MOLECULAR CONTROL OF HAEMOPOIESIS

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Abstract

Haemopoiesis is the process by which the circulating blood cells are generated from a limited number of primitive cells, so-called stem cells. In adult, it occurs within the bone marrow where haemopoietic cells are found in intimate association with a complex stroma. In this specific microenvironment, the stromal cells, extracellular matrix components, adhesion molecules and cytokines provide the appropriate balance of signals that preserves the stem cell pool while permitting controlled proliferation and differentiation.

Using *in vitro* systems for culturing haemopoietic cells, the effects of cytokines FGF3, SDF-1α and, particularly, MIP-1α on haemopoiesis were investigated. FGF3 is an embryonic protein and, although its expression coincides with the establishment of haemopoiesis, no information about its influence on haemopoiesis is available. In contrast, MIP-1α is known as an inhibitor of haemopoietic cell proliferation while SDF-1α is particularly involved in their migration.

The first part of this work describe the approach taken to investigate the possible participation of FGF3 on haemopoiesis. When tested in LTBMC, greater number of haemopoietic progenitors in the adherent layer was found in cultures treated with FGF3. In addition, some delay in granulocyte/macrophage differentiation was observed when tested on FDCP-mix cells. These preliminary results suggest a FGF3 supportive effect on haemopoiesis *via* stimulation of stromal function, and provide some baseline to further explore the potential of FGF3 in influencing haemopoiesis.

In the second part of this thesis, the influence of MIP-1α and SDF-1α on the behaviour of haemopoietic progenitors was further explored. First, the distribution of their receptors CCR1 and CCR5, both binding MIP-1α, and CXCR4, that binds SDF-1α, on a variety of haemopoietic cell lines, on mononuclear cells from several human sources as well as on isolated $CD34^+$ cell populations was examined. Subsequently, the functionality of these receptors on $CD34^+$ cells was evaluated using proliferation, migration and adhesion assays. Further, the participation of CCR1 in mediating the MIP-1α responses of $CD34^+$ cord blood cells was examined.

CCR1 and CXCR4 were detected in all primary cell subpopulations examined. CCR1 expression is heterogeneously distributed amongst $CD34^+$ progenitors: $CD34^+$ cells expressing higher levels of CCR1 are enriched for GM-CFC progenitors, while erythroid progenitors are more concentrated within the population expressing very low levels of CCR1. Furthermore, CCR1 expression on GM-CFC progenitors was maintained throughout their differentiated progeny, while the erythroid progeny loses it as maturation is reached.

MIP-1α affected the proliferation of $CD34^+$ cells, inhibiting myeloid progenitors from bone marrow, while stimulating the GM-CFC population from cord blood. By using an anti-CCR1 antibody to block these effects, the first evidence that CCR1 is at least one of the receptors that mediates the MIP-1α proliferation, but not inhibition, of $CD34^+$ progenitors was provided. MIP-1α is a weaker chemoattractant for $CD34^+$ cells when compared to SDF-1α, but induces migration of progenitors. Moreover, both chemokines showed ability to induce adhesion of progenitors to fibronectin. The data are likely to be relevant to the biological properties of these molecules, and provided further evidence that MIP-1α and SDF-1α have other and more complex roles in haemopoiesis that certainly warrants further investigation. Also, our results provide a base from which to explore their influence on the mechanisms that govern haemopoiesis exploiting specific ligand/receptor interactions.

Declaration

I declare that this thesis is my own composition, and that no portion of the work contained herein has been submitted in support of an application for another degree or qualification of this or any other University or Institute of learning.

Almeriane Maria Weffort Santos

Dedication

To Cid, Flora and Celso

"Let the word of Christ dwell in you richly as you teach and admonish
one another with all wisdom, and sing psalms, hymns
and spiritual songs with gratitude in your hearts to God.
And whatever you do, whether in word or deed,
do it all in the name of the Lord Jesus,
giving thanks to God, the Father, through him."

Colossians, 3:16-17.

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List of Abbreviations

Ab	antibody
ACDU	automatic cell deposition unit
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
AL	adherent layer
AMP	adenosine monophosphate
APAAP	alkaline phosphatase – anti-alkaline phosphatase
ATP	adenosine triphosphate
BFU-E	burst forming colonies – erythroid
BLC-1	B-lymphocyte chemoattractant 1
BM	bone marrow
BMT	bone marrow transplantation
bp	base pair
BrdU	5-Bromo-2'-deoxy-uridine
BSA	bovine serum albumin
CAFC	cobblestone-forming area cell
CB	umbilical/cord blood
C	γ chemokine
CC	β chemokine
CCR	β chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CFC	colony-forming cell
CFU-A	colony-forming units - agar
CFU-E	colony-forming units – erythroid
CFU-S	colony-forming units – spleen
Ci	Curie
CHO	Chinese hamster ovary
CIP	calf intestinal alkaline phosphatase
CM	conditioned medium
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CSF	colony stimulating factor
CTACK	cutaneous T cell-attracting chemokine
CXC	α chemokine
CXCR	α chemokine receptor
DAB	diaminobenzidine
DARC	Duffy antigen receptor for chemokines
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
DEAE	diethylaminoethyl
dGTP	2'-deoxyguanosine 5'-triphosphate
DG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	de-oxiribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate

DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetracetic acid
EGF	epithelial growth factor
EGTA	ethylene glycol-bis (ß-aminoethyl ether) N,N,N,N,'-tetracetic acid
ELTC	Extended stroma-dependent long-term cultures
EPO	erythropoietin
EPO-R	erythropoietin receptor
ES	embryonic stem cells
FACS	fluorescent-activated cell sorting
FCS	foetal calf serum
FDCP-mix	Factor Dependent Cells Paterson, Mixed Potential
FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
flk-2	foetal liver kinase, 2
Flt3	fms-like tyrosine kinase-3
f-MLP	N-formyl-methionyl-leucyl-phenylalanine
5-FU	5-fluorouracil
g	grams
G	protein G complex G; gravity force; needle gauge
GAM	goat anti-mouse immunoglobulins
G-CFC	granulocyte colony-forming cell
GCP	Granulocyte chemoattractant protein
GDP	guanidine diphosphate
GFP	green fluorescent protein
G-M	granulocyte/macrophage
GM-CFC	granulocyte/macrophage colony-forming cell
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor
GRO	Growth-related oncogene
GTP	guanidine triphosphate
HCC	Hemofiltrate CC chemokine
Hepes	N-(2-hydroxyethyl) piperazine-N'-(ethanesulfonic acid)
HIM	haemopoietic microenvironment
HIV	human immunodeficiency virus
HPC	haemopoietic progenitor cells
HPP-CFC	high proliferative potential-colony forming cell
HS	horse serum; heparan sulphate
HSC	haemopoietic stem cell
HSPC	haemopoietic stem/progenitor cells
IC	immunocytochemistry
ICAM-1	intracellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	interleukin
IL-3 CM	interleukin three conditioned medium
IL-8RB	interleukin-8 receptor, subtype B

IMDM	Iscove's modified Dulbecco's medium
IP-10	γ-interferon-inducible protein 10
IPTG	isopropylthio-β-D-galactoside
IRES	internal ribosome entry site
I-TAC	interpheron-inducible T cell alpha chemoattractant
IU	International Units
kb	kilobase
kDa	kiloDaltons
L	litre
LB	Luria Bertani
LFA-1	leukocyte function associated antigen 1
LIF	leukaemia inhibitory factor
LIX	LPS-induced CXC chemokine
LKN	leukotactin
LMP	low melting point
LPS	lipo-polyssacharide
LTBMC	long-term bone marrow culture
LTC	long-term culture
LTC-IC	long-term culture initiating cell
MAPK	Mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor
MDC	Macrophage-derived chemokine
MFI	mean fluorescence intensity
mg	milligrams
MIG	Monocyte induced γ-interferon
MIP	Macrophage Inflammatory protein
MIP-1α	macrophage inflammatory protein 1 alpha
MIPF-1	Myeloid progenitor inhibitor factor 1
ml	millilitre
MMTV	mouse mammary tomour virus
MNC	mononuclear cells
MoAb	monoclonal antibody
MoMuLV	Moloney Murine Leukaemia Virus
mOsm	milliosmole
MPIF	myeloid progenitor inhibitor factor
MRA	marrow repopulating assay
MRP-1	correspond to Murine C10 receptor
mRNA	messenger ribonucleic acid
NaOAc	sodium acetate
NAP	Neutrophil activating protein 1
NBCS	new born calf serum
NBME IV	normal bone marrow extract, fraction IV
ng	nanograms
NK cells	natural killer lymphocytes
NOD/SCID	non-obese diabetics/sever combined immunodeficiency
OD	optical density, absorbance measure
ON	overnight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PB	peripheral blood
PBS	phosphate buffered saline
PE	phycoerythrin
PEG	polyethylene glycol
PI_3	phosphatidylinositol 3 or inositol 1,4,5-triphosphate
PIP_2	phosphatidyl-inositol 4,5-bisphosphate
PKC	Protein Kinase C
PLC	phospholipase C
PLCβ2	phospholipase beta 2
QFACS	quantitative fluorescent activated cell sorting
RANTES	regulated on activation normal T, expressed and secreted
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SCF	stem cell factor
SCI	stem cell inhibitor
SDF-1	stromal-derived factor 1
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TAE	Tris-Acetate-EDTA
TARC	Thymus and activation-regulated chemokine
TBS	tris - buffered saline
TCA	tri-chloroacetic acid
TCA-4	T-cell activation gene 4
TE	tris-ethylene diamine tetra-acetate
TECK	Thymus expressed chemokine
TNF	tumour necrosis factor
TPO	thrombopoietin
U	unit/s
UV	ultra violet
V	voltage
VCAM-1	vascular cell adhesion molecule 1
XFGF3	fibroblast growth factor 3, from *Xenopus laevis*
X-gal	5-bromo-4-chloro-3 indolyl-β-D-galactopyroside
WT	wild type

List of Symbols

α	alpha
β	beta
°C	temperature at Celsius' degrees
γ	gamma
μF	microFaraday
μg	micrograms
μl	microlitre
%	percentage

Chapter 1

General introduction

The multicellular organisms are the result of simultaneous processes initiated from a single cell – the fertilised egg, which divides intensively to give rise to many millions of differentiated cells. The processes of cellular proliferation, differentiation, and progressive acquisition of specialised phenotype start during embryogenesis, where a limited number of primitive cells termed stem cells gives rise to a much larger population of more restricted progenitor cells that also proliferate to produce functionally mature cells (Wolpert *et al.*, 1998).

Although strictly regulated by mechanisms that are not entirely understood, the mammalian haemopoietic system is one of the best examples of such hierarchical nature. All the blood cells in the adult mammal originate from a population of pluripotent stem cells located in the bone marrow (BM). These stem cells are self-renewing and are the precursors of progenitor cells that become irreversibly committed to one of the haemopoietic lineages at a later stage. Haemopoiesis is in effect a complete developmental system in miniature, in which a single cell, the stem cell, gives rise to different cell types. Most of these are short-lived and must be replaced continuously throughout adult life (Klein, 1990; Metcalf, 1988).

Two main models have been proposed to account for the maintenance over haemopoiesis. In the first model, a fixed number of stem cells are laid down during embryogenesis. These cells are successively and randomly recruited into proliferation and differentiation to mature cells. As these cells proliferate, they undergo a progressive loss in their ability to self-renew to produce more stem cells (Brecher *et al.*, 1967; Hellman *et al.*, 1978). The second model suggests that a small number of stem cells develop during embryogenesis and these cells can reproduce and undergo extensive self-renewal, *i. e.* producing daughter cells with the same proliferative and

developmental potential as the parental cells. Inly a proportion of these cells will then undergo differentiation and maturation (Harrison, 1979). Clearly, the haemopoietic system is organised to ensure that this regenerative capacity persists throughout life and that sufficient flexibility exists to maintain normal steady state blood production while still allowing extra production for one or more types of cells required under stress (Orkin, 1995; Orkin, 1996). Therefore, haemopoiesis is a vigorous and dynamic *continuum* process of blood cell production that occurs primarily within bone marrow cavities and encompasses the formation, development, and specialisation of all formed elements (stem cells, and progenitor cells) into functional blood cells.

1.1. The Haemopoietic System

Development of haemopoiesis proceeds through two distinct stages, *ie.* primitive and definitive haemopoiesis. In the mammalian embryo, haemopoiesis changes from its initial location in the yolk sac blood islands to the foetal liver, and then to the BM (Lansdorp, 1995). This transitional process has been well characterised in mice, in which primitive haemopoiesis begins at 7.5 days *post coitum* (dpc) in the extraembryonic yolk sac and lasts for a few days. In contrast, definitive haemopoiesis takes place throughout the life (Dzierzak and Medvinsky, 1995; Robb, 1997; Yoder *et al.*, 1997; Zon, 1995).

Whilst primitive haemopoiesis generates mostly nucleated erythrocytes and macrophages, definitive haemopoiesis is distinguished by non nucleated erythrocytes, myelopoiesis, lymphopoiesis and generation of long-term repopulating haemopoietic stem cells (LTR-HSC). Although lymphocytic cells and multipotential haemopoietic progenitors are detected in the para-aortic splanchnopleura region of mouse embryos at 7.5-9.5 dpc (Cumano *et al.*, 1996; Delassus and Cumano, 1996; Godin *et al.*, 1995), LTR-HSC first appear in the aorta-gonad-mesonephros (AGM) region at 10.5-11.5 dpc (Dzierzak *et al.*, 1997; Medvinsky and Dzierzak, 1996; Muller *et al.*, 1994) and migrate to the foetal liver where massive expansion of haemopoietic cells takes place. LTR-HSC further migrate to the bone marrow.

In humans, the first detectable precursors of the myeloid cells are found in the yolk sac. From week 6 until 6-7 months of foetal life, the processes of blood cell formation predominantly occur in the liver. Migration of cells from foetal liver colonises the spleen and later the BM, which is fully active by the seventh to eighth

month (Wolpert *et al.*, 1998). Shortly after birth, haemopoiesis in the liver ceases, and practically the whole skeleton contains active marrow. During childhood and adolescence, a marked recession of marrow activity in the long bones occurs, and gradually, in the adults, such activity remains limited to the axial skeleton and skull, only a little remaining in the proximal ends of the humoral and femoral shafts. Also, during childhood, there is a progressive fatty replacement of marrow so that in the adult the marrow consists of islands of cellular activity separated by fat. Between the sinuses and sinusoids of the BM lie stem cells and precursor cells which, by the processes of multiplication, differentiation and maturation form the mature circulating cells (Rodeck and Whittle, 1999).

Mammalian blood cells comprise at least eight lineages of fully differentiated cell types, together with immature cells at various stages of differentiation. Thus, the haemopoietic system is best viewed as a *continuum* of cells in various stages of lineage-commitment, differentiation, and development. These cell types are derived from the myeloid and lymphoid lineages. The former yields the myeloid series, which include the red blood cells, the leukocytes, and the megakaryocytes. The lymphoid lineage produces the lymphoid cells, which comprise the two antigen-specific cell types of the immune system, the B and T lymphocytes. In mammals, the B and T precursors are progeny of the bone marrow stem cells, and while B lymphocytes develop in the BM, T cells develop in the thymus. Natural killer (NK) cells are found in small numbers (1-10%) in peripheral blood (Civin and Loken, 1987). NK cells were initially described on the basis of their ability to lyse certain tumor cells. NK cells have long been considered to be lymphocytes based on morphology. In addition, their functional activity most closely resembles T cells. Moreover, NK cells have Fc receptors and constitute an early innate immune response effector cell, more in common with macrophages, although NK cells are not phagocytic. The most definitive demon-stration that NK cells belong to the lymphocyte lineage was provided by study of the Ikaros gene that encodes a DNA-binding protein, selectively expressed in B and T cells. Targeted mutation in this gene by homologous recombination resulted in complete deficiency of B, T, and NK cells. There were no abnormalities in other bone marrow lineages, clearly indicating that NK cells are derived from lymphocyte precursors (reviewed in Yokoyama, 1997).

1.1.1. The functions of the blood cells

The mature erythrocytes of the human BM are non nucleated cells and contain no organelles. Their function is to mediate the exchange of respiratory gases, oxygen and carbon dioxide, between the lungs and the tissues, based upon the specialised properties of the haemoglobin molecule, the most important component of the erythrocytes. The thrombocytes, or platelets, are formed in the BM by their precursors, the megakaryocytes. Their main function is in the blood clotting mechanisms during normal haemostatic response to vascular injury. Central to this function are the platelet reactions of adhesion to the exposed sub-endothelial connective tissues after blood vessel injury, release of platelet granules, aggregation and fusion leading to the process of coagulation.

The remaining cells are often broadly classified as white blood cells or leukocytes, and in healthy conditions, at least five different leukocyte types are found circulating into the blood vessels. White cells are divided into granulocytes (also known as polymorphonuclear leukocytes, as they have non-uniform nuclear shape) and mononuclear cells (referring to the round shape of their nucleus). There are three types of granulocytes, all produced in the BM: neutrophils, eosinophils, and basophils. Neutrophils spend 6-9 hours in the blood stream before moving from capillaries into tissues (Springer, 1994). Their major function is as tissue phagocytes, moving preferentially to sites of infection or inflammation where they ingest, kill and break down bacteria (phagocytosis). The process of moving to sites of infection or inflammation is known as chemotaxis and occurs in response to activated complement components and chemical signals released by a variety of cells (reviewed in Carlos and Harlan, 1994). Eosinophils also respond to chemotactic stimuli, are phagocytic and kill ingested organisms. They play important roles in the body's defence against tissue parasites, being able to discharge their granule contents extracellularly, seriously damaging large parasites. They are involved in allergic reactions, along with basophils and mast cells. Basophils have a role in inflammatory responses and in allergic reactions.

Lymphocytes and monocytes are types of mononuclear leukocytes due to their usually round to oval nucleus shape. Lymphocytes are the second most numerous circulating white cells after neutrophils. They are produced from stem cells in the marrow and their function is performed particularly in tissues such as lymph nodes,

spleen, tonsils, and the lymphoid tissue associated with mucous membranes. They circulate in the blood stream, enter lymphoid tissues and emerge again from lymphoid tissues into lymphatic channels, where they form one constituent of the lymph. Lymphatics drain into thoracic duct and ultimately into blood stream. This process of continuing movement between tissues and blood stream is called lymphocyte re-circulation. Lymphocytes function in the body's immune responses, and are further divided into three functional categories:

- B cells, which develop and differentiate into plasma cells in the bone marrow environment, secreting antibodies, thereby providing humoral immunity;
- T cells, which develop in the thymus and function in cell-mediated immunity and also modulate B cell function;
- NK cells, or natural killer lymphocytes, which function in cell-mediated immunity alongside T cells.

Monocytes function mainly in tissues where they differentiate into long-lived macrophages. Both monocytes and macrophages respond to chemotactic stimuli and are phagocytic. They are part of the body's defence against bacterial and fungal infections and also ingest and break down dead and dying body cells. They present antigens to lymphocytes and secrete messengers, known as cytokines, which influence the behaviour of blood cells and their precursors. A number of specialised types of tissue macrophage are specific to different organs, such as the Kupffer cells of the liver, and the microglial cells of the brain (Bain, 1996; Lee *et al.*, 1993).

1.1.2. Hierarchy within the Haemopoietic System

For the purpose of understanding haemopoiesis as a whole, the cell populations involved in the generation of mature haemopoietic cells can be divided into at least three compartments based on their proliferating ability, differentiation potential and functional characteristics. These compartments are the stem cells, the committed progenitor cells and the morphological recognisable maturing cells. A general scheme of the haemopoietic system is shown in Figure 1.1.

1.1.2.1. Stem cells

The stem cells cannot be morphologically identified as yet, since a homogeneous population has not been isolated. Haemopoietic tissues contain very few stem cells. In

the bone marrow of adult mouse, they have been stimated as 500 MRA per 10^5 bone marrow cells or 10^5 per femour (Lord, 1997). Their existence was formally demonstrated using the murine *in vivo* spleen colony-forming assay, which detected a cell type with the properties of both self-renewal and multipotentiality (colony-forming units – spleen, CFU-S). It was shown that when haemopoietic cells were injected into potentially irradiated mice, some of the cells migrated into the spleen, where they lodged and proliferated, forming discrete nodules (Till and McCulloch, 1961), which consisted largely of maturing haemopoietic cells and their precursors (Lord and Schofield, 1985).

Subsequent work showed that these nodules, or colonies, were derived from single cells and contained multiple cell lineages (Wu *et al.*, 1967). In addition, some of the colonies contained primitive cells that could form more spleen colonies when infused into secondary irradiated mice. Taking together, these findings have demonstrated that the CFU-S derived from multipotent cells, were clonal, and some of them could undergo self-renewal. However, it was shown that there is heterogeneity among CFU-S, where not only multipotent stem cells which retain the multipotential nature are included, but also more mature cells that have lost the capacity to fully re-establish haemopoiesis (Magli *et al.*, 1982).

The functional approach described above led to the first definition of stem cells as cells with extensive self-maintaining capacity or self-renewal, *i.e* that have the ability to maintain their own numbers extending throughout the whole life span of the organism. In addition, stem cells are defined as pluripotent as they are capable of giving rise to all haemopoietic lineages (Lajtha, 1979). This *in vivo* technique is not applicable for assessing stem cells of human origin. However, in the haemopoietic tissues of many species, including man, there is a population of mixed differentiation potential that can be grown as colonies *in vitro* when appropriately stimulated (Fauser and Messner, 1978; Fauser and Messner, 1979; Metcalf *et al.*, 1979). These colonies contain all the different cells of myeloid lineages, and their growth requires the presence of stimulating molecules present in the culture medium. Thus, these mixed colony-forming cells (Mix-CFC or GEMM-CFC) can undergo some, albeit limited, self-renewal, *i.e* at least some of the Mix-CFC may be equivalent to a multipotential, self-renewing murine stem cells and that some overlap between the populations of CFU-S and Mix-CFC exists (Testa, 1985).

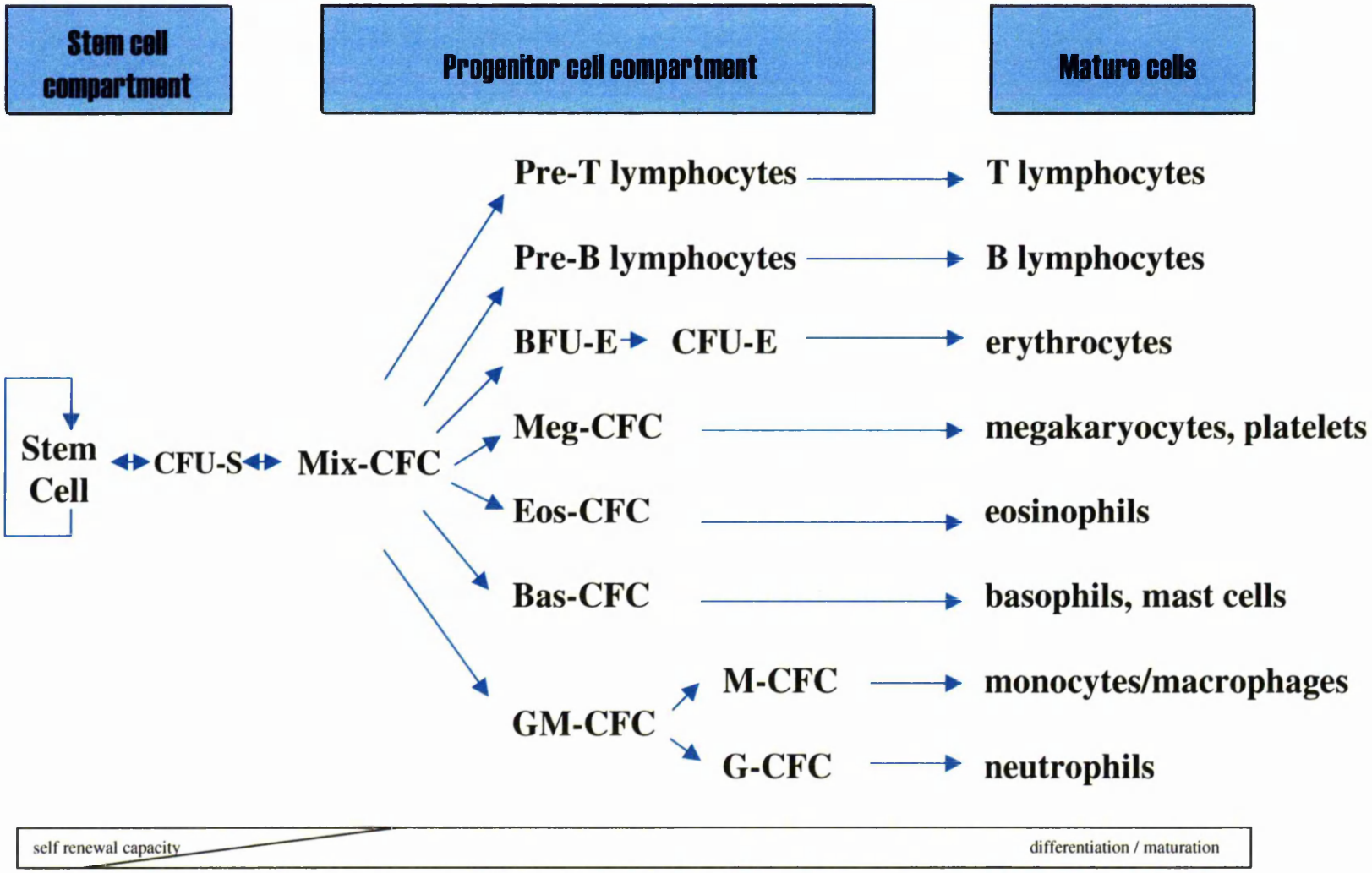


Figure 1.1. The structure of the haemopoietic system. Development of the haemopoietic tissue from the primary stem cell and through the progenitor cell compartment from which differentiation into various cell types arises.

The definition of a stem cell has become progressively more refined and is based on functional capabilities, which can be only assessed testing the abilities of these cells in specific experimental settings. Thus, stemness is a *spectrum* of capabilities and to define a cell as a stem cell of a particular tissue the following points should be satisfied:

- lack of certain specific differentiation markers (undifferentiated cell);
- capable of proliferation;
- able to self-maintain its population;
- able to produce a large number of differentiated, functional progeny;
- to regenerate the tissue after injury, and
- flexible use of these functions (Potten and Loeffler, 1990; Wright and Lord, 1992).

This concept to describe structured cell population in tissues involving stem cells, transit cells and mature cells is based on the assumption that the proliferation and differentiation/maturation processes are in principle independent in the sense that each may proceed without necessarily affecting the other. Stem cells may divide without maturation, while cells approaching functional competence may mature but do not divide. In contrast, transit cells divide and mature showing intermediate properties between stem cells and mature functional cells (Loeffler and Potten, 1977). In this context, a recent method has been described to assess the proliferative capacity of a cell population by measuring the telomere lengths of their chromosomes. Telomerase is an enzyme that avoids the loss of the terminal DNA segments when linear DNA is replicated and has been found in human testes, in ovarian follicles and in some somatic tissues, including human blood cells (Counter *et al.*, 1995; Kim *et al.*, 1994). It has been shown that primitive bone marrow haemopoietic cells express low but detectable levels of telomerase. Under cytokine stimulation, transient expression of telomerase activity was observed only in the early progenitor cells (Chiu *et al.*, 1996). These observations suggest that telomerase is active in high replicating tissues but not in differentiated cells. Its loss would result in a shortening of the telomeres such that progressive shortening should be expected along with the proliferation/differentiation/maturation pathways during haemopoiesis.

For several years the concept of haemopoietic stem cells as the progenitors for all blood cells was well established. Extending from this idea, researchers have provided evidence for stem cells for particular tissues such as satellite cells in muscle and neural

stem cells in brain (Orkin, 2000). However, the conventional ideas of the relationships between stem cells for specific tissues have been challenged by *in vivo* findings. Relevant in this context are data showing that populations of presumptive muscle stem cells contribute to the haemopoietic system upon transplantation into mice (Jackson *et al.*, 1999), and conversely, that purified haemopoietic stem cells contribute to muscle (Gussoni *et al.*, 1999). *In vitro* cultures of muscle cells seem to augment their ability to contribute to haemopoiesis, arguing that external cues might be able to redirect cell fates (Jackson *et al.*, 1999). A close relationship between haemopoietic stem cells and vascular progenitors is also supported by the presence of circulating endothelial precursors, which share with haemopoietic cells the cell surface antigen CD34 (Wood *et al.*, 1997). The common mesodermal origin of haemopoietic stem cells, muscle and endothelial cells can be conceptually reconciled with their inter-relationship.

More interesting, however, is the described generation of haemopoietic cells from cultured, clonal neural stem cells (Bjornson *et al.*, 1999), which are from ectodermal origin. Even more recently the contribution of purified haemopoietic stem cells to hepatic oval cells, possible hepatocyte precursors, and to hepatocytes in the mouse has been documented (Theise *et al.*, 2000a; Theise *et al.*, 2000b). The signals that elicit these cellular transformations are not known and for sure they will be the focus of much research in the coming years. Therefore, it will not be surprising if in a near future, the 'plasticity' of a cell will be included as one of the characteristics to fulfil the concept of a stem cell.

1.1.2.1.1. Enrichment of haemopoietic stem/progenitor cells

Much effort has been expended on attempts to physically isolate and characterise stem cells. Stem cells firstly emerged as typically small blast-like cells, few and small mitochondria, a nucleus with several nucleoli and little condensed chromatin (Bain, 1996; Carr and Rodak, 1999), with a relatively low buoyant density (Shortman, 1972) and counterflow centrifugal elutriation (Jones *et al.*, 1990). Using some of these characteristics, it has been demonstrated that primitive cells have a broad forward light scatter distribution occurring at detectable levels by flow cytometry in the region between the erythrocyte and small lymphocyte areas (Civin and Loken, 1987; Sutherland *et al.*, 1996).

Since there is a *continuum* between long-term repopulating stem cells and their more differentiated progeny, progenitor/transit cells possess certain characteristics in common with stem cells and some in common with mature functional cells. In this context, haemopoietic cells differentiate gradually and the changes are marked by the acquisition or loss of specific phenotypic characteristics. One of the most useful markers that has been used to characterise haemopoietic stem/progenitor cells is the CD34 molecule (Civin *et al.*, 1984). Early experiments have shown that after re-infusing human bone marrow $CD34^+$ cells into patients following myeloablative chemotherapy, haemopoiesis was re-established (Berenson *et al.*, 1991). Later, the demonstration that $CD34^+$ cells selected from peripheral blood progenitor cell harvests from cancer patients could achieve clinical engraftment (Brugger *et al.*, 1994; Shpall *et al.*, 1992) led to the conclusion that the cells responsible for the engraftment reside within the CD34 compartment.

The CD34 antigen is a 105- to 120kDa heavily glycosylated protein, member of the sialomucin family of surface molecules, encoded by a gene mapped to chromosome 1 in humans (Tenen *et al.*, 1990) and mice (Brown *et al.*, 1991). Approximately 1 to 5% of normal bone marrow cells (Berenson *et al.*, 1991; Civin *et al.*, 1990; Krause *et al.*, 1996) and less than 0.5% of peripheral blood cells (Civin *et al.*, 1987) express CD34, which can be detected using specific antibodies. At least 14 mouse monoclonal antibodies have been raised against the CD34 antigen, which identify at least nine different, but closely linked, epitopes whilst three of them are known to recognize non human, primate bone marrow cells (Krause *et al.*, 1996; Lansdorp *et al.*, 1989; Watt and Vissert, 1992). The binding epitopes of each monoclonal antibody on the heavily glycosylated CD34 protein are differentially sensitive to neuraminidase and to the glycoprotease from *Pasteurella haemolytica*. While neuraminidase cleaves sialic acid residues, the bacterial glycoprotease selectively cleaves sialylated *O*-linked glycans. Based on such distinct properties, the monoclonal antibodies have been grouped into three classes. Class I antibodies are directed against epitopes that are sensitive to the three enzymes (*e.g.*, MY10 or HPCA-1, B1.3C5, 12.8 and ICH3 clones); class II antibodies are represented by QBEND10, which detects an epitope sensitive to the bacterial glycoprotease, and class III antibodies, which epitopes are insensitive to all these enzymes and are represented by the 8G12 or HPCA-2 (Lansdorp *et al.*, 1990), TUK3 and 115.2 antibodies (Krause *et al.*, 1996; Lansdorp *et al.*, 1989; Watt and Vissert, 1992).

$CD34^+$ is first detected during human embryonic development at week 5 of gestation in the dorsal aorta region and in the embryonic liver (Tavian *et al.*, 1999). In adults, the $CD34^+$ cell population encompasses both multipotent and unipotent progenitors and its expression is downregulated as the immature cells undergo differentiation (Strauss *et al.*, 1986). The population of cells expressing CD34 antigen is therefore heterogeneous, and by itself the CD34 marker can not be used to distinguish stem from progenitor cells. However, in combination with expression (or lack of expression) of other markers, these different cell populations can be separated and the most primitive enriched. For example, $CD34^+CD38^-$ (Terstappen *et al.*, 1991; Visser and Van Bekkum, 1990), $CD34^+Thy\text{-}1^+$ (Spangrude *et al.*, 1988), and $CD34^+AC133^+$ (Yin *et al.*, 1997) populations all contain higher numbers of repopulating cells than the total $CD34^+$ population. Using fluorescence-activated cell sorter (FACS), physical isolation studies have shown that human stem cells express a variety of other cell surface antigens such as the c-*kit* receptor (c-*kit*-R), the IL-6 receptor (IL-6-R), CD45RA and Flt3. In contrast, they do not express lineage-specific markers such as HLA-DR, CD71, or CD33. Therefore, using a combination of CD34 antigen expression with these other markers it is possible to select a population of cells that may represent primitive haemopoietic stem cells.

The availability of the CD34 antibodies has greatly improved the development of techniques for the enrichment of primitive progenitor cells obtained from bone marrow, mobilised peripheral blood or cord blood samples for experimental use, including ongoing experiments to expand them *ex vivo*. They have been also useful for selecting primitive cells for clinical use in transplantation and gene therapy studies (Briddell *et al.*, 1997). Several commercial purification systems are currently available to isolate $CD34^+$ cells for experimental purpose using magnetic beads, avidin columns, or tissue culture flasks amongst others and they differ in relation to purity, cell yield and number of colony forming cells recovered (de Wynter *et al.*, 1995). Moreover, some of these technologies such as the use of magnetically cell sorting, have been recently used successfully in clinical scale to select $CD34^+$ populations for bone marrow transplantation (McNiece *et al.*, 1998; Richel *et al.*, 2000).

Despite the widespread clinical use of CD34 antibodies for the enumeration and purification of haemopoietic stem cells, the function of the CD34 molecule in these cells has only recently been addressed. Studies that have identified the L-selectin as a ligand for CD34 and CD34 overexpression in haemopoietic cells indicate a role in cell

adhesion (Greaves *et al.*, 1992). There is evidence for this in both homotypic (Gordon *et al.*, 2000; Majdic *et al.*, 1994; Traore and Hirn, 1994) and heterotypic (haemopoietic cells to bone marrow stromal layers) (Healy *et al.*, 1995) adhesion during haemopoiesis. In this context, it is interesting to note that the human chromosome 1q32 region contains several genes encoding adhesion matrix and complement cascade-binding molecules (Bruns and Sherman, 1988). The genetic co-localisation of CD34 with adhesion molecules suggests potential coordinated regulation of expression, and therefore reinforces its functional relevance in adhesion. CD34 antigen has been also involved in the mechanisms that govern haemopoietic cell proliferation (Fennie *et al.*, 1995; Gordon *et al.*, 2000) as well as in those that prevent terminal differentiation of myeloid cells (Fackler *et al.*, 1995).

Although the current acceptance of experimental and clinical strategies for the enrichment of human stem cells relies on the positive selection of the CD34 antigen, the dogma of the $CD34^+$ multipotent stem cell has been questioned for about 3 to 4 years. Recent evidence suggests that expression of CD34 on the cell membrane does not always correlate with stem cell activity (Brendel and Neubauer, 2000; Dao and Nolta, 2000). In the mouse, there is a highly quiescent population of stem cells that lacks CD34 expression, but has full reconstituting capacity (Goodell *et al.*, 1996; Osawa *et al.*, 1996). In addition, infusion of human $CD34^-$ cells resulted in long-term, multilineage human cell engraftment in a human/sheep model (Zanjani *et al.*, 1998). Bhatia and colleagues have reported an *ex vivo* culture system of bone marrow $CD34^-$ cells that induced SCID-repopulating cell expansion (Bhatia *et al.*, 1998). More recently, using 11 different markers to select human $CD34^-Lin^-$ cells from cord blood, Nakamura *et al.* have shown that these cells could engraft NOD/SCID mice and also generated $CD34^+$ cells *in vitro* (Nakamura *et al.*, 1999). These data collectively suggest that $CD34^+$ cells may be descendants of the $CD34^-$ population, and a new model has been proposed: as the CD34 antigen is an adhesion molecule, its presence or absence may represent two different states of activation of the same cell in the haemopoietic microenvironment (Brendel and Neubauer, 2000; Goodell, 1999). The significance of these findings in transplantation is not clear and must be pursued as several clinical and animal studies have clearly shown engraftment and haemopoietic reconstitution using $CD34^+$ cells.

The supravital fluorochrome dye Hoechst 33342 (Ho) alone or in combination with the vital fluorochrome Rhodamine-123 (Rh-123) have also been helpful in

haemopoietic stem cell isolation (Bertoncello *et al.*, 1985; Bertoncello *et al.*, 1988; Leemhuis *et al.*, 1996; McAlister *et al.*, 1990; Neben *et al.*, 1991; Nibley and Spangrude, 1998; Ratajczak *et al.*, 1998; Van Zant and Fry, 1983; Visser *et al.*, 1990). The former has specificity for nucleic acids (Baines and Visser, 1983) whilst the later binds specifically to mitochondria in viable cells (Johnson *et al.*, 1980). Stem cells are known to be relatively quiescent, having very little cellular activity. As such, they have relatively low numbers and/or inactivity of mitochondria and incorporate low levels of both dyes Ho and Rh-123. The cell sub fractions that stain most weakly with both dyes have been shown to be highly co-enriched for long-term repopulating cells and for *in vitro* high proliferative potential – colony forming cells (HPP-CFC) (Wolf *et al.*, 1993). Therefore, sub populations of very primitive haematopoietic cells can be isolated by FACS selection of density gradient-enriched, lineage-depleted marrow cells with small lymphocyte cell light scatter characteristics that bound low levels of the DNA binding dye Ho and retain differential amounts of the mitochondrial binding dye, Rh-123 in a single sorting operation.

The compound 5-fluorouracil (5-FU) has also been used to enrich populations of primitive haemopoietic cells based on their relatively quiescent, thus resistance to the effects of some anti-mitotic agents (Hodgson and Bradley, 1979; Lerner and Harrison, 1990; Rice *et al.*, 1993; Rice *et al.*, 1995; Van Zant, 1984). Actually, the method of choice for identifying and selecting a population enriched for stem cells will vary depending on their use, be it for experimental or clinical purposes.

1.1.2.1.2. Assays for stem/progenitor cells

Multipotent stem cells are measured by their ability to self-renewal and to completely repopulate all haemopoietic lineages *in vivo* over the lifetime of the recipient animal. The spleen colony technique remains a valuable *in vivo* assay for the multipotent progenitors in haemopoietic tissues. It is based on the fact that haemopoietic recovery (at least in mice) can be anticipated by the appearance of splenic nodules (or colonies) 8 (D8 CFU-S) to 12 (D12 CFU-S) days after transplant (Lord, 1993).

Another possible assay for repopulating cells, called long-term marrow repopulating assay (MRA) measures the ability of the cells to reconstitute the lympho-haemopoietic system of lethally irradiated recipient animals for over 6 months. This assay has been recently regarded as a definitive measure of stem cells in the mouse

(Eaves, 2000). In practice, the bone marrow from a mouse transplanted 13 days earlier is re-transplanted into secondary recipients and their spleen colonies counted 12 days later (Lord, 1993). The result gives a measure of the cells that are more primitive and cannot generate colonies within the time limits of a spleen colony assay. Thus, they are pre-CFU-S (Lord, 1993).

In order to assay primitive progenitor cells of human origin, several attempts have been made to achieve long-term reconstitution of human bone marrow progenitors in a mouse model. Using the severe combined immunodeficiency syndrome (SCID) mouse assay (Lapidot *et al.*, 1992; McCune *et al.*, 1988), grafted human haemopoietic stem cells proliferate and differentiate in the murine bone marrow to produce long-term culture – initiating cells (LTC-IC), immature $CD34^{+}Thy^{+}$ and $CD34^{+}CD38^{-}$ cells as well as more mature myeloid and erythroid cells (Baum *et al.*, 1992; Gan *et al.*, 1997; Nolta *et al.*, 1994; Pflumio *et al.*, 1993). Lymphoid cells are also produced in the bone marrow, and the human engraftment of murine thymus and spleen is almost exclusively lymphoid (Pflumio *et al.*, 1993). Measurements of human MRA, the cells responsible for engraftment in these recipients, occur in a frequency of the order of 1 in $9.3x10^{5}$ mononuclear cord blood cells and 1 in $3x10^{6}$ normal bone marrow cells as estimated by limiting diluting assays (Larochelle *et al.*, 1996; Wang *et al.*, 1997).

The sheep *in uterus* transplantation system also seems to be reliable as a model for studying human stem cells, because the human progenitor cells are transferred into the sheep foetus *in uterus* before the development of the ovine immune system (Zanjani *et al.*, 1998). These xenograft systems are promising and useful in the study of gene transduction of human hematopoietic stem cells, by tracing the development of individually marked BM stem cells into mature blood cells of different lineages (Dao and Nolta, 1999).

The supravital fluorochrome Rhodamine-123 (Rh-123) described earlier has been a powerful probe for the resolution and hierarchical ordering of transplantable haemopoietic cell populations (Bertoncello *et al.*, 1985). The majority of D8 and D12 CFU-S that actively incorporate Rh-123 ($RH\text{-}123^{bright}$) sustain haemopoiesis for only a short period of time when transplanted into irradiated recipients whilst $Rh\text{-}123^{dull}$ have an *in vivo* ability to sustain generation of D12 CFU-S and support long-term engraftment (Kim *et al.*, 1998; Spangrude and Johnson, 1990).

In practice, there are experimental limitations to define a stem cell and several methods and *in vitro* techniques to identify early stem/progenitor cells have been

described. These assays can be subdivided into two groups: (a) those that allow colony formation or clonal growth of single cells in semi-solid media containing growth-stimulating molecules, and (b) those that require a competent stromal layer for growth maintenance and are based on the Dexter long-term culture assay (Dexter *et al.*, 1977). Among the semi-solid based clonogenic assays, the mouse CFU-A assay (Graham *et al.*, 1990; Pragnell *et al.*, 1988) is equivalent to the D12 CFU-S and relies on the growth of macroscopic colonies >2mm diameter after 11 days in the presence of M-CSF, GM-CSF, IL-1, IL-6 and IL-3. This assay has been adapted for human bone marrow cells and detects macroscopic colonies >1mm after 23 days of culture.

The HPP-CFC assay (Bradley and Hodgson, 1979; McNiece *et al.*, 1988) has been recently referred as the one of the most informative, reliable, and versatile short-term *in vitro* assay for screening stem cell potential (Chang *et al.*, 2000). HPP-CFC are defined operationally by their relative resistance to near-lethal doses of 5-FU, their obligatory requirements for multiple cytokines, and formation of macroscopic colonies of at least 0.5mm diameter size, containing >50.000 cells/clone (Bertoncello and Kriegler, 1997; Kriegler *et al.*, 1994). HPP-CFC are heterogeneous and comprise a hierarchical order of at least four subpopulations of primitive haemopoietic cells characterised by their growth factor requirements (reviewed in Moore, 1991).

A primitive cell population can also be detected using the blast colony forming cell (Bl-CFC) assay (Leary and Ogawa, 1987; Nakahata and Ogawa, 1982b). The Bl-CFC originates small colonies of morphologically undifferentiated cells after 18 to 32 days in culture; these cells have high secondary recloning capacity, self-renewal potential, and the ability to generate various multilineage committed progeny. A similar assay to detect 5-FU resistant Bl-CFC has also been described (Brandt *et al.*, 1988). Re-plating colonies derived from Bl-CFC, in either assay, give rise to colonies containing variable mixtures of different mature myeloid cells.

Another primitive cell population previously mentioned in this chapter, the Mix-CFC or GEMM-CFC, was originally defined as a mouse multipotential colony forming cells that could develop into at least three haemopoietic lineages, one being erythroid (Johnson and Metcalf, 1977). The human Mix-CFC defines a cell population with the potential to form granulocytes, erythroid cells, monocytes, and megakaryocytes after 14 days of culture in the presence of several combinations of growth factors (Coutinho *et al.*, 1993). The culture conditions to assay Mix-CFC populations also allow the growth

of more restricted committed progenitors such as GM-CFC, G-CFC, M-CFC, Eos-CFC, BFU-E and CFU-E, discussed later in the present chapter.

Probably the most frequently used method for assessing primitive cells *in vitro* is the long-term culture initiating cells (LTC-IC) assay which involves seeding the test cells onto irradiated bone marrow stroma established in culture, and testing their capacity to generate haemopoiesis (Ploemacher *et al.*, 1989; Sutherland *et al.*, 1990). The LTC-IC form *foci* or cobblestone areas of primitive haemopoietic cells in the stroma and it has been estimated that in mouse, their frequency is about 1 in $1.6x10^4$ bone marrow cells (Ploemacher *et al.*, 1989). Human LTC-IC have been described as the primitive haemopoietic cells responsible for the output of clonogenic progenitors after five or more weeks in culture on a haemopoietic supportive stroma (Sutherland *et al.*, 1989). Its frequency has been estimated at 1 in $2x10^4$ bone marrow cells and 1 in 50 to 100 highly purified $CD34^+$ cells (Sutherland *et al.*, 1990).

Although the murine LTC-IC are able to regenerate haemopoiesis in an irradiated mouse (Ploemacher *et al.*, 1991), this ability has not been demonstrated for human LTC-IC. Larochelle and colleagues, using cell fractionation and gene marking techniques, have shown that human LTC-IC were different from cells with marrow repopulating ability as the gene-marked cells did not contribute significantly to the repopulation of engrafted NOD/SCID mice (Larochelle *et al.*, 1996). Therefore, human LTC-IC population represents a relatively more mature cell fraction within the stem cell compartment.

Extended stroma-dependent long-term cultures 8-14 weeks (ELTC, 8-14 weeks) have recently been described which may have revealed an even more immature human haemopoietic stem cell population than that detected in regular 5 weeks LTC-IC (Hao *et al.*, 1996). ELTC-initiating cells (ELTC-IC) differ from LTC-IC in several aspects. ELTC-IC produce most CFC after 60 days whereas LTC-IC do so earlier, and these differences are still not clear and they may be related to the use of different stromal support layers, cytokine combinations, sera *etc.* About 1% of ELTC are transduced in contrast to LTC-IC, which show high levels of gene marking (Hao *et al.*, 1996; Larochelle *et al.*, 1996). They are found in the $CD38^-$ fraction of the $CD34^+$ cells, as a human SCID-repopulating cells (Larochelle *et al.*, 1996) whilst LTC-IC are $CD38^{med}$ (Ploemacher, 1997). Most of the assays outlined describe cells with one or more stem cell attributes and it is likely that they identify similar or at least overlapping populations as depicted in Figure 1.2.

1.1.2.1.3. FDCP-mix cells: a model system for studying primitive haemopoietic stem cell development

The haemopoietic stem cell population represents a small percentage of the cells in haemopoietic tissues and as such it is difficult to isolate in large numbers to study. The generation of haemopoietic cell lines that can be grown continuously in culture provide an abundant, homogeneous source of cells and serve as useful models of haemopoiesis (Lubbert *et al.*, 1991). Moreover, they allow molecular and biochemical investigation of the regulatory mechanisms of haemopoiesis that currently cannot be approached using primary cells.

One appropriate model system for studying the mechanisms involved in cell self-renewal, lineage commitment and differentiation is represented by the multipotent haemopoietic cell line FDCP-mix (Factor Dependent Cells Paterson – mixed potential). These cells were isolated from long-term bone marrow cultures from $B_6D_2F_1$ mice injected with a molecular recombinant of Moloney Murine Leukaemia Virus (MMLV) containing the *src* oncogene from Rous Sarcoma Virus (Boettiger and Dexter, 1984). The *src* oncogene is not directly responsible for the maintenance of FDCP-mix, but the cells appear to have risen as a stable consequence of transformation, probably as a result of the helper virus integration. These cells are non-leukaemic, have a normal diploid karyotype and proliferate as multipotent haemopoietic progenitors in the continued presence of IL-3. The cells are IL-3-strictly factor-dependent, and undergo rapid apoptosis in its absence (Williams *et al.*, 1990b).

Initial studies in which FDCP-mix cells were grown in association with irradiated haemopoietic stromal cells have demonstrated their capacity to differentiate into all major blood cells, except those of the lymphoid lineage (Spooncer *et al.*, 1986). This model of lineage commitment and differentiation is presented in Figure 1.3. In liquid cultures, and in the presence of low levels of IL-3, appropriate combinations of G-CSF and GM-CSF support their differentiation into neutrophils and macrophages (Heyworth *et al.*, 1991), while haemin and erythropoietin support erythroid differentiation (Heyworth *et al.*, 1995a). Addition of thrombopoietin (TPO), stem cell factor (SCF) and IL-6 allows their differentiation into megakaryocyte lineage (Dr. C. M. Heyworth, personal communication). However, in the presence of high concentrations of IL-3, the cells undergo proliferation without differentiation and do not respond to these differentiation factors (Heyworth *et al.*, 1990a). Finally, FDCP-mix cells can generate

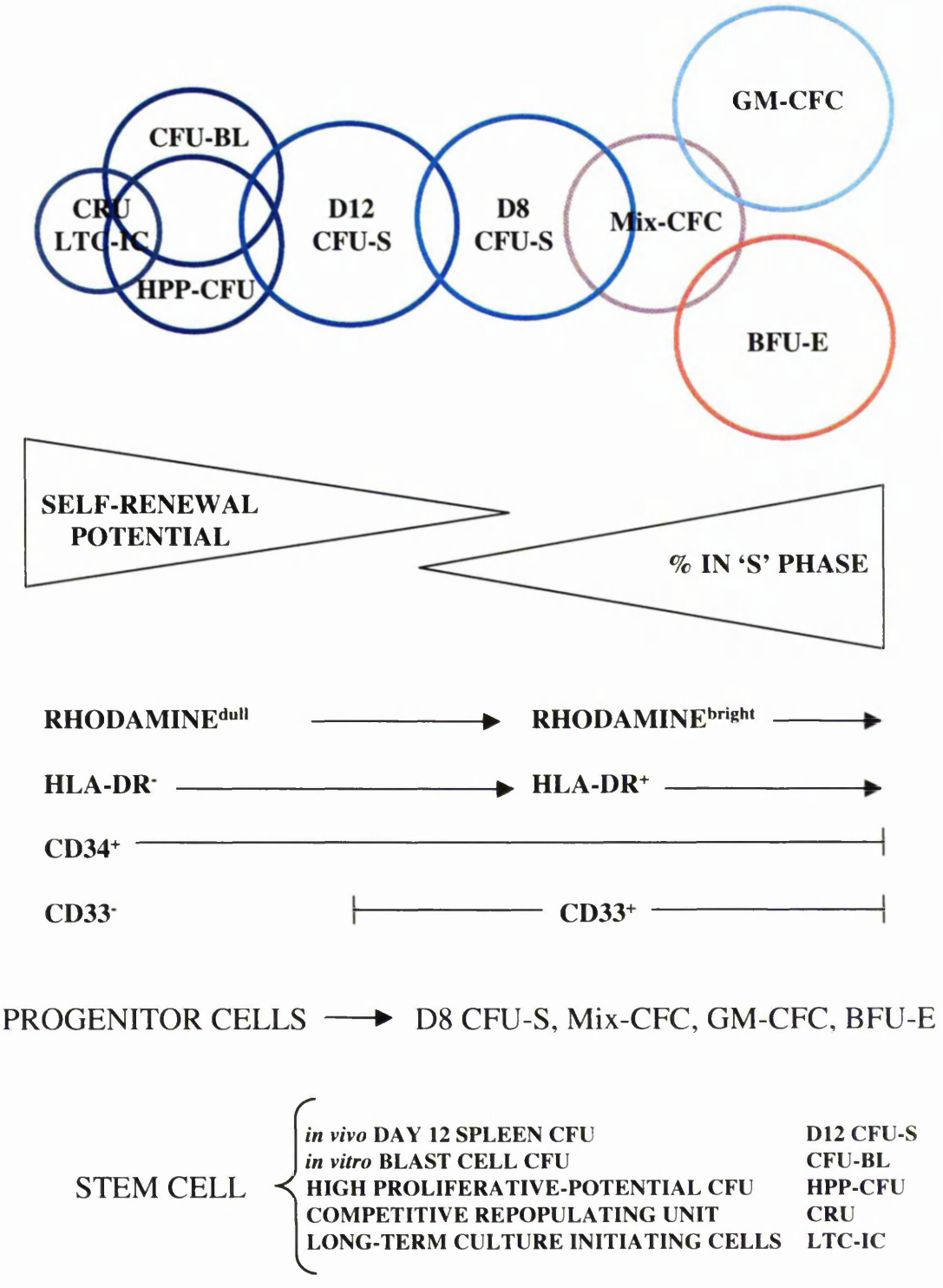


Figure 1.2. Diagram illustrating the hierarchical organisation of the stem and progenitor cell compartments as identified by currently available assays. The compartments are shown in relationship to proliferative potential and cell-cycle *status* in steady-state haemopoiesis. Stage-related phenotypic characteristics used for stem cell purification are also indicated (adapted from Moore, 1991).

mixed myeloid colonies *in vitro* and spleen colonies *in vivo*, although not capable of long-term re-population (Spooncer *et al.*, 1986). Taken together, these data suggest that while FDCP-mix cells may not represent the most ancestral haemopoietic cell type, they do display the features of self-renewal, commitment and multilineage differentiation. These qualities are relevant to early progenitors and thus provide a useful model system to study these features, and to complement studies of primary haemopoietic cells.

1.1.2.2. Progenitor cells

Haemopoietic progenitor cells are derived directly from the stem cells and are still capable of extensive proliferation. They are transit cells that differentiate under continuous regulation (Blau and Baltimore, 1991) and become eventually committed to a particular line of haemopoietic cell development. These cells produce progeny that become restricted to only one or two lineages and can undergo extensive proliferation and sequential development to produce the mature blood cells. As it is the case for the stem cell compartment, the number of haemopoietic committed progenitor cells is relatively small, and they remain morphologically undefined.

Most, if not all, of the progenitor cell populations can be cloned in soft-gel systems (Metcalf, 1977a; Metcalf, 1977b; Metcalf, 1977c). These clonogenic assays allow quantitative studies from multipotential cells not yet restricted to one lineage of differentiation, which give rise to colonies containing several thousands of recognisable different mature cells, to progenitor cells restricted to one line of differentiation, and which have very limited proliferation capacity. Pluznick and Sachs (Pluznik and Sachs, 1965), and Bradley and Metcalf (Bradley and Metcalf, 1966) were the first to grow mast cells and granulocyte and/or macrophage colonies *in vitro*. These early experiments led to the demonstration that neutrophils and mononuclear phagocytes are closely related populations, in many cases sharing a common progenitor cell. The clonogenic cells have been operationally defined as colony-forming cells (CFC) and prefixed by letters denoting the cell type to which they give rise.

Lineage-restricted progenitor cells are also classified on the basis of the progeny they produce in clonogenic soft-gel systems. Granulocyte-macrophage colony-forming cells (GM-CFC) are precursors of neutrophilic granulocytes and/or macrophages depending upon the presence of specific growth stimulus in the growth media. The proportion of granulocyte-macrophage, granulocyte or macrophage colonies depend not

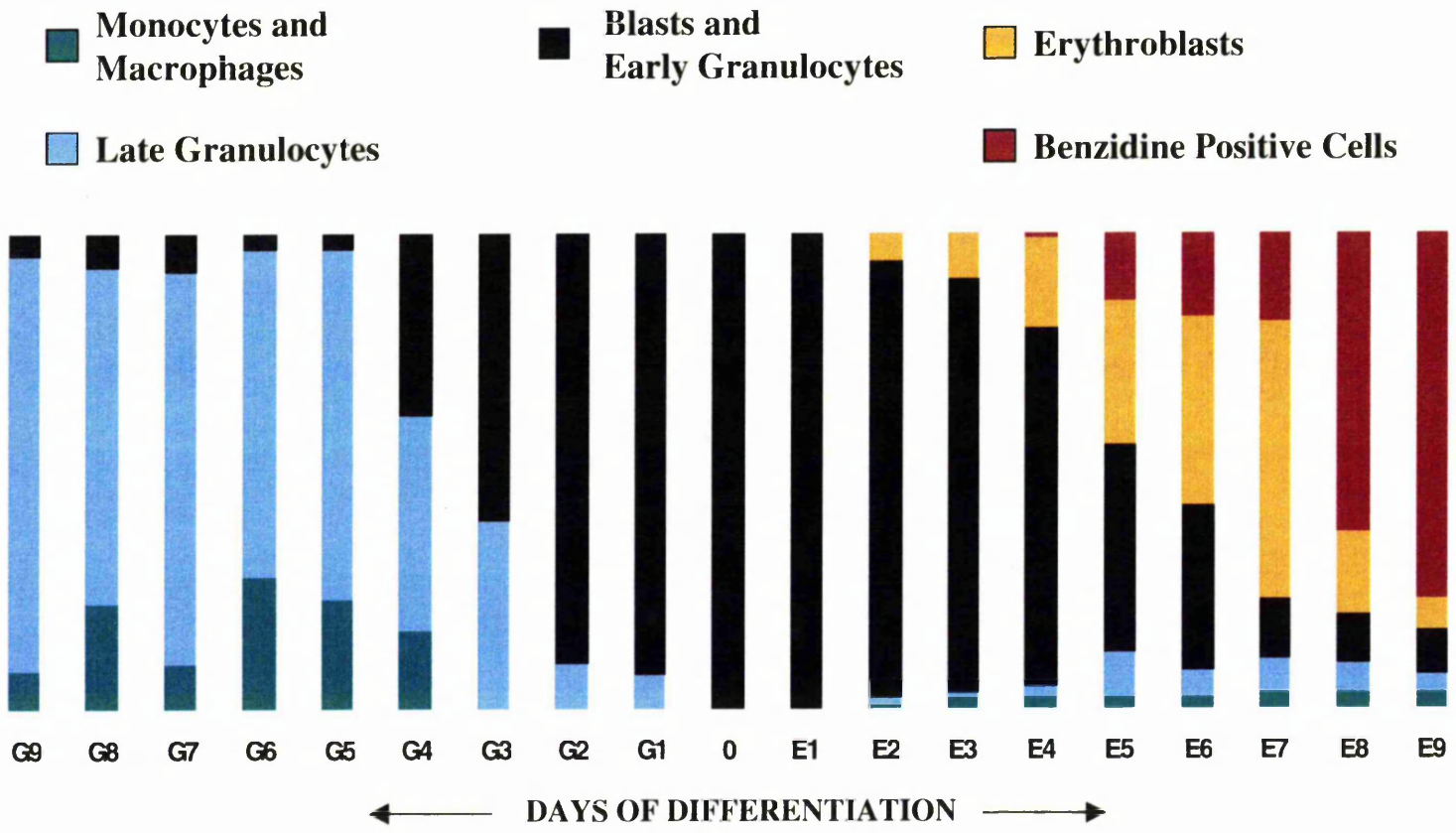


Figure 1.3. FDCP-mix differentiation morphology. The proportion of cell types at each day of Granulocyte-Macrophage (day 1 = G1 to day 9 = G9) and Erythroid (day 1 = E1 to day 9 = E9) differentiation are shown as coloured bars. In liquid culture 'day 0' undifferentiated cells are maintained in high concentration of IL-3. Granulocyte-Macrophage differentiation is induced with low IL-3 concentration plus G-CSF and GM-CSF. Erythroid differentiation requires low concentration of IL-3 and the presence of haemin and erythropoietin (adapted from Heyworth *et al.*, 1995).

only on the source of growth factor, but also on the concentration of the colony-stimulating factors used (Burgess and Metcalf, 1980b).

The most primitive erythroid progenitor cell detected in clonogenic assays is often called erythroid burst-forming units (BFU-E) due to the characteristic morphology of the colonies. After undergoing proliferation, colony cells begin to synthesise haemoglobin, generating typical erythroid bursts, which may contain from several hundreds to about 10^4 cells after 14-20 days of incubation (Testa, 1985). More mature erythroid progenitors are the erythroid colony-forming units (CFU-E) which give rise to small erythroid colonies of 8-60 cells after 7 days of incubation (Iscove *et al.*, 1974). Eosinophil (Metcalf *et al.*, 1974), basophil (Nakahata and Ogawa, 1982a), and megakaryocyte colony-forming cells (McLeod *et al.*, 1980) (Eo-CFC, Bas-CFC and Meg-CFC, respectively) can also be recognisable by their ability to undergo proliferation and development *in vitro* to produce mature progeny when appropriately stimulated.

1.1.2.3. Maturing/mature cells

After a series of divisions, the progenitor cells are no longer able to proliferate and finally take on the morphological characteristics of their cell types. As stated before, neither multipotent stem cells nor more committed progenitors are readily identified morphologically by traditional methods. Only the more mature forms of each cell type series can be distinguished from one another at various stages of maturation. The morphological boundaries of each mature cell compartment have been defined and *criteria* such as cell size, nucleus to cytoplasm ratio, fineness of nuclear chromatin, nuclear shape, the presence or absence of nucleoli, the presence and type of cytoplasm granules, affinity for specific dyes, and the composition of cytoplasmic enzymes are taken as the base of their recognition (Bain, 1996; Carr and Rodak, 1999). In normal circumstances, proliferation has ceased by the time a cell is fully mature and migrates to the blood circulation to carry out its function and to final senescence.

1.2. Regulation of Haemopoiesis

The choice between self-renewal and commitment, along with survival *versus* apoptosis, proliferation *versus* quiescence and periodic choice between alternative developmental lineages are some of a number of decisions that punctuate blood cell

production. The combination of these decisions leads to the number of mature cells emerging from each lineage, and tailors production to the changing demands imposed by stress regimens. Steady state haemopoiesis requires a fine control and co-ordination of regulatory mechanisms. Both positive and negative regulation is required to enable the system to respond to changes and to maintain production. Accumulated evidence has led to the concept that the regulation of cell production may be achieved at two levels: control of diffusible regulatory molecules including growth factor stimulators and inhibitors and control by the complex regulatory microenvironment of the marrow stroma (Dexter *et al.*, 1984).

1.2.1. Cytokines

The simultaneous discovery of the colony assays by two independent groups (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965) was the foundation for many advances in the knowledge of the cellular organisation of the haemopoietic system and its regulation by soluble factors. In these assays, as already mentioned, haemopoietic progenitor cells can be induced to proliferate and develop into mature cells if they are immobilised in a soft-gel matrix and supplied with a source of soluble factors which have been termed 'colony stimulating activities' (CSA) (Testa, 1985; Testa and Dexter, 1990). Later, the name of these factors became based on operational properties of the factors and, as an example, the molecule that in cultures of mouse cells resulted in the generation of colonies composed of neutrophil granulocytes was termed **g**ranulocyte **c**olony-**s**timulating **f**actor, or G-CSF.

Advances in biochemical purification techniques, and particularly in recombinant DNA technology, have led to the molecular cloning of the genes that encode these colony-stimulating factors and several other proteins or peptides released in the inflammatory response, many of which also affect haemopoietic cells. Collectively these molecules have been called cytokines, and they are broadly classified as growth factors (GF), colony stimulating factors (CSF), interleukins (IL), lymphokines, monokines, and interferons (Clemens, 1991). More recently, a family of structurally related cytokines recognised by their ability to recruit leukocytes during inflammatory process has been recognised and they have been grouped as chemokines, standing for **chemo**attractant cyto**kines** (reviewed in Baggiolini, 1998).

1.2.1.1. Haemopoietic growth factors

Haemopoietic growth factors are proteins that are ligands for specific cell surface receptors expressed on haemopoietic cells that influence their survival, proliferation and differentiation (Table 1.1) as well as the proliferation and functional activity of mature cells (Table 1.2) (Heyworth *et al.*, 1990b). They are produced in several body sites by a wide variety of cell types that include fibroblasts, macrophages, T cells, kidney cells, and endothelial cells, either in steady state haemopoiesis or in response to specific stimulation such as the stimuli provided by bacterial endotoxin. The widespread production of cytokines may reflect their participation in various regulatory processes. Thus, the same factor, acting at different stages of the developmental sequence, may exert different activities, starting with stimulation of proliferation and differentiation in the stem/progenitor cells, and inducing cell function on the maturing and fully mature cells. Consequently, the precise effects of any given factor is determined by the responding cell type, its concentration, and the presence of other stimuli, such that some growth factors may fulfil a variety of functions under different circumstances.

Most, if not all, genes for the GF have been molecularly cloned, sequenced and shown to be glycosylated polypeptides in their natural form (Copeland *et al.*, 1990; Flanagan and Leder, 1990; Fung *et al.*, 1984; Gough *et al.*, 1984; Huang *et al.*, 1990; Kawasaki *et al.*, 1985; Lyman *et al.*, 1994; Lyman *et al.*, 1993; Nagata *et al.*, 1986; Williams *et al.*, 1990a). The availability of recombinant molecules coupled with the advance in methodologies for cell selection has allowed studies at a mechanistic level. It has become clear that cytokines influence haemopoiesis in a complex and concerted fashion. Many of the GF are able to act on a variety of target cells that occur within different cell lineages and at different developmental stages (Figure 1.4), have a considerable overlap in their biological effects (Metcalf, 1993), and their actual names may not accurately indicate their specificity. This redundancy may allow compensation in the case of regulator deficiency (de Haan *et al.*, 1996). An example of such diversity is given by the IL-3, which has a broad spectrum of target cells in the haemopoietic system. It supports the survival, self-renewal and proliferation of multipotential progenitor cells and acts on committed progenitors of myeloid lineage, stimulating their development into colonies containing cells of various lineages such as neutrophils, macrophages, eosinophils, megakaryocytes, mast cells and erythroid cells (Schrader *et al.*, 1988; Spivak *et al.*, 1985). Such a multitude of actions probably is responsible for the name of IL-3 as multi-CSF (Metcalf *et al.*, 1987a). Administration of IL-3 to mice

Table 1.1. Haemopoietic Growth Factors that act upon Myeloid Progenitors *in vitro*.

CYTOKINE	MW (kDa)	CELL SOURCES	TARGET CELLS	REFERENCE	
				cDNA cloning	cellular source
IL-3	20-28 (m) 14-28 (h)	Activated T cells, endothelial cells, fibroblasts, mast cells, NK cells	HPP-CFC, CFC-Mix, GM-CFC Eos-CFC, Bas-CFC, Meg-CFC, BFU-E	Fung *et al.*, 1984	Ihle *et al.*, 1981 Prestidge *et al.*, 1984
GM-CSF	21-23 (m) 14-35 (h)	Activated T cells, endothelial cells, fibroblasts, macrophages	HPP-CFC GM-CFC Eos-CFC Meg-CFC BFU-E (s)	Gough *et al.*, 1984 Wong *et al.*, 1985 Gasson, 1991 (review)	Nicola *et al.*, 1979 Thorens *et al.*, 1987 Fibbe *et al.*, 1986 Burgess and Metcalf, 1980 Hermann *et al.*, 1988
G-CSF	25 (m) 18-22 (h)	Endothelial cells, fibroblasts, activated macrophages	HPP-CFC (s) Mix-CFC GM-CFC (g)	Tsuchiya *et al.*, 1986 Ikebuchi *et al.*, 1988 Nagata *et al.*, 1986	Yang *et al.*, 1988 Rennick *et al.*, 1987 Sieff *et al.*, 1987 Burgess and Metcalf, 1980
M-CSF	45-90 (m) 45-90 (h)	Endothelial cells, fibroblasts, activated macrophages	HPP-CFC (s) GM-CFC (m*)	Delamarter *et al.*, 1987 Kawasaki *et al.*, 1985	Metcalf, 1984
EPO	39 (m) 36 (h)	Kidney cells	BFU-E (late) CFU-E	Krantz and Jacobsen, 1970	Krantz, 1991 (review)

(Table 1.1 cont.)

SCF	30 (m) 30 (h)	Fibroblasts, liver cells	HPP-CFC (s) Mix-CFC (s) GM-CFC Bas-CFC	Zsebo *et al.*, 1990 Martin *et al.*, 1990 Witte, 1990 (review)	Copeland *et al.*, 1990 Flanagan & Leder, 1990 Huang *et al.*, 1990 Lyman & Jacobsen, 1998 (review)
IL-1	17 (m) 17 (h)	Endothelial cells, fibroblasts, monocytes	HPP-CFC (s)	Dinarello, 1991 (review)	
IL-4	20 (m) 20 (h)	T cells	GM-CFC (s) Bas-CFC (s) BFU-E (s)	Takatsu, 1988 (review)	Takatsu, 1988 (review)
IL-5	40-65 (m) 40 (h)	T cells	Eos-CFC	Sanderson *et al.*, 1988 (review)	Sanderson et al., 1988 (review)
IL-6	23-25 (m) 26 (h)	Fibroblasts, Monocytes, B and T lymphocytes	HPP-CFC (s) GM-CFC (s)	Leary *et al.*, 1988	Kishimoto, 1989 (review)
TPO	70 (m) 47 (h)	Kidney cells	BFU-E Meg-CFC	Foster *et al.*, 1994	Kaushansky, 1995 (review)
Flt-3	17-30 (h)	Primitive haemopoietic cells	Early haemopoietic progenitors	Hannun *et al.*, 1994 Lyman *et al.*, 1994	Lyman *et al.*, 1994

Abbreviations: IL, interleukin; SCF, stem cell factor; GM-CSF, granulocyte-macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; M-CSF, macrophage colony stimulating factor; EPO, erithropoietin; HPP-CFC, high proliferative potential colony forming cell; BFU-E, burst forming units, erythroid; CFU-E, colony forming units, erytrhoid; Eo-CFC, eosinophil colony forming cell; Bas-CFC, basophil colony forming cell; Mix-CFC, multipotential colony forming cell; TPO, thrombopoietin; m, mouse; h, human; s, synergistic activity only.

Table 1.2. Growth Factors that activate Mature Haemopoietic Cells.

GROWTH FACTOR	EFFECT	Reference
GM-CSF	Functional activation of eosinophils, neutrophils and macrophages; Increases cytotoxic activity of macrophages and eosinophils; Proliferation and migration of endothelial cells	Lorenzo *et al.*, 1987 Gasson *et al.*, 1984 Vadas *et al.*, 1983 Handman & Burgess, 1979 Cannistra *et al.*, 1987 Bussolino *et al.*, 1989 Lopez *et al.*, 1991
G-CSF	Functional activation of neutrophils; Proliferation and migration of endothelial cells	Kitagawa *et al.*, 1987 Vadas *et al.*, 1983 Begley *et al.*, 1986 Bussolino *et al.*, 1989
M-CSF	Functional activation of macrophages	Wing *et al.*, 1982 Weiser *et al.*, 1987
IL-1	T cell activation; chemotaxis of macrophages and neutrophils; stimulation of cytokine release by T cells	Bagby, 1989 Lee *et al.*, 1987
IL-3	Differentiation of eosinophils Priming of mast cells Activation of monocytes	Lopez *et al.*, 1987 Lopez *et al.*, 1991 Schneider *et al.*, 1987 Cannistra *et al.*, 1988
IL-4	Activation of T and B cells Activation of macrophages and inhibition of migration Eosinophil differentiation	Howard *et al.*, 1982 Yokota *et al.*, 1988 O'Garra *et al.*, 1986
IL-5	B cell differentiation Eosinophil production	Sanderson *et al.*, 1988 Lopez *et al.*, 1991 Lopez *et al.*, 1992
IL-6	Terminal differentiation of B cells, proliferation and differentiation of T cells in presence of IL-2; role in immune and acute phase responses	Kishimoto *et al.*, 1989

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; IL, interleukin.

causes a dose dependent increase in both progenitor cells and mature peritoneal macrophages, neutrophils and eosinophils (Lord *et al.*, 1986; Metcalf *et al.*, 1987b). In contrast with all the abilities of IL-3 described, transgenic mice that lack IL-3 receptor demonstrate little alteration in haemopoiesis, suggesting that the functions of IL-3 within bone marrow may be compensated for other cytokines (Nishinakamura *et al.*, 1996; Nishinakamura *et al.*, 1995).

GM-CSF is secreted by different cell types including macrophages, endothelial cells, and mast cells (Baldwin, 1992). It can act upon multilineage progenitors, stimulating the formation of colonies composed of neutrophils and macrophages (Metcalf, 1989), and can promote also proliferation and development of erythroid, eosinophils, and megakaryocyte progenitors (Baldwin, 1992; Metcalf, 1986). GM-CSF acts on mature macrophages and neutrophils, directly prolonging neutrophil lifespan, degranulation and up-regulation of receptors for G-CSF and M-CSF (Baldwin, 1992). Indirectly, GM-CSF primes neutrophils, enhancing superoxide production on consequent stimulation by the inflammatory molecules *f*-MLP, LTB_4 and C5a (Weisbart *et al.*, 1985). Administration of GM-CSF in mice results in a dose dependent increase in circulating leukocytes, including neutrophils and macrophages (Metcalf *et al.*, 1987a). Irradiated mice transplanted with cells expressing high levels of GM-CSF show hyperplasia of macrophages present in the peritoneal and lung cavities (Lang *et al.*, 1987). In contrast, GM-CSF knockout mice are normal, with no alterations in either granulocyte-macrophage progenitors or mature granulocytes and macrophages from blood, BM or spleen, but display alveolar proteinosis with surfactant accumulation asso ciated with dysfunction of alveolar macrophages (Stanley *et al.*, 1994). These studies suggest that GM-CSF does not appear to play an irreplaceable role in regulation of either granulocytes or macrophages, although elevated levels of GM-CSF increase the production of these cells and excessive production may be associated with certain disease states.

