MCO-17A1 CO_2 incubator at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Table 4.2 Cell lines description

Cell line	Purpose in study	Description
CEM	RNA isolation optimisation	T cell acute lymphoblastic leukaemia
MCF7	Positive control Cyclin D1, p21	Breast, adenocarcinoma cell line
Jurkat	Positive control Cyclin D2, p27	T cell acute leukaemia
Molt-4	Positive control Cyclin D3	Acute lymphoblastic leukaemia

4.2.2 Culture Media and cytokines

Culture conditions for CB and BM $CD34^+$ were as previously described in Chapter 3, section 3.2.7 for CB $CD34^+$ cells. Samples were cultured for up to 7 days with 20 ng/ml of SCF, MGDF and FL. RNA was extracted (see below) from cells at day 0 (unexpanded) and after 1, 3 and 7 days in culture.

4.2.3 RNA preparation

The RNA isolation method used was optimised to allow extraction of RNA from a small number of CB cells. Optimisation was first performed using the CEM cell line due to its availability in the laboratory. To examine the limitations of the method, RNA was extracted from samples varying between 200 to $2x10^6$ CEM cells, using TRIzol® Total RNA isolation reagent (Invitrogen) according to manufacturer's instructions with minor modifications. RNase-free glycogen (Invitrogen) was added later as a carrier to the aqueous phase. Below is the final optimised method. Samples were centrifuged at 375g for 5 min (Hettich Rotanta 96R, Tuttlingen, Germany). The pellets were lysed by repetitive pipetting in 800 µl of TRIzol® for $<10^6$ cells, or 1 ml of TRIzol® for samples over 10^6 and up to 10^7 cells. The amounts of reagent used for samples with over 10^6 cells are stated in parenthesis in this method. Samples were incubated for 5 min at room temperature and transferred to 1.5 ml microfuge tubes. To each tube with less than 10^6 cells, 100µg of RNase-free glycogen (Invitrogen) was added as a carrier to the aqueous phase. Then, 160 µl (200 µl) of chloroform was added to each sample, which was shaken vigorously, then incubated at room temperature for 2-3 min. Samples were centrifuged at 12,000g for 15min at 4°C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by adding 400 µl (500 µl) isopropyl alcohol. Samples were incubated at room temperature for 10 min and centrifuged at 12,000g for 10 min. The supernate was removed and the RNA pellet was washed with 800 µl (1 ml) of 75% ethanol. The samples were mixed and centrifuged at 7,500g for 5 min. The ethanol was removed and the RNA pellet allowed to air dry for 10 min. The RNA was re-dissolved in RNase-free water (Invitrogen) by pipetting and the samples were

incubated in a heating block at 60°C for 10 min, to aid dissolution. Samples were stored at -80°C until required.

4.2.4 Quantification of RNA samples

Prior to freezing, RNA concentration and purity were measured by spectrophotometry. The yield was calculated using absorbance at 260nm (A_{260}) as 1 A_{260} unit equals 40 µg/ml RNA. The ratio of A_{260}/A_{280} wavelengths was used to determine the purity of the RNA sample where expected ratios were >1.8.

4.2.5 cDNA synthesis

RNA was reverse transcribed as follows. Approximately 2µg of total RNA and 100 ng of random hexanucleotide primers (Amersham Biosciences, Buckinghamshire, UK) in a total volume of 5.34 µl, were denatured for 10 min at 70°C, followed by 5 min at 4°C. After incubation, the mix was made up to 10µL by adding first-strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen), 3.3 mM dithiothreitol (DTT; Invitrogen) and 13.2U Recombinant ribonuclease inhibitor (RNAsin; Promega, Madison, USA), 1 mM deoxyribonucleoside triphosphate mix (dNTP; Amersham Biosciences) and 200 U of Moloney murine leukaemia virus reverse transcriptase (M-MLV RT; Invitrogen). Samples were incubated at 37°C for 1 hour. For negative controls, duplicate samples were prepared without M-MLV RT. At the end of the reaction, 40 µl of RNAse-free water was added and the samples were stored at -20°C.

4.2.6 Reverse transcription - polymerase chain reaction (RT-PCR)

The gene expression of $p21^{WAF1/CIP1}$ (p21), $p27^{Kip1}$ (p27) and cyclin D1, D2 and D3 genes was examined by RT-PCR in unexpanded and cultured CB $CD34^+$ cells. β_2-microglobulin (β_2M) was used as an internal control. As part of the optimisation of RNA extraction of low numbers of cells, samples were tested by examining the gene expression of Bcl-2 by RT-PCR. p21, Bcl-2 and β_2M primers were kindly provided by Dr. R Lock (unpublished). The other oligonucleotide primers were designed using MacVector software (Oxford Molecular, Oxford, UK) and Primer express (Applied Biosystems, Foster City, CA) software using published sequence data from the NCBI online database. All primers were designed to span introns in the sequence. The primers were synthesised by Sigma-Genosys (Sydney, Australia).

The RT-PCR mixture required for the assay included PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 2.5 mM of $MgCl_2$, 1 U Ampli*Taq* Gold polymerase (all Applied Biosystems), 1 mM dNTPs, 500 nM of forward and reverse primers of the gene of interest (when multiplexing 500 nM of β_2M primers) and RNAse-free water, to a total volume of 24 µl per sample. All reagents were dispensed into sterile 0.2 ml thin walled-PCR tubes and 1 µl was added of the cDNA of sample in question. For a control, 1µl of RNAse-free water was used instead of cDNA. The PCR reactions were denatured at 94°C for 10 min, followed by 30-35 cycles (Table 4.3) consisting of 45 s of denaturation at 94°C, 45 s for primer annealing at 55°C and primer extension at 72°C for 1.5 min. The final extension step was at 72°C for 5 min. The reactions were carried out in a Biorad thermal cycler (BioRad, Regents Park, Australia). RT-PCR products were stored at 4°C.

Table 4.3 Primers for RT PCR assay

Gene	F 5' Primer 3' R 5' Primer 3'	Primer length (bp)	Amplicon size (bp)	Cycle No.
Cyclin D1	AGAGGCGGAGGAGAACAAACAG AGGGCGGATTGGAAATGAAC	22 20	95	35
Cyclin D2	CTCCAAACTCAAAGAGACCAGCC GCCAGGTTCCACTTCAACTTCC	23 22	129	30
Cyclin D3	CCATCGAAAAACTGTGCATC TGAAGGCCAGGAAATCATG	20 19	139	30
p21	AGACTCTCAGGGTCGAAAACGG AAGGCAGAAGATGTAGAGCGGG	22 22	161	30
p27	GCAGGAATAAGGAAGCGACC TTGGGGAACCGTCTGAAAC	20 19	94	30
Bcl-2	ACAACATCGCCCTGTGGATGAC AGCCAGGAGAAATCAAACAGAGG	22 23	133	35
β_2M	ACCCCCACTGAAAAAGATGA ATCTTCAAACCTCCATGATG	20 20	114	30

4.2.6.1 Electrophoresis of RT-PCR products

RT-PCR products (10 µl) were loaded onto 12% polyacrylamide (acrylamide/bis 19:1; 5C) electrophoresis gels (PAGE; BioRad). A 1kbp size ladder (Invitrogen) was used as a marker. PAGE gels were run at 150V for 2h. PAGE gels were stained with ethidium bromide and the images were captured using a Versadoc and analysed using Quantity One 4.2.2 program (both from Bio Rad Laboratories).

4.2.6.2 Sequencing of RT-PCR products

PCR products were sequenced to confirm the correct gene had been amplified. The PCR products were purified and sent to SUPAMAC (Royal Prince Alfred Hospital, Camperdown, Sydney) for sequencing. The results obtained were analysed using MacVector software. The ExoSAP-IT kit (USB, Ohio, USA) was used for purifying the PCR products as described by manufacturer. Briefly, 10 µl of PCR product was mixed with 2 µl of SAP (Shrimp Alkaline Phosphatase) and 2 µl of Exo1 (Exonuclease I) in a microfuge tube. The sample was incubated for 15 min at 37°C, followed by 15 min at 80°C in a thermal cycler (Applied Biosystems). In a fresh microfuge tube, 0.4 µl of each primer set was added to 7 µl of purified PCR product. Water was added to make a total of 16 µl.

4.2.7 Real time PCR (RQ-PCR)

The same primers from the RT-PCR section were used for the RQ-PCR assay, except for the β_2M primers and the reverse primers for cyclin D2 and p21. The β_2M primers and β_2M probe used were as published (Burkhart et al., 2003)) and were kindly provided by Mrs J Smith. Cyclin D2 and p21 reverse primers were redesigned to satisfy the criteria necessary for RQ-PCR. The criteria included melting temperature (Tm) between 58 to 60°C, GC content between 30 to 60% and amplicon length between 50-150 bp. TaqMan probe sequences (Table 4.4) were designed for each gene p21, p27 and the D cyclins using Primer Express software with criteria including 5'-3' orientation, Tm between 68 to 70°C and GC content between 30 to 80%. All probes were designed with the reporter dye FAM (5' label) except for β_2M where VIC was used. The quencher TAMRA (3' label) was used in all probes except for cyclin D2 (MGB probe). All probes were purchased from Applied Biosystems.

4.2.7.1 RQ-PCR assay conditions

The multiplex master mix was made up, per sample, of 12.5µl TaqMan Universal PCR master mix (Applied Biosystems), optimised concentrations of forward and reverse primers, 200 nM of the respective probes and water to a final volume of 24 µl. Aliquots of the mix were dispensed into Bio-Rad RQ-PCR 96 well plate and 1 µl cDNA of sample was added per well. Using the ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Foster City, CA, USA), the plates were processed with RQ- PCR cycling conditions including 50°C for 2 min, 95°C for 10 min followed by 40 cycles consisting

of 95°C for 15 s and 60°C for 1 min. The data acquired was analysed using Sequence Detection Systems v1.7 software (Applied Biosystems).

Table 4.4 Oligonucleotide primers and probes for RQ-PCR assay

Gene	5' Primer 3'	Probe
Cyclin D1	5'-AGAGGCGGAGGAGAACAAACAG-3' AGGGCGGATTGGAAATGAAC	ATCCGCAAACACGCGCAGACCTT
Cyclin D2	CTCCAAACTCAAAGAGACCAGCC AGTGACAGCTGCCAGGTTCC	AAGCTGTGCATTTAC
Cyclin D3	CCATCGAAAAACTGTGCATC TGAAGGCCAGGAAATCATG	TCTCTCCCCGCCAGTTGCGG
$p21^{WAF1}$	AGACTCTCAGGGTCGAAAACGG GGATTAGGGCTTCCTCTTGGAG	AGATTTCTACCACTCCAAACGCCGGC
$p27^{Kip1}$	GCAGGAATAAGGAAGCGACC TTGGGGAACCGTCTGAAAC	ACCGACGATTCTTCTACTCAAAACAAAAGAGCC
β_2 microglobulin	ACTGGTCTTTCTATCTCTTGTACTACAC TGATGCTGCTTACATGTCTCGAT	TGCCTGCCGTGTGAACCATGTGAC

4.2.8 Statistical Analysis

The results were expressed as mean ± SEM (range, n). The effect of cytokine-mediated expansion on gene expression was assessed using one-way ANOVA. When values were significant ($p<0.05$), t-test were performed to compare time points within an experiment. The GraphPrism program (GraphPad Software, San Diego, USA) was used for graphs and statistical analysis.

For the RT-PCR data, the fold increase was calculated by first calculating the ratio of gene interest and internal control. These values were then normalised to the ratio value of unexpanded cells.

Table 4.5 Key for p values used in figures

P value	Symbol	Comment
<0.001	***	Extremely significant
0.001 to 0.01	**	Very significant
0.01 to 0.05	*	Significant
>0.05	ns	Not significant

For the RQ-PCR data, the statistical analysis was performed using the ΔΔCt method. (Winer et al., 1999).This analysis uses the cycle threshold (Ct) value. The Ct is defined as the cycle number that a particular sample achieves when it crosses the set fluorescence threshold intensity (Rn). The Rn represents a normalised reporter signal based on the ratio of reporter and quencher dyes at any particular time point (Kwan et al., 2000). The quantification formula for the relative value required the normalisation of each Ct value of the gene of interest to the internal control, in this case, β_2M and it is then expressed relative to a calibrator (day 0 values). The steps followed to analyse the data are described below.

Firstly, the normalized Ct (ΔCt) was calculated by subtracting the Ct of β_2M from the Ct of gene of interest.

$$\Delta Ct = Ct_{\text{Gene of interest}} - Ct_{\beta 2M}$$

Secondly, ΔΔCt was calculated by subtracting the ΔCt of expanded samples to the ΔCt of unexpanded cells within each experiment

$$\Delta\Delta Ct = \Delta Ct \text{ day X} - \Delta Ct \text{ day 0}$$

Finally, the formula below was used to calculate the relative value of each time point per experiment per gene of interest.

$$\text{Relative value} = 2^{-\Delta\Delta Ct}$$

4.3 Results

4.3.1 Optimisation of RNA isolation for low cell numbers

In order to test and optimise the method for RNA extraction, the CEM cell line was used. As suggested by the manufacturers of TRIzol® and testing with CEM cells, a reduced volume of 800 µl was used for samples of less than 10^6 cells. However, for 200 and 2000 cells no RNA was recovered, hence, glycogen was added as a carrier for small amounts of RNA. This proved to be an advantage as a pellet was then visible when preparing the samples and a reading possible on the spectrophotometer at the end of the RNA extraction of smaller samples compared to the preliminary experiments where no glycogen was added. RNA was successfully extracted from 200, 2000, 20 000 and 200 000 CEM cells. For small number of cells, i.e. 200 and 2000 cells, RNA was not diluted for quantification so as to obtain a reliable reading with the spectrophotometer, where possible, and to then calculate 2ug of RNA for reverse transcription. Bcl-2 primers were used as part of the optimisation of RT-PCR of small cell numbers as the CEM cell line and CB $CD34^+$ cells are known to express this gene and the assay conditions were already established by other scientists in the lab. The lowest sample for which RNA extraction and RT-PCR of Bcl-2 were successful was 20 000 cells. Once the method was optimised on CEM cells, total RNA was isolated from CB $CD34^+$ cells. Since CB $CD34^+$ cells are smaller in size than CEM cells, the limit of detection had to be examined in these cells. RNA was extracted from 5000 to $1x10^6$ CB $CD34^+$. RNA could be successful extracted and detected using Bcl-2 and β_2M gene expression from $1x10^5$

CB $CD34^+$ cells. For lower cell number (not less than 20 000) the bcl-2 band was faint, but the internal control β_2M was still visible.

4.3.2 Optimisation of semi-quantitative RT-PCR conditions

For each gene used in this section, the RT-PCR conditions had to be optimised. Cell lines (Table 4.2) expressing the genes of interest were used as positive controls to test the conditions prior to using CB samples. Optimisation involved testing primers with respective positive controls, and choosing optimal $MgCl_2$ concentration (1.5-4 mM). The primers were then tested in multiplex reactions with β_2M primers and optimal primer concentrations were chosen. As a final test, a cDNA dilution of the respective positive control was examined, to ensure that multiplexing of primers did not affect the expression of the gene of interest and a linear relationship was observed between gene expression and a decrease in cDNA concentration (**Fig 4.1**). Once RT-PCR conditions were optimised and a single band for the gene of interest was obtained, the PCR products were sequenced. The reaction parameters varied for some primers; 30 cycles were used for all reactions except with cyclin D1 (35 cycles) and annealing temperature was decreased from 55°C to 50°C for p27 amplification.

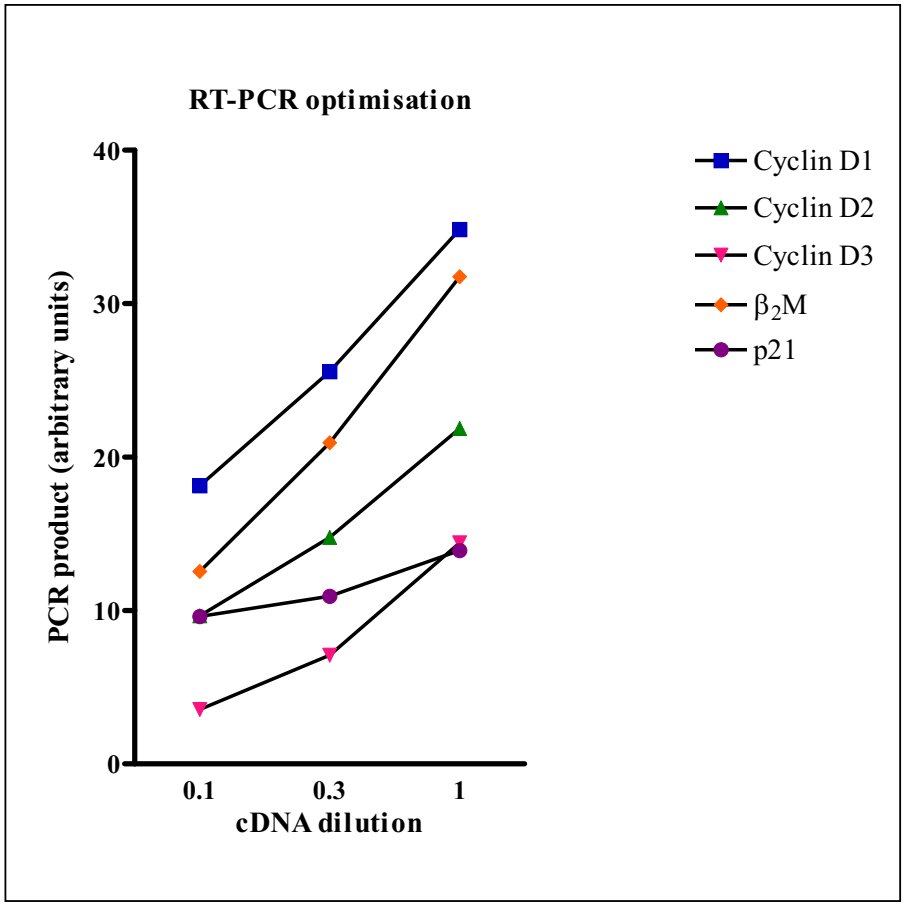

Figure 4.1 Linear relationship between PCR product and cDNA input

As a final step in the optimisation of RT-PCR conditions, a cDNA dilution of the respective positive control was examined to ensure that multiplexing of primers did not affect the expression of the gene of interest and a linear relationship was observed between gene expression and a decrease in cDNA concentration.

4.3.3 RT-PCR results for CB and BM samples

The gene expression of p21, p27 and the cyclin D1, D2 and D3 was examined by semi-quantitative RT-PCR in 4 separate pooled CB samples (CB# 1-4), one single CB (CB# 5) and one BM sample. These $CD34^+$ cells were cultured up to 7 days in serum free media with SCF, FL and MGDF cytokines. Gene expression was later quantified by real time PCR (see below) Gene expression was studied at least in triplicate, where possible, per sample per gene of interest in both assays. All results were statistically analysed using one-way ANOVA. If ANOVA was significant ($p < 0.05$) then t-test analysis was performed between the all time points. In the related Figures, the p values marked relate to significance between unexpanded cells versus day 1, day 3 and day 7.

4.3.3.1 D cyclin expression on $CD34^+$ cells cultured with SCF, MGDF and FL

Cyclin D1 expression was examined in 3 pooled CB samples, CB# 1-3 and 1 BM sample. Figure 4.2A shows representative images of CB# 1 and 2. Before normalising the data, the mean ratio of cyclin D1 expression to the internal control in unexpanded CB $CD34^+$ cells was 0.12 and in BM $CD34^+$ cells 0.40. ANOVA testing of CB samples showed changes to be statistically significant ($p < 0.0001$; Fig. 4.2A). T-test analyses were performed between unexpanded and day 1, 3 and 7 and between day 1 and 3, day 1 and 7 and day 3 and 7 to look more closely at the pairs. Cyclin D1 levels were initially low in CB $CD34^+$ unexpanded cells, increased significantly ($p = 0.0121$) at day 1, and then decreased on day 3. Expression of cyclin D1 in cells expanded for 3 days remained higher that unexpanded cells ($p = 0.02$) and then decreased further close to basal levels by day 7. The decrease between day 3 and day 7 was also significant ($p = 0.0005$). The

pattern of Cyclin D1 expression was consistent between CB samples. Cyclin D1 expression was also examined in one BM sample and in this case, expression appeared to peak on day 3 (Fig. 4.2B). However, the changes in expression were not as marked as those seen in the CB samples and they were not statistically significant as tested by ANOVA.

Five pooled CB samples (CB# 1-5) and 1 BM sample were examined for cyclin D2 expression, but due to the amount of sample available, only one run was performed for CB samples # 4 and # 5. Figure 4.2C shows representative images of CB# 1 and #3. The mean ratio of cyclin D2 expression to the internal control in unexpanded CB $CD34^+$ cells was 1.44 and 1.01 in unexpanded BM $CD34^+$ cells. Figure 4.3C shows cyclin D2 expression was high in CB $CD34^+$ unexpanded cells and in contrast to cyclin D1; cyclin D2 gene expression exhibited a gradual decrease throughout expansion. In some samples there was a slight increase in expression on day 1 but this was not statistically significant. ANOVA testing showed the changes in expression to be significant when performed for all 5 samples ($p< 0.0001$; Fig. 4.3C). T tests were performed and the changes in expression between unexpanded cells and day 3, and unexpanded and day 7 were significant ($p = 0.028$ and <0.0001 respectively). The decrease in cyclin D2 expression between day 1 and day 7 and also between day 3 and day 7 were both significant ($p <0.0001$ and $p = 0.0004$ respectively). For the BM $CD34^+$ sample, cyclin D2 expression was also high in unexpanded cells and the ANOVA test was significant ($p = 0.001$; Fig. 4.3D). When t tests were performed between the different time points, only the decrease in expression from day 0 to day 7 was statistically significant. In this sample there was a slight increase in expression between day 0 and day 1, however this was not statistically significant.

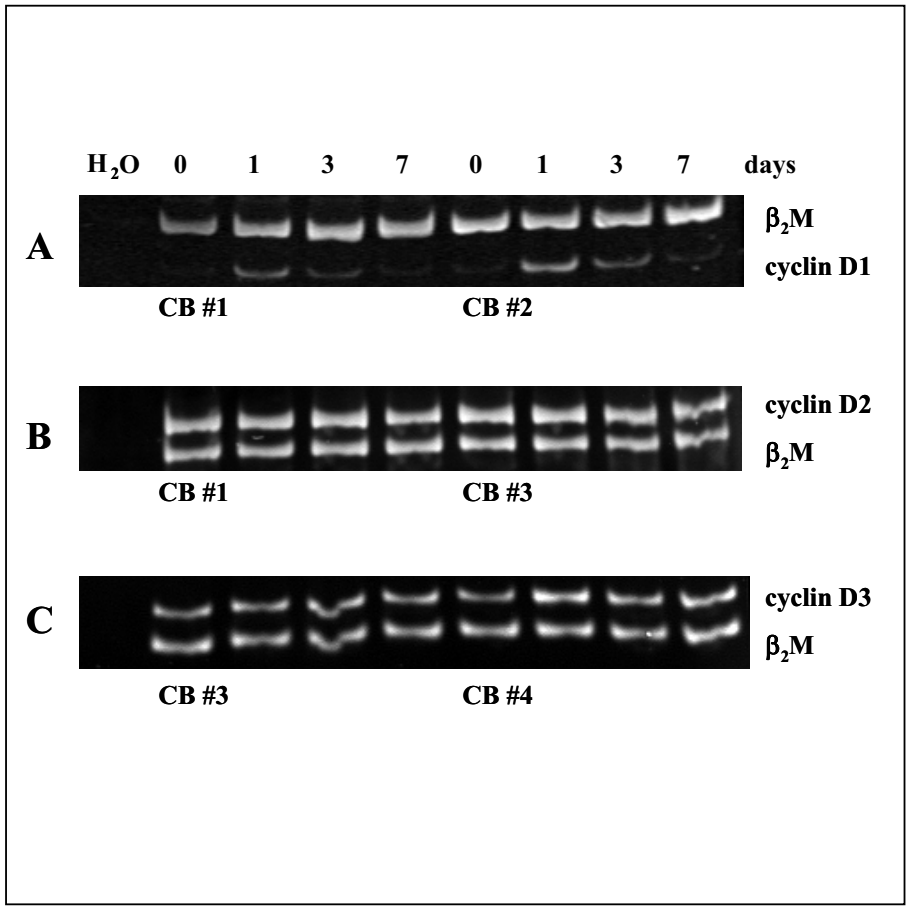

Figure 4.2 Expression of D cyclins in CB CD34-positive cells.
RT-PCR analysis of D cyclins was performed in CB $CD34^+$ cultured in the presence of SCF, MGDF and FL at the indicated time points. Untre ated cells (day 0) were also examined and β_2M was used as an amplification control.

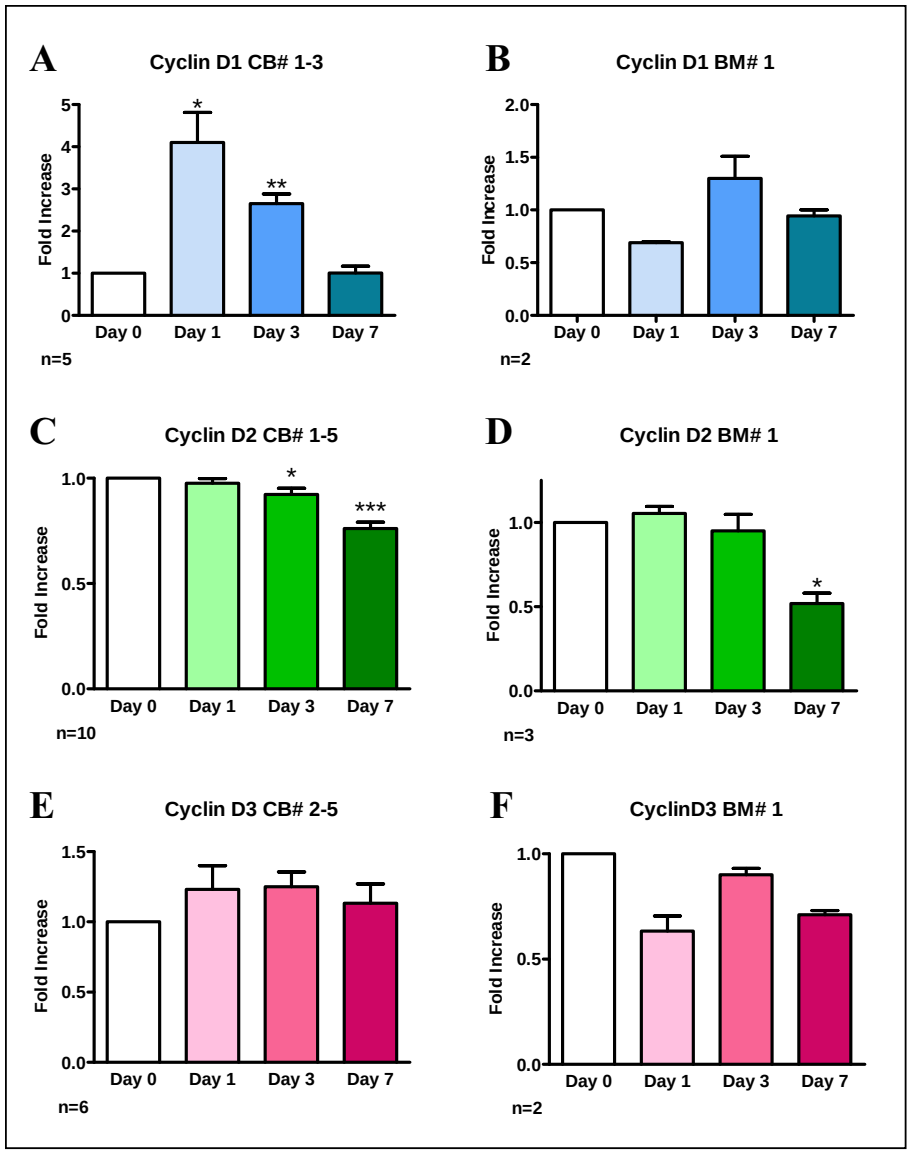

Figure 4.3 Mean expression of D cyclins in human CD34-positive cells analysed by RT-PCR

RT-PCR analysis of D cyclins was performed in CB (A,C,E) and BM (B,D,F) $CD34^+$ cells cultured in the presence of SCF, MGDF and FL. The results of the RT-PCR were quantified by normalizing data to β_2M expression and untreated cells values set at 1.0. For CB samples, data shown represent the mean of all samples. Means ± SEM. (n=number of times assayed was repeated).

Cyclin D3 expression was examined in 4 CB samples (CB# 2-5) and 1 BM sample. However, only CB# 3 was examined in triplicate. Figure 4.2C shows representative images of CB# 3 and #4. Cyclin D3 expression of the other 3 samples was only examined once. This was due to the decision to investigate gene expression of all genes by RQ-PCR and samples were then set aside for that purpose. The mean ratio of cyclin D3 expression to the internal control in unexpanded CB $CD34^+$ cells was 0.72 and in BM $CD34^+$ cells was 1.03. Cyclin D3 was low in unexpanded CB $CD34^+$ cells, peaked either on day 1 or day 3, depending on the CB sample and decreased after 1 week (Fig. 4.3E). Day 7 levels were higher than unexpanded cells. ANOVA testing of all samples showed no statistical significance. BM $CD34^+$ cells exhibited a decrease in cyclin D3 expression after expansion, with its highest down-regulation after 1 day in culture (Fig. 4.3F). ANOVA testing showed results to be significant (p 0.0089).

