EX VIVO EXPANSION OF HUMAN HAEMOPOIETIC PROGENITOR CELLS

by

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DEDICATION

This thesis is dedicated to my loving wife Ann and our three wonderful children, Kate, Benjamin and Thomas.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

In accordance with the University of Adelaide regulations, I give consent to this thesis being made available for photocopying and loan if accepted for the award of degree.

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ABSTRACT

The studies described within this thesis focus on the *ex vivo* growth of human haemopoietic progenitor cells (HPC) with the objective of defining culture conditions for generating myeloid post-progenitor cells for therapy.

Initial studies demonstrated that a combination of six recombinant human HGF including interleukin-1 (IL-1), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF) and stem cell factor (SCF) (136GGMS) provide a potent stimulus for *de novo* generation of myeloid cells and progenitors from mobilised blood $CD34^+$ cells. Subsequently, the four-factor combination of IL-3, IL-6, G-CSF and SCF (36GS) was shown to stimulate equivalent myeloid cell production from $CD34^+$ cells to that observed with 136GGMS. These studies demonstrated that sufficient myeloid post progenitor cells for clinical transplantation could be generated from $CD34^+$ cells cultured in 36GS. In addition, it is shown that generation of nucleated cells and nascent committed myeloid progenitor cells from $CD34^+$ cells during the first 14 days of *ex vivo* culture is mainly attributed to committed HPC with $CD34^+HLA\text{-}DR^+$ and $CD34^+CD38^+$ phenotypes. Analysis of HGF-receptor expression indicated that $CD34^+$ cells are heterogenous with respect to constitutive expression of receptors for IL-3, IL-6, G-CSF and GM-CSF and to a lesser extent SCF.

The fate of candidate haemopoietic stem cells during *ex vivo* culture was examined in studies designed to determine which HGF are required to maintain survival and stimulate the division of single HPC. As a single cytokine, thrombopoietin (TPO) was shown to be the most potent survival factor for $CD34^+CD38^-$ cells. These studies also suggested that the most primitive cells within the $CD34^+CD38^-$ fraction require simultaneous stimulation with

combinations of early acting HGF to induce cell division. Remarkably, the combination of IL-3, IL-6, G-CSF, SCF, FLT3L and TPO (36GSFT) was shown induce division in greater than 90% of single $CD34^+CD38^-$ cells. Subsequent studies demonstrated that this response was attributed to the combination of SCF, FLT3L and TPO (SFT). Finally, these studies suggested that primitive HPC cells within the $CD34^+CD38^-$ fraction could be hierarchically ordered according to their requirement for stimulation with combinations of HGF to induce cell division. It is proposed that the most primitive HPC within the $CD34^+CD38^-$ fraction are those that exhibit an obligate requirement for simultaneous stimulation with 36GSFT.

Collectively, the studies contained within this thesis lay a solid foundation for *ex vivo* manipulation of human haemopoietic progenitor cells under stroma-free cytokine dependent culture conditions.

ABBREVIATIONS

2ME	Beta-2-mercaptoethanol
4-HC	4-hydroxyperoxycyclophosphamide
5-FU	5-fluourouracil
ACDU	Automated Cell Deposition Unit
AGM	Aorta-gonad-mesonephros
ALL	Acute Lymphoblastic Leukaemia
β_2M	Beta-2 microglobulin
β_c	Beta-common chain of IL-3, IL-5 and GM-CSF receptors
BFU-E	Burst forming unit-erythroid
BM	Bone marrow
BSA	Bovine Serum Albumin
Bu	Busulphan
Ca-Br	Carcinoma of the breast
CAM	Cell Adhesion Molecule
CB	Cord Blood
CD	Cluster Designation
cDNA	Complementary Deoxyribonucleic Acid
CFC	Colony Forming Cell
CFU-C	Colony Forming Unit in Culture
CFU-GEMM	Colony Forming Unit-Granulocyte Erythroid Monocyte Megakaryocyte
CFU-GM	Colony Forming Unit-Granulocyte Macrophage
CFU-Meg	Colony Forming Unit-Megakaryocyte
CFU-S	Spleen Colony Forming Unit
CNTF	Ciliary Neurotrophic Factor
CRU	Competitive Repopulating Unit
CSA	Colony Stimulating Activity
CSF	Colony Stimulating Factor
CY	Cyclophosphamide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid

DPBS	Dulbecco's Phosphate Buffered Saline
ECD	Energy Coupled Dye, Tandom of PE and Texas Red
ECM	Extracellular Matrix
EPO	Erythropoietin
FACS	Fluorescent Activated Cell Sorting
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FLT3L	*flt3*Ligand
fMLP	N-formyl-methionyl-luecyl-phenylalanine
FSC	Forward Scatter
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GM-CSFR	Granulocyte Macrophage-Colony Stimulating Factor Receptor
gp	Glycoprotein
HBSS	Hanks Balanced Salt Solution
HEPES	N-2-Hydroxylethylpiperazine N'2-ethane sulphonic acid
HGF	Haemopoietic Growth Factor
HGFR	Haemopoietic Growth Factor Receptor(s)
HHF	HBSS/20mM HEPES/FBS
HIM	Haemopoietic Inductive Microenvironment
Ho	Hoechst 33342
HPC	Haemopoietic Progenitor Cell(s)
HP5b	Haemoregulatory Peptide-5b
HPP	High Proliferative Potential
HR	Haemopoietic Recovery
HSC	Haemopoietic Stem cell(s)
IFN	Interferon
IgG	Immunoglobulin isotype G
IgG_1	Immunoglobulin isotype G_1
IgG_{2a}	Immunoglobulin isotype G_{2a}
IgG_3	Immunoglobulin isotype G_3
IgM	Immunoglobulin isotype M
IL	Interleukin

IMDM	Iscove's Modification of Dulbecco's Media
I.U.	International Units
kDa	Kilo-dalton
Kg	Kilogram
LDL	Low Density Lipoprotein
Lin^-	Lineage Negative
LTBMC	Long-term Bone Marrow Culture
LTC-IC	Long-term Culture Initiating Cell
M	Molar
MAP	Mitogen Activated Protein
MBC	Metastatic Breast Cancer
McAB	Monoclonal Antibody
M-CSF	Monocyte/Macrophage-Colony Stimulating Factor
MGDF	Megakaryocyte Growth and Development Factor
MIP	Macrophage Inflammatory Protein
MM	Multiple Myeloma
mM	Milli Molar
MNC	Mononuclear Cell
MSC	Mesenchymal Stem Cell
NAE	No Adverse Events
Neo^r	Neomycin Resistance gene
NHL	Non-hodgkins Lymphoma
NGF	Nerve Cell Growth Factor
NGST	Normal Goat Serum and 0.5% tween-20
NOD/SCID	Non-obese Diabetic/severe Combined Immuno-deficient
OSM	Oncostatin M
PB	Peripheral Blood
PBPC	Peripheral Blood Progenitor Cell
PE	Phycoerythrin
Pre-CFU	Precursor to Colony Forming Unit
PSGL-1	P-selectin Glycoprotein Ligand-1
PI3	Phosphatidylinositol-3
Rh123	Rhodamine 123
RMPI	Rosewell Park Memorial Institute
RNA	Ribonucleic acid

RTK	Receptor Tyrosine Kinase
SBA	Soy-bean Agglutinin
Sca-1	Stem Cell Antigen-1
SCF	Stem Cell Factor
SCID	Severe Combined Immuno-deficient
SD	Standard Deviation
SDF-1	Stromal Derived Factor-1
SDM	Serum-deprived Media
SEM	Standard Error of Mean
SH2	Src homology-2
Sl	Steel locus
SOCS	Suppressors of Cytokine Signalling
SRC	SCID-repopulating cell
STAT	Signal Transducers and Activators of Transcription
SSC	Side Scatter
TβR	Transforming Growth Factor-beta Receptor
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TNFR	TNF Receptor
TPA	12-0-tetradecanoylphorbol-13-acetate
TPO	Thrombopoietin
UCB	Umbilical Cord Blood
UPN	Unique Patient Number
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA	Very Late Acting Antigen
v/v	Volume per Volume
w/v	Weight per Volume

PUBLICATIONS

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CONTENTS

CHAPTER 2 MATERIALS AND METHODS

CHAPTER 1. BACKGROUND AND INTRODUCTION

1.1 General Introduction

During the last decade, there has been great interest in the development of effective methods for manipulating the growth and development of haemopoietic stem and progenitor stem cells for therapy (reviewed by Emerson 1996, Haylock *et al* 1994). These initiatives include the generation of committed progenitor cells and myeloid precursors for transplantation (Haylock *et al* 1992, Brugger *et al* 1995, Williams *et al* 1996) and the use of haemopoietic stem cells (HSC) as vehicles for gene therapy (Nolta *et al* 1992, Cassel *et al* 1995). In general, *ex vivo* culture of haemopoietic precursors has involved growth of either unfractionated bone marrow or enriched populations of haemopoietic progenitor cells (HPC). In the former, haemopoietic cell proliferation is dependent on the development of a stromal cell layer to provide an appropriate microenvironment *in vitro* for haemopoietic cell growth. An alternative approach used by a number of investigators is to stimulate the growth of HPC not by co-culture with marrow stromal cells but by the provision of exogenous haemopoietic growth factors (HGF). Irrespective of the approach, an underlying objective is to generate sufficient haemopoietic cells utilising reproducible and safe methodologies that will enable a more widespread use of *ex vivo* generated cells for therapy. These general objectives were major considerations in the studies described within this thesis.

Accordingly, the studies described herein represent a detailed analysis of the culture conditions required to efficiently support the HGF-stimulated growth and development of human $CD34^+$ HPC in stromal-cell free suspension culture. The initial rationale for these studies was to develop a method for generation of myeloid cells, particularly granulocytic precursors for therapy. Subsequent studies concerned the survival, division and proliferation of primitive human HPC. In accord with these objectives, the following introduction reviews current knowledge concerning the organisation of the haemopoietic system and the cellular and molecular mechanisms that contribute to the regulation of blood cell formation.

1.2 Historical Perspectives: Eras in Experimental Haematology

1.2.1 The First Era: The Age of Morphology

For many centuries, mankind has had a strong appreciation of the link between blood and life. Blood has symbolised vitality, youth and immortality, yet knowledge of the constituents

of blood and regulation of the process of blood cell formation is relatively recent. The first era in experimental haematology was dominated by macroscopic and microscopic examination of haemopoietic tissues in order to define in morphological terms the components of the haemopoietic system. The initial discussion concerning the site of origin of haemopoiesis is attributed to William Hewson, who in 1774 proposed that blood cells originated in lymphatic tissues with the thoracic duct feeding cells directly into the peripheral blood (Gulliver 1846). This theory was largely unchallenged for over 70 years until in 1846, embryologists Weber and Kolliker suggested that the liver was the main site of adult haemopoiesis (Weber and Kolliker 1846). However, it was not until 1868 that Ernst Neumann and Giulio Bizzozero independently established a link between the bone marrow and blood (Neumann 1868, Bizzozero 1868). They described two important features of haemopoiesis: first relating to the cellular origin of blood (non-nucleated red cells arising from nucleated precursors) and secondly that haemopoiesis was a continuous process. In his report, Neuman states, "The present work intends to demonstrate the physiological importance of the bone marrow and that it is an important organ for blood formation which has not been recognised. It operates continually in a *de novo* formation of red blood cells" (Neumann 1869).

Within 10 years of describing these features of haemopoiesis, Paul Ehrlich developed methods for fixing and staining blood and marrow cells. He suggested that an agranular cell within the bone marrow (BM) - termed the *myelozyt* (marrow cell) was the precursor for granulocytes and that lymphocytes developed through an independent precursor cell located within the lymphatic tissue (Ehrlich 1879). These observations and suggestions were the beginning of the stem cell concept. Pappenheims' studies followed closely on from Ehrlich and using the newly developed Romanovsky stains, he described the various transitional forms according to their morphology and granulation and noted that these cells could be traced back to a relatively featureless "primitive" type of mononuclear cell - termed the *Lymphoidozyt*. He proposed the idea that because this cell was so primitive (on morphological grounds) that it could be considered as the common ancestor of all blood cells; ie it was the totipotent, undifferentiated HSC (Pappenheim 1898). Danchakoff made the first suggestion that red and white cells might originate from a common stem cell (Danchakoff 1916). It is notable that proof of this concept did not come until some 40 years later when transplantation experiments were performed. Thus, this era set the stage for those interested in determining how stem cells might function. The introduction of radiation biology heralded a new era in experimental haemopoiesis in which for the first time

experimental approaches were developed which allowed a structural and functional analysis of the organisation of the haemopoietic system.

1.2.2 The Second Era: Functional Analysis of Haemopoiesis

1.2.2.1 Characteristics of Haemopoietic Precursor cells

The concept of a hierarchy of cells arising from multipotent, self-renewing haemopoietic stem cells that give rise to progeny with reduced developmental potential and increased commitment is a well-established paradigm of haemopoiesis. Initial evidence for this model emerged primarily through functional studies in murine haemopoiesis. The existence of haemopoietic repopulating cells was established in the early 1950's in studies on lethally irradiated mice (Lorenz *et al* 1951, Ford *et al* 1956, Lindsley *et al* 1955). Subsequently, Till and McCulloch showed that mouse cells, when infused into irradiated syngeneic mice were capable of forming discrete haemopoietic nodules within the spleens of animals 8 –10 days after injection (Till and McCulloch 1961). These nodules consisted of granulocytic, erythroid, megakaryocytic and undifferentiated cells, either as pure populations or in varying mixtures (Lewis and Trobaugh 1964, Fowler *et al* 1967). With the use of radiation-induced chromosomal markers the clonality of spleen colonies (CFU-S) was demonstrated (Becker *et al* 1963, Wu *et al* 1967) and transplantation of cells harvested from a proportion of spleen colonies gave rise to secondary CFU-S indicating self-renewal of CFU-S (Siminovitch *et al* 1963, Cudkowicz *et al* 1964). In spite of these data there was controversy concerning the existence of a common, more primitive precursor cell with the ability to generate both lymphoid and myeloid cells. This issue was finally resolved by transplantation of chromosomal marked marrow (Abramson *et al* 1977), which demonstrated that a single marked cell could give rise to either myeloid, T-lymphoid or progeny of both lineages.

At this time, the CFU-S was considered to represent a candidate HSC. However, it was soon recognised that the CFU-S population is heterogeneous (Worton *et al* 1969, Schofield and Lajtha 1973), there is a pre-CFU-S pool (Hodgson and Bradley 1979) and that CFU-S represent transit cells rather than HSC with capacity for long-term (for the rest of life) haemopoietic reconstitution (Schofield 1978). For example, the work by Magli *et al* indicated that the majority of spleen colonies present 7-8 days after injection consisted mainly of developing erythroid cells whereas colonies appearing later (days 10-12) were more heterogenous and, as they contained multiple lineages (excluding lymphoid cells), were more representative of multipotent haemopoietic progenitors (Magli *et al* 1982).

Further studies of the CFU-S forming early and late colonies demonstrated differences between these two populations both in terms of proliferation (Chertkov and Drize, 1984) and cell surface phenotype (Harris *et al* 1984). Collectively, these studies provided some of the initial evidence to support a hierarchical organisation of haemopoietic stem and progenitor cells. Additional evidence for the hierarchical relationship between stem cells, progenitor cells, lineage committed precursors and mature blood cells came with the development of methods for cloning murine haemopoietic cells in semisolid media in 1966, independently but at the same time by groups in Australia (Bradley and Metcalf 1966) and Israel (Pluznik and Sachs 1966).

These initial murine studies involved culture of BM cells in agar and media supplemented with a range of stimuli. These included feeder layers of kidney and whole mouse embryo (Bradley and Metcalf 1966), conditioned medium from these cells (Bradley *et al* 1971, Pluznik and Sachs 1966) and animal and human urine (Robinson *et al* 1969). Metcalf was the first to describe activities within the supernatants of various organ cultures that stimulated colony growth and in 1972 coined the term colony-stimulating factor (CSF) and suggested that these factors might be humoral regulators of haemopoiesis. In addition, he introduced the term colony-forming unit in-culture (CFU-C) to describe those cells that give rise to colonies in semisolid media. The first report of growth of human CFU-C was published in 1967 where feeder layers of kidney tubule cells were used as a source of CSF (Senn *et al* 1967).

Subsequent studies established that human colony stimulating activity (CSA) could be provided by leukocyte feeder layers (Pike and Robinson 1970), human serum (Foster *et al* 1967, Metcalf and Wahren 1968), human urine (Metcalf and Stanley 1969), spleen cells (Paran *et al* 1970), conditioned medium from human leukocytes (Iscove *et al* 1971) and various tissue extracts. Additional studies demonstrated that erythroid clones could also be grown (Axelrad *et al* 1974), which lead to an appreciation of "committed" precursors or progenitors being an intercalated amplifying population between stem cells and mature blood cells which buffers the stem cell population from depletion by excess proliferation. Furthermore, these studies confirmed that colony formation by committed haemopoietic progenitors in semisolid assays is absolutely dependent on provision of exogenous CSF. Thus, a major contribution arising from the development of clonogenic assays was the ability to test the biological activity of CSF, which, were at that time being identified and purified from a variety of sources. The isolation of specific CSF is briefly discussed below in the

section on haemopoietic regulators. In addition, clonogenic assays proved to be an invaluable tool for investigating the physical characteristics of committed HPC and for testing methods for their purification and isolation (Jones *et al* 1990, Andrews *et al* 1989, Bertoncello *et al* 1991). However a major limitation of clonogenic assays was the inability to maintain production of cells for longer than 2-3 weeks, an observation that suggested that these systems were not suitable for investigating the biology of stem cells or their immediate progeny. Consequently, alternative assay systems such as the blast colony assay (Leary and Ogawa 1987) and pre-CFU cultures (Iscove *et al* 1989, Iscove *et al* 1990) were designed to examine the haemopoietic potential of primitive HPC. These *in vitro* assays were substantially enhanced by the development of a range of *in vivo* animal models that better test the developmental potential of human haemopoietic cells (Srour *et al* 1992a). A more detailed review of xenogeneic transplant models is provided below.

Our present understanding and concepts of haemopoiesis were shaped by two additional key observations during this functional era of haemopoiesis. The first of these arose from the studies of Lajtha and colleagues using thymidine suicide techniques (Lajtha *et al* 1962, Lajtha 1963). Lajtha showed that a high proportion of stem cells, measured as CFU-S, were in an "out of cell cycle" state he termed G_0 which was defined as "a state in which the cell does not prepare for, or is not in the process of DNA synthesis or mitosis. A cell in G_0 is not in the proliferative cell cycle but within a state where it can be triggered to enter the proliferative cycle". Lajtha also suggested that G_0 was important for stem cells in that it allowed time and conditions for repair and correction of accrued genetic defects. In addition, he proposed that exit from quiescence was a random process and in making this suggestion introduced the concept of haemopoiesis being stochastic.

Subsequent studies in both the murine (Hogson and Bradley 1979, Suda *et al* 1983) and human systems (Leary *et al* 1989, 1992) provided additional evidence for quiescent HSC. Several *in vivo* studies of animal haemopoiesis documented significant serial fluctuations of stem cell clones (Mintz *et al* 1984, Lemischka *et al* 1986, Snodgrass and Keller 1987, Capel *et al* 1989, Abkowitz *et al* 1990, Keller and Snodgrass 1990, Jordan and Lemischka 1990). In particular, transplantation studies using retroviral labelling of individual stem cell clones (Lemischka *et al* 1986, Snodgrass and Keller 1987, Capel *et al* 1989) provided experimental evidence for the "clonal succession" model of Kay (Kay 1965) and indirectly supported the concept of stem cell quiescence. More recent studies have since demonstrated that, the most primitive HSC, as characterised by their Rhodamine 123/Hoechst 33342 dull phenotype are

not completely dormant but cycle slowly in steady-state bone marrow (Bradford *et al* 1997). The ability of HSC to remain dormant has been exploited experimentally and used as the basis for selection of HSC from their progeny. Candidate HSC can be separated from more mature cells by the selective killing of the later cells by cell cycle specific anti-metabolites such as 4-hydroperoxycyclophosphamide (4-HC) and 5-fluorouracil (5-FU) (Rosendaal *et al* 1981, Beradi *et al* 1995, Gordon *et al* 1985, Smith *et al* 1991). Despite the considerable body of data demonstrating the non-cycling state of HSC, the physiological mechanisms responsible for regulating quiescence of primitive HPC and HSC remain poorly understood.

1.2.2.2 Stromal Cell Regulated Haemopoiesis

Another major observation made during this functional era concerned the role of non-haemopoietic cells within the bone marrow in regulation of haemopoiesis. Collectively, data from many investigators demonstrated that the BM microenvironment and its constituent population of stromal cells and their extracellular products are intimately involved in regulation of haemopoiesis.

There is now considerable evidence for control of haemopoiesis by the haemopoietic microenvironment (Metcalf and Moore 1971, Trentin 1975, 1978, Tavassoli 1975, Wolf 1979, Dexter 1982). Functionally, this regulation is considered to operate at close range and to be mediated by the same cellular and connective tissue elements that provide mechanical support for developing haemopoietic cells (Weiss 1976, Lichtman 1981). The most compelling evidence for a permissive haemopoietic inductive microenvironment (HIM) is the fact that despite the presence of HGF within blood and many tissues, adult haemopoiesis only occurs within the bone marrow. It is now clear that stromal cells mediate the regulatory effects of the HIM. The pivotal role of stromal cells in regulation of haemopoiesis is well illustrated by mutations of the white spotting (*W*) and *Steel* (*Sl*) loci in the mouse, which cause profound defects in haemopoiesis *in vitro* and *in vivo* (Russell 1979, Dexter and Moore 1977). The genetically anaemic Sl/Sl^{d} mice have normal stem cells, but the ability of the stroma to support haemopoiesis is defective. Transplantation studies have shown that the HSC from Sl/Sl^{d} mice can adequately repopulate lethally irradiated mice and that the anaemia of Sl/Sl^{d} mice can be treated by engraftment of stromal cells (McCulloch *et al* 1965). Subsequent molecular analysis demonstrated that the *W* mutations result in defects in the c-*kit* receptor in HSC (Chabot *et al* 1988, Geissler *et al* 1988), while *Sl* mutations are defective in production of its ligand, stem cell factor (SCF), by stromal cells (Huang *et al* 1990, Zsebo *et al* 1990, Flanagan *et al* 1991).

Additional evidence that stromal cells mediate haemopoiesis come from studies where severe damage or destruction of the BM stromal elements, induced by irradiation (Knopse *et al* 1966, Maloney and Patt 1972, Chamberlin *et al* 1974) or mechanical insult (Tavassoli *et al* 1973), results in a permanent failure of the organ to support haemopoiesis. Furthermore, experiments involving heterotopic transplantation indicate that regeneration of stromal elements is required before onset of haemopoiesis (Perla 1936, Tavassoli and Crosby 1968, Knopse *et al* 1978).

The first attempts to define the morphology and function of marrow stromal cells came from Friedenstein (Friedenstein *et al* 1970). He showed that "fibroblasts" cultured from murine BM, when implanted under the kidney capsule, could produce an environment that allowed haemopoiesis to develop. However, fibroblasts cultured *in vitro* with normal BM cells, failed to support haemopoiesis (Lubennikova and Domaratskay 1976). The possibility of extended *in vitro* haemopoiesis in adherent cell cultures was demonstrated by granulocyte production over several days, in association with "nurse" cells (Chang and Anderson 1971). The subsequent development of long-term (3-6 weeks) murine bone marrow culture (LTBMC) systems by Dexter demonstrated that active haemopoiesis was associated with the adherent layer, where more primitive cells were located and divided (Dexter and Lajtha 1974, Dexter *et al* 1977, Dexter *et al* 1980). In contrast, maturing, differentiated progeny migrated from the adherent layer and were found within the non-adherent cell fraction.

These *in vitro* studies supported the proposal that *in vivo*, HSC reside in specialised stromal cell domains or niches that preferentially facilitate their survival and proliferation; whereas other locations directed progenitor cell proliferation (Dexter *et al* 1984). This is in accord with an earlier concept of the HIM that defined the local developmental restriction of haemopoiesis mediated by stromal cells (Trentin 1970). The "stem cell niche" hypothesis was subsequently suggested by Schofield to describe the association between true HSCs and microenvironmental cells (Schofield 1978). In the stem cell niche model the HSC were considered as fixed tissue cells that resided in association with one or more stromal cells that collectively inhibited differentiation and thus maintained stem cell characteristics. It was considered that the number of stem cell niches were limited and that following division, one daughter cell would remain in the niche while the other would be displaced and thus be able to contribute to haemopoiesis. Schofield also proposed that the cell detected by CFU-S assay, which being free of bone marrow microenvironmental control, had limited self-

renewal potential. A fundamental premise of the stem cell niche model was that the cells comprising the niche regulated stem cell fate and therefore haemopoiesis.

Moreover, compelling additional, albeit indirect, evidence for the importance of stromal-cell-haemopoietic cell interactions (and perhaps a stem cell niche) in haemopoiesis is provided by the pattern of haemopoietic development during ontogeny. Haemopoietic stem cells reside at different anatomic sites during vertebrate development before they ultimately migrate to their final residence, the BM (Dzierzak and Medvinsky 1995, Zon 1995, Peault 1996). Early evidence suggested that the foetal yolk sac was the primordial location of HSC that seeded the foetal liver (Moore and Metcalf 1970). In addition, recent studies have shown the ability of yolk-sac-derived stem cells to reconstitute the BM of newborn recipients but not adult animals (Yoder *et al* 1997). More recently, however, an intraembryonic site, the para-aortic splanchnopleura, has been suggested to be the site of emergence of definitive HSC during ontogeny. In support of this notion, studies of chick yolk sac-embryo chimaeras demonstrated that HSC that seed the foetal liver and, ultimately, the BM are derived from an intraembryonic site and not the extraembryonic yolk sac (Martin *et al* 1978, Lassila *et al* 1978). The intraembryonic para-aortic splanchnopleura zone, which goes on to differentiate into the aorta-gonad-mesonephros (AGM) region, is therefore likely to be the site where definitive haemopoietic stem cells are first generated (Dieterlein-Lievre and LeDourain 1993, Pardanaud *et al* 1996).

In addition to these important findings, morphological studies have implicated the endothelium of the AGM-localised dorsal aorta as an *in vivo* site of HSC residence and, potentially, amplification. By using the sialomucin CD34 as a marker for both the endothelium and HPC, these studies revealed that a specific region of the dorsal aorta, contained within the AGM region, harbours small clusters of $CD34^+$ haemopoietic cells attached to the endothelium in both human and murine embryos (Tavian *et al* 1996, Wood *et al* 1997). Subsequent studies have revealed that endothelium-associated stem cell clusters emerging from both the dorsal aorta and the vitelline artery (Peault 1996, Tavian *et al* 1999) migrate to the foetal liver. Within weeks HSC from the liver then migrate to the BM and initiate haemopoiesis. It is presumed that migration of HSC to these specific sites is an active process, in part mediated by chemotactic factors. For instance, stromal derived factor-1 (SDF-1), secreted by stromal cells (Aiuti *et al* 1997) induces migration of HSC into the BM (Nagasawa *et al* 1996). Consequently, although mice lacking SDF-1 have severely reduced numbers of B-cell progenitors in the foetal liver and bone marrow, myeloid progenitors were

reduced only in the bone marrow. Thus failure of haemopoiesis in SDF-1 null mice has been attributed to the inability of primitive HPC to migrate to the BM microenvironment. It is inferred that the specific and sequential lodgement and subsequent proliferation of HSC within the liver and BM, rather than other tissues, is because the microenvironment of these respective sites is permissive, if not instructive, for haemopoiesis. Thus, stromal cells and ECM within the microenvironment not only serve to anchor HSC and their progeny but more importantly to stimulate and regulate blood cell production.

Many *in vitro* studies utilising LTBMC also demonstrate the critical role of stromal cells in regulating growth and development of HPC. Initial studies with LTBMC described the maintenance and production of CFU-S, myeloid progenitors (CFU-GM), neutrophils and monocytes but later studies demonstrated maintenance of megakaryocytic and erythroid progenitor cells (Williams *et al* 1978, Eliason *et al* 1979). Subsequent studies demonstrated that stromal layers could be developed from human BM but they did not support haemopoiesis for any length of time. A major improvement came from Gartner and Kaplan who used a combination of horse and foetal calf serum together with hydrocortisone and reported that cultures could be maintained for up to 20 weeks (Gartner and Kaplan 1980). Since this improvement, LTBMC assays have been used to characterise cells with the ability to initiate and sustain human haemopoiesis (Sutherland *et al* 1989, Moore 1980, Keating *et al* 1984, Brandt *et al* 1990) and also for measuring their incidence in various haemopoietic tissues (Sutherland *et al* 1990, Sutherland *et al* 1995).

1.2.2.3 Cell Adhesion Molecules and Haemopoiesis

Collectively, there are abundant data from many *in vivo* and *in vitro* studies to highlight the importance of physical interactions between stromal cells and the ECM in the regulation of HSC and their progeny (reviewed by Clark *et al* 1992). It is also evident that these physical interactions are mediated by cell adhesion molecules (or their counter-receptors) expressed by stem and progenitor cells. Cell adhesion molecules (CAM) expressed by haemopoietic cells are involved in two, seemingly distinct, but intrinsically linked functions. The first of these concern the role CAM as direct mediators of adhesion between haemopoietic cells and other cell types or the ECM. Such developmentally regulated adhesive interactions enable haemopoietic cells to migrate and home to sites of haemopoiesis, facilitate the retention of haemopoietic cells with these specialised sites and also mediate the extravasation and migration of mature haemopoietic cells into extramedullary tissues. The second function of CAM concerns their role as *bona-fide* signalling molecules and accordingly their ability to

regulate the survival, proliferation, activation and differentiation of haemopoietic cells (Yoder and Williams 1995, Levesque *et al* 1999a). In this respect, CAMs on haemopoietic cells exhibit identical functions to their counterparts expressed on non-haemopoietic cells, where signalling initiated by CAM-ligand interactions regulate cell adhesion, motility, cell growth (Hansen *et al* 1994, Symington 1995, Fang *et al* 1996, Zhu *et al* 1996), apoptosis (Zhang *et al* 1995), and specific gene regulation (Yurochko *et al* 1992).

It is now recognised that HSC and HPC express a large repertoire of CAM representing at least five superfamilies of molecules, including the immunoglobulin (Ig) gene superfamily, integrins, selectins, sialomucins, and the CD44 family of adhesion molecules (Kincade *et al* 1989, Clark *et al* 1992, Long 1992, Simmons *et al* 1994b, 1997). In some respects the diversity of molecules used by developing haemopoietic cells parallels that described for regulating cells within the immune system and the immune response (Springer 1990). Although HPC express such an extensive array of CAM, it remains unclear how, or where specific CAMs participate in specific adhesive interactions at different stages of haemopoietic cell development. However, recent studies show that foetal and adult HSC require β1 integrins for establishment of haemopoiesis in the foetal liver, spleen and BM (Potocnik *et al* 2000). In addition, the α integrins play distinct roles during *in vivo* development and trafficking of lymphoid and non-lymphoid cells (Arroyo *et al* 1996, 2000). In addition, *in vivo* studies with blocking monoclonal antibodies to the β1 or Very Late Antigen (VLA) integrins, VLA-4 or vascular cell adhesion molecule-1 (VCAM-1) indicate that the interaction of VLA-4 expressed on HPC with VCAM-1 expressed on marrow stromal and endothelial cells is critical for engraftment of HPC (Williams *et al* 1991, Papayannopoulou *et al* 1995). Moreover, perturbation of this single adhesive interaction can induce mobilisation of HPC from the BM into the peripheral circulation (Papayannopoulou *et al* 1993).

Apart from playing a critical role in the homing of HSC during embryonic development and retention of HSC within the BM, CAMs may also have important regulatory roles in adult haemopoiesis. Of particular note in this regard is the functional interplay or cross talk between HGF-receptors and signalling pathways utilised by β1-integrins (Levesque *et al* 1996) and the effects on HPC survival and proliferation induced following ligation of sialomucins (Levesque *et al* 1999b, Zannettino *et al* 1998, Bazil *et al* 1996).

Quiescent HPC express β1-integrins as nonactive, nonligand-binding receptors that can be rapidly, yet transiently activated by exposure of cells to cytokines such as IL-3, IL-6, GM-CSF, G-CSF and SCF. Exposure to combinations of these cytokines leads to specific activation of VLA-4 and VLA-5 without any change in the level of expression at the cell surface, resulting in the ability of these two integrin receptors to bind their cognate ligands, VCAM-1 and fibronectin, respectively, with high affinity. It is proposed that binding of HGF to their cognate receptors leads to activation of the integrins (so-called "inside-out" signalling), which in turn enables them to bind their counter-receptors such as fibronectin. This latter interaction results in a second signalling event or an "outside-in" signal. Whether the outside-in signal generated by ligation of VLA-4 and VLA-5 with fibronectin cooperates or antagonises the mitogenic signal generated by cytokine receptors is still unclear. Interestingly, studies with haemopoietic cell lines have demonstrated a spatial and functional convergence of the transduction pathways activated by cytokine receptors and by integrin-mediated outside-in signalling (Miyamoto *et al* 1995, Plopper *et al* 1995, Assoian and Zhu 1997). For example, both classes of receptor result in activation of common transducers such as MAP kinases (Chen *et al* 1994, Morino *et al* 1995, Renshaw *et al* 1997), the adapter Grb2 (Schlaepfer *et al* 1994), cytoskeleton-regulating proteins such as paxillin and tensin (Bockholt and Burridge, 1993) and the focal adhesion kinase, FAK (Takahira *et al* 1997). Moreover, integrin-mediated adhesion is a prerequisite for the synthesis of some cyclins and the activity of cell cycle dependent kinases (Fang *et al* 1996, Zhu *et al* 1996). These data are relevant to primitive human HPC: $CD34^+$ HPC and cytokine-dependent myeloid cells form adhesion plaques on ECM proteins in which integrins colocalise with cytokeletal proteins talin, α-actinin, and vinculin (Levesque and Simmons, 1999) leading to specific tyrosine phosphorylation (Gotoh *et al* 1997). These data thus suggest that signalling through the β1-integrins VLA-4 and VLA-5 cooperate to enhance cytokine mediated signalling of HPC. However, focal adhesion complexes within HPC are different in that they do not contain FAK or tensin, suggesting that signalling involves activation of a different repertoire of transducers.

In contrast, adhesion mediated by another family of CAMs the sialomucins results in signalling that antagonises cytokine receptor signalling and suppresses growth of HPC. Sialomucins are a family of glycoproteins expressed by cells of the haemopoietic and immune system (Baumheter *et al* 1993, Shimizu and Shaw 1993), which exhibit a common structural feature of multiple serine and threonine residues in their extracellular domains that act as sites for attachment of O-linked glycans. Of the multiple members of this family, at

least seven are expressed by human HPCs: CD34 (Krause *et al* 1996), CD43 (leukosialin: Shelley *et al* 1990, Moore *et al* 1994), CD45RA, CD162 (P-selectin glycoprotein ligand-1/PSGL-1: Sako *et al* 1993), CD164 (multiglycosylated core of 24kDa / MGC-24: Masuzawa *et al* 1992, Zannettino *et al* 1998), PCLP-1 (Sassetti *et al* 1998) and endoglycan (Sassetti *et al* 2000). Recent reports have also shown that sialomucins can also mediate outside-in signalling in HPCs. Expression of the full-length CD34 in murine M1 cells resulted in inhibition of cytokine induced differentiation (Fackler *et al* 1995) and antibody cross linking of CD43 resulted in apoptosis of a proportion of human $CD34^+$ cells (Bazil *et al* 1996). Notably, although expressing CD43 at high level, candidate HSC were refractory to the apoptosis-inducing effects of the anti-CD43 antibody. Similarly, adhesion of $CD34^+$ cells to P-selectin (CD62P) has been shown to markedly inhibit haemopoiesis in a stromal cell-free, cytokine dependent culture system (Levesque *et al* 1999). This response is due to induction of apoptosis in a subpopulation of primitive $CD34^+CD38^-$ cells and can be partially modulated by addition of thrombopoietin and *flt3*-ligand (FLT3L) to cultures. Similarly, addition of antibody to particular epitopes of CD164 markedly suppresses haemopoiesis in cytokine-dependent stroma-free suspension culture of $CD34^+$ cells (Zannettino *et al* 1998). Thus sialomucins on HPC appear to be potent negative regulators of haemopoiesis, at least *in vitro*. Their role *in vivo* requires further study although the phenotype of both the CD62P knockout ($CD62P^{-/-}$) mice and those deficient in both CD62P and E-selectin (CD62E) support this concept. In the case of $CD62P^{-/-}$ mice there is an increase in the number of megakaryocytes in the BM (Banu *et al* 1996) while there is an extreme leukocytosis and abnormally increased HPC numbers in the double deficient mice (Frenette *et al* 1996). Collectively these data suggest that sialomucin-mediated interactions *in vivo* might operate to dampen excessive expansion of HPC.

The cellular responses induced by adhesive interactions such as those mediated by integrins and members of the sialomucin family illustrate the importance of cellular interactions in the regulation of haemopoiesis. However, as described, soluble HGF are able to modulate adhesive interactions, suggesting that regulation of haemopoiesis *in vivo* is due to a dynamic interplay between these two classes of molecules.

In summary, much of the research performed during the functional era of haemopoiesis was descriptive in nature. Collectively, numerous studies provided a greater understanding of the characteristics of cells within the haemopoietic system, their hierarchical relationship and some insights as to the groups of molecules involved in regulation of haemopoiesis.

However, it was not until the introduction of molecular biological techniques that experimental haematologists began to investigate and appreciate the fundamental mechanism/s responsible for regulating haemopoiesis and the functions of haemopoietic cells. These studies form the basis of the third era of experimental haematology.

1.2.3 The Third Era: Molecular Analysis of Haemopoiesis

This era was heralded by the introduction of a broad range of biochemical and molecular techniques that enabled experimental haematologists to investigate the fundamental mechanisms that regulate haemopoiesis. Initial studies concerned the biochemical characterisation and purification of colony stimulating activity, the purification of colony stimulating factors, interleukins and cytokines (Price *et al* 1975, Burgess *et al* 1977, Nicola *et al* 1978, Nicola *et al* 1979, Clark and Kamen 1987). During the last 10 years the cDNAs and genes for many haemopoietic colony-stimulating factors in mouse and man were cloned, recombinant HGF produced in a variety of expression systems and preclinical and clinical trials initiated.

Based on the wealth of evidence that BM stromal cells and T lymphocytes secrete activities that influence (positively and negatively) haemopoietic cell development many investigators understandably chose to screen cDNA libraries prepared from these cell types. This approach was highly successful and led to cloning of a number of haemopoietic regulators including IL-11, SCF, and FLT3L, (Paul *et al* 1990, Flanagan *et al* 1991, Hannum *et al* 1994). Subsequent investigations, based on recombinant DNA technology led to the identification of genes that encode cell surface proteins on haemopoietic cells such as cytokine receptors, cell adhesion molecules, intracellular signalling molecules and transcription factors that regulate gene expression.

This era is also characterised by molecular analysis of the interactions between HGF and their cognate cell surface receptors on haemopoietic cells (Walker *et al* 1985, Metcalf 1989, Bazan 1990, Ihle *et al* 1994). These studies provided the basis for our current understanding of how HGF receptors interact with ligand(s) and induce signalling. As described below, there is now considerable data on the structure and function of HGF receptors and the signal transduction pathways used to induce biological responses. Similar experiments performed to establish the molecular basis for biological responses mediated by cell adhesion molecules (CAM) demonstrate a functional interplay between CAM and HGF receptors (Levesque *et al* 1996). In addition, the function of a broad range of gene products with putative involvement

in regulating haemopoiesis or haemopoietic cell function have been investigated by techniques including dominant negative mutants, gene knockout, gene over-expression in transgenic mice and site directed mutagenesis. In particular, murine gene-knockout models have proved to be important in defining the roles of regulators in steady state haemopoiesis. Mice nullizygous for genes encoding haemopoietic regulators or their cognate receptors have either normal peripheral blood counts or varying degrees of cytopenia but notably none show complete aplasia, indicating overlapping actions of multiple HGF in sustaining haemopoiesis. These models suggest that factors such as GM-CSF and IL-3, which have potent *in vitro* affects on HPC proliferation and differentiation, may not contribute to steady state haemopoiesis but have critical roles in enhancing haemopoiesis or the functional activity of haemopoietic cells during infection or antigenic challenges. In contrast, mice nullizygous for TPO or its receptor c-*mpl* demonstrate that factors initially considered as lineage specific may have wider roles in regulating the development and proliferation of primitive HPC.

This era has also seen the application of recombinant HGF in clinical haematology. In this regard, the contribution from experimental haematologist has been significant. The use of recombinant erythropoietin (EPO) for the treatment of anaemia associated with renal disease (Mohini *et al* 1989) and G-CSF or GM-CSF for stem cell mobilisation and treatment of neutropenia are two dramatic examples (Sheridan *et al* 1992, Gianni *et al* 1989, Gianni *et al* 1992).

The present era is also characterised by an unprecedented rate of advance in our understanding of the cellular and molecular mechanisms that regulate cell development and function. This is particularly apparent with studies of the haemopoietic system where technological advances together with the relatively easy access to haemopoietic tissues has allowed rapid progress in the characterisation of rare cells such as haemopoietic stem cells. Accordingly, a major focus for many experimental haematologists is the isolation, manipulation and use of haemopoietic cells for various clinical applications. In this respect, the work presented in this thesis concerning *ex vivo* culture of human haemopoietic stem and progenitor cells, represents a new and burgeoning era of experimental haematology. The following sections of the introduction review the characteristics of primitive haemopoietic stem and progenitor cells with particular emphasis on methodologies for their isolation and assay and also discusses the nature of regulatory molecules that influence their survival, proliferation and differentiation.

1.3 Characterisation of Haemopoietic Stem and Progenitor Cells

As stated previously, haemopoiesis is commonly represented as a hierarchical process where development and differentiation proceeds in a unidirectional fashion. The hierarchical model, proposed many years ago (Chervenick 1976, McCulloch 1983, Ogawa 1993) places haemopoietic cells within a series of compartments that represent cells identified by various *in vitro* and *in vivo* assay systems. Such a model is presented in figure 1.1.

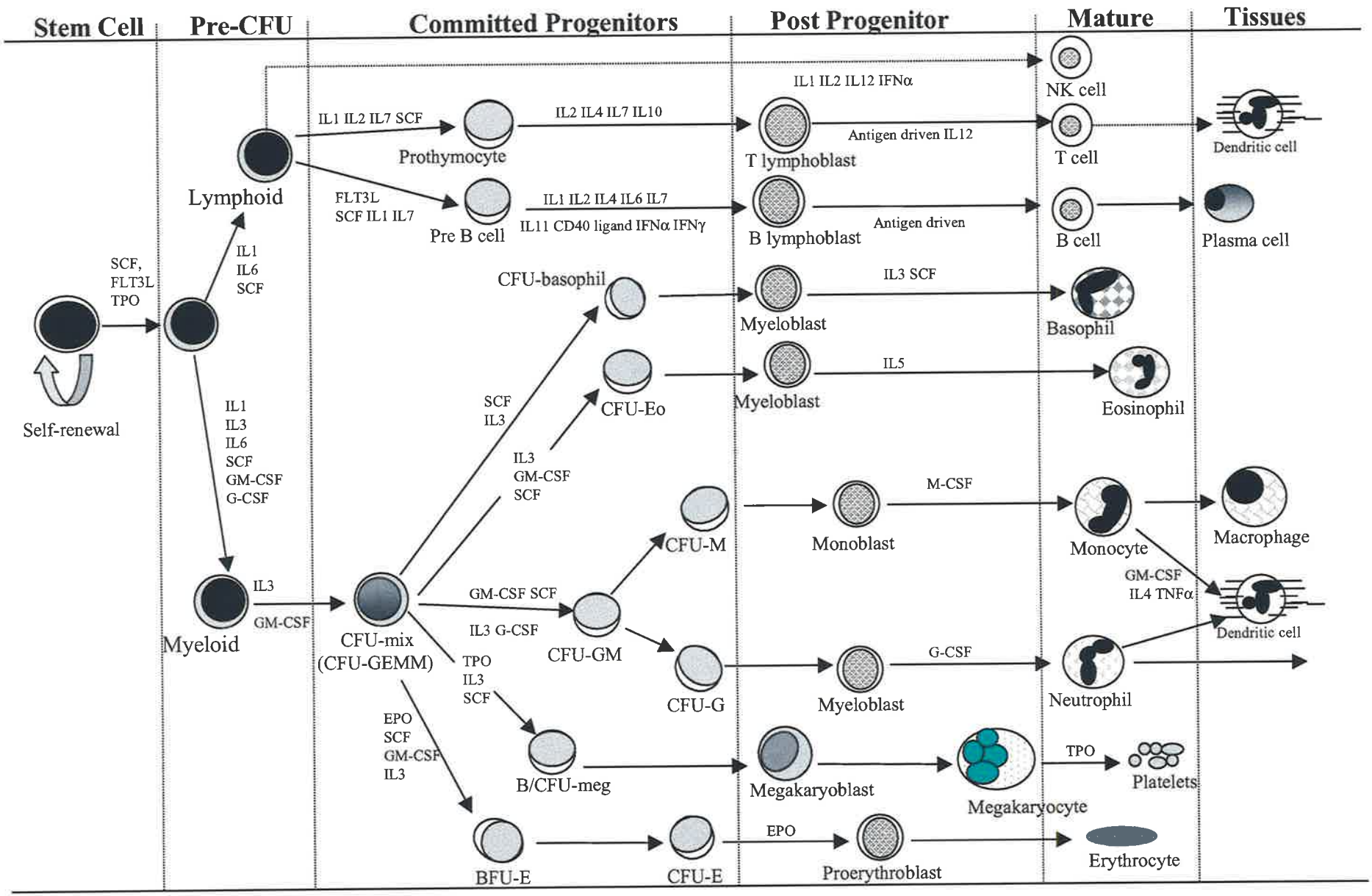
According to hierarchical models such as that presented in figure 1.1, HSC reside at the apex of the haemopoietic system and by definition are multipotent, capable of self-renewal and are responsible for long-term haemopoietic reconstitution after transplantation. It is generally accepted that during steady state haemopoiesis the majority of HSC are quiescent or transit through cell cycle very slowly (Bradford *et al* 1997), and do not contribute to haemopoiesis. It remains unclear if recruitment of HSC into the cell cycle is a random event (stochastic) or determined by a complex array of extrinsic signals mediated through CAMs, HGF and their receptors or other soluble regulators. Once recruited HSC give rise to progenitor cells with less developmental potential. The subsequent process of differentiation is dynamic and cells may be located at any level of development between the HSC and the mature, functional end cell.

Many investigations concerning the characteristics of HSC and progenitor cells are based on *in vivo* murine transplantation models. These models test the ability of purified populations of candidate HSC to reconstitute haemopoiesis. Such experiments have confirmed the presence of a common precursor for both lymphoid and myeloid cells (Lemischka *et al* 1986). In these studies, BM transplantation was performed with cells retrovirally transfected with the neomycin resistance gene (Neo^r). Following transplantation, myeloid and lymphoid cells were found to contain the same Neo^r integration site, suggesting that both lineages apparently derived from a single stem cell. Similar results have since been obtained in other laboratories (Snodgrass and Keller 1987, Capel *et al* 1989), so that the concept of a single pluripotent stem cell that gives rise to individual lineages is based on firm experimental evidence.

Spangrude *et al* first claimed the isolation of a purified population of marrow repopulating HSC from murine BM in 1988. They described a cell with a Thy-1^{lo}, $lineage^-$, stem cell

Figure 1.1 Schematic of human haemopoiesis

This figure depicts a conceptual framework for the relationship between cells within the haemopoietic system. The model is based on hierarchical ordering of haemopoietic cells with development and differentiation proceeding from the pluripotent haemopoietic stem cell (HSC), on the left, to mature functional cells on the right. Haemopoietic cells are arbitrarily placed within 6 compartments based on functional assays and parent - progeny relationships. The HSC is at the apex of haemopoiesis and gives rise to precursors to committed progenitor cells, collectively termed Pre-CFU. Cells within the committed progenitor compartment are identified by their ability to clone, in response to colony stimulating factors, in various *in vitro* assay systems. The post-progenitor compartment is characterised by extensive proliferation of cells within each lineage, which are now clearly identified by their unique morphology and cell surface antigen repertoire. Finally, the process of differentiation produces mature functional cells that move rapidly from the bone marrow into the peripheral circulation and in some cases migrate in response to chemotactic signals into the tissues. This schema also indicates some of the key positive regulators of haemopoiesis that induce proliferation and differentiation of cells at various stages of development.


Stem Cell
Pre-CFU
Committed Progenitors
Post Progenitor
Mature
Tissues
Self-renewal
SCF,
FLT3L
TPO
Lymphoid
IL1
IL6
SCF
Myeloid
IL3
GM-CSF
IL1
IL3
IL6
SCF
GM-CSF
G-CSF
CFU-mix
(CFU-GEMM)
IL1 IL2 IL7 SCF
Prothymocyte
FLT3L
SCF IL1 IL7
Pre B cell
EPO
SCF
GM-CSF
IL3
BFU-E
TPO
IL3
SCF
B/CFU-meg
IL3 G-CSF
CFU-GM
GM-CSF SCF
SCF
IL3
CFU-basophil
IL3
GM-CSF
SCF
CFU-Eo
CFU-M
CFU-G
CFU-E
IL2 IL4 IL7 IL10
IL1 IL2 IL4 IL6 IL7
IL11 CD40 ligand IFNα IFNγ
IL1 IL2 IL12 IFNα
T lymphoblast
Antigen driven IL12
B lymphoblast
Antigen driven
Myeloblast
IL3 SCF
Myeloblast
IL5
Monoblast
M-CSF
Myeloblast
G-CSF
Megakaryoblast
EPO
Proerythroblast
NK cell
T cell
B cell
Basophil
Eosinophil
Monocyte
Neutrophil
Megakaryocyte
TPO
Erythrocyte
Dendritic cell
Plasma cell
Macrophage
GM-CSF
IL4 TNFα
Dendritic cell
Platelets

antigen-1^+ (Thy-1^{lo}, Lin^-, Sca-1^+) phenotype which could reconstitute lymphoid and myeloid cells in lethally irradiated mice (Spangrude *et al* 1988). Following this, a number of laboratories reported the separation of subsets of primitive murine haemopoietic cells by fluorescent activated cell sorting (FACS) using either monoclonal antibodies (McAB) to specific surface antigens with restricted expression and/or specific supravital dye staining coupled with preparative fractionation steps to remove mature cells from BM (Visser and de Vries 1988, McAlister *et al* 1990, Li and Johnson 1992, Bertoncello *et al* 1991). Of note are studies where BM cells were sorted according to their ability to retain the DNA-binding dye hoechst 33342 (Ho) and the mitochondrial stain rhodamine 123 (Rh). As few as 20-30 cells with low retention of Ho and/or Rh were shown to completely and exclusively repopulate the myeloid and the lymphoid B and T cell compartments of recipients for months post transplant (Li and Johnson1992, Szilvassy *et al* 1989, Wolf *et al* 1993, Bradford *et al* 1997).

In humans, as in mice, the hemopoietic stem cell pool is estimated to represent a very small fraction (probably < 0.01%) of the nucleated cells within the BM (Harrison *et al* 1988). The identification and quantitation of primitive human haemopoietic cells with marrow repopulating ability has been problematic and remains a contentious issue. Until recently there was consensus that human HSC were restricted to the CD34 antigen positive ($CD34^+$) sub-fraction of human adult BM (Sutherland and Keating 1992). $CD34^+$ enriched BM cells reconstitute all lineages of the haemopoietic system in lethally irradiated baboons and rhesus monkeys whereas transplantation with CD34-depleated autologous BM leads to graft failure and recipient death (Berenson *et al* 1988, Wagemaker *et al* 1990). Successful autotologous (Berenson *et al* 1991, Shpall *et al* 1994a, 1994b) and allogeneic transplantation (Link *et al* 1996, Bensinger *et al* 1996) of $CD34^+$ cells has produced stable lymphohaemopoietic repopulation for more than 5 years in humans and 9 years in primates (Andrews *et al* 1992) suggesting that some $CD34^+$ cells have long-term marrow repopulating ability.

The CD34 antigen is a type I integral membrane glycoprotein of approximately 105kDa, present on 1-3% of normal human BM cells (Civin *et al* 1984, Andrews *et al* 1986, Lansdorp *et al* 1990). $CD34^+$cells within BM are now recognised to be a heterogeneous population containing virtually all unipotent burst-forming units-erythroid, colony-forming units-granulocyte macrophage, colony-forming units-megakaryocyte (BFU-E, CFU-G/M, CFU-Meg) and multipotent progenitors; colony-forming units granulocyte/erythroid/macrophage/megakaryocytic (CFU-GEMM) (Civin *et al* 1984, Debili *et al* 1992, Andrews *et al* 1986). Furthermore, B lymphoid committed progenitors, thymic T

cell precursors and long-term repopulating cells are also restricted to $CD34^+$ cells (Peault 1991, Galy 1993). A problem for experimental haematologists has been the delineation and separation of sub-fractions of $CD34^+$ cells that contain stem cells, precursors to committed progenitors and the committed progenitor cells.

A number of surrogate *in vitro* assay systems have been used to address this question. These include the standard semisolid clonogenic assays performed in agar, agarose or methycellulose (Metcalf and Burgess 1982, Metcalf 1984), LTBM culture (Dexter *et al* 1971, Moore *et al* 1979, Gordon *et al* 1987, Sutherland *et al* 1989), the CFU-blast assay (Leary and Ogawa 1987, Rowley *et al* 1987), and the Pre-CFU assay (Iscove *et al* 1989, Smith *et al* 1991). The latter 3 assays are designed to detect primitive haemopoietic cells by their ability to generate committed progenitor cells (as determined by clonogenic assays) and/or the production of mature cells of multiple lineages. In addition, as described below, *in vivo* transplantation experiments with immunodeficient animals (Reviewed by Srour 1992a, McCune *et al* 1988, Namikawa *et al* 1990) have proved to be useful for investigating the haemopoietic potential of defined populations of human BM.

A widely used assay, based on LTBMC is the so-called long-term culture initiating cell (LTC-IC) assay. This assay identifies and quantitates cells within haemopoietic tissues with the capacity to initiate and sustain haemopoiesis in a standard Dexter type stromal-cell dependent system. LTC-IC have been characterised as a distinct sub-fraction of $CD34^+$ cells which are small (Sutherland *et al* 1989), lack cell surface molecules expressed on mature leukocytes of the various lineages (Lin^-: Baum *et al* 1992) and also the transferrin receptor (CD71) (Srour *et al* 1991, Lansdorp *et al* 1992), the high molecular weight isoform of human leucocyte antigen (CD45RA) (Lansdorp *et al* 1992, Lansdorp *et al* 1990), CD33 (Andrews *et al* 1989, Buhring *et al* 1989, Bernstein *et al* 1991), and HLA-DR (Moore *et al* 1980, Keating *et al* 1984, Brandt *et al* 1990, Sutherland *et al* 1989). Further studies have shown that LTC-IC express low levels of Thy-1 (Craig *et al* 1993, Baum *et al* 1992), are c-kit^+ (Briddell *et al* 1992, Craig *et al* 1993, Simmons *et al* 1994a), Rhodamine 123 dull (Craig *et al* 1993, Udomsakdi *et al* 1992a) and quiescent as demonstrated by their relative insensivity to 4-HC (Winton *et al* 1989, Udomsakdi *et al* 1992b).

The blast colony assay devised by Ogawa allows for the growth of late-appearing undifferentiated blast colonies that have a high replating ability and the capacity to give rise to both differentiated and undifferentiated colonies (Nakahata and Ogawa 1982, Leary *et al*

1987). A modification of this system was used by Terstappen to show that blast colonies derived from $CD34^+CD38^-$ but not $CD34^+CD38^+$ cells were capable of extensive generation of new colonies after replating of the first generation colonies (Terstappen *et al* 1991).

Iscove and subsequently Smith *et al* developed a stromal-cell free suspension culture system in which cytokines alone supported the growth and development of purified populations of HPC. Termed the pre-colony forming unit (Pre-CFU) or delta assay, this culture system has been used to characterise the responses of primitive hemopoietic progenitors to purified recombinant HGF (Iscove *et al* 1989, Smith *et al* 1991). The Pre-CFU assay was based on the finding that precursors to myeloid progenitors (CFU-GM) that do not clone directly in semisolid clonogenic assays differentiate into CFU-GM following treatment in suspension phase with a combination of IL-1 and IL-3. A sub-fraction of $CD34^+$ cells which lack detectable surface markers for T-cell, B-cell, natural killer cell and myeloid lineage cells ($CD34^+Lin^-$) were found to yield comparable CFU-GM delta values to 4-HC resistant $CD34^+$ cells suggesting that these cells are closely related developmentally to long term marrow repopulating cells.

Collectively, the *in vitro* assay systems described above only provide indirect measures for the human HSC. An alternative approach that may provide more direct evidence for the identity of human HSC is xenogeneic transplantation of candidate human HSC-containing populations into immunodeficient mice (Srour *et al* 1992, Lapidot *et al* 1997). The bg/nu/xid and severe-combined immunodeficient (SCID) mouse strains have been used to test the transplant potential of haemopoietic cells isolated from human BM, foetal liver, thymus and lymph node (Kamel-Reid and Dick 1988, Namikawa *et al* 1990, McCune *et al* 1988). These studies show that SCID-hu mice with human thymus/liver co-implants contain stromal microenvironment(s) requisite for the long-term maintenance of human HPC. In further studies, the intravenous injection of human HPC in sublethally irradiated SCID and nonobese diabetic/SCID (NOD/SCID) mice result in the engraftment of human cells that proliferate and differentiate to multiple lineages in murine BM and spleen (Nolta *et al* 1994, Lowry *et al* 1996, Pflumio *et al* 1996, Cashman *et al* 1997). The transplanted human cells home to and engraft the BM, where they produce large numbers of LTC-IC, colony-forming cells (CFC), immature and mature myeloid, erythroid, and lymphoid cells without the influence of exogenous human HGF (Dick 1996). The primitive haemopoietic cells that give rise to human haemopoiesis in SCID mice have accordingly been termed SCID-repopulating

cells (SRC) (Conneally *et al* 1997) or in a similar quantitative assay, competitive repopulating units (CRU) (Larochelle *et al* 1996).

Although SRC represent a very primitive haemopoietic cell, their exact place in the stem cell hierarchy is not fully understood. For instance, SRC have been reported to be biologically distinct from CFC and LTC-IC: SRC are exclusively $CD34^+CD38^-$ in contrast to CFC and LTC-IC that are also found in the $CD34^+CD38^+$ fraction (Bhatia *et al* 1997, Cashman *et al* 1997). Furthermore, although the cells identified by the LTC-IC and the CRU assay may not necessarily represent identical cell populations, they are found within both the $CD34^+CD38^+$ and $CD34^+CD38^-$ subpopulations and seem to be expanded under the same culture conditions (Conneally *et al* 1997). In spite of this confusion, the NOD/SCID transplant model has been used recently to investigate the haemopoietic potential of *in vivo* generated cells from umbilical cord blood (Piacibello *et al* 1999).

Although each of the murine transplant models discussed above support human haemopoiesis, they are limited and may underestimate the haemopoietic potential of candidate HSCs. By their very nature these transplant models rely on homing and lodgement of human test cells within a murine BM microenvironment, a sequence of events that may be less effective than observed in human transplantation. Secondly, there is often a residual immunological barrier within the host mouse, which if not completely blocked, leads to graft resistance or graft failure. Both of these limitations are to a large extent overcome by transplantation in preimmune foetal sheep (Srour *et al* 1992, Zanjani *et al* 1992) where limited numbers (as low as 2000) of human haemopoietic cells can be directly injected into the developing sheep embryo. This approach has proved to be remarkably useful in comparing the haemopoietic potential of different haemopoietic cell populations. Recent studies indicate that human lymphohaemopoiesis can be sustained for months in the adult sheep following in-utero transplantation (Zanjani *et al* 1994, Kawashima *et al* 1996). In addition, human cells isolated from primary transplant recipients can give rise to long-term human haemopoiesis when injected into secondary foetal sheep. These serial transplantation experiments have demonstrated that this capacity resides with $CD34^+lin^-CD38^-$ cells isolated from adult human BM (Civin *et al* 1996).

Results from these various *in vivo* and *in vitro* assay systems suggest that primitive haemopoietic progenitor cells (perhaps stem cells) can be discriminated from lineage committed progenitors by the expression or absence of particular surface antigens. A

composite phenotype for the human haemopoietic stem cell could be $CD34^{+}Thy\text{-}1^{lo}$c-*kit*$^{+}$HLA-$DR^{-}CD38^{-}CD71^{-}CD45RA^{-}Rhodamine123^{dull}$ whereas committed progenitor cells would be $CD34^{+}$ but express lineage (eg CD33, CD10, CD19, CD2, CD7) or activation (eg HLA-DR, CD38) antigens and be non quiescent as demonstrated by their sensitivity to agents such as 4-HC and 5-FU and also their high retention of rhodamine 123. However, in spite of the vast amount of experimental data supporting the hypothesis that CD34 expression can be used to define HSC this needs to be re-examined in light of recent studies investigating the haemopoietic potential of $CD34^{low/-}$ cells (Osawa *et al* 1996, Goodell *et al* 1997, Morel *et al* 1998, Zanjani *et al* 1998, 1999, Bhatia *et al* 1998, Donnelly *et al* 1999, Sato *et al* 1999, Fujisaki *et al* 1999, Nakamura *et al* 1999, Andrews *et al* 2000, Gallacher *et al* 2000).

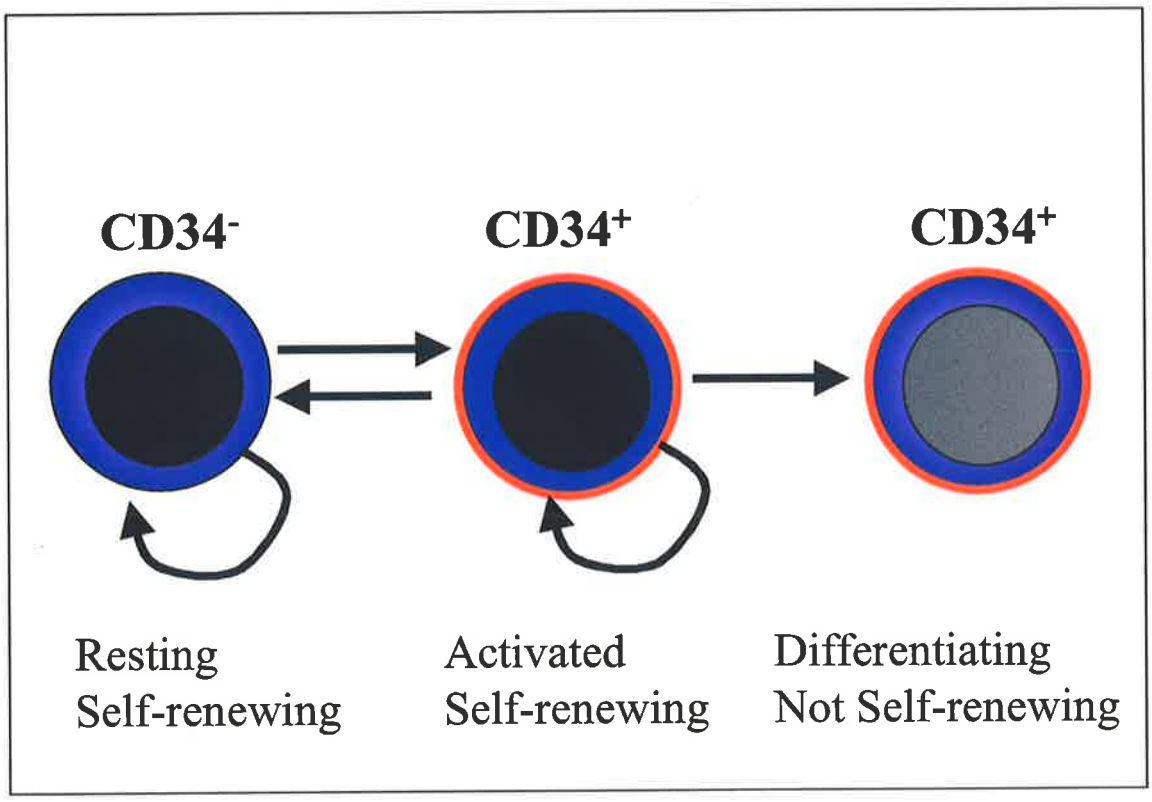
The first report of haemopoietic reconstitution potential of $CD34^{low/-}$ cells was made by Osawa *et al* who performed transplantation into Ly5.2 recipient mice with low numbers of c-*kit*$^{+}$ Sca-1^{+} Lin^{-} $CD34^{low/-}$ cells isolated from Ly5.1 donors. They observed a cell dose dependent level of donor reconstitution exhibiting both myeloid and lymphoid cells of donor origin for more than 3 months with 21% (9 out of 41) of recipients transplanted with single Ly5.1 $CD34^{low/-}$ cells (Osawa *et al* 1996). These data were confirmed and extended by Morel *et al* who used a similar transplantation strategy to compare the contribution made by $CD34^{+}$ and $CD34^{-}$ cells to haemopoiesis in primary and secondary recipient mice. These studies suggested that primitive competitive repopulating cells were distributed equally between $CD34^{+}$ and $CD34^{-}$, c-*kit*$^{+}$Sca-$1^{+}Lin^{-}$ cells (Morel *et al* 1998). In contrast, competitive repopulation experiments have also demonstrated that $CD34^{+}Lin^{-}$ HPC are better able to engraft the BM than are $CD34^{-}$ cells: the $CD34^{+}Lin^{-}$ cells providing both short and long-term engraftment but $CD34^{-}Lin^{-}$ cells being capable of only long-term engraftment (Donnelly *et al* 1999, Sato *et al* 1999). Of interest is the observation that CD34 expression of murine HSC may be linked to cell cycle status or activation state and that this may be reversible (Sato *et al* 1999). These data have been subsequently used to devise a model of CD34 expression on murine haemopoietic stem cells (Goodell 1999). The general concepts of populations of resting, activated and differentiating murine HSC are presented in figure 1.2. It remains to be determined if similar counterparts are found within human haemopoietic tissues.

Culture of human $Lin^{-}CD34^{-}$ umbilical cord blood cells on the murine marrow stromal cell line HESS-5 in the presence of exogenous TPO, FLT3L, SCF, G-CSF, IL-3 and IL-6 resulted in the acquisition of CD34 by up to 12% of cells during 7 days of culture

Figure 1.2 Model of CD34 expression on haemopoietic stem cells

This model is based from the data of Sato *et al* (1999) and discussed in detail by Goodell (1999). According to this model, under steady state haemopoiesis, quiescent $CD34^-$ stem cells are capable of self-renewal and also able to convert to $CD34^+$ stem cells upon activation. Furthermore $CD34^+$ stem cells can self-renew as well as give rise to resting stem cell that are $CD34^-$. Alternatively $CD34^+$ stem cells can differentiate, and lose the capacity for self-renewal in spite of maintaining CD34 expression.

CD34 expression on Haemopoietic Stem Cells



(Nakamura *et al* 1999). Although, the long-term multilineage repopulating ability of these cultured cells was demonstrated by transplantation into irradiated NOD/SCID mice, there was no direct evidence that repopulation could be attributed to nascent $CD34^+$ cells. A small fraction (approximately 1%) of $CD34^-Lin^-$ cells isolated from normal adult BM and G-CSF mobilised blood have also been shown to divide and differentiate in serum-free liquid suspension cultures stimulated by FLT3L, TPO, SCF, IL-3 and hyper-IL-6 (Fujisaki *et al* 1999). These cultures supported a 28-fold increase in total cells within 10 days and 5% of generated cells were $CD34^+$. Notably, transplantation of up to 1.7×10^5 Lin^-CD34^- adult BM cells failed to engraft sublethally irradiated NOD/SCID-$\beta_2M^{-/-}$ mice, contrasting with the demonstrated efficacy of the same population in contributing to human haemopoiesis in the NOD/SCID and pre-immune foetal sheep transplant models (Bhatia *et al* 1998, Zanjani *et al* 1998, 1999). The conflicting results of these studies may, in part be explained by the use of NOD/SCID-$\beta_2M^{-/-}$ mice rather than NOD/SCID recipients or by the subtle difference in the panel of monoclonal antibodies used to remove or discriminate cells expressing lineage associated antigens. Furthermore, the conflicting data from these studies suggest that further investigations are required to more accurately assess the potential of human Lin^-CD34^- cells. In addition, the extremely low incidence of these cells in adult human marrow and the low proportion that can be stimulated in *ex vivo* cultures suggest that the clinical use of this fraction of HPC may not be feasible.

Putting aside the question of whether HSC express CD34, there are abundant data from *in vitro* assays supporting the segregation of developing haemopoietic cells into discrete, although somewhat arbitrary, compartments. As shown in figure 1.1, haemopoietic cells can be considered to reside within one of 6 compartments comprising the HSC, Pre-CFU, committed progenitors or CFU, post progenitors, mature functional cells and tissue forms. Committed haemopoietic progenitor cells (HPC) are identified by clonogenic assays and as implied, cells within this compartment have made a commitment to a defined lineage that will only be revealed retrospectively. The Pre-CFU compartment (Smith *et al* 1991, Metcalf 1998) represents an intermediate pool of primitive cells connecting the quiescent stem cell pool to the committed progenitor compartment. It would be anticipated that cells within this pool have limited potential (if any) for self-renewal but have high proliferative potential and some capacity for differentiation along more than one lineage. In accord with this proposal, cells with Pre-CFU activity may contribute to early, rapid haemopoietic reconstitution following transplantation.

In contrast to cells within the Pre-CFU and progenitor compartments, post progenitor cells can be identified by virtue of their unique morphology associated with specific lineages. Haemopoietic progenitors within the Pre-CFU and progenitor compartments are morphologically indistinguishable by light microscopy, appearing as relatively small, agranular, blast-like cells when stained with Romanowsky dyes whereas post progenitor cells have unique morphology that enable them to be discriminated as erythroid, granulocytic or megakaryocytic cells. These morphological features provide a well-accepted means for identifying cells at various stages of maturation. For example, as shown in figure 1.3, the progression of myeloblast, promyelocyte, myelocyte, metamyelocyte to mature neutrophil follows a distinct pattern with each cell in the sequence displaying a unique morphology related to changes in nuclear and cytoplasmic shape and granulation. Similar but unique, morphological criteria are used to describe cells during erythroid and megakaryocytic maturation. In addition, when compared to committed progenitors, post progenitor cells have limited proliferative potential and give rise to mature functional cells within 1-3 days.

Apart from morphology, the pattern of cell surface antigen expression is a well-established means for identifying and isolating cells at particular stages of development. Numerous studies utilising monoclonal antibodies and multiparameter flowcytometric techniques have documented the sequence of antigen acquisition or loss during HPC differentiation (Loken and Stall 1982, Andrews *et al* 1983, Van den Engh and Visser 1984, Civin and Loken 1987). For example, as HPC differentiate into the granulo-monocytic lineage, CD34 is progressively lost whilst antigens such as CD64 (Olweus *et al* 1995), CD33 (Buhring *et al* 1989) and CD15 (Terstappen *et al* 1990) are expressed. Of particular relevance to this thesis was the study performed by Terstappen *et al* who identified a combination of monoclonal antibodies to CD11b, CD15, CD33, and CD16 that together with forward and orthogonal light scatter permitted isolation of neutrophilic BM cells from cells of other lineages with high purity (>95%) (Terstappen *et al* 1990). The subsequent immunophenotypic definition of myeloblasts, promyelocytes, early myelocytes, myelocytes, metamyelocytes and segmented neutrophils was to become the basis for multiparameter analysis of *ex vivo* generated myeloid cells (Bender *et al* 1991, Haylock *et al* 1992) and has been adopted within this thesis. The stage specific expression of a range of cell surface antigens during neutrophil development is presented in figure 1.4.

Figure 1.3 Stages of neutrophil maturation in human bone marrow

The schema represents the morphological appearance of neutrophil precursors following staining with Romanowsky dyes such as Jenner-Giemsa or Wrights stain. Under steady state haemopoiesis this process occurs within the bone marrow. Discrimination of cells during this dynamic sequence is based on the size and shape of the nucleus, the presence of nucleoli, the appearance of the cytoplasm and the type and number of cytoplasmic granules. Based on these criteria, cells progress through the sequence of myeloblast, promyelocyte, myelocyte, metamyelocyte, band form then finally mature into lobulated neutrophils. Myeloblasts are characterised by a high nucleus:cytoplasm ratio, prominent nucleoli and an agranular azurophilic cytoplasm. Granulocyte maturation is heralded by the appearance of large primary granules and an overall increase in the size of the cell that is attributed to an increase in the cytoplasm. Subsequent differentiation involves a progressive condensation in chromatin, an associated decrease in the size of the nucleus and an increase in the formation of granules including secondary granules within the cytoplasm. Myelocytes are distinguished from promyelocytes by the lack of nucleoli, an oval or bean shaped nucleus and a gradual pink staining of the cytoplasm corresponding to increased protein content. As the myelocyte differentiates its nucleus becomes more condensed and more bean shaped or elongated. Transition from myelocyte to the neutrophil involves further nuclear condensation and increased cytoplasmic granulation that are typical of the metamyelocyte and band form stages. Lobulation of the metamyelocyte and band-form stages results in generation of mature neutrophils.

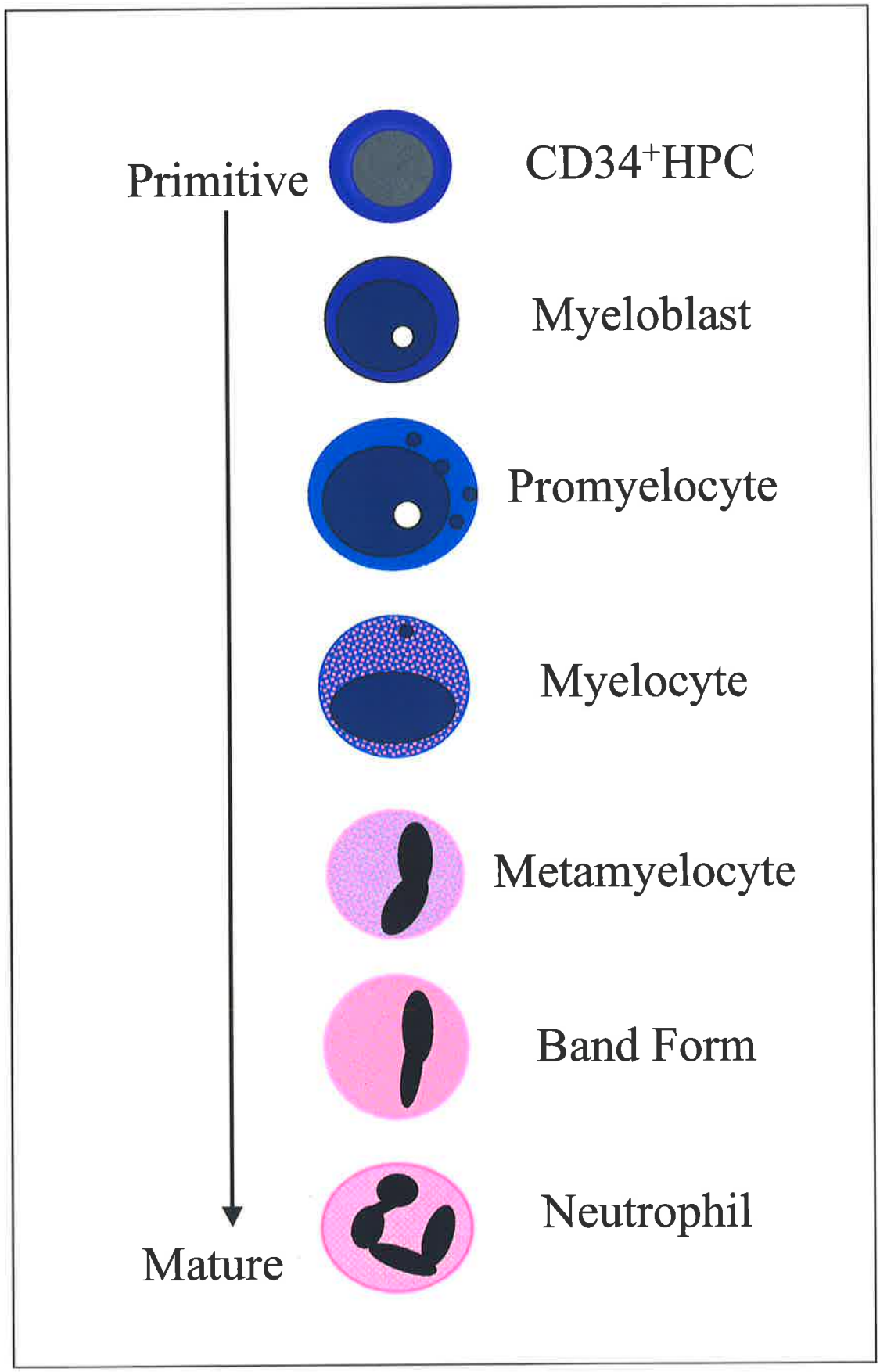

Primitive
CD34+HPC
Myeloblast
Promyelocyte
Myelocyte
Metamyelocyte
Band Form
Neutrophil
Mature

Figure 1.4 Antigen expression during neutrophil maturation

The schema illustrates the changes in expression of key cell surface antigens during neutrophil development. The composite immunophenotype at each stage is based on data published by Buhring *et al* 1989, Terstappen *et al* 1990, Olweus *et al* 1995, and Skubitz *et al* 1996. Primitive HPC are characterised by expression of high levels of CD34, Thy-1, and absence of CD33, CD38, HLA-DR and CD64. Upon differentiation, primitive haemopoietic progenitor cells gradually reduce expression of CD34 and express other cell surface antigens, such as CD33, CD64 and CD15. Although differentiation is a dynamic process, cells at specific stages of development and neutrophil maturation can be discriminated from each according to their unique composite immunophenotype. For example promyelocytes can be discriminated from their progeny, myelocytes, by CD11b, which is absent on the former but expressed by the latter cell type.

Primitive HPC		$CD34^{++}$, $CD38^{-}$, $CD33^{-}$, $Thy\text{-}1^{+}$, $HLA\text{-}DR^{-}$, $CD64^{-}$
Committed Myeloid Progenitor		$CD34^{+}$, $CD38^{+}$, $CD33^{+}$, $HLA\text{-}DR^{+}$, $CD64^{+}$
Myeloblast		$CD34^{+/-}$, $CD38^{++}$ $CD15^{+/-}$, $CD11b^{-}$
Promyelocyte		$CD34^{-}$, $CD15^{+/++}$, $CD11b^{-}$, $CD66b^{-}$
Myelocyte		$CD15^{++}$, $CD11b^{+}$ $CD66b^{+}$, $CD16^{-}$
Metamyelocyte		$CD15^{++}$, $CD11b^{++}$ $CD66b^{++}$, $CD16^{+/++}$
Neutrophil		$CD11b^{++}$, $CD15^{+}$ $CD16^{++}$

In spite of the large number of functional and immunophenotypic studies aimed at placing haemopoietic cells within a hierarchy there remains uncertainty concerning the discrimination between cells with stem cell function and Pre-CFU activity. However, one characteristic that appears to discriminate primitive HPC is their requirement for combinations of direct acting and synergistic HGF for maximal proliferation (Lowry *et al* 1991, Williams *et al* 1992, Muench *et al* 1992, Moore 1991, McNiece *et al* 1991, Bernstein *et al* 1991, Broxmeyer *et al* 1991). This aspect of haemopoietic cell biology was explored in greater detail during the current study.

1.4 Haemopoietic Regulators

1.4.1 Identification of Haemopoietic Regulators

The first haemopoietic regulator to be characterised, erythropoietin (EPO), which controls red cell production, was discovered as early as 1906 as a result of simple experiments with rabbits (Carnot and Deflandre 1906). However, it was not until 1953 that more definitive evidence for a humoral regulator of red cell production was published (Erslev 1953). Subsequent studies identified the kidneys as the site for oxygen sensing and for production of erythropoietin (Jacobson *et al* 1957). By comparison, the identification of humoral regulators for production of other haemopoietic cells was less successful and lagged behind the discovery of EPO.

Initial evidence for soluble haemopoietic regulators came during the late 1960's and early 1970's. The identification of colony-stimulating factors (CSF) was made possible by the development of semisolid clonogenic assays for haemopoietic progenitors cells in the mid 1960's, independently by Bradley, Metcalf and Sachs (Bradley and Metcalf 1966, Ichikawa *et al* 1966). These *in vitro* culture systems, which were effectively bioassays, showed that the survival, proliferation and differentiation of immature haemopoietic cells was dependent on the continued presence of factors which were collectively termed "colony-stimulating activity". The observed presence of CSA in serum, urine and tissue extracts suggested that the constituent CSFs might be genuine humoral regulators of myelopoiesis. Sheridan and Metcalf studied the synthesis of CSF from different mouse tissues; lung, spleen, kidney and salivary gland following endotoxin, poly AU and irradiation treatment: these lead to a dramatic increase in the levels of CSF in tissues and serum with 40 minutes to an hour. An analysis of electrophoretic mobilities and calcium phosphate chromatographic profiles indicated that different types of CSF's were produced but interestingly they all had similar

biological properties of being able to induce similar types of colonies (Sheridan and Metcalf 1972). Later, separate studies by Chervenick and Golde showed that the monocytes were the main source of CSA produced by human leucocytes (Chervenick and LoBuglio 1972, Golde and Cline 1972, Cline and Golde 1974).

Colony-stimulating factors were subsequently purified by a combination of biochemical means included ammonium sulphate precipitation, DEAE-cellulose chromatography, hydroxyapatite chromatography and gel filtration on G-150 Sephadex. The first CSF to be purified, macrophage colony-stimulating factor (M-CSF) had little or no direct colony-stimulating activity on human cells but effectively stimulated macrophage colonies from mouse BM cells (Stanley *et al* 1976). Granulocyte-macrophage colony-stimulating factor (GM-CSF) was first recognised as a small molecule (molecular weight of approximately 25 kDa) in lung-conditioned medium that could stimulate both granulocyte and macrophage colony formation (Sheridan and Metcalf 1973). Subsequently, granulocyte colony-stimulating factor (G-CSF) was identified as a distinct molecule that could only stimulate differentiated granulocytic colonies (Burgess and Metcalf 1980, Nicola and Metcalf 1981). Finally, Multi-CSF or interleukin (IL)-3 was identified as a T lymphocyte-derived factor that had the capacity to stimulate not only granulocyte/macrophage colonies but also the formation of eosinophil, megakaryocyte, erythroid and multipotential colonies (Burgess *et al* 1980, Bazill *et al* 1983, Ihle *et al* 1982).

Each of these CSFs was purified from murine tissues and in due course the corresponding human CSFs were purified from human tissue sources (Gasson *et al* 1984, Nomura *et al* 1986, Souza *et al* 1986). T lymphocytes were shown to be mainly responsible for production of Multi-CSF (Schreier and Iscove 1980, Staber *et al* 1982), a glycoprotein of 23 kDa. Due to the multiple biological activities of Multi-CSF, a number of synonyms evolved. In most instances, the names arose from the various bioassays used to detect and measure this factor. Multi-CSF obtained from either spleen conditioned media or WEHI-3 cells was variously termed P cell stimulating factor (Clark-Lewis and Schrader 1981), interleukin-3 (IL-3) (Ihle *et al* 1982a), burst-promoting activity (BPA) (Iscove *et al* 1982) or haemopoietic cell growth factor (Bazill *et al* 1983). Wagemakers group reported cloning of human IL-3 in 1987 (Dorssers *et al* 1987).

1.4.2 Common Characteristics of Haemopoietic Regulators

Since the discovery of CSFs many other cytokines and interleukins have been shown, in various bioassays to have CSA or to affect the *in vitro* proliferation or function of haemopoietic progenitors and their progeny. A non-exclusive list of these regulators and their primary tissue sources are shown in table 1.1. In general terms these regulators can be classified as either stimulators or inhibitors of HPC and in spite of the large number of molecules involved they share some common characteristics, including the following:

1. Pleiotropic biological activities.

CSF, cytokines or interleukins, even those with a high degree of lineage specificity have multiple biological activities (Metcalf 1993). For example, EPO, possibly the most restricted regulator has activity on megakaryocyte precursors (Ishibasi *et al* 1987) apart from its dominant affect on erythropoiesis. IL-3 is an extreme example with its documented affects on stem cells and precursors for most lineages apart from T cells (Kinashi *et al* 1988, Metcalf *et al* 1987). The possible consequences of "stimulating" HPC or differentiated progeny with these regulatory molecules include survival, apoptosis, division, differentiation, activation, migration, adherence, and induction of HGF synthesis. It is this broad range of biological effects elicited by these regulators that result in cells of any given lineage being regulated or influenced by multiple regulators. The reason for the significant redundancy amongst the haemopoietic regulators and the physiological consequences of this remains unclear although several possibilities have been considered (Metcalf 1993). One proposal is that a hierarchy of regulators exist, some which have an obligatory role in daily cell production whereas others have weaker actions or perhaps only affect haemopoiesis during emergencies. According to this model G-CSF may be viewed as a higher order regulator as animals nullizygous for G-CSF exhibit a severe reduction in daily cell production (Lieschke *et al* 1994). In contrast, IL-1 may be a lower order regulator, being called upon during severe antigenic challenge when cell production needs to be increased rapidly. Accordingly, the IL-1β knockout animal shows only minor effects to steady state haemopoiesis. An alternative to any of the proposed views is that regulation of haemopoiesis is independent of the vast number of redundant HGF but is determined more by the pattern of HGF receptor expression or downstream signalling molecules (Socolovsky *et al* 1998). Another consequence of the broad range of biological activities displayed by HGF is the ability to induce proliferation of HPC and also frequently the capacity to enhance the functional

Table 1.1 Legend

Macro refers to macrophages, Endo refers to endothelial cells, Fibro refers to fibroblasts Lympho refers to lymphocytes, aa refers to amino acids, LPS refers to lipopolysaccaride

References for data presented in Table 1.1

IL-1: (Thornberry 1994, March et al 1985, Le and Vilcek 1987)
IL-2: (Smith 1988, Taniguchi and Minami 1993)
IL-3: (Yang et al 1986, Niemeyer et al 1989)
IL-4: (Yokota et al 1986, Lewis et al 1988)
IL-5: (Campbell et al 1987, Yokata et al 1987)
IL-6: (Kishimoto 1989, Bagby 1989)
IL-7: (Namen et al 1988, Goodwin et al 1989)
IL-8: (Van Damme et al 1989, Van Damme 1994)
IL-9: (Van Snick et al 1989, Renault et al 1990)
IL-10: (Vieira P et al 1991, Benjamin et al 1992)
IL-11: (Paul et al 1990, Kawashima et al 1992)
IL-12: (Kobayashi et al 1989, Trinchieri and Gerosa 1996)
IL-13: (Obiri et al 1995, Zurawski et al 1994)
IL-14: (Ambrus et al 1996, Ford et al 1995)
IL-15: (Grabstein et al 1994, Mrozek et al 1996)
IL-16: (Zhang et al 1998, Scala et al 1997)
IL-17: (Yao et al 1995, Fossiez et al 1996)
IL-18: (Stoll et al 1997)
SCF: (Broudy 1997, Flannagan and Leder 1990)
TPO: (Lok et al 1994, Wendling et al 1994, Bartley et al 1994)
FLT3L: (Lyman et al 1994, Hannum et al 1994)
GM-CSF: (Wong et al 1985, Gasson et al 1984)
G-CSF: (Demetri and Griffin 1991, Souza et al 1986)
M-CSF: (Ralph et al 1986)
EPO: (Lin et al 1985, Browne et al 1986)
IGF1: IGF2 (Stewart and Rotwein 1990)
LIF: (Hilton and Gough 1991, Metcalf 1988)
OSM: (Miyajima et al 2000, Rose and Bruce 1991)
Leptin: (Mikhail et al 1997, Bennett et al 1996)
TNF-α: (Aggarwal et al 1995)
IFN-γ (Coutino et al 1986, Dexter and Moore 1986)
TGF-β1: (Sporn et al 1986, Hooper 1991)
MIP-1α: (Wolpe 1988, Graham et al 1990b, Thalmeier et al 1996)

Table 1.1

Name	Mwt (kDa)	Synthesis induced by	Cellular Source
IL-1	17	IL-1, GM-CSF, TNF, IL-2	Almost all cells
IL-2	23	antigen, IL-1, IL-2	T lymphocytes
IL-3	15-28	mitogens, IgE receptor activation	T lymphocytes
IL-4	18-20	antigens, lectins	$CD4^+$, $CD8^+$ lymphocytes
IL-5	45-50 dimer	antigens, mitogens	T lymphocytes
IL-6	21-29	IL-1, endotoxin, mitogens	Macro. Endo. Fibro, T lympho.
IL-7	22-25	constitutive	BM stroma, thymus
IL-8	6-8	LPS	various, Mono.
IL-9	32-39	IL-1, IL-2, IL-10	T lymphocytes
IL-10	17-21dimer	LPS, anti-CD3	TH2 lymph. Mono. B cells
IL-11	19-24	IL-1	BM Stroma, Fibro.
IL-12	heterodimeric 35, 40	LPS, pathogens	Mono/Macro.
IL-13	17	CD28 ligation, anti-IgE	T cells, BM stroma
IL-14	60	PHA	T cells, Lymphoma cells
IL-15	14	constitutive	Mono/Macro BM stroma
IL-16	16-18	Antigen, C5a	CD8+ T cells
IL-17	15, 22	CD3 ligation	TCR ab+/$CD4^-$/$CD8^-$
IL-18	18	LPS	BM Stroma
SCF	28-36	constitutive	BM Stroma
TPO	36	constitutive	Hepatocytes, BM Stroma
FLT3L	variable	constitutive, IL-1	Most tissues, BM stroma
GM-CSF	20-35	TNF-α, IL-1, LPS	T cells, Endo. Fibro.
G-CSF	18-20	IL-1, TNF-α,	Mono/Macro. Endo. Fibro
M-CSF	40-50, or 70-90	TNF-α, IL-3, IL-4,	Mono/ Macro. Endo. Fibro
EPO	34-39	hypoxia	Kidney (in adult)
IGF1	70aa	constitutive	Liver
IGF2	67aa	constitutive	Liver
LIF	32-45	IL-1, TGF-β, TNF	BM Stromal cells
OSM	28	IL-6	Mono. T lymphocytes
Leptin	16	food intake, insulin	Adipocytes
TNF-α	17	LPS	Activated Mono/Macro.
INF-γ	20-25	antigens, mitogens	T lymphocytes, NK cells
TGF-β1	25 dimer	constitutive	Many cells, BM Stroma, Bone
MIP1-α	8	steriods, neuropeptides	Macro. BM Stroma

activity of terminally differentiated progeny of responding precursor cells. In this respect, G-CSF provides an excellent example in being able to stimulate the neutrophil lineage precursor (CFU-G) but also activating mature neutrophil function *in vitro* and *in vivo* (reviewed by Demetri and Griffin 1991).

2. HGF influence biological outcomes by acting directly or indirectly.

As is indicated in table 1.1 stimulation by one regulator may result in a cascade of other regulators being produced by haemopoietic cells or other tissues. Thus it is difficult to determine if an observed biological response is due to the initial regulator or from those subsequently produced. For this reason, the role of any given factor in regulating *in vivo* haemopoiesis cannot be fully assessed by simply administration of the recombinant HGF to an animal. It is essential that loss of function analyses (gene knockout or gene disruption) be performed to establish if the regulator has any direct biological affect. In addition, the direct affects of a single regulator can be determined by cultures of single purified target cells under stringent conditions without the presence of other regulators. IL-1 is one regulator that induces production of many other cytokines from different haemopoietic cells, so it is impossible to ascribe direct effects on a target cell when cultured in the presence of other cells.

3. HGF can act synergistically or additively to deliver a biological response.

There are many examples of positive interactions between HGF when they are used for stimulating *in vitro* growth of HPC. This characteristic is exemplified by "early acting" HGF such stem cell factor (SCF), FLT3L or IL-6 that exhibit very limited ability to induce proliferation of primitive HPC when used alone. However, when such factors are used together or with late acting stimulators, there is a potent biological response. The simultaneous addition of two or more regulators can produce two effects *in vitro*. The first is an increase in the number of cells that can divide in the combination above that observed with single HGF. These additive interactions result in increased recruitment of cells into the cell cycle and as observed in semisolid clonogenic assays an increase in the number of colonies produced. The second effect is increased cell proliferation without an increase in the recruitment of HPC. This type of interaction between HGF has been referred to as synergy (Metcalf 1993) and results in an increase in the size of colonies in semisolid cultures. Knowledge of synergistic and additive interactions between haemopoietic regulators

is critical for optimal *ex vivo* expansion of haemopoietic cells. Although many positive interactions between HGF have been described, the molecular basis for this effect remains to be fully characterised. A large part of the current study involves assessment of the interactions between different HGF and how these affect recruitment and proliferation of committed and primitive HPC.

4. Many HGF are produced by BM stromal cells.

BM stromal cells produce a broad range of HGF including SCF, TPO, FLT3L, IL-6, IL-7, IL-8, IL-11, IL-13, IL-15, IL-18, TNF-α, M-CSF, G-CSF, and GM-CSF. The transcription and expression of genes for haemopoietic regulators by BM stromal cells can be either constitutive or inducible following stimulation with inflammatory mediators such as IL-1 or TNF-alpha. Production of haemopoietic regulators by BM stromal cells is in accord with the numerous *in vitro* studies demonstrating the importance of stromal cells in regulation of haemopoiesis and with the localisation of haemopoiesis to the BM in adult mammals. It is important to note that key regulators of primitive HPC such as SCF and FLT3L are produced by stromal cells as soluble and membrane bound isoforms (Anderson *et al* 1990, Broudy *et al* 1994, McClanahan *et al* 1996). This suggests that intimate contact between developing HSC and BM stromal cells is a critical component in the regulation of haemopoiesis; as evidenced by BM failure observed in Sl/Sl^{d} mice where membrane bound SCF is absent on stromal cells (Flanagan and Leder 1990). Lymphoid cells, particularly the T lymphocyte also produce other regulators, which serve to highlight the link between the cellular immune system and cells within the haemopoietic system and defence. In this respect the production of immunomodulators such as IL-1 and TNF-α during infection or antigenic challenge and the subsequent production of direct acting haemopoietic regulators by stromal cells or T lymphocytes results in a rapid production of effector cells.

5. The vast majority of HGF are glycoproteins.

Essentially all the HGF so far described have potential O and N linked glycosylation sites and are in fact naturally glycosylated. It is likely that carbohydrate decorations on native HGF protect them from degradation and thus increases their bioavailability and tissue distribution. The observed variation in molecular weights for the regulators listed in table 1.1 reflects variable glycosylation of the protein backbone. Depending on the protein expression system used, recombinant HGF synthesised for therapy can

be either glycosylated (as occurs when Chinese ovary hamster cells are used for expression) or non-glycosylated (when synthesised in bacteria).

6. HGF deliver their affects via specific receptor complexes.

All haemopoietic regulators deliver biological responses through specific cell surface receptors. As discussed below, ligand induced dimerisation of the receptor is a key first step toward initiation of intracellular signalling. Although there are as many receptors as there are regulators, there are a number of common structural features shared by the receptors that allow them to be grouped into families. In spite of the plethora of haemopoietic regulators there appears to be common signalling pathways involved in transmission of a proliferative signal.

Although each of these regulators affects some aspect of haemopoietic cell biology *in vitro* it is uncertain how they contribute to steady state haemopoiesis. Analysis of mice nullizygous for haemopoietic growth factor genes provides some insight but may be misleading. Deletion of a growth factor gene may induce no obvious abnormality or change in haemopoiesis until a particular challenge is applied, when a deficiency becomes evident. It as also possible that deficiencies may be missed if analysis is delayed, during which time compensatory systems are established.

The main objective of this thesis was to investigate the affect of HGF on the *ex vivo* manipulation of human HPC. Given the multiplicity of molecules that affect the regulation of human HPC, at least *in vitro*, there was a need to be pragmatic in deciding which molecules to study. Even if these studies were limited to those regulators available in recombinant form for clinical use this would mean analysis of at least 20 individual molecules. If these molecules were more effective in combination, then it presents a substantial logistic problem trying to meaningfully test the millions of possible combinations of HGF. Therefore, studies were performed with a restricted range of HGF, based on their known activities on primitive HPC and their potential availability within Australia for clinical *ex vivo* expansion trials. Accordingly, *ex vivo* manipulation studies of purified HPC populations were performed with IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF, FLT3L and megakaryocyte growth and development factor (MGDF, thrombopoietin, TPO). The following tables (tables 1.2 – 1.9) provide key information concerning each of these haemopoietic regulators and their known *in vitro* effects on HPC. It is of note that during the

Interleukin 1- beta (IL-1β)

Molecular weight and synonyms:

- 31kDa precursor cleaved by interleukin-1 converting enzyme to17kDa form (1).
- Lymphocyte Activating Factor (2), Mitogenic Protein (2), Haematopoietin-1 (3).

Main cellular source:

- Monocytes and macrophages.

Main biological functions:

- Is considered the prototype pro-inflammatory cytokine and exerts its effects on a wide variety of cell types (1).
- The varied biological effects of IL-1 are mediated by the induction of several genes and the suppression of others.
- IL-1 induces expression of several cytokines, including IL-6 and the entire chemokine family (4).

Effects on haemopoietic progenitors and HSC:

- No direct effects on HPC proliferation when used alone.
- IL-1 indirectly affects murine HPC by synergising with G-CSF, M-CSF, GM-CSF, IL-3 and IL-6 in development of HPP colonies (5-7).
- IL-1 synergises with IL-3 and EPO in development of large BFU-E and multi-lineage colonies (3,8).
- IL-1 synergises with SCF and IL-3 in "delta" assays of human HPC (9).
- IL-1 induces up-regulation of GM-CSF and IL-3 receptors when administered to mice (10).
- IL-1 induces up-regulation of receptors for GM-CSF, IL-3, IL-5 and EPO on TF1 cells (11)

Effects of the IL-1 knockout and implication for haemopoiesis:

- Mice nullizygous for IL-1 or the IL-1 receptor show only very minor effects in steady state haemopoiesis (12).

References:

1. Dinarello 1996
2. Blyden and Handschumacher 1977
3. Jubinsky and Stanley 1985
4. Dinarello 1994
5. McNiece et al 1989b
6. Ikebuchi et al 1988b
7. Moore et al 1987b
8. Moore 1989
9. Moore 1991
10. Hestdal et al 1992
11. Kitamura et al 1991b
12. Fantuzzi and Dinarello 1996

Table 1.2

Interleukin –3 (IL-3)

Molecular weight and synonyms:

- Human IL-3 is a 15-25 kDa glycoprotein (1).
- Multi-CSF (2).
- P cell stimulating factor (3).
- Interleukin-3 (IL-3) (4).
- Burst-promoting activity (BPA) (5).
- Haemopoietic cell growth factor (6).

Main cellular source:

- T lymphocytes induced by mitogens, and antigens (7).

Main biological functions:

- Action restricted to haemopoietic cells, both lymphoid and myeloid.
- IL-3 acts predominantly as an "early acting" synergistic factor. IL-3 and other lineage specific cytokines stimulate survival and proliferation of multi-lineage and committed HPC.

Effects on haemopoietic progenitors and HSC:

- In serum-free cultures IL-3 has little activity as a single cytokine on proliferation of CFU-GM (8,9,10).
- Is a survival factor for HPC (11,12).
- Stimulates CFU-G colonies from single $CD34^{+}CD33^{+}$ or $CD34^{+}CD33^{-}$ cells (13).
- Stimulates growth of mixed granulocyte, erythroid, megakaryocytic and macrophage colonies (14,15,16).
- Synergises with IL-1, G-CSF, GM-CSF, and SCF to increase the number and size of CFU-GM colonies (17 - 21).
- Synergises with EPO to induce BFU-E colony formation (5,14,19,20).
- Induces B-lymphocyte differentiation.

Effects of the knockout: implication of haemopoiesis:

- IL-3 is completely dispensable for normal steady state haemopoiesis because no haemopoietic defects have been found in IL-3 knockout mice (22).

References:

1. Yang et al 1986
2. Metcalf 1984
3. Clark-Lewis and Schrader 1981
4. Ihle et al 1982
5. Schreier and Iscove 1980
6. Bazill et al 1983
7. Niemeyer et al 1989
8. Sieff et al 1989
9. Sonada et al 1988
10. Migliaccio et al 1988
11. Bot et al 1989a
12. Brandt et al 1994
13. Ema et al 1990
14. Emerson et al 1988
15. Dorssers et al 1987
16. Bot 1988
17. Barlelmez et al 1989
18. Lopez et al 1987
19. McNeice et al 1991
20. Bernstein et al 1991
21. Lewis et al 1996
22. Mach et al 1998

Table 1.3

Interleukin – 6 (IL-6)

Molecular weight and synonyms:

- Human IL-6 is a 21-29 kDa glycoprotein (1).
- BCDF/BSF2 (B cell differentiation factor/B cell stimulating factor 2) (2).
- IFNβ_2 (3).
- 26-kDa protein (1).

Main cellular source:

- Multiple, including T cells, B cells, monocytes, BM stromal cells, endothelial cells, fibroblasts.
- IL-6 production is induced by IL-1, mitogens and endotoxin (4).

Main Biological Functions:

- Pleiotropic activities on the immune, haemopoietic and neural systems.
- Induces final maturation of B cells (2).
- Activates T cells and stimulates proliferation.
- Induction of acute phase proteins by hepatocytes.

Effects on haemopoietic progenitors and HSC:

- Has little direct activity on HPC growth when used alone.
- Early acting synergistic factor inducing proliferation of primitive murine HPC (5,6,7).
- IL-6 synergises with IL-3 to stimulate proliferation of human $CD34^+$ cells (8).
- IL-6 is an autocrine and paracrine growth factor for malignant lymphoid and myeloma cells (9,10).

Effects of the knockout: implication of haemopoiesis:

- IL-6-deficient animals show reduced survival of haemopoietic stem cells and multilineage progenitors, reduced T cell numbers, reduced proliferation and maturation of erythroid and myeloid cells (11).

References:

1. Haegeman et al 1986
2. Hirano et al 1986
3. Zilberstein et al 1986
4. Kishimoto T 1989
5. Ikebuchi et al 1987
6. Koike et al 1990
7. Lu et al 1990
8. Rennick et al 1989
9. Levy et al 1991
10. Klein et al 1990
11. Kopf et al 1997

Table 1.4

Granulocyte Colony-Stimulating Factor (G-CSF)

Molecular weight and synonyms:

- G-CSF is an 18-20 kDa hydrophobic glycoprotein (1).
- Granulocyte-macrophage differentiation factor (2).
- CSF-β (3).
- Pluripoietin (4).

Main cellular source:

- G-CSF production is highly regulated and not constitutive.
- Monocytes and Macrophages are the most prominent source.
- Induced expression in vascular endothelial, fibroblasts, mesothelial cells by IL-1, LPS, TNF, GM-CSF, IL-3, IL-4 and interferon-γ (5).

Main Biological Functions:

- The primary effect of G-CSF is limited to cells of the neutrophilic lineage.
- In vitro G-CSF stimulates proliferation and differentiation of committed neutrophil precursors and enhances the effector functions of mature neutrophils.

Effects on HSC, HPC and mature neutrophils:

- G-CSF as a single agent can stimulate neutrophil colony formation by $CD34^{+}CD33^{+}$ cells (6).
- Stimulates a single round of division of promyelocytes and myelocytes (7).
- Acts synergistically with GM-CSF and IL-3 to increase size of CFU-G colonies (8). and with IL-3 to hasten appearance of blast colonies (9).
- Acts synergistically with early acting factors such as IL-1, IL-3, IL-6, SCF (10, 11,12).
- G-CSF primes neutrophils and enhances superoxide production (13).
- Induces a delayed respiratory burst of adherent neutrophils (14)
- Stimulates antibody-dependent cellular cytotoxicity (15).

Effects of the knockout: implication of haemopoiesis:

- G-CSF deficient mice have chronic neutropenia with neutrophil counts only 20% of control mice.
- G-CSF is indispensable for maintaining the normal quantitative balance of neutrophil production during "steady-state" granulopoiesis in vivo and also required in "emergency" granulopoiesis during infections (16).