Stem Cell Factor, also termed mast cell growth factor (MGF), c-*kit* ligand (KL) or Steel factor (SLF), exists either as a soluble and as a membrane-bound form and is secreted by many tissues including bone marrow stromal cells (Anderson *et al.*, 1990; Lowry *et al.*, 1992). SCF shares with IL-3 a similar spectrum of targets within the haemopoietic system, but it does not affect mature macrophages as they lack the c-*kit* receptor. Alone, it stimulates the growth of haemopoietic progenitor cells only weakly and it is relatively ineffective as a colony stimulating factor (Schrader, 1992). *In vivo*

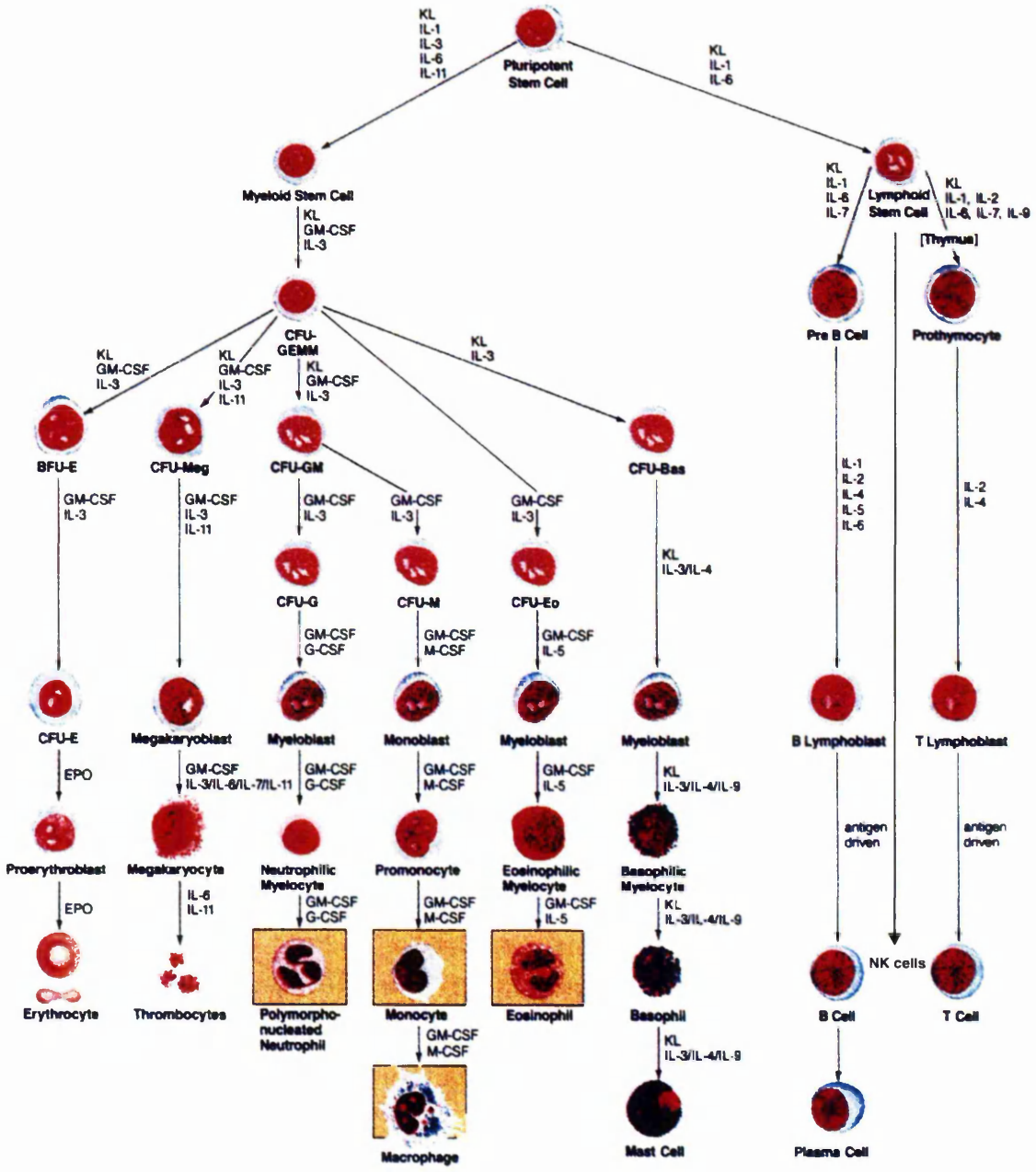


Figure 1.4. The structure of the haemopoietic system and its interacting cytokines. Relationship of haemopoietic precursors to each other and to the end cells into which they differentiate, and the sites of activity of some cytokines (adapted from Sandoz Pharmaceuticals Corporation and Schering-Plough, 1991).

administration of SCF to mice and primates induces neutrophilia, lymphocytosis and reticulocytosis, in parallel with increased numbers of immature myeloid and erythroid precursors in the marrow (Andrews *et al.*, 1994a; Andrews *et al.*, 1991).

Recently, another GF, flt3/flt2-ligand (Flt3), with many biological characteristics in common with SCF has been cloned (Lyman and Jacobsen, 1998; Lyman *et al.*, 1994; Lyman *et al.*, 1993). Flt3 has been demonstrated intracellularly and on cell membranes of bone marrow fibroblasts and in stromal cells of adherent layers of long-term bone marrow cultures (Lisovsky *et al.*, 1996). Flt3 stimulates the proliferation and colony formation of bone marrow, mobilised peripheral blood and cord blood progenitor cells (Broxmeyer *et al.*, 1995; Gabbianelli *et al.*, 1995; Jacobsen *et al.*, 1995; Rusten *et al.*, 1996), and has no species specificity regarding ligand binding or biological activity. Mice deficient in Flt3 receptor are healthy and viable, but their marrows have reduced potential to reconstitute the myeloid and lymphoid lineages of secondary recipients (Mackarehtschian *et al.*, 1995), suggesting that Flt3 may affect the most primitive haemopoietic cells.

G-CSF was originally characterised as a late acting, lineage specific factor, stimulating the proliferation and differentiation of the lineage committed G-CFC and potentiating the functional activities of mature neutrophils (Metcalf and Nicola, 1983). On its own, G-CSF has a weak activity on immature progenitors (Heyworth and Spooncer, 1993) and does not promote the survival of multipotential progenitors, but can synergise with IL-3, erythropoietin (EPO), GM-CSF and SCF to stimulate colony formation (McNiece *et al.*, 1991a). Sub-lethally irradiated mice injected with G-CSF have a dose dependent increase in granulopoiesis and the entry of neutrophils into circulation, indicating that G-CSF acts as a regulator of granulocyte production *in vivo* (Fujisawa *et al.*, 1986). In agreement with this, it has been shown that G-CSF knockout mice have profound neutropenia and reduction in GM-CFC progenitors, maturing macrophages and granulocytes (Lieschke *et al.*, 1994; Seymour *et al.*, 1997).

G-CSF has been used in clinical practice including repair of radiation and chemotherapy-associated suppression of normal haemopoiesis in cancer patients. G-CSF does relieve neutropenia in humans (Morstyn *et al.*, 1994), apparently by reducing the cell cycle time and increasing the number of cell divisions in the late granulocyte precursor compartment in the marrow (Lord *et al.*, 1989). It has been also used successfully for stimulation of normal granulocyte development in patients with infantile congenital agranulocytosis. The fact that G-CSF can mobilise primitive and

progenitor cells into the peripheral blood and that these mobilised cells are suitable for bone marrow reconstitution (Molineux *et al.*, 1990) has been largely explored as a treatment strategies for a number of other diseases (Pettengell *et al.*, 1993a; Pettengell *et al.*, 1993b; Weaver *et al.*, 1996b).

Erythropoietin (EPO) is the principal growth factor regulating the production of circulating erythrocytes. Knockout mice for EPO or EPO receptor (EPO-R) exhibit identical phenotype (Wu *et al.*, 1995), and both are crucial for definitive erythropoiesis *in vivo* as their null mutant mice die around embryonic day 13, owing to failure of foetal liver erythropoiesis. Committed erythroid BFU-E and CFU-E progenitors are present in both homozygous foetal livers, suggesting that neither EPO nor its receptor are required for erythroid lineage commitment or for the proliferation and differentiation of BFU-E to CFU-E progenitors. However, they are crucial *in vivo* for the proliferation and survival of CFU-E progenitors and their irreversible terminal differentiation. EPO in its recombinant form has been used for the treatment of patients with renal failure, anaemia of chronic disease and several other diseases that involve anaemia, and it has been shown to substantially improve health-related quality of life in these patients (Dunphy *et al.*, 1999; Foa, 1991; Klaesson, 1999; Peeters *et al.*, 1999; Van Damme Lombaerts and Herman, 1999).

When some GF are combined, as illustrated for G-CSF above, they can exert a proliferation/differentiation stimulus different from that showed by the individual GF themselves. Multipotent haemopoietic cells, for example, do not survive in the presence of either G-CSF or M-CSF in serum-free systems. However, when these two growth factors are used in combination, a powerful synergistic effect that induces both proliferation and differentiation is observed, leading to the development of mature neutrophils and macrophages (Heyworth *et al.*, 1988). Similar synergistic interactions are seen using GM-CSF plus G-CSF, or IL-3 plus IL-6 or IL-1 (Ikebuchi *et al.*, 1987; Jacobsen *et al.*, 1994). This suggests that progenitor cells that are not able to proliferate in response to one factor in isolation may respond to growth factor combinations, and implies that lineages of differentiation are not an intrinsic and predetermined property of the primitive cells, but may be determined by the balance of factors and their concentrationspresent in their environment (Testa and Dexter, 1990).

Examples of such synergism are also given by the SCF. By itself, SCF is a weak promoter of proliferation of primitive progenitors as already described, but when interacting with other GF such as IL-3, GM-CSF, G-CSF and EPO, it promotes myeloid

and erythroid colony development (Broxmeyer, 1992a; Heyworth *et al.*, 1992; Li and Johnson, 1994; McNiece *et al.*, 1991b; Migliaccio *et al.*, 1991a; Migliaccio *et al.*, 1991b). SCF synergises with cytokines such as IL-1, IL-4, IL-6 and IL-7 that have little or no colony-stimulating activity or direct ability to stimulate the division of progenitor cells (Schrader, 1992). *In vivo*, co-injection of SCF and G-CSF increases the number of peripheral neutrophils in a time- and dose-related manner, suggesting that synergy between these two molecules may be required for the optimal effects of G-CSF *in vivo* (Andrews *et al.*, 1994b).

Several combinations of GF that are more effective in inducing primitive cell proliferation have been demonstrated (Zandstra *et al.*, 1997), and the most striking was the combination of Flt3 and thrombopoietin (TPO), which stimulated expansion of LTC-IC over 20 weeks of culture (Piacibello *et al.*, 1997). TPO is the primarily regulator of megakaryocyte differentiation and induces the full developmental program required to produce platelets (Kaushansky, 1995). TPO alone promotes the growth of small megakaryocyte colonies (Meg-CFC) in numbers two to three times greater than those produced by IL-3. The combination of TPO and SCF exerts a significant synergistic effect on Meg-CFC formation. In the presence of TPO and IL-3 or GM-CSF, a significant number of Mix-CFC is also observed. The combination of TPO and EPO increases the number of CFU-Meg, and support BFU-E and Mix-CFC colony formation (Kaushansky *et al.*, 1996). The synergies between GF *in vitro* have potential clinical applications and some clinical trials have already shown that, after treating patients with a combination of SCF and G-CSF, a large number of progenitor cells and LTC-IC are released into peripheral circulation (Glaspy *et al.*, 1997; Weaver *et al.*, 1998; Weaver *et al.*, 1996a).

1.2.1.2. Haemopoietic growth factor receptors

Classically, growth factor stimuli are transmitted into cells *via* activation of specific receptors on their surface that modify key regulatory proteins in the cytoplasm. These, in turn, affect the decisions controlling proliferation and differentiation, including changes in gene expression and reactivity to other factors and stimulate the cell to respond in one of a number of different ways (Budel *et al.*, 1995; Cross and Dexter, 1991). There are two main groups of haemopoietic growth factor receptors that can be characterised by the presence or absence of a tyrosine kinase domain. The

tyrosine kinase receptors include the SCF receptor, c-*kit,* the Flt3 receptor, *flk*-2, and the M-CSF receptor, c-*fms*. The second group, the cytokine receptor superfamily, has no tyrosine kinase domain, but contains conserved sequences within their extracellular domain (Miyajima *et al.*, 1992).

Tyrosine kinase receptors contain a glycosylated extracellular ligand-binding domain, a single membrane-spanning domain, and a cytoplasmic domain linked to a tyrosine kinase. The M-CSF (c-*fms*), Flt3 (*flk*-2), and the SCF (c-*kit*) receptors belong to the class of receptor tyrosine kinase characterised by the presence of five immunoglobulin repeats, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain interrupted by an individual insert sequence. The receptors for G-CSF, GM-CSF, EPO, TPO and for the Interleukins 2, 3, 4, 5, 6 and 7 belong to the cytokine receptor superfamily. Most of these receptors only bind their ligand with low affinity and high affinity ligand binding involves the association of two or more receptor sub-units (di- or oligomerization). A common receptor sub-unit called 'beta common' (β_c) has been identified for the human IL-3, IL-5 and GM-CSF receptors. The α sub-unit binds the ligand with low affinity and associates with the β sub-unit to form the high affinity receptor (Chiba *et al.*, 1993; Devos *et al.*, 1991; Fukunaga *et al.*, 1990; Hayashida *et al.*, 1990; Hibi *et al.*, 1990; Sakamaki *et al.*, 1993; Takaki *et al.*, 1991; Tavernier *et al.*, 1991). Other receptor types found on haemopoietic primitive cells include the IL-1 type, member of the immunoglobulin superfamily (Sims *et al.*, 1988), which binds a cytokine that acts synergistically with other growth factors to stimulate proliferation and development of primitive cells. There are also instances of a cytokine binding to more than one type of receptor and this is well exemplified by the family of chemokines (Broxmeyer and Kim, 1999), discussed later in this chapter.

The simultaneous presence of a variety of growth factor receptors on specific cell types suggests that cell development and behaviour *in vivo* may be determined by a combination of interacting stimuli. The commitment of a cell to a particular growth factor used need not simply be the cumulative result of complementation between the growth factors present. It can also involve interference between stimuli, such that exposure of a cell to one growth factor compromises its ability to bind, and hence respond, to another. This may be achieved either by receptor modification or by downregulation of receptor expression (Walker *et al.*, 1985) An example of such negative interaction has been demonstrated using the haemopoietic cell line FDCP-mix.

High concentrations of IL-3 suppress the ability of these cells to respond to a mixture of GM-CSF and G-CSF. Lowering the concentration of IL-3 does not increase the number of GM-CSF receptors on the cell surface by itself, but with the addition of GM-CSF and G-CSF it does, and the cells develop along the granulocyte/macrophage lineage (Heyworth *et al.*, 1991).

1.2.1.3. Inhibitors of haemopoietic cell growth

As described previously, the growth of highly purified progenitor cells can be modulated *in vivo* and *in vitro* by the action of multiple cytokines, leading to the concept of 'growth factor synergy' (Lowry *et al.*, 1992). It is generally accepted that the majority of the stem cells, although having a high proliferative potential, are normally in a quiescent state, namely G_0/G_1 phase of the cell cycle (Berardi *et al.*, 1995b; Lajtha *et al.*, 1969; Lord and Woolford, 1993). It has been proposed that this quiescent state is important to minimise the potential for genetic damage during replication, which may lead to tumour induction (Lajtha, 1979). Also, it may be due in part to either the absence of stimulatory factors or the presence of negative regulators (Becker *et al.*, 1965).

The concept that haemopoietic progenitor cell growth can be negatively regulated emerged from early experiments in which an extract obtained from cultured bone marrow showed to specifically block the entry of CFU-S into DNA synthesis, having no effect on more mature populations (Lord *et al.*, 1976). This inhibitory effect was not present in conditioned medium prepared from fully active bone marrow and, by contrast, CFU-S were specifically triggered from G_0 phase directly into DNA synthesis (Lord *et al.*, 1977; Toksoz *et al.*, 1980). Later, it was demonstrated that two sub-populations of macrophages were responsible for the production of these distinct activities (Lord, 1988). Partial purification of the inhibitor, previously named normal bone marrow extract IV (NBME IV) (Lord *et al.*, 1976), showed a molecular weight of 50-100kDa (Tejero *et al.*, 1984). Subsequently, purification of a stem cell inhibitor (SCI) from the macrophage cell line J774.2, with biochemical properties similar to the previously described molecule named macrophage inflammatory protein 1 (MIP-1) (Sherry *et al.*, 1988) was described (Graham *et al.*, 1990).

Using the CFU-A assay, it has been shown that the inhibitory activity of NBME IV could be neutralised by an antibody raised against SCI/MIP-1α (Graham *et al.*, 1990), leading to the conclusion that the molecule present in the bone marrow extract

was MIP-1α. The block into DNA synthesis by MIP-1α leading to inhibition of proliferation is specific for CFU-S, with no inhibitory action over the committed progenitors. The inhibitory effect of MIP-1α on more primitive cells has led this cytokine to be used successfully as a protective chemotherapeutic agent from the cytotoxicity of cycle-specific drugs in animal models (Dunlop *et al.*, 1992; Lord *et al.*, 1992). These characteristics of MIP-1α have also been explored in human clinical trials (Broxmeyer *et al.*, 1998; Marshall *et al.*, 1998). The influence of MIP-1α on haemopoiesis is further discussed in this chapter under the chemokine section (section 1.4.2.1).

Transforming growth factor beta, TGF-β, is one of the most studied haemopoietic inhibitors. It is a member of a family of related multifunctional cytokines abundant in platelets and bone marrow (Wakefield *et al.*, 1988a; Wakefield *et al.*, 1988b) and three cloned isoforms, TGF-β1, TGF-β2 and TGF-β3, have overlapping functions and cross-reaction with shared receptors (Sporn *et al.*, 1987). TGF-β is a ubiquitous molecule and has been shown to have effects on a wide range of cell types. TGF-β1 exhibits both enhancing and suppressive effects in haemopoiesis and most of the studies have been performed with this isoform (Jacobsen *et al.*, 1991; Keller *et al.*, 1992). *In vitro*, TGF-β inhibits the cycling of primitive cells in the stroma of LTBMC (Cashman *et al.*, 1990), blocks the proliferation of normal and chronic myeloid leukaemia (CML) progenitors (Eaves *et al.*, 1993), reduces the number of HPP-CFC (Keller *et al.*, 1994), and inhibits the expansion of immature progenitor cells in suspension culture (Hatzfeld *et al.*, 1991). *In vivo*, TGF-β inhibits both CFU-S and cells with marrow repopulating ability (Keller *et al.*, 1994; Maltman *et al.*, 1993; Migdalska *et al.*, 1991). The effects of TGF-β on progenitor cells seem to depend on the maturation stage of the target cells and the presence of other cytokines. In contrast to its inhibitory effect, a co-stimulatory effect of TGF-β is observed in the presence of GM-CSF (Keller *et al.*, 1991; Piacibello *et al.*, 1991), M-CSF (Bursuker *et al.*, 1992; Celada and Maki, 1992), erythropoietin plus IL-3 and SCF (Krystal *et al.*, 1994) on more mature lineage committed progenitor cells.

Interferons, although originally identified as agents produced by virus-infected cells, also have some inhibitory effects on haemopoiesis. They are often classified into three groups according to their cellular source: IFN-α, leukocytes, IFN-β, fibroblast, and IFN-γ, activated T cells. IFN-γ, has been shown to decrease colony formation of GM-CFC, BFU-E and Mix-CFC in a dose-related manner and its exogenous addition as well

as constitutive production suppresses haemopoiesis in LTBMC (Coutinho *et al.*, 1986; Selleri *et al.*, 1996). Inhibition of colony formation is observed when IFN-γ synergises with IFN-α or TNF (Broxmeyer, 1992b). IFN-γ also modulates the expression of TNF, IL-4 and M-CSF receptors amongst others (Baccarini *et al.*, 1992).

Seraspenide, known also as the tetrapeptide AcSDKP (acetyl-N-Ser-Asp-Lys-Pro), is able to inhibit the *in vitro* growth of human GM-CFC and BFU-E and to decrease their proportion in cell cycle (Bonnet *et al.*, 1992; Bonnet *et al.*, 1993; Guigon *et al.*, 1990). *In vivo*, it has been shown to inhibit the entry into DNA synthesis of murine spleen colony-forming units (CFU-S) and has protected these cells during chemotherapy (Guigon *et al.*, 1990) (reviewed in Wright and Pragnell, 1992). The pentapeptide pEEDCK, pGlu-Glu-Asp-Cys-Lys) has also been described as an inhibitor of murine GM-CFC (Laerum and Paukovits, 1984) and CFU-S (Paukovits, 1989), with no effects on erythroid progenitors (Kreja *et al.*, 1986; Laerum and Paukovits, 1984). It antagonizes the stimulatory effects of IL-3 and synergises with the inhibitory activities of IFN-γ (Lu *et al.*, 1989).

1.2.2. Role of the stroma in haemopoiesis

Haemopoiesis only takes place if the BM offers an appropriate environment that permits the survival, self-renewal, proliferation, and differentiation of stem cells. The first evidence for the regulatory role of the stroma in haemopoietic cell development has been shown by the Sl/Sl^{d} mice carrying the Steel mutation, which have a macrocytic anaemia associated with a defective stromal cell environment. It has been shown that the anaemia could be cured by implantation of normal stromal spleen tissue, but not by infusion of normal stem cells (McCulloch *et al.*, 1965). The confirmation of these results was provided by the analysis of a 'defective haemopoiesis' using long-term bone marrow cultures (LTBMC) (Dexter and Moore, 1977). Adherent layers established with bone marrow from W/W^{v} mice, which have a defective stem cell population, could support long-term production of CFU-S from Sl/Sl^{d} cells and their progeny (Dexter and Moore, 1977).

The regulatory role of the microenvironment has indeed become clearer with the information provided by the LTBMC system, first described for mice by Dexter and colleagues (Dexter *et al.*, 1977), and subsequently adapted for human cells (Gartner and Kaplan, 1980). In these cultures, a hierarchy of haemopoietic cells initiated with bone

marrow cells, ranging from the LTC-IC through their clonogenic progeny to mature cells, is sustained in the absence of added growth factors, but is absolutely dependent upon the establishment of an adherent layer of bone-marrow derived stromal cells (Dexter, 1982). The stromal layer mimics structurally and functionally the *in vivo* haemopoietic microenvironment (Allen and Dexter, 1983) and its cellular composition includes at least endothelial cells, fibroblasts, fat cells, and macrophages. Primitive haemopoietic cells migrate beneath and between the stromal elements and haemopoiesis is then established in intimate association with the stroma. The production and maintenance of haemopoietic cells appears to be a function of the cells present in the adherent layer formed in this system when established with unseparated marrow or provided by pre-established feeder layers from a variety of sources (Breems *et al.*, 1994; Sutherland *et al.*, 1990). The mechanisms underlying the physiological regulation of haemopoiesis by stromal cells are poorly understood, and stromal functions probably include:

- synthesis, secretion, and presentation of the appropriate range and concentration of growth-stimulatory and growth-inhibitory factors that are responsible for regulating the proliferation and the development of haemopoietic progenitor cells;
- synthesis of extracellular matrix (ECM) molecules that play a variety of roles in maintaining the integrity of the haemopoietic inductive microenvironment (HIM) and also in presenting messengers to the developing haemopoietic cells;
- intercellular communication between the different types of stromal cells and between the stromal cells and haemopoietic cells.

The stromal cells, particularly the macrophages, have been shown to produce *in vitro* several cytokines, including M-CSF, GM-CSF, G-CSF, IL-1, IL-6, IL-7 (Fibbe *et al.*, 1988a; Fibbe *et al.*, 1988b; Gualtieri *et al.*, 1987; Gualtieri *et al.*, 1984; Henney, 1989), and SCF (Lyman and Jacobsen, 1998) as well as growth inhibitors as TGF-β and MIP-1α (de Wynter, 2000). Many of these molecules are produced in a soluble form and may specifically associate with certain components of the ECM (Gordon *et al.*, 1987). Haemopoietic progenitor cells have been shown to bind directly to ECM components influencing both myelopoiesis and lymphopoiesis (Clark *et al.*, 1992; Siczkowski *et al.*, 1992). Stromal cell adhesion to haemopoietic cells is in part mediated by a family of proteins such as integrins, selectins, and proteoglycans that are widely expressed on haemopoietic cells. They have a role not only in the adhesion of

haemopoietic progenitor cells to the stroma, but also in intracellular signalling *via* the occupation of their receptors. Haemopoietic cells, among the integrins, express VLA-4 ($\alpha4\beta1$) and VLA-5 ($\alpha5\beta1$) that bind to fibronectin, one of the most abundant components of the bone marrow extracellular matrix.

Haemopoietic growth factors can directly modulate cell adhesion molecules. IL-1 stimulation of stromal cells causes both increased VCAM-1 and ICAM-1 expression and increased attachment by $CD34^+$ bone marrow cells (Teixido *et al.*, 1992); IL-3 modulates VLA-4 action in haemopoietic progenitors (Hurley *et al.*, 1995). Recently, it has been reported that cytokines can interact with adhesion molecules synergistically to influence the developmental and proliferative *status* of primitive cells (Clark *et al.*, 1992; Levesque *et al.*, 1996). In this context, G-CSF has been shown to downregulate VLA-4 expression during neutrophil maturation (Kinashi and Springer, 1994). Therefore, the haemopoietic microenvironment could be viewed as a series of stromal domains or niches producing local islands of a number of growth modulators that synergise to influence stem and progenitor cell survival, proliferation and development.

1.3. Fibroblast Growth Factors

One of the major cell types that constitute the haemopoietic stroma is the fibroblast. Fibroblasts have long been recognised as playing an active role in the microenvironment of the haemopoietic system by providing a number of growth factors known to influence haemopoietic cell development. Accumulating evidence has demonstrated that fibroblasts are under the control of a closely related group of factors termed fibroblast growth factors (FGF) (Gospodarowicz *et al.*, 1986). The FGFs appear to play important roles in controlling cell proliferation and differentiation, particularly in cells of mesoderm origin (Gospodarowicz *et al.*, 1987).

During embryonic development, two kinds of signals are passed from the endoderm to the ectoderm to form a new germ layer, the mesoderm. The first signal directs the formation of the dorsal mesoderm, which forms the notochord; the other induces the formation of the ventral and lateral mesoderm, from which the blood is derived (Zon, 1995). The process of mesoderm induction is poorly understood, but there is evidence that members of the fibroblast growth factor family, currently comprising 22 members (FGF 1 to 22) in mammals (Coulier *et al.*, 1997; Hoshikawa *et al.*, 1998; Nobuyuki, 1999), play a role by stimulating mitogenic, differentiation, migration, and

survival responses, the precise nature of which depend on the target cell and its developmental history (Amaya *et al.*, 1993; Baird and Klagsbrun, 1991; Goldfarb, 1990; Slack, 1994; Slack *et al.*, 1987; Yamaguchi and Rossant, 1995).

Most of the FGF-family genes are expressed in various tissues during embryo development. The proteins encoded by the 22 FGF genes range from ~150 to 270 amino acids residues in length, and each contains a conserved 'core' sequence of ~120 amino acids with a common tertiary structure and the ability to bind heparin or heparan sulphate proteoglycans (Faham *et al.*, 1996; Zhu *et al.*, 1991). Consistent with their functions as intercellular signalling molecules, many FGFs are exported efficiently from the cells that produce them. Once released, they bind avidly to the heparan sulphate proteoglycans present on the cell surface and in the extracellular matrix.

The biological activity of FGFs is mediated through interaction with high affinity FGF-receptors (FGF-R). Cell surface heparan sulphate proteoglycans also play a role as low-affinity receptors, facilitating FGF signal transduction by oligomerising and presenting the ligands to high-affinity FGF-Rs (Yayon *et al.*, 1991). The high-affinity FGF-Rs belong to a family of transmembrane receptors with an extracellular ligand binding portion consisting of three immunoglobulin-like domains (D1, D2 and D3), a single transmembrane helix, and a cytoplasmic portion that contains protein tyrosine kinase activity (Szebenyi and Fallon, 1999). Ligand binding resides in D2, D3, and in the linker that connects them (Johnson and Williams, 1993; Stauber *et al.*, 2000). Specificity is achieved through interactions between the N-terminal and central regions of the FGF and the D2, D3 domains and the interdomain linker (Plotnikov *et al.*, 2000). To date, four cDNAs for such receptors have been isolated (FGF-R1 to 4) in mammalian species (Dionne *et al.*, 1990; Houssaint *et al.*, 1990; Keegan *et al.*, 1991a; Keegan *et al.*, 1991b; Partanen *et al.*, 1991), and numerous subtypes of alternatively spliced isoforms are thought to be responsible for differential responses (Johnson and Williams, 1993). Signal transduction by these receptors requires dimerisation followed by autophosphorylation of the receptors through their tyrosine kinase domain (Plotnikov *et al.*, 1999).

1.3.1. Fibroblast Growth Factors in haemopoiesis

The haemopoietic system derives from the mesoderm, but only recently has the expression of the different FGFs and their receptors on haemopoietic cells been studied

in more detail. Most of the studies investigating FGF in relation to the haemopoietic system have focused on FGF1 (acid FGF or aFGF), and on FGF2 (basic FGF or bFGF).

In vivo, pre-incubation of marrow cells with FGF2 has been shown to increase the number of D9 and D12 CFU-S (Gallicchio *et al.*, 1991). FGF2 is a potent mitogen for human bone marrow stromal cells *in vitro* (Oliver *et al.*, 1990), and it has been reported to improve the performance of human LTBMC (Wilson *et al.*, 1991) probably through an indirect mechanism, inducing the production of secondary cytokines such as IL-1, IL-6, GM-CSF or IL-3 (Allouche, 1995). Colony formation by $CD34^+CD33^-$ cells from peripheral blood is enhanced by the combination of FGF2, IL-3 and GM-CSF (Gabbianelli *et al.*, 1990). It synergises with GM-CSF, TPO and EPO or with SCF and GM-CSF to enhance GM-CFC, Meg-CFC and BFU-E colony formation (Bruno *et al.*, 1993; Gabrilove *et al.*, 1994; Gallicchio *et al.*, 1991). Single $CD34^+$HLA-DR^+CD38^- cells from foetal bone marrow can give rise to all haemopoietic cell lineages when cultured with IL-6, IL-3, GM-CSF and EPO in the presence of SCF, IGF-1 and FGF2 (Huang and Terstappen, 1994). FGF2 has been shown to antagonise the suppressive effects of TGF-β1 (Gabrilove *et al.*, 1993). Interestingly, FGF2 in combination with IL-3, IL-6 and SCF increased the transduction efficiency of $CD34^+$ cells from leukapheresis product (Dilber *et al.*, 1994), suggesting its potential use for improving gene transfer protocols. Recently, addition of FGF4 to human LTBMC has been reported to extend their capacity to support haemopoiesis for at least 8 months (Quito *et al.*, 1996).

The fact that haemopoietic progenitors respond to FGF2 suggests the presence of FGF-Rs. Most studies indicate that both FGF1 and FGF2 (Allouche and Bikfalvi, 1995), and possibly FGF4 (Basilico *et al.*, 1989; Bellosta *et al.*, 1993), bind to the same receptors. Basic FGF is a ligand for both FGFR-1 and FGFR-2 (Allouche and Bikfalvi, 1995). Using Northern blot and reverse transcript amplification (RT-PCR), FGFR-1 and FGFR-2 have been detected on murine bone marrow cells and on murine platelets, macrophages, granulocytes, T and B lymphocytes (Bikfalvi *et al.*, 1992). Human leukaemic cell lines have also shown to express FGF-R 1, 2, 3, and 4 (Allouche *et al.*, 1995; Armstrong *et al.*, 1992; Bikfalvi *et al.*, 1992; Chandler *et al.*, 1999). Three soluble forms of FGF-R proteins binding to bFGF and aFGF were detected in human serum and plasma (Hannenken *et al.*, 1994). Expression of FGFRs mRNA and protein level using RT-PCR and flow cytometry, respectively, could be demonstrated on sub populations of human peripheral blood mononuclear cells and on cells derived from day-6 GM-CFC

and CFU-E colonies (Ratajczak *et al.*, 1996). These data indicate a receptor-mediated effect for FGF2 that appears to be restricted to lineage-committed myeloid progenitor and mature cells. Therefore, the role of FGF2 as well as of the other members of the FGF family in regulating haemopoietic cell development, whether direct or indirect, remains to be elucidated.

1.4. Chemokines

Chemokines define a large family of molecules that have been implicated primarily in the recruitment of inflammatory cells into tissues in response to infection and inflammation (reviewed in Ben-Baruch *et al.*, 1995; Springer, 1994). However, they also exert a variety of effects on proliferation and differentiation of primitive haemopoietic cells. Chemokines are 8 to 12kDa proteins, with 70 to 80 amino acids in length, and 20 to 70% homology in amino acid sequences (Brown *et al.*, 1989). They have been subdivided into families based on whether or not an intervening amino acid is situated between the first two of four conserved cysteine motifs near the N-terminal portion of the molecule. For instances, in the CC subfamily, or β chemokines, the cysteine residues are adjacent; in the CXC subfamily, or α chemokines, cysteines are separated by one amino acid residue; in CX_3C chemokines, cysteines are separated by three residues; and C chemokines have only one cysteine. Members within a subfamily have a higher degree of structural homology and biological similarities with one another than with members of the other subfamily. Each subfamily is encoded by different chromosomes and homologous chemokine genes and products have been described in humans and mice (Oppenheim *et al.*, 1991).

The α-chemokines are further divided into those that contain or not the sequence glutamic acid-leucine-arginine (ELR) near the N-terminal preceding the CXC sequence. The formers are chemotactic for neutrophils, whereas those not-containing the sequence act on lymphocytes (Clark Lewis *et al.*, 1995). Structurally, the β-chemokines can be divided into two subfamilies. The monocyte-chemoattractant-protein-eotaxin family, which contains the five monocyte chemoattractant proteins (MCP-1 to 5) and eotaxin, and share approximately 65% homology, and the other β-chemokines (Luster and Rothenberg, 1997). Functionally, the β-chemokines are diverse, attracting and/or activating monocytes, different subsets of lymphocytes, basophils, eosinophils and dendritic cells, depending on the chemokine involved and on the conditions of the assay

performed (Rollins, 1997). Figure 1.5 illustrates the four chemokine families arranged by their amino-terminal cysteine motifs, some of the most studied chemokines within the families, and the receptors through which they target specific cells.

1.4.1. Chemokine receptors

Chemokines induce cell proliferation, migration, adhesion and activation by binding to specific seven transmembrane spanning, G-protein-coupled cell surface receptors on target cells (Premack and Schall, 1996). Chemokine receptors measure approximately 350 amino acids in length, have a short and overall acidic N-terminus and contain N-linked glycosylation sites. There is an intracellular C-terminus with serine and threonine residues that act as phosphorylation sites for receptor regulation. The seven α-helical transmembrane domains – with three intracellular and three extracellular connecting loops – are oriented perpendicularly to the plasma membrane; a disulphide bond links highly conserved cysteines in the extracellular loops 1 and 2. These receptors are generally coupled to Gαi proteins, making cellular responses to chemokines to be inhibited by pertussis toxin (Broxmeyer and Kim, 1999; Murphy, 1996).

To date, 18 human chemokine receptors have been identified (reviewed recently in Murdoch and Finn, 2000). Among the five receptors that bind the known CXC chemokines are the chemokine receptors CXCR1 to CXCR5, whereas the CC receptor family consists of 11 members, CCR1 to CCR11. Recently, receptors for fractalkine (CX_3CR1) (Imai *et al.*, 1997b) and lymphotactin (XCR1) (Yoshida *et al.*, 1998) have been identified. A further chemokine receptor, known as Duffy antigen receptor for chemokines (DARC) has been shown to bind both CXC and CC chemokines (Chaudhuri *et al.*, 1993; Chaudhuri *et al.*, 1994).

The precise mechanisms of coupling receptor activation to complex physiological responses are still not clear. The cellular responses to chemokines are strictly regulated mainly by a desensitisation process that is characteristic of G-protein receptors: after activation, chemokine receptors may become refractory to repeated stimulation with the same or other agonists (Baggiolini *et al.*, 1994; Savarese and Fraser, 1992). Therefore, desensitisation can be referred to as either 'homologous' or 'heterologous'. Homologous desensitisation is usually complete and occurs characteristically at high concentrations of ligand, is relatively transient, and results in diminished responsiveness

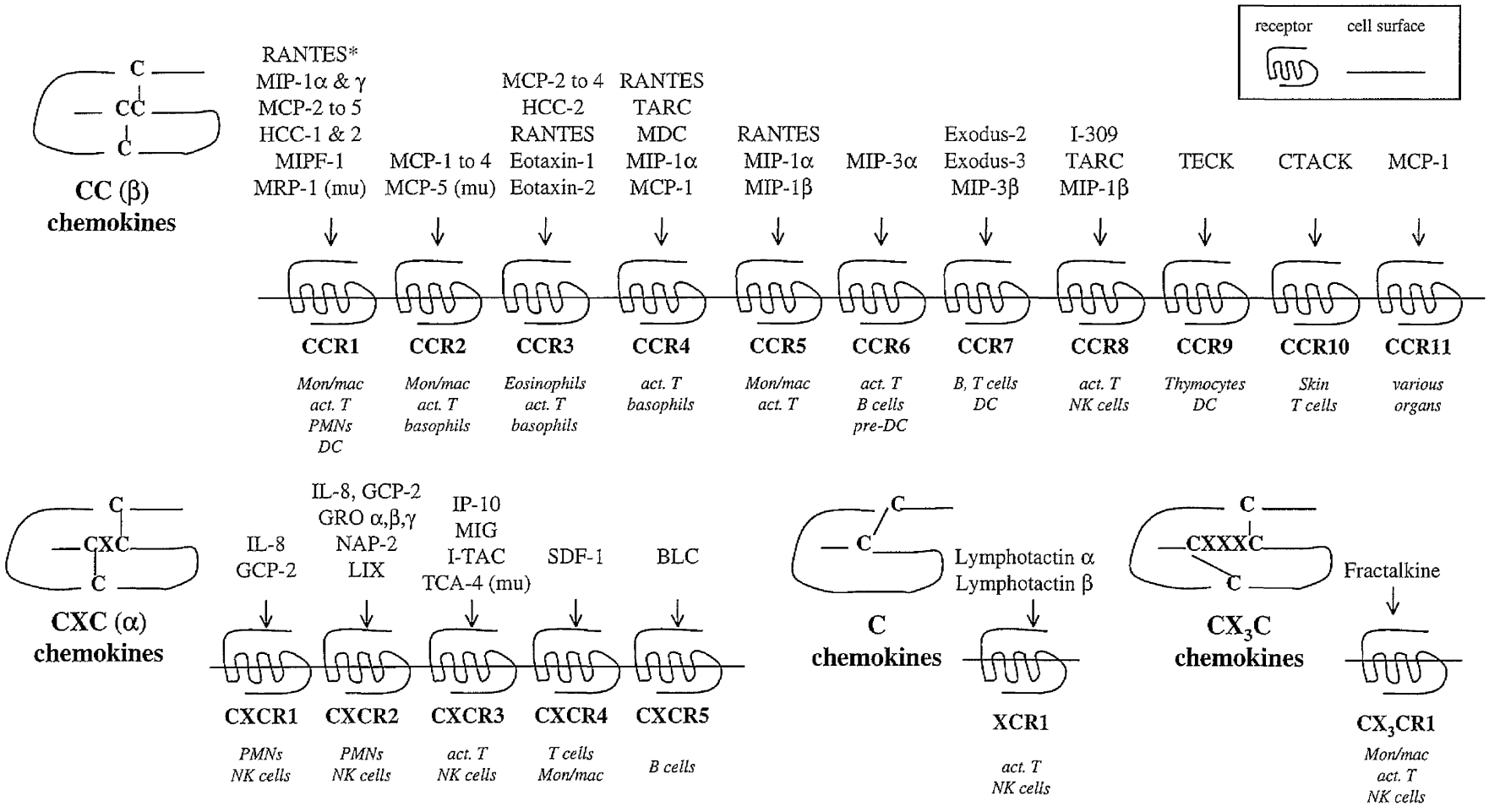


Figure 1.5. Chemokine families, their receptors and target cells. The families of the chemokines are shown, classified by the arrangement of amino-terminal cysteine residues. The seven transmembrane receptors are depicted. The chemokines, their receptors and the cellular expression of each receptor are indicated above and below, respectively, each group. Mon, monocytes; mac, macrophages; act. T, activated T cells; DC, dendritic cells; PMN, neutrophils; pre-DC, precursor or immature DC; mu, murine. Adapted from Luster, 1998; Kim and Broxmeyer, 1999; Mackay and Rosen, 2000, and Hancock *et al.*, 2000 (* for abbreviations of chemokines, see page 18).

specific for the original desensitising agent. Heterologous desensitisation is a reversible loss of responsiveness to multiple ligands.

During ligand binding, chemokine receptors associate with G-proteins. In the active state, an exchange occurs in the α sub-unit of the G protein from a GDP- to a GTP-bound state, resulting in a dissociation of the α sub-unit from the βγ sub-units. The free α sub-unit can activate both the membrane-associated enzymes phospholipase C (PLC) and PLCβ2, whereas the free βγ complex activates preferentially PLCβ2. The activation of PLCs results in hydrolysis of phosphatidil-inositol 4,5-bisphosphate (PIP_2) to generate two second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). IP_3 mobilises Ca^{+2} from intracellular stores leading to a transient rise in Ca^{+2} concentration, whereas DG stimulates Protein Kinase C (PKC) (Murphy, 1996; Rollins, 1997). The activation of PKC and of various calcium-sensitive protein kinases catalyses protein phosphorylation, which activates a series of coordinated signalling events that eventually lead to cellular responses (Bokoch, 1995; Kuang *et al.*, 1996; Murphy, 1996; Premack and Schall, 1996; Rollins, 1997). There is increasing evidence that chemokine receptors can also activate several others intracellular effectors downstream G coupling (Premack and Schall, 1996), including the low-molecular-weight proteins Ras and Rho (Bacon *et al.*, 1996; Bokoch, 1995), phospholipase A2, phosphatidylinositol-3-kinase (Turner *et al.*, 1995), tyrosine kinases (Ben-Baruch *et al.*, 1995; Ganju *et al.*, 1998b; Huang *et al.*, 1998; Mellado *et al.*, 1998), and the MAP kinase pathway (Kampten *et al.*, 2000).

Haemopoietic cells express unique, but overlapping subsets of chemokine receptors. Most receptors recognise more than one chemokine, and several chemokines bind to more than one receptor, indicating that redundancy and versatility are characteristic for the chemokine system. This is apparent from Figure 1.5. This redundancy in binding, also called promiscuity, may account for some of the chemokine overlapping responses and adds a complication in terms of interpreting how chemokines function individually. Some receptors and their cognate ligands have biologic functions significantly different from those of other chemokines and chemokine receptors. An example is given by the CXCR4 receptor which is the only receptor so far described to bind SDF-1α. Its importance in embryogenesis has been documented in mice lacking its expression that exhibit impaired B lymphopoiesis, myelopoiesis, derailed cerebellar

neurone migration, and defective formation of large vessels supplying the gastrointestinal tract (Ma *et al.*, 1998; Nagasawa *et al.*, 1996a; Tachibana *et al.*, 1998).

Chemokine receptors participate in several disease states, either by overexpressing receptors or by facilitating viral entry into permissive cells. For instance, DARC functions pathologically in malaria, acting as the invasin for *Plasmodium vivax, P. knowlesi,* and *P. falciparum* (Horuk *et al.*, 1993; Mason *et al.*, 1977; Miller *et al.*, 1976; Miller *et al.*, 1975; Miller *et al.*, 1977). African descendants who lack DARC on their erythrocytes are resistant to malaria. Since DARC lacks a known signalling function and is expressed on erythrocytes, it was originally proposed to act as a chemokine sink in the blood (Darbonne *et al.*, 1991). Regardless of the physiologic function of DARC, the fact that chemokines bind to it and block invasion of red cells by plasmodia *in vitro* could make them useful for the development of anti-malaric drugs.

Recently, MIP-1α, MIP-1β and RANTES were identified as the major HIV-suppressive factors in $CD8^+$ T cell supernatants as they inhibited HIV-1 replication in $CD4^+$ T cell clones and peripheral blood mononuclear cells (Cocchi *et al.*, 1995). These molecules have established the first connection between chemokines and HIV infection. Although the CD4 molecule has been identified as the major receptor for HIV-1 (Dalgleish *et al.*, 1984; Sattentau *et al.*, 1986), its expression alone is not sufficient to make mouse cells susceptible to HIV, suggesting the existence of co-receptors. Later, the receptor CCR5, which binds MIP-1α, MIP-1β and RANTES, was identified as the major co-receptor for M-tropic strains of HIV-1 (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996). These findings could then explain the earlier findings in which these chemokines could block HIV-1 membrane fusion mediated by the receptor.

At the same time, CXCR4 was also identified as a co-receptor for T-tropic HIV-1 virus (Feng *et al.*, 1996). Its ligand, SDF-1, was able to block completely infection of the virus in a dose dependent manner. Since these findings, other chemokine receptors such as CCR2, CCR3 (Doranz *et al.*, 1996), and CCR8 (Horuk, 1999; Horuk *et al.*, 1998) have been identified as a cofactor, in association with CD4, to permit infection of permissive cells either by T-cell tropic or by M-tropic HIV strains. CCR5 (de Wynter *et al.*, 1998; Lee *et al.*, 1999a; Ruiz *et al.*, 1998; Shen *et al.*, 1998) and CXCR4 (Aiuti *et al.*, 1999a; Aiuti *et al.*, 1999b; Lataillade *et al.*, 2000; Shen *et al.*, 1998; Viardot *et al.*, 1998; Wang *et al.*, 1998) have been shown to be expressed on human $CD34^+$ cells, a finding that make them likely to be targets for HIV infection. Although controversy

regarding the susceptibility of stem cells to HIV-1 infection exists (Deichmann *et al.*, 1997; Shen *et al.*, 1999; Weichold *et al.*, 1998), the presence of these receptors on progenitor cells may be correlated to the clinical reports showing that patients infected by HIV-1 exhibit several haematological abnormalities which some may be due to infection of more primitive haemopoietic cells and subsequent dysregulation of haemopoiesis.

As for chemokines, expression of chemokine receptors can be modulated by cytokines. For example, IL-4 and IL-13 up-regulate expression of IL-8R on normal B cells whilst IFN-γ, IL-2 and TNF-α downregulate them (Jinquan *et al.*, 1997). TNF-α also downregulates the expression of IL-8R on neutrophils (Khandaker *et al.*, 1999) while G-CSF stimulates them (Lloyd *et al.*, 1995). IFN-γ increases the expression of CCR1 and CCR3 on neutrophils (Bonecchi *et al.*, 1999). IL-2 and IL-15 induce expression of CCR1, CCR2, and CCR5 on activated T cells (Loetscher *et al.*, 1996; Perera *et al.*, 1999), whereas IL-10 up-regulates them on human monocytes (Sozzani *et al.*, 1998). It has been recently reported that IL-4 and IL-10 can up and downregulate, respectively, the expression of CXCR4 on $CD4^+$ cells (Jinquan *et al.*, 2000). Expression of MIP-1α receptors on $CD34^+$ cells can be modulated by TNF-α and IFN-γ (Durig *et al.*, 1998).

Genetically modified mouse models have been used to characterise the properties of chemokines. Most of the *in vitro* activities of chemokines have been recapitulated in these models, and this will certainly provide information for designing new drugs for the treatment of diseases ranging from inflammatory conditions to acquired immunodeficiency syndrome.

1.4.2. Chemokines in haemopoiesis

One of the first haemopoietic regulatory activities for chemokines in general and in particular for MIP-1α was that of the enhancement of colony formation *in vitro* by GM-CFC and M-CFC progenitors, respectively stimulated by GM-CSF and M-CSF (Broxmeyer *et al.*, 1989; Broxmeyer *et al.*, 1990). This was an effect observed only on more mature subsets of progenitors, those that responded to stimulation by a single growth factor. Later, it was demonstrated that MIP-1α has a suppressive activity on more immature subsets of normal murine and human bone marrow stem and progenitor cells *in vitro* and on murine myelopoiesis *in vivo*. In this setting, a number of other

chemokines have demonstrated to suppress progenitor cell proliferation *in vitro* to at least the same degree as MIP-1α (Broxmeyer *et al.*, 1999b).

Suppressive chemokines in the context of inhibition of myeloid progenitor cells stimulated to proliferate by a combination of growth factors have been extensively studied by Broxmeyer's group and include MIP-2α, MIP-3α, MIP-3β, MIP-1γ, MCP-1, MCP-4, MIP-5, Exodus 2, PF4, CKβ-6, CKβ-7, CKβ-8, Eotaxin-2, I-309, TECK, and MRP-1. Chemokines belonging to the CXC family that have suppressive effect in the same context include GRO-β, IL-8, GCP-2, PF4, IP-10, ENA 78, and Mig. Lymphotactin, the only member of the C chemokine family, also has a suppressive activity. CC chemokines without suppressive activity include MIP-1β, RANTES, MCP-2, MCP-3, Eotaxin-1, CKβ-1, TARC and MDC. Those CXC chemokines without suppressive activity include GRO-α, GRO-γ, NAP-2 and SDF-1. The only member of the CX_3C family, fractalkine, is also without suppressive activity (Broxmeyer *et al.*, 1999b).

Chemokine production can be regulated by cytokines. IL-8 expression, for example, can be induced by IL-1 and TNF-α (Cassatella *et al.*, 1993), while inhibited by IFN-α or IFN-γ (Cassatella *et al.*, 1993; Oliveira *et al.*, 1992); IL-10 has been shown to downregulate MIP-1α expression (Kasama *et al.*, 1994); IFN-γ inhibits early mRNA expression for MIP-1α, MIP-1β, and IL-8 (up to 8 hours post IFN-γ addition) in neutrophils, while augmenting their production at 24 hours post cytokine addition (Kasama *et al.*, 1995). Some cytokines with suppressive activity upon haemopoietic cells can regulate MIP-1α expression. For example, TGF-β downregulates both MIP-1α and MIP-1β expression (Maltman *et al.*, 1993); IFN-α stimulates MIP-1α production in marrow stroma (Bhatia *et al.*, 1995).

1.4.2.1. MIP-1α

Due to its involvement in haemopoiesis, we have been particularly interested in MIP-1α, one of the most studied members of the β family of chemokines. MIP-1, as originally characterised, is a heparin-binding protein doublet comprising the peptides MIP-1α and MIP-1β (Sherry *et al.*, 1992; Sherry *et al.*, 1988). MIP-1α was found to be the same molecule as the normal bone marrow extract fraction IV (NBME IV) and stem cell inhibitor (SCI) characterised by independent groups (Graham *et al.*, 1990; Lord *et al.*, 1976) as described earlier in this chapter (section 1.2.1.2). LD78 is the human

equivalent of the murine MIP-1α (Obaru *et al.*, 1986), sharing 74% amino acid sequence homology (Yamamura *et al.*, 1992). Subsequently, the cDNA sequences of the GOS19 gene expressed in human adult T lymphocytes was found to be homologous to that of the murine gene encoding MIP-1α (Blum *et al.*, 1990).

Produced mainly by macrophages (Christman *et al.*, 1992; de Wynter, 2000; Maltman *et al.*, 1993; Otsuka *et al.*, 1991; VanOtteren *et al.*, 1994), MIP-1α has been reported to act as an autocrine regulator for its cells of origin, causing their proliferation and promoting the secretion of TNF, IL-1, and IL-6 (Fahey *et al.*, 1992). Native MIP-1α has a tendency to aggregate, and this characteristic has been suggested as the basis for decreased specific activity (Wolpe *et al.*, 1988). This was later supported by the demonstration that the monomeric form of MIP-1α was up to 1000-fold more active in MIP-1α-suppression activity than the polymerised compound (Avalos *et al.*, 1994; Mantel *et al.*, 1993). However, it was found that the monomeric, dimeric and tetrameric mutant MIP-1α forms were equipotent, and disaggregation would spontaneously occur under assay conditions, where it acted as a monomer (Graham *et al.*, 1994). To overcome the aggregation problem, an active variant of LD78 with a single amino acid substitution (Asp26/Ala) has been developed and named BB10010 (Hunter *et al.*, 1995). This recombinant form has a reduced tendency to aggregate at physiological pH and ionic strength and has been shown to be active in receptor binding assays, calcium mobilisation, inhibition of colony formation and thymidine suicide assays (Hunter *et al.*, 1995). Its efficacy has been also demonstrated *in vivo*, where administration of BB10010 to mice resulted in mobilisation of early progenitors (Lord *et al.*, 1995) as does the native form.

In vitro, MIP-1α causes mouse neutrophil migration and influences the trafficking of distinct lymphocyte populations according to the dose used (Schall *et al.*, 1993). Basophils (Alam *et al.*, 1992) and eosinophils (Rot *et al.*, 1992) are responsive to MIP-1α resulting in migration and increased functional activity. However, the role of MIP-1α that has attracted widespread interest is as a haemopoietic stem cell proliferation inhibitor. As stated previously, MIP-1α has been described as a suppressor molecule for immature subsets of human GM-CFC, erythroid and multipotential progenitors that proliferate in response to multiple growth factors such as EPO, IL-3 and SCF (Broxmeyer *et al.*, 1990). These *in vitro* properties have been confirmed using a range of murine progenitor cells, including purified bone marrow progenitor cells and the

FDCP-mix A4 cell line (Clements *et al.*, 1992; Heyworth *et al.*, 1995b). In contrast, MIP-1α enhances the proliferation of the more mature members of the GM-CFC and M-CFC progenitor cell subsets that respond to stimulation by a single growth factor such as GM-CSF and M-CSF, respectively (Broxmeyer *et al.*, 1989; Broxmeyer *et al.*, 1990).

In vivo, mice receiving MIP-1α have been shown to have stem cell protection from subsequent *in vitro* killing by tritiated thymidine (Clements *et al.*, 1992). Also, pre-treatment of primitive haemopoietic cells with MIP-1α protected them from the cytotoxic effects of hydroxyurea and cytosine arabinoside (Dunlop *et al.*, 1992; Lord *et al.*, 1992; Maze *et al.*, 1992). Subsequently, it was demonstrated that the administration of MIP-1α to mice decreased the cycling rates and absolute numbers of myeloid progenitors from bone marrow and spleen (Cooper *et al.*, 1994; Mantel *et al.*, 1993). Therefore, MIP-1α seems to act as a regulator of stem cell proliferation through its ability to reduce the proportion of primitive haemopoietic cells in DNA synthesis specifically, thereby protecting them from the effects of cell-cycle-specific cytotoxic drugs. As such, MIP-1α would have important therapeutic applications in protecting haemopoietic cells from damage during cytotoxic therapies for cancer. Furthermore, as the effectiveness of chemotherapy correlates with the dose intensity and treatment is limited by myelotoxic effects, administration of MIP-1α would allow more intensive therapy. Indeed, the use of the mutant form BB10010 in human clinical trials has shown myelosuppressive effects similar to those observed in mice treated with MIP-1α (Broxmeyer *et al.*, 1998; Marshall *et al.*, 1998). On the other hand, mice treated with hydroxyurea have also shown to recover progenitor cell numbers faster than the untreated control group, suggesting that MIP-1α may shorten the cell cycle of the more mature progenitors (Lord *et al.*, 1992; Lord *et al.*, 1993). These findings indicate that the effects of MIP-1α on the *in vitro* proliferation of haemopoietic progenitors are not only inhibitory.

Of interest is that the clonogenic cells of chronic myeloid leukaemia (CML) are resistant to the *in vitro* growth inhibitory effects of MIP-1α (Broxmeyer *et al.*, 1993a; Chasty *et al.*, 1995; Eaves *et al.*, 1993; Holyoake *et al.*, 1993). At this time, it is not clear why CML clonogenic cells do not respond to inhibition by MIP-1α. It does not correlate to the lack of expression of MIP-1α receptors (Chasty *et al.*, 1995; Durig *et al.*, 1999b) or to the cell cycling status of these cells (Broxmeyer *et al.*, 1993a; Durig *et*

al., 1999b). However, it has been shown that whereas MIP-1α conferred protection to normal CFC against the cell cycle active drug cytosine arabinoside (Ara-C), it increased the sensitivity of CML CFC to the drug, probably as a result of an aberrant than an absent response in CML progenitor cells (Durig *et al.*, 1999b). Moreover, CML cells respond to MIP-1α induced adhesion to fibronectin in a similar manner to their normal bone marrow counterparts, indicating that MIP-1α signalling pathways are not completely uncoupled in these cells (Durig *et al.*, 1999b). Therefore, overexpression or lack of expression of receptors subtypes, defective receptors, faulty ligand-receptor interactions or disruptions of downstream signalling pathways may be some of the reasons for the MIP-1α unresponsiveness of CML progenitors.

CML is relatively resistant to chemotherapy and the disease is difficult to cure using conventional therapeutic routes (reviewed in Geary, 2000). The abrogation of the MIP-1α inhibitory responses may provide a rationale for treatment of CML patients and *in vitro* purging strategies. In such a leukaemic condition, in which patients have CML clonogenic cells not responsive to MIP-1α, the chemokine may be able to be used in clinical approach that would place the normal progenitor cells in those patients into a slow-proliferating and potentially more protected state so that chemotherapy can be used more efficaciously. It also could be used as a potential adjunct in the treatment of chronic phase CML patients receiving low dose Ara-C alone or in combination with interferon-α.

MIP-1α, after receptor binding, elicits its effects *via* activation of several different intracellular effectors. It has been shown to induce increase in cytosolic Ca^{++} levels *via* pertussis toxin G-coupled receptors in human neutrophils (Gao *et al.*, 1993), monocytes (Durig *et al.*, 1999a; Sozzani *et al.*, 1993), and in human 293 kidney cells expressing CCR1 (Neote *et al.*, 1993). It also stimulates inositol phosphate production in FDCP-mix cells through inositol lipid hydrolysis (Heyworth *et al.*, 1995b). Proliferation of MO7e cells promoted by co-addition of GM-CSF and SCF as a result of activation of MAP kinase pathway through Raf-1 activity is suppressed by pretreatment of MO7e cells with MIP-1α (Aronica *et al.*, 1997; Aronica *et al.*, 1995; Mantel *et al.*, 1995).

The *in vivo* and *in vitro* studies have shown that, directly or indirectly, MIP-1α affects early haemopoietic progenitors. However, homozygous mutant MIP-1α (-/-) mice have no overt abnormalities of peripheral blood or bone marrow cells, indicating that MIP-1α is not necessary for normal haemopoiesis (Cook, 1996). Due to the

presence of two MIP-1α related genes in humans (Nakao *et al.*, 1990), it is possible that the role of MIP-1α may be compensated for by another molecule. Therefore, plainly there are still many aspects of the effects of MIP-1α on primitive progenitor cell proliferation to be assessed in order to understand better its role as an *in vivo* regulator of haemopoiesis and explore its clinical potential as a protective chemotherapeutic agent.

1.4.2.2. SDF-1α

In contrast to the large number of chemokines that can affect haemopoietic cell growth, only three chemokines have been reported to direct the movement of myeloid progenitor cells: Exodus 2, Exodus 3 (Kim and Broxmeyer, 1999b; Kim *et al.*, 1999), and SDF-1 (Aiuti *et al.*, 1997). SDF-1, or pre-B-cell growth-stimulating factor – PBSF, was first implicated in the movement of these cells during foetal life through studies with SDF-1 null (-/-) mice (Nagasawa *et al.*, 1996a). These results were later confirmed through studies with the SDF-1 receptor, CXCR4, null mutant (-/-) mice (Zou *et al.*, 1998). In addition, it has several essential functions during embryonic development as SDF-1α knockout mice die perinatally, having hypoplasia of B cells in both liver and bone marrow, hypoplasia of myeloid cells in bone marrow, and abnormal cardiac organogenesis (Ma *et al.*, 1998; Nagasawa *et al.*, 1996a).

SDF-1 is a CXC chemokine cloned from mouse bone marrow stromal cells (Nagasawa *et al.*, 1994; Tashiro *et al.*, 1993), and occurs in two alternative splicing variants, SDF-1α and SDF-1β (Tashiro *et al.*, 1993). The human homologue gene coding for both SDF forms is located on chromosome 10, and its highly conserved amino acid sequence differs from the mouse only in one amino acid residue (Shirozu *et al.*, 1995). SDF-1α has several unique properties distinct from those of other chemokines. First, it is a potent chemoattractant for resting lymphocytes (Bleul *et al.*, 1996b) and in particular for $CD34^+$ haemopoietic progenitor cells (Aiuti *et al.*, 1997). Second, it is produced by bone marrow stromal cells constitutively (Nagasawa *et al.*, 1994; Tashiro *et al.*, 1993), whereas many other chemokines are in principle released by leukocytes upon pro-inflammatory conditions. Third, expression of the SDF-1 receptor, CXCR4, can be modulated *in vitro* by exposure to cytokines which are routinely used for the purpose of gene therapy or *ex vivo* expansion (Aiuti, 2000).

Movement/homing to certain organs also requires integrin-mediated adhesion to extracellular matrix components such as fibronectin (Campbell *et al.*, 1996). This adhesion is activated by certain cytokines such as GM-CSF, SCF (Takahira *et al.*, 1997), IL-3 (Shibayama *et al.*, 1999; Shibayama *et al.*, 1998), EPO and TPO (Gotoh *et al.*, 1997). Of potential relevance, SDF-1 has been shown to block or decrease growth factor-induced, integrin mediated adhesion of progenitor cells to fibronectin (Gotoh *et al.*, 1999). Recently, SDF-1 expressed on vascular endothelium has been shown to be crucial for translating rolling adhesion of $CD34^+$ progenitors into firm adhesion by up-regulating the expression of the integrins VLA-4 and LFA-1 and increasing their adhesiveness to their respective endothelial ligands, VCAM-1 and ICAM-1 (Peled *et al.*, 1999). Moreover, stromal-associated SDF-1α has been shown to trigger migratory and adhesion processes in re-populating SRC/stem cells that express functional integrins such as LFA-1, VLA-4 and VLA-5 (Peled *et al.*, 2000). Therefore, the possibility of up-modulating CXCR4 expression on $CD34^+$ cells by *in vitro* exposure to cytokines may be critical in improving the homing/adhesion of transduced haemopoietic stem/progenitor cells, especially when a limited number of cells is available.

1.5. Aims of the present work

As described throughout this chapter, the processes of proliferation, differentiation, and maturation of haemopoietic cells require a complex system of interacting regulatory mechanisms and can be influenced by physical parameters, stromal and accessory cells, adhesion molecules, extracellular matrix components, and by a largely complex network of cytokines. Little is known, however, about the interacting mechanisms that regulate the responses of haemopoietic progenitors to the many molecules to which they are exposed *in vivo*.

The embryonic FGF3 molecule has been associated with some defects in haemopoiesis in null mutant mice, and its influence on haemopoiesis has not been studied yet. Therefore, one of the aims of this thesis was to investigate the potential role of FGF3 in the regulation of haemopoiesis. Several *in vitro* culture assays were used to investigate the behaviour of haemopoietic cells after treatment with FGF3 purified from the conditioned medium obtained from transfected COS-7 cells with FGF3 cDNA. As haemopoietic cell models, we have chosen the FDCP-mix cells and selected primary haemopoietic cell populations from murine and human origins.

MIP-1α has been implicated in the regulation of myeloid stem and progenitor cell proliferation *in vivo* and *in vitro*, while SDF-1α is involved in their mobilisation. However, the underlying mechanisms involved are not clear. To gain additional insight into the roles of these two chemokines in haemopoiesis, we studied first the expression of their receptors on a variety of cell lines, and on several human primary haemopoietic cell populations from peripheral blood, bone marrow and cord blood. Subsequently, we explored further the effects of these chemokines on $CD34^+$ cells from cord blood and bone marrow in an attempt to establish the biological consequences of the activation of MIP-1α and SDF-1α receptors on their proliferation, migration and adhesion properties. Particularly for MIP-1α, we further correlated its biological effects on $CD34^+$ cells from cord blood to the CCR1 receptor, in an attempt to define the identity of the receptor responsible for its biological activities.

Chapter 2

Materials and Methods

Section 2.1 - Cell biology techniques

2.1.1. Mediapreparation

2.1.1.1. Iscove's Modified Dulbecco's Medium (IMDM)

Powdered medium (Gibco/BRL) was dissolved in double-distilled sterile water and supplemented with 3g/L sodium bicarbonate (Sigma), 500U/ml sodium benzylpenicillin (Glaxo/Crystapen), and 50μg/ml streptomycin sulphate (Sigma). Double strength medium was prepared by adding double-distilled sterile water to adjust the final osmolarity to 600mOsm/Kg (~500ml). Osmolarities of media were measured by depression of freezing point with the Gonotec Osmomat 030 cryoscopic osmometer, Clandon Scientific, calibrated with standard solution of 300mOsm/Kg and double-distilled sterile water (0mOsm/Kg). The medium was filtered through 0.22μm membrane filter (Nalgene) and stored at 4°C for 1-2 weeks (for longer period, it was stored at -20°C). Before being used, the medium was warmed at 37°C in water bath and diluted with double-distilled sterile water to adjust the final osmolarity as follows: 350mOsm/Kg for long-term cultures, and 320mOsm/kg for cell washings, maintenance of cell suspensions, and for clonogenic assays (Testa and Molineux, 1993).

2.1.1.2. RPMI 1640 Medium

The medium RPMI 1640 Medium (Gibco/BRL) was purchased ready made and was supplemented with 0.3g/L L-Glutamine, 500U/ml sodium benzylpenicillin and 50μg/ml streptomycin sulphate. It was used for the culture and maintenance of the 5637 bladder

carcinoma cells, NIH-3T3, HL-60, THP-1, TF-1, K562, Jurkat, and COS-7 fibroblast cell lines and for the proliferation assays.

2.1.1.3. Dulbecco's Minimum Essential Medium with Glutamax-I (DMEM)

DMEM-Glutamax Medium (Gibco/BRL) was purchased ready made and supplemented with twenty-two millilitres of MEM non-essential amino acids (Gibco/BRL) for each half litre of the purchased medium. The medium was filtered through a 0.22μm membrane filter and stored at 4°C up to a maximum of 10 days. It was used for phenotype changing studies, and for culturing and maintaining the X63 Ag8-653 cell line.

2.1.1.4. Fischer's Medium

Fischer's Medium was purchased as a 10X solution (Gibco/BRL) and used according to the manufacturer's instructions. Briefly, to prepare a litre of medium, 100ml of 10X medium was added to 881ml of double-distilled water supplemented with 1.13g/L sodium bicarbonate, 500U/ml of sodium benzyl penicillin and 50μg/ml of streptomycin sulphate. The medium was sterilised through a 0.22μm membrane filter and kept at 4°C. Prior its use, the medium was supplemented with 0.2g/L L-Glutamine and the final osmolarity adjusted to 320mOsm/kg with double-distilled water. It was used for the culture and maintenance of FDCP-mix cell line, for mouse long-term bone marrow cultures, and to obtain conditioned medium from murine lungs.

2.1.1.5. X-Vivo 10 medium

X-Vivo Medium was purchased from Biowhittaker UK LTD and used for the adhesion and migration assays. Prior its use, the medium was supplemented with 0.3g/L of L-Glutamine.

2.1.2. Stock solution of Bovine Serum Albumin at 10%

Bovine serum albumin (BSA, Sigma) was obtained as powder; stock solutions at 10% (w/v) were prepared as follows: for one litre, 100g of BSA were carefully poured onto the surface of 400ml of double-distilled sterile water and left at 4°C for 24 hours until totally dissolved. The solution was deionised by adding 15g of resin beads (AG 501-x8D, 20-50 mesh, medium porosity, analytical grade; Bio-Rad Laboratories, Richmond, California), and stirred slowly at 4°C until all beads had changed colour from yellow to

blue. Another 15g of resin beads were added and left stirring overnight* at 4°C. The solution was filtered by pouring it through a funnel lined with sterile gauze to catch the beads, and then spun at 3000rpm for 30 minutes. A volume of ~600mOsm/kg IMDM was added to the final volume of one litre. The BSA solution was sterilised by filtration consecutively through 0.45μm and 0.22μm membrane filters, and stored at -20°C (Testa and Molineux, 1993).

2.1.3. Preparation of Conditioned Media

2.1.3.1. Medium Conditioned by 5637 Bladder Carcinoma Cells

The 5637 cells were subcultured weekly. The adherent cells of a T-75 culture flask (Falcon, BD) were rinsed with RPMI 1640 twice; 1.5ml of 0.05% (w/v) trypsin in 5mM EDTA (Euro-Clone) were added and the flask placed in a incubator at 37°C for 6-7 minutes, under surveillance. Detached cells were washed and resuspended into 50ml of RPMI 1640 supplemented with 20% (v/v) FCS and plated in T-75 flasks (~10^6 cells/flask). The flasks were then placed at 37°C for 7 days. The 5637 conditioned medium (CM) was harvested by pouring off the supernatant medium from the culture flasks after one week of incubation and stored frozen at -20°C. It was filtered consecutively through a 0.45μm and 0.22μm filters and tested for its ability to support colony growth in Mix-CFC assay (section 2.1.12.1).

2.1.3.2. Murine Lung Conditioned Medium (LCM)

LCM was used as a source of growth factors (Burgess *et al.*, 1977) for the growth of FDCP-mix cells under granulocyte/macrophage differentiation condition (section 2.1.15). Lipo-polysaccharide (200μg/kg in 0.2ml of PBS) from *S. typhosa* (Difco/BRL) was injected intravenously into mice. After 3 hours, the mice were killed, their lungs removed and placed into 5ml Fischer's medium in a McCartney bottle (one pair per flask). The flasks were gassed with 5% CO_2 in air and incubated at 37°C. Two days later, the medium was collected, spun down at 1000g for 10 minutes and filtered consecutively through 0.45μm and 0.22μm filters. LCM was stored at 4°C and tested in murine clonogenic assay (section 2.1.12.2) to determine the optimal concentration for colony formation (Heyworth and Spooncer, 1993). In our experiments the concentration of LCM used was 10%.

* The overnight incubation and room temperature indicated throughout this work refer to 12-18 hours and 20-22°C, respectively.

2.1.3.3. Interleukin-3 Conditioned Medium (IL-3 CM)

The non-immunoglobulin-producing myeloma cell line X63 Ag8-653 transfected with the IL-3 gene was used as a source of IL-3 CM (Karasuyama and Melchers, 1988). Thawed cells were cultured in DMEM supplemented with 5% (v/v) FCS and 1μg/ml G418 (Geneticin), a neomycin analogue, to select resistant cells. G418 selected cells were subcultured twice a week at 1:20 and 1:50 dilutions. The conditioned medium was obtained from 2-4x10^5 cells/ml grown at 37°C for 2-3 days in the absence of G418. The harvested medium was then centrifuged, filtered through 0.22μm filter and kept frozen at -20°C. Before using, it was tested in murine clonogenic assay (section 2.1.12.2) to establish the optimal concentration to support colony formation (Heyworth and Spooncer, 1993). The concentration of IL-3 CM used varied between 2 and 5%.

2.1.4. Sera

Horse Serum (HS), New Born Calf Serum (NBCS), and Foetal Calf Serum (FCS) were purchased from Gibco-BRL, Bioclear UK LTD, PAA-Laboratories, Austria, Imperial-UK or TCS Biologicals, and were stored at -20°C. As there was variation both in the ability of FCS and HS sera to sustain cell growth and in the concentration required for optimal colony formation (Testa, 1985), the long-term bone marrow culture system (section 2.1.11) and clonogenic assays (section 2.1.12) were used to pre-test each serum batch. For LTBMC, the optimal concentration was found to be 10% (v/v) for both horse and foetal calf serum; for clonogenic assays, it was 30% (v/v) for human cells, and 20% (v/v) for FDCP-mix cells.

2.1.5. Matrices for colony growth

2.1.5.1. Methylcellulose

Carboxymethylcellulose powder (Dow Chemical Company, Kent, England) with a viscosity of 4000cps was prepared as a 2.7% (w/v) stock solution. For one litre, a conical flask with 500ml of double-distilled sterile water heated just below the boiling point was placed on a magnetic stirrer and 27g of methylcellulose powder was dispensed gently on the surface, stirring continuously. The mixture was boiled for 10 minutes and left to cool to ~37°C before adding 0.5 litre of pre-warmed 600mOsm/kg IMDM. The solution was again placed on a magnetic stirrer and stirred overnight at 4°C to prevent lumps forming. The mixture was then frozen. Two samples of 15ml each

were placed at 37°C for 7-10 days to check for fungal and bacterial contamination. The batch was thawed, aliquoted into 40ml size Oak Ridge centrifuge tubes (Nalgene), and enriched with 10^{-7}M Sodium Selenite (BHD), 200mM L-Glutamine, and $7.5x10^{-5}$M Thioglycerol (Sigma), allowing sufficient time for these compounds to diffuse through the viscous fluid. The preparation was then centrifuged for 2 hours at 4°C at approximately 25,000g to sediment any particulate matter. Aliquots used for experiments were kept at 4°C and warmed at 37°C before use. Each batch was assayed to determine the optimum viscosity concentration to support colony growth in human clonogenic assay (section 2.1.12.1). The useful concentration found varied between 1.2 and 1.3% (v/v).

2.1.5.2. Agar

Noble agar (Difco/BRL) was used as gelling agent for the semi-solid media and was prepared as 3.3% (w/v) stock solution by mixing agar powder with double-distilled water and heating the mixture in a water bath at about 90°C for one hour, stirring occasionally. The translucent solution obtained was dispensed in aliquots of 80ml in 100ml glass bottles, autoclaved and stored at room temperature until required. When needed for plating, the 3.3% (w/v) agar solution was melted in a bath of boiling water, and cooled to ~45°C before being added to the plating mixture to obtain a final concentration of 0.3% (w/v).

2.1.6. Metrizamide Density Gradient Solution

Metrizamide density gradient solution was used to separate dead cells and cell debris from the viable FDCP-mix cells and COS-7 cells after electroporation procedures. Metrizamide (Sigma) solution A was prepared as a 21% (w/v) solution in HEPES pH 6.7 (Sigma) supplemented with 1% (w/v) BSA (Sigma - Molecular Biology Grade). The density was adjusted to 1.1g/ml at 4°C. Metrizamide solution B was prepared as 11% (w/v) solution in HEPES pH 6.7 supplemented with 1% (w/v) BSA. The density was adjusted to 1.06g/ml at 4°C. The final solution was made by mixing solutions A and B to give a final density of 1.080g/ml at 4°C and was kept at -20°C till required. Ice-cold aliquots of 2.5ml of the final mixture were overlaid by 1ml of suspension cells containing up to $2x10^7$ cells, and centrifuged at 800g for 15 minutes at 4°C. The interface ring containing viable cells was then recovered, the cells washed twice with IMDM 300mOsm/kg and used for further procedures.

2.1.7. Human Cells

2.1.7.1. Bone marrow cells

Human bone marrow samples were obtained either from rib segments obtained from haematological normal individuals undergoing thoracotomy or as aspirates from informed and consenting normal individuals donating their marrows for transplantation. The rib segments were cut into segments 1-2cm in length, cleared from all extraneous connective tissue, immersed into 5-10ml of 320mOsm/kg IMDM supplemented with 10% FCS (v/v), and the cells extracted by repeated flushing using a 5ml syringe attached to a 21G needle. A single cell suspension was prepared by passing the cells through successively finer gauge needles down to 16G. Finally, the cell suspension was passed through a 40μm nylon strainer (Falcon/BD). To establish long-term bone marrow cultures, samples of human marrow aspirates which were highly diluted with blood were first subjected to 1:2 dilution with 320mOsm/kg IMDM and 0.1% (w/v) methylcellulose was added, mixed gently and left for 30-45 minutes at room temperature to sediment the red blood cells. The supernatant was collected, centrifuged at 800g for 10 minutes, the cells washed twice and resuspended in 320mOsm/kg IMDM supplemented with 10% (v/v) FCS, and kept on crushed ice until required.

2.1.7.2. Peripheral blood samples

Peripheral blood samples were obtained from normal individuals with informed consent and were collected aseptically into sterile tubes (Corning) containing preservative-free heparin (Monoparin/CP Pharmaceuticals Ltd, Wrexham, UK) as anticoagulant agent.