4.3.3.2 Cyclin-dependent kinase inhibitors

The expression of p21 was investigated in all five CB samples (CB #1-5) and 1 BM sample. Only CB samples 1 to 3 were examined in triplicate as CB #4 and #5 were set aside for RQ-PCR. Figure 4.4A shows representative images of CB #2 and #3. The mean ratio of p21 expression to the internal control in unexpanded CB 34^+ cells was 0.48 and in BM $CD34^+$ was 0.2. There were two types of patterns of p21 gene expression observed on the individual CB samples (Fig. 4.5). For one group, CB #1 and #5 (Fig. 4.5 A and E), p21 was expressed at high levels in unexpanded cells, decreased gradually with culture and then increased slightly on day 7. For the second group, CB #2, #3 and #4 (Fig. 4.5 B-D), expression increased after one day of culture, decreased on day 3 and again increased slightly after 7 days in culture. Statistical analysis using

ANOVA testing showed the differences in expression between time points to be statistically significant ($p = 0.0434$; Fig. 4.4B). *t*-tests were performed between time points and when comparing unexpanded cells to day 1, 3 and 7 only the difference in expression between unexpanded and day 3 was statistically significant ($p = 0.0073$). However, when comparing the expanded samples, that is day 1 and day 3, day 1 and day 7, and day 3 and day 7, the changes in expression were statistically significant ($p = 0.0002$, 0.008 and 0.0282 respectively). The gene expression of p21 in BM $CD34^+$ (Fig. 4.5F) cells was similar to the expression of CB samples in group 2, but the differences in expression between time points were not statistically significant when ANOVA test was performed.

Sufficient levels of RT-PCR product could not be achieved with multiplexing of p27 and β_2M primers for accurate quantitation, as the p27 expression was too low. The ratio between p27 and β_2M primers in CB $CD34^+$ cells was 0.12 for day 0 and 0.22 for day 7. As part of optimisation for all the primers used, a cDNA dilution was performed to ensure that a linear relationship is observed as the amount of cDNA is decreased. However, an acceptable linear graph could not be achieved when performing a cDNA dilution for p27 as the difference between the highest and lowest concentrations of cDNA was too small. The decision was made to try these primers (as they were suitable) in the RQ-PCR assay rather than redesign another set of primers and persist with RT-PCR.

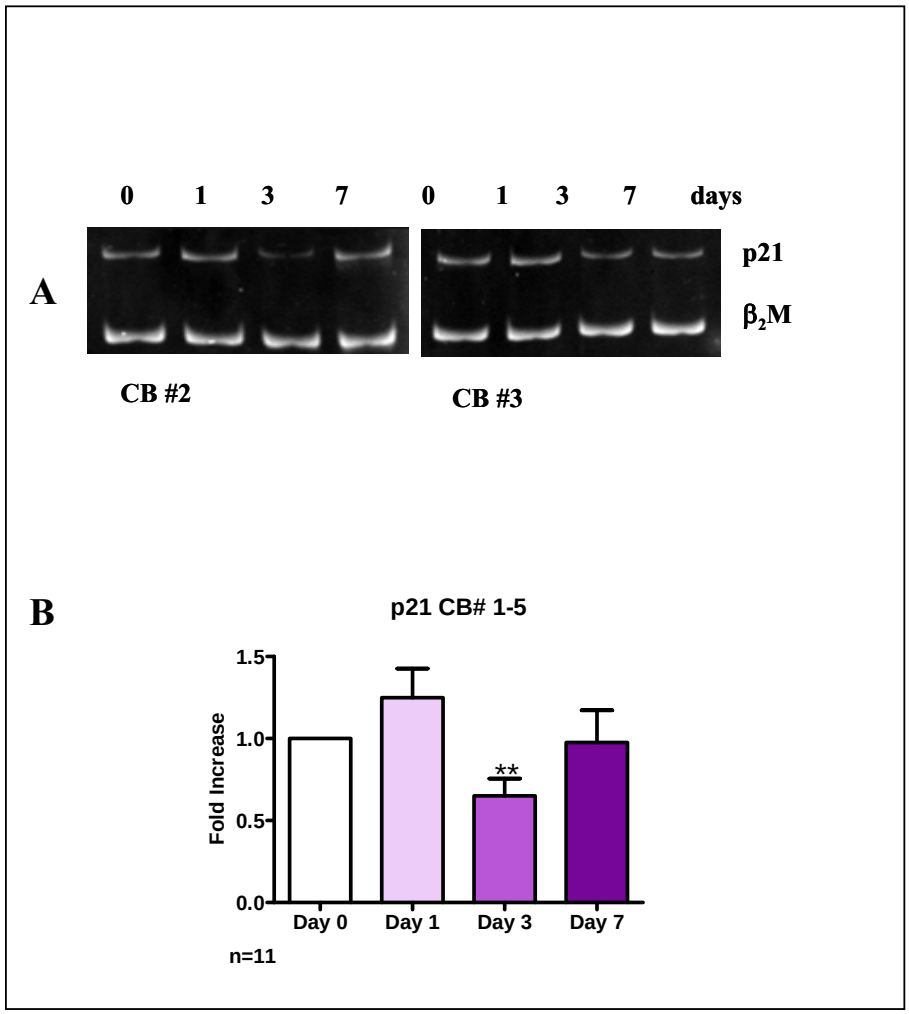

Figure 4.4 Expression of p21^{WAF1} in CB CD34-positive cells.

RT-PCR analysis of p21^{WAF1} was performed in CB CD34^{+} cultured in the presence of SCF, MGDF and FL at the indicated time points. Untreated cells (day 0) were also examined and β_2M was used as an amplification control. (A) Representative images of CB #2 and #3. (B) The results of the RT-PCR were quantified by normalizing data to β_2M expression and untreated cells values set at 1.0. The data shown represents the mean of all CB samples. Means ± SEM. (n=number of times assayed was repeated).

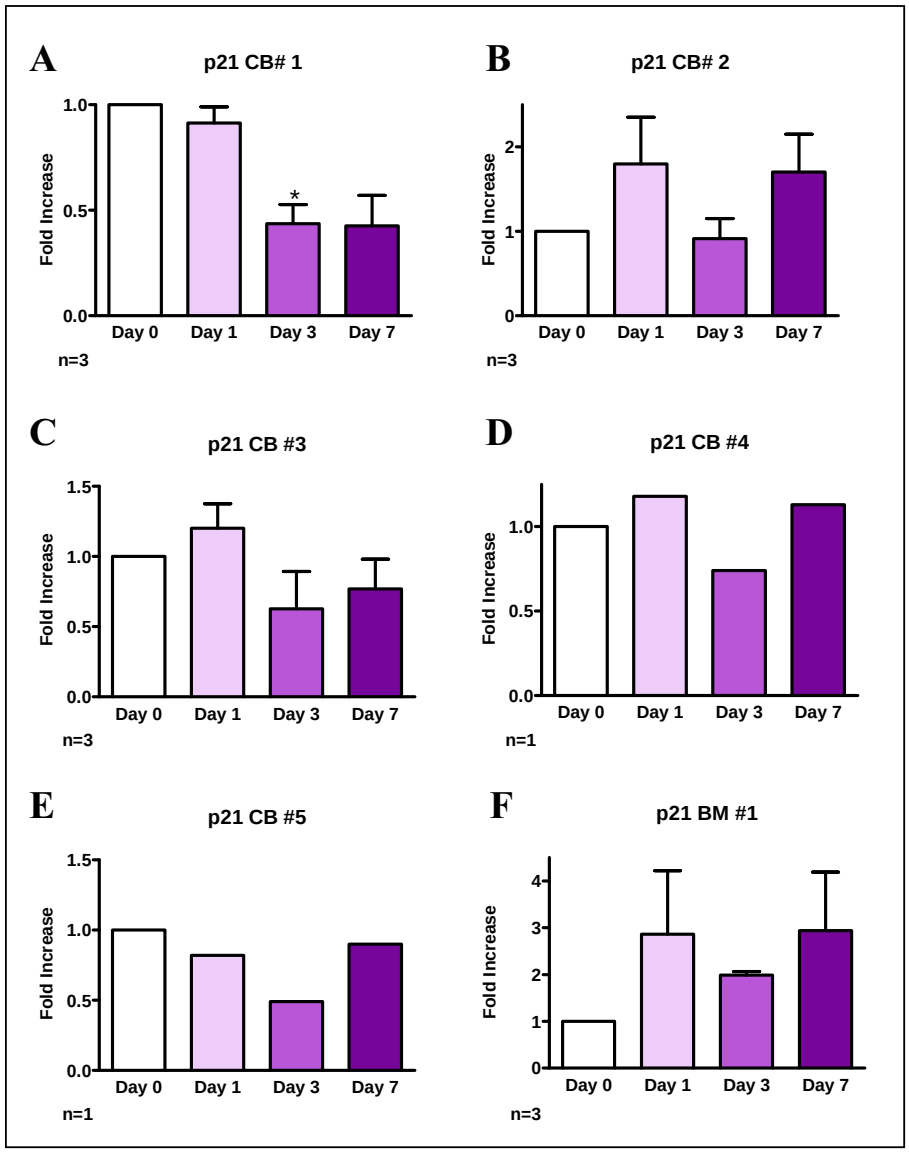

Figure 4.5 p21^{WAF1} expression differs between CB samples.

RT-PCR analysis of p21^{WAF1} was performed in five CB samples (A-E) and one BM sample (F). CD34^{+} cells cultured in the presence of SCF, MGDF and FL at the indicated time points. Means ± SEM. (n=number of times assayed was repeated per sample).

4.3.4 Optimisation of RQ-PCR assay

In order to achieve an optimal amplification reaction, all primer concentrations were optimised to ensure that no competition between different primer pairs occurred in the PCR reaction. The primer concentrations were assessed by examining the Ct and delta Rn values for each reaction. First, a range of primer concentrations (50 to 900 nM) of the genes of interest were tested without using an internal control (β_2M). From this test, it was identified that the optimal range, with lowest Ct and maximum delta Rn for all primers was between 50 to 300 nM for forward and reverse primers. A second test was performed with a smaller range of concentrations and the optimal concentration was found to be 50 or 100 nM for all genes. The next step in the optimisation was to test the primer concentrations of the gene of interest in a duplex reaction by including the internal control β_2M. The concentrations tested were 50 and 100 nM in all genes of interest and 100 nM were used for β_2M primers.

The role of the internal control was to ensure the integrity of the samples and reagents and it was also used to calculate the relative value of expression of each gene (see below). The optimal concentration was chosen where no major difference in Ct was observed between gene expression in single and duplex reactions, both in the gene of interest and β_2M. Once potential concentrations were chosen, the last step of the optimisation was to perform a cDNA dilution series (1/10 to 1/10000) for each set of primers in a duplex reaction with β_2M primers. The optimal primer concentrations chosen and used for all experiments were 100nM in both forward and reverse primers in all genes (cyclin D1, cyclin D3, p21, p27) except for cyclin D2 (50nM).

4.3.5 Real Time PCR of CB and BM

Cell cycle genes studied were as for RT-PCR, namely Cyclin D1, Cyclin D2, Cyclin D3, p21 and p27 (Fig. 4.6-10). Expression was determined for samples CB# 3 to 5 and BM# 1. Gene expression was studied in triplicate per sample per gene of interest. In some cases CB# 1 and #2 were able to be examined once but due to the small amount available it was not possible to repeat the assay. The results are mentioned below but are not included in the statistical analysis. The patterns of gene expression observed in the RT-PCR results were confirmed by the RQ-PCR assay, and differences were accurately quantified. RQ-PCR assay allowed examination of p27 which could not be studied using the RT-PCR. All results were analysed using ANOVA. If ANOVA was significant ($p<0.05$) then t-test analysis was performed between the different time points.

4.3.5.1 D cyclins

Cyclin D1 expression was examined in CB samples # 3 to 5 and MCF7 cells (positive control). Hence the overlap in results was only in CB sample # 3. The pattern of expression was as seen for RT-PCR but with some differences on day 3 and 7. Statistical analysis by ANOVA of all CB samples (Fig. 4.6A) identified changes in expression to be statistically significant ($p = 0.0258$). Cyclin D1 expression as seen previously, was greatly up-regulated on day 1 and then decreased by day 3 but still remained high to at least 10-fold the value of unexpanded cells. This increase observed was greater than seen RT-PCR but it was not statistically significant. Expression of cyclin D1, following 7 days in culture decreased to a value lower than seen in the

unexpanded cells in samples CB# 3 and # 5 ($p = 0.0288$ and $p = 0.0095$; Fig. 4.7A and 4.7C). However in CB# 4 (Fig. 4.7B), gene expression increased 3-fold compared to day 3 but this was not statistically significant. In the BM sample examined (Fig. 4.7D) the differences in expression with expansion were more marked than seen in RT-PCR but the peak occurred as with the CB samples on day 1. As seen in the previous assay the changes in expression were not as dramatic as observed in the CB samples. The results for the BM sample were not statistically significant when tested by ANOVA.

Cyclin D2 expression was examined in all five CB samples, but for CB# 1 and # 2 this was only done once. Jurkat cell line was used as the positive control in each run of RQ-PCR assay. As seen in the RT-PCR assay some samples had a small increase in expression on day 1 and decreased thereafter (CB# 1, 4 and 5; Fig. 4.8A, D-E) however this increase was only significant on sample # 4 ($p = 0.0155$). Figure 4.8B and C shows samples # 2 and #3 with the other pattern of expression where cyclin D2 was gradually down-regulated with culture. This down-regulation on day 1 was significant on CB# 3 ($p = 0.0063$). ANOVA test of CB# 1-5 was highly significant ($p < 0.0001$; Fig 4.6B). Analysis by *t*-test performed on CB samples # 1 to 5 as a group showed all changes in expression to be significant between all the time points studied, except between day 0 and day 1. That is, unexpanded cell versus day 3 ($p = 0.0007$), day 7 ($p < 0.0001$); between day 1 and day 3 ($p = 0.0001$), day 1 and day 7 ($p < 0.0001$) and between day 3 and day 7 ($p<0.0001$). Cyclin D2 expression in the BM sample (Fig. 4.8F) increased on day 1 and decreased thereafter as seen in CB samples # 1, 4 and 5. ANOVA ($p = 0.0001$) and t-tests were significant, except for t test between day 0 and day 1.

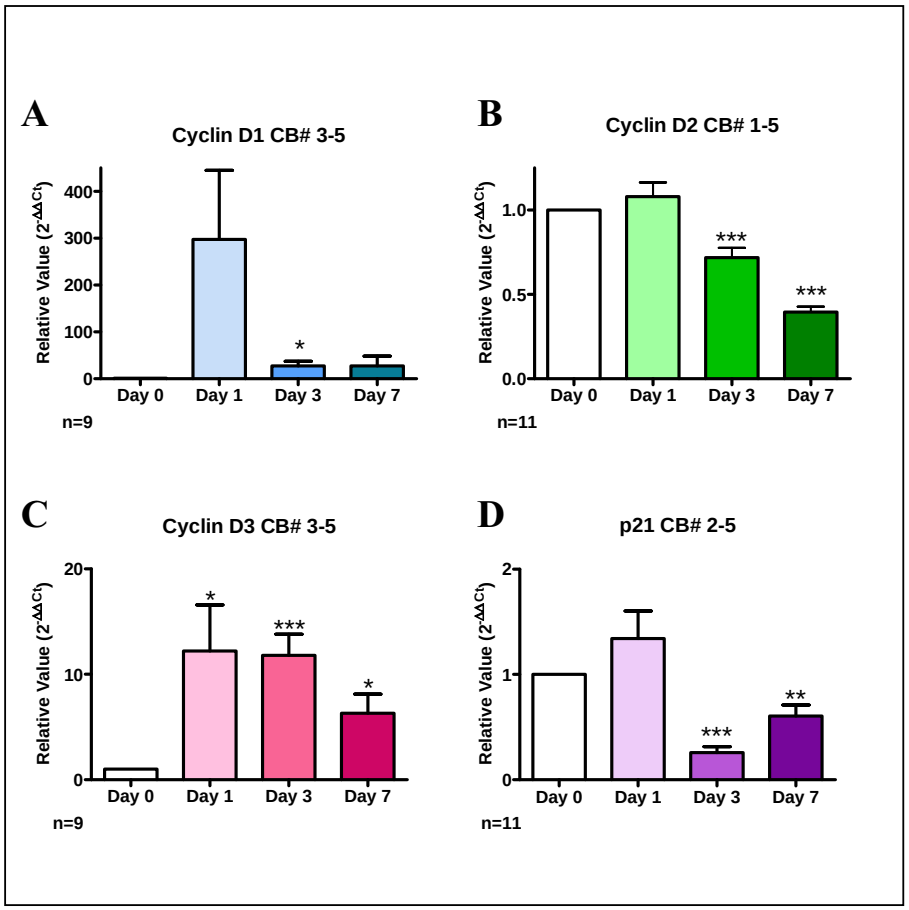

Figure 4.6 Mean expression of D cyclins and p21^{WAF1} in CB CD34-positive cells analysed by RQ-PCR.

RQ-PCR analysis of cell cycle related gene expression was performed in CB CD34^{+} cells cultured in the presence of SCF, MGDF and FL at the indicated time points. The results of the RQ-PCR assay were analysed using the ΔΔCt method. Means ± SEM. (n=number of times assayed was repeated).

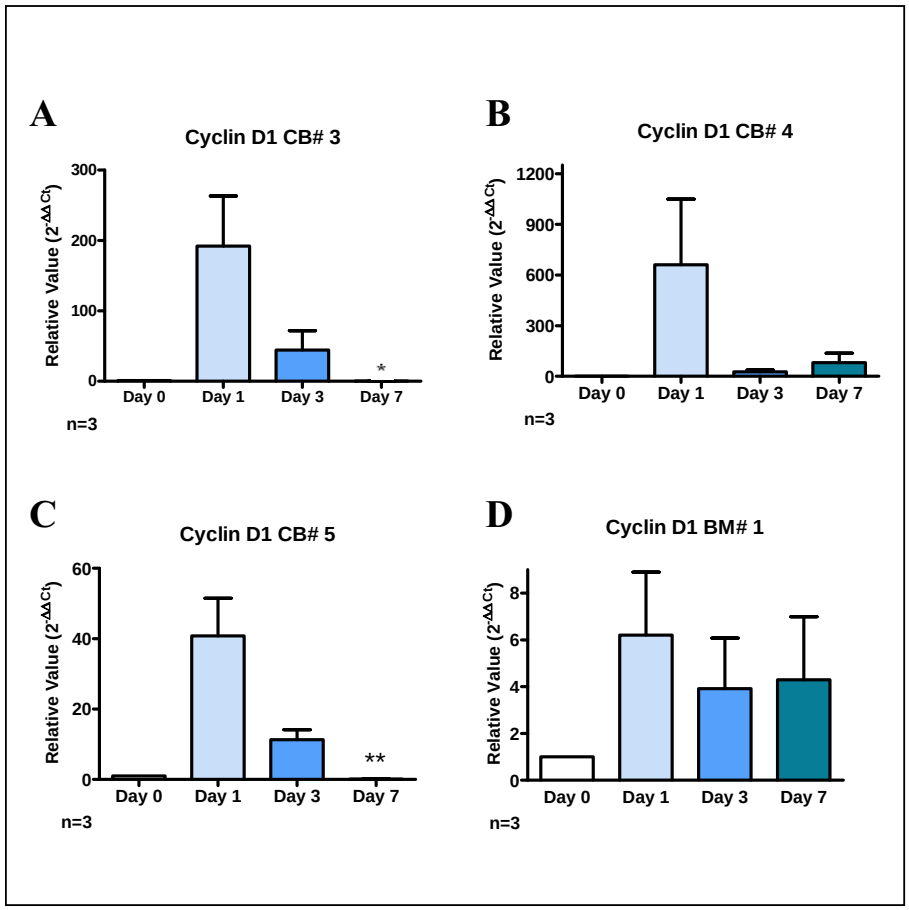

Figure 4.7 Cyclin D1 expression is initially upregulated in human CD34-positive cells following stimulation with SFM cytokines.

RQ-PCR analysis of cyclin D1 was performed in three CB samples (A-C) and one BM sample (D). $CD34^+$ cells cultured in the presence of SCF, MGDF and FL at the indicated time points. The results of the RQ-PCR assay were analysed using the ΔΔCt method. Means ± SEM. (n=number of times assayed was repeated per sample).

Cyclin D3 expression was examined in triplicate in CB# 3-5. Molt-4 cell line was used as the positive control. A pattern of expression for cyclin D3 was as seen in the RT-PCR assay where CB# 4 (Fig. 4.9B) peaked on day 1 but most peaked on day 3 (CB# 3 and # 5; Fig. 4.9A and C). In all samples cyclin D3 expression decreased by day 7 of culture. ANOVA test was significant ($p = 0.0125$) in all samples tested as a group (CB# 3-5. Fig. 4.6C). T tests showed significance when comparing changes in expression between unexpanded cells and samples that were 1 ($p = 0.0336$), 3 ($p = 0.0006$) and 7 ($p = 0.0192$) days in culture, The decrease in cyclin D3 expression between day 3 and 7 was also significant ($p = 0.0078$). For the BM sample studied (Fig 4.9D), cyclin D3 expression was first down-regulated on day 1 and peaked on day 7. ANOVA test showed that the changes observed were significant ($p = 0.0021$) T test between different time points were not significant.

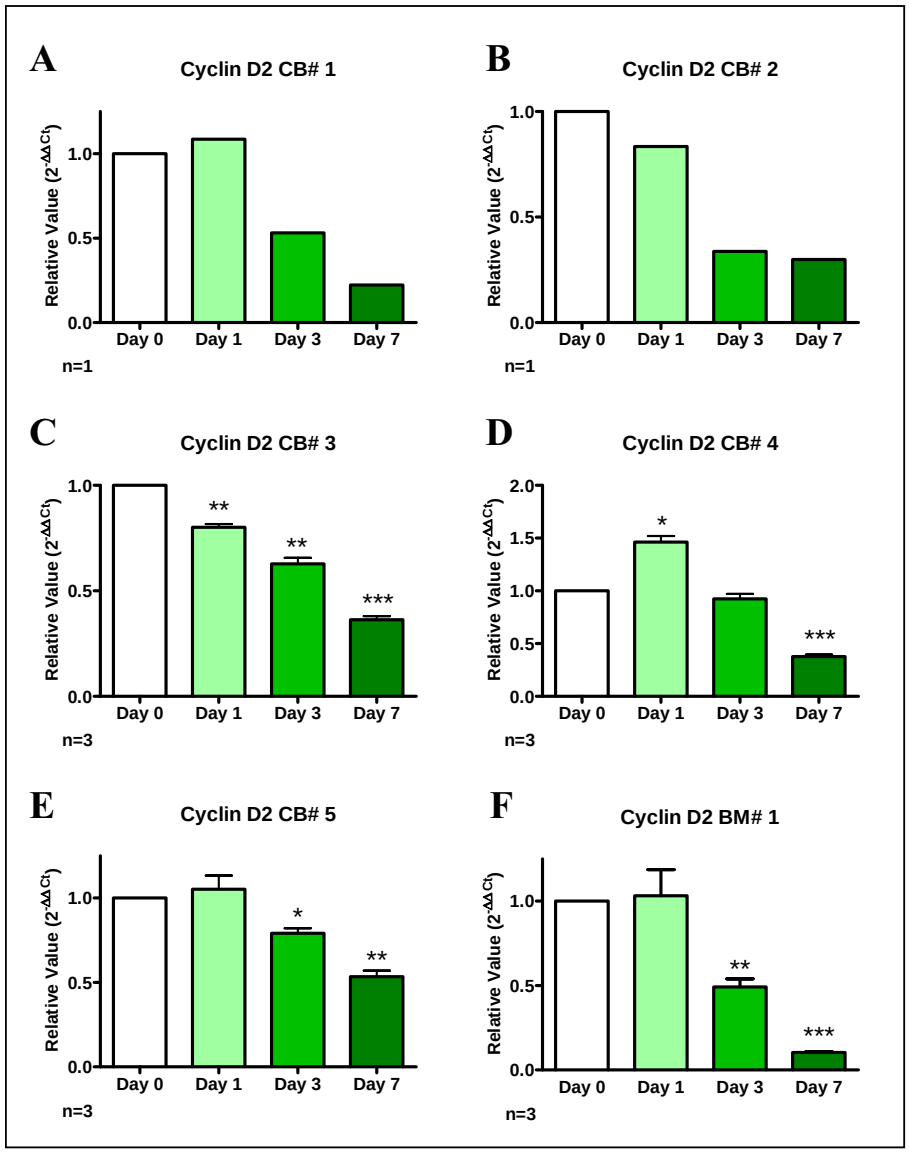

Figure 4.8 Cyclin D2 expression is down-regulated in CD34-positive cells following cytokine-mediated expansion.

RQ-PCR analysis of cyclin D2 was performed in 5 CB samples (A-E) and one BM sample (F). $CD34^+$ cells cultured in the presence of SCF, MGDF and FL at the indicated time points. The results of the RQ-PCR assay were analysed using the ΔΔCt method. Means ± SEM. (n=number of times assayed was repeated per sample).

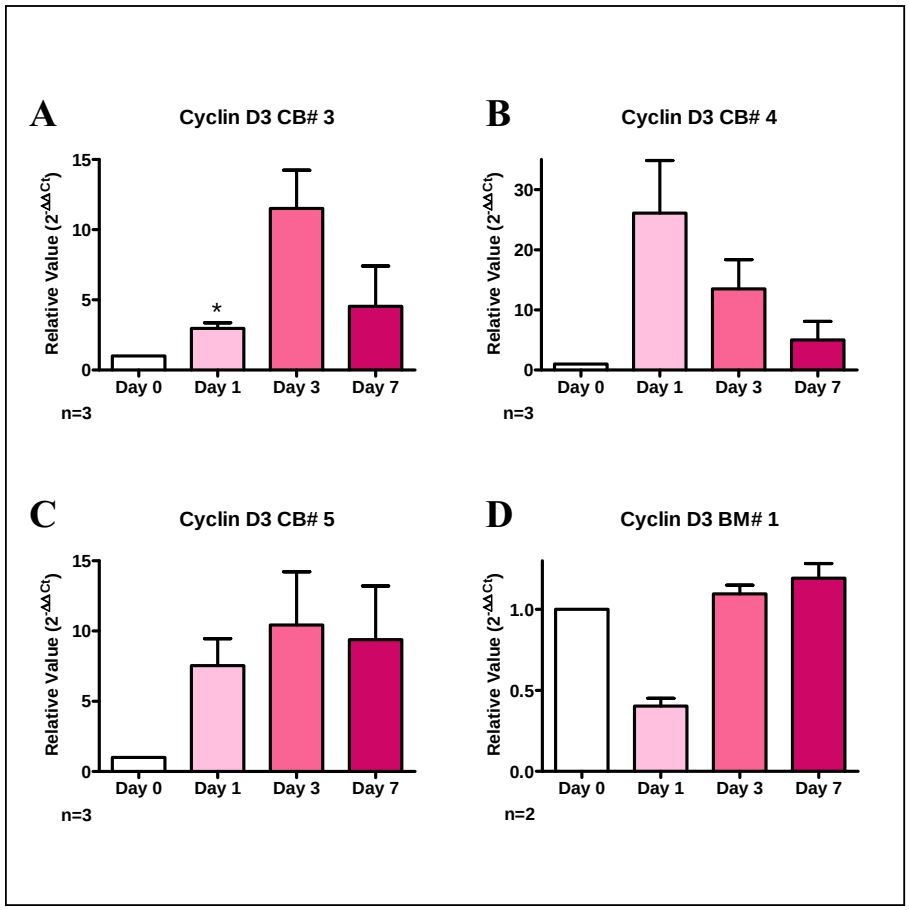

Figure 4.9 Expression of cyclin D3 differs between CB samples analysed by RQ-PCR.

RQ-PCR analysis of cyclin D3 was performed in three CB samples (A-C) and one BM sample (D). $CD34^+$ cells cultured in the presence of SCF, MGDF and FL at the indicated time points. The results of the RQ-PCR assay were analysed using the ΔΔCt method. Means ± SEM. (n=number of times assayed was repeated per sample).

4.3.5.2 Cyclin-dependent kinase inhibitors

p21 gene expression was examined in 3 CB samples (CB# 3-5) in triplicate and in CB# 2 in duplicate and MCF7 cell line was used as the positive control for the assay. As observed in the RT-PCR assay, there were two patterns of expression. In CB sample # 5 (Fig. 4.10D), p21 expression was down-regulated following cytokine culture whereas CB# 2, 3 and 4 (Fig. 4.10A-C), p21 peaked on day 1 initially. ANOVA test showed changes in expression to be significant when tested on the samples CB# 3-5 ($p = 0.0002$; when CB #2 was added to the group p value was <0.0001; Fig. 4.6D). Examining the pattern of expression of all samples combined, and comparing unexpanded cells to expanded cells, changes in p21 expression were significant after 3 ($p <0.0001$) and 7 ($p = 0.0044$) days in culture. The changes in p21 expression between expanded cells at different time points were all significant (d1 vs. d3 $p = 0.0009$; d1 vs. d7 $p = 0.0165$; d3 vs. d7 $p = 0.0076$). The gene expression of p21 in the BM sample (Fig. 4.10E) confirmed the RT-PCR results and for RQ-PCR the differences in expression between time points were statistically significant ($p = 0.0001$) when ANOVA test was performed.