References:

1. Souza et al 1986
2. Metcalf 1980
3. Nicola et al 1983
4. Platzer et al 1985
5. Demitri and Griffin 1991
6. Ema 1990
7. Begley et al 1988
8. McNiece et al 1989a
9. Ikebuchi et al 1988a
10. Rennick et al 1989
11. Ikebuchi et al 1988b
12. Martin et al 1990
13. Kitagawa et al 1987
14. Nathan et al 1989
15. Lopez et al 1983
16. Lieschke et al 1994

Table 1.5

Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)

Molecular weight and synonyms:

- Human GM-CSF is a 22-35kDa glycoprotein (1).
- CSF-α (2).

Main cellular source:

- Mast cells, T lymphocytes, endothelial cells, fibroblasts, stromal cells following induction with TNF-α, IL-1, LPS (3, 4).

Main Biological Functions:

- In vitro, GM-CSF stimulates pre-CFU and multipotent progenitors (5,6).
- In vivo and in vitro GM-CSF is a potent activator of phagocyte functions (7,8).
- In vivo GM-CSF is essential for normal pulmonary physiology and resistance to local infection (9,16).

Effects on haemopoietic progenitors and HSC:

- Stimulates proliferation of granulocyte, macrophage, bipotent granulocyte-macrophage and mutlipotential myeloid progenitors, and survival of BFU-E (6, 10,11).
- Acts synergistically with IL-3, G-CSF and SCF to enhance CFU-GM colony growth (6,12).
- Acts synergistically with EPO to enhance BFU-E growth (11-13).
- Acts with IL-4 and TNF-α to induce dendritic cell development and activate function of antigen presenting cells (14).

Effects of the GM-CSF knockout: implication of haemopoiesis:

- Mice nullizygous for the GM-CSF gene develop normally and show no major perturbation of hematopoiesis up to 12 weeks of age (9,15).
- GM-CSF is not essential for the maintenance of normal levels of the major types of mature hematopoietic cells and their precursors in blood, marrow, and spleen.
- All develop abnormal lungs with extensive and variable pathology including infection, implying that GM-CSF is essential for normal pulmonary physiology.

References:

1. Wong et al 1985
2. Gasson et al 1984
3. Gualtieri et al 1987
4. Rennick et al 1987
5. Bot et al 1989b
6. Emerson et al 1988
7. Moore 1991
8. Naccache et al 1988
9. Stanley et al 1994
10. Donahue et al 1985
11. Sieff et al 1985
12. Sonada et al 1988
13. Metcalf et al 1986
14. Hart 1997
15. Dranoff and Mulligan 1994

Table 1.6

Thrombopoietin (TPO)

Molecular weight and synonyms:

- TPO is a 36kDa protein (65-85kDa when fully glycosylated) (1).
- c-Mpl ligand (2,3,4).
- Megakaryocyte growth and development factor (5).

Main cellular source:

- TPO is constitutively produced by variety of organs/cell types including hepatocytes, bone marrow stromal cells, and spleen cells (6,7,8).
- Serum TPO levels is dependent on the platelet and megakaryocyte counts (9,10).

Main Biological Functions:

- TPO is a primary regulator of megakaryocyte and platelet development (11).
- TPO regulates HSC development.

Effects on haemopoietic progenitors and HSC:

- Alone, TPO stimulates megakaryocyte colony growth from BM HPC (4).
- Stimulates maturation of immature megakaryocytes and supports formation of functional platelets (12,13).
- TPO alone and in combination with SCF promotes proliferation and survival of megakaryocyte, erythroid and granulocyte/macrophage progenitors (14,15).
- TPO promotes survival and proliferation of primitive HPC (16,17,18).

Effects of the knockout: implication of haemopoiesis:

- Both c-mpl and TPO deficient mice show severe thrombocytopaenia and reduced levels of megakaryocyte progenitors (19,20).
- TPO and c-mpl also play critical roles in regulating HSC (21,22).

References:

1. Gurney et al 1995
2. Wendling et al 1994
3. Kaushansky 1994
4. Lok et al 1994
5. Bartley et al 1994
6. Nomura et al 1986
7. Sungaran et al 1997
8. Hirayama et al 1998
9. McCarty et al 1995
10. Nagata et al 1997
11. Kaushansky 1995
12. Cramer et al 1997
13. Hagiwara et al 1998
14. Rasko et al 1997
15. Ohmizono et al 1997
16. Young et al 1996
17. Ramsfjell et al 1997
18. Petzer et al 1996b
19. Alexander et al 1996b
20. Carver-Moore et al 1996
21. Solar et al 1998
22. Kimura et al 1998

Table 1.7

Stem Cell Factor (SCF)

Molecular weight and synonyms:

- SCF is a highly glycosylated 28-36 kDa protein (1).
- c-kit ligand (2).
- mast cell growth factor (3,4).
- steel factor (5).

Main cellular source:

- SCF exists as a soluble and transmembrane form and is constitutively produced by endothelial cells and bone marrow stromal cells and fibroblasts (6,7).
- Keratinocytes in normal skin (8).
- Epithelial cells in the gut (9) and sertoli cells (10).

Main Biological Functions:

- SCF is a key growth factor required for normal basal haemopoiesis (11).
- SCF stimulates melanocytes and mast cells.
- SCF influences the migration of germ cells, melanocytes and haemopoietic cells during development.

Effects on haemopoietic progenitors and HSC:

- SCF is an early acting synergistic growth factor affecting the growth of myeloid, erythroid, megakaryocytic and T lymphocyte precursors (11,12,13).
- In vitro SCF alone is a survival factor for primitive HPC (14).
- SCF acts synergistically with IL-3, IL-1, IL-6, G-CSF, GM-CSF, and EPO to increase the number and size of CFU-GM, BFU-E and CFU-GEMM (15,16,17).
- SCF acts synergistically with TPO and/or FLT3 to enhance division of primitive HPC (18,19).

Effects of the knockout: implication of haemopoiesis:

- Mutations at the Sl locus and the resultant macrocytic anaemia, abnormalities in megakaryocytopoiesis and granulocytopoiesis confirm that SCF has a critical role in steady state haemopoiesis (20,21).
- SCF is also essential for tissue mast cell development and function (22).

References:

1. Arakawa et al 1991
2. Huang et al 1990
3. Boswell et al 1990
4. Copeland et al 1990
5. Flannagan and Leder 1990
6. Heinrich et al 1993
7. Broudy et al 1994
8. Longley et al 1993
9. Klimpel et al 1995
10. Tajima et al 1991
11. Broudy 1997
12. Bernstein et al 1991
13. Rodewald et al 1995
14. Li and Johnson 1994
15. McNiece et al 1991
16. Lowry et al 1991
17. Migliaccio et al 1991
18. Ramsfjell et al 1997
19. Zandstra et al 1998
20. Russell 1979
21. Ruscetti et al 1976
22. Kitamura et al 1978

Table 1.8

Flt3-ligand (FLT3L)

Molecular weight and synonyms:

- Various isoforms are produced by alternative splicing but the predominant form is membrane bound and biologically active at the cell surface (1,2,3).
- Natural FTL3L from stromal cells is a 65 kDa non-disulphide linked homodimeric glycoprotein of 30kDa subunits, each with 12 kDa of N- and O-linked sugars (4).
- Ligand for flk2 (fetal liver kinase 2) (5).

Main cellular source:

- Although there is ubiquitous expression of the FLT3L mRNA, the main source of soluble protein is from T lymphocytes and stromal cells (4).

Main Biological Functions:

- Promotes survival and proliferation of primitive HPC including pro-B cells within the BM.

Effects on haemopoietic progenitors and HSC:

- FLT3L has little in vitro colony stimulating activity as a single agent but has potent synergism with a variety of other HGF including IL-3 (1), IL-6 (1), IL-7 (6), IL-11 (7,8), G-CSF (9,10), GM-CSF (9), and SCF (1), as well as in multi-HGF combinations (11,12).
- FLT3L supports in vitro proliferation of LTC-IC and long-term repopulating cells (13,14).
- FLT3L increases the recruitment of primitive HPC into cell cycle and inhibits apoptosis (12, 15, 16).
- In contrast to SCF, FLT3L has minimal effect on the cloning or proliferation of erythroid precursors (1,2,17).
- In combination with GM-CSF, TNF-α and IL-4, FLT3L increases production of dendritic cells from BM $CD34^+$ cells (18).

Effects of the knockout: implication of haemopoiesis:

- Mice with targeted disruptions of the flk2/flt3 gene have normal blood counts and BM cellularity. However there are subclinical deficiencies in B-lymphopoiesis and stem cell defects revealed by transplantation experiments (19).
- FLT3L is not essential for steady state haemopoiesis.

References:

1. Hannum et al 1994
2. Lyman et al 1994
3. Lyman et al 1995
4. McClanahan et al 1996
5. Matthews et al 1991
6. Lyman et al 1993
7. Jacobsen et al 1995
8. Hirayama et al 1995
9. Broxmeyer et al 1995
10. Piacibello et al 1995
11. Shah et al 1996
12. Haylock et al 1997
13. Yonemura et al 1997
14. Dooley et al 1997
15. Veiby et al 1996
16. Ohishi et al 1996
17. McKenna et al 1995
18. Maraskovsky et al 1997
19. Mackarehtschian et al 1995

Table 1.9

course of these studies both TPO and FLT3L were cloned and recombinant proteins were made available for investigation.

1.4.3 Negative Regulators of Haemopoiesis

In regenerating tissues such as bone marrow, skin, or intestinal epithelia the cells produced are just sufficient to replace the effete cells lost because of damage or ageing. The supply and demand of cells is finely balanced in such tissues. During normal steady state haemopoiesis, production of the required numbers of blood cells is achieved mainly through amplification of the committed progenitor cell population while the majority of stem cells remain quiescent. However, during severe infections or cytoreductive insults, HSC are rapidly recruited into the cell cycle, proliferate and differentiate to restore the blood counts to normal levels. Maintenance of this homeostatic balance involves both stimulators and inhibitors of HSC, which act in concert as a positive and negative feedback control system. Although many stimulators of haemopoiesis have been identified, cloned and produced in purified form much less is known about negative regulators or inhibitors of haemopoiesis and their mechanisms of action. Inhibitors of haemopoiesis include small peptides (Guigon and Bonnet 1995) or larger molecules that display pleiotropic effects on numerous target cells both within and outside the haemopoietic system (Graham and Pragnell 1990).

Likely candidates for inhibitors include molecules referred to as "chalones" which were first described in 1960 as tissue specific, species non-specific inhibitors of mitosis (Bullough and Lawrence 1960), produced by mature cells and acting on early cells of the same lineage. Such a concept rapidly became popular in haemopoiesis and early reports suggested the existence of a granulocyte-derived polypeptide that inhibited myeloid precursors without being cytotoxic (Paukovitz 1971, Bateman 1974). Subsequently a number of molecules were identified that in part have most of the characteristics of a chalone. The first of these was identified in extracts from normal BM cells, an activity capable of rendering cycling stem cells (CFU-S) quiescent (Lord *et al* 1976). This activity initially referred to as "Inhibitor" was subsequently found to be identical to macrophage inflammatory protein-1alpha (MIP-1α) (Wolpe *et al* 1988, Graham *et al* 1990). As the name suggests MIP-1α is produced by macrophages and at picomolar concentrations can induce quiescence of cycling CFU-S. As with other inhibitors, MIP-1α shows specificity for primitive human HPC over more committed HPC. In cultures of cord blood cells, addition of MIP-1α inhibits the numerical expansion of primitive ($CD34^+CD45RA^{low}CD71^+$) cells without inhibiting the proliferation of more mature subpopulations enriched for myeloid or erythroid progenitors

(Mayani *et al* 1995). Similar results have also been observed with primitive and committed HPC isolated from adult BM (Bonnet *et al* 1995).

One small inhibitory peptide, pEEDCK, originally isolated from normal human granulocytes can also be considered a chalone. This haemoregulatory peptide, also known as HP5b, was first shown to inhibit the *in vitro* growth of murine myeloid progenitors (Laerum and Paukovits 1984) in a dose dependent manner. Subsequent studies confirmed that the pentapeptide inhibited growth of human myeloid progenitors, mixed (CFU-GEMM), and BFU-E (Lu *et al* 1989). The *in vivo* administration of pEEDCK into normal mice led to a reversible decrease of myeloid progenitors and CFU-S. Furthermore, pEEDCK inhibits CFU-S from entering DNA synthesis induced by chemotherapy (Paukovits *et al* 1990). HP5b can dimerise through formation of a disulphide bond and interestingly the dimer is not inhibitory but stimulates both murine and human CFU-GM. This observation raises the possibility that the extent of inhibition or stimulation is dictated by the relative concentration of the monomer and dimer, which in turn could influence the overall proliferative status of HPC.

A second small inhibitory peptide containing only 4 amino acids, and hence termed "The Tetrapeptide" (Graham and Pragnell 1990), was first isolated from foetal bone marrow (Frindel and Guigon 1977) and has since been purified and shown to have the sequence AcSDKP (Lenfant *et al* 1989). In mice, the synthetic tetrapeptide, as well as the natural one, inhibits the entry into DNA synthesis of CFU-S after irradiation and or chemotherapy (Lenfant *et al* 1989). AcSDKP also inhibits the *in vitro* growth of human progenitors from normal BM and CB (Guigon *et al* 1990, Bonnet *et al* 1993) and their growth in methylcellulose cultures (Cashman *et al* 1994). In addition, studies performed with purified $CD34^+$ cells have confirmed that AcSDKP directly inhibits the proliferation and number of CFU-GM, BFU-E and HPP-CFC (Bonnet *et al* 1995). Studies on the mode of action of the tetrapeptide indicate that it is only capable of maintaining quiescent cells in G_o; actively cycling cells are refractory to the inhibitor. The peptide is also ineffective on cells at the G_1/S phase boundary, suggesting that it functions specifically in early G_1 or during transition from G_1 to G_o (Monpezat *et al* 1989). In spite of the data from the *in vitro* studies it is unclear if either the inhibitory pentapeptide or the tetrapeptide has any physiological role in regulating haemopoiesis.

Apart from MIP-1α, macrophages also produce tumour necrosis factor (TNF)-α, a pleiotropic cytokine released during inflammation (Rosenblum and Donato 1989), that can either inhibit or stimulate HPC (Lu *et al* 1993, Rusten *et al* 1994, Jacobsen *et al* 1992, Caux *et al* 1991, 1993, Bonnet *et al* 1995). The results from these reports indicate that TNF-α has double-edged biological functions: suppressing colony growth yet inducing growth factor gene expression (Vogel *et al* 1987, Kaushansky *et al* 1988). In this regard TNF-α has been shown to stimulate granulocytopoiesis and inhibit erythropoiesis (Ulich *et al* 1990). The recent studies by Snoeck *et al* clearly demonstrate that TNF-α is also a potent synergistic factor for the proliferation of primitive human HPC ($CD34^+CD38^-$ cells) and at low concentrations (0.1 ng/ml) abrogates the profound inhibitory effect of transforming growth factor-β (TGF-β) on the proliferation of these cells (Snoeck *et al* 1996). In accord with its double-edged biological functions TNF-α also inhibits 60-70% and 20-40% of CFU-GM and BFU-E, respectively, from mature HPC with a $CD34^+HLA\text{-}DR^{++}$ phenotype (Bonnet *et al* 1995).

Interferon (IFN)-γ has also been described as a potent direct inhibitor of primitive HPC (Broxmeyer *et al* 1986, Selleri *et al* 1995), and indirectly increases the inhibition by MIP-1α by upregulation of the MIP-1α receptor on $CD34^+$ cells (Durig *et al* 1998). In addition, recent studies have indicated that IFN-γ production by T lymphocytes and stromal cells results in direct, potent suppression of haemopoiesis and may be the underlying cause for some cases of aplastic anaemia (Selleri *et al* 1996, Yu *et al* 1999). In contrast, IFN-γ has also been shown to stimulate megakaryocytopoiesis, at least in vitro (Muraoka *et al* 1997).

Another series of immunomodulators with inhibitory effects on haemopoiesis is the TGF-β family of molecules (Sporn *et al* 1986, Hooper 1991). There are at least 5 different isoforms within this family of polypeptide growth factors. Although TGF-β was originally characterised by its ability to stimulate the proliferation of rodent fibroblasts (deLarco *et al* 1978, Roberts *et al* 1981), it is now evident that TGF-β is a multifunctional growth regulator whose effects depend on the cell type targeted (Sporn and Roberts 1988). TGF-β is a pleiotropic regulator of haemopoietic cell growth that can either inhibit (Ohta *et al* 1987, Keller *et al* 1988, Sing *et al* 1988, Hino *et al* 1988, Keller *et al* 1989, Ishibashi *et al* 1987b, Keller *et al* 1990, Goey *et al* 1989, Cashman *et al* 1990) or stimulate (Ottman and Pelus 1988, Aglietta *et al* 1989, Keller *et al* 1991, Jacobsen *et al* 1992b) the growth of murine and human HPC. A comprehensive review of the pleiotropic role of TGF-β in the regulation of

haemopoiesis has recently been published (Fortunel *et al* 2000). In brief, the effects of TGF-β are dependent on the presence of other growth factors (Keller *et al* 1988, Sing *et al* 1988, Ottman and Pelus 1988, Jacobsen *et al* 1992b), the differentiation state of the target cell (Keller *et al* 1988, Sing *et al* 1988, Keller 1990, Ottman and Pelus 1988, Jacobsen *et al* 1992b) and the concentration of TGF-β in culture. In particular, TGF-β1 is a potent inhibitor of primitive HPC whereas the growth of more committed progenitors is either unaffected or stimulated by TGF-β (Lardon *et al* 1994, Hatzfeld *et al* 1991, Cardoso *et al* 1993). Although there is evidence that human stromal cells constitutively express TGF-β1 it may also be upregulated by TNF-α and GM-CSF, suggesting a mechanism for dampening the stimulatory effect of these cytokines on haemopoiesis (Nemunaitis *et al* 1991). Notably, TGF-β is also produced by primitive HPC where it acts in an autocrine manner to maintain HSC quiescence (Hatzfeld *et al* 1991, Cardoso *et al* 1993, Li *et al* 1994). Interestingly, from the point of *ex vivo* manipulation of HSC, the use of antisense oligodeoxynucleotides or polyclonal antibodies to TGF-β can block the antiproliferative effect of TGF-β and thus increase the generation of primitive cells following stimulation in combinations of early acting and synergistic HGF (Li *et al* 1994, Van Ranst *et al* 1996, Fortunel *et al* 2000).

The pleiotropic effects of TGF-β are mediated by ligation with transmembrane or membrane-bound proteins known to interact with the TGF-βs, the type I and type II TGF-β receptors (TβR-1 and TβR-2). The mechanisms of signalling by these 2 receptors is now well established (Wrana *et al* 1994, Bruno *et al* 1998): in brief, it involves binding of TGF-β to TβR-2, formation of a phosphorylated tetrameric receptor complex and recruitment of the Smad protein family (Massague 1998). Subsequently, Smad complexes are translocated to the nucleus where they associate with DNA-binding partners and regulate the transcriptional response of target genes. Presently, the complete range of TGF-β responsive genes remains to be determined, so the exact molecular mechanisms underpinning the pleiotropic action of TGF-β are yet to be elucidated. However, one possible mechanism for the anti-proliferative effect of TGF-β on primitive HPC appears to involve down regulation of HGF receptors (Dubois *et al* 1990, Ruscetti *et al* 1992, McNiece *et al* 1992, Sansilvestri *et al* 1995). Treatment of progenitor cells with TGF-β decreases the number of IL-1 receptors without any significant reduction in receptor affinity (Dubois *et al* 1990) and also induces down-modulation of IL-3 and M-CSF receptor levels (Jacobsen *et al* 1993). In addition, TGF-β1 inhibits stromal cell production of SCF by repression of SCF gene transcription and represses HPC expression of c-*kit* by decreasing the stability of *c-kit* mRNA transcripts.

Taking into account the importance of SCF and *c-kit* in maintaining steady-state haemopoiesis *in vivo*, the dual effect of TGF-β_1 on both SCF and *c-kit* gene expression is likely to be one of the major mechanisms by which TGF-beta 1 inhibits haemopoiesis in vivo (de Vos *et al* 1993, Dubois *et al* 1994, Heinrich *et al* 1995). In addition, TGF-β has been shown to down-modulate *flt3* and the IL-6 receptor on human haemopoietic progenitors (Fortunel 1998).

In summary, MIP-1α, TNF-α, IFN-γ and TGF-β are pleiotropic immunoregulators that either stimulate or inhibit haemopoiesis. It has been postulated that in situations of increased demand for mature blood cells there is interplay between these immunomodulators to effect the desired response without compromising the size of the HSC pool. For example, TNF-α induces resistance to the inhibitory effects of TGF-β1 so that HSC become responsive to the proliferative effects of other cytokines. Exhaustion of the stem cell pool could be prevented by the selective inhibitory effect of another inflammatory cytokine, IFN-γ, which is not opposed by TNF-α (Snoeck *et al* 1996).

A similar paradigm has been proposed by Ruscetti *et al* to describe the opposing regulation of haemopoiesis by TGF-β1 and IL-1 (Ruscetti *et al* 1992). In their model, IL-1 is the prototype of the proinflammatory molecule while TGF-β is essentially anti-inflammatory. IL-1 is part of the cascade of cytokines that are produced during microbial invasion or bodily injury and enhance a variety of host responses, particularly in the immunological and haemopoietic systems, while TGF-β acts as an inhibitor of these responses. At several levels, IL-1 and TGF-β act in opposition to one another. IL-1 stimulates the expression of many genes in lymphoid and marrow stromal cells that stimulate haemopoietic cell growth and differentiation, while TGF-β inhibits these IL-1 mediated effects. In addition, IL-1 induces the cell surface expression of cytokine receptors on lymphoid and haemopoietic cells, while TGF-β dramatically inhibits the cell surface expression of these receptors, including c-*kit* and the IL-1 receptor. Finally, IL-1 augments lymphoid and haemopoietic cell growth and TGF-β1 potently inhibits this proliferation. The interactions of these cytokines serve to illustrate that the net balance of stimulatory and inhibitory signals, in part determines the fate of a given cell that may be responsible for homeostatic haemopoietic cell growth.

1.5 Haemopoietic Growth Factor Receptors

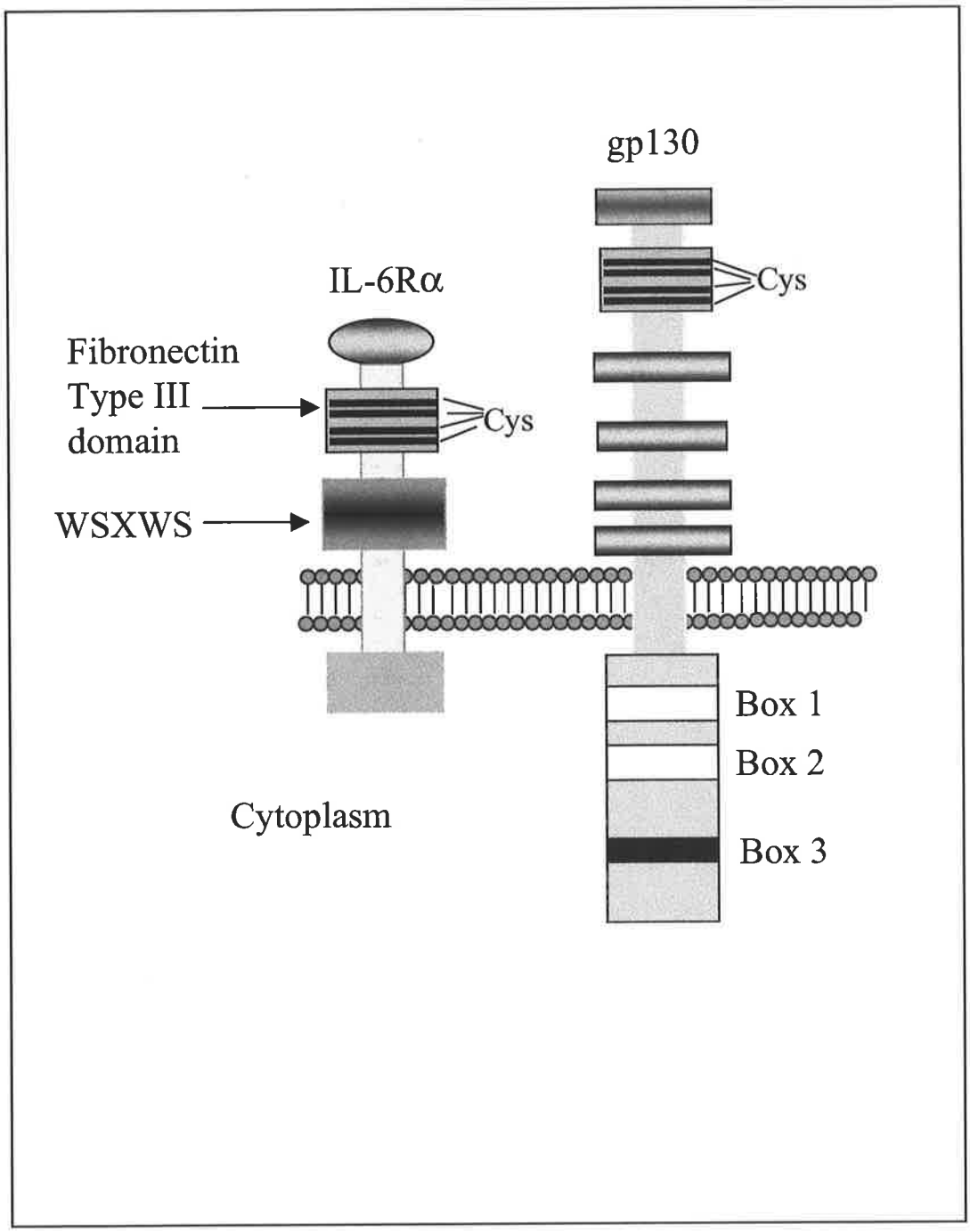
Haemopoietic growth factors are able to elicit a broad range of biological responses from haemopoietic target cells. These responses are mediated by messages transmitted via HGF receptors and a co-ordinated cascade of intracellular signalling molecules that ultimately lead to altered gene expression. The interaction between HGF and their specific receptors is the first event in this process and a key to appreciating how HGF regulate cell fates and function. Characterisation of cytokine and HGF receptors and the cloning of genes encoding these receptors have led to recognition that many HGF receptors can be grouped into families based on common structural features.

There are at least six receptor superfamilies involved in regulation of haemopoietic cell function. Most receptors of HGF are members of the Type 1 cytokine receptor family (or HGF receptor superfamily), which do not possess intrinsic tyrosine kinase activity. Instead, these receptors induce phosphorylation of cellular substrates by serving as docking sites for adaptor molecules that do have tyrosine kinase activity. The Haemopoietic Growth Factor Receptor Superfamily (or the cytokine receptor superfamily) includes receptors for leukaemia inhibitory factor (LIF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, IL-14, IL-18, GM-CSF, G-CSF, EPO, prolactin, growth hormone, ciliary neurotropic factor, and TPO (Nicola and Metcalf 1991, Bazan 1990, Vilcek 1998). As shown in figure 1.5, there are a number of repetitive structural themes in this superfamily including: i) four cysteine residues in the extracellular domain, ii) a W-S-X-W-S sequence in the ligand binding extracellular domain that may optimise the tertiary structure of the receptor (Hilton *et al* 1996), iii) a capacity for enhanced binding and or signal transduction when expressed as a heterodimer or homodimer (e.g. G-CSF and EPO receptors), iv) lack of a known catalytic domain in the cytoplasmic portion of the molecule and v) the presence of fibronectin type III domains (Patty 1990) in the extracellular regions. Another shared feature of many HGF receptors is their ability to prevent apoptosis, in some cases without inducing mitogenic signals. This is particularly true of the lineage specific factors, EPO, M-CSF, IL-5 and IL-7 (Yamaguchi *et al* 1991, Brach *et al* 1992, Muta *et al* 1994).

Notably, many of the receptors within the cytokine receptor superfamily can be classified into subfamilies according to common chains that comprise the heterodimeric or heterotrimeric receptor (Taga and Kishimoto 1995). These receptors typically contain one or

Figure 1.5 The haemopoietic growth factor receptor superfamily

The haemopoietic growth factor receptor or class I cytokine receptor superfamily includes receptors for EPO, G-CSF, GM-CSF, LIF, prolactin, growth hormone, and interleukins 2,3,4,5,6,7,9,11,12 and 15. There are a number of repetitive structural and functional themes in this superfamily including 1) four cysteine (cys) residues in the fibronectin type III regions of the extracellular domain, 2) a conserved Trp-Ser-any-Trp-Ser (WSXWS) box, each of which is essential for receptor function, 3) conserved residues in regions termed box-1 and box-2 located close to the membrane, 4) a capacity for enhanced binding and or signal transduction when expressed as a heterodimer or homodimer and 5) lack of a known catalytic domain in the cytoplasmic region of the molecule. Some of these features are represented in the figure showing the two components of the high affinity IL-6 receptor complex, IL-6Rα and gp130.


gp130
IL-6Rα
Cys
Fibronectin
Type III
domain
Cys
WSXWS
Box 1
Box 2
Box 3
Cytoplasm

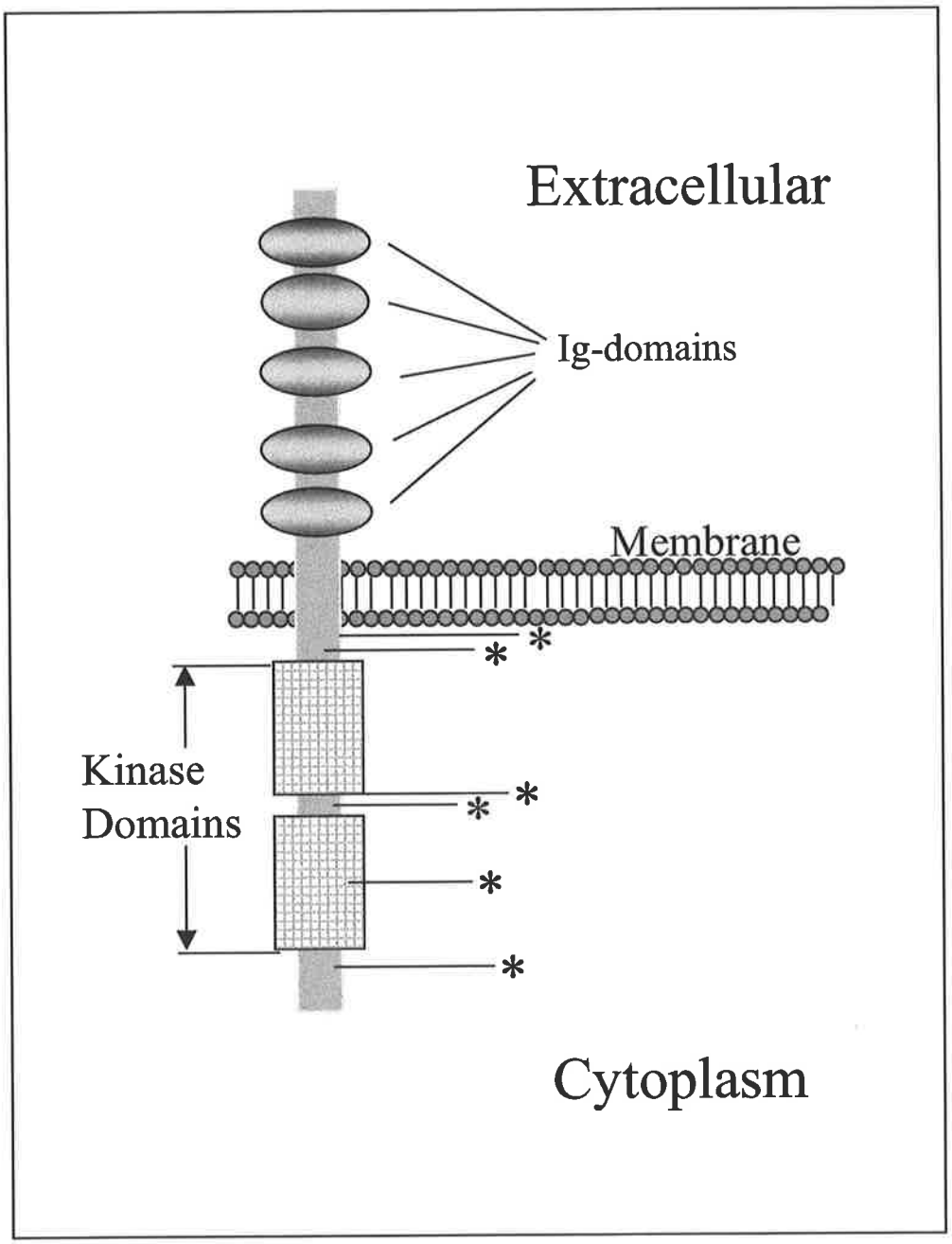
two unique subunits that act as specific binding components for a single cytokine, linked to a signal transducing chain that is shared with other members of the same family. Three such receptor families, the IL-6, GM-CSF and IL-2 group of receptors, utilise signalling components common to each of these families (the gp130 chain, β chain and γ chain, respectively). Thus although gp130 was originally identified as the signal transduction and affinity converting component of the IL-6 receptor (IL-6R) it has subsequently been shown to exhibit an identical function for LIF, Oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and IL-11 receptors (Ip *et al* 1992, Gearing *et al* 1992). Similarly, subunit promiscuity (Nicola and Metcalf 1991) as exhibited by the IL-3R, GM-CSFR and IL-5Rs, involves a communal β chain interacting with specific α chains to facilitate high affinity binding of their respective ligands (Hayashida *et al* 1990, Kitamura *et al* 1991a, Devos *et al* 1991) and subsequent signalling. Some members of this receptor superfamily also share sequences in the intracellular domains, termed box 1 and box 2 regions. These sequences found in the receptors for IL-2, IL-3, IL-4, IL-6, IL-7, EPO, G-CSF and in gp130 (Ihle 1995, Theze *et al* 1996) are important for activation of signalling pathways.

The second family of HGF receptors are those where the cytoplasmic domains exhibit intrinsic tyrosine kinase activity. Amino acid sequence analysis has revealed that the receptor tyrosine kinase (RTK) family can be split into 3 subclasses or Types. The nue/HER2 proto-oncogene and epidermal growth factor comprise subclass 1, the insulin and IGF-1 receptors belong to subclass 2 and the receptors for M-CSF, SCF, FLT3L and platelet derived growth factor are members of subclass 3 (or Type III RTKs, reviewed by Ullrich and Sclessinger 1990). C-*kit*, *flt*3 and c-*fms* are genes that encode three related HGF receptors, the ligands for which are SCF, FLT3L and M-CSF respectively. The structure of these receptors include an extracellular domain with five Ig-like motifs, a short membrane spanning domain, and a cytoplasmic domain containing two kinase domains separated by a kinase insert sequence (refer to figure 1.6). Although there are structural differences between the RTKs and the cytokine receptor superfamily there is now growing evidence that ligand binding and signalling through both involves di- or oligomerisation of receptor subunits (Cunningham *et al* 1991, Watowich *et al* 1992, Kitamura and Miyajima 1992, Taga *et al* 1989, Heldin 1995, Budel *et al* 1995).

The third family of receptors is the Class II subgroup, which includes the interferon receptors and the receptors for tissue factor and IL-10. Each of these receptors has an extracellular domain containing a region resembling the fibronectin III domain found in the

Figure 1.6 Structure of type III receptor tyrosine kinases

This figure shows c-*kit* as a protoype type III tyrosine kinase receptor. The extracellular domain contains no fibronectin like modules or WSXWS motifs but has five immunoglobulin like domains (oval shaped). There are 6 potential sites for tyrosine phosphorylation (shown by asterisks) in the cytoplasmic region, each of which may serve as a docking site for adaptor proteins containing SH2 domains. When growth factors (stem cell factor/kit ligand in this case) bind to this class of receptors, receptor chains dimerise, autophosphorylate, and then serve as docking sites for a range of adaptor proteins that serve as signal transducers.


Extracellular
Ig-domains
Membrane
Kinase
Domains
Cytoplasm

type I receptors, which may serve as the ligand binding site. A fourth family is the chemokine receptors, which includes the receptors for IL-8 and MIP-1α. The chemokine receptors are seven transmembrane spanning G protein linked receptors (Premack and Schall 1996, Murphy 1996) that are divided into three families based on their variability in cysteine residues; α or CXC, β or CC, and γ or C (Mackay 1997). The tumour necrosis factor receptor family (TNFR) makes up a fifth family and includes TNFR1, TNRF2, *fas*, CD40, nerve growth factor (NGF)-receptor, CD27, CD30, and OX40 (Wallach 1996). Signalling by TNFα and fas-ligand can suppress haemopoiesis by triggering programmed cell death in progenitor cells (Barcena *et al* 1996, Maciejewski *et al* 1995). The majority of these receptors contain cysteine-rich repeats in the extracellular domains and have cytoplasmic portions containing 80 amino acid "death domains" required for transducing apoptotic signals and for NF-κB activation. Finally, there is the family of transforming growth factor-β (TGF-β) receptors that are unique in that they contain intracellular serine-threonine kinase domains (Lui *et al* 1995, Attisano and Wrana 1996). The TGF-β receptors are divided, on the basis of structural features into type I and type II receptors and TGF-β signalling requires both a type II (the ligand binding component) and a type I (the signalling component) receptor.

Although molecular analysis provides information concerning the structure and function of receptors it is important to establish the distribution of HGFR on the cell surface of haemopoietic cells. This data may help explain or predict the biological responses of haemopoietic cells to specific HGF. The first studies investigating receptor expression by haemopoietic cells were performed with radioiodinated ligands (Byrne *et al* 1981, Pigoli *et al* 1982, Nicola *et al* 1983). These approaches showed that binding of M-CSF and G-CSF to murine BM cells was specific, saturable and of high affinity. Scatchard analysis of saturation binding revealed that post mitotic granulocytes exhibited a mean of 240 receptors for G-CSF per cell (Nicola and Metcalf 1985) and different mononuclear cells had between 3000-19,000 M-CSF receptors (Bryne *et al* 1981). In addition, further studies indicated that, in particular circumstances, all CSF receptors could exist in both high-affinity (K_D = 10-100p_M) and low-affinity (K_D = generally measured in ≥ nanomolar range) forms (Chiba *et al* 1990, Elliot *et al* 1989, Park *et al* 1989, Cannistra *et al* 1990). It is now appreciated, as predicted (Nicola 1991) that the low-affinity forms can be converted to high-affinity forms by the recruitment of additional receptor chains following ligand binding. IL-3, GM-CSF and IL-5 provide an excellent example where ligand binding to their specific receptor α chains then

binding to the common β chain leads to the formation of a high affinity ligand-receptor interaction.

Studies with radiolabelled ligands or analogues have also demonstrated that rapid internalisation of ligand-receptor complexes occurs within minutes of M-CSF (Rettenmier *et al* 1987), IL-3 (Hapel *et al* 1992), IL-6 (Dittrich *et al* 1996), G-CSF (Khwaja *et al* 1993), GM-CSF (Cannistra *et al* 1990), SCF (Broudy *et al* 1998, Zandstra *et al* 1999), FLT3L (Turner *et al* 1996) and TPO (Li *et al* 1999) binding to their respective receptors. There is accumulating evidence that this rapid internalisation of ligand-receptor complexes may be an important negative regulatory system for cytokine mediated signalling. Defective internalisation and the sustained activation of truncated granulocyte colony-stimulating factor receptor found in severe congenital neutropenia/acute myeloid leukaemia support this view (Ward *et al* 1999, Hunter *et al* 1999). HGF signalling can also result in either up-regulation or decreased expression of different HGFR: IL-1 increases the expression of IL-3, IL-5, GM-CSF and EPO receptors (Kitamura *et al* 1991b) and TGF-β1 results in down regulation of c-*kit* on primitive human HPC (Sansilvstri *et al* 1995). Furthermore, differentiation of HPC may be regulated by the hierarchical down-modulation of HGFR (Walker *et al* 1985).

Although the approaches outlined above provide data on the receptor density, affinity of ligand-receptor interactions and intracellular fate of these complexes they do provide information as to the distribution of receptors on particular cell types. This limitation can be overcome by using purified populations of cells together with cell autoradiography, and grain counting techniques (McKinstry *et al* 1997). However, when radiolabelled ligands or HGF analogues are used to examine receptor expression, cells cannot be used for functional studies. In contrast, HGFR expression can be investigated by using monoclonal antibodies and flow cytometry and cells can be isolated to high purity by FACS. Although cell autofluorescence and the inherent sensitivity of the laser excitation and sensing systems limit this approach it remains a powerful method for obtaining viable cells for functional studies. Most investigators have utilised combinations of McAB to investigate HGFR expression on sub-fractions of HPC that express the CD34 antigen (Wagner *et al* 1995). In the present study, a similar approach has been used for studies on expression of HGFR by adult human BM cells.

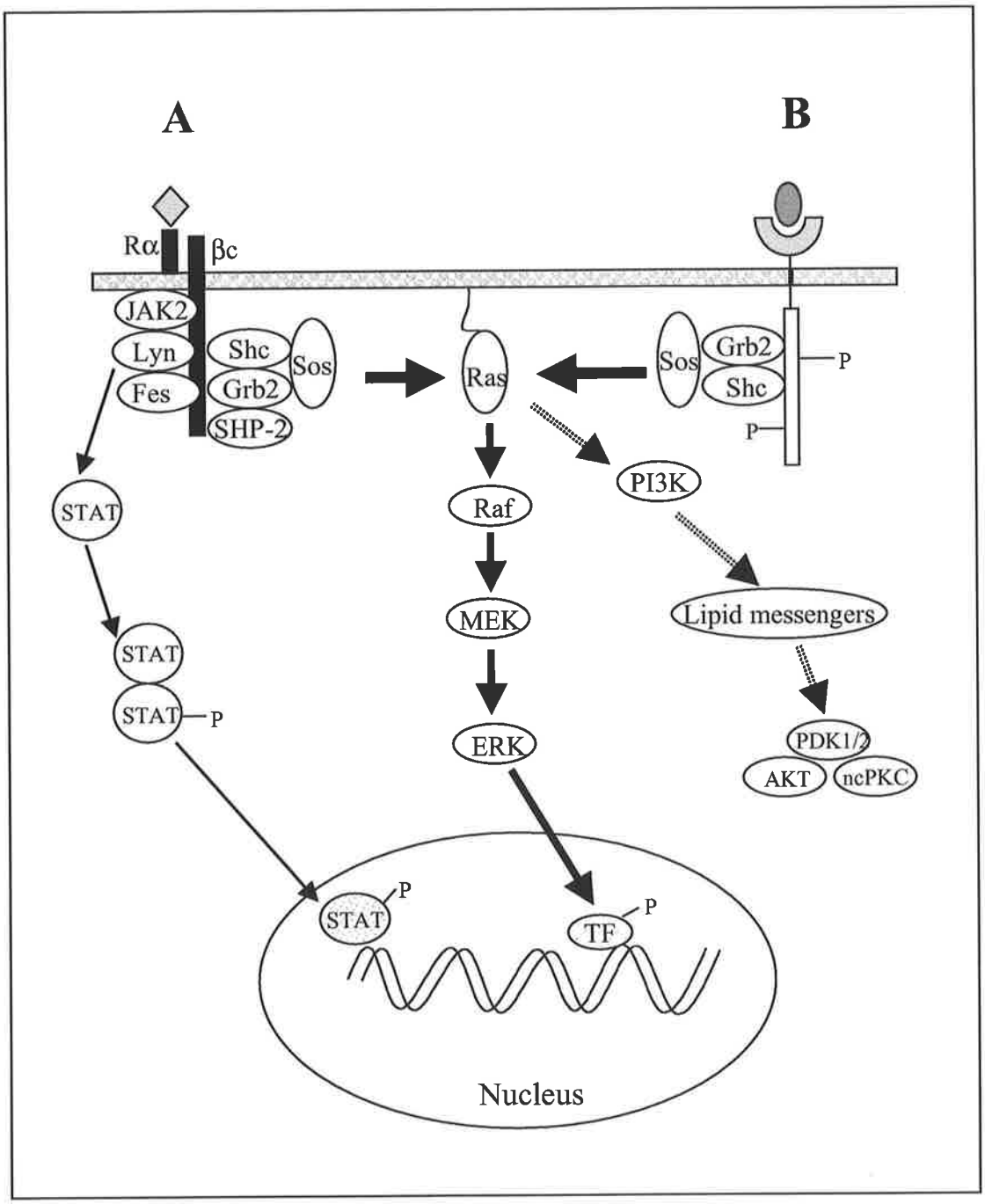
1.6 Haemopoietic Growth Factor Signalling

Following ligand binding, HGF receptors, in general, initiate a variety of cellular responses by altering intracellular biochemical events. As mentioned previously many of the haemopoietic regulators exhibit pleiotropic effects on different cell populations and considerable redundancy exists among the growth factors. These properties suggests that HGF may activate overlapping signal transduction pathways in a particular cell type and that cells at different stages of differentiation may differ in signalling events that are induced by a particular HGF. How is this achieved? There has been significant progress made in recent years concerning the mechanisms and pathways whereby signals are generated and transmitted from the HGFR to the inside of cells. Largely the structural characteristics and amino acid sequence of the cytoplasmic domains of the receptors determines the patterns of signal transduction. Thus, each of the families of HGFR described above exhibit distinct, characteristic patterns of signal transduction, although it is now evident that there is convergence of specific signalling pathways leading to cell proliferation. Since the current study concerns the *ex vivo* manipulation of HPC by HGF whose respective receptors have either intrinsic tyrosine kinase activity (c-*kit*, *flt3*) or are members of the Haemopoietic Growth Factor Superfamily (i.e.receptors for IL-1, IL-3, IL-6, G-CSF, GM-CSF and TPO), this discussion will only consider signalling for these specific receptors.

The receptors for SCF and FLT3L, c-*kit* and *flt3* respectively, are Type III receptor tyrosine kinases (RTKs) and exhibit intrinsic tyrosine kinase activity (Yarden *et al* 1987, Majumder *et al* 1988, Matthews *et al* 1991, Rosnet *et al* 1991). A general mechanism for the activation of receptor tyrosine kinases appears to involve ligand-induced dimerisation (Heldin 1995, Lemmon *et al* 1997), transient activation of tyrosine kinase activity (Linnekin *et al* 1997), and autophosphorylation (Ullrich and Schlessinger 1990, Maroc *et al* 1993). The resulting phosphotyrosines unmask the enzymatic activity and/or serve as docking sites for Src homology-2 (SH2) domain-containing cytoplasmic molecules such as phospholipase Cγ (Serve *et al* 1995), the p85 subunit of phospatidylinositol 3' (PI3) kinase or Grb2 (Pawson 1992, Van Der Geer and Hunter 1994). Thus ligand induced recruitment of such molecules to the cytoplasmic kinase domains of the receptor are initialising events that propagate downstream signals leading to both functional events and subsequent termination of signal (Yi and Ihle 1993). Downstream signalling may involve 3 pathways that ultimately lead to increases in gene transcription. As shown in figure 1.7, the first is activation of Ras (through multiple mechanisms) leading to activation of c-Raf-1 and the MAP kinase cascade

Figure 1.7 The classical Ras-to-MAPK cascade

The left hand side (A) of this figure shows signalling by members of the HGF receptor superfamily such as receptors for IL-3, IL-5 and GM-CSF. These receptors consist of ligand-specific α subunits and a common β-subunit (βc), which may already be bound by protein kinases such as JAK2, Lyn or Fes. After ligand binding, the α- and β-subunits are thought to dimerise, thus activating the receptor-bound protein kinases and subsequently leading to a cascade of tyrosine phosphorylations that result in docking of various signalling molecules including Shc, Grb2 and Sos. Extracellular signal-regulated kinases (ERK or MAP kinase) are subsequently activated via the classical Ras-Raf pathway. In addition, JAK2 phosphorylates the STAT (signal transducers and activators of transcription) family of nuclear factors which form heterodimers and homodimers, thus inducing their translocation to the nucleus and subsequent binding to activating sequences of the promoters of various genes. The right hand side (B) illustrates signalling by receptor tyrosine kinases through the classical Ras-Raf-1 pathway, subsequent phosphorylation of transcription factors (TF) and enhanced expression of immediate response genes in haemopoietic cells. This figure is adapted from Reuter *et al* (2000).


A
B
Rα
βc
JAK2
Lyn
Fes
Shc
Grb2
SHP-2
Sos
Ras
Sos
Grb2
Shc
P
P
STAT
STAT
STAT
P
Raf
MEK
ERK
PI3K
Lipid messengers
PDK1/2
AKT
ncPKC
STAT
P
TF
P
Nucleus

(Miyazawa *et al* 1991, McCormack 1994). The second pathway is induction of *c-Myc* through activation of Src or other Src family members (Barone and Courtneidge 1995, Eisenman and Cooper 1995). The third pathway involves activation of the *Janus* family of protein tyrosine kinases (Jaks), subsequently leading to activation of signal transducers and activators of transcription (STATs) (Ihle 1995, 1996b, Linnekin *et al* 1997). One important and interesting question with regards to signalling via *flt*3 and c-*kit* is the identity of the downstream signalling molecules and of the genes subsequently transcribed. Are these identical, partially overlapping or distinct for each receptor? Is there convergence of the signalling pathways to a common STAT and expression of the same genes? Are unique signal transduction molecules recruited if cells are simultaneously stimulated with both FLT3L and SCF? Is the pattern on signal transduction dependent on cell lineage? Although a number of studies have been performed with both haemopoietic cell lines and mast cells to investigate these questions (Dosil *et al* 1993, Deberry *et al* 1997, Timokhina *et al* 1998, Zhang *et al* 1999) there is a lack of data concerning signalling of RTKs in primitive human HPC.

However, the data from haemopoietic cell lines provide some clues. Stimulation with SCF leads to a rapid transient phosphorylation of ERK1 and ERK2 in FDCP-Mix A4 cells (Pearson *et al* 1998), the Src family member Lyn (Linnekin *et al* 1997) and JAK2 with subsequent activation of STAT1 in MO7e cells (Deberry *et al* 1997), and convergence of both the PI3-kinase and Src kinase signalling pathways to activate Rac1 and JNK in mast cells (Timokhina *et al* 1998). In contrast, $p115^{CBL}$ and $p52^{SHC}$ in myeloid cell lines and an unknown p115 protein in B cells (Lavagna-Sevenier *et al* 1998) are the predominant tyrosine-phosphorylated proteins following FLT3L stimulation. Additionally, SHIP (Liu *et al* 1997) is also autophosphorylated and associated with Shc after FLT3L stimulation of the murine IL-3 line Baf3, stably expressing human *flt*3 (Zhang *et al* 1999). Irrespective of the upstream components, MAP kinase appears to be ultimately activated. Notably these studies with cell lines have also shown that signalling by RTKs can be modulated by at least two mechanisms. Firstly, SOCS proteins (suppressors of cytokine signalling: Hilton *et al* 1998, Begley and Nicola 1999) which are rapidly induced following SCF stimulation can bind the signalling proteins such as Grb-2 and Vav (De Sepulveda *et al* 1999) and in doing so act as an inducible switch modulating proliferative signals in favour of cell survival signals. Secondly, tyrosine phosphatases such as SHP-1 are able to dephosphorylate tyrosines on *c-kit* and potentially *flt3* (Kozlowski *et al* 1998). Thus, there is an emerging theme that the response by haemopoietic cells to stimulation from ligands for RTKs is ultimately a balance

between the affects of STATS and suppressors of signalling. This theme also seems to hold for HGF that signal through the members of the HGF receptor superfamily (Begley and Nicola 1999).

There are also common aspects concerning signalling mediated by members of the HGF receptor superfamily. Firstly, ligand binding induces dimerisation of components of the receptor and formation of a ligand-receptor complex (reviewed by Ihle *et al* 1994). Both homo- and heterodimerisation occurs. For example, following binding of IL-6 to the IL-6 receptor α subunit, there is a rapid association of the complex with the β, gp130 subunit (Taga *et al*, 1989). Subsequently, disulphide-linked homodimerisation of gp130 occurs which leads to recruitment of a tyrosine protein kinase (Murakami *et al* 1993). In this case, the IL-6 receptor α subunit is not involved in recruitment of the tyrosine protein kinase. However, the situation is different for the IL-3/GM-CSF/IL-5 growth factor group. In these cases, ligands bind to the respective α subunits and this complex binds to the common β subunit. However, unlike the situation for IL-6, the cytoplasmic domain of α subunits seem to be essential for initiation of a proliferative response (Barry *et al* 1997, Orban *et al* 1999). Thus binding of IL-3, IL-5 and GM-CSF serves to stabilise the heterodimer and bring together the cytoplasmic domains, which recruit tyrosine protein kinases (Jenkins *et al* 1995).

A second common feature of the structure of cytokine receptors is that the cytoplasmic portion of these receptors contains at least 2 functional domains. For example, the membrane proximal domains of the EPO receptor, gp130, the β chains of the IL-2 and IL-3 receptors, c-*mpl* and the G-CSF receptor are essential for mitogenesis (D'Andrea *et al* 1989, 1991, Hatakeyama *et al* 1989, Sakamaki *et al* 1992, Drachman and Kaushansky 1997, Dong *et al* 1993, Fukunaga *et al* 1993). This domain consists of two loosely conserved regions, termed box 1 and box 2 and the mitogenic signals may be initiated by recruitment of common tyrosine protein kinases and activation of a common signalling pathway. In contrast the membrane distal domains of these receptors are not involved with mitogenic signalling but with induction of acute phase proteins (Ziegler *et al* 1993) or as is the case for the G-CSF receptor and c-*mpl*, differentiation (Dong *et al* 1993, Ziegler *et al* 1993, Fukunaga *et al* 1993, Alexander *et al* 1996a). The molecular basis for these different biological responses is not completely known although it seems that association of different signalling molecules with these distinct domains is in part responsible (De Koning *et al* 1998, Alexander *et al* 1996a).

A third common feature of signalling by the cytokine receptor superfamily is the range of signalling pathways used for induction of gene expression (reviewed by Ihle 1996a and Reuter *et al* 2000). At least 2 pathways, including the Ras-raf-MAP kinase and JAK-STAT systems predominate signalling that leads to cell proliferation (figure 1.6). Following binding of HGF there is a rapid induction of tyrosine phosphorylation and in most cases one of the substrates is the cytokine receptor itself. The latter can be accomplished by protein kinases (such as Jak2) that may already be associated with the cytoplasmic tail of the receptor. Following this event, many other adapter phosphoproteins become associated with the receptor. These proteins include Shc, Vav and the p85 subunit of PI3-kinase (Jenkins *et al* 1995, Lanfrancone *et al* 1995). A cascade of binding and phosphoylated proteins is established which eventually leads to activation of the Raf-1 kinase (Muszynski *et al* 1995) and MAP kinase. Subsequent phosphorylation of nuclear transcription factors by MAP kinase leads to enhanced expression of several immediate response genes in haemopoietic cells, including *fos*, *jun*, c-*myc*, c-*myb*, and *erg*-1, and enhanced formation of AP-1 complexes in target genes (Williamson and Boswell 1995, Nicholson *et al* 1995). Alternatively, phosphorylation and activation of Jaks (Jak1, Jak2, Jak3 or Tyk2) leads to phosphorylation of STAT molecules, which consequently dimerise, translocate to the nucleus and bind target genes. For example, G-CSF stimulation results in the activation of Jak1, Jak2, STAT1, STAT3 and STAT5 (Barge *et al* 1996, Dong *et al* 1998, Nicholson *et al* 1994, Tian *et al* 1994, 1996) and TPO stimulation induces rapid tyrosine phosphorylation of Shc, SHIP, Jak2, Tyk2, STAT3 and STAT5 (Drachman and Kaushansky 1997). However, although the Jak-STAT pathway is activated by HGF it does not necessarily mean that this event is responsible for proliferation (Dorsch *et al* 1997).

Overall, there appears to be overlap in the signal transduction pathways activated RTK and members of the HGF receptor superfamily. At present these include the Jak-STAT pathway and signalling through MAP kinase. As suggested by Brizzi *et al* the convergence of separate signalling pathways originating in two distinct receptors to a common signal transduction molecule may then be one mechanism underlying the synergistic actions of HGF on cell proliferation (Brizzi *et al* 1994). For example, a recent study has demonstrated that activation of the MAP kinase isoform ERK2, represents a point at which the two signalling pathways stimulated by IL-3 and SCF, interact synergistically (Pearson *et al* 1998). Although the current study does not investigate the molecular mechanisms underlying synergistic interactions between HGF it does highlight some particularly potent

combinations of HGF that enhance division and proliferation of primitive HPC. Obviously, a molecular analysis of these interactions would be logical extensions of the present study.

1.7 Summary and Aims

The controlled, ongoing production of mature functional blood cells in adults is ultimately the function of multipotent HSC located within the BM that give rise to erythroid, granulocytic, megakaryocytic, monocytic, dendritic, and lymphoid cells. In addition, HSC have the capacity to undergo self-renewal and in doing so give rise to daughter cells with similar if not identical developmental potential. It is generally proposed that during steady state haemopoiesis the majority of HSC are quiescent and do not contribute to haemopoiesis. Thus, the daily production of mature blood cells is attributed to a few HSC recruited from a pool of quiescent HSC. It remains unclear if recruitment of these HSC into the cell cycle is a random event or if it is solely determined by the interplay between a complex array of extrinsic signals mediated through cell adhesion molecules, HGF and their receptors or other soluble regulators. In this regard, the interaction between HSC and the constituents of the BM microenvironment, including stromal cells and their diverse biosynthetic products seem critical to regulating haemopoiesis.

Irrespective of which molecules are involved in regulating HSC quiescence, there are a host of soluble and membrane bound haemopoietic regulators which play a permissive, if not instructive role in subsequent haemopoietic cell differentiation. As discussed above these regulators are pleiotropic and act in concert to stimulate and/or inhibit HSC and their progeny to achieve exquisite control of blood cell levels. Although the exact mechanisms responsible for such precise regulation of steady state haemopoiesis remain to be determined, the *in vitro* affects of many haemopoietic regulators on HPC proliferation and differentiation are well characterised.

The challenge for *ex vivo* manipulation of haemopoiesis is to recreate this complex regulatory network so that a similar control over HSC and HPC survival, proliferation and differentiation can be effected, albeit under *ex vivo* conditions. Although it is clear that stromal cells play a critical role in the *in vivo* regulation of haemopoiesis, it is presently not possible to recreate a fully defined stromal microenvironment *in vitro*, where the responses of populations of HSC and HPC would be controllable or predictable. However, many

haemopoietic regulators provided by stromal cells, and other cells and tissues have been cloned and are available as soluble recombinant molecules.

Thus, the approach for the studies described within this thesis involves growth of purified populations of HPC under stromal-free culture conditions, where HPC development is dependent on provision of soluble recombinant HGF. An underlying hypothesis of these studies is that under such conditions, purified HPC would undergo a similar pattern of myeloid cell development to that observed *in vivo*. In addition, a major focus for this work was to develop culture conditions that could be used for generation of cells for therapy. One critical aspect of *ex vivo* culture of HPC for therapy is safety and reliability of the methodology. In this regard, it was envisaged that culture systems using adherent stromal cell layers would not be reliable, and in particular, not afford the required level of control over HPC proliferation and differentiation. In addition, a culture system should ultimately be based on a media that contains only human sources of serum, proteins, and mitogens and be completely chemically defined. With these criteria in mind a series of studies have been performed to investigate the following:

1. The use of combinations of HGF for *ex vivo* generation of neutrophil precursors from mobilised blood $CD34^+$ cells,
2. The effect of culture conditions on *ex vivo* expansion of $CD34^+$ cells,
3. The characteristics of $CD34^+$ cells that respond to combinations of HGF,
4. The fate of primitive HPC and candidate HSC under *ex vivo* culture conditions.

As indicated previously, in part for logistic reasons, these studies have been performed with a limited range of HGF that may be available for clinical use. Chapters 3 and 4 of this thesis comprise a systematic investigation of the effects of IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF alone and in combination for *ex vivo* expansion of mobilised blood $CD34^+$ cells. Chapter 5 addresses the identity and characteristics of cells within the $CD34^+$ fraction that respond to these HGF and give rise to neutrophil precursors during *ex vivo* culture. In addition, studies on HGF receptor expression by $CD34^+$ cells are presented within chapter 5. The final results chapter addresses the utility of FLT3L and MGDF in *ex vivo* expansion of $CD34^+$ cells with particular emphasis on the proliferation and fate of candidate HSC.

Collectively, the data presented within this thesis indicate that *ex vivo* culture of $CD34^+$ cells using stromal-free cytokine dependent systems can generate sufficient cells for clinical

therapy. In addition, the choice of HGF combinations for *ex vivo* culture is critical in determining the fate of HSC and the type of haemopoietic cells generated.

CHAPTER 2. MATERIALS AND METHODS

2.1 Subjects and Specimens

Peripheral blood (PB) and BM samples were collected from patients or normal adult volunteers as approved by the ethics committee of the Royal Adelaide Hospital and only after patients and donors signed informed consent forms. Before being accepted as suitable for donating BM, blood from normal adult volunteers was subject to the same range of microbiological and virological screening tests to that performed for blood donors at the Australian Red Cross Blood Transfusion Service. Only individuals with negative or normal results were accepted as appropriate BM and PB donors. Samples collected from patients being treated under company-sponsored clinical trials were only used after prior consent was obtained from both supervising medical staff and the sponsoring company.

2.1.1 Collection of Blood and Bone Marrow Samples

Unless otherwise stated, PB samples were collected by venepuncture into sodium heparin (Fisons Pharmaceuticals, Australia, 1,000 I.U./ml) evacuated tubes. Bone marrow was collected from young (less than 40 years of age) normal volunteers by aspiration from the sternum and/or the posterior iliac crest. Routinely, approximately 20 ml of BM was aspirated from each of two sites with syringes previously flushed with sodium heparin. The aspirated BM was transferred into a 50 ml conical centrifuge tube containing 500 units of sodium heparin (Fisons Pharmaceuticals). The BM and PB samples were routinely held at room temperature until processing for isolation of haemopoietic cell fractions, which was typically performed within an hour of collection. Bone marrow samples were also obtained from normal individuals donating marrow for allogeneic transplantation. In these cases the BM sample was a portion of the total BM collection taken in theatre and anticoagulated with sodium heparin.

2.1.2 Processing of Fresh Peripheral Blood and Bone Marrow Samples

Processing of human cells was performed within a class II rated biological safety cabinet (BH2000 series, Clyde Apac, Adelaide SA,) and unless otherwise stated in sterile polypropylene tubes (Falcon, Becton Dickinson Labware, New Jersey, USA). Peripheral blood or BM samples were routinely processed to provide mononuclear cells for immunolabelling or isolation of $CD34^+$ cells. In both cases, the initial processing involved separation of mononuclear cells (MNC) by centrifugation at 400 x g over a Ficoll-Hypaque

(Lymphoprep, 1.077g/dL; Nycomed Pharma, Oslo, Norway) density gradient for 30 minutes at room temperature. To minimise cell clumping and to aid in cell separation the BM samples were often pre-filtered with a 70 μ nylon cell strainer (Falcon, Cat. No. 2350). Mononuclear cells obtained by selecting the interface cells were washed twice by centrifugation at 400 x g (with the brake off to effect removal of platelets) at 4°C in "HHF" (Hanks Balanced Salt Solution, [HBSS; Gibco/BRL] supplemented with 20mM HEPES, pH 7.3 and 5% (v/v) foetal bovine serum), in preparation for immunolabelling or further cell isolation techniques. On some occasions MNC were held overnight in Iscoves Modified Dulbeccos Medium (IMDM) media and 20% FBS/DNase-I at 4°C on a rotating mixer.

2.1.3 Thawing and Preparation of Cryopreserved Cells

Patient and normal volunteer BM cells were cryopreserved as described below (section 2.3.8) in 10% (v/v) dimethyl sulphoxide (DMSO; BDH AnalaR®, BDH Chemicals, Klysth, Victoria, Australia; Cat. No. 10323). Cryopreserved PB or BM MNC were rapidly thawed at 37°C, and slowly diluted in a 10X volume of pre-warmed thaw solution (Ca^{++} Mg^{++} free Hanks Balanced Salt Solution, Gibco BRL, Victoria, Australia, Cat. No. 21250-089; 10mM acid citrate; 2% BSA and 50 kunitz units/ml of DNase-I). The cells were gently washed twice at 200 x g with the brake off during deceleration to effect removal of excess platelets. On occasions, a density gradient separation was performed over Ficoll-Hypaque (Lymphoprep; 1.077g/dl, Nycomed Pharma A/s, Oslo, Norway) to remove dead or aggregated cells as required. Mononuclear cells were harvested from the interface layer, washed twice in thaw solution and resuspended at 2 x 10^7 cells/ml.

2.1.4 Soybean Agglutination of BM MNC

This method, described by Reisner *et al* is based on the ability of the lectin, soybean agglutinin (SBA) to induce agglutination of red cells and leukocytes expressing the SBA receptor (Reisner *et al* 1980). The method enriches haemopoietic progenitor cells (HPC) by virtue of their relative inability to be agglutinated by SBA. Thus, HPC are located in the non-agglutinated fraction or the SBA^- fraction. Collection of SBA^- cells was performed by the following method:

i) Dilute a 25mg vial of SBA (Vector Laboratories, Burlingame, CA, USA) with 2.5mls of DPBS so that it is at a concentration of 10mg/ml.

ii) Wash BM MNC with DPBS / 1%BSA / 5mM HEPES and resuspend cells at concentration of 2 x 10^8 /ml.

iii) Add 140 µl of the 10 mg/ml SBA.

iv) Incubate at room temperature (21-23°C) for 10-20 minutes.

v) Gently layer the cells on top of a solution 5% BSA in IMDM in a 15ml conical bottom polystyrene (clear plastic) centrifuge tube. In doing this, the agglutinated fraction was transferred first so that it fell through the 5% BSA solution before the non-agglutinated fraction.

vi) Incubate at room temperature for 10-20 minutes.

vii) Remove the non-agglutinated fraction from the top of the 5% BSA interface and place into a 15 polypropylene centrifuge tube.

viii) Remove the agglutinated fraction from the bottom of the tube and transfer into a 15ml polypropylene centrifuge tube.

ix) Wash the non-agglutinated fraction (SBA negative: SBA^-) by centrifugation at 400 x g at 4°C, with DPBS / 1% BSA / 0.2M galactose (Sigma, St Louis, USA, Cat. No. G-0750) to remove residual lectin.

x) Wash the SBA^- cells with HBSS / 20 mM HEPES /5% FBS (HHF; pH 7.3) by centrifugation at 200 x g for 10minutes.

This method routinely resulted in a 5-10 fold enrichment of directly clonogenic cells and $CD34^+$ cells and recoveries consistently greater than 80%.

2.1.5 Enrichment of $CD34^+$ Cells by Dynal 561 Immunomagnetic Beads

Isolation of $CD34^+$ cells was performed from either light density PB, BM MNC or SBA^- BM by the use of immunomagnetic capture and a proprietary releasing reagent to provide cells devoid of surface immunoglobulin (Dynal, Oslo, Norway). The methodology was adapted from that recommended by the manufacturer and previous publications (Smeland *et al* 1992, Willems *et al* 1996). It should be noted that the 0.450 µm immunomagnetic beads used for $CD34^+$ cell selection were coated by a 2-layer (mouse anti-CD34 linked to the bead via a rat anti-mouse monoclonal antibody) method with the high affinity anti-CD34 monoclonal antibody 561 (Croockewit *et al* 1998). Previous studies (unpublished observations, David Haylock) confirmed that this bead was far more effective in capturing $CD34^+$ cells than first generation Dynal beads coated with anti-CD34 monoclonal antibodies with lower affinity (eg BI-C35 monoclonal antibody, Tindle *et al* 1985). Release of rosetted $CD34^+$ cells was achieved by incubation with an affinity purified sheep polyclonal serum

(DETACHaBEAD^{TM}reagent) against the Fab region of the 561 antibody. In brief, the following method was followed for isolation of $CD34^+$ cells.

i) Wash BM or PB MNC suspension by centrifugation at 200 x g then resuspend cells thoroughly in HHF at 2-4 x 10^7 cells/ml in the bottom of a 4.5 ml polypropylene tube.

ii) Add 1 Dynal 561 bead for each cell (eg. for 4 x 10^7 cells then 100 µl of beads @ 4 x 10^8 beads/ml)

iii) Keep the cell/bead mixture on ice and mix gently by rotation (not inversion) for 5 minutes to effect bead rosette formation on $CD34^+$ cells.

iv) To enhance capture of $CD34^+$ cells, add 1 ml of IMDM/1% BSA and place tube on rotator in 4°C cold room for 45 minutes.

v) Bring the volume of the cell suspension up to 4 ml by adding IMDM/1% BSA

vi) Place the tube on a magnetic particle collector (MPC) and leave for 2 minutes to effect capture of rosetted $CD34^+$ cells.

vii) Gently aspirate the bead-free supernatant, without disturbing the cell-bead slurry attracted to the magnet.

viii) Remove the tube from the MPC and add 4 ml of ice-cold IMDM/1% BSA.

ix) Mix vigorously by inversion or gentle vortexing. The bead-$CD34^+$ cell rosettes are strong and withstand quite vigorous mixing.

x) Repeat steps vi, vii, viii and ix for another 4 cycles then prior to removing the bead-free supernatant take a 10 µl sample and examine under a phase contrast microscope for evidence of free, non-rosetted cells which contaminate the rosetted $CD34^+$ cells. If the supernatant is cell free then capture and washing is complete. If not, repeat the washing until the supernatant is cell-free.

xi) Remove supernatant with the exception of approximately 100 µl.

xii) Remove the tube from the magnet and add 100 µl of DETACHaBEAD reagent by gentle pipetting down the side of tube.

xiii) Mix gently by rotation, and then float the capped tube in water contained within a 30 mL universal container.

xiv) Transfer to a reciprocating water bath at 37°C so that the tube containing the cell/beads and DETACHaBEAD remains vertical and will mix.

xv) Leave orbiting for 90 minutes at 200 oscillations/minute.

xvi) Bring the volume of the suspension up to 4ml with IMDM/1% BSA and place on the MPC.

xvii) Capture magnetic beads for 2 minutes.

xviii) Remove the released $CD34^+$ cells from the tube.

xix) Repeat the capture of beads and harvest of $CD34^+$ cells twice more with 4ml of IMDM on each occasion.

xx) Wash the enriched $CD34^+$ cells thrice with IMDM/5% FBS. It is essential that this be performed before immunolabelling with CD34 antibodies such as 8G12 (HPCA-2, Becton Dickinson, San Jose, CA) as any residual DETACHaBEAD reagent will block binding of these antibodies.

xxi) Cells are ready for immunolabelling or functional assays.

The $CD34^+$ cell purity with this method was routinely between 90-95% and resulted in 40-75% recovery and >95% cell viability.

2.1.6 Isolation of $CD34^+$ cells on the CEPRATE™ Device

Isolation of $CD34^+$ cells from apheresis products was performed with the CEPRATE™ device according to the manufacturer's instructions (CellPro, Bothell Washington, USA). In brief, the apheresis collection was washed with phenol red free RPMI-1640 (supplied by CellPro) in a COBE 2991, concentrated to 100 ml in RMPI-1640 with 0.1% human serum albumin (HSA) then incubated for 25 minutes at room temperature with biotinylated anti-CD34 monoclonal antibody (12.8, Berenson *et al* 1988). The cells were washed twice with RMPI-1640 to remove unbound antibody, the volume made up to 300 ml then processed on the CEPRATE™ device. Isolation of $CD34^+$ cells is achieved by binding of biotinylated 12.8 labelled cells to avidin coated beads. $CD34^-$ cells do not adsorb to the avidin column whereas the $CD34^+$ cells are adsorbed and are released by gentle agitation. The entire process is closed and computer controlled and results in approximately 75 ml of a $CD34^+$ cell enriched fraction. The proportion of $CD34^+$ cells in the initial apheresis collection, the adsorbed and unadsorbed fractions were determined by immunolabelling and flow cytometry. Isolation of $CD34^+$ cells with the CEPRATE™ device was performed on 5 apheresis collections and resulted in 54-105% recovery of $CD34^+$ cells with a purity ranging between 65-87%.

2.1.7 Lineage Depletion using Immunoadherence and Immunomagnetic Beads

PBMNC were incubated with a mixture of monoclonal antibodies to T-cells and monocytes (CD3, CD11b and CD14) for 40 minutes at 4°C. After 2 washes cells were added at 4×10^6 ml to a T175 tissue culture flask (Falcon, Becton Dickinson, New Jersey, USA) previously coated

with goat anti-mouse immunoglobulin (Caltag, San Francisco, California) and incubated for 60 minutes at 4°C. Unbound cells were removed and mixed with sheep anti-mouse coated Dynal M450 immunobeads (Dynabeads, Dynal, Oslo, Norway) at a 2:1 bead to cell ratio for a further 60 minutes by continuous rotation at 4°C. Mature cells bound to beads were removed using the Dynal magnetic particle concentrator. The remaining cells were washed and then resuspended at 2 x 10^7/ ml for immunolabelling and FACS.

2.2 Preparation of Buffers, Media and Media Additives

2.2.1 General Considerations

All water used to prepare non-tissue culture solutions was deionised and filtered using the Milli Q RO60 system (Millipore Corporation, USA). Tissue culture solutions were made with Milli-Q water prepared by further purification through two ion exchange resin beds, a carbon filter and an organic filter using the Milli-GF^{PLUS} system. Prepared solutions and buffers were sterilised by autoclaving under 2 atmospheres of pressure for 30 minutes or by using filter sterilisation through 0.22 µm pore size filters (Sterivex GS systems; Millipore Corporation, USA, Cat. No. SVGB 1010, bottle filters; Corning, USA, Cat. No. 25111, Spin-X filter; Costar, MA, USA, Cat. No. 8160). Following preparation and sterilisation, all solutions were stored at 4°C, -20°C or -80°C as indicated.

2.2.2 Hanks Balanced Salt Solution (HBSS)

Single strength (iso-osmolar; 270-300 mOsm), Ca^{++} and Mg^{++} free, Hanks balanced salt solution, pH 7.4 consisted of 0.14 M NaCl (Ajax Chemicals, Australia, Cat. No. 465), 5 mM KCl (Ajax, Cat. No. 383), 0.3 mM $Na_2HPO_4.12H_2O$ (BDH, Australia, Cat. No. 10248), 0.4 mM KH_2PO4 (BDH, Cat. No. 10203), 4.2 mM $NaHCO_3$ (BDH, Cat. No. 10247), 5.5 mM glucose (BDH, Cat. No. 10117), 1% phenol red (M&B, UK, Cat. No. P152/18/61, in 0.1 M NaOH) dissolved in Milli-Q water and subsequently autoclaved at 130°C for 20 minutes or filter sterilised as described above. Alternatively, powdered Ca^{++} and Mg^{++} free HBSS (Gibco/BRL, Cat. No. 21250-089) was reconstituted with 1 litre of Milli-Q water and 4.2 mM $NaHCO_3$, filtered then stored at 4°C.

2.2.3 Dulbecco's Phosphate Buffered Saline (DPBS)

DPBS was comprised of 0.14 M NaCl, 3 mM KCl, 8 mM $Na_2HPO_4.12H_2O$ and 1 mM KH_2PO4 dissolved in Milli-Q water. The pH was adjusted to 7.4 with 1 M HCl prior to sterilisation by autoclaving.

2.2.4 L-Glutamine

A 200 mM (100X) stock solution of L-glutamine (BDH, Cat. No. 37107) was prepared by dissolving 4gm of L-glutamine in 100 mL of water followed by incubation at 37°C to facilitate dissolution. The stock solution was aliquoted as 5 ml lots and stored at –20°C.

2.2.5 Sodium Pyruvate

Stock solutions of 100 mM sodium pyruvate (100X) were prepared by dissolving 1.1 gm of sodium pyruvate powder (Gibco/BRL, Cat. No. 890-1840) in 100 ml Milli-Q water, filter sterilised then stored at 4°C.

2.2.6 Penicillin –Streptomycin

20 ml of Milli-Q water was added to a vial of penicillin-streptomycin sulphate (Gibco/BRL, Cat. No. 600-5145AE, 20X) to give final concentrations of 500 I.U./ml and 500 mg/ml respectively, filter sterilised and stored at -20°C.

2.2.7 Insulin

A 1mg/ml solution was prepared by adding 2.7 ml of Actrapid HM (Neutral Insulin Injection; Novo Nordisk 100 units/ml = 3.7 mg/ml) to 7.3 ml of IMDM, filter sterilised and stored as 1 ml aliquots at –20°C.

2.2.8 Transferrin

A stock solution of 20 mg/ml of iron saturated human transferrin was prepared by adding 500 mg of transferrin (Sigma, St Louis, MO, USA, Cat. No T2158) to 25 ml of IMDM, filter sterilise and aliquot in 500 µl lots and store at –20°C.

2.2.9 2-betamercaptoethanol (2ME)

A 1 M stock solution of 2ME was prepared by diluting 0.7 ml of 14.7 M β-mercaptoethanol (Sigma, Cat. No. M-3148) into 9.3 ml HBSS and stored at –20°C. The 1 M stock solution was further diluted 1:10 (in HBSS) to prepare a 0.1 M working solution, filter sterilised and stored as 500 µl aliquots at –20°C.

2.2.10 Low Density Lipoprotein (LDL)

Low density lipoprotein from human plasma was prepared by Mr Neil Hime of the Lipid Laboratory, Hanson Centre for Cancer Research according to the following method.

i) Measure out 400-800 ml of plasma from blood bags (anticoagulated in either citrate-dextrose-phosphate or acid-citrate-dextrose) using a manual plasma extraction device.

ii) Adjust the density to 1.019 g/ml (do this by weighing 1 or 2 mls) with solid potassium bromide (KBr). Use a formula of no. of mls x 0.01839 = grams of KBr to add.

iii) Confirm the density by weighing the plasma.

iv) Centrifuge the plasma in a 55.2Ti rotor of a Beckman Ultracentrifuge at 55,000 rpm (55K) for 16 hours at 4°C. Use the specialised plastic Beckman centrifuge tubes for this.

v) Collect the bottom fractions, pool and adjust the density (by weight) to 1.055 g/ml

vi) Centrifuge in 55.2Ti rotor at 55K for 16 hours at 4°C.

vii) Collect the top fraction (approximately 10 ml), pool and dialyse against 1.055 g/ml density solution (water with KBr) for 2-3 hours.

viii) Centrifuge in 55.2Ti rotor at 55K for 16 hours.

ix) Collect the top fraction, pool and dialyse against 3 x 1 litres Tris buffered saline.

x) Store under N_2 in foil covered container.

xi) Measure the protein concentration by the Lowry method. The LDL fraction is normally around 6-10 mg/ml and is typically 8.8 mg/ml.

Following preparation, the ability of LDL to support growth of haemopoietic progenitor cells in Pre-CFU cultures was tested. A series of SDM were prepared with increasing amounts of LDL, up to a final concentration of 80ug/ml. FACS isolated $CD34^+$ cells were used as test cells and the effect of LDL concentration on cell and CFU-GM generation was investigated. During the course of this thesis 3 batches of LDL were used, and the LDL concentration within SDM ranged from 21-24 µg/ml.

2.2.11 Bovine Serum Albumin (BSA)

Batches of BSA were rigorously tested in both CFU-GM assays and Pre-CFU culture to identify material with the best growth promoting activity. During the course of the present studies, a single large batch of Sigma BSA (Batch No. A-2153) was used. The BSA powder was held in an airtight container at 4°C. A 10% (w/v) BSA solution was prepared by overlaying 20 gm of BSA to 88.4 ml of Milli-Q water in a small beaker, and refrigerating

overnight to facilitate complete dissolution. Once dissolved, 3 gm of Duolite mixed bed resin beads MB6113 (BDH, Cat. No. 55057) were added to remove trace elements and contaminants that could potentially inhibit cell growth in cultures. The suspension was refrigerated for 2 hours, with agitation every 15-20 minutes. After the bead colour changed from blue-green to yellow the solution was decanted and filtered through a Whatman No. 1 filter paper. The bead de-ionisation procedure was repeated until the beads did not turn yellow after 2 hours. The volume of BSA was measured and an equal volume of IMDM x 2 was added. The solution was slowly filtered through a 0.22μm filter and stored in 50 ml aliquots at –20°C.

2.2.12 Foetal Bovine Serum (FBS)

The FBS used during the course of these studies was selected after rigorous testing in various bioassays including haemopoietic clonogenic assays, Pre-CFU culture and hybridoma cultures. Three separate batches (PA Biologicals, Sydney, NSW Cat. No. 3000 lot No. 80693; MultiSer™, Trace Bioscience, Cat. No 15-010-0500V, batch No. 7052346; Gibco Life Technologies Cat. No. 200-6140PJ, batch 583) were provided in 500 ml bottles and stored at –20°C. The FBS was thawed, aliquoted into 50 ml conical centrifuge tubes and held at 4°C for routine daily use.

2.2.13 Deoxyribonuclease-I (DNase)

A DNase-I stock solution was prepared by reconstituting one vial of DNase-I (Sigma, Type IV, Cat. No. 61362, 3.37 x 10^5 Kunitz units), with 2 ml of 0.15 M NaCl, to give a final concentration of 1.875 x 10^5 Kunitz units/ml. The DNase-I solution was aliquoted into cryovials and stored at –80°C. DNase-I was routinely added to media to give a final effective concentration of 50 Kunitz units/ml.

2.2.14 Medium for Thawing Cryopreserved Cells

To 350 ml of Ca^{++}, Mg^{++} -free HBSS, 50 ml of acid citrate, 100 ml of 10% BSA (in HBSS), and 2 ampoules of DNase-I were added to give a final concentration of 10 mM, 2% and 50 kunitz units/ml respectively. DNase-1 was added to medium immediately prior to use.

2.2.15 Heat Inactivated Foetal Bovine Serum

Heat inactivation of FBS (various batches, refer to section 2.2.12) was achieved by incubating FBS at 56°C for 30 minutes in a water bath. Heat inactivation was performed to ensure inactivation of endogenous bovine complement factors.

2.2.16 Double-Strength Iscove's Modified Dulbecco's Medium (2 x IMDM)

Solutions of 2 x IMDM (Cytosystems, Castle Hill, NSW, Australia, Cat. No. 50-016-PA), were prepared according to the following protocol:

i) measure out 500 ml of Milli-Q water in a 2 litre beaker
ii) add 2 sachets of IMDM powder (1 litre sachets)
iii) rinse out sachets with a further 490 ml of Milli-Q water
iv) add 10 mls of penicillin/streptomycin solution (10,000 units of penicillin/ml and 10,000 µg/ml)
v) add 6.048 gm of $NaHCO_3$ (BDH, AnalaR, Cat. No. 10247.4V)
vi) add 0.4 gm L-glutamine (Gibco Cat. No. 21051-016)
vii) add 0.2 L-asparagine (Sigma Cat. No. A0884)
viii) mix by magnetic flea until all solids are dissolved
ix) adjust pH to 7.3
x) filter sterilise with a bottle filter (Costar Cat. No. 8380)
xi) perform a sterility check by placing 1 ml of medium into a 10 ml tryptone soy broth (MEDVET, Adelaide) and incubating for 5 days at 37°C.
xii) freeze in 50 mL conical polypropylene tubes at –20°C

2.2.17 Single Strength Iscove's Modified Dulbecco's Medium (IMDM)

Single strength IMDM was prepared by a 1:1 (v/v) dilution of 2 x IMDM in Milli-Q water. Prior to use the medium was supplemented with 2 mM L-glutamine (BDH, Cat. No. 37107). Alternatively, one sachet of IMDM powder (Gibco/BRL, Cat. No. 12200-036 or Cytosystems, Castle Hill, NSW, Australia Cat. No. 50-016-PA), 2 g $NaHCO_3$ and 2.5 ml 1 M HCL were dissolved in 1 litre of Milli-Q water. Sterile solutions of HEPES, streptomycin sulphate and penicillin were added to give final concentrations of 15 mM, 100 I.U./ml and 100 µg/ml, respectively. The solution was adjusted to a pH of 7.4 and the medium filter-sterilised with a sterivex GS 0.22µm filter. Prior to use the medium was supplemented with 2mM L-glutamine and FBS as required. Medium was replenished with 2 mM L-glutamine at weekly intervals.

2.2.18 Serum Deprived Medium (SDM)

Small batches (10-20 ml) of SDM were prepared freshly for all Pre-CFU cultures. Once prepared, SDM was held at 4°C and used within 1 week. The recipe for preparation of a 10 ml batch of SDM is as follows:

Component	Stock Concentration	Volume for 10 mL of SDM
BSA/IMDM	10% (w/v)	1 ml
Transferrin	20 mg/ml	100 µl
Insulin	1 mg/ml	100 µl
2ME	0.1 M	5 µl
LDL	8.8 mg/ml	24 µl
IMDM	x 1	8.8 ml

Each batch of SDM was sterile filtered through a 0.22 µm pore filter.

2.2.19 Delta Medium

This medium contains both FBS and BSA and was used for the studies described within Chapters 3, 4 and 5. A 300 ml batch of delta medium was prepared as follows:

Component	Volume
FCS	90 ml
10% BSA	30 ml
200 mM L-glutamine	3 ml
0.1 M 2ME	150 µl
IMDM	180 ml

Each batch was sterile filtered and stored at –20°C in 10 ml or 50 ml aliquots.

2.2.20 Alpha Modification of Eagles Medium (α-MEM)

To prepare 1 litre of single strength α-MEM, 10 gm of α-MEM (with glutamine, without sodium bicarbonate; Flow Laboratories, Australia, Cat. No. 10-311-26) was dissolved in 990 ml of Milli-Q water, supplemented with penicillin (100 I.U./ml), streptomycin (100 µg/ml) and buffered with 2 gm of sodium bicarbonate. The medium was filter sterilised and stored at 4°C.

2.2.21 Rosewell Park Memorial Institute (RMPI-1640) Medium

To prepare RMPI-1640, one sachet of RMPI-1640 powder (Life Technologies, Glen Waverley, Victoria, Australia, Cat. No. 31800-022), 2 g $NaHCO_3$ and 2.5 ml 1 M HCL was dissolved in 1 litre of Milli-Q water. Sterile solutions of N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES, pH 7.2; Boehringer-Mannheim, Australia, Cat. No. 737151), penicillin (Sigma, St Louis, MO, USA, Cat. No. P3032) and streptomycin sulphate (Sigma, Cat. No. S 9137) were added to give final concentrations of 15 mM, 100 I.U./ml and 100 µg/ml, respectively. A pH of 7.4 was attained and the medium was subsequently filter sterilised using a sterivex GS 0.22µm filter. Prior to use, the medium was replenished with 2mM L-glutamine and FBS as required.

2.2.22 IMDM/1% BSA

To prepare 1% BSA in IMDM (v/v), 10 ml of 10% BSA in IMDM was added to 90 ml of IMDM. The solution was filter sterilised and stored at 4°C.

2.2.23 Immunofluorescence Buffer (IF Buffer)

Cells labelled for flow cytometric analysis were washed with ice-cold IF buffer prepared by adding 10 ml of heat inactivated FBS and 0.2 g of sodium azide to 500 ml of HBSS. The IF buffer was sterile filtered and stored at 4°C.

2.2.24 Blocking Buffer for Flowcytometry Analysis

To prepare blocking buffer 20 ml of normal human serum, 20 ml of 10% BSA, 25 ml of FBS, and 5 ml of penicillin/streptomycin was added to 500 ml of HBSS. The normal human serum (NHS) was obtained as 250 ml frozen material from the Transfusion Department of the Royal Adelaide Hospital. Prior to use in the blocking buffer the NHS was thawed, centrifuged at 400 x g for 15 minutes then heat inactivated at 56°C for 30 minutes.

2.2.25 Flowcytometry Fixative (FACS FIX)

To prepare FACS FIX, add 10 ml of formalin (40% Formaldehyde solution, BDH Cat. No. 10113.60), 20 gm glucose (BDH Analar®, Cat. No. 10117) to 1 litre of Dulbecco's Phosphate Buffered Saline. The solution was subject to 0.22 µm filtration and stored at 4°C in 50 ml aliquots. If flow cytometric analysis of immunolabelled cells could not be performed on the same day as labelling, samples were resuspended in 300-400 µl of cold FACS FIX and held at 4°C until flow cytometric analysis was performed, which was always within 7 days of labelling.

2.2.26 Methylcellulose

To prepare methylcellulose, 8.1 gm of methylcellulose powder (A4M premium grade 4000 centipose, Dow Chemicals, Lake Jackson, Texas, USA) was added to a 500 ml Schott bottle, and autoclaved at 15 psi (121°C) for 15 minutes. To this, 270 ml of sterile 1 x IMDM was added, mixed vigorously and left stirring for 2 days with occasional shaking to assist mixing. When the medium was homogenous, 180 ml sterile FBS and 60 ml sterile BSA was added and the mixture allowed to sit for a further 3 hours. The methylcellulose was then stored at –20°C as 25-40 ml aliquots.

2.2.27 Cytokines and Haemopoietic Growth Factors

Haemopoietic growth factors and cytokines used for this study were either generously provided by biotechnology companies or purchased from commercial suppliers as indicated in the following table.

Name	Source	Stock Conc.	Working Conc.
IL-1β	AMGEN	0.8 mg/ml	1 µg/ml
IL-3	AMGEN	1.0 mg/ml	1 µg/ml
IL-6	AMGEN	2.2 mg/ml	1 µg/ml
G-CSF	AMGEN	0.5 mg/ml	10 µg/ml
GM-CSF	AMGEN	0.5 mg/ml	1 µg/ml
SCF	AMGEN	2.0 mg/ml	10 µg/ml
FLT3L	IMMUNEX	9.59 mg/ml	10 µg/ml
TPO (MDGF)	AMGEN	2.0 mg/ml	10 µg/ml
EPO	Jansenn Cilag	2,000 iu/ml	25 iu/ml

Lot numbers of HGF used in the present studies.
IL-1: unknown, IL-3: 1801K3, IL-6: 1901K3, G-CSF: 06166J8,
GM-CSF: 89819/2, MDGF: 11284 M4, FLT3L: 5479-071
Recombinant methylated human SCF: 03181D1

Erythropoietin (2,000 units/ml) was purchased as Eprex from Janssen-Cilag, Switzerland. A working solution of EPO at 160 units/ml was prepared by adding 1.0 mL of Eprex and 1.25 ml of 10% BSA in IMDM to 10.25 ml of IMDM. A volume of 25 µl of the 160 unit/ml

working solution was added to each dish (4 units of EPO/dish) to stimulate growth of erythroid progenitors. Stock solutions of the remaining HGF were provided in liquid formulation and stored at 4°C. Working solutions were routinely prepared by adding the calculated volume of stock HGF to 1% BSA in IMDM (v/v) to give the working concentrations shown. Routinely, only 5 ml of the working solutions were prepared, sterile filtered, aliquoted into external thread cryovials (Nalge Company, Rochester NY, Cat. No. 5000-0020) and stored at 4°C.

2.2.28 Fixative for Specific and Non-Specific Esterase Staining and Immuno-Histochemical Staining for P-Selectin

A solution of buffered formal acetone was prepared by adding 100 mg of Na_2HPO_4 and 500 mg of KH_2PO_4 to 150 ml of Milli-Q water then after their dissolution, addition of 225 ml of acetone and 125 ml of formalin (40% Formaldehyde, BDH Cat. No. 10113.60). The fixative solution was stored at 4°C.

2.2.29 Pararosanalin Solution

Dissolve 4 grams of pararosanalin hydrochloride (Sigma, St Louis, USA; Cat. No. P3750) in a mixture of 80 ml of Milli-Q water and 20 ml concentrated HCl in a conical flask. Place in a 56°C water bath for 30 minutes to aid in dissolution, cool then filter with a Whatman No. 1 paper. The solution was stored at room temperature.

2.2.30 0.07M Phosphate buffer, pH 7.4

This buffer was used for specific and non-specific esterase stains. Dissolve 0.87 gm of KH_2PO_4 and 3.84 gm of Na_2HPO_4 in 500 ml of Milli-Q water. Check and adjust the pH to 7.4, store at room temperature.

2.2.31 .07M Phosphate buffer, pH 6.3

Dissolve 3.5 gm KH_2PO_4 and 1.1 gm of Na_2HPO_4 in 500 ml of Milli-Q water. Check and adjust pH to 6.3, store at room temperature.

2.3 Cell Culture

2.3.1 General Considerations

All manipulations with cells were performed within class II biological safety cabinets to ensure sterility of cells and operator safety. In addition, samples were always contained

within capped tubes or flasks. All manipulations involving transfer or sampling of cells, addition of medium or additives were made with sterile individually wrapped pipettes or Gilson Pipettes (Gilson P1000, P200, P100, P20; Adelab, Adelaide, SA) fitted with sterile pipette tips. Adjustable Gilson Pipettes were tested for accuracy and reproducibility as part of the laboratory quality control programme. Unless otherwise stated, cell incubations, clonogenic assays and Pre-CFU cultures were incubated at 37°C in a 5% CO_2 incubator (Forma Stericult 200 CO_2 incubator, model No. 3035, Forma Scientific Instruments, USA) with a 97% relative humidity.

2.3.2 Clonogenic Assays

Day 14 CFU-GM and BFU-E assays were performed as described previously (Haylock *et al* 1992, Simmons *et al* 1990). Briefly, triplicate 1ml cultures were established in sterile, 35mm polycarbonate tissue culture grade petri dishes in 0.9% methylcellulose in IMDM supplemented with 30% FCS and 2mM L-glutamine. Unless otherwise stated 500 $CD34^+$ cells were plated for input clonogenic assays whereas cells generated in *ex vivo* culture were cultured at 5,000-10,000 cells/plate, depending on the age of the culture. In older cultures where less CFU-GM were anticipated then 10,000 nucleated cells were routinely cultured. For those experiments described in Chapter 3 the clonogenic assays were stimulated by 50 µl of 5637-conditioned medium, 1 ng IL-3 and 4 units of erythropoietin per plate. In subsequent studies, stimulation for clonogenic assays was provided by the combination IL-3, IL-6, G-CSF, GM-CSF, SCF (all used at 10 ng/ml) and EPO (4 units/ml). After 14 days incubation at 37°C in 5% CO_2, CFU-GM and BFU-E were scored using a dissecting microscope and standard criteria for their identification (Fauser and Messner 1979). Aggregates of greater than 40 translucent cells were scored as CFU-GM colonies and large uni-centric or multicentric aggregates of red cells were scored as BFU-E. The number of CFU-GM and BFU-E generated in Pre-CFU cultures was determined from 3 plates from each replicate Pre-CFU culture. The number of CFU-GM and BFU-E present in a culture was calculated by multiplying their incidence (CFU-GM or BFU-E / number of cells per plate) by the total number of nucleated cells in the culture.

2.3.3 Delta or Pre-Progenitor Cell Culture (Pre-CFU Culture)

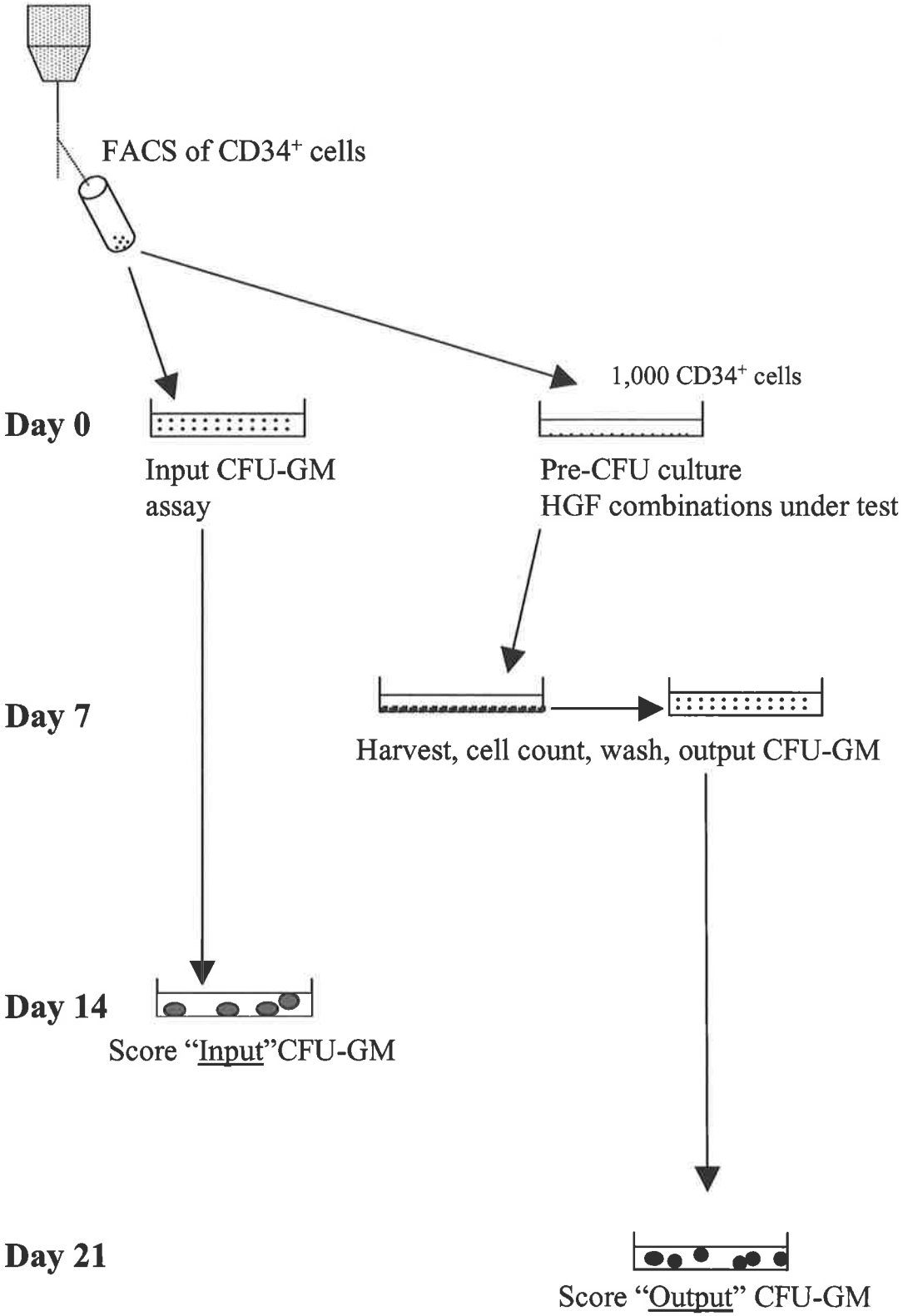
The Pre-CFU culture system is an adaptation of the 4-day suspension culture system described by Iscove *et al* and measures the generation of nascent CFU-GM as an index of precursors to CFU-GM (Iscove *et al* 1989). The system comprises culture of test cells in liquid media where growth is stimulated solely by provision of exogenous HGF. The culture

system used in these studies did not contain stromal cells. Thus, the culture is a cytokine dependent stromal-free system. Two media formulations were used for the cultures performed during the course of this thesis. The first contained 30% FBS and 1% BSA and was used for the studies reported in Chapters 3, 4 and 5. During the course of the study, it was realised that the presence of FBS in the medium was a concern. Firstly, FBS is an undefined material, containing both potential inhibitors of haemopoietic cell growth as well as a host of mitogens that could potentially interact with purified recombinant HGF being tested to stimulate growth. As such, the complex and variable nature of FBS presented a major concern for investigations on the affects of purified recombinant HGF on pure populations of HPC. A culture system containing FBS is not chemically defined and may not provide reproducible results. In addition, as one of the objectives for the study was to develop culture systems that could be applied clinically then FBS presented a problem. Patients transplanted with cells grown in the presence of FBS are at risk of allergic reactions to trace amounts of FBS but more importantly are a risk of viral and prion infections transmitted by FBS. Therefore a serum-deprived culture media devised by Dr Paul Simmons (Hanson Centre for Cancer Research, Adelaide) was used for studies described in chapter 6. The formulation for SDM was based on a medium used for liquid culture of primitive human haemopoietic cells (Lansdorp and Dragowska 1992).

Unless otherwise stated a "standard" Pre-CFU culture was initiated with 1,000 target cells, typically $CD34^+$ cells or subsets thereof, in 1.0ml of medium supplemented with single or combinations of HGF each at 10 ng/ml (illustrated in figure 2.1). These cultures were routinely prepared in triplicates or replicates of 6 in a 24 well tissue culture plate (Nunclon, Denmark Cat. No. 43982). Under these conditions, cells resided at the bottom of the well in contact with the plastic tissue culture plate. Growth of cells was monitored using an inverted microscope. Experiments were designed to test the growth of different cell populations or the affects of different combinations of HGF on the growth of the same target cells. Initial experiments confirmed that growth of HPC under these conditions was absolutely dependent on provision of HGF. In control cultures without HGF some cells remained viable after 7 or 14 days but the majority of HPC showed typical features of apoptosis within 3-7 days. Pre-CFU "assays" were routinely analysed after every 7 days of culture. Typically, this involved removal of the contents of a well, counting the number of viable cells, a clonogenic assay, immunophenotyping and occasionally preparation of a cytospin for staining and morphological examination.

Figure 2.1 Isolation and Pre-CFU culture of $CD34^+$ cells isolated by FACS

Immunolabelled bone marrow or peripheral blood cells were isolated following fluorescent activated cell sorting on the $FACStar^{PLUS}$. The initial number of CFU-GM within the isolated $CD34^+$ cell fraction is determined in a methylcellulose based clonogenic assay. Pre-CFU culture is performed in wells of 24 well plates where routinely 1,000 $CD34^+$ cells are cultured under stromal-free conditions with medium supplemented with combinations of haemopoietic growth factors. Pre-CFU cultures can be harvested after any time point to determine how many cells and nascent CFU-GM have been generated under the test conditions.



CFU-Delta value = Output CFU-GM/Input CFU/GM

In most experiments a triplicate of wells cultured under identical conditions were harvested and analysed. The mean and SEM of this data is shown in most data sets and figures. When cultures were extended beyond 7 days then additional HGF were added to sustain cell growth for the next 7 days. In some cases, especially when combinations of HGF were used, the cultures required "splitting" or dilution at day 14 (and later time points) with fresh medium and HGF to prevent cell death by consumption of HGF or production of toxic metabolites. For example a culture initiated with 1,000 $CD34^+$ cells stimulated by a combination of IL-3+IL-6+G-CSF+SCF+MGDF could generate 1 x 10^6 nucleated cells by day 14. These cultures would then be subject to a 1:10 split, with 100,000 cells being placed into a new tissue culture well with fresh medium and HGF. At day 21 the number of cells present in this well was multiplied by a factor of 10 to calculate the actual number of cells that could be generated by the initial 1,000 $CD34^+$ cells. Similar mathematics were used for cultures followed for longer periods; the actual number of cells generated was calculated by taking into account the cumulative culture dilution. Often an input clonogenic assay was included in these experiments to compare the number of nascent CFU-GM or BFU-E generated to that present at the beginning of culture.

The "delta" value represents the number of clonogenic cells detected after a period in liquid culture as a function of the input number of clonogenic cells (Moore 1990). The typical data collected from Pre-CFU cultures included the fold-increase of nucleated cells or CFU-GM, both being calculated from the actual number of cells or CFU-GM present at specific times divided by the original number of cells or CFU-GM. The following formula was also used.

F-I Cellsd7 = Number of cells at day 7/initial number of cells.

Where F-I Cellsd7 represents the fold increase of cells after 7 days of culture.

2.3.4 Pre-CFU Cultures in Terasaki Wells

Single HPC deposited by the automated cell deposition cell unit (ACDU) of the FACStar^{PLUS} were cultured in wells of Terasaki plates (Nunclon, Denmark, Cat. No. 63118) containing 10 µl of SDM supplemented with HGF. Within 6 hours after deposition, wells were examined under an inverted microscope to confirm that a single viable cell had been deposited. According to the experimental design, the growth of single cells was monitored microscopically and in some cases involved daily examination and counting of progeny from dividing cells. In some experiments, the contents of individual wells were removed and the

number of cells counted using a haemocytometer. This was performed by transferring the 10 µl from wells into 90 µl of IMDM and flushing the Terasaki well to ensure transfer of as many cells as possible. An aliquot of this diluted cell suspension was directly transferred to the haemocytometer chamber, a count performed and the number of cells in the original 10 µl culture calculated.