2.1.7.3. Cord blood samples

Human umbilical cord blood samples were obtained with informed consent from full-term normal deliveries and were collected aseptically into sterile tubes containing preservative-free heparin as anticoagulant agent.

2.1.7.4. Purification of Mononuclear cells (MNC) from human bone marrow, peripheral and cord blood samples

MNC were obtained using the density gradient centrifugation procedure as described elsewhere (Robinson, 1993). Briefly, bone marrow, peripheral blood or cord blood samples were diluted 1:2 in PBS, layered onto Lymphoprep, density 1.077g/ml (Nycomed Pharma, Norway), and centrifuged at 400g for 25 minutes at room

temperature. Mononuclear cells obtained from the interface ring were washed with PBS and resuspended into sorting buffer, composed of degassed PBS supplemented with 5mM EDTA and 0.5% (w/v) BSA. Cell suspensions were kept on ice till required.

2.1.7.5. $CD34^+$ selection from cord blood and bone marrow samples

MNC from cord blood or bone marrow samples obtained as described in section 2.1.7.4 were used as source of CD34 cells. $CD34^+$ cells were isolated using the Mini-MACS® immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, 1-2x10^8 cells were suspended into 100µl of cold sorting buffer and incubated with 50µl (2mg/ml) of human IgGFcR blocking antibody and 50µl of monoclonal microbead-conjugated (QBEND10) CD34 antibodies for 30 minutes at 4-8°C. Thereafter, the cells were washed in cold sorting buffer and passed through a 40µm nylon strainer and separated in a Mini-MACS® column type MS^+ exposed to the magnetic field of the Mini-MACS® device. The column was washed four times with 500µl of cold sorting buffer and removed from the separator. The retained cells were eluted by flushing the column with 1ml of cold sorting buffer, counted, and kept on ice until required.

2.1.7.6. Detection of markers on human haemopoietic cells

To verify the expression of the cell surface markers CD34 and CCR5, 10µl of commercially available antibodies CD34 (PE- or FITC-conjugated anti-HPCA-2 clone, Becton Dickinson, UK), or CCR5 (PE-conjugated mouse γ_{2a} clone 2D7, PharMingen, UK) were added to 0.2-5x10^5 cells in a final volume of 100µl and incubated in the dark for 30 minutes at 4°C. In some experiments, the anti-CCR5 clones 502, 523, 549, and 531 FITC-conjugated (RDI Research Diagnostics INC, Flanders, UK) were used. The cells were then washed with buffer, resuspended into 200-300µl of 1% formaldehyde and analysed by flow cytometry. For CCR1 and CXCR4 expression, 0.2-5x10^5 cells were resuspended into 100µl of DAKO Antibody Diluent (DAKO Corp., Carpinteria, CA) containing 50µg/ml of rabbit polyclonal antibody against MIP-1α receptor CCR1 (kindly provided by Dr. N. Mukaida, Japan) or mouse monoclonal antibody against human SDF-1α receptor CXCR4 (R&D Systems, Minneapolis, MN) supplemented with 1% (v/v) human AB serum and incubated in the dark, at 4°C for 30 minutes. After washing with buffer, the cells were labeled with a secondary antibody {FITC-

conjugated swine anti-rabbit immunoglobulins and FITC-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulins (DAKO A/S, Denmark) for CCR1 and CXCR4, respectively} for 30 minutes at 4°C. When double labelling was required, 10μl of the appropriate PE- or FITC-conjugated anti-CD34 antibody was added to the mixture 15 minutes after the incubation of the secondary antibody and the incubation continued for further 15 minutes. The cells were then washed with buffer, resuspended into 200-300μl of 1% (v/v) formaldehyde and analysed by flow cytometry on a FACS Vantage flow cytometer instrument (Becton Dickinson). As controls, the following isotype antibodies were used: PE- or FITC-conjugated mouse γ_1 for CD34, (Becton Dickinson); PE-conjugated mouse γ_{2a} for CCR5 (Becton Dickinson); mouse monoclonal γ_{2a} for CXCR4 (Becton Dickinson). In all flow cytometry experiments, fluorescence was measured using 530±15nm (FITC) or 575±12nm (PE) band pass filters in a FACS Scan or FACS Vantage flow cytometers, equipped with an Argon-Ion laser tuned at 488nm. Events were acquired for analysis and the data saved in list mode. All data list mode files were analysed with PC Lysis II software using a logical gating strategy.

2.1.7.7. Analysis of the purity of $CD34^+$ cells

The HPCA-2-PE conjugated antibody (Becton Dickinson UK Ltd, Cowley, Oxford) was used to analyse the purity of $CD34^+$ cells by flow cytometry according to the manufacturer's instructions. Briefly, two aliquots of $0.5\text{-}5x10^5$ cells were prepared. One aliquot was labeled with 10μl of HPCA-2-PE, and the second aliquot was labeled with 10μl of IgG_1 (γ_1) isotype matched PE-conjugated control antibody (Becton Dickinson UK Ltd, Cowley, Oxford). The cells were incubated for 15 minutes at room temperature, in the dark. The cells were then washed with PBS supplemented with 0.5% (w/v) BSA plus 0.5mM EDTA, the cell pellet resuspended into 200-500μl of 1% (v/v) formaldehyde solution and analysed on a FACS Scan flow cytometer as above.

2.1.7.8. Cell sorting

Immunomagnetically isolated $CD34^+$ cells labeled with the appropriate fluorescent markers were sorted under sterile conditions into positive and negative fractions using the ACDU of a FACS Vantage flow cytometer (Becton Dickinson). The cells were collected in flasks containing 3ml of X-Vivo 10 medium, centrifuged at 800g for 5 minutes, resuspended into appropriate fresh medium, counted and kept on ice till

required. After transfection and overnight incubation period, COS-7 cells (section 2.2.6.1) and FDCP-mix cells (section 2.2.10) transfected with cDNA FGF3 constructs were spun down at 800g for 5 minutes and resuspended into 320mOsm/kg IMDM (for FDCP-mix cells, IMDM was supplemented with 2% (v/v) IL-3 CM medium). Up to $2x10^7$ cells were loaded onto 2.5ml of Metrizamide density gradient solution (section 2.1.6) and spun down at 800g for 15 minutes, at 4°C. Cells at the interface ring were collected, washed and resuspended at $2x10^6$ cells/ml into PBS supplemented with 0.5% (w/v) heat-inactivated BSA. Cells were sorted using the fluorescence emission of the GFP reporter gene expression under sterile conditions as described for CD34 cells.

2.1.8. Mouse Bone Marrow Cells

Mouse bone marrow cells used for long-term bone marrow culture experiments (section 2.1.11) were obtained from femora of (C57B1 x DBA/2)F_1 mice 6 to 8 weeks old. Mice were killed by cervical dislocation and the femora dissected. The extremities of the bone were cut off and the content flushed into 10ml of culture medium composed by Fischer's Medium supplemented with 20% (v/v) horse serum, and 10^{-6}M hydrocortisone (Hydrocortisone 21-Hemisuccinate - Sigma). Usually one femur was sufficient to set up one T-25 culture flask with approximately $1.5\text{-}2x10^7$ cells.

2.1.9. Cell Lines and their Maintenance

2.1.9.1. FDCP-mix cells

FDCP-mix cells were maintained in suspension culture using Fischer's Medium 320 mOsm/kg supplemented with 20% (v/v) horse serum and 2-5% (v/v) IL-3 CM, condition that allowed the self-renewal and maintenance of the primitive phenotype of the cell line (Heyworth *et al.*, 1990a). Cells in logarithmic phase of growth were subcultured, (generally twice a week) by seeding $3\text{-}5x10^4$ cells/ml in 10ml of growth medium. The T-25 culture flasks were then gassed with 5% CO_2 in air and incubated at 37°C.

2.1.9.2. COS-7 cells

To maintain COS-7 cells, they were cultured in T-25 flasks. For passaging, the culture medium was removed and the monolayer rinsed twice with PBS; 1ml of 0.05% trypsin plus 5mM EDTA was added and the flasks incubated for 5-8 minutes at 37°C under surveillance. When the cells had detached, 100µl of FCS were added, and the cells

washed twice by centrifugation with RPMI 1640. The cell pellet was resuspended into RPMI 1640 supplemented with 10% (v/v) FCS (growth medium) and the cells counted. Culture flasks containing $5x10^4$ COS-7 cells/ml in 10ml of growth medium were gassed with 5% CO_2 in air and incubated at 37°C until the cells have grown to confluence, when they were then subcultured.

2.1.9.3. NIH-3T3 cells

Nearly confluent NIH-3T3 cells grown in medium supplemented with 5% (v/v) NBCS were rinsed twice with RPMI 1640 and the washings discarded; 1ml of 0.05% (w/v) trypsin plus 5mM EDTA was added and the flasks incubated for 5-8 minutes at 37°C under surveillance. When the cells had detached, 100µl of NBCS were added, and the cells washed twice by centrifugation with RPMI 1640. The cell pellet was resuspended into medium supplemented with 5% (v/v) NBCS and the cells counted. T-25 culture flasks containing $5x10^4$ NIH-3T3 cells/ml in 10ml of growth medium were gassed with 5% CO_2 in air and incubated at 37°C until the cells have grown to confluence, when they were then subcultured (generally every 3 days).

2.1.9.4. TF-1, THP-1, HL-60, K562, and Jurkat cells

These cell lines were subcultured every 2-3 days in 10ml of RPMI 1640 medium supplemented with 10% (v/v) FCS at a concentration of 10^5 cells/ml in a T25 culture flask. The flasks were gassed with 5% CO_2 in air and incubated at 37°C. TF-1 cultures were supplemented with 10ng/ml recombinant GM-CSF (Glaxo).

2.1.9.5. M2-10B4 cells

The mouse fibroblast cell line M2-10B4 was used to establish stromal layers for culturing haemopoietic cells in long-term culture system. Cells were cultured at $2\text{-}4x10^3$ cells/ml in RPMI 1640 medium supplemented with 20% (v/v) FCS, 0.3g/L L-Glutamine, 500U/ml of sodium benzylpenicillin and 50µg/ml streptomycin sulphate. When confluent, cells were detached by trypsin treatment as described in section 2.1.9.2 for COS-7 cells and re-plated into fresh medium.

2.1.9.6. Storage of cells in liquid nitrogen

Cells to be frozen ($1\text{-}2x10^6$/vial) were harvested by centrifugation at 800g for 5 minutes and washed twice with PBS. The pellet was resuspended into 1ml of 90% FCS (v/v) and 10% (v/v) DMSO (Sigma). The mixture was transferred to cryo tubes (Nalgene Nunc

International, Denmark) and placed into a controlled-freezing container at -70°C overnight. The following day, the tubes were transferred to a liquid nitrogen container.

2.1.9.7. Thawing of cells

Frozen cell vials removed from liquid nitrogen were thawed rapidly in a water bath at 37°C. The cells were transferred to a 15ml sterile tube and 10ml of complete medium were added slowly, with gentle mixing. The cell suspension was left at room temperature for 10 minutes, spun at 800g for 5 minutes and the pellet resuspended in fresh growth medium and cultured as usual. The cultures were checked for a few days to ensure normal cell development.

2.1.10. Cell Viability and Cell Counting

All cells used in the experiments had been tested for their viability determined by the Trypan Blue (INC Biomedicals, Inc.) dye exclusion test: 10µl of a cell suspension was diluted into Trypan Blue solution usually at 1:5 (or higher dilution, if necessary) and were observed and counted using an Improved Neubauer Haemocytometer, according to the technique described by Dacie and Lewis (Dacie and Lewis, 1984). Samples containing red blood cells were diluted 1 to 10 (or higher dilution, if necessary) and subsequently counted using the white cell diluting fluid, composed of 3% (v/v) glacial acetic acid solution (BDH, Analar) with a few drops of Gentian Violet. Only samples with less than 5% of the cells stained by the dye were used.

2.1.11. Long-Term Bone Marrow Cultures (LTBMC)

2.1.11.1. Establishment of LTBMC

The cultures were established according to the method first published by Dexter and colleagues (Dexter *et al.*, 1977) for murine cells and later modified by Gartner & Kaplan (Gartner and Kaplan, 1980). For LTBMC of mouse cells, the content of a femur obtained as described in section 2.1.8 was flushed into 10ml of Fischer's medium supplemented with 20% (v/v) horse serum, and 10^{-6}M hydrocortisone sodium succinate (Sigma). The flask was gassed with 5% CO_2 in air and incubated at 37°C. For human cells, 1.5-2x10^7 nucleated marrow cells obtained as described in section 2.1.7.1 were seeded in 10ml of growth medium in a T-25 culture flask (Falcon/BD) consisting of 10% (v/v) Horse Serum, 10% (v/v) Foetal Calf Serum, and 5x10^{-7}M hydrocortisone in IMDM 350mOms/Kg. Cells were grown in 5% CO_2 in air at 33°C.

2.1.11.2. Maintenance of LTBMC

Ten days following initiation and subsequently at weekly intervals, the LTBMC flasks were examined under an inverted microscope (Olympus) using phase contrast optics at low magnification to monitor the formation of the adherent layer. They were then gently agitated to suspend loosely adherent cells and the total volume of supernatant measured. Half of that volume was removed and replaced with equal volume of freshly prepared growth medium to feed the culture. The cells in the harvested medium were centrifuged at 800g for 10 minutes, the cell pellet resuspended into 320mOsm/kg IMDM supplemented with 10% FCS (v/v) for human cultures or Fischer's medium for mouse cultures and the cell number determined. This cell suspension was used to estimate the total number of non-adherent cells per culture flask, the number of progenitor cells (CFC) of the non-adherent fraction per culture in clonogenic assays (section 2.1.12) and for cytomorphology studies (section 2.1.13).

2.1.11.3. Enzymatic detachment of the adherent layer cells of LTBMC

Non-adherent cells were first removed from the culture vessel and the flask washed twice with fresh, warmed serum-free medium. The washings were pooled with the non-adherent cell suspension. To detach adherent cells, culture flasks were incubated at 37°C for 5-8 minutes with 1ml of 0.05% (w/v) trypsin plus 5mM EDTA; 0.1ml of serum was added to the flask to stop further trypsin action, and all adherent cells were detached by light pipetting. The harvested cell suspension was then centrifuged at 800g for 5 minutes, the supernatant discarded and the cell pellet resuspended and washed twice in sterile PBS supplemented with 20% (v/v) FCS. Finally, the cells were resuspended into medium, counted and kept on ice until required.

2.1.11.4. Adherent cell depletion

To enrich the haemopoietic adherent cell fractions of human LTBMC before plating them in clonogenic assays, $2x10^6$ cells/ml in 5ml of 320mOsm/kg IMDM supplemented with 20% (v/v) FCS were placed into T-25 culture vessel, gassed with 5% CO_2 in air and incubated at 37°C for 2-3 hours. After the incubation period, the non-adherent cells were transferred to a centrifuge tube. The flasks were rinsed twice and the washings pooled with the harvested medium. The cells were spun down at 800g for 5 minutes, resuspended into 320mOsm/kg IMDM supplemented with 10% (v/v) FCS, counted and kept on ice till required.

2.1.11.5. Establishment of long-term cultures onto pre-formed irradiated stroma

Human LTBMC were established and maintained as described in sections 2.1.11.1 and 2.1.11.2. When a fully confluent stroma was achieved, usually about 4 to 6 weeks after initiation, the culture flasks were irradiated with 80Gy using ^{137}Cs γ-rays at a dose rate of 4Gy/min. When required, the medium was removed and the adherent layer was gently washed twice with pre-warmed LTBMC medium before adding $2x10^3$ $CD34^+$ cord blood cells/flask. The cultures were then maintained as described in section 2.1.11.2.

2.1.11.6. Establishment and maintenance of LTC-IC cultures

The mouse fibroblast cell line M2-10B4 was used to establish stromal layers for culturing GFP-FGF3 transfected FDCP-mix cells and cord blood mononuclear cells in long-term culture system. Cells were cultured essentially as described in section 2.1.9.5. When confluent, cells were detached by trypsin treatment as described in section 2.1.9.2 for COS-7 cells and irradiated with 80Gy using ^{137}Cs γ-rays at a dose rate of 4Gy/min. Irradiated cells were plated at $1\text{-}1.4x10^6$ cells/T-12.5cm^2 culture flask for cord blood mononuclear cells or at 10^5 cells/well in a 24-well plate for FDCP-mix cells in growth medium, gassed with CO_2 in air and incubated at 37°C for no longer then 7 days. When necessary for plating, the medium was removed, the stromal cells washed with warm LTBMC medium (section 2.1.11.1) and cord blood MNC were added at a concentration of $5x10^4$ cells in 5 ml final volume/flask. The flasks were gassed and incubated at 33°C. Whereas indicated, $2x10^3$ GFP-FGF3 positively transfected cells were inoculated in 1ml of IMDM supplemented with 20% (v/v) FCS and 10ng/ml (self-renewal) of murine recombinant IL-3 (Minneapolis, MN system) onto a 24-well plate seeded with pre-irradiated stroma and incubated for 2 weeks at 37°C, 5% CO_2 in air in a humidified incubator. The maintenance of cultures was essentially as that described in section 2.1.11.2.

2.1.12. Assays for Colony-Forming Cells (CFC)

2.1.12.1. Multipotent Progenitor Cells Assay (Mix-CFC Assay) for Human Cells

Erythroid (BFU-E and CFU-E) and granulocytic-macrophage (GM-CFC) progenitor cells were assayed in semi-solid system as follows: $5x10^4$ mononuclear cells either from human cord blood, bone marrow or from non-adherent and adherent fractions of long-

term cultures, or $2x10^3$ $CD34^+$, $CD34^+CCR1^{high}$ or $CD34^+CCR1^{-/low}$ cells were cultured in the presence of 10% (v/v) 5637 Bladder Carcinoma Cell Conditioned Medium, 30% (v/v) FCS, 10% (v/v) BSA stock solution, 2IU of human recombinant EPO (Eprex/Cilag) and 1.2% methylcellulose in a final volume of 1ml plating mixture. Whereas indicated, MIP-1α as BB10010 (Lord *et al.*, 1995), SDF-1α and/or rabbit polyclonal anti-CCR1 antibody (Su *et al.*, 1996) at 5μg/ml were added to the mix plating medium just before the addition of the cells. The mixture was plated in 3 aliquots of 0.25ml each into 24-wells dish (Falcon/BD) and incubated at 37°C in a fully humidified atmosphere supplemented with 5% CO_2 in air. After 12-14 days, colonies of various sizes and cellular composition could be recognised by direct observation with an Olympus dissecting microscope with a zoom lens at 30x – 40x magnification.

2.1.12.2. Clonogenic Assay for FDCP-mix cells

A total of $2x10^3$/ml FCDP-mix cells were mixed in a final volume of 3.3ml plating mixture consisting of 20% (v/v) of HS, 10% (v/v) of BSA (Boehringer Mannheim), and 10ng/ml of murine recombinant IL-3 or 2-5% (v/v) of IL-3 CM in IMDM 320mOsm/Kg. Whereas necessary, the required concentrations of the purified fraction of XFGF3 were added. Then, 0.33ml of molten agar at 3.3% at about 45°C was added, and rapidly mixed. Three aliquots of 1ml were plated out in 35x10mm plastic tissue culture Petri dishes (Falcon). The plates were placed at room temperature for 5-10 minutes for gelling and incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 7 days. The colonies were scored using an Olympus Zoom Stereo Microscope.

2.1.12.3. Scoring of colonies

Clones of at least 50 or more cells were scored as colonies. The colonies were scored according to the criteria used in our laboratory which has been published elsewhere (Coutinho *et al.*, 1993). Briefly, GM-CFC, colonies containing granulocytes and/or macrophages; BFU-E, colonies containing three or more clusters of erythroid cells; CFU-E, colonies composed of two or less clusters or isolated clusters containing less than 50 cells; Mix-CFC, colonies containing more than one lineage of cells including erythroid.

2.1.13. Differential morphology of haemopoietic cells

2.1.13.1. Cytospins

Cells were resuspended in PBS containing 10% (v/v) of serum at 2-5x10^5 cell/ml, and 200µl were added to a plastic carrier and centrifuged against a slide with the edges covered with absorbent paper at 1000rpm for 5 minutes in a Shandon-Cytospin 2 centrifuge. The slides were air dried and stained as required.

2.1.13.2. May-Grunwald-Giemsa staining

This technique was used both for primary and cultured cells. May Grunwald (Gurr/BHD) stain was layered onto slides prepared as described in section 2.1.13.1 for 3 minutes and rinsed off with tap water. The slides were then coated with Giemsa stain (Gurr/BHD) diluted 1:20 in double distilled water for ~15 minutes. After washing with tap water, the slides were air dry and the films scored in an Olympus BH-2 microscope using oil immersion lenses (600-1000x). Whereas possible, 200-500 cells were scored and morphologies recorded as a percentage of the total.

2.1.13.3. O'-Dianisidine staining

Cytospun slides were fixed in methanol for 10 minutes and then placed in the dark for 10 minutes submersed into a solution containing 5 volumes of 0.2% (w/v) O'-Dianisidine in methanol, 1 volume of 3% hydrogen peroxide, and 1 volume of 1% (w/v) sodium nitroprusside. The slides were then washed with tap water and counter stained with May-Grunwald-Giemsa as described in section 2.1.13.2.

2.1.14. Survival and self-renewal of FDCP-mix cells

FDCP-mix cells were washed three times in IMDM 320mOsm/kg, and resuspended at 10^5 cells/ml in IMDM supplemented with 20% (v/v) FCS, 0.1ng/ml (survival) or 10ng/ml (self-renewal) of murine recombinant IL-3. In some experiments, different concentrations of purified XFGF3 (section 2.2.6.3) were added. The cells were plated in 12-well dishes at 1ml/well and incubated at 37°C for 6-8 days in a humidified incubator with 5% CO_2 in air. The number of cells was scored every two days and analysed for clonogenicity (section 2.1.12.2) and cytomorphology (section 2.1.13). Cells transfected with GFP-FGF3 were cultured in self-renewal conditions for 14 days.

2.1.15. Granulocyte/Macrophage differentiation of FDCP-mix cells

FDCP-mix cells were washed three times in IMDM 320mOsm/kg, and resuspended at 10^5 cells/ml in IMDM supplemented with 20% (v/v) FCS, 0.01ng/ml murine recombinant IL-3, 10% LCM, and for some experiments, the indicated concentrations of purified XFGF3 (section 2.2.6.3) were added. The cells were plated in 12-well dishes at 1ml/well and incubated at 37°C for 6-8 days in a humidified incubator with 5% CO_2 in air. The number of cells was scored daily and samples were analysed for clonogenicity (section 2.1.12.2) and cytomorphology (section 2.1.13).

2.1.16. Erythroid differentiation of FDCP-mix cells

FDCP-mix cells washed three times in IMDM 320mOsm/kg were resuspended at a concentration of 10^5 cells/ml in IMDM supplemented with 20% (v/v) FCS, 2mM haemin chloride (Calbiochem), 0.05ng/ml murine recombinant IL-3, 4IU/ml of recombinant EPO (Eprex-Cilag), and for some experiments the required concentrations (v/v) of purified XFGF3 (section 2.2.6.3) were added. The cells were plated in 12-well dishes at 1ml/well and incubated at 37°C for 6-8 days in a humidified incubator with 5% CO_2 in air. The number of cells was scored daily and the cells used for GM-CFC clonogenic assay (section 2.1.12.2) and cytomorphology (section 2.1.13).

2.1.17. Adhesion Assay

The adhesion assays were performed according to a technique previously reported (Durig *et al.*, 1999a). Briefly, 96-well flat-bottom microtiter plate wells (Costar, Cambridge) were coated with 50µl of 50µg/ml of human Fibronectin (Sigma, Poole, Dorset, UK) for 1 hour at room temperature, and the non specific binding sites blocked with 1mg of heat-inactivated BSA (10mg/ml in PBS) for 30 minutes at room temperature. Control wells were filled up with blocking solution and left for 30 minutes at room temperature. The blocking solution was removed and 100µl of $CD34^+$ or $CD34^+CCR1^{high}$ cell suspension at 1-1.5x10^6 cells/ml in X-Vivo 10 assay medium supplemented with 0.3g/L L-Glutamine was added. The cytokines MIP-1α (as BB10010, British Biotech Pharmaceuticals Ltd, Oxford, UK) or SDF-1α (R&D Systems, Minneapolis, MN) were diluted into assay medium and added to the wells at the indicated concentrations. The dishes were incubated at 37°C for 1 hour in a fully humidified incubator with 5% CO_2 and 5% O_2 in nitrogen. Non-adherent cells were

removed after placing the plates on a horizontal shaker at 100 revolutions/minute for 30 seconds followed by two washes with 100µl of PBS. The bound cells were recovered by aspiration with 100µl of assay medium. Both fractions were counted using a haemocytometer and adhesion was expressed as the percentage of bound cells/(bound + unbound cells). Aliquots of each fraction were assayed for their progenitor content using the Mix-CFC assay (section 2.1.12.1).

2.1.18. Migration Assay

$CD34^+$ or $CD34^+CCR1^{high}$ cells were resuspended at a concentration of 10^6 cell/ml in migration medium (X-Vivo 10 medium supplemented with 0.3g/L of L-Glutamine); 100µl of cell suspension were added to a 6.5mm Transwell, polycarbonate membrane, 5µm pore size cell culture chamber insert (Costar) which had been previously washed with migration medium and placed onto a 24-wells plate (Falcon) filled up with 600µl of migration medium supplemented with the indicated concentrations of MIP-1α or SDF-1α. The dish was placed at 37°C in a fully humidified incubator with 5% CO_2 in air for 4-5 hours. The medium of the lower chamber containing the migrated cells was transferred to eppendorf tube. The well was washed with migration medium to recover all migrated cells and the washing pooled to the migrated cell fraction. Cells were counted in a haemocytometer and migration was expressed as the percentage of migrated cells/input cells. Expontaneous migration (control) was measured by migration of the test cells towards medium alone. Input population and migrated cells were assayed for their progenitor content using the Mix-CFC assay (section 2.1.12.1).

2.1.19. Proliferation assay

NIH-3T3 cells detached by trypsin treatment as described in section 2.1.9.3 were spun down at 800g for 5 minutes and resuspended in DMEM; $2x10^4$ cells in 500µl of DMEM supplemented with 5% (v/v) NBCS were plated into each well of a 24-well plate and incubated for 6-8 hours in a fully humidified atmosphere of 5% CO_2 in air to allow the cells to adhere to the bottom of the flask. The medium was then harvested, the adherent cells rinsed 3 times with DMEM and 500µl of DMEM supplemented with 0.2% (v/v) NBCS were added and left for at least 30 hours in a fully humidified atmosphere of 5% CO_2 in air, to leave the cells in a quiescent state. The medium was then removed, the adherent cells rinsed with DMEM and 500µl of DMEM supplemented with 0.2% (v/v)

NBCS, 10μg/ml heparin, and different concentrations (v/v) of XFGF3 purified medium (5, 1, 0.5 and 0.1%) or with the conditioned medium from COS-7 cells transfected with XFGF3 plasmid (10, 20, and 30%, v/v) were added and the culture dish incubated in a fully humidified atmosphere of 5% CO_2 in air for 24 hours. Addition of 1μCi/well of tritiated thymidine (Methyl-thymidine, 20Ci/mmole-740GBq/mmole) and incubation of the plate in a fully humidified atmosphere of 5% CO_2 in air for 2.5 hours was followed by 3 washings of the adherent cells with PBS, and two washings with methanol (5 minutes each). The cells were then fixed with cold 5% (v/v) TCA for 20 minutes, and dissolved in 500μl of 0.3M NaOH. Two millilitres of scintillation fluid were added to each well and the incorporated thymidine assayed in a Beckmann's apparatus.

2.1.20. Phenotypic modification of NIH-3T3 cells

NIH-3T3 cells detached by trypsin treatment as described in section 2.1.9.3 were spun down at 800g for 5 minutes and resuspended in DMEM; $2x10^4$ cells in 200μl of DMEM supplemented with 10% (v/v) NBCS were plated in each well of a 48-well plate (Falcon/BD) and incubated overnight at 37°C in 5% CO_2 in air. After the incubation period, 200μl of DMEM supplemented with 10% (v/v) NBCS, 10μl/ml of heparin, and different concentrations (v/v) of the conditioned medium (10, 20, and 30%) and of the purified fraction (5, 1, 0.5, and 0.1%) were added. The plates were incubated at 37°C in 5% CO_2 in air for 24-48 hours and the morphology observed under an inverted microscope (Olympus). Purified medium from GFP transfected COS-7 cells and from wild type COS-7 cells were used as control of the experiments, as were blanks containing equivalent concentrations of 1M NaCl.

Section 2.2 - Molecular biology techniques

2.2.1. Source of Reagents

Agarose	Seakem GTG ultrapure agarose was obtained from Flowgen Instruments Ltd.
Bacterial cell culture medium	Media constituents were generally supplied by Difco Laboratories. Supplements: X-gal and isopropylthio-β-D-galactoside (IPTG) from Life Technologies. Antibiotics: tetracycline and carbenicillin supplied by Sigma Chemicals Co.

Chemicals	Chemical compounds were generally obtained from BDH Chemicals Ltd., Sigma Chemicals Co., or Boehringer-Mannheim Biochemicals, unless otherwise specified.
Enzymes	Restriction enzymes, calf intestinal alkaline phosphatase (CIP), T4 DNA ligase, Klenow (DNA polymerase I, large fragment), and T4 DNA polymerase were obtained from Boehringer-Mannheim Biochemicals or Promega.
Membranes	Hybond-C extra nitrocellulose membranes were supplied by Amersham.
Nucleotides	Deoxynucleotides were supplied by Boehringer-Mannheim Biochemicals.

2.2.2. Composition of Buffers, Media, and Solutions

Alkaline Lysis Solution I	50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, autoclaved before use
Alkaline Lysis Solution II	0.2M NaOH, 1% (w/v) SDS freshly prepared
Alkaline Lysis Solution III	3M KOAc, 5M Acetic Acid
Blocking Buffer	5% skimmed milk powder in PBS plus 0.2% Tween-20 (Bio-Rad)
Klenow Polishing Buffer 10X	0.1M Tris pH 7.9, 1M $MgCl_2$, 0.5M NaCl, 0.1M β-Mercaptoethanol in purified water
Loading Buffer	50% (w/v) glycerol, 0.1% (w/v) bromophenol blue, 60mM EDTA pH 8.0 in 1X TAE
LB agar	Luria Bertani Broth (LB) (Ron Lab PLC – BS611) supplemented with 15g per litre bacto-agar, autoclaved before use. The LB agar plates were prepared for blue-white colour screening as follows: 400µl of ampicillin (Sigma - 100mg/ml stock solution kept frozen at –20°C), 80µg/ml of X-gal, and 20mM IPTG were added to 400ml of autoclaved and cooled LB agar; about 20ml aliquots were asseptically poured into petri dishes and left to set at RT. The plates were then kept at 4°C till used

Luria Broth	10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl in 1 litre of distilled water, autoclaved before use
Lysozyme Solution	10mg/ml in 10mM Tris-HCl pH 8.0
NP40 Lysis Buffer pH 7.5	50mM Tris acetate buffer, 1mM EGTA, 1mM EDTA, 120mM NaCl, 50mM NaF, 10mM 2-Mercaptoethanol, 1% Nonidet P-40
Phenol Extraction Solution	0.1% hydroxyquinoline was added to liquid phenol and equal volume of 0.5M Tris-HCl pH 8.0; after mixing, the phases were allowed to separate and the upper aqueous phase removed. The equilibration was repeated using 0.1M Tris-HCl until the pH was < 7.8. Equilibrated phenol was stored at 4°C
SDS-PAGE Resolving Gel	15% gel consisting of 30% pre-dissolved stock of Acrylamide plus N-Methyl-bis-Acrylamide (Ultrapure Protogel, National Diagnostics, USA), 0.1% Sodium Dodecyl Sulphate, 0.375M Tris-HCl pH 8.8, 0.1% (w/v) amonium persulphate
SDS-PAGE Stacking Gel	5% stacking gel containing 0.1% SDS, 0.125M Tris-HCl pH 6.8 and 0.1% (w/v) amonium persulphate
SDS-PAGE Loading Buffer	10% β-mercaptoethanol, 20% glycerol, 2% Sodium Dodecyl Sulphate, 100mM Tris-HCl pH 8.8, 0.05% (w/v) Bromo-Phenol Blue
SDS-PAGE Running Buffer	0.15% SDS, 25mM Tris, 150mM Glycine pH 8.3
SDS-PAGE Transfer Buffer	25mM Tris, 192mM Glycine, 0.1% v/v SDS and 20% (v/v) methanol
SOC Medium	2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$, 10mM $MgCl_2$, 20mM glucose in distilled water, autoclaved before use
20x SSC	3M NaCl, 0.3M Tri-Sodium Citrate adjusted to pH 7.0 with 10M NaOH
10x TAE	0.4M Tris-HCl, 0.2M NaOAc, 20mM EDTA adjusted to pH 7.8 with glacial acetic acid
TBS	Tris buffered saline pH 7.6
TE	10mM Tris-HCl, 0.1mM EDTA pH 8.0

Terrific Broth	12g bacto-tryptone, 24g bacto-yeast extract, 4ml glycerol in 1 litre distilled water, autoclaved and supplemented with Terrific Salts 1:10 before use
Terrific Salts	23.5g Potassium di-hydrogen phosphate, 164.3g di-Potassium hydrogen phosphate

2.2.3. General molecular biology techniques

2.2.3.1. Agarose gel electrophoresis of DNA

0.9% agarose was prepared by adding 0.45g of agarose powder to 50ml of 1X TAE buffer and boiled in a microwave oven for 2 minutes at medium high power. After cooling it to ~45°C, 0.5μg/ml of Ethidium Bromide were added and the gel cast onto a gel box (8x10cm). On setting, this was transferred to a flat bed electrophoresis chamber containing 1X TAE buffer. DNA samples were resuspended into 1X loading buffer and loaded into wells. All gel electrophoresis were performed using 500ng-1kb DNA Ladder (New England Biolabs) as a molecular weight marker. The gels were usually run at 70-80V for about 1-2 hours and the separated fragments visualised under UV light using The Imager™ - Appligene Oncor.

2.2.3.2. Phenol/Chloroform extraction of DNA

DNA extracts were purified by adding an equal volume of equilibrated phenol, mixing thoroughly, and spinning at 10,000g for 1 minute. The upper aqueous phase was transferred to a clean eppendorf, and re-extracted once with 1:2 phenol/chloroform solution and once with chloroform. The upper phase was finally transferred to a clean eppendorf and the DNA ethanol precipitated (section 2.2.3.3).

2.2.3.3. Ethanol precipitation of DNA

Plasmid DNA was generally precipitated by the addition of 0.1 volume of 3M NaOAc pH 5.2 and 2 volumes of 95% ethanol. The sample was mixed thoroughly by vortexing and placed at –20°C for 2 hours or at –70°C for 20 minutes before spinning at 10,000rpm for 5 minutes. The supernatant was discarded and the pellet washed in 1ml of 70% (v/v) ethanol. The DNA was air dried and resuspended in 1X TE.

2.2.3.4. Digestion of plasmid DNA with restriction endonucleases

Conditions varied for each particular enzyme, according to the manufacturer's instructions. Generally, 10μg of plasmid DNA were incubated at 37°C for 1-2 hours in a 20μl reaction, containing 10 units (1μl) of the restriction enzyme (1 unit/μg of DNA), and 2μl of 10x Enzyme Buffer. Digestion was checked by electrophoresis on a 0.9% agarose gel in 1X TAE (section 2.2.3.1).

2.2.3.5. Dephosphorylation of linearised plasmid DNA

In order to prevent self-ligation, some DNA fragments were dephosphorylated with calf intestine alkaline phosphatase enzyme (CIP) following cleavage with restriction enzymes. The reaction was performed by resuspending the DNA in 100μl purified water containing 10% (v/v) of CIP buffer and 5μl of CIP enzyme and incubating at 37°C for 1 hour. The reaction was stopped by addition of 12.5mM EGTA and incubation at 65°C for 15 minutes. The DNA was purified by phenol/chloroform extraction and ethanol precipitation (sections 2.2.3.2 and 2.2.3.3, respectively).

2.2.3.6. Filling in recessed termini of DNA fragments

Overhanging ends of DNA were filled in using a mixture of Klenow and T4 DNA polymerase enzymes. After digestion of plasmid DNA with appropriate restriction enzyme, 5-10μg of DNA were resuspended into 30μl of TE and 4 μl of Klenow Polishing Buffer, 0.4mM dNTP (0.1mM of each dATP, dGTP, dCTP, and dTTP), 20IU of Klenow, and 1.3IU of T4 DNA polymerase were added and the mixture incubated at 37°C for 30 minutes. The reaction was heat inactivated at 65°C for 15 minutes. The DNA was phenol/chloroform extracted, ethanol precipitated (sections 2.2.3.2 and 2.2.3.3, respectively), and resuspended in 1X TE.

2.2.3.7. Ligation of DNA fragments

Ligations were carried out with insert and vector DNA at an equimolar ratio of 3:1 (~50ng of vector DNA) in a final volume of 10μl containing 1X Ligase buffer, and 0.1IU of T4 DNA Ligase. The reaction was carried out at 14-17°C overnight. To verify the efficiency of ligation, 5μl of the mixture was run on a 0.9% gel (section 2.2.3.1) and fragment sizes of vector, insert, and ligation products were compared.

2.2.3.8. Extraction of DNA fragments using QIAquick Gel Extraction Kit

Digested DNA was separated through a 0.9% agarose gel, visualised under UV light source and the desired band excised from the gel with a scalpel. The DNA was extracted from the gel using the QIAquick Gel Extraction Kit (QIAgen), according to the manufacturer's instructions. Briefly, the excised DNA fragment was placed into an eppendorf tube and weighed as an indication of volume (1mg=1ml). Three volumes of Buffer QG were added to 1 volume of gel and solubilised at 50°C. The sample was then applied to a QIAquick column already set up in a 2-ml collection tube kit and centrifuged at 10,000g for 1 minute. The filtrate was discarded and 750µl of Buffer E was added to the column. After standing for 5 minutes at room temperature, it was centrifuged at 10,000g for 1 minute. After repeating the centrifugation step once more, the column was placed onto a clean eppendorf tube and 30µl of TE was added, and centrifuged at 10,000g for 1 minute to elute the DNA, which was kept at –20°C until required.

2.2.3.9. Quantification of DNA fragments

Aliquots of 0.5, 2 or 5µl of purified DNA fragments were loaded onto a 0.9% agarose gel alongside with 5µl of HyperLadder I marker (Bioline) and run at 70-80V for 1-2 hours. The gel was exposed to and photographed under UV light and the intensity of the band fragment was compared to that of the weight marker to provide an approximation of final DNA concentration.

2.2.4. Vector preparation

2.2.4.1. pRSET B vector

To preserve the AUG start codon at the *Nco I* restriction site of plasmids pKC 4.12 and pKC4.16, vector pRSET B (Invitrogen) was used as a shuttle vector. It was first digested with *Eco RI*, blunt ended with Klenow fragment and further digested with *Nco I*. The linearised vector was then gel purified and frozen at -20°C till required.

2.2.4.2. CMVExpEGFP vector

Vector CMVExpEGFP (McIvor *et al.*, 2000) was linearised with *Spe I* restriction enzyme followed by dephosphorylation (section 2.2.3.5). It was then gel purified (section 2.2.3.1) and frozen at -20°C till required.

2.2.4.3. Use of Spe I linker

Spe I linker (New England Biolabs) was used to extend the pRSET B-4.12, pRSET B-4.16 and 3.2 fragment before ligation with the *Spe I* linearised CMVExpEGFP vector. *Spe I* linker (2μg) was ligated to 500ng of purified *Xho I* and *Hind III* fragments of pRSET B-4.12 and pRSET B-4.16, and to 500ng of the 3.2 insert in a 20μl reaction composed of 1% Ligation buffer, 5mM spermidine and 10 units T4 DNA Ligase. The mixture was incubated overnight at 14°C. After incubation of the mixtures at 65°C for 15 minutes to stop any further ligase action, 20 units of *Spe I* restriction endonuclease was added and digestion took place at 37°C for 4 hours. An excess (10 units) of *Spe I* were added with a further incubation of 1 hour. The resultant digested fragments were run in a 0.9% gel to remove unbound *Spe I* linker. The bands were then excised from the gel, extracted with phenol/chloroform, precipitated with ethanol, and frozen at -20°C till required.

2.2.4.4. DNA sequencing

Sequencing of the GFP-3.2, GFP-4.12, and GFP-4.16 constructs were performed using the fmol® DNA Cycle Sequencing System, according to the manufacturer's instructions (Promega). The primer used was mFGF-3 R1, based on data of the Genebank sequence of the entire gene (Y00848). Sequence 5'-3' used: CTT CCA CCG CAG TAA TCT C, complementary to bases 3745 – 3763 of exon II. Sequence analysis was performed on an ABI 377 automated sequencer.

2.2.5. Plasmid preparation

2.2.5.1. Transformation of competent cells

The Epicurian Coli® XL1 Blue *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA1 lac* {F' *proAB lacI*^{q}*ZΔM*15 Tn*10* (Tet^{r})} strain supplied by Stratagene Ltd was used as competent cells for all plasmid transformation. The required amount of pre-chilled DNA (10-50ng) in a volume of 2μl was added to 180μl of recent thawed competent cells on ice, mixed gently and incubated on ice for 30 minutes. The mixture was heat shocked at 42°C for 90 seconds and returned onto ice for 2 minutes; 800μl of LB Broth were then added to cells and incubated at 37°C for 1 hour under continuously gentle shaking. The cells were spun at 10,000g for 2 minutes, and the pellet was resuspended into 200μl of LB medium. Ten per cent of this volume was spread onto LB agar plate containing

ampicillin at 100μg/ml and the remaining bacterial suspension were pelleted (30 seconds at 10,000g), resuspended into 100μl of LB and also plated onto LB agar plate. The plates were incubated at 37°C overnight.

2.2.5.2. Small scale preparation of plasmid DNA - (QIAprep Spin Miniprep Kit - QIAGEN)

Individual bacterial colonies were picked and inoculated into 5ml Luria Broth culture with 100μg/ml ampicillin and incubated overnight at 37°C with continuous shaking (starter culture). Plasmids were isolated using the QIAprep Spin Miniprep Kit protocol, according to the manufacturer's instructions. Briefly, 1.5ml of bacterial suspension from the starter culture were harvested by centrifugation at 10,000g for 2 minutes. The pellet was resuspended in 350μl of Buffer P1 (NaOH/SDS) supplemented with RNAse and mixed until no visible clumps were present; 350μl of Buffer P2 were added and continuously mixed for 1 minute; 350μl of Buffer N3 were added, mixed and left on ice for 10 minutes. The lysate was spun down at 10,000g for 10 minutes and the supernatant placed onto a QIAprep column/collection tube set. The set was centrifuged at 10,000g for 1 minute and washed with 0.5ml of Buffer PB. A second wash was performed by adding 0.75ml of Buffer PE to the column, incubating for 5 minutes at room temperature, and spinning at 10,000g for 1 minute. After discarding the flow-through, an additional spin was performed for removing residual washing buffer. The column was placed onto a clean eppendorf tube, and the DNA was eluted with 50μl of TE by spinning the column at 10,000g for 1 minute.

2.2.5.3. Large scale preparation of plasmid DNA (lysis by alkali)

Glycerol stocks of *E. coli* XL1 Blue transformed with the different FGF3 DNA expression constructs were streaked onto Luria Broth (Ross Lab PLC - BS611) agar plate containing 100μg/ml of ampicillin (Sigma) and incubated overnight at 37°C. Cells from an individual colony were inoculated into 5ml of Luria Broth supplemented with ampicillin and left shaking at 37°C for 4-5 hours (starter culture). A mixture of 360ml of Terrific Broth, 40ml of Terrific salts, and 100μg/ml of ampicillin was then inoculated with 4ml of the starter culture, and incubated overnight at 37°C with continuous shaking. The culture was transferred to a 500ml Sorvall bottles and spun down at 5,000 rpm for 10 minutes at 4°C using a Sorvall GS3 rotor. The bacterial pellet was resuspended into 18ml of Solution I, and 2ml of a freshly prepared solution of lysozyme

were added. After 2-3 minutes at room temperature, 40ml of fresh Solution II were added. The contents were thoroughly mixed by gently inversion of the bottles and stored at room temperature for 5-10 minutes. Thirty millilitres of ice-cold Solution III were added, mixed well and left on ice for 10 minutes. The bacterial lysates were centrifuged at 4,000rpm for 15 minutes at 4°C (brake off). The supernatants were filtered through 4 layers of cheesecloth into a 250ml centrifuge bottle; 0.6 volume of isopropanol were added, mixed well, and stored at room temperature for 10 minutes. The nucleic acids were recovered by centrifugation at 5,000rpm for 15 minutes at room temperature. After discarding the supernatant and allowing the last drops to drain away by inverting the bottle, the pellet and the bottle wall were rinsed with 70% ethanol at room temperature. The ethanol was drained off and the pellet was left to air-dry for 5-10 minutes. It was then dissolved into 6ml of TE pH 8.0 and further purified by precipitation with polyethylene glycol (2.2.5.4).

2.2.5.4. Purification of plasmid DNA by precipitation with polyethylene glycol (PEG)

Nucleic acids were dissolved in 6ml of TE pH 8.0 and 6ml of 5M LiCl cold solution were added, mixed well and spun down at 10,000g for 10 minutes at 4°C. The supernatant was transferred to a fresh 30ml Corex tube; 12ml of isopropanol was added and mixed well. The precipitated nucleic acid was recovered by centrifugation at 10,000g for 10 minutes at room temperature. The pelleted DNA was rinsed with 70% ethanol and air dried. It was then dissolved in 500µl of TE pH 8.0 containing 2µl DNase-free pancreatic RNase (20mg/ml- Boeringer-Manheim), stored at room temperature for 30 minutes; 500µl of 1.6M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000) were added, mixed well and the plasmid DNA was recovered by centrifugation at 10,000g for 5 minutes at 4°C. The supernatant was removed by aspiration and the DNA was dissolved into 750µl of TE pH 8.0. Further purification was performed by phenol/chloroform extraction (section 2.2.3.2) followed by ethanol precipitation (2.2.3.3).

2.2.5.5. Quantification of nucleic acids

DNA preparations were quantified using a GeneQuant RNA/DNA Calculator - Pharmacia/Biotech. An UV absorbance (A) reading of 1.0 at 260nm corresponding to

50μg/ml of double-stranded DNA was used for all estimates of nucleic acid concentration. DNA preparations with an A_{260}:A_{280} ratio of 1.8 or greater were used.

2.2.6. Production of XFGF3 conditioned medium

2.2.6.1. COS-7 cell transfection

Adherent COS-7 cells grown to confluence were washed twice with PBS and the cells detached with trypsin 0.05% plus 5mM EDTA. Detached cells were washed once in PBS and once with RPMI supplemented with 10% FCS (full medium); $5x10^6$ cells in 400μl of medium were transfected with 10μg of *Xenopus laevis* FGF3 cDNA expression plasmid (XFGF3), mouse FGF3 cDNA plasmids, CMVExpEGFP (*McIvor et al.*, 2000) or pGreen Lantern (Life Technologies) at 260V and 1050μF capacitance using the Easy Ject apparatus. The cells were transferred to a T-75 culture flask containing 20ml full medium, gassed with 5% CO_2 in air and incubated overnight at 37°C.

2.2.6.2. Conditioned medium with XFGF3

After 12-18 hours of transfection of COS-7 cells with the XFGF3 cDNA plasmid, the growth medium was removed and replaced by 20ml of RPMI supplemented with 0.1% (v/v) of FCS and 10μg/ml of heparin (Monoparin). The flasks were gassed with 5% CO_2 in air and incubated for 48-60 hours at 37°C. After the incubation period, the transfected conditioned medium was collected, spun down at 800g for 5 minutes to be cleared from cell debris and 1mM (final concentration) of AEBSF hydrochloride (Calbiochem) was added. The medium was kept on ice until required. To control the transfection efficiencies of *Xenopus laevis* FGF3, COS-7 cells were also transfected with 10μg of the pGreen Lantern reporter gene, using similar conditions described above, and the percentage of fluorescent cells detected by flow cytometry.

2.2.6.3. Purification of XFGF3 proteins from the conditioned medium

Columns (PolyPrep/Bio-Rad) containing 1ml of a 50% slurry Heparin-agarose beads (Sigma) were washed and equilibrated with 10ml of 0.6M NaCl in PBS; 40ml of the conditioned medium obtained from transfected cell cultures were added to the column, followed by a wash with 0,6M NaCl in PBS. The FGF3 proteins were then eluted from the column using 1M NaCl in PBS, filtered through a 0.22μm Acrodisc filter and kept at -20°C until required. The conditioned medium from GFP transfected COS-7 cells and

the conditioned medium from non-transfected COS-7 cells (wild type) were submitted to the same purification procedures and were used as controls throughout.

2.2.7. Analysis of protein expression

2.2.7.1. Preparation of cell lysates

After removing the conditioned medium, T-75 (Falcon) flasks containing adherent COS-7 cells transfected with XFGF3 cDNA plasmid were washed twice with PBS and the cells detached by trypsin/EDTA treatment. Detached cells were washed twice with PBS and spun down. The pellet containing $2x10^6$ cells were resuspended in ice-cold 100µl of NP40 lysis buffer pH 7.5 plus 1mM Sodium Vanadate (phosphatase inhibitor) and protease inhibitors (Aprotinin at 6µg/ml, Trypsin Inhibitor, N-Tosyl-L-Phenylalanine Chloromethyl Ketone, Benzamidine, Antipain, and Tosyl-L-Lysine Phenylalanine Chloromethyl Ketone at 10µg/ml each, and AEBSF Hydrochloryde at 1mM). The cell lysates were rapidly vortexed and frozen at -20°C. Just before use, the cell lysates were thawed, spun down at 13,000g for 3 minutes, and the supernatant used for protein assay and/or Western blot.

2.2.7.2. Protein concentration assay

The protein content of cell lysates prepared as described in section 2.2.7.1 was quantified according to the Bradford methodology (Bradford, 1976) using the Bio-Rad protein dye concentrate assay according to the manufacturer's instructions. Briefly, 5µl of 1:10 dilution of the protein supernatant of the samples were added to 75µl of double-distilled water plus 20µl Dye Reagent (Bio-Rad) in a 96 well-plate and mixed well. Quantification of total protein was carried out by measuring the absorbances at 620 and 405nm (background colour) in a Pasteur Diagnostic LP400 plate reader, using a protein standard curve obtained by the preparation of serial dilutions of Bovine Serum Albumin-fraction V (Sigma) containing from 0 to 35µg/ml of BSA.

2.2.7.3. Protein fractionation by SDS-PAGE

To fractionate cell lysate proteins, 50-100µg of cell lysate proteins were loaded along with an appropriate size marker (Rainbow, Amersham, UK), on a 5% Stacking Gel and run at 20mA/90-100V during the stacking gel phase, and at 35mA/200V in the 15%

Resolving Gel. Following electrophoresis, the gel was transferred to a tray containing Transfer Buffer and kept until ready for electroblotting.

2.2.7.4. Electroblotting

Gels run on SDS-PAGE were transferred to a Hybond-C extra nitrocellulose membrane using a semidry apparatus (Biometra, UK) as follows: 4 pieces of 3MM paper (Whatmann, UK) cut slightly bigger than the gel size were soaked with transfer buffer; 2 of them were placed onto the blotter base. One piece of Hybond-C extra nitrocellulose membrane sized exactly as the gel size was placed onto the 2 first pieces of 3MM paper followed by the gel. Finally, the other 2 pieces of 3MM paper were placed onto the gel. The bubbles were then removed by rolling a clean pipette on the sandwich top and the top lid of apparatus was repositioned. The transfer was performed at 4°C for 60 minutes at 250mA, 17-18V using a Bio-Rad, mod. 250/2.5 power supply. The transferred membranes were then used for immunodetection or kept at -20°C until required.

2.2.7.5. Western Blotting

Hybond-C extra nitrocellulose membranes carrying blotted proteins were incubated for at least 2 hours at room temperature or overnight at 4°C, rocking (Rotatest shaker mod. R 100/TW, Luckham) gently with Blocking Buffer. They were then incubated for 12-18 hours at 4°C with the polyclonal rabbit antibody against the C terminal of *Xenopus laevis* FGF3 (1:500) or with 2μg/ml monoclonal mouse antibody MSD-1 (Santa Cruz Biotechnology, AutogenBioclear, UK) in Blocking Buffer. The membranes were washed 5 times, 10 minutes each washing, with PBS plus 0.2% Tween-20 and incubated for further 20-30 minutes with a peroxidase-conjugated swine anti-rabbit immunoglobulins or rabbit anti-mouse (DAKO A/S, Denmark) diluted 1:3000 and 1:1000, respectively, in blocking buffer. Another set of 5 washing steps with PBS plus 0.2% Tween-20 were followed by detection of the protein using the Enhanced ChemiLuminescence detection system (ECL Kit - Amersham) according to the manufacturer's instructions. Briefly, the kit reagents 1 and 2 were mixed in a 1:1 ratio and applied to the membrane for 2 minutes. The membrane was subsequently removed, gently dried, wrapped in Saran (Dow) cling film, exposed from 2 seconds to 10 minutes to a Kodak X-0MAT AR X-Ray film, and developed.

2.2.8. Immunostaining of COS-7 cells transfected with mouse FGF3 plasmids

After overnight incubation at 37°C in a fully humidified incubator with 5% CO_2 in air, COS-7 cells transfected with mouse FGF3 DNAs were detached from the culture flask with trypsin and EDTA, washed with medium and a maximum of $2x10^7$ cells in 1ml were loaded onto ice-cold 2.5ml of Metrizamide density gradient solution (section 2.1.6) and spun down at 800g for 15 minutes, at 4°C. Cells at the interface ring were collected, washed and resuspended at $2x10^6$ cells/ml into PBS supplemented with 0.5% (v/v) heat-inactivated BSA and sorted according to the fluorescence emission of the GFP reporter gene expression under sterile conditions using the ACDU on a FACS Vantage flow cytometer. The sorted cells were centrifuged at 800g for 5 minutes, resuspended, and cultured into full medium using a 4-chamber polystyrene vessel tissue culture treated glass slide (Falcon). After full attachment of the transfected cells at the bottom of the flask (6-8 hours) at 37°C in a fully humidified incubator with 5% CO_2 in air, the medium was washed off using PBS (3x 1 minute). The adhered cells were fixed with 4% (w/v) formaldehyde in PBS for 20 minutes. The cells were then permeabilised with 0.2% (v/v) Triton X-100 (Sigma) in PBS for 4 minutes and rinsed with PBS. Permeabilised cells were overlaid with mouse monoclonal MSD-1 (anti-FGF3) antibody (Santa Cruz Biotechnnology, USA) at 2μg/ml for 1 hour at 37°C. The antibody binding was detected using the APAAP method with Vector Red (Vector Laboratories, UK) as a colour substrate as follows: after the incubation period, the unbound antibody was washed off with TBS and goat anti-mouse immunoglobulins (GAM, DAKO, UK) solution at 1/25 was added for 30 minutes. Slides were washed with TBS and soluble complex of alkaline phosphatase and mouse anti-alkaline phosphatase (APAAP, DAKO, UK) at 1/50 was added for 10 minutes. After washing with TBS, GAM at 1/50 solution was added for 10 minutes, washed off with TBS, and APAAP at 1/100 dilution was added and left for 10 minutes. Slides were then washed with TBS, followed by an immersion in Buffer pH 8.3 for 5 minutes. Vector Red supplemented with 1mM levamisole and 0.1% (v/v) Tween solution was added and the slides incubated at room temperature in the dark for 20 minutes. After rinsing the slides with double-distilled water, the plastic walls of the chamber were removed with the supplied device, and Gills Haematoxilin activity I solution (Shandon, UK) was added for 1 minute and

washed off with double-distilled water. The slides were finally mounted with Glycergel Aqueous Montant (DAKO, UK) to prevent fading.

2.2.9. Detection of CCR1 receptor by immunocytochemistry

For the immunocytochemistry detection of CCR1 antigen on cell surface, titration of the polyclonal rabbit anti-CCR1 antibody was performed with fresh peripheral blood mononuclear cells, using 25, 10, and 5μg/ml of the antibody in Dako reagent supplemented with 2% human AB serum. The optimal working dilution obtained was 25μg/ml. Cytospin preparations of $CD34^{+}CCR1^{high}$ and $CD34^{+}CCR1^{-/low}$ cord blood cells grown in Mix-CFC assay (section 2.1.12.1) were fixed with 4% paraformaldehyde (w/v) in PBS for 15 minutes. Anti-CCR1 at 25μg/ml in Dako reagent supplemented with 2% human AB serum was added and the preparations incubated at room temperature for 2 hours in a humidified environment. After washing with PBS, the cells were covered with 15mM $NaNO_3$ plus 0.3% H_2O_2 in PBS for 30 minutes. Swine anti-rabbit biotinylated antibody (DAKO A/S, Denmark) was used a secondary antibody at 75μg/ml for 40 minutes. PBS washed preparations were covered with ABC solution using the Vectastain Elite ABC Kit according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame/USA) and subsequently developed in DAB (Sigma Fast 3,3'-Diaminobenzidine Tablet Sets, Sigma, Saint Louis/USA) for 10-15 minutes. The slides were counter stained with Gills Haematoxilin activity I solution (Shandon, UK) for 40 seconds and washed off with double-distilled water. The slides were mounted with Glycergel Aqueous Montant (DAKO, UK) to prevent fading.

2.2.10. Transfection of FDCP-mix cells

FDCP-mix cells in log phase of growth were washed twice with 320mOsm/kg IMDM and resuspended into medium at concentration of $1.4x10^7$cells/ml; $5x10^6$ cells in 400μl of medium were transfected with 10μg of mouse FGF3 cDNA expression plasmids using a 4mm electroporation cuvettes (Equibio) and the Easy Ject apparatus set at 280V and 1050μF capacitance. The cells were transferred to a 6-well plate containing 2.5ml of full growth medium and incubated for 12-18 hours at 37°C in a fully humidified incubator with 5% CO_2 in air.

Section 2.3. - Statistical Analysis

The results represent the mean ± standard error of mean (SEM) or ± standard deviation (SD) whenever applicable and statistic procedures were performed using the Student's *t*-test on data running on ORIGIN 5.0 for Windows.

Chapter 3

Effects of FGF3 on haemopoiesis

3.1. Introduction

As emphasised at the introduction of this work, most of the studies investigating the involvement of the FGFs in haemopoiesis have focused on FGF1 and, particularly, on FGF2. To date, the potential role of FGF3 in haemopoiesis has not been investigated. FGF3 is the protein product of the proto-oncogene *int-2*, a common site of mouse mammary tumour virus (MMTV) insertion in murine mammary tumours. It shows homology with FGF2, particularly in the 120-residue central core characteristic of the FGFs (Acland *et al.*, 1990; Dickson and Acland, 1990; Dickson *et al.*, 1990; Dickson *et al.*, 1989; Dixon *et al.*, 1989; Jakobovits *et al.*, 1986). The secreted form of FGF3 binds with high affinity to FGFR-2 and to a less extent to FGFR-1 (Mathieu *et al.*, 1995b).

In adult mice, *int-2* expression is not detected, with the possible exception of brain and testes (Stamp *et al.*, 1992). During embryonic development, *int-2* expression is restricted to specific sites in a tightly controlled temporal sequence from day 6 until parturition (Figure 3.1). The number and diversity of the sites of *int-2* expression suggest multiple roles consistent with functions such as mitogenesis, chemotaxis or induction of differentiation (Jakobovits *et al.*, 1986).

In mouse mammary tumorigenesis, the mechanism through which FGF3 exerts its effect is not clearly understood. *In vivo*, FGF3 has limited domain of tumorigenicity and in natural tumours, it is activated only by MMTV insertion. In transgenic mice containing the gene under control of the MMTV LTR, FGF3 acts as an epithelial growth factor inducing mammary and prostate hyperplasias (Muller *et al.*, 1990). It can effect morphological transformation of transfected NIH-3T3 cells (Goldfarb *et al.*, 1991) and also has been implicated in the stimulation of angiogenesis and cell prolifera-

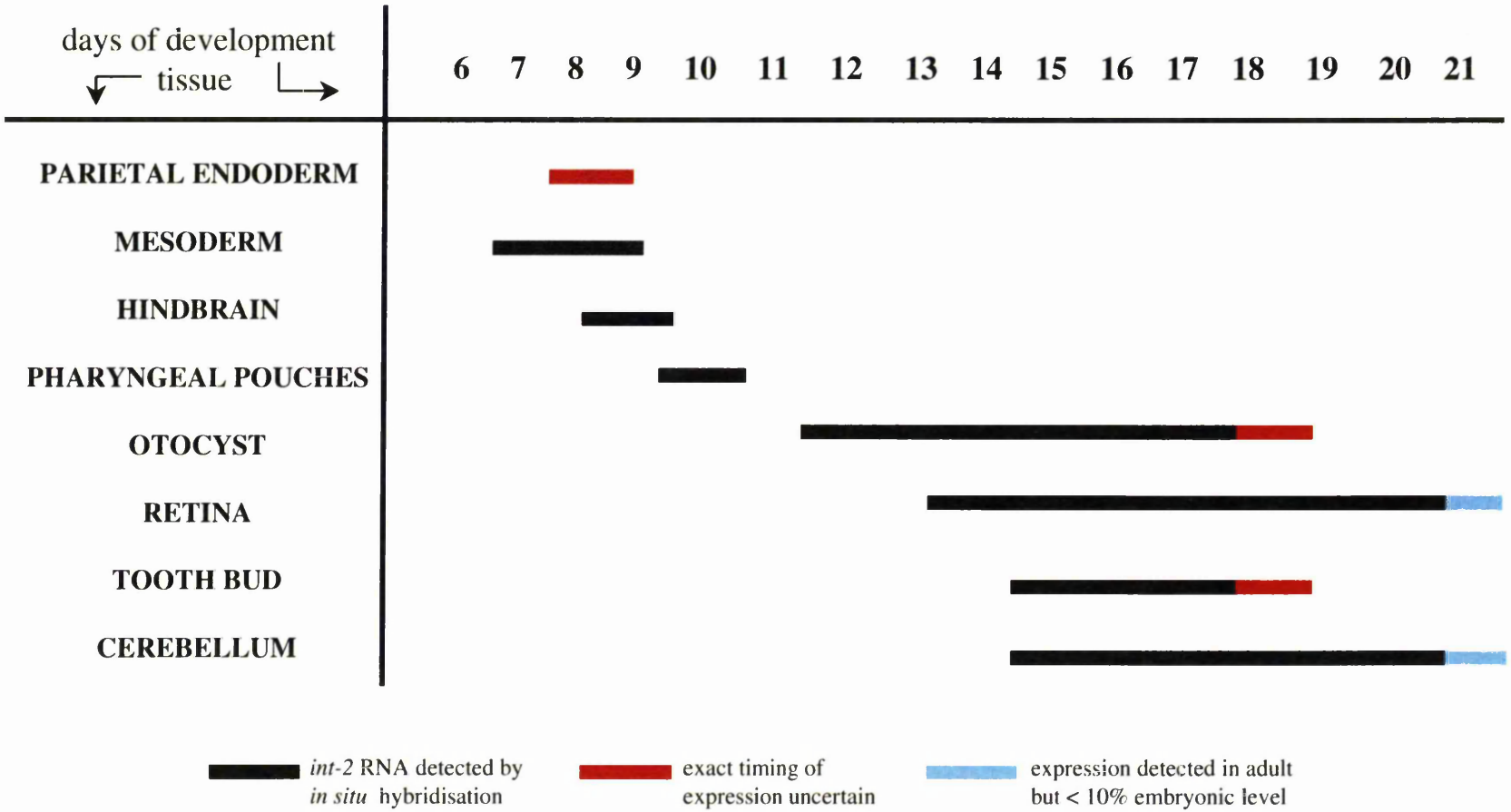


Figure 3.1. Expression of *int-2* in mouse embryo. The figure depicts the sites and times at which *int-2* expression has been detected in developing CBA mouse embryos by *in situ* hybridisation of RNA (adapted from Dickson *et al.*, 1989 and Dickson *et al.*, 1990).

tion during tumour development (Costa *et al.*, 1994). The human *FGF3* gene has been cloned, sequenced and mapped to chromosome 11q13 and shows similar intron-exon organisation to that of mouse and the sequence of the primary 27kDa protein product is highly conserved between the two species (Brookes *et al.*, 1989; Williams *et al.*, 1988).

A growing body of evidence implies a role for the *int-2* oncogene in human carcinomas. The gene has been found amplified in breast (Champeme *et al.*, 1994; Fantl *et al.*, 1990; Fantl *et al.*, 1993; Meyers and Dudley, 1992), ovarian (Rosen *et al.*, 1993), esophageal (Kitagawa *et al.*, 1991, Ikeda, 1996 #1425; Suzuki *et al.*, 1997) and endometrial (Esteller *et al.*, 1995) cancers and, also in Kaposi's sarcoma (Huang *et al.*, 1993). Recently, the potential tumorigenic and angiogenic roles of human FGF3 have been demonstrated in nude mice, where NIH-3T3 cells transformed by FGF3 were injected subcutaneously. Nodular lesions developed at the injection sites and histopathological features of these tumours contained alterations similar to those observed in human Kaposi's sarcoma lesions (Li *et al.*, 1998). Recent study of genomic imbalances in human nasopharyngeal carcinoma, showed that *int-2* amplification was present in more than 75% of the biopsy specimens studied (Fan *et al.*, 2000).

The presence of marked haemopoietic defects found in the $FGF3^{-/-}$ mouse model (Cross *et al.*, 1997) has been suggestive of the involvement of FGF3 in haemopoiesis. It has been shown that murine $FGF3^{-/-}$ foetuses were embryonic lethal, which the single exception of one pup born badly runt and unable to balance that survived for 8 days. The null mutant foetuses resembled littermates closely up to embryonic day 13 (E13). E14 null mutants had smaller livers which become very marked by E15 null mutants, by which stage they were anaemic and many were dead (Cross *et al.*, 1997). In addition, low levels of FGF3 expression have been detected by Northern blot analysis on murine FDCP-mix cells, specifically in the undifferentiated (multipotent) state and by RT-PCR in day 12 mouse foetal liver cells, at a stage during which haemopoiesis becomes established at this site (Dr. M. Cross, personal communication). As these characteristics were considered to be consistent with an haemopoietic defect, they prompted us to investigate the possible roles of FGF3 in haemopoiesis.

Thus, one of the aims of the present study was to investigate the potential biological effects of FGF3 on selected primitive haemopoietic cell populations from murine and human origins. FGF3 is not commercially available as a purified cytokine, due probably to the instability (Mathieu *et al.*, 1995b) and poor secretion of the murine and human forms from transfected cells (Dixon *et al.*, 1989; Kiefer *et al.*, 1993b). The

Xenopus laevis FGF3 (XFGF3), however, has been shown to be 82% identical to murine (Kiefer *et al.*, 1993a) (Figure 3.1.A) (murine/human homology is 90% (Dickson *et al.*, 1989)); to be more stable; to transduce signals through FGFR1 and FGFR2 receptors (Goldfarb *et al.*, 1991; Mathieu *et al.*, 1995a); to have transforming activity on a variety of mammalian cell lines (Kiefer *et al.*, 1993a), and to be efficiently secreted from transfected COS-1 cells (Kiefer *et al.*, 1993a). For these reasons, XFGF3 was chosen for the studies reported here. In the following sections, we first describe the methodology used to obtain the secreted form of XFGF3. Subsequently, we report the approaches taken to test FGF3 responsiveness of different cell populations from murine and human origins.

3.2. Results

3.2.1. Expression of XFGF3 products in COS-7 cells

Data have accumulated from several studies regarding the secretion, purification, characterisation, and some of the biological properties of FGF3 from mouse (Kiefer and Dickson, 1995; Kiefer *et al.*, 1991; Kiefer *et al.*, 1993b), *Xenopus laevis* (Kiefer *et al.*, 1993a), and zebrafish (Kiefer *et al.*, 1996a). In this study, we have used some of these reported approaches to obtain the purified XFGF3 protein.

To obtain XFGF3, the XFGF3.1 plasmid DNA (Kiefer *et al.*, 1993a), kindly supplied by Dr. Clive Dickson (Imperial Cancer Research Fund, London) was prepared from glycerol stocks of transformed *E. coli* XL1 Blue. Two independent maxi-preparations of this plasmid yielded 4.7 and 6.6μg of DNA protein/μl. Subsequently, we have used the COS-7 cells as XFGF3 cell producer. The COS-7 cell line is derived from African green monkey kidney cells by transformation with an origin-defective simian virus 40 (SV40) which has integrated into the chromosomal DNA (Gluzman, 1981). This cell line is used routinely to express efficiently secreted proteins (Old and Primrose, 1994). Furthermore, these proteins, although not normally produced by COS-7 cells, are usually correctly processed. The XFGF3.1 expression vector was introduced into COS-7 cells by electroporation as described in section 2.2.6.1 and a plasmid encoding the Green Fluorescent Protein (GFP) reporter gene was used as a control for monitoring transfection efficiency. The mean percentage of COS-7 cells showing

fluorescence after transfection with GFP vector was 30.5%±7.5% (range 11.6% to 72.9%, n=8).

Samples of the cell extracts (standardised by cell number) were prepared 48-72 hours after transfection (section 2.2.7.1), and 50-100μg of the total protein content were fractionated by SDS-PAGE in a 15% gel as described in section 2.2.7.3. The XFGF3 related proteins were detected by Western blot using the specific monoclonal antibody MSD–1, which recognises an epitope conserved in the mouse, *Xenopus*, and human FGF3 sequences (Kiefer *et al.*, 1993a) (section 2.2.7.5). Cell lysates from COS-7 cells transfected with the XFGF3 plasmid showed one specific FGF3 product with an apparent molecular mass of 31 kDa (Figures 3.2-A, line X and Figure 3.2-B, line 1), that was not present in the cell lysates from COS-7 wild type (Figure 3.2-A, line W).

In order to rule out responses to substances other than FGF3 that may be present in the culture medium, the transfected COS-7 cell culture medium was harvested and purified according to a reported procedure (Kiefer *et al.*, 1993a; Kiefer *et al.*, 1996a; Mathieu *et al.*, 1995b) that utilises the ability of FGF molecules to bind heparin when immobilised in agarose (Shing *et al.*, 1983). As described in section 2.2.6.3, the harvested medium from transfected COS-7 cells was loaded into a Heparin-agarose column and the purified XFGF3 protein, pX, eluted with 1M NaCl. The culture medium harvested 48-72 hours after transfection and the fraction pX obtained after Sepharose-heparin purification were also analysed by SDS-PAGE in a 15% gel. Their immunoblots showed the protein products p31 and p27 in the harvested medium (Figure 3.2-B, line 3) as expected, while only the p27 product was present in the purified fraction (Figure 3.2-B, line 5). No such products were detected in the cell extract, harvested medium or in the purified fraction from GFP transfected COS-7 cells (Figure 3.2-B, lines 2, 4 and 6, respectively).

The presence of the 31kDa product in the cell extracts from XFGF3 transfected COS-7 cells is in line with the reported biosynthesis of XFGF3, in which this product is efficiently secreted and becomes associated with the cell surface and extracellular matrix (Kiefer *et al.*, 1993a; Kiefer *et al.*, 1991). Also, it has been reported that proteolytic cleavage of p31 results in a more stable product, p27, and this takes place primarily in the extracellular compartment (Kiefer *et al.*, 1993a). In this study, we have detected the p27 product only in the medium harvested from the XFGF3 transfected COS-7 cells. Although we can not rule out that the conditions used in our experiments

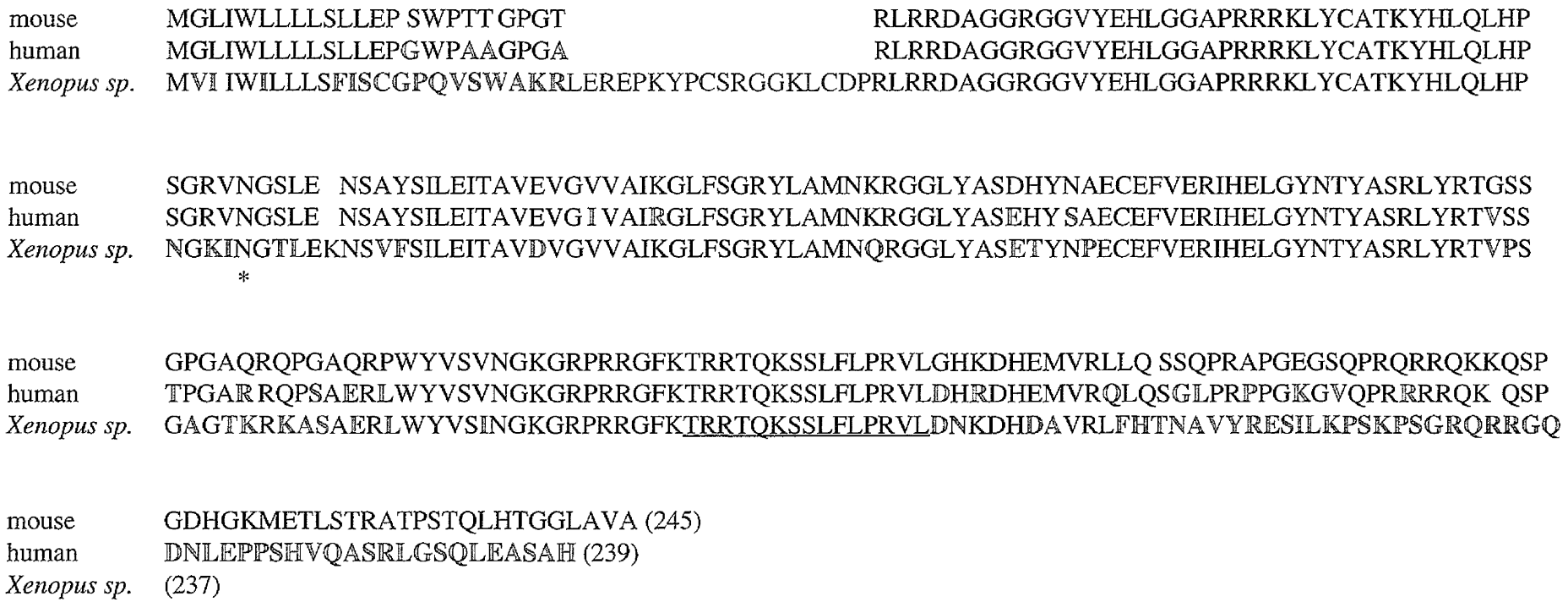


Figure 3.1.A. Amino acid sequence of FGF3. The sequence of the primary translation product of mouse FGF3 is shown in single letter amino acid code. The corresponding human and *Xenopus laevis* products are identical except at the indicated coloured positions. The asterisk indicates the common site of Asparagine-linked glycosylation and the underlined region delineates the peptides recognised by the monoclonal antibody MSD-1 (adapted from Dickson *et al.*, 1989 and Kiefer *et al.*, 1993).