Examination of p27 gene expression on CB samples # 3 to 5 proved to be difficult to interpret as there was no consistency between repeat experiments with the positive control and the expression was too low in CB samples. The jurkat cell line was used as a positive control. Changes in expression differed in all the CB samples examined. In CB #3 and # 4, expression was down-regulated following 1 day of culture. In CB #3 and # 5, p27 expression increased on day 7, whereas in CB# 4 it peaked on day 3.

Statistical analysis using ANOVA showed that changes between time points were not statistically significant.

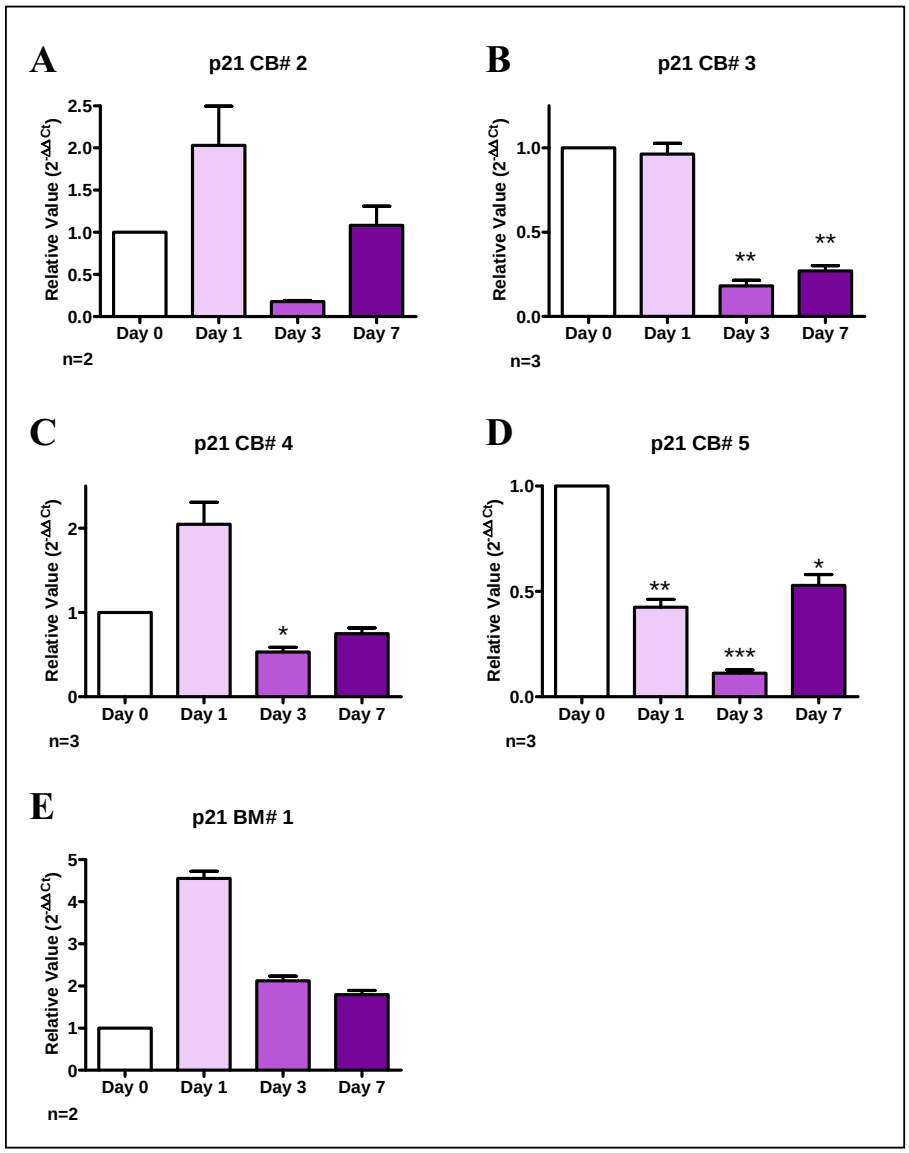

Figure 4.10 Expression of p21^{WAF1} differs between CB samples.

RQ-PCR analysis of p21^{WAF1} was performed in four CB samples (A-D) and one BM sample (E). CD34^{+} cells cultured in the presence of SCF, MGDF and FL at the indicated time points. The results of the RQ-PCR assay were analysed using the ΔΔCt method. Means ± SEM. (n=number of times assayed was repeated per sample).

4.4 Discussion

In previous studies, a relationship between the cell cycle and haematopoiesis has been established as reviewed by Steinman in 2002. The expression of cell cycle control genes is differentially regulated in a lineage specific manner in haematopoiesis (Furukawa et al., 2000). A better understanding of how ex vivo expansion affects cell cycle control in human haematopoietic stem cells and their progenitors would lead to improvement and advancement in current cell therapies. The role of G1 phase cell cycle regulators is of particular interest in haematopoiesis as they control the fate of cells following mitogen exposure. Hence, the main focus of this study was to characterise the changes in expression of genes associated with progression through G1 phase of the cell cycle in human haematopoietic cells following culture with the chosen cytokine combination.

4.4.1 Differential D cyclin expression in untreated and cultured CD34$^+$ cells

D cyclins are mitogen dependent and through activation of CDK4 and CDK6, they control the transition through G1 phase of cell cycle. D-type cyclin expression does not seem to be redundant but tissue specific. Previous studies have shown cyclin D2 and cyclin D3 to be predominant in haematopoietic cells (Steinman, 2002).

In the current study, untreated CB CD34$^+$ cells were found to express all D cyclins, albeit at different levels. CB CD34$^+$ cells highly expressed cyclin D2 and had moderate levels of cyclin D3 expression. This expression pattern correlated with other studies using untreated murine BM LT-HSCs (Cheshier et al., 1999) and human foetal BM cells (Fink and LeBien, 2001). However, in another study, the murine haematopoietic cell line 32Dcl3 was found to express cyclin D3 and to a lesser degree than cyclin D2 (Ando

et al., 1993). In untreated human BM $CD34^+$ cells, cyclin D2 and cyclin D3 gene expression was very low (Furukawa et al., 2000) and at the protein level, they express low cyclin D2 and high amounts of cyclin D3 (Della Ragione et al., 1997). In human non-stimulated lymphocytes, only 5% of the cells expressed cyclins D2 and D3 (Gong et al., 1995). In the current study, CB and BM untreated $CD34^+$ cells had low cyclin D1 expression. This correlates with another study where untreated human BM $CD34^+$ cells had also very low cyclin D1 gene expression (Furukawa et al., 2000) and had no cyclin D1 protein (Della Ragione et al., 1997).

Following culture of CB and BM $CD34^+$ cells in the presence of SCF, MGDF and FL cytokines, overall D cyclin expression was up-regulated but once again at different levels. Cyclin D1 expression was highly up-regulated after 1 day of culture but down-regulated thereafter in both CB and BM HSC sources. After 7 days of expansion, cyclin D1 expression in some CB samples remained at the same level as day 3 expression; in other CB samples it decreased further, whilst in the BM sample cyclin D1 expression was slightly higher than day 3 expression. Generally, day 7 expression was higher than the initial expression in unexpanded cells. Cyclin D3 expression in CB $CD34^+$ cells was up-regulated either on day 1 or day 3 of culture and down-regulated by day 7. As observed with cyclin D1 expression, cyclin D3 remained higher than the initial expression quantified in unexpanded cells. In the BM sample, the cyclin D3 expression pattern differed, as it was initially down-regulated on day 1 and then up-regulated by day 3. In both CB and BM, after a small increase in expression on day 1 in some samples, cyclin D2 expression was gradually down-regulated with culture. By day 7, expression was nearly half the initial value seen in unexpanded cells in CB samples and even lower in the BM sample.

The changes in expression in D cyclins in stimulated cells correlate with a study on human lymphocytes, where cyclin D2 and D3 expression rapidly increased to maximum levels early (8 to 24 h) after cell stimulation and then decreased after 48 h (Gong et al., 1995). However, in a study using human BM $CD34^+$ cells, cyclin D1 was up-regulated only in the late stages of myeloid differentiation, cyclin D3 was up-regulated in megakaryocytic differentiation and cyclin D2 was not induced in any lineages (Furukawa et al., 2000). In a study where murine BM subpopulations of HSC and progenitor cells were examined, cyclin D2 was expressed in all fractions (Zinovyeva et al., 2000). Recently it was shown that Cyclin D3 plays an important role in early B cell development (Cooper et al., 2006) and Cyclin D2 controls B cell progenitor numbers (Mohamedali et al., 2003). Thus, D cyclins expression levels vary in stimulated and differentiated cells.

When D cyclins are over-expressed, the time required for cell cycling is reduced and their over-expression can prolong the replicative life span of cells (Roussel et al., 1995). Overexpression of cyclin D2 enhances murine haematopoietic progenitor cell proliferative and granulocyte potential, leading to enhanced and accelerated short-term myeloid reconstitution and rescue of lethally myeloablated recipients (Sasaki et al., 2004). Cyclin D2 is expressed across different HSC sources and in many cases highly expressed as also observed in this study in CB and BM samples. Cyclin D2 is the most consistently expressed D cyclin in haematopoiesis and it has been shown to play a functional role in murine cells. Therefore, further investigation of the functional role of cyclin D2 in human HSC would be of great advantage to ascertain its possible role in the clinical setting.

The high up-regulation of cyclin D1 early after cytokine stimulation demonstrated in this study needs to be further investigated to determine whether it is a functional change. Expression of cyclin D1 seems to be HSC source and cell type specific but cyclin D1 has not been identified in the literature as a D-type cyclin involved in haematopoiesis. However it may have a role in human haematopoiesis, particularly in immature haematopoietic samples such as CB and foetal BM. In human foetal BM, sorted $CD34^{+}/CD19^{-}$ cells expressed cyclin D1 (Fink and LeBien, 2001) whereas T lymphocytes (Tam et al., 1994), the murine haematopoietic cell line 32Dcl3 (Ando et al., 1993) and murine BM LT-HSCs (Cheshier et al., 1999) did not express cyclin D1.

The difference in expression patterns of cyclin D3 between the samples studied correlated to that seen in the literature (Cheshier et al., 1999, Fink and LeBien, 2001, Ando et al., 1993, Furukawa et al., 2000, Della Ragione et al., 1997, Gong et al., 1995). Cyclin D3 may be a candidate marker for the molecular phenotype of the repopulating cell if the changes in expression are proved to be functionally significant. These results warrant further investigation of the functional role cyclin D3 plays in haematopoiesis and its correlation to engraftment ability.

During the course of this study, the knowledge about D cyclins and haematopoiesis focused mainly on their patterns of expression in different HSC sources and developmental lineages. Since completion of the experimental work and submission of this thesis there have been major breakthroughs in this field which will be discussed in the Section 4.4.4 of this discussion.

4.4.2 p21^{WAF1} expression is present in untreated cells but differs between CB samples following cytokine stimulation

The CDK inhibitors, p21 and p27 play an important role in controlling the progression of the cell cycle, particularly through the G1 phase. Monomeric p21 and p27 are involved in promoting the association of CDK4 or CDK6 to the D cyclins and targeting CDK4 and cyclin D1 to the nucleus (LaBaer et al., 1997). At higher ratios, p21 and p27 inhibit CDK activity (LaBaer et al., 1997) (Thron, 1997). A study investigating murine BM subpopulations of HSC and progenitors, suggested that p21 may have a role in maintenance of quiescent state of short term repopulating cells (Zinovyeva et al., 2000). Through animal studies, Cheng et al., concluded that p21 is the molecular switch governing the entry of SC into the cell cycle, and in its absence, increased cell cycling leads to stem cell exhaustion (Cheng et al., 2000).

In the current study, unexpanded cells and cells that had been cultured for 1 day expressed p21 and in some cases p21 was initially up-regulated while in other samples it was down-regulated as a result of expansion. After 3 days, p21 expression was down-regulated in all samples and following one week in culture, p21 expression was close to half the initial expression indicated in unexpanded cells. Due to problems with competition with the internal control and since p27 expression was very low, it was not possible to optimise the multiplex RT-PCR and RQ-PCR assays. When p27 was examined without the internal control, its expression differed between CB samples as it was either up-regulated on day 3 or day 7.

In the literature, the reported expression levels of p21 and p27 differ between studies. One study observed no p21 expression in untreated CB $CD34^+$ but up-regulation p21 expression with differentiation (Yaroslavskiy et al., 1999). Another study observed unexpectedly high levels of p21 mRNA in CB $CD34^+$ cells that decreased after 12h of culture (Taniguchi et al., 1999). In these studies, p27 expression in CB $CD34^+$ was low and remained constant with cytokine culture (Yaroslavskiy et al., 1999, Taniguchi et al., 1999). BM $CD34^+$ untreated cells only expressed a small amount of p21 and very small amounts of p27 at the mRNA level and only p21 was induced with differentiation (Furukawa et al., 2000). In foetal BM, the $CD34^+/CD19^-$ sorted population expressed both p21 and p27 expression (Fink and LeBien, 2001). Another group showed that untreated CB $CD34^+$ cells had high p27 expression and low p21 expression (mRNA and protein) but demonstrated progressive up-regulation of p21 expression and protein during maturation of haematopoietic progenitor cells (Steinman, 2002).

p27 has been reported to be involved in lymphocyte proliferation (Nourse et al., 1994) and in terminal erythroid differentiation (Hsieh et al., 2000). However, in the current study p27 levels were too low in all CB samples and it was not possible to optimise the PCR conditions to investigate accurately and quantify the effects of cytokine stimulation.

A number of studies have indicated that p21 has a 'universal' role in haematopoiesis particularly in mediating growth arrest in differentiated cells (Steinman, 2002) and as previously mentioned, p21 has also been identified as the molecular switch governing the entry of SC into the cell cycle (Cheng et al., 2000). Hence, the different pattern of p21 expression between CB samples may be of functional significance in ex vivo

expansion and also makes p21 a possible candidate for a molecular marker for primitive HSC. Therefore further investigation of the functional role of p21 in ex vivo expansion and also how it relates to engraftment capacity is essential.

Cytokine-mediated expansion of human haematopoietic cells caused changes in the expression of the cell cycle related genes investigated in this study. The current results warrant further investigation of the function of these changes so as to ascertain their specific role in ex vivo expansion and engraftment capacity of the human HSC.

4.4.3 Limitations of the study

The ultimate aim of this chapter was to determine the gene expression of candidate genes in undivided cells and within specific cell divisions to compare with the phenotypic and functional results described in Chapters 3 and 5 respectively. However, the main limitations in this section of the study were low cell numbers and the access to CB samples; an inherent problem when working with CB.

Two separate experiments were performed whereby individual divisions were sorted (as described in section 5.2.6). However, the cell numbers sorted were below the optimised limit and very low amounts of RNA were extracted from these populations and no results were obtained with real time PCR. Due to the high number of CB samples required for this type of sorting strategy (see section 5.2.2) this experiment could not be repeated. The availability of CB samples depended on samples been rejected by the Australian Cord Blood Bank; hence priority was given to the functional study described

in chapter 5 which required a large pool of samples. Alternatives to this approach are discussed below.

Low cell numbers led to the inability to further purify the $CD34^+$ population. The untreated population studied of CB $CD34^+$ cells was heterogenous (as discussed in Chapter 3, sections 3.3.1.5 and 3.4.4 and in Chapter 6) as it includes both HSC and its progenitors. Other populations of interest (if the amount of sample available was not a limitation) that better define a primitive human HSC are sorted $CD34^+$ $CD38^-$ Thy-1^+ or $CD34^+lin^-$Thy-1^+ cells. If these populations were used in the current study, they would have increased the current understanding of the role of cell cycle control in maintaining and expanding primitive HSC cells. Such a study could then be used as a baseline for studying changes after ex vivo expansion in cell cycle gene expression and their role in malignant transformations, in particular in cancers where a stem cell is thought to be the initiating cell.

4.4.4 Future directions and alternative approaches to this study

The hindrance of low cell numbers available from CB could be overcome by using a different experimental design. The main question of this project was to characterise human untreated haematopoietic cells and examine the changes in cell cycle-related genes after cytokine stimulation in their progeny. Hence, single cell sorting of the populations in question, followed by single cell RT-PCR would be an alternative approach to address this question. This technique was previously optimised to perform a molecular analysis of haematopoietic progenitors in the mouse embryo (Delassus, et al 1999, Hu, et al 1997).

Single cell experiments would be more powerful in allowing more parameters to be tested per cell. Since fewer cells are required for analysis (3 to 10 96 well plates depending on the size of the population investigated), pooling of samples would not be necessary and the use of single CB samples would more specifically address the issue of heterogeneity between samples. This set up would also allow more specific sorts to be investigated to better define the HSC population. The limit of the assay would depend on the initial phenotype used to sort the cells. Cell tracking may still be used as part of the experimental design to sort the expanded population into individual cell divisions and to identify the role of cell divisions on changes of gene expression. Another advantage of using single cell PCR is the ability to investigate a number of genes simultaneously on the same cell. Hence a molecular phenotype could be correlated to the surface marker phenotype of the cell under investigation.

A report just published identified an increase in D1 and D3 cyclins by endogenous and exogenous SDF-1 in PB $CD34^+$ (Chabanon et al. 2008). Hence it would be of interest to study, by an assay such an ELISA, whether cytokine-mediated expansion causes a change in SDF-1 levels in CB $CD34^+$ cells.

D cyclin and p21 expression were upregulated in cytokine-expanded $CD34^+$ cells that had undergone division; hence a point of interest would be to investigate the upregulation in expression of each gene more closely. Time course experiments would be beneficial to examine the changes in D cyclins and p21 expression before the 24 hour time point used in the current study, to determine how early the changes in gene expression occur in CB $CD34^+$ cells. Once a timeline is determined, other parameters such as cell cycle inhibitors may be used to further ascertain the role of these molecules

in cell proliferation. However, blocking D cyclins specifically may not be sufficient to cause an effect in proliferation as their function in cell cycle progression may not be as important as originally determined in previous studies as discussed below.

Since this study was completed new findings using the D cyclin knockout mouse have suggested that D cyclins are not as crucial to G1 cell cycle progression (reviewed by Sanchez and Dynlacht, 2005) as previously reviewed in section 2.10.3.1. Single D cyclin knockouts are viable. Cyclin D1 knockout mice are smaller in size but do not have a defective haematopoietic phenotype. Cyclin D2 knockout mice have impaired proliferation of B lymphocytes and the females are infertile. The cyclin D3 knockout mice have hypoplastic thymus with defective T cells. The most interesting phenotype is where all three D cyclins are knocked out (Kozar et al., 2004), haematopoiesis is ablated and mice are dead by E16.5. In these mice both the number and proliferative capacity of SC and multipotent progenitors are affected (reviewed by Sherr C and Roberts J, 2004). Hence, this may point to a yet undetermined role for D cyclins in haematopoiesis.

Another aspect that would complement the current study was to investigate the effect of cytokines on cell cycle status; hence experiments were designed to specifically address this question. Briefly, the aim was to develop a 3-colour flow cytometric assay to simultaneously study cell cycle status (Hoechst 33342 and Pyronin Y) and cell division (CFSE). Hoescht 33342 which incorporates into DNA and Pyronin Y, a RNA stain, have been previously used to examine the cell cycle status of HSCs and progenitors and to separate G0 from G1 (Gothot et al., 1997, 1998a). The addition of cell tracking to the combination of Hoescht 33342 and Pyronin Y would generate a novel technique that enables the separation of the G0 and G1 compartments of the cell cycle within each cell

division following cytokine-mediated expansion. The end point was to quantify cells at distinct phases of the cell cycle to provide unequivocal answers to the role of cell cycle status and expansion. The technique proved to be very challenging due to the interaction between the three dyes and after 10 separate experiments the technique was finally optimised. However, the technique was not able to be used as an artifact was discovered whereby a constant population of cells in G0 remained following numerous cell divisions. That is, after several divisions the same proportion of cells supposedly exited the cell cycle. Therefore, the combination of Hoescht 33342, Pyronin Y and CFSE staining was not compatible and the approach could not be pursued further.

Chapter 5
Engraftment capacity of CB stem cell progeny in the NOD-SCID mouse model

5.1 Introduction

5.1.1 Limitations of Cord blood transplantation

CB stem and progenitor cells have been used to restore function to the BM and stimulate haematopoietic reconstitution in both related and unrelated transplant recipients (Wagner et al., 1996, Rubinstein et al., 1998). CB has a number of advantages as a stem cell source for allogeneic transplants compared to BM. These include ease of collection with no risk to the donor, lower risk of transmitting infectious diseases (Donaldson et al, 2000), lower risk of both acute and chronic graft-versus-host disease (Kurtzberg et al., 1996, Rocha et al., 2000) and with the establishment of cord blood banking, better access of CB samples to transplantation centres (Case et al., 2001). However, CB samples are a one-off collection and due to the size of the samples a low dose of stem cells is obtained per collection. Hence, the use of CB for transplants may lead to delayed haematopoietic recovery (Kurtzberg et al., 1996, Wagner et al., 1996). The use of cytokine-mediated expansion has been proposed as a means of increasing the total cell dose for transplantation (Bertolini et al., 1994, Moore and Hoskins, 1994).

5.1.2 Cytokine-mediated expansion of CB CD34$^+$ cells

Numerous studies have been performed to investigate the effects of cytokine exposure on haematopoietic cells (Bryder and Jacobsen, 2000, Heike and Nakahata, 2002, Zandstra et al., 1997) and on the engraftment capacity of these cells (Bhatia et al., 1997, Novelli et al., 1999, Sitnicka et al., 2003) with diverse results as described in the literature review. In this study the cytokine combination SCF, MGDF, FL and IL-3 was chosen to study the effect of cytokine-mediated expansion on CB CD34$^+$ cells. Previous studies in our laboratory (Case et al., 2001) involving flow cytometry and factorial analysis confirmed that this cytokine cocktail is able to promote divisional recruitment and multilineage differentiation of CB CD34$^+$ cells. Hence this is a candidate cytokine combination for increasing the total cell number in CB samples.

5.1.3 The effect of cell division on engraftment

The promotion of divisional recruitment of CB CD34$^+$ cells by cytokine exposure is a desired effect, in order to expand the CB sample size available for transplant. However, the effect of cell division on the engraftment ability of HSC is controversial. Some reports suggest that stimulation of cell division in HSC by cytokines may lead to differentiation and loss of engraftment potential *in vivo* (Young et al., 1999). The cell tracking technology, optimised in Chapter 3, is a useful tool to investigate the relationship between cell division and engraftment potential. This technique allows the progeny of CB CD34$^+$ cells to be sorted by FACS based on the number of times the cells have divided in culture. The engraftment potential of the different cell populations can then be assessed in the NOD-SCID mice.

5.1.4 NOD-SCID mouse model

The immunodeficient NOD-SCID mouse model was selected to investigate the effect of cytokine-mediated expansion on the repopulation capacity of CB $CD34^+$ cells. The NOD/LtSz-*scid* (NOD-SCID) mouse strain (Shultz et al., 1995) is a well established model for the investigation of the effects of ex vivo expansion on the engraftment capacity of human haematopoietic cells (Bhatia et al., 1997, Denning-Kendall et al., 2002, Kollet et al., 1999, Piacibello et al., 1999, Bryder and Jacobsen, 2000, Ueda et al., 2000).

5.1.5 Aim

The hypothesis of this section of the study was that cell division following cytokine-mediated expansion reduces engraftment potential and causes stem cell exhaustion.

The aim was to determine the engraftment potential of CB $CD34^+$ cells with increased cell division following cytokine exposure. To achieve this, a method was developed to determine the ability of CB stem cell progeny generated *in vitro*, to expand *in vivo* using the NOD-SCID mouse model of human engraftment.

Specific Aim 1: To transplant into the NOD-SCID mouse, undivided and divided cell populations following cytokine-mediated expansion with SCF, MGDF, FL (± IL-3).

Specific Aim 2: To further fractionate the divided population and transplant individual divisions into the NOD-SCID mouse.

The findings of this study will lead to a greater understanding of the effect of cell division induced by cytokine-mediated expansion on human haematopoietic cells and their implication on engraftment potential.

5.2 Materials and Methods

5.2.1 Mice

Five to six week old female NOD-SCID (NOD/LtSz-scid/scid) mice were obtained from the Walter & Eliza Hall Institute (Melbourne, Victoria). The animals were housed and maintained under specific pathogen free conditions. They were given sterile food and water *ad libitum*. Animals were allowed to acclimatise for one week prior to commencement of experimental procedures. The Animal Care & Ethics Committee of the University of New South Wales approved all experimental procedures in this project.

5.2.2 CB requirements

The CB samples were processed as previously described in chapter 3, section 3.2.1-2.25. The experiments performed in this study necessitated a large number of CB $CD34^+$ cells. Cell loss during flow cytometric sorting was significant and a threshold number (10^5) of $CD34^+$ input cells were required to ensure engraftment in the NOD-SCID mouse (Rice et al., 2000).

Pooling of samples has been used in other studies where large number of cells were required (Rice et al., 2001) and also to decrease variability between individual CB (Leung et al., 1999). A minimum of $2x10^7$ CB $CD34^+$ cells were required per experiment to meet our experimental requirements. This number of cells was equivalent

to using between 9 to 25 CB samples, depending on total $CD34^+$ cell count per sample (Table 5.1).

5.2.3 CFSE staining of CB $CD34^+$ cells

CB $CD34^+$ cells were stained with CFSE, as previously described in Chapter 3, section 3.2.6.

5.2.4 Experimental design

Two types of experiments were performed in this study. For the first series, the aim was to transplant CB $CD34^+$ cells that had divided in culture, into NOD-SCID mice and compare the engraftment potential of this population to that of unexpanded cells. For the second set of experiments, the aim was to further separate the divided subpopulation into individual divisions to investigate the relationship between the number of cell divisions and engraftment potential. The sorting strategies required to achieve these aims are described in section 5.2.6.

5.2.4.1 NOD-SCID mice transplanted with sorted undivided and divided CB $CD34^+$ cells

The experimental design (I) for this section is described in Figure 5.1 and Table 5.1. Briefly, mice in group 1 were transplanted on day 0 with $1x10^5$ CB $CD34^+$ cells. Groups 2 and 3 received sorted CB $CD34^+$ cells that had not divided or had divided after 3 days of cytokine-mediated expansion, respectively. Group 4 mice were injected with bulk

(day 3, unsorted) expanded CB $CD34^+$ cells. The number of cells transplanted on day 3 was equivalent to the day 0 input cells after expansion (see statistical analysis below). The actual numbers of cells transplanted per group are shown in Table 5.4.

5.2.4.2 NOD-SCID mice transplanted with sorted undivided and individual divisions of CB $CD34^+$ cells

Experimental design II is as for section 5.2.4.1 but with modifications on day 3 (Table 5.2; Fig. 5.2). As above, mice in group 1 were transplanted on day 0 with $1x10^5$ CB $CD34^+$ cells. Group 2 received sorted CB $CD34^+$ cells that had not divided after 3 days of cytokine-mediated expansion. Group 3, 4, and 5 were injected with expanded CB $CD34^+$ cells sorted for division 1, 2 and 3. Group 6 mice were injected with day 3 bulk unsorted expanded CB $CD34^+$ cells (Figure 5.2, Table 5.2). The number of cells transplanted on day 3 was equivalent to day 0 input cells after expansion (see statistical analysis). The actual numbers of cells transplanted per group are shown in Table 5.4.