Experiments were also performed in Terasaki wells to assess the ability of individual HGF to maintain survival of $CD34^+CD38^-$ cells isolated from adult human BM. In these experiments, single cells were deposited into 10 µl of SDM supplemented with single HGF or a combination of 6 HGF, as a control, then left for 14 days. At the end of this time the wells were examined to determine how many single cells had divided in the presence of single HGF and also which cells appeared to be viable by morphological criteria (single, phase bright cells with intact cytoplasmic membranes and no evidence of nuclear pyknosis). Half the volume of the medium in all wells was removed without dislodging cells, and replaced with 5 µl of fresh SDM containing a combination of IL-3, IL-6, G-CSF, SCF, FLT3L and MGDF each at double the normal cytokine concentration. The wells were incubated for a further 7 days then examined by inverted microscopy to determine the proportion of cells that had divided and thus survived the original 14 day culture.

2.3.5 "Large Scale" Cultures of Mobilised Blood $CD34^+$ Cells

Cultures of mobilised blood $CD34^+$ cells, referred to in Chapter 3 (section 3.3.3) were initiated in Pre-CFU medium (containing 30% FBS and 1% BSA) in either tissue T75 culture flasks (Corning Costar Corp., Cambridge MA, USA; Cat. No. 430641) or in a 500ml PL269 Life Cell Bag (Fenwal, Travenol Laboratories, Deerfield, USA). In each culture, the starting $CD34^+$ cell concentration was approximately 4-5 x 10^3/ml. Recombinant human IL-1β, IL-3, IL-6, GM-CSF, G-CSF and SCF were added to Pre-CFU medium to give a final concentration of 10 ng/ml of each HGF. Large volume cultures were incubated at 37°C in 5% CO_2 for up to 21 days. At day 7 an additional 10ng/ml of each of the 6 HGF was added to each 50ml culture. On day 14, 25mls from the flask and bag cultures were removed for assessment of cell function. A further 75 ml of culture medium was added to both cultures but additional HGF was only added to the 500ml bag culture.

2.3.6 Exposure of Cells to 4-hydroxyperoxycyclophosphamide (4-HC)

Bone marrow MNC were treated with the cyclophosphamide derivative 4-HC as described by Ottmann *et al* (Ottmann *et al* 1991). The MNC were washed once and resuspended at a

final viable concentration of 1 x 10^7 cells/ml, in IMDM supplemented with 10% FBS. To two aliquots of this cell suspension were added 4-HC to give a final concentration of 120 µg/ml or a like volume of medium as a control. Both treatment and control tubes were incubated in a 37°C water bath for 30 minutes and agitated every 5 minutes. At the end of this period, the suspension in each tube was rapidly doubled in volume by addition of IMDM/10% FBS and then underlaid with a FBS "cushion". Tubes were centrifuged at 400 x g for 5 minutes at 4°C and washed three times to ensure removal of residual 4-HC. Cells within each treatment and control tube were resuspended in IMDM with 20% FBS and 50 Kunitz units of DNase-I, and held on ice until use.

2.3.7 Exposure of cells to 5-Fluorouracil (5-FU)

Bone marrow MNC were exposed to the cell cycle specific drug 5-FU for 24 hours to effect killing of dividing cells (Rice *et al* 1993). In brief, MNC were washed then resuspended at 1 x 10^7 cells/ml in IMDM/10% FBS. A volume of 5-FU (David Bull Laboratories, 500 mg in 10 ml injection. Cat. No. 2595A) was added so that an effective concentration of 25 µg/ml was achieved. The cell suspension was incubated at 37°C for 24 hours after which time, both the control cells (without 5-FU) and test cells were washed by centrifugation with IMDM/10% FBS at 200 x g 5 minutes. Three further washes were performed to remove residual 5-FU. The cells were finally resuspended in either SDM or IMDM/20% FBS for Pre-CFU culture or clonogenic assays.

2.3.8 Cryopreservation of Apheresis Samples

The method for cryopreservation of patient material has been published previously (To *et al* 1994). In brief, MNC were enriched from patient apheresis collections by density gradient separation over a Ficoll-Hypaque density gradient (Lymphoprep) then washed thrice in HBSS at 400 x g without braking to reduce platelet contamination of the cell suspension. The resultant MNC were resuspended in IMDM/20% FBS at 2-4 x 10^7 cells/ml then chilled on ice prior to cryopreservation. An equal volume of freeze solution comprising of 20% DMSO and 20% FBS in IMDM was prepared and held on ice until slowly added to the cell suspension. The cells in cryoprotective solution were aliquoted into 2 ml cryovials (Nalgene cryovials, Nalge Company Rochester, NY, USA; Cat. No. 5000-0020), held on ice then transferred into a rate controlled freezer (Kryo 10-16, Planer Biomed, Sunbury on Thames, England). The cell suspension was frozen at –1°C / minute and at –80°C transferred into the liquid phase of liquid nitrogen for storage.

2.3.9 Superoxide Release by Chemiluminescence

Studies to test the function of *ex vivo* generated neutrophil precursors were performed in collaboration with Dr Stephen Hardy, Department of Chemical Pathology, Adelaide Women's and Children's Hospital, Adelaide. Cells from flask and bag cultures were assessed for their ability to release superoxide anion when exposed to the agonists arachidonic acid (33 μM), N-formyl-methionyl-luecyl-phenylalanine (fMLP: 5 x 10^{-6} M or 12-0-tetradecanoylphorbol-13-acetate (TPA: 1 x 10^{-7} M). Superoxide release was measured by monitoring the chemiluminescence resulting from the oxidation of lucigenin (Gyllenhammar 1987). Cells from the cultures (100 μl; 1 x 10^7 cells/ml) in HBSS were preincubated for 5 minutes at 37°C. Lucigenin (0.25 mM) in HBSS was added, together with agonist and sufficient HBSS to bring the final assay volume to 1 ml. The cells were placed in a water-jacketed 37°C luminometer chamber (LBK-Wallac, Wallac, Finland) and the resulting chemiluminescence recorded at 10 second intervals. Triplicates for each cell source were assayed and the results expressed as the maximum recorded activity in millivolts. Neutrophils from a normal subject were used as a control.

2.4 Immunological Studies: Immunofluorescence Labelling and Fluorescence Activated Cell Sorting

2.4.1 General Considerations and Configuration of Flow Cytometers

Flow cytometric analysis was performed using either a Coulter Epics-Profile II or XL flow cytometer (Coulter Electronics Corp., Hialeah, Florida, USA). All fluorescent activated cell sorting (FACS) was performed on a FACStar^{PLUS} (Becton Bickinson, San Jose, CA, USA). Alignment of these instruments was checked daily and optimised with fluorescent beads (Flow-Check^{TM} Fluorospheres, Coulter Electronics, Hialeah, FL, USA, Cat. No. 6605359). In addition, both instruments were subject to a rigorous preventative maintenance programme including thorough cleaning and testing for carryover between samples, background particle counts and electronic interference. The Coulter flow cytometers were configured with air cooled, software controlled, 15 mW, argon ion lasers operating at 488 nm. Initial analysis with the Profile II was limited to collection of forward light scatter (FSC), side or perpendicular light scatter (SSC) and 3 fluorescent emission signals (FL-1 channel for Fluorescein isothiocyanate [FITC], FL-2 for Phycoerythrin [PE] and FL-3 for energy coupled dyes [ECD] or tandem conjugates such as PE with Texas red, respectively). The fluorescent signals for the 3 fluorescent channels were discriminated by the respective

use of 525 nm, 575 nm and 620 nm band pass filters in line before the photodetectors and photomultiplier tubes. The Epics-XL allowed simultaneous detection of an extra fluorescent signal with the inclusion of a fourth detector and a 675 nm band pass filter. This proved very useful for monoclonal antibodies conjugated with PE-cyanine5 tandem dyes (PE-Cy5). For immunophenotypic analysis of cells on the Coulter analysers, routinely, 10-50,000 events were collected as listmode files and analysed using Epics-Elite software. In addition, data was archived in "standard flow cytometry format" which enabled analysis by WinMDI version 2.7 and 2.8 software downloaded from Dr Joseph Trotters flowcytometry web site at the Scripps Clinic (www.scripps.edu).

The FACStar^{PLUS} was configured with a water-cooled argon ion laser that was operated between 200-300mW. This instrument was capable of collecting FSC and SSC signals and 3 fluorescent signals; FL1 (FITC), FL2 (PE) and FL3-1 (ECD or tri-color). Data acquisition and analysis was performed with Lysis II software. The standard approach to FACS of haemopoietic progenitors and $CD34^+$ fractions involved setting a FSC vs SSC region to select for lymphocytes and blasts (Andrews *et al* 1989) then additional sort regions based on expression of one or two antigens, typically chosen from the FL1 vs FL2 dot plot. Cell sorting was performed in the "normal R" sort mode selecting 3 drop packets and discriminating between 500-2,500 cells/second. All cell sorts were continually monitored by inspection of the droplet break-off position. If the break-off position changed then adjustments in amplitude and phase controls were made to maintain selection of the desired packet of drops containing the cell of interest. Prior to FACS the sample lines and the nozzle were sterilised with 70% ethanol for 10 minutes then back-flushed with 0.22 μm filtered sheath fluid. Sorted cells were collected into sterile polypropylene centrifuge tubes containing IMDM with 10% FBS. When sufficient cells were collected a post-sort purity check was performed. This involved at least 5 minutes of back-flushing with sterile sheath fluid, a background check, then analysis of approximately 5% of the cells sorted into the collection tube. The sort purity was typically greater than 98% and if this was not achieved the sample was re-sorted or discarded.

Single cell sorting into Terasaki wells was performed with the automated cell deposition unit of the FACStar^{PLUS}. The instrument was configured identically to that described above for sorting cells into tubes. Prior to single cell sorting the instrument was run in test mode and 50 drops of sheath were sorted into wells to confirm targeting. Adjustments to the plate home position were made until this packet of drops was reproducibly placed into the centre

of at least 20 Terasaki wells. During single cell sorting the sample flow rate was slowed so that only 1 cell of interest was detected/second. This was a critical adjustment as it allowed conservation of specimens and resulted in a high proportion of wells receiving only one cell, which was typically deposited in the same area of the well. Terasaki plates were placed directly into a 37^0C CO_2 incubator after FACS and examined within 6 hours under 200X magnification with an inverted phase microscope to record wells that contained single viable cells.

2.4.2 Immunophenotyping of Haemopoietic Cells

Immunophenotyping of PB MNC, BM MNC, cells generated in culture or freshly isolated $CD34^+$ cells was performed by one-colour, 2-colour or 3-colour immunolabelling procedures. The list of immunological reagents used for these studies is presented in table 2.1. Monoclonal antibodies (McAB) were directly conjugated to FITC, PE or energy coupled dyes (ECD) such as tri-colour and PE-Cy5 whereas binding of unconjugated McAB reagents was visualised by indirect immunolabelling with isotype specific secondary antibodies or by streptavidin linked to FITC, PE, or tri-colour.

Immunolabelling was routinely performed by incubating cells at 4°C (on ice) with saturating amounts of directly conjugated monoclonal antibodies, purified antibody or hybridoma supernatants. At least 1 µg of immunoglobulin was used for incubation with 5 x 10^6 cells for 30 minutes. Typically, immunolabelling was performed in 5 ml polypropylene tubes (Falcon, Becton Dickinson Labware, New Jersey, USA, Cat. No 2063) with cells and antibody comprising 100-150 µl. Removal of excess primary McAB or subsequent second label reagents was effected by washing the cells in ice cold IF buffer, twice by centrifugation at 400 x g in a refrigerated centrifuge (Beckman GS-6R with a GH-3.7 rotor, Beckman Instruments, Palo Alto, CA) set at 4°C. When the immunolabelled cells were to be analysed rather than sorted, the final cell suspension was resuspended in ice cold IF buffer or if held for greater than 4 hours, resuspended in FACS FIX. Immunolabelled cells for FACS were resuspended in IMDM/5% FBS and held on ice prior to sorting. Propidium iodide (Molecular Probes, Eugene Oregon, USA; 0.1 µg/ml) was added to those samples resuspended in IF buffer just prior to data acquisition, so that dead cells could be gated from analysis. All immunolabelling protocols included negative controls where cells were incubated with non-binding isotype matched McAB to test for Fc mediated or non-specific binding. In addition, 2 and 3-colour labelling protocols included cells labelled with the single McAB and pairs of McAB where necessary, to enable electronic compensation setting. For

Table 2.1 Identity, Source and Isotype of Antibodies

Source:

Professor A Lopez, Human Immunology, Hanson Centre for Cancer Research (HCCR), Adelaide, South Australia;
Dr L Ashman, Division of Haematology, HCCR, Adelaide, South Australia;
Dr J Leyton, Ludwig Institute for Cancer Research, Melbourne, Victoria;
Dr A Shimosaka, Kirin Brewery, Tokyo, Japan;
Dr K Bradstock, Haematology, Westmead Hospital, NSW,
Dr P Lansdorp, Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada;
Dr H-J Bűhring, Department of Medicine, University of Tubingen, Tubingen, Germany;
Dr H Zola, Adelaide Women's and Childrens Hospital, Adelaide South Australia;
Dr T Kishimoto, Osaka University, Osaka, Japan;
Serotec, Oxford, England,
Dako Corporation, Carpinteria, CA, USA;
Coulter/Immunotech, Miami, Florida, USA;
Becton Dickinson, San Jose, CA, USA;
PharMingen, San Diego, CA, USA;
S.B.A, Southern Biotechnology Associates Inc, Birmingham Al, USA.

n.a. = not applicable.

Table 2.1

CD	Antigen	Antibody/Clone Name	Supplier	Isotype
3	T cell receptor complex	WM48	Dr K Bradstock	IgG_1
		Leu-4	Becton Dickinson	IgG_1
11b	integrin alpha-M chain	2LPM19c	Dakopatts	IgG_1
		2MPM19	DAKO	IgG_1
14	55-57 kDa GPI linked protein	FMC 17	Dr H Zola	IgG_1
		MY4	Coulter Immunotech	IgG_{2b}
15	lacto-N-(neo) fucopentaose III	Leu-M1	Becton Dickinson	IgM
33	67 kDa glycoprotein	Leu-M9	Becton Dickinson	IgG_1
34	110 kDa sialomucin	43A1	Dr H-J Buhring	IgG_3
		HPCA-2	Becton Dickinson	IgG_1
		581	Coulter Immunotech	IgG_1
		8G12	Dr P Lansdorp	IgG_1
38	45 kDa glycoprotein	Leu-17	Becton Dickinson	IgG_1
		MCA971F	Serotec, Oxford, UK	
62-P	P-selectin	n.a.	Pharmingen	Polyclonal
71	Transferrin receptor	BerT9	Dakopatts	IgG_1
114	G-CSFR	711, 772, 775	Dr J Layton	IgG_1
116	GM-CSFR α	6E10, 4A5	Dr P Simmons	IgG_1
117	c-*kit*	YB5.B8	Dr L Ashman	IgG_1
123	IL-3R α	9F5, 6H6	Dr A Lopez	IgG_1
126	IL-6R	MT18, PM1	Dr T Kishimoto	IgG_1
130	gp130	AM64	Dr T Kishimoto	IgG_1
131w	β_c chain	4F3	Dr A Lopez	IgG_1
135	*flt*3	BV10	Dr H-J Buhring	IgG_1
n.a.	c-*mpl*	n.a.	Dr A Shimosaka	Polyclonal
n.a.	HLA-DR	L243	Becton Dickinson	IgG_{2a}
n.a.	IgG_1 isotype control	3D3	Dr P Simmons	IgG_1
		X40	Becton Dickinson	IgG_1
n.a.	IgG_3 isotype control	cat no 105-01	S.B.A.	IgG3

example, when labelling cells simultaneously with CD34-FITC and CD38-PE, tubes with only CD34-FITC and CD38-PE were included. The following sections provide details for specific key immunolabelling procedures.

2.4.3 Investigating Expression of c-*mpl* by Immunophenotyping

The expression of *c-mpl* on HPC was investigated by immunolabeling with affinity purified rabbit polyclonal antisera (Tahara *et al* 1996). Typically, $1x10^7$ BM MNC were incubated for 45 minutes at 4°C with 2 µg of rabbit anti-c-*mpl*, washed twice, incubated for 30 minutes with biotinylated goat-anti rabbit (Vector Laboratories, Burlingame, CA) then after washing, visualised by addition of streptavidin tri-color (Caltag, San Francisco, CA). During the final incubation, HPCA-2-FITC (anti-CD34) and Leu-17-PE (anti-CD38) were added. To assess non-specific reactivity of BM MNC with rabbit immunoglobulin the rabbit polyclonal anti-c-*mpl* antisera was replaced with 2 µg of purified rabbit IgG.

2.4.4 Immunolabelling for Haemopoietic Growth Factor Receptors

Expression of HGF receptors (HGFR) was examined by indirect immunolabelling with a panel of IgG_1 murine monoclonal antibodies to the IL-3R–α chain, GM-CSFR–α chain, the common IL-3 and GM-CSF β chain, gp130, IL-6R, G-CSFR and c-*kit* together with an IgG_3 murine McAB to CD34. Bone marrow MNC were resuspended at 2 x 10^7 cells/ml in blocking buffer and 100 µl of cells transferred to a 4 ml polypropylene tube (Falcon: Cat. No. 2063) to which was added 50 µl of both 43A1 (IgG_3, anti-CD34 McAB) and 50 µl of the respective IgG_1 HGFR monoclonal antibodies. The cells were incubated on ice for 45 minutes then excess monoclonal antibodies removed by 2 washes in HBSS/5 % FBS. A control tube containing a non-binding IgG_1 isotype murine monoclonal (3D3) together with 43A1 was also prepared. Following washing, goat anti-mouse IgG_1–PE (Caltag Laboratories, Burlinghame, CA. Cat. No. M32004) and goat anti-mouse IgG_3–FITC (Caltag, Cat. No. M32601) were added and cells incubated for 30 minutes on ice. Cells were washed twice in HBSS/5% FBS then resuspended in IMDM/5% FBS ready for analysis and FACS.

2.4.5 Rhodamine 123 (Rh123) Staining of Haemopoietic cells

Stock solutions of Rh123 (Molecular Probes Inc, Eugene, Oregon USA) were stored at 1 mg/ml in DPBS at –80°C with working solution used at 0.1 µg/ml in HBSS supplemented with 5 % FBS. Bone marrow cells were incubated at 1 x 10^7 cells/ml with 5 mls of 0.1 µg/ml Rh123 for 45 minutes at 37°C, 5% CO_2 and mixed every 15 minutes. Cells were washed twice in HBSS/5% FBS and incubated for a further 15 minutes at 37°C to effect efflux of

residual unbound Rh123. The cells were washed a further 2 times in HBSS/5% FBS prior to staining with PE conjugated monoclonal antibodies. The cell samples stained with Rh123 were not fixed but analysed or sorted on the same day of labelling. Excitation of Rh123 occurs with a 488 nm laser and its emission is similar to FITC so that Rh123 labelling of cells was detected via the FL-1 channel of the Coulter Profile II and XL flow cytometers and the Becton Dickinson FACStar^{PLUS}. Isolation of cells according to Rh123 expression was performed by placing sort regions on a Rh123 histogram. The Rh123^{low} fraction was taken as the 10% of cells with the lowest Rh123 expression. Rh123^{hi} or Rh123^{bright} cells were taken as the cells exhibiting greater Rh123 expression than the mean fluorescent intensity of the entire cell population.

2.5 Miscellaneous Techniques

2.5.1 Cytocentrifuge preparations

Cytocentrifuge preparations (cytospins) were used to concentrate cells onto a glass microscope slide for further staining. A Shandon Elliot cytocentrifuge was used for preparing cytospins. Between 10,000–25,000 cells were loaded into the plastic chamber in approximately 100 µl of medium together with 100 µl of PBS containing 40% FBS. The slides were centrifuged at 500 rpm for 5 minutes then the glass slide carefully separated from the filter paper support. Slides were left to air dry before Jenner-Giemsa or esterase staining.

2.5.2 Enumeration of Nucleated Haemopoietic Cells by Haemocytometer Counting

Counting of leukocytes in PB MNC and BM MNC suspensions was performed by dilution of the cell suspension into a leukocyte counting fluid (2% acetic acid in water tinged with methyl violet) so that contaminating erythrocytes were lysed and the nuclei of leukocytes were stained. Suspensions of purified haemopoietic cells obtained by FACS or generated in culture were counted directly on the haemocytometer. Dilution of these samples was performed with IMDM, if required. The number of cells over the 4 major corner squares of the haemocytometer were counted, then the number averaged to calculate the number of cells in 0.1 µl of the original cell suspension. The actual concentration of cells /µl was then calculated by multiplying the average cell count x 10 (correction for the volume of suspension counted) and the dilution factor. If more than 150 cells were present over one corner square then the sample was subject to a greater dilution. Counting was performed

under phase contrast microscopy at 200X magnification, which enabled discrimination of dead cells and non-cellular particulate matter from viable cells.

2.5.3 Non-Specific and Specific Esterase Staining of Ex vivo Generated Cells

Cytospin preparations of cultured cells were stained for non-specific and chloroacetate esterase to enable identification of monocyte/macrophages and cells of the neutrophil lineage, respectively. The basis for this discrimination lies in the substrate affinities of the different esterase isoenzymes within leukocytes (reviewed in the American Journal of Medical Technology, Volume 47, 6, June 1981). The first group of isoenzymes (3, 4, 5 and 6) occurs chiefly in monocytes and macrophages and is demonstrated by exposing cells to alpha-naphthyl butyrate as a substrate. The second group of isoenzymes (2, 7 and 9) occurs predominantly in granulocytes and their precursors and is demonstrated by the use of alpha-naphthyl AS-D chloroacetate as substrate and the diazonium salt Fast Blue BB as the capture reagent. The method for staining cells deposited on cytospins is as follows:

i) Fix cells on cytospin in cold esterase fixative for 30 seconds
ii) Wash in Milli-Q water and air dry
iii) Add 38 ml of 0.07M phosphate buffer pH 6.3 to a beaker
iv) In a plastic bijou mix 3 drops of α Napthyl-butyrate (Sigma, St Louis. Cat. No. N 8000) at –20°C with 3 ml of 2-ethoxyethanol and add to the phosphate buffer
v) Mix 0.3 ml of pararosanalin solution to 0.3 ml 4% sodium nitrite in a glass tube and allow to react for one minute, then add 0.5 ml to the solution in the beaker. Filter into a coplin jar
vi) Adjust pH to 6.1 with 5N NaOH (1-2 drops)
vii) Stain the cytospins for 45 minutes in this substrate mixture
viii) Wash in Milli-Q water
ix) Add 20 mg Fast Blue BB to 38 ml 0.7M phosphate buffer pH 7.4 in a clean, dry beaker
x) To 10 mg AS-D chloroacetate (Napthol AS-D chloroacetate Sigma, Cat. No. N 2875) add 5 ml N-N dimethyl formamide, mix and add 2 ml to beaker. Filter into a dry clean coplin jar
xi) Stain the cytospins or slides for 1 hour
xii) Wash slides well in Milli-Q water and counterstain in 2% methyl Green for 10 minutes

xiii) Wash slides in Milli-Q water and air dry

xiv) Mount slides or the cytospins in PIX and examine under both 400X and 1,000 times (oil immerson) magnification

Controls: Peripheral blood smears were fixed and stained with the same reagents used for test cytospins. The cytoplasm of neutrophils stain blue whereas monocytes exhibit red-brown staining.

2.5.4 Immunocytochemistry for P-selectin (CD62-P)

Immunocytochemistry for expression of CD62-P was performed on cytospin preparations that were either air dried overnight or stored longer term in an airtight container at –70°C. The following steps were followed:

i) Fix the cells for 30 seconds in buffered formal acetone, then quickly rinse in three changes of Milli-Q water and allow slides to dry.

ii) Rehydrate cells in 150 µl of NGST (PBS supplemented with 5% v/v normal goat serum [Vector Labs, Cat. No. S-1000] and 0.05% Tween 20) and allow to block for approximately 30 minutes at room temperature in a fully humidified chamber.

iii) Remove excess NGST by flicking and add primary antibody reagents (below) diluted in NGST to a final concentration of 5 µg/ml. Incubate for 1 hour at room temperature or overnight at 4°C.

Anti-P-selectin (CD62-P) (Pharmingen Cat. No. 09361A): affinity-purified rabbit polyclonal to human P-selectin.

Control Rabbit Immunoglobulin (Zymed Cat. No. 02-6102) chromatographically purified Rabbit IgG or Vector Labs Cat. No. I-1000.

iv) Wash 3 times with a 100ul/wash each at 5 minutes per wash in PBS + 0.05% Tween 20 (PBST).

v) After the last wash, cover cells with a drop of NGST and leave for approximately 5 minutes at room temp. Remove excess (do not wash) and add biotinylated Goat anti-Rabbit (Vector Laboratories Cat. No. BA-1000) diluted 1:200 in NGST. Incubate for 1 hour at room temperature.

vi) Prepare Avidin-Biotin-peroxidase Complex (ABC reagent: Vector Laboratories, Cat. No. PK-4000) according to the manufacturer's recommendations.

vii) Wash as in step iv above.

viii) Quench endogenous peroxidase activity by immersing slides for 30 minutes at room temperature in a freshly prepared mixture comprising 49 parts of 100% methanol: 1 part 30% hydrogen peroxide. Transfer quickly to an excess volume of PBST and wash several times in order to remove methanol/H_2O_2.

ix) Add ABC reagent and incubate for 30 minutes at room temperature.

x) Wash as in step iv above.

xi) Add substrate solution prepared according to the manufacturer's instructions (Vector Laboratories, Vector Red, Cat. No. SK-5100). Allow red reaction product to develop.

xii) Wash in Milli-Q water. Counterstain for 2 minutes in Mayers Haematoxylin (Sigma, Cat. No. MHS-1), wash in warm tap water and blue in Scott's buffer.

xiii) Mount in an aqueous mountant (VECTASHIELD^{TM,} Vector Laboratories Cat. No. H-1000).

The CD62-P antigen is distributed in the cytoplasm of megakaryocytic cells; typically between 5-8% of cells derived from $CD34^+$ cells grown in 36GS were $CD62P^+$ when stained under these conditions.

2.5.5 Jenner - Giemsa Staining of Cytocentrifuge Smears

Romanovsky stains are commonly used by haematologists for staining of blood films and bone marrow smears. The combined Jenner-Giemsa stain utilises the characteristics of both a Jenner stain, which reacts with cytoplasmic components and the nuclear staining activity of a Giemsa stain. Jenner-Giemsa staining was performed within the Diagnostic Services Laboratory of the Institute of Medical and Veterinary Science (IMVS) by use of an automated slide staining machine (Shandon Varistain 12, Shandon Elliot). Cytopsins were subject to sequential 2 minute staining in baths containing 100% Methanol (BDH, AnalaR, Cat. No. 10158), Jenner (BDH Cat. No. 35132), Giemsa (BDH, Cat. No. 35086), Giemsa, Giemsa, running water and phosphate buffer at pH 7.0. At completion of staining the cytospins were air-dried then mounted in PIX.

2.5.6 Photomicroscopy

Black and white photographs of cells in Terasaki wells were taken with an Olympus Camera (SLR, Olympus Optical Co, Japan) and a PM-10ADS automatic exposure system mounted to an Olympus Inverted Microscope (Model IMT-2). A Techpan film was used with the ASO set at 100, exposure adjustment set at 1 and reciprocity on 1. Colour photographs were taken by the same automatic exposure system mounted to an Olympus BH2 light microscope and

with an Ektachrome 64 Kodak colour film. The photographic unit of the IMVS developed negatives.

2.6 Data Analysis

Statistical analysis of primary data was performed using Statview 4.02 software (Abacus Concepts Inc, Berkerley, CA). Unless otherwise stated at least 3 replicates of test conditions were included in all experiments and the mean and standard deviation (SD) or standard error of mean (SEM) for data has been calculated and presented. Statistical tests for comparing data sets within experiments included one-way analysis of variance (ANOVA) and paired t tests (with 95% confidence limits).

CHAPTER 3. *EX VIVO* EXPANSION AND MATURATION OF PERIPHERAL BLOOD CD34$^+$ CELLS INTO THE MYELOID LINEAGE

3.1 Introduction

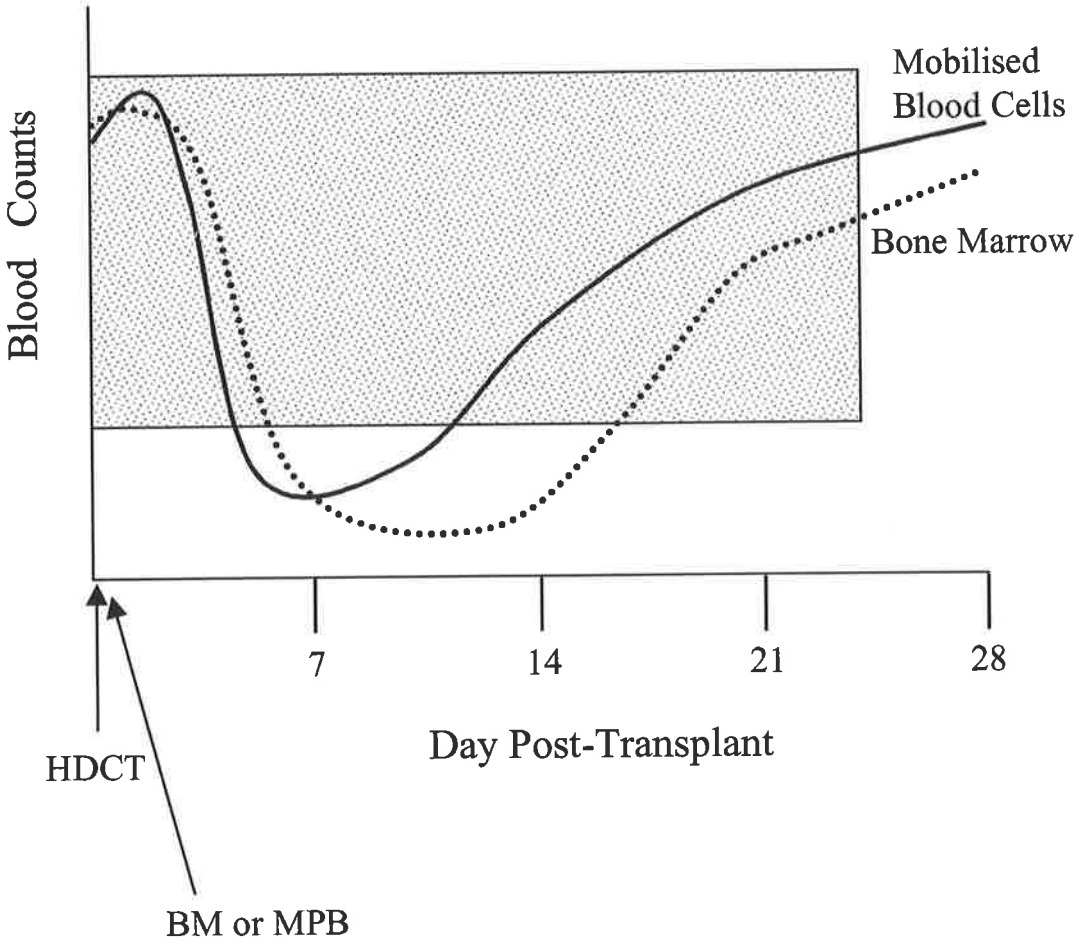
There is clear evidence from autologous and allogeneic haemopoietic transplantation in both non-human primates (Wagemaker *et al* 1990) and humans that CD34$^+$ cells from the bone marrow (BM) or peripheral blood (PB) can reconstitute the haemopoietic system following marrow ablative therapy (Berenson *et al* 1988, Vogel *et al* 2000). These results therefore indicate that both long-term and short term repopulating HPC are present within the CD34$^+$ cell fraction. In fact, CD34$^+$ cells represent a heterogeneous population containing not only primitive HPC but also progenitors of multiple lineages at various stages of commitment and proliferative potential. The early phase of complete tri-lineage (myeloid, megakaryocytic and erythroid) haemopoietic reconstitution (HR) after transplantation most likely results from the mature lineage committed progenitors within the CD34$^+$ population (Jones *et al* 1987, Naparestek *et al* 1992). Thus, CD34$^+$ cells that give rise to myeloid or megakaryocytic colonies (CFU-GM and CFU-Meg) in semisolid clonogenic assay are probably the same cells that yield neutrophils and platelets 2-3 weeks post graft. In contrast, sustained tri-lineage HR results from hierarchically more primitive progenitor/stem cells that are precursors to *in vitro* clonogenic haemopoietic progenitors. The compartment of cells that represent the precursors to clonogenic cells have been referred to as pre-progenitors or "Pre-CFU". Haemopoietic cells within the Pre-CFU compartment can be assayed *in vitro* by their ability to initiate and sustain haemopoiesis following co-culture with allogeneic BM stromal cells in long-term bone marrow culture (LTBMC) (Sutherland *et al* 1989, Winton *et al* 1987). Alternatively, Pre-CFU can be detected by their capacity for *de novo* generation of directly clonogenic progenitors following culture in the absence of marrow stromal cells in media supplemented with various combinations of haemopoietic growth factors (HGF) (Iscove *et al* 1989, Brandt *et al* 1990, Moore 1991).

A consistent feature of HR with any form of cellular and HGF rescue following marrow ablative therapy is the period of obligatory neutropenia and thrombocytopenia observed post graft (refer to figure 3.1). The most rapid HR following marrow ablative therapy occurs with transplantation of HPC collected from the blood during the recovery phase following high dose chemotherapy or following administration of HGF such as granulocyte-colony stimulating

Figure 3.1 Haemopoietic recovery following myeloablative therapy and haemopoietic cell transplantation

Myeloablative chemotherapy (high dose chemotherapy: HDCT) induces severe cytopenia within a few days following drug administration. The figure describes the typical changes in blood counts during the first 4 weeks following HDCT supported by BM or blood cell transplantation. The shaded area represents the range of blood counts for a healthy adult individual. In comparison to BM, transplantation with blood progenitor cells results in a reduced period of cytopenia and a more rapid return to safe, normal blood counts. This figure is adapted from various sources of data in particular that presented within "Blood Stem Cell Transplants: edited by Gale, Juttner and Henon 1994".

Haemopoietic Recovery after Bone Marrow and Mobilised Blood Stem Cell transplantation



factor (G-CSF) (To *et al* 1989, Sheridan *et al* 1992). This form of transplantation is commonly termed peripheral blood stem cell (PBSC) or blood progenitor cell (BPC) transplantation. For PBSC autografting a positive correlation exists between the number of CFU-GM infused and the rate of neutrophil and platelet recovery: a threshold dose of 2-4 $x10^4$ CFU-GM/Kg appears necessary for rapid HR following infusion of PBSC (Lowenthal *et al* 1988, To *et al* 1990b, Bender *et al* 1992). Importantly, however, the infusion of very high doses of CFU-GM or the administration of HGF, specifically G-CSF, post PBSC grafting does not significantly enhance the rate of HR (To *et al* 1992). In 1992, I proposed that this pattern of HR was a direct consequence of a lack of, or insufficiency in the numbers of post-progenitor cells infused (Haylock *et al* 1992). Accordingly, we and others (Muench and Moore 1992, Shapiro *et al* 1994, Smith *et al* 1993) postulated that a further enhancement in the rate of haemopoietic reconstitution might be achieved by adding a greatly increased number of post-progenitor cells to the transplant to provide mature functional cells within days of infusion. Such an approach, it was hypothesised, may lead to the amelioration or abrogation of severe cytopenia following high dose therapy resulting in shorter hospitalisation and a further improvement in the safety and efficacy of such treatment. Therefore, an objective of the studies described within this chapter was to determine if *ex vivo* culture of purified PBSC (essentially $CD34^+$ cells) could generate sufficient neutrophil precursors to offer a potential therapeutic benefit when infused.

Experiments were designed to assess the generation of neutrophil precursors and committed HPC from mobilised peripheral blood $CD34^+$ cells when cultured under static conditions without stromal cell support. Growth of HPC under these conditions is absolutely dependent on provision of exogenous mitogens or growth factors present in either serum or provided as soluble recombinant molecules. A primary objective of these studies was to identify a combination, or combinations of HGF that would support significant generation of neutrophil precursors and nascent myeloid progenitor cells (CFU-GM) from mobilised blood $CD34^+$ cells. An underlying hypothesis for these studies was that generation of large numbers of myeloid progenitor cells, and neutrophil precursors could not be achieved solely by proliferation of committed myeloid progenitor cells within the $CD34^+$ compartment but would require recruitment and proliferation of Pre-CFU. Studies from several independent groups had suggested that recruitment and proliferation of primitive HPC required stimulation by combinations of HGF (Ikebuchi *et al* 1987, Bartelmez *et al* 1989, Smith *et al* 1991, Kobayashi *et al* 1991). These reports also indicated that IL-3 was a key HGF required for growth of primitive cells: it was essential for survival and when used together with IL-1 (Smith *et al* 1991, Iscove *et al* 1989) or IL-6 (Leary *et al* 1988) was able to induce division of primitive

human HPC including CFU-Blasts, and also synergised with SCF, GM-CSF, G-CSF and EPO to generate human progenitor cells of the myeloid (Broxmeyer *et al* 1991), erythroid (Bernstein *et al* 1991, McNiece *et al* 1991) and megakaryocytic lineages (Avraham *et al* 1992). Therefore the studies described herein represent an attempt to systematically compare how combinations of specific HGF affect growth of $CD34^+$ HPC as determined by their generation of nascent myeloid progenitors (CFU-GM) and neutrophil precursors.

3.2 Experimental Details

3.2.1 Design

Experiments were performed in two stages using cryopreserved cells from three patients cultured in a stromal free, cytokine dependent, liquid culture system (Pre-CFU assay). Initial experiments assessed the ability of a combination of six recombinant cytokines (6HGF), ie interleukin (IL)-1beta (IL-1β), IL-3, IL-6, G-CSF, GM-CSF and SCF, either alone or in various combinations, to generate nascent progenitors from $CD34^+$ cells isolated from mobilised PBSC. Cultures were initiated with 1,000 $CD34^+$ cells in medium supplemented with foetal calf serum (FCS). Growth was stimulated by the addition of a range of different HGF combinations (as indicated in Figure 3.3) and assessed after 7 days. To limit the number of possible combinations of HGF to be tested, IL-3 was included as a common HGF for all combinations. This decision was based on the well-documented activity of IL-3 as a cytokine that interacts either additively or synergistically with both early and late acting HGF to induce proliferation and differentiation of primitive human HPC. This series of experiments identified a HGF combination that reproducibly supported a significant expansion of $CD34^+$ cells during 7 days of culture. Based on these data a second line of experimentation was initiated which involved larger volume (50 ml) Pre-CFU cultures of $CD34^+$ cells established in this HGF combination. Cultures were monitored for 3 weeks and generation of both nucleated cells and CFU-GM was measured to assess the feasibility of scaled-up *ex vivo* culture of $CD34^+$ cells for clinical transplantation. Additional endpoints measured were the morphology, immunophenotype and function of the *ex vivo* generated cells.

3.2.2 Peripheral Blood Mononuclear Cells for Study

Peripheral blood mononuclear cells (PBMNC) from 3 patients were studied. Patients UPN522 and UPN523 (advanced carcinoma of the ovary) received cyclophosphamide 4gms/m^2 intravenously as part of the treatment of their malignancy and PBMNC were collected by apheresis during haemopoietic recovery following the period of myelosuppression.

Cryopreserved PBMNC used for study were from aphereses on day 13 and 14 respectively following cyclophosphamide when the leukocyte count of both patients was 1.6 x 10^9/L. Patient UPN1197 (relapsed Hodgkin's disease) received recombinant human G-CSF, 12 μg/kg/day for 7 days by continuous subcutaneous infusion for mobilisation of PBSC. Peripheral blood MNC used for study were from the apheresis on day 5 of G-CSF administration when the leucocyte count was 52.8 x 10^9/L. The apheresis and cryopreservation methods have been reported previously (To *et al* 1989).

3.2.3 Processing of Cells for Study

Cryopreserved PBMNC were thawed and washed as described in section 2.1.3 of the materials and methods chapter. Because only 0.6% of UPN1197 PBMNC were $CD34^+$ a pre-enrichment step of mature cell depletion by immunoadherence followed by immunomagnetic bead separation was performed to facilitate flow cytometric sorting of $CD34^+$ cells. The $CD34^+$ enriched population from UPN1197 MNC contained 12% $CD34^+$ cells (78% recovery of $CD34^+$ cells) whereas the PBMNC from UPN522 and UPN523 contained 10% and 8% $CD34^+$ cells respectively. Immunophenotyping was performed on a small proportion of these cells to examine expression of activation and lineage antigens on $CD34^+$ cells. The remaining MNC were incubated with 8G12-FITC (anti-CD34, 1 μg/10^6 cells) for 40 minutes at 4^0C, washed twice and $CD34^+$ cells were then sorted into RPMI-1640/10% FBS using a Becton Dickinson FACStar^{PLUS}. $CD34^+$ cells within the lymphocyte/blast region (Andrews *et al* 1989) were collected and 1,000 $CD34^+$ cells were used for each 1ml Pre-CFU culture. A baseline CFU-GM assay was also performed on the input $CD34^+$ cells.

Subsequently, 50 ml Pre-CFU cultures of UPN522 $CD34^+$ cells at 5 x 10^3/ml were established in a T175 tissue culture flask (Falcon, Becton Dickinson) and a 500 ml PL269 Life Cell Bag (Fenwal, Travenol Laboratories, Deerfield, USA). In addition, a 50 ml culture of UPN1197 $CD34^+$ cells at 4.2 x 10^3/ml was established in a T175 flask. Recombinant human IL-1β, IL-3, IL-6, GM-CSF, G-CSF and SCF were added to Pre-CFU medium to give a final concentration of 10 ng/ml of each cytokine. Large volume cultures were incubated at 37^0C in 5% CO_2 for up to 21 days. At day 7 an additional 10 ng/ml of each of the 6 HGF was added to each 50 ml culture. On day 14, 25 mls from the UPN1197 flask and UPN522 bag cultures were removed for assessment of cell function. A further 75 mls of culture medium were added to both cultures but additional HGF was only added to the UPN522 bag culture.

3.2.4 Assessment of Cells in Large Volume Cultures

Cells (2 – 5 ml) were removed from the T175 flasks and the Life Cell bag on days 3, 5, 7, 10, 12, 14, 17 and 21 of culture. At each time point the number of viable nucleated cells were counted, cell morphology was assessed on Jenner-Giemsa and Esterase stained cytospin preparations, the immunophenotype of cells was analysed by flow cytometry and the incidence of day 14 CFU-GM and BFU-E determined. In calculating the number of cells present in cultures at each time point after day 3 a correction factor was applied to compensate for cells removed previously. The correction factor was applied as follows: Let a (ml) be the starting volume of culture at time t (0). At the first sampling time-point t (1), b ml was removed for testing. The correction factor, $CF_{(1)}$, to be applied at the next sampling time-point for cells removed at t (1) was (a/a-b). The culture was refed at t (1) and the reconstituted volume was c ml. At time-point t (2), the number of cells present was $n_{(t2)}$. Applying $CF_{(1)}$ the corrected number of cells was $n_{(t2)} \times CF_{(1)}$ or $n_{(t2)} \times a/(a-b)$. At time-point t (2) d ml was removed for testing so the correction factor, $CF_{(2)}$, to be applied at the next sampling time-point for cells removed at t (2) was (c/c-d). The culture was refed at t (2) and the reconstituted volume was e ml. At time point t (3) the number of cells present was $n_{(t3)}$ and by applying $CF_{(2)}$ the corrected number of cells was $n\ (t3) \times CF_{(2)} \times CF_{(1)}$ or $n_{(t3)} \times c/(c-d) \times a/(a-b)$. For subsequent time-points a similar formula was applied.

3.2.5 Immunophenotyping of Cells in Large Volume Cultures

Immunophenotyping of cultured cells at each time point was performed to assess myeloid maturation as determined by their expression of the CD34, CD33, CD15 and CD11b antigens (Terstappen *et al* 1990). Approximately 25,000 cells were labelled with appropriate antibodies as described in section 2.4.2 of the materials and methods (Chapter 2). Following labelling, cells were analysed within 1 hour on a Profile II flow cytometer (Coulter, Hialeah, Florida, USA) with standardised flow cytometer settings for forward and side scatter gains and fluorescence 1 and fluorescence 2 photomutiplier tube voltages for each day of analysis. This protocol allowed comparison of the level of expression of each myeloid antigen on each day that cells were sampled.

3.2.6 Quantitation of CFU-GM and BFU-E in Large Volume Cultures

CFU-GM and BFU-E assays were performed as described previously (Simmons *et al* 1990) and as detailed in materials and methods using 5×10^3 cells/plate. The number of CFU-GM and BFU-E present at each time point of the large volume cultures was calculated by

multiplying their incidence (CFU-GM or BFU-E/5 x 10^3cells) by the corrected number of nucleated cells present in the cultures.

3.2.7 Function Studies on Cells in Large Volume Culture

On day 14, cells from the UPN1197 flask and UPN522 bag cultures were assessed for their ability to release superoxide anion when exposed to the agonists arachidonic acid (33um), N-formyl-methionylluecylphenylalanine (fMLP) or 12-0-tetradecanoylphorbol-13-acetate (TPA). Superoxide release was measured by monitoring the chemiluminescence resulting from the oxidation of lucigenin as described in materials and methods (Gyllenhammar 1987). Triplicates for each cell source were assayed and the results expressed as the maximum recorded activity in millivolts. Neutrophils from a normal subject were used as a control.

3.3 Results

3.3.1 Characteristics of Cells Used for *Ex Vivo* Culture

The characteristics of the $CD34^+$ cells used for culture are shown in table 3.1. Ninety-nine percent of the cells used for culture were $CD34^+$ and the majority (96-100%) co-expressed HLA-DR and CD38 whereas CD71 was variably present on 7%, 17% and 46% of cells from patients UPN522, UPN523 and UPN1197 respectively. CD33 co-expression was also variable with 1%, 86% and 47% of cells positive respectively. In contrast, CD15 was expressed on 1-2% of $CD34^+$ cells while CD11b was co-expressed, albeit at low levels, on 32%, 45% and 47% of cells from patients UPN522, UPN523 and UPN1197. The $CD34^+$ cells from all 3 patients were small-intermediate sized cells with blast like morphology, high nuclear to cytoplasmic ratio, prominent nucleoli and basophilic, agranular cytoplasm (refer to panel A of figure 3.2).

3.3.2 Pre-CFU assay of Peripheral Blood $CD34^+$ Cells

The results of three experiments using sorted $CD34^+$ cells from cryopreserved PBSC collections are shown in figure 3.3. The combination of IL-1β+IL-3+IL-6+G-CSF+GM-CSF+SCF (136GGMS) was found to be significantly better than any of the other 31 combinations of these factors either alone or together ($p<0.0001$ for all combinations except 3, i.e., 6-factors vs IL-3+IL-6+GM-CSF+G-CSF+SCF: $p=0.02$, vs IL-1+IL-3+GM-CSF+SCF: $p=0.005$ and vs IL-1+IL-3+IL-6+GM-CSF+ SCF: $p=0.001$). The number of CFU-GM generated after 7 days in suspension culture, expressed as a fold increase from the number of CFU-GM present in the starting 1 x 10^3 $CD34^+$ cells was 29, 30 and 39 for patients UPN522, UPN523 and UPN1197 respectively. The input and maximum output CFU-GM (that observed

Figure 3.2 Morphology of *ex vivo* cultured $CD34^+$ cells

Cytospins of FACS isolated $CD34^+$ cells and *ex vivo* generated cells were prepared in a Shandon Elliot Cytocentrifuge then stained with Jenner-Giemsa as described in the materials and methods. Photomicrographs were taken of cells examined at a final magnification of 1000X using 100X oil immersion objectives. FACS isolated $CD34^+$ cells from mobilised blood are shown in Panel A. These cells have a "blast" like morphology with high nuclear to cytoplasmic ratio and nucleoli. Within 3 days of culture the $CD34^+$ cells increase in size, and as shown in Panel B exhibit basophilic cytoplasm and a prominent perinuclear non-stained area typical of active golgi. After 14 days, the culture contains a mixture of developing granulocytic precursors including myeloblast, promyelocytes, myelocytes, and macrophages as shown in panel C. In panel D a macrophage is located in the centre and is to the right of a promyelocyte, which exhibits typical azurophilic cytoplasm containing numerous primary granules.

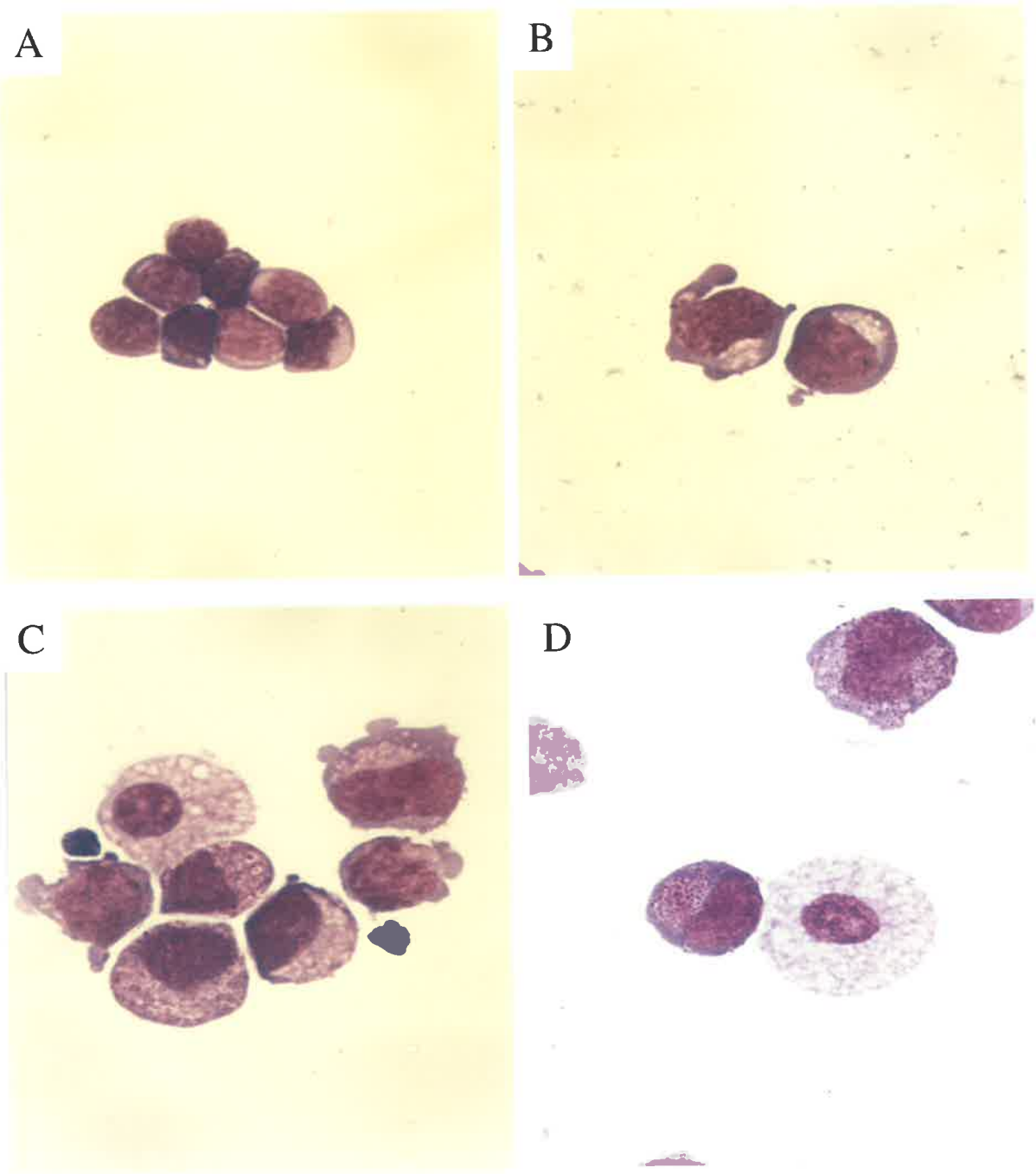

A
B
C
D

Table 3.1

Characteristics of cells used for ex vivo cultures.

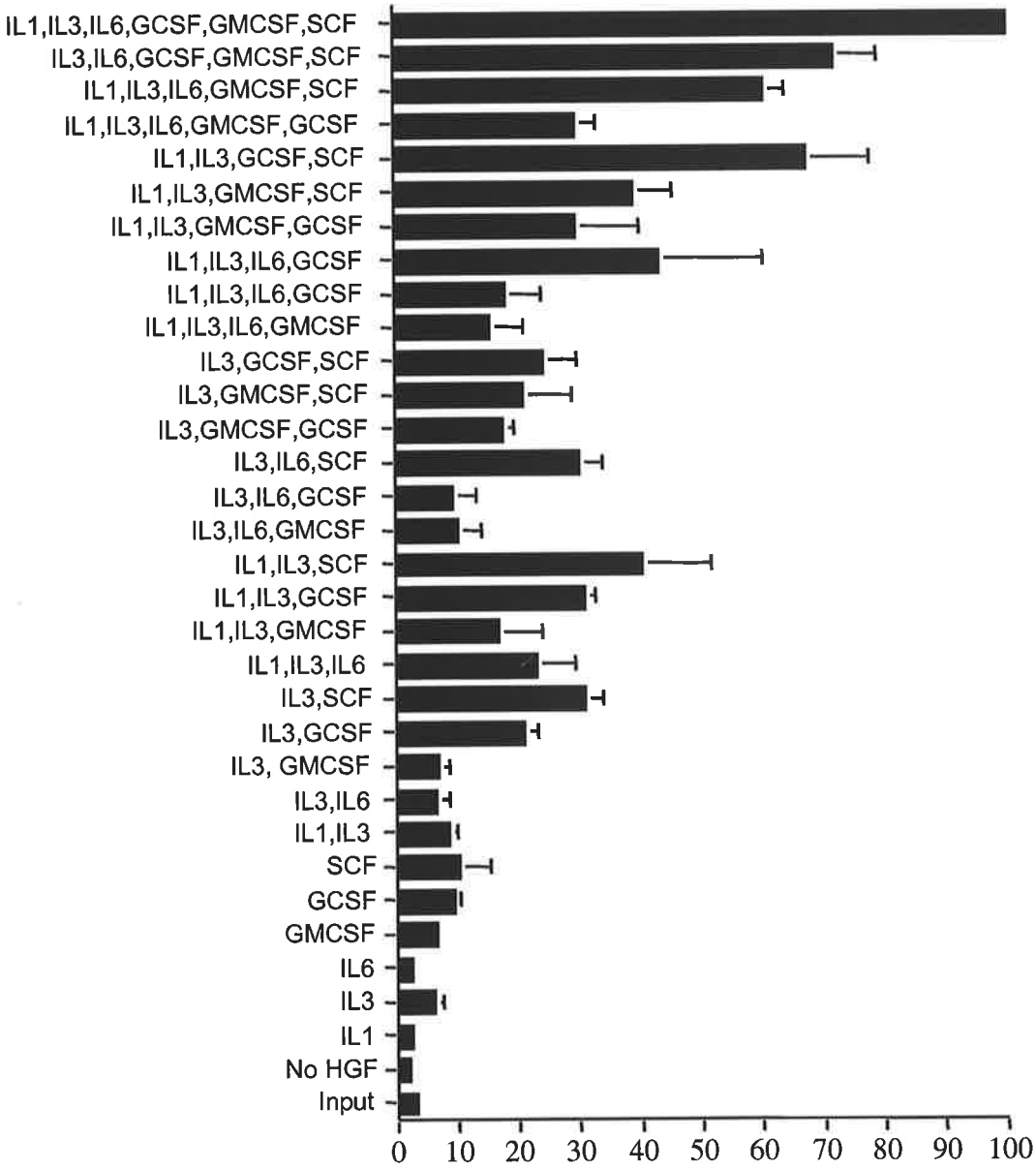
The numbers represent the percentage of cells expressing the designated cell surface antigen.

UPN represents the unique patient identification number for each patient sample.

Antigen	UPN522	UPN523	UPN1197
CD34	99	96	99
CD33	1	86	47
CD15	1	4	2
CD11b	32	45	47
CD38	100	37	98
CD71	7	17	46
HLA-DR	99	97	96

Figure 3.3 Haemopoietic growth factors affect generation of CFU-GM in Pre-CFU culture of $CD34^+$ cells

This figure shows the effect of single haemopoietic growth factor (HGF) or combinations of HGF on the generation of nascent CFU-GM after 7 days suspension culture of peripheral blood $CD34^+$ cells from 3 patients. Triplicate 1.0 ml cultures were initiated with 1 x 10^3 FACS sorted $CD34^+$ cells in 24 well plates. The following HGF were used at 10 ng/ml either alone or in combinations: IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. On day 7 the contents of each well were cultured to determine the number of CFU-GM present. For each HGF combination 9 replicates were scored. The data from each experiment was first normalised by expressing CFU-GM output for each HGF combination as a percentage of the maximum, which in each case occurred, with the 6-factor combination. The mean $\pm$ SEM of the three experiments are shown. The input and maximum output CFU-GM from 1 x 10^3 UPN522, UPN523 and UPN1197 $CD34^+$ cells were 99 and 2,916, 60 and 1,791, and 51 and 1,993 respectively.


IL1,IL3,IL6,GCSF,GMCSF,SCF
IL3,IL6,GCSF,GMCSF,SCF
IL1,IL3,IL6,GMCSF,SCF
IL1,IL3,IL6,GMCSF,GCSF
IL1,IL3,GCSF,SCF
IL1,IL3,GMCSF,SCF
IL1,IL3,GMCSF,GCSF
IL1,IL3,IL6,GCSF
IL1,IL3,IL6,GCSF
IL1,IL3,IL6,GMCSF
IL3,GCSF,SCF
IL3,GMCSF,SCF
IL3,GMCSF,GCSF
IL3,IL6,SCF
IL3,IL6,GCSF
IL3,IL6,GMCSF
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IL1,IL3,GCSF
IL1,IL3,GMCSF
IL1,IL3,IL6
IL3,SCF
IL3,GCSF
IL3, GMCSF
IL3,IL6
IL1,IL3
SCF
GCSF
GMCSF
IL6
IL3
IL1
No HGF
Input
0 10 20 30 40 50 60 70 80 90 100
Day 14 CFU-GM (% Maximum)

in the 136GGMS combination) from 1 x 10^3 UPN522, UPN523 and UPN1197 $CD34^+$ cells were 99 and 2,916, 60 and 1,791, and 51 and 1,993 respectively. Notably, there were less CFU-GM at day 7 than that present at the start of culture when $CD34^+$ cells were cultured in IL-1 or IL-6 alone. In contrast, cultures stimulated with IL-3, G-CSF, GM-CSF or SCF alone supported generation of CFU-GM above that present at the start of culture. Based on these results the larger volume cultures were established with the combination of 136GGMS each at 10 ng/ml.

3.3.3 Large Volume Cultures: Serial Changes in Nucleated Cells, CFU-GM and BFU-E

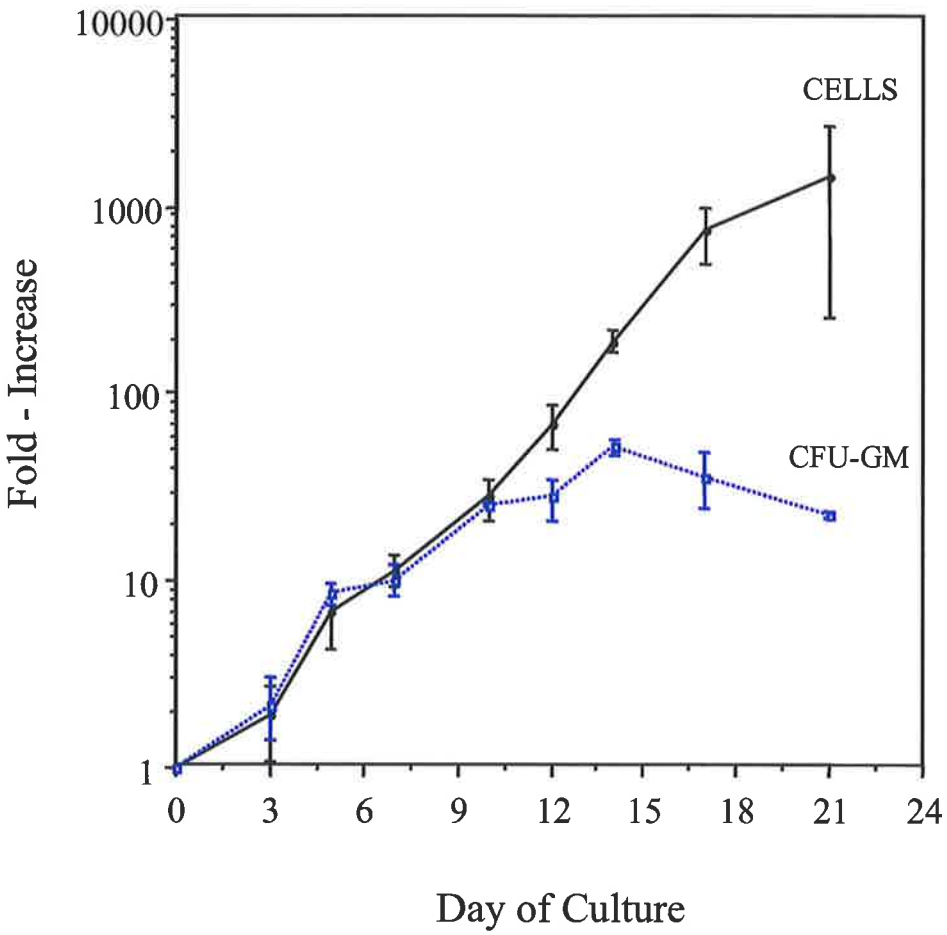
The serial fold increase (mean $\pm$ SEM) of nucleated cells and CFU-GM in culture is shown in figure 3.4. The pattern of nucleated cell and CFU-GM production was similar for each large volume culture. There was a parallel and exponential increase in nucleated cell and CFU-GM numbers until day 10. The rate of CFU-GM production then decreased although levels 22-57 fold above the starting number of CFU-GM were maintained even at day 21. The maximum fold-increase in CFU-GM for the UPN522 flask, UPN522 bag and UPN1197 flask were 97, 57 and 43 respectively and occurred at day 14 for each culture. In contrast, nucleated cell production continued exponentially and by day 21 an average of 1,324 fold more nucleated cells were present.

The UPN522 cultures were initiated with 2.5 x 10^5 $CD34^+$ cells in 50 mls of medium containing 1.7 x 10^4 CFU-GM and 1.2 x 10^4 BFU-E. After 21 days culture in the flask 6.33 x 10^7 cells (253 fold increase [FI]) and 36.7 x 10^4 CFU-GM (22-FI) were present. BFU-E increased to 6.2 x 10^4 (5-FI) on day 3 then decreased and were not detected after day 7. The bag culture contained 2.53 x 10^8 cells (1011-FI) and 97.3 x 10^4 CFU-GM (57-FI) on day 17. BFU-E increased to 13.1 x 10^4 on day 7 (11-FI) and thereafter decreased and were not detected at day 17.

The UPN1197 flask culture was initiated with 2.1 x 10^5 $CD34^+$ cells containing 2.4 x 10^4 CFU-GM and 2.1 x 10^4 BFU-E. This culture, which was not supplemented with additional HGF on day 14 generated 5.68 x 10^8 cells (2,706-FI) by day 21 containing 5.68 x 10^5 CFU-GM (24-FI). BFU-E increased to 5.4 x 10^4 on day 3 (2.6-FI) then decreased to 0.2 x 10^4 on day 5 and were not detected at or beyond day 7.

Figure 3.4 Nucleated cell and CFU-GM production in 50 ml Pre-CFU cultures

The pattern of cell production and differentiation in 3 large volume (50ml) Pre-CFU, suspension cultures of peripheral blood $CD34^+$ cells stimulated with IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF (each at 10 ng/ml). The vertical axis displays the fold increase (mean $\pm$ SEM) of nucleated cells and CFU-GM on each day of sampling, as shown on the horizontal axis.


10000
1000
100
10
1
Fold - Increase
CELLS
CFU-GM
0
3
6
9
12
15
18
21
24
Day of Culture

3.3.4 Large Volume Cultures: Serial Changes in Cell Type and Morphology

Figure 3.5 shows the proportion (mean ± SEM) of blasts, promyelocytes, myelocytes/metamyelocytes and monocytes/macrophages at each time point for the 3 cultures. The same pattern was seen in all 3 cultures. The mean percentage of blasts decreased from 100% at the start of culture to 87% on day 5, 39% on day 10, 17% on day 14 and 0.3% on day 21. Promyelocytes were slow to appear with 9% present on day 5 but thereafter increased to 30% on day 12 and peaked at 42% on day 17 before decreasing to 22% on day 21. The appearance of myelocytes and metamyelocytes lagged 2-3 days behind promyelocytes and continued to increase during culture to become the predominant (60%) cell type at day 21. Very few neutrophils (1-2%) were observed in any of the cultures and only on days 17 and 21. Monocytes or macrophages were not detected before day 7 but then increased in number to represent 21% and 19% of cells at days 17 and 21. The morphology of typical myeloblasts, promyelocytes, myelocytes, monocytes and macrophages at day 14 of culture is shown in figure 3.2. Megakaryocytes were rarely seen at days 3 or 5 but thereafter were present at low levels (2-5%) in all cultures. Megakaryocytes were confirmed by alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining of cytospin preparations using rabbit polyclonal sera to P-selectin (CD62P). An example of the typical appearance of megakaryocytic cells stained by P-selectin is shown in figure 3.6. These cells contained multiple nuclei and often exhibited cytoplasmic budding and an irregular cytoplasmic membrane edge, typical of mature megakaryocytes.

3.3.5 Large Volume Cultures: Serial Changes in Immunophenotype

Monitoring the serial expression of CD34, CD33, CD15 and CD11b antigens on cells during culture assessed myeloid cell differentiation. The same pattern of myeloid maturation was observed for each of the 3 cultures. The incidence of $CD34^+$ cells fell from 99% to 80% during the first five days, decreased rapidly to 1-2% by 12 to 14 days and could not be detected after this time. The incidence of cells expressing CD33 increased rapidly with more than 80% being $CD33^+$ by day 3 and day 7 in the UPN1197 and UPN522 cultures respectively. Thereafter expression of CD33 continued to increase with 100% and 96% of the UPN1197 and UPN522 cells being $CD33^+$ by day 14. Cells generated in culture progressively acquired both CD15 and CD11b antigens. The UPN522 cells cultured in the bag and the flask were 39% and 40% $CD15^+$ on day 7, 82% and 66% $CD15^+$ on day 14 and 90% and 85% $CD15^+$ at the end of culture respectively. The same cells were 42% and 46% $CD11b^+$ on day 7, 69% and 62% $CD11b^+$ on day 14 and 80% and 88% $CD11b^+$ at the end of culture in the bag and the flask respectively. On days 17 and 21 of each 50ml culture 98% of cells expressed either CD15 or

Figure 3.5 Characterisation of cells generated in 50 ml Pre-CFU cultures

The pattern of cell production and differentiation in 3 large volume (50 ml) Pre-CFU suspension cultures of peripheral blood $CD34^+$ cells stimulated with IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF (each at 10 ng/ml). The data is the mean $\pm$ SEM of the three cultures and depicts the proportion of blasts, promyelocytes (promye), myelocytes/metamyelocytes (mye/meta) and macrophages/monocytes (macro/mono) as assessed from Jenner-Giemsa and Esterase stained cytocentrifuge preparations.

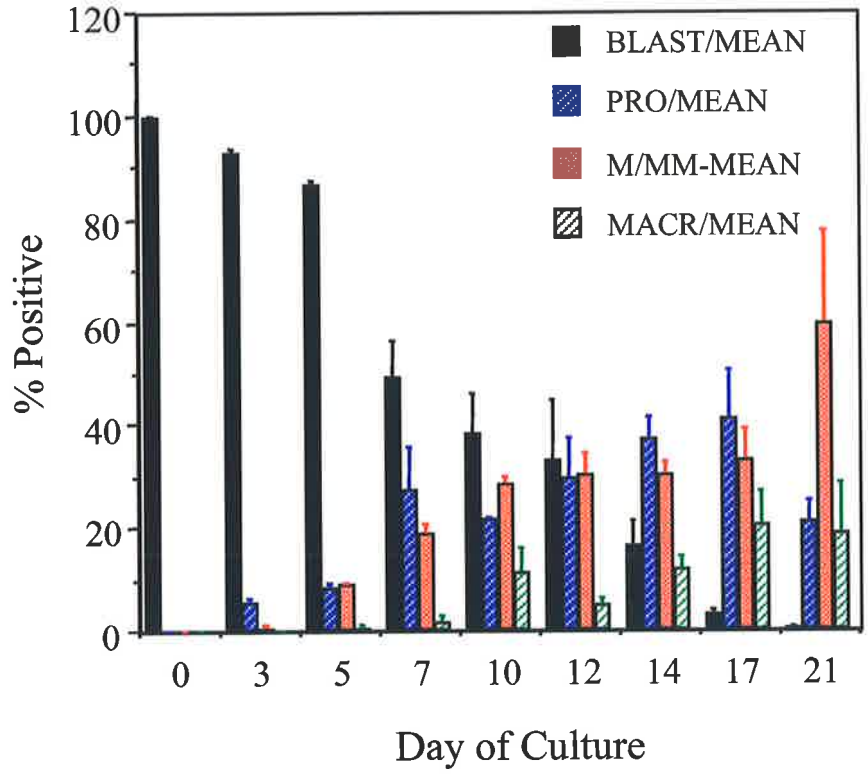
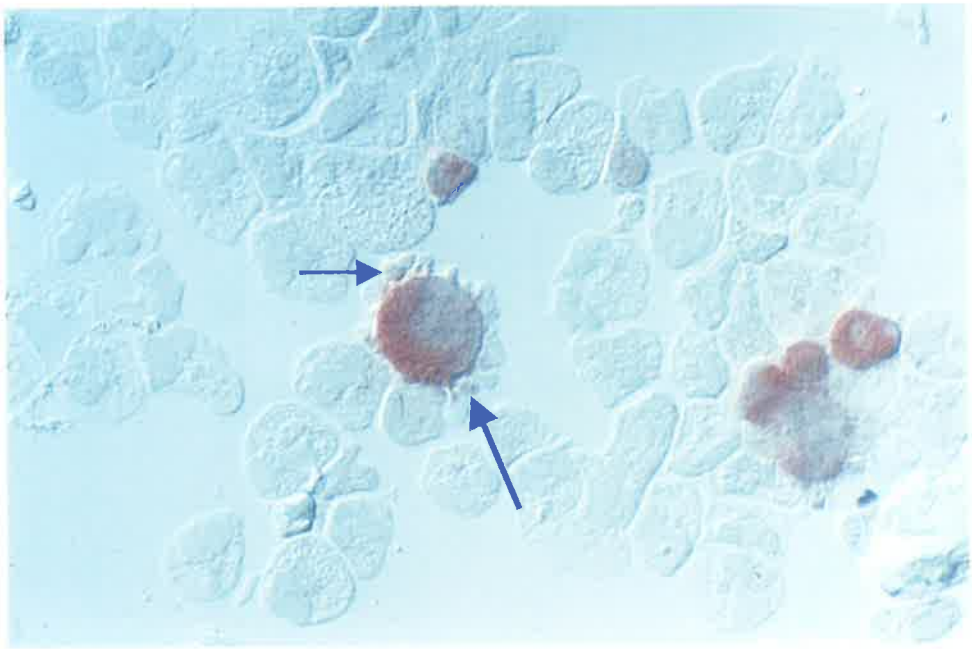

120
100
80
60
40
20
0
% Positive
BLAST/MEAN
PRO/MEAN
M/MM-MEAN
MACR/MEAN
0
3
5
7
10
12
14
17
21
Day of Culture

Figure 3.6 Immunocytochemistry for P-selectin in *ex vivo* generated cells

Mobilised blood $CD34^+$ cells were cultured in Pre-CFU medium containing 30% FCS and 1% BSA and supplemented with IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF (10 ng/ml of each). Cells were harvested at day 14, cytocentrifuge smears prepared then stained with the Anti-P-selectin (CD62-P) (Pharmingen Cat. No. 09361A): affinity-purified rabbit polyclonal to human P-selectin as described in the materials and methods. The cell in the centre of this photograph shows localisation of CD62-P to the cytoplasm and cell membrane pseudopodia (indicated by arrows), typical of megakaryocytic cells and platelet precursors. The photomicrograph is a Namarski interference contrast image taken of cells viewed at 60X magnification.



CD11b with more than 60% of cells expressing both antigens simultaneously. As a representative example of the pattern of cell maturation observed for the 3 cultures, the serial changes in the immunophenotype of cells generated in the UPN522 flask culture are shown in figure 3.7.

3.3.6 Large Volume Cultures: Functional Status of Cells

Both UPN522 and UPN1179 cells tested on day 14 consisted of blasts, promyelocytes and myelocytes but not neutrophils. These cells gave greater responses to arachidonic acid, fMLP and TPA than the control stimulus but less than that achieved from freshly prepared neutrophils (figure 3.8). Cells from the UPN522 bag culture consistently produced more superoxide anion in response to all agonists than UPN1179 cells.

3.4 Discussion

These studies demonstrated that a combination of 6 HGF namely, IL-1β + IL-3 + IL-6 + G-CSF + GM-CSF + SCF (136GGMS) produced a 29-39 fold increase in CFU-GM in a 7 day Pre-CFU assay of 1,000 peripheral blood $CD34^+$ cells collected during the recovery phase following high dose cyclophosphamide or after 5 days administration of G-CSF. Large volume suspension cultures with $CD34^+$ cells from the same patients, established with this 6-factor combination showed a parallel and exponential increase in both total nucleated cells and CFU-GM until day 10 (28 and 26 fold respectively). The number of CFU-GM peaked at day 14 (66 fold increase) and then remained stable until the end of culture. The number of nucleated cells increased exponentially, generating a 3-log increase by day 21 of culture. Cell production in this system consisted mainly of myeloid progenitor cells and neutrophil precursors which were morphologically normal and acquired myeloid antigens according to that described for neutrophil maturation in normal human bone marrow (Terstappen *et al* 1990). The functional maturity of these cells was further demonstrated by their ability to produce superoxide anion. Thus this combination of 6 HGF is capable of generating functional post progenitor cells and nascent myeloid progenitor cells.

The generation of large numbers of CFU-GM from $CD34^+$ cells under Pre-CFU culture conditions stimulated with a combination of 6 HGF is consistent with reports showing that primitive HPC require combinations of direct acting and synergistic HGF for maximal proliferation. Studies assessing the development of high proliferative potential CFU (HPP-CFU) and Blast-CFU in bone marrow from 24 hour post 5-FU treated mice have shown

Figure 3.7 Immunophenotype of cells generated from *ex vivo* culture of $CD34^+$ cells

Peripheral blood $CD34^+$ cells from UPN522 were cultured in a tissue culture bag with growth stimulated by IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF (each at 10 ng/ml). The immunophenotype of cells within the culture was assessed at the time points indicated. The graph depicts the proportion of viable cells expressing CD34, CD33, CD15 or CD11b at various times during *ex vivo* culture.

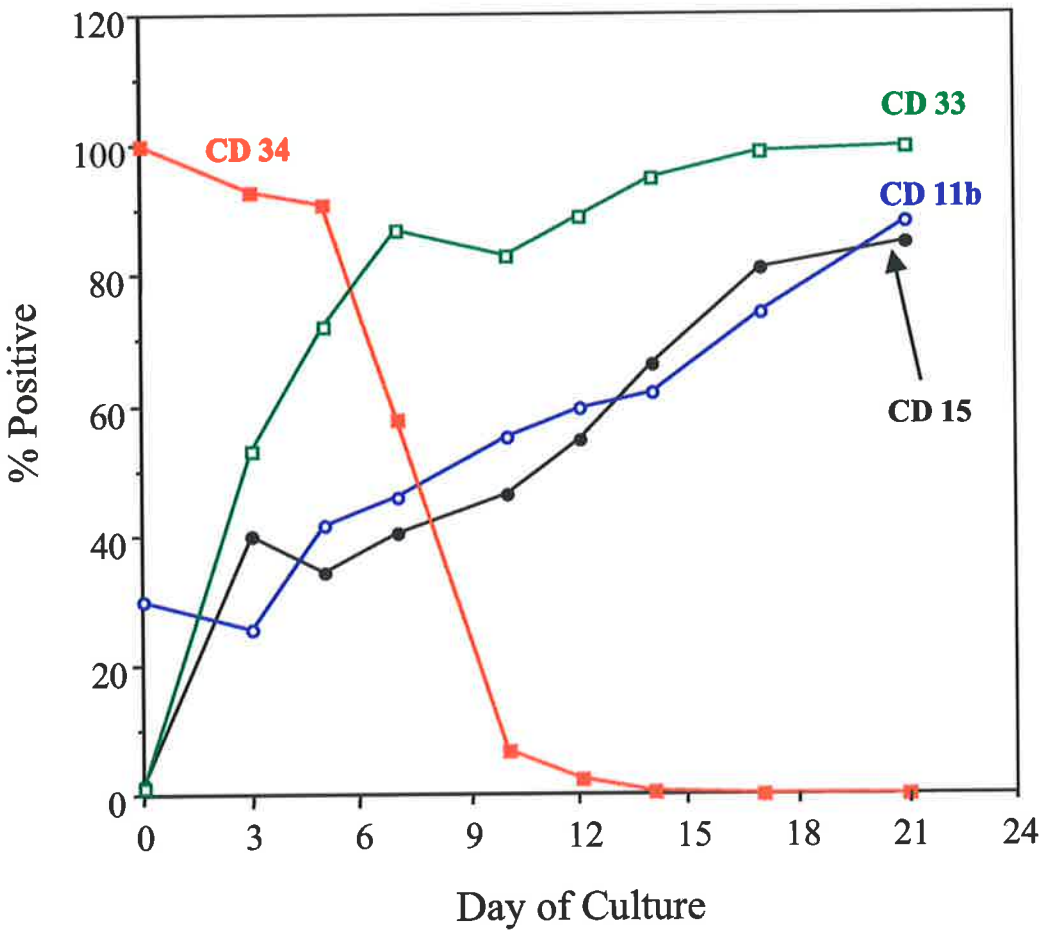
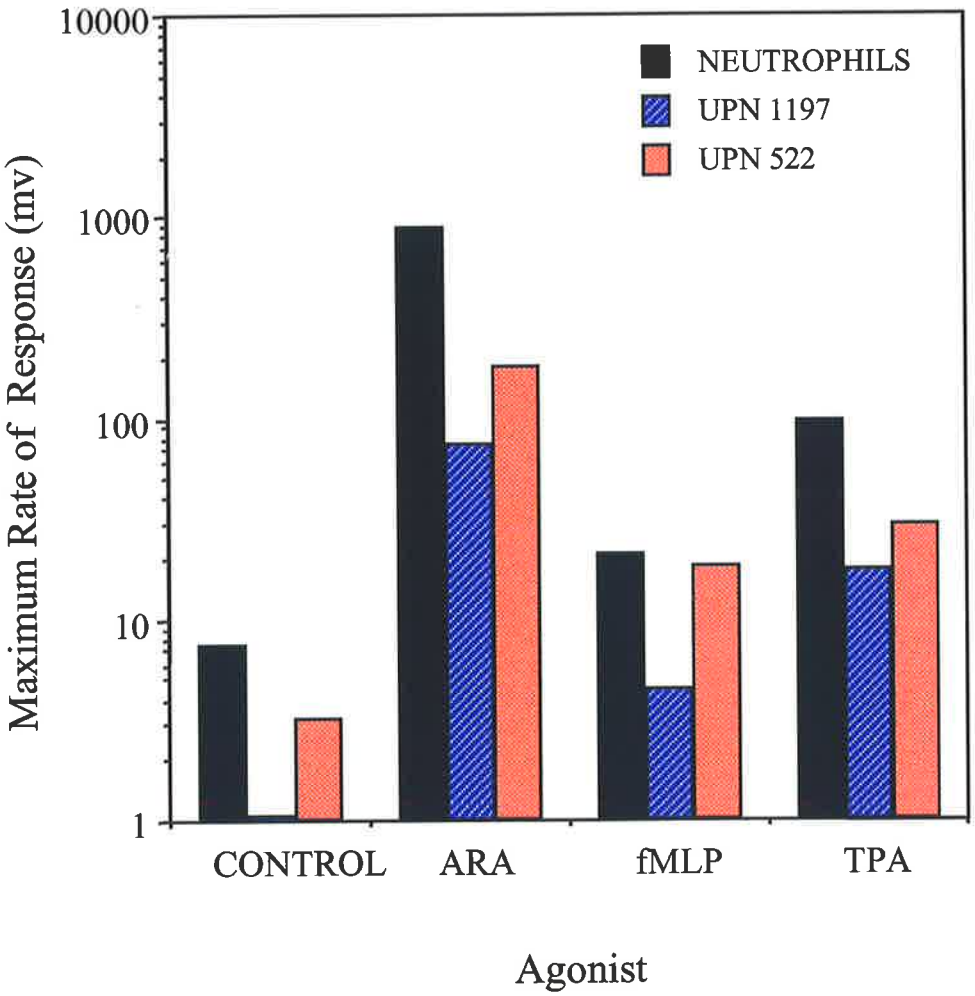

120
100
80
60
40
20
0
% Positive
CD 34
CD 33
CD 11b
CD 15
0
3
6
9
12
15
18
21
24
Day of Culture

Figure 3.8 Functional status of *ex vivo* generated myeloid cells

Function of cells generated in UPN522 bag and UPN1197 flask after 14 days of culture of peripheral blood $CD34^+$ cells stimulated by IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. The ability to produce superoxide anion in response to the phosphate buffer control or the agonists arachidonic acid (ARA: 33uM), N-formylmethionylleucylphenylalanine (FMLP: 5 x 10^{-6}M) and 12-O-tetradecanoylphorbol-13-acetate (TPA: 1 x 10^{-7}M) was measured by monitoring the chemiluminescence resulting from the oxidation of lucigenin and expressed as the maximum rate of response in millivolts. The response of cultured cells was compared to that of fresh neutrophils from a normal individual.


10000
1000
100
10
1
Maximum Rate of Response (mv)
NEUTROPHILS
UPN 1197
UPN 522
CONTROL
ARA
fMLP
TPA
Agonist

synergy between IL-1 and IL-3 (Ikebuchi *et al* 1987, Ikebuchi *et al* 1988) and with IL-3, G-CSF, GM-CSF and M-CSF (Moore and Warren 1987, Moore *et al* 1990). Further studies show that SCF synergises with IL-1, IL-3 or IL-6 in the generation of secondary HPP-CFU (Zsebo *et al* 1990) and that recombinant rat SCF synergises with three and four factor combinations of CSF-1, G-CSF, GM-CSF, IL-1alpha and IL-3 to increase HPP-CFU and total colony production by Sca-1^+ lin^- cells (Lowry *et al* 1991, Williams *et al* 1992). Short term Pre-CFU suspension cultures of murine bone marrow cells enriched for early stem cells also demonstrated that maximum output of both lineage restricted progenitors and HPP-CFU is achieved when the four factor combination of SCF, IL-1 and IL-6 with either IL-3 or GM-CSF are used (Moore 1991). In addition IL-3 and IL-6 were synergistic for human CFU-Blast colony formation from $CD34^+$ bone marrow cells (Leary *et al* 1988). Smith *et al* also reported that generation of secondary CFU-GM from 4-HC resistant $CD34^+$ human bone marrow cells was highest when IL-1α and IL-3 were used in a 7 day suspension culture (Smith *et al* 1991). HGF combinations including SCF, IL-3, GM-CSF, G-CSF and Erythropoietin are also synergistic in the stimulation of human progenitor cells of the myeloid (Broxmeyer *et al* 1991), erythroid (Bernstein *et al* 1991) and megakaryocytic (McNeice *et al*, 1991) lineages. It is thus not surprising that the combination of IL-1β+IL-3+IL-6+G-CSF+GM-CSF+SCF used in the large volume cultures of peripheral blood $CD34^+$ cells provides an excellent synergistic combination of HGF for the expansion and maturation of primitive and committed HPC, both those present initially and those generated during culture.

The large volume cultures showed two phases of growth. In the first 7-10 days there was a rapid increase in the total number of blasts, $CD34^+$ cells and CFU-GM with very few maturing myeloid cells. This suggests that the predominant effect of the 6-factor combination in the first phase of culture is recruitment of Pre-CFU and expansion of committed progenitor cell numbers. From 10 days onwards cell production continued exponentially with increasing numbers of mature myeloid cells at all stages of differentiation whereas the rate of CFU-GM production decreased. This suggests that the predominant effect in the second phase of growth is myeloid maturation although the pre and committed progenitor cell compartment is by no means exhausted. Even after 21 days in culture there were still a significant number (10-fold above the number in the input) of CFU-GM present (Fig 3.5), suggesting that pre-progenitors were present in the second week of culture. Definitive proof of the presence of pre-progenitors in the *ex vivo* stimulated cells will require secondary cultures of sorted $CD34^+$ cells because their frequency is likely to be too low to be detectable in culture of unsorted cells.

This study represents the first report of a systematic analysis of the effect of different HGF combinations on *ex vivo* expansion of mobilised blood $CD34^+$ cells (Haylock *et al* 1992). Two previous studies, based on culture of bone marrow reported that the combination of IL-1 and IL-3 was effective for the generation of secondary CFU-GM in either a 4 or 7 day suspension culture of unfractionated murine bone marrow (Iscove *et al* 1989) or 4-$HC^{resistant}/CD34^+$ human cells (Smith *et al* 1991) respectively. Iscove showed an 8-12 fold increase in CFU-GM over 4 days while Smith *et al* reported a secondary CFU-GM cloning efficiency of 0.2% from 1×10^5 cells generated after 7 days. In addition, Brandt *et al* measured total cell and CFU-GM production from $CD34^+DR^-CD15^-$ bone marrow cells in 1 ml suspension cultures stimulated by SCF and an IL-3/GM-CSF fusion protein (Brandt *et al* 1992). These cultures were fed additional growth factors and demi-depopulated every 48 hours and produced a mean 4,340 fold increase in total cells after 3 weeks. The present study shows that similar increases in total cells and CFU-GM can be achieved over 3 weeks using total unfractionated peripheral blood $CD34^+$ cells and with less frequent refeeding of HGF. This may be due to both synergism and a stronger proliferative stimulus provided by the 6-factor combination although it is also possible that PB $CD34^+$ cells have a higher proliferative potential. Peripheral blood $CD34^+$ cells mobilised by high dose cyclophosphamide or G-CSF have low CD71 expression (Bender *et al* 1991), are virtually all rhodamine 123^{dull} and in G_0 phase as assessed by staining with acridine orange (To *et al* 1994) indicating that they are comparable to primitive bone marrow $CD34^+$ cells.

The magnitude of cell and CFU-GM production and the pattern of myeloid maturation were similar for peripheral blood $CD34^+$ cells collected either during the recovery phase following high dose cyclophosphamide or during G-CSF administration. This result is not unexpected given the similar pattern of haemopoietic recovery following PBSC transplantation using cells collected from either of these stem cell mobilisation protocols (To *et al* 1989, Sheridan *et al* 1992).

The central aim of these studies was to determine whether *ex vivo* culture of peripheral blood $CD34^+$ cells could generate sufficient numbers of progenitor and post-progenitor cells to potentially abrogate cytopenia following transplantation. Peripheral blood stem cell (PBSC) transplantation is ideally suited to assess the efficacy of *ex vivo* cultured cells. Firstly, the rapid post graft haemopoietic recovery minimises the period of cytopenia to be covered by cultured cells to only 7-10 days. Secondly PBSC collections during the recovery phase following high

dose cyclophosphamide or during administration of G-CSF are a richer source of $CD34^+$ cells than bone marrow.

It was postulated that the cultured cells need to be capable of maintaining a neutrophil count of $>0.5 \times 10^9$/litre for 10 days following infusion and should contain enough progenitor and post-progenitors to maintain a steady supply of granulocytes from 1-3 days of infusion (Haylock *et al* 1992). Assuming a) a neutrophil half life of 6 hours, b) a blood volume of 5 litres and c) that neutrophil consumption is increased (doubled) in the neutropaenic state, then approximately 2×10^{11} neutrophils would be required over 10 days. Using the CFU-GM number as an index to determine the degree of *ex vivo* expansion and assuming that one day 14 CFU-GM gives rise to 500 mature granulocytes (based on 9 symmetric divisions in 14 days) then 4×10^8 CFU-GM would be required. After either cyclophosphamide or G-CSF mobilisation a single PBSC collection yields on average 5.0×10^6 CFU-GM. Thus a 80-fold ($4.0 \times 10^8 / 5.0 \times 10^6$) CFU-GM expansion from one PBSC collection should provide enough precursors to maintain the target neutrophil production. Alternatively, the number of promyelocytes or myelocytes generated by the *ex vivo* culture of $CD34^+$ cells could be used as a target. This may be more appropriate as these cells are the immediate precursors to mature neutrophils, although defining the actual number required to maintain the desired neutrophil count is just as empirical as using CFU-GM generation as an index of *ex vivo* expansion and maturation. Irrespective of how a target is determined the *ex vivo* cultured cell suspension needs to be rich in blasts, promyelocytes and myelocytes to produce neutrophils rapidly. The described culture system generates this mix of cells between days 14-17 and the magnitude of CFU-GM expansion (mean of 66) is close to the calculated target. Furthermore these *ex vivo* cultured cells may also be effective in reducing the period of thrombocytopenia since between 2 and 8% of cells are megakaryocytic on day 14 of culture.

A number of issues need to be considered before full-scale clinical application of this approach. Firstly, since it is possible that *ex vivo* cultured cells will not provide long-term marrow repopulating ability, only a proportion of PBSC should be cultured. These cells could then be infused with the remaining unmodified PBSC or bone marrow to ensure that long-term marrow reconstitution is not compromised. Secondly, it will be impractical to FACS sort enough $CD34^+$ cells to initiate clinical scale *ex vivo* cultures and therefore, methods for large scale enrichment of $CD34^+$ cells to high purity and high recovery (yields) will need to be used developed. Thirdly, it will be important to determine the optimal system for large volume cultures. A number of parameters including starting cell density, refeeding with HGF or

medium and the source or requirement for serum is likely to affect cell growth. Whether flasks or bags are used for culture may also influence yields although the present studies show that nucleated cell and CFU-GM generation is similar for 50ml volumes. Fewer adherent cells were observed in the life cell bag, which may result in decreased activation of maturing cells and contribute to better viability and function after infusion. In addition, greater cell production may be achieved by using rapid medium perfusion, as recently shown to be effective in long-term *in vitro* Dexter type cultures of human bone marrow (Schwartz *et al* 1991).

Despite these practical issues, which may lead to further optimisation of *ex vivo* cultures, the method described here clearly demonstrates the feasibility of this approach as an adjunct to haemopoietic stem cell transplantation. There is great potential for the generation of mature functional cells from all lineages by using different combinations of HGF and cytokines. It is of note that since the results presented in this chapter were published (Haylock et 1992), a number of clinical studies have since demonstrated that infusion of *ex vivo* generated cells may improve haemopoietic recovery (Williams *et al* 1996, Reiffers *et al* 1999, McNiece *et al* 1999). These studies, described and discussed in detail in chapter 7 essentially validate my original hypothesis that infusion of *ex vivo* generated neutrophil precursor cells will improve the rate of neutrophil recovery following blood stem cell auto-transplantation.

CHAPTER 4. OPTIMISING *EX VIVO* CULTURE CONDITIONS FOR GENERATION OF NEUTROPHIL PRECURSORS

4.1 Introduction

The studies described in Chapter 3 demonstrated that a combination of 6 HGF, comprising IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF (136GGMS) was superior to any other single HGF or combination of HGF in supporting generation of myeloid progenitor cells (CFU-GM) from mobilised blood $CD34^+$ cells following 7 days of culture. Additional studies with 50 ml cultures suggested that *ex vivo* culture of $CD34^+$ cells with the HGF combination of 136GGMS has the potential to generate sufficient neutrophil precursors to be of clinical benefit in augmenting haemopoietic recovery. However, a number of issues are raised by the data reported in chapter 3. An important issue is in regard to the apparent requirement for 6 recombinant cytokines to achieve optimal expansion of myeloid cell numbers *in vitro*. What is the contribution of each of the 6 HGF in *ex vivo* generation of neutrophil precursors from $CD34^+$ cells? Could any of the growth factors be omitted without compromising growth of $CD34^+$ cells? In addition, could growth of $CD34^+$ cells be improved by altering either the concentrations of HGF, the concentration of $CD34^+$ cells or by re-feeding cultures with fresh media and HGF? The present chapter addresses a number of these questions and provides data towards optimisation of HGF mediated *ex vivo* generation of neutrophil precursors from $CD34^+$ cells.

4.2 Experimental Design and Methods

Experiments to determine if further improvements in production of neutrophil precursors could be achieved were conducted as follows. Firstly, studies were performed to establish the contribution of each of the six HGF in generation of maturing myeloid cells and CFU-GM from $CD34^+$ cells. A series of experiments were performed to examine the consequences of omission of each of the single HGF in turn from the 6HGF combination. These studies identified 4 HGF (IL-3, IL-6, G-CSF and SCF: 36GS) as the most important and suggested that omission of IL-1 and or GM-CSF would not compromise cell generation. Based on the results of these initial experiments, dose response studies were performed for IL-3, IL-6, G-CSF and SCF and finally a dose response matrix was tested for G-CSF and

SCF. This series of studies suggested that the combination of 36GS could support the generation of an equivalent number of nascent CFU-GM and neutrophil precursors from $CD34^+$ cells to that achieved by cultures stimulated with the 6 HGF combination of 136GGMS. This possibility was subsequently tested. In the course of these studies it became apparent that other variables, apart from the choice and concentration of HGF, affect the *ex vivo* expansion of $CD34^+$ cells. The latter sections of this chapter discuss how the initial concentration of $CD34^+$ cells affects growth, how growth in static cultures is limited by cell density, and how pseudo-perfusion affects growth. Collectively, the experiments presented in this chapter provide a systematic basis for selection of HGF needed for *de novo* generation of neutrophil precursors from $CD34^+$ cells and also describe the major issues affecting large scale cultures for the clinic.

4.2.1 Source of Cells

$CD34^+$ cells were obtained from either fresh or cryopreserved apheresis collections from patients undergoing therapeutic BSC mobilisation induced by myelosuppressive chemotherapy with or without HGF or by administration of HGF alone. One patient (UPN659) with carcinoma of ovary received 7 gms/m^2 of cyclophosphamide as previously reported (To *et al* 1990a). One patient (UPN1543) with stage 2 carcinoma of the breast (Ca-Br) received recombinant human G-CSF (Filgrastim, Amgen, Thousand Oaks, Ca) at 12 µg/kg/day in a continuous 24-hour subcutaneous infusion over 6 days as previously reported (Sheridan *et al* 1992). A second patient (UPN1520) with Ca-Br received IL-3 at 5 µg/kg/day for 7 days and GM-CSF at 5 µg/kg/day from day 5 to day 14. Two patients (UPN 1387, 1394) with non-hodgkins lymphoma received DHAP (Philip *et al* 1991) and G-CSF at 5 µg/kg/day as a subcutaneous injection. Three patients (UPN1472, 1444, 1534) with Ca-Br received cyclophosphamide 4 gm/m^2 with epirubicin 200 mg/m^2 and GM-CSF at 5 µg/kg/day as a subcutaneous injection. The details of apheresis and cryopreservation have been reported previously (To *et al* 1990a) and are described briefly in the materials and methods.

4.2.2 Processing and Isolation of Cells for Study

Cryopreserved peripheral blood mononuclear cells (PBMNC) from apheresis collections were thawed at 37^0C, washed, immunolabelled with anti-CD34 antibody HPCA-2-PE (or isotype matched non-binding control) and $CD34^+$ cells isolated by FACS as described in the materials and methods. $CD34^+$ cells were similarly obtained from fresh apheresis collections following lymphoprep separation, immunolabelling and FACS. As with the experiments

described in Chapter 3, $CD34^+$ cells within the lymphocyte/blast region (Andrews *et al* 1989) were collected after comparing the staining obtained with HPCA-2-PE with that observed with the IgG_1-PE control antibody. In all experiments the $CD34^+$ cells could be discriminated from non-labelled cells on the Fluorescence 2 vs side scatter dot-plot. Post sort purity checks confirmed that 98.7 ± 1.2% of cells collected were $CD34^+$.

4.2.3 Pre-progenitor (Pre-CFU) Culture

The method for Pre-CFU culture is described in Chapter 2 and was published in 1992 (Haylock *et al* 1992). Briefly, 500-1500 $CD34^+$ cells obtained by FACS were re-suspended in 1 ml of Pre-CFU medium together with the appropriate HGF in 24 well tissue culture plates. Interleukin-1 beta (IL-1β), IL-3, IL-6, G-CSF, GM-CSF and Stem Cell Factor (SCF) were all supplied by Amgen (Thousand Oaks, CA) and unless otherwise stated were used at 10 ng/ml. In most experiments, six replicate 1 ml cultures were prepared for each variable tested. Three cultures were harvested on day 7, the remaining 3 were refed with HGF then harvested on day 14 and, if required, a proportion of the cells from each replicate re-cultured for another 7 days for harvest on day 21. After incubation at 37^0C in 5% CO_2 for the prescribed time the contents from each well were re-suspended and diluted in IMDM. The number of viable nucleated cells was determined using trypan blue staining and counting with a haemocytometer under phase contrast microscopy. Cell morphology was assessed on Jenner-Giemsa (BDH Ltd, Poole, UK) stained cytocentrifuge preparations (Dacie 1984). Specific and non-specific esterase staining was performed in parallel on replicate slides and at least 300 cells were examined at 1,000X under oil immersion. Ten percent of the cells present on days 7, 14 and 21 were plated in triplicate in a day 14 CFU-GM assay to determine the number of CFU-GM generated from the input $CD34^+$ cells.

4.2.4 Clonogenic Assays

Myeloid progenitor (CFU-GM) and burst-forming unit-erythroid (BFU-E) assays were performed as described in Chapter 2. Briefly, triplicate 1 ml cultures were established in 35mm plates in 0.9% methylcellulose in IMDM supplemented with 30% FCS and 3mmol/L L-glutamine. Cultures were stimulated with 10 ng of each of IL-1, IL-3, IL-6, G-CSF, GM-CSF, SCF and 4 I.U. of erythropoietin per plate. After 14 days of incubation at 37^0C in 5% CO_2, CFU-GM and BFU-E were scored using a dissecting microscope and standard criteria for their identification as described in section 2.3.2. The absolute number of CFU-GM and BFU-E generated in a Pre-CFU assay was determined by scoring 3 plates from each of the triplicate wells then correcting for the cell dilution performed before the clonogenic assay

and the total number of nucleated cells present in Pre-CFU culture.

4.2.5 Large Scale Cultures

An entire apheresis collection from each of two patients (UPN's 659 & 1472) was processed on the CEPRATE device to enrich $CD34^+$ cells. The CEPRATE device isolates $CD34^+$ cells by an avidin-biotin immunoadsorption system. In brief, cells were separated over a lymphoprep density gradient, the mononuclear cells at the interface collected, washed by centrifugation 3 times to remove platelets then incubated with the biotinylated anti-CD34 monoclonal antibody 12.8. The cell suspension was washed twice then MNC were processed on the CEPRATE device according to the manufacturer's recommendation (Heimfeld 1992). The percentage of $CD34^+$ cells present in the adsorbed cell fraction was determined after incubating cells with HPCA-2-PE then analysis on a Profile II flow cytometer (Coulter, Hialea, Florida). A proportion of the isolated $CD34^+$ cells from each of the "stem" cell fractions were cultured in a 3 litre PL732 life cell flask (Baxter Healthcare Corporation, Deerfield, IL) at 5,000 cells/ml in 1 litre of Pre-CFU media. Cultures were stimulated with IL-3 (10 ng/ml), IL-6 (10 ng/ml), G-CSF (100 ng/ml) and SCF (100 ng/ml) and incubated at 37^0C in 5% CO_2 for 14 days without addition of fresh media or HGF.

4.2.6 Statistics

Where appropriate, Anova, two-tailed t-tests and Fisher's PLSD were applied to data using the Statview 4.0 analysis software. A 5% significance level was set for tests.

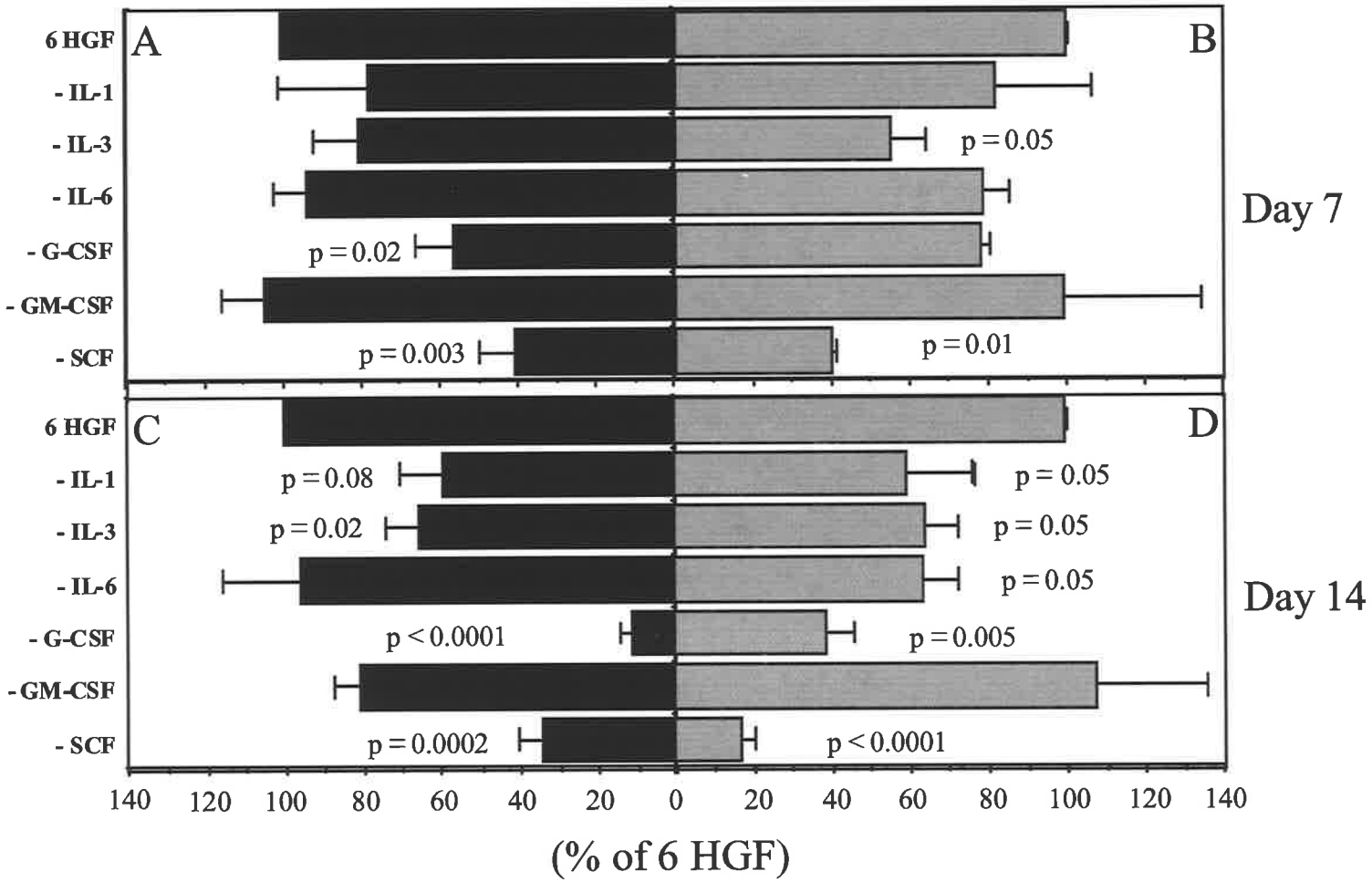
4.3 Results

4.3.1 Contribution of HGF in Culture of PB $CD34^+$ cells

In order to assess the relative contribution of the six cytokines in 6 HGF, PB $CD34^+$ cells were placed into Pre-CFU cultures and their growth stimulated either by the addition of 6 HGF or with various combinations of 5 HGF. The latter comprised six combinations of 5 HGF in which each of the six component cytokines were omitted in turn from 6 HGF. Cultures were assayed for total cell and CFU-GM production at day 7, and 14 (Fig 4.1). At day 7, PB $CD34^+$ cells cultured without G-SCF or SCF produced significantly less nucleated cells ($p = 0.02$ and 0.003 respectively) than cultures stimulated with 6 HGF. Omission of IL-1, IL-3, or IL-6 from the 6 HGF combination resulted in decreased nucleated cell production although this was not statistically significant. CFU-GM production from PB $CD34^+$ after 7 days was maximal with 6 HGF but was reduced significantly by the omission of IL-3 or SCF

Figure 4.1 Effect of individual HGF on growth of $CD34^+$ cells

Production of nucleated cells (panels A & C) and CFU-GM (panels B &D) from 1,000 PB $CD34^+$ cells grown in Pre-CFU cultures with 6 HGF (IL-1, IL-3, IL-6, G-CSF, GM-CSF, SCF) or 5 HGF combinations (the HGF omitted from 6 HGF is indicated on the axis). The graph shows the mean fold increase ($\pm$ SEM, n=3) in cells and CFU-GM at day 7 and 14 as a proportion of that generated with 6 HGF. The p values refer to the significant difference in growth between the respective 5 HGF combination and the 6 HGF combination. At day 14, a mean of 1.25 x 10^6 nucleated cells (range 1.15 - 1.4 x 10^6) and 7,664 CFU-GM (range 2,937 - 10,825) were generated from 1,000 $CD34^+$ cells when cultured with 6 HGF. Cells from patients UPN659, UPN1543 and UPN1520 were used for these experiments.