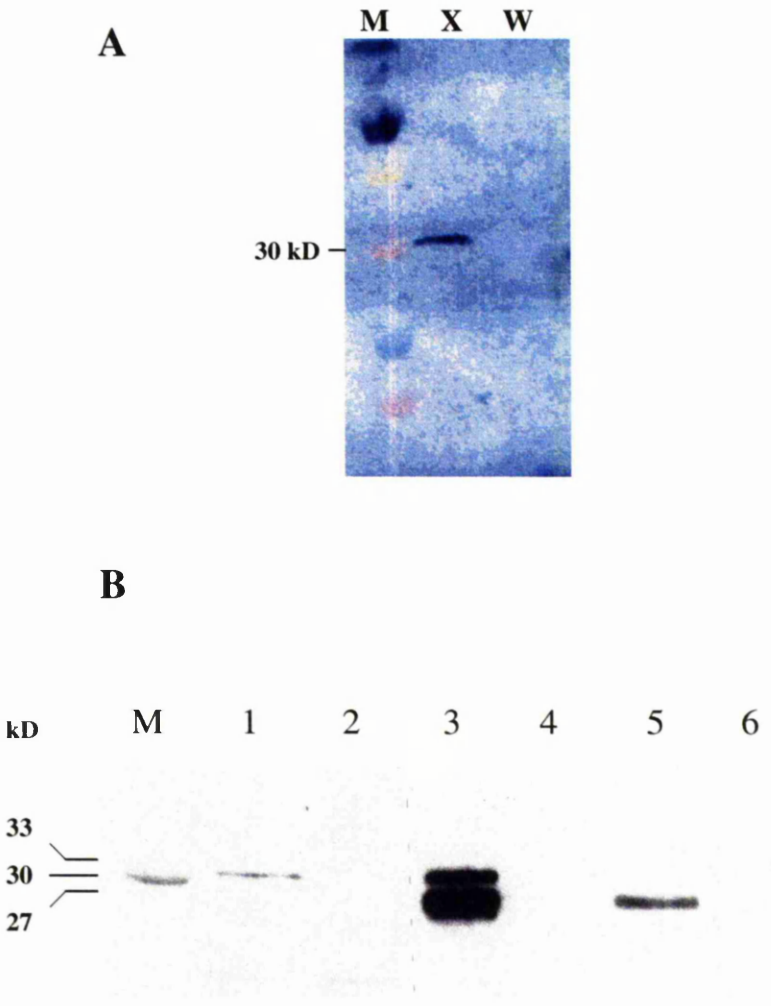


Figure 3.2. Presence of XFGF3 in COS-7 cell lysates, culture medium, and purified fraction. COS-7 cells transfected with XFGF3 cDNA plasmid or with the GFP control vector were harvested after 48 h and the culture medium recovered. Cell lysates were prepared as described in section 2.2.7.1. The culture medium was purified through a heparin-agarose column as described in section 2.2.6.3. Samples of the cell extract, culture medium, and purified fraction were fractionated by SDS-PAGE at 15% gel and transferred to nitrocellulose membrane. The XFGF3-related proteins were detected with the monoclonal antibody MSD-1. The sizes of the FGF3-related proteins p31 and p27 were estimated relative to molecular weight marker standard **M**. (**A-X**, **B-1**) XFGF3 transfected COS-7 cell extract; (**A-W**) COS-7 cell wild type cell extract; (**B-2**): GFP vector transfected COS-7 cell extract; (**B-3**) conditioned medium from transfected XFGF3 COS-7 cells; (**B-4**) conditioned medium from GFP transfected COS-7 cells; (**B-5**) purified fraction from transfected XFGF3 COS-7 cells; (**B-6**) purified fraction from GFP transfected COS-7 cells.

were sub-optimal to allow its detection, the lack of p27 in the cell extracts from transfected COS-7 cells with XFGF3 plasmid and its presence in the conditioned medium are in agreement with the concept that the presence of soluble heparin in the medium can dislodge the protein from the cell surface and/or from the ECM and displace it into the medium (Kiefer *et al.*, 1993a).

3.2.2. Biological properties of the secreted XFGF3

3.2.2.1. Induction of DNA synthesis

The above results showed that with the technical approaches taken, we were able to obtain XFGF3, the protein product from *Xenopus laevis*. Subsequently, we investigated whether this product was secreted in an active form. The ability to promote DNA synthesis and cellular proliferation *in vitro* in a variety of cell types is one of the clearest biological properties of the FGF family of proteins (Burgess and Maciag, 1989). To investigate whether the XFGF3 protein products released into the growth medium of transfected COS-7 cells would have mitogenic activity, different concentrations of the harvested medium (v/v) were added to cultures of NIH-3T3 rendered quiescent by placing them in 0.2% NBCS prior to the (^{3}H)-thymidine incorporation assay (section 2.1.19). To monitor the minimum and maximum responses to the induction of DNA synthesis, NIH-3T3 cells were cultured in the presence of 0.2 and 5% (v/v) of NBCS, respectively, under the same testing conditions.

As depicted in Figure 3.3-A, the XFGF3 protein products released into the medium harvested after transfection of COS-7 cells could induce DNA synthesis in quiescent NIH-3T3 cells in a dose-related manner. The purified fraction pX obtained after treatment of the transfected COS-7 conditioned medium through a Heparin-agarose column and elution at a salt concentration of 1M was also mitogenic (Figure 3.3-B). Moreover, this effect was more pronounced than the effect observed with the conditioned medium alone. These results indicate that the process of purification has increased the concentration of the protein, as would be expected. Also, this effect was not related to the salt present in the purified fraction since equivalent concentrations of 1M NaCl added to quiescent NIH-3T3 cells showed very limited induction of DNA synthesis when compared to the purified protein fraction.

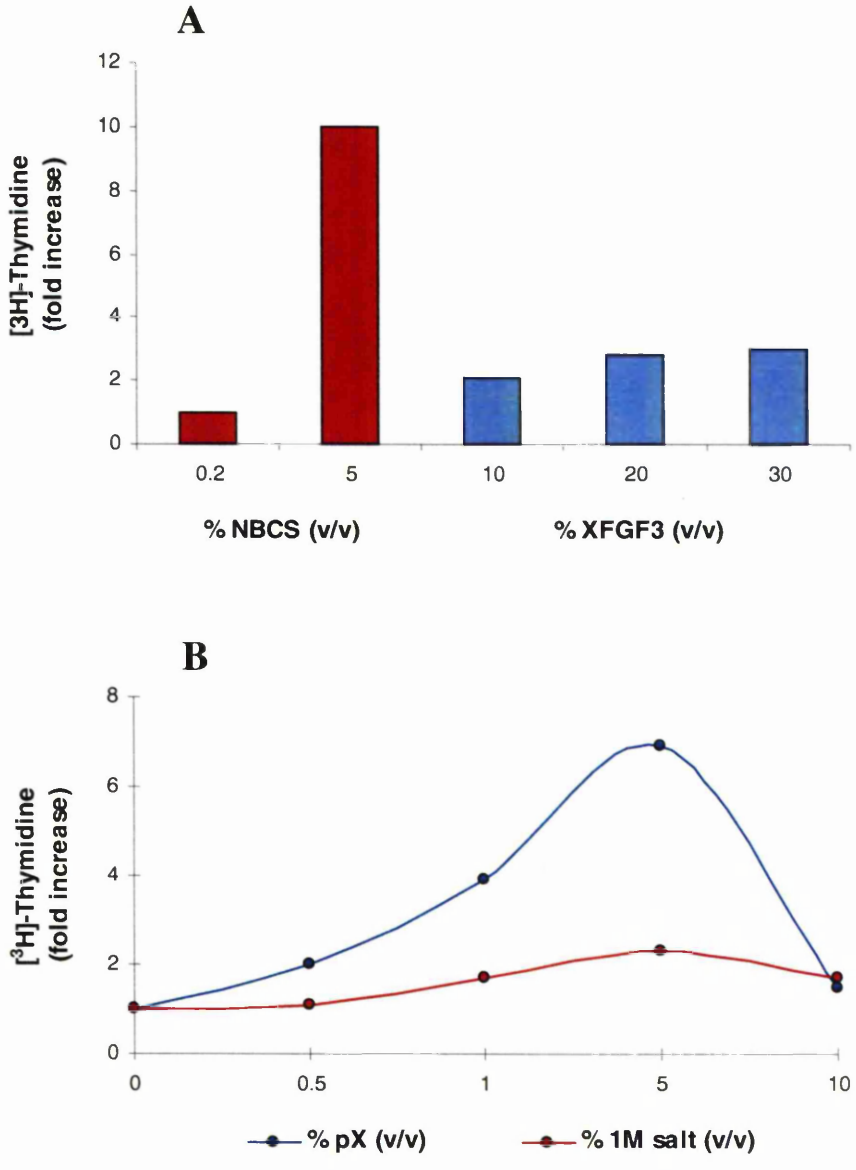


Figure 3.3. ^{3}H-thymidine incorporation in response to secreted XFGF3. (**A**) NIH-3T3 cells were plated in medium containing 5% (v/v) serum (section 2.1.9.3), and rendered quiescent by reducing the serum concentration to 0.2% (v/v). The cultures were then exposed to different concentrations (v/v) of conditioned medium from COS-7 cells transfected with XFGF3 cDNA plasmid. After 24 hours, the cultures were labelled for a further 3 hours with 1µCi/well of ^{3}H-thymidine. The cells were then lysed with 0.3M NaOH and the ^{3}H-Thymidine incorporation determined by liquid scintillation counting. The columns represent the concentration of newborn calf serum (NBCS - red bars) and conditioned medium (blue bars) added to the cultures. (**B**) Samples of the conditioned medium (v/v) from transfected COS-7 cells purified through heparin-sepharose column and eluted in 1M NaCl were also tested for their ability to induce proliferation on quiescent NIH-3T3 cells (pX - blue line). The 1M NaCl salt solution (red line) was used to monitor the experimental conditions used. Each point represents the proportion of the number obtained in the experiment over control values (fold increase), where no salt or XFGF-3 was added. Data representative of one experiment.

3.2.2.2. Phenotypic modification of NIH-3T3 cells

It has been shown that the protein products secreted and released into the medium harvested after transfecting COS-1 cells with XFGF3 cDNA can induce transient morphological transformation of NIH-3T3 cells, and this effect is the result of the binding of FGF3 to its receptors FGFR-1 and/or FGFR-2 IIIc isoform (Kiefer *et al.*, 1993a). As the secreted XFGF3 has shown to induce DNA synthesis, we sought to investigate its transforming potential. Therefore, the conditioned medium obtained from transfected COS-7 cells and the pX fraction were tested for their ability to induce similar effects. As described in section 2.1.20, different concentrations of both conditioned medium and purified fraction pX were added to near-confluent cultures of NIH-3T3 cells in the presence of heparin. The culture dishes were then incubated at 37°C in CO_2 atmosphere and cell transformation observed after 24 hours.

Figure 3.4-A shows the typical phenotype of spindle-shaped (S) NIH-3T3 cells grown as a monolayer in cultures where DMEM was supplemented with 10% (v/v) NBCS and heparin. In the cultures where the conditioned medium from transfected COS-7 cells (Figure 3.4-B) or the XFGF3 purified fraction (Figure 3.4-C) was present, two different types of cells could be observed along with the typical ones. Some cells were smaller, had several sharply ended processes, and resembled stars (T) while others were bigger and smoother in appearance (G). Also, the monolayer appeared to be disrupted by the overgrowth of the transformed cells. These results suggested that the XFGF3 proteins produced by COS-7 cells after transfection with the appropriate vector and secreted into the conditioned medium as well as its purified form were functional, able to induce DNA synthesis and morphological changes in the phenotype of NIH-3T3 cells in agreement with published reports (Kiefer *et al.*, 1993a; Kiefer *et al.*, 1991; Kiefer *et al.*, 1996b).

Subsequently, the purified fraction of XFGF3 eluted through a heparin-Sepharose column in 1M NaCl solution (pX) was used to investigate whether this protein could induce biological responses in haemopoietic progenitor cells of murine and human origins. In all experiments, the appropriate salt concentration was used as control.

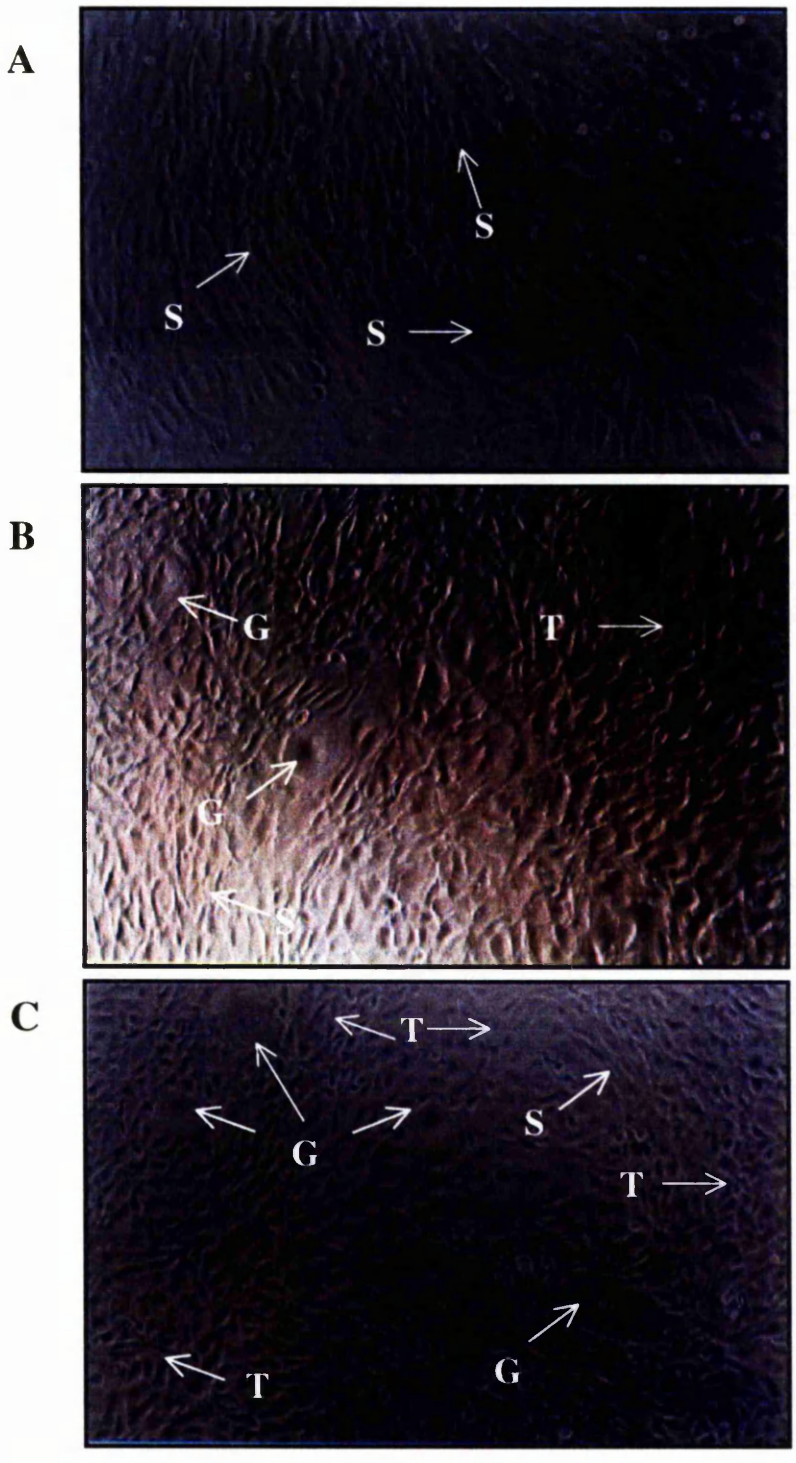


Figure 3.4. Phenotypic modification of NIH-3T3 cells induced by XFGF3. Samples of the NIH-3T3 cells grown in DMEM supplemented with 10% NBCS and 10µg/ml heparin (**A, control**) are compared with those where conditioned medium from COS-7 transfected with the XFGF3 vector before (**B, conditioned medium**) and after purification through a Heparin-agarose column (**C, pX fraction**) were diluted with the growth medium and added to near-confluent cultures of NIH-3T3 cells. The cell cultures were photographed after 24 hours under phase contrast. (S) spindle-shaped typical appearance; (T) star-shape cells; (G) bigger and smoother cells (phase contrast - magnification x40).

3.2.3. Effects of XFGF3 on murine FDCP-mix cells

3.2.3.1. Clonogenic potential

As previously described, FDCP-mix cells are non-leukaemic, IL-3 dependent and can undergo both self-renew and differentiation under suitable conditions. As such, they represent a physiologically relevant system in which the effects of cytokines on both self-renewal and development can be studied. Therefore, we examined the direct effects of the purified fraction of XFGF3 protein alone and in combination with other cytokines on the survival, proliferation and development of FDCP-mix cells using different approaches such as soft-gel and liquid culture systems.

Colony-Forming Cell (CFC-) assays were used in these studies to investigate the direct biological effects of XFGF3 on FDCP-mix progenitor cells, alone and in combination with IL-3 and EPO. While IL-3 supports the survival, self-renewal and proliferation of multipotential progenitor cells and stimulates their development into colonies containing a mixture of cells from various lineages (Metcalf *et al.*, 1987a), EPO is necessary for the late stages of erythroid growth, both in pure and in mixed colonies (Testa, 1985). Therefore, to investigate the effects of XFGF3 on the clonogenic potential of FDCP-mix cells, we have used the clonogenic assay described in section 2.1.12.2. The dose range of the purified fraction of XFGF3 (pX) that showed mitogenic activity for NIH-3T3 cells (Figure 3.3-B) was added as indicated. To facilitate comparison between the groups, the results are expressed as the mean percentage of the number of colonies produced in the control experiment plus or minus one standard error of the mean (SEM).

In the absence of other growth factors, XFGF3 was not able to support colony formation. However, as illustrated in Figure 3.5, its combination with IL-3 and EPO resulted in a concentration-dependent increase in the number of colonies formed. The maximum increase observed was at the XFGF3 concentration of 1%, with 19.4%±4.3 more colonies formed when compared to colony numbers obtained in the salt control (p=0.004, n=4). The size and the morphology of the colonies were similar to those seen in the control. No colony growth was observed at 5% XFGF3 concentration, although that was the concentration that showed better response when tested for promoting NIH-3T3 cells DNA synthesis.

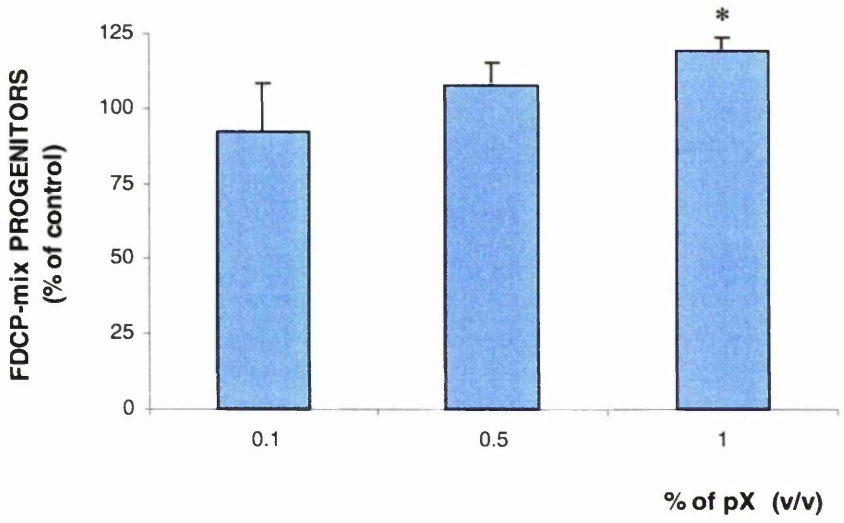


Figure 3.5. Effect of XFGF3 in combination with cytokines on FDCP-mix colony formation. FDCP-mix cells were seeded in IMDM supplemented with horse serum, murine recombinant IL-3, EPO, and XFGF3 purified fraction (pX) as indicated (section 2.1.12.2). Each column represents the mean percentage ± SEM of the number of colonies from 4 experiments performed under similar conditions in proportion to the colony number obtained in the salt control. Control numbers for colonies ranged from 27 to 83. * $p<0.01$.

3.2.3.2. Survival and self-renewal

It has been reported that IL-3 promotes a dose-dependent increase in the survival and proliferation of FDCP-mix cells, and that doses as low as 0.01ng/ml, although non-mitogenic, are capable of supporting their survival (Spooncer *et al.*, 1986). As XFGF3 was found to influence the clonogenic potential of FDCP-mix cells, we sought to investigate its ability in influencing their survival. To achieve this, liquid cultures supplemented with different concentrations of XFGF3 were established as described in section 2.1.14. As a control of survival, FDCP-mix cells were cultured in the presence of 0.1ng/ml of IL-3. Cell numbers and viability were assessed by trypan blue exclusion (section 2.1.10). As shown in Figure 3.6, FDCP-mix cells survived and proliferated in low concentrations of IL-3 as expected. In contrast, the cultures in which only XFGF3 was added, the cells showed poorer ability to survive or proliferate when compared to the control, and after 4 days, the FGF3-cultured cells were all dead.

To test the ability of XFGF3 to influence the self-renewal and proliferation of FDCP-mix cells, different concentrations of XFGF3 were added to liquid cultures in the presence of 10ng/ml of IL-3. As illustrated in Figure 3.7-A, a similar growth profile of FDCP-mix cells was observed for all conditions tested during the first three days of culture. After this period, the XFGF3 treated cultures showed a decline in proliferation when compared to the control values. Analysis of these cells on cytospin preparations stained with May-Grunwald-Giemsa (section 2.1.13.2) at days 4 and 7 showed that more than 65% of the cells retained the morphology of immature granulocytes (blasts, promyelocytes, and myelocytes), indicating that the cells had little ability to undergo differentiation in the presence of a high concentration of IL-3 as expected. The removal of cells from each time point into colony forming assays revealed an unexpected decrease in colony forming activity by day 4, even in the control cultures to which only salt solution had been added (Figure 3.7-B). Although the addition of 1% (v/v) of a 1M NaCl solution represents an increase of approximately 10% in the NaCl already present in the medium, it is feasible that this is sufficient to disturb the maintenance of CFC.

An additional effect appeared to result from the addition of XFGF3, in that loss of colony forming units was accelerated. However, this effect was more marked with lower concentrations of XFGF3, and was not significant (may be because of the low number of replicates). While it remains possible that relatively low levels of XFGF3 have an inhibitory effect on FDCP-mix proliferation and colony forming ability that is compensated by higher concentrations, a more through analysis would be necessary to

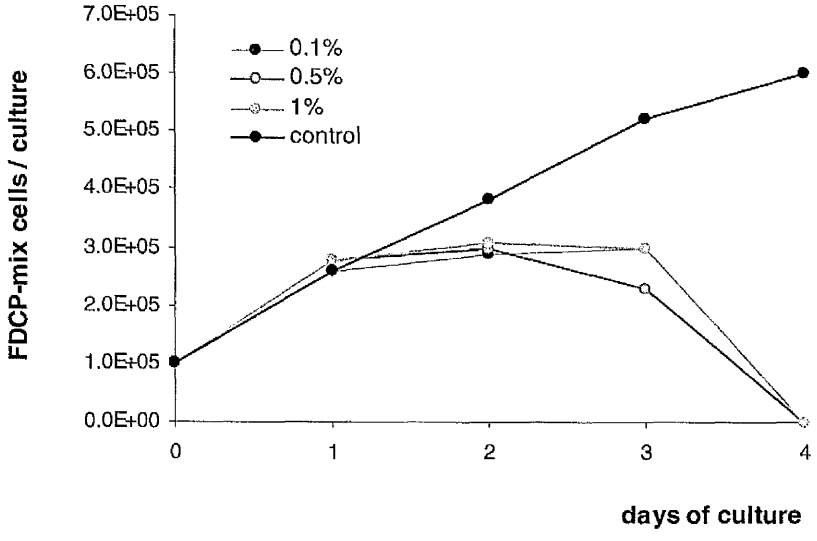


Figure 3.6. Survival of FDCP-mix cells in the presence of XFGF3. FDCP-mix cells were grown in IMDM supplemented with horse serum, BSA, and different concentrations of purified XFGF3 as indicated; 0.1ng/ml of IL-3 was used in the salt control. Each point represent the number of cells assessed by trypan blue for each condition in one experiment.

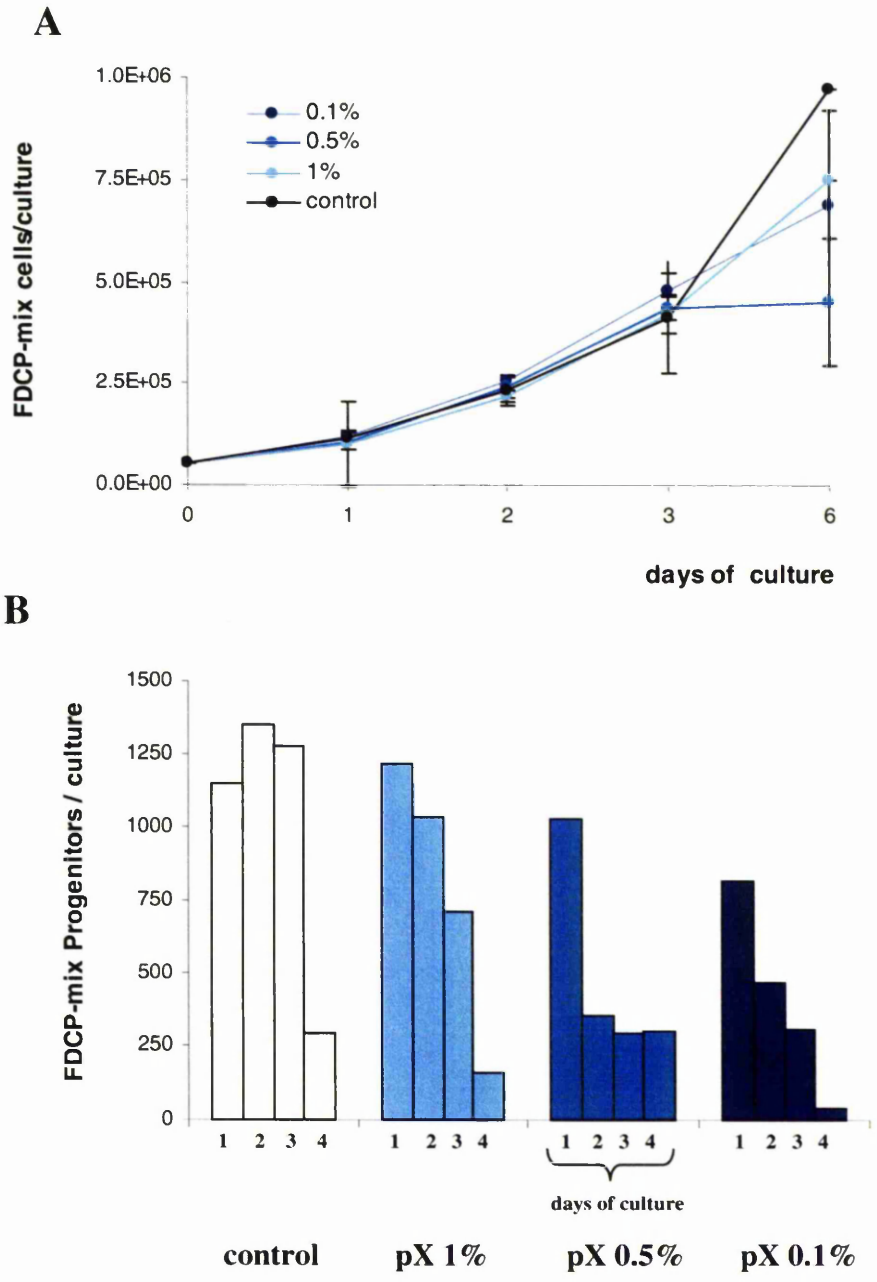


Figure 3.7. Self renewal of FDCP-mix cells in the presence of XFGF3. FDCP-mix cells were incubated with IL-3, GM-CSF, G-CSF and different concentrations of purified XFGF3 as indicated, during 6 days in liquid culture (section 2.1.14). 1M NaCl a 1% (v/v) was used as a control. The cells were counted daily (**A**) and plated out in soft agar colony-forming assays (section 2.1.12.2) (**B**). Each point represent the mean number ± SEM of cells assessed by trypan blue for each condition from 3 experiments. Each bar represents the average from two independent experiments.

justify this conclusion, particularly in consideration of the non-specific effects seen in the control cultures.

3.2.3.3. Granulocyte/Macrophage differentiation

The addition of XFGF3 appeared to have no clear effects either on FDCP-mix cell survival in the absence of IL-3 or on their proliferation in the presence of high IL-3 concentration. It was next decided to test for FGF3 effects on the processes of lineage commitment and differentiation by including it in standard granulocyte/macrophage (section 2.1.15) and erythroid differentiation (section 2.1.16) cultures.

As shown in Figure 3.8-A, the presence of IL-3 and growth factors such as G-CSF and GM-CSF present in the lung conditioned medium (Burgess *et al.*, 1977) stimulated the proliferation of FDCP-mix cells as expected, but the co-addition of XFGF3 had no effect on this proliferation. Proliferation started at day 2, continued up to day 6 and then stopped as the cells matured. Nor was there any clear difference either in the balance of granulocytes to macrophages generated, or the rate at which they appeared (Table 3.1). The cloning efficiency of the cultured FDCP-mix cells induced to undergo granulocyte/macrophage differentiation in the presence of XFGF3 was similar to that observed in the controls. A steady decline in the number of CFCs generated after 3 days of culture was observed, although some colony forming activity remained (Figure 3.8-B), probably as a result of the maturation of the progenitors.

3.2.3.4. Erythroid differentiation

We have also investigated whether XFGF3 could have any influence on FDCP-mix cells induced to undergo erythroid differentiation in the presence of EPO and haemin (section 2.1.16). XFGF3 was added as indicated. Cells were counted daily and cultured in soft gel colony-forming assays as described in section 2.1.12.2. At days 4 and 7, the morphology of the cells grown in these cultures was assessed after O'-Dianisidine and May-Grunwald-Giemsa stained of cytospin preparations (sections 2.1.13). As illustrated in Figure 3.9-A, similar proliferation profiles were again found in all cultures, irrespective of the conditions used. The clonogenic potential of these cells remained relatively high for 3 days, followed by a steady decline, until after 5 days in liquid culture no further colonies were formed when the cells were transferred to soft gel assays (Figure 3.9-B).

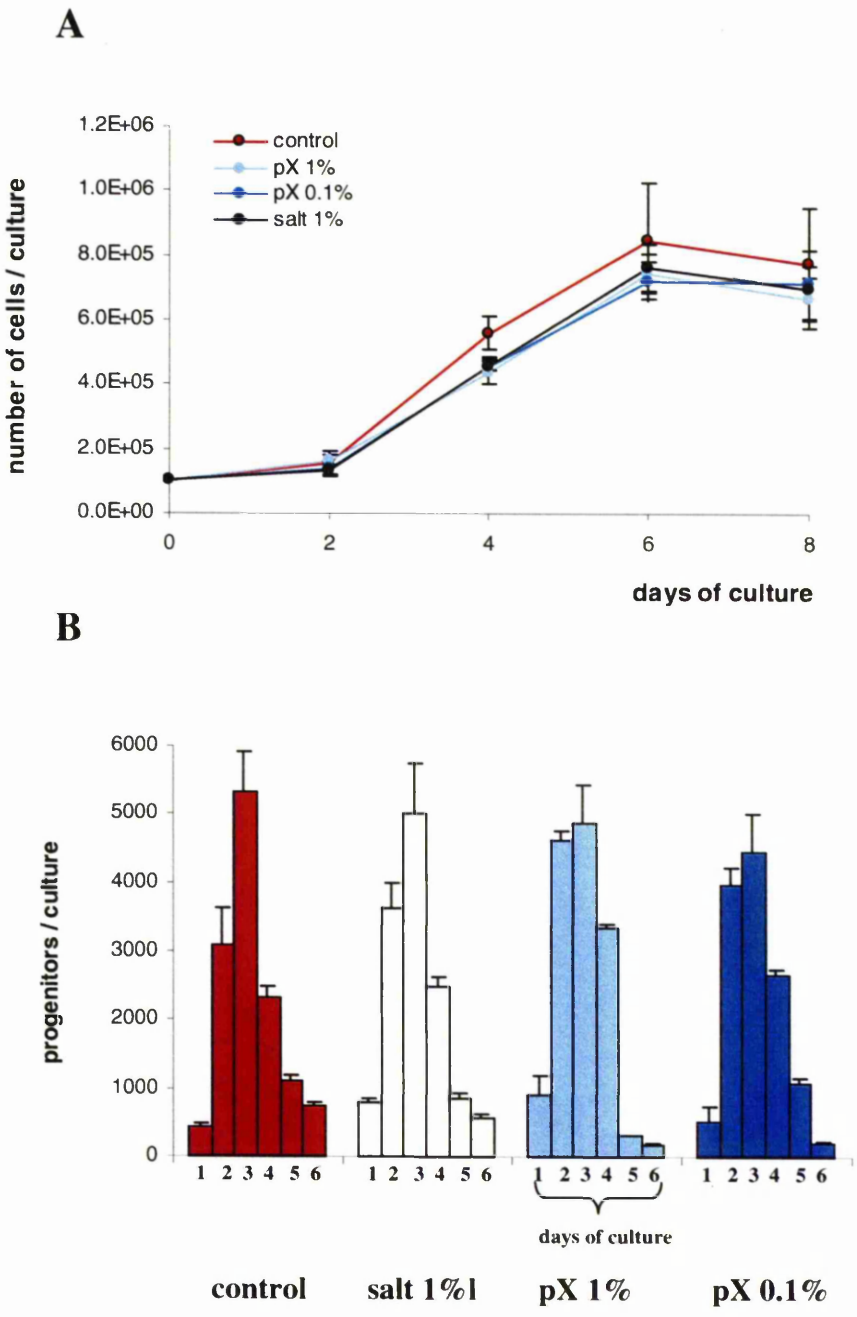


Figure 3.8. Effects of XFGF3 on development of FDCP-mix cells: granulocyte/macrophage differentiation. (**A**) FDCP-mix cells were cultured in conditions to undergo granulocyte-macrophage differentiation (section 2.1.15) and XFGF3 was added as indicated. Total cell numbers for each condition were determined daily by trypan blue exclusion test (section 2.1.10). (**B**) Cells were plated out in clonogenic assay (2.1.12.2) for assessing their colony-forming potential. Each point/bar represents the mean ± SEM of cells/colonies obtained from at least 4 experiments.

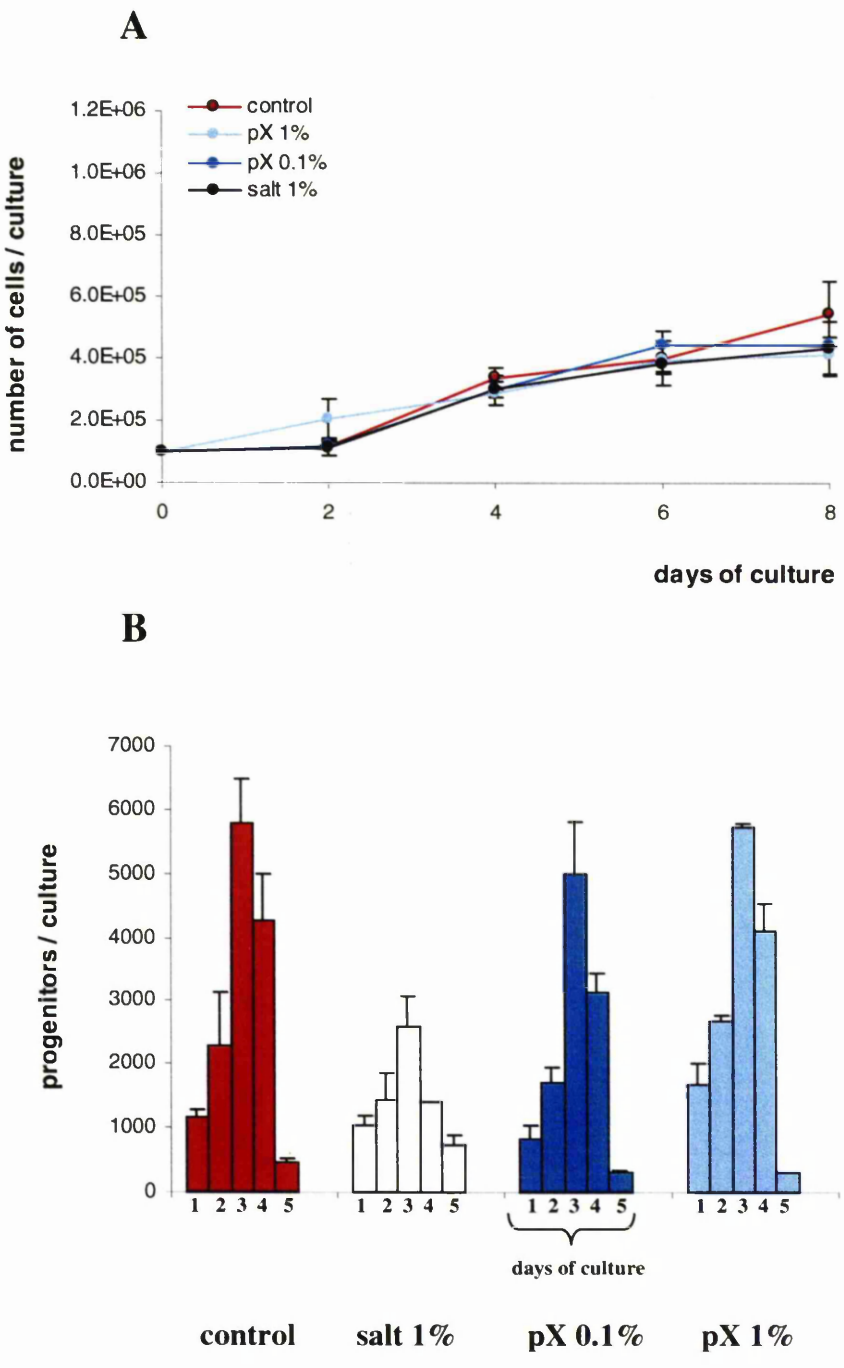


Figure 3.9. Effects of XFGF3 on development of FDCP-mix cells: erythroid differentiation. (**A**) FDCP-mix cells were seeded at 10^5 cells/ml in conditions to undergo erythroid differentiation (section 2.1.16) and XFGF3 was added as indicated. Total cell numbers present in each condition were determined daily. (**B**) Cells were harvested daily and seeded in clonogenic assays to evaluate their progenitor content. Each point/bar represents the mean ± SEM of cells/colonies obtained from at least 4 experiments.

Table 3.1. Morphological development of FDCP-mix cell population induced to granulocyte and macrophage differentiation in the presence of secreted XFGF3.

Day of culture	test condition	Morphology (%)			
		B	EG	LG	M
4	pX 1%	0	72	28	0
	pX 0.1%	0	75	25	0
	salt 1%	0	76	23	1
	control	0	67	33	0
7	pX 1%	0	7.4	85	7.6
	pX 0.1%	0	8	83	9
	salt 1%	0	12	75	13
	control	0	14	77	9

Morphological development of FDCP-mix cells induced to differentiate in medium containing IL-3, GM-CSF, G-CSF, and XFGF3 as indicated (section 2.1.15). Cells were seeded at 10^5 cells/ml and morphology determined at days 4 and 7 of culture after May-Grunwald-Giemsa staining (section 2.1.13.2). Results shown are the means of at least two experiments. B, blast cells; EG, early granulocytes (promyelocytes and myelocytes); LG, late granulocytes (metamyelocytes and mature granulocytes); M, macrophages.

The morphology of the cultured cells at day 4 revealed the presence of a relatively high incidence of late granulocytes in these cultures. By day 7, however, cells of the erythroid lineage dominated all cultures as expected (Table 3.2). No effects of XFGF3 were seen in any parameter analysed.

3.2.4. Effects of XFGF3 on human haemopoietic cells

To investigate the possible direct effects of XFGF3 on primitive human haemopoietic cells, $CD34^+$ cells from cord blood (CB) and bone marrow (BM) were used, and were monitored both for GM-CFC and Erythroid progenitor production in colony-forming assays. Mononuclear cells from CB and BM cells obtained by density

gradient separation were submitted to Mini-MACS® binding system to select the $CD34^+$ population (section 2.1.7.5). Then, the selected cells were cultured in the presence of different concentrations of XFGF3 purified fraction (pX) in the presence of EPO and 5637 conditioned medium (section 2.1.12.1), which has been reported to have GM-CSF, G-CSF, and IL-1 (Coutinho *et al.*, 1993; Myers *et al.*, 1984).

Table 3.2. Morphological development of FDCP-mix cell population induced to erythroid differentiation in the presence of secreted XFGF3.

Day of culture	test condition	Morphology (%)					
		B	EG	LG	M	E	(b^+)
4	pX 1%	1	51	23	4	18	(3)
	pX 0.1%	1	42	32	2	17	(6)
	salt 1%	0	54	29	0	11	(6)
	control	1	56	25	2	13	(3)
7	pX 1%	0	2	16	2	40	(40)
	pX 0.1%	1	13	21	2	28	(35)
	salt 1%	0	2	19	4	46	(29)
	control	0	5	22	5	29	(39)

Morphological development of FDCP-mix cells induced to differentiate in medium containing IL-3, EPO, haemin, and XFGF3 as indicated (section 2.1.16). Cells were seeded at 10^5 cells/ml and morphology determined at days 4 and 7 of culture after O'-dianisidine (section 2.1.13.3) and May-Grunwald-Giemsa (section 2.1.13.2) stainings. Results shown are the mean percentage of at least two experiments. B, primitive blast cells; EG, early granulocytes (promyelocytes and myelocytes); LG, late granulocytes (metamyelocytes and mature granulocytes); M, macrophages; E, erythroblasts; (b^+), percentage of erythroblasts that were benzidine positive.

3.2.4.1. Cord blood $CD34^+$ cells

As illustrated in Figure 3.10, the most noticeable effect on cord blood $CD34^+$ cells was an increase of 15 and 18% in the frequency of erythroid colonies grown in the presence of 0.5 or 0.1%, but not 1%, XFGF3, together with a slight decrease in GM-CFC coloni-

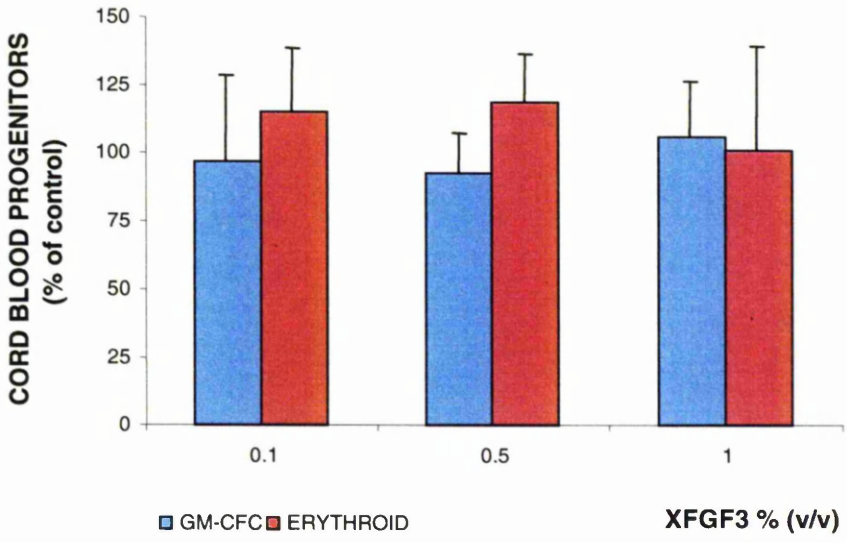


Figure 3.10. Effect of XFGF3 on proliferation of cord blood CD34⁺ cells. CD34⁺ cord blood cells were plated in clonogenic assay section (2.1.12.1) in the presence of XFGF3 as indicated. Results are expressed as the mean percentage ± SEM of the number of colonies obtained from 5 to 7 experiments in proportion to the colony numbers obtained in the 1% 1M NaCl control. Control numbers for colonies ranged from 50 to 127 for GM-CFC, and from 60 to 283 for Erythroid progenitors.

es. Neither effect was significant, however. The size of both the erythroid and GM-CFC colonies in the presence of pX was generally smaller than that of the controls and the BFU-E colonies were also less haemoglobinized when compared with the typical reddish colour of the control colonies.

3.2.4.2. Bone marrow $CD34^+$ cells

Addition of XFGF3 to $CD34^+$ bone marrow cells resulted in a slight increase in the number of GM and erythroid progenitors in all concentrations tested (Figure 3.11), but this effect was not significant when compared to control values. The size and morphology of the colonies grown in the presence of XFGF3 were similar to that of controls. Together, these results showed that XFGF3 had a weak, if any, effect on the clonogenic potential of haemopoietic progenitors derived from cord blood and bone marrow.

3.2.5. Effects of XFGF3 in long-term bone marrow cultures

The results so far showed that XFGF3 had little effect on $CD34^+$ cells from human cord blood or bone marrow in clonogenic assays, while being more effective in stimulating mouse cells, as demonstrated by the ability to improve colony formation by FDCP-mix cells. Haemopoiesis in clonogenic assays is limited to the quantification of primitive progenitor cells present in the starting population that possess the ability to proliferate, differentiate, and develop into phenotypically and functionally mature cells in an environment defined by the presence of growth-promoting substances (Heyworth and Spooncer, 1993).

In the long-term culture system, in contrast, haemopoiesis occurs in the absence of added growth factors and is sustained by the stromal cells, which provide the appropriate environment to promote survival, self-renewal and differentiation of the most primitive cells for several weeks (Dexter *et al.*, 1977). Previous studies have indicated the usefulness of this culture system for investigation of the effects of added haemopoietic regulators on haemopoiesis as they may cause significant changes in the growth of haemopoietic progenitors and mature cells. Therefore, the long-term bone marrow culture (LTBMC) system was used in these studies to investigate the effects of XFGF3 in haemopoiesis *in vitro* using primary human and mouse haemopoietic cells.

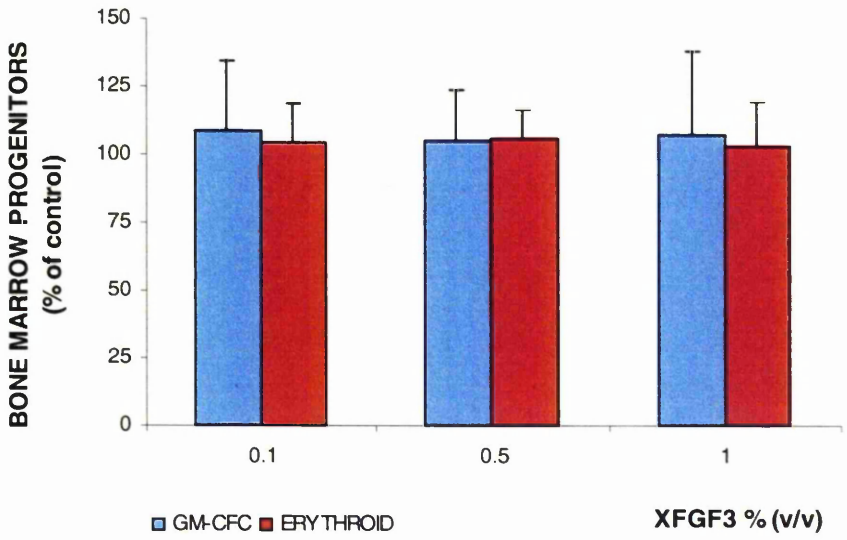


Figure 3.11. Effects of XFGF3 on proliferation of bone marrow CD34⁺ cells. CD34⁺ cord blood cells were plated in clonogenic assay (section 2.1.12.1) in the presence of XFGF3 as indicated. Results are expressed as the mean percentage ± SEM of the number of colonies obtained from 7 to 9 experiments in proportion to the colony numbers obtained in the salt control. Control numbers for colonies ranged from 33 to 178 for GM-CFC, and from 46 to 231 for Erythroid progenitors.

3.2.5.1. Human LTBMC

Bone marrow cells from bone marrow aspirates or rib segments were used for setting up the cultures as described in section 2.1.11. To test for its effects, XFGF3 at 1% (v/v) concentration was added at the beginning of the cultures; 1% (v/v) 1M NaCl solution was used to monitor salt effect. Cultures were fed weekly by replacing 50% of supernatant with fresh medium containing XFGF3 where appropriate. The harvested medium was used to monitor the number of cells and progenitors present in the non adherent fraction.

Development of cultures. The time course of cultures was monitored weekly by phase contrast using an inverted microscope. LTBMC established with BM cells supplemented with 1% (v/v) XFGF3 or 1% (v/v) 1M NaCl had similar growth pattern as the control vessels. By the end of the first week, most flasks contained islands of cells where a scant number of cells resembling fibroblast was attached to the bottom of the flask along with flattened cells. Small and well haemoglobinized clusters of erythroid cells loosely attached to adherent cells and could easily be dislodged by shaking. After the first three weeks in culture, these clusters were no longer found. At the end of week two, the number of cells adhering to the flask had increased, and at the end of week four, confluence of the adherent layer (AL) was achieved with rare patchy areas remaining until the end of the culture period. Also, a small number of cells began to accumulate small droplets of lipid and were located in different regions of the culture flask. Over the subsequent weeks, increasing numbers of lipid-containing cells, or adipocytes, became evident. The stable phase of LTBMC was characterised by an AL with areas of confluent fibroblast-like cells, many lipid-laden cells, and islands of haemopoietic activity (*foci*) (Figure 3.12-A). These *foci* are referred as the areas where progenitors as well as mature cells are generated, and subsequently released into the supernatant (Coutinho *et al.*, 1993). The progenitors could then be recovered as non adherent cells and were detected in clonogenic assays.

Non adherent cells. The production of non adherent cells in the cultures presented two distinct phases. The first phase, between initiation and the first 4 weeks, was marked by a decline in the number of the non-adherent cells probably as result of attachment of cells to the culture flask, feeding-depopulation, and cell death (Figure 3.13-A). In the second phase, there was evidence that proliferation and differentiation were occurring since the number of cells in suspension was kept at reasonable constant levels between each feeding, although half of the non-adherent cell population had been

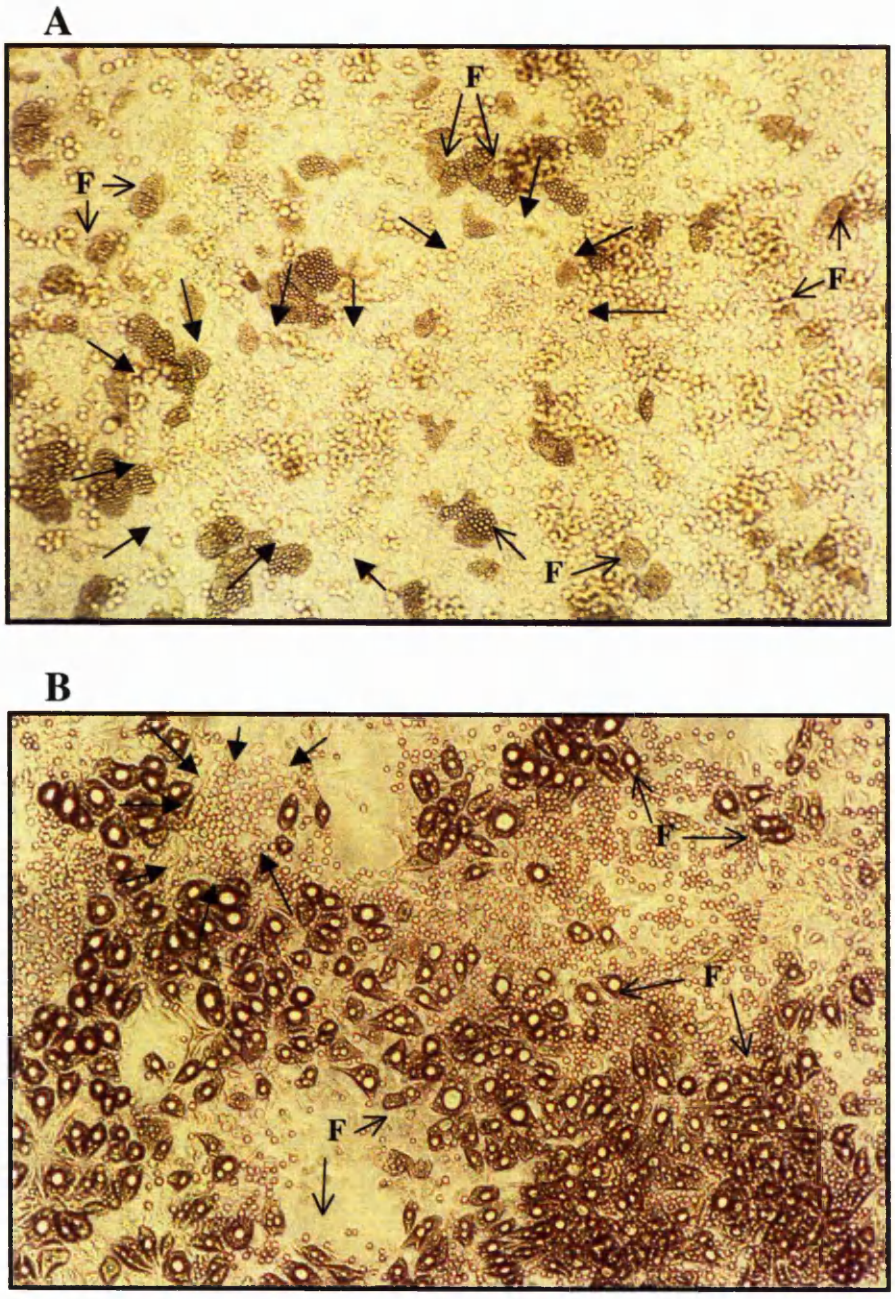


Figure 3.12. Adherent layer of long-term bone marrow cultures. Adherent layers of human (**A**) and mouse (**B**) long-term culture at weeks 8 and 7, respectively, showing areas of active haemopoiesis (cobblestone areas - surrounded by closed arrows) and fat cells (F, open arrows). Mouse adherent layers were patchy and thicker than that observed for human cultures (light microscopy, x80).

weekly removed. The changes in the number of the total cells from the non-adherent fraction observed each week were probably influenced by the release of cells from the AL. As no detachment or disruption of the stromal layer was observed throughout the duration of the experiments, this increased number of cells in the supernatant represented a true proliferation rather than simple detachment of the AL population. As the cultures aged, decline in the number of the non-adherent cells was generally related to the reduction in the proportion of the granulocytes, leaving macrophages as the major component of the cultures as indicated by morphological analysis of the non adherent cells at weeks 4 and 8. The results regarding to the composition of the non-adherent fraction cell population were similar to those previously reported (Coutinho *et al.*, 1993; Gartner and Kaplan, 1980; Moore and Dexter, 1978; Potter *et al.*, 1981).

Non adherent progenitors. The progenitor cells generated by the non-adherent cells removed during the feeding process were detected by colony growth in methylcellulose cultures. The number of progenitors generated weekly had the same profile as the number of harvested cells and, although not statistically significant, an increased number of progenitors was present in the first 5 weeks in the cultures treated with XFGF3 (Figure 3.13-B).

Adherent layer components. The number of adherent cells increased after the establishment of the *plateau* phase, between weeks 4 and 8, as judged by the number of cells released after trypsin treatment at weeks 4 and 8. The results are shown in Table 3.3. The number of total progenitors recovered at week 4 was higher in the cultures in which XFGF3 was present. However, the salt control cultures also had higher number of progenitors, suggesting a salt effect. At week 8, the increase in the number of progenitors present in the cultures treated with XFGF3 was significantly more evident: 7.8- and 2.5-fold increase, respectively for the XFGF3 and salt control cultures, when compared to their values at week 4.

3.2.5.2. Mouse LTBMC

Mouse long-term cultures were established as described in sections 2.1.8 and 2.1.11. The development of the adherent layer and the changes observed during its formation were similar to that just described for human bone marrow cells, with some peculiarities. One of the differences between the two systems was related to the adipocyte formation. In mouse cultures, adipocytes appeared earlier and in higher numbers when compared to human cultures. Also, as the cultures aged, the fat droplets

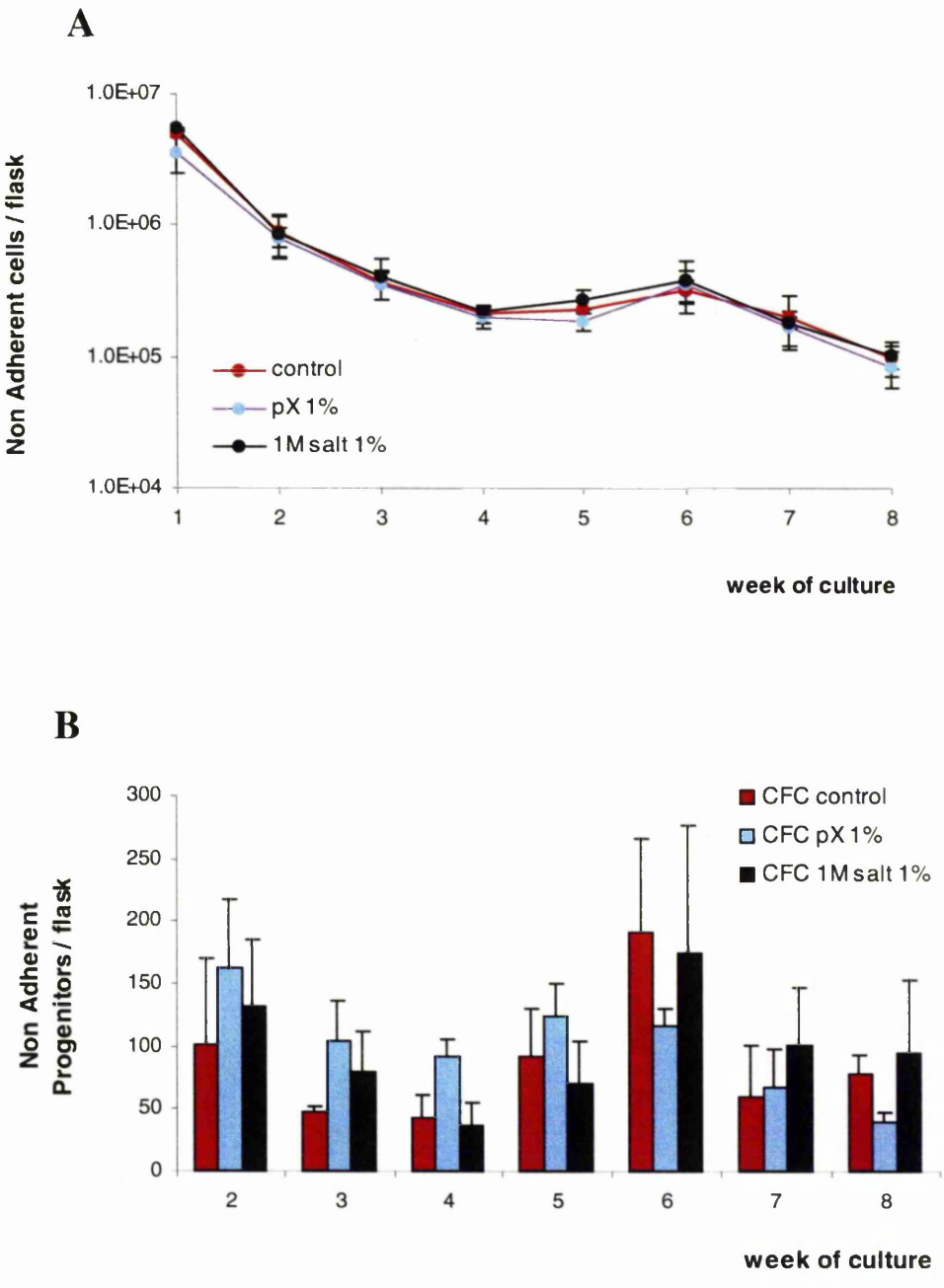


Figure 3.13. Effects of XFGF3 on total cellularity and progenitors from the non adherent fraction generated in human long-term bone marrow cultures. LTBMC was established as described in section 2.1.11, and were treated with 1% (v/v) of purified fraction of XFGF3. (**A**) Non adherent cells recovered at weekly intervals were counted and plated in clonogenic assays for assessing their progenitor content (**B**). Each line/bar represents the mean ± SEM or SD of the number of cells/colonies from 2 to 5 experiments kept under similar conditions.

started to fuse and, by the end of culture period, adipocytes became the predominant stromal cell type observed, sometimes obscuring the cobblestone areas.

Table 3.3. Effects of the purified fraction of XFGF3 on cell content and progenitors generated from the adherent layer of human Long-Term Bone Marrow Cultures.

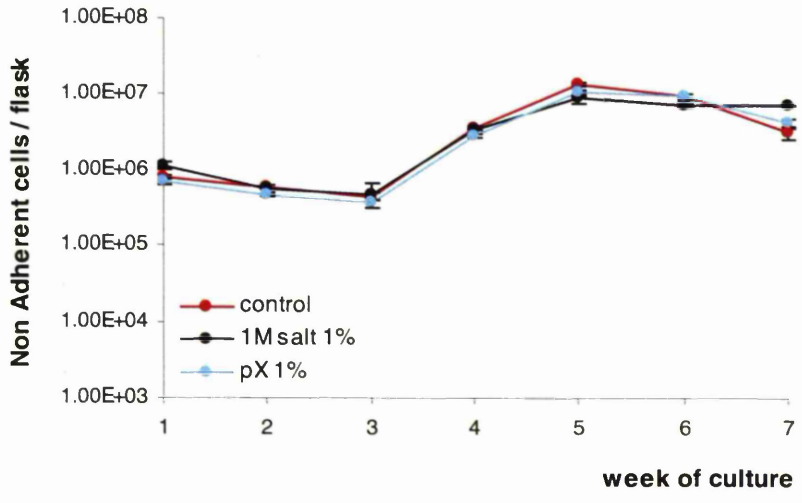
week of culture	culture condition	number of adherent cells / flask ($x10^6$)	Number of progenitors per flask
4	pX 1%	1.53 ± 0.35	4,211 ± 1,213
	salt 1%	1.79 ± 0.71	6,121 ± 5,754
	control	1.72 ± 0.72	1,784 ± 1,446
8	pX 1%	1.88 ± 0.43	32,951 ± 3,545*
	salt 1%	1.88 ± 0.35	15,604 ± 15,099
	control	2.08 ± 0.88	8,406 ± 4,410

Adherent layers of LTBMC were detached by trypsin treatment at weeks 4 and 8 of culture period and their cells counted and analysed for progenitor content in human clonogenic assay (section 2.1.12.1). The values represent the mean ± SEM or SD from 2 to 5 experiments. (*) denotes significant difference in the number of progenitors generated from the adherent layer at week 4 when compared to salt control (p=0.022).

The adherent layer was thicker and not as confluent as observed in human cultures (Figure 3.12-B). Large patchy areas were irregularly present in the culture vessels. The cobblestone areas were also larger than those of human cultures.

As shown in Figure 3.14-A, the number of non adherent cells had a slight decrease during the first three weeks, probably as a result of the formation of the stromal layer, demi-population during feedings, and cell death as suggested for human cultures. Thereafter and up to week 7, a *plateau* of non adherent cell production was reached. Morphological analysis of the non adherent cells at week 7 showed the predominant presence of late granulocytes (71%, 76.7%, and 75.6%, for control, salt 1%, and pX 1%, respectively) followed by macrophages/monocytes (20%, 17.9%, and

A



B

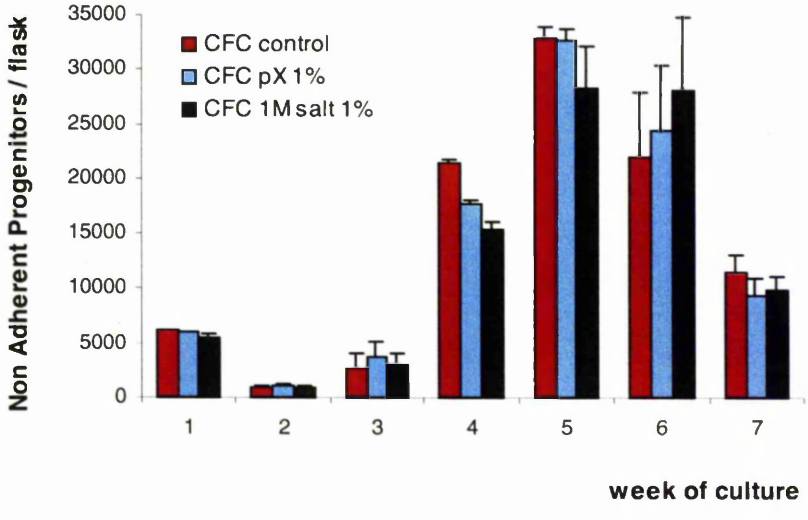


Figure 3.14. Effects of XFGF3 on total cellularity and progenitors from the non adherent fraction generated in mouse long-term bone marrow cultures. LTBMC was established as described in section 2.1.11, and were treated with 1% (v/v) of purified fraction of XFGF3. (**A**) Non adherent cells recovered at weekly intervals were counted and plated in clonogenic assays (**B**) for assessing their progenitor content. Each line/bar represents the mean ± SEM or SD of the number of cells/colonies from 2 to 6 experiments kept under similar conditions.

13.8% for control, salt 1%, and pX 1%, respectively), and early granulocytes (9%, 5.4%, and 10.6%, for control, salt 1%, and pX 1%, respectively). These results are in agreement with the cell types present in a steady state phase of mouse long-term cultures as reported (Spooncer *et al.*, 1993b).

During the *plateau* phase, the number of progenitors harvested from the supernatant increased probably as the result of active haemopoiesis, and their numbers, independent of the culture condition, were comparable. By week 7, the adherent layer started showing signals of detachment. Decrease in the number of progenitors was also observed at that time (Figure 3.14-B). Detachment of adherent layers can be due to contamination, mechanical disruption of the stroma during feedings or overgrowth (Coutinho *et al.*, 1993). In our study, the detachment of the adherent layer was probably due to culture overgrowth. This is supported by the increase in the numbers of cells and, particularly, of the progenitors present in the supernatant observed after the 4^{th} week of culture. The number of cells and progenitors recovered from the adherent layer after terminating the cultures at weeks 4 and 7 are presented in Table 3.4.

Although the number of adherent cells decreased during the duration of culture, active *in vitro* haemopoiesis was taking place as an increase in the progenitor cell population were noted between weeks 4 and 8. The progenitor content recovered from XFGF3 treated cultures was significantly higher than that of the controls at week 4 and remained so until the termination of the experiment.

3.2.5.3. Human LTBMC onto pre-established stroma

Cord blood cannot be established in primary long-term culture because it does not contain sufficient stromal precursor cells to provide the microenvironment necessary to support haemopoiesis. However, when a pre-formed stroma is provided, both progenitor cell production and lifespan of cultures can be significantly enhanced (Hows *et al.*, 1992; Mayani *et al.*, 1998). To further investigate the effects of XFGF3 on cord blood cells, $CD34^+$ cord blood cells selected as described in section 2.1.7.5 were seeded onto pre-formed irradiated bone marrow stroma as described in section 2.1.11.5 and 1% (v/v) of the XFGF3 purified fraction was added. As control, 1M salt at 1% (v/v) was used. As above, the cultures were weekly fed by replacing 50% of supernatant with fresh medium added of the appropriate amount of XFGF3. The cells recovered after feeding were counted and assayed for their progenitor content.

Table 3.4. Effects of the purified fraction of XFGF3 on cellular content and progenitors generated from the adherent layer of mouse Long-Term Bone Marrow Cultures.

week of culture	Culture Condition	number of adherent cells / flask ($x10^6$)	Number of progenitors per flask
4	pX 1%	3.55 ± 0.15	20,683 ± 1,495*
	salt 1%	3.02 ± 0.19	14,394 ± 270
	control	3.07 ± 0.25	11,623 ± 538
7	pX 1%	1.66 ± 0.26	49,224 ± 10,814
	salt 1%	1.93 ± 0.07	28,205 ± 3,095
	control	1.98 ± 0.34	30,690 ± 5,397

Adherent layers of LTBMC were detached by trypsin treatment at weeks 4 and 7 of culture period and their cells counted and analysed for progenitor content in clonogenic assay (section 2.1.12.2). The values represent the mean ± SEM or SD from 2 to 6 experiments. (*) denotes significant difference in the number of progenitors generated from the adherent layer when compared to salt control (p=0.048).

In cultures set up with pre-formed adherent layers derived from normal marrow, continuous growth could be maintained, resulting in the establishment of a *plateau* in cell numbers in these cultures from the 3rd week onwards. The results indicated that haemopoiesis in cultures treated with XFGF3, measured by the level of non adherent (Figure 3.15-A) and progenitor cell (Figure 3.15-B) production *in vitro*, was maintained at close levels to those of control cultures. For the entire duration of experiment, the number of non adherent progenitors recovered at each feeding did not differ among the experimental groups. At week 7, when the cultures were terminated, no progenitors could be recovered from the control cultures, in contrast to the cultures in which XFGF3 and salt had been added (Table 3.5).

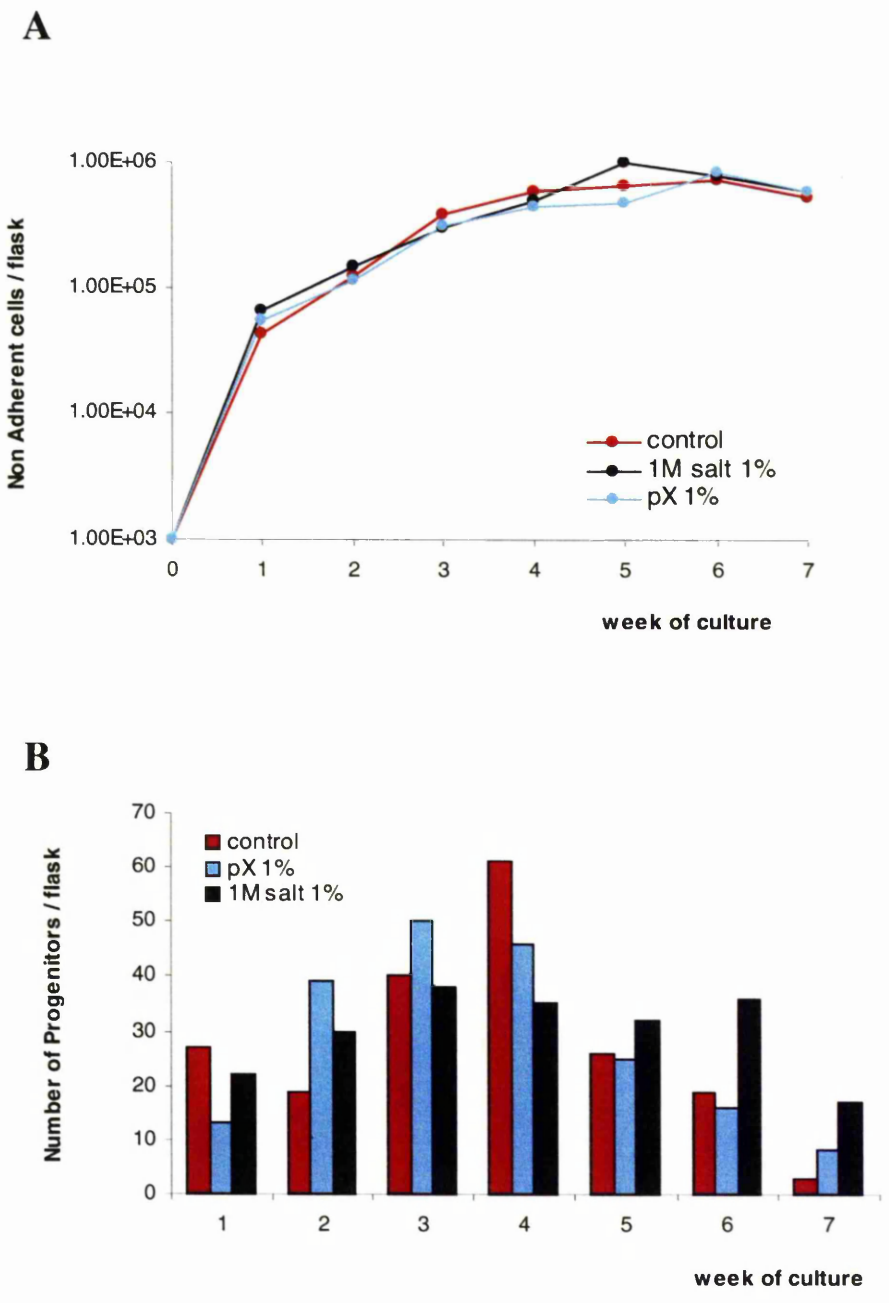


Figure 3.15. Effects of XFGF3 on total cellularity and number of progenitors from the non adherent fraction generated in long-term bone marrow cultures. LTBMC onto irradiated stroma was established as described in section 2.1.11.5, and treated with XFGF3 as indicated. Non adherent cells recovered at weekly intervals were counted (**A**) and plated in clonogenic assays (**B**) for assessing their progenitor content. Each line/bar represents the number of cells/colonies obtained in one experiment.

Table 3.5. Effects of the purified fraction of XFGF3 on cell content and progenitors generated from the adherent layer of human Long-Term Bone Marrow Cultures established onto irradiated pre-formed stroma.

week of culture	culture condition	number of adherent cells / flask ($x10^6$)	Number of progenitors per flask	
			GM-CFC	Erythroid
3	pX 1%	1.45	406	346
	salt 1%	1.46	448	594
	control	0.83	89	198
7	pX 1%	1.24	21	0
	salt 1%	2.98	50	0
	control	1.28	0	0

Adherent layers of LTBMC were detached by trypsin treatment at weeks 3 and 7 of culture period and their cells counted and analysed for progenitor content in clonogenic assay (section 2.1.12.1). Values are from 1 experiment.

3.3. Discussion

FGF3 is a time-regulated protein which expression during embryonic development coincides with the establishment of haemopoiesis. Previous work with FGF3 (-/-) knockout mice has shown that the E14 and E15 null mutants had small livers and were particularly anaemic (Cross *et al.*, 1997). This phenotype could be explained either by a requirement for FGF3 on behalf of the haemopoietic cells in order to establish efficient haemopoiesis, or by a requirement for FGF3 in the stroma, in order to establish a supportive environment. The other possibility is that FGF3 produced by haemopoietic stem cells may have an inductive role in establishing supportive stroma. These properties need not be mutually exclusive. Subsequently, Northern blot analysis of murine FDCP-mix cells has shown expression of FGF3 specifically in the undifferentiated state. Since FDCP-mix cells constitute an evidently heterogeneous population, of which only about 5-10% has clonogenic potential, it was considered that the low levels of FGF3 expression could indicate signalling between cells at different stages of maturation. The dual signalling phenomenon whereby FGF3 mRNA encodes

both a secreted protein (signalling to neighbouring cells) and a nuclear protein (intrinsic signalling) could be a way of establishing identity in this way.

In order to explore these possibilities, purified XFGF3 was added to murine and human haemopoietic cells under a range of standard culture conditions. However, no strong or reproducible effects were observed on the parameters of survival, proliferation, self-renewal, commitment or differentiation, although in some cases there were tendencies towards potentially interesting effects. For instance, inclusion of XFGF3 in the semi-solid medium of EPO-containing colony forming assays of both FDCP-mix (Figure 3.5) and primary cord blood progenitors (Figure 3.10) appeared to increase colony formation frequency. The effect on FDCP-mix cells even reached statistical significance. In contrast, the inclusion of XFGF3 in standard FDCP-mix self-renewal culture appeared to result in a rapid decrease in colony forming efficiency (Figure 3.7). While it is possible that these effects are real, and are due to differences in the culture conditions (semi-solid medium and low IL-3 concentration in the first case; liquid medium and high IL-3 concentration in the second case), the fact that the salt control often showed effects similar to those of XFGF3 fraction raises doubts about the results.