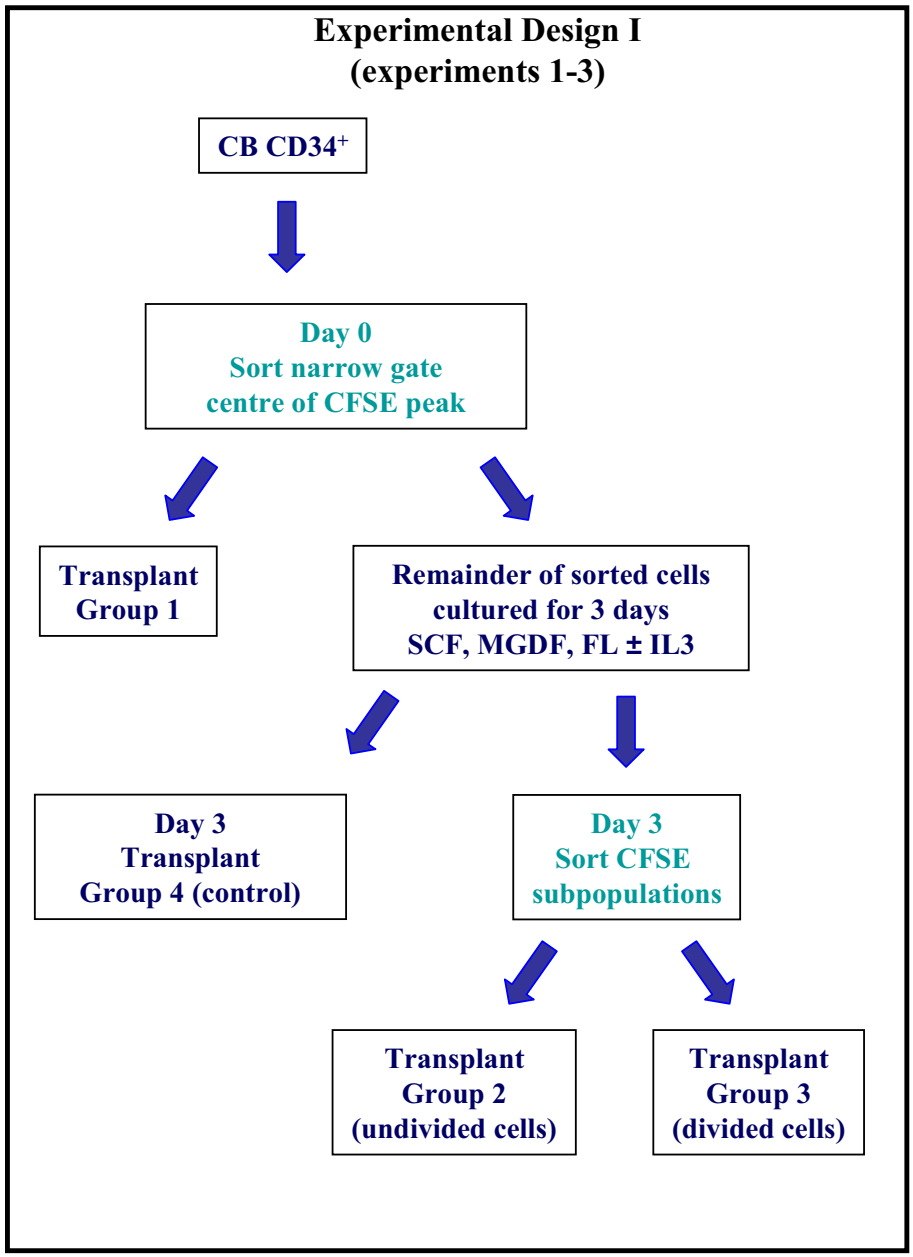

Figure 5.1 Experimental design I

For experiment 1 to 3, CB CD34$^+$ cells were stained with CFSE and sorted on day 0 for a narrow gate centre of the CFSE peak. Group 1 was transplanted with 1x10^5 cells. The remainder of the sample was cultured with SCF, MGDF, FL ± IL3 and sorted on day 3 to fractionate cells into undivided (group 2) and divided (group 3) cell populations. Group 4 was transplanted with cells cultured for 3 days not sorted on day 3.

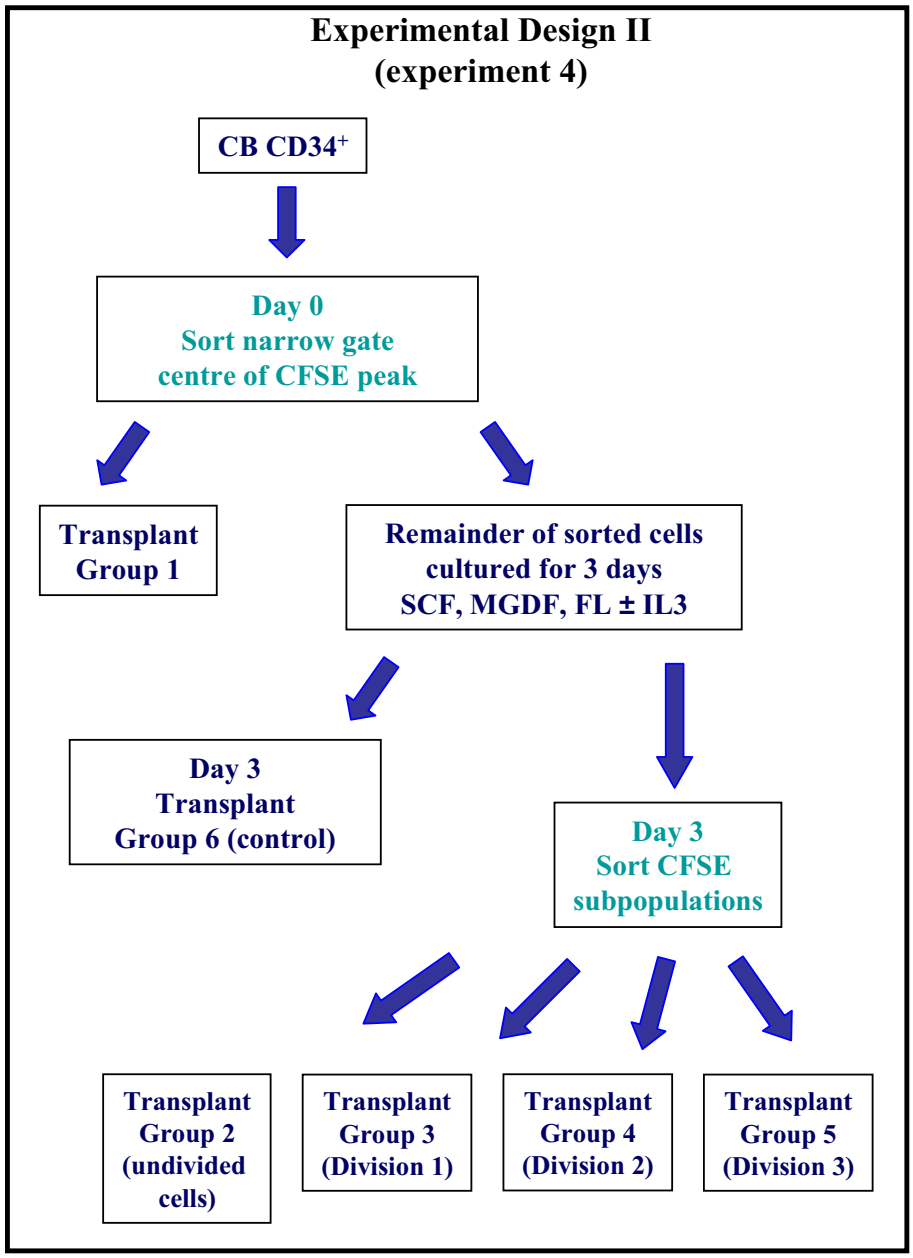

Figure 5.2 Experimental design II

For experiment 4, CB $CD34^+$ cells were stained with CFSE and sorted on day 0 for a narrow gate centre of the CFSE peak. Group 1 was transplanted with $1x10^5$ cells. The remainder of the sample was cultured with SCF, MGDF, FL ± IL3 and sorted on day 3 to fractionate cells into undivided (group 2) and individual divisions. Group 6 was transplanted with cells cultured for 3 days not sorted on day 3.

Table 5.1 Experimental design I

NOD-SCID mice transplanted with sorted undivided and divided CB $CD34^+$ cells

Transplant Group	Cell population injected	Duration of cytokine treatment	Day of transplant
1	$CD34^+$	-	0
2	Sorted $CD34^+$ undivided	3 days	3
3	Sorted $CD34^+$ divided	3 days	3
4	Sorted $CD34^+$ unsorted	3 days	3

Table 5.2 Experimental design II

NOD-SCID mice transplanted with sorted undivided and individual divisions of CB $CD34^+$ cells

Transplant Group	Cell population injected	Duration of cytokine treatment	Day of transplant
1	$CD34^+$	-	0
2	Sorted $CD34^+$ undivided	3 days	3
3	Sorted $CD34^+$ division 1	3 days	3
4	Sorted $CD34^+$ division 2	3 days	3
5	Sorted $CD34^+$ division 3	3 days	3
6	Unsorted $CD34^+$	3 days	3

5.2.5 Cytokine-mediated expansion of CB CD34$^+$ cells

CB CD34$^+$ cells were cultured for 3 days as previously described in Chapter 3, section 3.2.8.

5.2.6 Sorting strategy

For all mice experiments, CB CD34$^+$ cells were initially sorted, on day 0, for a narrow peak of CFSE as previously described in Chapter 3, section 3.2.6 (Figure 3.4). This strategy allowed for cell divisions to be individually distinguished after 3 days of culture and therefore, sorting of CFSE subpopulations.

5.2.6.1 Sorting of undivided and divided CFSE subpopulations

After 3 days of culture (see below for details), cells were washed in PBS and resuspended in a small volume with serum free media in a 4ml sterile glass tube. The sample was sorted into undivided and divided fractions as shown in Figure 5.3.

5.2.6.2 Sorting of CFSE populations into undivided and individual divisions.

As above, on day 3 cells were removed from culture, washed and resuspended in serum-free media. The sample was then divided into 2 separate 4ml sterile glass tubes. Since the flow cytometer was only a two-way sorter, 2 separate sorts had to be performed. One sort allowed separation of undivided and division 2 cell fractions, the other sorted division 1 and division 3 (Fig. 5.4).

5.2.7 Irradiation of mice

On the day of experimental procedure, the NOD-SCID mice were placed in a sterile calibrated radiation jig prior to irradiation. The animals received 250 cGy whole body irradiation at a dose rate of 325 cGy/minute, using parallel opposed fields 4 MV x-rays (Rice et al., 2000).

5.2.8 Cell preparation for transplantation

CB $CD34^+$ cells to be transplanted were washed after sorting in IMDM/10% FCS and resuspended in PBS with the volume adjusted so 100μl of sample would be injected per mouse. All animals were co-transplanted with 10^7 irradiated $CD34^-$ filler cells to facilitate engraftment (Bonnet et al., 1999, Rice et al., 2000). $CD34^-$ filler cells, matched to $CD34^+$ cells used within each experiment were thawed using IMDM/20 % FCS with 20 μL/ml DNAse. Samples were centrifuged at 300*g* for 8 min at 4 °C and resuspended in a 50 ml solution of IMDM, 10 % FCS and 20 μL/mL DNAse and irradiated (25 Gy/3.14min) at Prince of Wales Hospital, Haematology department. Samples were then centrifuged as above and resuspended in IMDM/10 % FCS at a concentration of 10^8 cells per ml and stored at 4 °C until use. $CD34^+$ and fillers cells were not mixed until immediately before transplant to avoid clumping of cells.

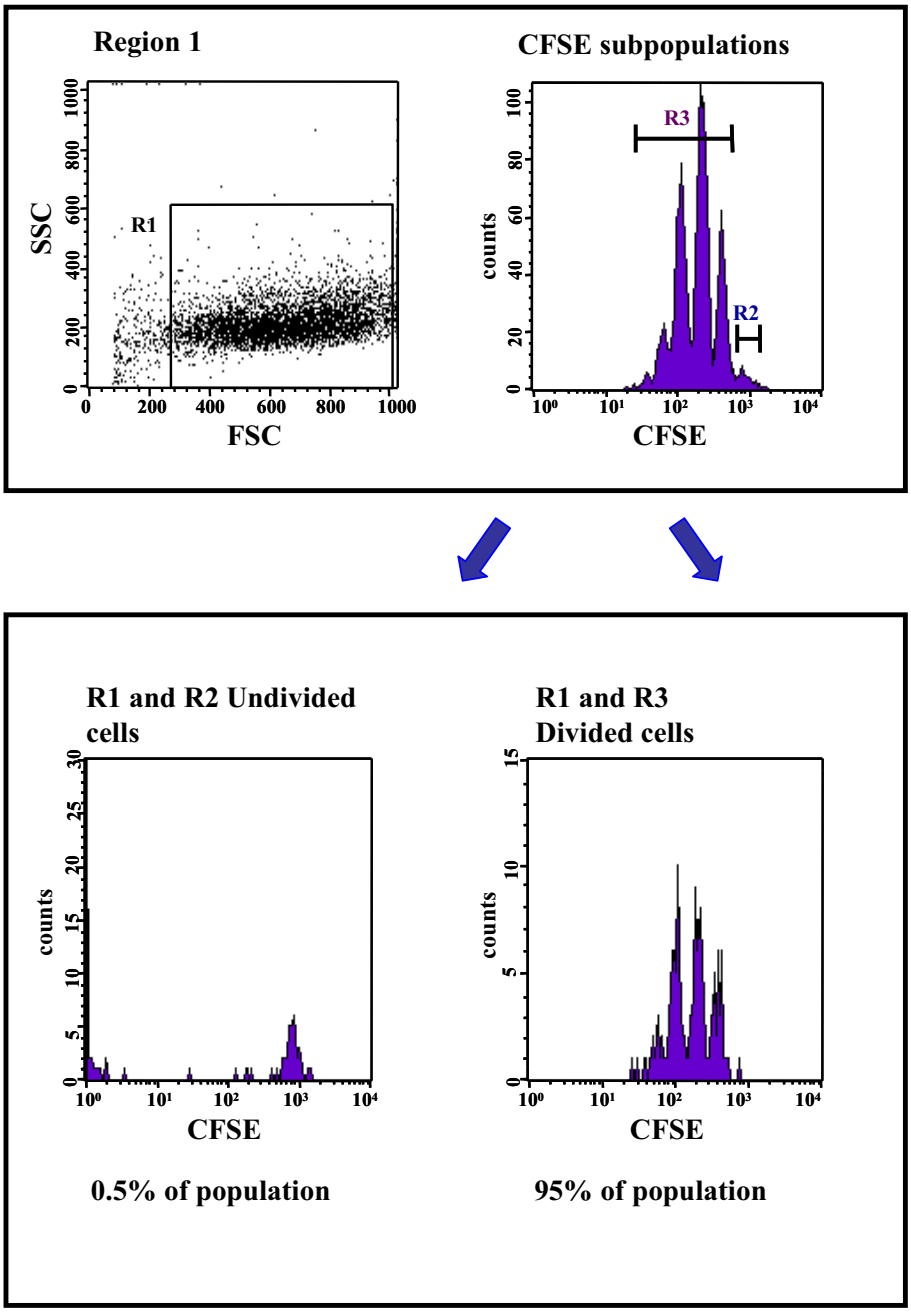

Figure 5.3 Sorting of subpopulations of CFSE

For experiment 1 to 3, CB CD34+ cells were stained with CFSE and sorted on day 0 for a narrow gate (36 channels) centre of the CFSE peak (figure 2.2a). Cells cultured and sorted on day 3 to fractionate cells into undivided and divided populations.

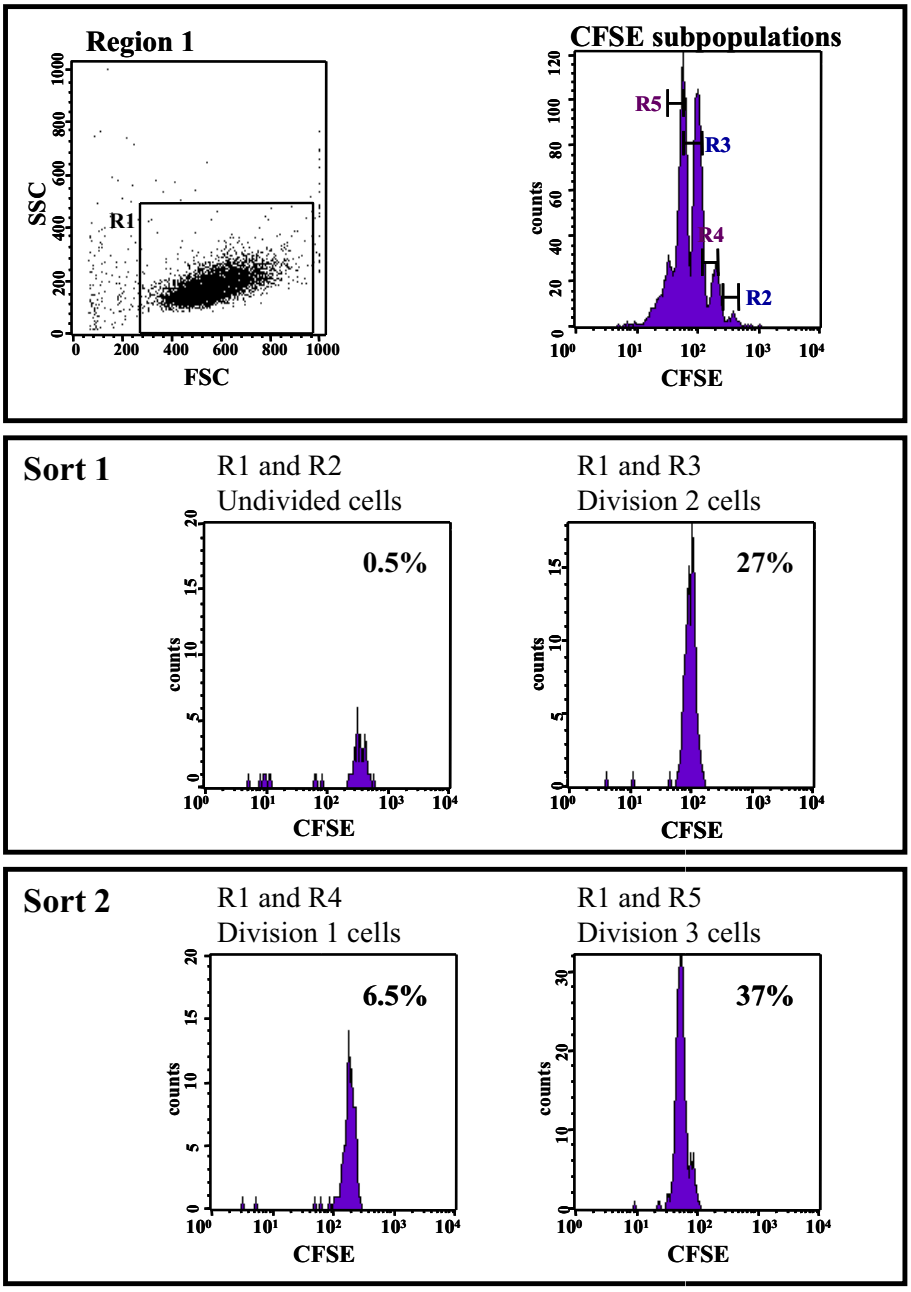

Figure 5.4 Sorting of subpopulations of CFSE

For experiment 4, cells were sorted after 3-day culture into undivided cells and individual divisions. As a two way sorter was used, the sample was halved and sorted for undivided and division 2, followed by a second sort for division 1 and division 3. Percentages represent the proportion of the sorted cells from the total population.

5.2.9 Transplantation

Mice were transplanted by intravenous injection with a maximum volume of 200 µl of sample, within 1-5 hours following irradiation. The actual numbers of cells transplanted per group are shown in Table 5.4. The animals were warmed by an infrared lamp just prior to the injection, causing vasodilation, aiding tail-vein injection. The animals were monitored daily, for general well-being, until sacrifice 6 weeks post transplant.

5.2.10 Assessment of human engraftment

Expression of the pan leukocyte human CD45 antigen was used to identify human cells and assess the level of engraftment in murine BM, spleen and PB (Fig. 5.5a). The samples were co-stained with anti-murine (mo) CD45-PE and anti-human (hu) CD45-allophycocyanin (APC; BD Biosciences, San Diego, CA) for 30 minutes in the dark. Samples were washed in PBS with 0.2 % BSA and 0.2 % sodium azide. To examine multilineage human engraftment in the BM and spleen, a panel of MoAbs were used including hu CD3 CyChrome (T lymphoid) and hu CD61 PerCp (megakaryocytic) engraftment. Hu CD34 PE Cy5 was also included in BM samples to asses the quality of engraftment (see section 5.2.11; Fig. 5.5b). Appropriate isotype controls were also included. The debris was excluded using a FSC threshold and the samples were acquired using a BD Biosciences FACSCalibur and analysed using CellQuest software. 50,000 events were acquired per sample to obtain a sensitivity level that could reliably detect 0.01 % human cells in murine PB and 0.1 % human cells in murine BM (Rice et al., 2000, Rice et al., 2001).

5.2.10.1 Peripheral blood

To monitor the level of engraftment per mouse, peripheral blood (PB) was collected from the tail vein weekly from 2 weeks post transplant until mice were sacrificed at 6 weeks. Following incubation with hu CD45 APC and mo CD45 PE, ammonium chloride (0.15 M) was added to lyse erythrocytes. After a 10 minute incubation, samples were washed in PBS. Immediately after preparation, 20, 000 events were acquired by flow cytometry.

5.2.10.2 Processing of BM and spleen samples

Mice were sacrificed by cervical dislocation 6 weeks post transplant. BM cells were collected by flushing both femurs from each animal, with a 1 ml (27 gauge needle) syringe filled with PBS. One femur is considered to be approximately 6.7% of total BM, 2 femurs in this case were analysed, that is 13.4% of total BM. Hence, the total number of human cells in mouse BM was calculated by multiplying by 7.4 (Brecher *et al.*, 1982). The spleen was also removed and macerated to obtain a single cell suspension. Samples were incubated with 50µl of normal mouse serum (Institute of Medical and Veterinary Science, Veterinary Services Division, Gilles Plains, SA) for 15 minutes at 4 °C prior to the addition of antibodies. In the case of BM and spleen, 50,000 events were acquired by flow cytometry.

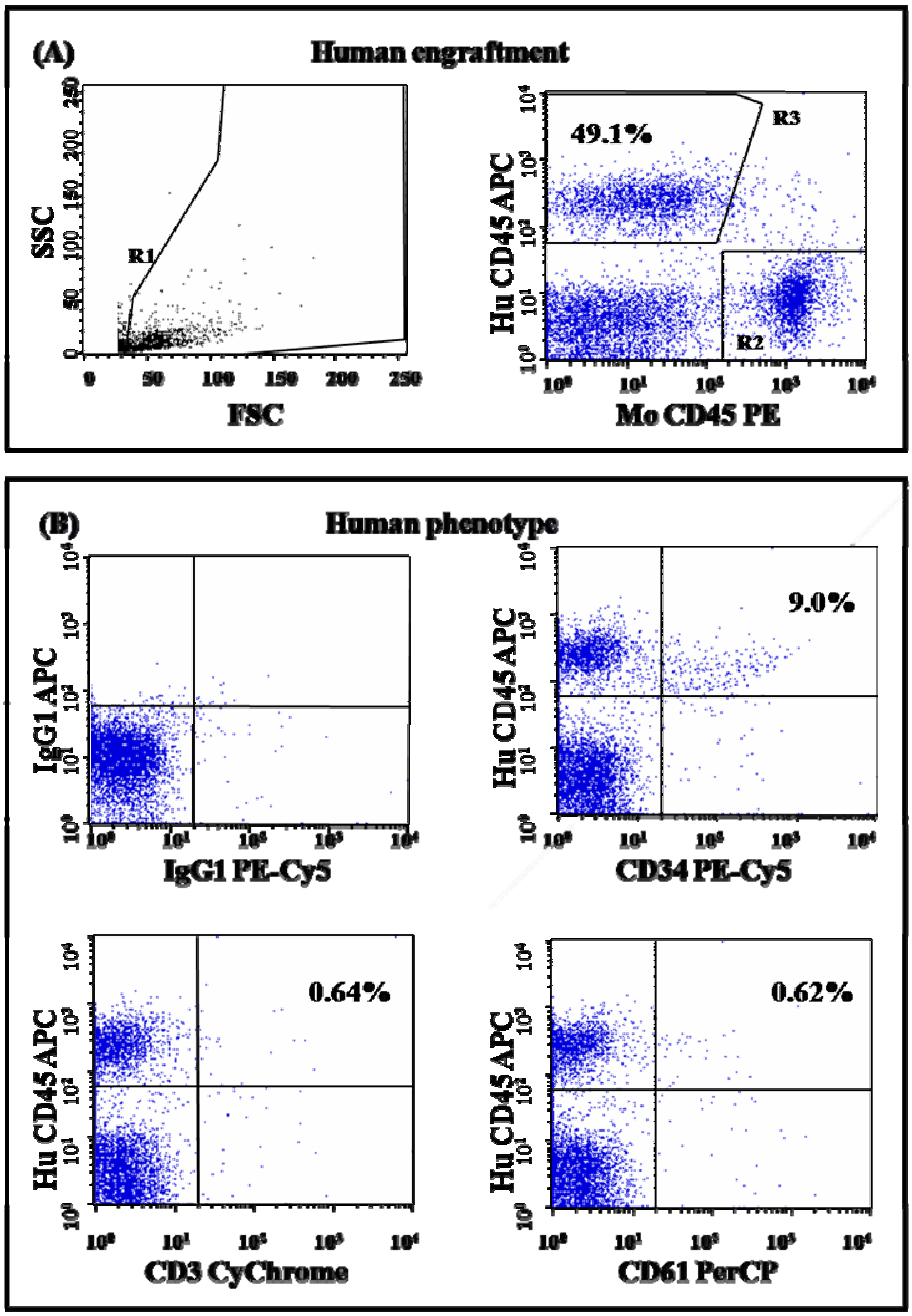

Figure 5.5 Human engraftment of CB CD34$^+$ in BM of NOD/SCID mouse
(a) The level of human engraftment in the NOD/SCID mouse was quantified as the percentage of human CD45$^+$ (APC) cells over total CD45 (human and murine - PE) expression in the BM, spleen and PB. (b) Multilineage engraftment was also examined in BM and spleen by adding the markers CD34 (BM only), CD3 and CD61 to CD45 human and CD45 mouse.

5.2.11 Statistical analysis

Statistical analysis was performed using GraphPrism software. Results were expressed as mean ± SEM and the paired Student *t*-test was used to evaluate statistical significance. A p-value ≤ 0.05 was considered statistically significant.

Input cells were defined as the number of $CD34^+$ cells used to initiate the culture on day 0 (10^5 cells per mouse). Mice were transplanted with unexpanded cells on day 0 or on day 3 with sorted cells from cultures initiated with 10^5 input cells cultured for 3 days with SCF, MGDF, FL and IL-3.

The fold expansion, *Delta value* (Δ), was calculated by subtracting the number of $CD34^+$ cells put into the system (input) on day 0 from the number of cells acquired after 3 days in culture (output), and divided all by input (Rice et al., 1995).

$$\textbf{Delta value } (\Delta) = \frac{\textbf{Output - Input}}{\textbf{Input}}$$

The number of cells transplanted per mouse on day 3, was calculated using the formula:

$$\textbf{Input cells} = \frac{\textbf{Number of cells transplanted}}{(\Delta + 1)}$$

Level of engraftment, that is percentage of human $CD45^+$ cells in the animal, was calculated by dividing the number of human $CD45^+$ cells by the total number of $CD45^+$ cells and expressing the ratio as a percentage:

$$\textbf{Level of engraftment} = \frac{\textbf{CD45}^+_{\textbf{human}}}{\textbf{CD45}^+_{\textbf{human}} + \textbf{CD45}^+_{\textbf{mouse}}} \textbf{ x 100}$$

The percentage of $CD34^+$ cells in the animal may be used as a surrogate to the *quality of engraftment* (Rice et al., 2001) and it was calculated by dividing the number of human $CD34^+$ by the total number of $CD45^+$ cells:

$$\textbf{Quality of engraftment} = \frac{\textbf{CD34}^+_{\textbf{human}}}{\textbf{CD45}^+_{\textbf{human}} + \textbf{CD45}^+_{\textbf{mouse}}} \textbf{ x 100}$$

SCID engraftment potential (SEP) is an indirect assessment of the potential of the expanded input cell, irrespective of the total number of expanded cells transplanted (Rice et al., 2001). SEP was calculated by multiplying the total number of human cells (BM cell count x 7.4) by hu $CD45^+$ cells and divided by input cells transplanted (Novelli et al., 1999). SEP was defined as the average number of human $CD45^+$ cells collected from each NOD-SCID BM, per human $CD34^+$ cell transplanted.

$$\textbf{SEP} = \frac{\textbf{Total number of human cells x CD45}^+_{\textbf{human}}}{\textbf{Number of input cells transplanted}}$$

5.3 Results

5.3.1 In vitro culture of CB CD34$^+$

The relationship between cell division and engraftment of CB CD34$^+$ cells was assessed after 3 days of culture with SCF, MGDF, FL and IL-3. As described in Chapter 3, closer inspection of cells cultured with IL-3 added to the cytokine cocktail, reduced the number of cells in the undivided peak. This made it difficult to sort this subpopulation. For this reason, IL-3 was only used in experiment 1 and for the rest of the study cells were cultured with SCF, MGDF and FL only.

Table 5.3 Characteristics of CB samples used for NOD-SCID experiments. Experiment # 1 samples were cultured with 4 factors (SCF+MGDF+FL+IL-3). Experiment 2, 3 and 4, 3 factors (SCF+MGDF+FL) were used for 3 days of culture.

Experiment No.	No. of CB samples	Cell No. pre-freeze	Cell No. Post-thaw	Cell No. Post-sort day 0	Fold expansion (Δ)
1	18	2.12 x10^7	1.84 x10^7	2.5 x10^6	1.5
2	9	1.82 x10^7	1.15 x10^7	3.2 x10^6	0.7
3	25	3.13 x10^7	1.73 x10^7	1.5 x10^6	0.9
4	9	2.36 x10^7	2.1 x10^7	3.45 x10^6	1.64

Nucleated cells expanded 1.5 fold (Δ) when cultured with 4 factors and 0.7, 0.9, 1.64 fold with 3 factors (experiment 2, 3 and 4, respectively) after 3 days of cytokine culture. Experiments 1 to 3 used sorting strategy I, where expanded cells were re-sorted after 3 days of culture to separate undivided cells from the divided population. In experiment 4, sorting strategy II was used to further separate the divided population into individual divisions. To successfully sort CB $CD34^+$ cells on day 0 and again on day 3, a large number of samples were used in each experiment. The CB samples required are listed on Table 5.3. The number of input cells on day 0 was $1x10^5$, in all experiments. The number of cells transplanted after 3 days of culture was calculated using Δ value as previously described (Table 5.3 -4).