6 HGF
- IL-1
- IL-3
- IL-6
- G-CSF
- GM-CSF
- SCF
A
B
p = 0.05
p = 0.02
p = 0.003
p = 0.01
Day 7
C
D
p = 0.08
p = 0.05
p = 0.02
p = 0.05
p = 0.05
p < 0.0001
p = 0.005
p = 0.0002
p < 0.0001
Day 14
140 120 100 80 60 40 20 0 20 40 60 80 100 120 140
(% of 6 HGF)

($p< 0.05$ and < 0.01 respectively) but not by the omission of other single HGF. This data is also in agreement with the data presented in figure 3.3, where CFU-GM generation from $CD34^+$ cells was assessed for different HGF combinations.

After 14 days, PB $CD34^+$ cells cultured without IL-1, IL-3, G-CSF or SCF yielded significantly less nucleated cells ($p = 0.08$, 0.02, <0.0001 and 0.0002 respectively) compared to the 6 HGF cultures. In contrast, the omission of either IL-6 or GM-CSF from the 6 HGF combination did not result in a significant reduction in the number of nucleated cells generated. In accord with the nucleated cell data, omission of IL-1, IL-3, IL-6, G-CSF or SCF resulted in a significant reduction of CFU-GM ($p = 0.006$, 0.014, 0.01, 0.003 and <0.001 respectively) compared to the 6 HGF cultures. Omission of GM-CSF resulted in a slight increase in CFU-GM over that observed with 6 HGF, after both 7 and 14 days of culture.

These results suggested that significant growth of $CD34^+$ cells could be achieved with less than 6 HGF, provided at least G-CSF, SCF and IL-3 were included in the HGF combination. In addition, the pattern of myeloid cell maturation and the proportion of myeloblasts, promyelocytes and metamyelocytes present at day 14 were very similar in each 5 HGF combination with the exception of those cultures containing GM-CSF or IL-1 which always had a greater number of monocytes and macrophages. Since the main purpose of this study was to optimise culture conditions for generation of neutrophil precursors, further experiments were performed to investigate whether the combination of IL-3, IL-6, G-CSF and SCF (4 HGF) could generate qualitatively and quantitatively equivalent cell populations from PB $CD34^+$ cells to those stimulated with 6 HGF. A series of studies were therefore initiated in order to investigate this possibility. Firstly, HGF dose response experiments were performed to determine the optimal concentrations for each of the 4 HGF ie IL-3, IL-6, G-CSF and SCF.

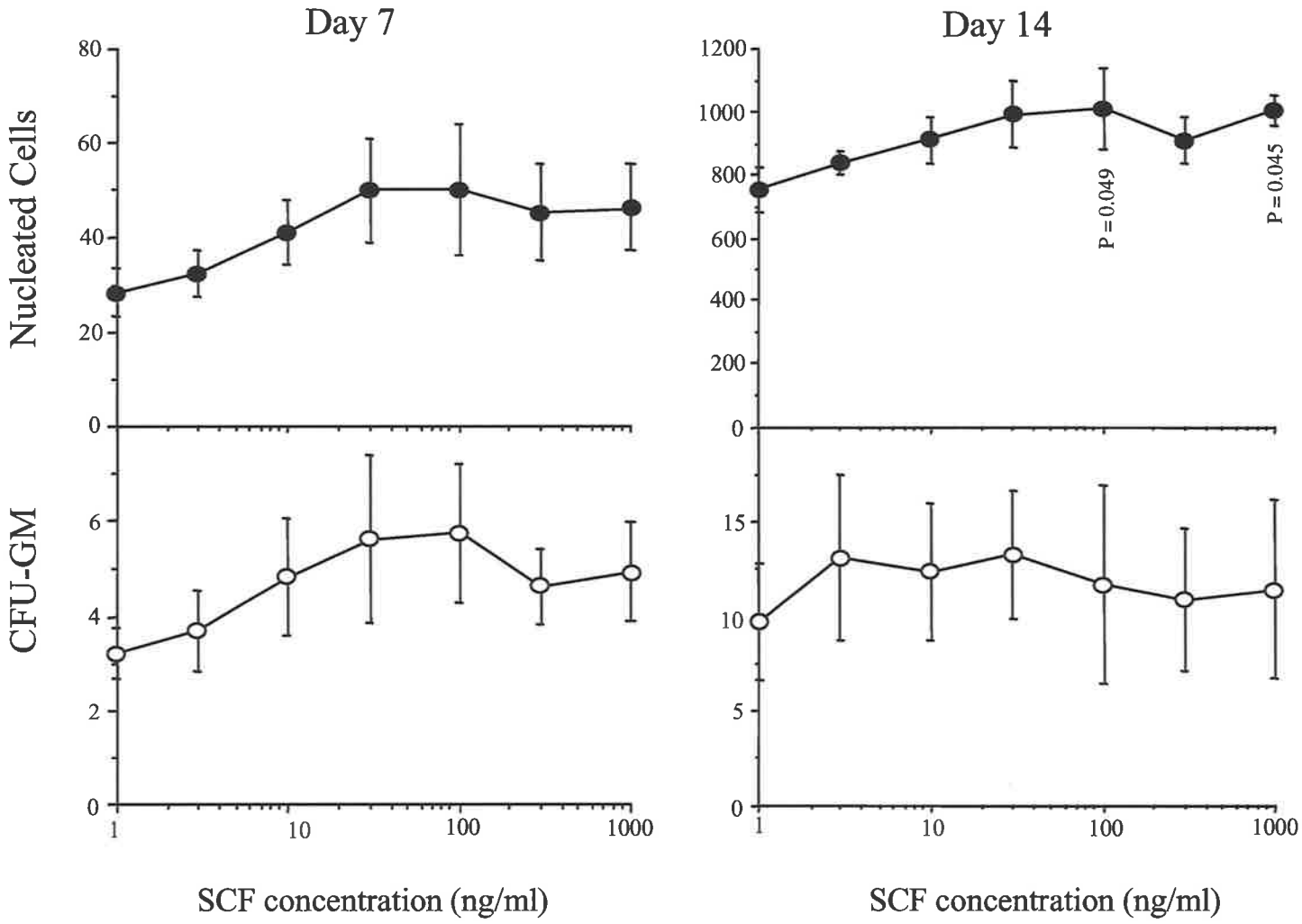
4.3.2 4 HGF (IL-3, IL-6, G-CSF, SCF): Optimisation of Concentrations

4.3.2.1 SCF

The previous "omission" experiments indicated that omission of SCF from the 6 HGF combination resulted in the greatest reduction in generation of nucleated cells and CFU-GM. Accordingly, SCF was the first cytokine to be titrated. Cultures of $CD34^+$ cells were initiated with concentrations of G-CSF, IL-3 and IL-6 at 10 ng/ml while SCF was added over

Figure 4.2 Dose response for stem cell factor (SCF) in Pre-CFU culture

Cultures of 1,000 PB $CD34^+$ cells (UPN: 1387, 1394, 1444) were established with 10 ng/ml of G-CSF, IL-3 and IL-6 and increasing concentrations of SCF over the range of 1 ng/ml to 1 µg/ml. These cultures were initiated with Pre-CFU medium containing 30% FBS and 1% BSA. The mean fold increase (± SEM, n=3) in cells and CFU-GM at day 7 and 14 are shown for each concentration of SCF. The p values represent significant difference from cells grown in 1 ng/ml of SCF.


Day 7
Day 14
Nucleated Cells
CFU-GM
80
60
40
20
0
1200
1000
800
600
400
200
6
4
2
15
10
5
P = 0.049
P = 0.045
1
10
100
1000
SCF concentration (ng/ml)
SCF concentration (ng/ml)

the range of 1 ng/ml to 1 μg/ml. The number of nucleated cells and CFU-GM present after 7 and 14 days of culture were recorded in 3 separate experiments using different sources of PB $CD34^+$ cells. The results of these studies are shown in Figure 4.2. At day 7, the number of nucleated cells generated from 1,000 PB $CD34^+$ cells ranged from 20,700 to 76,500. There was no significant difference in nucleated cell production at the SCF concentrations tested. Maximal cell production occurred at SCF concentrations greater than 30 ng/ml. A similar trend was observed for CFU-GM at day 7 and no single SCF concentration was significantly better than any other. At day 14, PB $CD34^+$ cells cultured in either 100 ng/ml or 1000 ng/ml of SCF generated significantly more nucleated cells than those cultured in 1 ng/ml ($p = 0.049$ and $p = 0.045$ respectively), but not significantly more than those cultured in 10 ng/ml or 30 ng/ml. CFU-GM production at day 14 was not significantly different between any SCF concentrations. Based on these data a concentration of 100 ng/ml was chosen for SCF in all subsequent experiments.

4.3.2.2 G-CSF

Titration of G-CSF was performed in an analogous manner to that described for titration of SCF with IL-3, and IL-6 at 10 ng/ml and SCF at 100 ng/ml. As shown in figure 4.3 there was a trend towards greater cell numbers with increasing concentrations of G-CSF. However this was only significant on day 14 where the nucleated cell production was greater at the 100 ng/ml dose than with 1.0 ng/ml G-CSF ($p = 0.03$). These data also demonstrated that there was no benefit in terms of increasing production of nucleated cells from using G-CSF at concentrations greater than 100 ng/ml. Similarly the fold increase of CFU-GM at day 7 or day 14 was independent of G-CSF concentration. In fact, there was a trend toward decreased generation of CFU-GM after 14 days with G-CSF concentrations above 3 ng/ml. Based on these data, unless otherwise stated G-CSF was used at 100 ng/ml for subsequent experiments.

4.3.2.3 IL-3

The effect of IL-3 concentration was similarly determined in 3 separate experiments by titration of IL-3 from 0.1 ng/ml to 300 ng/ml. For these studies IL-6 was used at 10 ng/ml and both G-CSF and SCF at 100 ng/ml. The maximum number of cells at day 7 was obtained with IL-3 at 100 ng/ml (2 experiments) or 10 ng/ml. At day 14 the maximum number of cells generated in the 3 experiments occurred at different IL-3 concentrations ie 10, 30 and 300 ng/ml. Overall, nucleated cell production was proportional to IL-3 concentration up to 10 ng/ml (Fig 4.4). There were significantly more nucleated cells

Figure 4.3 Dose response for granulocyte colony-stimulating factor (G-CSF) in Pre-CFU culture

The figure shows the dose response for G-CSF under Pre-CFU culture conditions with medium containing 30% FBS and 1% BSA. Cultures of PB $CD34^+$ cells (UPN: 1387, 1394, 1472) were established with 10 ng/ml of IL-3 and IL-6, 100 ng/ml of SCF and increasing concentrations of G-CSF over the range of 1 ng/ml to 300 ng/ml. The mean fold increase ($\pm$ SEM, n=3) in cells and CFU-GM at day 7 and 14 are shown for each concentration of G-CSF.

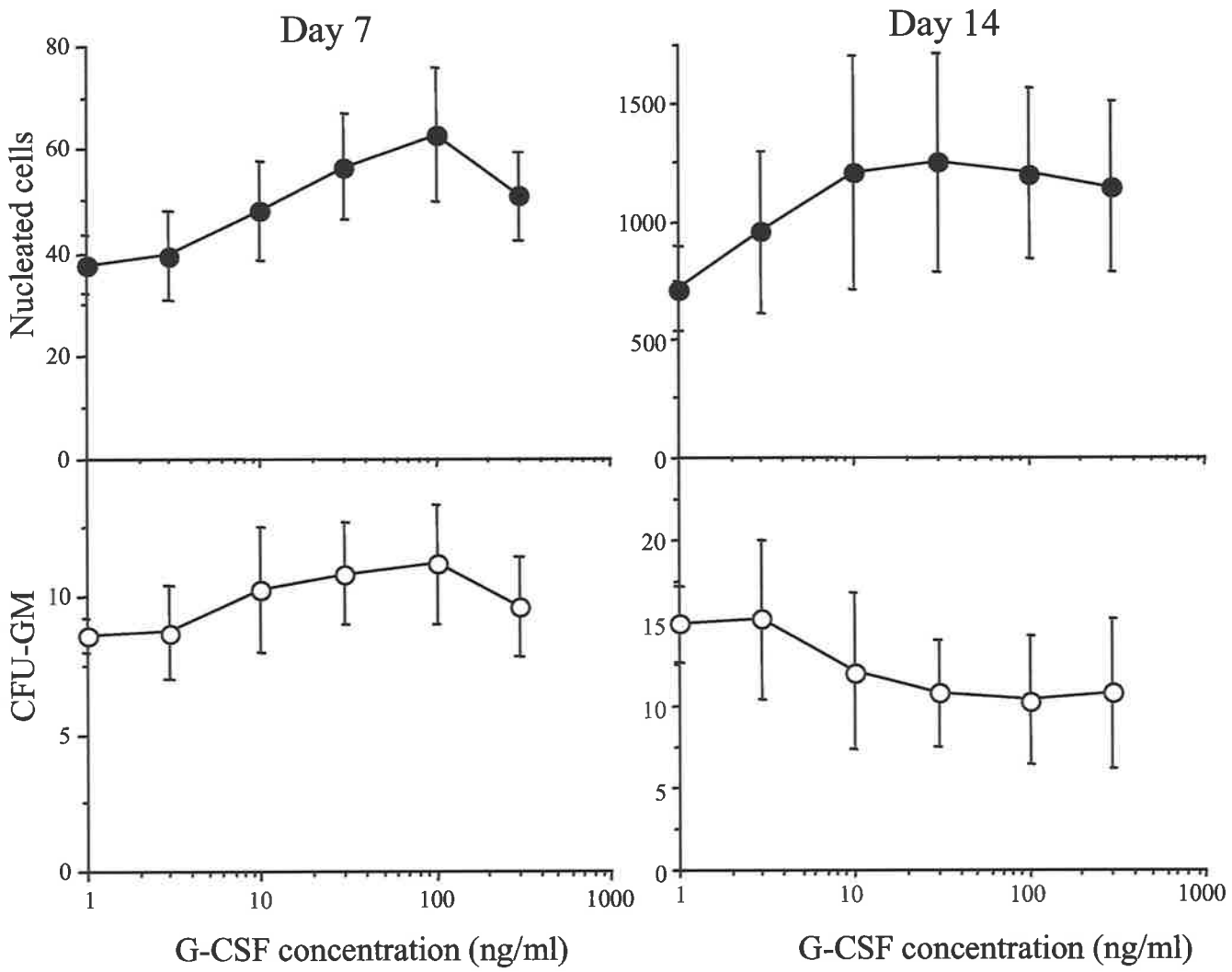
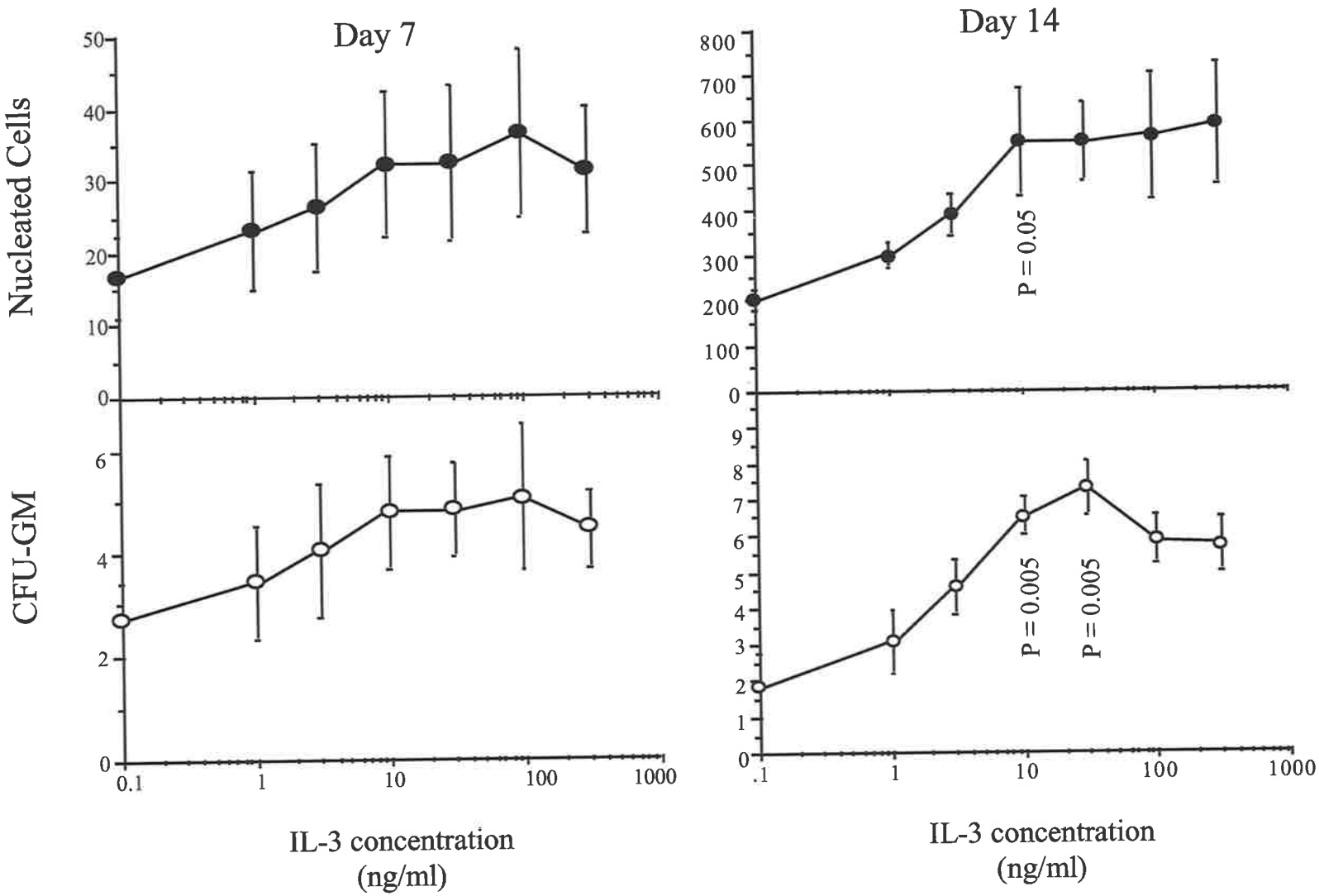

Day 7
Day 14
Nucleated cells
CFU-GM
80
60
40
20
0
1500
1000
500
10
5
15
1
10
100
1000
G-CSF concentration (ng/ml)
G-CSF concentration (ng/ml)

Figure 4.4 Dose response for interleukin-3 (IL-3) in Pre-CFU culture

Dose response for IL-3 under Pre-CFU culture conditions with medium containing 30% FBS and 1% BSA. Cultures of PB $CD34^+$ cells (UPN: 1472, 1444, 1534) were established with 10 ng/ml of IL-6, and 100 ng/ml of SCF and G-CSF and increasing concentrations of IL-3 over the range of 0.1 to 300 ng/ml. The mean fold increase ($\pm$ SEM, n=3) in cells and CFU-GM at day 7 and 14 are shown for each concentration of IL-3.


Day 7
Day 14
Nucleated Cells
CFU-GM
50
40
30
20
10
0
800
700
600
500
400
300
200
100
0
6
4
2
0
9
8
7
6
5
4
3
2
1
0
P = 0.05
P = 0.005
P = 0.005
0.1
1
10
100
1000
.1
1
10
100
1000
IL-3 concentration
(ng/ml)
IL-3 concentration
(ng/ml)

generated at 10 ng/ml than 1 or 3 ng/ml of IL-3 ($p = 0.05$). Notably, nucleated cell production did not increase when IL-3 concentrations of greater than 10 ng/ml were used. A similar pattern was observed for CFU-GM production at day 14. At 10 ng/ml and 30 ng/ml significantly ($p = 0.005$) more CFU-GM were produced than at 1 ng/ml of IL-3 but there was no increase in CFU-GM production at higher concentrations. It was therefore concluded that addition of IL-3 at 10 ng/ml was sufficient for growth of PB $CD34^+$ cells under the Pre-CFU culture conditions described.

4.3.2.4 IL-6

The effect of IL-6 concentration was similarly determined in 3 separate experiments by using IL-6 at concentrations of 0.01, 0.1, 1, 5, 10, 20, 40 and 100 ng/ml. For these studies IL-3 was used at 10 ng/ml and both G-CSF and SCF at 100 ng/ml. The results are shown in figure 4.5. There was no significant difference in generation of nucleated cells or CFU-GM at either day 7 or day 14 at the concentrations of IL-6 tested. This suggested that with "optimised" concentrations of IL-3, G-CSF and SCF then the concentration IL-6 does not influence *ex vivo* expansion of neutrophil precursors from $CD34^+$ blood cells. However, for all subsequent cultures IL-6 was used at 10ng/ml, a concentration often used by others (Rebel *et al* 1994, Tsujino *et al* 1993) for clonogenic and suspension culture of haemopoietic cells.

4.3.2.5 Cross Titration of G-CSF and SCF

The dose response experiments performed above were designed to determine the effect of changing the concentration of one HGF while the concentrations of others were kept constant. This approach is limited in that it may not identify concentrations of HGF that result in synergistic interactions between HGF and increased growth of $CD34^+$ cells. Given the major importance of G-CSF and SCF indicated by the previous omission experiments, the possible interactions between these 2 factors was therefore investigated by means of a series of concentration matrix experiments. In these assays, G-CSF and SCF were used at concentrations of 10 ng, 30 ng and 100 ng/ml while IL-3 and IL-6 were provided at 10 ng/ml. The results at day 7 and day 14 were similar (Fig. 4.6). At day 7 significantly more nucleated cells were produced in cultures where G-CSF was used at 100 ng/ml rather than 30 or 10 ng/ml (p values ranged from .0036 to .0203, Fisher's PLSD, comparing growth with 100 ng SCF + 100 ng G-CSF). Maximal CFU-GM production at day 7 occurred with 100 ng/ml of both SCF and G-CSF. All other G-CSF/SCF concentration combinations yielded significantly less CFU-GM (p values ranged from .0003 to .0413, Fisher's PLSD). At day 14

Figure 4.5 Dose response for interleukin-6 (IL-6) in Pre-CFU culture

Dose response for IL-6 under Pre-CFU culture conditions with medium containing 30% FBS and 1% BSA. Cultures of PB $CD34^+$ cells were initiated with IL-3 at 10 ng/ml and both G-CSF and SCF at 100 ng/ml and increasing concentrations of IL-6 over the range of 0.001 ng/ml to 100 ng/ml. The mean fold-increase increase ($\pm$ SEM, n=3) in cells and CFU-GM at day 7 and 14 are shown for each concentration of IL-6.

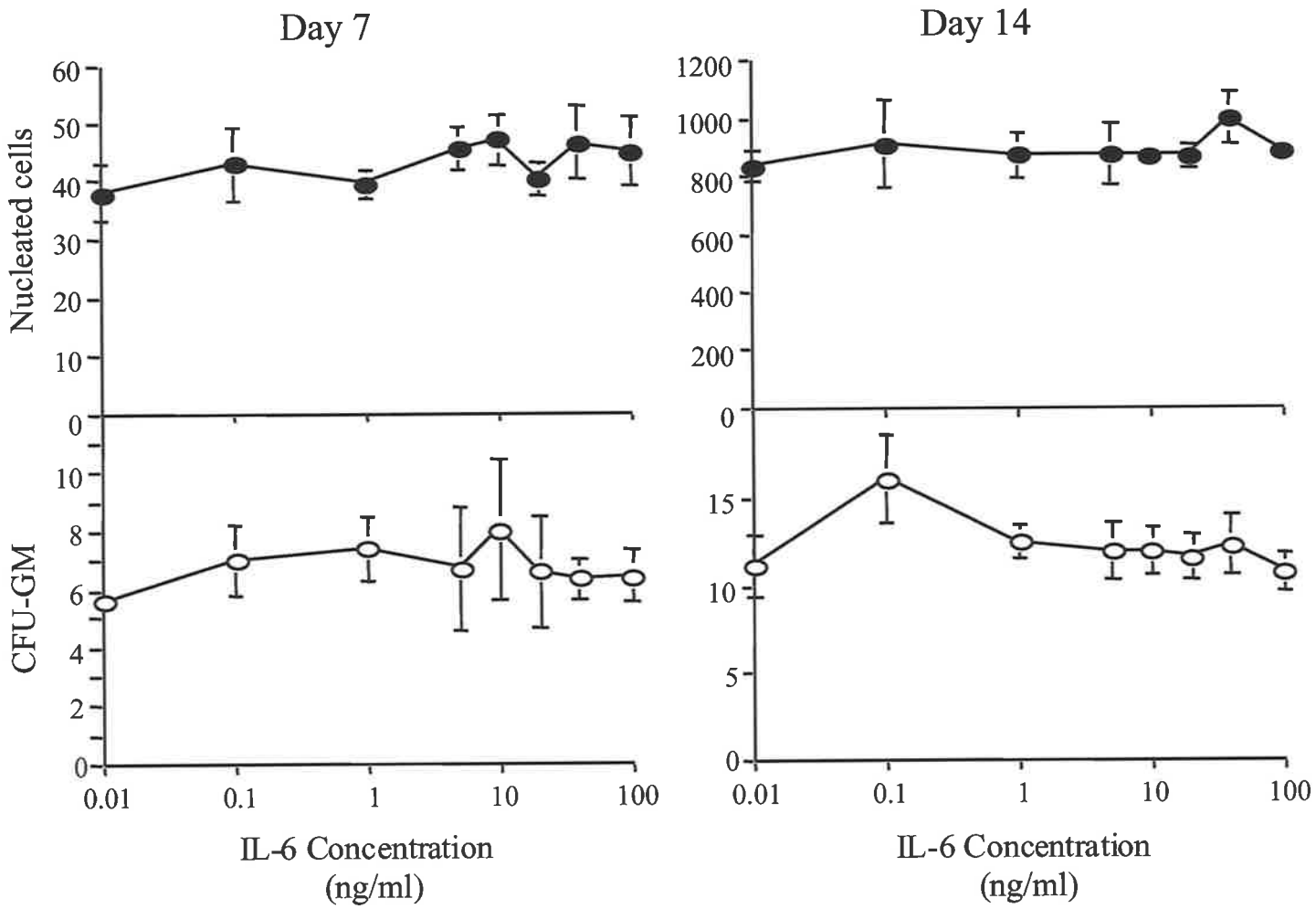
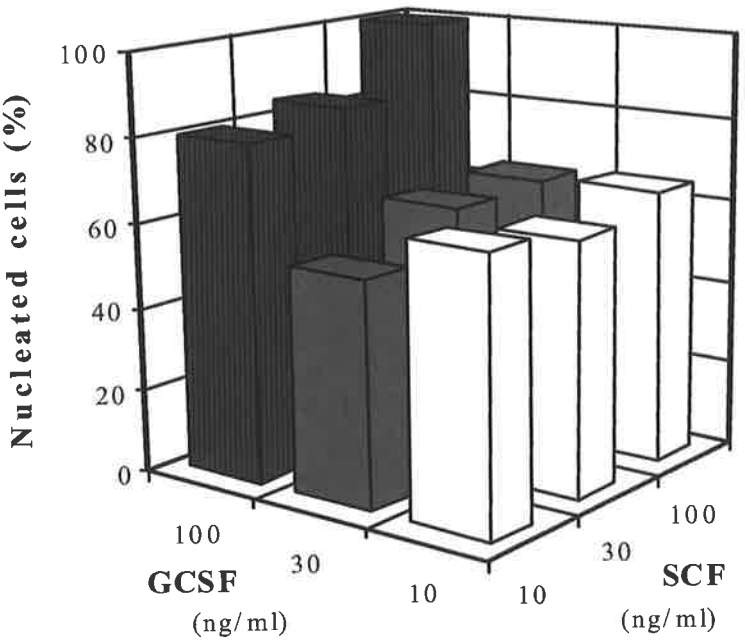
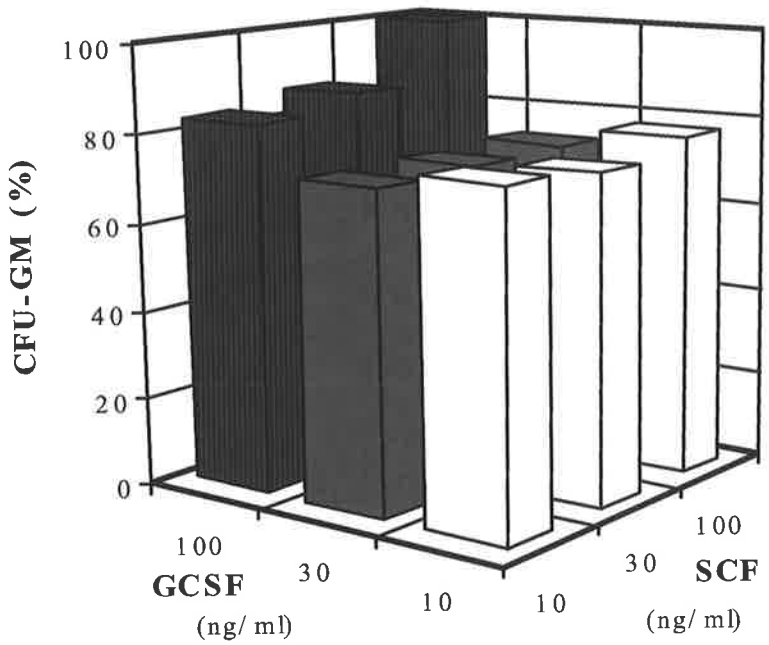

Day 7
Day 14
Nucleated cells
CFU-GM
60
50
40
30
20
10
0
1200
1000
800
600
400
200
0
10
8
6
4
2
0
15
10
5
0
0.01
0.1
1
10
100
IL-6 Concentration
(ng/ml)
IL-6 Concentration
(ng/ml)

Figure 4.6 Titration of G-CSF and SCF in Pre-CFU culture

This figure shows the cross-titration of G-CSF and SCF under Pre-CFU culture conditions with medium containing 30% FBS and 1% BSA. $CD34^+$ cells (UPN: 659, 1520, 1534) were cultured in 10 ng/ml of IL-3 and IL-6 and either 10 ng, 30 ng or 100 ng/ml of both G-CSF and SCF. The number of nucleated cells (top panel) and CFU-GM (bottom panel) at day 7 is shown for the 9 different HGF combinations. The data is taken from 3 separate experiments and is expressed as a proportion of the number of cells or CFU-GM generated when G-CSF and SCF were used at 100 ng/ml. Significantly more nucleated cells were produced in cultures where G-CSF was used at 100 ng/ml rather than 30 or 10 ng/ml (p values ranged from 0.0036 to 0.0203, Fisher's PLSD, comparing growth with 100 ng SCF + 100 ng G-CSF). Maximal CFU-GM production occurred with 100 ng/ml of both SCF and G-CSF. All other concentration combinations yielded significantly less CFU-GM (p values ranged from 0.0003 to 0.0413).


Nucleated cells (%)
100
80
60
40
20
0
100
30
10
GCSF
(ng/ml)
10
30
100
SCF
(ng/ml)


CFU-GM (%)
100
80
60
40
20
0
100
30
10
GCSF
(ng/ml)
10
30
100
SCF
(ng/ml)

the pattern of nucleated cell production for the different concentrations was identical to that at day 7: significantly more cells were generated when G-CSF was used at 100 ng/ml, irrespective of the SCF concentration (p values ranged from .0126 to .0014, Fisher's PLSD). In contrast, at day 14, there was no significant difference between the numbers of CFU-GM present with any of the G-CSF/SCF concentrations. There was however a reproducible trend for greater numbers of CFU-GM at day 14 when both SCF and G-CSF were used at 100 ng/ml. Despite this trend, these data suggest that CFU-GM production is independent of the concentration of G-CSF and SCF, provided at least 10 ng/ml of each factor is added. Furthermore, provided G-CSF is added at 100 ng/ml then SCF could be used at 10 ng/ml without compromising either CFU-GM or nucleated cell production.

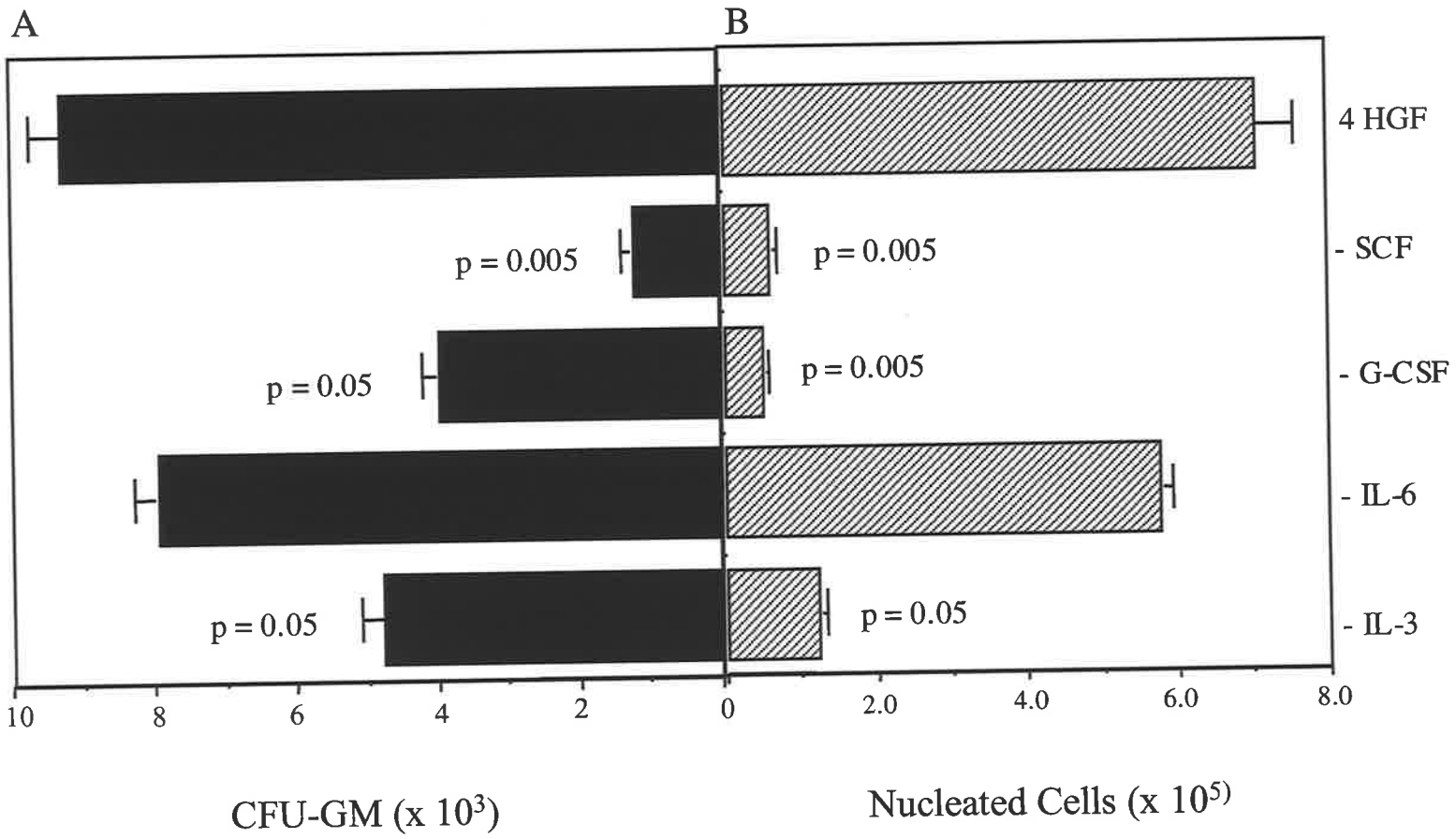
Collectively, the data described above suggested that the combination of IL-3 + IL-6 (both at 10 ng/ml) + G-CSF + SCF (both at 100 ng/ml) would be as effective as the original 6 HGF combination of 136GGMS (each HGF at 10 ng/ml) for *ex vivo* generation of neutrophil precursors from blood $CD34^+$ cells. However, it was also possible that a lesser combination of HGF could also be as effective as 136GGMS. This proposal was subsequently investigated.

4.3.3 Can Combinations of 3 HGF Replace 4 HGF?

Having determined that a combination of IL-3+IL-6+G-CSF+SCF provides an efficient stimulus for myeloid cell development in *ex vivo* culture of $CD34^+$ cells then experiments were performed to examine whether this 4 HGF represented the minimal combination of HGF required to support cell production in this culture system. This was investigated by using an experimental approach identical to that described above which involved culture of peripheral blood $CD34^+$ cells for 14 days in a series of 3-cytokine combinations resulting from the sequential omission of IL-3, IL-6, G-CSF and SCF from 4 HGF. As shown in Fig 4.7, omission of either G-CSF, SCF or IL-3 from the 4 HGF combination resulted in a significant reduction in both nucleated cells ($p = 0.005, 0.006, 0.05$ respectively) and CFU-GM ($p = 0.05, 0.005, 0.05$ respectively). Culture of $CD34^+$ cells without IL-6 ie with IL-3 + G-CSF + SCF was the least deleterious. However the decreased production of nucleated cells and CFU-GM (approximately 20%), although not statistically significant was highly reproducible from experiment to experiment. As the efficacy of *ex vivo* expanded $CD34^+$ cells in post transplant cytopenia would most likely depend on the total number of maturing myeloid cells transplanted a reduction of 20% in the total number of cells generated by *ex vivo* culture would be an important consideration. Therefore, these results strongly justify

Figure 4.7 The effect of omitting single HGF from 36GS when cells are cultured under Pre-CFU conditions

Production of CFU-GM (panel A) and nucleated cells (panel B) from 1,000 PB $CD34^+$ cells (UPN's: 1387, 1394, 1534) cultured in 4 HGF (IL-3, IL-6, G-CSF, SCF) or 3 HGF combinations (the HGF omitted from 3 HGF is indicated on the axis). The graph shows the total number (mean $\pm$ SEM, n=3) of cells and CFU-GM at day 14. Cultures without G-CSF, SCF or IL-3 produced significantly less nucleated cells and CFU-GM ($p>.05$, p values shown adjacent to the respective bars) than those stimulated with 4 HGF.


A
B
4 HGF
p = 0.005
p = 0.005
- SCF
p = 0.05
p = 0.005
- G-CSF
- IL-6
p = 0.05
p = 0.05
- IL-3
10
8
6
4
2
0
2.0
4.0
6.0
8.0
CFU-GM (x 10³)
Nucleated Cells (x 10⁵)

the use of 4 HGF (IL-3, IL-6, G-CSF and SCF: 36GS) as the least combination of cytokines supporting the efficient generation of neutrophil precursors from PB $CD34^+$ cells.

4.3.4 Comparison of 4HGF to 6HGF

Collectively the preceding series of experiments resulted in identification of a minimal combination of HGF (36GS) each at "optimised" concentrations that would potentially support growth of $CD34^+$ cells equivalent to that supported by the combination of 136GGMS. This proposal was tested by comparing the growth of $CD34^+$ cells cultured in either 36GS or 136GGMS. As shown in figure 4.8, nucleated cell or CFU-GM production for cells cultured in either HGF combination were very similar with no significant differences between the 4 HGF and 6 HGF cultures at 7, 14 or 21 days. However, there were differences in the type of myeloid cells present after 14 and 21 days of culture. As indicated in Table 4.1 there were significantly ($p = 0.03$) more neutrophils present in the 36GS cultures at day 14 and conversely, significantly ($p = 0.029$) more monocytes/macrophages at day 21 when cells were cultured in 136GGMS. These data are in accord with previous observations on the effects of various 5 HGF combinations.

4.3.5 Large Scale Separation and Culture of PB $CD34^+$ Cells

All the experiments described previously were performed with 1,000 $CD34^+$ cells cultured in 1 ml. To determine whether equivalent cell production would occur in large "clinical" scale cultures $CD34^+$ cells were isolated from apheresis collections and cultured in 1 litre of media supplemented with 36GS. Mononuclear cells were obtained from the second and third apheresis collections, respectively, from 2 patients, treated with cyclophosphamide for mobilisation of HPC and $CD34^+$ cells. In each case, 1×10^{10} MNC were immunolabelled with the anti-CD34 monoclonal 12.8 then processed on a CEPRATE column according to the manufacturers recommended method. Initially, 0.37% and 0.5% of MNC were $CD34^+$ and after processing the final cell suspension contained 64% (UPN 659; yield of 76%) and 73% (UPN 1472; yield of 105%) $CD34^+$ cells. In both cases, 1 litre cultures were initiated in PL732 tissue culture flasks (Baxter, Fenwal Division, Deerfield, IL, USA) containing Pre-CFU media supplemented with 36GS and 5×10^3 $CD34^+$ cells/ml, after adjusting the number of cells according to the $CD34^+$ cell purity. Nucleated cell counts, CFU-GM assays and morphological examination were performed on samples taken from cultures at days 5, 7, 10, 12 and 14. The pattern of cell and CFU-GM generation during the 2 weeks of culture is shown in figure 4.9. The kinetics and extent of nucleated cell and CFU-GM growth was similar for $CD34^+$ cells isolated from both samples. In cultures initiated with $CD34^+$ cells

Figure 4.8 Growth of mobilised PB $CD34^+$ cells in 4 HGF versus 6 HGF

1,000 $CD34^+$ cells were cultured in Pre-CFU medium containing 30% FBS and 1% BSA supplemented with either 4 HGF (10 ng/ml IL-3, 10 ng/ml IL-6, 100 ng/ml G-CSF, 100 ng/ml SCF) or 6 HGF (IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF; each at 10 ng/ml). Nucleated cells and CFU-GM were recorded at days 7, 14 and 21 and plotted as the mean (± SEM, n=3) fold increase. There was no significant difference, at any time point, in numbers of cells or CFU-GM generated by culture in 4 HGF or 6 HGF.

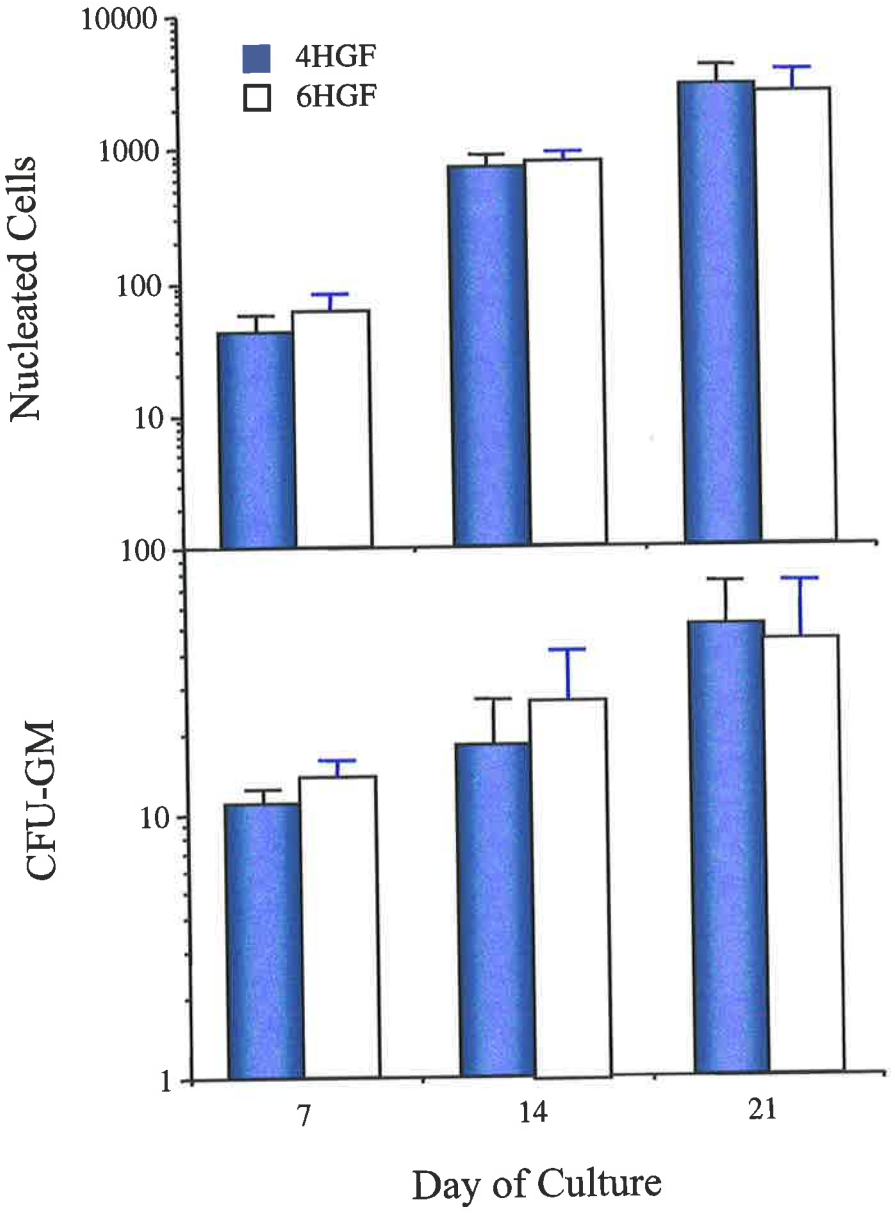

10000
1000
100
10
100
10
1
4HGF
6HGF
Nucleated Cells
CFU-GM
7
14
21
Day of Culture

Table 4.1

Cell types present on days 14 and 21 after culture of $CD34^+$ cells with 4 HGF (36GS) and 6 HGF (136GGMS)[a].

	Day 14		Day 21	
	4 HGF[b]	6 HGF[b]	4 HGF[b]	6 HGF[b]
Myeloblast	16.8 ± 2.4	19.0 ± 3.1	1.0 ± 0.3	1.6 ± 0.9
Promyelocytes	35.6 ± 3.9	32.5 ± 4.0	21.5 ± 0.3	20.3 ± 1.7
Myelo/Metamye[c].	39.1 ± 3.5	40.2 ± 6.8	62.5 ± 1.0	52.1 ± 3.2
Neutrophils	3.8 ± 0.6	1.0 ± 0.3	7.5 ± 4.3	2.66 ± 0.3
Monocytes/Macro[d].	4.7 ± 2.3	3 ± 2.3	7.5 ± 3.7	23.3 ± 1.4

[a] On day 14 there were significantly more neutrophils generated in 4 HGF (IL-3, IL-6, G-CSF, SCF) than in 6 HGF (IL-1β, IL-3, IL-6, G-CSF, GM-CSF, SCF) ($p = 0.03$), and on day 21 there were significantly more monocytes/macrophages present in the 6 HGF cultures ($p = 0.029$).

[b] Percent of each cell type (mean ± SEM, n =3)

[c] Myelo refers to myelocytes and Metamye refers to metamyelocytes.

[d] Macro refers to macrophages.

from UPN659, cell production peaked at day 14 when 1.91 x 10^9 nucleated cells (382-fold expansion) were present. In the second culture (UPN1472, 73% $CD34^+$), cell production was greatest at day 12 when 2.11 x 10^9 nucleated cells (422-fold expansion) were present. In both cultures, the peak in CFU-GM generation occurred after 10 days with 444.2 x 10^5 (18-fold expansion) and 512.5 x 10^5 (21-fold expansion) CFU-GM present from UPN659 and UPN1472 $CD34^+$ cells respectively. A spectrum of myeloid cells at different stages of maturation, was generated in both cultures. At day 12, 13% and 14% of cells were myeloblasts, 37% and 39% were promyelocytes, 43% and 39% were myelocytes, metamyelocytes or neutrophils and 6% and 9% were monocytes or macrophages in cultures from UPN659 and UPN1472 $CD34^+$ cells, respectively. At day 14, 22% and 38% of cells in these cultures had pyknotic nuclear forms typical of dead cells. A further concern was the lower fold-increase in production of nucleated cells from these bag cultures as compared to the average fold-increase observed for $CD34^+$ cells grown in 1 ml well cultures. This finding highlights the problems associated with large volume cell culture and indicates that before systems are considered for clinical studies they should be validated. In spite of these concerns, the results suggest that a 5 – 10 litre culture of $CD34^+$ cells could provide sufficient neutrophil precursors for clinical studies.

4.3.6 Other Variables Affecting Stroma-free, Cytokine Dependent Static Culture of $CD34^+$ Cells

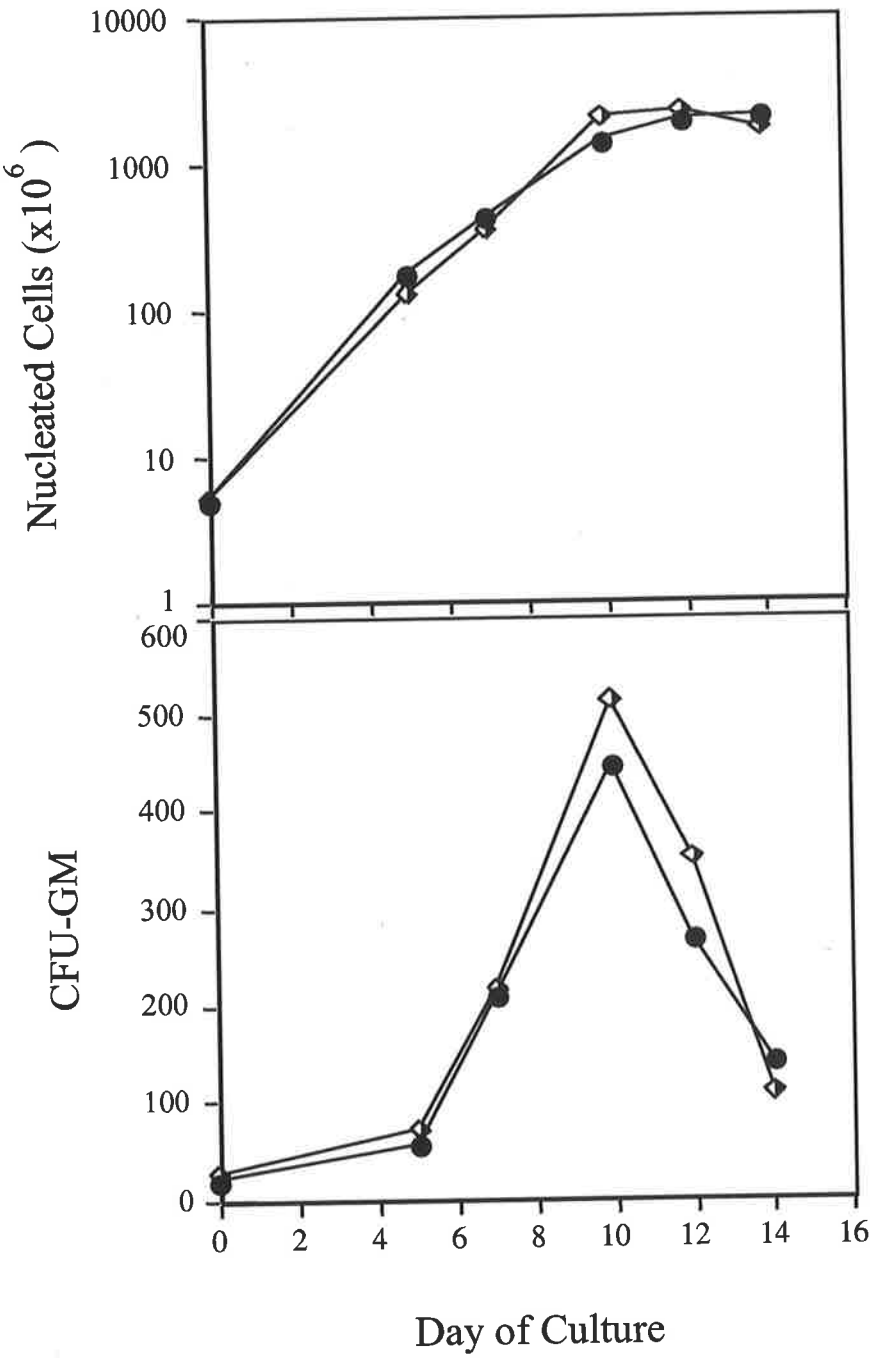
The results of large-scale cultures initiated with 5,000 $CD34^+$ cells/ml suggested that under static conditions, sustained cell proliferation may not be possible at cell concentrations in excess of 1-2 x 10^6 /ml. This was considered a major limitation for *ex vivo* culture of HPC and therefore a series of studies were initiated to further investigate this issue. Experiments were performed to determine if the cell concentration at the start of cultures affected cell proliferation, to explore the metabolic basis for any effects and to assess if regular replacement of spent media with fresh media and cytokines could improve HPC growth.

4.3.6.1 Initiating Cultures at Different Concentrations of $CD34^+$ cells

To investigate the influence of the initial cell concentration on growth, $CD34^+$ cells isolated from 3 separate mobilised blood samples were cultured in triplicate at 300, 1000, 3000 or 5,000 cells/ml with 36GS and monitored regularly over 16 days. The kinetics of nucleated cell generation at the 4 different $CD34^+$ cell concentrations is shown in figure 4.10. The pattern of cell growth was similar for each cell concentration and appeared to consist of 3 phases i.e. lag, exponential and inhibitory phases. The lag phase represents the time required

Figure 4.9 ***Ex vivo*** **expansion of CD34$^+$ cells in 1 litre cultures**

Large-scale culture of cyclophosphamide mobilised blood CD34$^+$ cells isolated by the CEPRATE^{TM} device. A total of 5 x 10^6 CD34$^+$ cells (5 x 10^3 cell/ml) were cultured in 1 litre of Pre-CFU medium containing 30% FBS, 1% BSA and IL-3, IL-6, G-CSF and SCF at 10 ng, 10 ng, 100 ng and 100 ng/ml, respectively. These cultures were performed in PL732 tissue culture flasks generously provided by Baxter Fenwal (Baxter Healthcare, Round Lake, IL, USA) and assessed on days 5, 7, 10, 12, and 14 to determine the number of nucleated cells and CFU-GM present. The CFU-GM content was assayed as described in the materials and methods (section 2.3.2) with 5637 conditioned medium and IL-3 used to stimulate myeloid colony growth. The figure shows the actual number of viable nucleated cells and CFU-GM present in each culture at these time points.


10000
1000
100
10
1
Nucleated Cells (x10^6)
600
500
400
300
200
100
0
CFU-GM
0
2
4
6
8
10
12
14
16
Day of Culture

for $CD34^+$ cells to respond to HGF stimulation, exit from quiescence and start cell division. At each cell concentration, there was growth of cells during the first 4 days, although as indicated by figure 4.10 the lag phase in cultures initiated at 300 and 1,000 $CD34^+$ cells/ml seemed to be longer than that in those initiated with 3,000 or 5,000 $CD34^+$ cells/ml. Unfortunately, cell counts were not performed at day 2 of culture so there was no data to indicate if the length of the lag phase was dependent on cell concentration. The exponential phase of growth continued until the cell density in culture reached or exceeded approximately 1 x 10^6 cells/ml. At this stage, cells start to die at a greater rate than new cells were generated. The inhibitory phase resulted in a rapid decrease in the number of viable cells. Cultures initiated with 5,000 $CD34^+$ cells/ml reached the inhibitory phase at day 10 or 11, whereas cultures initiated with only 300 $CD34^+$ cells/ml were still in exponential phase after 16 days. Notably, during the exponential phase of growth, the rate of cell production seemed to be independent of the initial $CD34^+$ cell concentration. This is indicated by panel B of figure 4.10, where the kinetics of cell production are plotted on a log scale. A line of best fit for each culture shows an almost identical slope and the relationship between time in culture (X) and the number of nucleated cells (Y) was similar and could be defined by the equation, $\log_{10}Y = 0.25X$. By extrapolation, the doubling time for cells, during the exponential growth phase, was calculated to be 1.204 days (approximately 28.9 hours) and independent of the initial concentration of $CD34^+$ cells in culture.

Similar results were obtained for generation of CFU-GM during the exponential phase of growth. As with nucleated cell production, after 9-11 days of culture, there was a trend for decreasing CFU-GM generation in cultures initiated at higher cell density (refer to figure 4.11). Analysis of both, the maximum fold-increase of nucleated cells and maximum fold-increase of CFU-GM revealed an inverse relationship between the initial cell concentration and each these two variables (panels C and D respectively of figure 4.11). For example, the higher the initial $CD34^+$ cell concentration the lower the maximum fold-increase of nucleated cells and CFU-GM. Conversely, cultures initiated at lower concentrations of $CD34^+$ cells (300 and 1,000 $CD34^+$ cells/ml) resulted in a greater maximum fold-increase of both nucleated cells and CFU-GM.

Based on these data, proliferation of $CD34^+$ cells, stimulated by 36GS in static culture conditions can be represented by the model growth curve shown in figure 4.12. As indicated, $CD34^+$ cells cultured under these conditions with the 4 HGF combination of 36GS have a doubling time of approximately 1.2 days. Moreover the data presented above indicates that

Figure 4.10 The effect of initial $CD34^+$ cell concentration on nucleated cell production

Triplicate, 1ml Pre-CFU cultures were initiated with 300, 1000, 3000 or 5000 $CD34^+$ cells in Pre-CFU medium containing 30% FBS and 1% BSA supplemented with IL-3, IL-6, G-CSF and SCF at 10, 10, 100 and 100 ng/ml respectively. Three identical experiments were performed from 3 separate sources of mobilised blood $CD34^+$ cells obtained by FACS. At various times throughout the cultures the contents of wells were mixed by gentle aspiration with a Gilson P1000 pipette, a 20 µl sample removed and a cell count performed under phase contrast microscopy. Panel A shows the actual number of viable cells present (mean $\pm$ SEM, n=3 experiments) at each time of analysis for the cultures initiated at each cell concentration. Panel B depicts the mean cell count at each time point from cultures initiated at different cell concentration, plotted on a log scale. This data is censored in that it only includes the cell counts during the growth phase of the cultures. For example, in cultures initiated with 5,000 $CD34^+$ cells, the number of viable cells decreased after day 12, thus the cell numbers on days 13, 14 and 15 have been omitted. The line of best fit for each of these cultures was generated by Cricket Graph III software and demonstrates an almost identical rate of cell production during the exponential phase of growth.

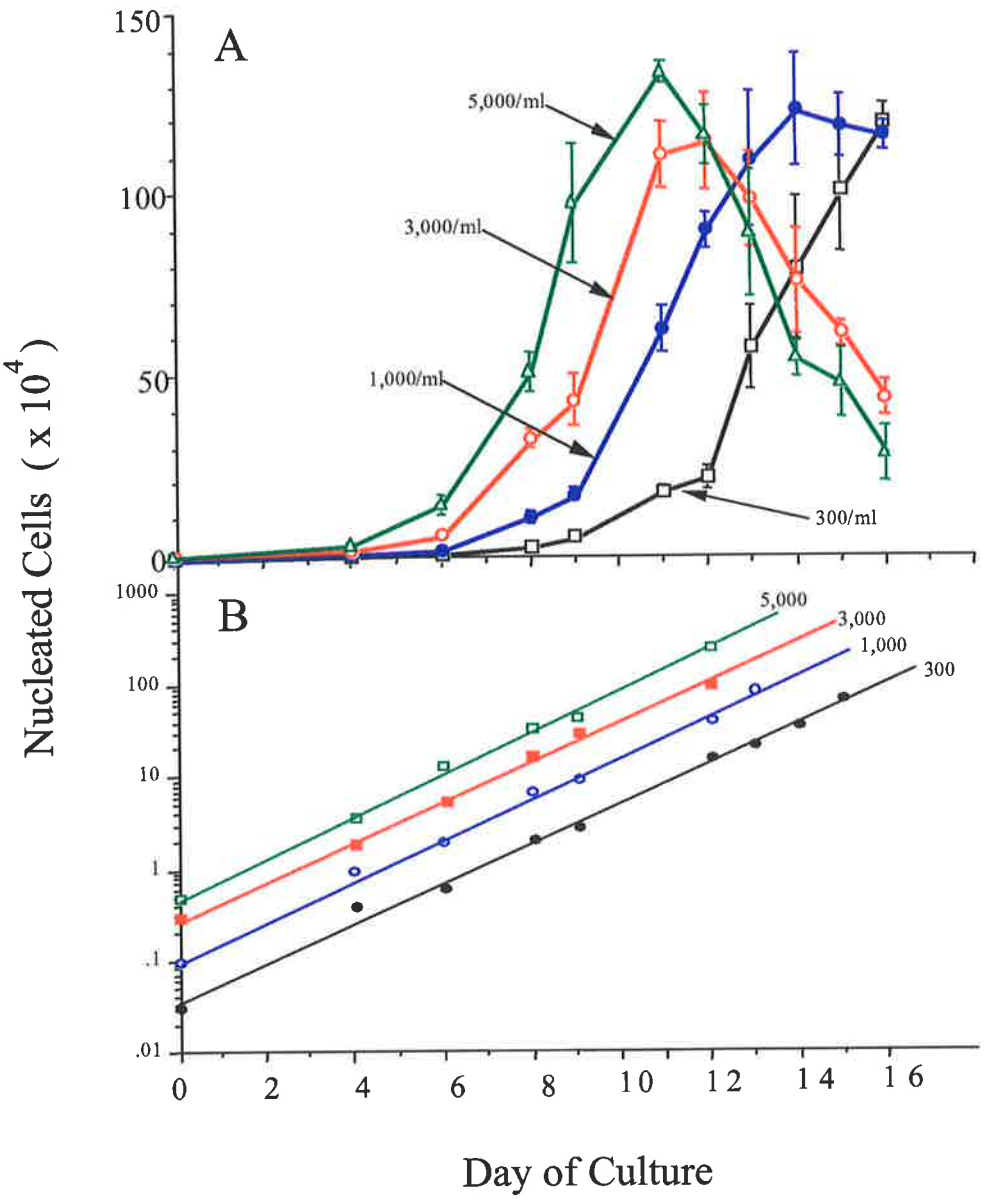

A
B
5,000/ml
3,000/ml
1,000/ml
300/ml
5,000
3,000
1,000
300
150
100
50
0
1000
100
10
1
.1
.01
0
2
4
6
8
10
12
14
16
Nucleated Cells (x 10^4)
Day of Culture

Figure 4.11 The effect of initial $CD34^+$ cell concentration on nucleated cell and CFU-GM production

The data presented in this figure is based on the same three experiments described in figure 4.10. This figure also includes information on CFU-GM generation. Panels A and B depicts the actual number of nucleated cells and CFU-GM, respectively, on a log scale, as a function of days in culture for the different initial cell concentrations. Panels C and D depict the fold-increase in nucleated cells and CFU-GM, respectively, and both show an inverse relationship between initial $CD34^+$ cell concentration and fold-increase.

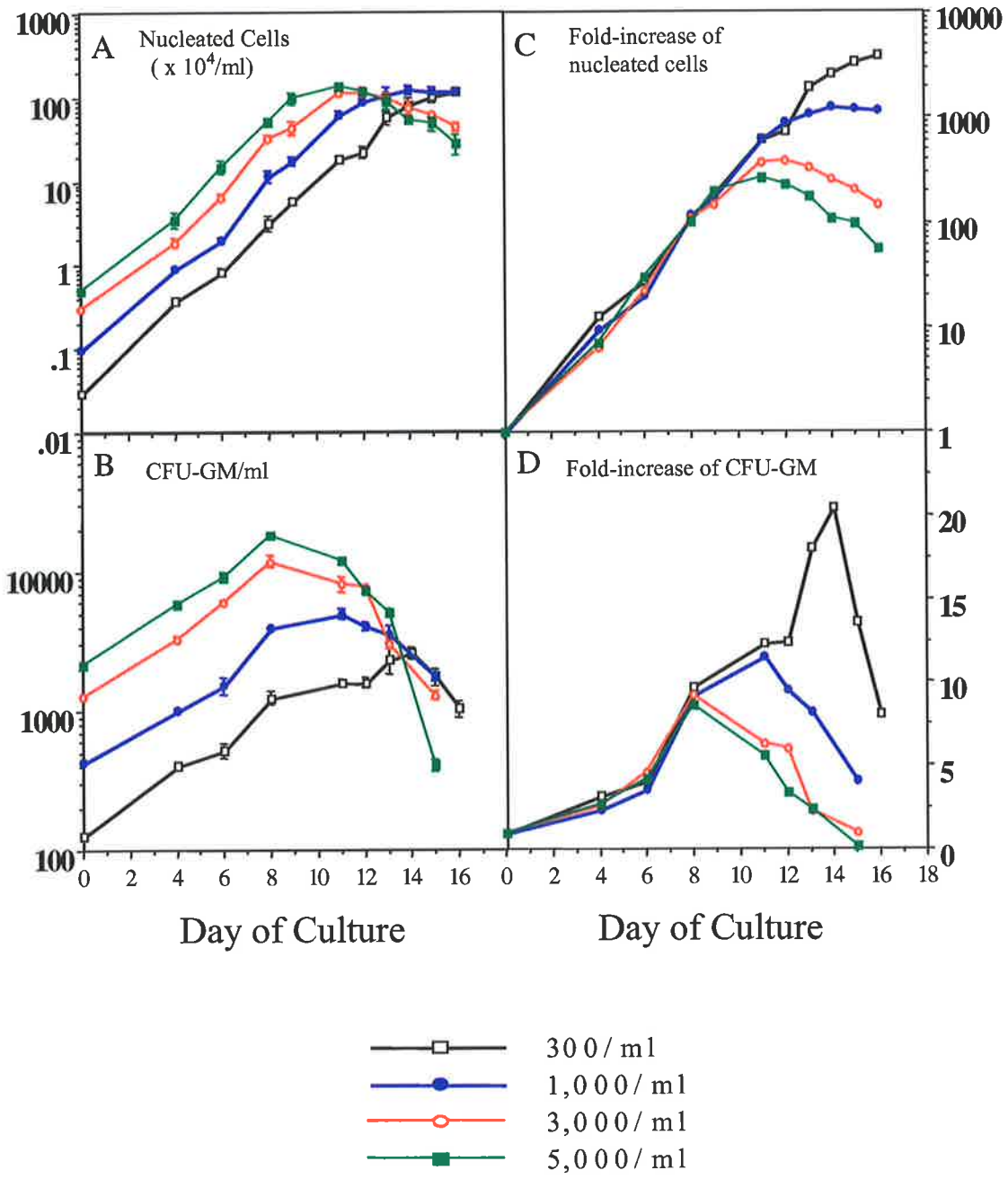
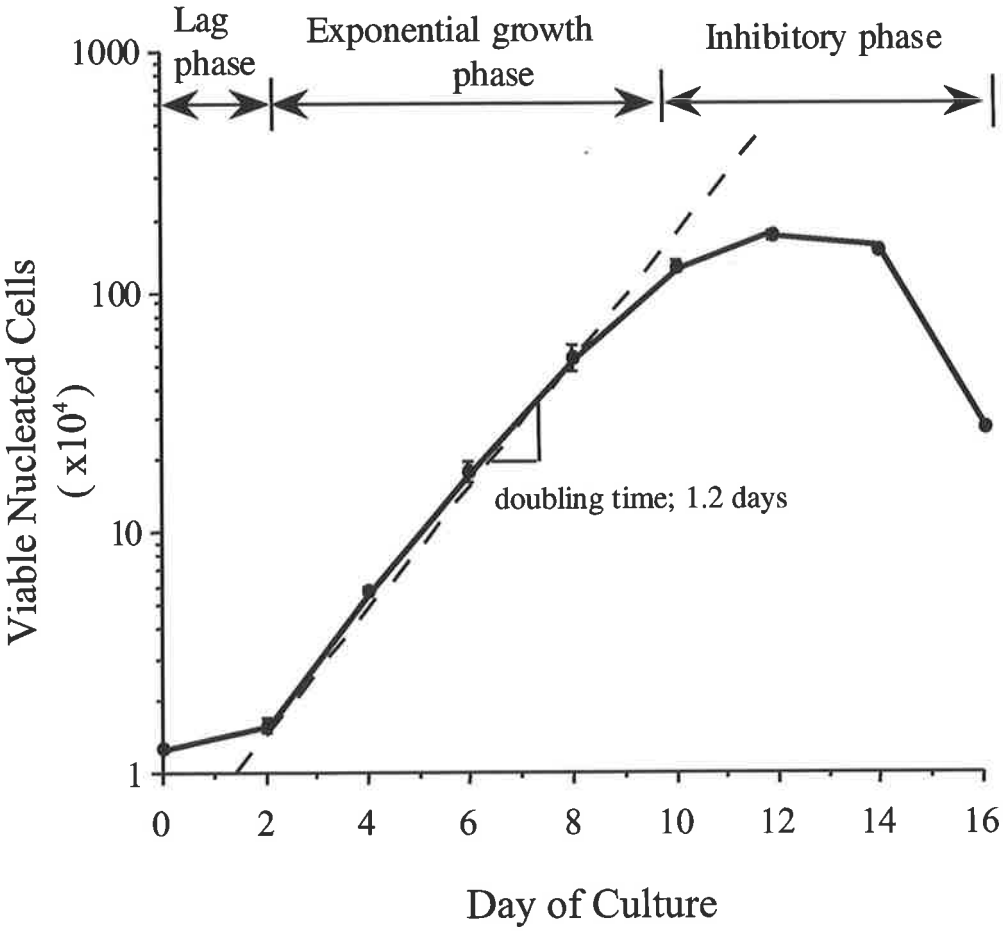

A
Nucleated Cells
(x 10⁴/ml)
C
Fold-increase of
nucleated cells
B
CFU-GM/ml
D
Fold-increase of CFU-GM
1000
100
10
1
.1
.01
10000
1000
100
10
1
10000
1000
100
20
15
10
5
0
0 2 4 6 8 10 12 14 16
0 2 4 6 8 10 12 14 16 18
Day of Culture
Day of Culture
300/ml
1,000/ml
3,000/ml
5,000/ml

Figure 4.12 Model for *ex vivo* proliferation of $CD34^+$ cells

This figure presents a model for the growth of $CD34^+$ cells cultured in stromal-free Pre-CFU culture conditions stimulated by a combination of IL-3, IL-6, G-CSF and SCF. This model is based on the data presented within figures 4.10 and 4.11 and predicts 3 phases in nucleated cell generation. The first phase or lag phase is characterised by limited increase in total cell numbers, which may persist for 1-3 days. The second phase or exponential growth phase is characterised by a rapid increase in cells with the number of viable cells doubling every 24-26 hours. This phase continues until the cell density approaches or exceeds 1×10^6 cells/ml at which stage the rate of cell death exceeds the rate of new cell production. At this stage, the culture is in decline or inhibitory phase.


Lag phase
Exponential growth phase
Inhibitory phase
1000
100
10
1
Viable Nucleated Cells (x10⁴)
doubling time; 1.2 days
0
2
4
6
8
10
12
14
16
Day of Culture

the length of the exponential phase and the start of the inhibitory phase is directly related to the concentration of $CD34^+$ cells used to initiate cultures. An understanding of the mechanisms that limit growth of maturing myeloid cells during the exponential phase has important implications for *ex vivo* culture of $CD34^+$ cells for therapy. Therefore, the next series of experiments were performed to investigate this issue.

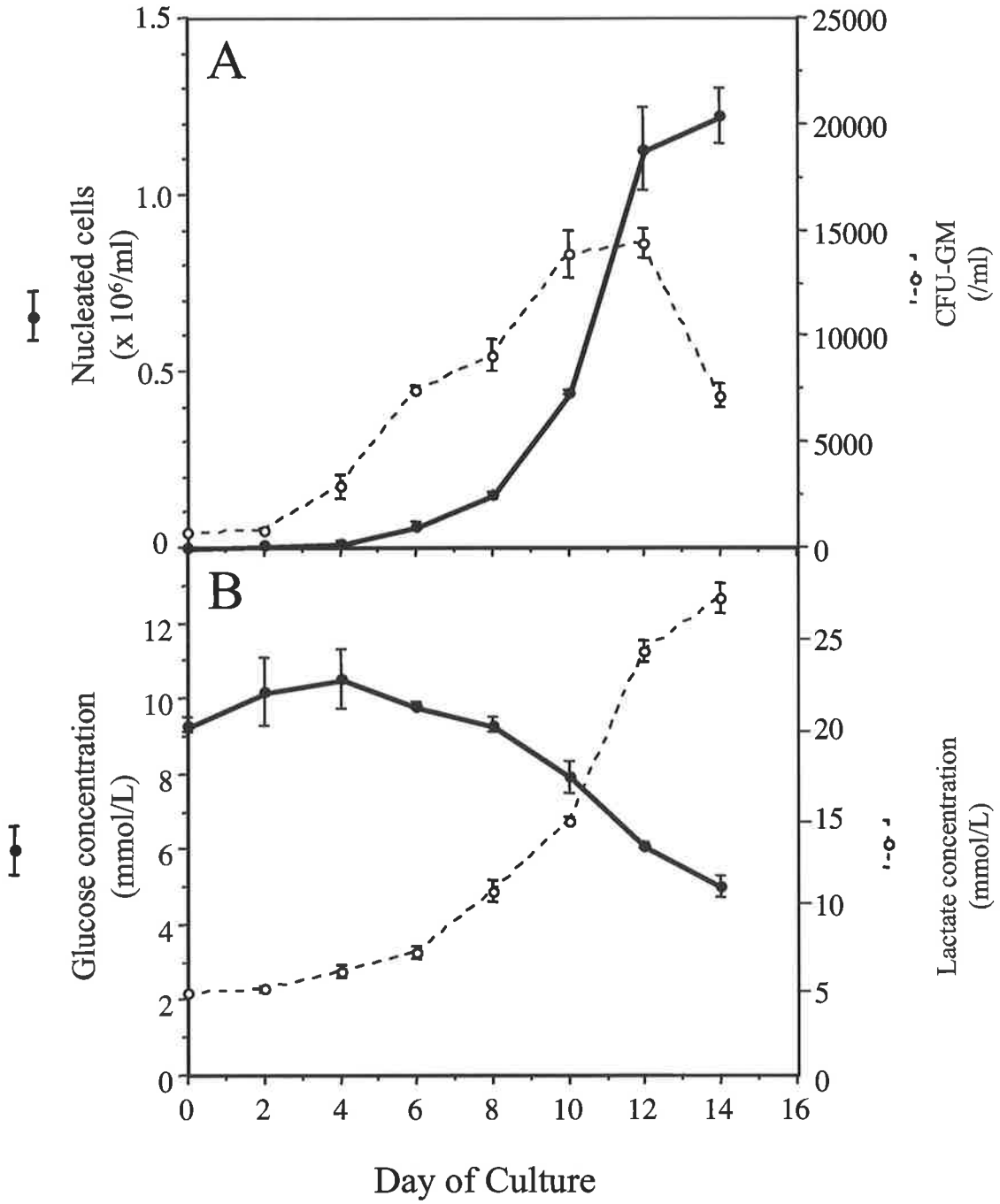
4.3.6.2 Changes in Lactate and Glucose Levels During Cytokine Dependent, Static Culture of $CD34^+$ cells

Possible reasons for death of cells during static culture include exhaustion of energy sources and/or HGF or inhibition of cell growth by cell metabolites and/or inhibitory cytokines released by maturing cells. A common feature of HPC growth at high cell density was the obvious acidity of cultures heralded by the yellow colour change of the indicator dye, phenol red. This suggested that cell death during the late stages of culture might be directly related to consumption of glucose and accumulation of lactic acid. A single experiment was therefore performed to analyse the change in glucose and lactate levels during culture of $CD34^+$ cells and to relate the level of these analytes to cell proliferation. The results of this experiment, shown in figure 4.13 represent the mean (± SEM) values from triplicate cultures of 1,000 PB $CD34^+$ cells cultured in Pre-CFU medium supplemented with the HGF combination of 36GS.

At the start of culture, the concentration of glucose in fresh media supplemented with 36GS was 9.4 mmol/L. During the first 8 days of culture the glucose concentration did not alter significantly and was assayed at 9.3 mmol/L on day 8. However during the remaining 6 days there was a progressive decline in the glucose concentration. At day 14, glucose was 5.0 mmol/L representing a 46.8% reduction from that at the start of culture. In contrast, the lactate concentration increased throughout the period of culture with the most rapid increase coinciding with the exponential phase of growth between days 6 and 12. Overall the lactate level increased 5-fold during culture: from an initial concentration of 5.1 mmol/L to 27.1 mmol/L at day 14. When the cell density reached 1 x 10^6/ml the lactate level was approximately 22.5 mmol/L (extrapolated from figure 4.14). This single experiment suggested that hypoglycaemic acidotic conditions might be a reason for increased cell death at high cell density. In addition the consumption or degeneration of HGF may also contribute. Accordingly then, replacement of spent media with fresh media and cytokines may abrogate the toxicity associated with lactic acidosis and therefore increase myeloid cell generation from $CD34^+$ cells.

Figure 4.13 Changes in lactate and glucose levels during Pre-CFU culture

Pre-CFU cultures were initiated with 1,000 $CD34^+$ cells in 1 ml of medium containing 30% FBS and 1% BSA supplemented with a combination of IL-3, IL-6, G-CSF and SCF. The cultures were sampled every 2 days for 14 days to record the number of nucleated cells and CFU-GM and to collect supernatant for lactate and glucose determinations. Panel A shows the change in nucleated cell and CFU-GM during the course of this culture whereas panel B depicts the levels of lactate (broken line) and glucose (solid line). Each data point shows the mean $\pm$ SEM, from triplicate wells.


A
B
Nucleated cells
(x 10⁶/ml)
CFU-GM
(/ml)
Glucose concentration
(mmol/L)
Lactate concentration
(mmol/L)
Day of Culture
0
0.5
1.0
1.5
5000
10000
15000
20000
25000
2
4
6
8
10
12
14
16

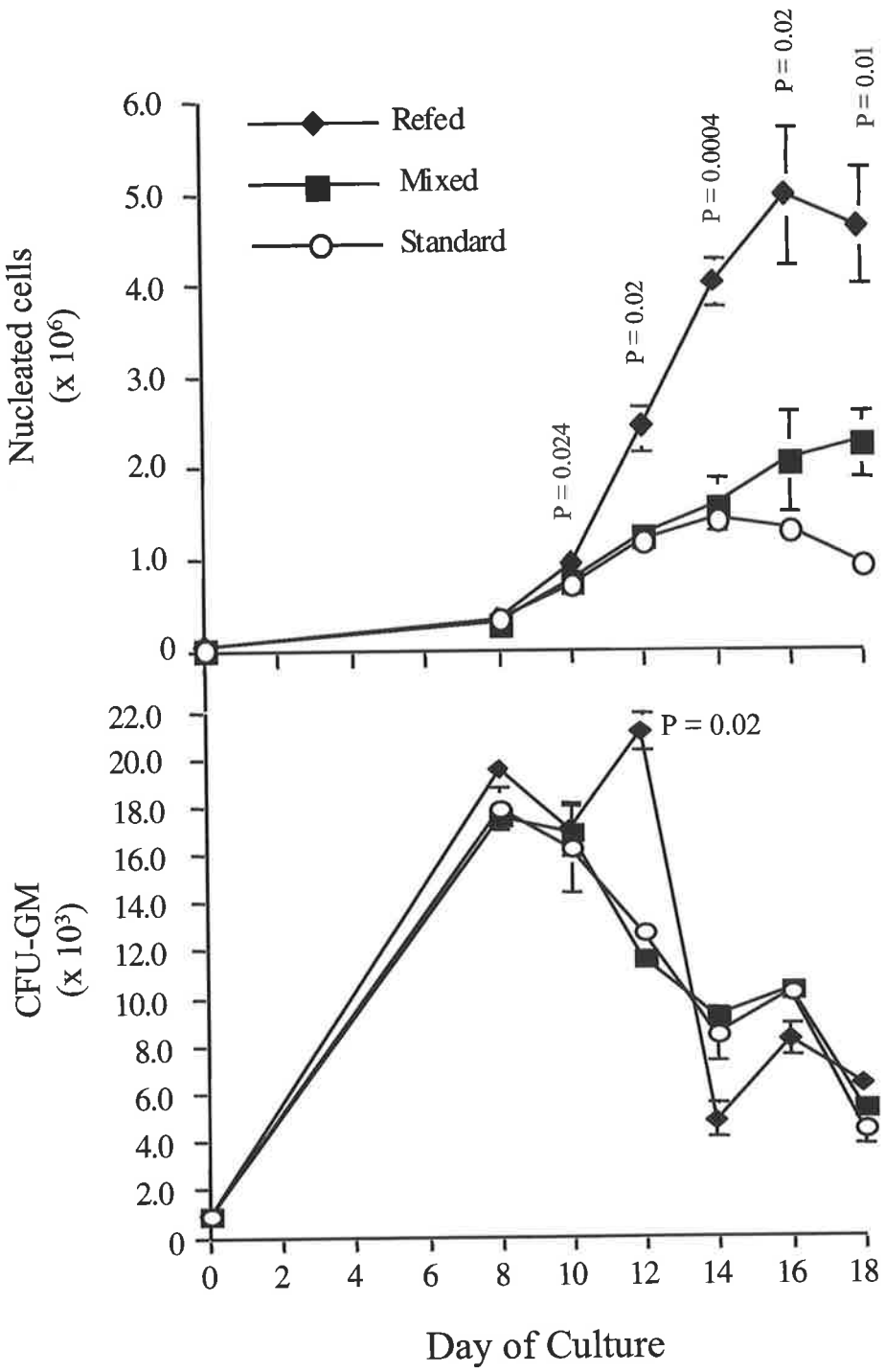
4.3.6.3 Media and HGF Replenishment Improves Growth of $CD34^+$ Cells

Three experiments to determine whether re-feeding cultures with fresh media and cytokines might increase generation of neutrophil precursors were performed with PB $CD34^+$ cells obtained by FACS. Cultures were initiated with 3,000 $CD34^+$ cells in 1 ml of Pre-CFU media and the HGF combination 36GS. These cultures were (i), left for 18 days without any further manipulation (standard static culture conditions), (ii) fed by removal and replenishment of media/HGF (pseudo-perfusion) or (iii) mixed without removal of media. The media replacement schedule was started after 7 days and involved the daily removal of 500 µL of media and replacement with 500 µL of fresh media and HGF for the next 10 days. Cultures that were mixed each day from day 7 – 16 served as a control for the pseudo-perfusion group. Viable cell counts and CFU-GM assays were performed at days 8, 10, 12, 14, 16 and 18 on a triplicate of wells harvested at each time from each culture condition. Thus the data shown in figure 4.14 represents the mean (± SEM) of 9 wells from 3 separate experiments.

In accord with previous experiments, standard static cultures that were not subject to media/HGF replenishment sustained exponential growth until a cell concentration of 1.2 x 10^6 /ml was reached at day 12. After this time the number of viable cells declined. In contrast, in the cultures subjected to daily, half volume media and HGF replacement from day 7 there was a significant increase in the number of nucleated cells generated. This increase in cell growth was observed as early as day 10 when 32% more cells were present in the fed cultures than in those maintained under at standard static culture conditions ($p = 0.024$). The ability to generate more cells in fed cultures was sustained until day 18. The average, maximum number of nucleated cells generated from fed cultures was 4.96 x 10^6 and was reached after 16 days of culture (in all experiments). Thus cultures subject to daily media and HGF replacement from day 7 were able to sustain cell growth at a significantly ($p = 0.0004$) higher cell density (approximately 5-fold greater) than standard static cultures. Although there was an increase in cell generation under pseudo-perfusion conditions the mix of myeloid cells was similar to standard static cultures and at day 16 consisted of a mean of 4% blasts, 36% promyelocytes, 38% myelocytes, 15% metamyelocytes, 3% neutrophils and 4% monocytes/macrophages. Of interest was the extended exponential growth phase observed in those cultures that were mixed but not fed fresh media. This resulted in approximately twice as many viable cells present at days 14 and 16 as compared to $CD34^+$ cells cultured under standard static culture conditions.

Figure 4.14 Media and HGF replenishment improves *ex vivo* expansion from $CD34^+$ cells

Three independent experiments were initiated with 3,000 $CD34^+$ cells isolated by FACS. Cells were cultured in 1 ml of Pre-CFU medium containing 30% FBS and 1% BSA supplemented with the 36GS HGF combination and subject to 3 different culture experiences. One set of wells were cultured for 18 days without any further manipulation (referred to as "standard" on the legend), a second set were subject to daily removal of half the tissue culture medium/HGF and replenishment with fresh medium/HGF from day 7- day 18 (referred to as "refed"), while the culture from the third group served as an additional control and were mixed (referred to as "mixed") from day 7 – day 18, but not subject to replenishment of medium/HGF. The effect of these manipulations was assessed by measuring nucleated cell and CFU-GM content from day 7 onwards. The top panel represents the change in nucleated cell numbers during the culture period for each of the experimental conditions. The p values refer to the level of significance difference (paired t test, c.i. of 95%) between the numbers of cells observed in standard compared to refed cultures. The lower panel shows the absolute number of CFU-GM present at sampling times for the different culture conditions. Again, the p value represents the difference between the standard and refed cultures.

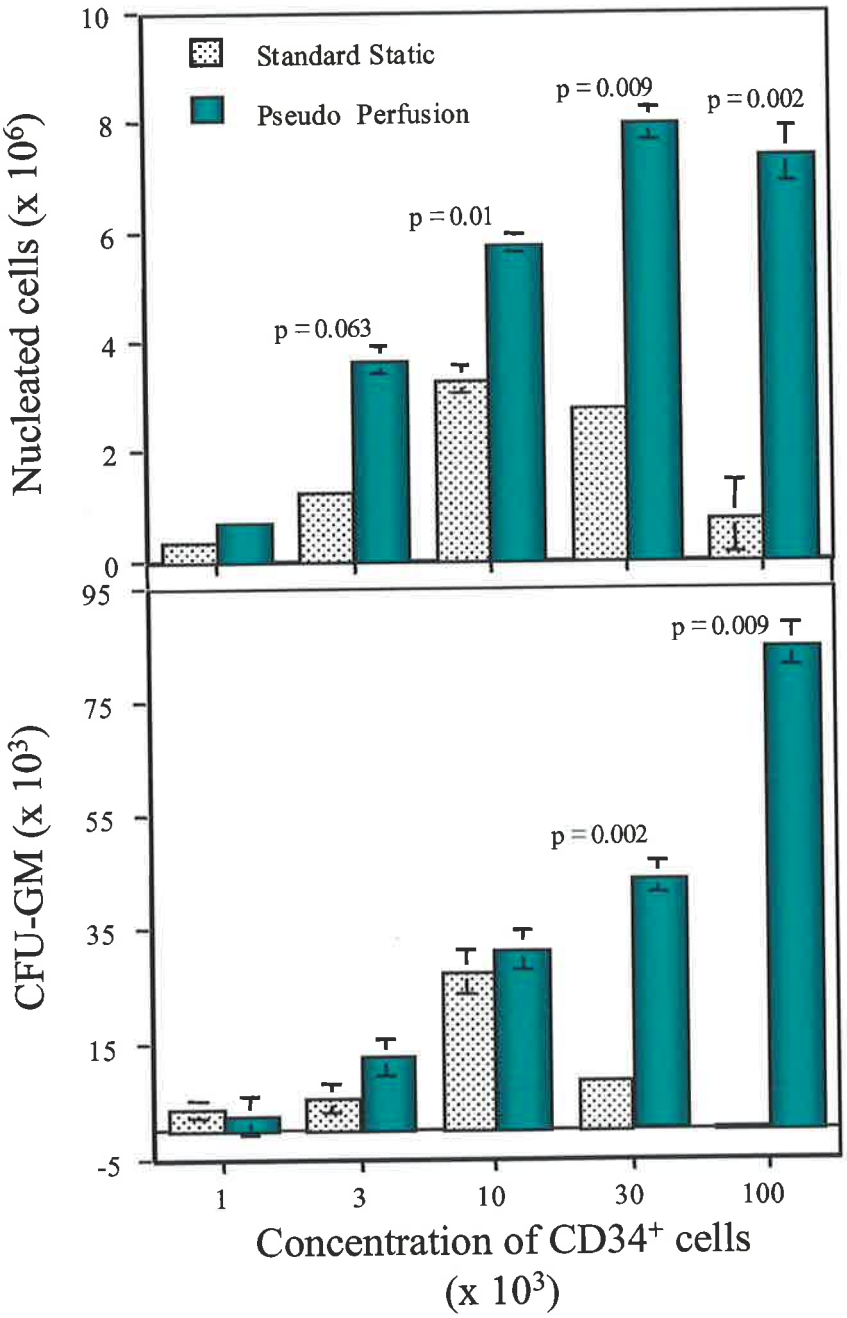

Refed
Mixed
Standard
Nucleated cells
(x 10⁶)
6.0
5.0
4.0
3.0
2.0
1.0
0
P = 0.024
P = 0.02
P = 0.0004
P = 0.02
P = 0.01
CFU-GM
(x 10³)
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
P = 0.02
0
2
4
6
8
10
12
14
16
18
Day of Culture

The bottom panel of figure 4.14 shows the number of CFU-GM present at each time point for the different cultures. The average number of CFU-GM present in 3,000 $CD34^+$ cells at the start of culture was 982. Although replacement of media and HGF increased significantly the number of neutrophil precursors generated this did result in a dramatic increase in the number of nascent CFU-GM produced. There was however, 2-fold more CFU-GM present at day 12 in the refed cultures as compared to the standard static conditions. Collectively, this series of experiments supported the proposal that growth inhibition at high cell density can be partially, if not completely overcome by frequent removal of spent media and replenishment by fresh media and cytokines. This idea was further investigated by testing the effects of pseudo-perfusion on cultures initiated at a range of $CD34^+$ cell concentrations up to 1×10^5 /ml.

For these studies, $CD34^+$ cells were isolated by FACS from cryopreserved mononuclear cells obtained following apheresis. Triplicate cultures were initiated with 1, 3, 10, 30 and 100 x 10^3 $CD34^+$ cells/ml in Pre-CFU media supplemented with 36GS. One set of triplicates at each cell concentration were to be maintained under standard static culture conditions for 14 days whilst the other set of triplicate cultures were to have half volume media/HGF replacement on each day from day 3 until day 14. However, from day 10 onwards there was considerable cell death in the static cultures initiated with 30 and 100 x 10^3 $CD34^+$ cells. Thus these experiments were completed at day 12 when viable cell counts and CFU-GM assays were performed. The pooled results of 2 experiments are shown in figure 4.15. Production of nucleated cells was increased at all $CD34^+$ cell concentrations when cultured under the pseudo-perfusion conditions. This effect was more noticeable and significant at higher $CD34^+$ cell concentrations (p values from 0.063 – 0.002, paired t test). Cultures initiated with 100,000 $CD34^+$ cells and subjected to the daily re-feeding schedule from day 3 had an average of 10 times more viable nucleated cells at day 12 than the corresponding cultures maintained under static conditions. A similar trend between the static and pseudo-perfused cultures was observed for generation of nascent CFU-GM although the differences were not significant in cultures initiated with 1, 3 and 10 x 10^3 $CD34^+$ cells. This result is in accord with the previous experiments represented in figure 4.14. However, in contrast, when cultures were initiated with 30,000 or 100,000 $CD34^+$ cells there was a significant ($p = 0.002$ and 0.009, respectively) increase in CFU-GM in the pseudo-perfused cultures as compared to those in standard static conditions (refer to lower panel of figure 4.15). In particular, in the cultures established with 100,000 $CD34^+$ cells there were very few (an average of 313) CFU-

Figure 4.15 Growth of $CD34^+$ cells under static culture and pseudo perfusion conditions

The figure shows the number of nucleated cells (top panel) and CFU-GM (bottom panel) generated by FACS isolated $CD34^+$ cells after 12 days growth in static and pseudo perfusion culture conditions. The cultures were initiated with either 1, 3, 10, 30, 100 x 10^3 $CD34^+$ cells in 1 ml and subject to either standard static culture for 12 days or half volume medium/HGF replacement from day 3 to day 12 (pseudo perfusion). Three identical experiments were established with different sources of $CD34^+$ cells isolated by FACS from cryopreserved apheresis collections. The columns represent the number of viable nucleated cells or CFU-GM present at day 12 (mean $\pm$ SEM, n=3 experiments) for each culture condition. A paired t test was used to test the difference between the two experimental conditions.


Standard Static
Pseudo Perfusion
Nucleated cells (x 10⁶)
10
8
6
4
2
0
p = 0.063
p = 0.01
p = 0.009
p = 0.002
CFU-GM (x 10³)
95
75
55
35
15
-5
p = 0.009
p = 0.002
1
3
10
30
100
Concentration of CD34⁺ cells
(x 10³)

GM present after 12 days of static culture whereas 84,460 (± 3,663) CFU-GM were present after 12 days of pseudo-perfusion culture. This result suggests that survival and/or proliferation of primitive HPC and Pre-CFU may be preferentially inhibited under lactic acidotic conditions typically observed in the late stages of static cultures. The encouraging aspect of the data is that it supports the notion that even for cultures initiated at quite high $CD34^+$ cell concentrations then the limitations of metabolic inhibition can be partially overcome by simple media replacement schedules. One conclusion, to be discussed further, is that bio-reactors or perfusion culture systems may substantially improve production of myeloid cells from $CD34^+$ cells.

4.4 Discussion

There has been considerable interest in the development of methodologies for the *ex-vivo* growth of primitive haemopoietic cells for subsequent use in clinical applications. Particular interest has focused on the development of culture systems that optimally expand haemopoietic progenitors in the absence of marrow stromal cells where the sole stimulus for growth is haemopoietic cytokines. There is consensus amongst many groups performing these studies that a combination of HGF is required to ensure efficient *ex vivo* expansion. Nevertheless, considerable empiricism is still evident in the choice of cytokines required to ensure adequate *ex vivo* expansion of HPC. The studies described in this chapter have been devoted largely toward the development of an efficient culture system for the generation of neutrophil post progenitor cells (Haylock *et al* 1994a, Haylock *et al* 1994b). Previous studies (Haylock *et al* 1992) have shown that significant production of nascent myeloid progenitor cells from PB $CD34^+$ cells after 7 days culture, occurred with a combination of 6 growth factors, ie IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF (6 HGF or 136GGMS). The present studies demonstrate that SCF, G-CSF and to a lesser extent IL-3 are critical for generation of both neutrophil precursors and CFU-GM from $CD34^+$ cells. In addition, a combination of IL-3, IL-6, G-CSF and SCF (4 HGF or 36GS) each at optimised concentrations provides an equivalent stimulus to 6 HGF for production of neutrophil precursors from PB $CD34^+$ cells.

That SCF and IL-3 were identified, as important cytokines in this culture system is perhaps not surprising. Numerous previous studies have described the potent synergistic effects of SCF on the growth of both primitive (Pre-CFU) and committed haemopoietic progenitors when used with other growth factors such as G-CSF, GM-CSF, IL-3, and erythropoietin (Tsujino *et al* 1993, Brand *et al* 1994, Migliaccio *et al* 1992, McNiece *et al* 1991). The

significant decrease in production of nascent CFU-GM and maturing myeloid cells that results from omission of SCF from HGF combinations is consistent with these reports. Similarly, IL-3 is a synergistic cytokine with well-documented effects on both primitive and committed haemopoietic cells. These effects include the maintenance of cell survival through suppression of apoptosis and the stimulation of proliferation and differentiation of haemopoietic progenitors for multiple haemopoietic lineages (Iscove *et al* 1989, Metcalf 1991). In addition, IL-3 when used in combination with SCF is particularly effective in stimulating the proliferation of very primitive haemopoietic progenitor cell populations as evidenced by the fact that this particular combination is frequently used in gene therapy protocols in order to increase efficiency of retroviral integration into stem and progenitor cells (Cassel *et al* 1993, Crooks *et al* 1993, Nolta *et al* 1992, Lusky 1992).

An unanticipated observation that emerged from the current study was the great importance of G-CSF in this culture system. The role of G-CSF in regulating the production of neutrophilic granulocytes has been well described (Metcalf 1983, Demetri *et al* 1991). A considerable body of data derived from *in vitro* and *in vivo* studies suggests that G-CSF is a late acting HGF, supporting not only the proliferation and differentiation of lineage restricted neutrophil clonogenic progenitor cells but also stimulating functions of mature neutrophils. In contrast, the current studies suggest that G-CSF has a major role in this *ex vivo* culture system as an early acting HGF. This is shown by the dramatic reduction in the *de novo* generation of CFU-GM that occurs following omission of G-CSF from either 6 HGF or 4 HGF. The importance of G-CSF is also supported by the SCF/G-CSF matrix experiments, which demonstrate that the concentration of G-CSF rather than that of SCF limits the production of neutrophilic cells in this culture system. The role of G-CSF as an early acting HGF are consistent with the observations of Ogawa and colleagues who previously showed that G-CSF in addition to IL-3 was required to allow progression of $CD34^+$, $HLA\text{-}DR^-$, SBA^-, Lin^- cells from Go into S phase of the cell cycle (Leary *et al* 1992, Ikebuchi *et al* 1988a).

However, the results differ from those of Brugger who were unable to demonstrate a further additive effect of G-CSF on nucleated cell production from peripheral blood $CD34^+$ cells cultured in IL-1, IL-3, IL-6, EPO and SCF (Brugger *et al* 1993): an understandable finding given that this combination has been shown to be very efficient for promoting proliferation and differentiation of erythroid cells rather than neutrophil generation (Lansdorp *et al* 1992). Moreover, the authors reported that addition of 100 ng/ml of GM-CSF to this HGF

combination resulted in increased nucleated cell production but significantly less CFU-GM. The reason for the difference in the effects of G-CSF in these *ex vivo* culture systems remain to be determined but one possibility is restriction of erythroid differentiation when G-CSF is added to the basic combination of IL-3, IL-6 and SCF (36S). This possibility implies the concept of competition between G-CSF and EPO in regulating differentiation of cells stimulated by 36S. The data of Brugger *et al* suggest that addition of G-CSF may block erythroid differentiation and therefore there is no net increase in cell production in the culture. Another possible mechanism may be related to the very high concentration of IL-1 (100 ng/ml) used in their study. Interleukin-1 has been shown to modulate the expression of HGF receptors, notably the G-CSF receptor (G-CSFR) and therefore may indirectly regulate myeloid cell differentiation. In addition, IL-1 is also a potent inducer of production of other cytokines, which may indirectly affect the type of cells produced. Sato also compared growth of PB $CD34^+$ cells in the 3 HGF combinations of IL3+IL6+IL11, 36S and IL3+IL11+SCF and identified the combination of 36S as the most effective for production of total cells and non-erythroid progenitors after 7 days of culture (Sato *et al* 1993b). They also reported that addition of GM-CSF to 36S resulted in greater production of nucleated cells and progenitors than in cultures where the 3 HGF were supplemented with G-CSF. It should be noted however that, unlike this study, their experiments were not performed for 14 days. The present study demonstrates that the effects of G-CSF are not evident by 7 days but are clearly so after 14 days which may therefore explain the apparent discordance between the present data and those reported by Sato and colleagues.

Collectively, the data presented here provide a sound rationale for choosing combinations of HGF for *ex vivo* expansion of $CD34^+$ cells and also to predict the outcome of cultures established in different HGF combinations. Consequently, the type and number of cells to be generated by an *ex vivo* culture should dictate the choice of HGF combination. For example, GM-CSF should be included in the HGF combination when the objective is to generate both neutrophil precursors and monocytes but should be omitted from cultures designed specifically for production of neutrophil precursors. This idea is supported by the data of Steen who showed that $CD34^+$ cells cultured with SCF, IL-3 and GM-CSF generated mainly monocytic cells as determined by the number of cells expressing CD33, CD13 and CD14 (Steen *et al* 1994). In contrast, Smith *et al* reported that BM $CD34^+$ cells cultured in SCF, IL-3, G-CSF and GM-CSF generated mainly neutrophil precursors as determined by the number of cells co-expressing CD15 and CD11b although increasing numbers of monocytes and macrophages were reported in the non adherent cell fraction up until day 10-

13 of culture (Smith *et al* 1993).

Apart from identifying the critical HGF for generation of neutrophil precursors from $CD34^+$ cells the optimal concentrations for each of these factors in static cultures has been determined. Although these recommendations are based on cultures established in media supplemented with 30% foetal calf serum, initiated with 1,000 $CD34^+$ cells they are suitable for cultures initiated at higher concentrations of $CD34^+$ cells. Similar findings have been reported for culture of $CD34^+$ cells in media supplemented with 10% autologous plasma (Purdy *et al* 1995). These investigators also concluded that a combination of IL-3, IL-6, G-CSF and SCF is adequate for generation of a target number of 1×10^7 CFU-C, although no justification was given for the choice or the concentration of each cytokine.

Herein it is demonstrated that the 4 HGF combination of IL-3, IL-6, G-CSF and SCF, with each HGF used at its optimal concentration is better than a 6 HGF combination of 136GGMS. A 1 litre culture containing 5×10^6 $CD34^+$ cells established with 4 HGF generated 2×10^9 myeloid cells at all stages of maturation and 5×10^7 CFU-GM. Based on these data, a 5 litre culture initiated with 2.5×10^7 $CD34^+$ cells could potentially generate 1×10^{10} myeloid cells which could represent a clinically effective cell dose. In the study of Williams *et al* where $CD34^+$ cells isolated from mobilised blood of 8 patients were cultured in the presence of the GM-CSF/IL-3 fusion protein PIXY123 for 12 days, an average of 3.35×10^9 myeloid cells were generated from an average 1.2×10^8 $CD34^+$ cells (Williams *et al* 1996). Infusion of the *ex vivo* expanded cells did not improve the rate of neutrophil or platelet recovery although in the 1 patient infused with 1.2×10^{10} cells the neutropenia was less severe and the nadir abbreviated. These data suggested that infusion of at least $1\text{-}5 \times 10^{10}$ maturing myeloid cells might be required to show improved hemopoietic recovery and clinical benefit. Evidence that infusion of this number of *ex vivo* generated cells can improve haemopoietic recovery following myelosuppressive chemotherapy was provided by the recent study reported by Reiffers (Reiffers *et al* 1998, Reiffers *et al* 1999)

In this landmark study, $CD34^+$ cells were cultured for 10 days under static conditions in Teflon bags containing serum free medium supplemented with 100 ng/ml each of SCF, G-CSF and megakaryocyte growth and development factor (MGDF). After high dose conditioning therapy, expanded cells and unprocessed PB MNC were infused on day 0 and day 1, respectively, and patients received G-CSF until a normal neutrophil count was

reached. Each of the 14 patients treated showed an increased rate of both neutrophil and platelet recovery and 3 patients did not experience a neutrophil nadir less than 0.5 x 10^9/L. Notably these patients received between 0.86 and 1.77 x 10^{10} *ex vivo* expanded cells, suggesting that a dose in the range of 1.0 x 10^{10}, as was predicted earlier by Haylock (Haylock *et al* 1992, Haylock *et al* 1994b), would be required to abrogate neutropenia. Unfortunately Reiffers did not report data on the mix of myeloid cells in the *ex vivo* expanded product so it is unclear exactly what cells contributed to the rapid neutrophil and platelet recovery. In spite of this minor limitation, the Reiffers' study confirms that infusion of *ex vivo* expanded PB $CD34^+$ cells can dramatically improve HR following myelosuppresive therapy. Based on the results presented in this chapter, it seems that the very promising results reported by Reiffers were accomplished with sub-optimal conditions for *ex vivo* expansion of PB $CD34^+$ cells. Apart from the effects of a different HGF combination, *ex vivo* culture of $CD34^+$ cells could be significantly improved by taking into consideration some of the variables examined in the present work.