The addition of 1% (v/v) of 1M NaCl is equivalent to an increase in about 10% in the total NaCl in IMDM medium, and a much lower percentage of the total salt. It was originally considered that this was unlikely to affect *in vitro* haemopoiesis on its own. The results, however, suggest otherwise, with the salt control occasionally showing marked effects, probably as a result of the alteration of the physical-chemical conditions of the system. Relevant to this is the report in which the presence of 1M salt concentration in binding assays dramatically decreased the binding of bFGF to its receptors in HepG2 cells and that the ligand-binding activity could be restored after exposure of the receptors to heparin (Kan *et al.*, 1993). Moreover, Swiss 3T3 cells bFGF-mediated growth was inhibited by chlorate and this activity could be reversed by the addition of heparin (Rapraeger *et al.*, 1991). In addition, it has been demonstrated that free heparin and heparin sulphate added to culture conditions can function as a low affinity receptor that is, in turn, required for the high affinity binding of bFGF (Yayon *et al.*, 1991). In support of this, it has been shown that FDC-P1 mouse myeloid progenitor cell line, which lacks FGF receptors and depends on IL-3 or GM-CSF for proliferation and survival, when induced for expressing FGFR-1 binds aFGF and bFGF with high affinity only in the presence of heparin (Bernard *et al.*, 1991). Moreover, the presence of

high affinity receptors for bFGF on K562 and U937 cell lines could be demonstrated only when heparin was present (Allouche *et al.*, 1995). In our experiments, we have used heparin in two occasions: first, with the purpose of displacing XFGF3 into the conditioned medium after transfecting COS-7 cells with cDNA XFGF3 plasmid; secondly, when we tested the biological activities of XFGF3 on NIH-3T3 cells (sections 3.2.2.1 and 3.2.2.2). Therefore, the presence of salt and the absence of heparin in our experimental conditions could have interfered with the binding of FGF3 to its receptor(s) on tested cells, affecting the results. It is feasible that a FGF3 response may be amplified by the co-addition of a more purified salt-free form of FGF3 and extra heparin in the experiments performed.

One of the strongest and potentially most interesting results from this series of experiments was the increase in the number of progenitors recovered from the adherent layer from both mouse and human LTBMC. In murine cultures, the salt control showed a similar (although weaker) tendency, but in the human cultures the increase in the number of progenitors in the adherent layer appeared specific to the cultures treated with XFGF3. Moreover, this effect was more evident in cultures where XFGF3 was added before the formation of the stromal layer. The osmolarities of the harvested media in this case were 323.6±1.2, 348.4±2.6 and 352.0±1.5 for the control, XFGF3- and salt-treated cultures, respectively, suggesting that the presence of the salt was not interfering with the performance of the cultures. Taken together, these observations would be consistent with XFGF3 having a supportive effect on haemopoiesis in LTBMC *via* stimulation of stromal function. In line with such hypothesis, bFGF has been described as a potent mitogen for human bone marrow stromal cells and also as a stimulator of myelopoiesis *in vitro* (Oliver *et al.*, 1990; Wilson *et al.*, 1991). It has been suggested that these effects are the result of bFGF-induced production of secondary cytokines by stromal cells such as M-CSF (Abboud and Pinzani, 1991), IL-1, G-CSF and GM-CSF (Hamilton *et al.*, 1992), which would in turn improve myelopoiesis. Alternatively, bFGF has been suggested to suppress the action of growth inhibitors such as TGF-β (Le Bousse Kerdiles *et al.*, 1996; Wilson *et al.*, 1991). Similar results using FGF4 have been reported (Quito *et al.*, 1996). Therefore, it is possible that FGF3 may modulate haemopoiesis acting on stromal cells through a complex mechanism involving regulation of proteins with both proliferative and inhibitory effects as suggested for other members of the FGF family.

The fact that addition of FGF3 to LTBMC could stimulate haemopoiesis also suggests the presence of FGF-Rs on haemopoietic progenitors and/or stromal cells. Basic FGF and FGF3 are both ligands for FGFR-1 and FGFR-2 (Allouche and Bikfalvi, 1995; Mathieu *et al.*, 1995b). Using Northern blot and RT-PCR, FGFR-1 and FGFR-2 have been detected on murine bone marrow cells and on murine platelets, macrophages, granulocytes, T and B lymphocytes (Bikfalvi *et al.*, 1992). Human leukaemic cell lines have also shown to express the FGF-R 1, 2, 3, and 4 receptors (Allouche *et al.*, 1995; Armstrong *et al.*, 1992; Bikfalvi *et al.*, 1992). Three soluble forms of FGF-R proteins binding to bFGF and aFGF were detected in human serum and plasma (Hannenken *et al.*, 1994). Expression of FGFRs mRNA and protein level using RT-PCR and flow cytometry, respectively, could be demonstrated on sub populations of human peripheral blood MNC and on cells derived from day-6 GM-CFC and CFU-E colonies (Ratajczak *et al.*, 1996). In contrast, the expression of FGFRs on haemopoietic $CD34^+$ cells is not clear. Le Bousse Kerdiles and colleagues have reported low expression of FGFR-1 and FGFR-2 detected by PCR and immunocytochemistry in normal bone marrow and peripheral blood $CD34^+$ cells (Le Bousse Kerdiles *et al.*, 1996), while no cell surface receptors for both FGF1 and FGF2 could be detected by flow cytometry by other two independent groups (Berardi *et al.*, 1995a; Ratajczak *et al.*, 1996). These data collectively indicate a mediated effect for FGF2 that appears to be more restricted to lineage-committed progenitors and mature cells, and may explain the marginal effects of XFGF3 observed in our clonogenic assays when we tested its direct effect on $CD34^+$ from cord blood and bone marrow.

On the other hand, FDCP-mix cells responded to XFGF3 as a significant increase in the number of progenitors in clonogenic assays was observed (Figure 3.5). There are no reports concerning FGF receptor expression on FDCP-mix cells. So, it is uncertain whether any of the cells in an undifferentiated FDCP-mix population has the capacity to respond through the known FGF receptors. Therefore, a more detailed investigation of the expression of FGF3 receptors on primitive haemopoietic cells and on FDCP-mix cells, which have already shown the presence of mRNA for FGF3, would be of interest to clarify this issue and may provide new insight into the potential role of FGF3 in influencing the behaviour of these cells.

Important of note is that XFGF3 has shown to be mitogenic (Figure 3.4) and a transforming agent (Figure 3.5) for NIH-3T3 cells. These cells are derived from mouse embryos and have shown to express both FGFR-1 and FGFR-2 (Goldfarb *et al.*, 1991;

Mathieu *et al.*, 1995a). As FGF3 is a time-regulated protein expressed during embryonic development, it may interact with highly specific receptors that are abundantly expressed in embryonic lineages. Embryonal stem cells (ES) are totipotent, can transmit genes through the germ line and have recently been shown the ability to generate all the mature components of the lymphohaemopoietic system under appropriate stimuli (reviewed in Hole and Graham, 1997). This suggest that at some point between being an ES with no direct haemopoietic potential and becoming a differentiated embryonal body containing a range of mature haemopoietic lineages, some of the ES within the embryonal body must commit to the haemopoietic system, *i.e* they become haemopoietic stem cells. Therefore, ES differentiation system represents an attractive model of haemopoietic stem cell differentiation to test the ability of FGF3 in modulating haemopoiesis.

Finally, it should be borne in mind that the addition of FGF3 to the growth medium as reported here does not address the possible role of the protein directed to the nucleus, and that any contribution of this form of FGF3 to haemopoiesis would not be revealed by this approach.

Chapter 4

Effects of the overexpression of FGF3 in FDCP-mix cells

4.1. Introduction

The studies reported in the previous chapter investigated the effects of the secreted form of FGF3. However, the mammalian FGF3 genes, in addition to a conserved AUG initiation codon, have an in-frame CUG codon positioned 87 (mouse) (Figure 4.1-A) or 78 (human) nucleotides upstream (Acland *et al.*, 1990). By mutating these codons and the adjacent nucleotides in the mouse gene, it has been shown that AUG-initiated products are found predominantly in the endoplasmic reticulum and Golgi apparatus, while CUG-initiated products are located extensively in the nucleus (Acland *et al.*, 1990). This suggests a dual function for FGF3 – on the one hand, as a secreted, paracrine growth factor signalling to neighbouring cells *via* interaction with membrane bound receptors, and on the other hand, as a nuclear protein with autocrine function.

Since the addition of the secreted recombinant FGF3 to the culture medium as reported in Chapter 3 only tests for possible paracrine functions, it was decided to take a separate approach: the manipulation of FGF3 expression in FDCP-mix system that should reveal also any effects due to endocrine function. Therefore, derivatives of FGF3 cDNA engineered to produce either the secreted, the nuclear, or both forms of the protein were incorporated into plasmid expression vectors designed to direct co-expression of FGF3 protein with GFP, thus allowing purification and assay specifically those cells that had taken up and expressed transfected DNA.

4.2. Results

4.2.1. Expression plasmids construction

FGF3 cDNA derivatives optimised for secretion, nuclear targeting or both were a kind gift of Dr. Clive Dickson, ICRF, London. The pKC 3.2 vector lacks the upstream CUG translation start site of the wild-type gene (Figure 4.1-A), has the sequences surrounding the ATG initiation codon converted to the Kozak consensus optimised for translation initiation (Kozak *et al.*, 1987), and carries a mutation in an out-of-frame ATG in the -8 to -6 position (Figure 4.1-B). The product of pKC 3.2 lacks an effective nuclear localisation signal and is secreted (Dixon *et al.*, 1989; Smith *et al.*, 1988). The pKC 4.12 is optimised for initiation at the upstream CUG by mutation of this sequence to an ATG embedded in an optimised Kozak sequence (Figure 4.1-C). Mutation of the downstream ATG ensures that pKC 4.12 can only produce full-length protein, which segregates between secreted and nuclear fates. The pKC 4.16 is similar to pKC 4.12, but carries a deletion that removes the signal sequence necessary for effective targeting to the endoplasmic reticulum (Figure 4.1-D). The product of this plasmid is directly solely towards the nucleus (Acland *et al.*, 1990; Kiefer *et al.*, 1994).

Schematic illustration of the steps taken to construct the FGF3 expression plasmids GFP-3.2, GFP-4.12 and GFP 4.16 as well as the gels showing the restriction fragments obtained during their construction are sequentially detailed and presented in Figures 4.3 to 4.7. Briefly, the FGF3 coding sequences from pKC 3.2 were excised on a *Bgl II – Bgl II* fragment (Figure 4.3), blunt-ended, and ligated *via Spe I* linkers (section 2.2.4.3) into the *Spe I* site of the expression vector pCMVExpEGFP (section 2.2.4.2, Figure 4.2) (McIvor *et al.*, 2000) (Figure 4.4). In order to preserve the upstream ATG in both of the pKC 4.12 and pKC 4.16 derivatives, the cDNA fragment were excised with *Sph I* (blunt-ended) and *Nco I*, then inserted between the *Eco RI* (blunt-ended) and *Nco I* sites of plasmid pRSET B (section 2.2.4.1) (Figure 4.5). This permitted re-excision of the fragments on *Xho I – Hind III* fragments that were then *Spe I* linkered and inserted into the *Spe I* site of pCMVExpEGFP as above (Figures 4.6 and 4.7). The identity of all constructs was confirmed by restriction digestion on miniprep DNA (section 2.2.5.2), and the presence of the appropriate FGF3 derivatives in the final constructs was confirmed by sequencing the FGF3/vector junctions (section 2.2.4.4). The size of the several restriction fragments expected at each step followed during the construction of the FGF3 plasmids is presented in Table 4.1.

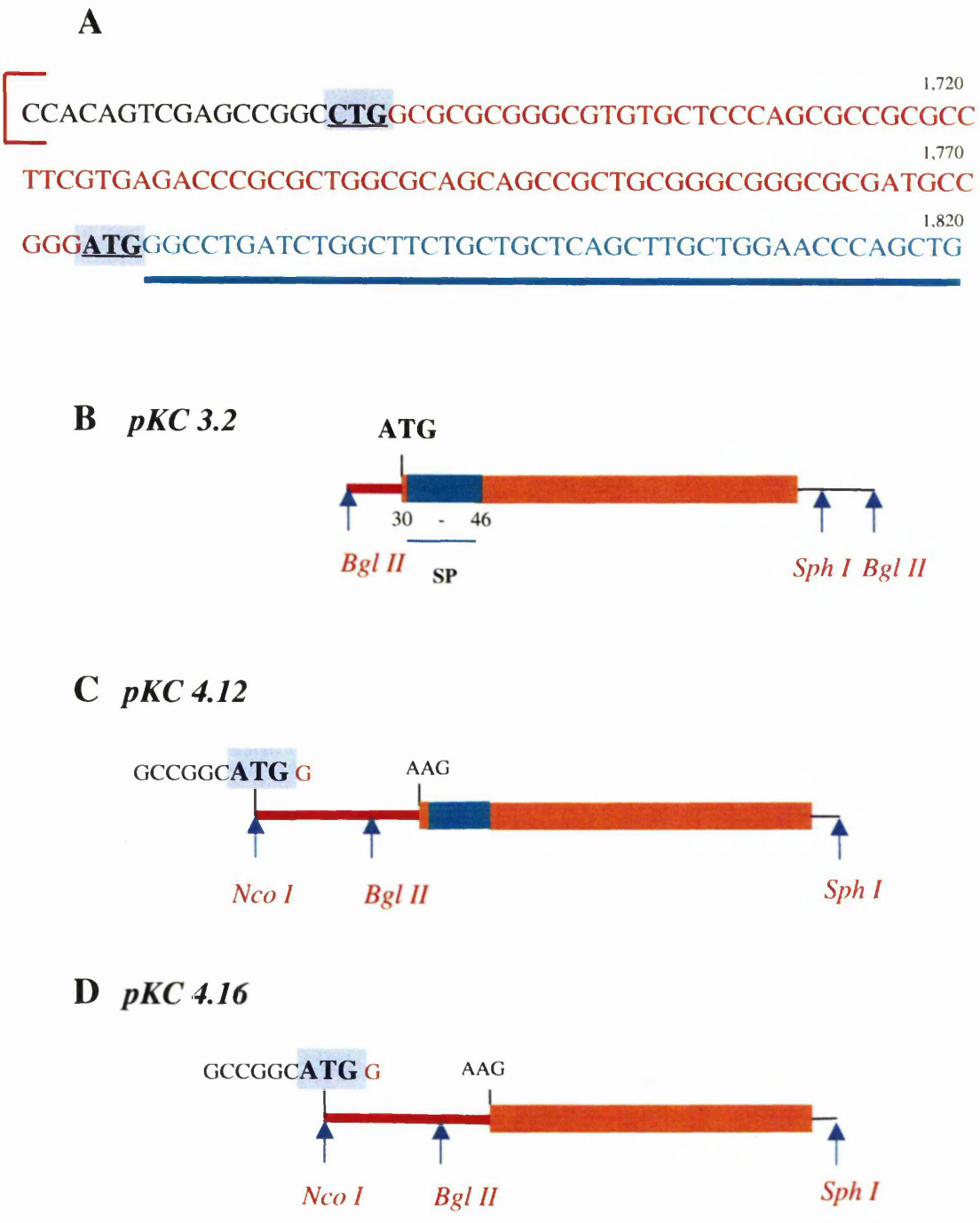


Figure 4.1. Schematic representation of the structure of the three cDNA used in the construction of FGF3-GFP expressing plasmids. (A) The sequence of mouse *int-2* cDNA is shown beginning at the 5' end of exon Ib, indicated by the open box, and extending to nucleotide 1,820. The green line constitute the signal peptide sequence predicted for *int-2* protein, assuming ATG-directed initiation. Orange boxes indicate sequences encoding FGF3 and light green boxes indicate sequences of the signal peptide. (**B**) pKC 3.2; (**C**) pKC 4.12, in which the upstream CTG has been mutated to ATG for optimal translation while the downstream ATG triplet has been mutated to AAG; (**D**) pKC 4.16, in which the core (residues 30 to 46) of the signal peptide has been deleted (adapted from Acland *et al.*, 1990 and Kiefer *et al.*, 1994).

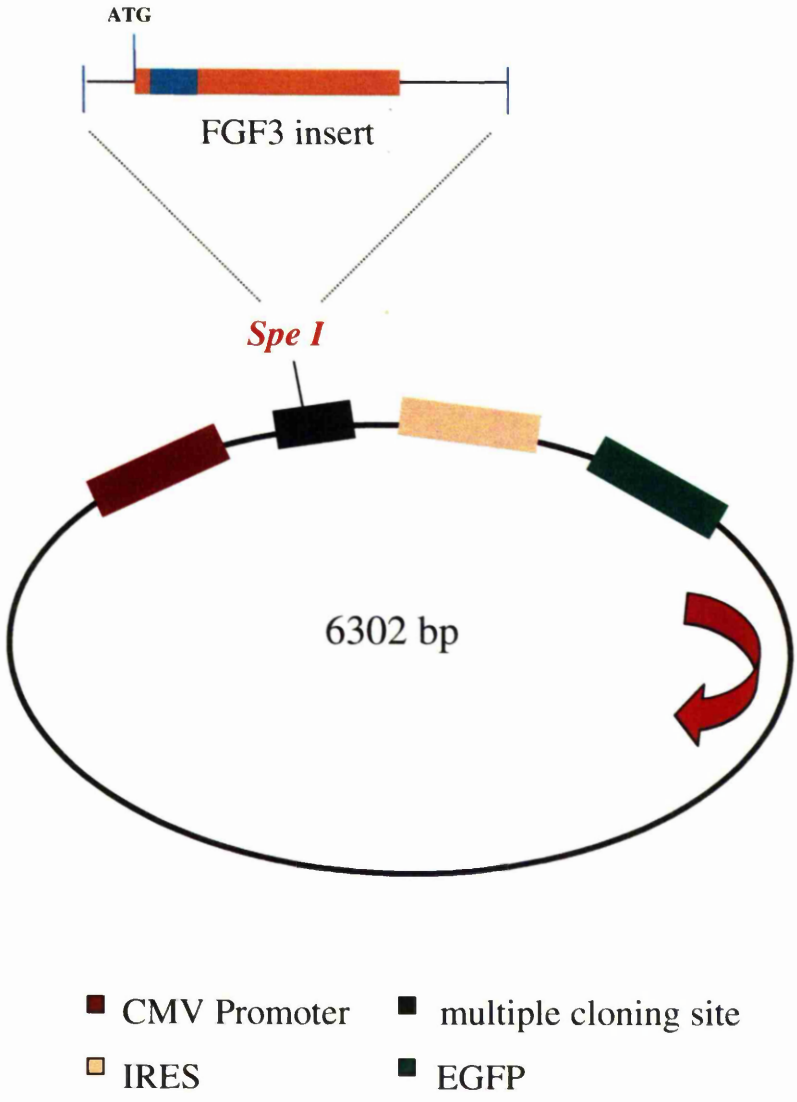


Figure 4.2. CMVExpEGFP vector. The diagram illustrates the structure of the vector used for insertion of the FGF3 inserts at the *Spe I* restriction site. CMV, cytomegalovirus; IRES, internal ribosome binding site; EGFP, enhanced Green Fluorescent Protein. (Adapted from McIvor *et al.*, 2000).

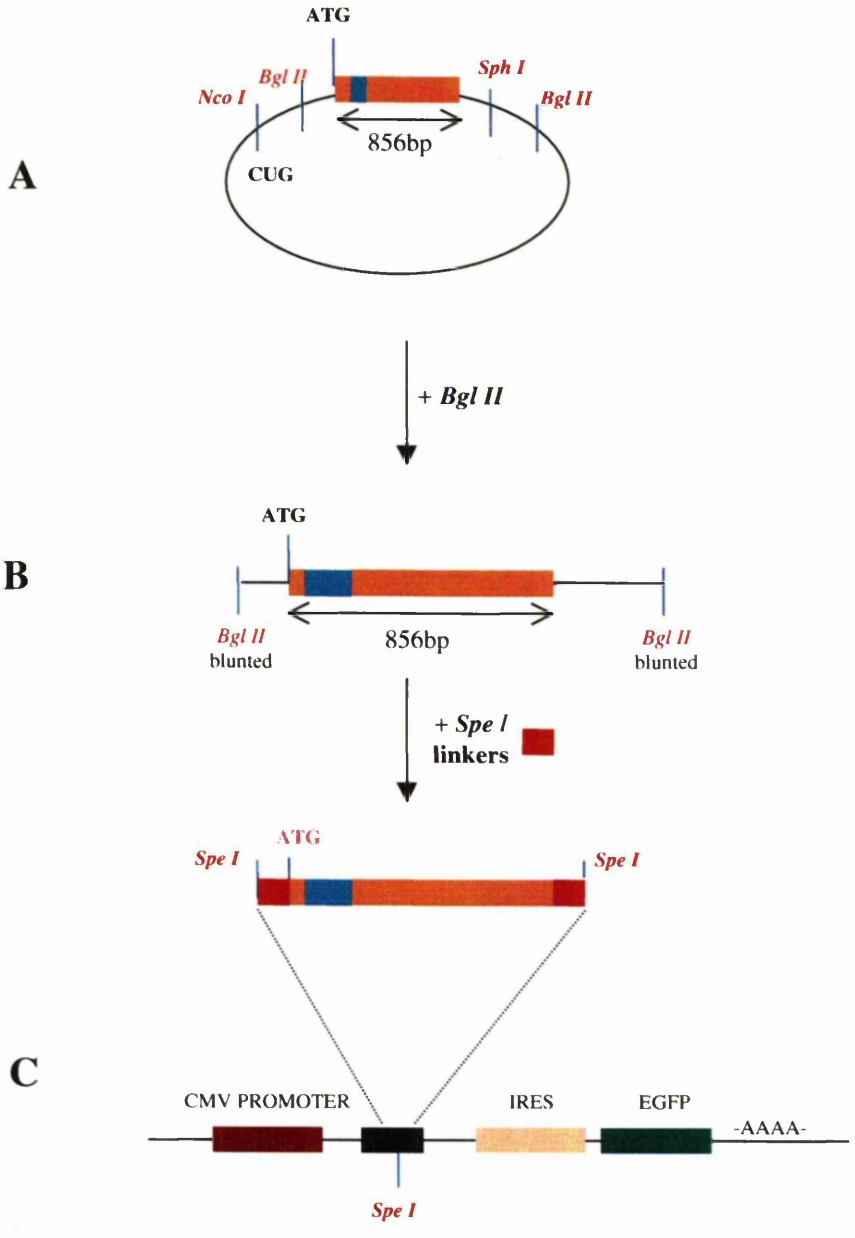


Figure 4.3. Design of the GFP-3.2 construct. The diagrams illustrate the design of GFP-3.2 construct. Only the restriction sites that are relevant to the cloning strategy described in the text have been included. The FGF3 cDNA fragment was excised from the pKC 3.2 plasmid and inserted into the *Spe I* restriction site of CMVExpEGFP vector, generating the GFP-3.2 vector. (**A**) pKC 3.2 plasmid; (**B**) FGF3 insert excised from pKC 3.2 after digestion with *Bgl II*; (**C**), CMVExpEGFP vector.

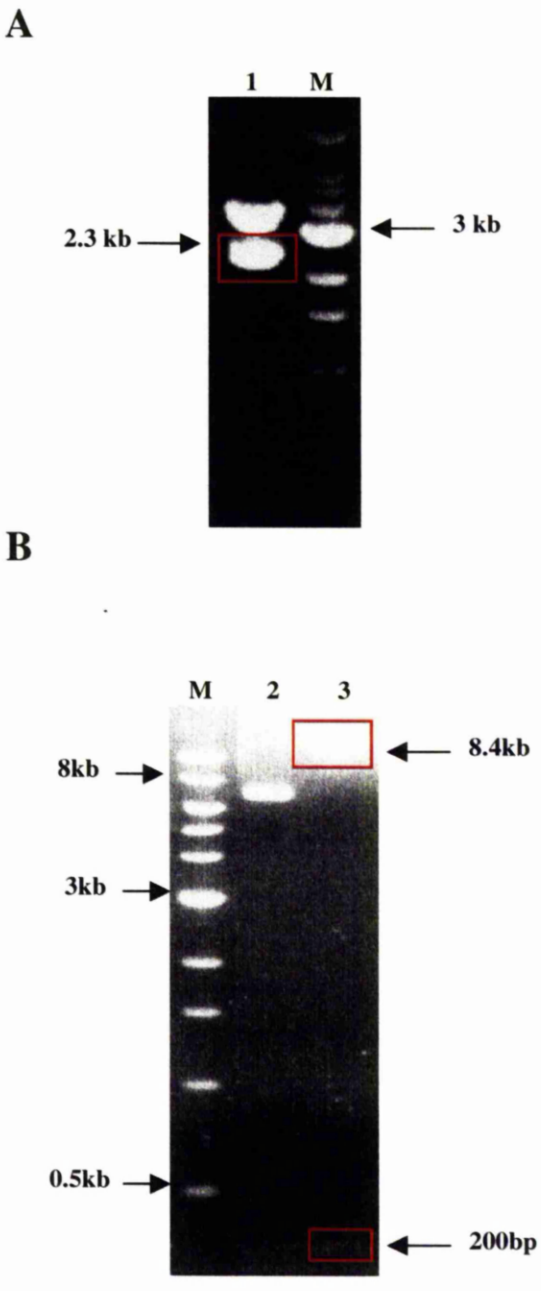


Figure 4.4. GFP-3.2 plasmid. (**A**) The pKC 3.2 plasmid was digested with *Bgl II* restriction enzyme and the 2.3kb FGF3 cDNA fragment (lane 1) was inserted into CMVExpEGFP vector, resulting in the GFP-3.2 plasmid. (**B**) Insert sense orientation was checked by digesting the plasmid with *Not I*, yielding the 200 and 8440bp fragments (lane 3). Lane 2 shows the uncut plasmid. Some of the sizes of the molecular weight marker (M) have been included.

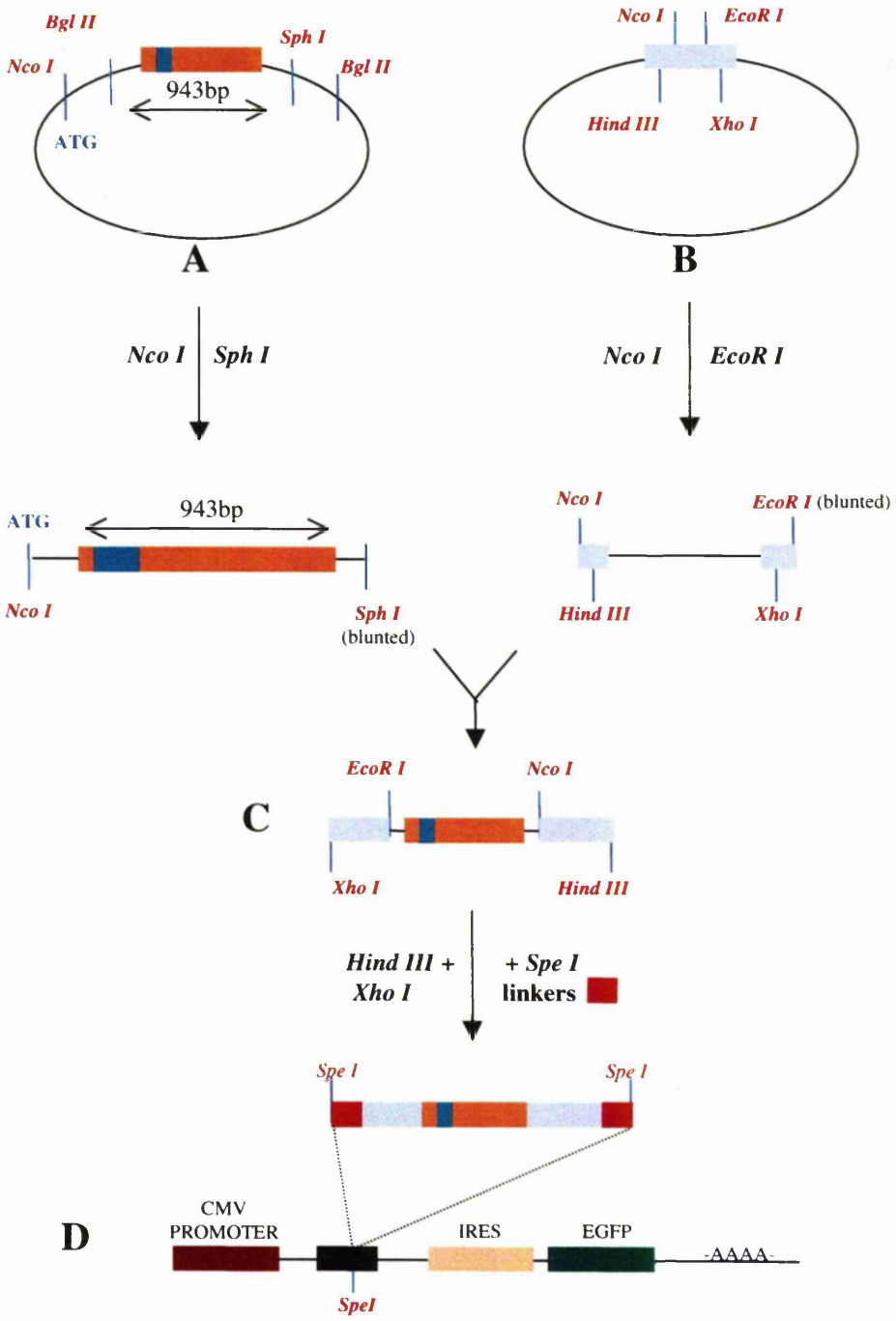


Figure 4.5. Design of GFP-4.12 construct. The diagrams detail the steps followed to obtain the GFP-4.12 construct. Only the restriction sites that are relevant to the cloning strategy described in the text have been included. The FGF3 cDNA was excised from pKC4.12 and linked to the CMVExpEGFP vector using the pRSET B vector as a shuttle vector. (**A**) pKC 4.12 plasmid; (**B**) pRSET B vector; (**C**) FGF3 fragment inserted into *Eco RI* and *Nco I* restriction sites of the pRSET B vector; (**D**) CMVExpEGFP vector. The GFP-4.16 vector was constructed as described for GFP-4.12.

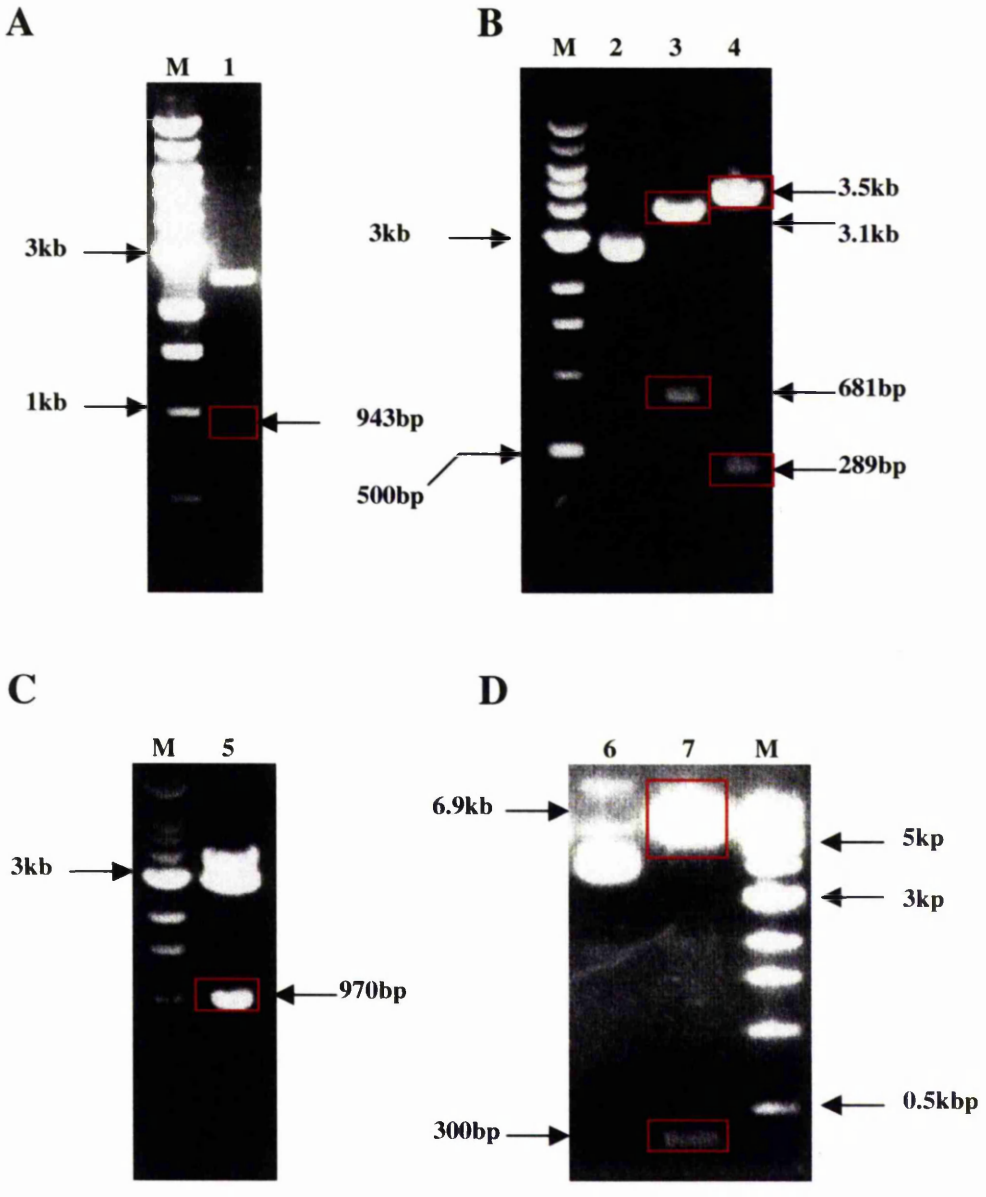


Figure 4.6. GFP-4.12 plasmid. (**A**) The pKC 4.12 plasmid was digested with *Sph I* and *Nco I* restriction enzymes and the 943bp FGF3 cDNA fragment (lane 1) was inserted into pRSET B shuttle vector. (**B**) Sense orientation of the insert was confirmed by digestion with *Hind III/Not I* (lane 2, uncut plasmid; lane 3, 681 and 3170bp fragments) and *Xho I/Not I* (lane 4, 289 and 3562bp). (**C**) The FGF3 cDNA 970bp fragment obtained after digestion with *Hind III* and *Xho I* restriction enzymes (lane 5) was excised from the shuttle vector and inserted into CMVExpEGFP vector, resulting in the GFP-4.12 plasmid. (**D**) Insert sense orientation was confirmed after digestion with *Not I* (lane 6, uncut plasmid; lane 7, 300 and 6995bp fragments). Some of the sizes of the molecular weight marker (M) have been included.

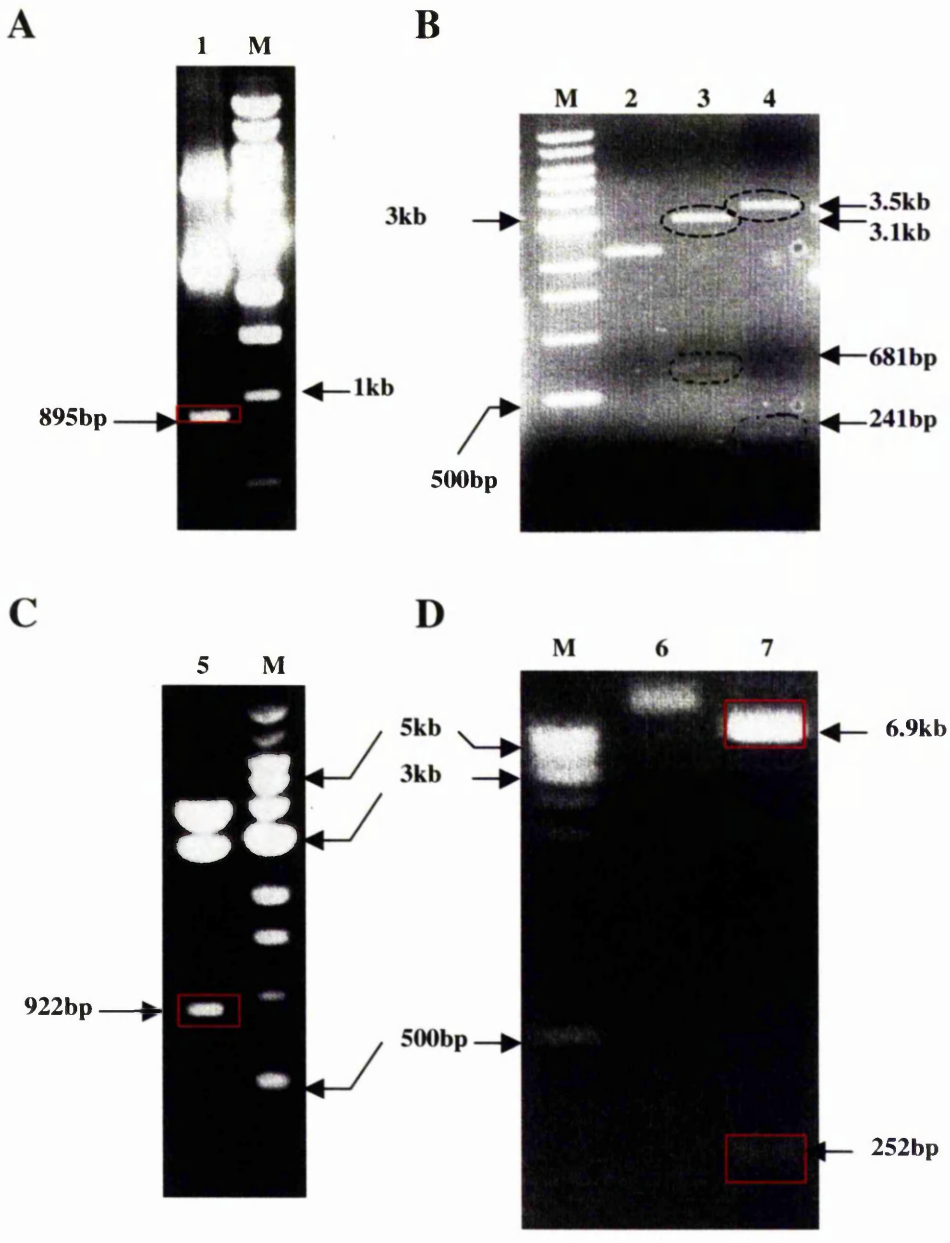


Figure 4.7. GFP-4.16 plasmid. (**A**) The pKC 4.16 plasmid was digested with *Sph I* and *Nco I* restriction enzymes and the 895bp FGF3 cDNA fragment (lane 1) was inserted into pRSET B shuttle vector. (**B**) Sense orientation of the insert was checked by double digestion with *Hind III/Not I* (lane 3, 681 and 3122bp) and *Xho I/Not I* (lane 4, 241 and 3562bp) restriction enzymes. (**C**) The 922bp FGF3 cDNA fragment excised from the shuttle vector after digestion with *Hind III* and *Xho I* (lane 5) was inserted into CMVExpEGFP vector, resulting in the GFP-4.12 plasmid. Insert sense orientation was confirmed after digestion with *Not I* (lane 7, 252 and 6995bp fragments). Lanes 2 and 6 represent uncut plasmids. Some of the sizes of the molecular weight marker (M) have been included.

Table 4.1. Expected sizes of mouse FGF3 constructs according to sense and anti-sense orientation after digestion with restriction endonucleases.

PLASMID	size of full plasmid (bp)	fragment size sense orientation (bp)		fragment size anti-sense orientation (bp)	
		Hind III /Not I	*Xho I/Not I*	*Hind III / Not I*	*Xho I/Not I*
PRSET B- 4.12	3851	681 3170	289 3562	276 3575	694 3157
PRSET B- 4.16	3803	681 3122	241 3562	228 3575	694 3109
		Not I		*Not I*	
GFP-4.12	7295	300 6995		693 6602	
GFP-4.16	7247	252 6995		645 6602	
GFP-3.2	8640	200 8440		2150 6490	

Gel electhrophoresis of DNA samples (section 2.2.3.1) were run at 70-80V for 1-2 hours, along with molecular weight markers. Separated fragments were visualised and photographed under UV light using The Imager™ – Appligene Oncor.

4.2.2. Transfection of COS-7 cells

Previous studies have shown that COS-7 cells are relatively easy to transfect, allowing characterisation of expressed products (Old and Primrose, 1994). Therefore, maxiprep DNA of each of the FGF3-GFP co-expression constructs (section 2.2.5.3), and of the CMVExpEGFP vector were used to transiently transfect COS-7 cells by electroporation (section 2.2.6.1), in an attempt to demonstrate the expression of the appropriate protein products and their location in the expected cellular compartments using immunostaining. After overnight recovery, adherent cells were detached by trypsin treatment and viable cells separated using Metrizamide density gradient centrifugation (section 2.1.6). COS-7 cells expressing GFP were FACS sorted (section 2.1.7.8), cultured and immunostained essentially as described in section 2.2.8. The percentage of surviving COS-7 cells that were detectably fluorescent was relatively low:

10%±2.3 for the CMVExpEGFP control plasmid, and 2%±0.2, 17.5%±1.5 and 4.5%±0.5 for GFP-3.2, 4.12 and 4.16 (n=2), respectively. The histograms of the FACS data revealed a spread of positive signals into the third decade (Figure 4.8-A). Immunostaining of the GFP^+ population with an antibody to murine FGF3 revealed that, while the cellular localisation of the various forms were as expected, only very few cells showed detectable levels of staining with the specific antibody (Figure 4.8-B to E).

4.2.3. Transfection of FDCP-mix cells with FGF3-GFP constructs and study of the effects of their expression

As FGF3 could be expressed in COS-7 cells, we sought to study the potential effects of the expression of the different forms of mouse FGF3 on haemopoiesis. To address this, FDCP-mix cells were transfected transiently with GFP-3.2, GFP-4.12, GFP-4.16 constructs using electroporation (section 2.2.10), and also with CMVExpEGFP vector, which was used as a control of GFP expression. To monitor the effects of the electroporation procedure, FDCP-mix cells were submitted to the same transfection protocol, but without addition of foreign DNA (mock). Following overnight recovery, viable cells were separated after Metrizamide density gradient centrifugation (section 2.1.6) and FACS sorted according to the GFP expression (section 2.1.7.8). Despite repeated attempts, the frequency of productively transfected FDCP-mix cells following electroporation using these plasmid DNAs remained very low as can be observed in Table 4.2. Figure 4.9 illustrates the data obtained during FACS analysis of FDCP-mix transfected cells, in which a very low number of GFP^+ cells could be observed spreading over the first decade in all conditions used, thus recapitulating the results obtained with COS-7 cells.

Despite these low transfection efficiencies, it was decided to perform some initial experiments to test for any obvious effect of FGF3 expression on FDCP-mix cell behavior. Green fluorescent (*i.e*, productively transfected) cells from electroporations with CMVExpEGFP control and with each of the three FGF3-GFP co-expression vectors were FACS sorted and entered into assays for short-term survival in the presence of a low concentration of IL-3 (section 2.1.14), self-renewal in the presence of high concentration of IL-3 (section 2.1.14), granulocyte-macrophage (section 2.1.15) and erythroid (section 2.1.16) differentiation, colony forming ability (section 2.1.12.2), and ability to establish growth on pre-formed stroma (section 2.1.11.6).

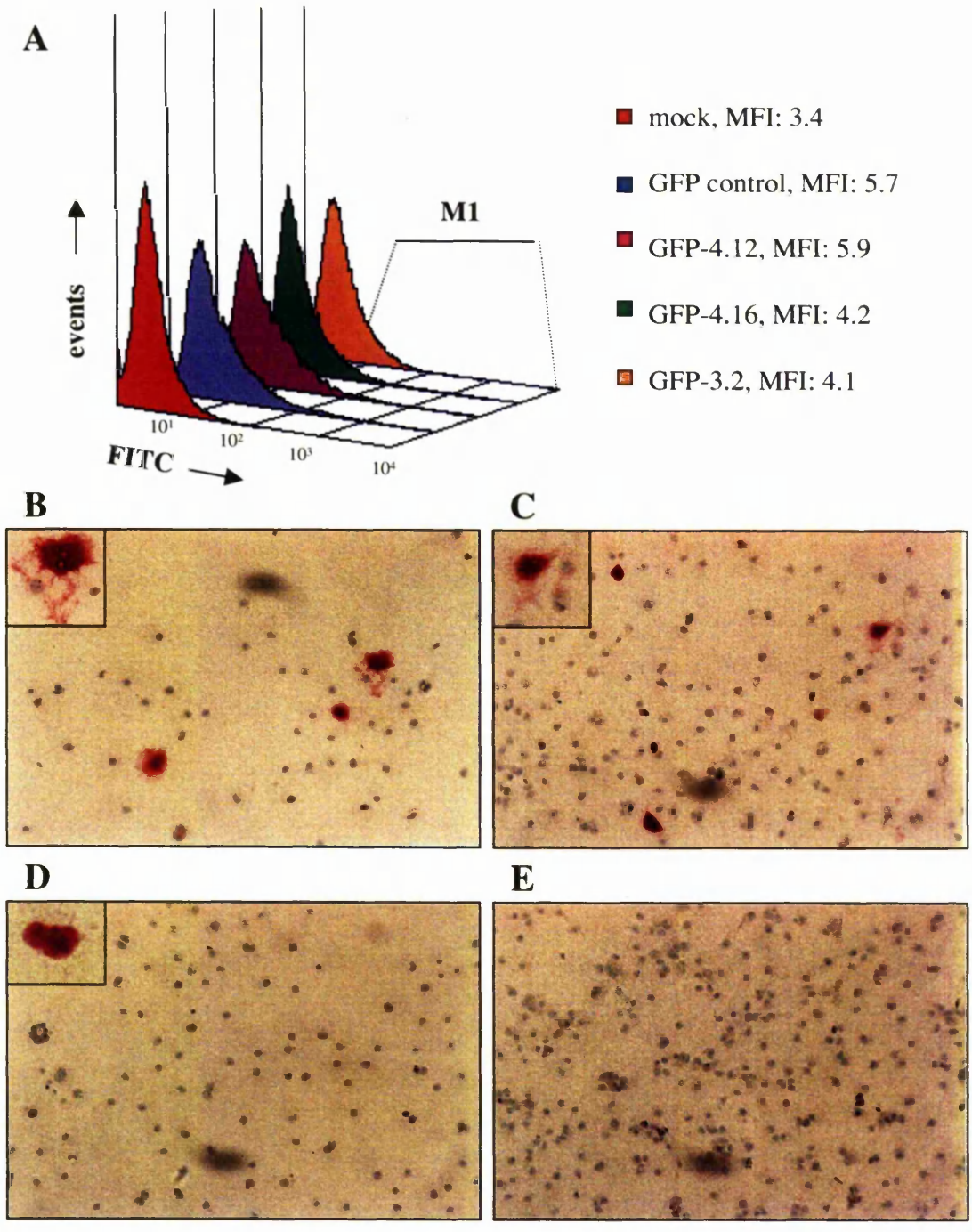


Figure 4.8. Expression of FGF3 by COS-7 cells. COS-7 cells were transfected with FGF3-GFP plasmids as described in section 2.2.6.1. After overnight recovery, cells were detached by trypsin treatment, washed and FACS sorted according to GFP expression. (**A**) Each histogram represents the population of cells transfected with the different FGF3-GFP plasmids as indicated and M1 represents the region of the GFP^+ cells. Transfected cells with (**B**) GFP-3.2, (**C**) GFP-4.12, (**D**) GFP-4.16 plasmid and (**E**) CMVExpEGFP plasmids were immunostained with mouse anti-FGF3 antibody as described in section 2.2.8. The insets show some of the positively stained cells with anti-FGF3 antibody in more detail. MFI: mean fluorescence intensity.

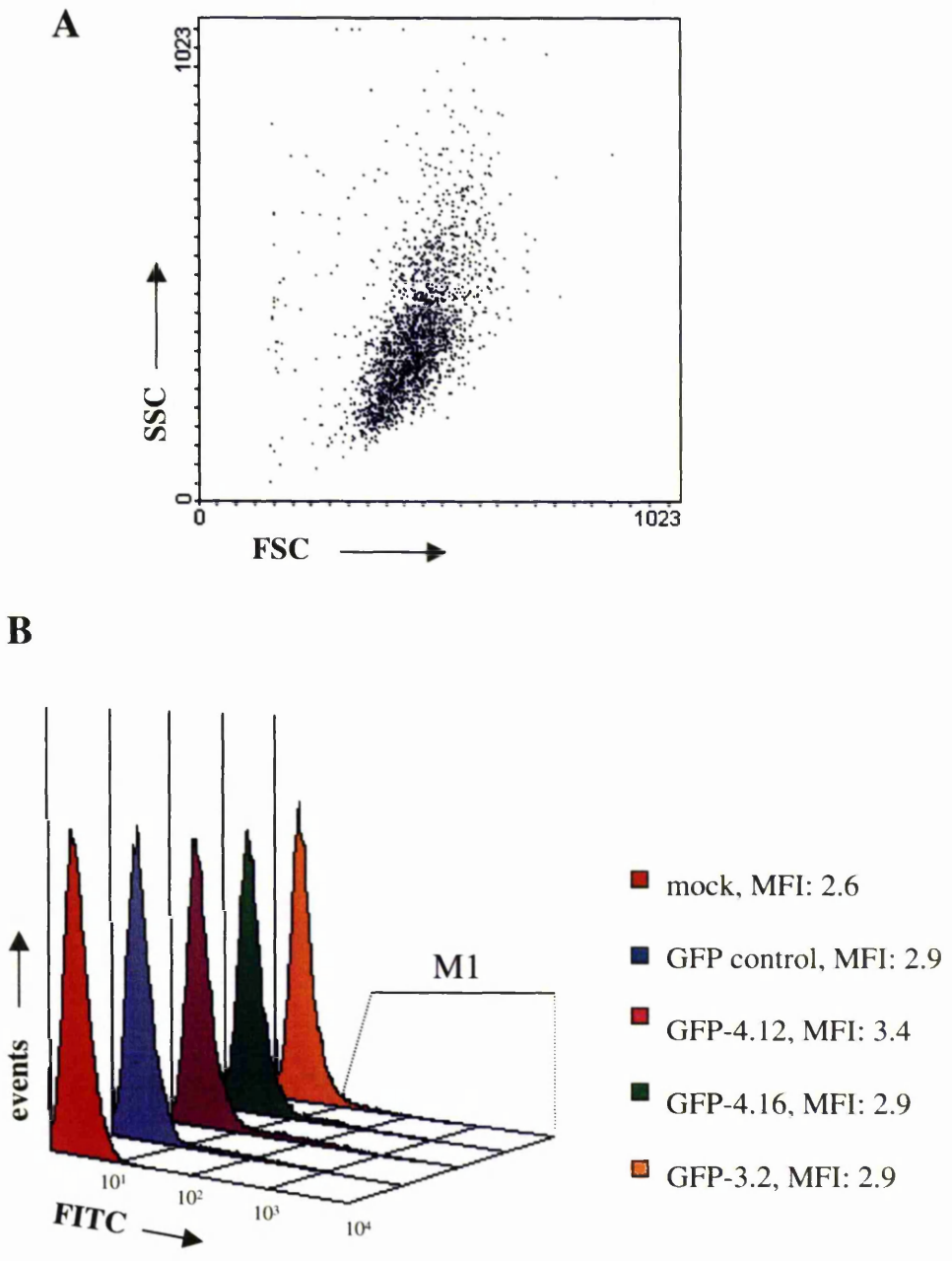


Figure 4.9. FACS data of FDCP-mix cell expressing FGF3-GFP. FDCP-mix cells were transfected with the FGF3-GFP constructs as described in section 2.2.6.1. (**A**) The forward *versus* side scatter dot plot shows control cells used for selecting FDCP-mix cells according to the expression of GFP. (**B**) Each histogram represents the population of cells transfected with the different FGF3-GFP plasmids as indicated and M1 represents the region of the GFP^+ cells. The MFI (mean fluorescence intensity) value of each peak is also showed.

Table 4.2. Responsiveness of FDCP-mix cells to electroporation at standard parameters (280V, 1050μF, 10μg FGF3-GFP plasmids) after overnight recovery.

FGF3 PLASMID	CELL SURVIVAL (%)•	GFP^+ SORTED CELLS (%)••
GFP-3.2	4.94 ± 0.71	5.4 ± 2.7
GFP-4.12	7.33 ± 1.33	8.6 ± 5.7
GFP-4.16	5.98 ± 0.79	9.6 ± 3.5
CMVExpEGFP (control)	12.7 ± 6.04*	11.9 ± 5.4
FDCP-mix mock	45.9 ± 1.65	-

FDCP-mix cells were transfected with FGF3-GFP plasmids as described in section 2.2.10. After overnight recovery, viable cells were selected using Metrizamide density gradient centrifugation (section 2.1.6), counted using trypan blue stain (2.1.10) and the percentage of survival established (•). Transfected cells were then FACS sorted according to GFP expression (section 2.1.7.8). (••) values express the mean percentage of fluorescent cells detected by flow cytometry over the viable surviving population. (*) p=0.021, n=2-5.

4.2.3.1. Survival and proliferation assays

Cells receiving each of the FGF3 encoding plasmids survived marginally better in the presence of 0.1ng/ml IL-3 than those transfected with CMVExpEGFP control vector over a period of one week (Figure 4.10), but the differences were not significant (p>0.05, n=2). The mock transfected (unsorted) control population behaved similarly over this period and, although non-transfected cells were not included as controls, the decrease in viability of these cells over the first two days indicated electroporation damage from which many cells fail to recover. High concentrations of IL-3 reduced the severity of this damage, as shown by the maintenance of cell numbers in the proliferation assay (Figure 4.11-A). However, there was little proliferation during the following 8 days after electroporation, even though the CMVExpEGFP control used was identical to that reported elsewhere to yield substantial proliferation under similar conditions (McIvor *et al.*, 2000). Examination of the cell morphology at day 4 following electroporation (Figure 4.11-B) revealed an unusually high frequency of differentiated cells in all populations that had been electroporated in the presence of plasmid DNA but

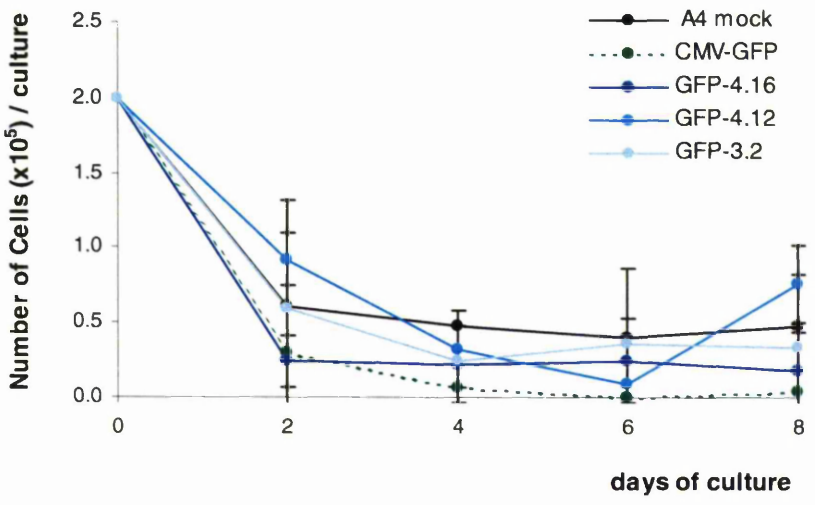


Figure 4.10. Effects of overexpression of FGF3 on survival of FDCP-mix cells. FDCP-mix cells were transfected with plasmid vectors expressing different forms of FGF3 (section 2.2.10). After overnight recovery, viable cells were separated using metrizamide density gradient centrifugation as described in section 2.1.6 and FACS sorted using GFP as a marker. GFP$^+$ cells were cultured in IMDM supplemented with 20% FCS, and 0.1ng/ml IL-3. FDCP-mix cells submitted to electroporation conditions (mock) and transfected with vector CMVExpEGFP alone were used as controls. Each point represents the mean ± SD of cells obtained from 2 independent experiments at indicated days. CMV-GFP, plasmid control; GFP-4.16, plasmid expressing the nuclear form of FGF3; GFP-4.12, plasmid expressing both secreted and nuclear forms of FGF3; GFP-3.2, plasmid expressing only the secreted form of FGF3.

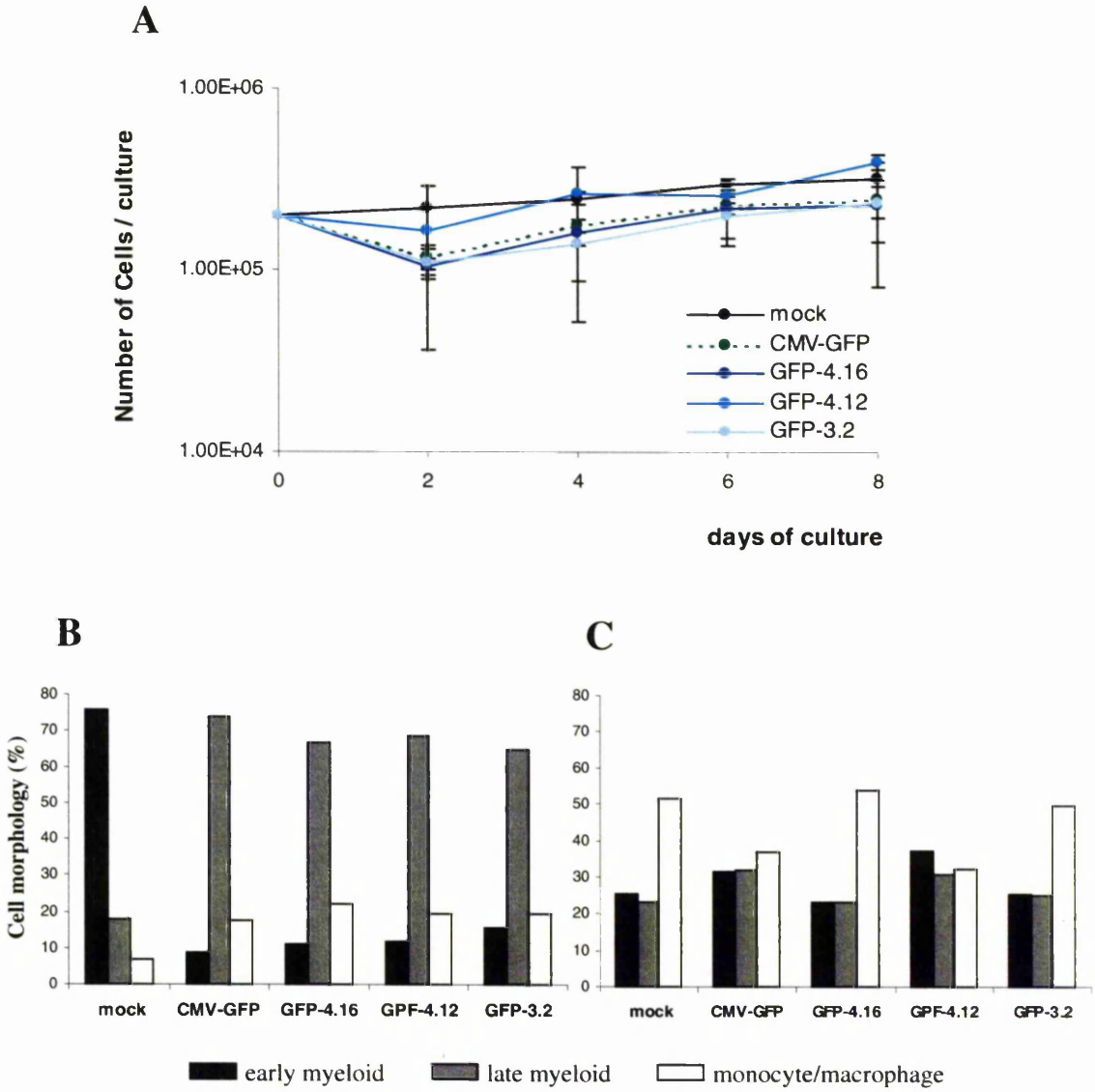


Figure 4.11. Effects of the overexpression of FGF3 on self renewal and proliferation of FDCP-mix cells. (**A**) Viable FDCP-mix cells transfected with FGF3 constructs were FACS sorted using GFP as a selection marker. GFP expressing cells were cultured in self renewal conditions as described in section 2.1.14. Cells submitted to electroporation conditions (mock) and transfected with the CMVExpEGFP vector were used as controls. Cell counts were performed at indicated days and each point represents the average±SD of 2 independent experiments. Harvested cells at days 7 (**B**) and 14 (**C**) of culture were stained with May-Grunwald-Giemsa and their cellular content analysed. CMV-GFP, plasmid control; GFP-4.16, plasmid expressing the nuclear form of FGF3; GFP-4.12, plasmid expressing the secreted and nuclear forms of FGF3; GFP-3.2, plasmid expressing only the secreted form of FGF3; early myeloid: blast, promyelocytes and myelocytes; late myeloid: metamyelocytes and neutrophils.

not in the mock-transfected control. This difference had gone by day 7 (Figure 4.11-C), and seemed to indicate a wave of commitment following electroporation.

4.2.3.2. Differentiation assays

In order to investigate whether expression of FGF3 products could influence the process of differentiation and maturation of FDCP-mix cells, we performed two series of experiments. First, viable FGF3-GFP transfected and FACS sorted cells were cultured for 8 days in conditions to undergo granulocyte-macrophage differentiation (section 2.1.15). The proliferation profile and the clonogenic potential of transfected cells were monitored by counting the cells every two days using trypan blue solution (section 2.1.10) and assaying them in colony-forming cell assay (section 2.1.12.2), respectively. Second, we repeated the experiments using conditions to undergo erythroid differentiation as described in section 2.1.16. FDCP-mix cells submitted to electroporation without DNA (mock) and also transfected with CMVExpEGFP vector were used as controls.

In contrast to the poor proliferation pattern observed in high IL-3 concentration, the mock-transfected cells showed a near-normal pattern of growth under granulocyte-macrophage differentiation conditions after the electroporation recovery period (Figure 4.12-A). Cells transfected with the CMVExpEGFP empty vector, with GFP-4.16 or with GFP-4.12 constructs failed to proliferate. However, those transfected with GFP-3.2 vector proliferate as extensively as the mock-transfected controls. GFP-3.2 transfected cells also yielded a higher proportion of early myeloid cells after this period (Figure 4.12- B and C). GFP-3.2 produces the secreted form of FGF3, and the observation that cells transfected with GFP-4.12 (secreted plus nuclear forms of FGF3) also contained more early than late myeloid cells suggests that there may be a delay or block in granulocyte/macrophage commitment associated with expression of the secreted form of FGF3 under these conditions. The GFP-3.2 transfected cells also grew slightly better than the other populations under erythroid differentiation conditions (Figure 4.13-A). Although there were no obvious differences in the cell morphologies (Figure 4.13, B and C), the very poor yield of erythroid cells in this experiment allows no firm conclusions to be drawn.

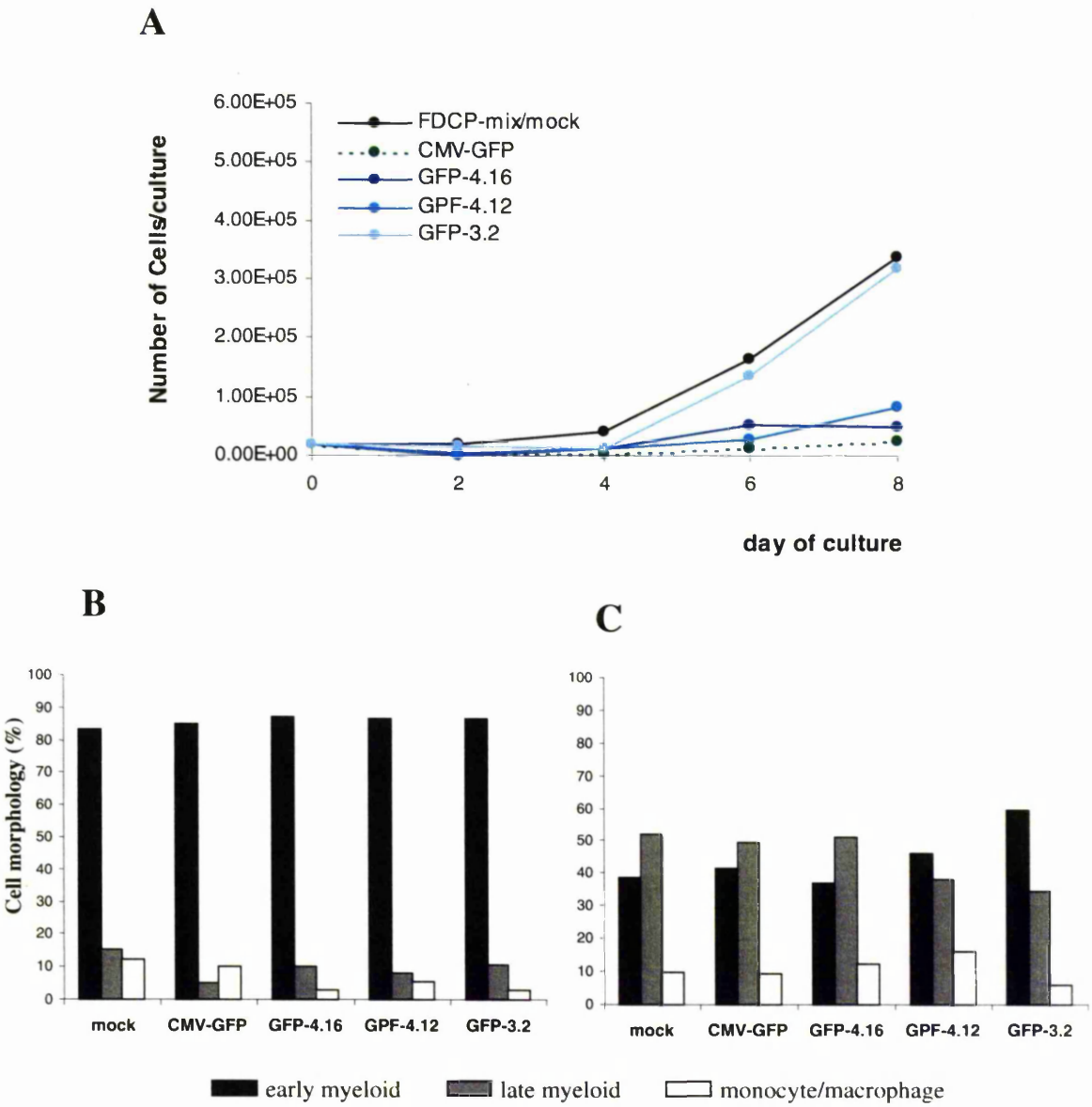


Figure 4.12. Effects of the overexpression of FGF3 on granulocyte-macrophage differentiation of FDCP-mix cells. FDCP-mix cells transfected with FGF3 plasmids were FACS sorted according to GFP expression. (**A**) GFP^+ cells were cultured in conditions to promote granulocyte-macrophage differentiation as described in section 2.1.15 and their proliferation rate assessed by trypan blue dye counting every two days. Cells submitted to electroporation conditions (mock) were used as control. Each point represents the number of cells obtained in 1 experiment at indicated days. Harvested cells at days 4 (**B**) and 7 (**C**) of culture were stained with May-Grunwald-Giemsa and their cellular content analysed. CMV-GFP, plasmid control; GFP-4.16, plasmid expressing the nuclear form of FGF3; GFP-4.12, plasmid expressing the secreted and nuclear forms of FGF3; GFP-3.2, plasmid expressing only the secreted form of FGF3; early myeloid cells include blast, promyelocytes and myelocytes; late myeloid, metamyelocytes and neutrophils.

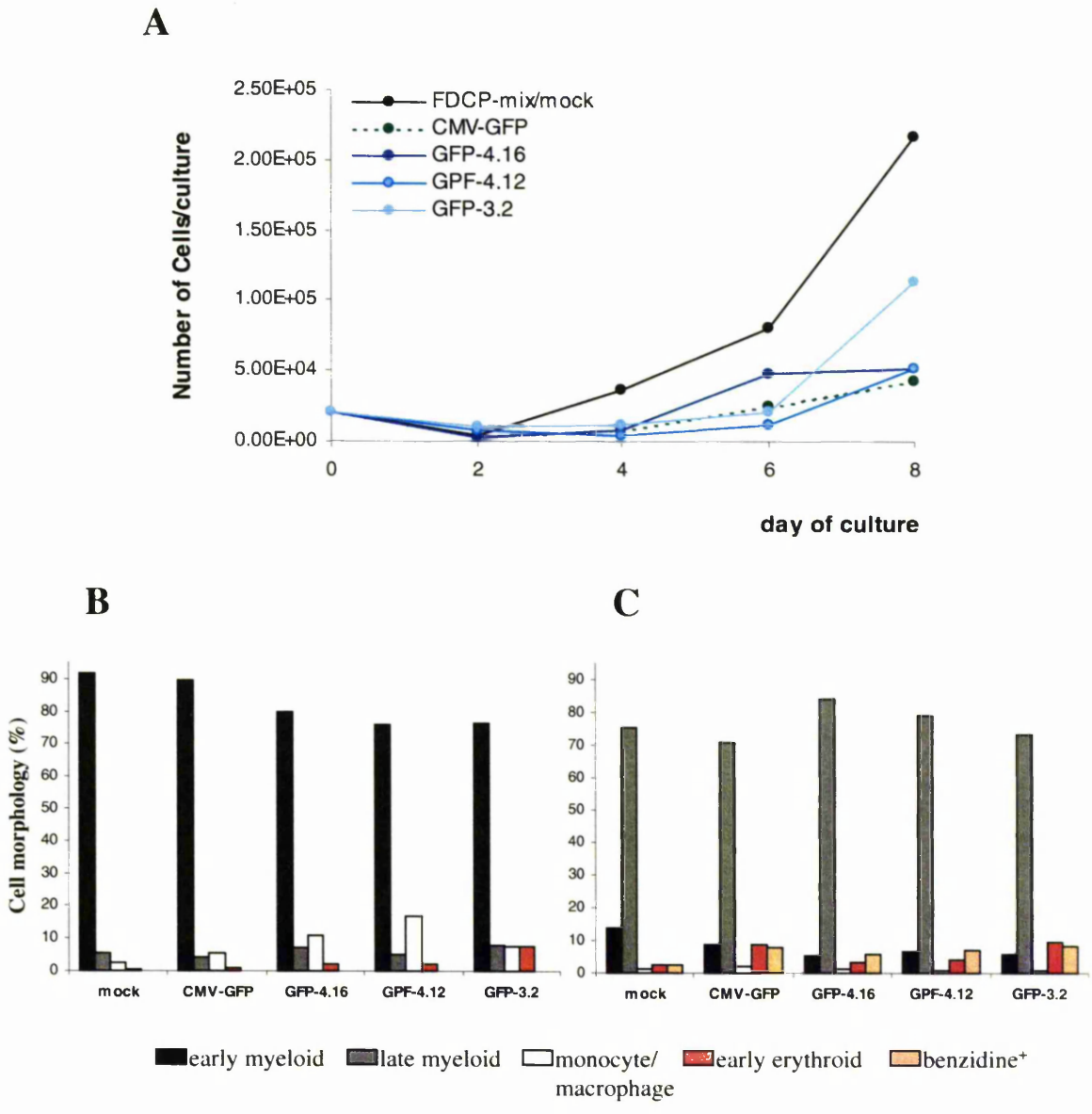


Figure 4.13. Effects of the overexpression of FGF3 on erythroid differentiation of FDCP-mix cells. FDCP-mix cells transfected with FGF3 plasmids were FACS sorted according to GFP expression. (**A**) GFP$^+$ cells were cultured in conditions to promote erythroid differentiation as described in section 2.1.16, and their numbers counted every two days. Cells submitted to electroporation conditions (mock) were used as control. Each point represents the average of cells obtained from 2 independent experiments at indicated days. Harvested cells at days 4 (**B**) and 7 (**C**) of culture were stained with May-Grunwald-Giemsa and their cellular content analysed. CMV-GFP, plasmid control; GFP-4.16, plasmid expressing the nuclear form of FGF3; GFP-4.12, plasmid expressing the secreted and nuclear forms of FGF3; GFP-3.2, plasmid expressing only the secreted form of FGF3; early myeloid cells include blast, promyelocytes and myelocytes; late myeloid, metamyelocytes and neutrophils.

4.2.4. Ability of FGF3 transfected FDCP-mix cells to establish haemopoiesis on pre-formed stroma

When cultured onto irradiated pre-formed stroma, FDCP-mix cells establish haemopoiesis and their haemopoietic activity is maintained for several months as measured by the increase in the number of cells present in the supernatant, in the number of colonies generated in clonogenic assays and by their ability to undergo differentiation to produce mature progeny (Spooncer *et al.*, 1993a; Spooncer *et al.*, 1986). We have used this approach to test the ability of FGF3 transfected cells to adhere and further establish areas of active haemopoiesis. To achieve this, FDCP-mix cells were transfected with the different FGF3-GFP vectors. Sorted GFP^+ cells were seeded onto irradiated stroma as described in section 2.1.11.5. The cultures were fed every three days by removing half of the growth medium volume and adding an equal volume of fresh medium for 2 weeks. Culture flasks were observed under an inverted phase contrast microscope to monitor changes in the adherent layer.

Cobblestone areas were established in all cases (Figure 4.14). However, the haemopoietic activity was low (Table 4.3). Furthermore, in no case were colony forming cells recovered either from the supernatants or from the trypsinised stromal layers of these cultures. This is in accordance with the lack of colony forming activity found from both transfected and mock-transfected cells maintained in high concentrations of IL-3.

4.3. Discussion

The transfer of foreign genes into haemopoietic cells has become an important tool for studying the effects of the expression of their products on the development and differentiation profiles of transfected cells. Amongst the methods to transiently transfer genes into cells, electroporation has been widely used and, in the previous chapter, we showed its feasibility as a means of obtaining biologically active FGF3 products purified from the conditioned medium of transfected COS-7 cells. Furthermore, studies of the internal ribosome entry site (IRES) have made it possible to develop bi-cistronic or policistronic vectors in which a fusion transcript can be obtained from two or more genes linked together by one or more IRES sequences (Schmid and Wimmer, 1994).