Table 5.4 - Input cells transplanted into NOD-SCID mice

(A) Number of cells transplanted into NOD-SCID mouse for experiments #1 to #3. On day 3 cultured cells were sorted into undivided and divided populations **(B)** Number of cells transplanted into NOD-SCID mouse for experiment #4. The day 3 divided population was sorted into individual divisions. (Div = number of divisions cell population underwent in culture; n= number of mice transplanted)

(A)

NC input			
Experiment	Day 0 unexpanded $CD34^+$ cells	Day 3 expanded $CD34^+$ cells	
		Divided population	Expanded Unsorted
1	$1x10^5$ (n=2)	$2.9x10^5$ (n=4)	$2.5x10^5$ (n=2)
2	$1x10^5$ (n=3)	$1.9\ x10^5$ (n=5)	$1.7\ x10^5$ (n=7)
3	$1x10^5$ (n=3)	$1.8\ x10^5$ (n=3)	$1.9\ x10^5$ (n=3)

(B)

NC input						
Experiment	Day 0 $CD34^+$ cells	Day 3 expanded $CD34^+$ cells				
		Undivided	Div 1	Div 2	Div 3	Expanded unsorted
4	$1x10^5$ (n=2)	17860 (n=1)	55300 (n=2)	$3x10^5$ (n=2)	$4.1x10^5$ (n=2)	$4x10^5$ (n=2)

5.3.2 Evaluation of human engraftment in NOD-SCID mice transplanted expanded CB CD34$^+$ cells sorted for undivided and divided populations

In experiments 1 to 3, irradiated NOD-SCID mice were transplanted with 10^5 unexpanded CB CD34$^+$ sorted CFSE stained cells on day 0 (group 1). The remaining cells were expanded with SCF, MGDF, FL and IL-3 for experiment 1 and SCF, MGDF and FL for experiment 2 and 3. After 3 days of culture, the undivided (group 2) and divided cells (group 3) were sorted by flow cytometry. The undivided fraction was between 0.5 - 2% of the total population and it could not be transplanted into the mice in experiment 1 and 3 as cell numbers were too small. In addition, the purity of the cells was not reliable. Two mice were transplanted in experiment 2 with undivided cells, one with the equivalent of 2% of the population (3,500 cells) and the second with 26,500 cells. Group 4 received expanded cells not sorted on day 3 as a control.

5.3.2.1 Kinetics of engraftment

The kinetics of human engraftment in each mouse was monitored weekly from 2 weeks post transplant until the mice were sacrificed (Fig. 5.6 and 5.7). Engraftment levels in expanded cells varied between mice. The divided fraction had mostly lower engraftment compared to the expanded unsorted population except for experiment 1 where all mice engraftment levels in PB were low.

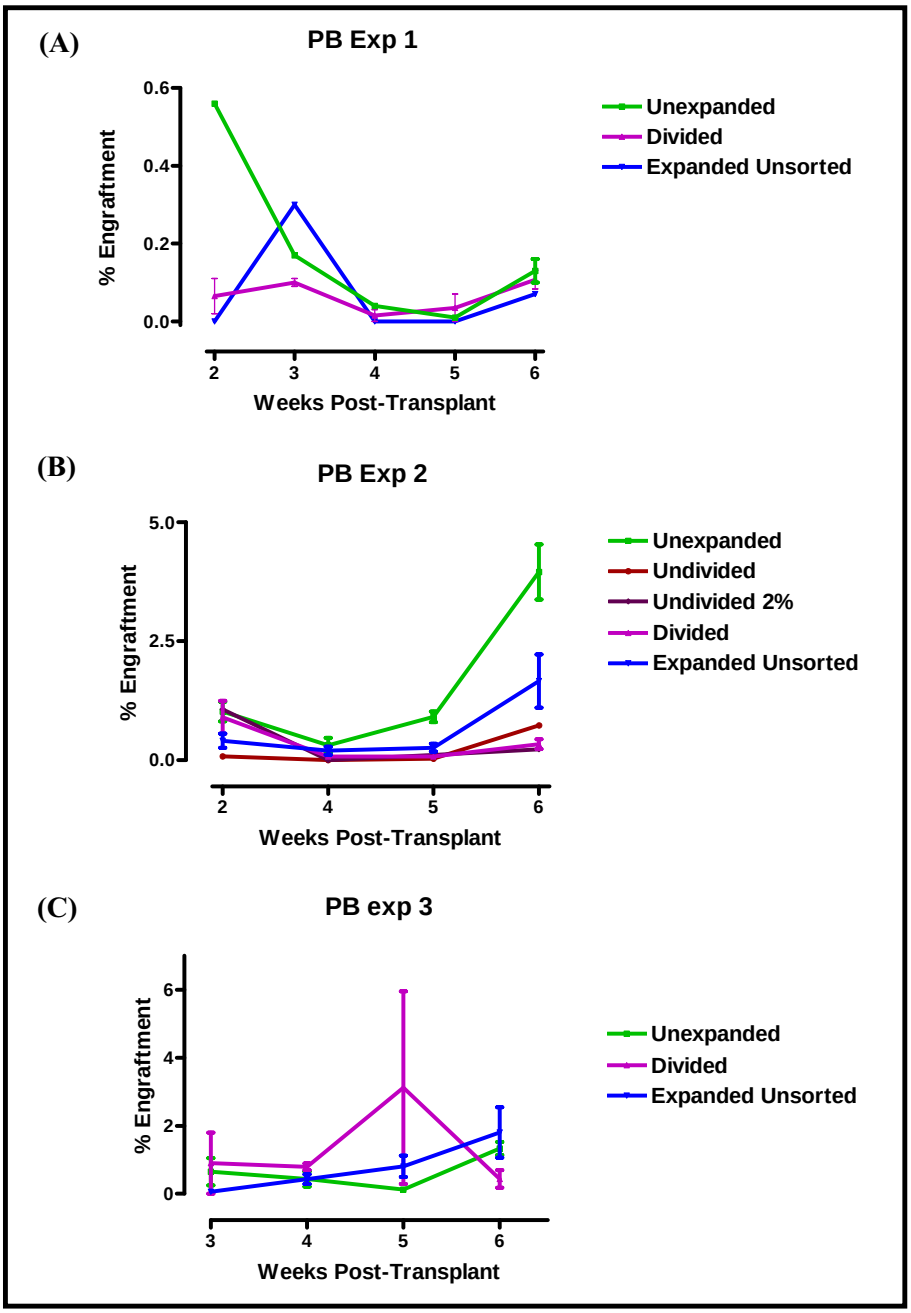

Figure 5.6 Kinetics of human engraftment in undivided and divided cell populations

PB was collected weekly from week 2 from each mouse to monitor the level of engraftment. Limit of sensitivity of the assay was 0.01%.

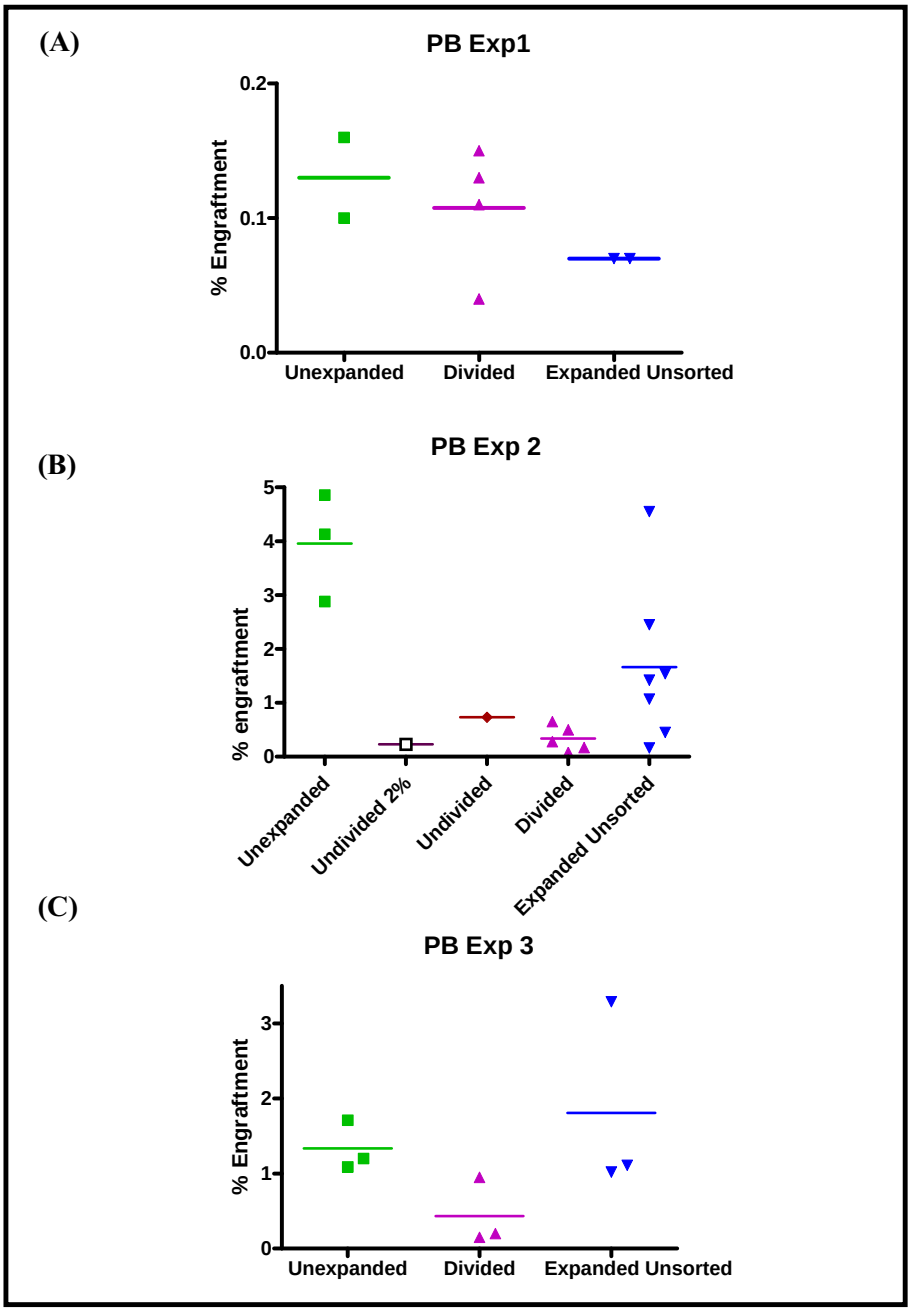

Figure 5.7 Human engraftment in PB at week 6 in undivided and divided cell populations.

PB was collected from each mouse, 6 weeks post transplant at the time of BM and spleen harvest.

5.3.2.2 Detection of human engraftment in BM and splenocytes

Human cells were detected in the BM of all NOD-SCID mice transplanted with unexpanded cells (Fig. 5.8 and 5.9). Engraftment levels were >37.6% in 5 out of 8 mice transplanted. Undivided cells were only able to be transplanted in experiment 2, at two different doses. The animal transplanted with 3,500 (equivalent to 2% of total population) did not engraft. The mouse transplanted with 7 times more undivided cells engrafted at 11.8%. The engraftment levels ranged for divided cells from 0.11 - 17.3% when 4 factors were used for *in vitro* expansion (experiment 1) and 0.2 – 13.1% when 3 factors were used in experiments 2 and 3. Expanded cells that were not sorted on day 3 engrafted at low levels in experiment 1 (0.10 - 0.36) and ranged between 2.6 - 53.9% in experiments 2 and 3. Only one animal did not engraft (below 0.1%) in this group. Human engraftment in splenocytes was also examined (Fig. 5.9). Similar trends were observed between levels of engraftment of spleen and BM but at lower levels in the splenocytes.

SEP was used to measure the average number of human $CD45^+$ cells collected from the BM of each NOD-SCID mouse per human $CD34^+$ cell transplanted (Novelli et al., 1999). SEP is an indirect assessment of the potential of the expanded input cell, irrespective of the total number of expanded cells transplanted (Rice et al., 2001). The SEP of unexpanded cells was higher than both expanded populations in experiments 1 and 3 (Table 5.5)

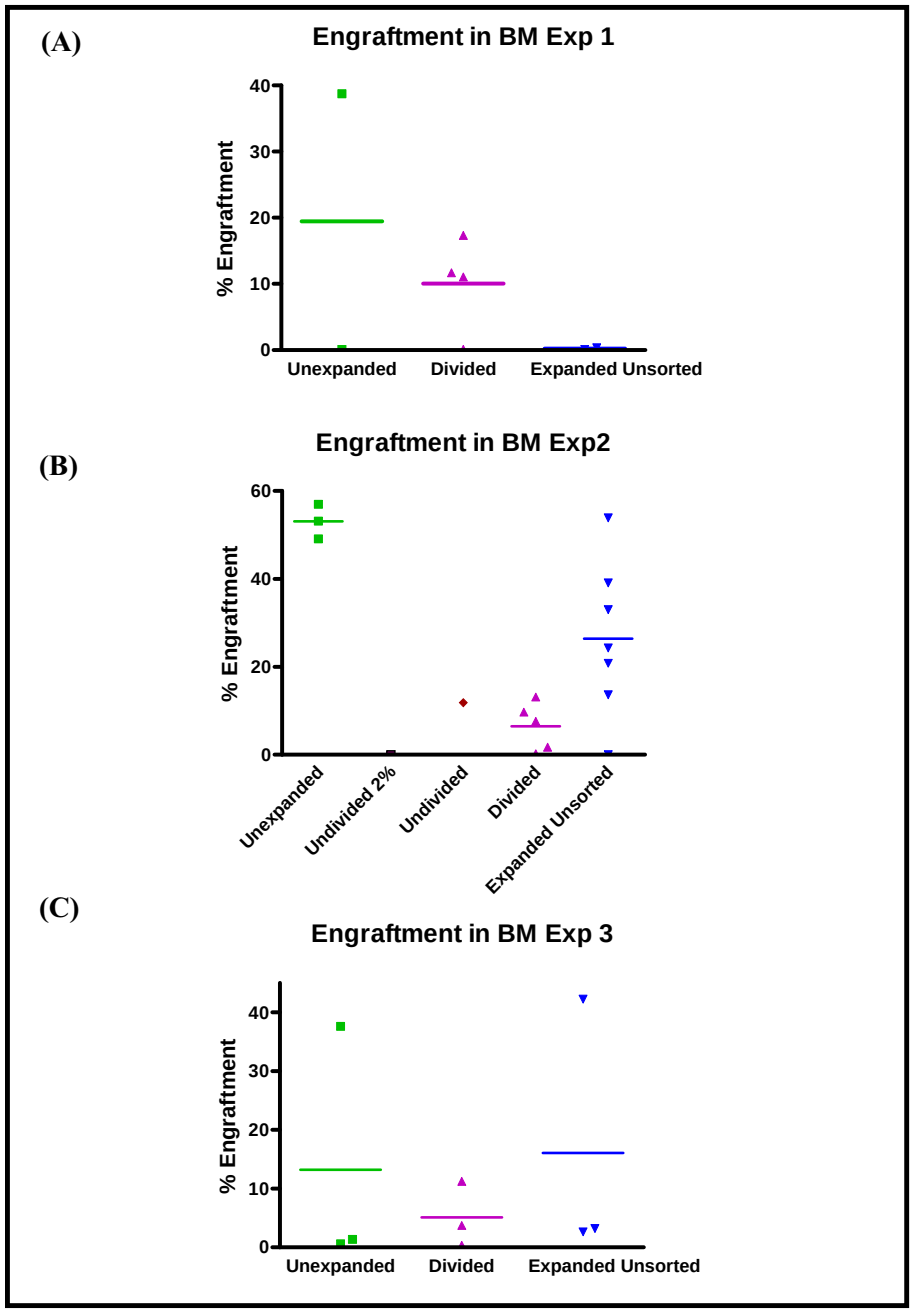

Figure 5.8 Human engraftment of CB CD34$^+$ cells sorted after 3-day culture into undivided and divided cell populations.

Murine BM was harvested after cervical dislocation 6 weeks post transplant. The level of engraftment was assessed as % of human CD45+ cells in the BM.

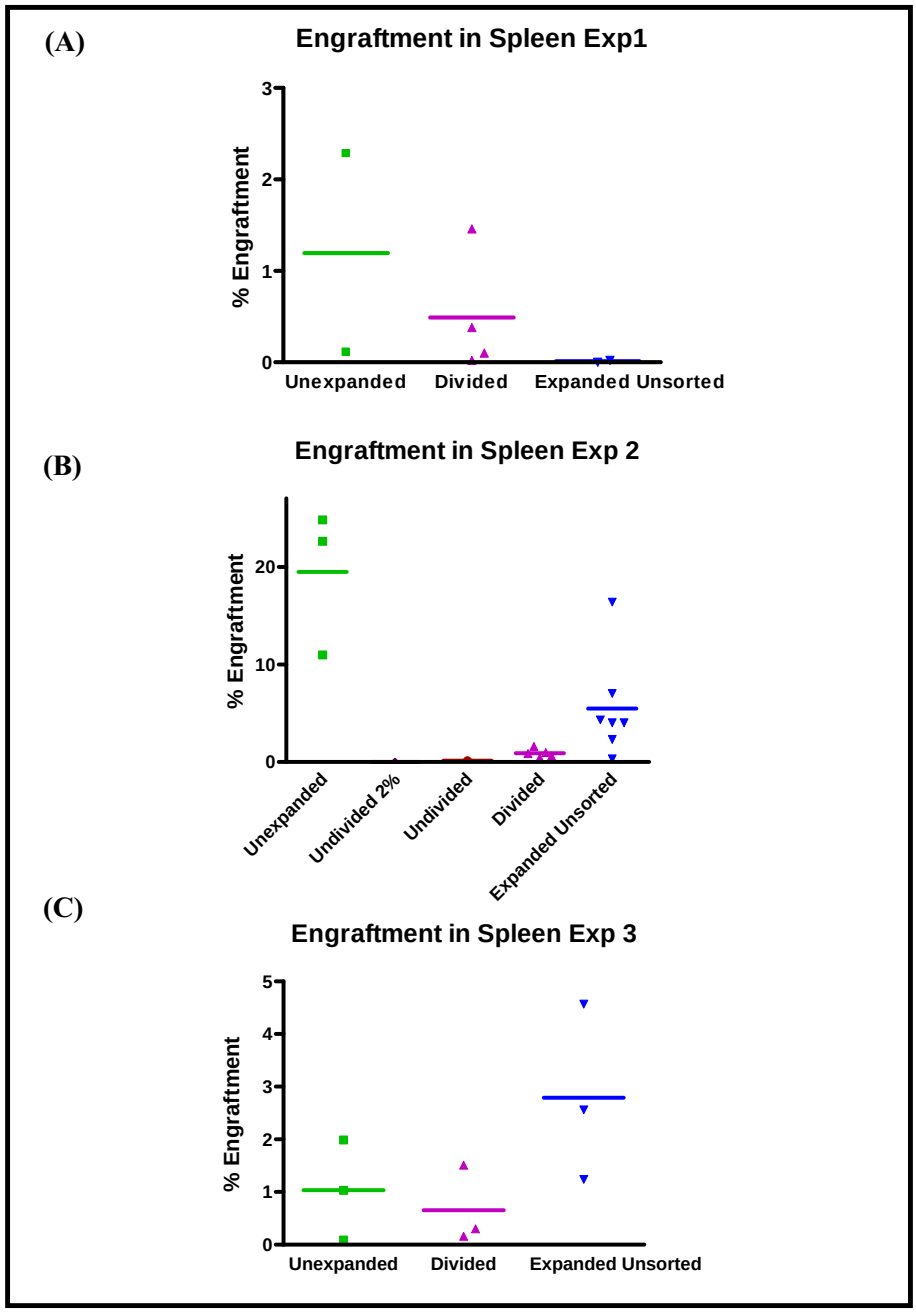

Figure 5.9 Human engraftment in spleen in undivided and divided cell populations

Splenocytes were harvested after cervical dislocation 6 weeks post transplant. The level of engraftment was assessed as % of human CD45+ cells of total CD45 population (human and mouse) in the spleen.

Unexpanded cells had a ratio of 0.27 and 0.21 to divided cells expanded with 4 and 3 factors respectively. That is, unexpanded cells had a 3.7 and 4.7 fold higher SEP than divided cells in experiment 1 and 3 respectively. The ratio of expanded cells unsorted on day 3 to unexpanded cells was 0.43 in experiment 3, that is unexpanded cells had a 2.3 fold higher SEP than expanded cells. Engraftment of the expanded cells unsorted on day 3 was unusually low in experiment 1, hence ratios were not calculated for this group.

Table 5.5 SEP values

SEP				
Experiment	Day 0 unexpanded $CD34^+$ cells	Day 3 expanded $CD34^+$ cells		
		Divided population	Expanded Unsorted	Divided/ Unexpanded
1	58.18	15.83	0.19	0.27
3	58.3	12.47	24.96	0.21

5.3.2.3 Multilineage engraftment

Three-colour flow cytometric analysis of BM was performed to examine multilineage engraftment. Cells were originally stained with CFSE and its fluorescence is detected on FL1 channel, however, when samples were examined, no CFSE fluorescent was detected in the PB, BM or splenocytes. T lymphoid and megakaryocytic engraftment was low in all groups transplanted (< 7.3% for CD3 and < 3.6% for CD61) as seen in

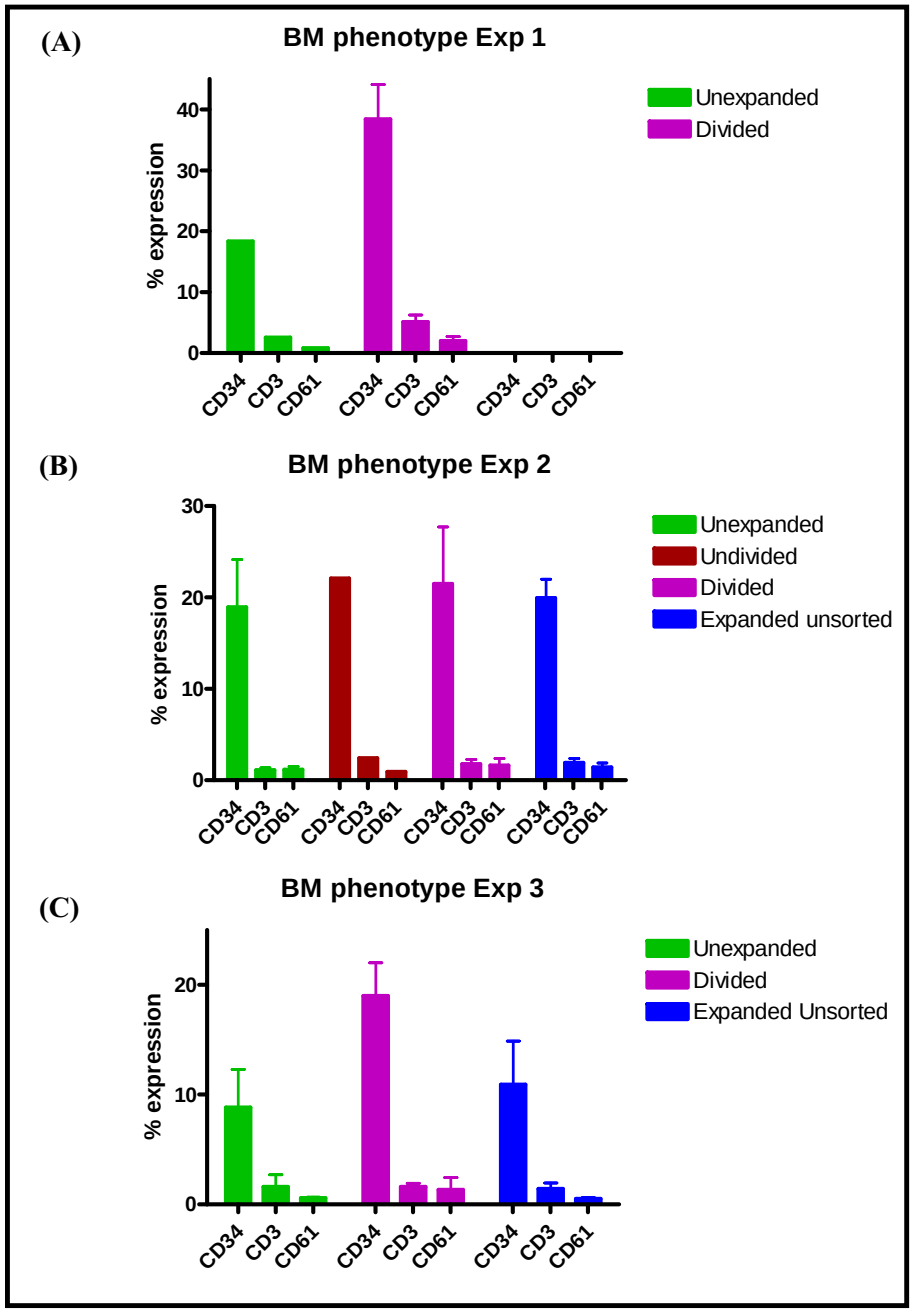

Figure 5.10 Multilineage engraftment in undivided and divided cell populations

Phenotype of engraftment was assessed in murine BM concurrent with human CD45 engraftment. CD3 and CD61 expression was below 7.3% and 3.6%, respectively in all experiments

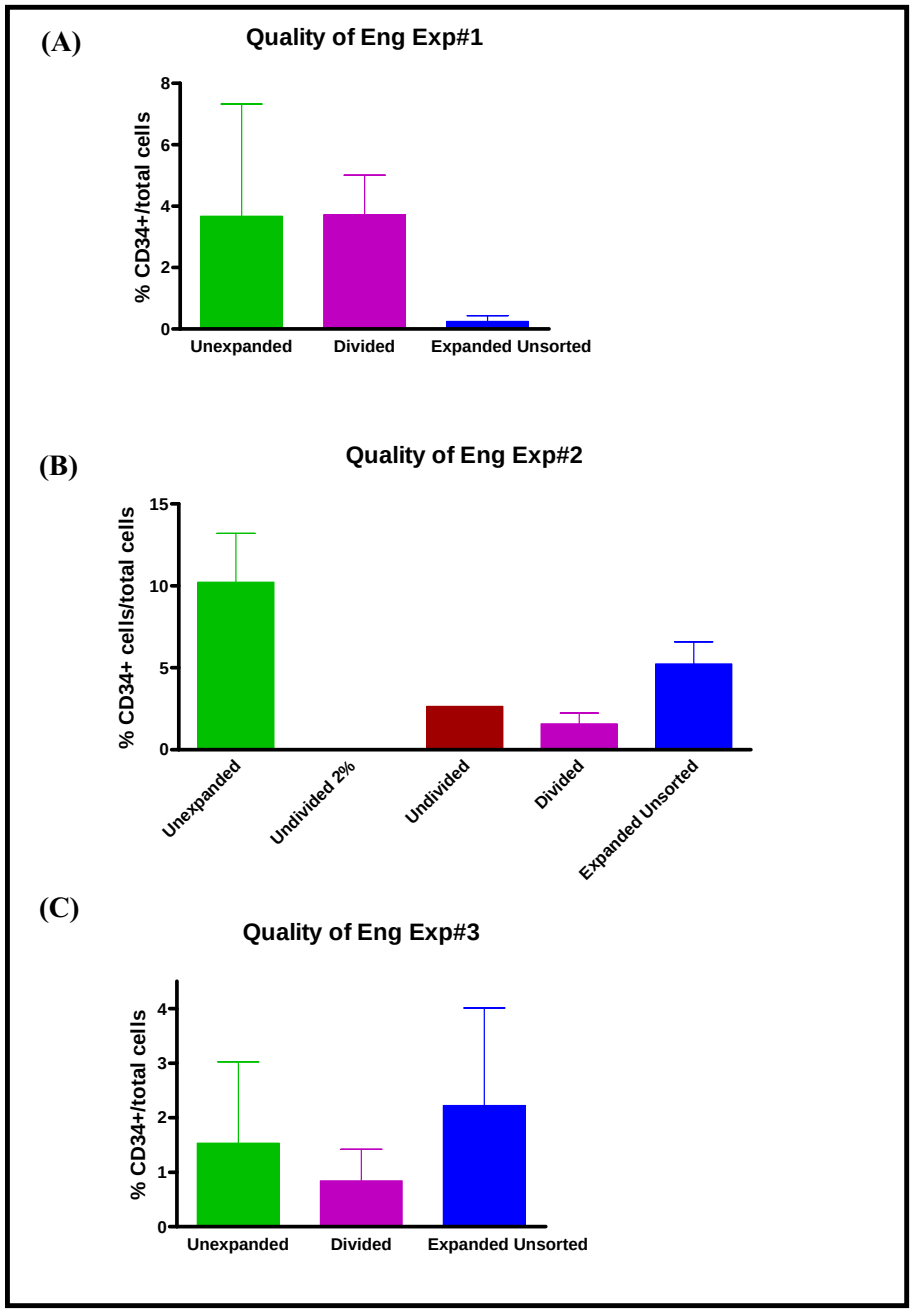

Figure 5.11 Quality of engraftment in undivided and divided cell populations

Quality of engraftment was assessed by the number of $CD34^+$ of the total number of CD45 cells in BM and expressed as a percentage.