Firstly, generation of myeloid cells from $CD34^+$ cells cultured under static conditions appears to be self-limiting. As the cell density in culture approaches 1 – 2 x 10^6/ml there is a decrease in cell viability and proliferation that is associated with consumption of glucose and increased production of lactate. The rapid rise in lactate levels were to be expected, given that $CD34^+$ cells grew at the bottom of a tissue culture well under 4-5mm of media that effectively created an anaerobic environment. Under these conditions, cellular energy is predominantly generated by glycolysis and 2 lactate molecules are produced for every glucose molecule consumed. Moreover, lactate cannot be converted back to pyruvate and therefore would rapidly accumulate in growth media. Similar findings have been observed in bone marrow stromal cultures (Caldwell *et al* 1991) where the lactate levels in cultures with 1 x 10^6 adherent cells/ml were also in the range of 20-25mmol/L. The present studies have not determined if high levels of lactate and low pH directly inhibit growth of $CD34^+$ cells or their progeny so it remains unclear as to the actual mechanism/s responsible for growth inhibition at high cell density. Additional, potential contributing factors include consumption of other essential metabolites apart from glucose, production of metabolic inhibitors or inhibitory cytokines or consumption/degradation of HGF during culture. The latter issue was not investigated as part of the present series of experiments to optimise *ex vivo* culture of PB $CD34^+$ cells but have since been addressed by other groups working in the field. Immunoassays on HGF supplemented media held at 37°C has shown a 50% concentration decline for IL-3 and < 10% for both IL-6 and SCF after 12 days. In contrast, the

concentration of G-CSF decreases to 1% of its original level within 5 days (Martinson *et al* 1998). This data suggests that decreased G-CSF levels may, in part, contribute to deceased cell proliferation and increased cell death during the late stage of cultures. Although the present studies have not determined the exact mechanism/s responsible for decreased cell viability at high cell density the studies described clearly show that this issue is a major limitation for *ex vivo* expansion of $CD34^+$ cells.

A potential means to overcome this limitation in *ex vivo* cell generation is suggested by the current data: that is to initiate cultures at lower concentrations of $CD34^+$ cells. This means that cells grow for longer before reaching the limiting "terminal" cell density, the extra time in culture translating into a greater fold increase in the number of CFU-GM and maturing neutrophil precursors. Based on this principle and with knowledge of the kinetics of $CD34^+$ cell growth in Pre-CFU media supplemented with 4 HGF a model describing the relationship between initial cell concentration and cell generation at particular days of culture can be constructed (Table 4.2). The data in table 4.2 is based on the following: i) there is a lag phase of 1-2 days before $CD34^+$ cells divide and a net increase in the number of viable cells, ii) an assumption that during the exponential growth phase all divisions are symmetric and iii) that the cell doubling time, as derived from the proliferation data (figure 4.13) is approximately 24 hours. The table shows the expected number of viable cells on each day for cultures initiated with different concentrations of $CD34^+$ cells. For example, in a culture initiated with 300 $CD34^+$ cells in 1 ml of Pre-CFU media supplemented with 4 HGF it is predicted that a "terminal" density of 1.22×10^6 cells/ml will be reached after 14 days. In comparison, a culture started with 20,000 $CD34^+$ cells will reach exactly the same cell density in just 8 days. The $CD34^+$ cells cultured at 300 cells/ml would undergo a 4066-fold expansion whereas the 20,000 $CD34^+$ cells a 61-fold expansion. Implications of these projections for clinical *ex vivo* expansion of $CD34^+$ cells are shown in table 4.3. The data presented within this table is based on the calculations in table 4.2 and shows how generation of a target number of nucleated cells could be achieved in different ways depending on how many $CD34^+$ cells are available and how long the cells are cultured. With a limited number of $CD34^+$ cells, generation of 6×10^9 nucleated cells could be achieved by preparing a 5 litre culture of 5×10^6 $CD34^+$ cells at 1×10^3 cell/ml and waiting for 11-12 days. If however more $CD34^+$ cells were available, then a similar number of nucleated cells could be generated by establishing a 5 litre culture of 50×10^6 $CD34^+$ cells at 10,000 cells/ml and waiting for 9 days. It is important to note that in these 2 scenarios, cultures may contain a different mix of myeloid cells. In the case of the 1,000 $CD34^+$cells/ml cultured for 12 days there may be a

Table 4.2

Projection of nucleated cell production from $CD34^+$ cells cultured under static conditions in Pre-CFU media supplemented with IL-3 (10 ng/ml) + IL-6 (10 ng/ml) + G-CSF (100 ng/ml) + SCF (100 ng/ml).

Assumptions: This projection is based on the assumption that there is a 2-day lag period before $CD34^+$ cells start dividing, there is asymmetric division and that the doubling time during the exponential phase of growth is 24 hours.

Days in culture →

**$CD34^+$	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.3	.3	.3	.3	.6	1.2	2.4	4.8	9.6	19	38	76	154	307	614	1220
1	1	1	1	2	4	8	16	32	64	128	256	512	1024		
3	3	3	3	6	12	24	48	96	192	384	768	1536			
5	5	5	5	10	20	40	80	160	320	640	1220				
10	10	10	10	20	40	80	160	320	640	1220					
20	20	20	20	40	80	160	320	640	1220						
30	30	30	30	60	120	240	480	960	1920						
50	50	50	50	100	200	400	800	1600							
100	100	100	100	200	400	800	1600								

The numbers within the table represents the concentration of cells (x 10^3/ml) at that day of culture.

** Represents the concentration of $CD34^+$ cells used to initiate culture (x 10^3/ml).

greater proportion of late stage neutrophil precursors whereas the culture initiated with 10,000 $CD34^+$cells/ml may have a higher proportion of blasts after 9 days.

Another consideration is the loss of HGF activity during longer cultures. As is the case for G-CSF, HGF may need to be added at regular intervals during longer cultures. Thus while the information presented in table 4.3 may seem to be a blueprint for performing clinical scale *ex vivo* generation of neutrophil precursors the predicted outcomes need to be validated. Clearly the result of a culture depends on at least the interplay between the starting concentration of $CD34^+$ cells, the length of the culture, the HGF combination and HGF replenishment schedules.

The data presented in section 4.3.6.3 indicates that a second way to overcome the limiting effect of high cell density on myeloid cell proliferation is by perfusion culture. This approach has been proposed by a number of investigators for culture of haemopoietic cells in stromal-supported culture systems (Schwartz *et al* 1991, Caldwell *et al* 1991, Koller *et al* 1993). The basis for artificial perfusion is that the culture conditions would more closely resemble haemopoiesis *in vivo* where there is a constant, steady supply of nutrients and removal of metabolites. Plasma perfusion rates through bone marrow *in vivo* have been reported to be on the order of 0.1 ml per gram of bone marrow per minute (Martait *et al* 1987). Assuming a typical BM cell density of about 5×10^8 cells per ml, this perfusion rate approximately corresponds to a complete daily exchange of medium containing 20% serum for cells grown at 10^6 per ml. However, the standard exchange schedules for liquid bone marrow cultures call for a weekly exchange (Dexter *et al* 1977, Gartner *et al* 1980). These exchanges rates are thus at least 7-fold lower than that experienced by cells *in vivo*. For liquid culture cell densities that are higher than 10^6 cells/ml, the departure from *in vivo* conditions would be even higher. Perfusion with half-volume medium exchanges per day has been shown to significantly increase the productivity and longevity of human bone marrow cultures (Schwartz *et al* 1991). In accord with this, the data presented in 4.3.7.3 indicates that a similar rate of medium/HGF exchange is also able to significantly increase the growth of haemopoietic cells in stromal-free HGF dependent cultures. Furthermore it demonstrates that perfusion culture systems more effectively exploit the proliferative potential of $CD34^+$ cells *in vitro*. This suggestion is supported by other investigators who have demonstrated increased expansion of LTC-IC and progenitors in stroma-replete bone marrow cells cultured in stirred bioreactors (Zandstra *et al* 1995) or in perfusion cultures (Sandstrom *et al* 1995). Artificial hollow-fibre perfusion systems have also been developed or proposed for

Table 4.3

Summary of the relationship between the concentration of $CD34^+$ cells at the beginning of a 5 litre clinical scale *ex vivo* expansion culture, the length of culture until the terminal cell concentration is reached and the total number of cells generated at this time. This data is based on the information and assumptions shown for table 4.2.

Starting $CD34^+$ cell density ($\times 10^3$)/ml	Volume of Culture (Litres)	Initial number of $CD34^+$ cells ($\times 10^6$)	Length of Culture (Days)	Number of cells generated ($\times 10^9$)
0.3	5	1.5	14	6.1
1	5	5	12	5.12
3	5	15	11	7.68
5	5	25	10	6.1
10	5	50	9	6.1
20	5	100	8	6.1
30	5	150	7	4.8
50	5	250	6.5	6

larger scale expansion of haemopoietic cells (Kidwell 1996, Davis *et al* 1994, Nordon *et al* 1996). Perfusion culture has also been proposed for generation of other mammalian cell types for therapy. Expansion of mammalian neural stem cells at high cell density has been achieved in suspension bioreactors (Kallos *et al* 1999). However, at present there are no commercial devices for stroma-free perfusion culture of purified $CD34^+$ cells. In spite of this, culture of $CD34^+$ cells in bags or flasks could be improved significantly by frequent exchange of media and HGF.

In conclusion, the studies within this chapter represent the basis for optimising *ex vivo* generation of neutrophil precursors from $CD34^+$ cells cultured under stromal free, cytokine dependent, static conditions. A combination of IL-3 + IL-6 + G-CSF + SCF (36GS), each at optimum concentration represents the minimum combination of HGF for generating sufficient post-progenitor cells for therapy. A key finding of the studies is that static culture is self-limiting and severely compromises the proliferative potential of $CD34^+$ cells. Perfusion culture of $CD34^+$ cells may overcome this limitation. The advantage of perfusion systems is that they will allow much better control over culture conditions including levels of glucose and other essential metabolites, lactate, pH and oxygen tension. Cultures performed with perfusion of media and cytokines either in hollow fibre cartridges or in stirred bioreactors have the potential to generate many more cells, which means that less $CD34^+$ cells will be needed for expansion. As shown in the present study despite optimisation of the combination of HGF, changes in the culture environment significantly impact upon cell survival and proliferation. Although the present study has not addressed the affect of variation in oxygen tension, there is evidence that cultures at low O_2 tension may further improve growth of HPC (Bradley *et al* 1978, Maeda *et al* 1986, Rich 1986, Broxmeyer *et al* 1990). In addition, further improvements in expansion of $CD34^+$ cells may be expected by adding newly identified HGF that stimulate the growth and development of HPC. In this regard, Chapters 6 concern the affects of *flt3*-ligand (FLT3L) and thrombopoietin (TPO) on HPC growth.

Finally there are 2 important issues that arise from the data presented within this chapter. Firstly, although a potent combination of HGF has been identified for stimulating growth of HPC it is unclear which cells within the $CD34^+$ fraction respond to this combination of cytokines. The $CD34^+$ cell fraction is heterogeneous, containing committed haemopoietic progenitors and primitive HPC including Pre-CFU and cells responsible for long-term haemopoietic reconstitution. Culture of $CD34^+$ cells with 36GS is able to give rise to nascent

CFU-GM, indicating that a proportion of Pre-CFU are stimulated by this combination of HGF. However it remains to be determined exactly which cells within the $CD34^+$ fraction are stimulated by 36GS? Furthermore, which cells are responsible for the greatest *de novo* generation of CFU-GM and nucleated cells?

The second issue arising from the present study is related to the first and concerns the fate of long-term repopulating cells (stem cells) in *ex vivo* cultures stimulated with the 36GS combination. If *ex vivo* expanded cells are used alone for transplantation following truly myeloablative conditioning then it is essential that long-term haemopoiesis is not compromised. There is an obligation to determine whether the number of stem cells, their homing function and proliferative potentials are altered during *ex vivo* culture. The difficulty is that most *in vitro* assays of haemopoiesis at best, provide a surrogate measure of stem cell activity. The following chapters of this thesis deal with these issues. Chapter 5 investigates exactly which cells within the $CD34^+$ fraction are stimulated by 36GS and secondly examines cytokine receptor expression by $CD34^+$ cells so as to investigate the mechanism(s) responsible for HGF mediated proliferation of $CD34^+$ cell fractions. Chapter 6 concerns the response of primitive HPC to combinations of HGF and in particular the role of FLT3L and TPO in *ex vivo* manipulation of these cells.

CHAPTER 5. CHARACTERISATION OF CD34$^+$ HAEMOPOIETIC PROGENITOR CELLS AND PRE-CFU

5.1 Introduction

The data presented in the previous chapters indicate that combinations of HGF including IL-3, IL-6, G-CSF, GM-CSF and SCF provide a potent proliferative stimulus to CD34$^+$ cells and lead to significant production of myeloid cells and neutrophil precursors cells in *ex vivo* culture. Although these data suggest that a sufficient number of neutrophil precursors for clinical purposes could be generated from cultures of CD34$^+$ cells some critical questions remain unresolved. For example, which cells within the CD34$^+$ population respond to these HGF and give rise to neutrophil precursors? Do combinations of HGF stimulate growth of both primitive and committed HPC within the CD34$^+$ population? In particular, does culture with combinations of HGF such as 36GS induce division of candidate human haemopoietic stem cells? In addition, does HGF receptor (HGFR) expression by CD34$^+$ cells predict their response to combinations of HGF? Such questions concern the underlying mechanism/s responsible for the HGF stimulated proliferation of CD34$^+$ cells and also the fate of HSC under *ex vivo* culture conditions. The studies presented within this and the following chapter seek to address these questions.

A central issue relevant to these questions is the discrimination between committed and primitive HPC including HSC. Although it is evident that human HSC, their immediate multipotential progeny and lineage committed precursors within adult haemopoietic tissues express the CD34 antigen it remains contentious as to which additional markers best discriminate committed HPC from primitive HPC within this heterogeneous population. The standard approach for such investigations has been to test the haemopoietic potential of sub-populations of cells isolated according to expression of cell surface antigens or their biological properties. In lieu of a definitive *in vivo* assay for human HSC with marrow repopulating ability, a number of surrogate *in vitro* assay systems have been developed and used for such studies.

These systems include the standard semisolid clonogenic assays performed in agar, agarose or methylcellulose (Metcalf 1984), long-term BM culture (Dexter *et al* 1971, Moore *et al* 1979, Gordon *et al* 1987), the CFU-blast assay (Leary *et al* 1987, Rowley *et al* 1987) and the Pre-CFU culture system (Iscove *et al* 1989, Smith *et al* 1991). As distinct from the

semisolid clonogenic assay, the latter 3 systems have been used to identify primitive HPC. Long-term bone marrow culture (LTBMC), assays for cells (long-term culture-initiating cells: LTC-IC) with the ability to initiate and sustain haemopoiesis in a standard Dexter type stromal-cell dependent system. The blast colony assay devised by Ogawa and colleagues assays for late-appearing undifferentiated blast colonies which have a high replating ability and the capacity to give rise to both differentiated and undifferentiated colonies (Nakahata and Ogawa 1982, Leary and Ogawa 1987). A modification of this system, which involved a liquid/suspension culture instead of semi-solid media, was used to isolate blast colonies capable of extensive generation of new colonies after replating of the first generation colonies (Terstappen *et al* 1991). Similarly, the Pre-CFU culture system was designed to identify precursors of cells that clone in semisolid media and is based on the rationale that Pre-CFU-GM would, when stimulated by HGF, give rise to nascent CFU-GM (Iscove *et al* 1989, Smith *et al* 1991). The ability to generate nascent CFU-GM was considered an index of Pre-CFU activity in the starting cell population. Each of these surrogate *in vitro* assays/culture methods have been used to compare the haemopoietic potential of sub-populations of $CD34^+$ cells defined according to expression of lineage, activation or functional antigens

Collectively, a considerable body of data obtained with these different assays has demonstrated that the $CD34^+$ cell population is quite heterogeneous. It contains virtually all BFU-E, CFU-G/M, CFU-Meg and CFU-GEMM (Watt *et al* 1987, Civin and Loken 1987, Debili *et al* 1992, Andrews *et al* 1989). Furthermore, B lymphoid committed progenitors, thymic T cell precursors (Pontvert-Deucq *et al* 1993, Peault *et al* 1991, Galy *et al* 1995b) and NK cell precursors (Galy *et al* 1995a, Miller *et al* 1992, Latzova *et al* 1993) are also restricted to $CD34^+$ cells. In addition, LTC-IC have been characterised as a distinct sub-fraction of $CD34^+$ cells which are small (Sutherland *et al* 1989) and lack various lineage markers (Baum *et al* 1992), transferrin receptor (Brandt *et al* 1992, Lansdorp *et al* 1992), the high molecular weight isoform of human leukocyte antigen (CD45RA) (Lansdorp *et al* 1990, Lansdorp *et al* 1992), CD33 (Andrews *et al* 1989, Buhring *et al* 1989, Bernstein *et al* 1991), and HLA-DR (Moore *et al* 1980, Keating *et al* 1984, Brandt *et al* 1990, Sutherland *et al* 1989). Further studies demonstrated that LTC-IC express low levels of Thy-1 (Craig *et al* 1993, Baum *et al* 1992), are c-*kit*$^+$ (Briddell *et al.* 1992, Simmons *et al* 1994a, Craig *et al* 1993), Rhodamine 123 dull (Chaudhary *et al* 1991, Udomsakdi *et al* 1991, Uchida *et al* 1996) and quiescent as judged by their relative insensitivity to 4-HC (Winton *et al* 1987, Eaves *et al* 1992). Terstappen and colleagues also demonstrated that blast colonies derived

from $CD34^+CD38^-$ but not $CD34^+CD38^+$ cells are capable of extensive generation of new colonies after replating of the first generation colonies (Terstappen at al 1991). Further studies have demonstrated that Pre-CFU activity is greater in a sub-fraction of $CD34^+$ cells which lacked detectable surface markers for T-cell, B-cell, natural killer cell and myeloid lineage ($CD34^+lin^-$ cells) (Iscove *et al* 1990).

A consistent finding and emerging theme from these studies was that committed HPC cells were enriched within the $CD34^+$ fraction expressing lineage associated or activation antigens whereas primitive HPC and most likely HSC, were enriched within the $CD34^+$ fractions lacking these markers. In this regard, expression of CD38 or HLA-DR on $CD34^+$ cells has been well established as particularly useful markers for discriminating committed HPC from their precursors (Terstappen *et al* 1991, Rusten *et al* 1994b, Huang *et al* 1994). Results from both LTBMC (Sutherland *et al* 1989, Srour *et al* 1991) and blast colony assays (Brandt *et al* 1988) indicate that committed HPC are enriched within the $CD34^+HLA\text{-}DR^+$ or $CD34^+CD38^+$ fractions whereas primitive HPC are enriched in the $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^-$ fractions.

In order to optimise generation of neutrophil precursors from unfractionated $CD34^+$ cells it is essential to establish how HGF's affect the growth of the constituent primitive and committed HPC within the $CD34^+$ population. Experiments were therefore initiated to firstly investigate the affects of single HGF or combinations thereof on the growth of CFU-GM from $CD34^+$ cell fractions. Further experiments were also performed to compare generation of neutrophil precursors from the same $CD34^+$ fractions when grown in Pre-CFU cultures stimulated by combinations of HGF. Similarly, Pre-CFU cultures were also used to determine if HGF receptor (HGFR) expression on $CD34^+$ cells predicted the ability of cells to produce neutrophil precursors when cultured in combinations of HGF including 36GS.

5.2 Experimental Design and Methods

The first series of experiments within this chapter were performed to assess the affect of single or combinations of HGF on cloning of CFU-GM within $CD34^+$ cell sub-populations representing committed and primitive HPC. Bone marrow MNC were immunolabelled with anti-CD34 and anti-CD38 or anti-HLA-DR monoclonal antibodies then $CD34^+$, $CD34^+CD38^+$, $CD34^+CD38^-$, $CD34^+HLA\text{-}DR^+$ and $CD34^+HLA\text{-}DR^-$ cell fractions were isolated by FACS. CFU-GM assays were performed with cells from each fraction and

stimulated with either single HGF or combinations thereof. In addition, replicate 1 ml Pre-CFU cultures were initiated in media containing 30% FCS and 1% BSA supplemented with the HGF combination of 136GGMS. Cultures were fed with additional HGF after 7 days and thereafter split weekly by dilution with fresh media and HGF. Nucleated cell counts and CFU-GM assays were performed at days 14, 21 and 28 from cultures initiated with unfractionated $CD34^+$ cells, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$, $CD34^+CD38^+$ and $CD34^+CD38^-$ cells.

The second aspect of studies within this chapter concerns examination of haemopoietic growth factor receptor (HGFR) expression on $CD34^+$ cells. This was achieved by dual colour immunostaining with a panel of IgG_1 murine monoclonal antibodies to the IL-3R – alpha chain (IL-3Rα), GM-CSFR–alpha chain (GM-CSFRα), the common IL-3 and GM-CSF β chain (β_c), gp130, IL-6R, G-CSFR and c-*kit* in combination with McAB, 43A1 (Buhring *et al* 1995), an IgG_3 murine monoclonal antibody to CD34. It was anticipated that some receptors might be expressed at very low levels and would be at the limit of detection by flow cytometry. Therefore, PE-conjugated anti-mouse IgG_1 reagents were routinely used to enhance receptor detection. The sorted cells were assayed for their content of clonogenic progenitor cells (CFU-GM and BFU-E) and plated in Pre-CFU culture.

5.3 Results

5.3.1 Expression of CD38 and HLA-DR on BM $CD34^+$ cells

The expression of CD38 and HLA-DR antigens was investigated on BM $CD34^+$ cells isolated from normal adult volunteer donors. The pattern of staining was highly reproducible over many BM samples and allowed for easy discrimination of $CD34^+$ cell subsets according to the expression of CD38 and HLA-DR antigens. Representative examples of the typical pattern of expression of these antigens on BM $CD34^+$ cells are shown in figures 5.1, 5.2 and 5.3. As shown in figure 5.1 and 5.2 the pattern of CD38 expression on $CD34^+$ cells is quite distinctive and is a continuum ranging from no CD38 expression to high level expression (panel D, figure 5.2). The majority of $CD34^+$ cells express high levels of CD38 whereas only 6-8% were found to be $CD38^-$. These $CD34^+CD38^-$ cells express uniformly higher levels of the CD34 antigen than their $CD34^+CD38^+$ counterparts and represent a discrete population of small cells with distinctive low forward (FSC) and side scatter (SSC) (panel D, figure 5.1). In contrast $CD34^+CD38^+$ cells are more heterogeneous in size and generally larger which is in accord with the more variable and generally greater FSC profile (panel E,

Figure 5.1 Expression of CD34 and CD38 on adult bone marrow cells

The cell surface expression of CD34 and CD38 on light density MNC isolated from adult human BM was assessed following immunolabelling with monoclonal antibodies directly conjugated to FITC and PE, respectively. Panel A represents the forward scatter (FSC) versus side scatter (SSC) distribution of MNC and panel B the expression of CD34 as compared to SSC. The percentages shown within the quadrant of panel B indicate the proportion of cells within the respective quadrants (in this example 7.38% of MNC were $CD34^+$). Panel C is a two-colour dot plot with CD34 expression shown on the horizontal axis and CD38 expression on the vertical axis. Cells with a $CD34^+CD38^+$ phenotype are shown in blue and are located within the R2 sort window whereas $CD34^+CD38^-$ cells are shown in red and are located within the R1 sort window. Cells defined by R1 and R2 regions were sorted and used for the functional studies described in sections 5.3.2 and 5.3.3. Panels D and E represent the FSC versus SSC distribution of $CD34^+CD38^-$ (red dots) and $CD34^+CD38^+$ (blue dots) cells, respectively. The rectangular window within panels D and E is located in an identical position and allows comparison of the FSC and SSC characteristics of each cell population.

Adult Bone Marrow: Expression of CD34 and CD38

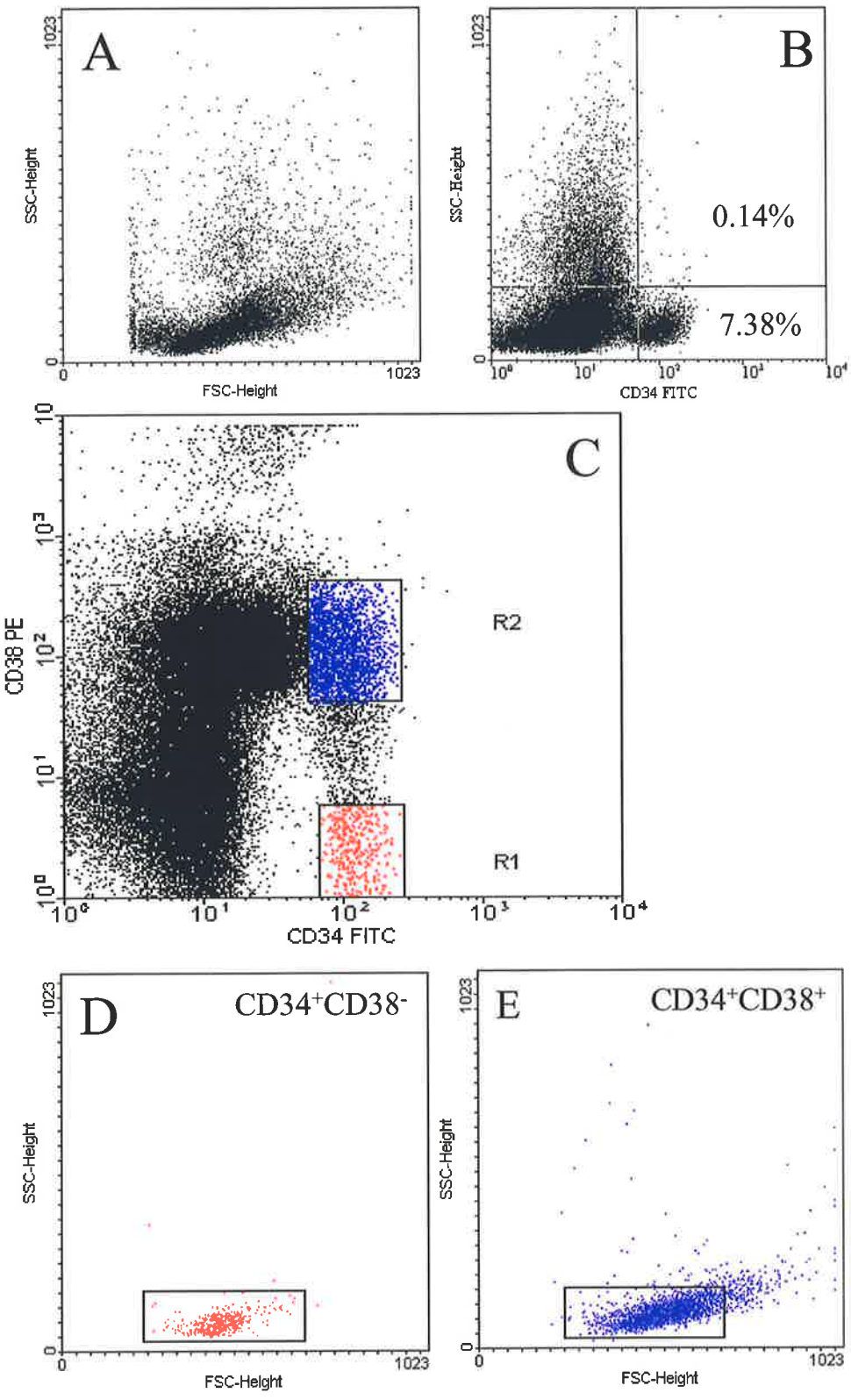


figure 5.1). The typical regions used for sorting $CD34^+CD38^-$ and $CD34^+CD38^+$ cells for functional studies are shown as regions R1 and R2, respectively, in panel C of figure 5.1. The $CD38^-$ and $CD38^+$ cells defined by these sort region, comprised 7.2 ± 2.1% and 68.7 ± 12% (mean ± SEM, n=12), of the $CD34^+$ cells, respectively.

The pattern of HLA-DR expression by $CD34^+$ cells was also consistent between BM samples but noticeably different to that of CD38. These differences are evident in the 2-colour dot plots of figure 5.2, which show Dynal bead purified BM $CD34^+$ cells labelled with HPCA-2-FITC (anti-CD34) and either anti-CD38-PE or anti-HLA-DR-PE. As with expression of CD38 on $CD34^+$ cells, there is also a continuum of HLA-DR expression, with approximately 10-12% being $HLA\text{-}DR^-$ and the majority expressing intermediate levels of HLA-DR. Only a small proportion of $CD34^+$ cells express high levels of HLA-DR whereas the majority of $CD34^+$ cells express high levels of CD38. There is also a difference in expression of these two antigens by $CD34^{dim}$ cells within BM (compare panels C with D, figure 5.2). Essentially all $CD34^{dim}$ cells express high levels of CD38 whereas HLA-DR splits the $CD34^{dim}$ fraction into a $HLA\text{-}DR^+$ fraction which is more abundant than the $HLA\text{-}DR^{++}$ fraction. The $CD34^+HLA\text{-}DR^-$ cells have lower forward and side scatter profiles than $CD34^+HLA\text{-}DR^+$ cells but they do not cluster as tightly on FSC and SSC as compared to $CD34^+CD38^-$ cells (panel D figure 5.3). The regions used for sorting $CD34^+HLA\text{-}DR^-$ and $CD34^+HLA\text{-}DR^+$ cells for functional studies are shown as R2 and R3, respectively on panel C of figure 5.3. The $HLA\text{-}DR^-$ and $HLA\text{-}DR^+$ cells defined by these sort region, comprised 11.6 ± 4.3% and 80.6 ± 8.2% (mean ± SEM, n=12), of the $CD34^+$ cells, respectively.

Three-colour immunolabelling of Dynal purified BM $CD34^+$ cells for CD34, HLA-DR and CD38 revealed that $CD34^+$ cells could be separated into 4 discrete populations based on the expression of CD38 and HLA-DR. These populations are arbitrarily defined by the location of the quadstat marker shown in figure 5.4. Approximately 90-95% of $CD34^+$ cells co-express both CD38 and HLA-DR, 2-4% are $CD38^+HLA\text{-}DR^-$, 4-7% are $CD38^-HLA\text{-}DR^+$ and less than 0.5% are $CD38^-HLA\text{-}DR^-$. Thus expression of CD38 and HLA-DR on $CD34^+$ cells appears to be differentially regulated and mutually exclusive.

5.3.2 Cloning of CFU-GM Within the $CD34^+CD38^-$ Fraction is Dependent on Combinations of HGF

The effects of single HGF or combinations thereof on growth of $CD34^+$ fractions enriched in primitive and committed HPC was assessed by performing CFU-GM assays on purified cell

Figure 5.2 Expression of HLA-DR and CD38 on adult bone marrow $CD34^+$ cells

The cell surface expression of HLA-DR and CD38 on Dynal enriched $CD34^+$ cells from adult human BM was assessed following immunolabelling with monoclonal antibodies directly conjugated to PE. Panel A represents the FSC and SSC characteristics of cells obtained following enrichment with Dynal 561 beads and detach-a-bead polyclonal antisera. The two colour dot-plot in panel B serves as a control for panels C and D and shows CD34 expression (horizontal axis) together with staining of an irrelevant IgG_1-PE conjugate (isotype control antibody) on the vertical axis. In this example, 91.3% of cells were $CD34^+$. Panels C and D show expression of HLA-DR and CD38 respectively, as a function of CD34 expression. As shown, in this example, 9.3% of cells were $CD34^+HLA\text{-}DR^-$ as compared to 4.53% $CD34^+CD38^-$.

Expression of CD38 and HLA-DR on BM CD34^{+} Cells

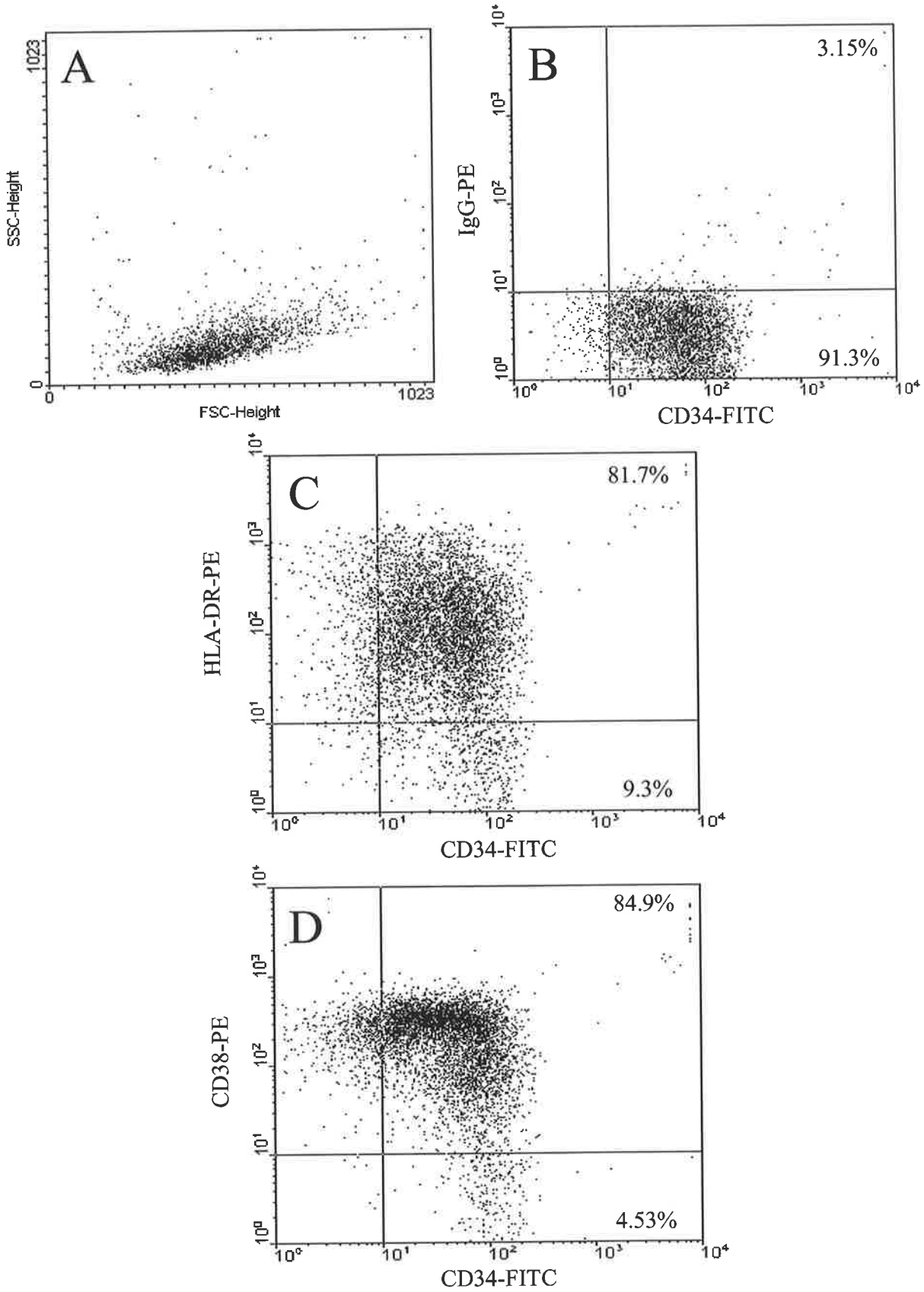


Figure 5.3 Expression of HLA-DR on adult bone marrow cells

The cell surface expression of HLA-DR and CD34 on light density MNC isolated from adult human BM was analysed by flowcytometry. Panel A represents the forward scatter (FSC) versus side scatter (SSC) distribution of MNC and panel B the expression of CD34 (antibody HPCA-2-FITC) as compared to SSC. Panel C is a two-colour dot plot with CD34 expression shown on the horizontal axis and HLA-DR on the vertical axis. Cells with a $CD34^{+}HLA\text{-}DR^{+}$ phenotype are shown in red and are located within the R3 sort window, whereas $CD34^{+}HLA\text{-}DR^{-}$ cells are represented by blue dots within the R2 sort window. Panels D and E represent the FSC versus SSC distribution of $CD34^{+}HLA\text{-}DR^{-}$ cells (blue dots) and $CD34^{+}HLA\text{-}DR^{+}$ cells (red dots), respectively. The rectangular window in panels D and E is arbitrarily placed to allow comparison of FSC and SSC characteristics of each cell population. Cells defined by R2 and R3 regions were used for sorting cells used in the functional studies described in sections 5.3.2 and 5.3.3. The rectangle labelled as $CD34^{+}$ within panel B represents a typical sort region for isolation of $CD34^{+}$ cells used in functional studies.

Adult Bone Marrow: Expression of CD34 and HLA-DR

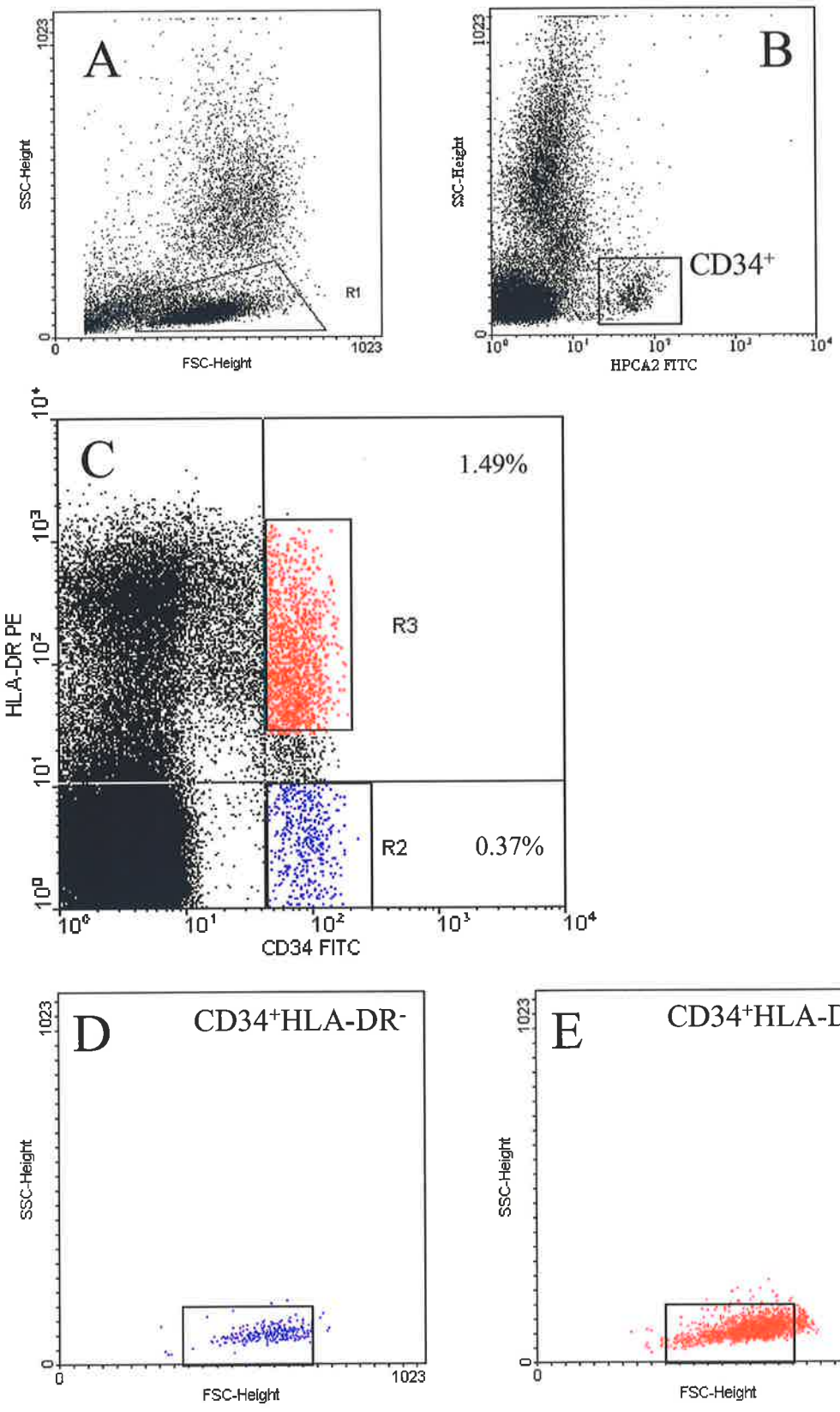
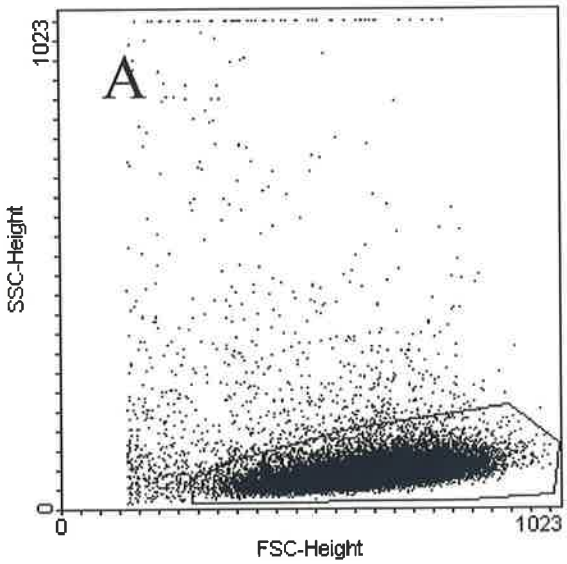
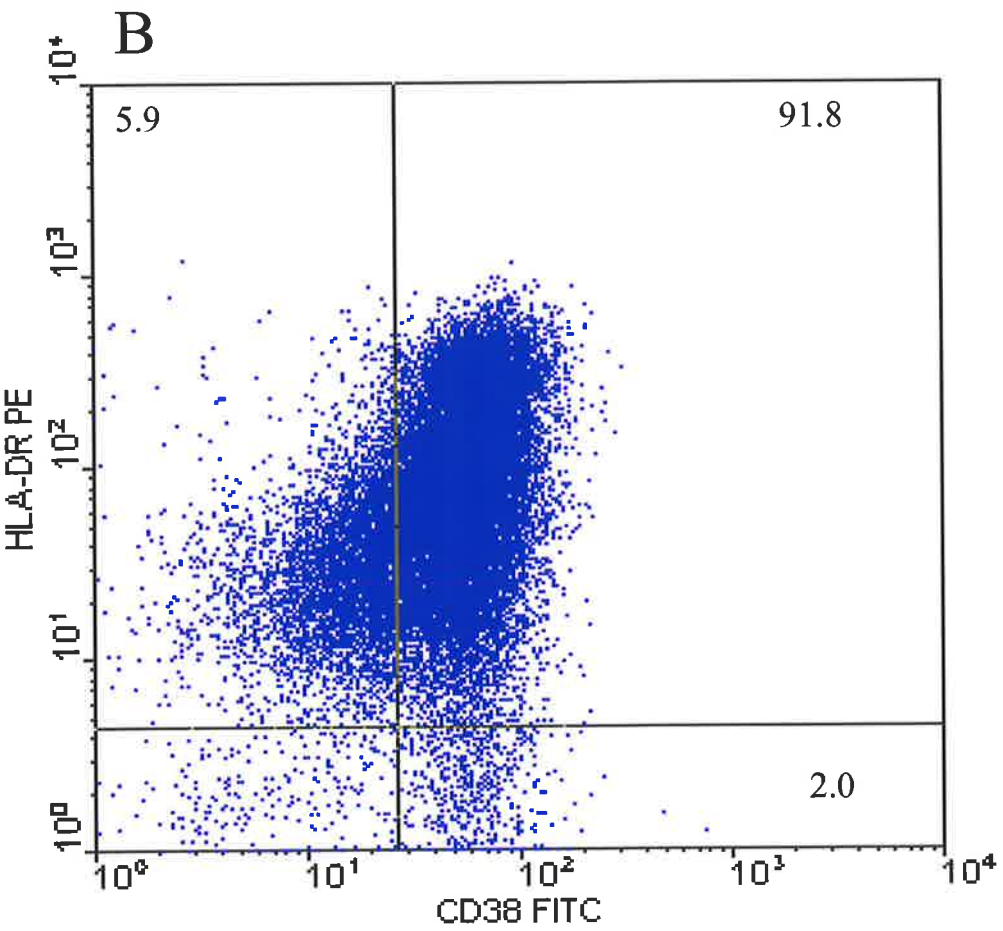


Figure 5.4 Expression of CD38 and HLA-DR on $CD34^+$ BM cells

$CD34^+$ cells were enriched from adult human BM by the 561-Dynal bead and detach-a-bead method. Cells were labelled with 43A1 (IgG_3, anti-CD34), biotinylated goat anti-mouse IgG_3, streptavidin-tricolour, CD38-FITC (IgG_1 isotype) and HLA-DR-PE (IgG_1 isotype) conjugates. Panel A shows the forward scatter (FSC) versus side scatter (SSC) characteristics of enriched cells and panel B expression of CD38 and HLA-DR on $CD34^+$ cells within the sort window displayed in panel A. In this representative example, 2.0% of $CD34^+$ cells were $CD38^-HLA\text{-}DR^+$, 5.9% were $CD38^-HLA\text{-}DR^+$ and 0.3% were $CD38^-HLA\text{-}DR^-$.


A
1023
SSC-Height
0
0
1023
FSC-Height


B
5.9
91.8
2.0
HLA-DR PE
CD38 FITC
10⁰
10¹
10²
10³
10⁴

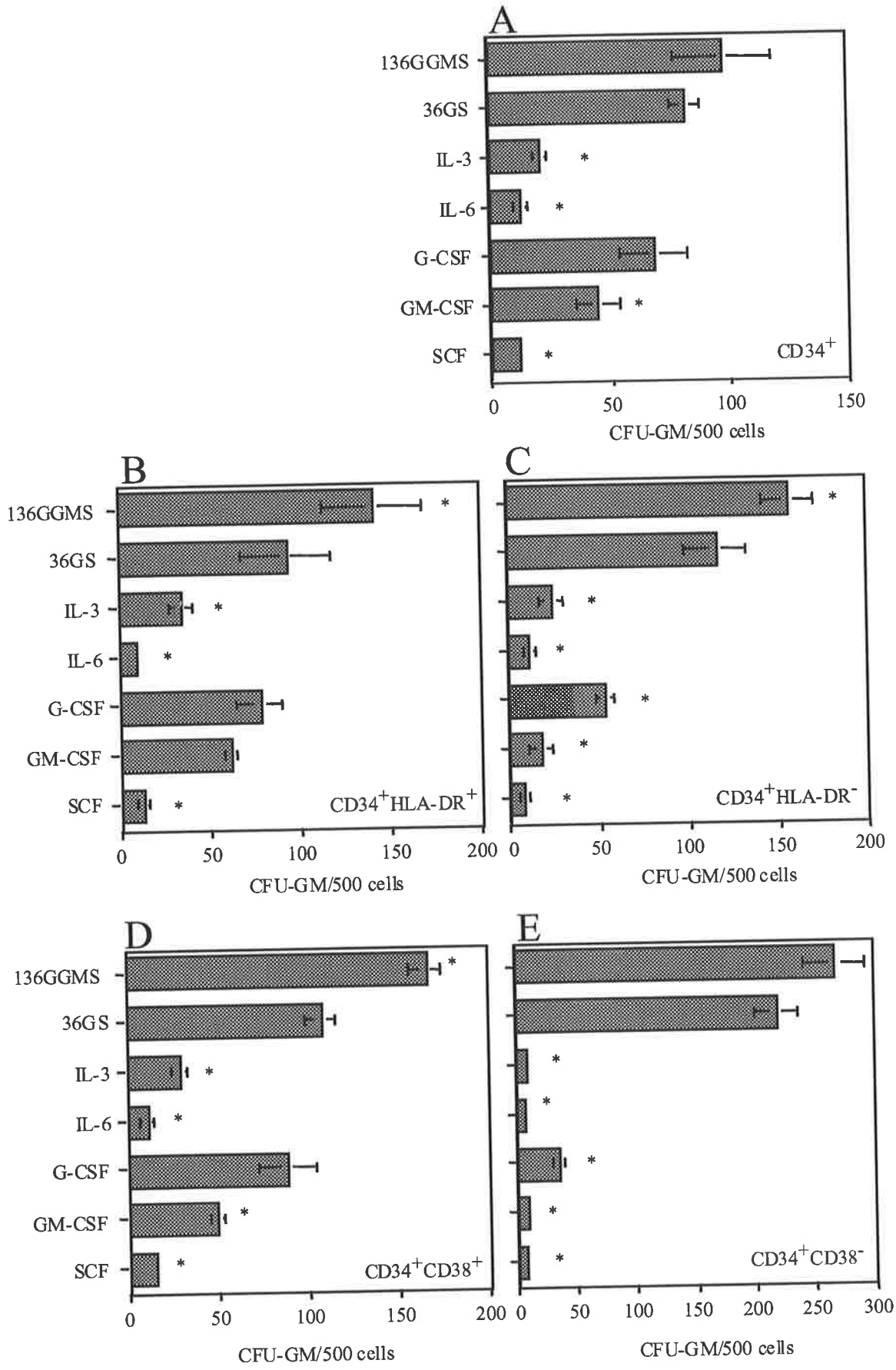
populations cells isolated by FACS. These studies were performed on 5 cell fractions (unfractionated $CD34^+$, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$, $CD34^+CD38^+$ and $CD34^+CD38^-$ cells) sorted according to the regions previously described above. Following FACS, CFU-GM assays were initiated with 500 cells/plate in methylcellulose and stimulated with IL-3, IL-6, G-CSF, GM-CSF, SCF, (each at 10 ng/ml) the combination of 36GS or the 6 factor combination of 136GGMS. Plates were examined after 14 days incubation and colonies counted using standard criteria for identification of granulocyte-macrophage clones. The combined results of 4 independent investigations are presented in figure 5.5.

The greatest numbers of CFU-GM from each cell fraction occurred when the 136GGMS combination was used as the source of colony stimulating activity. The cloning efficiency of $CD34^+HLA\text{-}DR^+$ (panel B), $CD34^+HLA\text{-}DR^-$ (panel C) and $CD34^+CD38^+$ (panel D) cells was significantly greater when 136GGMS was used for stimulation rather than the 4 HGF combination of 36GS (p values of 0.03, 0.08 and 0.015). In contrast, significantly less CFU-GM ($p \leq 0.05$) were observed when cells were stimulated by single HGF. This result was independent of the $CD34^+$ cell fraction although it was most striking for $CD34^+CD38^-$ cells Panel E). A culture of 500 $CD34^+CD38^-$ cells, stimulated with 36GS yielded 218 ± 18.5 CFU-GM (mean $\pm$ SD) but only 9.7 ± 2.7, 6.4 ± 1.7, 34 ± 5.6, 10 ± 1.2 and 6 ± 1.9 CFU-GM when stimulated by only IL-3 or IL-6 or G-CSF or GM-CSF or SCF, respectively. G-CSF and GM-CSF were the most potent single HGF for stimulating CFU-GM from $CD34^+$, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^+$ cells. Cultures stimulated with G-CSF alone resulted in 69.9%, 55.3%, 33.9% and 53%, respectively of the number of CFU-GM produced from $CD34^+$, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^+$ cells when stimulated by 136GGMS. SCF and IL-6 were the least active single HGF for promoting CFU-GM growth, irrespective of the cell fraction.

As indicated in the previous section (5.3.1) approximately 90% -95% of the $CD34^+$ cells within BM and a similar proportion of mobilised blood $CD34^+$ cells (To *et al* 1994) express both CD38 and HLA-DR antigens. It is therefore clear from the data presented that a combination of 36GS would be quite effective in stimulating proliferation of CFU-GM within this population of cells. Moreover, the data suggests that a large proportion of the cell expansion during a 14 day *ex vivo* culture of $CD34^+$ cells could be attributed to proliferation of committed myeloid progenitors within the $CD34^+CD38^+$ and $CD34^+HLA\text{-}DR^+$ fraction that respond to single HGF such as G-CSF or combinations such as 36GS. This proposal was

Figure 5.5 CFU-GM cloning in $CD34^+$ cell subsets: effect of HGF combinations

$CD34^+$, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$, $CD34^+CD38^+$ and $CD34^+CD38^-$ cells were isolated by FACS from normal adult human BM and placed into methylcellulose CFU-GM assays stimulated by either single HGF including SCF, GM-CSF, G-CSF, IL-3, IL-6 or combinations of HGF comprising 36GS or 136GGMS. Typically, total $CD34^+$ cells were isolated from a sort region created from the CD34 versus side scatter (SSC) dot-plot as shown in panel B of figure 5.3. Regions used for sorting $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^+$, $CD34^+CD38^-$ cells are shown in figures 5.3 and 5.1, respectively. CFU-GM colonies were counted after 14 days incubation at 37^0C, 5% CO_2 and the average CFU-GM of triplicate plates was normalised to CFU-GM/500 input cells. The results (mean ± SEM, n = 4 independent BM samples) for $CD34^+$, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$, $CD34^+CD38^+$ and $CD34^+CD38^-$ cells are shown in panels A, B, C, D and E respectively. The * indicates stimulation conditions where CFU-GM incidence was either significantly ($p < 0.05$, paired t test) greater or less than that observed when the target cells were incubated with the HGF combination of 36GS.


A
136GGMS
36GS
IL-3
IL-6
G-CSF
GM-CSF
SCF
CD34+
0
50
100
150
CFU-GM/500 cells
B
136GGMS
36GS
IL-3
IL-6
G-CSF
GM-CSF
SCF
CD34+HLA-DR+
0
50
100
150
200
CFU-GM/500 cells
C
CD34+HLA-DR-
0
50
100
150
200
CFU-GM/500 cells
D
136GGMS
36GS
IL-3
IL-6
G-CSF
GM-CSF
SCF
CD34+CD38+
0
50
100
150
200
CFU-GM/500 cells
E
CD34+CD38-
0
50
100
150
200
250
300
CFU-GM/500 cells

further investigated by assessing nucleated cell and CFU-GM production from the same $CD34^+$ cells fractions grown under Pre-CFU culture conditions.

5.3.3 Committed and Primitive HPC Generate Neutrophil Precursors in Pre-CFU Culture

A series of studies were performed to determine if neutrophil precursor production in HGF stimulated *ex vivo* cultures results from growth of both committed and primitive HPC. Three experiments were performed to compare the generation of myeloid cells and CFU-GM in Pre-CFU culture conditions from sub-populations of BM $CD34^+$ cells sorted according to immunophenotype. Total $CD34^+$ cells, $CD34^+CD38^+$, $CD34^+CD38^-$, $CD34^+$HLA-DR^+ and $CD34^+$HLA-DR^- cells were isolated by FACS according to the sort regions shown in figures 5.1 – 5.3. Triplicate cultures with up to 1,000 cells of each phenotype were initiated in 1 ml of Pre-CFU medium containing 30% FCS and 1% BSA supplemented with IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF (each at 10 ng/ml). Where less than 1000 cells were available for replicate cultures the results were corrected accordingly so that nucleated cell and CFU-GM generation was normalised for 1,000 cells.

The pattern of nucleated cell and CFU-GM generation from each $CD34^+$ cell fraction was monitored for up to 4 weeks. Nucleated cell production over this period is shown in figure 5.6. After 14 days 1,000 $CD34^+$ cells generated $1.71 \pm 0.14 \times 10^6$ (mean ± SEM) nucleated cells. A similar number of nucleated cells were produced by $CD34^+$HLA-DR^- and $CD34^+CD38^+$ cells whereas significantly more were produced by $CD34^+$HLA-DR^+ cells (p= 0.086). In contrast, significantly fewer cells were produced by $CD34^+CD38^-$ cells (p= 0.0012). These data suggest that during the first 2 weeks of *ex vivo* culture the majority of nucleated cell production can be attributed to committed HPC that express HLA-DR or CD38 antigens. As seen in figure 5.4, the majority of BM $CD34^+$ cells co-express both these antigens so it is likely that a high proportion of nucleated cell production at 14 days is due to proliferation of $CD34^+CD38^+$HLA-DR^+ cells. After 21 days of Pre-CFU culture, total unfractionated $CD34^+$ cells produced $13.3 \pm 2.6 \times 10^6$ nucleated cells. Cell production was independent of HLA-DR expression as both $CD34^+$HLA-DR^+ and $CD34^+$HLA-DR^- cells produced similar numbers of cells. Surprisingly, at this time point, cell production was significantly less from either of the $CD34^+CD38^+$ or $CD34^+CD38^-$ fractions (p= 0.083, 0.073 respectively) as compared to that achieved by unfractionated $CD34^+$ cells. However, after 28 days of Pre-CFU culture significantly more nucleated cells were produced from those cultures initiated with $CD34^+CD38^-$ cells as compared to unfractionated $CD34^+$ cells (p=

Figure 5.6 Generation of nucleated cells by $CD34^+$ cells and sub-fractions thereof in Pre-CFU culture

The same $CD34^+$ cells and sub-fractions thereof as used for CFU-GM assays presented in figure 5.5 were cultured in 1 ml of Pre-CFU medium containing 30% FCS and 1% BSA supplemented with IL-1, IL-3, IL-6, G-CSF, GM-CSF, SCF (each at 10 ng/ml). At least 1,000 cells of each immunophenotype were cultured in triplicate. At day 14 the number of cells in each culture was counted by phase contrast microscopy and if required the culture split for analysis at day 21 and day 28. The results (mean ± SEM, n=3) represent the number of nucleated cells present after 14, 21 and 28 days of Pre-CFU culture. After 14 days, significantly ($p = 0.086$) more nucleated cells were generated from 1,000 $CD34^+HLA\text{-}DR^+$ cells than unfractionated $CD34^+$ cells whereas significantly less were generated from $CD34^+CD38^-$ ($p = 0.0012$). At day 21, cell production was significantly less from $CD34^+CD38^+$ and $CD34^+CD38^-$ cells as compared to total $CD34^+$ cells ($p = 0.08$ and 0.073, respectively). After 28 days of Pre-CFU culture significantly ($p = 0.03$) more nucleated cells were produced from $CD34^+CD38^-$ cells than unfractionated $CD34^+$ cells.

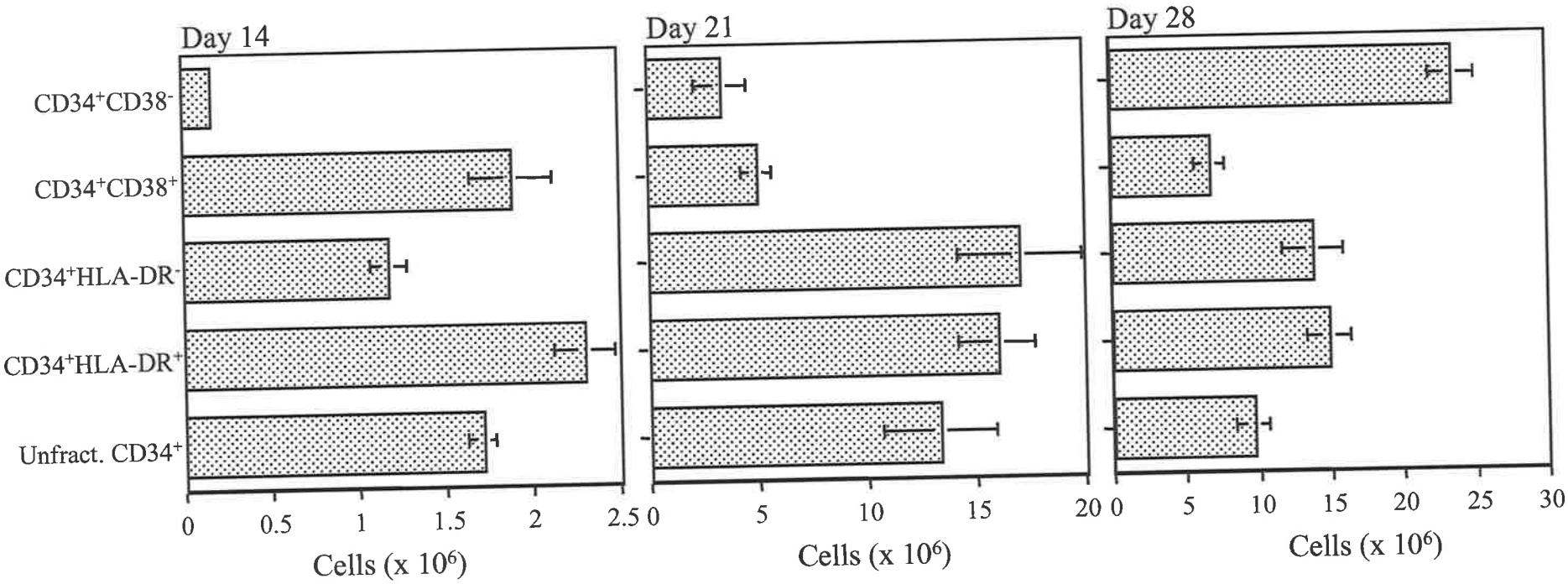
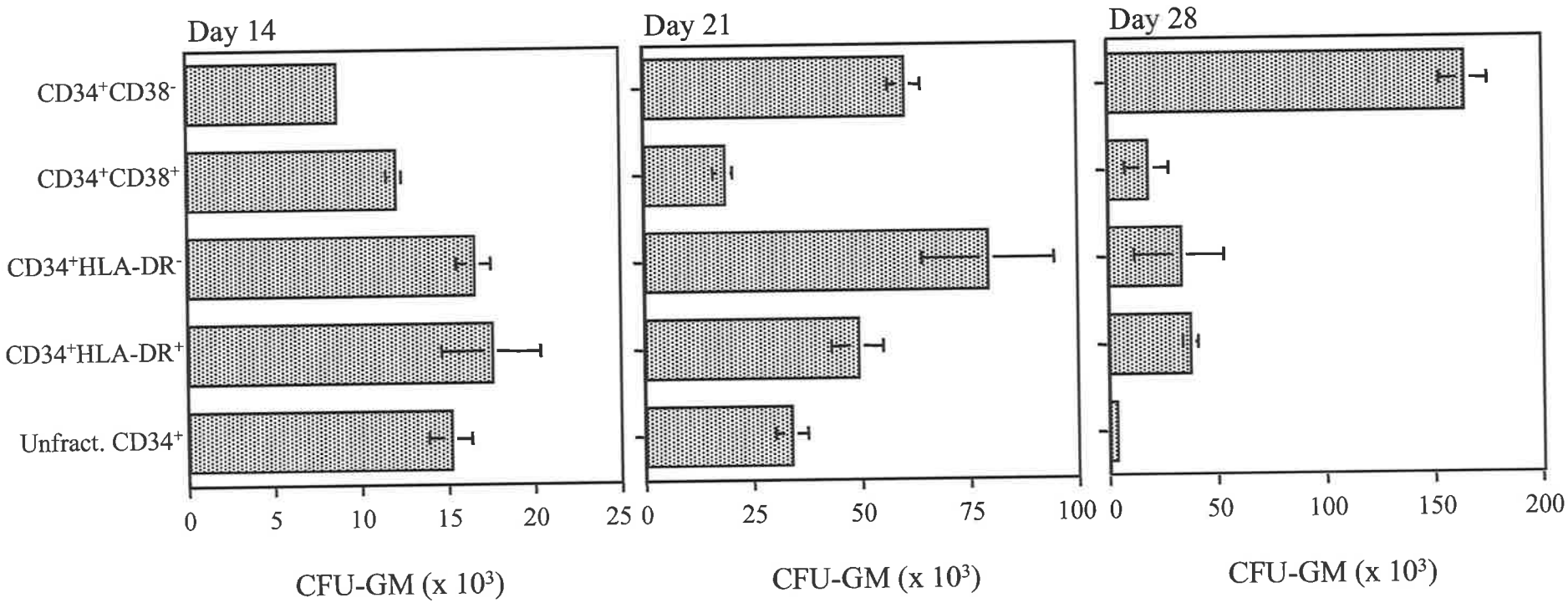

Day 14
Day 21
Day 28
CD34+CD38-
CD34+CD38+
CD34+HLA-DR-
CD34+HLA-DR+
Unfract. CD34+
0
0.5
1
1.5
2
2.5
0
5
10
15
20
0
5
10
15
20
25
30
Cells (x 10^6)
Cells (x 10^6)
Cells (x 10^6)

Figure 5.7 Generation of CFU-GM by $CD34^+$ cells and subsets thereof in Pre-CFU culture

FACS isolated cells were cultured in pre-CFU conditions as described in figure 5.6. Cultures were sampled at days 14, 21 and 28 and CFU-GM cultures were initiated to determine the number of nascent CFU-GM generated at these time points. The results (mean ± SEM, n = 3) represent the number of CFU-GM derived from 1,000 cells of each starting cell type after 14, 21 and 28 days Pre-CFU culture stimulated by 136GGMS. After 14 days, $CD34^+CD38^-$ cells generated significantly less ($p = 0.04$) nascent CFU-GM than unfractionated $CD34^+$ cells whereas there was similar CFU-GM production from the other $CD34^+$ cell fractions. At day 21, both the $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^-$ cells generated significantly more ($p = 0.09$ and 0.05, respectively) CFU-GM than that observed with unfractionated $CD34^+$ cells. After 28 days of Pre-CFU culture, generation of nascent CFU-GM was significantly greater from $CD34^+CD38^-$ cells than any other cell fraction (p values from 0.001- 0.05).


Day 14
Day 21
Day 28
CD34+CD38-
CD34+CD38+
CD34+HLA-DR-
CD34+HLA-DR+
Unfract. CD34+
0
5
10
15
20
25
0
25
50
75
100
0
50
100
150
200
CFU-GM (x 10³)
CFU-GM (x 10³)
CFU-GM (x 10³)

0.03). In contrast, there was a decline in the number of cells generated from each of the other fractions, including total, unfractionated $CD34^+$ cells.

As indicated in figure 5.7 generation of CFU-GM after 14 days of Pre-CFU culture followed a similar pattern to that observed for production of nucleated cells. A similar number of nascent CFU-GM were generated by unfractionated $CD34^+$ cells, $CD34^+HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^+$ cells. In contrast, significantly less CFU-GM ($p= 0.04$) were produced in those cultures initiated with 1,000 $CD34^+CD38^-$ cells. However, after 21 days of Pre-CFU culture, significantly more CFU-GM were present in cultures initiated with $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^-$ cells as compared to unfractionated $CD34^+$ cells ($p= 0.09, 0.05$ respectively). Notably, a large number of CFU-GM was also produced in cultures initiated with either $CD34^+HLA\text{-}DR^+$ or $CD34^+CD38^+$ cells. However these $CD34^+$ fractions and $CD34^+HLA\text{-}DR^-$ cells could not sustain production of nascent CFU-GM at day 28. In contrast there was a sustained and increased level of CFU-GM generation from the $CD34^+CD38^-$ cells after 28 days of Pre-CFU culture.

Taken together, the data on cell and CFU-GM production suggests that generation of nucleated cells and nascent myeloid progenitors during the first 2-3 weeks of HGF stimulated Pre-CFU culture can be attributed to cells with phenotypes associated with both committed and primitive HPC. Furthermore these data suggest that sustained generation of cells and CFU-GM beyond 3 weeks in Pre-CFU culture may in large part be a function of the $CD34^+CD38^-$ fraction of bone marrow.

5.3.4 HGF Receptor Expression on Bone Marrow $CD34^+$ Cells

Cytokines and HGF deliver signals to cells via their cognate receptors that are typically located at the cell surface as transmembrane molecules. Therefore, before a cell is able to respond to a given cytokine it must express its receptor. Analysis of mean receptor numbers per cell and the affinity of the interaction between a cytokine and its corresponding receptor have classically been performed by Scatchard analysis after the binding of radiolabelled ligands (Scatchard 1949, Stanley and Guilbert 1981, Nicola and Metcalf 1985, Nicola and Metcalf 1986). However the binding of the radiolabelled ligand by specific cell types can only be determined by using either pre-enriched populations or by autoradiography of cell populations (McKinstry *et al* 1994, McKinstry *et al* 1997). Apart from the safety concerns associated with handling of radioactive ligands, these methods are not ideal, as they do not enable isolation of viable cells for subsequent functional studies according to receptor

expression. This limitation was overcome by indirect immunolabelling of BM cells with monoclonal antibodies to HGFR then FACS. A number of stains were performed on separate BM samples with single monoclonal antibodies to particular HGFR, as pilot studies to validate the immunolabelling method. Subsequently, studies were performed to examine expression of a range of HGFR on 3 separate BM samples. Each sample was prepared identically and BM MNC were labelled with the same monoclonal antibodies and isotype-specific second layer fluorochromes. The typical staining involved incubation with an IgG_3 anti-CD34 monoclonal (43A1) together with an IgG_1 monoclonal to one of the HGFR. Visualisation of these cell surface antigens was achieved by incubation with an anti-mouse IgG_3 reagent conjugated to FITC (for detection of CD34) and an anti-mouse IgG_1 reagent conjugated to PE (for detection of HGFR). This approach gave specific, reproducible staining with easy visualisation of HGFR expression on the $CD34^+$ fraction of BM.

In each BM sample the pattern of staining was remarkably similar. As shown in panel A of Figure 5.8, the pattern of staining with 43A1 enabled BM cells to be separated into 3 populations: 43A1 bright (FITC signals of 3-log intensity) which represented $CD34^+$ cells, 43A1 positive (FITC signal of 2-log intensity) which represented $CD34^{dim}$ cells and 43A1 negative (FITC signal of 1-log intensity) representing $CD34^-$ cells. This pattern of immunoreactivity did not change in spite of blocking with human serum or irrelevant mouse monoclonal antibodies. Importantly, when cells were labelled with an IgG_1 isotype control antibody (3D3) there was very little background PE staining on the $CD34^+$ cell population. Therefore, any increases in the PE signal (above that seen with 3D3) for the $CD34^+$ cells, following labelling with receptor antibodies represented true expression of the receptor.

A composite figure of 2-colour dot-plots of CD34 and HGFR expression for one of the 3 BM samples analysed is shown in figure 5.8. In addition, the series of histograms in figures 5.9A and 5.9B represent HGFR expression on $CD34^+$ cells from one of the BM samples. Both the dot plots and the histograms show that $CD34^+$ cells express detectable levels (by flow cytometry) of all the receptors investigated apart from the common beta chain (βc-chain) for the IL-3R, IL-5R and GM-CSFR. Table 5.1 summarises the data from the 3 independent BM samples. The numerical value assigned to each of the HGFR represents the proportion of $CD34^+$ cells that express that receptor as compared to the isotype control. To calculate these figures a quadstat marker was placed according to the background fluorescence of cells labelled with the isotype control monoclonal antibody (3D3) and 43A1, as shown in panel A of figure 5.8. To determine the proportion of $CD34^+$ cells expressing a particular HGFR the

Figure 5.8 Expression of HGF Receptors on BM MNC and $CD34^+$ cells

Each panel of this composite figure shows a two-colour flow cytometry dot-plot of BM MNC immunolabelled with an anti-CD34 monoclonal antibody (43A1; IgG_3 isotype) and an IgG_1 antibody to a HGFR. Detection of CD34 was achieved by incubation with a FITC-conjugated anti-mouse IgG_3 secondary antibody whereas detection of the HGFR was achieved by incubation with PE-conjugated anti-mouse IgG_1 secondary antibody. Panel A, shows the binding or an irrelevant non-binding control IgG_1 monoclonal antibody (3D3). The following monoclonal antibodies were used for immunolabelling and detection of HGFR: YB5 (c-kit), 4F3 (β_c-chain), 9F5 (IL-3R α-chain), 6E10 and 4A5 (GM-CSFR α-chain), 711, 772 and 775 (G-CSFR), MT18 (IL-6R) and AM64 (gp130). In panels B through to H, the quadrant marker is identical to that set with the isotype control antibody 3D3.

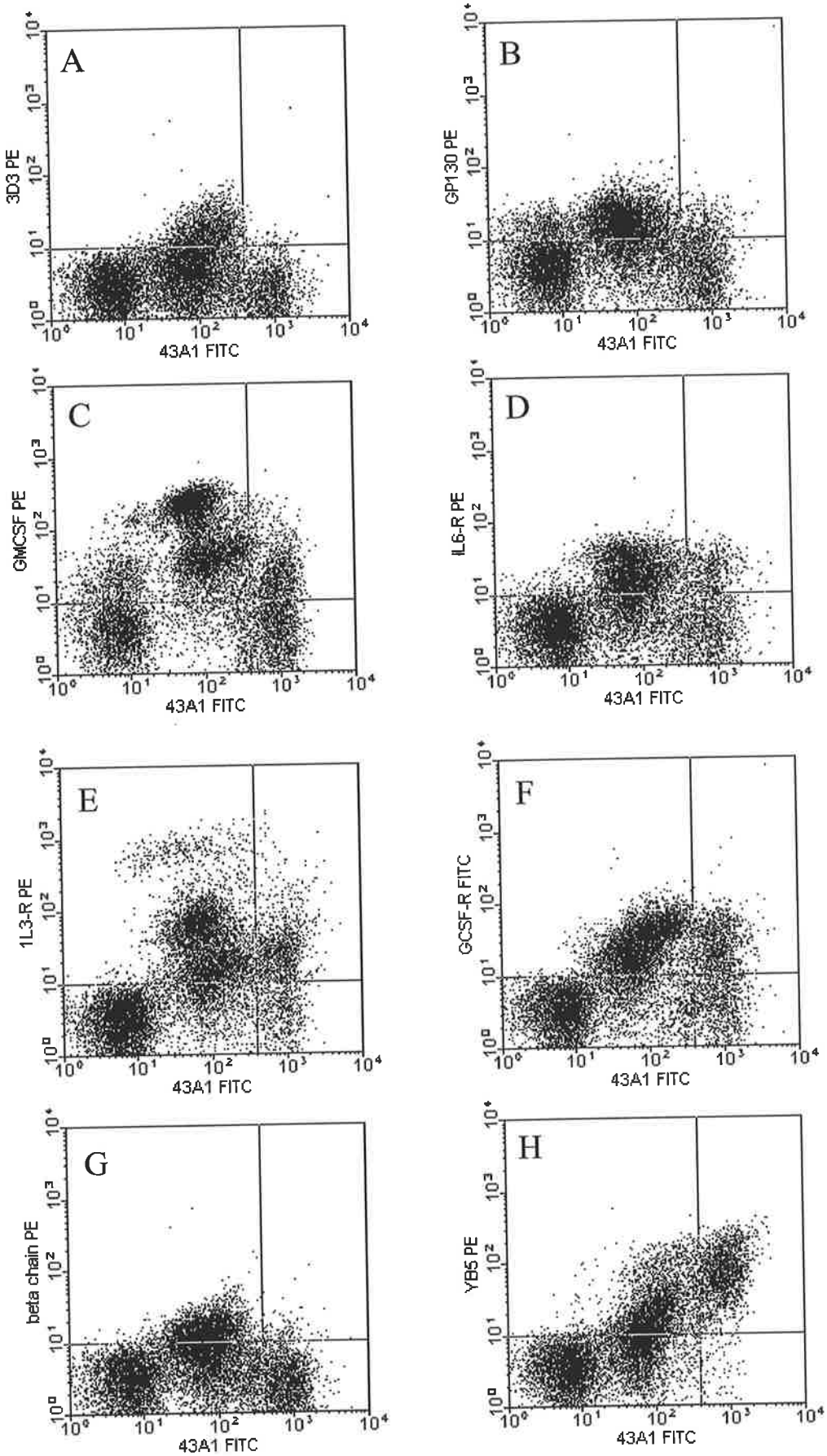

A
3D3 PE
43A1 FITC
B
GP130 PE
43A1 FITC
C
GMCSF PE
43A1 FITC
D
IL6-R PE
43A1 FITC
E
1L3-R PE
43A1 FITC
F
GCSF-R FITC
43A1 FITC
G
beta chain PE
43A1 FITC
H
YB5 PE
43A1 FITC

Table 5.1

Expression of HGF receptors on BM $CD34^+$ cells.

The results of analyses on 3 BM samples from normal adult volunteers are shown. The numerical values represent the % of $CD34^+$ cells expressing the respective receptors above that observed with the 3D3 isotype control antibody. This data was calculated from similar dot plots to that shown in figure 5.8. The following monoclonal antibodies were used for these studies: 43A1 (CD34), Yb5 (c-*kit*), 4F3 (βc-chain), 9F5 (IL-3Rα-chain), 6E10 and 4A5 (GM-CSFRα-chain), 711, 772, 775 (G-CSFR), MT18 (IL-6R), and AM64 (gp130).

	Isotype Control	**c-*kit***	**βc- chain**	**IL-3Rα chain**	**GM-CSFRα**	**G-CSFR**	**IL-6R**	**gp130**
BM 1	6.4	87.9	1.6	70.5	41.1	53.8	34.5	18.6
BM 2	3.5	87.9	0.7	61.8	41.4	49.6	27.9	16.8
BM 3	2.2	74.6	-1.1	60.7	24.8	38.7	23.4	21.9
Mean	***4.0***	***83.5***	***0.4***	***64***	***35.8***	***47.4***	***28.6***	***19.9***
SD	2.1	7.7	1.37	5.4	9.5	7.8	5.5	2.6

number of events in the upper right quadrant ($CD34^+HGFR^+$) was calculated as a percentage of the total $CD34^+$ fraction (ie sum of upper right and lower right quadrants). Prior to performing this calculation the number of cells classified as $CD34^+HGFR^+$ was corrected for non-specific staining by subtracting that number of cells exhibiting staining with the isotype (3D3) control monoclonal antibody.

Of all the HGFR examined, c-*kit* is the most abundant on BM $CD34^+$ cells with an average of 83.5% of $CD34^+$ cells expressing high levels of this receptor (panel H, figure 5.8 and histogram I, figure 5.9B). In contrast, as shown in Panel G of figure 5.8 and histogram C of figure 5.9A, very few $CD34^+$ cells express the βc-chain for the IL-3 and GM-CSF receptors although its partnering IL-3Rα and GM-CSFRα (panels E and C, respectively figure 5.8) are present on many $CD34^+$ cells. Further analysis revealed that expression of the GM-CSFRα effectively splits the $CD34^+$ cells into 2 populations with distinct forward and side scatter characteristics (figure 5.10). The $CD34^+$GM-$CSFR\alpha^-$ cells have lower FSC and SSC than their $CD34^+$GM-$CSFR\alpha^+$ counterparts. Subsequent analysis demonstrated that the latter population is enriched for promyelocytes that form colonies in methylcellulose in 7 –10 days (data not shown). In contrast, there is little difference between the FSC and SSC characteristics of $CD34^+$IL-$3R\alpha^+$ and $CD34^+$IL-$3R\alpha^-$ cells (figure 5.11). The G-CSFR is also expressed by 38-54% of $CD34^+$ cells and $CD34^+$G-$CSFR^+$ cells can be easily resolved from $CD34^+$G-$CSFR^-$ cells (histogram J, figure 5.9B).

In addition, both the IL-6R and its signalling chain, gp130 are also expressed by 20-35% of $CD34^+$ cells. Histogram analysis of IL-6Rα and gp130 on $CD34^+$ cells revealed that the level of expression of both is similar: it is a continuum from undetectable, to levels well above the isotype control (histograms G and H of figure 5.9B). Given the limited sensitivity of the immunolabelling and flow cytometry method used, it is most likely that a greater proportion of $CD34^+$ cells express both gp130 and the IL-6R. Supporting this view, comparison of histogram G of figure 5.9B (gp130) with that of histogram F (isotype control) shows a peak shift in fluorescence for the entire cell population. A similar population peak shift is apparent for IL-6 expression. Interestingly, as indicated by panels B and D of figure 5.8 a population of $CD34^-$ cells express gp130 but not the IL-6R suggesting that within these cells gp130 may be associated with the oncostatin M or LIF receptors.

The 2-colour dot-plots shown in figure 5.8 also highlight the distribution of each receptor on $CD34^{dim}$ and $CD34^-$ cells. A significant proportion of $CD34^{dim}$ BM MNC express both IL-

Figure 5.9A HGFR expression on $CD34^+$ BM cells

Bone marrow mononuclear cells were immunolabelled with a combination of an anti-CD34 monoclonal antibody (43A1: IgG_3 isotype) and a monoclonal(s) to specific HGFR (IgG_1 isotype) as described in the legend to figure 5.8. The data presented in this figure is representative of 3 normal BM samples processed, immunolabelled and analysed identically. Panel A shows CD34 versus SSC. $CD34^+$ cells typically exhibit low SSC and form a discrete population, which are easily discriminated from $CD34^-$ cells. The subsequent panels (C – E and G through to J) in figure 5.9A and 5.9B represent histograms of HGFR expression on $CD34^+$ gated cells (shown in blue in panel A). Panels B and F show staining of $CD34^+$ cells with the IgG_1 isotype control monoclonal antibody 3D3. In each histogram, the marker M1 is located in an identical position and the number over the marker indicates the percentage of $CD34^+$ cells included within the marked region.

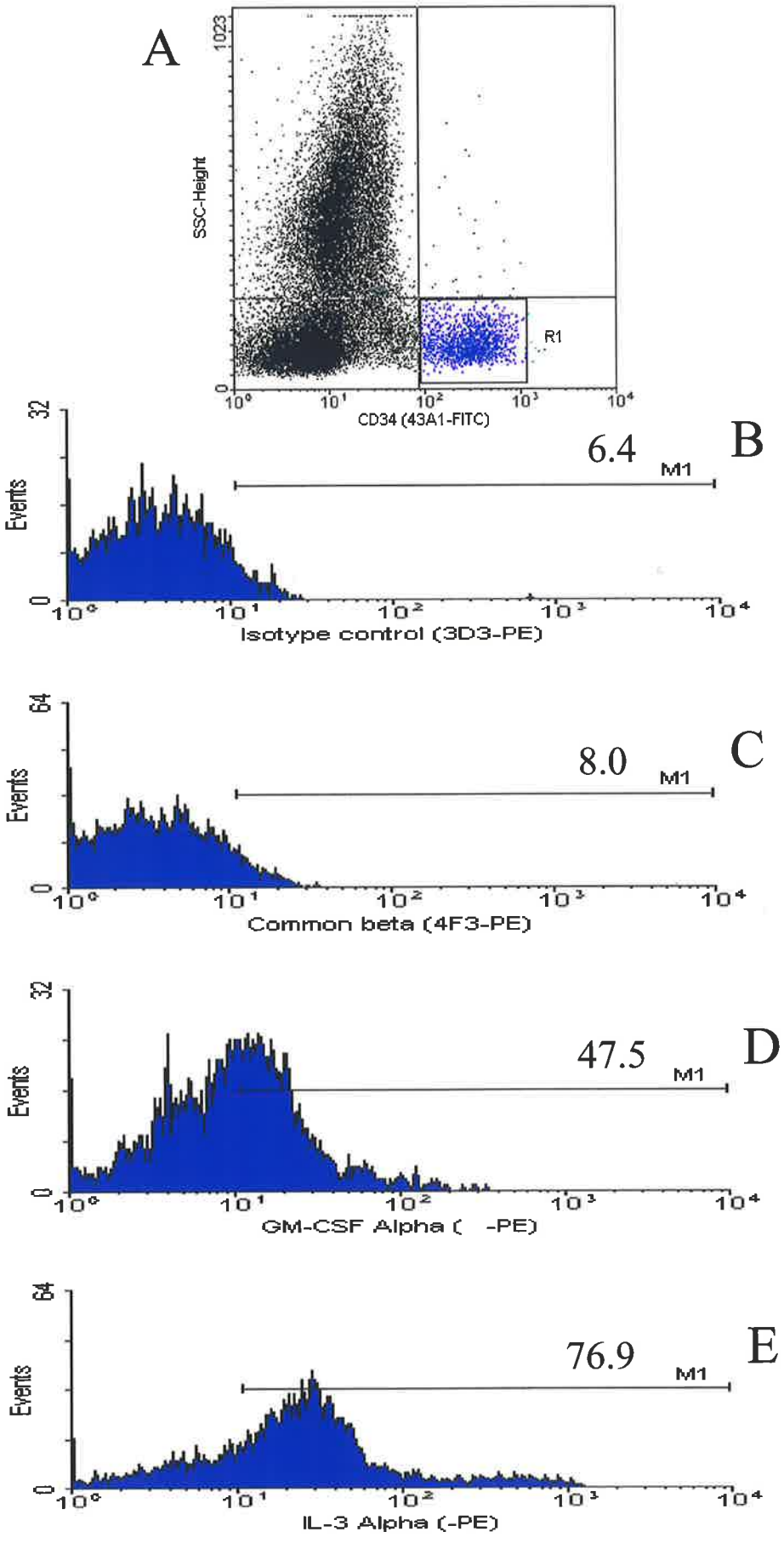
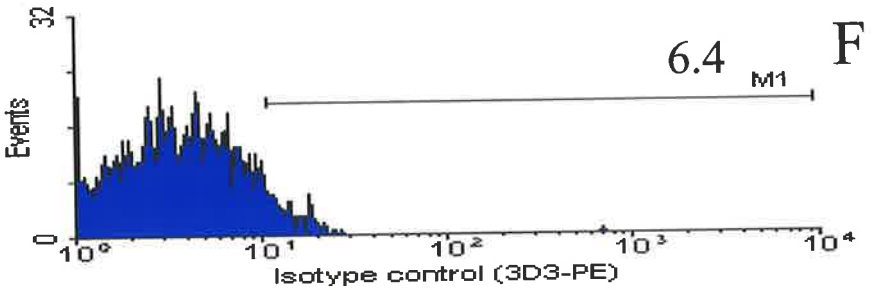
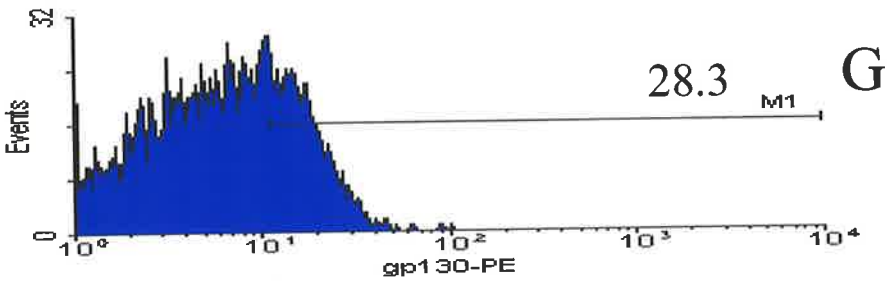
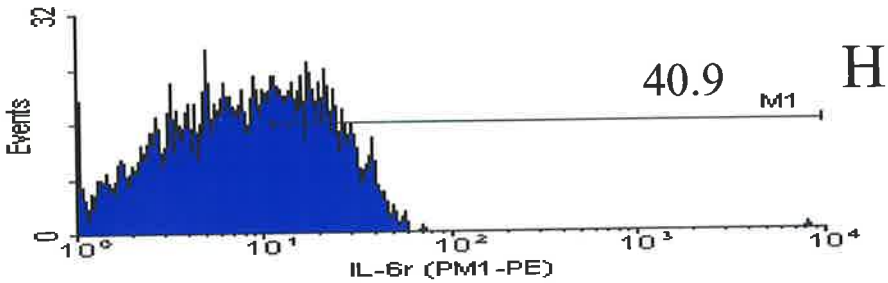

A
SSC-Height
1023
0
R1
10^0 10^1 10^2 10^3 10^4
CD34 (43A1-FITC)
B
6.4
M1
Events
32
0
Isotype control (3D3-PE)
C
8.0
M1
Events
64
0
Common beta (4F3-PE)
D
47.5
M1
Events
32
0
GM-CSF Alpha (-PE)
E
76.9
M1
Events
64
0
IL-3 Alpha (-PE)

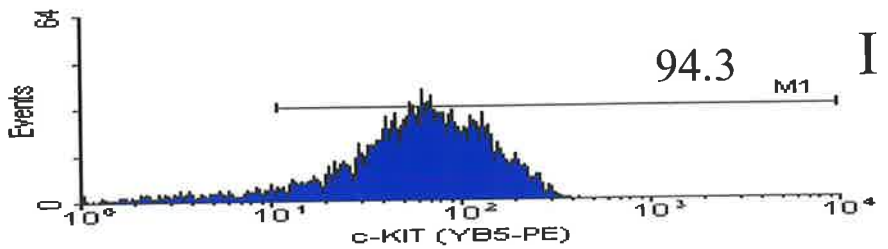
Figure 5.9B HGFR expression on $CD34^+$ BM cells

The expression of HGFR on $CD34^+$ cells is shown in comparison to labelling observed with an IgG_1 isotype control monoclonal antibody (3D3). Bone marrow mononuclear cells were immunolabelled with a combination of an anti-CD34 monoclonal antibody (43A1: IgG_3 isotype) and a monoclonal(s) to specific HGFR (IgG_1 isotype) as described in the legend to figure 5.8. The data presented in this figure is representative of 3 normal BM samples processed, immunolabelled and analysed identically. Panel A shows CD34 versus SSC. $CD34^+$ cells typically exhibit low SSC and form a discrete population, which are easily discriminated from $CD34^-$ cells. The subsequent panels (C – E and G through to J) in figure 5.9A and 5.9B represent histograms of HGFR expression on $CD34^+$ gated cells (shown in blue in panel A). Panels B and F show staining of $CD34^+$ cells with the IgG_1 isotype control monoclonal antibody 3D3. In each histogram, the marker M1 is located in an identical position and the number over the marker indicates the percentage of $CD34^+$ that are $HGFR^+$ cells.


32
Events
0
6.4
M1
F
10°
10¹
10²
10³
10⁴
Isotype control (3D3-PE)


32
Events
0
28.3
M1
G
10°
10¹
10²
10³
10⁴
gp130-PE


32
Events
0
40.9
M1
H
10°
10¹
10²
10³
10⁴
IL-6r (PM1-PE)


64
Events
0
94.3
M1
I
10°
10¹
10²
10³
10⁴
c-KIT (YB5-PE)

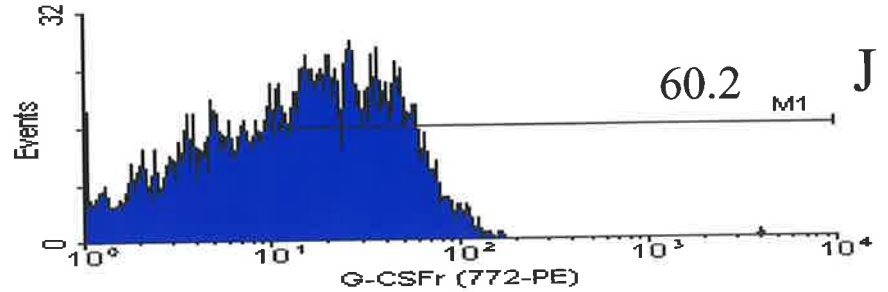
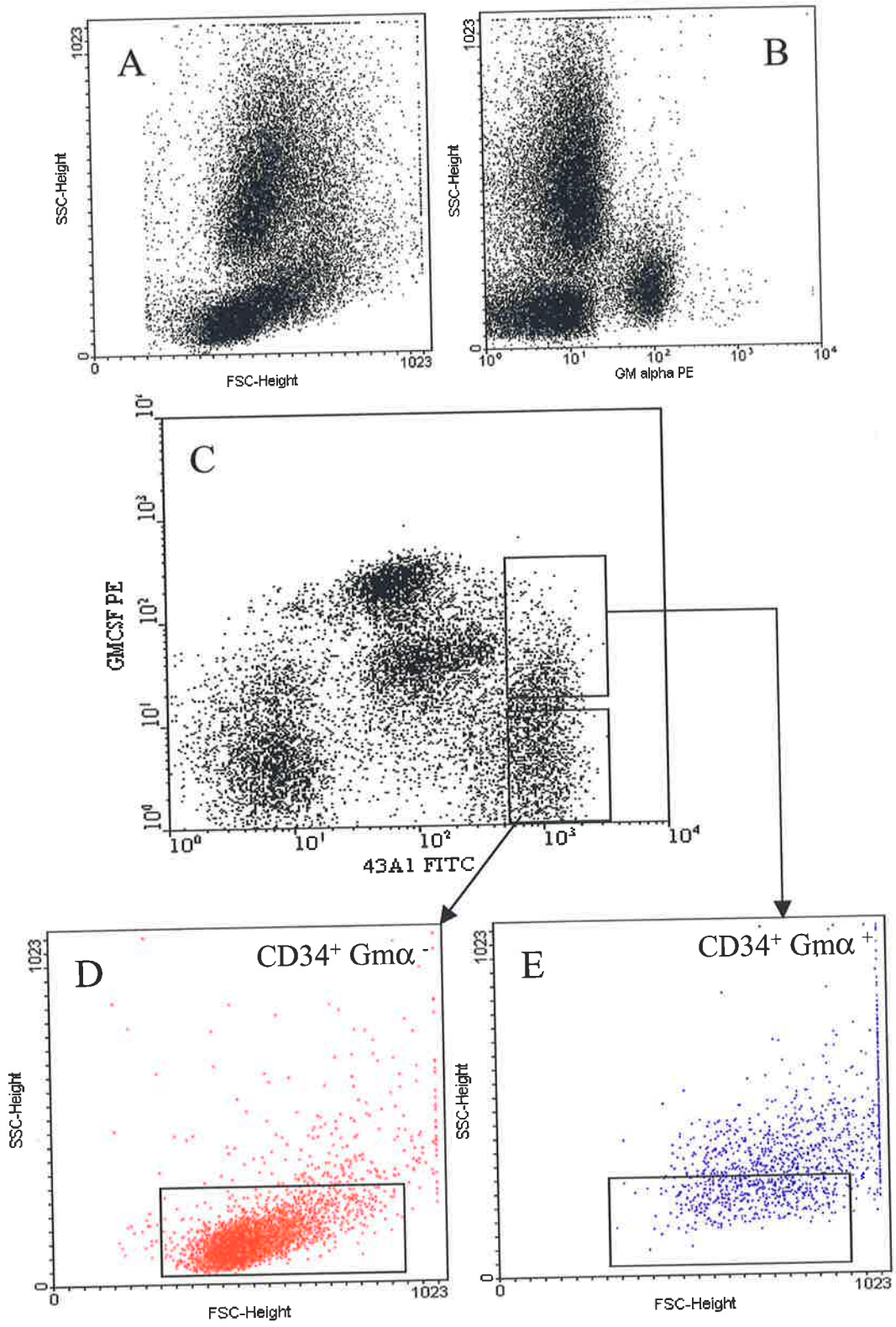

32
Events
0
60.2
M1
J
10°
10¹
10²
10³
10⁴
G-CSFr (772-PE)

Figure 5.10 Expression of GM-CSFR α-chain on BM MNC

This composite figure is representative of the pattern of GM-CSFR α-chain expression observed with 3 BM samples immunolabelled as described in the legend to figure 5.8. Panel A shows the FSC versus SSC characteristics of the BM MNC and panel B the GM-CSFR α-chain expression as compared to SSC. Panel C displays the typical pattern of CD34 (43A1-FITC) and GM-CSF α-chain expression. The majority of $CD34^+$ cells do not express the GM-CSFR α-chain and represent cells with low FSC and SSC characteristics, as shown in panel D. In contrast those $CD34^+$ cells (average of 35.8%) that express the GM-CSFR α-chain exhibit higher FSC and SSC characteristics, as shown in panel E.

Adult Bone Marrow: Expression of GM-CSF^{r} α-chain



3Rα and GM-CSFRα which when shown in relation to CD34 expression allows discrimination of at least 2 populations of cells with different levels of IL-3Rα expression (panel B, figure 5.11) and 2 populations with different levels of the GM-CSFRα expression (figure 5.10). The nature of these discrete sub-populations has not been completely analysed in the current study although examination of Jenner-Giemsa stained cytospins of sorted IL-3Rα^{bright} cells revealed enrichment for basophils and a population of blast-like cells (figure 5.12). Approximately 25% of CD34$^-$ cells also express low levels of the GM-CSFRα-chain (panel C, figure 5.8). These cells exhibit a distinct forward and side scatter distribution and following FACS and Jenner-Giemsa staining were shown to be neutrophils. A similar analysis has identified the CD34^{dim}GM-CSFR^{++} cells as monocytes.

Taken together, these data on expression of HGFR indicates that a proportion of CD34$^+$ cells have the necessary receptors to immediately respond to a combination of HGF including, IL-3, IL-6, G-CSF, GM-CSF, and SCF. However, the analysis does not reveal which HGFR are expressed by committed HPC, primitive HPC or Pre-CFU. This question was therefore investigated by performing clonogenic assays and Pre-CFU cultures on sub-populations of CD34$^+$ cells isolated by FACS according to HGFR expression. It was anticipated that these studies would also provide further insight as to the role of individual HGF in *ex vivo* expansion of CD34$^+$ cells.

5.3.5 HGFR Expression on CFU-GM and BFU-E

Assays for myeloid and erythroid progenitor cells (CFU-GM and BFU-E) were performed on CD34$^+$ sub-populations isolated according to HGFR expression from 3 normal bone marrow samples. The same, sorted cell fractions were also used to initiate parallel pre-CFU cultures (described below). In all cases, except for the IL-3R/GM-CSFR common beta chain (β_c) it was possible to discriminate and sort CD34$^+$ cells on the basis of HGFR expression. The regions used for sorting of CD34$^+$HGFR$^+$ and CD34$^+$HGFR$^-$ cells were based on the quadstat position shown in figure 5.8. For each HGFR the CD34$^+$ cells expressing HGFR were those cells in the upper right quadrant and CD34$^+$HGFR$^-$ were cells within the lower right quadrant. As there was very little expression of the β_c on CD34$^+$ cells it was not possible to discriminate CD34$^+\beta_c{}^+$ and CD34$^+\beta_c{}^-$ cells. Therefore a fraction of CD34$^+$ cells expressing less than the median PE-fluorescence for the β_c chain was sorted as putative CD34$^+\beta_c{}^-$ cells. CFU-GM and BFU-E assays were performed in methylcellulose and colony growth was stimulated by a combination of IL-3, IL-6, G-CSF, GM-CSF, SCF (each at 10 ng/ml) and 4 I.U. EPO.

Figure 5.11 Expression of IL-3R α-chain on BM MNC

This composite figure is representative of the pattern of antigen expression observed with 3 BM samples immunolabelled as described in the legend to figure 5.8. Panel A shows the FSC versus SSC characteristics of the BM MNC and panel B, CD34 and IL-3R α-chain expression. Panels C – F show the FSC and SSC characteristics of discrete populations of cells discriminated according to CD34 and IL-3R α-chain expression. Thus panels C, D, E and F shows $CD34^{dim}$IL-3Rα^{+} (coloured turquoise, $CD34^{dim}$IL-3Rα^{bright} (pink), $CD34^{+}$IL-3Rα^{-} (blue) and $CD34^{+}$IL-3Rα^{+} cells (red), respectively.

IL-3Rα-chain expression on Bone Marrow Cells

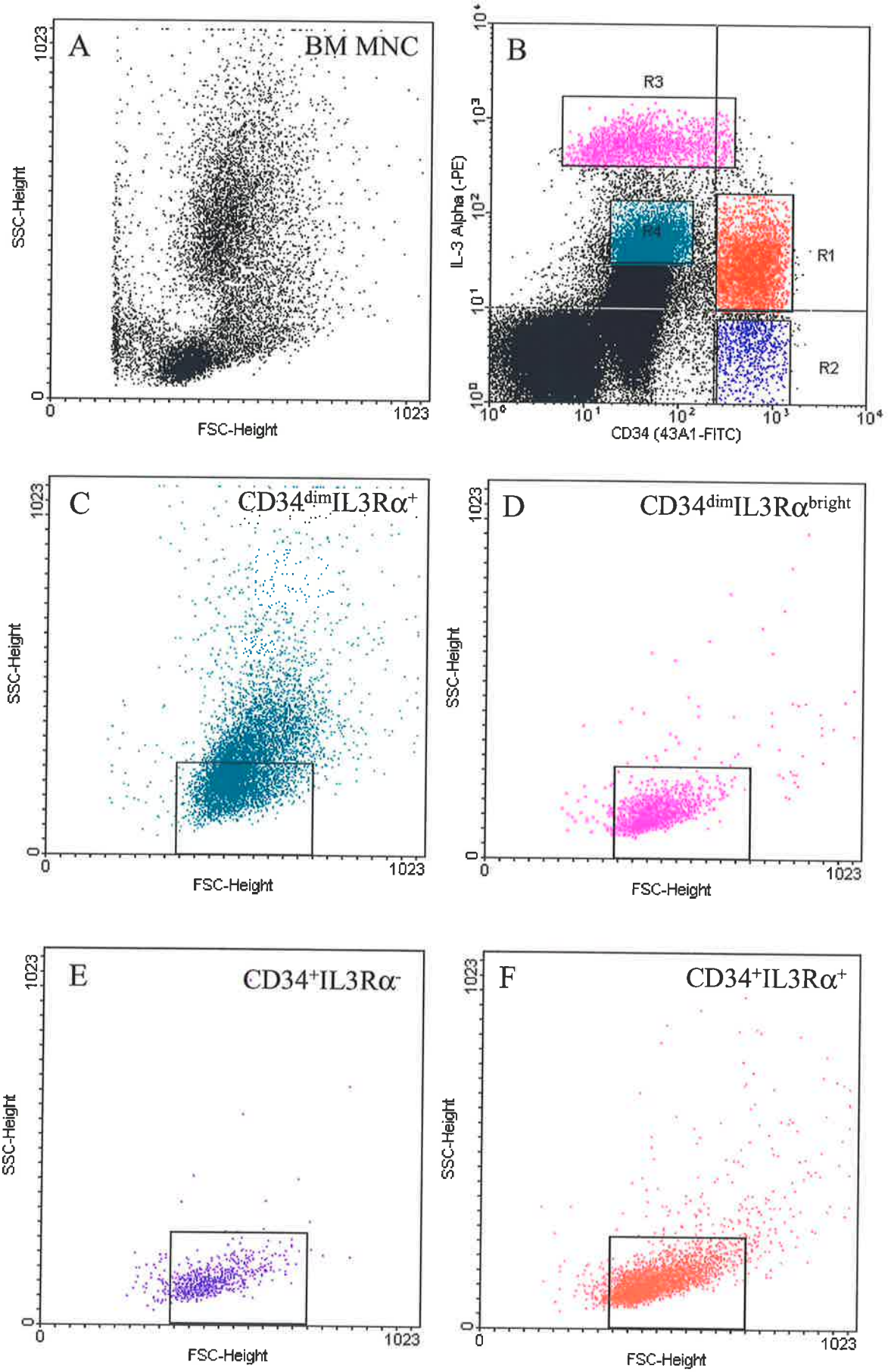
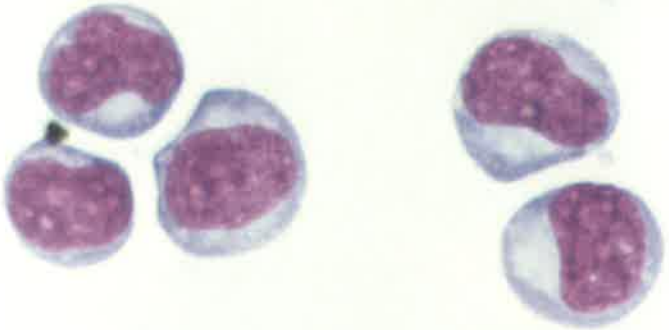


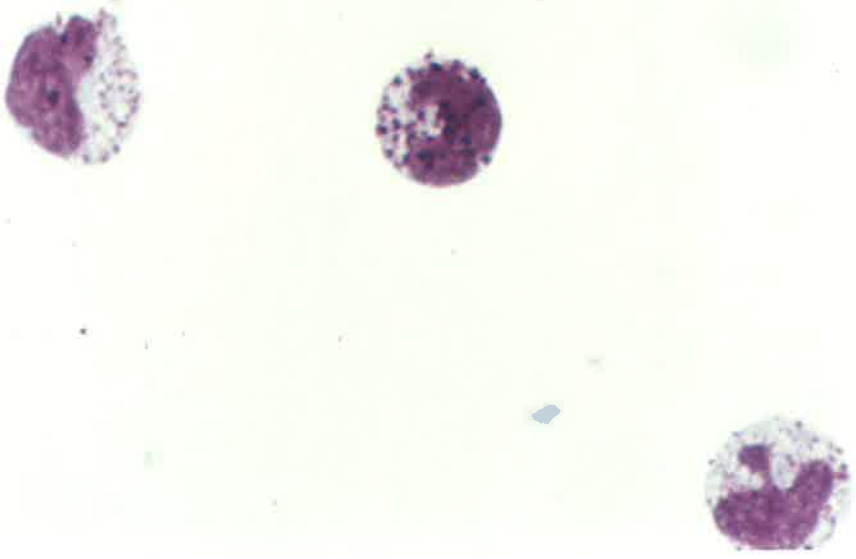
Figure 5.12 Morphology of $CD34^{dim}$IL-3Rα-$chain^{bright}$ cells isolated from adult human bone marrow

Cells within region 3 (R3: shown in panel B of figure 5.11) were isolated by FACS, a cytospin prepared with a Shandon Elliot cytocentrifuge and stained with Jenner-Giemsa stain. Panel A shows cells with a distinctive blast-like morphology, including a prominent nucleolus, agranular, azurophilic cytoplasm and prominent perinuclear non-stained region typical of the golgi. Panel B shows cells with prominent, large, dark blue stained cytoplasmic granules typical of basophils.