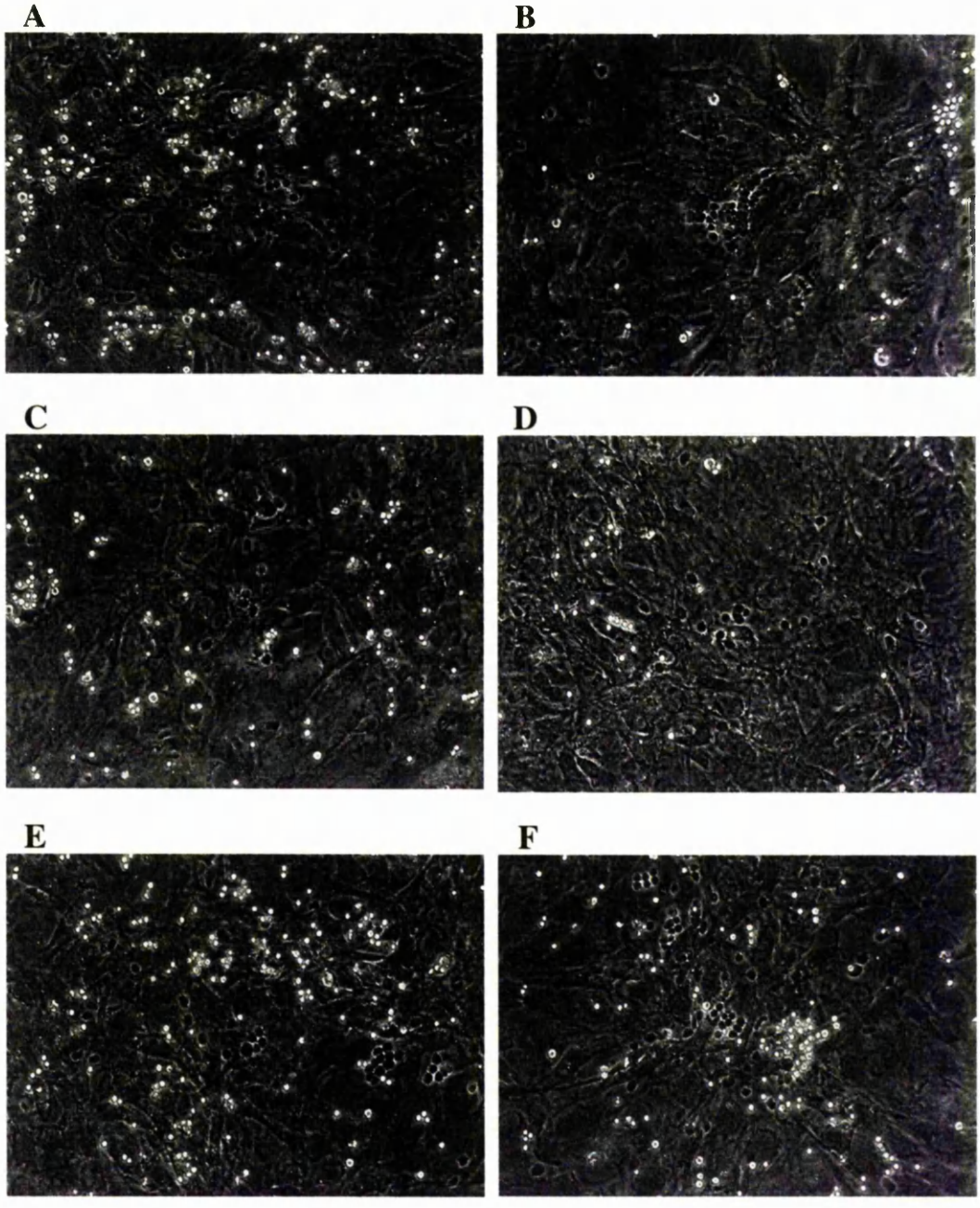


Figure 4.14. Ability of FDCP-mix cells expressing different forms of FGF3 to establish haemopoiesis. FDCP-mix cells transfected with mouse FGF3-GFP constructs were seeded onto irradiated stroma and the number of cells and progenitors produced assessed as described in sections 2.1.10 and 2.1.12.2, respectively. The figures show cobblestone areas at week 2 of culture of mock-transfected FDCP-mix cells (**A** and **B**) and of FDCP-mix cells transfected with the different FGF3-GFP constructs: (**C**) CMVExpEGFP control vector; (**D**) GFP-4.12; (**E**) GFP-4.16; (**F**) GFP-3.2 (phase contrast, 80x magnification).

Table 4.3. Growth of FGF3-GFP transfected FDCP-mix cells onto pre-established stroma.

Transfectant cell type	Supernatant cells ($x10^3$)	Fold Increase*
FDCP-mix/mock	11.7	5.85
GFP-4.12	3.9	1.95
GFP-4.16	3.9	1.95
GFP-3.2	5.0	2.50
CMVExpEGFP	2.8	1.40

FGF3-GFP transfected FDCP-mix cells were seeded onto irradiated stroma as described in section 2.1.11.6. Cultures were fed every three days. At day 14, cells from the supernatant were recovered, counted and assayed for their clonogenic potential. (*) values represent the ratio between the number of supernatant cells and the input cell number.

We have constructed a series of plasmids in which the FGF3 cDNA was transcriptionally linked by a viral IRES to the GFP gene to test the effects of FGF3 on haemopoiesis. This configuration allows coordinated expression of the FGF3 and GFP proteins, the later of which would facilitate the selection of genetically modified cells. FDCP-mix cells were chosen as a testing system not only because they have characteristics similar to those of normal haemopoietic cells, but also because of that the key decisions for their survival, proliferation and lineage commitment all occur in a short period of time. The combination between the transient expression of FGF3 (as the CMV promoter activity generally lasts 2-3 days) with the time necessary to induce development of FDCP-mix cells would provide the opportunity to study events associated with commitment and differentiation.

COS-7 cells were transfected with the FGF3-GFP constructs to confirm the expression characteristics of FGF3 products by flow cytometry and immunocytochemistry (Figure 4.8). The results clearly showed that COS-7 transfected cells expressed FGF3 products. However, only a few of the GFP-expressing cells were visibly labeled with the specific anti-FGF3 antibody (Figure 4.8, B to E). In our experience, the MSD-1 antibody used to detect FGF3 proteins seemed to work only

when relatively high levels of protein were present. The use of a bi-cistronic vector ensures that the expression of the target gene remains coupled to the expression of the marker gene and it has been shown that the expression of a test gene placed upstream IRES is markedly higher when compared to the expression of the same gene inserted downstream IRES (McIvor *et al.*, 2000). Since the FGF3 cDNAs were located upstream from the GFP coding region in the expression vectors (Figure 4.2), it should therefore have been translated at a higher efficiency than that of GFP. It seems fair to assume that at least low levels of FGF3 were being made by the cells under assay. Even given the low frequency of detectable FGF3 expression in COS-7 cells, it was therefore considered worthwhile progressing to transfection of FDCP-mix cells.

Overall, it did not prove possible to reproduce the observations reported by McIvor and colleagues, in which cells transfected with the CMVExpEGFP expression vector were able to survive and proliferate in the presence of IL-3, and also to form colonies in clonogenic assays (McIvor *et al.*, 2000). Since even the mock-transfected cells failed to form colonies under these conditions, it seems likely that this was due to differences in the behaviour of the FDCP-mix cells in each case. Indeed, the results obtained in some of the experiments performed here indicate atypical behaviour of the cells assayed. First, the tendency of the cells to cease proliferation and to differentiate in the presence of high concentrations of IL-3 following electroporation even in the absence of DNA (Figure 4.11). Second, the unusually high proportion of granulocyte-macrophage cells produced under erythroid differentiation conditions (Figure 4.13). Thirdly, the very low yield of erythroid cells from the erythroid differentiation assay.

Other possible differences may be related to the electroshock parameters used and/or, particularly, on the quality of the DNA. Although evidence that electroporation did harm the cells, the wave of differentiation observed in high concentration of IL-3 following electroporation was restricted to those samples in which plasmid DNA was present (Figure 4.11-B). This suggests a general effect either of something contaminating the DNA preparations, or of GFP expression, or of the presence of CMV promoter. High concentrations of CMV promoter-containing plasmids have been found previously to be toxic to FDCP-mix cells, an effect that may be due to the titration of limiting amounts of certain transcription factors that bind strongly to the transfected promoter DNA (Dr. M. A. Cross, personal communication). The severity of this effect could vary also with the state of the cells.

The most interesting observation from this series of assays was the possible delay or block in commitment under granulocyte-macrophage conditions associated with production of the secreted form of FGF3 (Figure 4.12-C). This effect was not observed when purified XFGF3 was added to similar system (Chapter 3, Table 3.1). This may be due to species differences in ligand/receptor interactions (mouse FGF3 and *Xenopus* FGF3, respectively) or to higher local concentrations expected from endogeneous production of FGF3. This effect certainly warrants further investigation, but not until the assay conditions reflect more closely those reported by McIvor *et al.* (2000), in which electroporated cells retained proliferation and colony forming abilities as well as differentiation potential.

Chapter 5

Expression of MIP-1α and SDF-1α receptors on haemopoietic cells

5.1. Introduction

During the last decade, more than 50 chemokines and over 25 chemokine receptors have been identified, but their precise expression and biological functions remain to be defined. Chemokines play diverse roles in the inflammatory process, particularly in mediating leukocyte trafficking. In this situation, leukocyte subsets respond preferentially to unique but overlapping subsets of chemokines as determined by the receptor distribution (Murphy, 1996). Chemokines have also been involved in a variety of mechanisms that regulate haemopoiesis and angiogenesis (reviewed in Baggiolini, 1998; Baggiolini *et al.*, 1997; Kim and Broxmeyer, 1999a; Rollins, 1997). Of considerable interest is the recent discovery that chemokines such as MIP-1α and SDF-1α function as HIV-suppressive factors by interacting with chemokine receptors which, together with CD4, are recognised as binding sites by HIV-1 (Bleul *et al.*, 1996a; Bleul *et al.*, 1997; Cocchi *et al.*, 1995; Garzino Demo *et al.*, 1998; Garzino Demo *et al.*, 1999).

As reviewed at the introduction of this thesis, chemokine receptors have complex ligand binding patterns, displaying both specificity and promiscuity (Broxmeyer and Kim, 1999). Although the chemokine signalling network is highly complex, the primary task of each receptor is conceptually simple: to bind a chemokine and to relay its signal to a heterotrimeric guanine nucleotide-binding regulatory protein (G protein) (Baggiolini, 1998; Murphy, 1994; Murphy, 1996; Rollins, 1997). An understanding of the expression patterns and the biochemistry of these receptors is therefore central to the overall understanding of the roles played by chemokines in both physiological and pathological processes.

One of the aims of this study was to better understand how MIP-1α and SDF-1α can influence stem cell proliferation, migration, and adhesion through interactions with their receptors, and try to correlate these biological functions with specific receptor sub-types. MIP-1α has been reported to bind the receptors CCR1, CCR4, CCR5, and D6 (Gao *et al.*, 1993; Neote *et al.*, 1993; Nibbs *et al.*, 1997b; Power *et al.*, 1995; Samson *et al.*, 1996). Subsequently, it has been shown that CCR4 is a receptor that binds the chemokines TARC (Thymus and Activation Regulated Chemokine) and MDC (Monocyte Derived Chemokine) (Imai *et al.*, 1997a; Imai *et al.*, 1998; Yoshie *et al.*, 1997) and does not bind MIP-1α with any degree of specificity. The D6 receptor binds MIP-1α and also a variety of other chemokines. However, its function is currently unclear since there is no signalling response detected to chemokine binding in CHO (Chinese Hamster Ovary) or HEK 293 (Human Kidney) cells expressing D6 (Nibbs *et al.*, 1999). In contrast to MIP-1α receptors, CXCR4 is the only receptor so far described that binds SDF-1α (Bleul *et al.*, 1996a; Nagasawa *et al.*, 1996b; Oberlin *et al.*, 1996).

This chapter describes the approach taken to investigate the expression of the MIP-1α receptors CCR1 and CCR5, and the SDF-1α receptor, CXCR4, using flow cytometry on mononuclear cells freshly isolated from peripheral blood, bone marrow, and cord blood samples. CD34 is a cell-surface glycophosphoprotein expressed by only a minority of marrow mononuclear cells that contain precursors for all lymphohaemopoietic lineages (Krause *et al.*, 1996). As described in more detail in Chapter 1, this antigen has been currently used in a variety of clinical settings such as the quantification of the stem/progenitor cells after mobilisation with haemopoietic growth factors, in enrichment procedures such as the ones necessary for haemopoietic rescue after high-dose myeloablative therapy, transplantation, and gene therapy trials. Also, it has been used as a marker for the purification of stem/progenitor cell in experimental procedures (reviewed in Krause *et al.*, 1996; Sutherland and Keating, 1992). As such, we further investigated the distribution of the CCR1 receptor on progenitor cells bearing the CD34 marker from bone marrow and particularly from cord blood. In order to characterise the binding capacity of the antibodies and for technical optimisation, we used the human leukaemia haemopoietic cell lines HL60 (Gallagher *et al.*, 1979), Jurkat (Gillis and Watson, 1980), K562 (Koeffler and Golde, 1980), THP-1 (Tsuchiya *et al.*, 1980), and TF-1 (Kitamura *et al.*, 1991), which have been reported to bind or to respond to MIP-1α or SDF-1α. As it has been recently reported that Chronic Myelogenous Leukaemia (CML) $CD34^+$ cells bind biotinylated MIP-1α (Durig *et al.*,

1999b; Nicholls *et al.*, 1999), we have also investigated whether the chemokine receptors CCR1, CCR5, and CXCR4 are expressed on K562 cells, a cell line derived from a patient with CML (Koeffler and Golde, 1980).

5.2. Results

5.2.1. Expression of CCR1, CCR5, and CXCR4 receptors

5.2.1.1. Haemopoietic cell lines

Expression of CCR1, CXCR4 and CCR5 on haemopoietic cells lines were detected using flow cytometry after labelling the cells as described in section 2.1.7.6. Figure 5.1 illustrates the gating strategy used for analysis of CCR1 expression on cell lines, depicting as an example the Jurkat cell line. Gates were set on the forward *versus* side scatter characteristics of each cell line (Figure 5.1-A). The histogram represents analysis of at least 5,000 events acquired in the gated population R1, where the solid red peak denotes the isotype control used and included ~99% of the population analysed. The green peak represents the cells that were labeled with the fluorescent specific antibody and percent positivity was calculated beyond marker M1 (Fig. 5.1-B). The data was acquired with PC Lysis II software (Becton Dickinson Immunocytometry Systems) and analysed using the WinMDI 2.8 software. The results are presented as the mean percentage of positive cells plus or minus one standard error of the mean (SEM).

All cell lines analysed in this study stained clearly and brightly with the anti-CCR1 antibody, demonstrating homogeneous expression of this MIP-1α receptor on their cell surface. A representative example of the expression of the CCR1 on the surface of THP-1, TF-1, K562, and HL60 cells is shown in Figure 5.2. We examined the expression of the MIP-1α receptor CCR5 on the same cell lines, using a panel of 5 antibodies of different clones from two different sources (see section 2.1.7.6). In contrast to the clear expression of CCR1, expression of CCR5 was not detected in any of the cell lines tested. We then investigated the expression of the SDF-1α receptor CXCR4, which was highly expressed by Jurkat, and THP-1 cells, with the percentage of positive cells being 98.7%±0.1 and 96.4%±0.8, respectively. At the same time, HL60 cells showed a moderate level of expression (61.1%±28.0), while K562 cells showed a much more restricted pattern of expression, ranging from 1.7 to 20.8% (mean 8.1%±4.3,

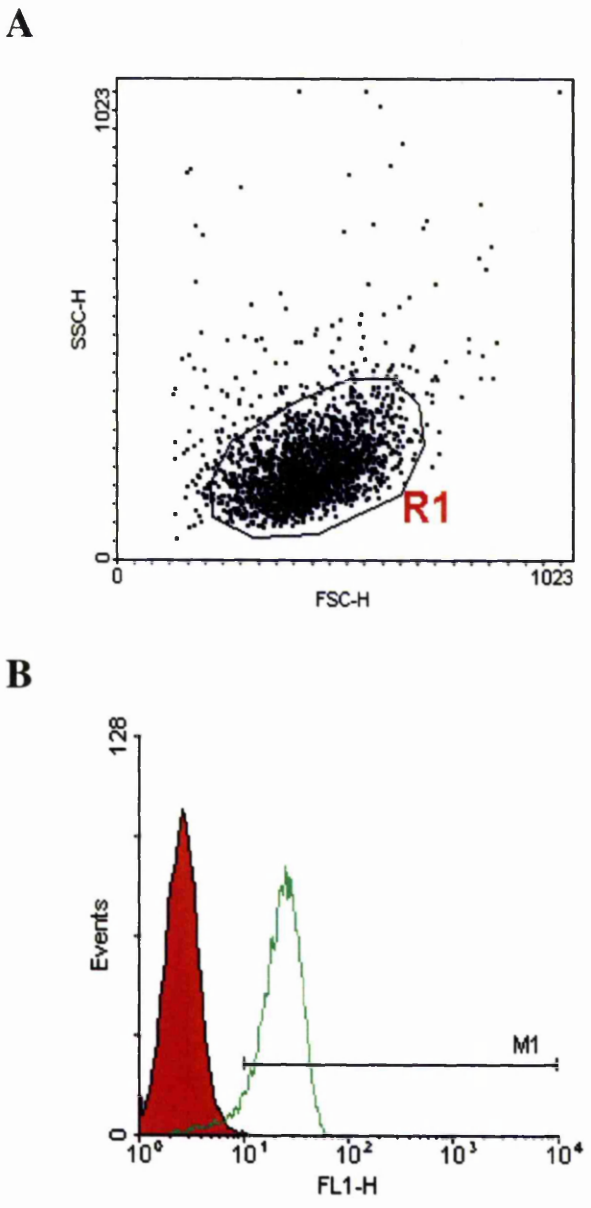


Figure 5.1. Gating strategy and single colour immunofluorescence of cell lines labeled with chemokine receptor antibodies. (**A**) Forward *versus* side scatter dot plot of Jurkat cells labeled with anti-CCR1 antibody as described in section 2.1.7.6. R1 indicates the gate set to analyze the expression of CCR1 on a more homogeneous cell population. (**B**) Histogram of the R1 gated population, indicating the fluorescence intensity of cells stained positively for CCR1 (green line). The red peak denotes the control staining and M1 indicates the percentage of positive cells. In the example shown, 91.8% of the Jurkat cells are positively labeled for CCR1.

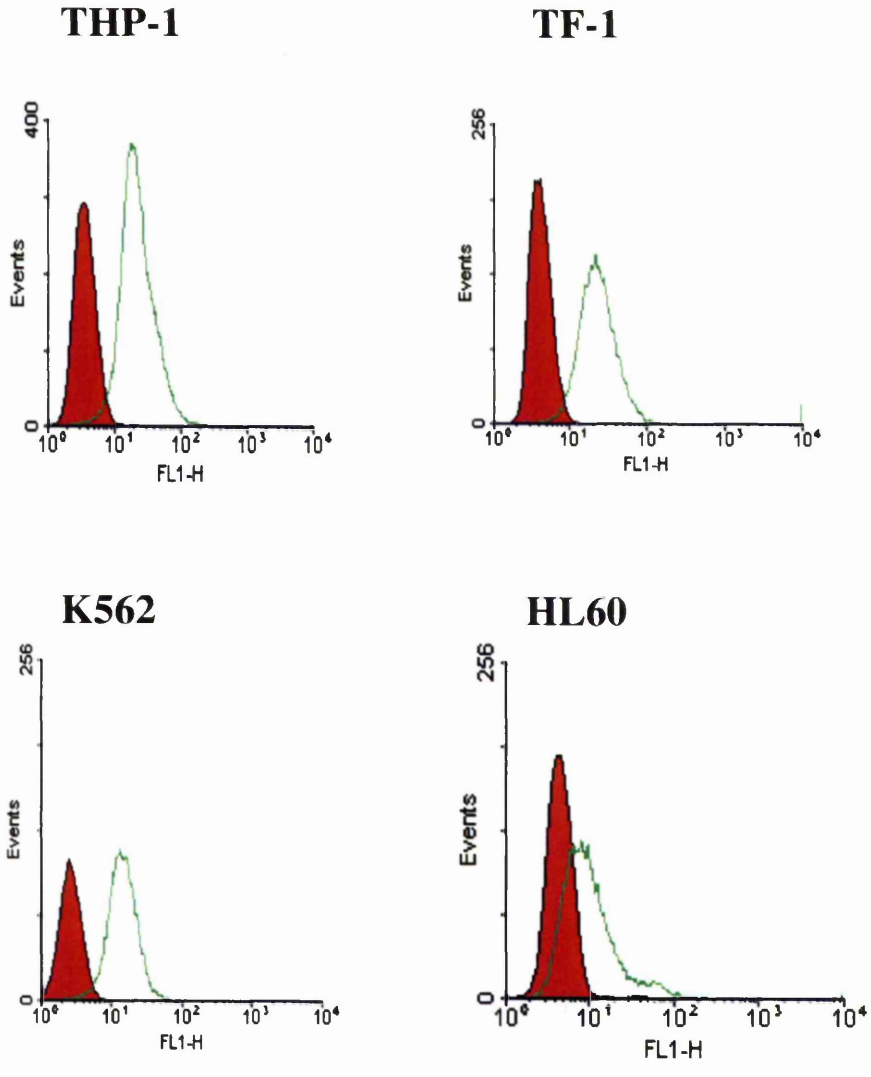


Figure 5.2. Single colour immunofluorescence of cell lines labeled with anti-CCR1 antibody. Cells from each cell line were labeled with the anti-CCR1 antibody as described in section 2.1.7.6. Histograms indicate uniform cell populations with fluorescence intensity (FL1) of cells labeled with anti-CCR1 (green peak) and control staining for FITC (solid red peak).

n=4). TF-1 cells were found not to express CXCR4. A representative example of the CXCR4 expression on the cell lines tested is shown in Figure 5.3. Table 5.1 summarises the percentage of positive cells from the different cell lines stained with the MIP-1α CCR1 and the SDF-1α CXCR4 receptor antibodies.

Table 5.1. Expression of the MIP-1α receptor CCR1 and the SDF-1α receptor CXCR4 on human leukaemic cell lines.

Human Cell Line (type)	Expression of Receptors (%)	
	CCR1	CXCR4
K562 (Chronic Myelogenous Leukaemia)	85.9±0.4	8.1±4.3
HL60 (Acute Pro-Myelocytic Leukaemia)	80.4±13.0	61.1±28.0
TF-1 (Erythroleukaemia)	84.8±3.1	<1%
THP-1 (Acute Monocytic Leukaemia)	96.5±1.5	96.4±0.8
Jurkat (Acute T Cell Leukaemia)	94.4±0.3	98.7±0.1

Values represent the mean percentage ± SEM of cells positively stained for the expression of the indicated receptor. Data obtained from 3 to 8 independent experiments.

5.2.1.2. Human mononuclear cells

Having validated the antibodies for the detection of CCR1 and CXCR4 receptors on different cell lines, we sought to characterise the expression of these receptors on different human haemopoietic cells freshly isolated from steady state peripheral blood (PB), adult bone marrow (BM), and cord blood (CB). Although CCR5 expression could not be demonstrated on any of the cell lines tested, we investigated its expression on different human haemopoietic cells using the anti-CCR5 antibody clone 2D7 based on recent reports that have shown its expression on human haemopoietic cells after labelling them with this antibody (Hariharan *et al.*, 1999; Lee *et al.*, 1999c).

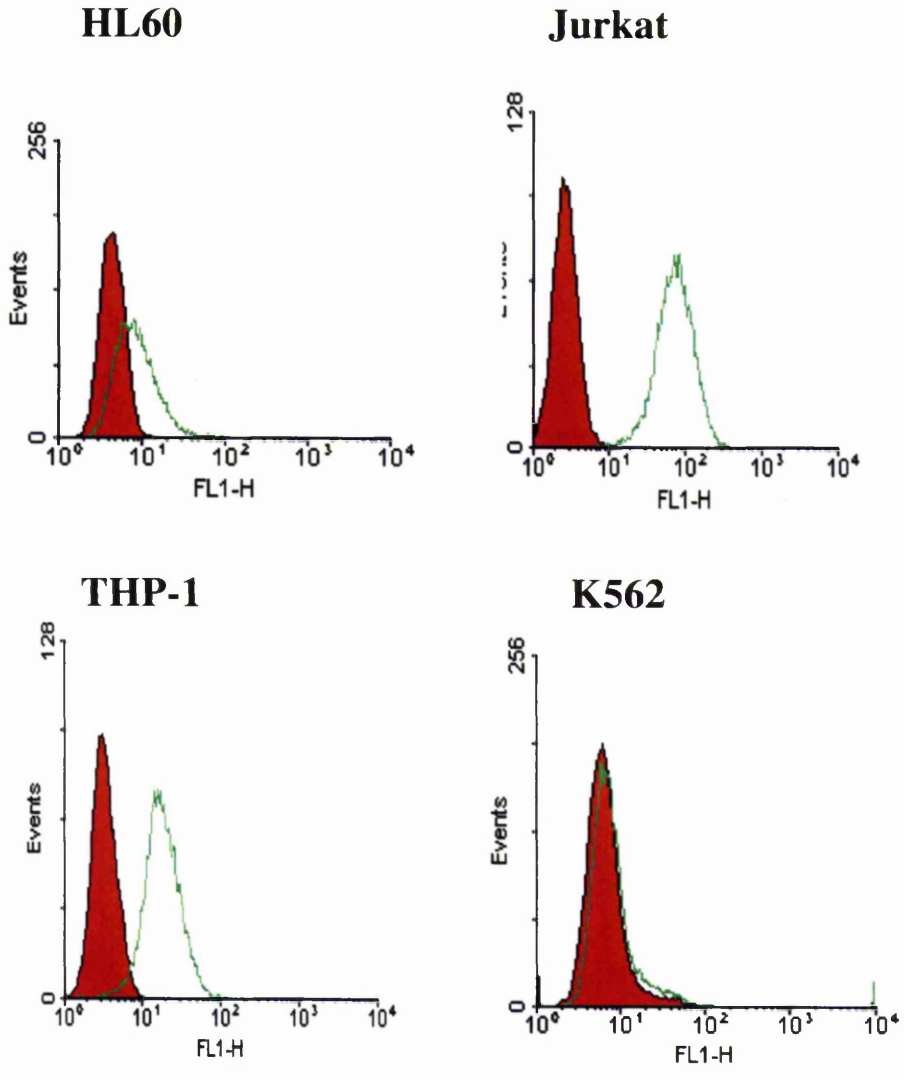
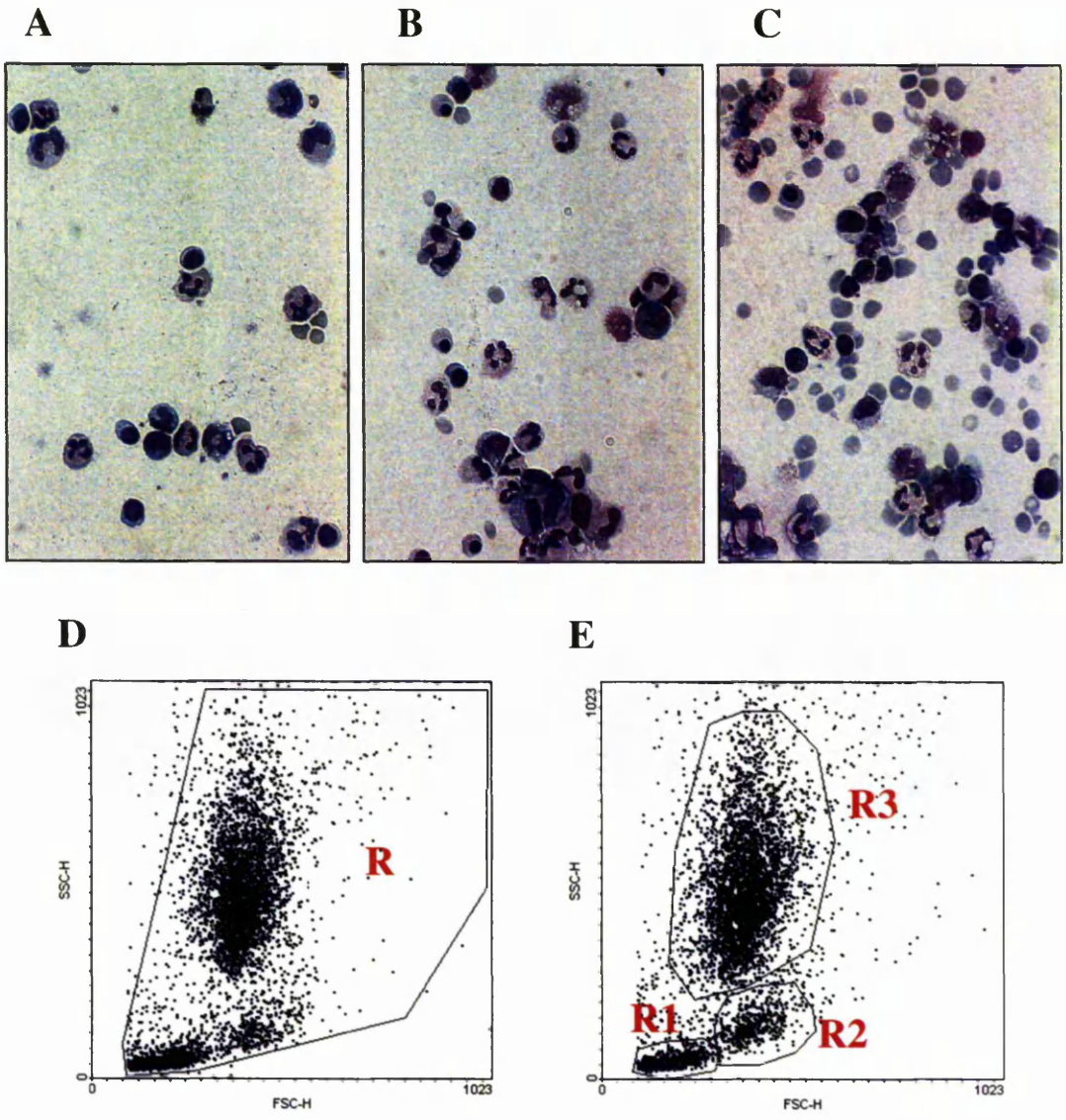


Figure 5.3. Single colour immunofluorescence of cell lines labeled with anti-CXCR4 antibody. Cells from each cell line were labeled with the CXCR4 antibody as described in section 2.1.7.6 of Materials and Methods. Histograms indicate the population with fluorescence intensity (FL1) of cells treated with anti-CXCR4 antibody (green peak) and with isotype control staining for FITC (red peak).

Human mononuclear cells (MNC) obtained after density gradient centrifugation (section 2.1.7.4) were washed and labeled with anti-CCR1, anti-CCR5, or anti-CXCR4 antibodies as described in section 2.1.7.6. For the cytofluorimetric analysis of the expression of the chemokine receptors, one-colour FACS analysis was first performed on the whole population 'R' using the gating strategy for obtaining positive cells as depicted in a forward *versus* side scatter dot plot presented in Figure 5.4-D. To investigate further the expression of these receptors on the sub populations of the mononuclear cells, specific gates were then set on the basis of the forward *versus* side light scatter characteristics of each distinct population as follows: R1, for a lymphocyte gate; R2, for a monocyte gate, and R3, for a granulocyte gate (comprising neutrophils, eosinophils, and basophils) (Civin and Loken, 1987). This gating strategy was used to analyse the MNC sub populations from PB, BM, and CB samples and is illustrated in Figure 5.4-E. Unless otherwise indicated, at least 5,000 events were acquired for each analysis. The threshold line was based on the maximum staining of a matched isotype control antibody with irrelevant specificity. This threshold line for defining positive and negative cells was set such as that ~1% of isotype positive cells was present above the threshold line. Labeled cells brighter than those stained with the isotypic antibody were defined as positive for the specific antibody. The results are presented as the mean percentage plus or minus one SEM of positive cells for the indicated receptor.

5.2.1.2.1. Peripheral blood

To investigate the chemokine receptor expression at steady state levels on peripheral blood cells, we used fresh PBMNC from healthy individuals (n=6). The average number of MNC recovered after density gradient centrifugation was $1.0x10^6 \pm 1.1x10^5$ cells/ml of peripheral blood. Flow cytometric analysis of PBMNC demonstrated variable degree of labelling with the antibodies tested. The overall expression of the receptors CCR1, CXCR4, and CCR5 were 35.0%±1.3, 44.9%±3.4, and 7.5%±1.8, respectively. Closer analysis of the sub populations showed that CCR1 was highly expressed on the cells of the monocyte (88.5%±3.4) and granulocyte (57.5%±10.6) gates, whereas the cells of the lymphocyte region showed lower expression (38.8%±6.5). CXCR4 was also readily discerned on the surface of the cells from the monocyte (51.5%±6.3), and lymphocyte (55.9%±11.7) gates, with the granulocyte gated cells having a more moderate expression pattern (37.7%±6.3). CCR5 expression, although in much lower levels when compared to the expression of the two



GATES: R, positive cells; R1, Lymphocyte; R2, Monocyte, R3: Granulocyte

Figure 5.4. Morphology and gating strategy for the flow cytometric analysis of chemokine receptors expression on MNC obtained from human samples. Mononuclear cells from peripheral blood (**A**), bone marrow (**B**), and cord blood (**C**) samples were stained with May-Grunwald-Giemsa. Each photo represents the mononuclear cell population analysed by flow cytometry obtained from peripheral blood, bone marrow and cord blood of one experiment. The forward *versus* side scatter characteristics of the total positive cells (**D**) and of the each distinct population (**E**) of the MNC were used to define gates for the analysis of the expression of the CCR1, CCR5 and CXCR4 receptors using one-colour immunofluorescence as described. R1, for lymphocyte, R2, for monocyte, and R3, for granulocyte gated cells. (magnification: 400x).

other receptors, could be detected on all the sub populations except of the granulocyte gate (<1% expression), with 15.6%±1.9% and 6.4%±2.5% expression on lymphocyte and monocyte gated cells, respectively. Morphological analysis of PBMNC stained with May-Grunwald-Giemsa (Figure 5.4-A) showed that lymphocytes were the major white cell population present (60.4%), followed by granulocytes (24.2%) and monocytes (15.4%). Figure 5.5 shows histograms of the expression of CCR1, CCR5, and CXCR4 on the cell surface of the PBMNC from one representative experiment.

5.2.1.2.2. Bone marrow

To investigate the chemokine receptor expression on bone marrow cells, we used bone marrow aspirates (n=6) and rib sections (n=3) obtained from healthy individuals. The number of mononuclear cells recovered after density gradient centrifugation was $2.4\pm1x10^6$ cells/ml of bone marrow aspirates. BMMNC expressed the receptors CCR1, CCR5, and CXCR4, and their overall expression were 37.3%±7.2, 4.2%±1.2, and 7.7%±3.1, respectively. When the gated sub populations were examined, CCR1 was highly expressed on the cells of the monocyte (79.2%±7.7) and of the lymphocyte (67.2%±7.0) regions, while only 15.4%±3.8 of the cells of the granulocyte region were positively labeled. CCR5 receptor was expressed at low levels on all three sub populations examined: 9.9%±2.6, 10.2%±4.4, and 5.4%±3.7 for lymphocyte, monocyte, and granulocyte regions, respectively. It is worthy of note that CCR5 expression was <1% in 8, 5, and 1 samples on the granulocyte, monocyte, and lymphocyte regions, respectively, in a total of 9 samples analysed. As for CCR5, CXCR4 expression was generally low on all BMMNC lymphocyte (6.5%±1.4), monocyte (20.3%±5.6), and granulocyte (5.5%±1.2) gated sub populations. A representative experiment of the expression of the receptors CCR1, CCR5, and CXCR4 on the surface of BMMNC detected by flow cytometry is illustrated in Figure 5.6.

Morphological analysis of cells stained with May-Grunwald-Giemsa (Figure 5.4-B) showed that BMMNC were composed of immature granulocytes (comprising blasts, promyelocytes, and myelocytes – 13.3%), mature granulocytes (comprising metamyelocytes, band and segmented neutrophils, basophils, and eosinophils - 28.9%), lymphocytes (32.9%), monocytes (9.3%), plasmocytes (1.2%), and nucleated erythroid

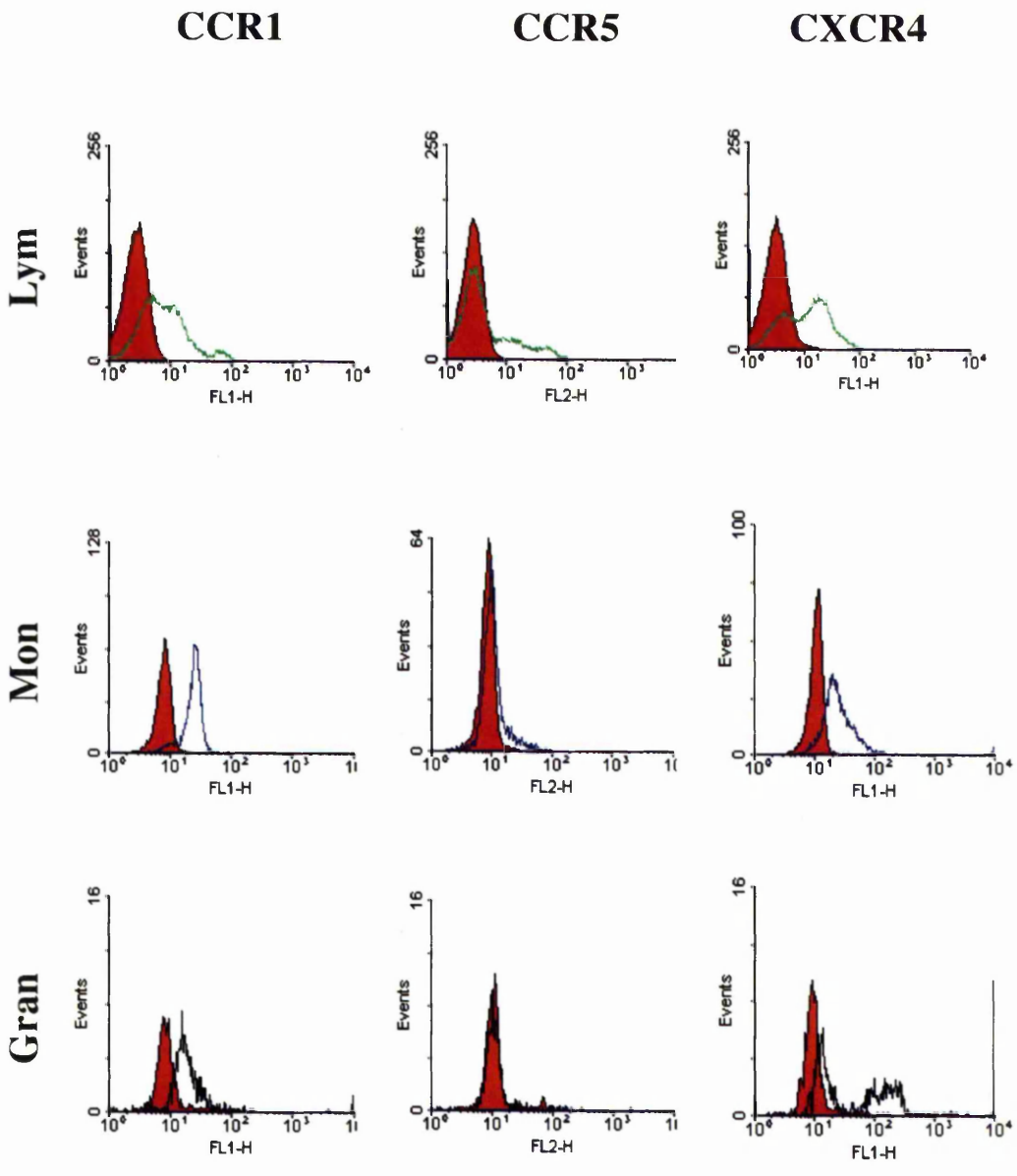


Figure 5.5. Expression of MIP-1α and SDF-1α receptors on PBMNC. MNC were isolated from peripheral blood by density gradient centrifugation, stained with antibodies to CCR1, CCR5 and CXCR4 receptors and subjected to FACS analysis. The histograms represent analysis of >3,000 events acquired in the lymphocyte (Lym, green line) and monocyte (Mon, blue line) regions and of 400-700 events acquired in the granulocyte (Gran, black line) region. The red peaks represent cells labeled with isotype matched antibody. Data from one representative experiment are presented.

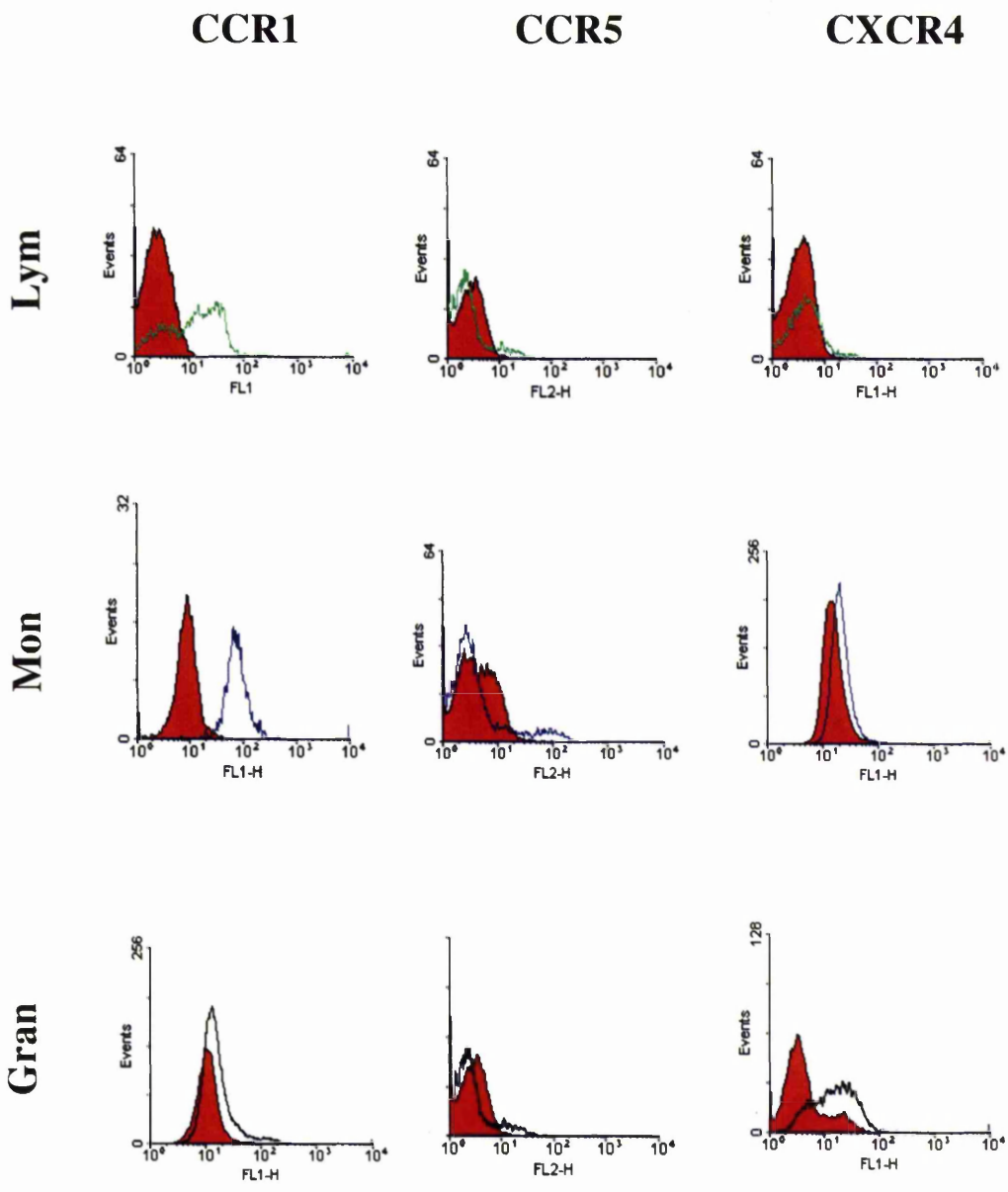


Figure 5.6. Expression of MIP-1α and SDF-1α receptors on BMMNC. MNC were isolated from bone marrow samples by density gradient centrifugation, stained with anti-CCR1, anti-CCR5 and anti-CXCR4 antibodies as described in section 2.1.7.6, and subjected to FACS analysis. The histograms represent analysis of 5,000 events acquired in the lymphocyte (Lym), monocyte (Mon) and granulocyte (Gran) regions as indicated. Data from one representative sample are shown.

cells (14.4%). More than 75% of these erythroid cells were mature haemoglobin-synthesising cells that stained positive for benzidine.

5.2.1.2.3. Cord blood

Cord blood mononuclear cells (CBMNC) obtained from full-term neonates were examined for the expression of the CCR1, CCR5, and CXCR4 receptors . The number of MNC recovered after density gradient treatment was $5.9x10^6 \pm 7.9x10^5$ cells/ml cord blood (n=11). As for PB and BM, CBMNC showed expression of the three chemokine receptors, and their overall expressions were: 72.0%±1.5 for CCR1, 1.6%±0.5 for CCR5, and 46.0%±6.7 for CXCR4. Gated sub populations showed high expression of CCR1: 57.1%±7.0, for lymphocyte, 72.7%±2.3, for monocyte, and 43.5%±5.0 for granulocyte gated cells. In contrast, CCR5 was weakly expressed, with 1.1%±0.21, for lymphocyte, 15.6%±3.3, for monocyte, and 1.3%±0.9 for granulocyte gated cells. CXCR4 was expressed by 43.5%±7.3, 51.5%±4.5%, and 26.1%±2.4 of the lymphocyte, monocyte and granulocyte gated cells, respectively. Figure 5.7 shows the expression of these receptors on the cell surface of CBMNC. Morphological analysis of MNC cells stained with May-Grunwald-Giemsa showed that CBMNC were composed of immature myeloid cells (comprising blasts, promyelocytes, and myelocytes, 1.6%), mature granulocytes (comprising metamyelocytes, band and segmented neutrophils, eosinophils, and basophils – 12.9%), lymphocytes (76%), monocytes (7.7%), and a few nucleated red blood cells (1.8%) (Figure 5.4-C).

Interestingly and worthy of note is that when CBMNC were gated in the lymphocyte region, a *doublet* for CCR1 expression was observed. This *doublet* is depicted in Figures 5.8-A1 and it is more detailed in Figure 5.9-A. This pattern of expression was consistent in all samples examined. It has been reported that density gradient solutions such as ficoll may interfere with the analysis of antigen expression by flow cytometry (Lee *et al.*, 1999b). In our studies, Lymphoprep solution was used to select MNC by density gradient centrifugation (section 2.1.7.4) from all samples. The use of density gradient solution is particularlly indicated in circumstances in which enriched populations of monocytes and lymphocytes are required (Robinson, 1993). Using this technical procedure, the presence of cell types other then monocytes and lymphocytes amongst the MNC recovered was often observed, including cells from the

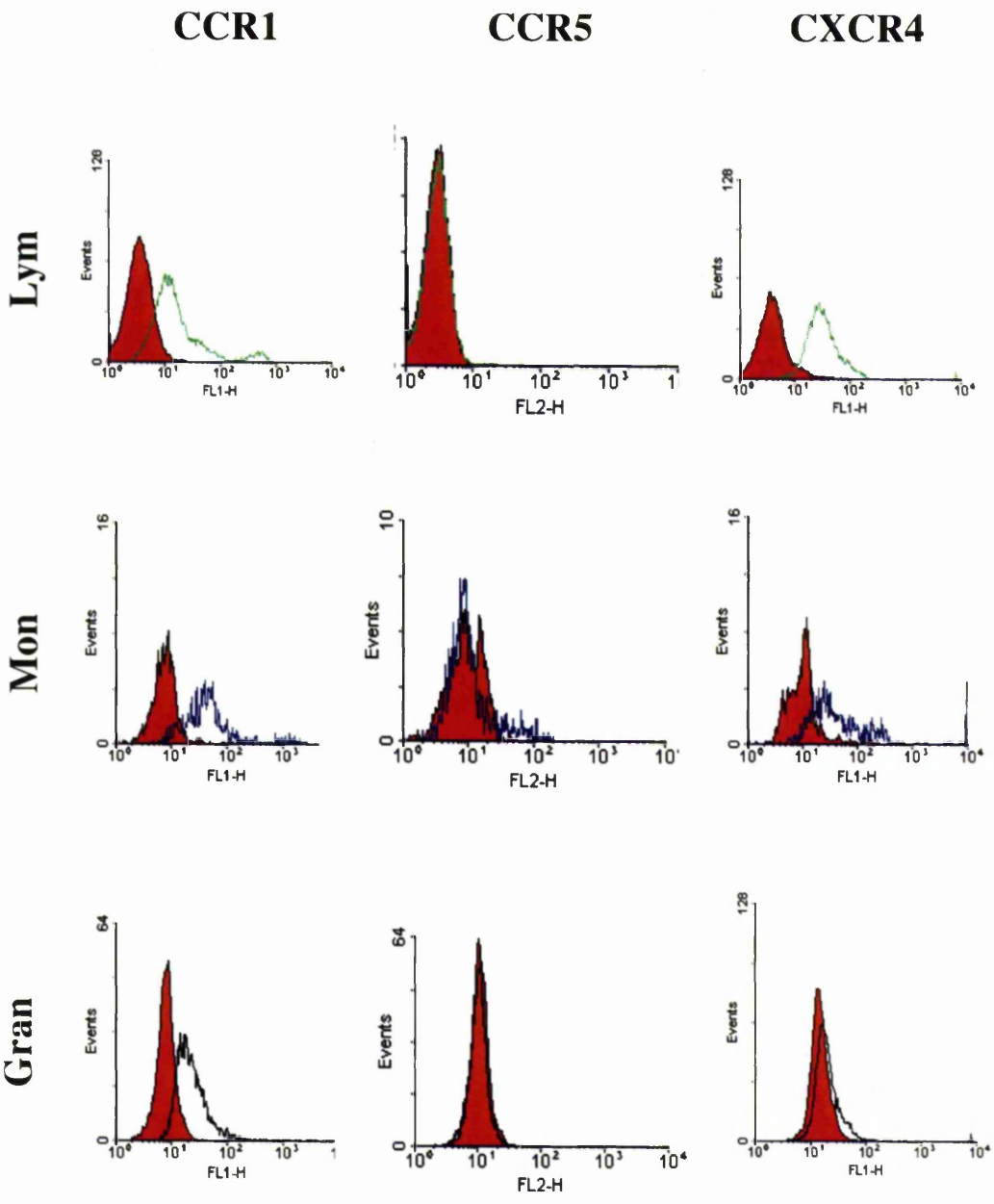


Figure 5.7. Expression of MIP-1α and SDF-1α receptors on CBMNC. MNC were isolated from cord blood by density gradient centrifugation, stained with antibodies to CCR1, CCR5 and CXCR4 receptors, and analysed by flow cytometry. The histograms represent analysis of >4,000 events acquired in the lymphocyte (Lym, green line), and granulocyte (Gran, black line) regions and of ~600 events for the monocyte (Mon, blue line) region. The red peaks represent cells labeled with isotype matched antibody. Data from a representative experiment are presented.

erythroid lineage. This could be confirmed by the morphological analysis of the MNC from PB, BM, and CB (Figure 5.4-A, B, and C, respectively). To investigate whether this *doublet* could be an artfact due to the density gradient separation procedure, we used 0.1% (w/v) methylcellulose to obtain CBMNC as described in section 2.1.7. This procedure can be used when red blood cells need to be depleted from the samples. Therefore, MNC collected from the supernatant were washed, labeled with anti-CCR1 antibody, and FACS analysed.

When comparing the two procedures, the number of cells present in each gate was different. As depicted in Figure 5.8, higher concentration of cells in the lymphocyte region (Figure 5.8-A, R1 and A1) was evident when using Lymphoprep solution whereas granulocyte gated cells were more abundant when using methylcellulose solution (Figure 5.8-B, R3 and B3). In contrast, the cell population present in the monocyte gate was not affected by any of the enrichment procedures (Figure 5.8-A, R2, and A2, or B, R2 and B2). Although different cell populations were selected by the two procedures, the expression of CCR1 on lymphocyte, monocyte and granulocyte gated cells were slightly but not significantly ($p>0.05$, n=4) decreased when methylcellulose treatment was used as can be observed by the reduction of the Mean Fluorescence Intensity (MFI) over the baseline values. With these results, it was clear that the density gradient solution was not affecting the CCR1 expression of these cells.

As the same *doublet* profile of CCR1 expression observed for density gradient separated MNC on lymphocyte gated cells was found when using methylcellulose (Figure 5.8- A1 and B1, respectively), we further investigated whether this phenomenon could be related to the heterogeneity and/or to a different stage of maturation of these cells. Thus, according to the intensity of CCR1 single labelling, we sorted the CBMNC cells into $CCR1^{dim}$, for low CCR1 binding population (Figure 5.9-A, R2), and $CCR1^{bright}$, for high CCR1 binding population (Figure 5.9-A, R3), following the gating/sorting strategy presented in Figure 5.9-B.

Morphological analysis of these two sorted populations using cytospin preparations stained with May-Grunwald-Giemsa showed that the $CCR1^{dim}$ population was composed of lymphocytes (59.6%±13.3), monocytes (35.6%±13.1), and a few granulocytes (4.8%±1.9) (Figure 5.9-C), whereas the $CCR1^{bright}$ cells mostly contained lymphocytes (98.4%±0.8) (Figure 5.9-D). To investigate the progenitors that might be present in these two populations, the $CCR1^{dim}$ and the $CCR1^{bright}$ sorted cells, along with

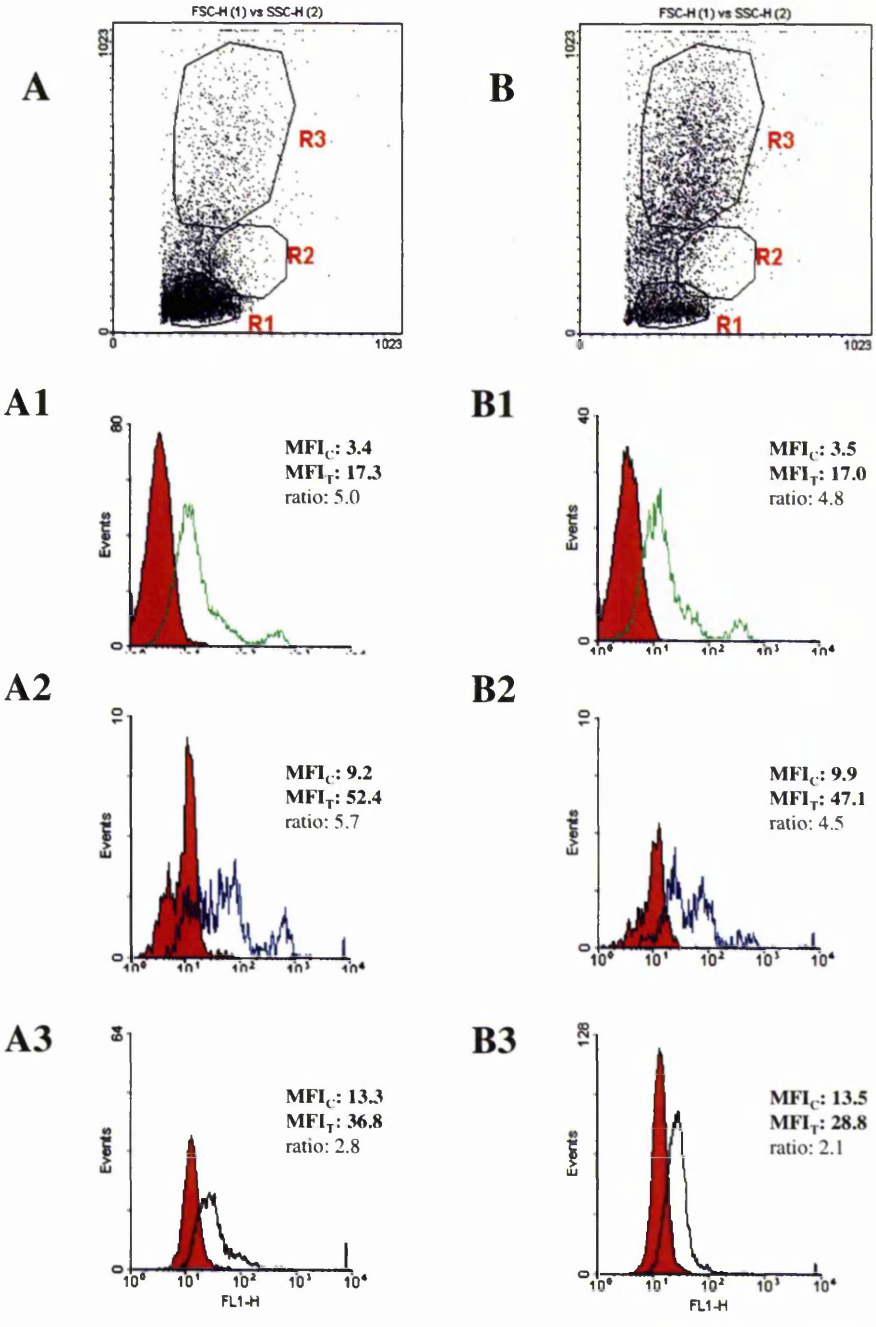


Figure 5.8. Levels of expression of CCR1 receptor on cord blood mononuclear cells (CBMNC) after density gradient centrifugation and methylcellulose treatments. CBMNC obtained using density gradient centrifugation (**A**) or sedimentation with 0.1% (v/v) methylcellulose (**B**) were labeled with anti-CCR1 antibody and FACS analysed, using the LYM (**R1**), MON (**R2**), and GRAN (**R3**) gates defined in a forward *versus* side scatter dot plots (**A** and **B**). Histograms represent analysis of 5000 events and indicate the fluorescence intensity (FL1-H) of LYM (**A1, B1**), MON (**A2, B2**), and GRAN (**A3, B3**) gated cells for anti-CCR1 (coloured lines). Red solid peaks denote control staining. The mean fluorescence intensities of the controls (MFI_C) and of the test samples (MFI_T) are indicated.

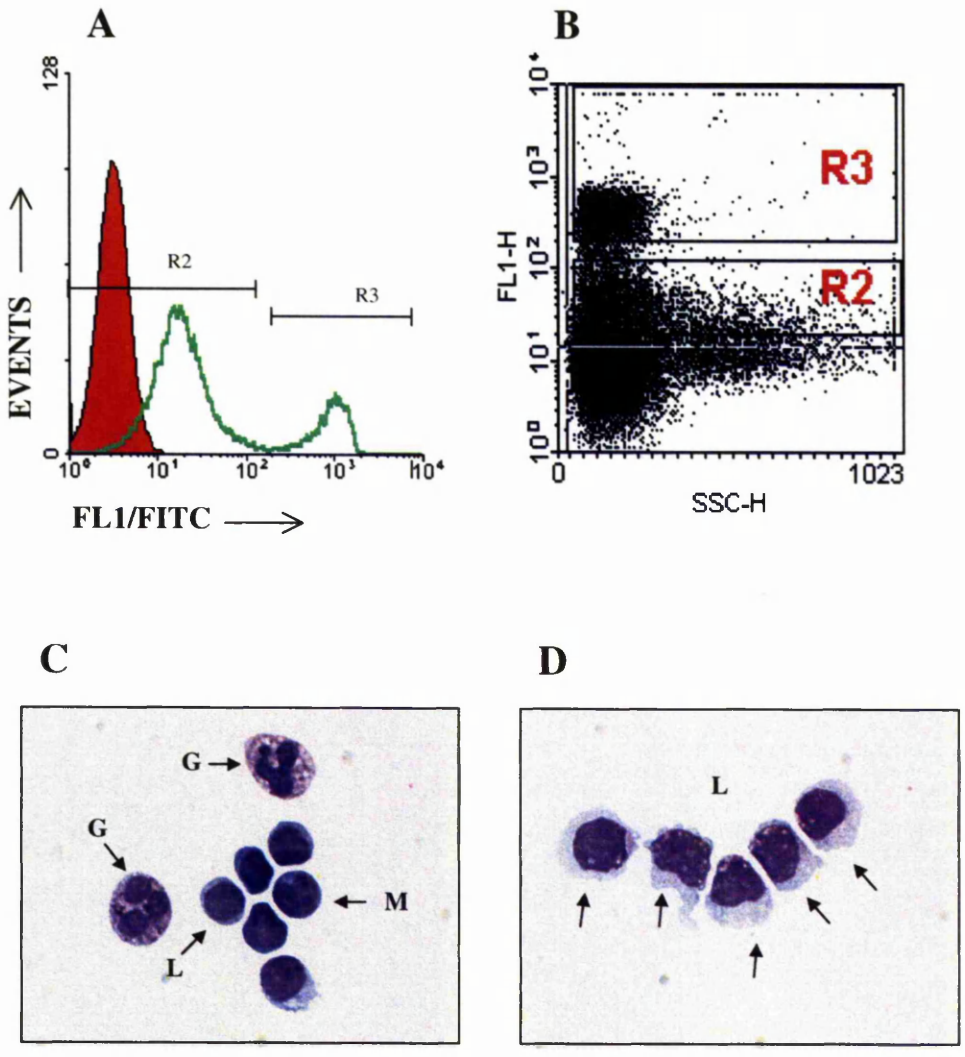


Figure 5.9. FACS sorting and morphological analysis of CBMNC cells binding to anti-CCR1 antibody. (**A**) Cord blood MNC labeled with anti-CCR1 antibody were FACS sorted as CCR1^{dim} (R2) and CCR1^{bright} (R3) according to the gating strategy showed in (**B**) and stained with May-Grunwald-Giemsa for morphological analysis. CBMNC-CCR1^{dim} cells (**C**) were composed of lymphocytes (L), granulocytes (G), and monocytes (M), while the CBMNC-CCR1^{bright} (**D**) cells were mostly composed of lymphocytes. The results are representive of one experiment from 4 performed under similar conditions.

the unsorted MNC, were plated in human multipotent progenitor cell assay (section 2.1.12.1). After 14 days incubation period, the unsorted MNC cells generated colonies of progenitors of both granulocyte/macrophage and erythroid lineages (40.6%±7.2 and 59.4%±7.2, respectively). The $CCR1^{dim}$ population also generated colonies of both lineages, of which 40.2%±4.6% were of the granulocyte/macrophage lineage, while 59.8%±4.6 belonged to the erythroid lineage. In contrast, only a few GM-CFC colonies was observed in the $CCR1^{bright}$ population (7.9%±6.5 of the unsorted input colonies). Thus, 92.1%±6.6 of the unsorted input colony-forming cell population was present in the $CCR1^{dim}$ fraction. These results indicated that most of the progenitors capable of generating colonies in soft gel systems found in the unsorted population were contained, after sorting, in the $CCR1^{dim}$ population.

We, then, further investigated the expression of CD34 antigen on the $CCR1^{dim}$ and $CCR1^{bright}$ populations. CBMNC were double stained for CCR1 and CD34 expression and FACS analysed. Indeed, as presented in Table 5.2, CD34 expression was found on both the $CCR1^{dim}$ and $CCR1^{bright}$ population, but CD34 expression was 2- to 22-fold higher on $CCR1^{dim}$ population than that observed for $CCR1^{bright}$. These results could in part explain the higher incidence of colony-forming cells present in the $CCR1^{dim}$ fraction. Because the CD34 antigen was detected on a small percentage of the $CCR1^{bright}$ cells and only a few GM-CFC colonies were present in this fraction, we questioned whether LTC-IC cells could be present in this population. LTC-IC cells have been described as the primitive haemopoietic cells that are responsible for the output of clonogenic progenitors after five or more weeks in culture on a haemopoietic supportive stroma (Ploemacher *et al.*, 1989; Sutherland *et al.*, 1989). Therefore, LTC-IC cells do not generate colonies when assayed directly into clonogenic assays but will generate them after being cultured for at least 5 weeks in the LTBMC system, where they proliferate and become eventually colony-forming cell progenitors capable of detection by soft gel systems.

Table 5.2. Expression of CCR1 and CD34 antigens on cord blood mononuclear cells (CBMNC) gated in the lymphocyte region.

experiment	CBMNC lymphocyte gated cells		$CD34^+$ lymphocyte gated cells (%)	
	CCR1 Expression (%)	$CCR1^+CD34^+$ expression (%)	CCR1 DIM	CCR1 BRIGHT
I	39.5	3.4	7.7	0.6
II	54.3	6.5	10.5	6.5
III	40.2	2.9	15.4	0.7
IV	16.7	2.0	10.6	3.3

CBMNC obtained after density gradient treatment were double stained with anti-CCR1 and anti-CD34 antibodies as described in section 2.1.7.6 and FACS analysed using the gating strategies described in Figure 5.4 for CCR1 expression, and Figure 5.11 for CCR1/CD34 expression. Results are the percentage of positively labeled cells for the indicated antigens.

To address this question, CBMNC cells were labeled with anti-CCR1 antibody, FACS sorted into $CCR1^{dim}$ and $CCR1^{bright}$ fractions and cultured in long-term bone marrow cultures as described in section 2.1.11.6. The results are presented in Figure 5.10. The number of cells weekly recovered from the supernatant of the $CCR1^{dim}$ cultures increased up to week 6 of the culture period, slowly declining thereafter (Figure 5.10-A). The number of colony-forming cells (CFC) generated in soft-gel system from this population increased after the second week of culture, declining subsequently up to week 4. At week 5, another increase in the number of CFC was observed (Figure 5.10-B). The first wave of CFC production, detected between weeks 2 and 4 of culture, is more likely to represent the progenitors already present in the input population. The second wave, between week 5 up to the end of the experiment, may represent the CFC produced by the LTC-IC population. At week 8, no progenitors were detected in the adheren layer cells after trypsin treatment, implying exhaustion of the system, thereby justifying the decline of progenitors observed at the end of the culture period. In contrast, a slow and progressive decline in the number of non adherent cells of the

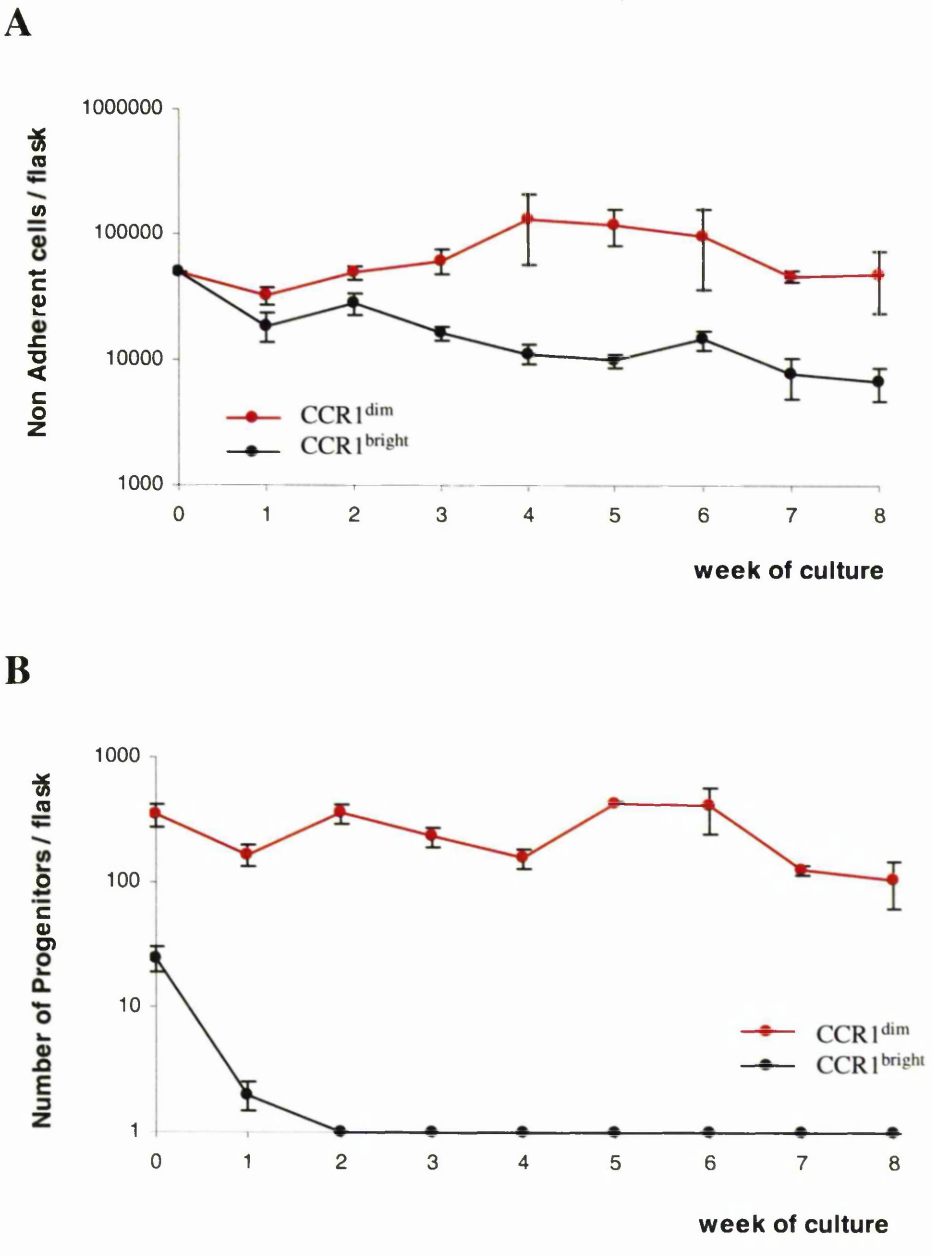


Figure 5.10. LTC-IC in CCR1^{dim} and CCR1^{bright} cells. Cord blood MNC labeled with anti-CCR1 were sorted as CCR1^{dim} and CCR1^{bright} fractions according to the gate strategy depicted in Figure 5.9-B. Sorted populations were plated in long-term cultures for evaluating their LTC-IC content (section 2.1.11.6). The number of cells harvested from the supernatant after the weakly feedings was enumerated and plated in clonogenic assays (section 2.1.12.1). Each data point represents the mean ± SEM of the number of cells (**A**) and progenitors (**B**) in culture supernatants as a function of time from 3 to 9 experiments.

$CCR1^{bright}$ population occurred after the second week of culture. Colony formation by this population showed similar pattern, with CFC detected only during the first two weeks of culture. No progenitors were further generated by either the non adherent cells from the weekly harvested supernatant or from the adherent cells released after trypsin treatment at the end of the esperiment (week 8). These data suggest that not only the majority of the cells with clonogenic potential, but also the LTC-IC population reside within the CBMNC expressing lower levels of CCR1.

5.2.1.3. $CD34^+$ haemopoietic cells

We have used the CD34 marker to select a population that contains the stem/progenitor cells in the BMMNC and CBMNC, and investigated the pattern of expression of the receptors CCR1, CCR5, and CXCR4 on the surface of these purified cells using flow cytometry. $CD34^+$ cells from bone marrow and cord blood MNC obtained after density gradient treatment were positively selected using the Mini-MACS™ immunoseparation system as described in section 2.1.7.5. The selected $CD34^+$ cells were labeled first with the anti-CCR1, anti-CCR5 or anti-CXCR4 antibodies followed by labelling with anti-CD34 antibody (section 2.1.7.6). Figure 5.11 shows the gating strategy of the two-colour flow cytometric analysis used to investigate the expression of these receptors on the surface of $CD34^+$ cells. The gating strategy involved setting a gate based on the forward *versus* low side scatter characteristics of the cells (R1), which are similar to that of the lymphocyte population (Carr and Rodak, 1999). The region boundary R1 was set to include all potential $CD34^+$ cells (Figure 5.11-A). Then, $CD34^+$ cells present in R1 were used to set a second gate, R2 (Figure 5.11-B). In order to determine the percentage of CD34 positive cells which also label with the antibodies for the MIP-1α and SDF-1α receptors, a third gate, R3, was specified to include the R1 (lymphocytes) and R2 ($CD34^+$ cells) regions. CCR1-FITC, CCR5-PE, and CXCR4-FITC expression on the $CD34^+$ cells were analysed using the R3 region (Figure 5.11-D). Cells stained with the isotype control were used to define any non-specific staining events and the quadrants were set to include ~99% of these events (Figure 5.11-C).

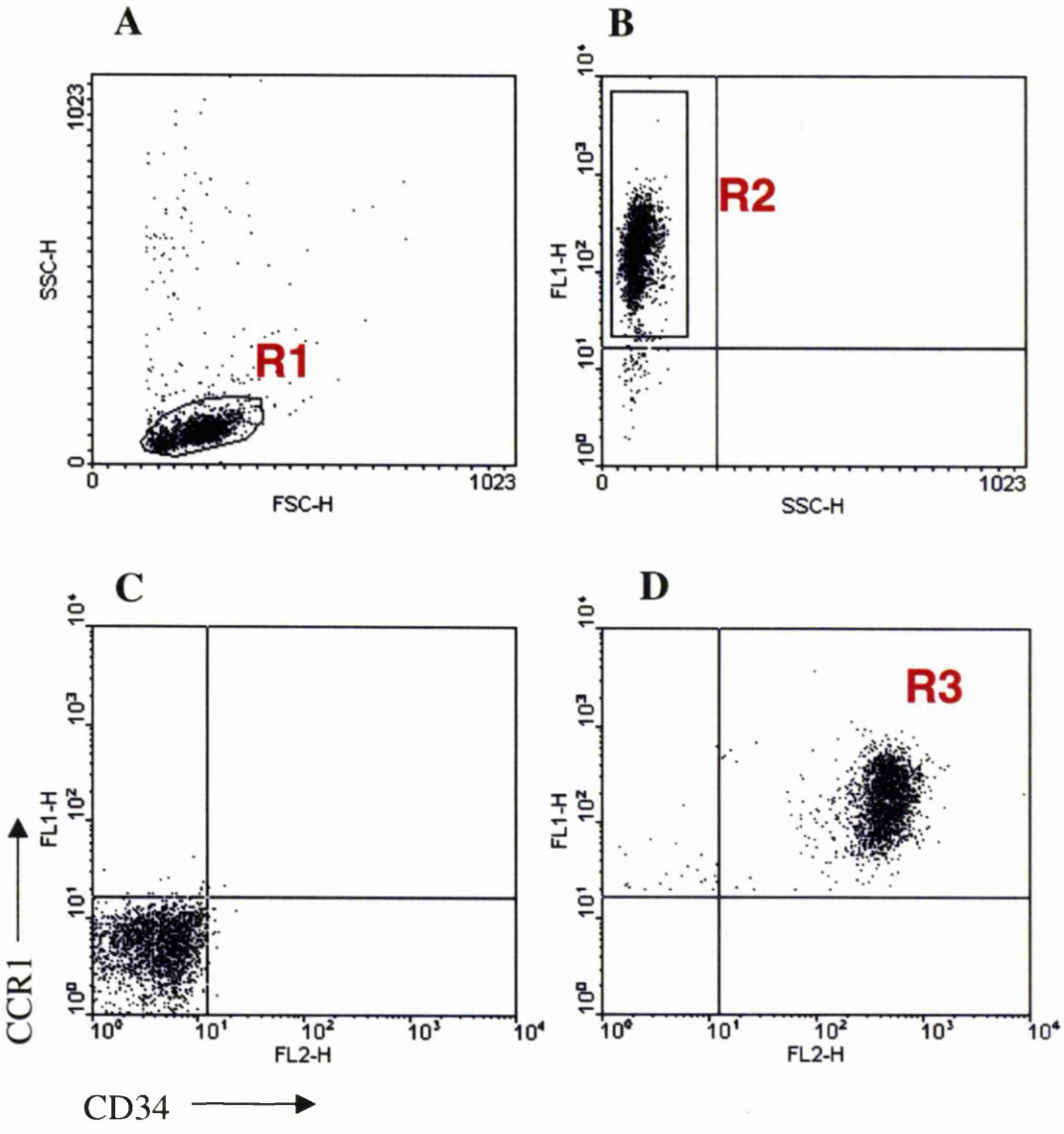


Figure 5.11. Gating strategy for flow cytometry analysis of chemokine receptors on isolated CD34⁺ cells. Two colour fluorescence staining with anti-CCR1/FITC and anti-CD34/PE antibodies from a representative experiment. (**A**) Forward *versus* low side scatter profile of the CD34 cells isolated from cord blood. The gate R1 was set around the lymphocytes region to select all potential CD34 cells; (**B**) CD34⁺ cells gated in the lymphocyte region (R2) were stained with isotype controls for PE (FL2) (**C**) and FITC (FL1); (**D**) CD34⁺ cells in specified region (R3), showing CD34-PE *versus* CCR1-FITC binding.