Figure 5.10. Expression of CD3 and CD61 in the unexpanded population ranged between 0.49 - 2.69% and 0.61 - 1.73% respectively. Expression of these markers in expanded (unsorted) and divided populations did not vary greatly from the unexpanded cells. Quality of engraftment (Fig. 5.11) varied between experiments. Experiment 2 had the highest percentage of $CD34^+$ cells in unexpanded cells and the divided population compared to experiment 2 and 3. Unexpanded cells had a higher quality of engraftment than other populations in experiment 1 and 2, whilst expanded cells had a higher quality of engraftment in experiment 3.

5.3.3 Evaluation of human engraftment in NOD-SCID mice transplanted with expanded CB $CD34^+$ cells sorted for undivided and individual divisions

To evaluate the impact of cell division on engraftment potential, the divided cell compartment was examined more closely. The divided cells were sorted into their individual divisions and transplanted into mice. From the regions set up for sorting the CFSE peaks, the proportion of each fraction was calculated as a percentage of the total population. For experiment 4, undivided cells were 0.5%, division 1 6.5%, division 2 27%, division 3 37%. The same controls were used as for the previous experiments (day 0 unexpanded sorted cells and day 3 expanded unsorted cells).

5.3.3.1 Kinetics of Human engraftment in the PB

Engraftment of human cells in the PB was low for all mice in experiment 4 (Fig.5.12a). Only 4 mice were above limit of detection by flow cytometry (0.01%).

5.3.3.2 Detection of Human engraftment in BM and splenocytes

All mice transplanted with expanded cells that had undergone at least two divisions engrafted. There were very low levels of human engraftment (limit of sensitivity 0.1%) in undivided and division 1 groups of mice. SEP values are listed on Table 5.6. The levels of T lymphoid and megakaryocytic engraftment were below the sensitivity of detection by flow cytometry in this experiment.

This type of experiment proved to be very difficult to replicate, mainly due to the large number of cells required at the start point of the experiment in order to ensure engraftment (10^5). Hence, it was only successfully completed once, despite 3 separate attempts to repeat it and it is therefore difficult to draw conclusions from these results.

Table 5.6 SEP values

SEP						
Experiment	Day 0	Day 3 expanded $CD34^+$ cells				
	$CD34^+$ cells	Undivided	Div 1	Div 2	Div 3	Expanded unsorted
4	0.02	0.45	0.01	0.02	0.05	0.14

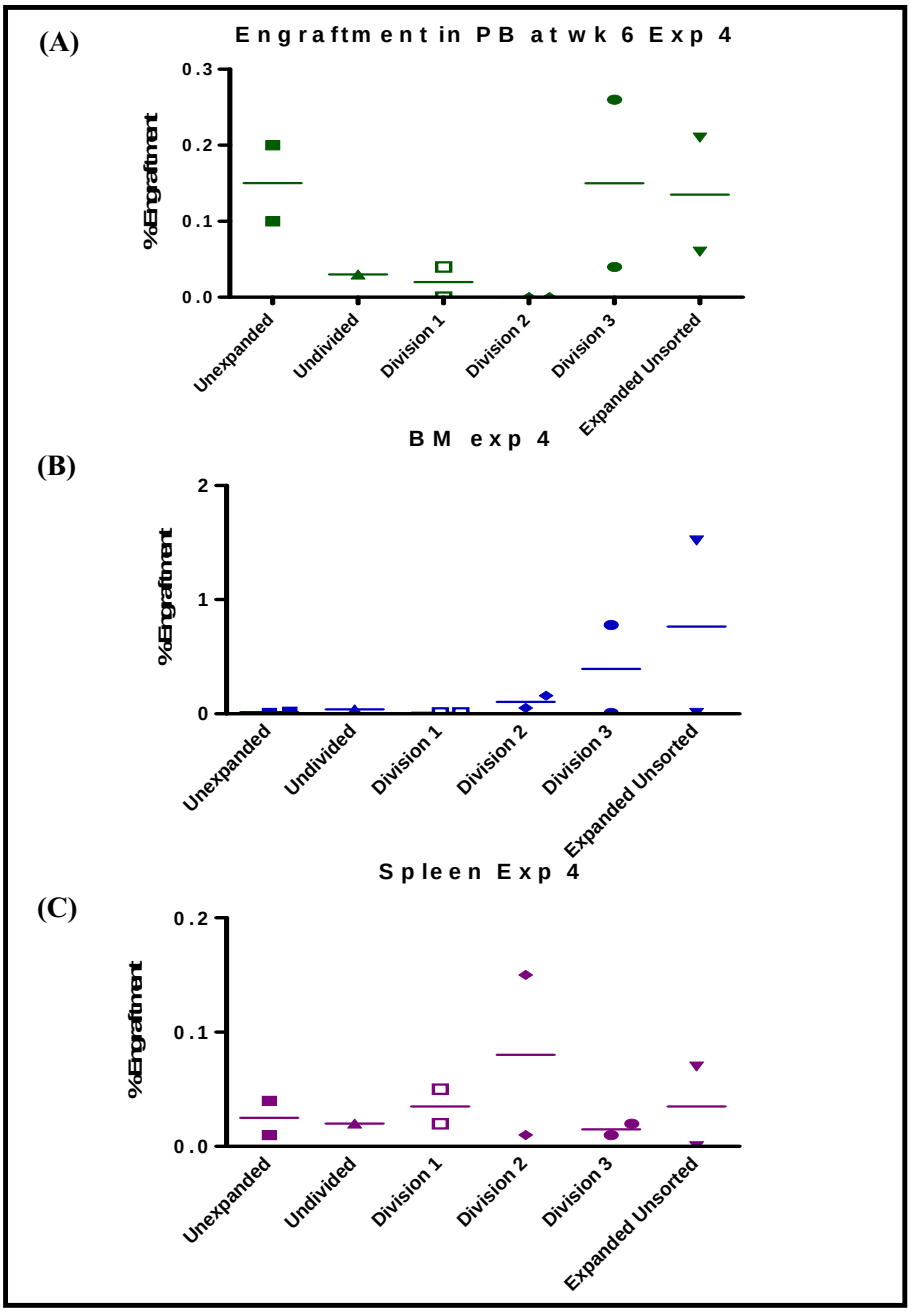

Figure 5.12 Human engraftment in CB CD34$^+$ expanded for 3 days and sorted for undivided cells and individual divisions.

The level of human engraftment was examined in BM, spleen and PB at 6 weeks post transplant. The limit of detection of the assay was 0.1% for BM and spleen and 0.01% for PB.

5.4 Discussion

5.4.1 Engraftment potential of CB CD34$^+$ cells and their progeny

In this study, the cell tracking dye CFSE was used to examine the relationship between cell division and engraftment potential in CB CD34$^+$ after a 3 day culture with SCF, MGDF, FL ± IL-3. Addition of IL-3 to the culture increased the number of cell divisions but it did not affect the phenotype distribution (Chapter 3). Rice et al. found no significant difference in engraftment levels by including IL-3 to the culture of cells transplanted in NOD-SCID mice (unpublished results). Addition of IL-3 also decreased the number of cells in undivided and early divisions (as seen in Chapter 3 also). Hence, in the experiments # 2 to # 4 in this section, IL-3 was not used for expansion of CB CD34$^+$ cells.

Our lab previously performed a study to determine the effects of the duration of cytokine-mediated expansion on CB CD34$^+$ cells (Rice et al., 2001). This study directly compared the duration of expansion using SCF, MGDF and FL for 7 and 14 days on the engraftment capacity of CB CD34$^+$ cells in the NOD/SCID mouse model. The overall finding was that prolonged exposure to the cytokine cocktail facilitates the rate and level of human engraftment in the NOD/SCID mouse. This suggests that longer expansion of the CB CD34$^+$ may be beneficial in the clinical setting. However, the purpose of the current study was not to determine whether 3 days expansion provided a substantial fold increase in the population to affect the engraftment capacity of CB CD34$^+$ but to determine the early changes caused by cytokine-mediated expansion on these cells and to correlate them to cell division and function.

Differences in fold expansion were observed between experiments, where delta values ranged from 0.7-1.64. This could be due to the inherent variability between CB samples. Usage of pooled CB samples has been proposed as a way to decrease the variability between single CB (Leung et al., 1999). However, in this study the large number of CB collections used per experiments (9 to 25) may have impacted on the quality of the expansions.

The highest level of engraftment was detected in unexpanded cells in experiment 1 and 2 and in 2/3 of mice in experiment 3 (Fig 5.7). In experiments #1-3, results showed that cells that have divided were still able to engraft the NOD-SCID mouse but at lower levels compared to unexpanded cells and the population of expanded cells, unsorted on day 3. These results are contrary to other reports were cytokine-mediated expansion of $CD34^+$ cells was detrimental to engraftment potential (Denning-Kendall et al., 2002, Novelli et al., 1999, Bhatia et al., 1997, Conneally et al., 1997) as a proportion of expanded and divided cell populations still retained their ability to engraft the NOD-SCID mouse.

An important report using the SCID-repopulating cell (SRC) assay showed that culture of CB $CD34^+CD38^-$ cells for up to 4 days with SCF, FL, G-CSF and IL-3 did not exhaust SRC capability, and increased cell numbers, CFC and the $CD34^+CD38^-$ population (Bhatia et al., 1997). Another report also showed that *ex vivo* expansion of CB $CD34^+CD38^-$ with SCF, FL, G-CSF, IL-3 and IL-6 for 5-8 days produced 100-fold expansion of CFC, 4-fold expansion of LTC-IC and 2-fold expansion of competitive repopulating units (CRU; NOD/SCID based assay) compared to its unexpanded counterpart (Conneally et al., 1997). These report highlight that the use of CB $CD34^+$

cells is still a relevant population to study as the current study confirms the finding that cytokine-mediated expansion does not ablate the engraftment capacity of CB $CD34^+$.

Another important question was whether CB $CD34^+$ cells that had undergone more than one cell division retained their engraftment capacity following cytokine-mediated expansion. In experiment 4 the divided cells were sorted into individual divisions but low levels of human cell engraftment were detected in all mice (limit of sensitivity 0.1%) particularly in the undivided and division 1 cohorts of mice. This observation may due to the low number of transplanted cells. This experiment could not be repeated due to the large numbers of $CD34^+$ cells required for this type of experiment and the low availability of CB samples as discussed in Section 5.4.2. However, it was still determined that cells that had undergone at least two divisions were able to engraft the NOD-SCID mouse.

Maintenance of engraftment capacity in cells that have undergone multiple divisions was also reported in the literature. However their approach differed to the current study as one group used the SCID-hu bone engraftment assay and the other did not sort individual divisions in the expanded population. Young et al. (1999) showed that MPB expanded over 5 days using 2 combinations of cytokines (TPO, FL, IL6 and TPO, FL, SCF) underwent between 1 to 4 divisions. HSC function as defined by CAFC and SCID-hu assays was maintained up to the 3^{rd} division in Thy-1^+ cells after culture. The SCID-hu assay however does not examine the ability of cells to home; it only measures the ability to engraft. Low levels of engraftment were observed in the study by Glimm and Eaves (1999) where CB cells were sorted for CFSE subpopulations after a 5-day culture (FL, SCF, IL-3, IL6 and G-CSF) and a comparison in engraftment levels was

made between cells that had divided ≤2 divisions and ≥3 divisions (0.2-5% and 1-24%, respectively). This study also found that most repopulating cells in both foetal liver and CB were stimulated to undergo cell division after cytokine expansion and that a proportion retained their engraftment potential.

5.4.2 Limitations of the study

As mentioned in previous Chapters, the main limitations of this study were low cell numbers and the number of samples required for these experiments. These problems were particularly detrimental to the experiments described in this Chapter as experiments required between 9 to 25 CB samples. These issues also limited the number of cells that could be injected per mouse, the number of mice per group and the number of times the experiments could be repeated.

A technical limitation was the cell sorter used in this study. At the time the experiments were performed a two-way cell sorter was available, the FACStar^{plus} flow cytometer. For the sorting strategy used on day 3 of culture, the sample had to be divided and two separate sorts were performed, one for undivided and division 2 cells and another for division 1 and division 3. Hence, in both sorts the populations not sorted were not collected and precious sample was wasted. An ideal machine for this study was the BD FACS Vantage SE Cell Sorter with DiVa option as it had the option to perform 4-way sorting. However this machine was not optimised for 4-way sorting in time to be used for the experiments described here.

5.4.3 Future directions and alternative approaches to the study

Recent advancements in technology would have greatly benefited the experimental work performed in this study if available at the time. The NOD/SCID mouse model has residual murine NK cell activity in these mice affecting the engraftment levels of cells transplanted. Two alternative approaches to improve engraftment in the NOD/SCID mouse model were developed including the use of intrafemoral injections (McKenzie et al., 2005) and administering *in vivo* injections of anti-IL-2Rβ (anti-CD122) in transplanted NOD/SCID mice (McKenzie et al., 2005, Shultz et al., 2003). Intrafemoral injections are a good alternative to tail vein injections to overcome any homing defects created by culture or expansion of the CB $CD34^+$ cells. *In vivo* injections of anti-CD122 reduce NK cell activity, thereby improving the engraftment of the samples in the animal. Another improvement in the field is the better models available for transplanting small number of cells (as low as 10^2). The NOD/Shi-*scid* γ_c^{null} or NOD/LtSz-*scid* IL2Rγ^{null} mouse models (Ito et al., 2002, Shultz et al., 2005) lack NK cell activity and $CD34^+$ cells engraft this mice at a higher level (up to 6 fold higher) in the BM, spleen and PB compared to NOD/SCID injected with anti-NK cell antibody and NOD/SCID/β2m^{null}.

Improvement in engraftment levels from low numbers of transplanted cells would have then allowed serial transplantation studies to be performed, a more specific test for HSC function following cytokine-mediated expansion. The self-renewal and serial engraftment capacity of CB $CD34^+$ cells following cytokine-mediated expansion would be examined as secondary and tertiary transplants provide a more definitive assay and functional measure of the quality of engraftment of transplanted cells.

Chapter 6

6.1 Conclusions

In this thesis the relationship between cell division, phenotype and engraftment potential of CB $CD34^+$ cells following cytokine-mediated expansion was investigated to determine if cytokine-mediated expansion is detrimental to the long-term engraftment capacity of CB $CD34^+$ cells. The results to each section of the study are discussed in details in Chapter 3, 4 and 5. Below I will discuss the overall findings, provide conclusions for the current study and future directions.

High resolution cell division tracking technique was shown to be a powerful tool to monitor changes in phenotype and function in CB $CD34^+$ cells as a consequence of cytokine-mediated expansion. In this study, cytokine-mediated expansion promoted divisional recruitment and induced up-regulation of lineage and proliferation markers and down-regulation of putative stem cell markers with concomitant cell division. However, when drawing conclusions on these phenotype changes caused by ex vivo expansion and their relationship to function, two points need to be highlighted; phenotype dissociation with function following culture with cytokines and the still uncertain phenotype of the human engrafting cell (Section 2.10.3).

The changes and in some cases maintenance of adhesion molecule expression determined in this study would suggest that cytokine-mediated expansion may be beneficial to the engraftment capacity of CB $CD34^+$ cells as seen in a study published after the completion of the experimental work in this thesis (Herrera et al., 2004). The

study compared different sources of $CD34^+$ cells and also showed an increase in adhesion molecule expression following cytokine-mediated expansion. However the changes induced in CXCR4 and adhesion molecule expression after expansion of human $CD34^+$ cells did not cause significant differences in the homing efficiency of these cells. Interestingly a trend was seeing towards higher homing efficiency in the NOD/SCID mouse in cells with positive CXCR4 and CD62L compared to the negative counterparts.

Cytokine-mediated expansion also caused changes in cell cycle related gene expression of G1 phase regulators. D cyclins were upregulated following stimulation with cytokines suggesting a positive effect considering their role in cell proliferation. p21 expression was also upregulated following cytokine-mediated expansion, an important finding considering its role promoting the association of CDK4 or CDK6 to the D cyclins (LaBaer et al., 1997) and as the molecular switch governing the entry of SC into the cell cycle while its absence may lead to increased cell cycling and hence to stem cell exhaustion (Cheng et al., 2000).

In summary, ex vivo expansion with cytokines caused a number of changes that according to the literature would improve the engraftment capacity of CB $CD34^+$ cells: an increase in the number of cells to be inoculated, an increase in adhesion molecule and CXCR4 expression, and an increase in D cyclin and p21 expression.

All these findings may explain why a proportion of expanded and divided cell populations still retained their ability to engraft the NOD-SCID mouse after cytokine-mediated expansion. Since the mean repopulation capacity was lower in cytokine

expanded CB $CD34^+$ cells compared to unmanipulated cells, the next question to address is whether the net retention of engraftment is due to a balance of factors that decrease versus others that increase the engrafting capacity of CB $CD34^+$ cells. Hence it is important to further investigate the determined changes in this study and examine which are functionally relevant.

Overall, cytokine-mediated expansion of CB $CD34^+$ cells was not completely detrimental to engraftment potential as has been seen in other reports where CB $CD34^+$ $CD38^-$ cells were used (Bhatia et al., 1997, Conneally et al., 1997). These reports showed that *ex vivo* expansion of haematopoietic cells with cytokines supported both short- and long-term engraftment and were not detrimental to serial transplantation, suggesting that culture for up to 4 days did not exhaust SRC capability. Another report highlighted the importance of using the right combination of cytokines in *ex vivo* expansion. MPB $CD34^+$ cells were expanded with SCF, TPO and FL and showed engraftment of NOD/SCID mice was at levels similar to that seen with unexpanded cells. However, addition of IL-3 and IL-6 completely abrogated the engraftment ability of these cells (Herrera et al., 2001).

6.2 Future directions

The limitations affecting each section of the study and specific future directions have been addressed in the discussion section of each respective chapter. A summary of future directions is given below including other general future directions and alternative approaches now available to this study due to advancements in the field and technology.

In vitro assays such as LTC-IC were suggested to further characterise the differences between untreated and expanded populations of CB $CD34^+$ cells, in particular the undivided and divided cells, and also the resulting engrafted cells following transplantation in the recently generated NOD/SCID/γ_c^{null} mouse model. This mouse model would improve engraftment of small number of cells and allow serial transplantation experiments to be performed, giving further insight on the self renewal capacity of expanded CB $CD34^+$ that have divided in culture. Since it was determined that expansion did not ablate engraftment in the divided cells, the next step is to investigate why engraftment capacity was diminished in these cells.

Migration studies are of interest due to the changes in adhesion molecule expression determined in this study. Chemotaxis assays could be used to assess the ability of cultured cells to migrate following cytokine-mediated expansion. Further studies could include blocking with CXCR4 antagonists such as AMD3100 and TC14012 (Broxmeyer et al., 2008) to investigate the role of CXCR4 and SDF-1 in these cells and whether migration is affected in the different populations, untreated compared to undivided cells and divided cells. Another approach would be sorting populations based on their phenotype (eg. CD62L) and comparing the positive versus negative populations by *in vitro* assays and transplanting them into *in vivo* models.

It would also be of interest to determine the levels of SDF-1 in untreated and treated cells by an assay such as ELISA as it was recently identified that endogenous and exogenous SDF-1 increased D1 and D3 cyclins in PB $CD34^+$ (Chabanon et al. 2008).

Investigating the gene expression of G1 cell cycle markers is not clear cut, as a number of signalling pathways are continuously interacting with one another. Their role in haematopoiesis may be attenuated by other molecules compensating or interacting, hence the use of cell cycle inhibitors in sorted divided populations may be more specific.

Advancement in cell sorting technology has broadened the capacity to perform complex and more accurate single cell studies. Single cell experiments, described in more detail in Chapter 4 (page 142) were suggested as a more powerful tool to investigate a number of genes simultaneously on the one sorted cell and further characterise the engrafting cell. In the experiment suggested, the phenotype and the specific cell division of the progeny of the sorted cell would be chosen and more than 9 molecular markers would be able studied on the same cell. Hence a molecular phenotype could be correlated to the surface marker phenotype of the cell under investigation and address the aims of this study more efficiently.

Another approach would be to investigate the effect cytokine-mediated expansion on other pathways such as apoptosis. Suppression of apoptosis plays an important role in SC self renewal (Domen and Weissman 2000). Viability in this study was assessed by Trypan blue in all experiments and no changes were recorded. However as a preliminary assay the different population could be assessed by Annexin V and propidium iodide to determine whether the cytokine cocktail used in this study protected or promoted apoptosis in these cells.

Other areas of interest that have only surfaced recently in the literature are the roles of PTEN and the phosphatidylinostol-3-OH kinase (PI(3)K) - Akt pathway in cell cycle progression. The PI(3)K-Akt pathway has a crucial role in cell proliferation, survival, differentiation and migration while PTEN has been shown to be a negative regulator of this pathway (Stiles et al., 2004, Cully et al., 2006). That is, PTEN controls cell cycle entry through inhibition of the PI(3)K-Akt pathway and it is expressed mostly by cells in the G1 phase of the cell cycle that are Cyclin D1 positive (Zhang et al., 2006). This study also determined that Cyclin D1 is a target of the PI(3)K-Akt pathway. D cyclins may not play a direct role in cell cycle progression, but further investigation of their function in other related pathways is central to a better understanding of cell proliferation. This knowledge is crucial for the ex vivo expansion field and may provide further insight to the D cyclins role in cancer.

Bone Morphogenesis Protein (BMP) signalling pathway is also relevant to this study due to its involvement in maintaining SC properties, particularly self renewal (Zhang and Li 2005) and its control over the stem cell niche size (Zhang et al., 2003). Neither the specific function of BMP in HSC regulation nor the effects of ex vivo expansion on of HSC on BMP are currently known.

6.3 Significance of the study

This study confirms reports in the literature that cytokine-mediated expansion compromises HSC function as engraftment in expanded cells was lower as compared to untreated cells. Even though extensive research has focused on expansion of haematopoietic progenitors and stem cells prior to transplantation by cytokine driven

systems, overall cytokine-mediated expansion has been a disappointing strategy (Hofmeister et al., 2007). The cytokines used in this study still remain relevant in the literature (Zheng et al., 2005, Levac et al., 2005, Gammaitoni et al., 2004) as candidate cytokines for ex vivo expansion of human haematopoietic stem and progenitor cells but other factors may need to be included in the ex vivo expansion strategy for a successful outcome. The increased knowledge of the haematopoietic niche and cell cycle regulation in recent years (Myatt and Lam, 2007, Hofmeister et al., 2007, Ballen, 2007, Scadden, 2006) together with improvement of current in vivo assays may lead to a better understanding of the human engrafting cell and to the discovery of new strategies for ex vivo expansion.

Characterisation of the effects of cytokine stimulation on stem cell progeny, as performed in this study, will lead to the development of clinically relevant cytokine-mediated expansion protocols to further improve the safety of haematopoietic stem cell transplantation.

References

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

Alcorn, M.J., Holyoake, T.L., Richmond, L., Pearson, C., Farrell, E., Kyle, B., Dunlop, D.J., Fitzsimons, E., Steward, W.P., Pragnell, I.B. & Franklin, I.M. (1996) CD34-positive cells isolated from cryopreserved peripheral-blood progenitor cells can be expanded ex vivo and used for transplantation with little or no toxicity. *J Clin Oncol,* **14,** 1839-1847.

Allsopp, R.C., Cheshier, S. & Weissman, I.L. (2001) Telomere shortening accompanies increased cell cycle activity during serial transplantation of hematopoietic stem cells. *J Exp Med,* **193,** 917-924.

Allsopp, R.C. & Weissman, I.L. (2002) Replicative senescence of hematopoietic stem cells during serial transplantation: does telomere shortening play a role? *Oncogene,* **21,** 3270-3273.

Almasan, A., Linke, S.P., Paulson, T.G., Huang, L.C. & Wahl, G.M. (1995) Genetic instability as a consequence of inappropriate entry into and progression through S-phase. *Cancer Metastasis Rev,* **14,** 59-73.

Ando, K., Ajchenbaum-Cymbalista, F. & Griffin, J.D. (1993) Regulation of G1/S transition by cyclins D2 and D3 in hematopoietic cells. *Proc Natl Acad Sci U S A,* **90,** 9571-9575.

Andrews, R.G., Singer, J.W. & Bernstein, I.D. (1986) Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. *Blood,* **67,** 842-845.

Andrews, R.G., Singer, J.W. & Bernstein, I.D. (1989) Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *J Exp Med,* **169,** 1721-1731.

Andrews, R.G., Singer, J.W. & Bernstein, I.D. (1990) Human hematopoietic precursors in long-term culture: single CD34+ cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells. *J Exp Med,* **172,** 355-358.

Baines, P. & Visser, J.W. (1983) Analysis and separation of murine bone marrow stem cells by H33342 fluorescence-activated cell sorting. *Exp Hematol,* **11,** 701-708.

Ballen, K. (2007) Targeting the stem cell niche: squeezing blood from bones. *Bone Marrow Transplant,* **39,** 655-660.

Banu, N., Wang, J.F., Deng, B., Groopman, J.E. & Avraham, H. (1995) Modulation of megakaryocytopoiesis by thrombopoietin: the c-Mpl ligand. *Blood,* **86,** 1331-1338.

Barker, J.N., Weisdorf, D.J., DeFor, T.E., Blazar, B.R., McGlave, P.B., Miller, J.S., Verfaillie, C.M. & Wagner, J.E. (2005) Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood,* **105,** 1343-1347.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M. & Peault, B. (1992) Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A,* **89,** 2804-2808.

Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamasz, D., Knitter, G., Buckner, C.D. & Bernstein, I.D. (1988) Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J Clin Invest,* **81,** 951-955.

Berrios, V.M., Dooner, G.J., Nowakowski, G., Frimberger, A., Valinski, H., Quesenberry, P.J. & Becker, P.S. (2001) The molecular basis for the cytokine-induced defect in homing and engraftment of hematopoietic stem cells. *Exp Hematol,* **29,** 1326-1335.

Bertolini, F., Lazzari, L., Lauri, E., Corsini, C. & Sirchia, G. (1994) Cord blood plasma-mediated ex vivo expansion of hematopoietic progenitor cells. *Bone Marrow Transplant,* **14,** 347-353.

Bertoncello, I., Hodgson, G.S. & Bradley, T.R. (1985) Multiparameter analysis of transplantable hemopoietic stem cells: I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. *Exp Hematol,* **13,** 999-1006.

Bhatia, M., Bonnet, D., Kapp, U., Wang, J.C., Murdoch, B. & Dick, J.E. (1997) Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. *J Exp Med,* **186,** 619-624.

Bhatia, M., Bonnet, D., Murdoch, B., Gan, O.I. & Dick, J.E. (1998) **A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med,* **4,** 1038-1045.

Bonnet, D. (2002) Haematopoietic stem cells. *J Pathol,* **197,** 430-440.

Bonnet, D., Bhatia, M., Wang, J.C., Kapp, U. & Dick, J.E. (1999) Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. *Bone Marrow Transplant,* **23,** 203-209.

Brandt, J., Srour, E.F., van Besien, K., Briddell, R.A. & Hoffman, R. (1990) Cytokine-dependent long-term culture of highly enriched precursors of hematopoietic progenitor cells from human bone marrow. *J Clin Invest,* **86,** 932-941.

Breems, D.A., Blokland, E.A., Neben, S. & Ploemacher, R.E. (1994) Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. *Leukemia,* **8,** 1095-1104.

Broudy, V.C. (1997) Stem cell factor and hematopoiesis. *Blood,* **90,** 1345-1364.

Broudy, V.C., Lin, N.L. & Kaushansky, K. (1995) Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. *Blood,* **85,** 1719-1726.

Broxmeyer, H.E. (2008) Chemokines in hematopoiesis. *Curr Opin Hematol,* **15,** 49-58.

Broxmeyer, H.E., Cooper, S., Lu, L., Hangoc, G., Anderson, D., Cosman, D., Lyman, S.D. & Williams, D.E. (1991) Effect of murine mast cell growth factor (c-kit proto-oncogene ligand) on colony formation by human marrow hematopoietic progenitor cells. *Blood,* **77,** 2142-2149.

Broxmeyer, H.E., Hangoc, G., Cooper, S., Ribeiro, R.C., Graves, V., Yoder, M., Wagner, J., Vadhan-Raj, S., Benninger, L., Rubinstein, P. & et al. (1992) Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults. *Proc Natl Acad Sci U S A,* **89,** 4109-4113.

Broxmeyer, H.E., Gluckman, E., Auerbach, A., Douglas, G.W., Friedman, H., Cooper, S., Hangoc, G., Kurtzberg, J., Bard, J. & Boyse, E.A. (1990) Human umbilical cord blood: a clinically useful source of transplantable hematopoietic stem/progenitor cells. *Int J Cell Cloning,* **8 Suppl 1,** 76-89; discussion 89-91.

Brugger, W., Heimfeld, S., Berenson, R.J., Mertelsmann, R. & Kanz, L. (1995) Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo. *N Engl J Med,* **333,** 283-287.