A



B



Under these conditions, 318 ± 60.9 (mean ± SEM) CFU-GM and 82.7 ± 5.6 (mean ± SEM) BFU-E /1000 $CD34^+$ cells cloned. There was significant enrichment of CFU-GM in the $CD34^+$G-$CSFR^+$ fraction (p= 0.06) with 497 ± 65 CFU-GM/1000 cells and significantly less CFU-GM in the $CD34^+$c-*kit*$^-$ fraction (29 ± 5.6 CFU-GM/1000 cells, p= 0.037) as compared to total $CD34^+$ cells. CFU-GM were enriched in the $CD34^+$c-*kit*$^+$ fraction as compared to the $CD34^+$c-*kit*$^-$ fraction (p= 0.0085) and also in $CD34^+$G-$CSFR^+$ fraction compared to $CD34^+$G-$CSFR^-$ (p= 0.012). There was no significant difference in the incidence of CFU-GM in any of the other paired fractions isolated according to expression or absence of HGFR expression (Figure 5.13). In contrast BFU-E were consistently enriched in those $CD34^+$ fractions that lacked HGFR expression. As shown in figure 5.14 the incidence of BFU-E was significantly higher in the $CD34^+$gp130$^-$ (c.f. gp130$^+$, p= 0.01), $CD34^+$IL-6R$^-$ (c.f. IL-6R$^+$, p= .004), $CD34^+$IL-3Rα^- (c.f. IL-3Rα^+, p= 0.05), $CD34^+$G-$CSFR^-$ (c.f. G-$CSFR^+$, p= 0.0015), $CD34^+$GM-CSFRα^- (c.f. GM-CSFRα^+, p= 0.04) and the $CD34^+$c-*kit*$^-$ (c.f. c-*kit*$^+$, p= 0.02) cell fractions. Although this data describes the relative enrichment of myeloid and erythroid progenitor cells within discrete $CD34^+$ subsets it does not reflect the proportion of these progenitors within these immunophenotypically-defined subpopulations. This can be calculated by taking into account the proportion of cells that express or lack a particular HGFR together with the incidence of progenitors within this population. The following equation was used for these calculations.

$$\text{Relative number (RN) of BFU-E in } R^+ = \frac{(\%R^+ \times IR^+)}{(\%R^+ \times IR^+) + (\%R^- \times IR^-)}$$

Where

i) RN of BFU-E in R^+ represents the relative number of BFU-E within a $HGFR^+$ fraction

ii) $\%R^+$ represents the % of $CD34^+$ cells that express the HGFR (as taken from table 5.1)

iii) IR^+ represents the incidence of BFU-E within the $HGFR^+$ fraction

iv) $\%R^-$ represents the % of $CD34^+$ cells that lack the HGFR

v) IR^- represents the incidence of BFU-E within the $HGFR^-$ fraction.

Thus for distribution of BFU-E, based on c-*kit* expression the following can be calculated.

Relative number of BFU-E in the c-*kit*$^-$ fraction = 83.5 x 5.1/(83.5 x 5.1) + (16.5 x 9.44)

= 0.736

Figure 5.13 CFU-GM assay of $CD34^+$ cells fractionated according to HGFR expression

Incidence of CFU-GM in different fractions of $CD34^+$ cells discriminated according to HGFR expression. CFU-GM assays were performed with cells isolated by FACS according to CD34 and HGFR expression. The sort windows used for selecting $CD34^+HGFR^+$ and $CD34^+HGFR^-$ cells were based on the quadrant positions shown in figure 5.8 with $CD34^+HGFR^+$ cells located in the upper right quadrant and $CD34^+HGFR^-$ cells in the lower right quadrant. Post-sort purity checks were performed to ensure that fractions were at least 97% pure. If this did not occur, the sample was re-sorted. Typically 500 cells were cultured in triplicate, each in 1 ml of methylcellulose supplemented with IL-3, IL-6, G-CSF, GM-CSF and SCF (each at 10 ng/ml) and 4 I.U. of EPO. The results represent the CFU-GM/1000 target cells (mean ± SEM, n = 3 adult human BM) after 14 days of culture.

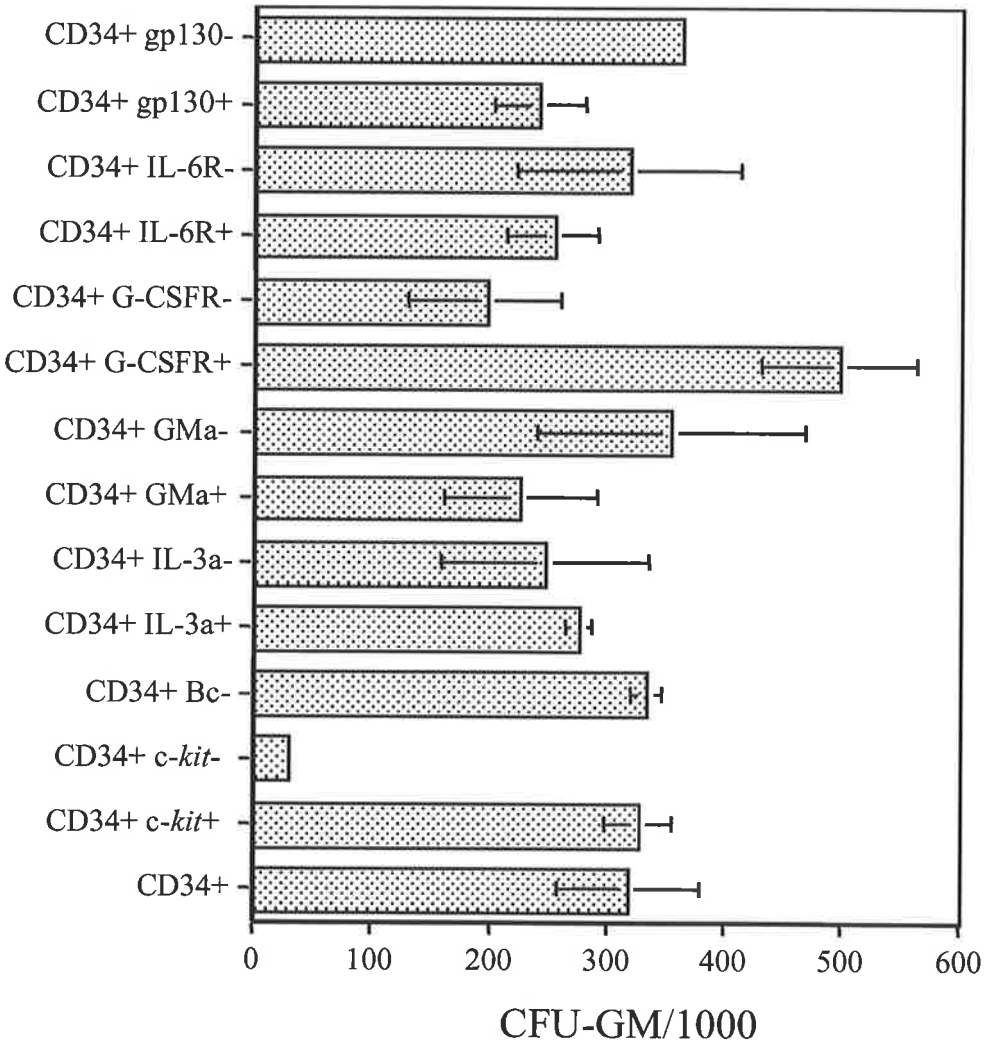
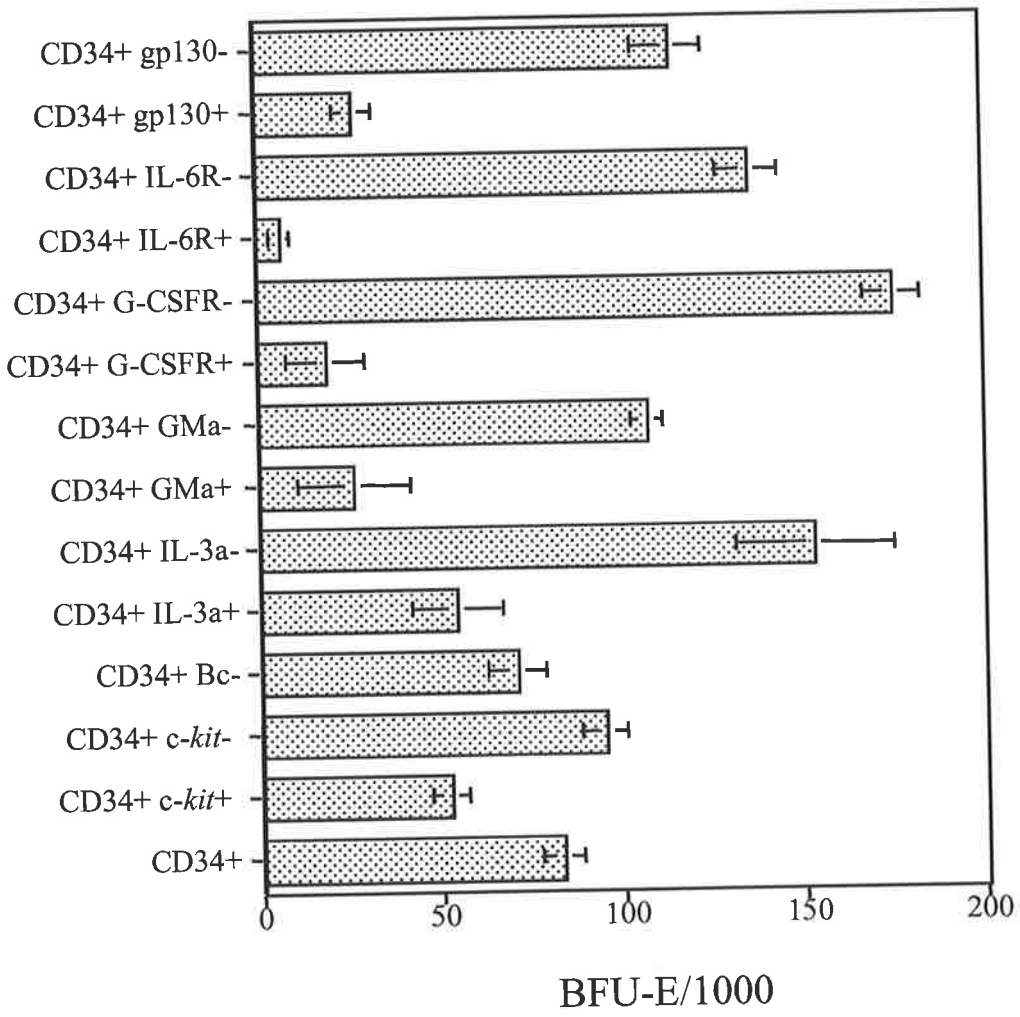

CD34+ gp130-
CD34+ gp130+
CD34+ IL-6R-
CD34+ IL-6R+
CD34+ G-CSFR-
CD34+ G-CSFR+
CD34+ GMa-
CD34+ GMa+
CD34+ IL-3a-
CD34+ IL-3a+
CD34+ Bc-
CD34+ c-*kit*-
CD34+ c-*kit*+
CD34+
0
100
200
300
400
500
600
CFU-GM/1000

Figure 5.14 Incidence of BFU-E in different fractions of $CD34^+$ cells discriminated according to HGFR expression

The same, populations isolated as described in the legend to figure 5.13 were assayed for BFU-E. The results represent BFU-E/1000 $CD34^+HGFR^+$ or $CD34^+HGFR^-$ cells (mean ± SEM, n = 3).


CD34+ gp130-
CD34+ gp130+
CD34+ IL-6R-
CD34+ IL-6R+
CD34+ G-CSFR-
CD34+ G-CSFR+
CD34+ GMa-
CD34+ GMa+
CD34+ IL-3a-
CD34+ IL-3a+
CD34+ Bc-
CD34+ c-*kit*-
CD34+ c-*kit*+
CD34+
0
50
100
150
200
BFU-E/1000

Thus 73.6% of BFU-E are located within the $CD34^+$c-kit^+ cell fraction and 26.4% are located within the $CD34^+$c-kit^- cell fraction. This is in contrast to the data on incidence of BFU-E that suggests that most of the BFU-E would be located in the $CD34^+$c-kit^- fraction. Similar calculations were performed for all $CD34^+$HGFR fractions and are shown below in table 5.2.

This analysis confirms that CFU-GM are highly enriched within the $CD34^+$c-kit^+ fraction and that there is at least a 2-fold enrichment of CFU-GM within $CD34^+$ cells that express the G-CSFR or IL-3Rα but lack gp130, the IL-6R or GM-CSFRα. However, it is important to note that CFU-GM are also present within $CD34^+$ cell fractions that lack or express these respective HGFR. In addition, these calculations confirm that BFU-E are highly enriched in $CD34^+$ fractions that lack gp130, the IL-6R, G-CSFR and GM-CSFRα. However, BFU-E are present within both the c-kit^+ (73.6%) and c-kit^- (26.4%) fractions and also the IL-3Rα^+ (38.7%) and IL-3Rα^- fractions. Based on this data it would be predicted that in *ex vivo* cultures designed for generation of neutrophil precursors a high proportion of $CD34^+$ myeloid progenitors (CFU-GM) would divide and proliferate when stimulated by combinations of HGF containing SCF, G-CSF and IL-3.

5.3.6 Pre-CFU Culture of Sub-populations of $CD34^+$ cells Based on HGFR Expression

These experiments were performed for two reasons. Firstly, to determine if cell and CFU-GM production in Pre-CFU culture (*ex vivo* expansion conditions) was attributed to particular $CD34^+$ cell fractions discriminated on the basis of HGFR expression and secondly, to provide further insight as to why particular HGF were required in *ex vivo* expansion cultures of $CD34^+$ cells. Pre-CFU cultures were initiated in parallel to the CFU-GM and BFU-E assays described above using an aliquot of the same fractions of $CD34^+$ cells isolated according to HGFR expression. The gating strategy used for FACS was described above in section 5.3.5. Examining generation of cells and nascent CFU-GM after 14, 21 and 28 days of culture, assessed Pre-CFU activity within the different $CD34^+$HGFR fractions. The results of these studies are presented in figures 5.16-5.21. Pre-CFU cultures initiated with 1,000 $CD34^+$ cells generated 0.514 x 10^6, 4.47 x 10^6 and 9.86 x 10^6 cells and 4852, 9030 and 14,879 CFU-GM at 14, 21 and 28 days, respectively.

Separation of $CD34^+$ cells according to expression of the IL-3Rα chain or gp130 did not discriminate pre-CFU activity as there was no significant difference in the production of total cells and CFU-GM between the $CD34^+$ IL-3Rα^+ and $CD34^+$ IL-3Rα^- cells or between

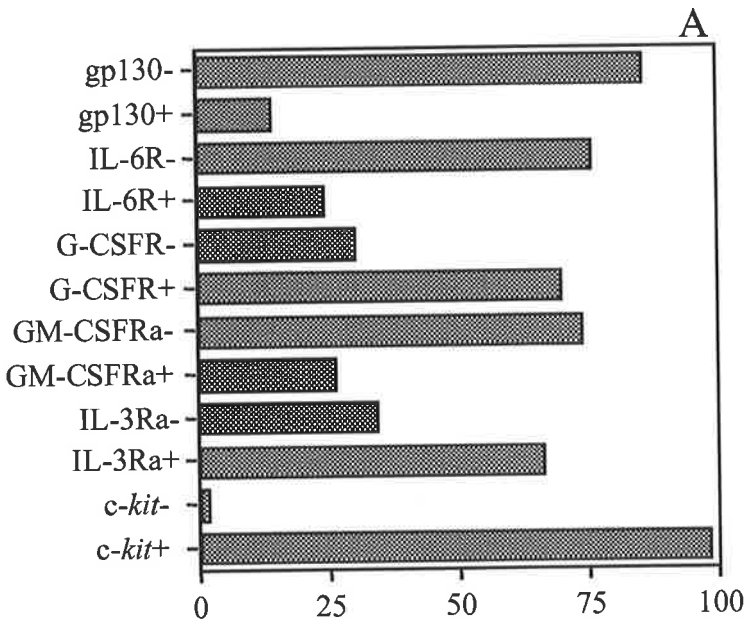
Table 5.2

Proportion of CFU-GM and BFU-E within $CD34^+$ cell fractions according to HGFR expression. * Refers to the calculated percentage of CFU-GM or BFU-E that are present within each cell fraction. These data are graphed in figure 5.15.

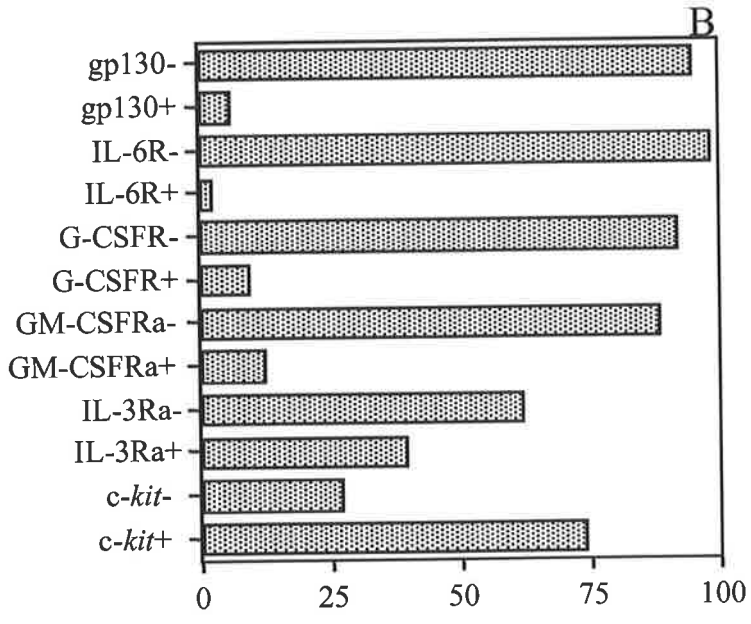
$CD34^+$ fraction	**CFU-GM***	**BFU-E***
c-*kit*$^+$	98.3	73.6
c-*kit*$^-$	1.7	24.4
IL-3Rα^+	66	38.7
IL-3Rα^-	34	61.3
GM-CSFRα^+	26.3	12
GM-CSFRα^-	73.7	88
G-CSFR$^+$	69.6	8.7
G-CSFR$^-$	30.4	91.3
IL-6R$^+$	24.2	1.9
IL-6R$^-$	75.8	98.1
gp130$^+$	14.2	5.6
gp130$^-$	85.8	94.4

Figure 5.15 Absolute number of CFU-GM and BFU-E within sub-fractions of $CD34^+$ cells discriminated according to HGFR expression

Panels A and B are graphs of the absolute numbers of CFU-GM and BFU-E, respectively, found within subsets of $CD34^+$ cells either expressing or lacking the HGFR examined. The derivation of values used to for these graphs is described in the text. In brief, the absolute number of CFU-GM or BFU-E within a particular subset was calculated by considering both the incidence of the clonogenic cell type and also the proportion of $CD34^+$ cells that express or lack the particular HGFR. For example, if 5.2% of $CD34^+$c-*kit*$^+$ cells are BFU-E and 83.5% of $CD34^+$ cells express c-*kit* then 73.6% of all BFU-E are present within the $CD34^+$c-kit^+ fraction (refer to the calculation shown on page 120). By extrapolation, 26.4% of BFU-E are present within the $CD34^+$c-*kit*$^-$ fraction of adult BM.


A
gp130-
gp130+
IL-6R-
IL-6R+
G-CSFR-
G-CSFR+
GM-CSFRa-
GM-CSFRa+
IL-3Ra-
IL-3Ra+
c-kit-
c-kit+
0
25
50
75
100

CFU-GM


B
gp130-
gp130+
IL-6R-
IL-6R+
G-CSFR-
G-CSFR+
GM-CSFRa-
GM-CSFRa+
IL-3Ra-
IL-3Ra+
c-kit-
c-kit+
0
25
50
75
100

BFU-E

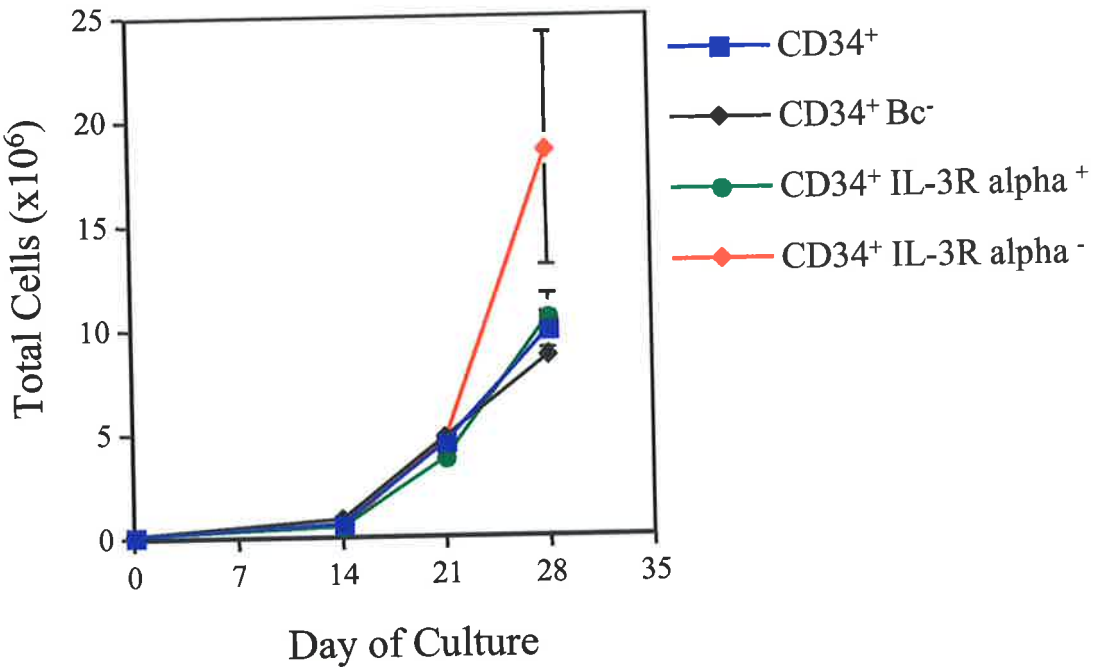
the $CD34^+gp130^+$ and $CD34^+gp130^-$ cells (figures 5.16 and 5.17). In contrast, separation of $CD34^+$ cells according to expression of GM-CSFRα, c-*kit*, G-CSFR and to a lesser extent the IL-6R allowed discrimination of cells with different activity in Pre-CFU culture. For example, significantly more nucleated cells and CFU-GM were generated by $CD34^+$GM-$CSFR\alpha^-$ cells than $CD34^+$GM-$CSFR\alpha^+$ cells after both 21 and 28 days of culture (p values of 0.061, 0.03 for cells and .06 and .08 for CFU-GM, figure 5.18). Similarly, as shown in figure 5.19, $CD34^+$c-kit^+ cells produced significantly more cells and CFU-GM than their $CD34^+$c-kit^- counterparts (p = 0.08, 0.08 and 0.06 for cells and p = 0.048, 0.06, 0.04 for CFU-GM after 14, 21 and 28 days respectively). After 14 days of Pre-CFU culture there was no significant difference in the number of nucleated cells or CFU-GM produced from $G\text{-}CSFR^+$ and $G\text{-}CSFR^-$ cells (figure 5.20). However, significantly more CFU-GM were produced by $G\text{-}CSFR^-$ cells than $CD34^+GCSFR^+$ cells at both 21 and 28 days of Pre-CFU culture (p = 0.06 and 0.08 respectively). A similar trend was observed for Pre-CFU activity within $CD34^+$ cell fractions discriminated according to expression of the IL-6R. At 14 and 21 days of culture there was no significant difference in cell or CFU-GM production between $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ cells although significantly more cells and CFU-GM were generated at day 28 from the $CD34^+IL\text{-}6R^-$ fraction (p= 0.03 and 0.025 respectively, figure 5.21).

5.4 Discussion

The studies described within this chapter address fundamental questions concerning the mechanisms underlying the production of neutrophil precursors from $CD34^+$ cells in HGF stimulated *ex vivo* culture. The first issue addressed concerned the identity of cells within the $CD34^+$ population that proliferates following stimulation by combinations of HGF. Experiments were designed to indicate whether combinations of HGF comprising IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF stimulated growth of either committed or primitive HPC or both. Discrimination of these two haemopoietic cell compartments was based on expression of CD38 and or HLA-DR antigens on $CD34^+$ cells. Flow cytometric analysis indicated that the majority of adult BM $CD34^+$ cells expressed both CD38 and HLA-DR antigens but for the remaining $CD34^+$ cells, expression of these antigens was essentially mutually exclusive. That is, the majority (76%) of $CD34^+CD38^-$ cells expressed intermediate levels of HLA-DR and the majority (86%) of $CD34^+HLA\text{-}DR^-$ cells expressed high levels of CD38. Of note was the finding that a minor sub-population of $CD34^+$ cells did not express either CD38 or HLA-DR. These $CD34^+CD38^-HLA\text{-}DR^-$ cells represented less than 0.5% of the $CD34^+$

Figure 5.16 Pre-CFU cultures of total $CD34^+$, $CD34^+\beta_c^-$, $CD34^+$IL-3Rα^+ and $CD34^+$IL-3Rα^- BM cells

The same sorted fractions of $CD34^+HGFR^+$ and $CD34^+HGFR^-$ BM cells as used for clonogenic assays presented in figures 5.13 and 5.14 were placed into Pre-CFU culture and total nucleated cell and CFU-GM generation assessed after 14, 21 and 28 days. Typically, triplicate 1 ml cultures containing 1,000 target cells were initiated in pre-CFU medium containing 30% FCS and 1% BSA supplemented with IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF each at 10 ng/ml. The top and bottom panels show total nucleated cells and CFU-GM (mean ± SEM, n = 3) present, respectively, at each day of analysis.


Total Cells (x10⁶)
25
20
15
10
5
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ Bc-
CD34+ IL-3R alpha +
CD34+ IL-3R alpha -

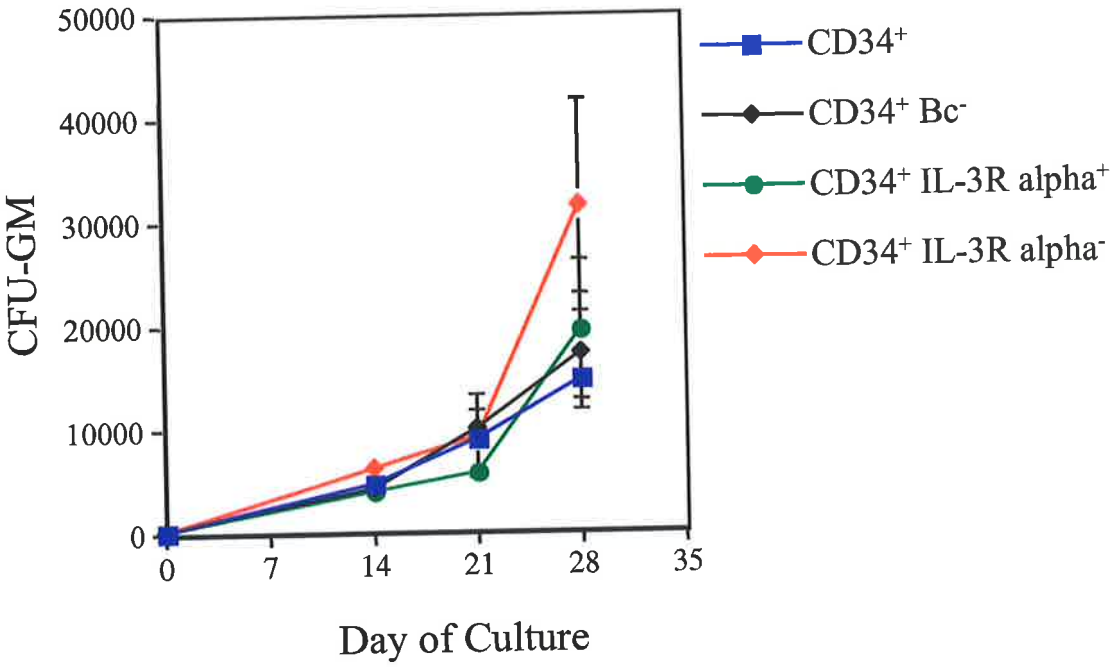
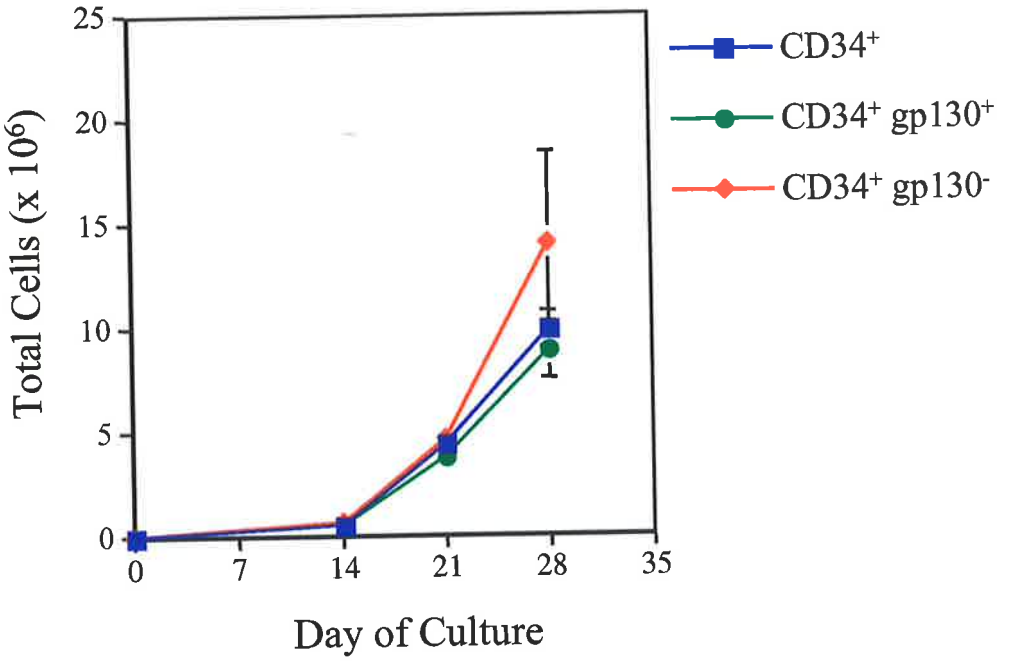

CFU-GM
50000
40000
30000
20000
10000
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ Bc-
CD34+ IL-3R alpha+
CD34+ IL-3R alpha-

Figure 5.17 Pre-CFU cultures of total $CD34^+$, $CD34^+$ $gp130^+$ and $CD34^+gp130^-$ BM cells

The same sorted fractions of $CD34^+HGFR^+$ and $CD34^+HGFR^-$ BM cells as used for the clonogenic assays described in figures 5.13 and 5.14 were placed into Pre-CFU culture and total nucleated cell and CFU-GM production assessed after 14, 21 and 28 days. Typically, triplicate 1 ml cultures containing 1,000 target cells were initiated in Pre-CFU medium containing 30% FCS and 1% BSA supplemented with IL-1, Il-3, IL-6, G-CSF, GM-CSF and SCF each at 10 ng/ml. The top and bottom panels show total nucleated cells and CFU-GM (mean ± SEM, n = 3) present, respectively, at each time of analysis.


Total Cells (x 10⁶)
25
20
15
10
5
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ gp130+
CD34+ gp130-

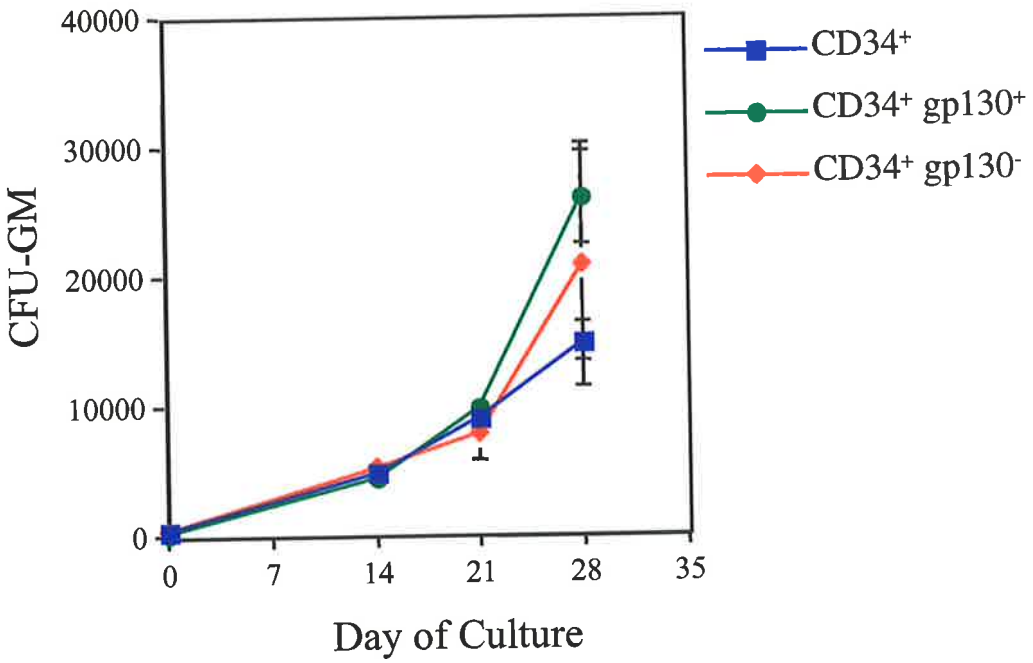
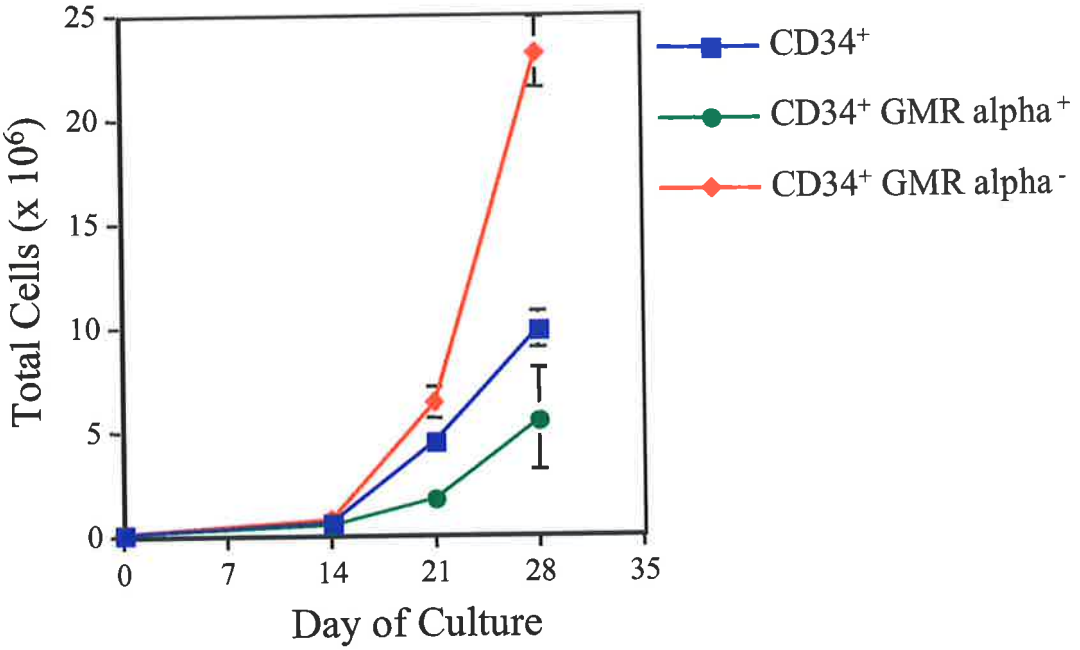

CFU-GM
40000
30000
20000
10000
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ gp130+
CD34+ gp130-

Figure 5.18 Pre-CFU cultures of total $CD34^+$, $CD34^+$GM-CSFRα^+ and $CD34^+$GM-CSFRα^- BM cells

The same sorted fractions of $CD34^+$GM-CSFRα^+ and $CD34^+$GM-CSFRα^- cells as used for the clonogenic assays described in figures 5.13 and 5.14 were placed into Pre-CFU culture. Typically, triplicate 1 ml cultures containing 1,000 target cells were initiated in Pre-CFU medium containing 30% FCS and 1% BSA supplemented with IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF, each at 10 ng/ml. The top and bottom panels show total nucleated cell and CFU-GM (mean ± SEM, n = 3) present, respectively, at each day of analysis.


Total Cells (x 10⁶)
25
20
15
10
5
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ GMR alpha+
CD34+ GMR alpha-

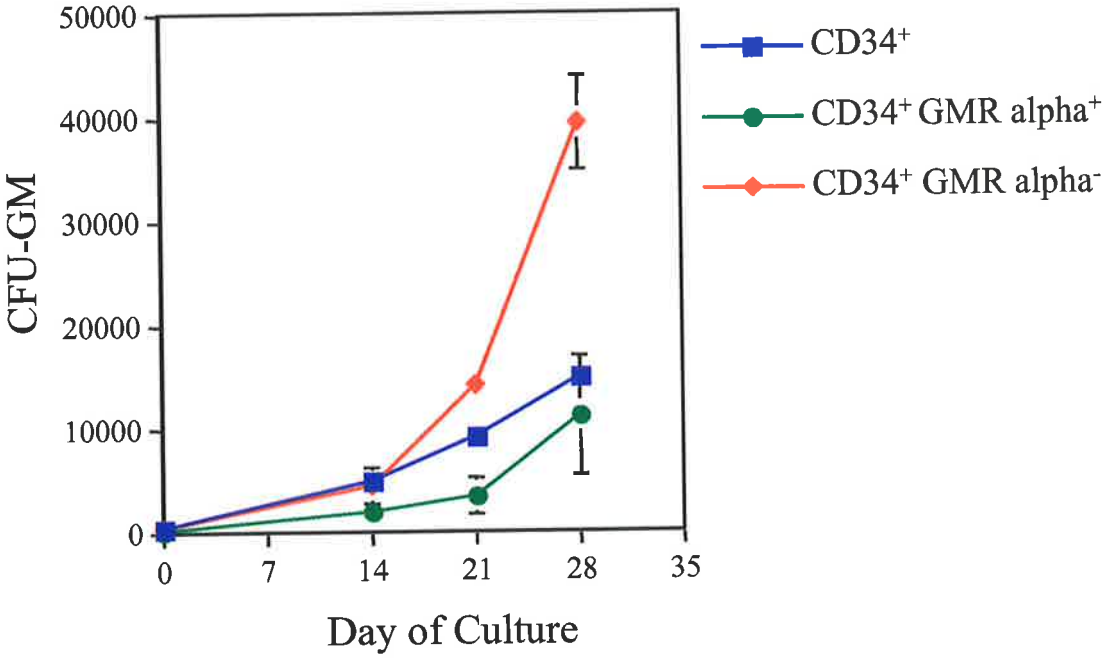
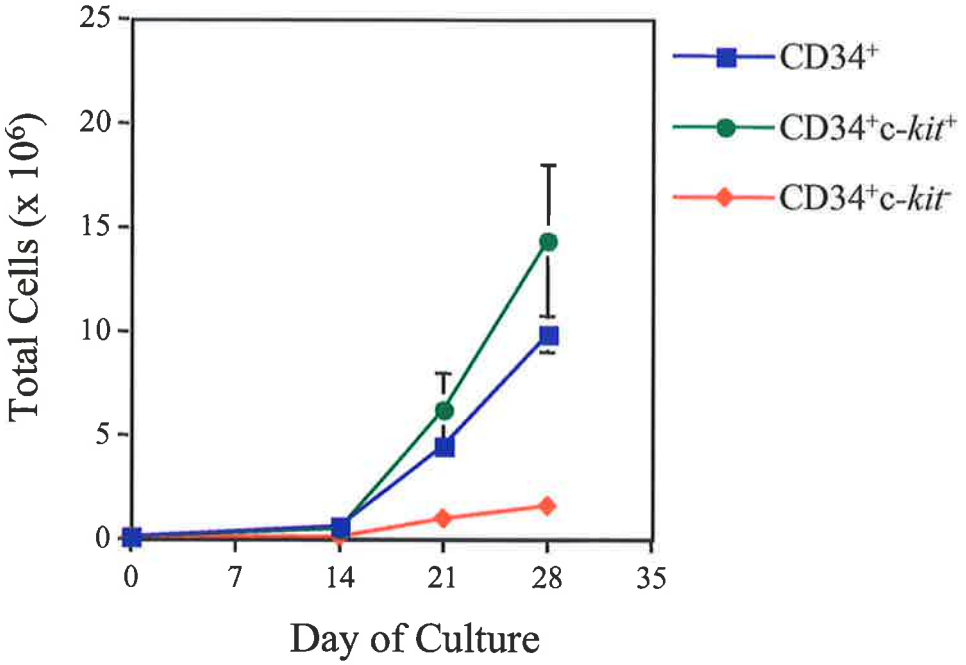

CFU-GM
50000
40000
30000
20000
10000
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ GMR alpha+
CD34+ GMR alpha-

Figure 5.19 Pre-CFU culture of total CD34$^+$, CD34$^+$c-*kit*$^+$ and CD34$^+$c-*kit*$^-$ BM cells

The same sorted fraction of CD34$^+$c-*kit*$^+$ and CD34$^+$c-*kit*$^-$ cells as used for the clonogenic assays described in figures 5.13 and 5.14 were placed into Pre-CFU culture. Typically, triplicate 1 ml cultures containing 1,000 FACS isolated target cells were initiated in pre-CFU medium containing 30% FCS and 1% BSA supplemented with IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF, each at 10 ng/ml. The top and bottom panels show total nucleated cell and CFU-GM (mean ± SEM, n = 3) present, respectively, at each day of analysis.


Total Cells (x 10⁶)
25
20
15
10
5
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+c-kit+
CD34+c-kit-

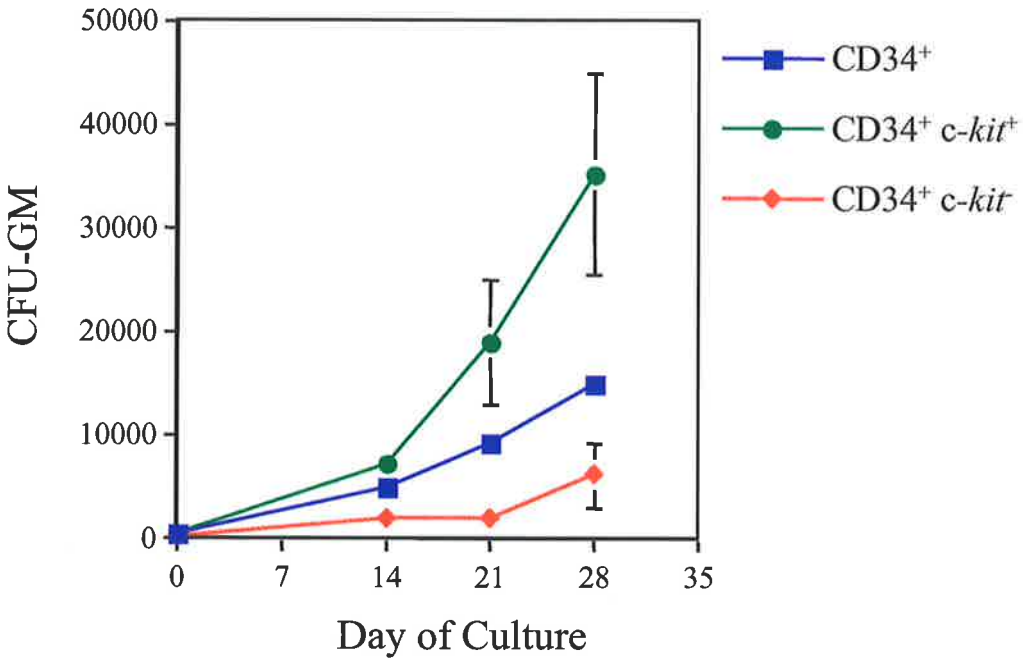
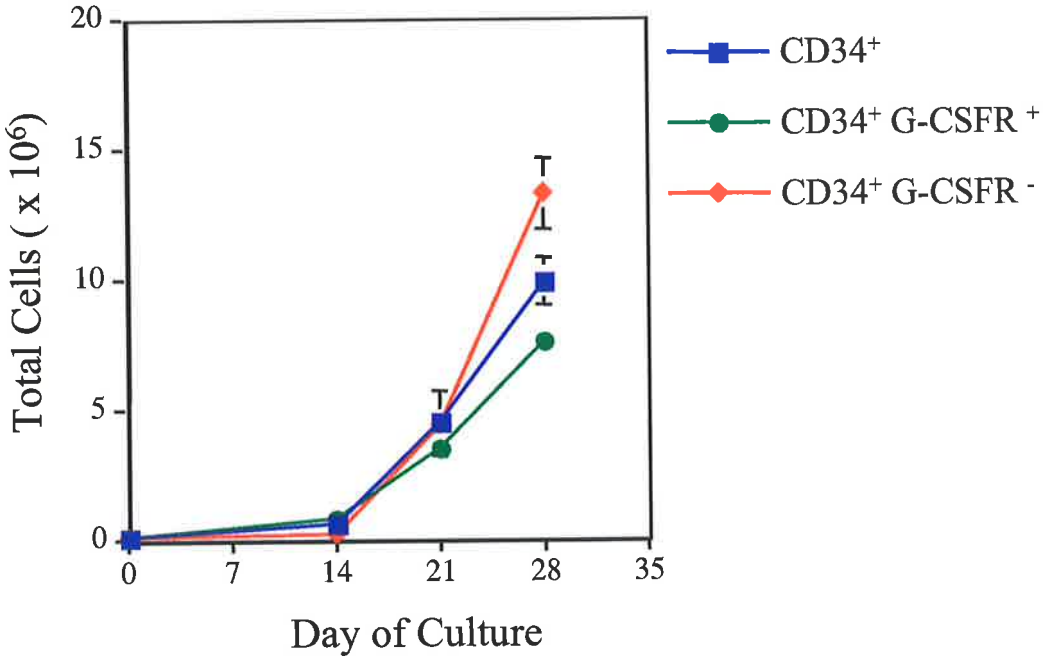

CFU-GM
50000
40000
30000
20000
10000
0
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ c-kit+
CD34+ c-kit-

Figure 5.20 Pre-CFU cultures of total $CD34^+$, $CD34^+$G-$CSFR^+$ and $CD34^+GCSFR^-$ BM cells

Sorted fractions of $CD34^+$G-$CSFR^+$ and $CD34^+$G-$CSFR^-$ cells identical to that used for the clonogenic assays described in figures 5.13 and 5.14 were placed into Pre-CFU culture. Typically, triplicate 1 ml cultures containing 1,000 FACS isolated target cells were initiated in pre-CFU medium containing 30% FCS and 1% BSA supplemented with 10 ng/ml each of IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF. The top and bottom panels show total nucleated cell and CFU-GM (mean ± SEM, n = 3) present, respectively, at each day of analysis.


20
15
10
5
0
Total Cells (x 10⁶)
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ G-CSFR +
CD34+ G-CSFR -

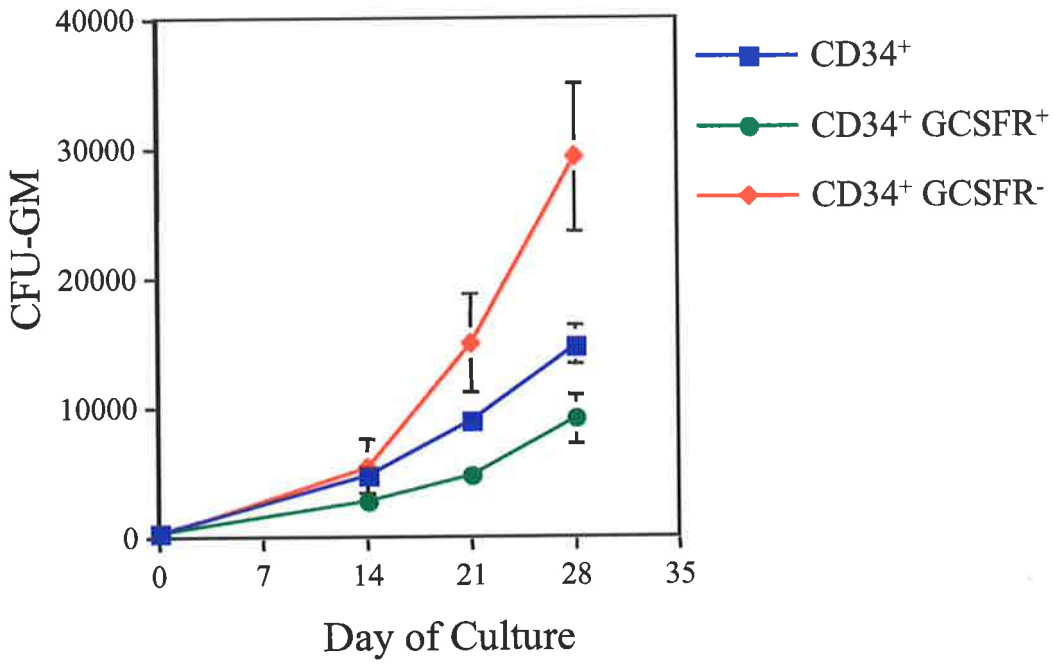
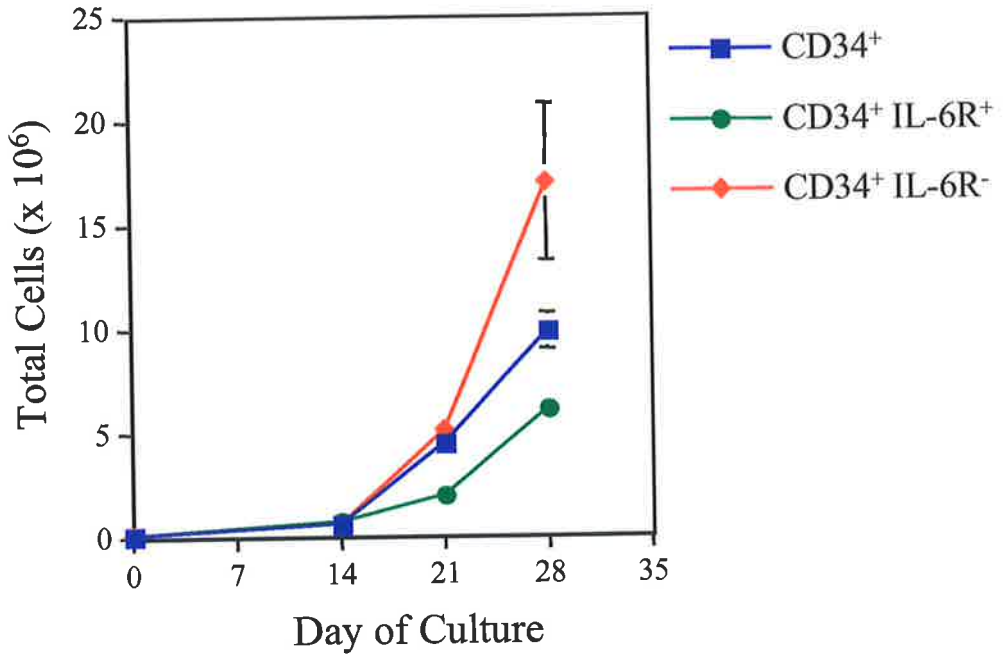
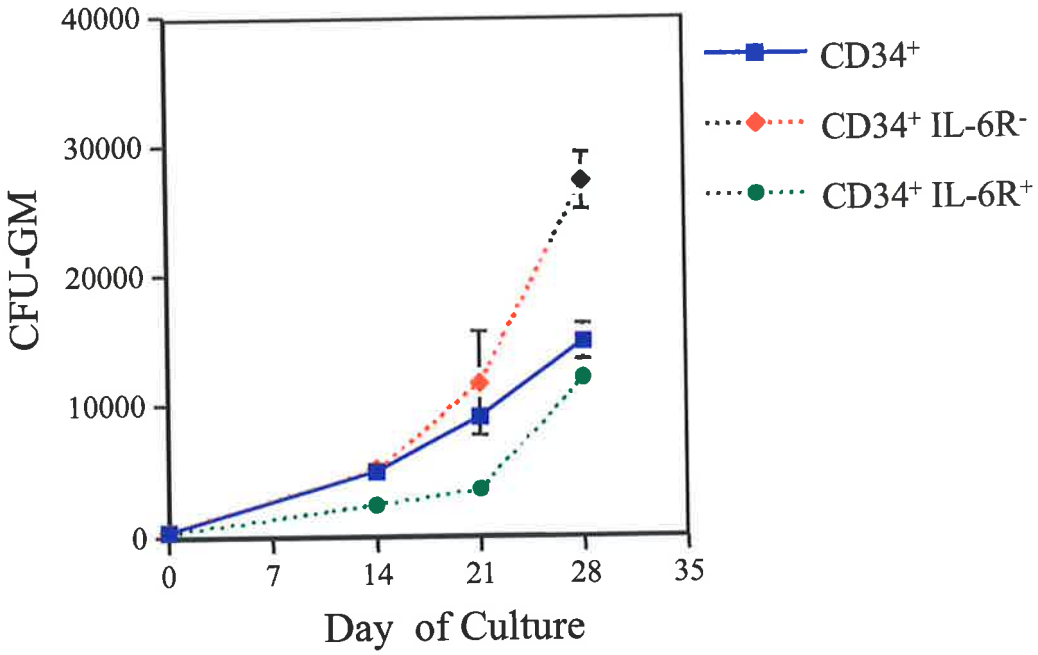

40000
30000
20000
10000
0
CFU-GM
0
7
14
21
28
35
Day of Culture
CD34+
CD34+ GCSFR+
CD34+ GCSFR-

Figure 5.21 Pre-CFU cultures of total $CD34^+$, $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ BM cells

Sorted fractions of $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ cells identical to those used for the clonogenic assays described in figures 5.13 and 5.14 were placed into Pre-CFU culture. Typically, triplicate 1 ml cultures containing 1,000 FACS isolated target cells were initiated in Pre-CFU medium containing 30% FCS and 1% BSA supplemented with 10 ng/ml each of IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF. The top and bottom panels show total nucleated cell and CFU-GM (mean ± SEM, n = 3) present, respectively, at each day of analysis.


25
20
15
10
5
0
Total Cells (x 10⁶)
0
7
14
21
28
35
Day of Culture
CD34⁺
CD34⁺ IL-6R⁺
CD34⁺ IL-6R⁻


40000
30000
20000
10000
0
CFU-GM
0
7
14
21
28
35
Day of Culture
CD34⁺
CD34⁺ IL-6R⁻
CD34⁺ IL-6R⁺

population in adult bone marrow. Based on this pattern of antigen expression it is tempting to speculate that the most primitive HPC, perhaps HSC, are found within the $CD34^+HLA\text{-}DR^-CD38^-$ cell fraction and that these cells follow alternative pathways of differentiation. Some $CD34^+HLA\text{-}DR^-CD38^-$ cells acquire CD38 before HLA-DR whereas others acquire HLA-DR before CD38. Irrespective of the initial differentiation, $CD34^+$ cells eventually express both CD38 and HLA-DR.

These observations are in accord with other flow cytometric analyses of adult BM (Snoeck *et al* 1993, Rusten *et al* 1994b, Harvey *et al* 1997), umbilical cord blood (Cicuttini *et al* 1994, De Bruyn *et al* 1995) and foetal BM (Huang and Terstappen 1994), although the proportion of $CD34^+CD38^-HLA\text{-}DR^-$ cells in the latter tissues was reported to represent between 1 - 4% of $CD34^+$ cells. Clonogenic assays performed on $CD34^+$ cell subsets (figure 5.5) demonstrated that CFU-GM were present not only in the $CD34^+HLA\text{-}DR^+$ and $CD34^+CD38^+$ fractions but also in $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^-$ fractions and that maximal CFU-GM numbers for each cell fraction was observed in cultures stimulated by combinations of HGF, including 136GGMS and 36GS. This result is consistent with previous work performed in our laboratory where we demonstrated a correlation between the HGF combination and the cloning efficiency of target $CD34^+$ cells (Lewis *et al* 1996). The data presented in figure 5.5 is also consistent with the results observed by Ema *et al* who found single factors such as IL-3, G-CSF but not IL-6 were capable of stimulating colony formation of single sorted $CD34^+$ and $CD34^+CD33^+$ bone marrow cells (Ema *et al* 1990). However it is important appreciate that because approximately 90% of $CD34^+$ cells express both CD38 and HLA-DR, then a high proportion of clonogenic cells within the $CD34^+$ fraction will grow when stimulated by single factors such as G-CSF and GM-CSF.

The requirement for multiple HGF to stimulate CFU-GM was most apparent in cultures of $CD34^+CD38^-$ cells and to a lesser extent for $CD34^+HLA\text{-}DR^-$ cells. The greatest incidence of CFU-GM was observed from these cells when combinations of 36GS or 136GGMS were used as stimulus. In contrast, whereas single HGF, particularly G-CSF and GM-CSF were able to stimulate significant CFU-GM growth from $CD34^+HLA\text{-}DR^+$ and $CD34^+CD38^+$ cells these same HGF stimulated few CFU-GM from $CD34^+CD38^-$ cells. These findings are in accord with previous studies performed with murine and human BM indicating committed haemopoietic progenitors respond well to single HGF, whereas more primitive progenitors typically require multiple HGF's for optimal proliferation (Heimfeld *et al* 1991, Ikebuchi *et al* 1987, Ikebuchi *et al* 1988b, Iscove *et al* 1989)

Pre-CFU cultures initiated with the same $CD34^+$ fractions revealed that nucleated cell and nascent CFU-GM production at day 14 of cytokine-dependent liquid culture could be mainly attributed to committed HPC with $CD34^+HLA\text{-}DR^+$ and $CD34^+CD38^+$ phenotypes. Based on the data from CFU-GM assays and Pre-CFU cultures it is reasonable to speculate that during the first 14 days of *ex vivo* culture with combinations of HGF, generation of nucleated cells and nascent CFU-GM is the result of proliferation from a large number of committed HPC that express CD38 and HLA-DR antigens (approximately 90% of the $CD34^+$ fraction) as well as proliferation from a small number of primitive HPC cells that lack CD38 or HLA-DR. Notably, for cultures that are continued for greater than 3 weeks, an increasing proportion of cell and CFU-GM production can be attributed to $CD34^+CD38^-$ cells, as shown in figures 5.6 and 5.7, respectively. However, generation of nascent CFU-GM from total $CD34^+$ cells after 4 weeks is not only attributed to $CD34^+CD38^-$ cells but also to the $CD34^+CD38^+$ and $CD34^+HLA\text{-}DR^+$ fractions. This point is better appreciated by taking into account that the latter 2 cell fractions comprise 80-90% of total $CD34^+$ cells and although relatively less nascent CFU-GM are generated from these cell fractions, on a per cell basis, the total number of CFU-GM generated from these cells is almost equivalent to the total number of CFU-GM generated by $CD34^+CD38^-$ cells. Thus when unfractionated $CD34^+$ cells are cultured for 3-4 weeks, committed HPC ($CD34^+CD38^+$ and $CD34^+HLA\text{-}DR^+$) as well as primitive HPC contribute significantly to generation of cells and CFU-GM.

The data also indicate that cells with the greatest Pre-CFU activity are $CD34^+CD38^-$ rather than $CD34^+HLA\text{-}DR^-$. If hierarchically more primitive HPC have an ability to generate greater numbers of nascent CFU-GM at later times in *ex vivo* culture then the data suggests that $CD34^+CD38^-$ cells represent a hierarchically distinct and more primitive fraction of HPC than $CD34^+HLA\text{-}DR^-$ cells. The question of whether the most primitive HPC are $CD34^+CD38^-$ or $CD34^+HLA\text{-}DR^-$ has been the subject of numerous studies over many years. Initial data provided by long-term marrow culture, suggested that primitive HPC were $HLA\text{-}DR^-$ (Moore *et al* 1980, Keating *et al* 1984), and resided within the $CD34^+HLA\text{-}DR^-$ fraction (Sutherland *et al* 1989, Brandt *et al* 1990, Verfaille *et al* 1993, Srour *et al* 1993). However more recent data suggests that primitive HPC and most likely HSC are enriched in the $CD34^+CD38^-$ fraction of fetal liver (Muench *et al* 1994, Huang and Terstappen 1994), cord blood (Cardoso *et al* 1993, Conneally *et al* 1997) and BM (Issaad *et al* 1993, Civin *et al* 1996, Bhatia *et al* 1997). The most compelling evidence supporting the proposal that HSC reside within the $CD34^+CD38^-$ fraction comes from *in vivo* transplantation studies in the pre-

immune fetal sheep model (Civin *et al* 1996) and NOD/SCID immune deficient mice (Bhatia *et al* 1997). In spite of the large number of investigations examining the haemopoietic potential of different $CD34^+$ sub-fractions there have been none that directly compare the potential of $CD34^+CD38^-$ cells with $CD34^+$HLA-DR^-cells isolated from adult haemopoietic tissue. The present studies indicate that both of these cell populations have significant activity within a Pre-CFU culture. $CD34^+$HLA-DR^- cells are able to sustain production of nascent CFU-GM for at least 4 weeks, which is in accord with studies showing that this fraction of cells sustains cell production during a 5 week stroma-non contact culture (Verfaille 1993). However, production of nascent CFU-GM at 4 weeks of pre-CFU culture in 136GGMS by $CD34^+CD38^-$ cells is significantly greater than that observed by $CD34^+$HLA-DR^- cells. Although generation of nascent CFU-GM under pre-CFU culture conditions is a surrogate index of haemopoietic potential this result is in keeping with the accumulating evidence that the most primitive HPC reside within the $CD34^+CD38^-$ cell fraction. It remains to be determined if HSC activity within adult haemopoietic tissues is a function of all $CD34^+CD38^-$ cells or whether this is exclusively restricted to $CD34^+CD38^-$ HLA-DR^- cells.

The results presented in this chapter describing HGFR expression need to be considered in the context of the limitations of indirect immunofluorescence as a method for detection of cell surface molecules. Firstly, the sensitivity of this approach is dependent on the affinity of the monoclonal antibodies used for detection. Secondly, it is well accepted that the sensitivity of commercial flow cytometers such as the FACStar^{PLUS} are limited by instrument design and cell auto- fluorescence so that for conventional indirect two-layer labelling, 1500-2000 molecules of fluorescein and or phycoerythrin per cell is the detection limit (Shapiro 1988, Hulett *et al* 1989). Therefore in the current study, cells that are referred to as $HGFR^-$ may in fact express low levels of HGFR albeit below the limit of detection by flow cytometry. In situations where functional bioassays indicate that cells do express low levels of HGFR then alternative, more sensitive methods need to be used to assess and confirm receptor expression. Such methods include optimised, multi-layer immunofluorescence, which is capable of detecting as few as 100 molecules cell (Zola *et al* 1990) or by toxins linked either chemically or genetically to cytokines or receptor antibodies (Uckun and Frankel 1993). These more sensitive, flow cytometric approaches represent very useful alternatives to the use of radiolabelled ligands for assessing HGFR expression especially where the HGFR- ligand interaction is of low affinity. In spite of the inherent limitations of

flow cytometry the present study provides useful information concerning HGFR expression on HPC and BM MNC.

The 2-colour labelling strategy of CD34 together with antibodies to HGFR allowed clear discrimination of HGFR expression on $CD34^+$ cells for all HGFR tested with the exception of the β_c chain of the IL-3 and GM-CSF receptors. Overall the data demonstrate that a high proportion of $CD34^+$ cells express different classes of HGFR including those with intrinsic tyrosine kinase activity (such as c-*kit*) and those that non-covalently associate with intracellular tyrosine kinases. Subfamilies of the latter include those that signal through gp130, and members of the hematopoietin family such as the IL-3R, GM-CSFR and G-CSFR. The data presented herein confirm previously published information by this laboratory (Cambareri *et al* 1988, Ashman *et al* 1991, Simmons *et al* 1994a) and of others (Broudy *et al* 1992, Papayannopoulou *et al* 1991, Buhring *et al* 1991, Briddell *et al* 1992) concerning the expression of the c-*kit* protein by haemopoietic cells. Approximately 83% of BM $CD34^+$ cells express c-*kit* and essentially all myeloid progenitors fractionated with $CD34^+$c-*kit*$^+$ cells whereas BFU-E were present in both the $CD34^+$c-*kit*$^+$ and $CD34^+$c-*kit*$^-$ fractions, findings that were consistent with previous studies (Broudy *et al* 1992, Simmons *et al* 1994a). In addition, the current study also confirmed that Pre-CFU activity was much greater in $CD34^+$c-*kit*$^+$ cells than $CD34^+$c-*kit*$^-$ cells. Although a very high proportion of $CD34^+$ cells express high levels of c-*kit* it is notable that its ligand, SCF, when used as a single HGF is a very poor stimulator of cell proliferation or colony formation. However the high level expression of c-*kit* by the majority of $CD34^+$ cells is consistent with the synergistic interactions observed between SCF, and other HGF in stimulating both HPC proliferation and colony formation from myeloid, erythroid and megakaryocytic progenitors (Bernstein *et al* 1991, Broudy 1997).

The expression of IL-6R and gp130 and the biological activity of its ligand, IL-6 are similar to that for c-*kit* and SCF. Although there is low level expression of both the IL-6R and gp130 components of the IL-6R signalling complex on $CD34^+$ cells there is little CFU-GM stimulating activity observed when IL-6 is used alone to stimulate $CD34^+$ cells or the putative committed and primitive fractions thereof. Thus, although approximately 20% of BM $CD34^+$ cells express both gp130 and IL-6R, less than 4% of $CD34^+$ cells clone when cultured in IL-6 alone. This data is consistent with other studies indicating that IL-6 is a synergistic or permissive HGF that acts together with other early acting and lineage specific cytokines such as SCF, IL-1, IL-3 and G-CSF to stimulate HPC proliferation (Leary *et al*

1988, Moore *et al* 1990, Ikebuchi *et al* 1987). The finding that a mean of 28.6% of $CD34^+$ cells express the IL-6R is consistent with the reported proportion of cord blood $CD34^+$ cells that are $IL\text{-}6R^+$ (Tajima *et al* 1996), which is notable given that same anti-IL-6R monoclonal (MT18) was also used by Tajima *et al*. However, unlike the observations made in the current study, Tajima observed that essentially all $CD34^+$ cells expressed gp130. The discrepancy between my data and that of Tajima may be explained by the use different monoclonal antibodies to gp130, and also by the Tajima's use of a biotinylated anti-gp130, which would result in increased sensitivity of detection. Irrespective of the reason for these differences it seems that gp130 may be far more abundant than IL-6R in the membrane of HPC. As a consequence $CD34^+IL\text{-}6R^-$ cells may have other ligand-specific receptor(s) requiring gp130 as a signal transducer. The IL-11R, LIF receptor, Oncostatin M receptor, or another unknown receptor may fill this role. Another interesting and useful consequence of this observation, especially for *ex vivo* manipulation of HPC is the enhanced stimulation provided to HPC by the combined use of a soluble IL-6R/IL-6 complex, an effect mediated through interaction with membrane anchored gp130 on target cells (Nakahata *et al* 1996, Kimura *et al* 1997, Fischer *et al* 1997, Zandstra *et al* 1998).

The results of Pre-CFU culture indicate that the greatest Pre-CFU activity resides with the $CD34^+IL\text{-}6R^-$ fraction although considerable activity is also present with $CD34^+IL\text{-}6R^+$ cells. In comparison there is no difference in the Pre-CFU activity of $CD34^+$ cells discriminated somewhat arbitrarily on the basis of gp130 expression, a result which is consistent with the possibility that all $CD34^+$ cells may express low levels of gp130. The receptor expression studies for c-*kit* and the IL-6R complex together with the clonogenic assays performed with $CD34^+$ cells and fractions thereof isolated according to CD38 or HLA-DR expression serve to highlight a critical aspect of the actions of some single HGF on HPC. That is; it does not necessarily follow that a single HGF will be able induce a biological response in a cell that expresses its receptor. Often a biological response will only be observed if the HGF activates its receptor at the same time as other signal pathways are activated. In this respect IL-6 and SCF typify synergistic HGF.

One intriguing finding from the current study was the low to undetectable levels of surface expression of the β_c chain on $CD34^+$ cells isolated from adult BM. Given the vast excess of IL-3Rα and GM-CSFRα chains over β_c chain on $CD34^+$ cells then this suggests that the majority of alpha chains on surface of HPC are monomeric. The very low level of β_c chain surface expression was not due to inactive monoclonal antibody to the β_c chain as it clearly

bound to $CD34^{dim}$ cells including a fraction enriched in basophils. Similar studies based on indirect immunofluorescence with antibodies to the human IL-3Rα and β_c chains also demonstrated low level expression of the β_c chain on cord blood and BM $CD34^+$ cells (Sato *et al* 1993a, Kurata *et al* 1995). These observations are in direct contrast to a recent study of cytokine receptor expression on human foetal BM where the β_c chain was detected on both $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (Lund-Johansen *et al* 1999). However the studies of Lund-Johansen were performed with an experimental, high sensitivity flow cytometer (Olweus *et al* 1996) capable of detecting as few as 50-100 molecules of fluorescein/cell and unfortunately expression of the β_c chain was not assessed on adult human bone marrow. It is therefore difficult to compare the results of Lund-Johansen *et al* with those of the present study. It is possible that the expression of the β_c chain on foetal BM $CD34^+$ cells may reflect an overall increased level of cytokine receptor expression on developmentally more primitive haemopoietic tissues. However, with the current study, in comparison to the very low proportion of $CD34^+$ cells expressing the β_c chain, a relatively high proportion of $CD34^+$ cells expressed both IL-3Rα and GM-CSFRα chains. This data therefore raises some interesting issues concerning the assembly of both the IL-3R and GM-CSFR complexes on the surface of HPC.

Firstly, the lower relative incidence of β_c chains to GM-CSFRα chains suggests that a ligand-independent pre-formed complex of GMCSFRα/β_c may not form within HPC (Woodcock *et al* 1997). Secondly, the lower abundance of β_c chains relative to both GM-CSFRα and IL-3Rα chains suggests that the alpha chains exist at the cell surface in a monomeric form and thus may have an alternative function on HPC. For instance GM-CSFRα may be involved in directly signalling for hexose transport (Ding *et al* 1994). In addition it implies competition between the GM-CSFRα and IL-3Rα chains for a limited number of partner β_c chains. It is also possible that there is a large intracellular pool of β_c chain that can be translocated to the surface of HPC following activation by other cytokines. Support for this idea comes from RNase protection assays for cytokine receptor mRNA in $CD34^+$ cells. Unpublished data by Dr E Korpelainen (Professor A Lopez laboratory, Hanson Centre for Cancer Research, Adelaide) indicates that a high level of mRNA for the β_c chain exists in $CD34^+$ cells. The lack of β_c cell surface protein would indicate that this mRNA species is either rapidly degraded, not translated or the protein is not immediately translocated to the membrane. Although only a mean of 0.4% of $CD34^+$ cells expressed the β_c chain approximately 5-7% of these cells produced CFU-GM colonies when cultured with

only IL-3. This suggests that IL-3 binding to a proportion of HPC with a very low number of IL-3 receptors is sufficient to induce proliferation. As with early acting HGF the affect of IL-3 on HPC proliferation is more apparent when it is used in conjunction with other HGF. For differentiating myeloid cells including basophils where the IL-3 receptor complex is expressed at higher levels then IL-3 may have more potent biological activity when used alone.

Although $CD34^+$ cells can be clearly separated into $CD34^+$IL-3Rα^+ and IL-3Rα^- fractions they have similar biological properties. Both fractions contain CFU-GM and BFU-E although there is a slight enrichment for CFU-GM within the IL-3Rα^+ fraction and conversely enrichment for BFU-E in the $CD34^+$IL-3Rα^- fraction. This implies that expression of the IL-3R may not restrict development of HPC to either myeloid or erythroid differentiation. It is however, in accord with IL-3's action as a multi-CSF stimulating both CFU-GM and BFU-E progenitors (Schrader 1986, Clark and Karmen 1987, Egeland *et al* 1991, Moore 1991). Similarly, Pre-CFU activity is comparable in the 2 fractions although the greater production of nascent CFU-GM at day 28 by $CD34^+$IL-3Rα^- cells implies that this fraction may be enriched for primitive HPC.

Although GM-CSF has been shown to synergise with IL-3, G-CSF and SCF to enhance growth of HPC (McNiece et 1989a, McNiece *et al* 1991, Lewis *et al* 1996) there are few studies investigating the expression of its receptor on human HPC. There is only 1 previous study reporting GM-CSFR expression on primitive HPC from foetal BM cells (Lund-Johansen *et al* 1999) and a single report where binding of radiolabelled GM-CSF has demonstrated GM-CSFR expression on mobilised PB $CD34^+$ cells (Roberts *et al* 1997). A further study based on flowcytometry with anti-GM-CSFR monoclonal antibodies also indicated that GM-CSFRα was expressed more abundantly on $CD34^+CD33^{high}$ cells than on $CD34^+CD33^{low}$ cells (Kurata *et al* 1995). In comparison, a collection of reports suggest that primitive HPC lack receptors for GM-CSF (McKinstry *et al* 1997, Jubinsky *et al* 1994, Wognum *et al* 1994). The data presented herein provides no definitive evidence that primitive HPC express or lack the GM-CSFR although the enhanced generation of nascent CFU-GM in Pre-CFU culture by $CD34^+$GM-CSFRα^- cells implies that primitive HPC may not constitutively express the GM-CSFR. This issue remains to be resolved and will require testing of GM-CSFR$^+$ and GM-CSFR$^-$ cells in suitable *in vivo* transplantation models. Irrespective of whether the GM-CSFR is expressed on primitive HPC the subsequent expression of this receptor in conjunction with other lineage associated antigens on cells

produced in *ex vivo* culture appears to be a valuable means of mapping monocyte and neutrophil maturation (Ericson *et al* 1994, Lund-Johansen *et al* 1999). For example, immunolabelling with antibodies to CD64, CD24 and CD14 together with CD116 (GM-CSFRα chain) would be an alternative method to the use of CD11b and CD15 for discriminating stages of neutrophil development. This strategy could be improved by also using antibodies to the IL-3Rα chain in conjunction with those mentioned above.

Data presented within chapter 4 indicated that apart from its well-recognised function as the main cytokine responsible for promoting neutrophil development (reviewed in Demetri and Griffin 1991), G-CSF might directly affect the proliferation of Pre-CFU. This view is supported by studies that show G-CSF commonly exhibits synergistic haemopoietic activity with other cytokines *in vitro* to promote blast colony formation and the growth of highly purified multipotential haemopoietic precursors (Ikebuchi *et al* 1988a, Ikebuchi *et al* 1988b, Takaue *et al* 1990, McNeice *et al* 1989a). Furthermore, in the present study, G-CSF alone was able to promote granulocyte-macrophage colony formation from a proportion of $CD34^+$, $CD34^+CD38^+$, $CD34^+HLA\text{-}DR^+$, $CD34^+CD38^-$ and $CD34^+HLA\text{-}DR^-$ cells. Collectively this data implies that the G-CSFR should be expressed on a proportion of primitive as well as committed HPC. The present studies indicate that this is indeed the case with approximately 45-50% of $CD34^+$ cells expressing the G-CSFR (panel D of figure 5.8). This result is consistent with the finding that a high proportion of murine stem cells ($Rh^{lo}lin^-Sca\text{-}1^+c\text{-}kit^+$) (McKinstry *et al* 1997) and 25.8% of BM $CD34^+$ and 29% of cord blood $CD34^+$ cells express low levels of the G-CSFR (Shimoda *et al* 1992). Recent studies with ^{125}I-labeled G-CSF also demonstrated that 25% of $CD34^+CD38^{-/dim}$ cells from mobilized blood exhibit low-level (estimated mean of 120 G-CSFR/cell) expression of the G-CSFR. Shinjo *et al*, using a newly-devised quantitative flow-cytometric assay (Shinjo *et al* 1995) has also reported a maturation dependent increase in G-CSFR expression during differentiation of neutrophil precursors from BM $CD34^+$ cells (Shinjo *et al* 1997). In spite of these flow cytometric data there is not firm evidence to demonstrate that the G-CSFR is present on HSC. In fact, the data from pre-CFU cultures indicate that cells with greatest Pre-CFU activity are found within the $CD34^+G\text{-}CSFR^-$ fraction of BM. However this activity is not exclusively associated with these cells as the counterpart $CD34^+G\text{-}CSFR^+$ fraction also has significant capacity for production of cells and nascent CFU-GM.

One intriguing aspect of G-CSFR biology is the potential for different receptor isoforms to deliver qualitative different signals to HPC. In addition to the normal wild type form, at least

6 different forms of the human G-CSFR, resulting from alternative splicing of G-CSFR mRNA, have been cloned from human placenta, myeloid leukaemia cells (Fukunaga *et al* 1990, Larsen *et al* 1990) and normal neutrophils (Bernard *et al* 1996). The majority of these isoforms have differences within the cytoplasmic regions of the receptor where distinct domains appear to have a determinative role in G-CSF induced proliferation and maturation of myeloid progenitor cells (Dong *et al* 1993, Fukunaga *et al* 1993). Localization of different functions to distinct regions of the receptor tail has been demonstrated by creating deletion mutants (Fukunaga *et al* 1991, Ziegler *et al* 1993). The membrane proximal region including boxes 1 and 2 is both necessary and sufficient for proliferation whilst the more distal region containing box 3 is required for differentiation (Bernard *et al* 1996). Thus while analysis of G-CSFR distribution by techniques such as autoradiography and flowcytometry provides information on receptor expression they do not indicate which G-CSFR forms are present. It is therefore possible that preferential expression of a receptor isoform(s) at different stages of HPC development may contribute to primitive cell expansion (proliferation) without lineage commitment. Such a proposal can only be examined by a molecular analysis of highly purified populations of cells representing different stages of HPC development and mapping alterations in receptor isoform usage during cytokine induced maturation.

In summary, the results presented within this chapter demonstrate that generation of nucleated cells and CFU-GM during the first 2 weeks of HGF stimulated *ex vivo* expansion culture is mainly, but not exclusively, attributed to proliferation of committed, $CD34^+$ HPC which express CD38 and HLA-DR antigens. With combinations of HGF comprising IL-3, IL-6, G-CSF and SCF then primitive cells including $CD34^+CD38^-$ and $CD34^+HLA\text{-}DR^-$ cells also contribute to cell and CFU-GM production.

Analysis of HGFR expression indicates that $CD34^+$ cells are heterogeneous with respect to constitutive expression of receptors for IL-3, IL-6, G-CSF, and GM-CSF and to a lesser extent SCF. A proportion of $CD34^+$ cells may express receptors for many HGF whereas others may express only a few such as c-*kit* or gp130. This observed pattern of HGFR expression is consistent with the ability for combinations of HGF to induce division of a proportion of $CD34^+$ within 1-2 days of *ex vivo* culture. Expression of the HGFR tested was also observed to show a strong inverse correlation with commitment to the erythroid lineage. Thus, BFU-E activity was greatly enriched in $CD34^+$ cells that did not express receptors to factors that promote leukocyte production ie IL-6, G-CSF, and GM-CSF. This observation is easily reconciled on the basis of cells not requiring receptors for factors that are not required

to complete their lineage differentiation. For example, following commitment of a $CD34^+$ cell to erythroid development then there is no further need for cells to express the receptors for G-CSF or GM-CSF. However, it seems that a proportion of developing erythroid cells require both IL-3 and SCF as BFU-E express both IL-3R and c-*kit*. Conversely, apart from c-*kit*, expression of other HGFR on $CD34^+$ cells did not predict for CFU-GM growth. Interestingly, results from Pre-CFU cultures performed with $CD34^+$ cells isolated according to HGFR expression suggest that cells with the ability to generate nascent CFU-GM after 4 weeks culture may not constitutively express IL-6R, G-CSFR, GM-CSFR, or the β_c chain. Although $CD34^+$ cells may not express particular HGFR at the start of culture it has been postulated that upregulation of HGFR following stimulation with one of the component HGF may be one mechanism underlying synergistic interactions between HGF (Jacobsen *et al* 1992a, Sato *et al* 1993a, Testa 1993).

The data presented within this chapter also support an emerging paradigm concerning growth and differentiation of primitive HPC. That is, in spite of expressing an extensive repertoire of HGFR, primitive HPC do not divide in response to single HGF but require simultaneous signalling with a number of early acting and lineage restricted cytokines to induce proliferation. Although this paradigm is mainly based on the results of *in vitro* assays it is interesting to speculate that the requirement for simultaneous signalling mediated through HGFR is a key mechanism used by primitive HPC *in vivo* to maintain quiescence. This issue is investigated in further detail in chapter 6. Finally, studies presented within this chapter support the view that primitive HPC and HSC are located within the $CD34^+CD38^-$ fraction of adult BM. The following chapter of this thesis explore the biology of these cells in greater detail and describe how additional HGF affect their survival, recruitment and proliferation.

CHAPTER 6. THE EFFECT OF FLT3 LIGAND AND THROMBOPOIETIN ON *EX VIVO* MANIPULATION OF HAEMOPOIETIC PROGENITOR CELLS.

6.1 Introduction

A major influence on haemopoiesis is affected by HGF many of which are produced by stromal cells of the bone marrow microenvironment and exert either stimulatory or suppressive effects on haemopoietic cell growth (Flanagan *et al* 1991, Heinrich *et al* 1993, Roberts *et al* 1987, Simmons *et al* 1997, Eaves *et al* 1991, Verfaille 1993). Of considerable interest are those HGF, which influence the proliferation and development of primitive HSC and their immediate progeny. Such HGF are likely to be of importance in cellular therapies that involve *ex vivo* manipulation of the haemopoietic system, such as gene therapy or the expansion of either transplantable cells or their mature myeloid progeny (Crooks and Kohn 1993, Haylock *et al* 1992, Brugger *et al* 1993, Muench *et al* 1993, Brandt *et al* 1994, Smith *et al* 1993). Many of these HGF, including IL-1, IL-6, IL-11, leukaemia inhibitory factor (LIF) and SCF are regarded as synergistic HGF, which act to enhance the stimulatory activity of other HGF such as IL-3, the colony-stimulating factors or erythropoietin (EPO) and exhibit little or no effect alone on proliferation *in vitro* (Leary *et al* 1992, Verfaille and McGlave 1991, Ploemaker *et al* 1993, Bernstein *et al* 1991). Importantly, the most primitive HPC exhibit an almost obligatory requirement for combined stimulation by multiple synergistic HGF in order to elicit proliferation (McNeice *et al* 1991, Moore 1991, Metcalf 1993, Brandt *et al* 1990). This is in contrast to committed HPC populations which although demonstrating synergistic responses to combinations of HGF are generally stimulated to proliferate by single HGF (Moore 1991).

A particularly potent synergistic HGF is SCF whose actions have been documented on both very primitive HPC and their immediate lineage restricted clonogenic progeny (Bernstein *et al* 1991, McNeice *et al* 1991, Lowry *et al* 1991, Broxmeyer *et al* 1991, Muench *et al* 1992). The actions of SCF are mediated by the product of the *c-kit* proto-oncogene, a receptor tyrosine kinase (RTK) which is a member of the platelet derived growth factor (PDGF) receptor superfamily that includes *c-fms* (Ullrich and Schlessinger 1990, Sherr 1990). More recently, an additional member of this receptor superfamily has been identified, *flt3/flk2*

(Matthews *et al* 1991). The highly restricted expression of the *flt3/flk2* RTK in both murine and human HPC demonstrated by initial studies (Rosnet *et al* 1991, Rosnet *et al* 1993, Zeigler *et al* 1994) suggested that the ligand for this receptor, once identified, would play an important role in regulating the growth and development of primitive haemopoietic cells. The ligand (*flt3*-ligand; FLT3L), when identified, exhibited striking structural homology with SCF and monocyte colony stimulating factor (M-CSF) and in keeping with this, likewise demonstrated synergy with a range of other HGF including IL-3, IL-6, IL-7, IL-11, IL-12, G-CSF and GM-CSF (Lyman *et al* 1994, Hunte *et al* 1996).

At approximately the same time, thrombopoietin (TPO), also known as megakaryocyte growth and development factor (MGDF: Bartley *et al* 1994) was identified as the ligand for the *c-mpl* proto-oncogene (Lok *et al* 1994, Wendling *et al* 1994) and initially considered to be a lineage specific factor affecting the proliferation and maturation of megakaryocytes (Kaushansky *et al* 1994). However, subsequent studies including the administration of TPO to myeloablated non-human primates (Hokom *et al* 1995, Farese *et al* 1996) and the impaired haemopoiesis observed in both *c-mpl* (Alexander *et al* 1996b) and TPO knockout mice (Murone *et al* 1998) strongly suggests that TPO/MGDF also acts on primitive HPC. Thus within a relatively short period, two new cytokines with potential for use in *ex vivo* manipulation of human haemopoietic cells were available for investigation. Initial studies confirmed that these cytokines, when added with SCF, exhibited profound effects on growth of lineage negative, Sca-1^+ murine bone marrow progenitor cells (Ramsfjell *et al* 1996), and moreover promoted extensive expansion of human HPC following culture of umbilical cord blood derived $CD34^+$ cells (Piacibello *et al* 1997). A further study demonstrated a key role of TPO in promoting survival of primitive human BM cells (Borge *et al* 1997). However, in spite of these findings, the combined action of TPO and FLT3L with other HGF had not been fully investigated on primitive human HPC from adult bone marrow. The studies presented within this chapter investigate the affects of both TPO and FLT3L on survival and proliferation of adult human BM $CD34^+CD38^-$ cells. Herein, it is shown that both these cytokines increase recruitment of $CD34^+CD38^-$ cells, and when used together with 36GS induce division in greater than 90% of these cells. In addition, the combination of 36GS+FLT3L+TPO (36GSFT) proved to be an extremely potent mixture for stimulating proliferation of BM $CD34^+CD38^-$ cells in *ex vivo* culture.

6.2 Experimental Design and Methods

All Pre-CFU cultures described in this chapter were performed with cells grown in serum deprived medium (SDM) consisting of IMDM supplemented with human transferrin, recombinant human insulin, 1% bovine serum albumin and human low density lipoprotein. The exact formulation of this media is described in chapter 2, section 2.2.18. Under these culture conditions, HPC growth is absolutely dependent on provision of exogenous HGF.

Initial experiments were performed to determine whether addition of FLT3L and or TPO to 36GS, effected the generation of nucleated cells and nascent CFU-GM from BM $CD34^+CD38^-$ grown in 1 ml Pre-CFU cultures. These studies were complimented by a series of investigations where single HPC were deposited by the ACDU of the FacStar^{PLUS} into Terasaki wells containing 10 µl of SDM supplemented by combinations of HGF. The rationale for these experiments was that, under such conditions, the response of single target cells would be directly attributed to the combination of HGF used for stimulation. Sorting of single $CD34^+CD38^-$ cells was facilitated by prior enrichment of $CD34^+$ cells with Dynal 561 beads. This method routinely yielded a suspension containing >90% $CD34^+$ cells that were subsequently stained with HPCA-2-FITC (anti-CD34) and Leu17-PE (anti-CD38). As mentioned previously, the sort gate for the $CD34^+CD38^-$ fraction was positioned to contain between 5-8% of the $CD34^+$ cells. Megakaryocyte growth and development factor, at 100 ng/ml (generously provided by Amgen, Inc, Thousand Oaks, CA) was used as the source of TPO for these studies. FLT3L, (generously provided by Immunex, Seattle, WA, USA), was also used in cultures at 100 ng/ml. Single cell cultures, in SDM, were used to investigate the effects of individual or combinations of HGF on recruitment and proliferation of sub-populations of HPC. Cells cultured in Terasaki wells were assessed at regular intervals under inverted phase contrast microscopy to monitor the time to the first division (recruitment) and the number of cells present (cell proliferation). At day 14, the contents of individual wells were removed for cell counts and immunophenotyping (to assess differentiation). Single cell cultures were also used to assess the ability of single HGF to support survival of $CD34^+CD38^-$ cells. For these studies, single cells were deposited into Terasaki wells containing SDM supplemented with either the combination of all cytokines or each of the 6 individual cytokines within 36GSFT. The cultures were incubated for 14 days, scored using inverted phase contrast microscopy, 5 µl of media carefully removed without cells then replenished with 5 µl of SDM supplemented with 36GSFT. Cultures were incubated for a

further 7 days and then examined to determine the proportion of cells that had survived as indicated by cell division in the combination of 36GSFT.

In addition to single cell cultures, 1 ml, Pre-CFU cultures were initiated with various numbers of $CD34^+CD38^-$ cells to investigate the potential for *ex vivo* generation of nascent HPC by combinations of HGF, specifically, 36GSF, 36GST and 36GSFT. These experiments involved secondary and tertiary cultures of $CD34^+CD38^-$ cells isolated by FACS, from the primary cultures.

Collectively, these studies demonstrated that TPO was important for survival, recruitment and proliferation of $CD34^+CD38^-$ cells. Accordingly, a final aspect of the studies in this chapter was to investigate expression of *c-mpl*, the TPO receptor on HPC. A three colour immunostaining protocol with an affinity purified polyclonal rabbit anti-*mpl* anti-sera (Tahara *et al* 1996) was used to examine *c-mpl* expression on $CD34^+$ cells as a function of CD38 expression. Subsequently, $CD34^+mpl^+$ and $CD34^+mpl^-$ cells were isolated by FACS and cultured in SDM supplemented with 36GSFT. Unless otherwise stated, all studies described in this chapter were performed with bone marrow obtained from normal adult volunteer donors.

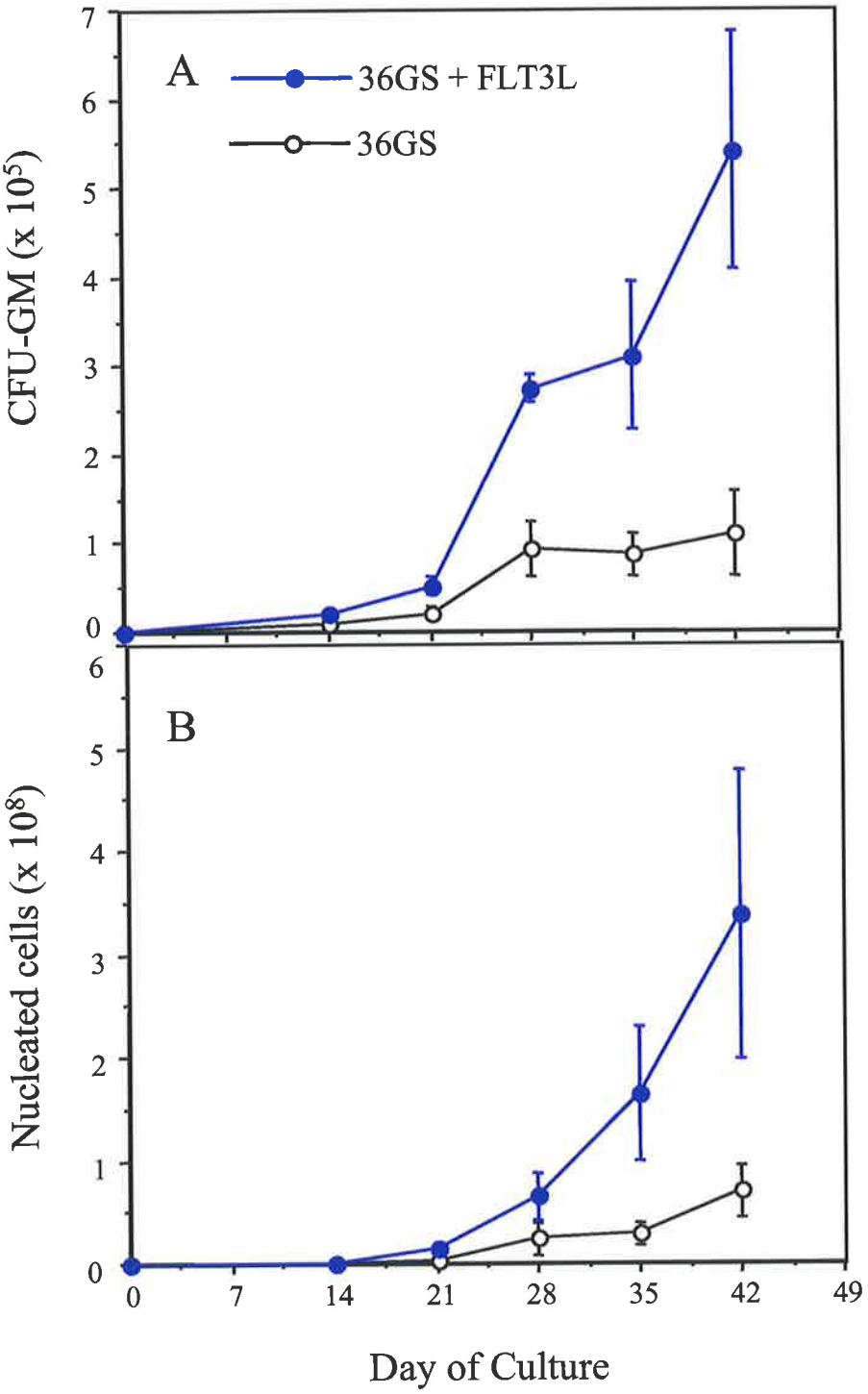
6.3 Results

6.3.1 FLT3 Ligand Increases Production of Nucleated Cells and Nascent CFU-GM from $CD34^+CD38^-$ Cells

The data presented within chapter 4 show that the combination of IL-3, IL-6, G-CSF and SCF, when used at 10 ng/ml, 10 ng/ml, 100 ng/ml and 100 ng/ml respectively (36GS) is superior to 136GGMS for generation of nascent CFU-GM from $CD34^+$ cells (Makino *et al* 1997). Therefore, to determine if FLT3L might effect proliferation of primitive HPC, Pre-CFU cultures were established with BM $CD34^+CD38^-$ cells in either 36GS or 36GS+FLT3L (36GSF). Production of nucleated myeloid cells and CFU-GM were monitored weekly for 6 weeks. The results shown in figure 6.1 represent the data from 5 separate experiments performed with BM cells isolated from different normal adult donors. The generation of CFU-GM at weekly time points is shown in panel A of figure 6.1. Culture of $CD34^+CD38^-$ cells with 36GSF resulted in significantly more CFU-GM generated at days 14, 21, 28, 35 and 42 ($p= 0.0026$, 0 .0094, 0.005, 0.06 and 0.07 respectively) than with cultures stimulated with 36GS. Of note was the absolute number of CFU-GM present at day 42. An average of

Figure 6.1 Effect of FLT3L on growth of $CD34^+CD38^-$ cells in Pre-CFU culture

Generation of CFU-GM (panel A) and nucleated cells (panel B) from 1,000 $CD34^+CD38^-$ bone marrow cells in Pre-CFU culture stimulated with either IL-3, IL-6, G-CSF and SCF: 36GS) or 36GS + FLT3L. At each time point, the mean and standard error from 5 separate experiments with different sources of bone marrow are presented. $CD34^+CD38^-$ cells were cultured in wells of a 24 well plate. At day 14 and at weekly intervals thereafter the cultures were subjected to 1 in 5 splits and refed with fresh media and HGF.


A
36GS + FLT3L
36GS
CFU-GM (x 10^5)
Nucleated cells (x 10^8)
B
0
1
2
3
4
5
6
7
0
7
14
21
28
35
42
49
Day of Culture

541,200 (range 251,700 to 893,000) CFU-GM were generated at day 42 from 1,000 $CD34^+CD38^-$ cells when cultured with 36GSF. In comparison the same number of $CD34^+CD38^-$ cells generated an average of 109,200 CFU-GM (range 0 to 220,000) at day 42 after culture in 36GS.

The corresponding production of nucleated cells in the same cultures is shown in panel B of figure 6.1. At each time point there were significantly more nucleated cells present in 36GSF stimulated cultures (p values ranging from 0.06 to 0.0058, paired t test, 95% confidence limits) than in cultures stimulated by 36GS. At day 42 an average of 3.3×10^8 cells were generated from 1,000 cells when cultured in 36GSF compared to 6.9×10^7 with 36GS alone. Thus FLT3L was found to markedly potentiate the *de novo* generation of mature myeloid cells from $CD34^+CD38^-$ cells, consistent with its proposed action on a minor subpopulation of very primitive progenitors. Therefore, a series of experiments were performed to investigate how FLT3L might mediate this effect.

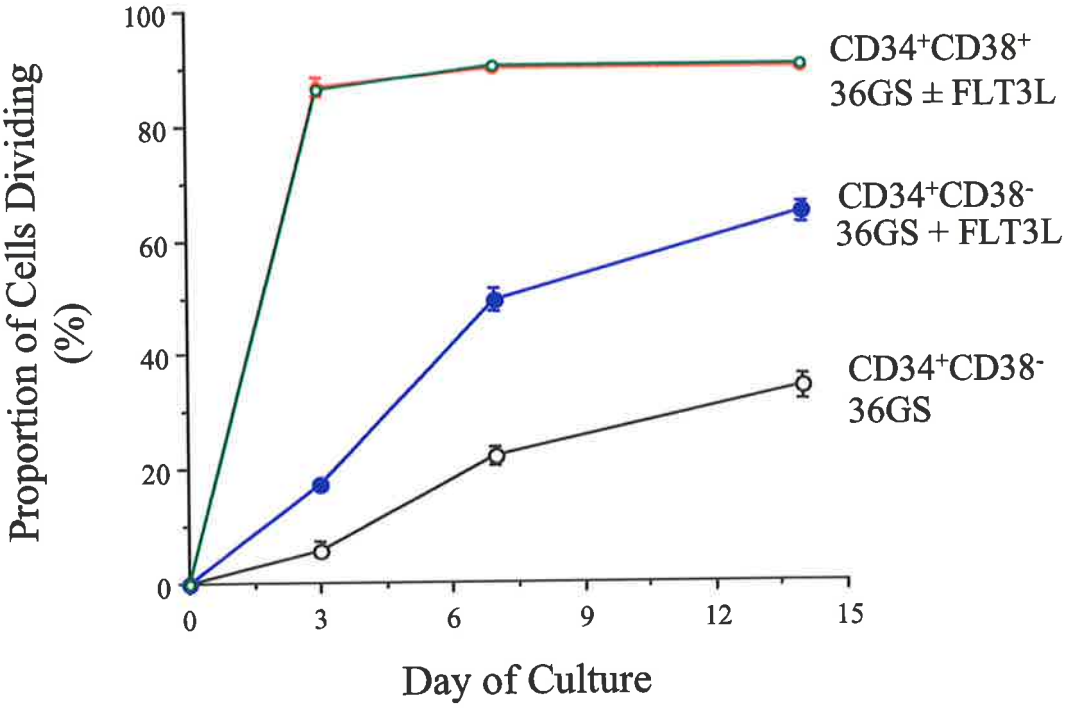
6.3.2 FLT3L Increases Recruitment of Single $CD34^+CD38^-$ Cells into Cell Cycle

To investigate the basis for the capacity of FLT3L to augment *ex vivo* expansion of myeloid progenitors from $CD34^+CD38^-$ cells single bone marrow $CD34^+CD38^+$ or $CD34^+CD38^-$ cells were sorted into Terasaki wells containing serum deprived media supplemented with 36GS with or without FLT3L. Wells were scored microscopically on days 3, 7 and 14 to determine the proportion of cells of each phenotype that divide in response to the different HGF combinations. The responses of single $CD34^+CD38^+$ and $CD34^+CD38^-$ were very different (figure 6.2). Single $CD34^+CD38^+$ cells responded rapidly to either 36GS or 36GSF as evidenced by the high proportion of cells dividing within 3 days (89.1% and 88.7% respectively). During the remainder of culture only a few more single $CD34^+CD38^+$ cells were recruited into cell cycle. At day 14, 90.3% and 89.7% of cells cultured in 36GS or 36GSF respectively had divided.

In contrast, a significantly greater proportion of $CD34^+CD38^-$ cells divided when cultured in 36GSF as compared to those cultured in 36GS (p= 0.015, 0.0003 and 0.001 for days 3, 7 and 14 respectively). The enhanced proliferative response in the presence of FLT3L was evident by day 3 and was maintained at all subsequent times examined. Thus at day 14, 62.5% of single $CD34^+CD38^-$ cells had divided when stimulated with 36GSF as compared to 34.7% with 36GS. At day 14, in the first 2 experiments those wells containing single $CD34^+CD38^-$

Figure 6.2 FLT3L enhances division of single $CD34^+CD38^-$ cells

Single $CD34^+CD38^+$ and $CD34^+CD38^-$ cells were deposited into wells of a Terasaki plate each containing10 μL of serum-free Pre-CFU media supplemented with either IL-3+IL-6+G-CSF+SCF (36GS) or 36GS+FLT3L. Wells containing a single cell were examined at 3, 7 and 14 days to determine the proportion of single cells undergoing division. The figure shows the proportion of cells dividing at each time point. The results are the means and standard error of three experiments performed with different sources of adult human bone marrow.


Proportion of Cells Dividing (%)
100
80
60
40
20
0
0
3
6
9
12
15
Day of Culture
CD34+CD38+ 36GS ± FLT3L
CD34+CD38- 36GS + FLT3L
CD34+CD38- 36GS

cells cultured in 36GSF appeared to contain many more progeny as compared to those stimulated with 36GS. Therefore, in a further experiment the number of cells generated at day 14 from single $CD34^+CD38^-$ or $CD34^+CD38^+$ cells cultured in either 36GS or 36GSF were counted. The results of this analysis are shown in figure 6.3. Proliferation of single $CD34^+CD38^+$ cells was not enhanced by the addition of FLT3L to the 36GS combination. However, the number of cells generated from single $CD34^+CD38^-$ cells was increased by the addition of FLT3L to the cultures. Culture with 4 HGF resulted in a median of 23.4 cells/well (range 2-120, n=48 wells). In contrast, stimulation with 36GSF resulted in a median of 875 cells/well (range 2-9,000, n=96 wells), representing 35-fold more cells/well. This increase in the average number of cells/well is primarily explained by a small proportion (11.9% of responding cells) of single $CD34^+CD38^-$ cells generating greater than 6,000 cells when stimulated with 36GSF. The difference in cell production between the 2 groups was highly significant (p= 0.0010, paired t test).

The results of these experiments indicate that increased recruitment of primitive HPC cells into cell cycle and thereafter increased proliferation of recruited cells underlies the *ex vivo* expansion potential of FLT3L. The data also demonstrates that all but 38% of adult BM $CD34^+CD38^-$ cells are recruited into division by the HGF combination of 36GS + FLT3L. Several issues arise from this observation and each concern the nature of the $CD34^+CD38^-$ cells that do not respond to this 5-HGF combination. Firstly, would addition of TPO to the combination of 36GS induce division of these cells? Secondly, would these 5-HGF non-responsive $CD34^+CD38^-$ cells have different haemopoietic potential than their 5-HGF responsive counterparts? Furthermore, if this fraction of $CD34^+CD38^-$ cells could be stimulated to divide then what is the implication for *ex vivo* manipulation of haemopoiesis and cellular therapy? The remaining studies presented in this thesis address these issues.

6.3.3 Thrombopoietin Augments Cytokine Induced Division and Proliferation of $CD34^+CD38^-$ Cells

A series of studies, analogous to those described previously in section 6.3.2, were performed to determine if TPO could also effect recruitment of single BM $CD34^+CD38^-$ cells. These studies were performed with cells deposited into Terasaki wells containing 10 µl of serum-deprived Pre-CFU media supplemented with combinations of HGF, including, 36GS, 36GSF, 36GS+TPO (36GST) and 36GS+FLT3L+TPO (36GSFT). In contrast to the studies investigating the effect of FLT3L on single $CD34^+CD38^-$ cells, the growth of single $CD34^+CD38^-$ cells was assessed daily for the first 7 days then again at day 14.

Figure 6.3 FLT3L enhances proliferation of $CD34^+CD38^-$ cells

Proliferative response of single $CD34^+CD38^+$ (panel A) or $CD34^+CD38^-$ cells (panel B) cultured in serum-free Pre-CFU media stimulated with either 36GS or 36GS+FLT3L (shown as the solid columns). The number of viable cells present after 14 days of culture was determined for each single cell that divided. The results are from one of the experiments performed to generate data shown in figure 6.2.