5.2.1.3.1. Bone marrow

The majority of BMCD34$^+$ cells gated in R3 and analysed by two-colour flow cytometry stained positively for CCR1 (90.7%±8.5, n=3), while CXCR4 was expressed by 30.3%±2.5 (n=3) of the BMCD34$^+$ cells. CCR5 was weakly expressed and its average expression was 4.6%±1.51 (n=4).

5.2.1.3.2. Cord blood

Flow cytometry of the R3 gated population showed that CBCD34$^+$ cells, as for BMCD34$^+$ cells, were clearly and distinctly stained with the anti-CCR1 antibody, with 95.5%±1.0 (n=6) of cells positively labeled. CXCR4 was also detected on the surface of the CBCD34$^+$ cells (71.2%±9.9, n=6), but its pattern of expression varied widely from sample to sample (range: 31.9% - 99.9%). The expression of the CCR5 receptor was also very low, with only 4.4%±1.1 (n=16) of cells staining. Figure 5.12 illustrates the pattern of expression of CCR1, CCR5, and CXCR4 receptors on CBCD34$^+$ cells analysed by flow cytometry of one representative experiment. Figure 5.13 shows an overview of the results of the expression of the chemokine receptors CCR1, CCR5, and CXCR4 detected by flow cytometry on the surface of all MNC sub populations and CD34$^+$ cells obtained from human steady state peripheral blood, bone marrow and cord blood cells used in this work.

5.2.2. CCR1 as a marker for distinguishing lineage committed progenitors

It was clear from the previous experiments that the CCR1 receptor has a level of expression which varies according to the haemopoietic cell population analysed, and to their ontogenic source. To identify the cell types within the sub populations that express CCR1, MNC from peripheral blood, bone marrow and cord blood samples were labeled with anti-CCR1 antibody and sorted according to the intensity of CCR1 expression into positive (CCR1$^+$) and negative (CCR1$^-$) populations. It is worth noting that the designation of cells as 'negative' for any given antigen is arbitrary, since the limit for cellular fluorescence detection is approximately 1,000 molecules per cell and depends upon the settings established for the analysis (Civin and Loken, 1987)(J. Barry, personal

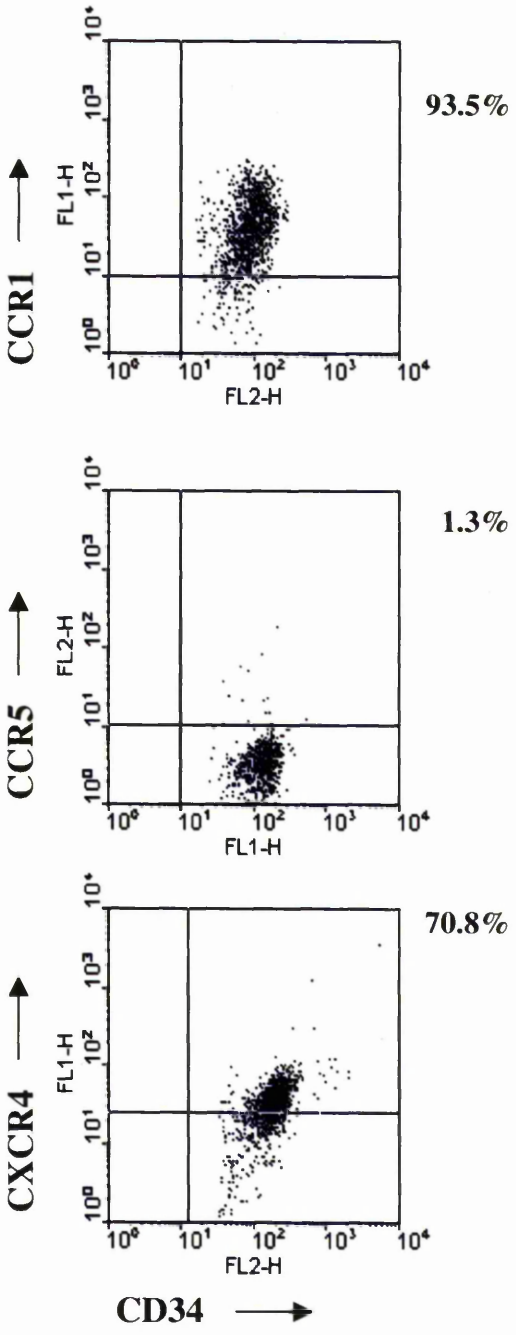


Figure 5.12. Flow cytometry analysis of CCR1, CCR5, and CXCR4 receptor expression on CD34⁺ cells from cord blood. CBCD34⁺ cells were double stained with PE or FITC antibodies for CD34 and CCR1, CCR5 or CXCR4, and analysed by flow cytometry using the gating strategy shown in Figure 5.11. Values represent the percentage of CD34⁺ cells positive for the indicated receptor. Data from one representative sample are presented.

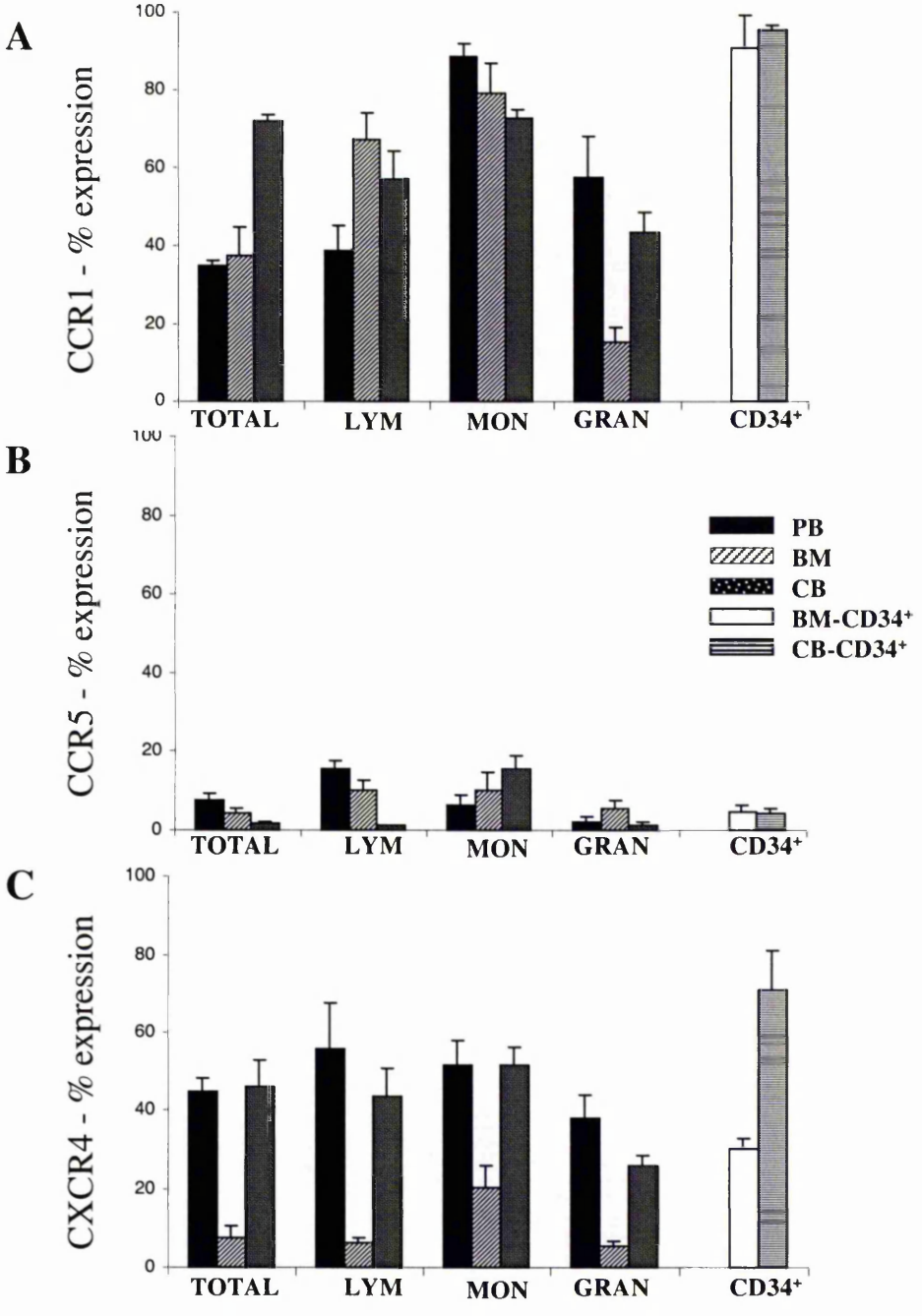


Figure 5.13. Expression of the chemokine receptors CCR1, CCR5, and CXCR4 on cell surface of mononuclear (MNC) and CD34⁺ obtained from peripheral blood (PB), bone marrow (BM) and cord blood (CB), detected by flow cytometry. MNC were labeled with anti-CCR1 (**A**), anti-CCR5 (**B**), and anti-CXCR4 (**C**) antibodies as described in section 2.1.7.6. One-colour FACS analysis was performed on the total cell population (**TOTAL**) and on the subsets gated as lymphocytes (**LYM**), monocytes (**MON**), and granulocytes (**GRAN**) using the gating strategy depicted in Figure 5.4-D and E. CD34⁺ cells were double labeled with anti-CCR1, anti-CCR5, and anti-CXCR4 antibodies followed by anti-CD34 and FACS analysed using the gating strategy detailed in Figure 5.11. The values represent the mean percentage ± SEM from 3 to 16 experiments performed under similar conditions.

communication). Thus, cells designated as "CCR1$^-$" might actually express, for example, 50 or perhaps 500 CCR1 molecules, but would not be distinguishable from cells which completely lack CCR1. The morphology of each sorted population was then analysed on cytospin preparations after May-Grunwald-Giemsa staining (section 2.1.13). Cord blood and bone marrow cytospin preparations were stained first with O'-Dianisidine as described in section 2.1.13.3 prior May-Grunwald-Giemsa staining. The results are detailed in Table 5.3.

Lymphocytes and monocytes were distributed in both CCR1$^+$ and CCR1$^-$ fractions from PB, BM, and CB. Monocytes were found mainly distributed in the CCR1$^+$ population from PB. The majority of mature cells of myeloid origin (band and segmented neutrophils) from PB were present in the CCR1$^+$ fraction. In BM, immature myeloid cells such as blasts, promyelocytes, and myelocytes were distributed in both fractions and metamyelocytes and band form neutrophils were more abundant in the CCR1$^-$ fraction. CB myeloid cells were found in both CCR1$^+$ and CCR1$^-$ populations. Immature and benzidine positive erythroid cells from BM were observed in both fractions, while CB erythroid cells were only found in the CCR1$^-$ cells. Basophils, although present in low numbers, were consistently found in the CCR1$^-$ fraction. Bone marrow plasmocytes were present in both CCR1$^+$ and CCR1$^-$ populations.

As CD34$^+$ cells from BM and CB have shown high expression of CCR1, we went on to investigate its expression on the surface of CD34$^+$ cells with clonogenic potential from these two sources. CD34$^+$ cells were labeled with anti-CCR1 antibody and sorted according to the strategy depicted in Figure 5.14. As CD34$^+$ cells have similar forward and side scatter characteristics to lymphocytes, in a forward *versus* side scatter profile, the gate R1 was set to select the lymphocyte population (Figure 5.14-A). Cells expressing CD34 were then gated as shown in Figure 5.14-B (R2). As the CCR1 expression on CD34$^+$ cells was usually high, and due to the difficulties of sorting enough cells as "negative" for CCR1 expression, we FACS sorted CD34$^+$ cells into CCR1^{high} and CCR1$^{-/low}$ fractions as demonstrated in Figure 5.14-D, R3 and R4, respectively. The two sorted cell fractions were then finally plated in a clonogenic assay (section 2.1.12.1).

After the 14 days incubation period, unsorted CBCD34$^+$ cells generated colonies of progenitors, of which greater than 65% were of the granulocyte-macrophage lineage and ~34% of the erythroid lineage. For BM, ~75% and 25% of progenitors were GM-CFC and erythroid progenitors, respectively. In contrast, colonies generated from the

Table 5.3. Morphological analysis of FACS sorted CCR1$^+$ and CCR1$^-$ cell populations obtained from peripheral blood, bone marrow, and cord blood mononuclear cells.

MORPHOLOGY OF MONONUCLEAR CELLS*	PERIPHERAL BLOOD		BONE MARROW		CORD BLOOD	
Erythroid lineage:	CCR1 +	CCR1 -	CCR1 +	CCR1 -	CCR1 +	CCR1 -
Pronormoblast	/	/	0.5	0.9	0	0
Basophilic normoblast	/	/	1.4	0.3	0	0
Polychromatic normoblast	/	/	3.4	2.4	0	0.4
Orthochromic normoblast	/	/	5	8.8	0	2.8
Benzidine positive	/	/	8.4	11.2	0	3.2
Myeloid lineage:						
Blast	/	/	1.9	1.7	0.4	0.5
Promyelocyte	/	/	0.5	0.2	0.2	0
Myelocyte	/	/	11.7	10.2	7.7	4.5
Metamyelocyte	/	/	2.3	15.7	0.3	0.5
Band neutrophil	9	0	3.1	22.2	7.4	6.5
Segmented neutrophils	18	2	8.4	8	13	13.5
Eosinophils	0	0	0	0.6	0.7	0.7
Basophils	0	3	0	0.4	0	0.7
Monocytes	36	1	5.9	2.6	4.5	0.7
Lymphocytes	37	94	53.3	25.7	65.8	72.1
Plasmocytes	0	0	2.6	0.3	0	0

Mononuclear cells obtained after density gradient centrifugation were labelled with anti-CCR1 antibody and sorted according to the intensities of CCR1 expression into CCR1$^+$ and CCR1$^-$ populations. Cytospin preparations of each sorted population were stained with O'-Dianisidine and May-Grunwld-Giemsa stainings and analysed for cell morphology. The values represent the mean percentage of each cell type after morphological examination of 300 to 600 cells/experiment in each sorted population of 3 independent experiments for peripheral blood and bone marrow samples, and 4 for cord blood. (*) Cells were classified according to the morphological characteristics of their maturational stage as described by Carr, J.H & Rodal, B.F. *Clinical hematology atlas*. Philadelphia, W.B. Saunders Co., 1999.

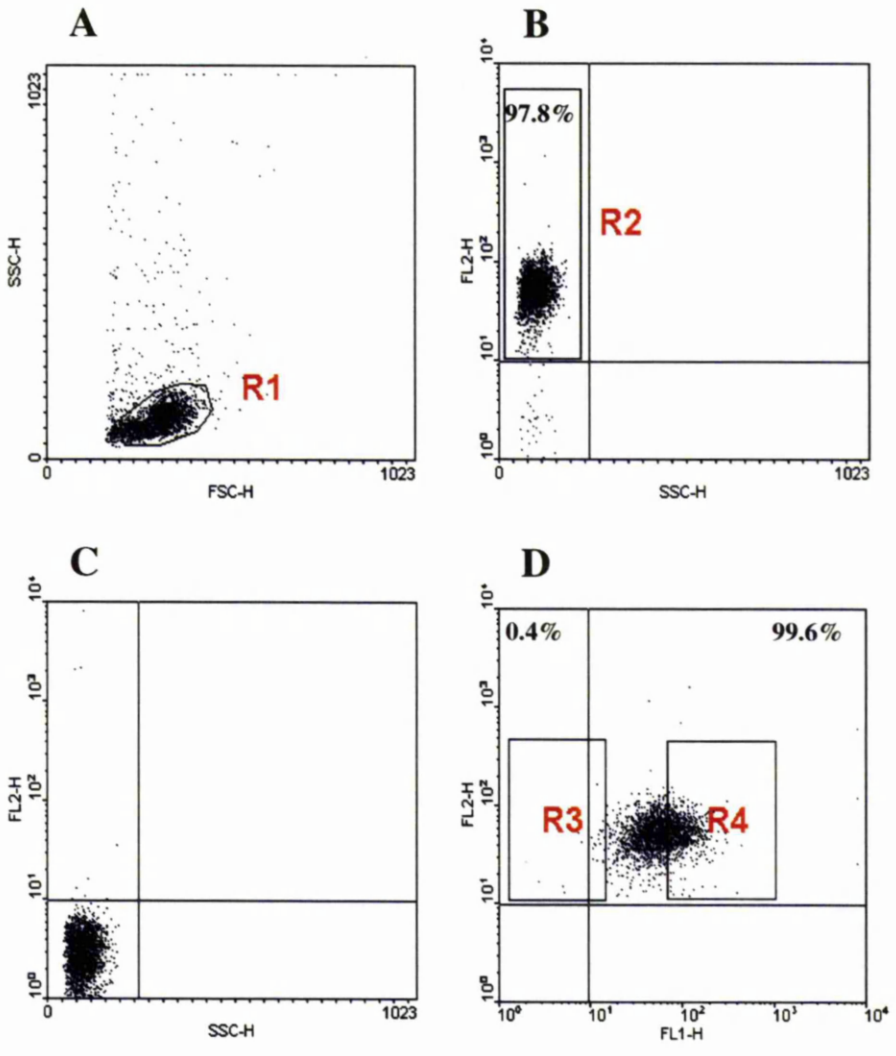


Figure 5.14. FACS sorting of CD34⁺ cord blood cells according to the expression of the CCR1 receptor. CD34⁺ cells isolated using the Mini-MACS procedure were incubated with anti-CCR1 antibody and the bound ligand detected by conjugating with swine anti-rabbit FITC antibody. CD34 cells were subsequently labeled with anti-CD34-PE antibody and sorted by FACS into $CD34^{+}CCR1^{high}$ and $CD34^{+}CCR1^{-/low}$ populations. (**A**) Forward *versus* side scatter profile of the potential $CD34^{+}$ cells in the lymphocyte region; (**B**) 97.8% of cells of the R1 gated population are $CD34^{+}$; (**C**) isotype control for FITC and PE staining used to include ~99% of the population under selection; (**D**) CD34-PE (FL2) *versus* CCR1-FITC (FL1) profile of the $CD34^{+}$ cells from the R2 region, which were sorted as $CCR1^{high}$ (R3) and $CCR1^{-/low}$ (R4) fractions.

CBCD34$^+$CCR1^{high} and BMCD34$^+$CCR1^{high} sorted fractions were enriched in more than 82 and 88%, respectively, for GM-CFC progenitors. The majority of the CBCD34$^+$CCR1$^{-/low}$ and of the BMCD34$^+$CCR1$^{-/low}$ fractions were composed of erythroid progenitors (77 and >89%, respectively). Table 5.4 shows the percentage distribution of total GM-CFC and BFU-E derived from both CCR1^{high} and CCR1$^{-/low}$ sub populations from CB and bone BM CD34$^+$ cells.

Table 5.4. Distribution of progenitors in the CCR1^{high} and CCR1$^{-/low}$ populations obtained from CD34$^+$ cells isolated from human cord blood and bone marrow samples.

CD34$^+$ cells		Number of progenitors (/2000 CD34$^+$ cells)		CFC in sorted populations(%)*	
		GM-CFC	BFU-E	GM-CFC	BFU-E
unsorted	CB	199 ± 28	102 ± 21	65 ± 6	35 ± 6
	BM	52 ± 10	24 ± 18	74 ± 15	26 ± 15
CCR1^{high}	CB	192 ± 32	37 ± 9	82 ± 5	18 ± 5
	BM	94 ± 11	11 ± 9	89 ± 8	11 ± 8
CCR1$^{-/low}$	CB	67 ± 21	174 ± 33	23 ± 6	77 ± 6
	BM	9 ± 3	110 ± 73	11 ± 5	89 ± 5

CD34$^+$ cord blood (CB) and bone marrow (BM) cells were sorted into CCR1^{high} and CCR1$^{-/low}$ cell fractions and plated in colony-forming assays as described in Materials and Methods. (*) Values represent the mean percentage ± SEM (or SD) of the distribution of the total GM-CFC and BFU-E in the indicated populations. (GM-CFC: granulocyte/macrophage colony-forming cells; BFU-E: burst forming units – erythroid). Cord blood results are from 11 experiments where control numbers for GM-CFC ranged from 52 to 328 and from 37 to 346 for BFU-E. Bone marrow results from 2 experiments where the control numbers for GM-CFC ranged from 43 to 62 and from 5 to 42 for BFU-E colonies/2000 cells.

The differences between the types of colonies generated by CD34$^+$CCR1^{high} and CD34$^+$CCR1$^{-/low}$ progenitor cells were easily discerned by direct observation of the

culture dishes (Figure 5.15-A1, and A2), where abundant small red dots of the erythroid colonies could be observed in the $CCR1^{-/low}$ population (Figure 5.15-A2). This was clearly demonstrated by centrifugation of the colonies from each fraction which gave a white pellet (Figure 5.15-B1) as a result of the $CCR1^{high}$ pooled colony cells whilst a bright red pellet was formed by the $CCR1^{-/low}$ colony cells (Figure 5.15-B2). Despite the differences in the number of colonies generated from $CCR1^{high}$ and $CCR1^{-/low}$ progenitor cells, the morphology and the size of their GM-CFC colonies were similar. In contrast, the majority of the erythroid colonies from $CCR1^{-/low}$ progenitors consisted of small but numerous reddish-orange clusters scattered around the dish (Figure 5.15-C2). The few $CCR1^{high}$ erythroid colonies were usually composed of larger clusters situated in closer proximity to one another (Figure 5.15-C1). Morphological analysis of the cells from $CBCD34^{+}CCR1^{high}$ pooled colonies after staining with O'-Dianizidine and May-Grunwald-Giemsa showed the presence of macrophages and granulocytes in different stages of maturation (Figure 5.15-D) while cells from $CBCD34^{+}CCR1^{-/low}$ pooled colonies were mostly of erythroid lineage, with occasional macrophages seen amongst them (Figure 5.15-E).

In addition, we investigated whether the expression profile of CCR1 in $CBCD34^{+}$ progenitors could change during their *in vitro* differentiation/maturation. $CBCD34^{+}$ cells sorted into $CCR1^{high}$ and $CCR1^{-/low}$ fractions were plated in clonogenic assay and, after 10-14 days of incubation, colonies generated from both sorted populations were pooled, their cellular content washed, labeled with anti-CCR1 antibody and FACS analysed for the expression of CCR1. CCR1 expression was detected on cells descendant from the $CD34^{+}CCR1^{high}$ population, but hardly observed on cells from the $CD34^{+}CCR1^{-/low}$ fraction as depicted in Figure 5.16, A and B, respectively. Because the cells within both $CCR1^{high}$ and $CCR1^{-/low}$ populations showed different levels of CCR1 expression, they were further sorted into $CCR1^{high}$ and $CCR1^{-/low}$ sub populations for morphological analysis. May-Grunwald-Giemsa staining of cytospin preparations of the cells from the $CBCD34^{+}CCR1^{high}$ colonies that had higher CCR1 expression (marker C, Figure 5.16-A) showed the presence of macrophages and granulocytes mainly at the differentiation stages of metamyelocyte and band form (Figure 5.16-C), while cells with lower CCR1 expression (marker E, Figure 5.16-A) were composed of mature granulocytes (segmented neutrophils) and benzidine positive cells, representing mature erythroid cells (Figure 5.16-E). Morphological analysis of the very few cells sorted from $CBCD34^{+}CCR1^{-/low}$ colonies having CCR1 expression (marker D, Figure 5.16-B) were

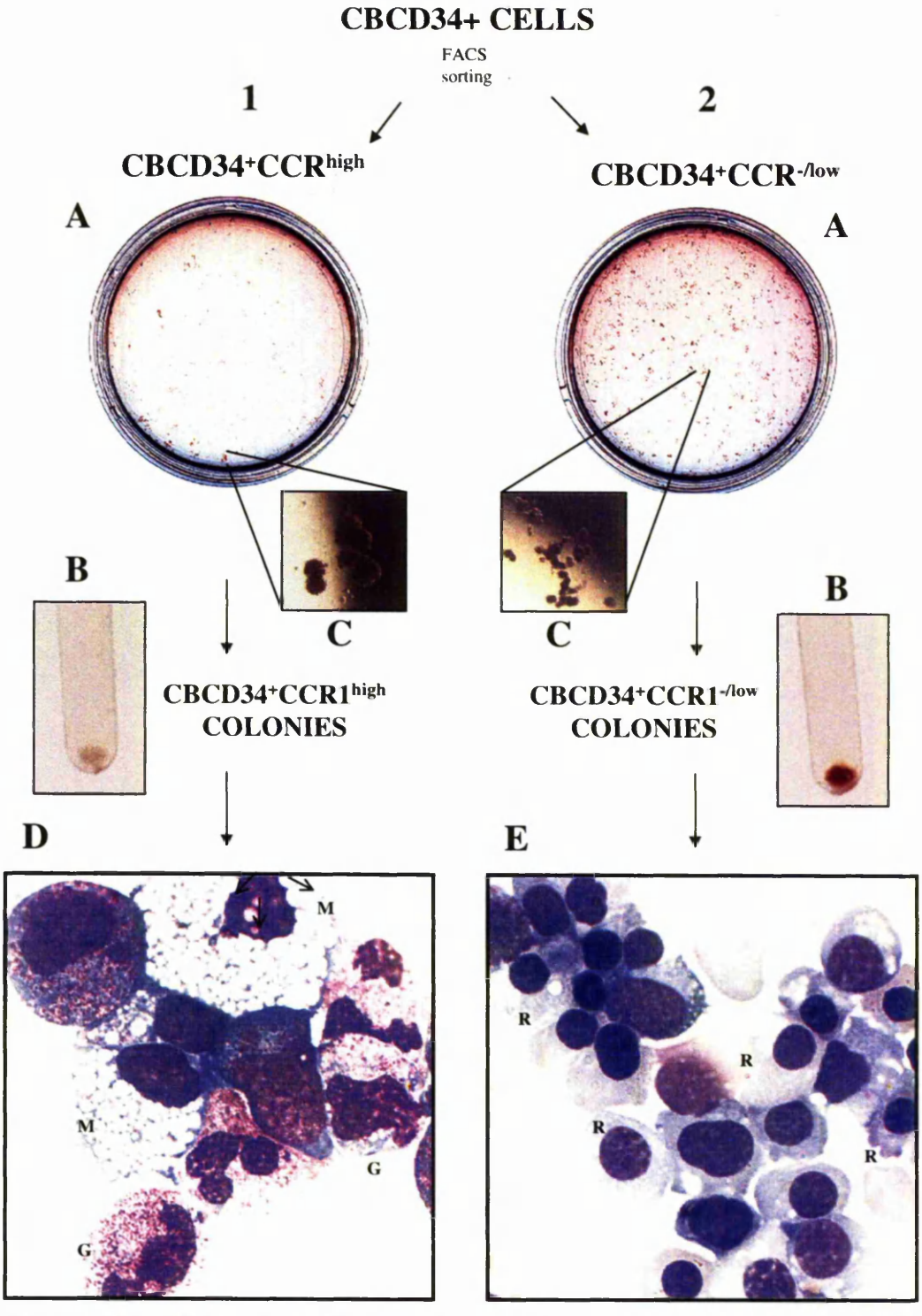


Figure 5.15. CCR1 selects different lineages of progenitors in cord blood cells. $CD34^+$ cord blood cells were sorted according to the expression of CCR1 into $CCR1^{high}$ (**1**) and $CCR1^{-/low}$ (**2**) fractions and cultured in clonogenic assays (**A**). Morphology of the erythroid colonies generated from the $CBCD34^+CCR1^{high}$ and $CBCD34^+CCR1^{-/low}$ fractions is described in the text (**B**). Grown colonies of each fraction were pooled (**C**), and their cell content stained with May-Grunwald-Giemsa. Morphological analysis of each population showed mainly the presence of macrophages (**M**) and granulocytes (**G**) in the $CCR1^{high}$ fraction (**D**). Erythroid cells (**R**) in different stages of maturation were present in the $CCR1^{-/low}$ fraction (**E**) (1000x magnification).

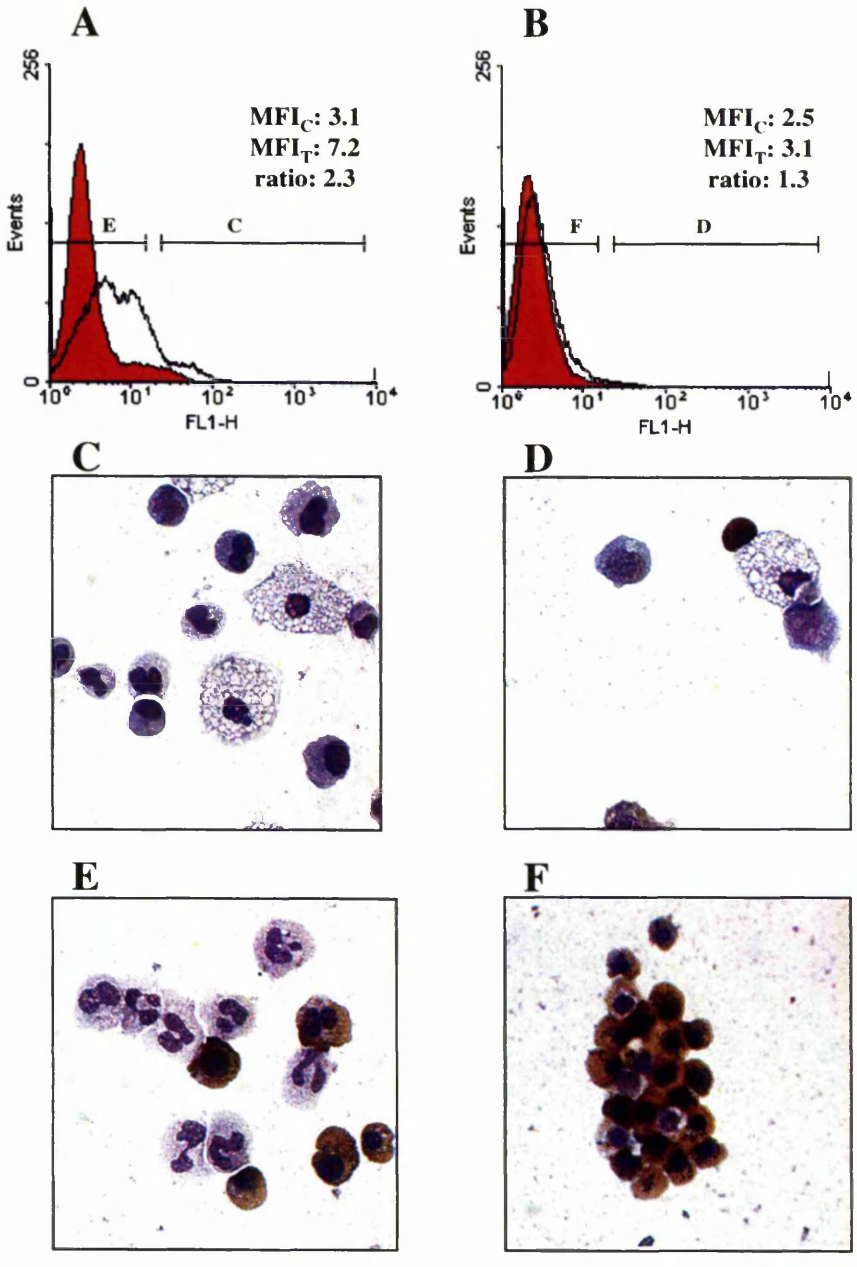


Figure 5.16. CCR1 expression during *in vitro* differentiation/maturation of CD34$^+$ cord blood progenitors. CBCD34$^+$ cells FACS sorted into 'positive' and 'negative/low' populations according to CCR1 expression were plated in clonogenic assays. The mature colonies of each population were pooled, their cells labeled with anti-CCR1 antibody and FACS analysed and sorted according to the expression of CCR1. Histograms depict CCR1 expression on cells from the CBCD34$^+$CCR1^{high} (**A**) and CCBCD34$^+$CCR1$^{-/low}$ (**B**) colonies. Macrophages and immature granulocytes (**C**) were present in the high CCR1 expression fraction (marker C) of the CBCD34$^+$CCR1^{high} colony cells while neutrophils and eryhtrocytes (**E**) were found in the CBCD34$^+$CCR1^{high} colony cells expressing low levels of CCR1 (marker E). Similar cell composition was found in the high (**D**) and low (**F**) CCR1 expressing cells from CBCD34$^+$CCR1$^{-/low}$ colony cells (see text for details). Mean Fluorescence Intensity for the isotype control (MFI$_C$) and for the test (MFI$_T$) are shown. (600x magnification)

composed of immature myeloid cells and macrophages (Figure 5.16-D), whilst the majority of the cells with '-/low' CCR1 expression (marker F, Figure 5.16-B) showed the presence of erythroid cells in different stages of maturation, with most of them positively stained for benzidine (Figure 5.16-F). These results suggested that flow cytometry could, according to the level of CCR1 expression on the surface of $CBCD34^+$ cells, select progenitor cells that were functionally different. Cells characterised and selected as 'high' for CCR1 expression according to the flow cytometry settings were able to give rise to colonies mostly of the granulocyte-macrophage lineage, while cells characterised as 'low' for CCR1 expression generated mostly erythroid colonies.

Our results for the expression of CCR1 on MNC and $CD34^+$ cells from bone marrow are in agreement with the expression of CCR1 on bone marrow-derived erythroid cells that has been recently reported (Su *et al.*, 1997). In contrast, according to the CCR1 sorting selection, erythroid cells from cord blood MNC were found only in the $CCR1^-$ fraction (Table 5.3). We then hypothesised that the level of expression of CCR1 on these MNC cells was below the limit of detection for flow cytometry. In order to clarify this issue, we have investigated the presence of the CCR1 antigen on MNC from CB using immunocytochemistry (IC) as an alternative procedure.

IC can be defined as a method of detecting molecular components within tissues or cells by microscopy using specific antigen-antibody reactions. The antibody's site within tissues or on cell surface is demonstrated by labelling it either with enzymes, fluorochromes or visible particles. The indirect method used in these studies (section 2.2.9) is particularly more sensitive than the direct method because further amplification can be achieved by using complexes of enzyme-anti-enzyme or other bridging methods such as avidin-biotin-enzyme complexes. As MNC from PB have shown high expression of CCR1 (Figure 5.13-A) we have used them as positive control for IC. Cytospin preparations of freshly isolated PBMNC and CBMNC were fixed with 4% (v/v) paraformaldehyde and labeled with the same rabbit polyclonal anti-CCR1 antibody used for flow cytometry analysis, and CCR1 expression was microscopically visualised by the peroxidase staining. Invariably, all nucleated cells present were stained for CCR1 in both PB (Figure 5.17-B) and CB (Figure 5.18-B) samples examined. Platelets were also positively stained for CCR1, showing for the first time that platelets express CCR1 on their surface (Figure 5.17-B, arrows). Worthy of note was also the finding that some of the non nucleated erythrocytes, bigger than the normal sized erythrocytes present in cord blood (Figure 5.18-B) showed a reticular pattern of staining

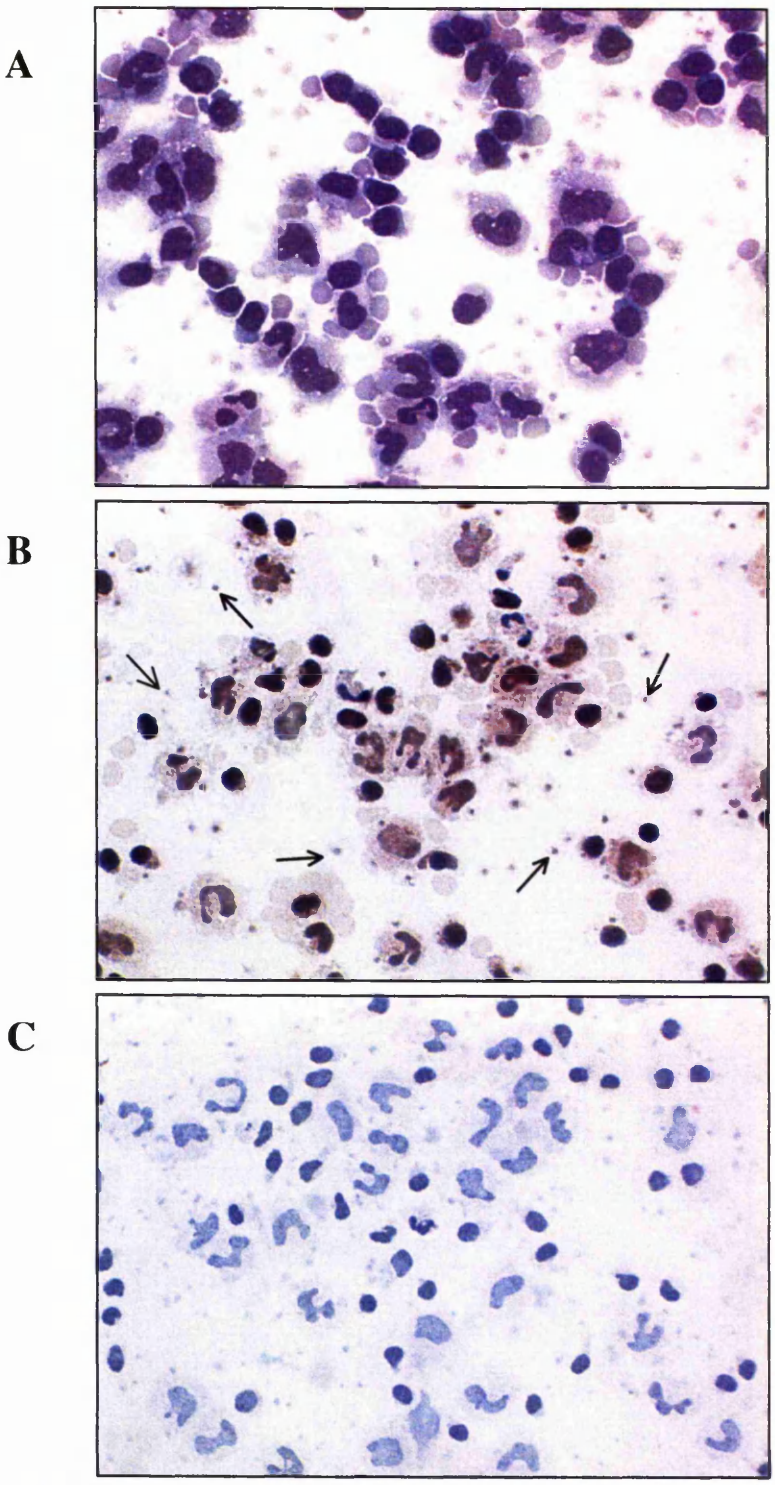


Figure 5.17. CCR1 antigen detection on peripheral blood mononuclear cells (PBMNC). Cytospin preparations of PBMNC were stained with May-Grunwald-Giemsa or immunostained with anti-CCR1 antibody as described in sections 2.1.13.2 and 2.2.8, respectively. Morphological analysis of cells showed the presence of monocytes, lymphocytes and granulocytes (**A**). Immunocytochemistry showed the presence of the CCR1 antigen on the cell surface of all nucleated cells and on platelets (arrows) (**B**) which were distinct from their negative controls (**C**) (400x magnification).

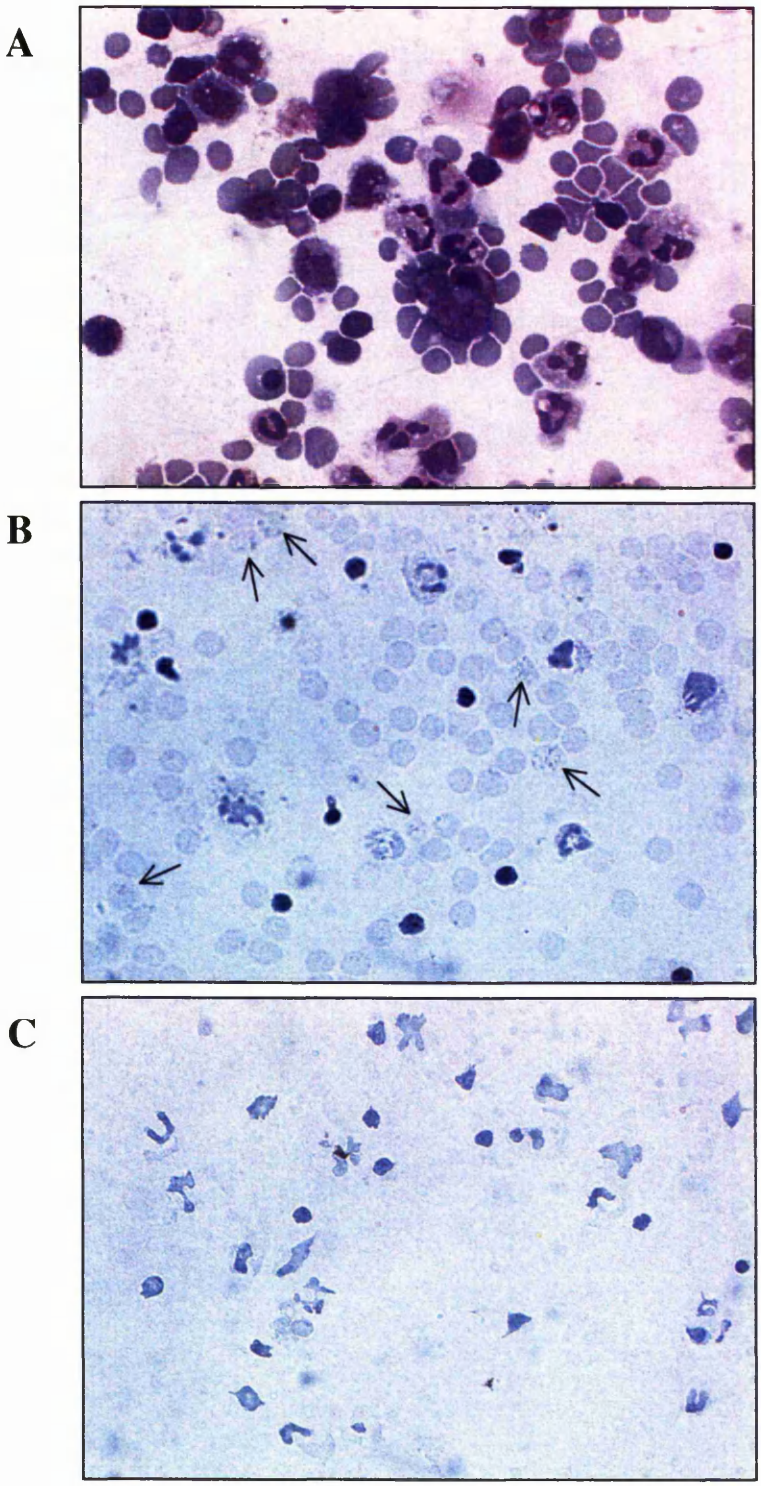


Figure 5.18. CCR1 antigen detection on cord blood mononuclear cells (CBMNC). Cytospin preparations of CBMNC were stained with May-Grunwald-Giemsa or immunostained with anti-CCR1 antibody as described in sections 2.1.13.2 and 2.2.8, respectively. Morphological analysis of cells showed the presence of immature myeloid cells, monocytes, lymphocytes and granulocytes (**A**). Immunocytochemistry showed the presence of the CCR1 antigen on the cell surface of all nucleated cells and on macroerythrocytes (reticulocytes/arrows) (**B**), which were distinct from their negative controls (**C**). (400x magnification).

which was not present on erythrocytes from PB samples. May-Grunwald-Giemsa staining of cord blood MNC cells showed that these erythrocytes were polychromatic (Figure 5.18-A), and probably represent circulating reticulocytes. Reticulocytes are immature non nucleated erythrocytes present in higher quantity in cord blood when compared to their values in adult peripheral blood or bone marrow (3-7%, 0.5-2%, and 1%, respectively) (Carr and Rodak, 1999; Lanzkowsky, 1999). Although at low levels as showed by the FACS data, these results suggest that erythroid cells express CCR1 on their surface throughout their differentiation/maturation, but loose it as the erythrocytes approach full maturation.

Using IC, we also investigated the expression of CCR1 antigen during the granulocyte-macrophage and erythroid differentiations *in vitro* using cytospin preparations of CBCD34^{+}CCR1^{high} and CBCD34^{+}CCR1$^{-/low}$ colony cells (Figure 5.19). Cells bearing the surface CCR1 antigen were observed in both CCR1^{high} (Figure 5.19-C) and CCR1$^{-/low}$ populations (figure 5.19-D). Morphological analysis of these two populations stained with May-Grunwald-Giemsa showed their distinct cell composition as macrophages and neutrophils in the CCR1^{high} population (Figure 5.19-A), and erythroid cells in different stages of differentiation in the CCR1$^{-/low}$ population (Figure 5.19-B), confirming previous findings. All together, these results suggest that both erythroid- and granulocyte-macrophage-derived cells express CCR1 on their surface throughout their differentiation/maturation pathway, but the level of CCR1 expression is different for each lineage. They also indicate that IC is a more sensitive procedure than flow cytometry for detecting CCR1, particularly in populations expressing very low levels of this receptor.

5.3. Discussion

Until recently, only a few techniques such as binding studies or mRNA analyses were used to investigate the expression of chemokine receptors. Radioactively labeled ligands in binding studies have shown the presence of high (Avalos *et al.*, 1994; Graham *et al.*, 1993; Wang *et al.*, 1993; Yamamura *et al.*, 1992) and low (Gao *et al.*, 1993; Neote *et al.*, 1993; Oh *et al.*, 1991) affinity receptors for MIP-1α in different murine (Graham *et al.*, 1993) and human (Avalos *et al.*, 1994) leukaemia haemopoietic cell lines. These binding studies address the chemokine-receptor interaction more

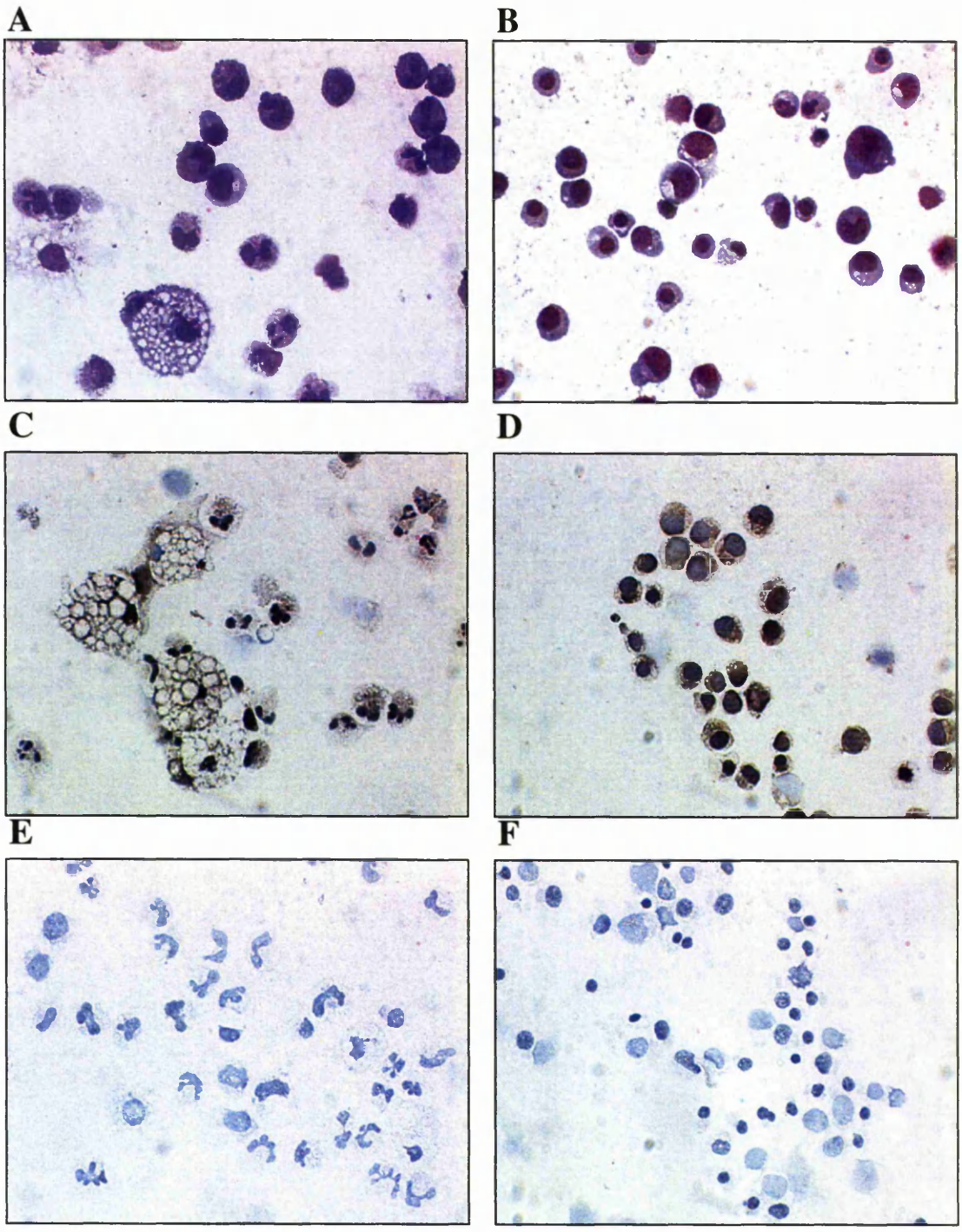


Figure 5.19. CCR1 antigen detection on colony cells from CCR1^{+} and CCR1$^{-/low}$ sorted cord blood CD34^{+} cells. Cord blood CD34^{+} cells labelled with anti-CCR1 antibodies and sorted into CCR1^{+} and CCR1$^{-/low}$ fractions were plated in clonogenic assay and incubated for 14 days. Cytospin preparations of cells from CCR1^{+} and CCR1^{-} colonies were stained with May-Grunwald-Giemsa and immunostained with anti-CCR1 antibody as described in sections 2.1.13.2 and 2.2.8, respectively. Morphological analysis of CCR1^{+} cells showed the presence of macrophages and neutrophils (**A**) while CCR1$^{-/low}$ cells were composed of erythroid cells in different stages of maturation (**B**). Immunocytochemistry showed the presence of the CCR1 antigen on the cell surface of both CCR1^{+} (**C**) and CCR1$^{-/low}$ (**D**) cells, which were distinct from their negative controls (**E** and **F**, respectively). (400x magnification).

directly but necessitate chemical modification of the chemokine by iodination or other label. Reverse transcriptase-polymerase chain reaction (RT-PCR) has been used to demonstrate the presence of mRNA of a variety of chemokine receptors in different human cell lines (Deichmann *et al.*, 1997; Feng *et al.*, 1996; Herzog *et al.*, 1993; Samson *et al.*, 1996) as well as in human primary cells (de Wynter *et al.*, 1998). Although this technical approach demonstrates the presence of transcripts for a specific receptor, it does not reflect its presence at the protein level. The use of biotinylated cytokines to study the cytokine receptor expression pattern by flow cytometry on different subsets of human haemopoietic cells has been successfully demonstrated in recent reports (Chasty *et al.*, 1995; de Wynter *et al.*, 1998; Durig *et al.*, 1998; Durig *et al.*, 1999a; Wognum *et al.*, 1990; Wognum *et al.*, 1994). However, this technique did not identify the specific sub-types of receptors present. Although the use of biotinylated molecules and demonstration of mRNA transcripts, particularly in small cell populations, remain powerful tools to study the expression of receptors, they have some limitations. The most important limitation of these techniques is that they do not preserve the cells for further studies such as functional assays or cell culture.

Recently, a range of specific antibodies to detect cell surface chemokine receptor proteins by flow cytometry has become available. As such, flow cytometry has become a valuable tool for the elucidation of the mechanisms that govern chemokine functions as observed in calcium efflux and chemotactic assays, actin polymerisation, modulation of receptor expression by cytokines, *etc* (Murphy, 1996). Flow cytometry is an automatic counting method that uses some cell characteristics such as size and internal structure complexity as discriminating elements for their analysis. The fluorescent-activated cell sorter, or FACS, is an instrument based on flow cytometry that can select cells that are labeled with an antibody, which is in turn linked to a fluorescent dye. The labeled cells can then be conveniently gated and separated from other cells when they emit fluorescence in the FACS. A sequential gating is usually required in order to select the population of interest and simultaneously minimise interference from debris and cells to which antibodies can bind non-specifically (Macey, 1994; Shapiro, 1985). The major advantage of this technique is that the cells sorted are preserved, and can be used in a variety of functional assays including cell culture.

In this study, we have combined the high specificity of some antibodies with the flow cytometric methodology to investigate the expression of the MIP-1α, CCR1 and CCR5 receptors, and the SDF-1α CXCR4 receptor on specific haemopoietic cell lines

and on primary haemopoietic cells obtained from PB, BM, and CB. To investigate the presence of these receptors on the surface of these cells, the following antibodies were used: for CCR1, we used a polyclonal antibody obtained from immunised rabbits with the NH_2-terminal portion of the receptor, which has been shown to be specific for CCR1 (Su *et al.*, 1996). In addition, the specificity and binding characteristics of this anti-CCR1 antibody to human haemopoietic cells have been recently checked and confirmed (de Wynter, 2000). For CCR5, we used the anti-CCR5 PE-conjugated antibody derived from the clone 2D7. It has been reported that the monoclonal antibody 2D7 binds to a much greater fraction of cell surface CCR5 molecules than any of the 18 other monoclonal antibodies tested. This suggests that its binding site is present and accessible on a large fraction of CCR5 molecules (Lee *et al.*, 1999b). Moreover, analysis of the antigenic structure of CCR5 has shown that the epitopes recognised by 2D7 monoclonal antibody overlap with the chemokine binding sites (Doranz *et al.*, 1999; Wu *et al.*, 1997). The cell surface expression of CXCR4 was investigated using the monoclonal antibody anti-CXCR4 clone 12G5, the binding site of which has been reported to recognise and bind specifically to the SDF-1α epitope on the CXCR4 receptor (Endres *et al.*, 1996).

As commonly observed when primary cells obtained from different donors are used for experiments, a high degree of assay variability cannot be avoided. Cell lines have been useful in studying gene expression and the modulation of haemopoietic cell proliferation and differentiation. Cell lines may have different degrees of commitment depending on the 'stemness' or precursor *status* of the cells from which they were derived. But unless the correct environmental conditions are re-established and proliferation is encouraged, they will remain at the same positions in the lineage. Therefore, we have used the well established human leukaemic cell lines HL60, K562, Jurkat, THP-1, and TF-1 not only to characterise the binding capacity of the chosen antibodies, but also to investigate the chemokine receptor expression on these cells in more detail using flow cytometry.

Each one of these cell lines is already committed to one of the haemopoietic lineages. HL60 cells are derived from peripheral blood leukocytes of a patient with acute promyelocytic leukaemia (Gallagher *et al.*, 1979), therefore committed to the myeloid lineage. The K562 cell line is derived from a CML patient and is composed of undifferentiated blast cells that are rich in glycophorin and may be induced to produce fetal and embryonic hemoglobin in the presence of haemin, leading to differentiation

down the erythroid lineage (Koeffler and Golde, 1980). Jurkat cells are derived from a human T cell leukaemia (Gillis and Watson, 1980), thus belonging to the lymphoid lineage. THP-1 and TF-1 cells are both derived from patients with acute monocytic leukaemia (Tsuchiya *et al.*, 1980), but only TF-1 cells are growth factor dependent for survival (Kitamura *et al.*, 1991). Both of these latter cell lines are committed to the monocytic lineage. Therefore, using these cell lines we also investigated the pattern of expression of the CCR1, CCR5, and CXCR4 receptors in different cell subsets that are related to the myeloid, erythroid, monocytic, and lymphoid subsets of haemopoietic cells. This would provide an overview of the expression of these receptors on a wide *spectrum* of cell types of the haemopoietic system.

Using flow cytometry, all cell lines tested in this study showed high expression of CCR1 (Table 5.1 and Figures 5.1 and 5.2), in agreement with other reports of MIP-1 chemokine receptor expression on these cells lines. Although the identity of the receptors was not known, expression of high and low affinity receptors for MIP-1α on HL60 (Avalos *et al.*, 1994; Gao *et al.*, 1993; Yamamura *et al.*, 1992), Jurkat (Avalos *et al.*, 1994; Yamamura *et al.*, 1992), and THP-1 cells (Avalos *et al.*, 1994; Gao *et al.*, 1993; Hunter *et al.*, 1995) has been reported. TF-1 and THP-1 cells have also been demonstrated to bind significant levels of biotinylated MIP-1α by flow cytometry (Nicholls *et al.*, 1999). As already described for the promyelocytic leukaemic cell line HL-60, the CML K562 cells have also been shown to constitutively express high levels of CCR1, providing the first evidence for the expression of at least one specific MIP-1α receptor on these cells.

Unlike the clear expression of CCR1, none of the cell lines tested showed expression of CCR5. In a recent report, using Northen blot hybridisation, HL60 and K562 cell lines were also found to be negative for CCR5 mRNA expression (Samson *et al.*, 1996). We were unable to demonstrate CCR5 expression even on the Jurkat cell line that has recently been reported to express CCR5 (Lee *et al.*, 1999c; Shen *et al.*, 1999). This discrepancy between our results and of the others may be due to the sensitivity of the methodology employed. Lee and colleagues have used QFACS (Quantitative Fluorescent Activated Cell Sorting) to detect cell surface expression of CCR5, while Shen and colleagues have used a immunomagnetic bead rosette assay. The former technique quantifies the number of antigen binding sites on a cell surface using a fixed number of labeled microbeads while the later uses antibody-conjugated beads that form rosettes with the positive cells, enhancing the ability to detect antibody binding by

microscopy. We have used flow cytometric analysis, which analyses each cell individually, but expresses the results for the entire population analysed. As it measures physical number of cell surface molecules, the number of receptors expressed on the cell surface of Jurkat cells may be under the threshold of detection using this technique.

The SDF-1α receptor CXCR4 was highly expressed on THP-1 and Jurkat cells (Table 5.1 and Figure 5.3). These cell lines have been reported to express CXCR-4 transcripts (Loetscher *et al.*, 1994) and CXCR4 cell surface receptors detected by flow cytometry (Hori *et al.*, 1998; Majka *et al.*, 2000b). In contrast, this receptor was not detected on TF-1 cells in agreement with a recent report (Majka *et al.*, 2000b). Although variable levels of expression of CXCR4 from experiment to experiment were observed (range 33.1 to 89.1%), CXCR4 was expressed on HL60 cells, in agreement with high levels of its mRNA (Deichmann *et al.*, 1997; Feng *et al.*, 1996; Gupta *et al.*, 1999; Herzog *et al.*, 1993; Loetscher *et al.*, 1994), and cell surface expression that have been reported recently (Hori *et al.*, 1998; Majka *et al.*, 2000b; Mohle *et al.*, 1998). In K562 cells, CXCR4 had a more restricted pattern of expression, ranging from 1.7 to 20.8% (mean 8.1%±4.4%, n=4).

This high variation observed for CXCR4 receptor expression on HL60 and K562 cells may be in part related to the culture conditions in which cell lines are maintained. The cell lines analysed in this study were obtained from different sources, and in some cases, they were not subcultured under restricted protocols. We observed, for example, that TF-1 cells only expressed CCR1 receptors at high levels after at least four passages, with the cells being routinely subcultured at 10^5cells/ml every three days. Thus, the culture conditions in which the cell lines are kept may affect the expression of the chemokine receptors analysed. In summary, the haemopoietic cell lines used in these studies expressed CCR1 as well as CXCR4. Thus, they can be used as a model for CCR1 and CXCR4 regulation. Moreover, not only can these cell lines be used to follow expression of these receptors, but they can also be useful for studying receptor antagonists as they become available particularly for chemokines. Also, these cell lines can be used for detailed studies of gene regulation and signal transduction for CCR1 and CXCR4.

Next, we examined the distribution of CCR1, CCR5 and CXCR4 receptors on MNC and $CD34^+$ cells obtained from PB, BM and CB. Although with some variability on the expression level, CCR1 was detected by flow cytometry in all MNC subsets examined (Figure 5.13-A) and its presence was confirmed by positive immunostaining

of PB and CB cells (Figures 5.18 and 5.19, respectively). Monocyte gated cells demonstrated the highest levels of CCR1 expression, and the presence of monocytes was confirmed, particularly in PBMNC, by morphological analysis of $CCR1^+$ sorted MNC (Table 5.3). These results are in agreement with the high levels of CCR1 expression on monocytes reported by others (Su *et al.*, 1996). Also, they support the concept that human monocytes constitutively express CCR1 and that chemotaxis (Su *et al.*, 1996; Wang *et al.*, 1993) and signalling responses (Rollins *et al.*, 1991) of some ligands such as MIP-1α can be mediated through CCR1.

CCR1 was also expressed on lymphocyte gated cells from all sources, particularly from CB. Morphological analysis of sorted MNC populations showed presence of lymphocytes in both $CCR1^+$ and $CCR1^-$ fractions from PB, BM and CB (Table 5.3). It has been reported that activated $CD4^+$ and $CD8^+$ T cells migrated towards MIP-1α (Taub *et al.*, 1993) and that most of the lymphocytes bearing the CD3, CD4, CD8, and CD16 antigens were stained with anti-CCR1 antibodies, whereas $CD19^+$ was not (Su *et al.*, 1996). This indicates that T cells and NK cells, but not B cells, express CCR1 on their surface. This also may explain our morphological findings, where lymphocytes were found in both positive and negative CCR1 populations (Table 5.3). Sorting cells double stained for CCR1 and lymphocyte-specific subset antibodies can specify which lymphocyte subsets express CCR1.

Furthermore, CCR1 expression on CB lymphocyte gated cells has shown different binding patterns (Figure 5.9-A). Based on these findings, we have sorted CBMNC according to the CCR1 expression intensity as $CCR1^{dim}$ and $CCR1^{bright}$ fractions. As a result, we have separated a population from the CBMNC-$CCR1^{dim}$ fraction that is composed of LTC-IC and able to sustain haemopoiesis for several weeks when seeded in long-term cultures as well as more differentiated progenitor cells able to generate colonies of granulocyte/macrophage and erythroid progenitors when plated directly in clonogenic assays. Together, these results indicate that the different reactivity of the anti-CCR1 antibody used and detected by flow cytometry can be related to the stage of maturation of the cells, with immature precursors exhibiting lower labelling when compared to less immature progenitor cells within a given population.

As for monocyte and lymphocyte gated cells, cells present in the granulocyte region in all samples examined showed some degree of CCR1 expression. More than 56% and 43% of the gated granulocytes from PB and CB, respectively, stained with anti-CCR1 antibody, in contrast to only 15% found in BM granulocyte region (Figure

5.13-A). Morphological analysis of unsorted myeloid cells from PBMNC showed that they were composed only of mature neutrophils (Figure 5.4-B), while both mature and immature neutrophils were present in BM as wells as in CB cells (Figure 5.4-C and D, respectively). Myelopoiesis may be followed both immunologically and morphologically by flow cytometry. In the late, in a plot of the low density mononuclear cell BM population, early myeloid precursors cells and myeloblasts are found in the blast gate, which overlaps the monocyte region. Metamyelocytes, band and segmented neutrophils are found in the granulocyte region, while promyelocytes and myelocytes (immature myeloid cells) are variably split between the monocyte and the granulocyte gates (Civin and Loken, 1987). Due to the irregular distribution of the myeloid cells in various stages of differentiation/maturation in different regions, it is possible that their presence may have interfered with the levels of CCR1 expression on MNC, particularly from BM where these intermediate forms were abundant according to our morphological data.

In an attempt to clarify this issue, we labeled MNC from PB, BM, and CB with anti-CCR1 antibody and FACS sorted them according to the CCR1 expression into 'positive' and 'negative' populations (Table 5.3). Morphological analysis of the cell types present in the $CCR1^+$ and $CCR1^-$ MNC sorted fractions revealed that the majority of the PB circulating neutrophils were present in the $CCR1^+$ population while immature and mature neutrophils from CB were present in both $CCR1^+$ and $CCR1^-$ fractions. BM immature myeloid cells were evenly distributed between $CCR1^+$ and $CCR1^-$ fractions while the more mature forms such as metamyelocytes and band neutrophils were more abundant in the $CCR1^-$ fraction. The cause of the discrepancy for CCR1 expression detected by flow cytometry on neutrophils in different stages of differentiation/maturation from different ontogenic sources is unknown. They indicate that the levels of CCR1 expression on neutrophils may be related to their maturational stage. Reports about the expression of CCR1 on neutrophils have also been controversial. While some reports have shown evidence for CCR1 expression on PB neutrophils using binding studies (Crisman *et al.*, 1999), flow cytometry (Forster *et al.*, 1998), or demonstration of CCR1 transcripts (Nomura *et al.*, 1993), others have not been able to detect its presence (Hori *et al.*, 1998; Su *et al.*, 1996).

Although technical limitations for detecting CCR1 expression cannot be ruled out, our flow cytometry and IC data of MNC from PB, BM, and CB have clearly shown the presence of CCR1 antigen on neutrophils independent of their maturational stage. Cell

differentiation leads to distinct cell types whose specialised character and properties are determined by their pattern of gene activity and thus the proteins they produce. It is possible that CCR1 receptors on neutrophils are modulated as these cells mature and become receptive to traffic signals that enable selective migratory behaviour, as suggested for leukocyte trafficking (Springer, 1994). PB neutrophils are circulating cells that do not return to the BM environment, having a turn over of 6-9 hours after release into circulation (Springer, 1994). CB cells are transit cells between the peripheral circulation and the marrow, while BM cells are confined into marrow cavities. In this context, as neutrophils mature, CCR1 expression may be modulated according to specific triggering signals that are involved in the different biological properties of these cells.

Peripheral blood leukocytes migrate from the bloodstream into tissues in response to molecular changes on the surface of blood vessels that signal injury or infection. The nature of the inflammatory stimulus determines whether lymphocytes, monocytes, neutrophils, or eosinophils predominate. Recent reports have shown that chemokine receptors can be modulated by cytokines. IFN-γ, for example, has been shown to cause a rapid and concentration-dependent increase of CCR1 and CCR3 mRNA in human neutrophils, which also migrated efficiently towards MIP-1α (Bonecchi *et al.*, 1999). IFN-γ has been also shown to increase the expression of CCR1 on monocytes and on monocytoid U937 cells as well as calcium mobilisation and cell migration to specific ligands such as RANTES (Zella *et al.*, 1998; Zella *et al.*, 1999).

CCR1 expression has been up regulated during the neutrophil-dependent phase of mouse glomerulonephritis and its expression was kept during the subsequent mononuclear cell infiltration (Topham *et al.*, 1999). Recently, Jarmin and colleagues have shown that IL-3 and GM-CSF can up-regulate the expression of CCR1 mRNA on monocytes as well as to increase the number of their CCR1 cell surface receptors (Jarmin *et al.*, 1999). D1 DC cell line is a murine model of dentritic cells that is maintained in an immature state in presence of GM-CSF and can be induced to maturation by adding inflammatory stimulus or bacterial products (Winzler *et al.*, 1997). Recently, it has been shown that MIP-1α is an efficient chemoattractant for D1 DC cells during LPS treatment, and CCR1 was the main receptor expressed and modulated during the early stages of dentritic cells maturation (Foti *et al.*, 1999). Therefore, CCR1 receptor expressed by D1 DC cells correlated with their maturational

stage and correspondent biological activity. Thus, neutrophil maturation and acquisition of biological properties such as migration may be induced by cytokines, chemokines or other molecules such as bacterial products through modulation of CCR1 receptor expression as reported by other leukocyte sub types.

In addition, as stated earlier, density gradient solutions have the purpose of selecting mononuclear cells such as lymphocytes and monocytes (Robinson, 1993). However, as often observed and can be exemplified by our morphological findings on unsorted populations (Figure 5.4), it also retains other cell types such as immature myeloid cells, or mature cells such as neutrophils, eosinophils, basophils, or even erythroblasts. Our morphological analysis data have shown that different types of cells are recovered depending on the treatment used to enrich MNC. Comparing both density gradient centrifugation and methylcellulose sedimentation procedures, the former has enriched the lymphocyte population (Figure 5.8-A1), while impoverishing the cells present in the granulocyte gate. Thus, expression of CCR1 on granulocytes, particularly on neutrophils, should be investigated on cells from different ontogenic sources obtained by a technical procedure that would allow the recruitment of all neutrophils present in the sample, avoiding the possible partial selection or interferences that may occur when density gradient separation is used.

In addition to the variable expression pattern found on MNC from different ontogenic sources, CCR1 was highly expressed on $CD34^+$ cells from CB and BM, corroborating with previous reports demonstrating CCR1-specific transcripts (de Wynter *et al.*, 1998; Su *et al.*, 1997) and specific anti-CCR1 antibody bindings by flow cytometry (Lee *et al.*, 1998; Lee *et al.*, 1999a; Su *et al.*, 1996) on these cells. Because of the heterogeneity of $CD34^+$ expressing cells, we have used clonogenic assays to assess the distribution of CCR1 on progenitor cells derived from BM and CB. Owing the difficulties of obtaining enough FACS sorted cells lacking CCR1 expression, $CD34^+$ cells labeled with anti-CCR1 antibody were sorted into $CCR1^{high}$ and $CCR1^{-/low}$ expressing fractions. In contrast with the findings that have been published for BM cells (Su *et al.*, 1997), the majority of the GM-CFC was distributed in the $CCR1^{high}$ fraction while most of the erythroid progenitors were present in the $CCR1^{-/low}$ population (Table 5.4). These findings were consistent in all cord blood samples examined and suggested, at first, that CCR1 expression levels could be lineage specific or be dependent upon developmental stage of these progenitors.

To clarify this issue, we have first labeled the colony cells generated from $CBCD34^{+}CCR1^{high}$ and $CBCD34^{+}CCR1^{-/low}$ populations grown in clonogenic assay with anti-CCR1 antibody and assessed their CCR1 expression by flow cytometry. As a result, CCR1 expression was kept throughout differentiation/maturation of $CCR1^{high}$ cells which were mostly composed of macrophages and mature neutrophils (Figure 5.16-A), while very low expression, if any, of CCR1 was detected by flow cytometry on cells grown from the $CCR1^{-/low}$ population, composed mainly of erythroid cells (Figure 5.16-B). We, then, further investigated the CCR1 expression on these populations using IC. As a result, immunostaining showed that both populations expressed the CCR1 receptor on their cell surface and that IC is a more sensitive method for detecting low levels of CCR1 expression than flow cytometry. Moreover, the latter results were in agreement with our previous data obtained when CBMNC cells sorted as a low binding population ($CCR1^{dim}$) were able to generate erythroid colonies as well as granulocyte-macrophage ones. All together, these results suggest that, at least for $CBCD34^{+}$ progenitor cells:

- the CCR1 receptor expression is associated with the differentiation/maturation stage which includes the clonogenic progenitor cells;
- it is heterogeneously distributed amongst the progenitors committed to the myeloid and erythroid lineages;
- its level of expression is high on cord blood GM progenitor cells, and its expression is maintained throughout their differentiation/maturation;
- it is expressed at low levels on cord blood erythroid progenitor cells and their progeny, and fully mature erythrocytes are devoid of its expression.

Therefore, the distinct pattern of CCR1 expression on GM and erythroid progenitors observed seems to be related to the functional abilities of the progeny of these cells as they approach maturation and can be influenced by different combinations of growth and differentiation factors acting to produce the adequate cell types for the individual's physiological needs. This singular pattern of expression of CCR1 on progenitor cells is somewhat advantageous in a way that flow cytometry can be used to discriminate and select these populations whenever studies involving commitment and differentiation are required.

In contrast to CCR1, expression of CCR5 was restricted to monocyte and lymphocyte gated cells (Figure 5.13-B) as described by others (Chelucci *et al.*, 1999;

Hariharan *et al.*, 1999; Lee *et al.*, 1999c), and is in agreement with the well-defined functions of CCR5 natural ligands (*i.e*, MIP-1α, MIP-1β, and RANTES), involved mostly in the activation and recruitment of leukocyte chemoattractant receptors (Murphy, 1994). Cells of the granulocyte region always showed less than 5% expression and confirmed the poor expression of this receptor on other cell types under steady state conditions. Also, less than 5% of $CD34^+$ cells from CB and BM were labeled with CCR5, confirming the low levels of expression of CCR5 on these progenitor cells recently reported by others (de Wynter *et al.*, 1998; Lee *et al.*, 1999a; Ruiz *et al.*, 1998; Shen *et al.*, 1998).

CXCR4 was highly expressed on all sub populations of MNC from PB and CB examined (Figure 5.13-C). These results corroborated with earlier experiments using Northern blot analysis that showed expression of CXCR4-specific transcripts in primary PB monocytes and neutrophils (Loetscher *et al.*, 1994), and with the demonstration of high affinity specific binding sites on neutrophils, monocytes, and T lymphocytes using binding assays (Ueda *et al.*, 1997). Also, they are in agreement with the demonstration of this cell surface receptor on neutrophils, monocytes, and T and B lymphocytes from PB by flow cytometry (Chelucci *et al.*, 1999; Forster *et al.*, 1998; Hori *et al.*, 1998; Lee *et al.*, 1999c). Although we did not use specific antibodies to discriminate the different lymphocyte sub populations, the high expression of CXCR4 on lymphocytes and monocytes coincides with the well known responses of these cells induced by SDF-1α (Bleul *et al.*, 1996a; Bleul *et al.*, 1996b; Oberlin *et al.*, 1996), so far the only ligand described for CXCR4.

In contrast to the high expression found in PB and CB mononuclear cells, BM mononuclear cells showed lower levels of CXCR4 expression, particularly on cells of the granulocyte fraction. Also, BM samples showed great variability on the expression of CXCR4 between samples. These differences in the level of CXCR4 expression between PB and CB cells and BM cells may be explained by their cell type content. In contrast with the presence of mostly mature cells in PB and CB, BMMNC had more than 25% of their cellular content represented by immature myeloid and erythroid nucleated cells. Mature erythrocytes and reticulocytes have a characteristic low forward and side scatter and are predominantly found next to the lymphocyte region in a flow cytometry chart. As they can be easily gated out, they usually do not interfere with the analysis. Less mature nucleated erythroid precursors such as pronormoblasts as well as the more mature orthochromatic normoblasts are usually present in the lymphocyte

region. The other two least mature erythroid precursors identifiable by morphology (basophilic and polychromatic normoblasts) are found somewhere between the monocyte and granulocyte gates (Loken *et al.*, 1987a; Loken *et al.*, 1987b). Moreover, CXCR4 mRNA transcripts or its protein expression by flow cytometry have not been detected in erythroid cells during their differentiation *in vitro*, leading to the conclusion that erythrocytes do not express CXCR4 at any differentiation/maturation stages (Chelucci *et al.*, 1999). Therefore, the presence of immature and mature nucleated erythroblasts within BMMNC might have contributed to lower the levels of CXCR4 detection in all gated sub populations as their different sizes and cell complexity could facilitate their distribution irregularly amongst lymphocyte, monocyte and granulocyte gated cells.