Brummendorf, T.H., Dragowska, W. & Lansdorp, P.M. (1999) Asymmetric cell divisions in hematopoietic stem cells. *Ann N Y Acad Sci,* **872,** 265-272; discussion 272-263.

Brummendorf, T.H., Rufer, N., Baerlocher, G.M., Roosnek, E. & Lansdorp, P.M. (2001) Limited telomere shortening in hematopoietic stem cells after transplantation. *Ann N Y Acad Sci,* **938,** 1-7; discussion 7-8.

Bryder, D. & Jacobsen, S.E. (2000) Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. *Blood,* **96,** 1748-1755.

Buhring, H.J., Asenbauer, B., Katrilaka, K., Hummel, G. & Busch, F.W. (1989) Sequential expression of CD34 and CD33 antigens on myeloid colony-forming cells. *Eur J Haematol,* **42,** 143-149.

Burkhart, C.A., Cheng, A.J., Madafiglio, J., Kavallaris, M., Mili, M., Marshall, G.M., Weiss, W.A., Khachigian, L.M., Norris, M.D. & Haber, M. (2003) Effects of

MYCN Antisense Oligonucleotide Administration on Tumorigenesis in a Murine Model of Neuroblastoma. *J. Natl. Cancer Inst.,* **95,** 1394-1403.

Burns, C.E. & Zon, L.I. (2006) Homing Sweet Homing: Odyssey of Hematopoietic Stem Cells. *Immunity,* **25,** 859-862.

Cairo, M.S. & Wagner, J.E. (1997) Placental and/or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. *Blood,* **90,** 4665-4678.

Cardoso, A.A., Watt, S.M., Batard, P., Li, M.L., Hatzfeld, A., Genevier, H. & Hatzfeld, J. (1995) An improved panning technique for the selection of CD34+ human bone marrow hematopoietic cells with high recovery of early progenitors. *Exp Hematol,* **23,** 407-412.

Case, J., Rice, A., Wood, J., Gaudry, L., Vowels, M. & Nordon, R.E. (2001) Characterization of cytokine interactions by flow cytometry and factorial analysis. *Cytometry,* **43,** 69-81.

Cashman, J., Bockhold, K., Hogge, D.E., Eaves, A.C. & Eaves, C.J. (1997a) Sustained proliferation, multi-lineage differentiation and maintenance of primitive human haemopoietic cells in NOD/SCID mice transplanted with human cord blood. *Br J Haematol,* **98,** 1026-1036.

Cashman, J.D., Lapidot, T., Wang, J.C., Doedens, M., Shultz, L.D., Lansdorp, P., Dick, J.E. & Eaves, C.J. (1997b) Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood,* **89,** 4307-4316.

Challen, G.A. & Little, M.H. (2006) A side order of stem cells: the SP phenotype. *Stem Cells,* **24,** 3-12.

Chan, J.Y. & Watt, S.M. (2001) Adhesion receptors on haematopoietic progenitor cells. *Br J Haematol,* **112,** 541-557.

Chaudhary, P.M. & Roninson, I.B. (1991) Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell,* **66,** 85-94.

Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M. & Scadden, D.T. (2000) Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science,* **287,** 1804-1808.

Cheshier, S.H., Morrison, S.J., Liao, X. & Weissman, I.L. (1999) In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A,* **96,** 3120-3125.

Christopherson, K., 2nd & Hromas, R. (2001) Chemokine regulation of normal and pathologic immune responses. *Stem Cells,* **19,** 388-396.

Chute, J.P., Saini, A.A., Kampen, R.L., Wells, M.R. & Davis, T.A. (1999) A comparative study of the cell cycle status and primitive cell adhesion molecule

profile of human CD34+ cells cultured in stroma-free versus porcine microvascular endothelial cell cultures. *Exp Hematol,* **27,** 370-379.

Civin, C.I., Banquerigo, M.L., Strauss, L.C. & Loken, M.R. (1987) Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of My-10-positive progenitor cells in normal human bone marrow. *Exp Hematol,* **15,** 10-17.

Civin, C.I., Strauss, L.C., Brovall, C., Fackler, M.J., Schwartz, J.F. & Shaper, J.H. (1984) Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol,* **133,** 157-165.

Civin, C.I., Trischmann, T., Kadan, N.S., Davis, J., Noga, S., Cohen, K., Duffy, B., Groenewegen, I., Wiley, J., Law, P., Hardwick, A., Oldham, F. & Gee, A. (1996) Highly purified CD34-positive cells reconstitute hematopoiesis. *J Clin Oncol,* **14,** 2224-2233.

Conneally, E., Cashman, J., Petzer, A. & Eaves, C. (1997) Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc Natl Acad Sci U S A,* **94,** 9836-9841.

Cooper, A.B., Sawai, C.M., Sicinska, E., Powers, S.E., Sicinski, P., Clark, M.R. & Aifantis, I. (2006) A unique function for cyclin D3 in early B cell development. *Nat Immunol,* **7,** 489-497.

Coulombel, L. (2004) Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. *Oncogene,* **23,** 7210-7222.

Cowling, G.J. & Dexter, T.M. (1994) Apoptosis in the haemopoietic system. *Philos Trans R Soc Lond B Biol Sci,* **345,** 257-263.

Craig, W., Kay, R., Cutler, R.L. & Lansdorp, P.M. (1993) Expression of Thy-1 on human hematopoietic progenitor cells. *J Exp Med,* **177,** 1331-1342.

Cully, M., You, H., Levine, A.J. & Mak, T.W. (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer,* **6,** 184-192.

Cumano, A. & Godin, I. (2001) Pluripotent hematopoietic stem cell development during embryogenesis. *Curr Opin Immunol,* **13,** 166-171.

Cumano, A. & Godin, I. (2007) Ontogeny of the Hematopoietic System. *Annual Review of Immunology,* **25,** 745-785.

Cutler, C. & Antin, J.H. (2001) Peripheral blood stem cells for allogeneic transplantation: a review. *Stem Cells,* **19,** 108-117.

Danet, G.H., Lee, H.W., Luongo, J.L., Simon, M.C. & Bonnet, D.A. (2001) Dissociation between stem cell phenotype and NOD/SCID repopulating activity

in human peripheral blood CD34+ cells after ex vivo expansion. *Experimental Hematology,* **29,** 1465-1473.

de Bruijn, M.F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M.J. & Dzierzak, E. (2002) Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity,* **16,** 673-683.

De Felice, L., Di Pucchio, T., Mascolo, M.G., Agostini, F., Breccia, M., Guglielmi, C., Ricciardi, M.R., Tafuri, A., Screnci, M., Mandelli, F. & Arcese, W. (1999) Flt3LP3nduces the ex-vivo amplification of umbilical cord blood committed progenitors and early stem cells in short-term cultures. *Br J Haematol,* **106,** 133-141.

Delassus, S., Titley, I. & Enver, T. (1999) Functional and molecular analysis of hematopoietic progenitors derived from the aorta-gonad-mesonephros region of the mouse embryo. *Blood,* **94,** 1495-1503.

Della Ragione, F., Borriello, A., Mastropietro, S., Della Pietra, V., Monno, F., Gabutti, V., Locatelli, F., Bonsi, L., Bagnara, G.P. & Iolascon, A. (1997) Expression of G1-phase cell cycle genes during hematopoietic lineage. *Biochem Biophys Res Commun,* **231,** 73-76.

Denning-Kendall, P.A., Evely, R., Singha, S., Chapman, M., Bradley, B.A. & Hows, J.M. (2002) In vitro expansion of cord blood does not prevent engraftment of severe combined immunodeficient repopulating cells. *Br J Haematol,* **116,** 218-228.

Denning-Kendall, P.A., Horsley, H., Donaldson, C., Bradley, B. & Hows, J.M. (1999) Different behaviour of fresh and cultured CD34+ cells during immunomagnetic separation. *Br J Haematol,* **105,** 780-785.

Dick, J.E., Bhatia, M., Gan, O., Kapp, U. & Wang, J.C. (1997) Assay of human stem cells by repopulation of NOD/SCID mice. *Stem Cells,* **15 Suppl 1,** 199-203; discussion 204-197.

Dick, J.E., Lapidot, T. & Pflumio, F. (1991) Transplantation of normal and leukemic human bone marrow into immune-deficient mice: development of animal models for human hematopoiesis. *Immunol Rev,* **124,** 25-43.

Ding, Z., Issekutz, T.B., Downey, G.P. & Waddell, T.K. (2003) L-selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: implications for cellular activation during adhesion and migration. *Blood,* **101,** 4245-4252.

Domen, J., Cheshier, S.H. & Weissman, I.L. (2000) The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. *J Exp Med,* **191,** 253-264.

Domen, J. & Weissman, I.L. (1999) Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Mol Med Today,* **5,** 201-208.

Dorrell, C., Gan, O.I., Pereira, D.S., Hawley, R.G. & Dick, J.E. (2000) Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood,* **95,** 102-110.

Dravid, G. & Rao, S.G. (2002) Ex vivo expansion of stem cells from umbilical cord blood: expression of cell adhesion molecules. *Stem Cells,* **20,** 183-189.

Eapen, M., Rubinstein, P., Zhang, M.J., Stevens, C., Kurtzberg, J., Scaradavou, A., Loberiza, F.R., Champlin, R.E., Klein, J.P., Horowitz, M.M. & Wagner, J.E. (2007) Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet,* **369,** 1947-1954.

Eaves, C., Miller, C., Cashman, J., Conneally, E., Petzer, A., Zandstra, P. & Eaves, A. (1997) Hematopoietic stem cells: inferences from in vivo assays. *Stem Cells,* **15 Suppl 1,** 1-5.

Ema, H., Suda, T., Miura, Y. & Nakauchi, H. (1990) Colony formation of clone-sorted human hematopoietic progenitors. *Blood,* **75,** 1941-1946.

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J. & Livingston, D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell,* **73,** 487-497.

Fink, J.R. & LeBien, T.W. (2001) Novel expression of cyclin-dependent kinase inhibitors in human B-cell precursors. *Experimental Hematology,* **29,** 490-498.

Fleming, W.H., Alpern, E.J., Uchida, N., Ikuta, K., Spangrude, G.J. & Weissman, I.L. (1993) Functional heterogeneity is associated with the cell cycle status of murine hematopoietic stem cells. *J Cell Biol,* **122,** 897-902.

Ford, C.E., Hamerton, J.L., Barnes, D.W. & Loutit, J.F. (1956) Cytological identification of radiation-chimaeras. *Nature,* **177,** 452-454.

Furukawa, Y., Kikuchi, J., Nakamura, M., Iwase, S., Yamada, H. & Matsuda, M. (2000) Lineage-specific regulation of cell cycle control gene expression during haematopoietic cell differentiation. *Br J Haematol,* **110,** 663-673.

Gammaitoni, L., Weisel, K.C., Gunetti, M., Wu, K.D., Bruno, S., Pinelli, S., Bonati, A., Aglietta, M., Moore, M.A. & Piacibello, W. (2004) Elevated telomerase activity and minimal telomere loss in cord blood long-term cultures with extensive stem cell replication. *Blood,* **103,** 4440-4448.

Glimm, H. & Eaves, C.J. (1999) Direct evidence for multiple self-renewal divisions of human in vivo repopulating hematopoietic cells in short-term culture. *Blood,* **94,** 2161-2168.

Glimm, H., Oh, I.L.H. & Eaves, C.J. (2000) Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G2/M transit and do not reenter G0. *Blood,* **96,** 4185-4193.

Gluckman, E., Rocha, V., Boyer-Chammard, A., Locatelli, F., Arcese, W., Pasquini, R., Ortega, J., Souillet, G., Ferreira, E., Laporte, J.P., Fernandez, M. & Chastang, C. (1997) Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. *N Engl J Med,* **337,** 373-381.

Godin, I., Garcia-Porrero, J.A., Dieterlen-Lievre, F. & Cumano, A. (1999) Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J Exp Med,* **190,** 43-52.

Godin, I.E., Garcia-Porrero, J.A., Coutinho, A., Dieterlen-Lievre, F. & Marcos, M.A. (1993) Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature,* **364,** 67-70.

Gong, J., Bhatia, U., Traganos, F. & Darzynkiewicz, Z. (1995) Expression of cyclins A, D2 and D3 in individual normal mitogen stimulated lymphocytes and in MOLT-4 leukemic cells analyzed by multiparameter flow cytometry. *Leukemia,* **9,** 893-899.

Gothot, A., Pyatt, R., McMahel, J., Rice, S. & Srour, E.F. (1997) Functional Heterogeneity of Human CD34+ Cells Isolated in Subcompartments of the G0 /G1 Phase of the Cell Cycle. *Blood,* **90,** 4384-4393.

Gothot, A., Pyatt, R., McMahel, J., Rice, S. & Srour, E.F. (1998a) Assessment of proliferative and colony-forming capacity after successive in vitro divisions of single human CD34+ cells initially isolated in G0. *Exp Hematol,* **26,** 562-570.

Gothot, A., van der Loo, J.C., Clapp, D.W. & Srour, E.F. (1998b) Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood CD34(+) cells in non-obese diabetic/severe combined immune-deficient mice. *Blood,* **92,** 2641-2649.

Greiner, D.L., Hesselton, R.A. & Shultz, L.D. (1998) SCID mouse models of human stem cell engraftment. *Stem Cells,* **16,** 166-177.

Gurney, A.L., Carver-Moore, K., de Sauvage, F.J. & Moore, M.W. (1994) Thrombocytopenia in c-mpl-deficient mice. *Science,* **265,** 1445-1447.

Haar, J.L. & Ackerman, G.A. (1971) A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat Rec,* **170,** 199-223.

Habibian, H.K., Peters, S.O., Hsieh, C.C., Wuu, J., Vergilis, K., Grimaldi, C.I., Reilly, J., Carlson, J.E., Frimberger, A.E., Stewart, F.M. & Quesenberry, P.J. (1998) The fluctuating phenotype of the lymphohematopoietic stem cell with cell cycle transit. *J Exp Med,* **188,** 393-398.

Hao, Q.L., Thiemann, F.T., Petersen, D., Smogorzewska, E.M. & Crooks, G.M. (1996) Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood,* **88,** 3306-3313.

Harrison, D.E. (1980) Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood,* **55,** 77-81.

Haylock, D.N., Horsfall, M.J., Dowse, T.L., Ramshaw, H.S., Niutta, S., Protopsaltis, S., Peng, L., Burrell, C., Rappold, I., Buhring, H.J. & Simmons, P.J. (1997) Increased recruitment of hematopoietic progenitor cells underlies the ex vivo expansion potential of FLT3 ligand. *Blood,* **90,** 2260-2272.

Heike, T. & Nakahata, T. (2002) Ex vivo expansion of hematopoietic stem cells by cytokines. *Biochim Biophys Acta,* **1592,** 313-321.

Heinrich, M.C., Dooley, D.C., Freed, A.C., Band, L., Hoatlin, M.E., Keeble, W.W., Peters, S.T., Silvey, K.V., Ey, F.S., Kabat, D. & et al. (1993) Constitutive expression of steel factor gene by human stromal cells. *Blood,* **82,** 771-783.

Herrera, C., Sanchez, J., Torres, A., Pascual, A., Rueda, A. & Alvarez, M.A. (2004) Pattern of expression of CXCR4 and adhesion molecules by human CD34+ cells from different sources: role in homing efficiency in NOD/SCID mice. *Haematologica,* **89,** 1037-1045.

Herrera, C., Sanchez, J., Torres, A., Bellido, C., Rueda, A. & Alvarez, M.A. (2001) Early-acting cytokine-driven ex vivo expansion of mobilized peripheral blood CD34+ cells generates post-mitotic offspring with preserved engraftment ability in non-obese diabetic/severe combined immunodeficient mice. *Br J Haematol,* **114,** 920-930.

Hickey, M.J., Forster, M., Mitchell, D., Kaur, J., De Caigny, C. & Kubes, P. (2000) L-selectin facilitates emigration and extravascular locomotion of leukocytes during acute inflammatory responses in vivo. *J Immunol,* **165,** 7164-7170.

Hirsch, E., Iglesias, A., Potocnik, A.J., Hartmann, U. & Fassler, R. (1996) Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. *Nature,* **380,** 171-175.

Ho, A.D. & Wagner, W. (2007) The beauty of asymmetry: asymmetric divisions and self-renewal in the haematopoietic system. *Curr Opin Hematol,* **14,** 330-336.

Hofmeister, C.C., Zhang, J., Knight, K.L., Le, P. & Stiff, P.J. (2007) Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. *Bone Marrow Transplant,* **39,** 11-23.

Hogan, C.J., Shpall, E.J., McNulty, O., McNiece, I., Dick, J.E., Shultz, L.D. & Keller, G. (1997) Engraftment and development of human CD34(+)-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. *Blood,* **90,** 85-96.

Holyoake, T.L., Alcorn, M.J., Richmond, L., Farrell, E., Pearson, C., Green, R., Dunlop, D.J., Fitzsimons, E., Pragnell, I.B. & Franklin, I.M. (1997) CD34 positive PBPC expanded ex vivo may not provide durable engraftment following myeloablative chemoradiotherapy regimens. *Bone Marrow Transplant,* **19,** 1095-1101.

Hsieh, F.F., Barnett, L.A., Green, W.F., Freedman, K., Matushansky, I., Skoultchi, A.I. & Kelley, L.L. (2000) Cell cycle exit during terminal erythroid differentiation is associated with accumulation of p27Kip1 and inactivation of cdk2 kinase. *Blood,* **96,** 2746-2754.

Hu, M., Krause, D., Greaves, M., Sharkis, S., Dexter, M., Heyworth, C. & Enver, T. (1997) Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev,* **11,** 774-785.

Huang, S., Law, P., Francis, K., Palsson, B.O. & Ho, A.D. (1999) Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. *Blood,* **94,** 2595-2604.

Hunt, P., Li, Y.S., Nichol, J.L., Hokom, M.M., Bogenberger, J.M., Swift, S.E., Skrine, J.D., Hornkohl, A.C., Lu, H., Clogston, C. & et al. (1995) Purification and biologic characterization of plasma-derived megakaryocyte growth and development factor. *Blood,* **86,** 540-547.

Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., Heike, T. & Nakahata, T. (2002) NOD/SCID/gamma cnull mouse: an excellent recipient mouse model for engraftment of human cells. *Blood,* **100,** 3175-3182.

Jackson, K.A., Majka, S.M., Wulf, G.G. & Goodell, M.A. (2002) Stem cells: a minireview. *J Cell Biochem Suppl,* **38,** 1-6.

Jacobsen, K., Kravitz, J., Kincade, P.W. & Osmond, D.G. (1996) Adhesion receptors on bone marrow stromal cells: in vivo expression of vascular cell adhesion molecule-1 by reticular cells and sinusoidal endothelium in normal and gamma-irradiated mice. *Blood,* **87,** 73-82.

Jacobsen, S.E., Okkenhaug, C., Myklebust, J., Veiby, O.P. & Lyman, S.D. (1995) The FLT3 ligand potently and directly stimulates the growth and expansion of primitive murine bone marrow progenitor cells in vitro: synergistic interactions with interleukin (IL) 11, IL-12, and other hematopoietic growth factors. *J Exp Med,* **181,** 1357-1363.

Johnson, L.V., Walsh, M.L. & Chen, L.B. (1980) Localization of mitochondria in living cells with rhodamine 123. *Proc Natl Acad Sci U S A,* **77,** 990-994.

Jones, R.J., Collector, M.I., Barber, J.P., Vala, M.S., Fackler, M.J., May, W.S., Griffin, C.A., Hawkins, A.L., Zehnbauer, B.A., Hilton, J., Colvin, O.M. & Sharkis, S.J. (1996) Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. *Blood,* **88,** 487-491.

Ju, Z., Choudhury, A.R. & Rudolph, K.L. (2007) A dual role of p21 in stem cell aging. *Ann N Y Acad Sci,* **1100,** 333-344.

Katz, F.E., Tindle, R., Sutherland, D.R. & Greaves, M.F. (1985) Identification of a membrane glycoprotein associated with haemopoietic progenitor cells. *Leuk Res,* **9,** 191-198.

Kaushansky, K. (1995) Thrombopoietin: the primary regulator of platelet production. *Blood,* **86,** 419-431.

Kobayashi, M., Laver, J.H., Kato, T., Miyazaki, H. & Ogawa, M. (1996) Thrombopoietin supports proliferation of human primitive hematopoietic cells in synergy with steel factor and/or interleukin-3. *Blood,* **88,** 429-436.

Kolb, H.J. & Holler, E. (1997) Hematopoietic transplantation: state of the art. *Stem Cells,* **15 Suppl 1,** 151-157; discussion 158.

Kollet, O., Aviram, R., Chebath, J., ben-Hur, H., Nagler, A., Shultz, L., Revel, M. & Lapidot, T. (1999) The soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro maintenance and proliferation of human CD34(+)CD38(-/low) cells capable of repopulating severe combined immunodeficiency mice. *Blood,* **94,** 923-931.

Kondo, M., Wagers, A.J., Manz, M.G., Prohaska, S.S., Scherer, D.C., Beilhack, G.F., Shizuru, J.A. & Weissman, I.L. (2003) Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol,* **21,** 759-806.

Kondo, M., Weissman, I.L. & Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell,* **91,** 661-672.

Kozar, K., Ciemerych, M.A., Rebel, V.I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R.T., Akashi, K. & Sicinski, P. (2004) Mouse development and cell proliferation in the absence of D-cyclins. *Cell,* **118,** 477-491.

Krause, D.S. (2002) Regulation of hematopoietic stem cell fate. *Oncogene,* **21,** 3262-3269.

Ku, H., Yonemura, Y., Kaushansky, K. & Ogawa, M. (1996) Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. *Blood,* **87,** 4544-4551.

Kurtzberg, J., Laughlin, M., Graham, M.L., Smith, C., Olson, J.F., Halperin, E.C., Ciocci, G., Carrier, C., Stevens, C.E. & Rubinstein, P. (1996) Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N Engl J Med,* **335,** 157-166.

Kwan, E., Norris, M.D., Zhu, L., Ferrara, D., Marshall, G.M. & Haber, M. (2000) Simultaneous detection and quantification of minimal residual disease in childhood acute lymphoblastic leukaemia using real-time polymerase chain reaction. *Br J Haematol,* **109,** 430-434.

LaBaer, J., Garrett, M.D., Stevenson, L.F., Slingerland, J.M., Sandhu, C., Chou, H.S., Fattaey, A. & Harlow, E. (1997) New functional activities for the p21 family of CDK inhibitors. *Genes Dev,* **11,** 847-862.

Lajtha, L.G. (1975) Haemopoietic stem cells. *Br J Haematol,* **29,** 529-535.

Lansdorp, P.M. & Dragowska, W. (1992) Long-term erythropoiesis from constant numbers of CD34+ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. *J Exp Med,* **175,** 1501-1509.

Lansdorp, P.M. & Dragowska, W. (1993) Maintenance of hematopoiesis in serum-free bone marrow cultures involves sequential recruitment of quiescent progenitors. *Exp Hematol,* **21,** 1321-1327.

Lansdorp, P.M., Schmitt, C., Sutherland, H.J., Craig, W.H., Dragowska, W., Thomas, T.E. & Eaves, C.J. (1992) Hemopoietic stem cell characterization. *Prog Clin Biol Res,* **377,** 475-484; discussion 485-476.

Lansdorp, P.M., Sutherland, H.J. & Eaves, C.J. (1990) Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. *J Exp Med,* **172,** 363-366.

Lapidot, T., Pflumio, F., Doedens, M., Murdoch, B., Williams, D.E. & Dick, J.E. (1992) Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science,* **255,** 1137-1141.

Laughlin, M.J., Eapen, M., Rubinstein, P., Wagner, J.E., Zhang, M.J., Champlin, R.E., Stevens, C., Barker, J.N., Gale, R.P., Lazarus, H.M., Marks, D.I., van Rood, J.J., Scaradavou, A. & Horowitz, M.M. (2004) Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med,* **351,** 2265-2275.

Leung, W., Ramirez, M. & Civin, C.I. (1999) Quantity and quality of engrafting cells in cord blood and autologous mobilized peripheral blood. *Biol Blood Marrow Transplant,* **5,** 69-76.

Levac, K., Karanu, F. & Bhatia, M. (2005) Identification of growth factor conditions that reduce ex vivo cord blood progenitor expansion but do not alter human repopulating cell function in vivo. *Haematologica,* **90,** 166-172.

Levesque, J.P., Haylock, D.N. & Simmons, P.J. (1996) Cytokine regulation of proliferation and cell adhesion are correlated events in human CD34+ hemopoietic progenitors. *Blood,* **88,** 1168-1176.

Levesque, J.P., Leavesley, D.I., Niutta, S., Vadas, M. & Simmons, P.J. (1995) Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *J Exp Med,* **181,** 1805-1815.

Liu, B., Liao, C., Chen, J., Gu, S., Wu, S. & Xu, Z. (2003) Significance of increasing adhesion of cord blood hematopoietic cells and a new method: platelet microparticles. *Am J Hematol,* **74,** 216-217.

Lu, L., Walker, D., Broxmeyer, H.E., Hoffman, R., Hu, W. & Walker, E. (1987) Characterization of adult human marrow hematopoietic progenitors highly

enriched by two-color cell sorting with My10 and major histocompatibility class II monoclonal antibodies. *J Immunol,* **139,** 1823-1829.

Lucotti, Malabarba, Rosti, Bergamaschi, Danova, Invernizzi, Pecci, Ramajoli, Perotti, Torretta, Amici, D., Salvaneschi & Cazzola (2000) Cell cycle distribution of cord blood-derived haematopoietic progenitor cells and their recruitment into the S-phase of the cell cycle. *Br J Haematol,* **108,** 621-628.

Lyman, S.D., Brasel, K., Rousseau, A.M. & Williams, D.E. (1994) The flt3 ligand: a hematopoietic stem cell factor whose activities are distinct from steel factor. *Stem Cells,* **12 Suppl 1,** 99-107; discussion 108-110.

Lyman, S.D. & Jacobsen, S.E. (1998) c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood,* **91,** 1101-1134.

Lyman, S.D., James, L., Vanden Bos, T., de Vries, P., Brasel, K., Gliniak, B., Hollingsworth, L.T., Picha, K.S., McKenna, H.J., Splett, R.R. & et al. (1993) Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell,* **75,** 1157-1167.

Lyons, A.B. & Parish, C.R. (1994) Determination of lymphocyte division by flow cytometry. *J Immunol Methods,* **171,** 131-137.

Mahmud, N., Devine, S.M., Weller, K.P., Parmar, S., Sturgeon, C., Nelson, M.C., Hewett, T. & Hoffman, R. (2001) The relative quiescence of hematopoietic stem cells in nonhuman primates. *Blood,* **97,** 3061-3068.

Makinodan, T. (1956) Circulating rat cells in lethally irradiated mice protected with rat bone marrow. *Proc Soc Exp Biol Med,* **92,** 174-179.

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J. & Kato, J.Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol Cell Biol,* **14,** 2066-2076.

McCune, J.M. (1996) Development and applications of the SCID-hu mouse model. *Semin Immunol,* **8,** 187-196.

McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Lieberman, M. & Weissman, I.L. (1988) The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science,* **241,** 1632-1639.

McKenzie, J.L., Gan, O.I., Doedens, M. & Dick, J.E. (2005) Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. *Blood,* **106,** 1259-1261.

McNiece, I. (2000) The CD34+Thy1+ cell population: are they all stem cells? *Exp Hematol,* **28,** 1312-1314.

McNiece, I., Jones, R., Bearman, S.I., Cagnoni, P., Nieto, Y., Franklin, W., Ryder, J., Steele, A., Stoltz, J., Russell, P., McDermitt, J., Hogan, C., Murphy, J. & Shpall,

E.J. (2000) Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer. *Blood,* **96,** 3001-3007.

McNiece, I.K., Langley, K.E. & Zsebo, K.M. (1991) Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. *Exp Hematol,* **19,** 226-231.

Medvinsky, A. & Dzierzak, E. (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell,* **86,** 897-906.

Medvinsky, A.L., Samoylina, N.L., Muller, A.M. & Dzierzak, E.A. (1993) An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature,* **364,** 64-67.

Metcalf, D. (1993) Hematopoietic regulators: redundancy or subtlety? *Blood,* **82,** 3515-3523.

Miller, J.S., McCullar, V., Punzel, M., Lemischka, I.R. & Moore, K.A. (1999) Single adult human CD34(+)/Lin-/CD38(-) progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. *Blood,* **93,** 96-106.

Mirabelli, P., Di Noto, R., Lo Pardo, C., Morabito, P., Abate, G., Gorrese, M., Raia, M., Pascariello, C., Scalia, G., Gemei, M., Mariotti, E. & Del Vecchio, L. (2008) Extended flow cytometry characterization of normal bone marrow progenitor cells by simultaneous detection of aldehyde dehydrogenase and early hematopoietic antigens: implication for erythroid differentiation studies. *BMC Physiol,* **8,** 13.

Miyake, K., Weissman, I.L., Greenberger, J.S. & Kincade, P.W. (1991) Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J Exp Med,* **173,** 599-607.

Mohamedali, A., Soeiro, I., Lea, N.C., Glassford, J., Banerji, L., Mufti, G.J., Lam, E.W. & Thomas, N.S. (2003) Cyclin D2 controls B cell progenitor numbers. *J Leukoc Biol,* **74,** 1139-1143.