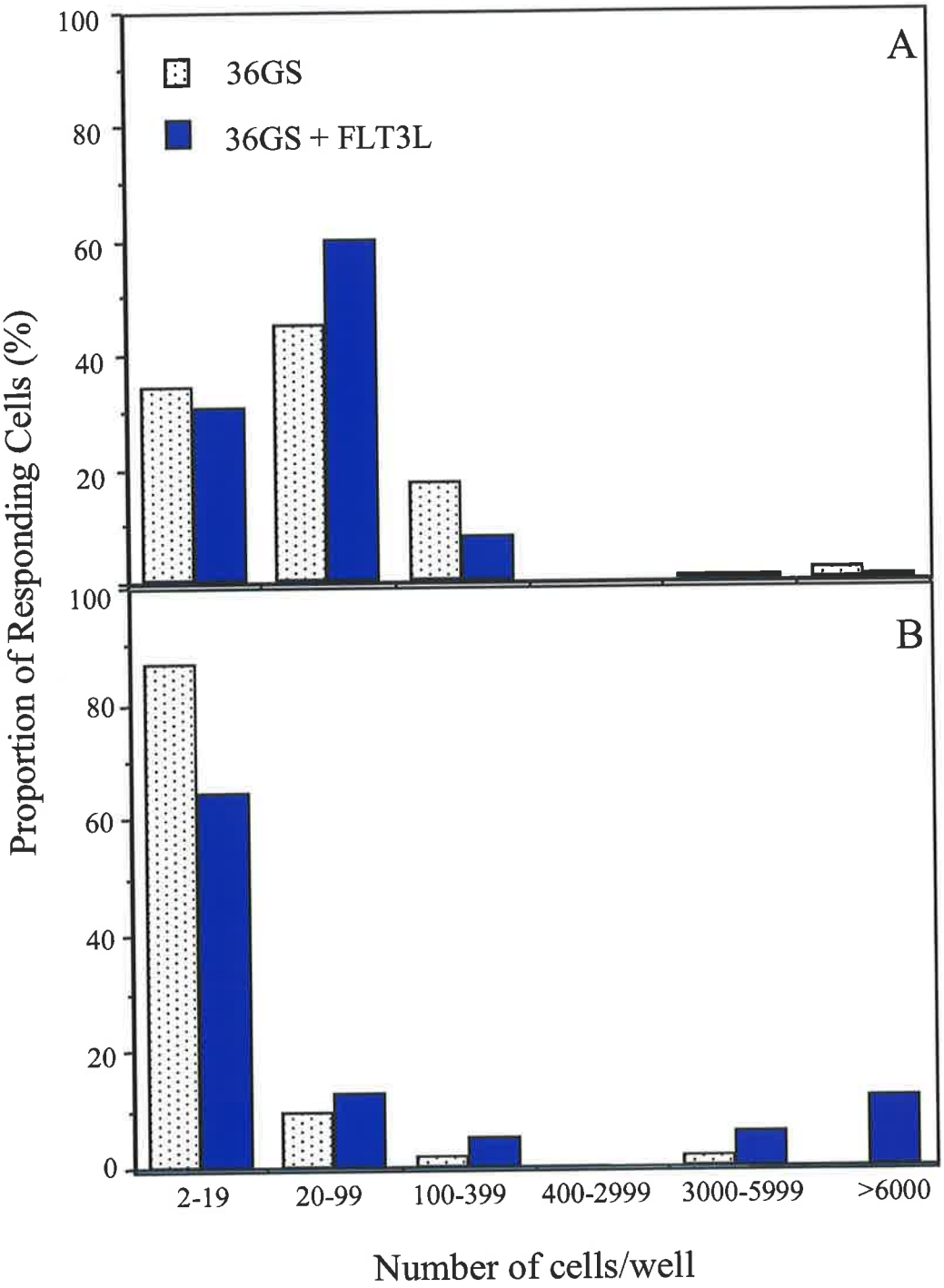

A
B
36GS
36GS + FLT3L
Proportion of Responding Cells (%)
100
80
60
40
20
0
2-19
20-99
100-399
400-2999
3000-5999
>6000
Number of cells/well

Figure 6.4 Morphology of $CD34^+CD38^-$ cells and their progeny cultured in Terasaki wells

This composite figure shows the appearance of a single $CD34^+CD38^-$ cell (plate A) and representative progeny of different single $CD34^+CD38^-$ cells after various periods of culture. Single BM $CD34^+CD38^-$ cells were deposited into Terasaki wells containing SDM supplemented with 36GSFT to stimulate cell growth. Photographs were captured with an Olympus SLR camera and an Olympus PM10 automatic exposure system mounted on an Olympus inverted microscope. In each plate the cells were examined under phase contrast at an effective magnification of 200X. Plate A shows the typical appearance of $CD34^+CD38^-$ cells soon after deposition within the Terasaki well. As shown, these cells are relatively small and phase bright. Plate B shows a pair of daughter cells derived from a single $CD34^+CD38^-$ cell after 3 days culture. In contrast to the uniform and spherical appearance of daughter cells in plate B the daughter cells shown in Plate C exhibit extended pseudopodia and irregular cell shape, suggesting these cells may be motile. Accordingly, plate D shows 2 sets of doublets where the first generation daughter cells moved apart before completing a second division. Plate E shows a collection of 8 cells, representing 3 symmetrical divisions from the original $CD34^+CD38^-$ cell. Plate F shows the typical appearance of a colony of greater than 120 cells generated after 7 days culture. Plate G also shows the progeny of a single cell after 7 days but the colony contains less than 24 cells that are large, irregularly shaped and translucent. Plate H illustrates the heterogeneous nature of cells present within a clone after 14 days of culture. Cells often exhibited large variations in size and cytoplasmic granulation and were adherent as characterised by a phase dull appearance and extended cytoplasmic projections.

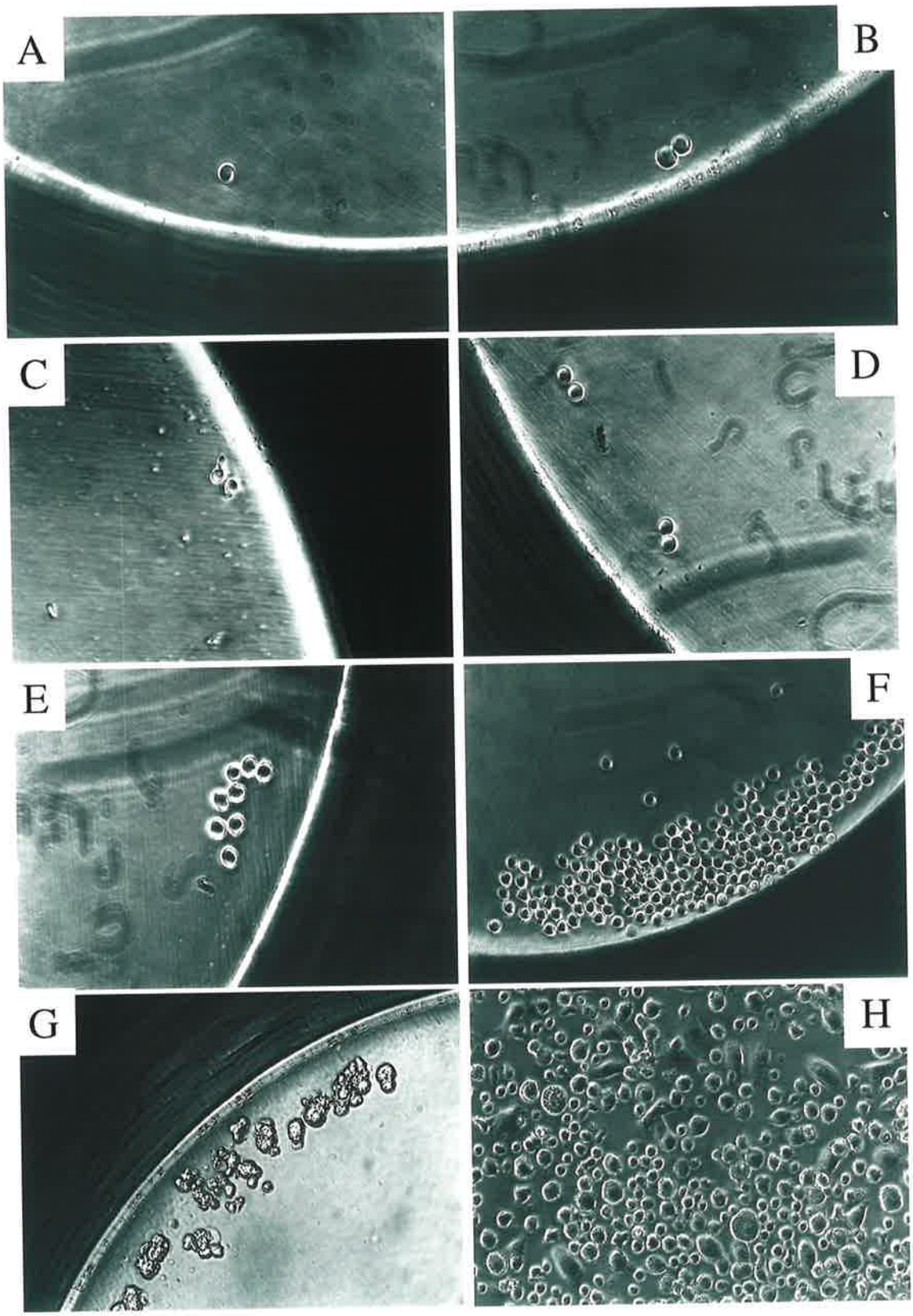

A
B
C
D
E
F
G
H

Figure 6.4 shows representative examples of single $CD34^+CD38^-$ cells and their progeny at various days in culture. As can be seen it was relatively straightforward to accurately enumerate cell numbers by microscopy, at least for the first 7 days of culture. At later time points, where wells frequently contained many cells, the contents were removed, diluted, and cells counted on a haemocytometer using phase contrast microscopy. The pooled results from 11 experiments are shown in Figure 6.5. Two phases of cell growth were observed for $CD34^+CD38^-$ cells cultured with 36GS. There was an initial lag period of 2 days where only a small proportion (2.4 ± 0.7%, mean ± SEM) of cells divided. The second phase occurred from day 3 to day 7 and involved a gradual recruitment of $CD34^+CD38^-$ cells so that by day 14, 33.8% of cells had divided. In contrast, when FLT3L or TPO or both FLT3L and TPO were added to 36GS a different pattern of growth was observed. The 2 day lag period characterised by minimal cell division was preserved but between day 3 and day 7 there was rapid recruitment of $CD34^+CD38^-$ cells. The rate of recruitment was greatest with the 6 HGF combination of 36GSFT and at each time point significantly more cells had divided than with any other HGF combination. For example, after 5 days, 81 ± 2.2% (mean ± SEM) of cells had divided in 36GSFT as compared to 57 ± 2.0% in 36GST, 43 ± 1.1% in 36GSF and 19.7 ± 4.8% in 36GS (p values from 0.015 to 0.0062). The addition of FLT3L or TPO to 36GS resulted in incremental increases in the rate of recruitment with greater rates of recruitment observed with 36GST. This was not significant at all time points but the general trend was observed in all experiments. Irrespective of the HGF combination, the final 7 days of culture could be considered as a plateau phase where very few additional cells divided. When $CD34^+CD38^-$ cells were cultured with 36GSFT, 91.5 ± 1.76% (mean ± SEM, n=11 experiments) of cells divided within 14 days compared to 63.3 ± 9.8% with 36GST, 61.5 ± 2.7% with 36GSF and 33.8 ± 5.1% with 36GS. Of the $CD34^+CD38^-$ cells that did not divide when cultured in 36GSFT, half (approximately 2-3% of the initial cells) were phase dull and appeared to be dead. The remaining 2-3% of $CD34^+CD38^-$ cells were small and phase bright and considered to be viable but unresponsive to the 36GSFT combination.

6.3.4 $CD34^+CD38^-$ Cells Undergo Extensive Proliferation in 36GSFT

During the assessment of single $CD34^+CD38^-$ cells it was frequently observed that when cultured in 36GSFT a single cell would generate enough progeny to completely cover the surface of a Terasaki well (panel B and D, figure 6.6). To document this response the number of cells generated after 7 and 14 days in the various HGF combinations were counted. These data are presented in Figure 6.7. After 7 days each dividing cell generated 53

Figure 6.5 Division of single $CD34^+CD38^-$ cells cultured in Terasaki wells

This figure shows the effect of FLT3L and Thrombopoietin (T) on recruitment of single $CD34^+CD38^-$ BM cells. Single BM $CD34^+CD38^-$ cells were deposited by the ACDU of the FACStar^{PLUS} into Terasaki wells containing 10 µl of SDM supplemented with different combinations of HGF. The following HGF were added: IL-3 (3; 10 ng/ml), IL-6 (6; 10 ng/ml), G-CSF, SCF, FLT3L, MGDF (G; S; F; T, respectively: each at 100 ng/ml) in combinations as shown. Wells were examined within 12 hours of sorting to confirm deposition of single viable cells, then daily for the first 7 days, and again at day 14. On each examination, the number of single cells that had divided was recorded. The presented data (mean ± SEM) is from 11 separate experiments with different adult BM samples with at least 100 single $CD34^+CD38^-$ cells for each experiment.

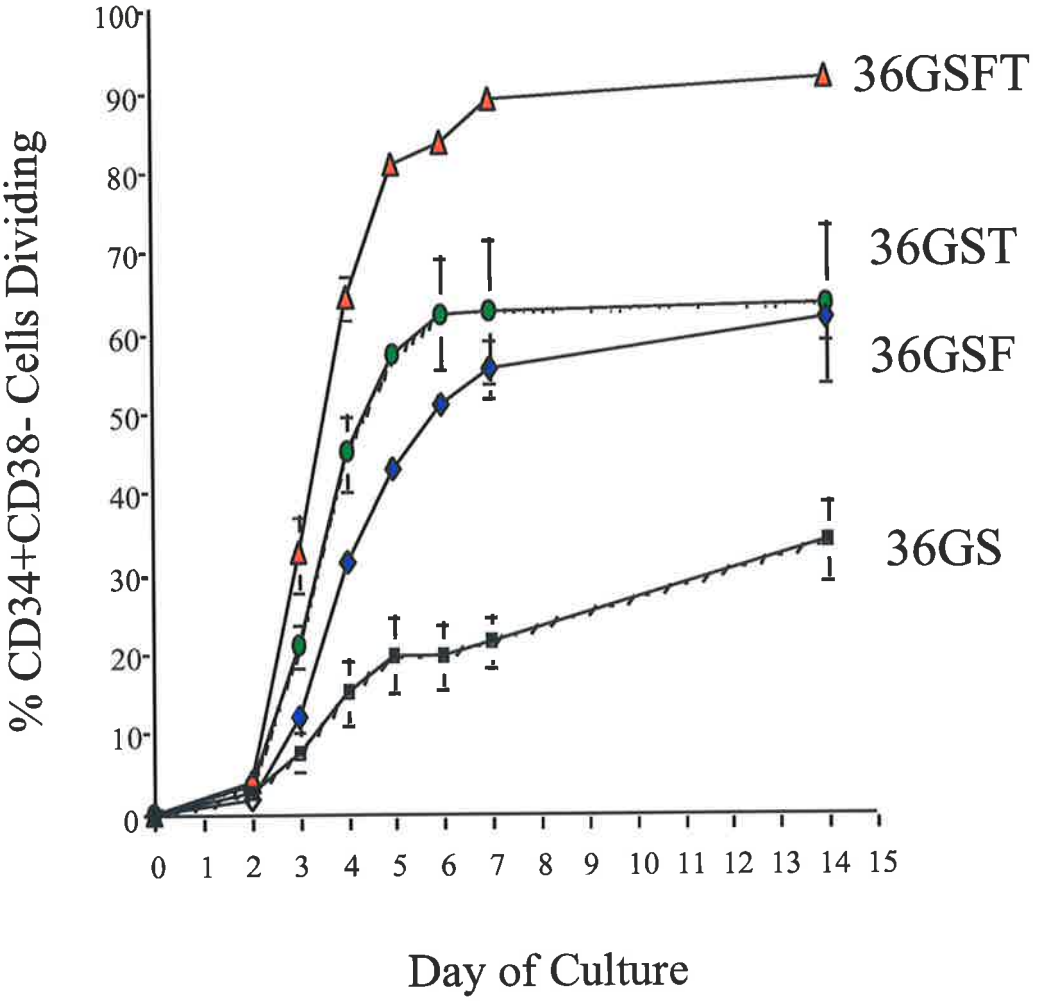

% CD34+CD38- Cells Dividing
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day of Culture
36GSFT
36GST
36GSF
36GS

Figure 6.6 Day 14 cultures of $CD34^+CD38^-$ cells in Terasaki wells

This composite figure depicts wells of a Terasaki plate containing the progeny of single $CD34^+CD38^-$ cells after 14 days culture in SDM with growth stimulated by the 36GSFT growth factor combination. Photographs were captured with an Olympus SLR camera and an Olympus PM10 automatic exposure system mounted on an Olympus inverted microscope. In each plate the cells were examined under phase contrast at an effective magnification of 40X. In plate A, half the surface area of the Terasaki well contains cells that have a uniform size and shape. Plates B and D are representative of clones that covered the entire surface of a Terasaki well. In these cases, 12,300 and 21,600 cells were present in wells shown in plates B and D respectively. A high proportion of clones also showed formation of discrete secondary clones by 14 days of culture. This feature is shown in plates C and D.

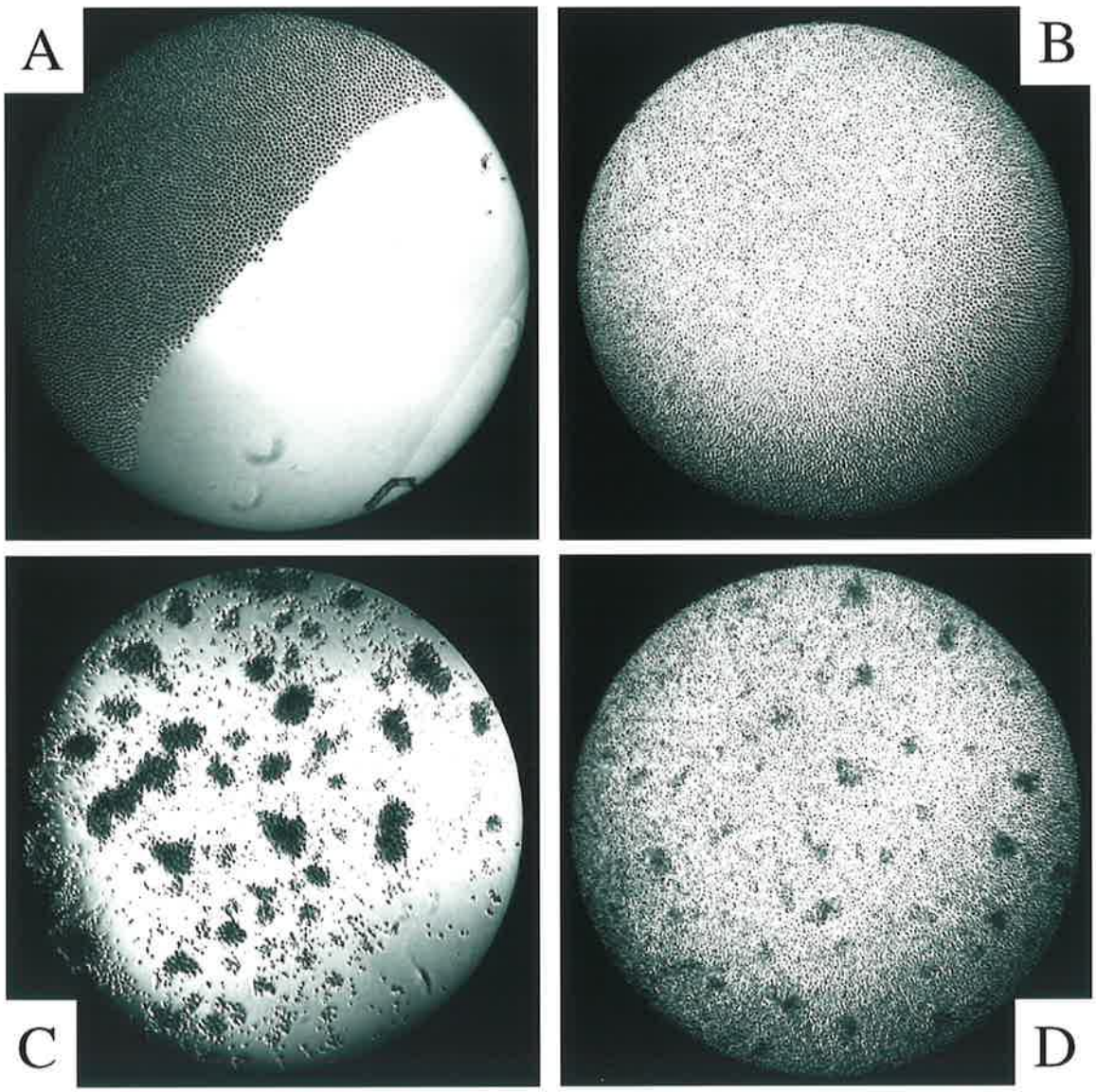
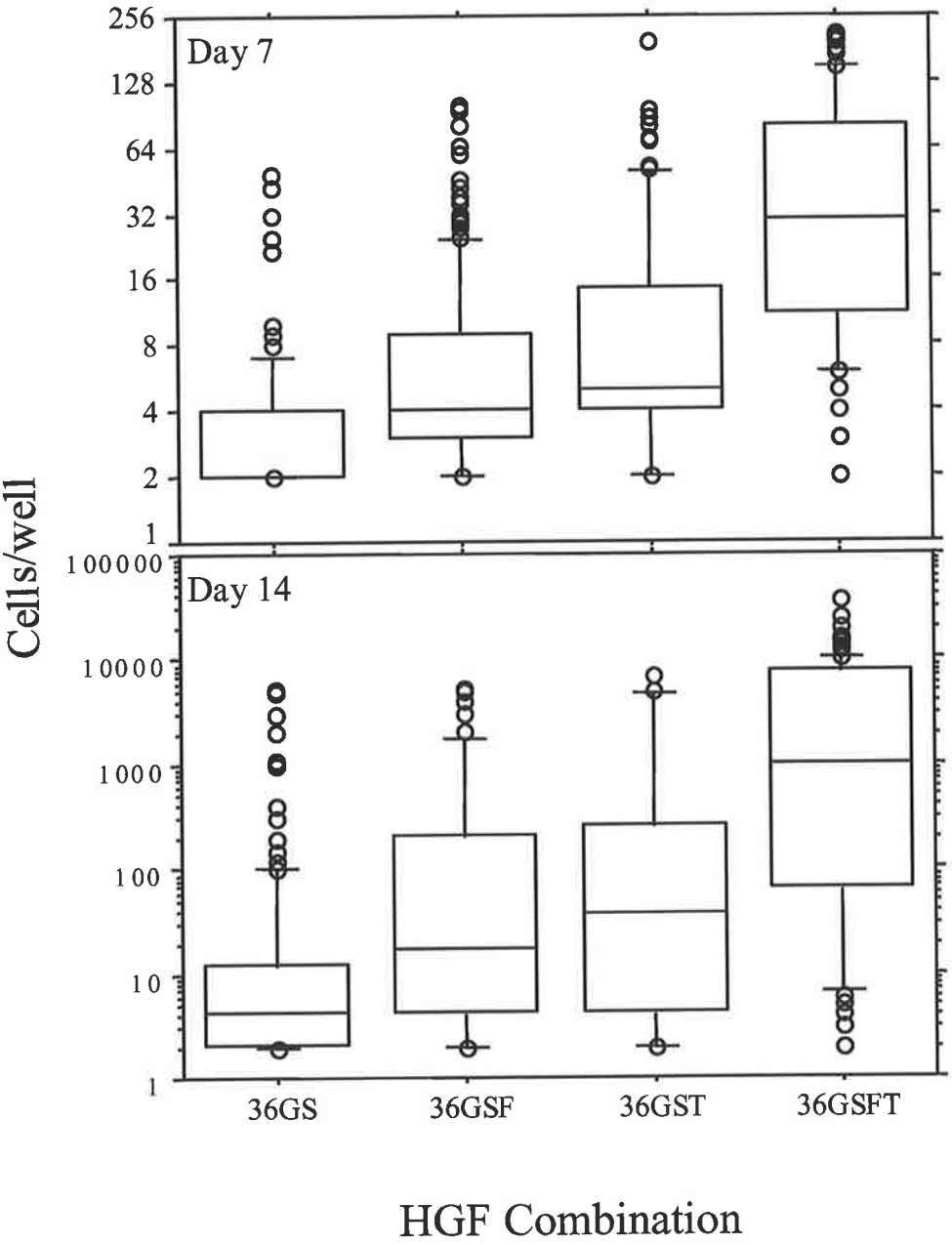

A
B
C
D

Figure 6.7 Proliferation of single BM $CD34^+CD38^-$ cells in different combinations of HGF

Cultures of $CD34^+CD38^-$ cells were initiated in Terasaki wells as described in Chapter 2. The number of cells generated from a single cell was determined after 7 and 14 days of culture. The results from 6 independent experiments have been pooled and box plots represent the response of 115, 188, 93 and 406 single $CD34^+CD38^-$ cells cultured in 36GS, 36GSF, 36GST and 36GSFT, respectively. Each box displays the distribution in the number of cells generated at these time points: the five horizontal lines on each of the box plots represent the 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles of the number of cells for each HGF combination. A significantly greater number of cells ($p = 0.03 - < 0.0001$) were generated in cultures stimulated by 36GSFT as compared to cultures stimulated by any other HGF combination.

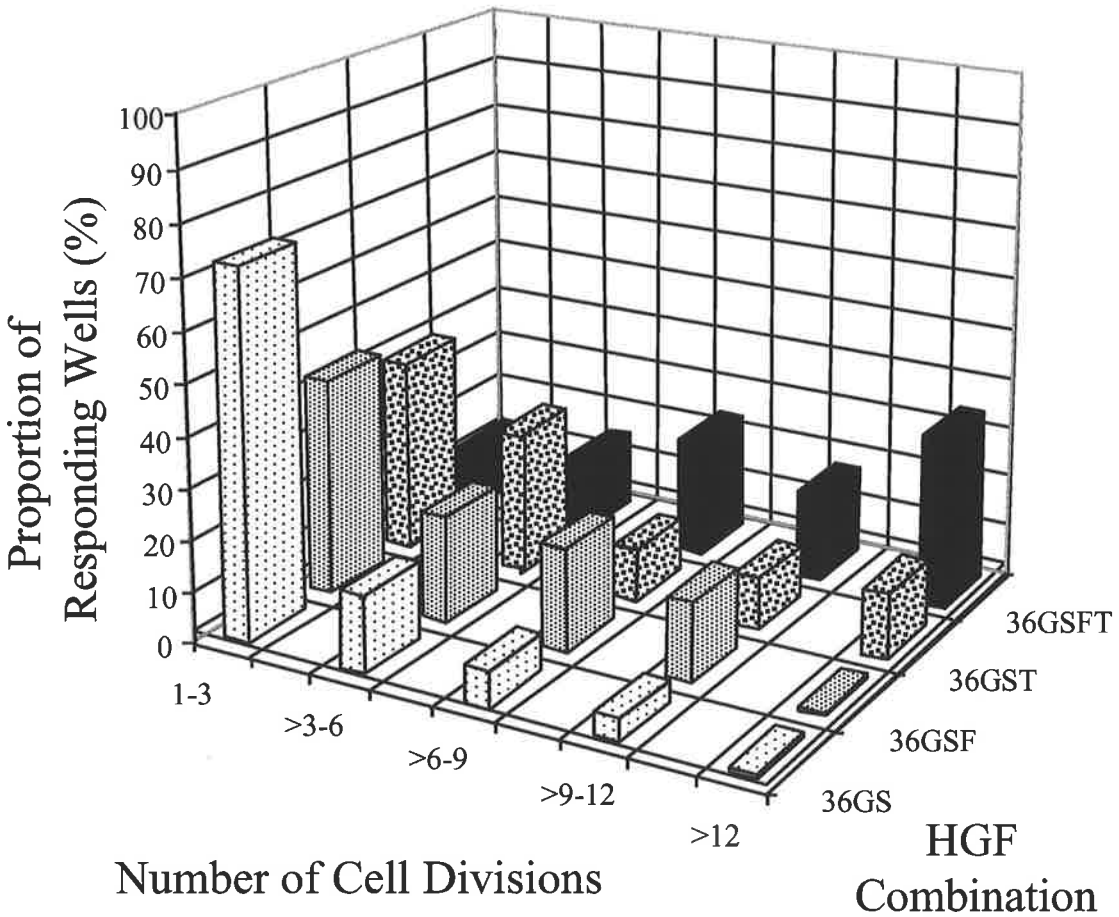

Day 7
256
128
64
32
16
8
4
2
1
Day 14
100000
10000
1000
100
10
1
Cells/well
36GS
36GSF
36GST
36GSFT
HGF Combination

± 2.8 cells (mean ± SEM, progeny of 406 single cells analysed) when cultured in 36GSFT, which was significantly greater (p= 0.0003 - < 0.0001) than the number of cells generated in either 36GS (4.7 ± .75 cells/cell, n=115), 36GSF (mean of 9.8 ± 1.1 cells/cell, n=188) and 36GST (mean of 16.4 ± 2.9 cells/cell, n=93). A similar trend was observed at day 14 where again significantly more cells were generated by 36GSFT (p= 0.03 - < 0.0001) than with any other HGF combination. An average of 3,670 ± 370 cells were generated from single $CD34^+CD38^-$ cells when cultured in 36GSFT compared to 931 ± 317 with 36GST, 382 ± 52.8 with 36GSF and 138 ± 45 cells from 36GS cultures. The number of symmetrical divisions of a parent cell can also represent the number of cells it generates. For example, 4 symmetrical divisions of a single $CD34^+CD38^-$ cell is equivalent to 16 cells, 8 divisions represents 256 cells and 12 divisions 4096 cells. This method of analysis was used to generate figure 6.8 and illustrates that when grown in 36GS, 72% of responding $CD34^+CD38^-$ cells undergo only 1-3 divisions. The addition of FLT3L or TPO or both of these HGF to 36GS resulted in greater proliferation as demonstrated by the lower proportion of cells undergoing 1-3 divisions. This result is in accord with the data shown previously in figure 6.3 where the addition of FLT3L to 36GS resulted in a greater proportion of cells undergoing 3-12 divisions. The addition of TPO to 36GS also resulted in a greater proportion of cells undergoing 3-12 divisions and notably 12.8% generated more than 4,096 cells (> 12 divisions). Of particular note was the potent proliferative stimulus provided by the combination of 36GSFT. More than half of the 91% of recruited $CD34^+CD38^-$ cells produced more than 512 cells (ie 9 divisions) in 14 days culture and remarkably, 33.7% of cells produced more than 4,096 cells and 2.6% produced 15,000 cells or more.

As can be seen in figures 6.4 and 6.9 there was considerable heterogeneity in the morphology of cells within clones. The progeny of an individual $CD34^+CD38^-$ cell could consist of small agranular cells, adherent macrophages, large refractile cells resembling megakaryocytes or large cells with long thin cytoplasmic projections resembling those of dendritic cells. In addition, a proportion of the clones contained agranular cells with each cell displaying a comma-like appearance suggesting that they were actively motile (refer to panel of figure 6.9). Subsequent studies with time-lapse photography taken over 7 days have confirmed that many cells grown under these conditions, including those with the comma-like morphology, are highly mobile and engage in multiple contact with neighbouring cells (data not shown). Commonly at day 14 there was evidence of cell maturation within clones as indicated by the wide variation in cell size and cytoplasmic granulation. A proportion of clones also appeared to undergo apoptosis (as evidenced by the appearance of nuclear and

Figure 6.8 Proliferation of $CD34^+CD38^-$ cells after 14 days, represented as symmetric divisions

Single $CD34^+CD38^-$ cells were cultured in SDM and stimulated by HGF combinations of 36GS, 36GSF, 36GST, and 36GSFT. The data shown is derived from the same experiments used for generation of figure 6.7. However, in this figure, cell proliferation is presented as the number of symmetric divisions giving rise to the total number of cells present at day 14 of culture. For example, if a single cell gave rise to 512 – 1023 cells then it was recorded as undergoing 9 divisions. The proportion (%) of single cells undergoing the listed number of symmetric divisions is shown for each HGF combination.


Proportion of Responding Wells (%)
100
90
80
70
60
50
40
30
20
10
0
1-3
>3-6
>6-9
>9-12
>12
Number of Cell Divisions
36GSFT
36GST
36GSF
36GS
HGF Combination

cytoplasmic blebing and apoptotic bodies) during the 14 days of culture. In these situations the onset of cell death was highly variable. Some clones began dying after only a few divisions when the total number of cells was 16-32, whereas in others this occurred much later when several thousand cells were present.

In spite of the extensive proliferation shown by a high proportion of $CD34^+CD38^-$ cells cultured in 36GSFT, a considerable number of clones also exhibited either slower sustained growth or only produced 16-32 cells over 14 days culture. In the case of the former, the progeny typically consisted of agranular blast-like cells whereas the progeny of the latter either died or differentiated into large, granular cells or adherent macrophages. After the first or second division of a $CD34^+CD38^-$ cell the progeny either remained in close contact with each other, producing a cobblestone appearance (panels A and B of figure 6.9) or moved considerable distances apart. It addition, some clones contained large aggregates of 50-100 cells. Figure 6.9 shows a representative view of the range of cell morphologies observed in clones derived from single $CD34^+CD38^-$ cells cultured in 36GSFT.

6.3.5 Increased Generation of Nascent $CD34^+$ Cells from $CD34^+CD38^-$ Cells Stimulated by 36GSFT

The results of experiments thus far indicate that the majority of $CD34^+CD38^-$ cells divide when cultured in the combination of 36GSFT and furthermore a high proportion of recruited cells exhibit extensive proliferative potential. These observations suggested that culture of $CD34^+CD38^-$ cells in 36GSFT may result in generation of nascent $CD34^+$ and $CD34^+CD38^-$ cells. This possibility was examined by 2 complementary approaches. Firstly, immunophenotyping was performed on the contents of Terasaki wells to determine if single $CD34^+CD38^-$ cells cultured in 36GSFT for 14 days gave rise to an increased number $CD34^+$ cells. To ensure that sufficient cells were available for flow cytometry only those wells containing at least 2,000 cells were harvested and analysed. The pooled results from three independent experiments are shown in figure 6.10. Seventy-six clones from 232 wells (32.7% of the dividing cells) containing single $CD34^+CD38^-$ cells that had divided were harvested (18, 28 and 30 clones from respective experiments). As shown in Panel A of figure 6.10 the number of cells present in wells was highly variable and ranged from 2,200 to 50,000 (14,065 ± 1,098; mean ± SEM). The proportion of $CD34^+$ cells within any one clone was also highly variable and ranged from 0 – 67.3% (3.14 ± 0.94%) although most clones had between 0.5 and 5% $CD34^+$ cells (panel B figure 6.10). Furthermore, the total number of $CD34^+$ cells generated from a single $CD34^+CD38^-$ cell was variable, ranging from 0 to 1,615

Figure 6.9 Appearance of progeny of $CD34^+CD38^-$ cells

The morphology of progeny of single $CD34^+CD38^-$ cells cultured in SDM supplemented with 36GSFT. Plates A and B (200X and 400X, respectively) show progeny from single $CD34^+CD38^-$ cells growing as a monolayer sheet of small, round, agranular cells that remain in close contact and collectively have a cobblestone appearance. In contrast, the cells in plates C and D (200X and 400X respectively) are generally not in contact, have variable shapes and often exhibit cytoplasmic projections and have a "comma"-like appearance. The arrow placed on plate E points to a single large, phase dull, adherent macrophage present among numerous other adherent and non-adherent differentiated myeloid cells. Plate F shows adherent cells that exhibit irregular shaped cytoplasmic projections resembling those observed on dendritic cells.

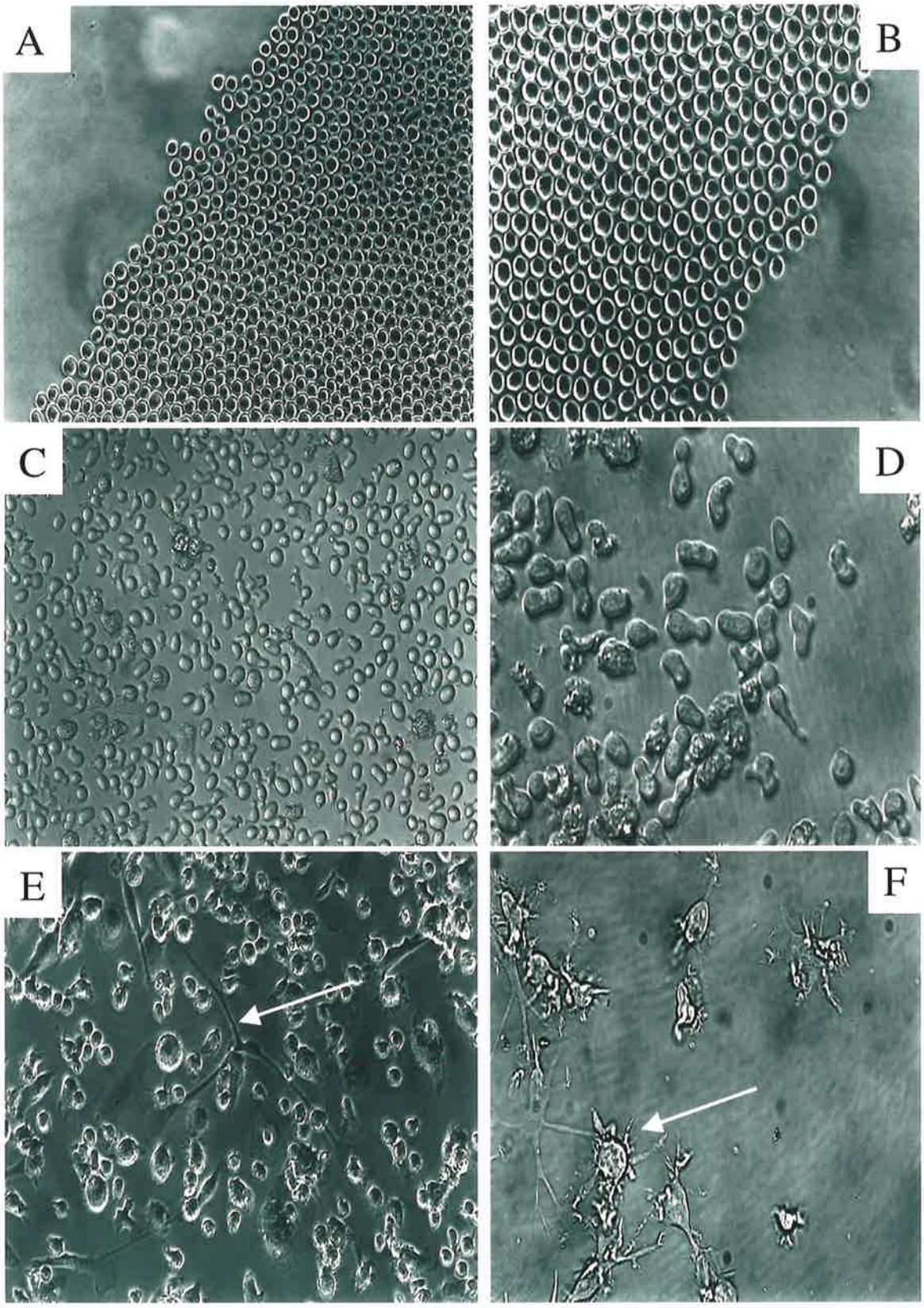
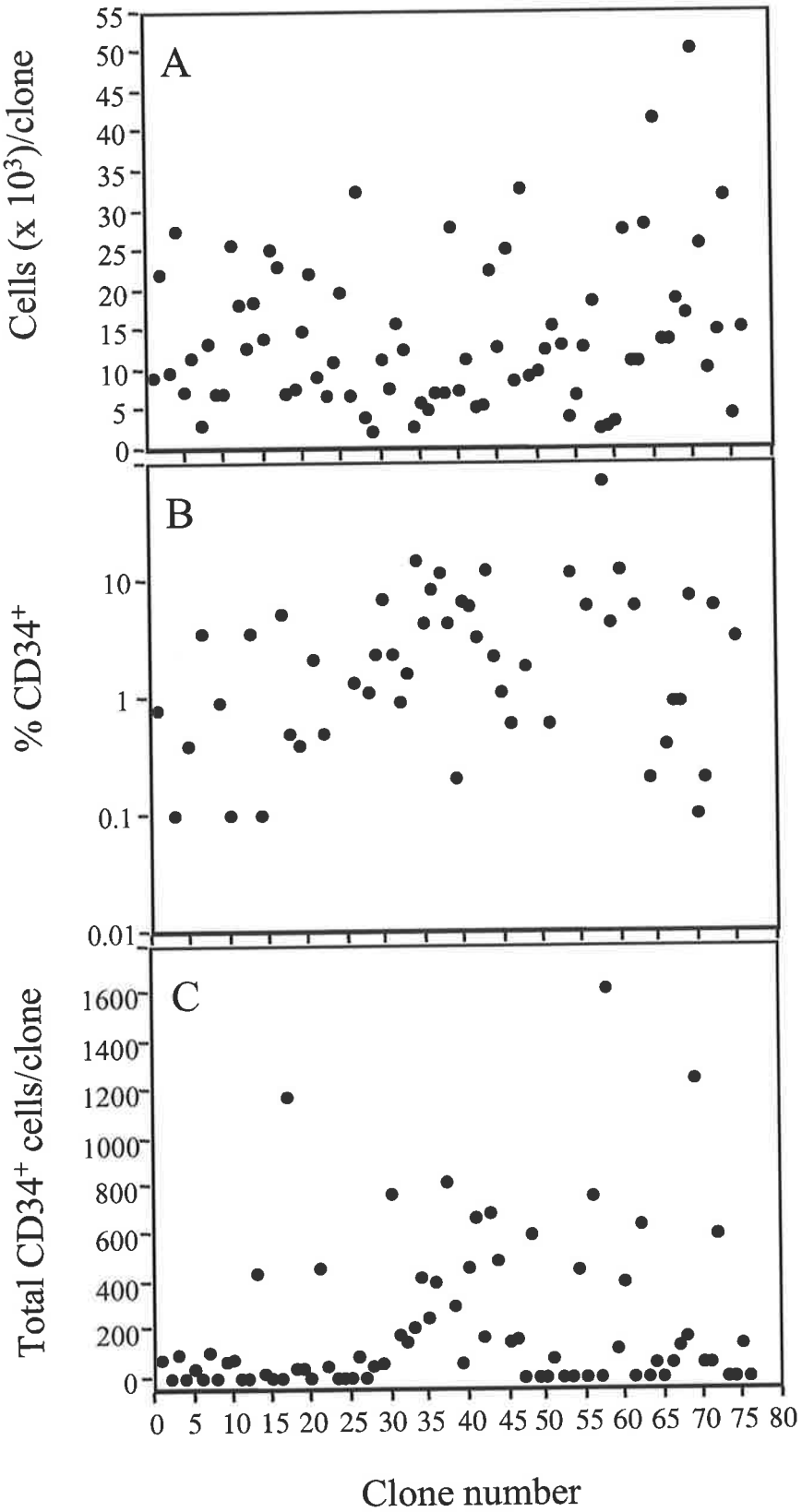

A
B
C
D
E
F

Figure 6.10 Analysis of clones derived from single $CD34^+CD38^-$ cells

This figure depicts the analysis of clones generated from single $CD34^+CD38^-$ cells after 14 days culture in SDM supplemented with 36GSFT. The pooled results from 3 independent experiments are shown. To facilitate cell counting and flow cytometric analysis only those clones estimated to contain at least 2000 cells were analysed. Seventy-six clones from 232 single $CD34^+CD38^-$ cells fulfilled this criterion. The number of nucleated cells, percentage of $CD34^+$ cells and the total number of $CD34^+$ cells present within these 76 clones is shown in panels A, B and C, respectively.


A
B
C
Cells (x 10³)/clone
% CD34⁺
Total CD34⁺ cells/clone
Clone number
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
0 5 10 15 20 25 30 35 40 45 50 55
0.01 0.1 1 10
0 200 400 600 800 1000 1200 1400 1600

with a mean of 212 (SEM = 37.2; panel C figure 6.10). A total of 26 of the 76 clones (34%) did not contain $CD34^+$ cells whereas 15 of the 76 clones (19%) contained 5% or more $CD34^+$ cells at day 14. The value of 212 mentioned above represents the average fold-increase of $CD34^+$ cells over the 76 clones analysed. Considering that only 32.7% of all wells with responding cells were used for this analysis, and assuming that the remaining 67.3% of responding wells had less than 2,000 progeny and that these contained low levels of $CD34^+$ cells (this may not be a valid assumption) then the expected overall fold-increase of $CD34^+$ cells at day 14 from $CD34^+CD38^-$ cells cultured in 36GSFT would be approximately 71 (212÷3). This prediction was subsequently confirmed in larger scale experiments (see below). Regression analysis of the single cell culture data revealed no statistical correlation (r^2 = 0.089) between the total cells generated and the percentage of $CD34^+$ or total $CD34^+$ cells present. However there was a slight inverse relationship between the number of cells in clones and the proportion of $CD34^+$ cells present. Notably, for every clone containing less than 6,000 cells, at least 1% of the cells was $CD34^+$. Conversely, $CD34^+$ cells could not be detected in 39% of clones containing greater than 6,000 cells.

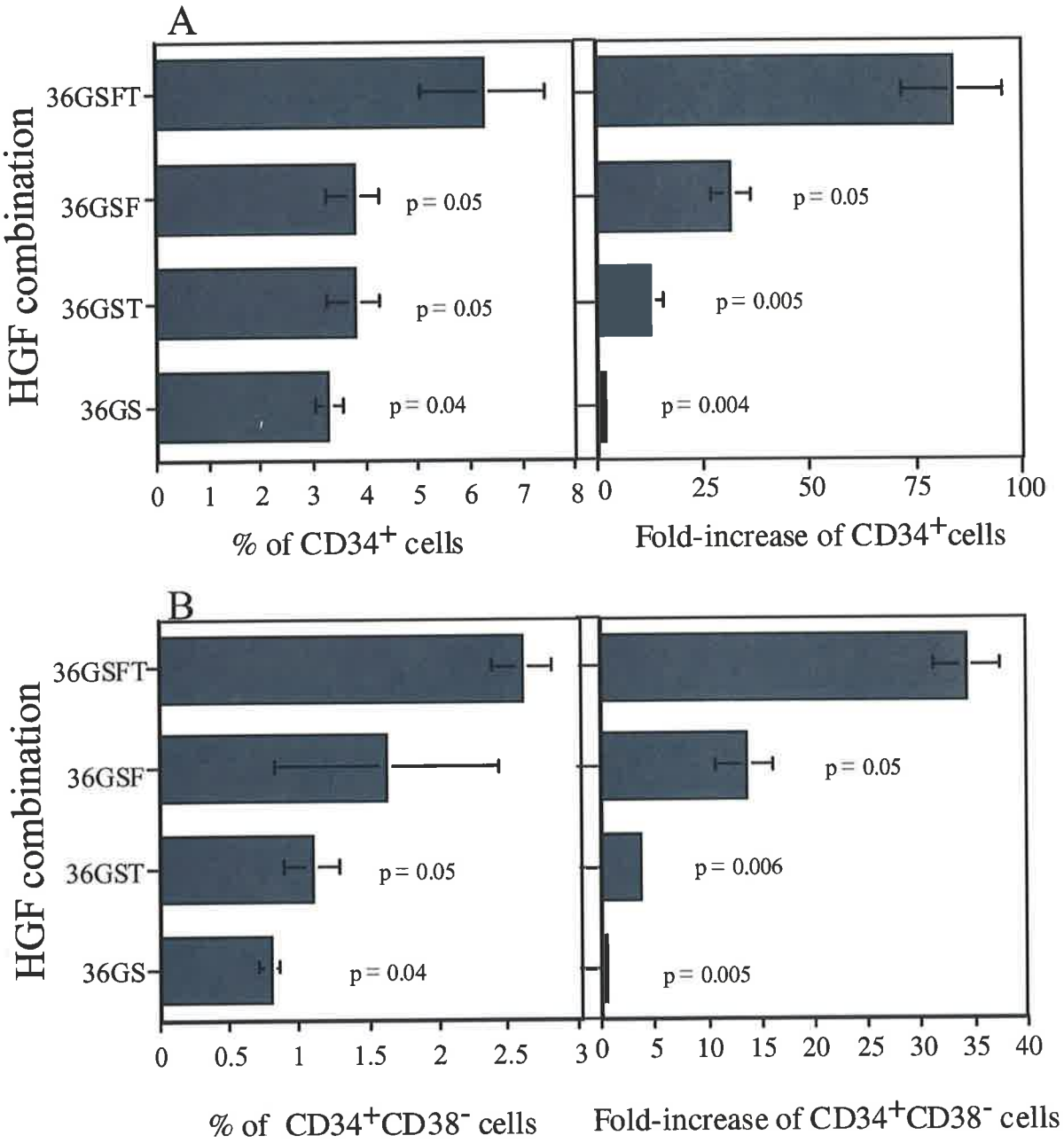
Unfortunately, owing to the limited number of cells produced by individual clones it was difficult to assess by flow cytometry the incidence of $CD34^+CD38^-$ cells within the nascent $CD34^+$ population. This limitation was overcome by initiating cultures with 1,500 $CD34^+CD38^-$ cells in 1ml of SDM and monitoring growth for 3 weeks. These experiments were also used to compare the generation of nascent $CD34^+$ cells from $CD34^+CD38^-$ cells when cultured in 36GS, 36GSF, 36GST or 36GSFT. Fresh HGF was added on day 7 and again on day 14 when cultures were also split by dilution with SDM. Quantitation of $CD34^+$ cells and $CD34^+CD38^-$ cells was performed on days 14 and 21 using 2-colour immunolabelling with HPCA-2-PE and LeuM1-FITC for CD34 and CD15 or 581-PECy5 and Leu17-PE for CD34 and CD38, respectively.

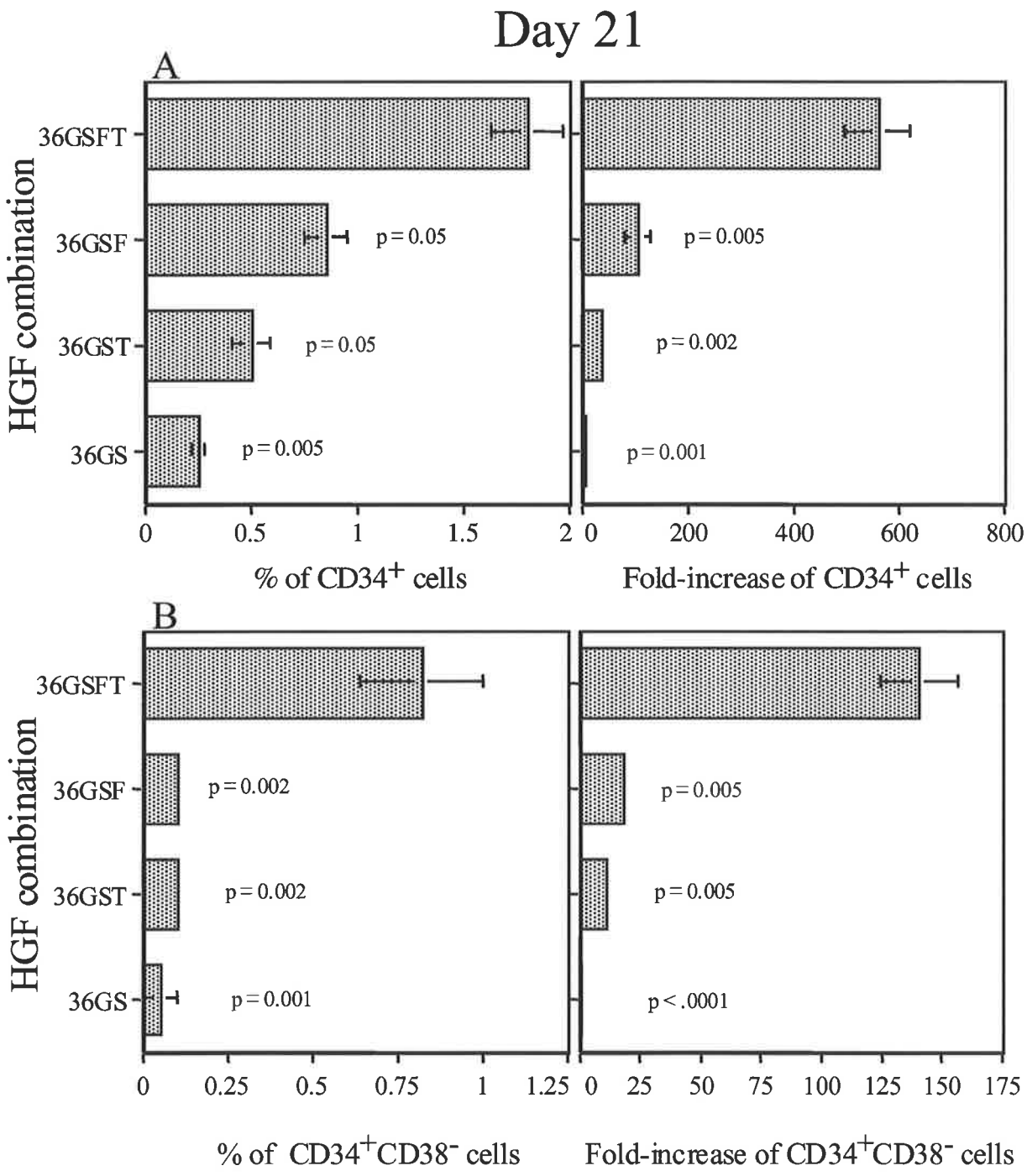
At both day 14 and day 21, significantly (p values ranged from 0.05 to 0.001) more $CD34^+$ and $CD34^+CD38^-$ cells were generated in cultures stimulated with 36GSFT as compared to any other combination of HGF tested (figures 6.11 and 6.12). At day 14, 6.3 ± 0.8% (mean ± SEM, n=3 experiments) of cells were $CD34^+$ representing an 84 ± 12 fold-increase above input: a level slightly higher than that predicted from the previous studies performed with single $CD34^+CD38^-$ cells cultured in Terasaki plates. In cultures stimulated with 36GS, 36GSF, or 36GST, the incidence of $CD34^+$ cells was 3.3 ± 0.27%, 3.8 ± 0.5% and 3.8 ± 0.4% (mean ± SEM, n = 3) respectively: approximately half that observed with 36GSFT.

Figure 6.11 Day 14 generation of nascent $CD34^+$ and $CD34^+CD38^-$ cells

Generation of nascent $CD34^+$ and $CD34^+CD38^-$ cells after 14 days culture in different combinations of HGF. Triplicate, 1 ml cultures of 1,000 FACS isolated BM $CD34^+CD38^-$ cells were initiated in SDM and cell growth stimulated by 36GS, 36GSF, 36GST or 36GSFT combinations of HGF. Cultures were harvested on day 14 and total nucleated cell counts and immunophenotyping for $CD34^+$ and $CD34^+CD38^-$ cells was performed. The results in panel A show the percentage of $CD34^+$ cells and fold-increase of $CD34^+$ cells (mean ± SEM, n = 3) in cultures stimulated with different HGF combinations. Panel B shows the percentage of $CD34^+CD38^-$ cells and the fold-increase of $CD34^+CD38^-$ cells according to the different HGF stimulation. A paired t test was applied to determine if the results obtained with the 36GSFT combination were significantly different to that obtained to other HGF combinations (p values are shown).

Day 14


A
HGF combination
36GSFT
36GSF
36GST
36GS
p = 0.05
p = 0.05
p = 0.04
p = 0.005
p = 0.004
0 1 2 3 4 5 6 7 8
0 25 50 75 100
% of CD34+ cells
Fold-increase of CD34+cells
B
HGF combination
36GSFT
36GSF
36GST
36GS
p = 0.05
p = 0.05
p = 0.04
p = 0.006
p = 0.005
0 0.5 1 1.5 2 2.5 3
0 5 10 15 20 25 30 35 40
% of CD34+CD38- cells
Fold-increase of CD34+CD38- cells

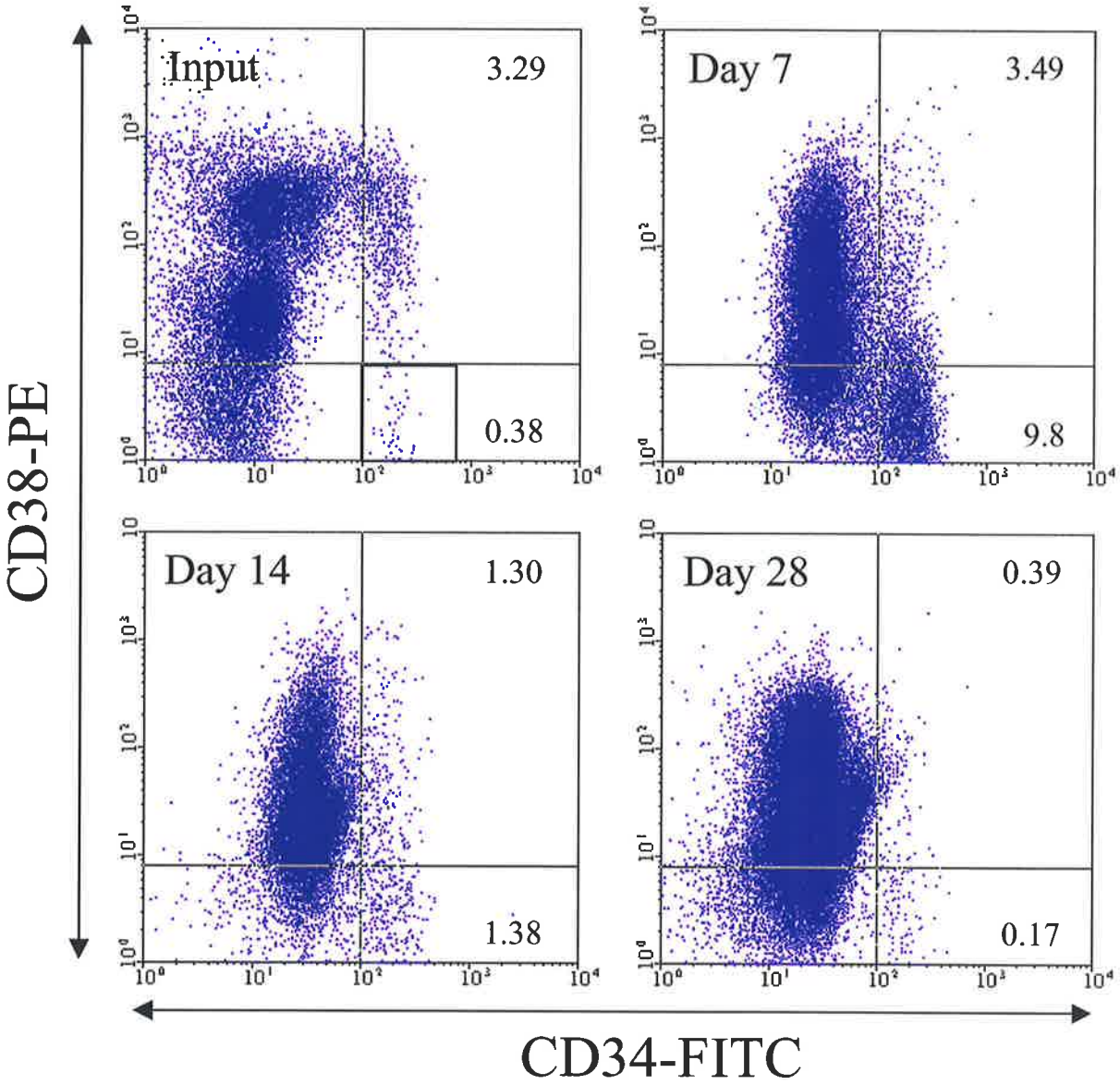

Day 21
A
HGF combination
36GSFT
36GSF
36GST
36GS
p = 0.05
p = 0.05
p = 0.005
p = 0.005
p = 0.002
p = 0.001
0
0.5
1
1.5
2
0
200
400
600
800
% of CD34+ cells
Fold-increase of CD34+ cells
B
HGF combination
36GSFT
36GSF
36GST
36GS
p = 0.002
p = 0.002
p = 0.001
p = 0.005
p = 0.005
p < .0001
0
0.25
0.5
0.75
1
1.25
0
25
50
75
100
125
150
175
% of CD34+CD38- cells
Fold-increase of CD34+CD38- cells

However, as shown in the right hand panel of figure 6.11A, the fold-increase of $CD34^+$ cells was substantially greater in cultures stimulated by 36GSFT than that in other HGF combinations, a result attributed to the greater proliferation of cells in these conditions. Notably, when $CD34^+CD38^-$ cells were stimulated with 36GS, a combination previously shown to very effective for generation of neutrophil precursors from $CD34^+$ cells, there was only marginal expansion of $CD34^+$ cells (1.65-fold increase). As shown in panel B of figure 6.11 production of nascent $CD34^+CD38^-$ cells in the different combinations of HGF followed a similar pattern to that for production of $CD34^+$ cells. Cultures stimulated by 36GSFT produced significantly more nascent $CD34^+CD38^-$ cells than any other HGF combination (p values 0.05 – 0.005). In those cultures stimulated by 36GS, less $CD34^+CD38^-$ cells were present than at the start of culture, suggesting that this combination of HGF was unable to generate cells of this phenotype or maintain the viability of these cells.

At day 21 there was an even greater difference in the incidence and absolute number of $CD34^+$ cells and $CD34^+CD38^-$ cells between cultures stimulated by the different HGF combinations. In accord with the findings at day 14, maximal numbers of both $CD34^+$ cells and $CD34^+CD38^-$ cells were observed in cultures stimulated with 36GSFT (figure 6.12). Cultures stimulated with 36GS, 36GSF and 36GST contained 0.23 ± 0.03%, 0.85 ± 0.1% and 0.5 ± 0.09% $CD34^+$ cells (mean ± SEM) respectively, as compared to 1.8 ± 0.17% in 36GSFT stimulated cultures. As shown in panel B of figure 6.12 when the number of cells in a culture was taken into consideration, the fold-increase of $CD34^+CD38^-$ cells between the 4 groups was even more disparate. At day 21 of the 36GSFT stimulated cultures there were 140 ± 16.1 more $CD34^+CD38^-$ cells present than at the start i.e. 210,000 $CD34^+CD38^-$ cells were generated from the initial 1,500 $CD34^+CD38^-$ cells. In comparison, significantly less $CD34^+CD38^-$ cells were produced from those cultures stimulated by 36GST, 36GSF or 36GS. A representative example of the flow cytometry data from a culture of $CD34^+CD38^-$ cells stimulated by 36GSFT is shown in figure 6.13. In this particular experiment 3.67% of the BM MNC were $CD34^+$ and the sorted $CD34^+CD38^-$ cells represented 10.3% of the $CD34^+$ cell fraction. After 7 days of culture 13.3% of cells were $CD34^+$ and 73.7% of these cells were $CD38^-$ as defined by the sort region used initially for isolation of $CD34^+CD38^-$ cells. At day 14, 2.7% of cells were $CD34^+$ and 51.5% of these were $CD38^-$. At day 28, only 0.56% of cells were $CD34^+$ with 30.3% of these being $CD38^-$. In addition, a high proportion of nascent $CD34^+$ cells also expressed Thy-1, a cell surface antigen commonly associated

Figure 6.13 Flow cytometric analysis of cells stimulated by 36GSFT

This series of flow cytometry dot-plots display CD34 and CD38 expression of adult BM MNC and cells generated after 7, 14 and 28 days culture of $CD34^{+}CD38^{-}$ cells. CD34 and CD38 was detected by labelling with HPCA-2-FITC and Lue-17-PE, respectively. $CD34^{+}CD38^{-}$ cells used for culture were selected according to the rectangular sort region shown in the top left hand dot plot (Input). Cultures were initiated with 1,500 cells/ml in SDM and growth stimulated by the combination of 36GSFT. The figure displays the representative data from one of three identical experiments. The values in the upper and lower right quadrants of each dot plot indicate the proportion of total cells within these quadrants, ie $CD34^{+}CD38^{+/-}$ or $CD34^{+}CD38^{-}$ cells, respectively.


Input
3.29
0.38
Day 7
3.49
9.8
Day 14
1.30
1.38
Day 28
0.39
0.17
CD38-PE
CD34-FITC

with haemopoietic stem cells. Figure 6.14 shows the dot-plots from 1 representative experiment where 2.06% and 0.3% of cells were $CD34^+Thy1^+$ at days 7 and 21, respectively.

Collectively the data presented within this section indicates that, as well as stimulating >90 % of $CD34^+CD38^-$ cells into division, the combination of 36GSFT results in substantial *de novo* generation of $CD34^+$ cells with a composite phenotype previously attributed to haemopoietic stem cells. Do these *de novo* generated cells have stem cell characteristics? At present the most rigorous way to test this possibility would be to examine the capacity of the nascent $CD34^+CD38^-$ cells to initiate human haemopoiesis following transplantation in irradiated immunocompromised NOD/SCID mice (Lapidot *et al* 1997, Bhatia *et al* 1997, Conneally *et al* 1997) or preimmune fetal sheep (Srour *et al* 1992a, Civin *et al* 1996). However, since these models were not available for these studies, 2 alternative, surrogate methods were used to assess the potential of *ex vivo* generated cells. Firstly, 36GSFT stimulated cultures of $CD34^+CD38^-$ cells were grown for extended periods to establish how many myeloid, $CD34^+$ and $CD34^+CD38^-$ cells could be generated. Secondly, FACS isolated nascent $CD34^+CD38^-$ cells from primary cultures were re-cultured under identical conditions to determine if these cells exhibited similar growth potential to the initial $CD34^+CD38^-$ cells.

6.3.6 Culture in 36GSFT Supports *Ex vivo* Expansion of Adult BM $CD34^+CD38^-$ Cells for up to 11 Weeks

Transplantation studies in the NOD/SCID mouse model show that human long-term repopulating HPC represent a small fraction of $CD34^+CD38^-$ cells (Bhatia *et al* 1997). A hallmark of these primitive progenitors is their extensive proliferative potential, which enable them to sustain haemopoiesis for long periods. In view of the fact that 36GSFT stimulated division of approximately 92% of $CD34^+CD38^-$ cells then it was possible that some long-term repopulating cells may divide in this combination of cytokines. Once recruited these cells may also undergo extensive proliferation. Culturing BM $CD34^+CD38^-$ cells for long periods and assessing nucleated cell, $CD34^+$ and $CD34^+CD38^-$ cell generation tested this hypothesis. Three experiments were initiated with 1500, 20,000 and 660 $CD34^+CD38^-$ cells and monitored for 10, 8, and 11 weeks respectively. The results of these studies are shown in figure 6.15 and details of the experimental procedures are included in the figure legend. In brief, 1ml cultures were initiated in SDM supplemented with 36GSFT or 36GS and split weekly to control the cell concentration and to ensure that either lack of cytokines or accumulation of toxic metabolites did not compromise growth. A typical, weekly culture split involved removing the contents of a well, performing a cell count and

Figure 6.14 Expression of Thy-1 by $CD34^+CD38^-$ cells stimulated by 36GSFT

$CD34^+CD38^-$ cells were isolated by FACS according to the sort region shown in figure 6.13. These cells were cultured in SDM and stimulated by 36GSFT. Expression of Thy-1 was examined on cells generated after 7, 14, 21 and 28 days of culture. Analysis of CD34 was performed with a PE/Cy-5 conjugate that resulted in bright staining and excellent discrimination of $CD34^+$ cells. Panel A shows the expression of Thy-1 and CD34 on cells after 7 days culture. Panel B represents the labelling observed with an IgG-PE isotype control antibody whereas panel C shows the expression of Thy-1 and CD34 on cells generated after 21 days of culture. The values in the upper right and lower right quadrants of each dot plot indicate the proportion of total cells within these quadrants, ie $CD34^+Thy\text{-}1^+$ or $CD34^+Thy\text{-}1^-$ cells, respectively.

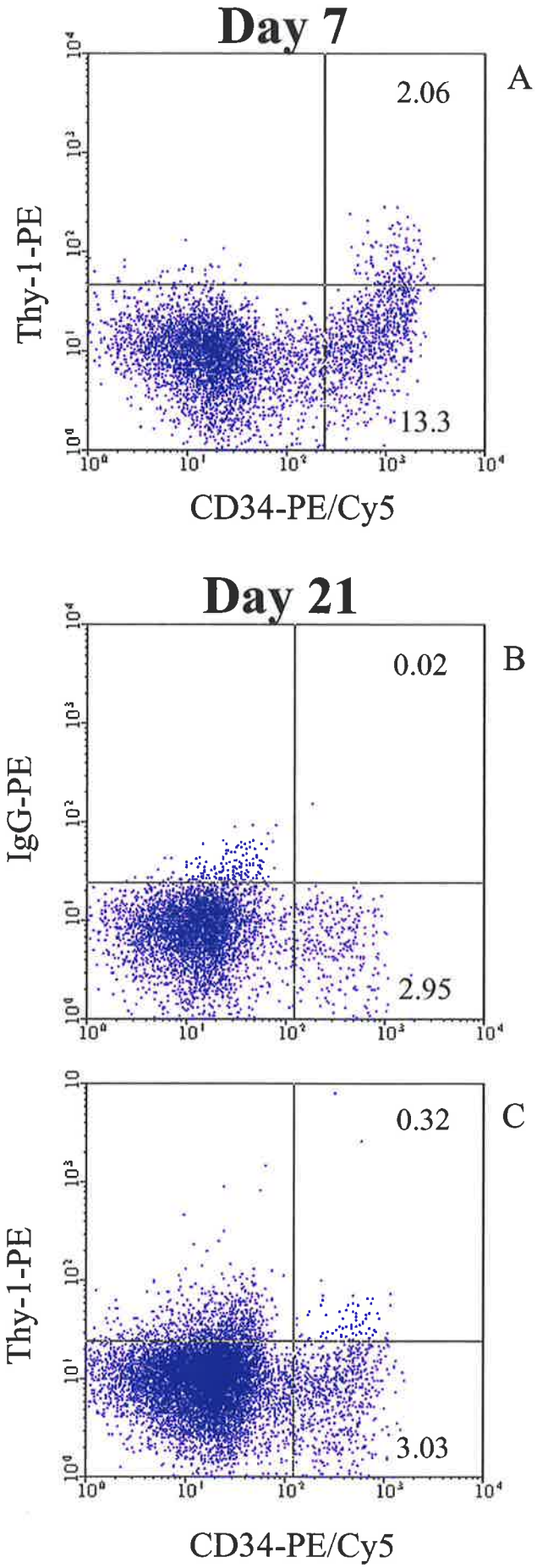
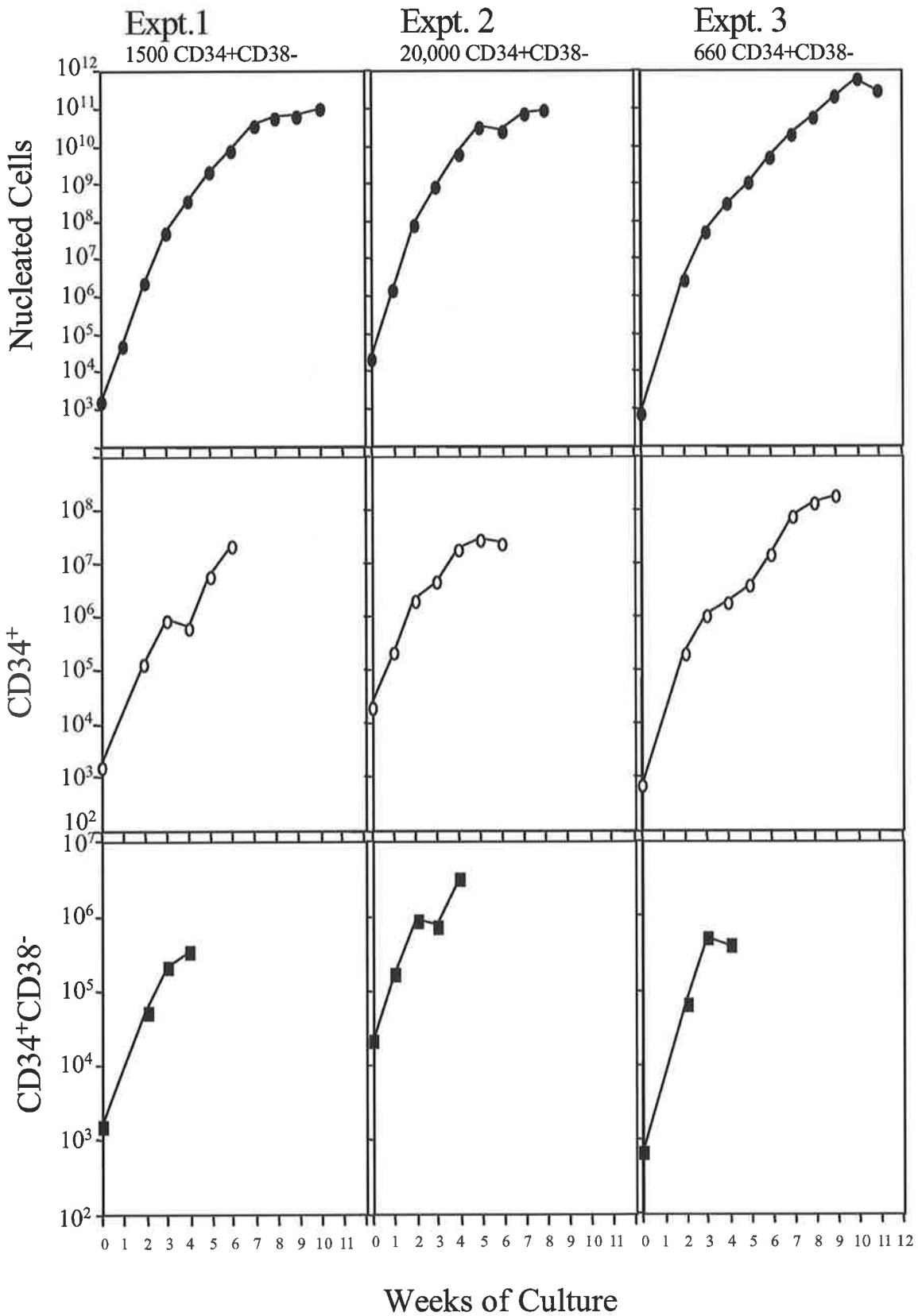

Day 7
A
2.06
13.3
Thy-1-PE
CD34-PE/Cy5
Day 21
B
0.02
2.95
IgG-PE
C
0.32
3.03
Thy-1-PE
CD34-PE/Cy5

Figure 6.15 Generation of total nucleated cells, $CD34^+$ and $CD34^+CD38^-$ cells after long-term culture in 36GSFT

$CD34^+CD38^-$ cells isolated from 3 normal adult BM samples were cultured in SDM supplemented with 36GSFT for up to 11 weeks. In each experiment, cultures were assessed at weekly intervals: a viable cell count was performed, a sample was removed for immunophenotyping and if required were "split" by dilution with fresh SDM and HGF so that the cell concentration was reduced to approximately 100,000 cells/ml. Cultures were terminated when the cell count did not double from that of the previous week or contained predominantly macrophages. Each of these cultures were used for the resorting studies described in section 6.3.7 The top, middle and bottom panels of the figure shows the cumulative number of viable nucleated, $CD34^+$ and $CD34^+CD38^-$ cells at each week of culture. Experiment 1 was initiated with 1,500 $CD34^+CD38^-$ cells in 1ml of SDM and continued for 10 weeks. Experiment 2 was initiated, with 20 x 10^4 $CD34^+CD38^-$ cells cultured in 4 x 1 ml lots at 5,000 cells/ml and was followed for 8 weeks. The final experiment was initiated with 660 $CD34^+CD38^-$ cells cultured in 1 ml of SDM and was followed for 11 weeks. At any week the cumulative total cell number was calculated after taking into consideration the preceding culture splits. For example, in an experiment where the culture was diluted 1/20, 1/20, and 1/5 on weeks 2, 3 and 4 respectively, the cumulative number of cells present after 5 weeks was calculated by multiplying the number of cells present on that day x 2000 (20 x 20 x 5). A similar calculation was used for all time points analysed. The cumulative dilution factor for the 3 experiments was 1 in 1,920,000, 1 in 691,200 and 1 in 2,560,000 respectively and the cumulative number of cells generated was 9.6 x 10^{10} and 8.29 x 10^{10} and 25.6 x 10^{10}. A similar approach was used for calculating the number of $CD34^+$ and $CD34^+CD38^-$ cells at each time point. The percentage of cells with each phenotype was determined by flowcytometry following labelling with a PE-Cy5 anti-CD34 conjugate and a PE-anti-CD38 direct conjugate and the absolute number of $CD34^+$ and $CD34^+CD38^-$ cells calculated by multiplying the incidence of the cell type by the total number of cells. At least 20,000 cells were analysed and an arbitrary threshold of 0.1% $CD34^+$ cells was applied before the sample was classified as containing $CD34^+$ cells. For this analysis, cultures with less than 0.1% $CD34^+$ cells were considered not to contain $CD34^+$ cells.


Expt.1
1500 CD34+CD38-
Expt. 2
20,000 CD34+CD38-
Expt. 3
660 CD34+CD38-
Nucleated Cells
CD34+
CD34+CD38-
Weeks of Culture

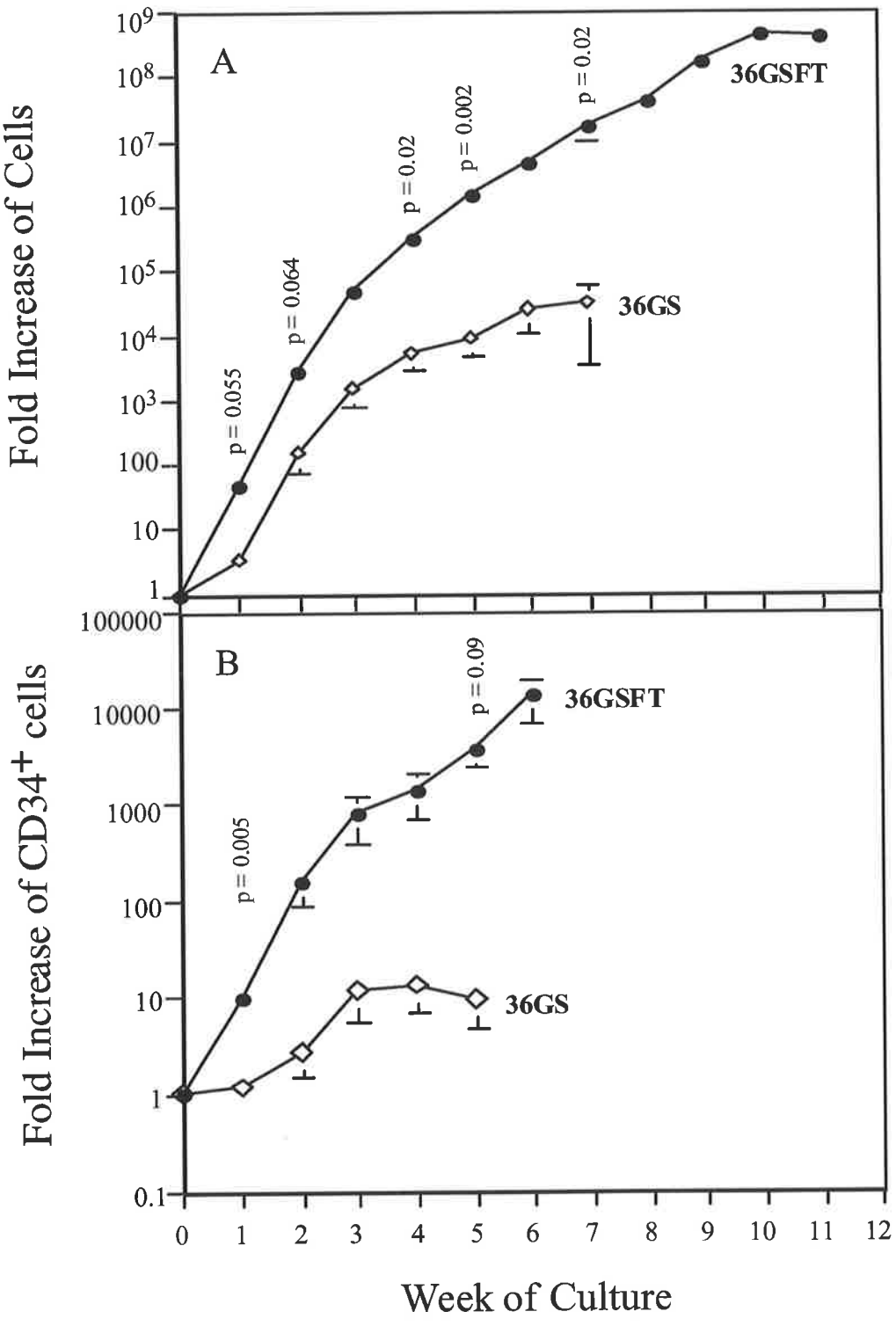
immunophenotyping, then continuation of culture with 100,000 cells in fresh SDM+HGF. Often, the cell concentration on the day of a split was 1.5-2 x 10^6 /ml so that a 1 in 15 or 1 in 20 split was required to continue the culture with 100,000 cells. The cumulative dilution of cells over the course of the 3 cultures stimulated by 36GSFT was 1 in 1,920,000, 1 in 686,400, and 1 in 2,560,000 respectively. The cumulative number of cells present on any given day was a product of the actual viable cell count multiplied by the cumulative dilution factor. An example of a typical calculation is provided in the legend to figure 6.15. As shown in the top 3 panels of this figure the pattern of cell growth was very similar for each culture. During the first 5 weeks there was a 6-log fold-increase in the number of cells generated. Thereafter, cell expansion continued at a slightly reduced rate for another 3-5 weeks. In each experiment, the latter stages of culture in 36GSFT were associated with a gradual increase in the proportion of macrophages and a decrease in the number of small agranular non-adherent cells. Cultures were terminated when there was less than a 2-fold increase in cell production in any week period. These 3 cultures gave rise to 9.6 x 10^{10}, 8.3 x 10^{10} and 2.5 x 10^{11} nucleated cells representing a 64 x 10^6, 4.14 x 10^6 and 387 x 10^6– fold increase in cells, respectively.

The pattern of $CD34^+$ cell expansion was similar to that for total nucleated cells and is shown in the middle panel of figure 6.15. Although there was a slow progressive decline in the incidence of $CD34^+$ cells during the culture period, the absolute number of $CD34^+$ cells rose considerably. In 2 experiments there was a 3-4 log increase in total $CD34^+$ after 6 weeks with 22.5 x 10^6 and 23 x 10^6 $CD34^+$ cells generated from 1500 and 20,000 $CD34^+CD38^-$ cells respectively. In the third experiment, which was initiated with 660 $CD34^+CD38^-$ cells, 0.1% of cells present at week 9 of culture in 36GSFT were $CD34^+$, which translates into 1.79 x 10^8 $CD34^+$ cells. In all cultures stimulated with 36GSFT, in spite of persistence of $CD34^+$ cells for 5-9 weeks, $CD34^+CD38^-$ cells could only be detected during the first 4 weeks (bottom panel of figure 6.15). After 2 weeks $CD34^+CD38^-$ cells represented 2.0%, 1.2% and 2.5% of total cells in each experiment respectively. At week 4, 0.1%, 0.1% and 0.15% of total cells were $CD34^+CD38^-$ which by extrapolation represented a total of 2.1 x10^5, 6.19 x10^6 and 3.78 x 10^5 $CD34^+CD38^-$ cells respectively, in these cultures.

In contrast, as shown in figure 6.16 there was significantly less cell production and generation of $CD34^+$ cells from $CD34^+CD38^-$ cells when cultured with 36GS. During the first 3 weeks the rate of cell production from $CD34^+CD38^-$ cells cultured in 36GS was similar to that of cells cultured in 36GSFT. However, during the next 3 weeks, cell growth in

Figure 6.16 Stimulation by 36GSFT enhances total and $CD34^+$ cell expansion

The generation of nucleated cells and $CD34^+$ cells (expressed as fold-increase) from $CD34^+CD38^-$ cells cultured in either 36GS or 36GSFT was compared. The results for the cultures stimulated with 36GSFT are the mean ± SEM of the experiments described and shown in figure 6.15. In each of these experiments, cells were also stimulated with the alternative combination of 36GS. The top panel (panel A) shows fold-increase of nucleated cells and the bottom panel (panel B) the fold-increase of $CD34^+$ cells. A paired t-test was applied to determine if generation of total cells and $CD34^+$ cells was significantly different between cultures stimulated with 36GS and 36GSFT (p values are shown).


A
B
Fold Increase of Cells
Fold Increase of CD34+ cells
Week of Culture
36GSFT
36GS
p = 0.055
p = 0.064
p = 0.02
p = 0.002
p = 0.02
p = 0.005
p = 0.09
10^9
10^8
10^7
10^6
10^5
10^4
10^3
100
10
1
100000
10000
1000
100
10
1
0.1
0 1 2 3 4 5 6 7 8 9 10 11 12

the 36GS cultures was slow and significantly less than that observed in 36GSFT stimulated cultures. After 6 weeks the 36GS cultures declined and consisted mainly of macrophages and were terminated at the seventh week whereas the 36GSFT cultures continued producing cells for a further 3-5 weeks. Panel B of figure 6.16 compares $CD34^+$ cell generation from $CD34^+CD38^-$ cells cultured in either 36GSFT or 36GS. In accord with the data on cell generation there were significantly more $CD34^+$ cells generated by $CD34^+CD38^-$ cells when cultured with 36GSFT. The difference between the two HGF combinations was evident after 1 week. There were approximately 10-fold more nascent $CD34^+$ cells generated in 36GSFT than 36GS ($p = 0.005$). Over the remainder of the culture period the difference between the 36GSFT and 36GS stimulated cultures increased. The 36GSFT stimulated cultures exhibited greater expansion of $CD34^+$ cells over a longer time than did 36GS stimulated cultures. These data highlight the potent synergistic interactions between SCF and FLT3L and TPO and also demonstrate the limitations of the 36GS combination for *ex vivo* culture of primitive HPC.

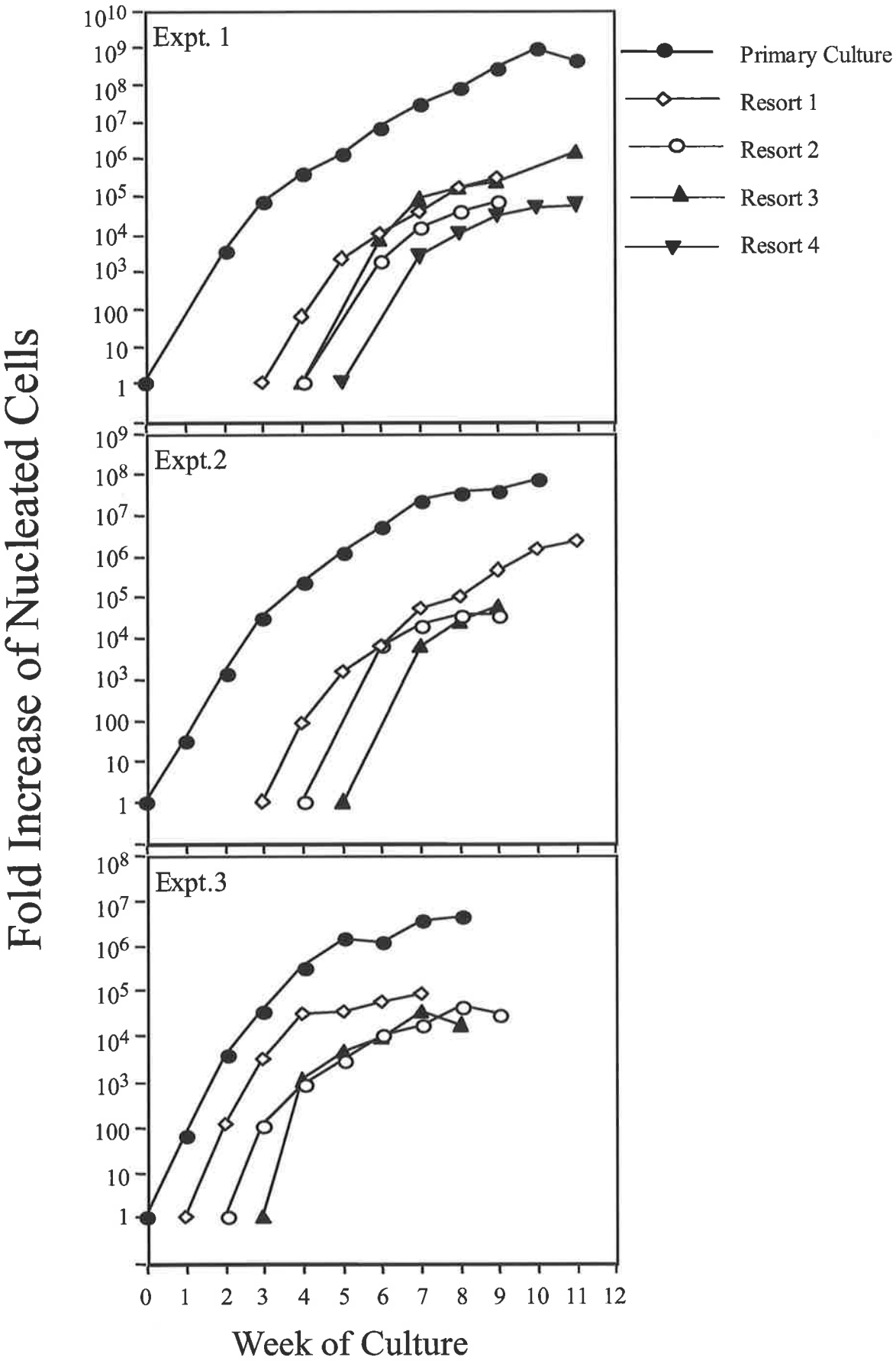
The extent of *de novo* generation of $CD34^+$ and $CD34^+CD38^-$ cells observed in these experiments supported the thesis that primitive HPC could be expanded when $CD34^+CD38^-$ cells are cultured in a HGF combination of 36GSFT. It was therefore important to determine if these nascent $CD34^+CD38^-$ cells had similar proliferative potential to their parent cells.

6.3.7 Nascent $CD34^+CD38^-$ Cells have Similar Proliferative Potential to Primary $CD34^+CD38^-$ Cells

As previously shown in 2 independent studies with murine HPC, amplification or generation of cells with a phenotype associated with primitive HPC does not necessarily indicate that these cells have equivalent haemopoietic potential as primary cells with the same phenotype (Rebel *et al* 1994, Spangrude *et al* 1995). This issue was examined by performing Pre-CFU cultures with nascent $CD34^+CD38^-$ cells present at various times after culture in 36GSFT. Isolation of $CD34^+CD38^-$ cells from cultures was performed by FACS with sorting gates identical to those used to define $CD34^+CD38^-$ cells in the initial BM sort. Overall, the pattern of growth from these resorted $CD34^+CD38^-$ cells was similar to primary $CD34^+CD38^-$ cells isolated from adult bone marrow. As shown in figure 6.17 the rate of cell production paralleled that observed with the original $CD34^+CD38^-$ cells and was independent of the time at which these cells were isolated from the primary culture. That is, $CD34^+CD38^-$ cells obtained after 3 or 4 weeks had similar rates of growth to those obtained after 7 days in the primary culture. Each resort culture was continued until a doubling in cell numbers between

Figure 6.17 Resorting and culture of nascent $CD34^+CD38^-$ cells

$CD34^+CD38^-$ cells were isolated at various times from 36GSFT stimulated *ex vivo* cultures and re-cultured in 1 ml of SDM supplemented with the same HGF combination. In each experiment $CD34^+CD38^-$ cells were collected by FACS using identical FACStar^{PLUS} laser power, colour compensation and amplification settings to that used for sorting the original $CD34^+CD38^-$ cells. In addition, the sorting gates used for defining $CD34^+CD38^-$ cells were identical to that used in the original sort. In experiment 1, $CD34^+CD38^-$ cells used for resort 1 (2,100 $CD34^+CD38^-$ cells) and resort 2 (370 $CD34^+CD38^-$ cells) cultures were obtained from the primary culture after 3 and 4 weeks respectively. Resort 3 and resort 4 cultures were initiated with 150 and 132 $CD34^+CD38^-$ cells obtained after 1 and 2 weeks from the resort 1 culture, respectively. In the second experiment resort cultures 1, 2 and 3 were performed with 3,300, 430 and 205 $CD34^+CD38^-$ cells isolated from the primary culture after 3, 4 and 5 weeks respectively. Similarly, each resort culture in the 3rd experiment was performed with $CD34^+CD38^-$ cells isolated from the primary culture. Resort cultures 1, 2 and 3 were performed with 3,000, 3,000 and 2,500 cells collected from the primary culture after 1, 2 and 3 weeks, respectively.


Fold Increase of Nucleated Cells
Expt. 1
Expt.2
Expt.3
Primary Culture
Resort 1
Resort 2
Resort 3
Resort 4
Week of Culture

successive weeks was not sustained. At this time, as was the case for the primary cultures there were very few non-adherent, small agranular myeloid cells but many macrophages. In all cases the longest period of growth for the resort cultures was less than that observed for the original $CD34^+CD38^-$ cells. Although only a limited number of $CD34^+CD38^-$ cells could be collected from the original cultures after 3, 4 or 5 weeks, these cells were capable of extensive cell production. For example, in the 2nd experiment the resort 2 culture, initiated with 430 $CD34^+CD38^-$ cells produced 15 x 10^6 cells after 5 weeks.

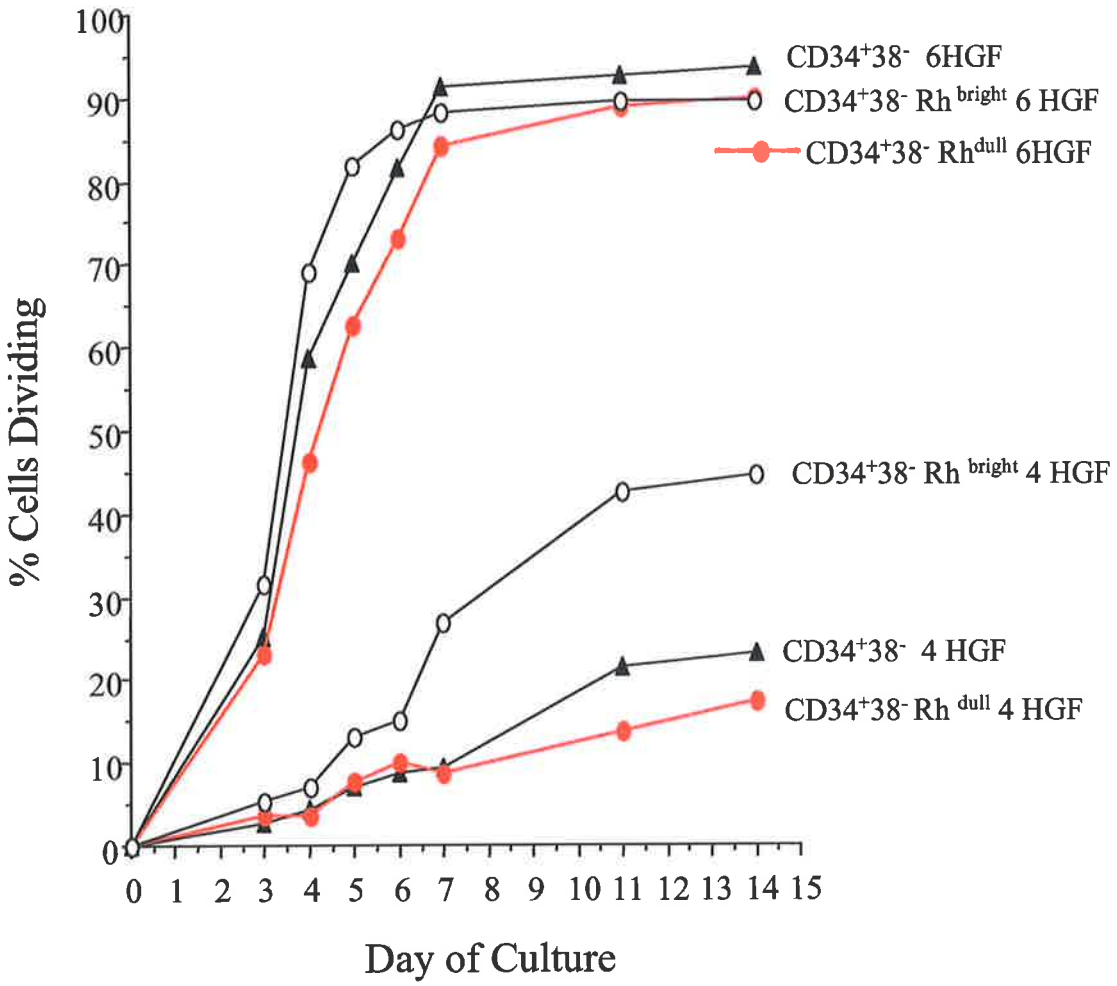
Taken together, the data from this set of experiments demonstrates that the 36GSFT combination is capable of generating $CD34^+CD38^-$ cells with similar proliferative potential to $CD34^+CD38^-$ cells isolated from fresh BM. Do all $CD34^+CD38$- cells recruited by this combination of cytokines have the ability to give rise to further $CD34^+CD38^-$ cells? The data from section 6.3.5 shows that there is heterogeneity in the ability of single $CD34^+CD38^-$ cells to give rise to nascent $CD34^+$ cells. This suggests that only a proportion of $CD34^+CD38^-$ cells stimulated by 36GSFT may also only generate nascent $CD34^+CD38^-$ cells. This issue could be addressed by investigating the growth response of sub-populations of $CD34^+CD38^-$ cells discriminated on the basis of additional cell surface antigens or functional properties. In this regard, the vital mitochondrial dye Rhodamine 123 has previously been shown to separate both murine and human HPC cells into fractions that support either short or long-term haemopoiesis (Spangrude et al 1995, Leemhuis et al 1996, Li 1992). Accordingly, Rh123 staining was used to further subdivide the $CD34^+CD38^-$ fraction and cells exhibiting high or low retention of Rh123 were isolated and cultured with 36GSFT. This approach would determine if these distinct subfractions of $CD34^+CD38^-$ cells divided in 36GGSFT or if they required additional stimuli to induce division.

6.3.8 $CD34^+CD38^-Rh123^{dull}$ Cells Divide in 36GSFT

Single $CD34^+CD38^-Rh123^{dull}$ (ie 10% of cells exhibiting the lowest retention of Rh123) and $CD34^+CD38^-Rh123^{bright}$ cells were sorted and cultured with either 36GSFT or 36GS. The response of these cell fractions is shown in figure 6.18. When cultured in 36GSFT, there was no significant difference between the response of $CD34^+CD38^-Rh^{dull}$, $CD34^+CD38^-Rh^{bright}$ and total $CD34^+CD38^-$ cells: the proportion of dividing cells was similar, as was the rate of cell recruitment. However, when cultured in 36GS more $CD34^+CD38^-Rh^{bright}$ cells divided than total $CD34^+CD38^-$ or $CD34^+CD38^-Rh^{dull}$ cells. Therefore these experiments indicate that $CD34^+CD38^-Rh^{dull}$ cells which represent a metabolically less active, ostensibly quiescent population of HPC also requires combinations of HGF, including FLT3L and

Figure 6.18 $CD34^+CD38^-Rh^{dull}$ cells divide in 36GSFT

Recruitment of single $CD34^+CD38^-$, $CD34^+CD38^-Rh^{dull}$ and $CD34^+CD38^-Rh^{bright}$ BM cells was assessed during culture in Terasaki wells. Single cells of each $CD34^+$ cell fraction were deposited into wells of Terasaki plates containing 10 μL of SDM supplemented with either 36GSFT (6HGF) or 36GS (4HGF). Wells were examined within 12 hours of deposition to identify which wells contained a single cell. Cultures were subsequently examined on each day for 7 days then again after 14 days with the number of viable cells per well recorded. The figure shows the pattern of cell recruitment for each cell fraction (expressed as the percentage of cells dividing) at each time point.


% Cells Dividing
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day of Culture
CD34+38- 6HGF
CD34+38- Rh bright 6 HGF
CD34+38- Rh dull 6HGF
CD34+38- Rh bright 4 HGF
CD34+38- 4 HGF
CD34+38- Rh dull 4 HGF

thrombopoietin, to induce cell division. In spite of this finding, additional studies were not performed to compare the *de novo* generation of $CD34^+CD38^-$ cells between $CD34^+CD38^-Rh^{dull}$ and $CD34+CD38^-Rh^{bright}$ cells following stimulation with 36GSFT.

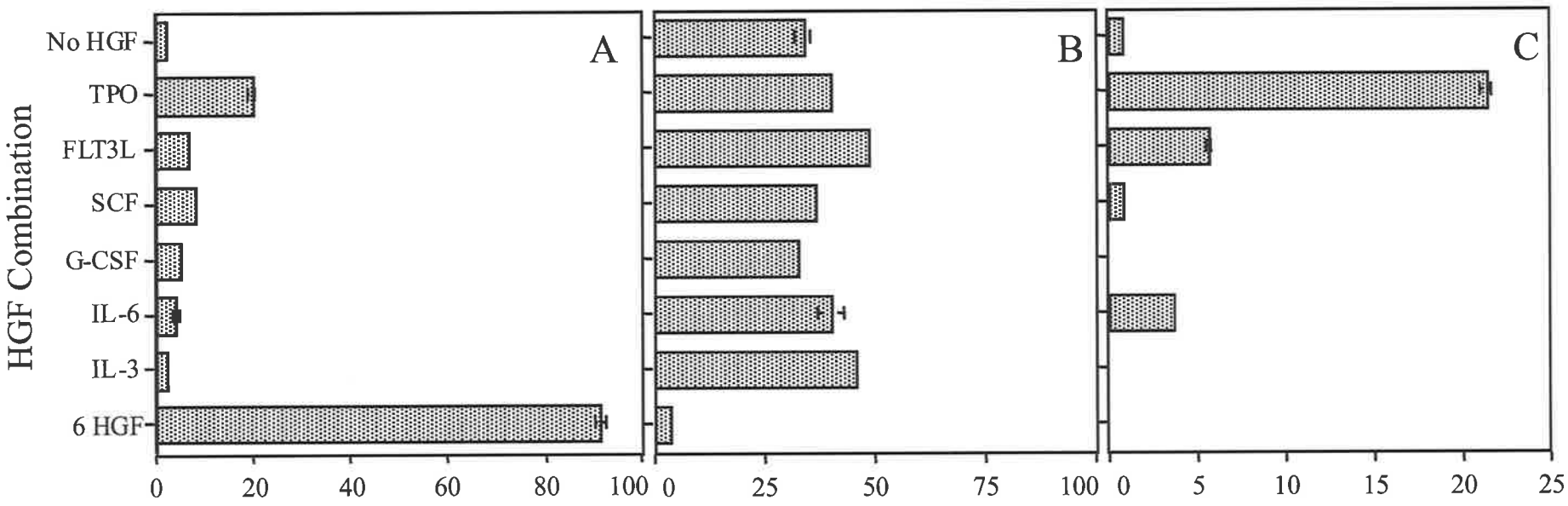
6.3.9 Thrombopoietin Maintains Survival of $CD34^+CD38^-$ cells

The data presented so far suggest that TPO interacts in an additive if not synergistic fashion with other HGF to induce division of primitive HPC. The increased responsiveness of these cells in the presence of TPO may, in part, be due to the ability of this cytokine to maintain survival of HPC. To investigate the role of TPO as a survival factor a series of experiments where single $CD34^+CD38^-$ cells were cultured in SDM for 14 days with each of the 6 HGF comprising 36GSFT were initiated. After 2 weeks in Pre-CFU media and single HGF, the cells were examined under a phase contrast microscope to decide whether i) they had divided, ii) they were non-viable (phase dull, nuclear condensation, cytoplasmic membrane disruption), or iii) viable (phase bright, intact, smooth cytoplasmic membrane). Half the culture media from each well was removed and without dislodging single cells, 5 µl of fresh Pre-CFU media containing 36GSFT (at 2x the normal concentrations) was added. Cultures were incubated for a further 7 days then examined by phase contrast to determine the number of dividing cells as an index of $CD34^+CD38^-$ cell survival during the preceding 14 days of culture.