Mohle, R., Bautz, F., Rafii, S., Moore, M.A., Brugger, W. & Kanz, L. (1998) The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood,* **91,** 4523-4530.

Moore, M.A. (1991) Review: Stratton Lecture 1990. Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood,* **78,** 1-19.

Moore, M.A. & Hoskins, I. (1994) Ex vivo expansion of cord blood-derived stem cells and progenitors. *Blood Cells,* **20,** 468-479; discussion 479-481.

Morrison, S.J., Prowse, K.R., Ho, P. & Weissman, I.L. (1996) Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity,* **5,** 207-216.

Morrison, S.J., Uchida, N. & Weissman, I.L. (1995) The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol,* **11,** 35-71.

Morrison, S.J. & Weissman, I.L. (1994) The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity,* **1,** 661-673.

Morrison, S.J., Wright, D.E., Cheshier, S.H. & Weissman, I.L. (1997a) Hematopoietic stem cells: challenges to expectations. *Curr Opin Immunol,* **9,** 216-221.

Morrison, S.J., Wright, D.E. & Weissman, I.L. (1997b) Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci U S A,* **94,** 1908-1913.

Muller, A.M., Medvinsky, A., Strouboulis, J., Grosveld, F. & Dzierzak, E. (1994) Development of hematopoietic stem cell activity in the mouse embryo. *Immunity,* **1,** 291-301.

Murray, L.J., Young, J.C., Osborne, L.J., Luens, K.M., Scollay, R. & Hill, B.L. (1999) Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division. *Exp Hematol,* **27,** 1019-1028.

Myatt, S.S. & Lam, E.W. (2007) Promiscuous and lineage-specific roles of cell cycle regulators in haematopoiesis. *Cell Div,* **2,** 6.

Namikawa, R., Weilbaecher, K.N., Kaneshima, H., Yee, E.J. & McCune, J.M. (1990) Long-term human hematopoiesis in the SCID-hu mouse. *J Exp Med,* **172,** 1055-1063.

Neben, S., Redfearn, W.J., Parra, M., Brecher, G. & Pallavicini, M.G. (1991) Short- and long-term repopulation of lethally irradiated mice by bone marrow stem cells enriched on the basis of light scatter and Hoechst 33342 fluorescence. *Exp Hematol,* **19,** 958-967.

Nocka, K., Buck, J., Levi, E. & Besmer, P. (1990) Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *Embo J,* **9,** 3287-3294.

Nordon, R.E., Ginsberg, S.S. & Eaves, C.J. (1997) High-resolution cell division tracking demonstrates the FLt3-ligand-dependence of human marrow CD34+CD38- cell production in vitro. *Br J Haematol,* **98,** 528-539.

Nourse, J., Firpo, E., Flanagan, W.M., Coats, S., Polyak, K., Lee, M.H., Massague, J., Crabtree, G.R. & Roberts, J.M. (1994) Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature,* **372,** 570-573.

Novelli, E.M., Cheng, L., Yang, Y., Leung, W., Ramirez, M., Tanavde, V., Enger, C. & Civin, C.I. (1999) Ex vivo culture of cord blood CD34+ cells expands progenitor cell numbers, preserves engraftment capacity in nonobese diabetic/severe

combined immunodeficient mice, and enhances retroviral transduction efficiency. *Hum Gene Ther,* **10,** 2927-2940.

Nowell, P.C., Cole, L.J., Habermeyer, J.G. & Roan, P.L. (1956) Growth and continued function of rat marrow cells in x-radiated mice. *Cancer Res,* **16,** 258-261.

Ogawa, M. (1993) Differentiation and proliferation of hematopoietic stem cells. *Blood,* **81,** 2844-2853.

Ogawa, M. (1999) Stochastic model revisited. *Int J Hematol,* **69,** 2-5.

Ogawa, M. (2002) Changing phenotypes of hematopoietic stem cells*1. *Experimental Hematology,* **30,** 3-6.

Ogawa, M. & Matsunaga, T. (1999) Humoral regulation of hematopoietic stem cells. *Ann N Y Acad Sci,* **872,** 17-23; discussion 23-14.

Ogden, D.A. & Mickliem, H.S. (1976) The fate of serially transplanted bone marrow cell populations from young and old donors. *Transplantation,* **22,** 287-293.

Orkin, S.H. & Zon, L.I. (2002) Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nat Immunol,* **3,** 323-328.

Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science,* **273,** 242-245.

Pallavicini, M.G., Summers, L.J., Dean, P.N. & Gray, J.W. (1985) Enrichment of murine hemopoietic clonogenic cells by multivariate analyses and sorting. *Exp Hematol,* **13,** 1173-1181.

Papayannopoulou, T., Craddock, C., Nakamoto, B., Priestley, G.V. & Wolf, N.S. (1995) The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proc Natl Acad Sci U S A,* **92,** 9647-9651.

Papayannopoulou, T. & Nakamoto, B. (1993) Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. *Proc Natl Acad Sci U S A,* **90,** 9374-9378.

Papayannopoulou, T., Priestley, G.V. & Nakamoto, B. (1998) Anti-VLA4/VCAM-1-induced mobilization requires cooperative signaling through the kit/mkit ligand pathway. *Blood,* **91,** 2231-2239.

Parish, C.R. (1999) Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunol Cell Biol,* **77,** 499-508.

Parry, D., Mahony, D., Wills, K. & Lees, E. (1999) Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. *Mol Cell Biol,* **19,** 1775-1783.

Peled, A., Kollet, O., Ponomaryov, T., Petit, I., Franitza, S., Grabovsky, V., Slav, M.M., Nagler, A., Lider, O., Alon, R., Zipori, D. & Lapidot, T. (2000) The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood,* **95,** 3289-3296.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D. & Lapidot, T. (1999) Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science,* **283,** 845-848.

Peters, S.O., Kittler, E.L., Ramshaw, H.S. & Quesenberry, P.J. (1996) Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood,* **87,** 30-37.

Petzer, A.L., Zandstra, P.W., Piret, J.M. & Eaves, C.J. (1996) Differential cytokine effects on primitive (CD34+CD38-) human hematopoietic cells: novel responses to Flt3-ligand and thrombopoietin. *J Exp Med,* **183,** 2551-2558.

Pflumio, F., Izac, B., Katz, A., Shultz, L.D., Vainchenker, W. & Coulombel, L. (1996) Phenotype and function of human hematopoietic cells engrafting immune-deficient CB17-severe combined immunodeficiency mice and nonobese diabetic-severe combined immunodeficiency mice after transplantation of human cord blood mononuclear cells. *Blood,* **88,** 3731-3740.

Piacibello, W., Sanavio, F., Garetto, L., Severino, A., Dane, A., Gammaitoni, L. & Aglietta, M. (1998a) Differential growth factor requirement of primitive cord blood hematopoietic stem cell for self-renewal and amplification vs proliferation and differentiation. *Leukemia,* **12,** 718-727.

Piacibello, W., Sanavio, F., Severino, A., Dane, A., Gammaitoni, L., Fagioli, F., Perissinotto, E., Cavalloni, G., Kollet, O., Lapidot, T. & Aglietta, M. (1999) Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. *Blood,* **93,** 3736-3749.

Piacibello, W., Sanavio, F., Severino, A., Garetto, L., Dane, A., Gammaitoni, L. & Aglietta, M. (1998b) Ex vivo expansion of cord blood progenitors. *Vox Sang,* **74 Suppl 2,** 457-462.

Ploemacher, R.E. & Brons, R.H. (1989) Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. *Exp Hematol,* **17,** 263-266.

Ploemacher, R.E., van der Sluijs, J.P., Voerman, J.S. & Brons, N.H. (1989) An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. *Blood,* **74,** 2755-2763.

Potocnik, A.J., Brakebusch, C. & Fassler, R. (2000) Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. *Immunity,* **12,** 653-663.

Punzel, M., Wissink, S.D., Miller, J.S., Moore, K.A., Lemischka, I.R. & Verfaillie, C.M. (1999) The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. *Blood,* **93,** 3750-3756.

Quesenberry, P., Becker, P., Nilsson, S., Stewart, M., Zhong, S., Grimaldi, C., Reilly, J., Hababian, H., Dooner, M., Peters, S. & Ramshaw, H. (1999) Stem cell engraftment and cell cycle phenotype. *Leukemia,* **13 Suppl 1,** S92-93.

Ramsfjell, V., Borge, O.J., Veiby, O.P., Cardier, J., Murphy, M.J., Jr., Lyman, S.D., Lok, S. & Jacobsen, S.E. (1996) Thrombopoietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone marrow progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3. *Blood,* **88,** 4481-4492.

Ramsfjell, V., Bryder, D., Bjorgvinsdottir, H., Kornfalt, S., Nilsson, L., Borge, O.J. & Jacobsen, S.E. (1999) Distinct requirements for optimal growth and In vitro expansion of human CD34(+)CD38(-) bone marrow long-term culture-initiating cells (LTC-IC), extended LTC-IC, and murine in vivo long-term reconstituting stem cells. *Blood,* **94,** 4093-4102.

Reems, J.A., Mielcarek, M. & Torok-Storb, B. (1997) Differential modulation of adhesion markers with ex vivo expansion of human umbilical CD34+ progenitor cells. *Biol Blood Marrow Transplant,* **3,** 133-141.

Rice, A., Boiron, J.M., Barbot, C., Dupouy, M., Dubosc-Marchenay, N., Dumain, P., Lacombe, F. & Reiffers, J. (1995) Cytokine-mediated expansion of 5-FU-resistant peripheral blood stem cells. *Exp Hematol,* **23,** 303-308.

Rice, A.M., Wood, J.A., Milross, C.G., Collins, C.J., Case, J., Vowels, M.R. & Nordon, R.E. (2001) Prolonged ex vivo culture of cord blood CD34(+) cells facilitates myeloid and megakaryocytic engraftment in the non-obese diabetic severe combined immunodeficient mouse model. *Br J Haematol,* **114,** 433-443.

Rice, A.M., Wood, J.A., Milross, C.G., Collins, C.J., McCarthy, N.F. & Vowels, M.R. (2000) Conditions that enable human hematopoietic stem cell engraftment in all NOD-SCID mice. *Transplantation,* **69,** 927-935.

Rocha, V., Wagner, J.E., Jr., Sobocinski, K.A., Klein, J.P., Zhang, M.J., Horowitz, M.M. & Gluckman, E. (2000) Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. *N Engl J Med,* **342,** 1846-1854.

Rossmanith, T., Schroder, B., Bug, G., Muller, P., Klenner, T., Knaus, R., Hoelzer, D. & Ottmann, O.G. (2001) Interleukin 3 improves the ex vivo expansion of

primitive human cord blood progenitor cells and maintains the engraftment potential of scid repopulating cells. *Stem Cells,* **19,** 313-320.

Rosu-Myles, M., Gallacher, L., Murdoch, B., Hess, D.A., Keeney, M., Kelvin, D., Dale, L., Ferguson, S.S., Wu, D., Fellows, F. & Bhatia, M. (2000) The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression. *Proc Natl Acad Sci U S A,* **97,** 14626-14631.

Roussel, M.F., Theodoras, A.M., Pagano, M. & Sherr, C.J. (1995) Rescue of defective mitogenic signaling by D-type cyclins. *Proc Natl Acad Sci U S A,* **92,** 6837-6841.

Rubinstein, P., Carrier, C., Scaradavou, A., Kurtzberg, J., Adamson, J., Migliaccio, A.R., Berkowitz, R.L., Cabbad, M., Dobrila, N.L., Taylor, P.E., Rosenfield, R.E. & Stevens, C.E. (1998) Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med,* **339,** 1565-1577.

Rubinstein, P., Dobrila, L., Rosenfield, R.E., Adamson, J.W., Migliaccio, G., Migliaccio, A.R., Taylor, P.E. & Stevens, C.E. (1995) Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci U S A,* **92,** 10119-10122.

Rusten, L.S., Lyman, S.D., Veiby, O.P. & Jacobsen, S.E. (1996) The FLT3 ligand is a direct and potent stimulator of the growth of primitive and committed human CD34+ bone marrow progenitor cells in vitro. *Blood,* **87,** 1317-1325.

Sanchez, I. & Dynlacht, B.D. (2005) New insights into cyclins, CDKs, and cell cycle control. *Semin Cell Dev Biol,* **16,** 311-321.

Sasaki, Y., Jensen, C.T., Karlsson, S. & Jacobsen, S.E. (2004) Enforced expression of cyclin D2 enhances the proliferative potential of myeloid progenitors, accelerates in vivo myeloid reconstitution, and promotes rescue of mice from lethal myeloablation. *Blood,* **104,** 986-992.

Scadden, D.T. (2006) The stem-cell niche as an entity of action. *Nature,* **441,** 1075-1079.

Schweitzer, K.M., Drager, A.M., van der Valk, P., Thijsen, S.F., Zevenbergen, A., Theijsmeijer, A.P., van der Schoot, C.E. & Langenhuijsen, M.M. (1996) Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. *Am J Pathol,* **148,** 165-175.

Shah, A.J., Smogorzewska, E.M., Hannum, C. & Crooks, G.M. (1996) Flt3 ligand induces proliferation of quiescent human bone marrow CD34+CD38- cells and maintains progenitor cells in vitro. *Blood,* **87,** 3563-3570.

Shay, J.W. & Wright, W.E. (2000) Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol,* **1,** 72-76.

Sherr, C.J. (1993) Mammalian G1 cyclins. *Cell,* **73,** 1059-1065.

Sherr, C.J. & Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev,* **13,** 1501-1512.

Sherr, C.J. & Roberts, J.M. (2004) Living with or without cyclins and cyclin-dependent kinases. *Genes Dev,* **18,** 2699-2711.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., Greiner, D.L. & Handgretinger, R. (2005) Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R{gamma}null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells *J Immunol,* **174,** 6477-6489.

Shultz, L.D., Banuelos, S.J., Leif, J., Appel, M.C., Cunningham, M., Ballen, K., Burzenski, L. & Greiner, D.L. (2003) Regulation of human short-term repopulating cell (STRC) engraftment in NOD/SCID mice by host CD122+ cells. *Experimental Hematology,* **31,** 551-558.

Shultz, L.D., Lang, P.A., Christianson, S.W., Gott, B., Lyons, B., Umeda, S., Leiter, E., Hesselton, R., Wagar, E.J., Leif, J.H., Kollet, O., Lapidot, T. & Greiner, D.L. (2000) NOD/LtSz-Rag1null mice: an immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabetogenic T cells. *J Immunol,* **164,** 2496-2507.

Shultz, L.D., Schweitzer, P.A., Christianson, S.W., Gott, B., Schweitzer, I.B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T.V., Greiner, D.L. & et al. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol,* **154,** 180-191.

Siminovitch, L., McCulloch, E.A. & Till, J.E. (1963) The Distribution of Colony-Forming Cells among Spleen Colonies. *J Cell Physiol,* **62,** 327-336.

Simmons, P.J., Masinovsky, B., Longenecker, B.M., Berenson, R., Torok-Storb, B. & Gallatin, W.M. (1992) Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. *Blood,* **80,** 388-395.

Simmons, P.J., Zannettino, A., Gronthos, S. & Leavesley, D. (1994) Potential adhesion mechanisms for localisation of haemopoietic progenitors to bone marrow stroma. *Leuk Lymphoma,* **12,** 353-363.

Sitnicka, E., Buza-Vidas, N., Larsson, S., Nygren, J.M., Liuba, K. & Jacobsen, S.E.W. (2003) Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. *Blood,* **102,** 881-886.

Sitnicka, E., Lin, N., Priestley, G.V., Fox, N., Broudy, V.C., Wolf, N.S. & Kaushansky, K. (1996) The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. *Blood,* **87,** 4998-5005.

Smith, C. (2003) Hematopoietic stem cells and hematopoiesis. *Cancer Control,* **10,** 9-16.

Spangrude, G.J., Heimfeld, S. & Weissman, I.L. (1988) Purification and characterization of mouse hematopoietic stem cells. *Science,* **241,** 58-62.

Spence, S.E., Keller, J.R., Ruscetti, F.W., McCauslin, C.S., Gooya, J.M., Funakoshi, S., Longo, D.L. & Murphy, W.J. (1998) Engraftment of ex vivo expanded and cycling human cord blood hematopoietic progenitor cells in SCID mice. *Exp Hematol,* **26,** 507-514.

Spradling, A., Drummond-Barbosa, D. & Kai, T. (2001) Stem cells find their niche. *Nature,* **414,** 98-104.

Srour, E.F., Abonour, R., Cornetta, K. & Traycoff, C.M. (1999) Ex vivo expansion of hematopoietic stem and progenitor cells: are we there yet? *J Hematother,* **8,** 93-102.

Srour, E.F., Jetmore, A., Wolber, F.M., Plett, P.A., Abonour, R., Yoder, M.C. & Orschell-Traycoff, C.M. (2001) Homing, cell cycle kinetics and fate of transplanted hematopoietic stem cells. *Leukemia,* **15,** 1681-1684.

Srour, E.F., Zanjani, E.D., Brandt, J.E., Leemhuis, T., Briddell, R.A., Heerema, N.A. & Hoffman, R. (1992) Sustained human hematopoiesis in sheep transplanted in utero during early gestation with fractionated adult human bone marrow cells. *Blood,* **79,** 1404-1412.

Steinman, R.A. (2002) Cell cycle regulators and hematopoiesis. *Oncogene,* **21,** 3403-3413.

Steinman, R.A., Huang, J., Yaroslavskiy, B., Goff, J.P., Ball, E.D. & Nguyen, A. (1998) Regulation of p21(WAF1) Expression During Normal Myeloid Differentiation. *Blood,* **91,** 4531-4542.

Stiles, B., Groszer, M., Wang, S., Jiao, J. & Wu, H. (2004) PTENless means more. *Dev Biol,* **273,** 175-184.

Sutherland, H.J., Eaves, C.J., Eaves, A.C., Dragowska, W. & Lansdorp, P.M. (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood,* **74,** 1563-1570.

Sutherland, H.J., Lansdorp, P.M., Henkelman, D.H., Eaves, A.C. & Eaves, C.J. (1990) Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci U S A,* **87,** 3584-3588.

Szilvassy, S.J., Meyerrose, T.E. & Grimes, B. (2000) Effects of cell cycle activation on the short-term engraftment properties of ex vivo expanded murine hematopoietic cells. *Blood,* **95,** 2829-2837.

Tam, S.W., Theodoras, A.M., Shay, J.W., Draetta, G.F. & Pagano, M. (1994) Differential expression and regulation of Cyclin D1 protein in normal and tumor human cells: association with Cdk4 is required for Cyclin D1 function in G1 progression. *Oncogene,* **9,** 2663-2674.

Taniguchi, T., Endo, H., Chikatsu, N., Uchimaru, K., Asano, S., Fujita, T., Nakahata, T. & Motokura, T. (1999) Expression of p21(Cip1/Waf1/Sdi1) and p27(Kip1) cyclin-dependent kinase inhibitors during human hematopoiesis. *Blood,* **93,** 4167-4178.

Terstappen, L.W., Huang, S., Safford, M., Lansdorp, P.M. & Loken, M.R. (1991) Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood,* **77,** 1218-1227.

Thoren, L.A., Liuba, K., Bryder, D., Nygren, J.M., Jensen, C.T., Qian, H., Antonchuk, J. & Jacobsen, S.E. (2008) Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol,* **180,** 2045-2053.

Thron, C.D. (1997) Bistable biochemical switching and the control of the events of the cell cycle. *Oncogene,* **15,** 317-325.

Till, J.E. & Mc, C.E. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res,* **14,** 213-222.

Toyoshima, H. & Hunter, T. (1994) p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell,* **78,** 67-74.

Traycoff, C.M., Cornetta, K., Yoder, M.C., Davidson, A. & Srour, E.F. (1996) Ex vivo expansion of murine hematopoietic progenitor cells generates classes of expanded cells possessing different levels of bone marrow repopulating potential. *Exp Hematol,* **24,** 299-306.

Uchida, N., Jerabek, L. & Weissman, I.L. (1996) Searching for hematopoietic stem cells. II. The heterogeneity of Thy-1.1(lo)Lin(-/lo)Sca-1+ mouse hematopoietic stem cells separated by counterflow centrifugal elutriation. *Exp Hematol,* **24,** 649-659.

Uchida, N., Tsukamoto, A., He, D., Friera, A.M., Scollay, R. & Weissman, I.L. (1998) High doses of purified stem cells cause early hematopoietic recovery in syngeneic and allogeneic hosts. *J Clin Invest,* **101,** 961-966.

Uchida, N. & Weissman, I.L. (1992) Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *J Exp Med,* **175,** 175-184.

Udomsakdi, C., Lansdorp, P.M., Hogge, D.E., Reid, D.S., Eaves, A.C. & Eaves, C.J. (1992) Characterization of primitive hematopoietic cells in normal human peripheral blood. *Blood,* **80,** 2513-2521.

Udomsakdi, C., Eaves, C.J., Sutherland, H.J. & Lansdorp, P.M. (1991) Separation of functionally distinct subpopulations of primitive human hematopoietic cells using rhodamine-123. *Exp Hematol,* **19,** 338-342.

Ueda, T., Tsuji, K., Yoshino, H., Ebihara, Y., Yagasaki, H., Hisakawa, H., Mitsui, T., Manabe, A., Tanaka, R., Kobayashi, K., Ito, M., Yasukawa, K. & Nakahata, T. (2000) Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest,* **105,** 1013-1021.

van der Kooy, D. & Weiss, S. (2000) Why stem cells? *Science,* **287,** 1439-1441.

Vaziri, H., Dragowska, W., Allsopp, R.C., Thomas, T.E., Harley, C.B. & Lansdorp, P.M. (1994) Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc Natl Acad Sci U S A,* **91,** 9857-9860.

Verfaillie, C.M. (1993) Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. *Blood,* **82,** 2045-2053.

Visser, J.W. & De Vries, P. (1990) Identification and purification of murine hematopoietic stem cells by flow cytometry. *Methods Cell Biol,* **33,** 451-468.

Vlach, J., Hennecke, S. & Amati, B. (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *Embo J,* **16,** 5334-5344.

Vormoor, J., Lapidot, T., Pflumio, F., Risdon, G., Patterson, B., Broxmeyer, H.E. & Dick, J.E. (1994) Immature human cord blood progenitors engraft and proliferate to high levels in severe combined immunodeficient mice. *Blood,* **83,** 2489-2497.

Wagers, A.J., Allsopp, R.C. & Weissman, I.L. (2002) Changes in integrin expression are associated with altered homing properties of Lin(-/lo)Thy1.1(lo)Sca-1(+)c-kit(+) hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. *Exp Hematol,* **30,** 176-185.

Wagner, J.E., Rosenthal, J., Sweetman, R., Shu, X.O., Davies, S.M., Ramsay, N.K., McGlave, P.B., Sender, L. & Cairo, M.S. (1996) Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. *Blood,* **88,** 795-802.

Watanabe, T., Dave, B., Heimann, D.G., Jackson, J.D., Kessinger, A. & Talmadge, J.E. (1998) Cell adhesion molecule expression on CD34+ cells in grafts and time to myeloid and platelet recovery after autologous stem cell transplantation. *Exp Hematol,* **26,** 10-18.

Watt, F.M. & Hogan, B.L. (2000) Out of Eden: stem cells and their niches. *Science,* **287,** 1427-1430.

Watt, S.M., Katz, F.E., Davis, L., Capellaro, D., Gordon, M.Y., Tindle, R.W. & Greaves, M.F. (1987) Expression of HPCA-1 and HLA-DR antigens on growth factor- and stroma-dependent colony forming cells. *Br J Haematol,* **66,** 153-159.

Watt, S.M. & Visser, J.W. (1992) Recent advances in the growth and isolation of primitive human haemopoietic progenitor cells. *Cell Prolif,* **25,** 263-297.

Weissman, I.L. (2000) Stem cells: units of development, units of regeneration, and units in evolution. *Cell,* **100,** 157-168.

Weissman, I.L. (2002) The road ended up at stem cells. *Immunol Rev,* **185,** 159-174.

Whetton, A.D. & Spooncer, E. (1998) Role of cytokines and extracellular matrix in the regulation of haemopoietic stem cells. *Curr Opin Cell Biol,* **10,** 721-726.

Williams, D.A., Rios, M., Stephens, C. & Patel, V.P. (1991) Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature,* **352,** 438-441.

Wilpshaar, J., Bhatia, M., Kanhai, H.H., Breese, R., Heilman, D.K., Johnson, C.S., Falkenburg, J.H. & Srour, E.F. (2002) Engraftment potential of human fetal hematopoietic cells in NOD/SCID mice is not restricted to mitotically quiescent cells. *Blood,* **100,** 120-127.

Wilpshaar, J., Falkenburg, J.H.F., Tong, X., Noort, W.A., Breese, R., Heilman, D., Kanhai, H., Orschell-Traycoff, C.M. & Srour, E.F. (2000) Similar repopulating capacity of mitotically active and resting umbilical cord blood CD34+ cells in NOD/SCID mice. *Blood,* **96,** 2100-2107.

Winer, J., Jung, C.K., Shackel, I. & Williams, P.M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem,* **270,** 41-49.

Wright, D.E., Bowman, E.P., Wagers, A.J., Butcher, E.C. & Weissman, I.L. (2002) Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. *J Exp Med,* **195,** 1145-1154.

Yaroslavskiy, B., Watkins, S., Donnenberg, A.D., Patton, T.J. & Steinman, R.A. (1999) Subcellular and cell-cycle expression profiles of CDK-inhibitors in normal differentiating myeloid cells. *Blood,* **93,** 2907-2917.

Yonemura, Y., Ku, H., Hirayama, F., Souza, L.M. & Ogawa, M. (1996) Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. *Proc Natl Acad Sci U S A,* **93,** 4040-4044.

Yonemura, Y., Ku, H., Lyman, S.D. & Ogawa, M. (1997) In vitro expansion of hematopoietic progenitors and maintenance of stem cells: comparison between FLT3/FLK-2 ligand and KIT ligand. *Blood,* **89,** 1915-1921.

Young, J.C., Lin, K., Hansteen, G., Travis, M., Murray, L.J., Jaing, L., Scollay, R. & Hill, B.L. (1999) CD34+ cells from mobilized peripheral blood retain fetal bone

marrow repopulating capacity within the Thy-1+ subset following cell division ex vivo. *Exp Hematol,* **27,** 994-1003.

Yui, J., Chiu, C.P. & Lansdorp, P.M. (1998) Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. *Blood,* **91,** 3255-3262.

Zandstra, P.W., Conneally, E., Petzer, A.L., Piret, J.M. & Eaves, C.J. (1997) Cytokine manipulation of primitive human hematopoietic cell self-renewal. *Proc Natl Acad Sci U S A,* **94,** 4698-4703.

Zandstra, P.W., Conneally, E., Piret, J.M. & Eaves, C.J. (1998) Ontogeny-associated changes in the cytokine responses of primitive human haemopoietic cells. *Br J Haematol,* **101,** 770-778.

Zanjani, E.D., Flake, A.W., Almeida-Porada, G., Tran, N. & Papayannopoulou, T. (1999) Homing of human cells in the fetal sheep model: modulation by antibodies activating or inhibiting very late activation antigen-4-dependent function. *Blood,* **94,** 2515-2522.

Zanjani, E.D., Flake, A.W., Rice, H., Hedrick, M. & Tavassoli, M. (1994) Long-term repopulating ability of xenogeneic transplanted human fetal liver hematopoietic stem cells in sheep. *J Clin Invest,* **93,** 1051-1055.

Zanjani, E.D., Mackintosh, F.R. & Harrison, M.R. (1991) Hematopoietic chimerism in sheep and nonhuman primates by in utero transplantation of fetal hematopoietic stem cells. *Blood Cells,* **17,** 349-363; discussion 364-346.

Zhang, J., Grindley, J.C., Yin, T., Jayasinghe, S., He, X.C., Ross, J.T., Haug, J.S., Rupp, D., Porter-Westpfahl, K.S., Wiedemann, L.M., Wu, H. & Li, L. (2006) PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature,* **441,** 518-522.

Zhang, J. & Li, L. (2005) BMP signaling and stem cell regulation. *Dev Biol,* **284,** 1-11.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y. & Li, L. (2003) Identification of the haematopoietic stem cell niche and control of the niche size. *Nature,* **425,** 836-841.

Zheng, Y., Sun, A. & Han, Z.C. (2005) Stem cell factor improves SCID-repopulating activity of human umbilical cord blood-derived hematopoietic stem/progenitor cells in xenotransplanted NOD/SCID mouse model. *Bone Marrow Transplant,* **35,** 137-142.

Zhou, S., Schuetz, J.D., Bunting, K.D., Colapietro, A.M., Sampath, J., Morris, J.J., Lagutina, I., Grosveld, G.C., Osawa, M., Nakauchi, H. & Sorrentino, B.P. (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med,* **7,** 1028-1034.

Zhu, J. & Emerson, S.G. (2002) Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene,* **21,** 3295-3313.

Zinovyeva, M.V., Zijlmans, J.M., Fibbe, W.E., Visser, J.W. & Belyavsky, A.V. (2000) Analysis of gene expression in subpopulations of murine hematopoietic stem and progenitor cells. *Exp Hematol,* **28,** 318-334.