The results of these studies are shown in figure 6.19. After 14 days of culture, TPO alone was able to stimulate division of 19.6 % of $CD34^+CD38^-$ cells, and was significantly better at inducing cell division than any other single cytokine (p values ranged from 0.0034 for IL-3 to 0.0076 for SCF). Of note was the observation that the majority of the clones stimulated by TPO were not megakaryocytic but small agranular blasts typical of early myeloid cells. As found in earlier experiments the combination of 36GSFT stimulated 91.4% of $CD34^+CD38^-$ cells to divide. Panel B of figure 6.19 represents the proportion of cells that were scored as "viable" on day 14. This score ranged between 32 and 48.6% and there was no significant difference between any single HGF. Many of the cells (34 ± 1.73, mean ± SEM) cultured without HGF also appeared viable as judged by their appearance under phase microscopy. However, the addition of fresh media containing the potent 6-factor combination of 36GSFT revealed the true identity of any viable cells. This result is shown in panel C of figure 6.19. Remarkably, the greatest numbers of viable cells were recorded when TPO was added for the first 14 days of culture. In this respect, TPO/MGDF was significantly (p values from 0.005 to 0.0002, paired t test) better at maintaining the viability of

Figure 6.19 Thrombopoietin is a potent survival factor for $CD34^+CD38^-$ cells

Single $CD34^+CD38^-$ cells were deposited into wells of Terasaki plates containing 10 μL of SDM supplemented with single HGF, including IL-3 (10 ng/ml), IL-6 (10 ng/ml), G-CSF (100 ng/ml), SCF (100 ng/ml), FLT3L (100 ng/ml), TPO (100 ng/ml) or the combination of these HGF. Wells were examined within 12 hours of deposition to identify those containing a single cell. Cultures were subsequently examined after 14 days to determine whether cells had divided or remained as single phase bright, and potentially "viable". Five μL of medium was removed from each well and replaced with 5 μL of SDM supplemented with 36GSFT. The cultures were incubated for a further 7 days at 37°C then examined to determine the proportion of single cells that had divided. Panel A represents the proportion of single $CD34^+CD38^-$ cells dividing after 14 days of culture (mean ± SEM, n = 3). Panel B indicates the proportion of single cells that were classified as potentially viable after 14 days and panel C the fraction of $CD34^+CD38^-$ cells that divided in the subsequent 7 days ie representing cells surviving the first 14 days of culture in single HGF.


HGF Combination
No HGF
TPO
FLT3L
SCF
G-CSF
IL-6
IL-3
6 HGF
A
B
C
0
20
40
60
80
100
0
25
50
75
100
0
5
10
15
20
25
% Cells dividing at day 14
% Single "viable cells at day 14
% Viable CD34+CD38- cells

$CD34^+CD38^-$ cells than other single cytokine. In spite of 45.8% and 32% of cells appearing viable at day 14 after culture in IL-3 and G-CSF respectively, none of the cells cultured in these HGF were able to divide following addition of 36GSFT. Although, TPO/MGDF was by far, the most effective HGF for maintaining survival of $CD34^+CD38^-$ cells in serum deprived media, under these experimental conditions only 21% of the input $CD34^+CD38^-$ cells were found to be viable ie capable of responding to 36GSFT. An important caveat to this conclusion is that HGF may not remain biologically active over the initial 2 weeks of culture. If the HGF concentration decreased due to degradation then this may result in decreased action on the target cell population and thus indicate the HGF to be a poor survival factor for $CD34^+CD38^-$ cells. This concern was addressed by incubating cytokine containing Pre-CFU media without cells for 2 weeks at 37°C then using this media for culture of $CD34^+CD38^{++}$ cells. The ability to stimulate growth of the test cells confirmed that each of the 6 HGF remained bioactive during 2 weeks of culture.

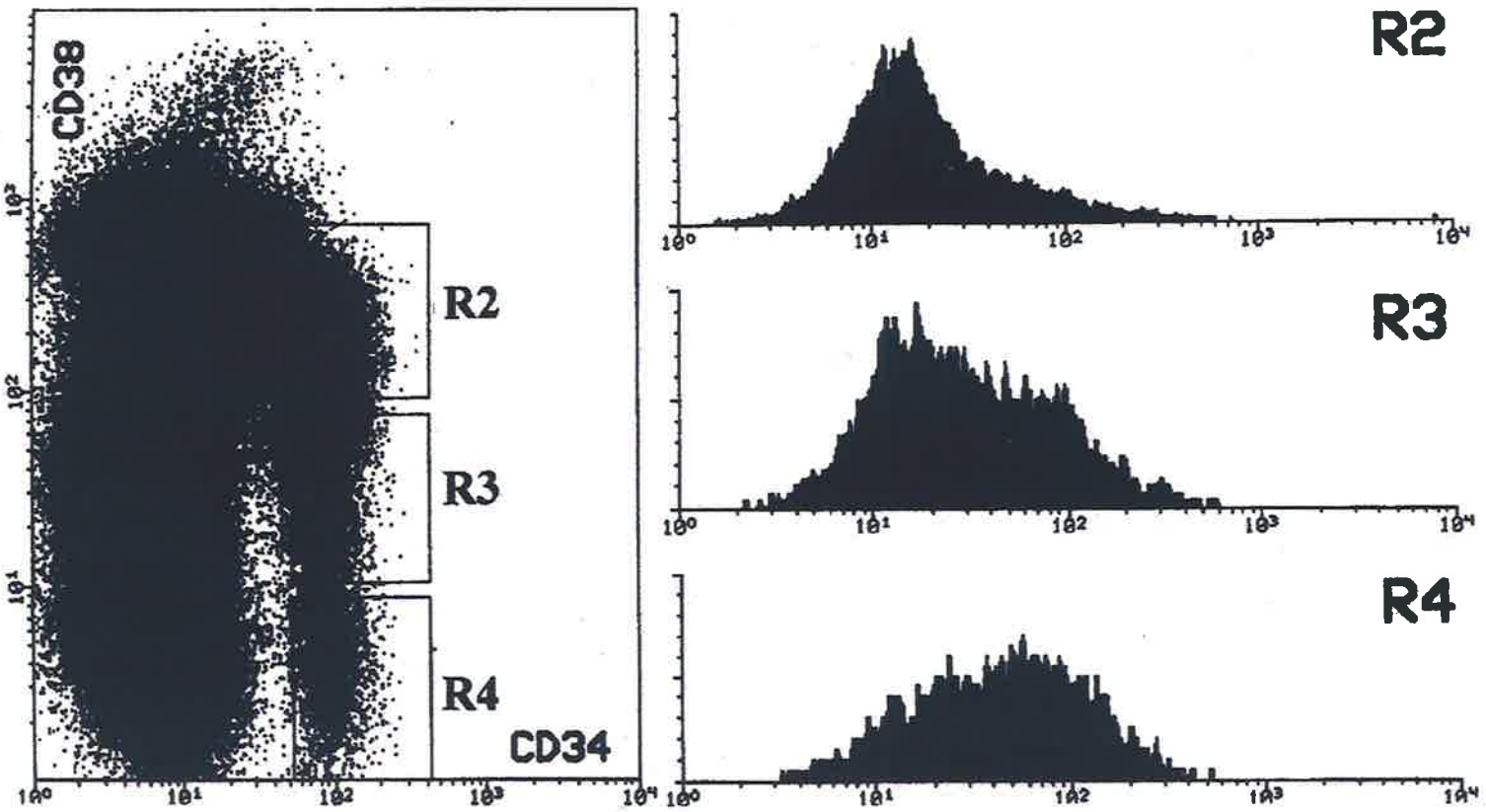
6.3.10 c-*mpl* is Expressed at High Levels on $CD34^+CD38^-$ Cells

Taken together, the above data indicate that TPO has a critical role in supporting the survival and proliferation of primitive HPC. To further investigate the basis for this effect of TPO, the expression of the TPO receptor on BM $CD34^+$ cells was examined using an affinity purified polyclonal antibody (Tahara *et al* 1996). Three colour flow cytometric analysis using anti-c-*mpl* polyclonal in combination with CD34 and CD38 was performed on BM MNC from 5 normal donors. As shown in figure 6.20, c-*mpl* was expressed at readily detectable levels on $CD34^+$ cells. The proportion of $CD34^+$ cells expressing c-*mpl* varied between 30.1% and 60.1% with a mean of 41.5%. Of note was the correlation between expression of c-*mpl* and CD38 on $CD34^+$ cells. The highest levels of c-*mpl* expression were observed consistently on $CD34^+CD38^-$ cells. c-*mpl* expression gradually diminished as the $CD34^+$ cells acquired CD38. The histograms in figure 6.20 show the distribution of c-*mpl* on $CD34^+CD38^+$ (R2), $CD34^+CD38^{+/-}$ (R3) and $CD34^+CD38^-$ (R4) cells.

Based on these observations, $CD34^+$ cells were sorted according to c-*mpl* expression and $CD34^+mpl^+$ and $CD34^+mpl^-$ cells were cultured in methycellulose clonogenic assays and Pre-CFU cultures. Significantly more ($p = 0.028$, paired t test) CFU-GM were present in the $CD34^+mpl^+$ fraction (373 ± 82.4/1,000 cells; mean ± SEM) as compared to the $CD34^+mpl^-$ fraction (279 ± 70.3/1,000 cells). In contrast, there was no significant difference in the number of erythroid progenitors (BFU-E) present in the 2 populations. In addition, $CD34^+mpl^+$ cells had the greatest capacity for *de novo* generation of haemopoietic cells and

Figure 6.20 Expression of c-*mpl* on bone marrow $CD34^+$ cells

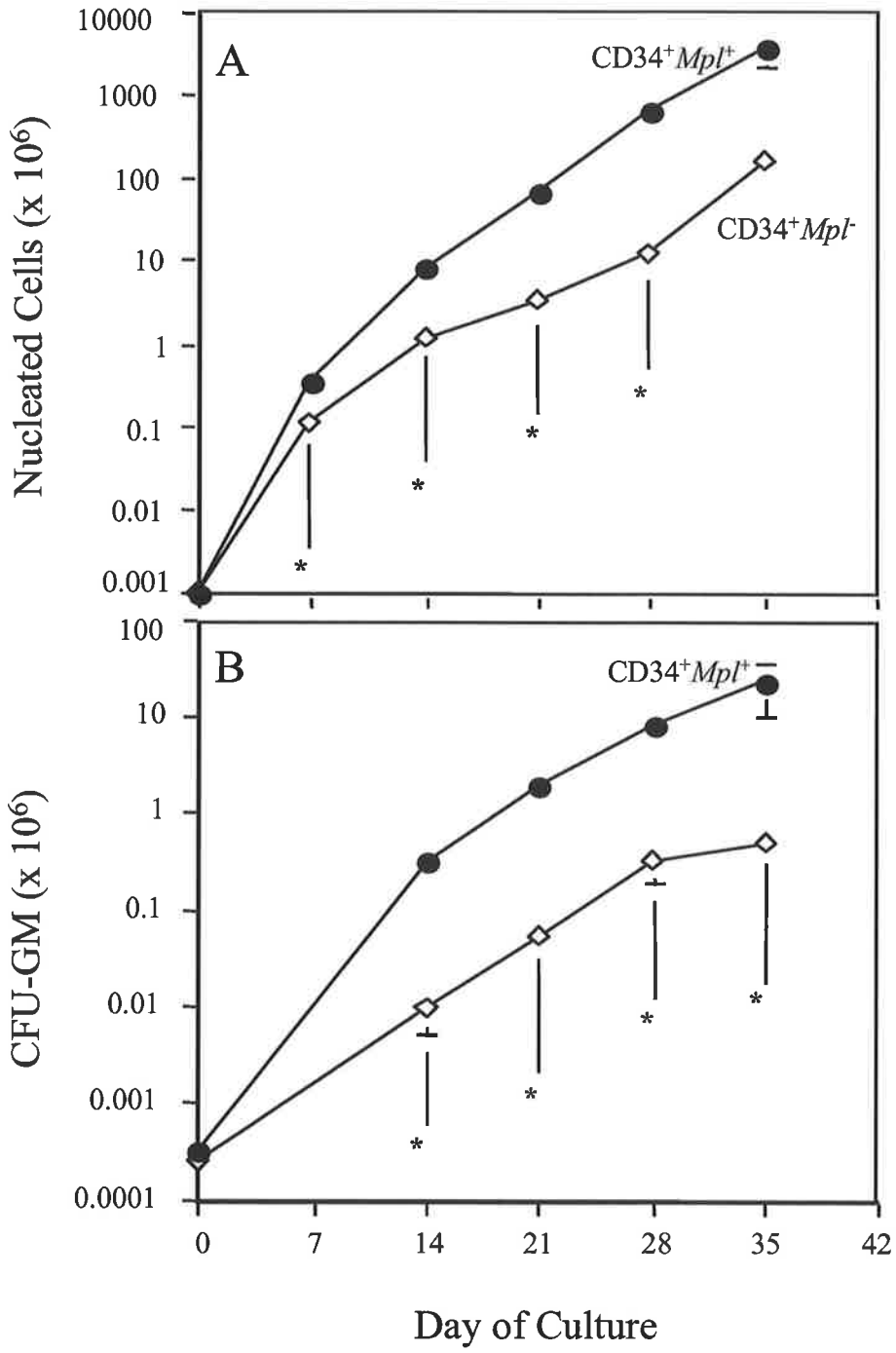
Bone marrow MNC's were immunolabelled with an affinity purified rabbit polyclonal antisera to c-*mpl*, CD34-FITC and CD38-PE as described in the materials and methods. The result of one representative experiment of the 4 independent BM samples studied is shown. The left panel shows the expression of CD34 and CD38 on a two-colour dot-plot. Regions R2, R3 and R4 define $CD34^+CD38^+$, $CD34^+CD38^{+/-}$ and $CD34^+CD38^-$ cells, respectively. The right panel displays histograms of c-*mpl* expression on cells defined by these respective regions. Thus the highest level of c-*mpl* expression is observed on cells within R4, ie $CD34^+CD38^-$ cells.



c-mpl Expression

Figure 6.21 Growth of bone marrow CD34$^+$c-*mpl*$^+$ and CD34$^+$c-*mpl*$^-$ cells in Pre-CFU culture

Bone marrow CD34$^+$c-*mpl*$^+$ and CD34$^+$c-*mpl*$^-$ cells were cultured in SDM stimulated with 36GSFT. Panels A and B represent the production of nucleated cells and CFU-GM, respectively, from 1,000 CD34$^+$c-*mpl*$^+$ or CD34$^+$c-*mpl*$^-$ cells over 5 weeks of culture (mean ± SEM, n = 3). Significantly (assigned by *, p values all < 0.05, paired t-test) more cells were generated by CD34$^+$c-*mpl*$^+$ cells than CD34$^+$c-*mpl*$^-$ cells after 1, 2, 3 and 4 weeks of culture. Significantly more CFU-GM were generated by CD34$^+$c-*mpl*$^+$ than CD34$^+$c-*mpl*$^-$ cells at 2, 3, 4 and 5 weeks.


A
CD34+Mpl+
CD34+Mpl-
10000
1000
100
10
1
0.1
0.01
0.001
B
CD34+Mpl+
100
10
1
0.1
0.01
0.001
0.0001
0
7
14
21
28
35
42
Nucleated Cells (x 10^6)
CFU-GM (x 10^6)
Day of Culture

clonogenic cells in Pre-CFU culture. Shown in figure 6.21 are data from three experiments demonstrating production of nucleated cells and CFU-GM from $CD34^+mpl^+$ and $CD34^+mpl^-$ cells over 5 weeks when stimulated by 36GSFT. Within 7 days of culture the $CD34^+mpl^+$ cells had generated significantly more cells (p= 0.05) than $CD34^+mpl^-$ cells. This trend continued and became more significant at later time points. For example, after 35 days 1,000 $CD34^+mpl^+$ cells generated 3.73 x 10^9 nucleated cells and 22.2 x 10^6 CFU-GM whereas $CD34^+mpl^-$ cells generated 0.16 x 10^9 nucleated cells and only 0.46 x 10^6 CFU-GM. The proliferative response of $CD34^+mpl^+$ in Pre-CFU culture is equivalent to that observed with $CD34^+CD38^-$ cells and is in accord with the high level of c-*mpl* expression on $CD34^+CD38^-$ cells.

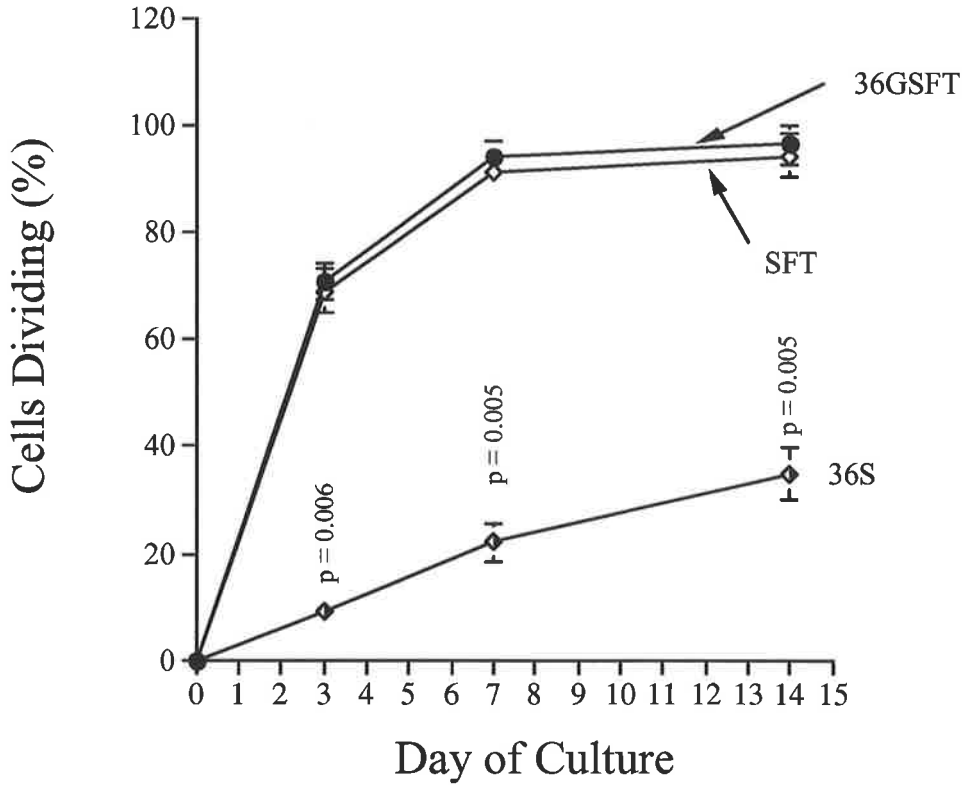
6.3.11 Can SFT Replace 36GSFT?

One key finding from chapter 4 was the importance of SCF in the *ex vivo* expansion of peripheral blood $CD34^+$ cells. Omission of this cytokine from combinations of HGF resulted in a significant reduction in generation of CFU-GM from $CD34^+$ cells. This finding was in accord with the well-described role for SCF as a potent synergistic stimulator of haemopoiesis and particularly the growth and development of primitive haemopoietic cells. Results presented earlier in this chapter demonstrated that the ligand for *flt3*, another receptor tyrosine kinase is also a potent synergistic cytokine affecting growth of primitive HPC. The data presented in this chapter now demonstrate that apart from its role in megakaryocytopoiesis, thrombopoietin directly affects the survival and proliferation of primitive HPC. In brief, SCF, FLT3L and now TPO have been shown to be potent regulators of primitive HPC. This raises two relevant questions. Firstly, since the presence of cytokines such as G-CSF and IL-3 may conceivably promote differentiation and maturation can these be dispensed with? Secondly, would a combination of these 3 cytokines (SFT) be as effective as the 6-factor combination of 36GSFT for inducing division and proliferation of $CD34^+CD38^-$ cells? These questions were addressed by comparing the growth of single $CD34^+CD38^-$ cells in each of these cytokine combinations and also with a HGF combination commonly used for retroviral mediated gene transfer into haemopoietic progenitor cells, namely IL-3+IL-6+SCF (36S). The results of these studies are shown in figure 6.22. The combination of SFT was found to be just as effective as 36GSFT for inducing division of $CD34^+CD38^-$ cells. After 3 days of culture, 71 ± 2.3% (mean ± SEM, n = 3 experiments) and 69 ± 4.1% of $CD34^+CD38^-$ cells divided when cultured in 36GSFT or SFT, respectively. In contrast, significantly less (9 ± 2.0%; p = 0.006) $CD34^+CD38^-$ cells divided when cultured with 36S. At both day 7 and 14 the proportion of cells dividing was above 90% when

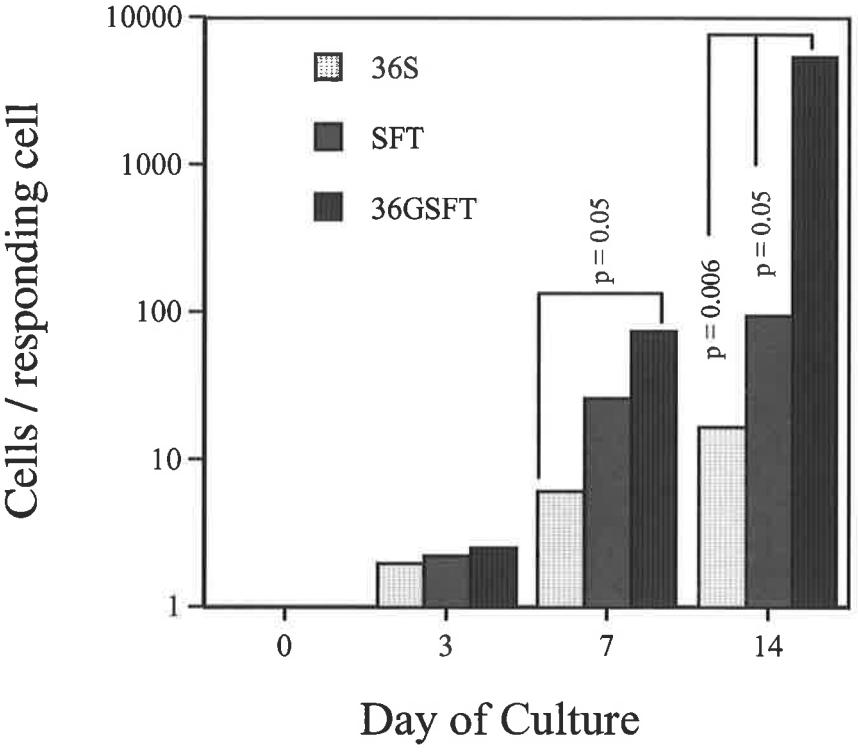
Figure 6.22 Can SFT replace 36GSFT?

Single bone marrow $CD34^+CD38^-$ cells were deposited by the ACDU of a FACStar^{PLUS} into Terasaki wells containing 10 µL of SDM supplemented with different combinations of haemopoietic growth factors (HGF). The following HGF were added: IL-3 (10ng/ml), IL-6 (10ng/ml), G-CSF, SCF, FLT3L, TPO/MGDF (each at 100 ng/ml) in combinations shown. Wells were examined by phase contrast microscopy within 12 hours of sorting, to confirm deposition of single cells, then after 3, 7 and 14 days. On each examination the number of single cells that had divided and the number of cells generated from each recruited cell was recorded. The presented data (mean ± SEM) is from 3 separate experiments with different adult BM samples with at least 100 single $CD34^+CD38^-$ cells for each experiment. Panel A represents recruitment of $CD34^+CD38^-$ cells in the HGF combinations tested and panel B the average number of cells generated from each recruited $CD34^+CD38^-$ cell. A paired t test was applied to compare the proportion of recruited cells and the average number of cells generated from recruited cells at each time for each HGF combination.

A


120
100
80
60
40
20
0
Cells Dividing (%)
36GSFT
SFT
36S
p = 0.006
p = 0.005
p = 0.005
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day of Culture

B


10000
1000
100
10
1
Cells / responding cell
36S
SFT
36GSFT
p = 0.05
p = 0.006
p = 0.05
0
3
7
14
Day of Culture

cultured in 36GSFT or SFT but significantly less ($p = 0.005$) in 36S. However, as shown in panel B of figure 6.22 although the rate and extent of recruitment of $CD34^+CD38^-$ cells was equivalent for cultures stimulated by 36GSFT and SFT there was a significant difference in proliferation from recruited cells. This effect was not observed after 3 days where dividing cells, irrespective of the HGF combination, produced 2-3 cells i.e. 1 or 2 divisions. However, at day 7, cells that had divided in 36GSFT, SFT or 36S produced an average of 75, 26 and 6 progeny, respectively. The differences at day 14 were more pronounced: an average of 5415, 236 and 17 cells produced for each dividing cell when cultured in 36GSFT, SFT or 36S respectively. These results are in accord with similar studies performed with single primitive HPC isolated from adult BM, that also demonstrate the synergistic interaction between TPO, SCF and FLT3L (Kobayashi *et al*1997, Young *et al* 1996).

6.3.12 Isolation of 36GS Non-responsive $CD34^+CD38^-$ Cells by 5-FU

The data presented in sections 6.3.3 and 6.3.4 suggest that primitive HPC within the $CD34^+CD38^-$ fraction might be hierarchically ordered according to the combination of HGF necessary to induce division. It is proposed that HPCs dividing in 36GS are hierarchically less primitive than HPC that require additional stimulation with FLT3L or TPO to induce division. In addition, the most primitive HPC within the $CD34^+CD38^-$ fraction will only divide when stimulated with 36GSFT or SFT. A model of this proposed hierarchy is shown in figure 6.23. If this model were correct it would be expected that removal of 36GS responsive cells from the $CD34^+CD38^-$ fraction would lead to enrichment of more primitive HPC that only respond to 36GST, 36GSF, 36GSFT or SFT. This prediction was investigated by using an approach similar to that described previously for isolation of HPC with lymphoid-myeloid potential from BM $CD34^+$ cells (Beradi *et al* 1995). In brief, BM $CD34^+CD38^-$ cells were cultured in the Pre-CFU assay and stimulated initially with 36GS. After 4 days, cells were incubated for 24 hours with increasing concentrations of the anti-metabolite 5-FU to affect selective killing of dividing cells. The 5-FU was removed by extensive washing and cells were then cultured in either 36GS or 36GSFT for 7 days. At this time, cell counts were performed and fresh 36GSFT was added to both sets of cultures. After a further 7 days the cultures were examined to determine if cells spared by 5-FU had divided in either 36GS or 36GSFT. Two experiments with different normal BM samples were performed according to this protocol.

The results of both experiments were very similar. During the initial 4 days of culture, there was a 3.6 and 4.3-fold expansion of nucleated cells from the $CD34^+CD38^-$ cells grown in

Figure 6.23 Functional hierarchy within the $CD34^+CD38^-$ fraction of bone marrow cells

This figure depicts a proposed hierarchical organisation of BM $CD34^+CD38^-$ cells according to their requirements for HGF to initiate cell division. The figure is based on the data presented in figure 6.5. In this model, HPC within the $CD34^+CD38^-$ fraction of cells can be discriminated according to the combinations of HGF required to initiate cell division. The most primitive HPC reside at the top of the hierarchy and require simultaneous stimulation with combinations of early acting HGF including SCF+FLT3L+TPO to induce cell division. It is proposed that these ancestral HPC will not divide in lesser combinations of HGF that do not include these 3 HGF. In contrast, committed HPC are located at the bottom of the hierarchy and will divide in combinations that do not contain each or all of these early acting HGF. Thus, HPC dividing in the combination of 36GS are considered hierarchically more committed than those cells dividing in HGF combinations such as 36GSF or 36GST.

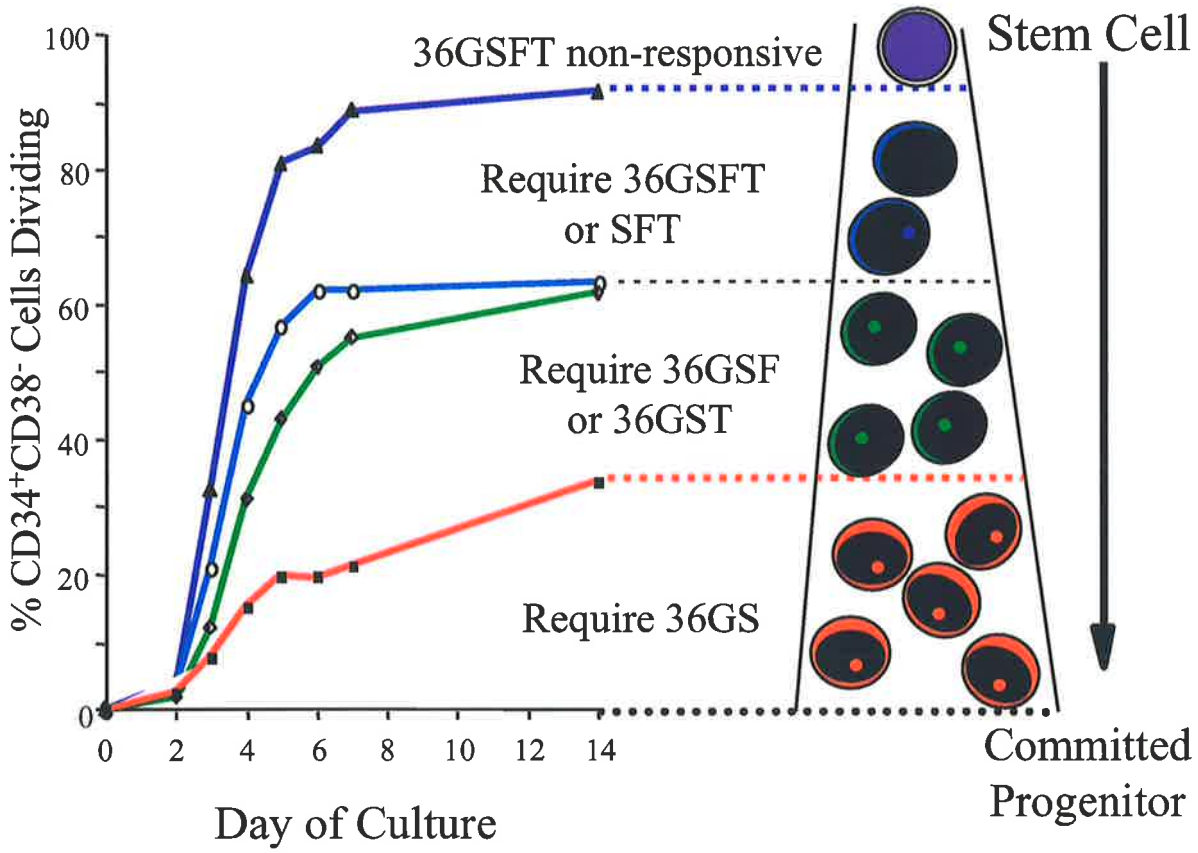
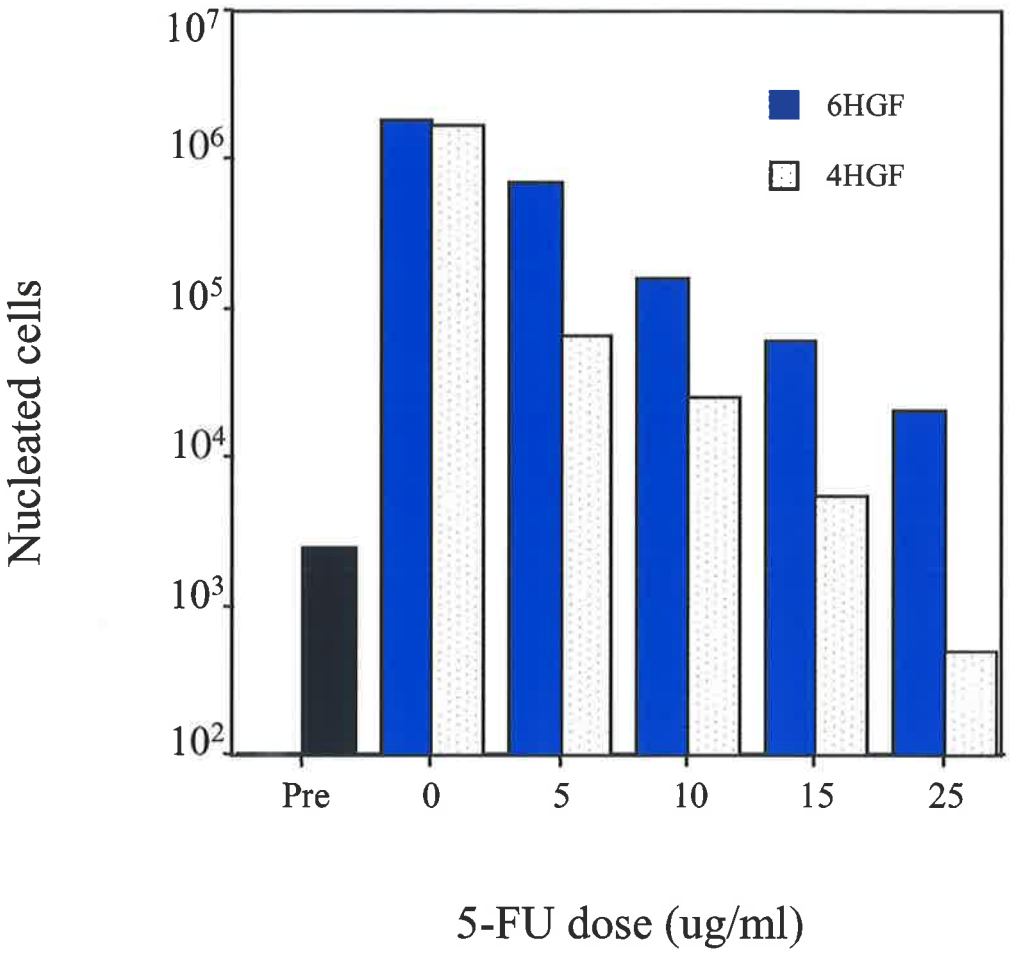

% CD34+CD38- Cells Dividing
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Day of Culture
36GSFT non-responsive
Require 36GSFT
or SFT
Require 36GSF
or 36GST
Require 36GS
Stem Cell
Committed
Progenitor

Figure 6.24 Isolation of primitive haemopoietic progenitor cells by 5-FU

Bone marrow $CD34^{+}CD38^{-}$ cells were cultured in 36GS for 4 days then exposed to increasing concentrations of 5-FU for 24 hours at 37°C. After washing to remove residual 5-FU, cells were split and equal numbers cultured with either 36GS or 36GSFT for 7 days. At the end of this period, 36GSFT was added to each set of cultures, which were then incubated for a further 14 days. Two experiments were conducted where replicates of 2,500 cells were incubated with increasing doses of 5-FU (5, 10, 15 and 25 μg/ml). The figure shows the number of cells present at the beginning of cultures (2,500) and the number generated after exposure to increasing concentrations of 5-FU and rescue with 36GSFT following incubation for the first 7 days post 5-FU with either 36GS or 36GSFT. The bars represent the mean values obtained from two experiments.


Nucleated cells
10^7
10^6
10^5
10^4
10^3
10^2
6HGF
4HGF
Pre
0
5
10
15
25
5-FU dose (ug/ml)

36GS. A series of 1ml cultures, each containing 5,000 cells were then incubated with increasing concentrations of 5-FU (5, 10, 15 or 25 μg/ml) for 24 hours at 37° C. At the end of this incubation there was evidence of dose dependent killing, as indicated by an increasing number of apoptotic cells and nuclear remnants observed with increasing doses of 5-FU. From the data presented in section 6.3.3 it was anticipated that only 15-20% of $CD34^+CD38^-$ cells would divide in 36GS after 4-5 days and accordingly, only 15-20% of cells would be killed by 5-FU. However, it was obvious, although not documented by cell counts, that a far greater and increasing proportion of cells were killed in 10, 15 and 25 μg/ml of 5-FU suggesting that cell death was independent of cell division. Incubation with a dose of 5 μg 5-FU/ml resulted in approximately 30-40% cell death. In contrast, there were very few dead or dying cells in the control cultures not exposed to 5-FU. Each culture was washed extensively to remove 5-FU, split into 2, with one culture stimulated with 36GS and the other by 36GSFT and then examined daily until day 7. For cells exposed to 5-FU then maintained with 36GS there was no evidence of cell division, although small agranular viable cells were present at all concentrations of 5-FU tested. In contrast, in cultures stimulated with 36GSFT immediately after 5-FU treatment, cell doublets were observed within 2 days and rapid cell growth continued until day 7. Since there were so few viable cells present in the 36GS stimulated cultures, cell counts were not performed at day 7. Addition of fresh 36GSFT to these cultures at day 7 resulted in cell division within 2-3 days. Cell counts performed on all cultures 14 days after 5-FU confirmed that 36GSFT was able to initiate division in viable cells spared by 5-FU treatment that had not responded during the previous 7 day culture in 36GS. These data are presented in figure 6.24.

A dose of 25 μg/ml of 5-FU was very toxic and spared only a few $CD34^+CD38^-$ cells. Those cells that survived in 25 μg/ml of 5-FU did not divide when cultured in 36GS for 7 days but responded when cultured for a further 7 days in 36GSFT although less viable cells were present at this time (2,000 cells less than the number originally incubated with 5-FU). In contrast, the few $CD34^+CD38^-$ cells remaining after incubation with 25 μg/ml of 5-FU divided rapidly when cultured in 36GSFT and produced an average of 21.1×10^3 nucleated cells representing an 8.4-fold increase over input. Incubation of $CD34^+CD38^-$ cells in lower concentrations of 5-FU resulted in a less cell death as was demonstrated by the greater number of cells generated following rescue with 36GSFT. At each concentration of 5-FU, fewer cells were produced when addition of 36GSFT was delayed by 7 days. These data support the use of this approach for isolation of HPC fractions that have distinct HGF requirements for initiating cell division.

6.4 Discussion

The data presented within this chapter describe the effect of FLT3L and TPO, alone and in combination with 36GS, on the survival, recruitment and proliferation of primitive human HPC. The data concerning the effect of FLT3L is in accord with a series of studies previously performed within our laboratory, demonstrating that FLT3L acts preferentially on a minor subpopulation of clonogenic $CD34^+$ cells characterised by their low retention of rhodamine 123, resistance to 4-HC and low/absent expression of the CD38 antigen (Haylock *et al* 1997). These are all characteristics of hierarchically primitive haemopoietic progenitors as demonstrated by the capacity of such cells to initiate and sustain haemopoietic cell development in various *in vitro* and *in vivo* assay systems (Simmons *et al* 1990, Sutherland *et al* 1989, Terstappen *et al* 1991). These data prompted an examination of the affect of FLT3L on the growth of BM $CD34^+CD38^-$ cells in cytokine driven, stromal cell-free suspension cultures. Previous studies (chapter 4) from this and other laboratories have shown that the combination of 36GS provides a potent stimulus for growth of primitive human haemopoietic progenitors (Pre-CFU) under these conditions (Makino *et al* 1997, Purdy *et al* 1995).

In accord with these data, 36GS in the present study consistently supported mean 500-fold and 80,000-fold increases in the numbers of CFU-GM and total cells, respectively, at 7 weeks following initiation of cultures with 1,000 $CD34^+CD38^-$ cells (figure 6.1). Despite this considerable expansion of haemopoietic cells, the further addition of FLT3L resulted in a significant enhancement of cell generation over that obtained in cultures stimulated with 4 HGF alone, corresponding to a 330,000 fold expansion of total cells numbers at this 7 week time point.

These data clearly demonstrate the remarkable capacity of FLT3L to potentiate the *ex-vivo* expansion of human HPC and are in accord with, although considerably more impressive than the previous observations of the effects of this cytokine on primitive human and murine HPC (McKenna *et al* 1995, Gabbianelli *et al* 1995, Hunte *et al* 1996, Hirayama *et al* 1995, Piacibello *et al* 1995). Moreover, these studies provide a further example of the potent synergy demonstrated by FLT3L with other cytokines, an observation in agreement with a number of previous reports documenting the synergistic enhancement of colony formation effected by FLT3L when added in combination with cytokines such as IL-3, IL-6, G-CSF and SCF (Broxmeyer *et al* 1995, McKenna *et al* 1995, Rusten *et al* 1996).

Single cell deposition studies performed under serum deprived conditions to investigate the basis for the potent enhancement of haemopoiesis by FLT3L confirmed the preferential action of this cytokine on primitive ($CD34^+CD38^-$) HPC and in addition demonstrated that the activity of FLT3L on these largely quiescent progenitors was direct and not dependent upon either serum components or accessory cell populations. Most significantly, the single cell studies demonstrated that FLT3L when added to 36GS, recruits approximately 2 fold more $CD34^+CD38^-$ cells than in cultures stimulated by the combination of 36GS alone (figure 6.2). In a complementary series of studies performed in conjunction with Dr Ramshaw (Hanson Centre for Cancer Research, Adelaide) we demonstrated that retroviral transduction of $CD34^+CD38^+$ and $CD34^+CD38^{+/-}$ cells was independent of FLT3L, but in contrast, the transduction of $CD34^+CD38^-$ cells, was significantly increased by the addition of FLT3L to a combination of IL-3, IL-6, G-CSF and SCF (Haylock *et al* 1997). In accord with the increased recruitment of single $CD34^+CD38^-$ cells, a 2-fold increase in the proportion of $CD34^+CD38^-$ cells transduced was observed when these cells were exposed to LAPSN retrovirus in the presence of FLT3L. These studies clearly indicate that retroviral transduction of primitive HPC would benefit by the inclusion of FLT3L in combinations of HGF used for co-culture of virus and cells.

Furthermore, the primitive HPC recruited by FLT3L demonstrated, at a single cell level, significantly greater proliferation on average than those stimulated to proliferate by 4 HGF. This was in large part due to the specific recruitment by FLT3L of a minor subpopulation compromising approximately 12% of $CD34^+CD38^-$ cells which, over the two week time course of the assay, were identified by their capacity to generate greater than 6,000 cells per input HPC (figure 6.3). Cells with this proliferative potential were only rarely detected in cultures stimulated with 4 HGF.

Collectively, therefore these data suggest that the marked potentiation of HPC expansion promoted by FLT3L is at least partially due to the increased recruitment of primitive progenitor cells with enhanced proliferative potential. This explanation is supported by the studies of Shah *et al* who demonstrated similar results with single $CD34^+CD38^-$ cells cultured on preformed irradiated BM stromal cells supplemented with HGF (Shah *et al* 1996). Additional evidence for the action of FLT3L on primitive HPC within the $CD34^+CD38^-$ compartment comes from a study reported by Petzer *et al* where FLT3L alone or in combination with other HGF including SCF, IL-3, IL-6, G-CSF and β-nerve growth factor was found to expand long term culture initiating cells (LTC-IC) within the $CD34^+CD38^-$ cell population (Petzer *et al* 1996a). In a further study they reported that in

serum deprived, stroma free cultures of 200 $CD34^+CD38^-$ cells, aside from thrombopoietin, FLT3L was the only factor that, on its own was able to stimulate expansion of LTC-IC numbers to above input levels (Petzer *et al* 1996b). Also relevant in this regard are recent studies showing that FLT3L accelerates the cycling of 5-flurouracil resistant murine HPC by shortening the G_1 phase of the cell cycle (Ohishi *et al* 1996). To date, comparable studies have not been performed using human HPC but if a similar acceleration of cycling is subsequently demonstrated, then this additional activity of FLT3L must also be considered in assessing the overall contribution of FLT3L to the *ex vivo* growth of human HPC.

As with FLT3L, addition of TPO to a combination of 36GS also resulted in a 2-fold increase in the proportion of $CD34^+CD38^-$ cells dividing. More significant however was the finding that greater than 90% of single BM $CD34^+CD38^-$ cells divided when TPO and FLT3L were added together with the combination of 36GS (figure 6.5). Furthermore, this combination of six HGF (36GSFT) was able to induce division in greater than 90% of single $CD34^+CD38^-$ Rh^{dull} cells which represent a more quiescent population of primitive HPC. In each of these experiments it was also observed that addition of TPO to cultures resulted in a more rapid recruitment of cells, which was significantly greater than that observed when FLT3L was added to 36GS. This result is in accord with previous studies demonstrating that synergistic cytokines accelerate cell cycle entry of primitive HPC (Ogawa *et al* 1993, Ku *et al* 1996) and that TPO, in particular leads to an increased rate of cell division by $CD34^+CD38^-$ BM cells (Ramsfjell *et al* 1997). This finding suggests that TPO may further enhance retroviral-mediated transduction of primitive HPC and also allow shorter periods for HPC-retrovirus culture without reducing the efficacy of retroviral transduction. Of particular interest in this regard was the observation that a combination of SCF, FLT3L and TPO (SFT) was also able to induce division rapidly in greater than 90% of $CD34^+CD38^-$ cells. Culture of primitive HPC in this combination of cytokines, without late acting HGF such as G-CSF and IL-3 may lead to high levels of retroviral transduction without compromising the ability of transduced cells to contribute to long-term haemopoiesis. Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with IL-6, SCF, FLT3L and MGDF have confirmed this speculation (Kiem *et al* 1998).

Although the single cell cultures indicate that both FLT3L and TPO can interact synergistically with SCF and 36G to effect a similar increase in recruitment of $CD34^+CD38^-$ cells it was notable that TPO was far superior to FLT3L in supporting survival of these cells. This finding is in accord with that of Borge *et al* who also performed delayed addition experiments and reported that TPO was significantly better than either SCF or FLT3L at

maintaining survival of $CD34^+CD38^-$ cells over 5-6 days, an affect attributed to the ability of TPO to suppress apoptosis (Borge *et al* 1997). In addition, TPO has also been shown to support survival of megakaryocyte, erythroid and granulocyte/macrophage progenitors (Rasko *et al* 1997) and together with SCF to support the survival of thymocyte precursor cells (Amado *et al* 1998). Moreover, it has recently been shown that TPO as a single factor can support the survival of cord blood (CB) $CD34^+Thy\text{-}1^+Lin^-$ cells (Goff *et al* 1998).

The present studies also show that, in comparison to other HGF, especially SCF and FLT3L, TPO is uniquely able to induce division in 20% of single $CD34^+CD38^-$ cells. Notably, the majority of single cells that divide in response to TPO produced clones of cells with blast-like morphology, but only rarely cells with morphology characteristic of megakaryocytes. It remains to be determined if these TPO-responsive cells are developmentally different to those cells that require stimulation with multiple HGF before undergoing division. In addition to demonstrating that TPO is a potent survival factor for $CD34^+CD38^-$ cells the studies show the remarkable capacity of TPO to synergise with other HGF, particularly SCF and FLT3L, and potentiate the recruitment of primitive HPC.

The present studies also demonstrate the ability of TPO to synergise with other early and late acting HGF to affect a potent proliferative stimulus to recruited $CD34^+CD38^-$ cells. This is highlighted by the data (figures 6.7 and 6.8) that show that when cultured in 36GSFT, a high proportion of single $CD34^+CD38^-$ cells undergo extensive cell production. Remarkably, greater than 30% of recruited $CD34^+CD38^-$ cells complete 12 or more divisions within 14 days when cultured in the 36GSFT combination. This may be a direct consequence of recruitment of more primitive cells which have implicitly increased proliferative potential, or may be because TPO when added together with other early acting cytokines such as SCF and FLT3L produce a shortening of the G_1 phase of the cell cycle (Tanaka *et al* 1995, Ohishi *et al* 1996). In addition, the combination of 36GSFT was significantly better than 36GS, 36GSF or 36GST for generating nascent $CD34^+$ and $CD34^+CD38^-$ cells from primary $CD34^+CD38^-$ cells (figure 6.11 and 6.12).

The potency of the 36GSFT combination for *ex vivo* expansion of cells was further highlighted by the immunophenotypic analysis of single $CD34^+CD38^-$ cell cultures and the three larger scale cultures initiated with 660, 1,500 and 20,000 $CD34^+CD38^-$ cells. Analysis of the progeny from single $CD34^+CD38^-$ cells stimulated by 36GSFT revealed that 66% of clones with greater than 2,000 cells, contained nascent $CD34^+$ cells. Furthermore, the larger

scale cultures demonstrated that stimulation of $CD34^+CD38^-$ cells by 36GSFT resulted in sustained production of nascent $CD34^+$ cells for up to 9 weeks and moreover generation of significant numbers of nascent $CD34^+CD38^-$ (figure 6.15) and $CD34^+Thy\text{-}1^+$ cells for 3-4 weeks. Presumably, these nascent HPC are responsible for the extensive nucleated cell generation observed for up to 11 weeks. It is indeed remarkable that in one experiment, 660 $CD34^+CD38^-$ cells gave rise to 179 x 10^6 $CD34^+$ cells after 9 weeks and generated 25.6 x 10^{10} nucleated myeloid cells at week 11. If these nascent $CD34^+$ or $CD34^+CD38^-$ cells have equivalent long-term repopulating potential developmental potential to their counterparts in freshly isolated BM, this number of cells would be sufficient to transplant a 70 kg individual.

Unfortunately, during the present studies, the haemopoietic potential of *ex vivo* generated nascent $CD34^+$ and $CD34^+CD38^-$ cell populations could not be tested in a suitable transplantation model. However the results of cultures initiated with resorted nascent $CD34^+CD38^-$ cells suggest that newly derived HPC have significant proliferative potential (figure 6.16). In spite of these data, it is uncertain if HSC numbers are increased by *ex vivo* culture in 36GSFT and, as shown by other investigators, the notion of *ex vivo* expansion of HSC remains controversial. For example, despite amplification of $Sca\text{-}1^+$ lin^- WGA^+ cells in serum free cultures containing SCF, IL-6, EPO with or without IL-3, this was not accompanied by a parallel increase in the number of cells with both lymphoid and myeloid repopulating potential *in vivo* (Rebel *et al* 1994). A further murine study demonstrated *in vitro* expansion of $Thy\text{-}1.1^{low}Lin^{neg}Ly\text{-}6A/E^+$ cells but these cells were vastly inferior in radioprotection and long-term reconstitution assays when compared to cells of exactly the same phenotype isolated from normal animals (Spangrude *et al* 1995).

Ex vivo expansion studies with human BM or CB cells has produced conflicting results. A study from the Eaves laboratory showed that incubation of purified CB $CD34^+CD38^-$ CB cells in serum-free medium containing 36GSF for 5-8 days resulted in 100-fold expansion of CFU-GM, a 4-fold expansion of LTC-IC and a 2-fold expansion in NOD/SCID competitive repopulating units (Conneally *et al* 1997). Similarly, Bhatia *et al* showed a 4-fold increase in SCID repopulating cells (SRC) after a 4 day incubation of $CD34^+CD38^-$ CB cells in stroma-free conditions; however SRC were lost after 9 days of culture (Bhatia *et al* 1997). More recently, and of relevance to the present study was the report of human BM $CD34^+Thy\text{-}1^+Lin^-$ cells cultured in SFT retaining their *in vivo* repopulating ability in the SCID/hu bone assay (Luens *et al* 1998). In this study, the membrane bound, fluorescent cell tracking dye PKH26 was used to monitor division of BM $CD34^+Thy\text{-}1^+$Lin- cells. Assuming that there is

considerable, if not complete overlap in BM cells defined by the $CD34^+Thy\text{-}1^+Lin^-$ and $CD34^+CD38^-$ phenotypes, then the data presented by Luens *et al* are in accord with the data presented within this chapter. Luens *et al* observed that > 90% of $CD34^+Thy\text{-}1^+Lin^-$ cells divided within 6 days and at this time 76% of progeny expressed high levels of the CD34 antigen. These $CD34^+$ cells also retained expression of Thy-1 and the ability to give rise to both B-lymphoid and myeloid engraftment at 8 weeks in the SCID-hu bone graft model. This observation suggests that expression of CD34 or Thy-1 on nascent HPC may predict engraftment potential. However it remains to be determined if nascent $CD34^+$ or $CD34^+Thy\text{-}1^+$ cells generated after weeks in *ex vivo* culture are equivalent to their parent cells and also retain the ability to engraft. A recent report of *ex vivo* expansion of CB $CD34^+$ cells for up to 12 weeks, stimulated by either the FLT3L+TPO, SFT or 6SFT combinations suggests that this may be possible (Piacibello *et al* 1999).

In their study, Piacibello *et al* used engraftment in myeloablated NOD-SCID mice as a measure of haemopoietic potential of both unmanipulated CB $CD34^+$ cells and *ex vivo* expanded cells. Mice were sacrificed 6-8 weeks after injection and evidence of human engraftment determined by flow cytometry for human cell surface markers and southern analysis using a human chromosome 17 specific probe. Transplantation with 10 or 20,000 unmanipulated CB $CD34^+$ cells resulted in low levels (< 0.6 %) of human engraftment in only 3 of 12 mice tested. In contrast, there was substantial improvement in the proportion of animals with human engraftment (15 of 17 transplanted mice) in those mice transplanted with $CD34^+$ cells cultured in either of the cytokine combinations. This data demonstrated that a proportion of cells within the *ex vivo* expanded cell product have the ability to repopulate a NOD-SCID mouse and contribute to human haemopoiesis for 6-8 weeks. Unfortunately, these investigators did not determine which cells within the *ex vivo* expanded cell product might be contributing to engraftment. In addition, the data do not demonstrate the ability of these *ex vivo* expanded cells to sustain human haemopoiesis for longer times and to reconstitute secondary recipient mice. Therefore it remains to be determined if HSC were expanded or persisted under their culture conditions.

In spite of their limitations, these reports (Conneally *et al* 1997, Bhatia *et al* 1997, Luens *et al* 1998, Piacibello et al 1999) indicate that both the NOD-SCID and SCID-hu transplant models are suitable for testing the haemopoietic potential of ex vivo expanded human haemopoietic cells. In addition these studies have confirmed and extended the findings presented within this chapter with regard to the potency of 36GSFT and SFT for ex vivo

expansion of human HPC. There is now accumulating evidence that culture of primitive HPC in combinations of cytokines including TPO, FLT3L and SCF results in phenotypic self- renewal of HPC. Do all HPC, be they $CD34^+CD38^-$ or $CD34^+Thy\text{-}1^+lin^-$ cells, from adult BM or CB or foetal liver have the capacity to give rise to nascent cells of an identical phenotype? Although this question has not been specifically addressed in the current work, the results of the studies with single $CD34^+CD38^-$ cells provide some clues.

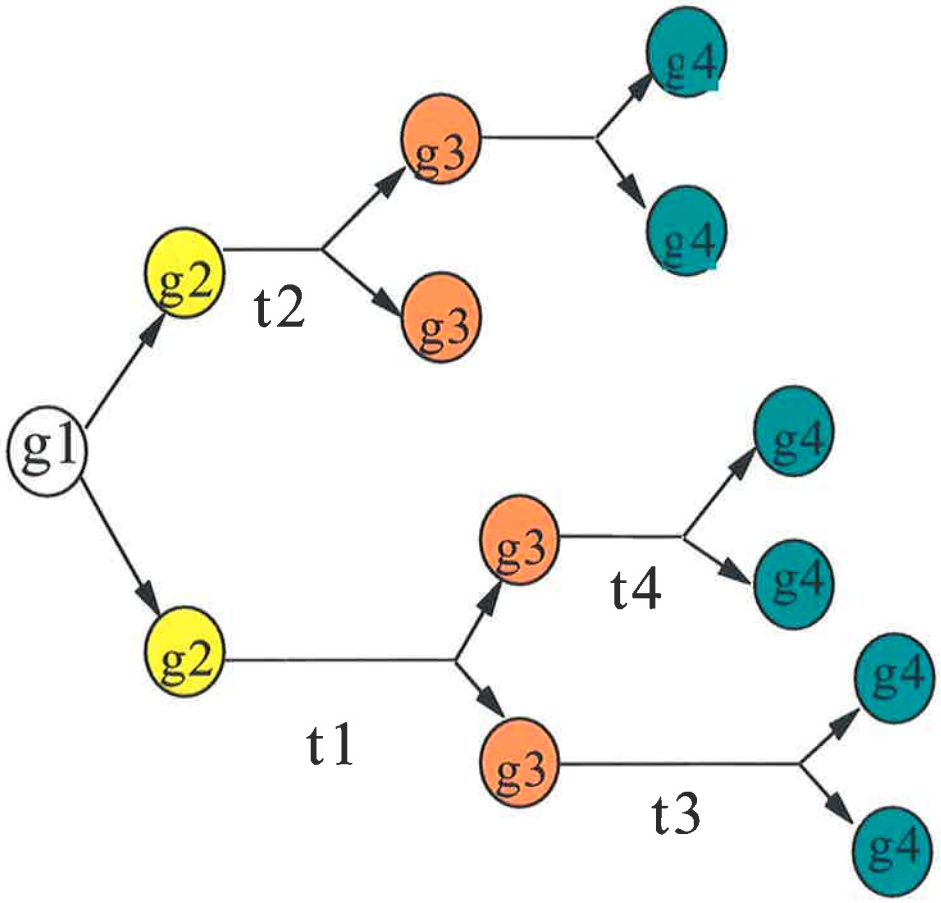
The analysis performed on the progeny of single $CD34^+CD38^-$ cells after 14 days culture in 36GSFT revealed considerable heterogeneity in the ability of these cells to give rise to nascent $CD34^+$ cells (figure 6.10). Although 66% of clones contained $CD34^+$ cells, the proportion of $CD34^+$ cells within any clone was highly variable and only 19% contained 5% or more $CD34^+$ cells at day 14. As mentioned previously this analysis is biased because it ignores slow growing clones of less than 2,000 cells that potentially contain nascent $CD34^+$ cells. In this regard, the report from the Lansdorp group provides important insights into the nature of slow growing $CD34^+CD38^-$ cells (Brummendorf *et al* 1998). In their study, single foetal liver $CD34^+CD38^-$ cells were cultured in the HGF combination of IL-3+IL-6+G-CSF+SCF+FLT3L (analogous to the 36GSF combination used in the present studies) and analysis at day 9 revealed that 60% of clones were fast growing (generated 1,000 progeny) and 16% were slow growing (50-200 cells). As expected the slow growing cells exhibited a longer average cell cycle time (32 hours) as compared to that for fast growing clones (20.8 hours). The fast growing clones produced nascent $CD34^+CD38^-$ cells for only 16 days whereas the slow growing clones had the unique capacity for continued generation of $CD34^+CD38^-$ cells for up to 129 days indicating that the proliferative potential and cell cycle characteristics in primitive HPC are linked. Furthermore the heterogeneity with regard to slow and fast growing clones, observed within the pool of $CD34^+CD38^-$ cells at the start of culture was preserved in the progeny of slow growing clones. That is, with successive generations from slow growing clones, isolated, single nascent $CD34^+CD38^-$ cells produced similar proportions of slow and fast growing clones.

The intrinsic timetable model (figure 6.25) of stem cell function was proposed by Brummendorf *et al* to describe this behaviour of primitive HPC. This model is underpinned by the proposal that each stem cell division is asymmetric and results in daughter cells that differ in cell cycle properties. As a result, the time interval between successive generations is variable between clones of the same generation and the interval between division is subject to both intrinsic (developmental) and extrinsic (microenvironment and growth factors)

Figure 6.25 Intrinsic timetable model of haemopoiesis

The intrinsic timetable model of haemopoiesis as described by Brummendorf *et al* 1998 describes the pattern of cell division exhibited by primitive HPC in *ex vivo* cultures stimulated by combinations of HGF. Cell generations are depicted by the "g" prefix and a number corresponding to the number of cell divisions the cell is from the parent cell. Thus, g3 corresponds to a third generation cell. In a similar fashion the time taken for division of daughter cells is indicated by the "t" prefix and a number corresponding to the time. A feature of this model is the asymmetric cell division displayed by daughter cells. This is manifest by the different times taken for daughter cells to undergo their next division. According to this model, Brummendorf and colleagues proposes that the most primitive cells exhibit longer times between division, a characteristic that is independent of the combination of HGF used to stimulate division but more importantly an intrinsic property of stem cells.

The Intrinsic Timetable Model of Haemopoietic Stem Cell Biology



Adopted from Brummendorf et al. J. Exp. Med. 188; 1998

control. Furthermore, asymmetric cell divisions would result in one daughter cell being similar to the mother cell (functionally as well as phenotypically) and the other daughter cell more committed to terminal differentiation. Direct evidence for asymmetric divisions in early haemopoiesis has been reported by at least 2 independent groups using time-lapse video recordings of cultures (Denkers *et al* 1993, Huang *et al* 1999).

It is of note that Brummendorf reported that most slow growing $CD34^+CD38^-$ cells were small, motile and appeared as "commas". This morphology, as described earlier in section 6.3.4 and shown in figure 6.9, panels C and D, was observed in a proportion of the progeny of single BM $CD34^+CD38^-$ cells cultured in 36GSFT. In addition, asymmetric divisions, represented by either the difference in time taken for daughter cells to undergo second or subsequent divisions or the inability of one of the daughter cells to divide have also been observed during the present study. It is proposed that asymmetric division of adult BM $CD34^+CD38^-$ following stimulation with 36GSFT underlies the sustained generation of $CD34^+$, $CD34^+CD38^-$ and nucleated cells in Pre-CFU cultures. This proposal implies that a small proportion of recruited $CD34^+CD38^-$ cells are slow growers and that these sustain production of nascent $CD34^+$ and $CD34^+CD38^-$ cells whereas the majority of recruited cells grow rapidly and reach terminal differentiation much sooner. Thus, there is considerable functional heterogeneity even within the $CD34^+CD38^-$ fraction of adult BM. It remains a challenge to identify which cells within this fraction of BM might be slow growers or fast growers.

If asymmetric division occurs within the HSC compartment then another expected finding would be conservation of the stem cell phenotype during division. Goff *et al*, who analysed the immunophenotype of daughter cells generated following stimulation of single CB $CD34^+Lin^-$ cells, addressed this issue (Goff *et al* 1998). It was observed that although there was evidence for differentiation at the first division a proportion of daughter cells exhibited a conserved stem cell phenotype ($CD34^+lin^-$). Moreover, the phenotype of daughter cells at first division was regulated by the combination of HGF. Stimulation of $CD34^+lin^-$ cells with a combination FLT3L and TPO resulted in 56.8% of the doublets exhibiting a conserved phenotype ($CD34^+lin^-$), whereas stimulation in hepatocyte growth factor + IL-3 + IL-6 + G-CSF + GM-CSF resulted in only 6.7% of doublets expressing a conserved phenotype. Interestingly, there was no correlation observed between the time when cells divided and the phenotype of the daughters. This finding is in contrast to an earlier report describing lineage commitment in HPC involving asymmetric division of multipotent progenitors that did not

appear to be influenced by cytokines (Mayani *et al* 1993). Furthermore, Goff's observations are difficult to reconcile with the previous data and model proposed by Brummendorf since it would be predicted that the initial daughters of slow growing clones might exhibit a higher conservation of the stem cell phenotype. Perhaps the different proportion of primitive cells recruited by the HGF combinations being tested can explain the different results between these reports. In the case of the Goff study, only 68% of $CD34^+Thy\text{-}1^+lin^-$ cells divided whereas 85% of $CD34^+CD38^-$ cells divided in the HGF combination used in the Brummendorf study. These two studies suggest that regulation of differentiation is complex and mediated by interactions between intrinsic regulators and extrinsic regulators such as HGF.

The present data demonstrating differential recruitment of single $CD34^+CD38^-$ cells in different HGF combinations suggest the possibility that primitive HPC may be hierarchically ordered according to their HGF requirements. Figure 6.23, based on the data presented in figure 6.5 provides a model for the hierarchical relationship of HPC within the $CD34^+CD38^-$ cell fraction. It is proposed that the most primitive HPC require simultaneous exposure to multiple HGF including SCF, FLT3L and TPO in order to induce cell division whereas less primitive HPC divide in HGF combinations lacking either FLT3L or TPO. Therefore, cells that have an obligatory requirement for stimulation by 36GSFT, before cell division is initiated could be considered more primitive than cells of the same phenotype that respond to 36GSF or 36GST. It follows that $CD34^+CD38^-$ cells responding to a four-factor combination of 36GS represent more committed progenitors that display restricted developmental and proliferative potential in comparison to those cells requiring 36GSFT to initiate division.

This model was investigated by using an approach similar to that described by Beradi and involved stimulation of $CD34^+CD38^-$ cells with 36GS then incubation with the anti-metabolite 5-FU (Beradi *et al* 1995). It was hypothesised that 5-FU would kill cells that need only 36GS to initiate division and spare those HPC that require stimulation with additional HGF. The data presented in section 6.3.12 suggests that this approach is effective for elimination of cells with specific HGF requirements, although the extent of cell death was greater than expected and not in accord with the anticipated proportion of dividing cells. In spite of this problem the experiments demonstrated that cells spared by 5-FU were unable to respond to the initial combination of HGF (36GS) but would divide in 36GSFT. Therefore this approach could be used to isolate cells at various positions within the proposed hierarchy. For example, culture of $CD34^+CD38^-$ cells with 36GSF or 36GST then incubation

with 5-FU would spare 36GSFT responsive cells. This approach could be improved by tracking cell fate and division with dyes such as CFSE (Nordon *et al* 1997, Lyons *et al* 1994) or PKH26 (Samlowski *et al* 1991, Lansdorp *et al* 1993) to identify fractions of HPC that respond or do not respond to specific combinations of HGF. In this respect $CD34^+CD38^-$ cells could be labelled with CFSE at the beginning of an experiment, cell fate monitored after the initial culture, following 5-FU, then during culture in the combinations of HGF designed to "rescue" spared, hierarchically more primitive cells.

To determine if the proposed hierarchy has any physiological relevance then cells with different HGF requirements could be tested in a suitable xenogeneic transplant model (Zanjani *et al* 1982, Srour *et al* 1992b, Lapidot *et al* 1997). In addition it may be possible to determine if a composite antigenic phenotype can be ascribed to cells at various levels in the hierarchy.

Another implication of the proposed hierarchical ordering model is that those few cells, which do not divide in 36GSFT or SFT (7-10% of the $CD34^+CD38^-$ fraction of BM), may represent the most primitive HPC and perhaps haemopoietic stem cells? This question can be addressed by the approaches described above. If these investigations indicate that the 36GSFT-nonresponsive fraction contains HSC then it raises the issue of what additional stimuli are required to maintain survival and induce division of these cells in *ex vivo* culture? There are at least 2 possible mechanisms or interventions that may result in recruitment of these candidate haemopoietic stem cells. Firstly, these cells may remain quiescent due to the overriding influence of negative regulators of haemopoiesis, including TGF-β1 and MIP-1α. In particular TGF-β1, a well-described potent negative regulator of haemopoiesis that acts in an autocrine manner on primitive HPC (Hatzfeld *et al* 1991, Li *et al* 1994) may regulate the recruitment of 36GSFT- nonresponsive cells. The use of antisense TGF-β1oligonucleotides or monoclonal antibodies to TGF-β1 may block the affect of TGF-β1 and therefore enable $CD34^+CD38^-$ cells to enter cell cycle in response to HGF (Cardoso *et al* 1993, Li *et al* 1994).

A second possible means of recruiting 36GSFT-nonresponsive cells involves culture with stromal cells and or ligands for specific CAMs. The rationale here is that primitive HPC may require the combined intracellular signalling mediated through CAMs, together with those provided through cytokine receptors to initiate entry into cell cycle. Normal HPC express at their surface a wide variety of cell adhesion molecules representing at least five superfamilies of CAMs, including integrins, immunoglobulin, selectin, sialomucin, and the

CD44 family of adhesion molecules (Kincade *et al* 1989, Clark *et al* 1992, Long *et al* 1992, Simmons *et al* 1994b, Simmons *et al* 1997). It is now recognised that there is significant functional interaction (cross-talk) between cytokine receptors and CAM signalling pathways, particularly those utilised by the $\beta_1$integrins (Levesque *et al* 1995, 1996). Fibronectin, (Fn) a major component of the extracellular matrix synthesised by BM stromal cells is a ligand for both the $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5) integrins and apart from its role in anchoring HPC within the BM microenvironment (Williams *et al* 1991, Papayannopoulou *et al* 1995) it also functions as an activating ligand for these integrins (Simmons *et al* 1994, Levesque *et al* 1995). As a consequence of the latter function, Fn has been shown to stimulate, in co-operation with IL-3, the formation of mixed granulocyte, erythroid, monocyte and megakaryocytic colonies (Weinstein *et al* 1989). In addition, there is evidence that culture of $CD34^+$ cells with either Fn or fragments of Fn improves retroviral transduction of reconstituting haemopoietic stem cells by retroviral vectors: a result presumed to be mediated by increased recruitment of candidate HSC (Moritz *et al* 1994, Nolta *et al* 1995, Moritz *et al* 1996, Dao *et al* 1998). Therefore, these studies suggest that recruitment of $CD34^+CD38^-$ cells may be further enhanced in cultures where cells are exposed to the collective stimulation provided by combinations of soluble cytokines and ligands for CAMs.

Another possible means for increasing the proportion of $CD34^+CD38^-$ cells recruited is to present HGF in an immobilised form rather than as a collection of soluble molecules. In this case, stimulating molecules would be presented in an analogous fashion to the *in vivo* situation where HGF such as SCF and FLT3L are presented by stromal cells as transmembrane molecules, (Flanagan *et al* 1991, Toksoz *et al* 1992, McClanahan *et al* 1996) or in the case of molecules such as GM-CSF and IL-3, complexed to proteoglycans (Roberts *et al* 1988). Presentation of immobilised HGF has the potential to increase the local concentration of each cytokine and to deliver a qualitatively different intracellular signal. This concept is supported by the observation that membrane-bound steel factor (SCF) has been shown to induce more persistent tyrosine kinase activation and longer life span for surface c-*kit* than its soluble form (Miyazawa *et al* 1995). As might be predicted, membrane-bound SCF synergises with soluble FLT3L in supporting early haemopoietic cells in long-term culture of normal and aplastic bone marrow (Slanika-Krieger *et al* 1998). Additionally, immobilised monoclonal antibodies to tyrosine kinase receptors could be used as surrogate ligands and deliver signals identical of those provided by transmembrane bound ligands (Kurosawa *et al* 1996).

The affects of any of the above manipulations can be examined ideally in the stroma-free, serum deprived Pre-CFU culture system where both survival and growth of individual HPC is absolutely dependent on provision of exogenous stimuli. Apart from adding soluble recombinant cytokines, this system can be modified so that tissue culture wells are coated with purified recombinant ECM proteins, such as fibronectin, or other proteins known to modulate haemopoiesis. Under these conditions it will be possible to determine if such molecules, either alone or together with HGF, directly affect the survival or growth of 36GSFT non-responsive $CD34^{+}CD38^{-}$ cells.

It is worth noting that the intrinsic timetable model proposed by Brummendorf and the hierarchical ordering model proposed as a result of the present studies address different but linked aspects of the early events in haemopoiesis. The two models are not exclusive of each other but may overlap. The intrinsic timetable model suggests that primitive HPC use asymmetric division as a method for maintaining the stem cell compartment and regulating differentiation. It is feasible that asymmetric division may be restricted to only the most primitive cells within the haemopoietic hierarchy, for example only those dividing in response to HGF combinations such as SFT or 36GSFT. Alternatively, asymmetric division may occur with all $CD34^{+}CD38^{-}$ cells irrespective of their position within the proposed hierarchy. Thus although different combinations of HGF are required to initiate division other intrinsic regulators determine the fate of dividing cells.

The proposed model for hierarchical ordering of HPC according to HGF requirements may however, be misleading. The present studies with single $CD34^{+}CD38^{-}$ cells were performed with a culture system that places cells in an entirely artificial environment. These cells do not have contact with other developing haemopoietic cells, the stromal elements nor the ECM components normally found within the bone marrow microenvironment. Therefore cells under investigation are not exposed to the range of molecules and cellular interactions that normally regulate haemopoiesis. As a result, isolated purified HPC are not subject to inhibitors of haemopoiesis that would otherwise modulate the positive affects of haemopoietic growth factors or cytokines. Therefore, culture of single $CD34^{+}CD38^{-}$ in an overwhelming excess of positive haemopoietic regulators is unlikely to accurately reflect the *in vivo* role of these factors in haemopoiesis. In spite of this limitation the culture system does provide some valuable insights into which molecules may have a role in regulating quiescence. Maintenance of quiescence and slow rates of cell division are fundamental

characteristics of stem cell populations, although the mechanisms underlying these properties remain to be completely defined.

The data presented within this chapter indicate that for both the $CD34^+CD38^-$ and the less metabolically active $CD34^+CD38^-Rhodamine123^{dull}$ cell fractions that are both predominantly quiescent, then simultaneous activation of *c-kit*, *flt3* and *c-Mpl* by their respective ligands results in exit from quiescence and progression through the cell cycle. Given these target cells are cultured in isolation *in vitro* and are not subject to extrinsic negative regulatory influences, the combined signalling mediated by SCF + FLT3L + TPO (SFT) could be considered as an event necessary to overcome an intrinsic control system regulating quiescence. As mentioned earlier, intrinsic control of cell cycle status can be mediated by TGF-β1, which acts as potent autocrine inhibitor to entry into the cell cycle for primitive HPC (Keller *et al* 1988, Hatzfeld *et al* 1991). Future studies that identify the signalling pathways activated by SFT may give some insights as to how stimulation with this combination of HGF affects quiescence. It should be noted that primitive HPC from different haemopoietic tissues such as foetal liver and cord blood might require fewer cytokines to induce cell division. Therefore different mechanisms for regulation of quiescence may be operational in haemopoietic tissues at different stages of ontogeny. In this regard, the results of Piacibello *et al* would suggest that a significant proportion of primitive HPC from cord blood divide in a combination of just 2 HGF, namely FLT3L and TPO (Piacibello *et al* 1997).

In keeping with the biological activity of TPO/MGDF on primitive HPC it has been demonstrated that expression of the TPO receptor, *c-mpl*, is inversely correlated to expression of CD38. The lowest level of *c-mpl* was observed on $CD34^+CD38^{++}$ cells and the highest level of *c-mpl* was found on $CD34^+CD38^-$ cells. Since, the most primitive HPC require TPO to maintain survival and to induce cell division it is not surprising to find high levels of *c-mpl* on $CD34^+CD38^-$ cells. In contrast, committed HPC, which express higher levels of the CD38 antigen are able to divide in absence of TPO and accordingly express less *c-mpl*. These results are in complete agreement with those reported by Takeshita *et al* who used flow cytometric analysis with biotinylated MGDF to investigate *c-mpl* expression on BM cells and various hematopoietic cells line (Takeshita *et al* 1997). It is notable that in contrast to *flt3* (Haylock *et al* 1997), *c-mpl* was expressed at high levels on essentially all $CD34^+CD38^-$ cells. This finding suggested that primitive HPC within the $CD34^+$ cells fraction could be identified according to *c-mpl* expression. Culturing $CD34^+mpl^+$ and

$CD34^+mpl^-$ fractions in Pre-CFU assays tested this proposal. Under these conditions it was demonstrated that $CD34^+mpl^+$ cells generate more CFU-GM and nucleated cells than $CD34^+mpl^-$ cells, suggesting that more primitive HPC fractionate with the $CD34^+$ cells expressing high levels of *c-mpl*. As very few other nucleated cells within BM express high levels of *c-mpl* then is conceivable that HPC could be isolated solely on the basis of *c-mpl* expression.

Collectively the data presented within this chapter provides strong evidence that TPO is not only required for megakaryocyte growth and platelet production but is important for the growth and development of primitive HPC. In this regard my studies confirm and extend the findings from a number of other investigators who have studied the action of TPO/MGDF on both murine (Ramsfjell *et al* 1996, Era *et al* 1997, Itoh *et al* 1996) and human HPC (Piacibello *et al* 1997, Ramsfjell *et al* 1997, Leuns *et al* 1998, Petzer *et al* 1996a, Young *et al* 1996, Kobayashi *et al* 1997, Birkmann *et al* 1997, Tanimukai *et al* 1997). Moreover, the data presented herein describing the affects of combinations of HGF containing SCF, TPO and FLT3L have a number of important implications for *ex vivo* manipulation of cells for therapy.

Firstly, HGF combinations containing SFT induce division in > 90% of primitive HPC. Once recruited these cells have the potential for extensive generation of myeloid cells and nascent HPC over extended periods of culture. Importantly, from the point of view of *ex vivo* generation of myeloid cells for cellular therapy the combination of 36GSFT results in 3 - 4,000-fold expansion of cells within 14 days. Secondly, *ex vivo* cultures initiated in 36GSFT or SFT might lead to expansion of HSC, which may facilitate the use of these cells for gene therapy or generation of specific haemopoietic lineages for specific applications. Thirdly, the data indicates that in *ex vivo* cultures performed without TPO or FLT3L then HSC might not divide but may survive. Thus, when HGF combinations such as 36GS or 36S stimulate the *ex vivo* growth of $CD34^+$ cells the majority of primitive HPC ($CD34^+CD38^-$ cells) may not respond but should remain viable and able to contribute to long-term haemopoiesis.

CHAPTER 7 CONCLUSIONS AND DISCUSSION

This thesis describes the *ex vivo* manipulation of purified populations of human HPC under stromal-free, cytokine dependent culture conditions. A major objective for this work was to develop conditions suitable for generation of myeloid cells for clinical use. An underlying hypothesis to be tested was that culture of purified human HPC would result in a similar pattern of cell development and differentiation to that observed *in vivo*. The data presented herein, particularly that of chapters 3 and 4 support this hypothesis. Specifically, the data demonstrate that culture of purified $CD34^+$ cells or subpopulations thereof, in combinations of HGF results in generation of cells that appear morphologically, immunophenotypically and functionally equivalent to normal neutrophil precursors within the BM. In this respect, under the stromal-free culture conditions used throughout the thesis, the number and type of myeloid cells generated from $CD34^+$ cells was shown to be dependent on the choice of HGF used for stimulating growth and differentiation. The effects of individual and combinations of HGF on generation of neutrophil precursors were a major focus for the present series of investigations and are summarised in the following section.

7.1 Summary of Data and Implications.

In brief, the major findings and implications from the studies presented within this thesis include the following:

a) Stem cell factor, G-CSF and to a lesser extent IL-3 and IL-6 are essential HGF for stimulating growth of neutrophil precursors from $CD34^+$ cells *in vitro*. The combination of 10 ng/ml IL-3, 10 ng/ml IL-6, 100 ng/ml G-CSF and 100 ng/ml of SCF (36GS) was identified as a potent stimulus for *ex vivo* generation of neutrophil precursor cells from mobilised blood or BM $CD34^+$ cells. Based on the data presented within chapters 3 and 4, it is therefore predicted that culture of 100-200 x 10^6 $CD34^+$ cells stimulated with 36GS would generate sufficient neutrophil precursors that, when infused, would shorten neutropenia associated with chemotherapy induced myelosuppression. Although this could not be tested in the present work, subsequent studies from other investigators have essentially proven this hypothesis. These clinical studies are discussed below in greater detail.

b) *Ex vivo* generation of myeloid progenitors and specifically neutrophil precursors from $CD34^+$ cells under static culture systems is limited by cell density. The data presented in chapter 4 demonstrate that when the concentration of cells in static culture exceeds $1 - 1.5 \times 10^6$ cells/ml then cell death exceeds cell proliferation, resulting in a dramatic decline in the number of viable cells. This feature of static culture systems is a major concern for investigators wishing to generate sufficient viable haemopoietic cells for therapy. The main implication is that large numbers of $CD34^+$ cells ($100 - 200 \times 10^6$) need to be cultured in large volumes (5-10 litres) of media to ensure the "terminal" cell density is not exceeded and cell viability remains high. Therefore, cultures must be established in multiple bags or flasks, and washing and concentration of the final cellular product is required to yield a safe volume for infusion into patients. The data presented in chapter 4 also suggest that culture systems where pH, lactic acid, glucose and HGF concentrations are monitored and controlled would most likely yield significant improvements in the growth of cells. Thus, culture of HPC under perfusion conditions where toxic metabolites can be removed, and HGF and nutrients regularly replenished should result in greater generation of cells. It is predicted that such cultures would be more reproducible and generate a greater number of cells from fewer HPC. This prediction has since been confirmed with cultures of haemopoietic cells in a flat-bed perfusion chamber (Horner *et al* 1998).

c) The generation of neutrophil precursors during a 14-day *ex vivo* culture of $CD34^+$ cells is mainly attributed to committed HPC that express high levels of CD38 and HLA-DR antigens. The data presented in chapter 5 demonstrate that $CD34^+CD38^+$/$CD34^+$HLA-DR^+ cells proliferate in response to single, late acting HGF such as G-CSF or GM-CSF. Furthermore, when combinations of HGF such as 36GS are used, committed HPC generate significantly greater numbers of myeloid cells at day 14 than their primitive ancestral precursors ($CD34^+CD38^-$ or $CD34^+$HLA-DR^- cells). These data strongly suggest that isolation and *ex vivo* culture of the total $CD34^+$ cell fraction from mobilised blood or BM is sufficient for *ex vivo* generation of neutrophil cells for therapy: the data does not support isolation and culture of primitive HPC within the total $CD34^+$ cell fraction for this purpose.

d) In accord with the results from transplantation studies in both NOD/SCID mice and pre-immune foetal sheep, the data from Pre-CFU cultures in chapter 5 suggest that primitive HPC are highly enriched within the $CD34^+CD38^-$ fraction of BM cells. Subsequently, experiments investigating the effect of FLT3L and MGDF/TPO (chapter 6) on survival, division and proliferation of primitive HPC were performed with this cell population. Notably, a key finding from these studies was that $CD34^+CD38^-$ cells, (representing 5-10% of $CD34^+$ cells) were heterogenous with respect to their response to HGF and their proliferative potential. For example, although greater than 90% of $CD34^+CD38^-$ cells divide following stimulation in a combination 36GSFT, only a limited proportion of dividing cells display the ability to generate nascent $CD34^+$ or $CD34^+CD38^-$ cells.

e) Analysis of HGFR expression indicates that $CD34^+$ cells exhibit heterogeneous expression of receptors for IL-3, IL-6, G-CSF, GM-CSF and to a lesser extent SCF. In spite of this, the pattern of HGFR expression on $CD34^+$ cells is of some value in discriminating between myeloid and erythroid progenitors and committed HPC from primitive HPC. Firstly, there is a strong inverse correlation between expression of receptors for IL-6, G-CSF, and GM-CSF and commitment to the erythroid lineage: there is significant enrichment of BFU-E in fractions of cells that do not express the IL-6R, G-CSFR and GM-CSFR. Secondly, as indicated by the generation of nascent CFU-GM and nucleated cells after 4 weeks in Pre-CFU culture, primitive HPC may not constitutively express the IL-6R, G-CSFR, GM-CSFR or the β_c chain. In contrast, expression of *flt*3 and c-*mpl* appears restricted to a minor fraction of $CD34^+$ cells enriched for primitive HPC, an intriguing observation given the demonstrated need for simultaneous stimulation with the ligands for these receptors, (FLT3L and MGDF/TPO respectively), to induce cell division. In this respect, the studies on HGFR expression also highlight an interesting biological difference between committed HPC and primitive HPC: both fractions of HPC express a broad, albeit different, range of HGFR yet committed HPC divide and proliferate when stimulated by single HGF such as G-CSF or GM-CSF whereas primitive HPC display very limited proliferation following stimulation by single cytokines. The repertoire of HGFR expression does not necessarily predict the biological response.

f) Primitive human HPC within the $CD34^+CD38^-$ fraction of BM require stimulation by combinations of early acting HGF to induce cell division. In this respect both FLT3L and TPO were shown to be key cytokines, as demonstrated by the observation that addition of either to a combination of 36GS results in a doubling in the proportion of primitive HPC that divide. Furthermore, when both FLT3L and TPO are added together to 36GS, greater than 90% of single primitive HPC (defined as $CD34^+CD38^-$ cells) divide. Subsequent studies demonstrated that this response is attributed to the combined effects of simultaneous stimulation with the early acting factors SCF, FLT3L and TPO. In accord with these observations I propose that primitive HPC can be hierarchically ordered according to their growth factor requirements when cultured under stromal-free cytokine dependent conditions. As presented in figure 6.20 the model suggests that recruitment of primitive cells from quiescence is directly related to the combination of HGF used for stimulation. Thus, the most primitive HPC are suggested to be in deep Go and display an obligate need for stimulation by the combination of IL-3, IL-6, G-CSF, SCF, FLT3L and TPO/MGDF before they divide.

7.2 Outstanding Questions

Although the present studies have provided new information and a greater understanding of the role of HGF in regulating the *ex vivo* growth of human HPC, a number of important questions remain.

7.2.1 Will Infusion of Ex *Vivo* Expanded Cells Provide Therapeutic Benefit?

Unfortunately, during the course of this work this question could not be addressed. Nevertheless, there have been a number of informative studies published within the last 5 years demonstrating that *ex vivo* generated cells can contribute to haemopoietic recovery in human patients. Since 1995 there have been 14 published reports of patients being transplanted or infused with *ex vivo* cultured HPC. Collectively, these studies, listed in table 7.1, have recorded 164 patients receiving cultured cells with no reports of toxicity or adverse events. However there is a wide discrepancy in the documented effects on haemopoietic recovery (HR) attributed to the infused cells. Nine of the studies were performed with mobilised blood cells with the majority (8 of the 9) involving cultures initiated with $CD34^+$ -

Table 7.1

Clinical studies performed with ex vivo cultured human haemopoietic progenitor cells.

Abbreviations: NAE represents No Adverse Events, MM represents multiple myeloma, HR refers to haemopoietic recovery, MBC represents metastatic breast cancer, NHL represents non-hodgkins lymphoma, MSC represents mesenchymal stem cells.

Year	Author	Cell Source	Culture Conditions	Patient Group	Outcome
1995	Gluck	Apheresis MNC	7 days, IL-3, IL-6, G-CSF, GM-CSF, SCF, 20% FCS	3 with metastatic breast cancer (MBC)	NAE No improvement in HR
1995	Brugger	Autologous PBPC, selected $CD34^+$	12 days, IL-1, IL-3, IL-6, SCF, EPO, Autologous plasma	10 with advanced cancer	NAE No improvement in HR
1996	Alcorn	Autologous PBPC Selected $CD34^+$	8 days, IL-1, IL-3, IL-6, SCF, EPO, 5-10% autologous serum	10 with non-haemopoietic malignancies	NAE No improvement in HR
1996	Williams	Autologous PBPC Selected $CD34^+$	12 days, PIXY321, XVIVO-10 + 1% Human Serum Albumin	8 with MBC	NAE 2 patients with improved neutrophil recovery
1997	Holyoake	Autologous PBPC Selected $CD34^+$	8 days, IL-1, IL-3, IL-6, SCF, EPO, 5-10% autologous serum	4 patients, (2 MM, 2 NHL) conditioned with CY/TBI and Bu/Melphalan	Graft failure Infusion of unmanipulated MNC
1997	Bertolini	Autologous PBPC Selected $CD34^+$	IMDM + 5% AB serum, IL-3, IL-6, IL-11, SCF, FLT3L, MGDF and MIP-1α	10 patients (8 with breast cancer, 2 NHL) infused	NAE Prompt HR Reduced platelet support
1998	Kogler	UCB	10 days, XVIVO-10, 10% CB plasma, G-CSF, TPO, FLT3L	1 child with c-ALL	ANC of 410 at day 14, delayed platelet recovery
1999	Reiffers	Autologous Selected $CD34^+$ PBPC	12 days in G-CSF, SCF, MGDF	14 patients with newly diagnosed MM	Median duration of severe neutropaenia 1.5 days, Median <20,000 platelets of 1 day
1999	Bachier	BM MNC	Aastrom device, 12 days, EPO, Pixy321, FLT3L	5 patients with high-risk stage II or MBC	Equivalent HR to unmanipulated BM
2000	Pecora	Unrelated UCB	Aastrom Replicell System	2 adults with CML	Engrafted and in remission
2000	McNiece	UCB unrelated donors	Stericell Flask, G-CSF, SCF, MGDF	19 patients; 17 haemopoietic malignancies, 2 with breast cancer	500 ANC in 25 days Delayed platelet recovery (58 days to 20,000)
2000	Koc	Mobilised PB MNC	20-50 days for MSC	28 patients with advanced and breast cancer	NAE. ANC of 500 on day 8; 20,000 platelets on 8.5 days
2000	Stiff	BM	12 days, Aastrom device, EPO + Pixy321 + FLT3L	19 adults, breast cancer	500 ANC at 16 days
2000	McNiece	Autologous PB $CD34^+$ cells	10 days, G-CSF, SCF, MGDF	21 patients with breast cancer	Improved neutrophil recovery, no change in platelet

selected cells from apheresis collections. Bone marrow was used in 2 studies and 3 were performed with umbilical cord blood.

A common feature of these reported studies was the use of combinations of recombinant human HGF to stimulate growth and proliferation of cultured cells. In this respect, IL-1, IL-3, IL-6, IL-11, G-CSF, GM-CSF, SCF, FLT3L, MGDF, EPO, MIP1-alpha and the IL-3/GM-CSF fusion molecule PIXY321 have been used either alone or in various combinations in culture. It is also interesting to note that 11 of the 14 published studies were based on *ex vivo* culture of HPC in flasks or bags under static conditions similar to that described and used within this thesis. The remaining 3 reports were based on the use of the Aastrom perfusion bioreactor (Bachier *et al* 1999, Pecora *et al* 2000, Stiff *et al* 2000) where cell growth is dependent on the generation of an adherent stromal layer (Koller *et al* 1993 and 1998). The main objective of the *ex vivo* expansion culture in each of these published reports was to generate a fraction of cells that would enhance haemopoietic recovery. Of relevance to the present thesis, 10 of the published studies were performed with the objective of producing cells that could increase the rate of neutrophil or platelet recovery post transplant or high dose chemotherapy.

The first of these studies was performed in 1995, where 3 patients with breast cancer received apheresis MNC expanded for 7 days in a combination of IL-3, IL-6, G-CSF, GM-CSF, SCF and 20% foetal calf serum. An average of 3.5 x 10^9 cells were infused without toxicity or improved haemopoietic recovery (Gluck *et al* 1995). A similar study was performed with purified autologous $CD34^+$ cells cultured for 12 days in IL-1, IL-3, IL-6, SCF, EPO and autologous plasma. Ten patients were infused with the cultured cells yet HR was equivalent to that observed with unmanipulated PBPC (Brugger *et al* 1995). A further 2 studies involved culture of $CD34^+$-selected cells in combinations of HGF with either autologous serum (Alcorn *et al* 1996) or human serum albumin (Williams *et al* 1996). In retrospect, the study from a group in Chicago (Williams *et al* 1996) was ground breaking, in that it provided the first indication that infusion of sufficient *ex vivo* generated neutrophil precursors could improve the rate of neutrophil recovery in patients following chemotherapy; a remarkable outcome given that cells were stimulated with only the IL-3/GM-CSF fusion protein PIXY321. Of the 8 patients transplanted with *ex vivo* cultured cells, 2 received almost 1 x 10^{10} cells and these patients showed a slight reduction in the period of neutropenia. This study also described the use of two-colour immunophenotyping with CD11b and CD15 to monitor neutrophil precursor production in culture.

Until this stage no attempt had been made to use *ex vivo* culture cells following intensive myeloablative conditioning therapy. However, this was tested in 4 patients, following cyclophosphamide/total body irradiation or busulphan/melphalan conditioning. $CD34^+$ cells were cultured for 8 days and infused without unmanipulated PBPC. Unfortunately these patients failed to engraft and the unmanipulated, backup mobilised blood had to be infused (Holyoake *et al* 1997).

The first study designed specifically to improve platelet recovery by infusion of *ex vivo* generated cells was reported in 1997 and involved culture of mobilised blood $CD34^+$ cells in the combination of IL-3, IL-6, IL-11, SCF, FLT3L, MGDF and MIP-1alpha supplemented with 5% AB serum (Bertolini et 1997). A total of 10 patients received unmanipulated cells then 3 hours later the *ex vivo* generated cells. No adverse events were recorded and although HR was prompt in all patients the rate of platelet recovery was not improved.

Convincing evidence that infusion of *ex vivo* generated myeloid post-progenitor cells could significantly improve the rate of neutrophil and possibly platelet recovery was provided by the group in Bordeaux (Reiffers *et al* 1999). Autologous $CD34^+$ cells isolated from patients mobilised by chemotherapy and G-CSF were cultured for 12 days in large plastic tissue culture flasks containing 1 litre of a propriety media, supplemented with G-CSF, SCF and MGDF each at 100 ng/ml. Fourteen newly diagnosed multiple myeloma patients were infused with *ex vivo* expanded cells on day 0 then un-manipulated cells one day later. Patients were conditioned with 200 mg/m^2 melphalan or 140 mg/m^2 melphalan plus 8-10Gy of total body irradiation. G-CSF was administered following infusion of cells until the neutrophil count returned to safe levels. Haemopoietic recovery was compared to an age and sex matched historical control group of patients treated with an identical conditioning regimen and infusion of unmanipulated mobilised blood progenitor cells.

Patients receiving the *ex vivo* expanded cells had a median duration of severe neutropenia of 1.5 days as compared to 9.5 days in the historical control group transplanted with mobilised blood progenitors. In addition, there was only 1 day where the median platelet count was less than 20,000 per μL. Apart from demonstrating that neutropenia could be abrogated by infusion of *ex vivo* expanded neutrophil post-progenitors, this landmark study also provided evidence of a cell-dose effect in predicting the rate of haemopoietic recovery. As evidenced by their data, it was suggested that at least 1×10^{10} expanded maturing neutrophil and

megakaryocytic precursors were required to affect enhanced neutrophil and platelet recovery. This proposed cell dose is in accord with my initial hypothesis made some 7 years earlier (Haylock *et al* 1992) and with that predicted in a computer simulation of HR based on blood progenitor cell transplantation (Scheding *et al* 1999).

Subsequent studies based on culture of $CD34^+$ cells with G-CSF, SCF and MGDF have since confirmed and extended the data published by Reiffers *et al* (McNiece *et al* 1999, 2000 and 2000b). Notably, infusion of *ex vivo* expanded cells without unmanipulated mobilised blood progenitors has been shown to restore haemopoiesis following high dose myelosuppressive chemotherapy (McNiece *et al* 2000b). Infusion of *ex vivo* generated haemopoietic cells has also been tried to improve the rate of neutrophil and platelet recovery in patients being transplanted with umbilical cord blood (UCB) cells. Although UCB is a rich source of primitive HPC, the limited volume obtained from a single UCB collection has resulted in it being used predominantly for transplantation of children and small adults. Even then, transplantation with UCB is typically associated with prolonged periods of neutropenia and thrombocytopenia post engraftment (Rubinstein *et al* 1998). *Ex vivo* expansion of UCB $CD34^+$ cells could thus be performed with two objectives in mind. Firstly, to generate an increased number of long-term repopulating cells, thus enabling the tissue to be used for transplantation of larger recipients and secondly to generate progenitors and post progenitor cells capable of contributing to rapid neutrophil and platelet recovery. At present there is no convincing data demonstrating an increase in long-term repopulating HSC following *ex vivo* culture of UCB cells: this remains as a major objective for the proponents of *ex vivo* cellular therapy. In respect of the second of these objectives, infusion of *ex vivo* expanded cord blood $CD34^+$ cells has resulted in only a slight improvement in neutrophil recovery without a concomitant improvement in platelet recovery (Kogler *et al* 1998, McNiece 2000a) and also remains as a major problem and an ongoing objective for *ex vivo* expansion protocols.

Improved HR has also been attempted by infusion of a population of *ex vivo* generated mesenchymal precursor cells together with mobilised PBPC (Koc *et al* 2000). The mesenchymal cells were generated from a small volume of BM cultured over 20-50 days. Twenty-eight breast cancer patients were infused with 1 to 2.2 x 10^6 autologous mesenchymal cells/Kg over 15 minutes. There were no adverse events during infusion of these cells but no improvement in the rate of HR post PBPC transplantation. As is the case with all the clinical studies reported to date, the fate of infused, *ex vivo* generated cells was not assessed. It remains unclear whether these mesenchymal cells lodged within the

extravascular compartment of the bone marrow or were trapped in the vasculature of other organs.

Collectively, the clinical studies performed with *ex vivo* generated haemopoietic cells demonstrate that infusion of neutrophil precursor cells can improve neutrophil recovery following myelosuppressive chemotherapy. However, the mechanism(s) responsible for this improved HR remains to be elucidated. Although infused *ex vivo* expanded $CD34^+$ cells contains a mixture of maturing myeloid cells it is uncertain which cell types within this mix is responsible for the enhanced rate of neutrophil recovery. Unfortunately, none of the published studies include a comprehensive breakdown of cells generated in *ex vivo* culture and infused into patients. For example, by combining immunophenotyping with morphological examination of stained cytospin preparations the number of $CD34^+$ cells, myeloblasts or promyelocytes could have been determined and correlated to neutrophil recovery. Furthermore, none of the studies have attempted to track the fate of infused *ex vivo* expanded cells. Thus, it is uncertain what proportion of the infused cells actually lodge in the marrow. This issue may be particularly relevant where expanded cells are being cultured specifically for generation of platelet precursors. In this case, megakaryocytes or their precursors may be trapped within small blood vessels and never lodge within the BM.

A common feature of those studies demonstrating efficacy of infused *ex vivo* generated cells is the administration of G-CSF post graft, a practice presumably based on the perceived need to stimulate the continued differentiation of neutrophil precursors after infusion. However, this notion has not been formally proven, even though anecdotal reports suggest this to be the case (Prof Jossy Rieffers and Dr Ian McNiece, personal communications). Subsequent clinical studies should be designed to address this and other questions related to the mechanism(s) underlying the ability of *ex vivo* generated cells to abrogate neutropenia and to contribute to HR.

The data presented within thesis suggests that optimal expansion of myeloid progenitors and specifically neutrophil precursors from $CD34^+$ blood or BM cells could be achieved by combinations of HGF including IL-3, IL-6, G-CSF, SCF, FLT3L and TPO/MGDF. However, it is unlikely that this range of HGF will be available for clinical studies. In reality, this will depend on supply and support from competing pharmaceutical companies and registration of each HGF by regulatory agencies such as the United States Food and Drug Administration and the Australian Therapeutic Goods Administration. There I anticipate that

future clinical studies will be performed with cells stimulated by a limited range of HGF that can be supplied from a single manufacturer and under conditions that guarantee a safe and reproducible cellular product. Additionally, in Australia, *ex vivo* expansion cultures are required to be performed within GMP accredited laboratories where the procedures comply with the Blood and Tissue Act.

7.2.2 Can Ex *Vivo* Culture Result in Numerical Expansion of HSC?

The data presented in chapter 6 demonstrate that the majority of $CD34^+CD38^-$ cells from adult BM, divide in combinations of HGF including the key factors, SCF, FLT3L and TPO. Furthermore, a low proportion of the dividing cells generate nascent $CD34^+CD38^-$ cells. Does this mean that cells with haemopoietic stem cell function are being produced and found within the $CD34^+CD38^-$ fraction of cultured cells? This question was not addressed in the present study but remains highly controversial. Evidence to support this notion comes from studies where virtually all new cells produced within 4 days in expansion cultures initiated with UCB $CD34^+CD38^-$ cells retained this phenotype and the capacity to establish human haemopoiesis in NOD/SCID mice (Bhatia *et al* 1997). In contrast, expansion of UCB $CD34^+CD38^-$ cells in *ex vivo* culture during retroviral transduction was not accompanied by retention of NOD/SCID repopulating function (Dorrell *et al* 2000). This observation was in part explained by down-regulation of CD38 on $CD34^+$ cells during culture and the expression of CD13 and CD33 on nascent $CD34^+CD38^-$ cells. Thus the majority of $CD34^+CD38^-$ cells derived from cultured cells appeared to be fundamentally different compared with uncultured input cells because they showed evidence of significant myeloid maturation. In my experiments, similar multiparameter flowcytometric analysis was not performed on nascent $CD34^+$ cells generated by Pre-CFU culture of $CD34^+CD38^-$ BM cells stimulated with 36GSFT or the combination of SFT. These studies together with transplantation into NOD/SCID mice would significantly enhance the value of my observations and provide some insight as to whether culture of primitive human HPC in SFT or 36GSFDT can lead to numerical expansion of cells with stem cell function. Although these studies were not performed, my data suggest that stimulation of primitive human HPC with combinations of HGF including SCF, FLT3L and TPO may enhance retroviral mediated gene transfer into HSC and provide a potent means of expanding various haemopoietic cell types.

7.2.3 Why do Primitive HPC Require Simultaneous Stimulation With Multiple Cytokines to Induce Division?

A considerable amount is known concerning the specific intracellular biochemical pathways that are activated following ligation of SCF, FLT3L and MGDF/TPO with their cognate receptors c-*kit*, *flt*3 and c-*mpl*, respectively. However, as with most cytokine receptors, there is little evidence for a causal relationship between activation of these pathways and biological responses such as proliferation, survival and differentiation. The same applies in understanding the molecular basis for synergistic interactions of different cytokines on HPC. Thus while, the data presented in this thesis provide further examples of synergistic interactions of HGF on HPC growth and development, I have not sought to address the fundamental question of mechanism. This is particularly so with the interaction of SCF, FLT3L and MGDF/TPO on primitive HPC. The data presented within chapter 6 add to the abundant body of literature demonstrating that primitive HPC require simultaneous stimulation with multiple HGF to induce cell division. The molecular basis for this requirement remains to be determined but might involve overcoming the dominant effect of inhibitors such as TGF-β, the need for recruitment of novel signalling molecules following ligation of c-*kit*, c-*mpl* and *flt3*, or enhanced strength of signalling as a result of simultaneous stimulation via these 3 receptors. Most likely, genetic manipulation of signalling pathways will be important tools to elucidate the complexity and interactions between different receptors and their independent signalling pathways.

7.2.4 Will $CD34^-$ Cells Proliferate in Pre-CFU culture?

During the course of this thesis the presence of $CD34^-$ cells with *in vivo* haemopoietic activity were identified (Osawa *et al* 1996, Goodell *et al* 1997, Morel *et al* 1998, Zanjani *et al* 1998). This subset of cells can be identified in human UCB (Nakamura *et al* 1999), adult BM and mobilised peripheral blood (Fujisaki *et al* 1999) where they comprise between 0.3% and 0.58% of low density or total nucleated cells from these haemopoietic tissues. Assuming $CD34^-$ cells are hierarchically ancestral to $CD34^+$ HPC then *ex vivo* expansion of this population may lead to generation of $CD34^+$ progeny for clinical transplantation. Although this possibility was not tested in my current work, it has been investigated by at least 2 independent groups. Serum free cultures, similar to the serum-deplete Pre-CFU culture system used in this thesis demonstrated that less than 1% of $lin^-CD34^-CD38^-$ cells isolated from BM and mobilised blood proliferate in a combination of IL-3, FLT3L, SCF, TPO and hyper-IL-6 (Fujisaki *et al* 1999). In spite of the low proportion of dividing cells, approximately 10% of their progeny expressed CD34 and gave rise to colonies in

methylcellulose containing erythroblasts, granulocytes, macrophages and megakaryocytes, exclusively or mixtures of these. In contrast, in a separate study, lin^-CD34^- cells did not proliferate when cultured under similar serum deplete conditions following stimulation by 36GSFT (Nakamura *et al* 1999): the combination of HGF shown in the present study to be a potent stimulus for growth of human $CD34^+CD38^-$ cells. However, a small number of lin^-CD34^- cells survived and proliferated when cultured on the murine bone marrow stromal line HESS-5 in the presence of foetal calf serum and 36GSFT. Taken together, the results from these recent studies suggest that *ex vivo* growth and manipulation of $CD34^-$ cells may be dependent on yet to be defined stimulatory signals mediated by cell contact with stromal cells. Furthermore stimulation with the existing repertoire of soluble purified recombinant HGF will not be sufficient to support growth of $CD34^-$ cells. The definition of the cell adhesive interactions that might be involved in supporting growth of $CD34^-$ cells remain to be elucidated and is an obvious area for future investigation. *Ex vivo* manipulation of $CD34^-$ haemopoietic precursor cells for potential therapeutic application is therefore impractical at present.

7.3 Summary

The studies presented in this thesis describe the variables affecting *ex vivo* expansion of human HPC under stromal-free, cytokine dependent culture conditions. In accord with an abundant body of published literature, the data presented herein demonstrate that the choice of HGF or combinations of HGF determine in large part the type of cells generated in *ex vivo* culture of HPC. Thus, generation of sufficient neutrophil precursor cells for potential therapeutic use, from $CD34^+$ cells, can be achieved by a combination of IL-3, IL-6, G-CSF and SCF. Moreover, growth of primitive HPC, was shown to depend on combinations of early acting cytokines including IL-3, IL-6, SCF and FLT3L. Notably, TPO, a factor initially recognized for its ability to stimulate growth of megakaryocytic cells and production of platelets was demonstrated to be a potent survival factor and to interact synergistically with SCF and FLT3L in stimulating division of primitive HPC. Collectively, these studies lay a solid foundation for *ex vivo* manipulation of human haemopoietic stem and progenitor cells for clinical therapies including generation of cells for transplantation and gene therapy.

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