Several studies have reported the presence of CXCR4 on primitive haemopoietic cells from CB (Rollins, 1997; Shen *et al.*, 1998), BM (Shen *et al.*, 1998; Wang *et al.*, 1998) and from mobilised (Shen *et al.*, 1998) and steady state PB (Lataillade *et al.*, 2000), while little attention has been given to its expression on more differentiated cells. However, the effects of SDF-1α on mature cells such as monocytes, lymphocytes and neutrophils have been demonstrated through Ca^{+2} mobilisation, chemotactic (Oberlin *et al.*, 1996), and binding assays (Ueda *et al.*, 1997), indicating that these cells express the ligand receptor. Immature cells are usually confined within marrow spaces and changes in CXCR4 expression may facilitate their retention within stromal niches and their release and emigration into circulation upon maturation. This hypothesis is supported by the fact that SDF-1α, the natural ligand for CXCR4, is produced by bone marrow stromal cells (Bleul *et al.*, 1996b; Tashiro *et al.*, 1993) and has been shown to modulate the expression of its receptor (Forster *et al.*, 1998). Also, it has been suggested that SDF-1α released from stromal cells exerts its critical haemopoietic function by selectively attracting and confining early precursors within the bone marrow microenvironment that provides the necessary factors for their growth and differentiation (Aiuti *et al.*, 1999a; D'Apuzzo *et al.*, 1997). Therefore, whilst BMMNC are still non-circulating cells and are in intimate contact with marrow stroma they would express lower levels of CXCR4, which would keep them within marrow spaces. As they mature and are ready to migrate to peripheral circulation, acquisition of migration properties would be accompanied by modulation of CXCR4 expression. In this case, our morphological analysis data of the BMMNC showing the presence of immature myelocytes (Figure 5.4-B), cells that are not found normally circulating in the PB,

would be of value. Moreover, CXCR4 was expressed on $CD34^+$ cells from both CB and BM, in agreement with previous reports (Aiuti *et al.*, 1999a; Aiuti *et al.*, 1999b; Lataillade *et al.*, 2000; Shen *et al.*, 1998; Viardot *et al.*, 1998; Wang *et al.*, 1998). Also, its expression on CB cells was higher than that on BM cells (30,3%±2.5% and 71.2%±9.9%, respectively). These results also support our hypothesis that circulating or migrating cells for instance, would express higher numbers of CXCR4 receptors than immobilised cells. Studies of CXCR4 expression on haemopoietic cells during differentiation/maturation *in vitro* and their exposition to conditions similar to those *in vivo* would therefore be useful to clarify these findings.

Chapter 6

Biological effects of MIP-1α on haemopoietic progenitors and involvement of CCR1

6.1. Introduction

MIP-1α has been implicated in many aspects of *in vivo* and *in vitro* haemopoiesis primarily as an inhibitor of primitive haemopoietic cell proliferation (Clements *et al.*, 1992; Graham *et al.*, 1990; Lord *et al.*, 1992; Maze *et al.*, 1992). Studies at the single cell level have shown that the effects of MIP-1α on stem/progenitor cells are the result of its direct interaction with the target cell (Keller *et al.*, 1994; Lu *et al.*, 1993a), indicating that responsive cells must bear the appropriate receptor. Thus, an understanding of the mechanisms by which this chemokine elicits its diverse biological effects requires characterisation of its specific receptors on responsive cells. In the previous chapter, the expression of the MIP-1α receptors CCR1 and CCR5 was demonstrated by flow cytometry on human haemopoietic cell lines and on primary haemopoietic cells from peripheral blood, bone marrow and cord blood samples. Their presence on haemopoietic progenitors and on maturing/matured cells may in part explain some of the reported biological effects of MIP-1α, but very little is known about the identity of the receptor(s) mediating these effects.

Cytokines that induce chemotaxis of haemopoietic progenitor cells may play important roles in their retention within and trafficking from bone marrow niches. Moreover, identification of a chemokine that could attract the earliest subsets of stem cells with long-term marrow repopulating capacity would be particularly useful in the context of stem cell transplantation. MIP-1α has been shown to mobilise stem and progenitor cells from bone marrow to blood (Lord *et al.*, 1995). Recently, using

transgenic mice lacking functional CCR1 receptor as a model, it has been demonstrated that MIP-1α failed to induce *in vivo* mobilisation of progenitor cells (Broxmeyer *et al.*, 1999a; Gao *et al.*, 1997). In addition, as GM-CFC and M-CFC bone marrow progenitors from the null mutants did not respond to the enhancing effect of MIP-1α when compared to the wild-type controls, it has been suggested that the dominant receptor associated with MIP-1α stimulation of GM-CFC progenitors is CCR1 (Broxmeyer *et al.*, 1999a). Collectively, these data suggest the involvement of the MIP-1α receptor CCR1 not only in the mechanisms involving proliferation, but also in migration of early and late subsets of myeloid progenitor cells.

MIP-1α binds to CCR1 with high affinity (Neote *et al.*, 1993). In the previous chapter, we demonstrated that $CD34^+$ cells express CCR1. Moreover, the level of CCR1 expression on $CD34^+$ progenitors could be correlated with different types of progenitors. The experiments in this chapter describe the effects of MIP-1α on proliferation, migration and adhesion properties of $CD34^+$. The possible involvement of CCR1 mediating these functional MIP-1α responses was also investigated using sorted populations expressing high ($CD34^+CCR1^{high}$) and very low ($CD34^+CCR1^{-/low}$) levels of CCR1 expression. SDF-1α has recently been reported to influence haemopoietic stem cell behaviour as reviewed in Chapter 1 (section 1.4.2.2), particularly the mechanisms involving cell adhesion/migration/homing (Aiuti *et al.*, 1997), where this activity is manifest on most of the haemopoietic progenitor subsets (Kim and Broxmeyer, 1998). Therefore, the corresponding effects of SDF-1α on proliferation, migration and adhesion of progenitor cells were also examined in parallel.

6.2. Results

6.2.1. Effects of MIP-1α on proliferation of haemopoietic cells

One of the aims of the present study was to assess the influence of MIP-1α on proliferation of haemopoietic progenitor cells. To achieve this, we have used the clonogenic assay which allows the *in vitro* quantification of primitive progenitor cells that possess the ability to directly proliferate, differentiate and develop into phenotypically and functionally mature myeloid cells according to the growth stimuli present in the medium (Heyworth and Spooncer, 1993). $CD34^+$ cells from cord blood

and bone marrow were isolated using the Mini-MACS® immunomagnetic separation system as described in section 2.1.7.5. The purity of the $CD34^+$ population was analysed as described in section 2.1.7.7, and ranged from 68.6% to 96.4%, with an average of 87.5%±1.5 (n=30) for cord blood, and from 71.3% to 98.7%, with an average of 91.8%±2.3 (n=5) for bone marrow samples. Because of the tendency of some forms of MIP-1α to polymerise (Wolpe *et al.*, 1988) and become inactive *in vitro* (Avalos *et al.*, 1994; Lord *et al.*, 1993; Mantel *et al.*, 1993), the recombinant non aggregating BB10010 form of MIP-1α (Hunter *et al.*, 1995; Lord *et al.*, 1995) was used and its effect on $CD34^+$ cells evaluated by adding a range of concentrations directly to the clonogenic assay plating mixture (section 2.1.12.1). The results are expressed as the mean percentage of increase or decrease of colony numbers over untreated controls (mean ± SEM, for n≥3, or SD, for n=2).

6.2.1.1. $CD34^+$ cells

Figure 6.1-A illustrates the effects of adding increasing concentrations of MIP-1α on $CD34^+$ cells from cord blood. MIP-1α stimulated the formation of GM-CFC colonies above the control numbers to a maximum of 29%±11 at 50ng/ml, at which concentration it reached significance (p=0.008). In contrast, MIP-1α had little, if any, effect on cord blood BFU-E colony formation, and only at a dose of 500ng/ml was a significant suppressive effect on erythroid colony formation observed (22%±8, p=0.024). A dose-related suppressive activity to MIP-1α under similar conditions was found for $CD34^+$ bone marrow myeloid progenitors (Figure 6.1-B). There was a significant suppressive effects of MIP-1α on GM-CFC colony formation at 15 (10.5%±1, p<0.001) to 50ng/ml (28%±8, p=0.027), with inhibition increasing from 10%±15 at 1.5ng/ml to 45%±16 of control values at 500ng/ml of MIP-1α. Only a slight suppression of bone marrow erythroid progenitors was seen at lower MIP-1α doses, in contrast with higher concentrations (36%±17, p=0.027 at 500ng/ml MIP-1α).

When SDF-1α was added under the same culture conditions, erythroid progenitors from cord blood (Figure 6.2-A), but not from bone marrow (Figure 6.2-B), were significantly affected only at higher concentrations, with a decrease of 24%±7 (p=0.009) in colony formation at 150ng/ml SDF-1α. Very little effect of SDF-1α on the GM-CFC population was observed for both cord blood and bone marrow samples.

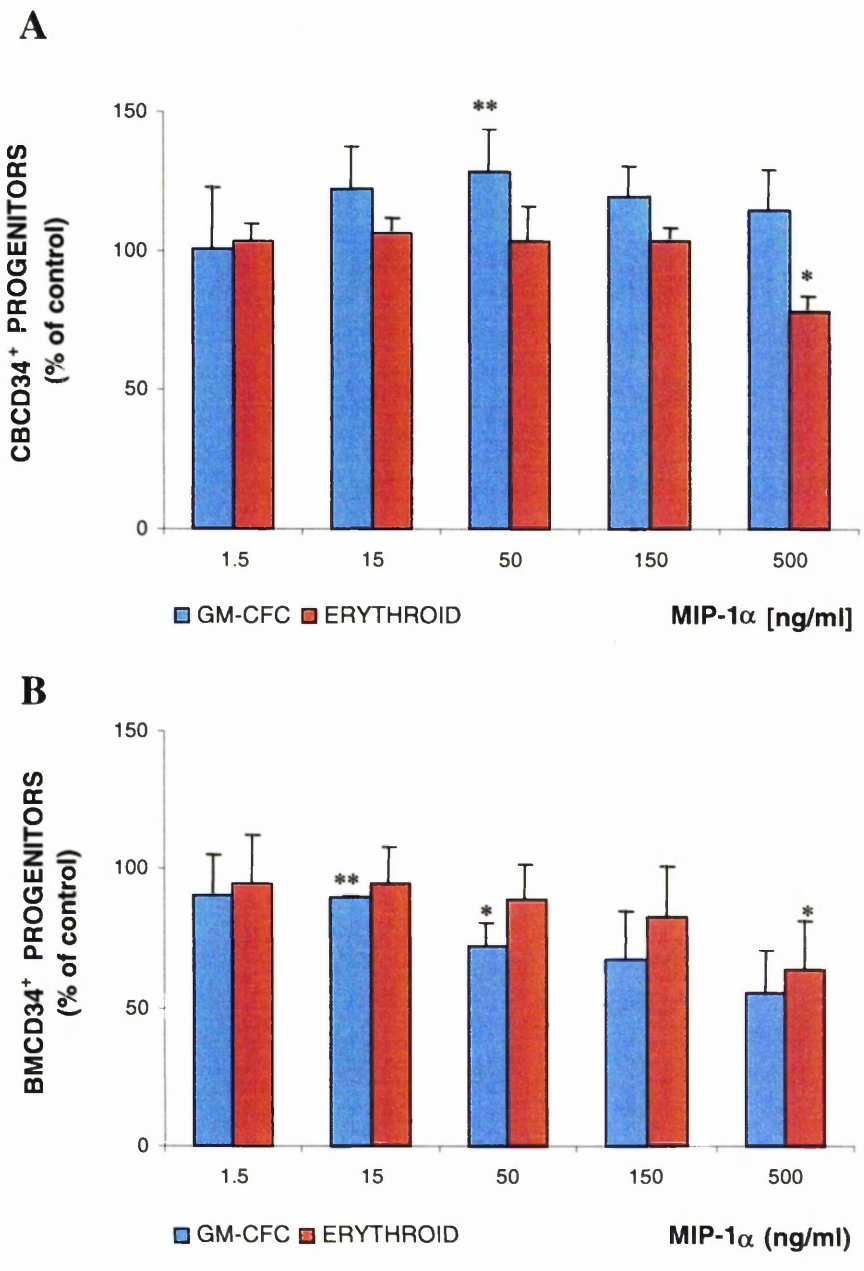


Figure 6.1. Effects of MIP-1α on proliferation of CD34⁺ cells. $CD34^+$ cells from cord blood (CB) (**A**) or bone marrow (BM) (**B**) obtained after immunomagnetic separation (section 2.1.7.5) were cultured in clonogenic assay (section 2.1.12.1) in continuous presence of MIP-1α at the indicated concentrations. Colonies of the granulocyte-macrophage (GM-CFC) and erythroid (BFU-E) lineages were scored after 14 days. The results are expressed as the mean percentage ± SEM of the total number of colonies generated from treated cultures over control experiments. Control colony numbers ranged from 13 to 223 for GM-CFC, and from 12 to 192 for BFU-E for cord blood cells (n=3-13), and from 23 to 166 for GM-CFC, and from 20 to 86 for BFU-E for bone marrow (n=3-4). * $p<0.05$; ** $p<0.01$

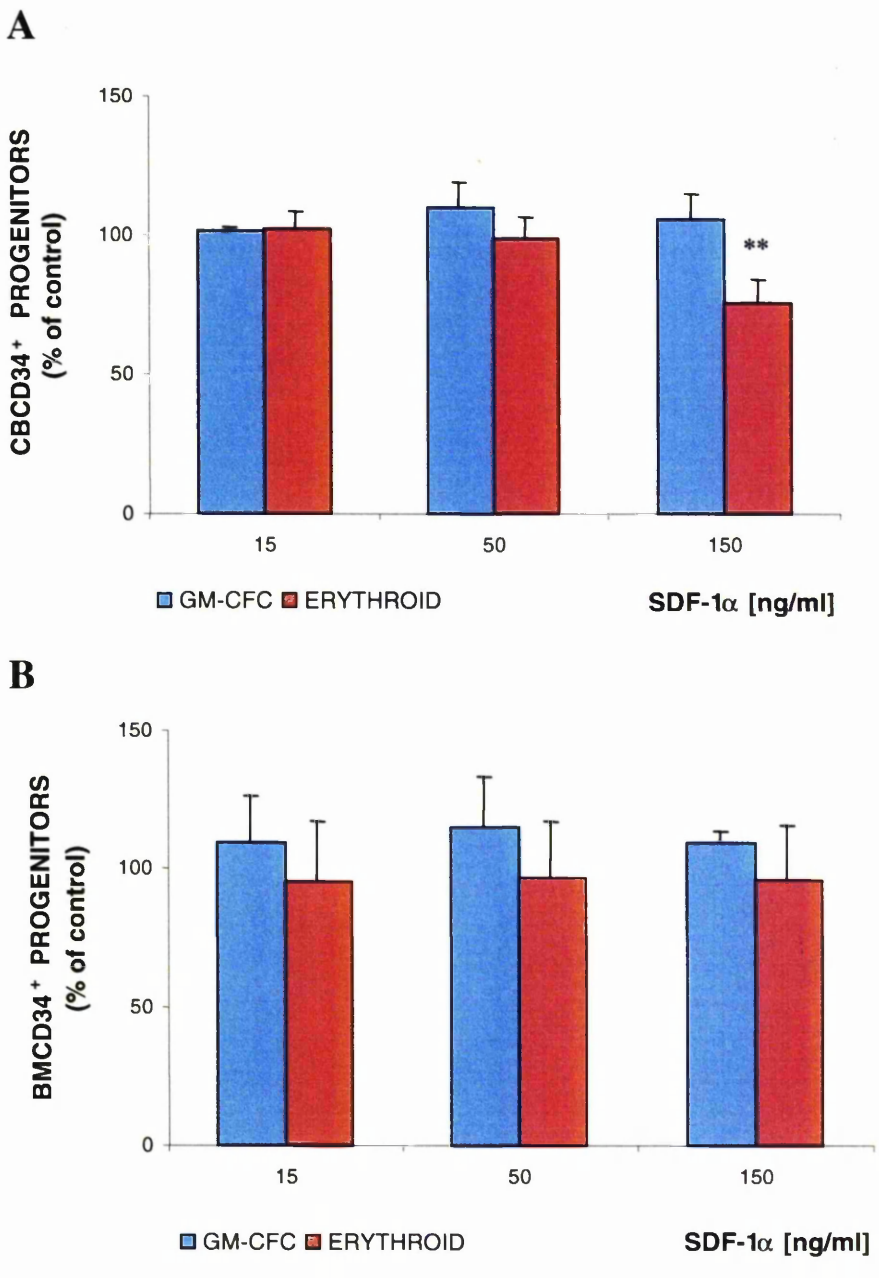


Figure 6.2. Effects of SDF-1α on proliferation of CD34⁺ cells. CD34⁺ cells from cord blood (CB) (**A**) and bone marrow (BM) (**B**) obtained after immunomagnetic separation (2.1.7.5) were cultured in clonogenic assay as described in section 2.1.12.1 in continuous presence of SDF-1α at the indicated concentrations. GM-CFC and erythroid (BFU-E) colonies were scored after 14 days. The results are expressed as the mean percentage ± SEM of the total number of colonies generated in SDF-1α treated cultures over untreated controls. The number of colonies in control cultures ranged from 81 to 393 for GM-CFC, and from 43 to 167 for BFU-E for cord blood cells (n=3-9), and from 23 to 119 for GM-CFC, and from 20 to 86 for BFU-E for bone marrow (n=3-5) ** $p<0.01$

6.2.1.2. $CD34^{+}CCR1^{high}$ cells

The results in the previous section indicated that, depending on the cell ontogenic source, $CD34^{+}$ cells respond differently to MIP-1α. Since CCR1 is one of the receptors that binds MIP-1α with high affinity and it is present on the surface of these cells, MIP-1α–mediated proliferation effects on progenitor cells expressing CCR1 were further examined. $CD34^{+}$ cells obtained after immunomagnetic separation (section 2.1.7.5) were labeled with the anti-CCR1 antibody (section 2.1.7.6), sorted into $CCR1^{high}$ and $CCR1^{-/low}$ populations by flow cytometry using the gating strategy depicted in Figure 5.14, and cultured in clonogenic assays (section 2.1.12.1).

Exposure of $CD34^{+}CCR1^{high}$ population from cord blood (which has been shown to contain mostly GM-CFC progenitors) to varying concentrations of MIP-1α in clonogenic assays resulted in an increased dose-related number of GM-CFC colonies (Figure 6.3-A). This reached maximum significance at 150ng/ml MIP-1α (20%±7, p=0.025). In contrast, the addition of MIP-1α to $CD34^{+}CCR1^{high}$ sorted cells from bone marrow resulted in a concentration-dependent reduction in the number of GM-CFC colonies formed (Figure 6.3-B), from 8%±4 to 31%±24 over the dose range used (p>0.05).

6.2.1.3. $CD34^{+}CCR1^{-/low}$ cells

The majority of the progenitors present in the $CD34^{+}CCR1^{-/low}$ cell population belong to the erythroid lineage. As for $CD34^{+}$ cells, addition of MIP-1α had little effect on cord blood erythroid colony formation (Figure 6.4-A), with significant inhibition found only at 500ng/ml of MIP-1α (31%±5, p=0.001). In contrast, for $CD34^{+}CCR1^{-/low}$ bone marrow cells, the formation of erythroid colonies was suppressed in a dose-related manner (Figure 6.4-B), reaching maximum significance at 50ng/ml (34%±2, p=0.001).

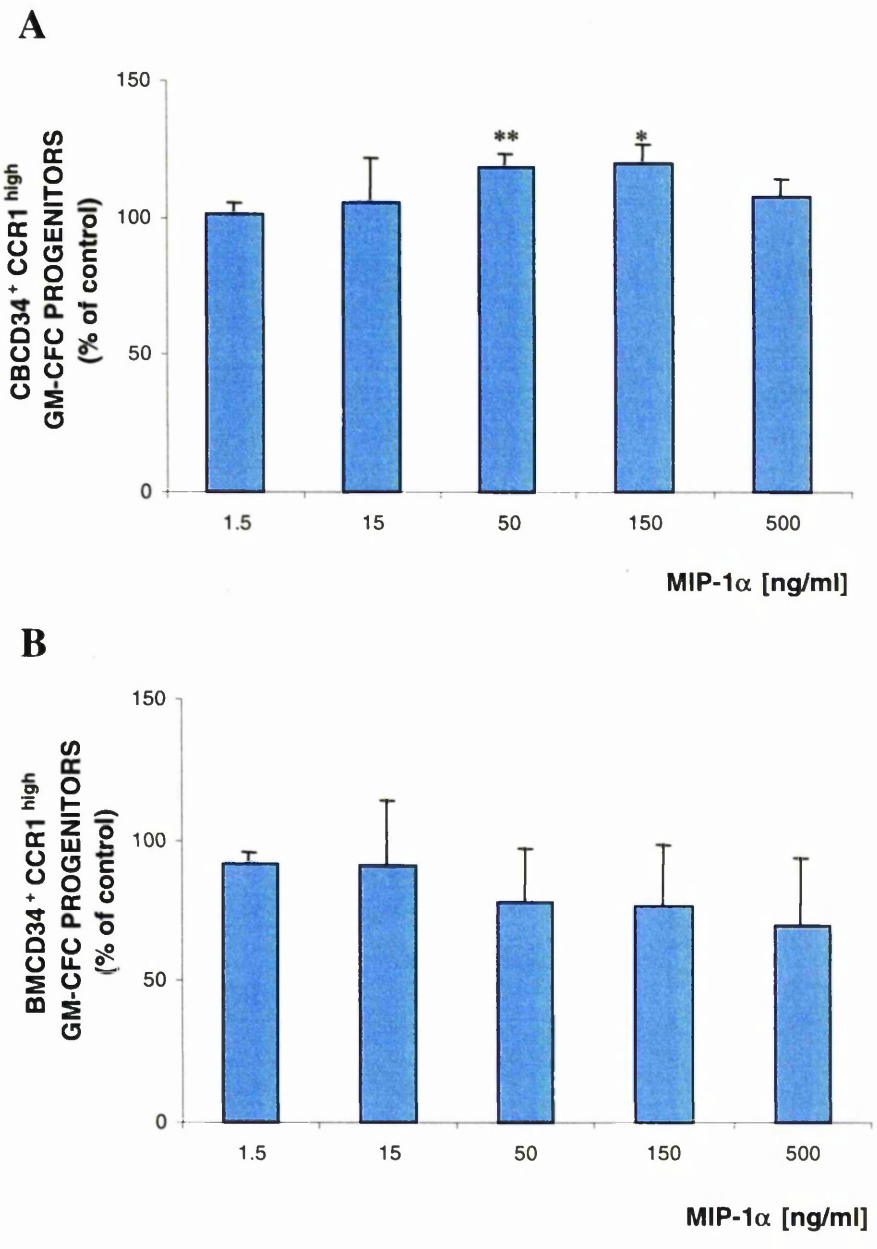


Figure 6.3. Effects of MIP-1α on proliferation of CD34+CCR1^{high} cells. CD34+ cells from cord blood (**A**) or bone marrow (**B**) obtained after immunomagnetic separation (section 2.1.7.5) were labeled with anti-CCR1 antibody as described in section 2.1.7.6 and sorted into CCR1^{high} population by flow cytometry according to the expression of CCR1 using the gating strategy depicted in Figure 5.14. Sorted cells were cultured in clonogenic assay (section 2.1.12.1) in continuous presence of MIP-1α at the indicated concentrations. Colonies of the granulocyte-macrophage (GM-CFC) lineage were scored after 14 days. The results are expressed as the mean percentage ± SEM (or SD, where n=2) of the total number of colonies generated from treated cultures over control experiments. The number of colonies in control cultures ranged from 120 to 246 for cord blood (n=3-6) and from 67 to 105 for bone marrow samples (n=2-3). * $p<0.05$; ** $p<0.01$

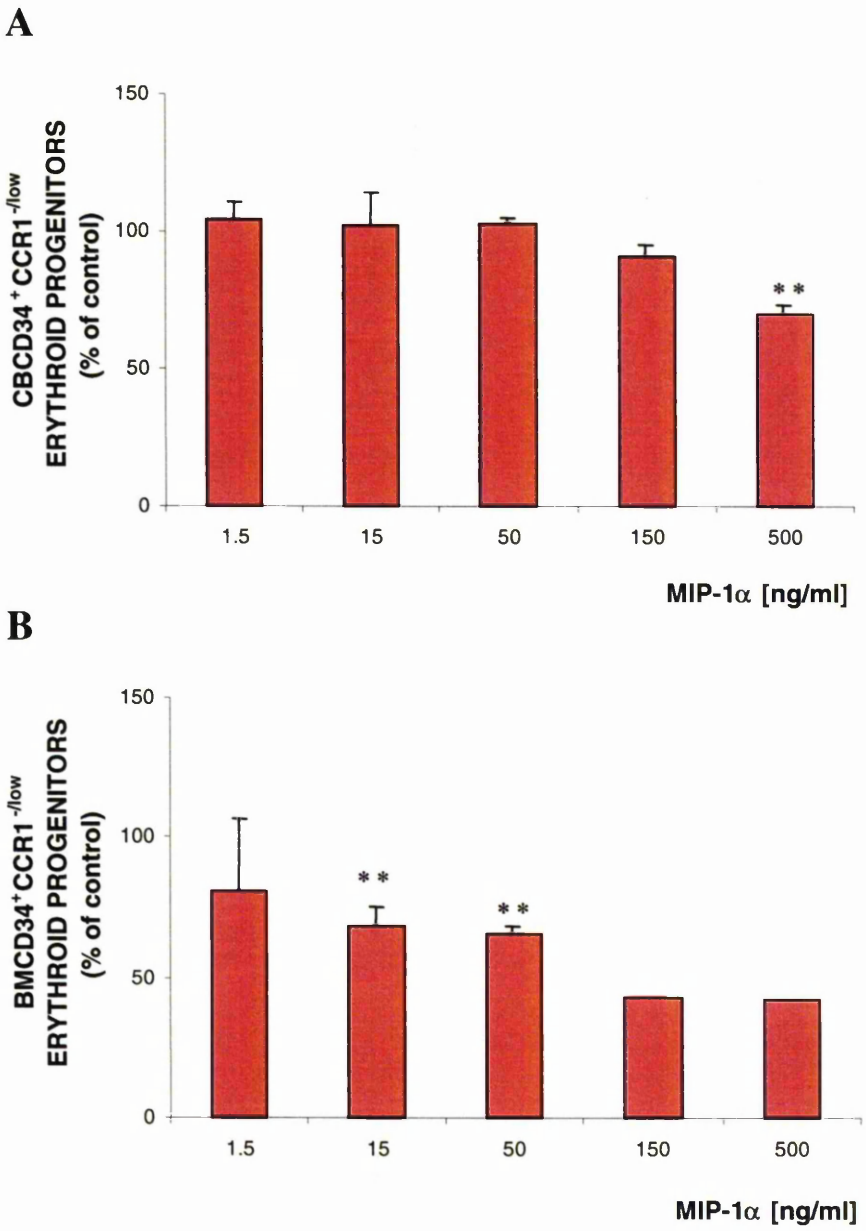


Figure 6.4. Effects of MIP-1α on proliferation of CD34$^+$CCR1$^{-/low}$ cells. CD34$^+$ cells from cord blood (**A**) or bone marrow (**B**) obtained after immunomagnetic separation (section 2.1.7.5) were labeled with anti-CCR1 antibody as described in section 2.1.7.6 and sorted into CCR1$^{-/low}$ population by flow cytometry according to the expression of CCR1, using the gating strategy depicted in Figure 5.14. Sorted cells were cultured in clonogenic assay (section 2.1.12.1) in continuous presence of MIP-1α at the indicated concentrations. Colonies of the erythroid lineage (BFU-E) were scored after 14 days. The results are expressed as the percentage ± SEM (or SD, where n=2) of the total number of colonies generated from treated culture over control the experiment. The number of colonies in control cultures ranged from 28 to 323 for cord blood (n=5-7) and from 20 to 132 for bone marrow (n=1-3). ** $p<0.01$

6.2.2. Effects of the anti-CCR1 antibody on MIP-1α-mediated proliferation of haemopoietic cells

6.2.2.1. $CD34^+$ cord blood cells

The polyclonal antibody used throughout this study has been shown to have specificity for CCR1. The antibody blocked the increase in MIP-1α-mediated intracellular calcium in the human embryonic kidney cell line 293 transfected with human CCR1 cDNA, and interfered with MIP-1α-induced monocyte chemotaxis (Su *et al.*, 1996). In order to investigate whether this antibody could block the MIP-1α stimulatory effects observed in cord blood progenitor cells, immunomagnetically enriched $CD34^+$ cord blood cells were plated in clonogenic assays (section 2.1.12.1) in the presence of MIP-1α and/or anti-CCR1 antibody.

The addition of anti-CCR1 antibody alone (5μg/ml) had little effect on the GM-CFC colony formation from $CD34^+$ cord blood cells (Figure 6.5-A) when compared to control cultures. In agreement with the data generated in section 6.2.1.1, addition of MIP-1α at 50ng/ml to the cultures led to a significant increase in the number of GM-CFC colonies generated (24%±9, p=0.043) in comparison with untreated controls. When the $CD34^+$ population was cultured in the presence of both MIP-1α and anti-CCR1 antibody, no proliferative response in GM-CFC-derived colony formation to MIP-1α was observed. On the contrary, a significant reduction in the number of GM-CFC colonies (38%±7, p=0.048) was found when compared to the values obtained in cultures treated with anti-CCR1 antibody alone. In contrast, the addition of MIP-1α and anti-CCR1 antibody, alone or in combination, to the cultures had little effect on erythroid colony formation when compared to their respective controls (Figure 6.6-A), recapitulating the lack of effects observed previously.

6.2.2.2. $CD34^+CCR1^{high}$ cord blood cells

Using a similar approach, the effects of the anti-CCR1 antibody on the MIP-1α-mediated response of $CD34^+CCR1^{high}$ cord blood cells were further explored and the results are illustrated in Figure 6.5-B. As observed for $CD34^+$ cells, the addition of anti-CCR1 antibody (5μg/ml) alone to $CD34^+CCR1^{high}$ cells had little influence on the number of GM-CFC progenitors generated. However, addition of MIP-1α at 50ng/ml

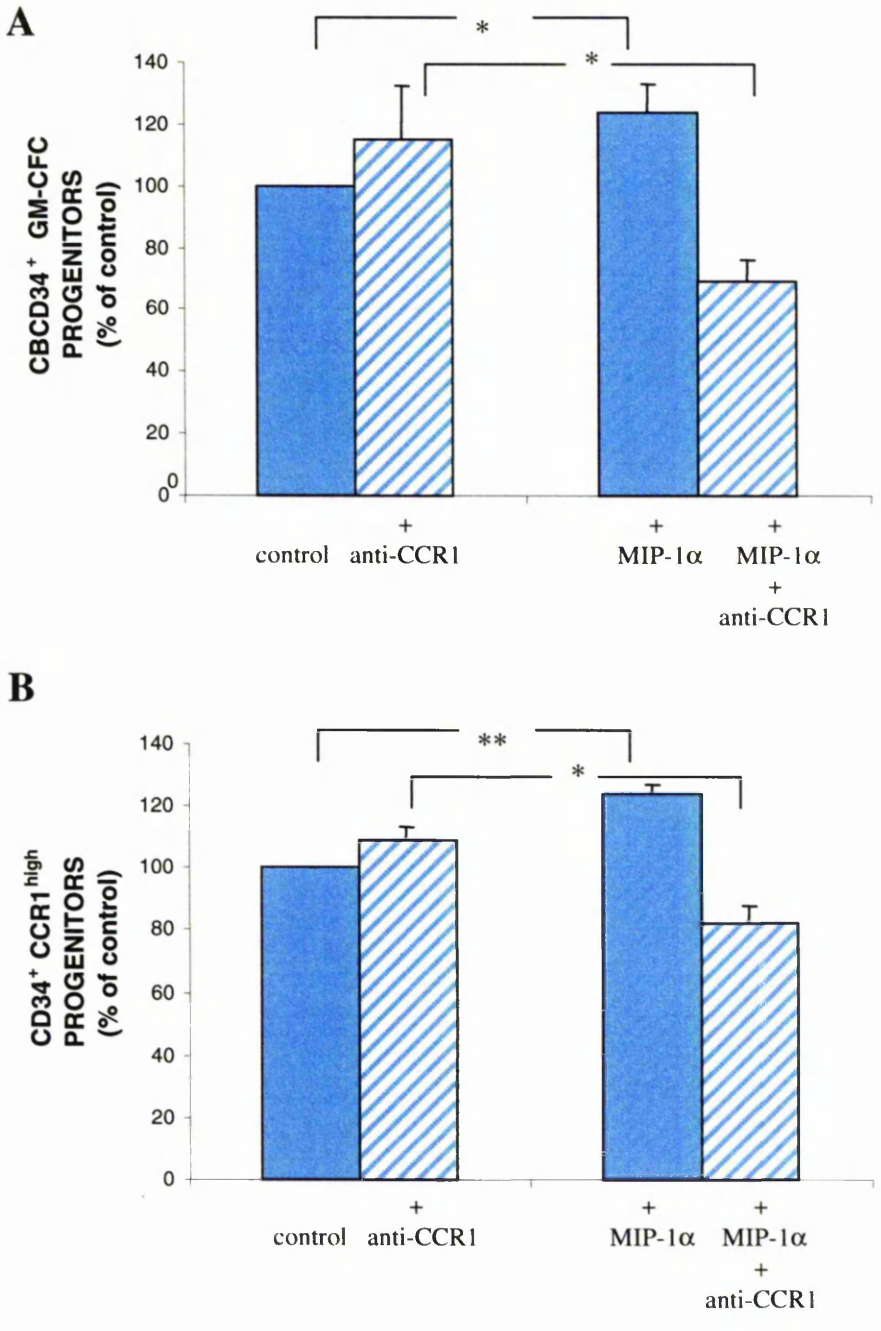


Figure 6.5. Effects of the anti-CCR1 antibody on MIP-1α–mediated proliferation of GM-CFC. $CD34^+$ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were labeled with anti-CCR1 antibody (section 2.1.7.6) and flow cytometry sorted into $CD34^+CCR1^{high}$ fraction using the gating strategy depicted in Figure 5.14. Unsorted (**A**) and sorted (**B**) cells were cultured in clonogenic assay (section 2.1.12.1) with or without MIP-1α at 50ng/ml and anti-CCR1 antibody at 5μg/ml as indicated. Colonies were scored after 14 days. The results are expressed as the mean percentage ± SEM of the total number of colonies generated from treated cultures over control experiments. The number of colonies obtained in control cultures ranged from 123 to 265 for $CD34^+$ cells (n=4) and from 107 to 328 for $CD34^+CCR1^{high}$ cells (n=8). * $p<0.05$; ** $p<0.01$

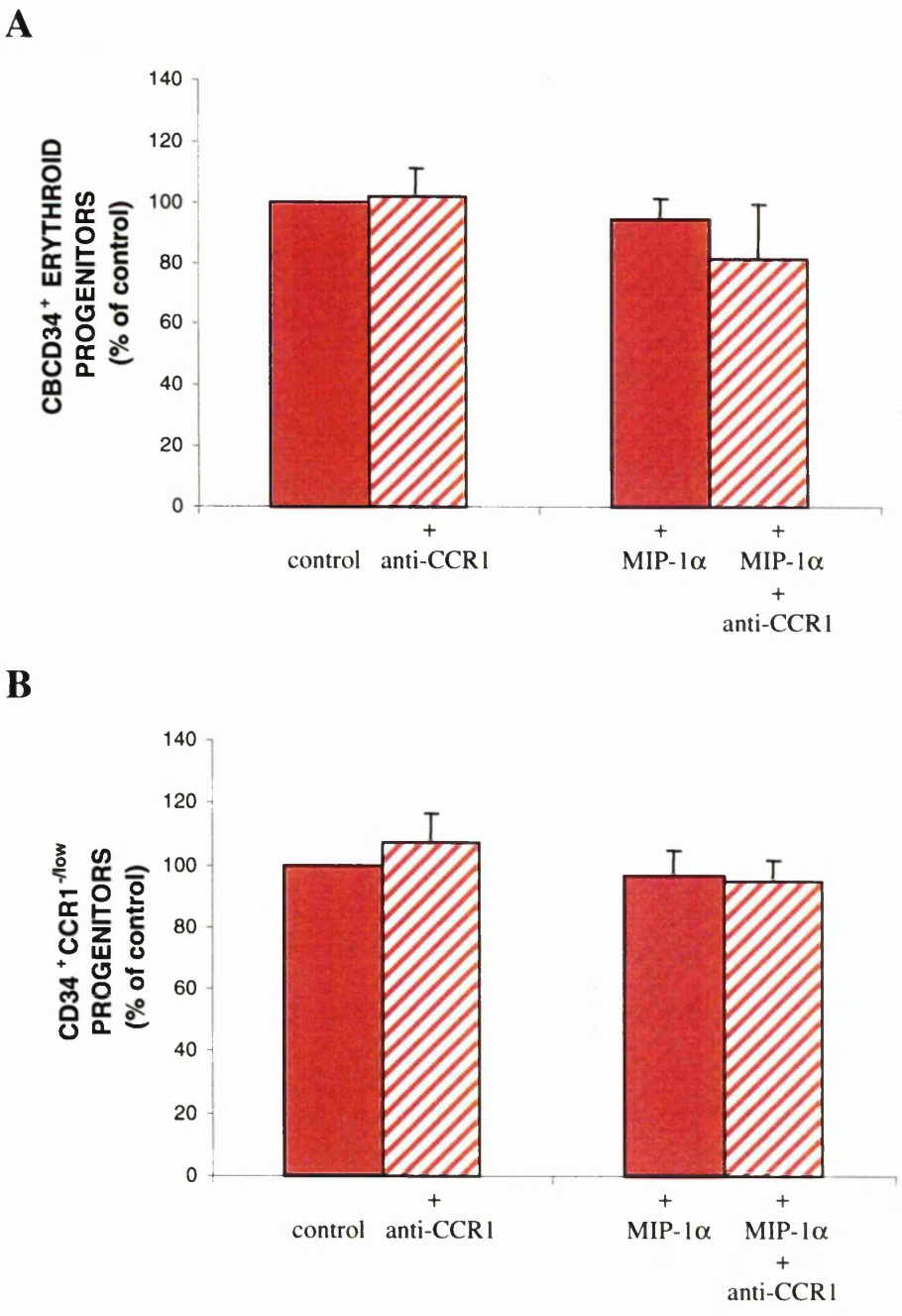


Figure 6.6. Effects of the anti-CCR1 antibody on MIP-1α–mediated proliferation of erythroid progenitors. $CD34^+$ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were labeled with anti-CCR1 antibody (section 2.1.7.6) and sorted into $CD34^+CCR1^{-/low}$ fraction by flow cytometry using the gating strategy depicted in Figure 5.14. Unsorted (**A**) and sorted (**B**) cells were cultured in clonogenic assay (section 2.1.12.1) with or without MIP-1α at 50ng/ml and anti-CCR1 antibody at 5μg/ml as indicated. Colonies were scored after 14 days. The results are expressed as the mean percentage ± SEM of the total number of colonies generated from treated cultures over control experiments. The number of colonies obtained in control cultures ranged from 40 to 107 for $CD34^+$ (n=4) and from 63 to 210 for $CD34^+CCR1^{-/low}$ (n=4).

resulted in a significant increase in the number of GM-CFC colonies generated (24%±3, $p<0.001$). The co-addition of MIP-1α and anti-CCR1 antibody blocked the stimulatory MIP-1α effect on colony formation. Moreover, it resulted in a significant reduction of 23%±8 ($p=0.025$, n=8) in the number of GM-CFC colonies generated when compared to the cultures in which only anti-CCR1 antibody was present.

6.2.2.3. $CD34^+CCR1^{-/low}$ cord blood cells

For the erythroid progenitors generated from the $CD34^+CCR1^{-/low}$ cord blood fraction, the addition of MIP-1α and anti-CCR1 antibody, alone or in combination, followed similar response patterns to those observed for unsorted $CD34^+$ cells. As Figure 6.6-B illustrates, there was no significant effect on colony formation when compared to cultures treated with anti-CCR1 alone (11%±5, $p=0.295$).

6.2.3. Effects of MIP-1α on migration of haemopoietic cells

During their maturation, haemopoietic progenitor cells have been postulated to occupy physically separated niches surrounded by particular stromal cell types (Uchida *et al.*, 1993). It has been suggested that migration of progenitor cells from one niche to another within marrow spaces may involve gradients of chemotactic factors (Aiuti *et al.*, 1998). However, little is known about the regulation of haemopoietic cell migration. To study the migratory responses of cord blood haemopoietic cells to MIP-1α, an assay in which the ability of the cells to migrate through a membrane in response to different stimuli was used (Kim and Broxmeyer, 1998; Mohle *et al.*, 1999). The cells that have transmigrated and were collected in the lower chamber of the system after a pre-established period of time assay were enumerated. Migratory cells were evaluated both phenotypically by the expression of $CD34^+$ (input cells) and functionally by their ability to form colonies in progenitor cell assays. As SDF-1α has been considered a model chemokine for studying the migration properties of haemopoietic cells *in vitro* (Aiuti *et al.*, 1997; Kim and Broxmeyer, 1998), this chemokine was used as a positive control in the present experiments.

6.2.3.1. $CD34^+$ cord blood cells

Chemokine-dependent chemotaxis of $CD34^+$ cord blood cells (section 2.1.7.5) was determined using transwell inserts as previously described in section 2.1.18. Varying concentrations of MIP-1α were used in the assay in order to determine the optimal concentration for migrating cells. As illustrated in Figure 6.7-A, MIP-1α had little chemoattractant effect on $CD34^+$ cells after 5 hours incubation. However, progenitors of both GM-CFC and erythroid lineages were amongst the $CD34^+$ cells that responded to the chemoattractant effects of MIP-1α over the concentration range used (Figure 6.7-B). The significant increase in the migration of GM-CFC progenitors reached a maximum at 50ng/ml, with 4.9%±1.1 (p=0.006) of the input GM-CFC progenitors responding to the induced migratory effect of MIP-1α. Erythroid progenitors responding to MIP-1α reached maximum significant migration values of 3.6%±0.7 over input (p=0.038) at 1.5ng/ml MIP-1α.

When $CD34^+$ cells (Figure 6.8-A) as well as GM-CFC and erythroid progenitors (Figure 6.8-B) were exposed to SDF-1α gradient, there was significant migration towards SDF-1α in a dose-related fashion, with 11%±5 to 40%±6 (p<0.001) of the input cells migrating at doses 15 and 150ng/ml, respectively. Progenitor cells had a migration profile similar to that exhibited by $CD34^+$ cells, and, at 150ng/ml, the maximum dose used in these experiments, 41%±10 (p=0.031) and 35%±9 (p=0.019) of the input GM-CFC and erythroid progenitors, respectively, were found in the SDF-1α migrated fraction.

6.2.3.2. $CD34^+CCR1^{high}$ cord blood cells

To examine the possible involvement of CCR1 in the mechanisms of progenitor cell migration mediated by MIP-1α, $CD34^+$ cells expressing the CCR1 receptor were selected from cord blood by flow cytometry using the gating strategy depicted in Figure 5.14. Migration assays were performed using the conditions described previously. Based on the data in which efficient and significant migration of progenitors towards SDF-1α at 50ng/ml was observed (section 6.2.2.1), we used a similar SDF-1α concentration as a control of these experiments. MIP-1α induced migration of $CD34^+CCR1^{high}$ sorted cells at 15ng/ml, at which 1.6%±0.2 (p=0.008) of input cells responded efficiently to the chemokine (Figure 6.9-A).

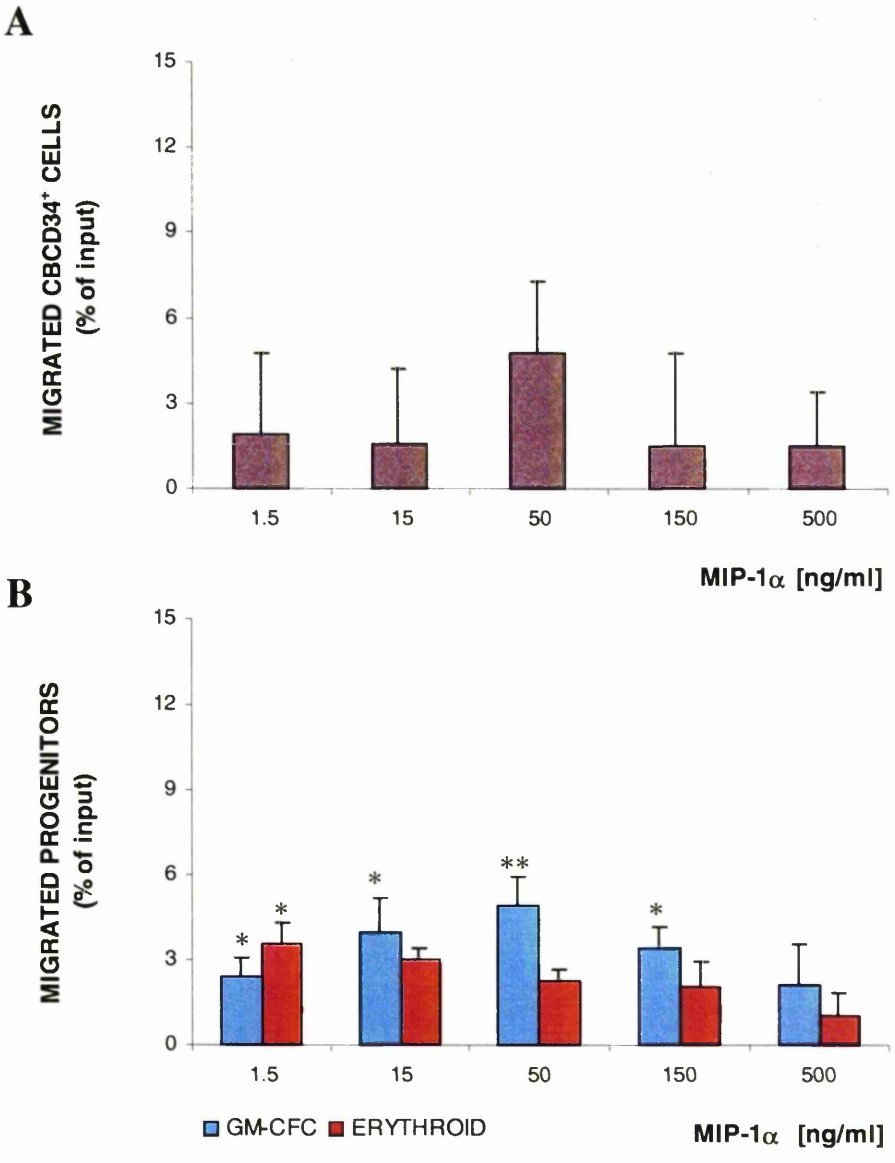


Figure 6.7. Response of CD34⁺ cells to MIP-1α–induced chemotactic activity. CD34⁺ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were added to the upper chamber of a transwell insert and migration assay performed as described in section 2.1.18. MIP-1α was added to the lower chamber at indicated concentrations. After 5 hours, transmigrated cells recovered from the lower chamber were enumerated using an haemocytometer (section 2.1.10). Input and migrated cells were plated in clonogenic assays (section 2.1.12.1) and grown colonies of GM-CFC and erythroid progenitors scored after 14 days. The results are expressed as the mean percentage ± SEM of cells (**A**) or colonies (**B**) that migrated over the input cell population after subtracting the percentage of cells that migrated towards medium alone. The number of colonies obtained in control cultures ranged from 41 to 194 for GM-CFC and from 44 to 155 for BFU-E (n=3-5). * $p<0.05$; ** $p<0.01$

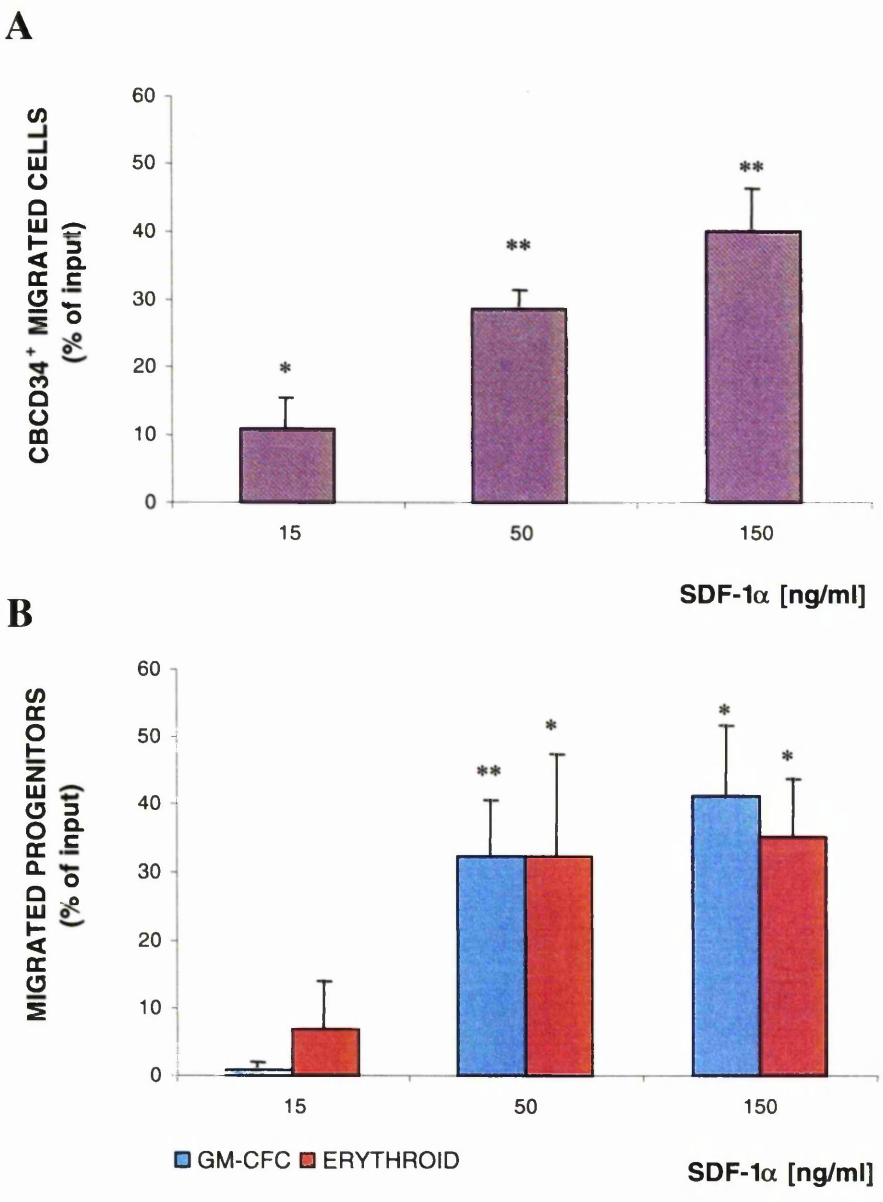


Figure 6.8. Chemotactic activity of SDF-1α on CD34⁺ cord blood cells. CD34⁺ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were added to the upper chamber of a transwell insert and SDF-1α was added to the lower chamber at indicated concentrations. The migration assay was performed as described in section 2.1.18. After 5 hours, transmigrated cells recovered from the lower chamber were enumerated using a haemocytometer (section 2.1.10). Input and migrated cells were plated in clonogenic assays a described in section 2.1.12.1. Grown colonies of GM-CFC and erythroid progenitors were scored after 14 days. The results are expressed as the mean percentage ± SEM of cells (**A**) and colonies (**B**) that migrated over input cell population after subtracting the percentage of cells that migrated spontaneously towards medium alone. The number of colonies of control cultures ranged from 92 to 411 for GM-CFC and from 44 to 313 for BFU-E (n=3-8). * $p<0.05$; ** $p<0.01$

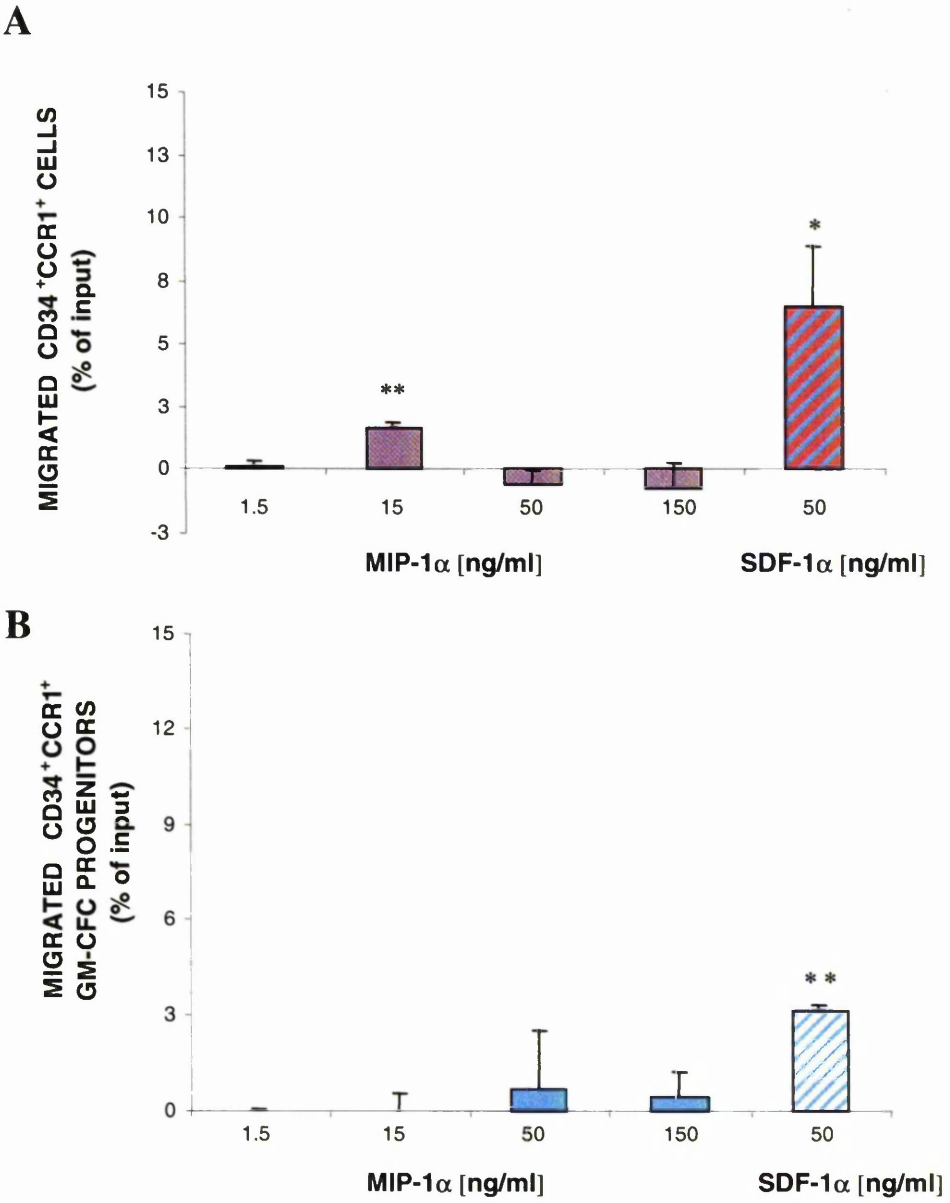


Figure 6.9. Chemotactic activity of MIP-1α on CD34$^+$CCR1^{high} cells. CD34$^+$ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were labeled with anti-CCR1 antibody (section 2.1.7.6) and the CD34$^+$CCR1^{high} cells sorted by flow cytometry using the gating strategy depicted in Figure 5.14. CD34$^+$CCR1^{high} sorted cells were added to the upper chamber of the migration insert and the migration assay was carried out as described in section 2.1.18. MIP-1α was added to the lower chamber at indicated concentrations. After 5 hours, transmigrated cells recovered from the lower chamber were enumerated using a haemocytometer (section 2.1.10). Input and migrated cells were plated in clonogenic assays and the grown colonies were counted after 14 days. The results are expressed as the mean percentage ± SEM of the total number of cells (A) or progenitors (**B**) migrated over input after subtracting the percentage of cells that migrated towards medium alone. SDF-1α was used as a control. The number of GM-CFC colonies generated in control cultures ranged from 68 to 493 (n=3-5). * $p<0.05$; ** $p<0.01$

When migrated cells were plated in clonogenic assays, maximum MIP-1α chemoattraction on GM-CFC progenitors was observed at 50ng/ml chemokine, with few progenitors responding to MIP-1α (Figure 6.9-B). As observed for $CD34^+$ cells, $CD34^+CCR1^{high}$ sorted cells also migrated towards SDF-1α more efficiently. This effect was at least 4-fold higher than that observed for MIP-1α at optimal concentration, with 6.5%±2.4 (p=0.024) of input cells recovered in the lower chamber.

However, in contrast to the SDF-1α migration effect observed for the unsorted GM-CFC progenitors, only 3.1%±0.3, (p=0.001) of input GM-CFC from the $CD34^+CCR1^{high}$ population migrated efficiently and significantly towards SDF-1α than to MIP-1α. This reduction in the number of GM-CFC colonies migrating towards SDF-1α is probably due to the sorting procedures with the aim of selecting the $CD34^+CCR1^{high}$ population, which may have selected a population that do not express similar levels of CXCR4 as the unsorted cells. Indeed, when the same $CD34^+$ population was analysed for CCR1 and CXCR4 co-expression (Figure 6.10, A and B), only ~87% of the population analysed expressed CXCR4 while more than 96% were positively labeled for CCR1.

6.2.4. Effects of MIP-1α and SDF-1α on adhesion of haemopoietic cells

Adhesive interactions between haemopoietic progenitors and the bone marrow microenvironment are necessary to control localisation, retention, and proliferation of stem and progenitor cells (Levesque *et al.*, 1996; Williams *et al.*, 1991). Like haemopoietic cells, cytokines bind to specific extracellular matrix components (Verfaillie, 1998), and selective adhesion of progenitors and cytokines to extracellular matrix components or stromal cells is involved in the co-localisation of progenitors at a specific stage of differentiation (Lemischka, 1997). $CD34^+$ cells express α4β1 and α5β1 integrin receptors that function as receptors for fibronectin, the main component of the extracellular matrix in bone marrow (Clark *et al.*, 1992; Kerst *et al.*, 1993). Our results have shown that MIP-1α and SDF-1α can affect the proliferation status of haemopoietic progenitor cells. As adhesion-stimulated signalling pathways may be an important component of the regulation of cell division during normal haemopoiesis, we, next, investigated the ability of these chemokines to induce adhesion of cord blood $CD34^+$ cells to immobilised fibronectin.

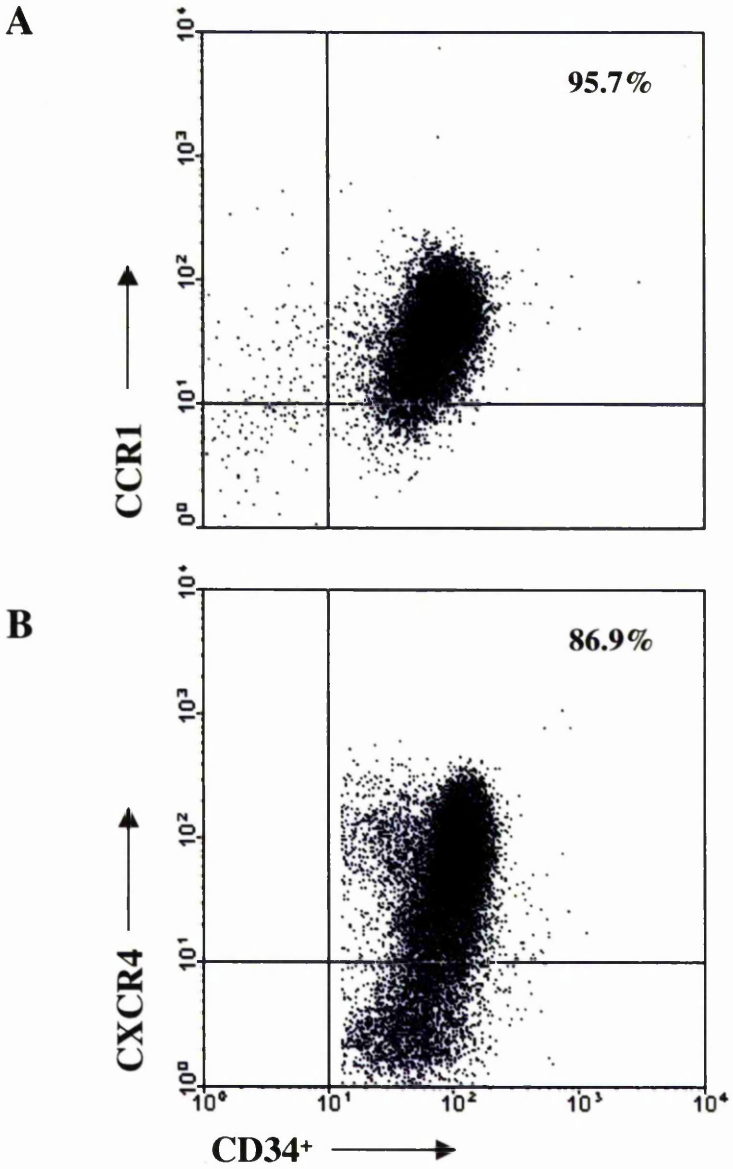


Figure 6.10. Expression of CCR1 and CXCR4 on cord blood CD34⁺ cells. CD34⁺ cells obtained after Mini-MACS separation (section 2.1.7.5) were double stained with PE or FITC antibodies for CD34 and CCR1 (**A**) or CXCR4 (**B**) (section 2.1.7.6) and analysed by flow cytometry using the gating strategy depicted in Figure 5.11. Values represent the percentage of CD34⁺ cells positive for the indicated receptor. Data from one representative experiment are shown.

6.2.4.1. $CD34^+$ cord blood cells

As for the previous studies, we have used the Mini-MACS® immunomagnetic separation system to obtain $CD34^+$ cells from cord blood (section 2.1.7.5) and tested the ability of MIP-1α to trigger their adhesion to fibronectin over a dose range of the chemokine as described in section 2.1.17. Adhesion of $CD34^+$ cells to fibronectin-coated wells was significantly higher than their adhesion to BSA-treated wells. On average, 7.2%±1.5 of $CD34^+$ cells adhered to BSA-treated wells whilst 32%±3 of this population adhered to fibronectin ($p<0.001$). These results indicate that a high percentage of $CD34^+$ cells has the ability to adhere spontaneously to immobilised fibronectin. Exposure of cord blood $CD34^+$ cells to MIP-1α increased the adhesive capacity of these cells to fibronectin (Figure 6.11-A), with maximum response observed at 1.5ng/ml MIP-1α, with 18%±2 ($p=0.002$) increase over fibronectin alone.

Aliquots of both adherent and non adherent populations were plated in clonogenic assays in order to investigate whether GM-CFC and erythroid subsets within the $CD34^+$ population also bind to fibronectin. As for $CD34^+$ cells, both GM-CFC and erythroid progenitors showed significant increased adherence to fibronectin when compared to the BSA control (21%±2 *versus* 43%±5, $p=0.014$, for GM-CFC, and 19%±4 *versus* 36%±3.2, $p=0.027$, for erythroid progenitors). MIP-1α enhanced the adhesion of both GM-CFC and erythroid progenitors to fibronectin (Figure 6.11-B). The maximum enhancing adhesion effect for GM-CFC was achieved at 50ng/ml MIP-1α, at which dose 11%±4 ($p=0.154$) more progenitors were found in the fibronectin-adherent fraction. For erythroid progenitors, significant enhancement was found at 15-50ng/ml (9%±1.5, $p=0.043$) of chemokine, reaching maximum adhesion at 50ng/ml (16%±5, $p=0.062$).

We, next, examined whether SDF-1α could influence adhesion of $CD34^+$ cells to fibronectin. As depicted in Figure 6.12-A, SDF-1α showed also capacity to induce adhesion of $CD34^+$ cells to fibronectin, with maximum cell adhesion attained at 50ng/ml (14%±1.1 over fibronectin alone, $p=0.008$). In addition, GM-CFC and erythroid progenitors showed a similar adhesion profile (Figure 6.12-B), and maximum effect was observed at 15ng/ml SDF-1α, with more than 20% adhesion for GM-CFC ($p=0.016$) and erythroid ($p=0.002$) progenitors compared to fibronectin control values.

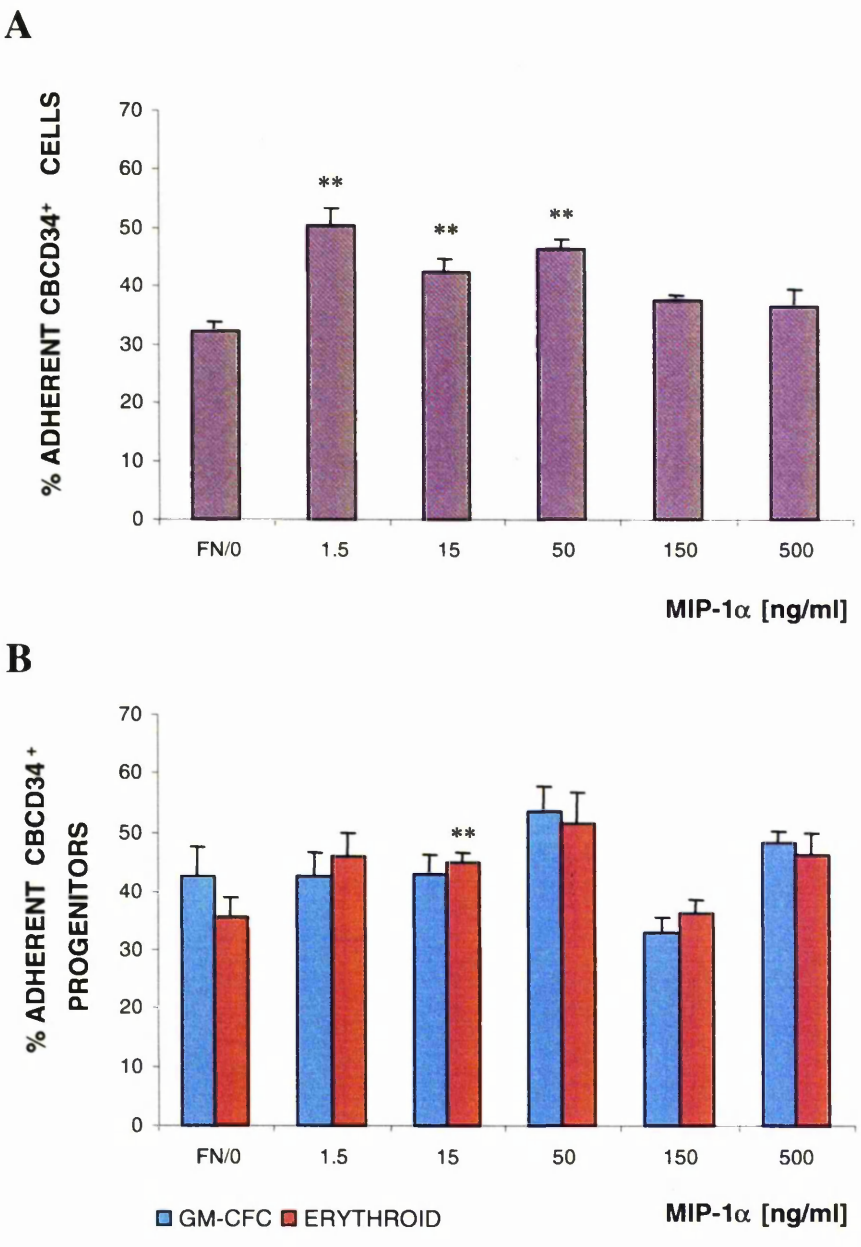


Figure 6.11. MIP-1α-mediated adhesion of CD34⁺ cells. Adhesion of CD34⁺ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) to immobilised fibronectin was assayed as described in section 2.1.17. After 1 hour in at 37°C, recovered adherent and non-adherent cells were enumerated using a haemocytometer (section 2.1.10) and plated in clonogenic assays as described in section 2.1.12.1. Colonies of GM-CFC and erythroid progenitors were scored after 14 days. Adhesion is expressed as the mean percentage ± SEM of bound cells/(unbound+bound cells) (**A**) and bound colonies/(unbound+bound colonies) (**B**). The number of colonies of control cultures ranged from 80 to 276 for GM-CFC and from 72 to 168 for BFU-E (n=3-5). ** $p<0.01$

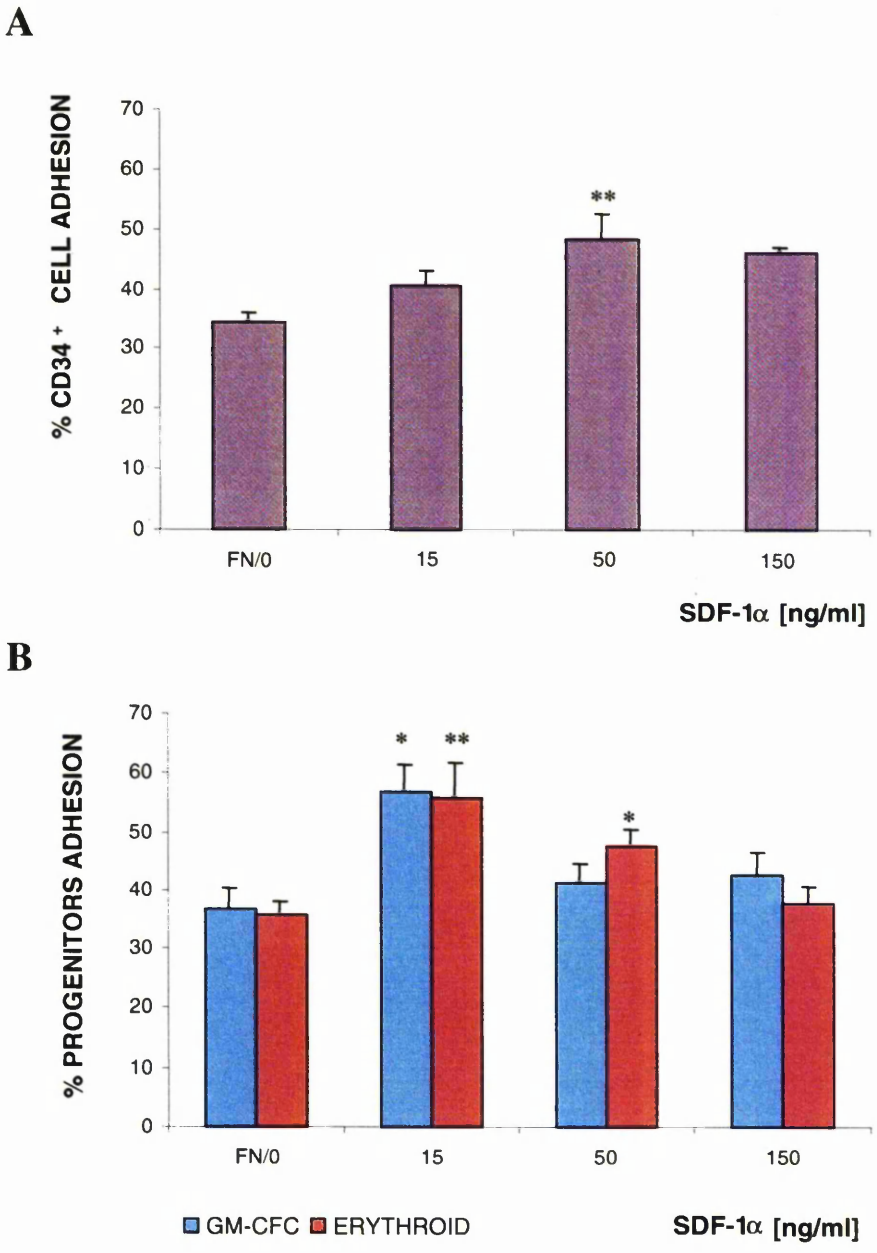


Figure 6.12. SDF-1α–mediated adhesion of CD34⁺ cells. 10^5 $CD34^+$ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were assayed in adhesion assay as described in section 2.1.17. After 1 hour incubation at 37°C in 5% CO_2 and O_2 in air, recovered adherent and non-adherent cells were enumerated using a haemocytometer (section 2.1.10) and plated in clonogenic assays as described in section 2.1.12.1. Colonies of GM-CFC and erythroid progenitors were scored after 14 days. Adhesion is expressed as the mean percentage ± SEM of bound cells/(unbound+bound cells) (**A**) and bound colonies/(unbound+bound colonies) (**B**) that adhered to fibronectin. The number of colonies of control cultures ranged from 80 to 276 for GM-CFC and from 72 to 168 for BFU-E (n=3-5). * $p<0.05$; ** $p<0.01$

6.2.4.2. $CD34^+CCR1^{high}$ cord blood cells

The data generated from the previous experiments showed that MIP-1α significantly enhanced adhesion of $CD34^+$ haemopoietic cells to immobilised fibronectin. To correlate whether this effect was related to CCR1 expression, $CD34^+CCR1^{high}$ sorted cells were assessed for MIP-1α fibronectin-adhesion induced effects.

Surprisingly, $CD34^+CCR1^{high}$ cells showed a significant increase in their capacity to adhere to BSA when compared to unlabeled $CD34^+$ cells (38%±5 *versus* 7%±1, $p<0.01$). Similarly, $CD34^+CCR1^{high}$ cells also adhered more to fibronectin than their $CD34^+$ counterpart (48%±8 versus 32%± 3, $p=0.014$). However, there was no significant difference in the adherence of $CD34^+CCR1^{high}$ cells when adhesion to BSA and fibronectin was compared. The addition of MIP-1α to the assays had no effect on the adhesion to fibronectin profile for both $CD34^+CCR1^{high}$ cells (Figure 6.13-A) and their GM-CFC progenitors (Figure 6.13-B).

To investigate whether the anti-CD34 antibody used for the double labelling could have any participation in these events, in some experiments $CD34^+$ cells obtained after immunomagnetic separation were labeled only with the anti-CD34 antibody (section 2.1.7.7), FACS sorted and used in the adhesion assay. No differences in the results between unsorted and sorted populations were detected, indicating that this mechanism was specific for the anti-CCR1 antibody.

6.3. Discussion

The underlying mechanisms through which MIP-1α influences haemopoiesis remain obscure. As these activities may be regulated at a cellular receptor level and $CD34^+$ cells constitutively express high levels of CCR1, part of this study was undertaken to increase our understanding of the role of CCR1 in mediating MIP-1α responses. In this chapter, we investigated the biological effects of MIP-1α on *in vitro* proliferation, migration and adhesion of $CD34^+$ cells and, where possible, correlated these biological functions with the expression of CCR1 using sorted populations. Unfortunately, because of the rarity of $CD34^+$ cells expressing low levels of CCR1 receptor and the difficulties of getting sufficient cell numbers to pursue similar studies,

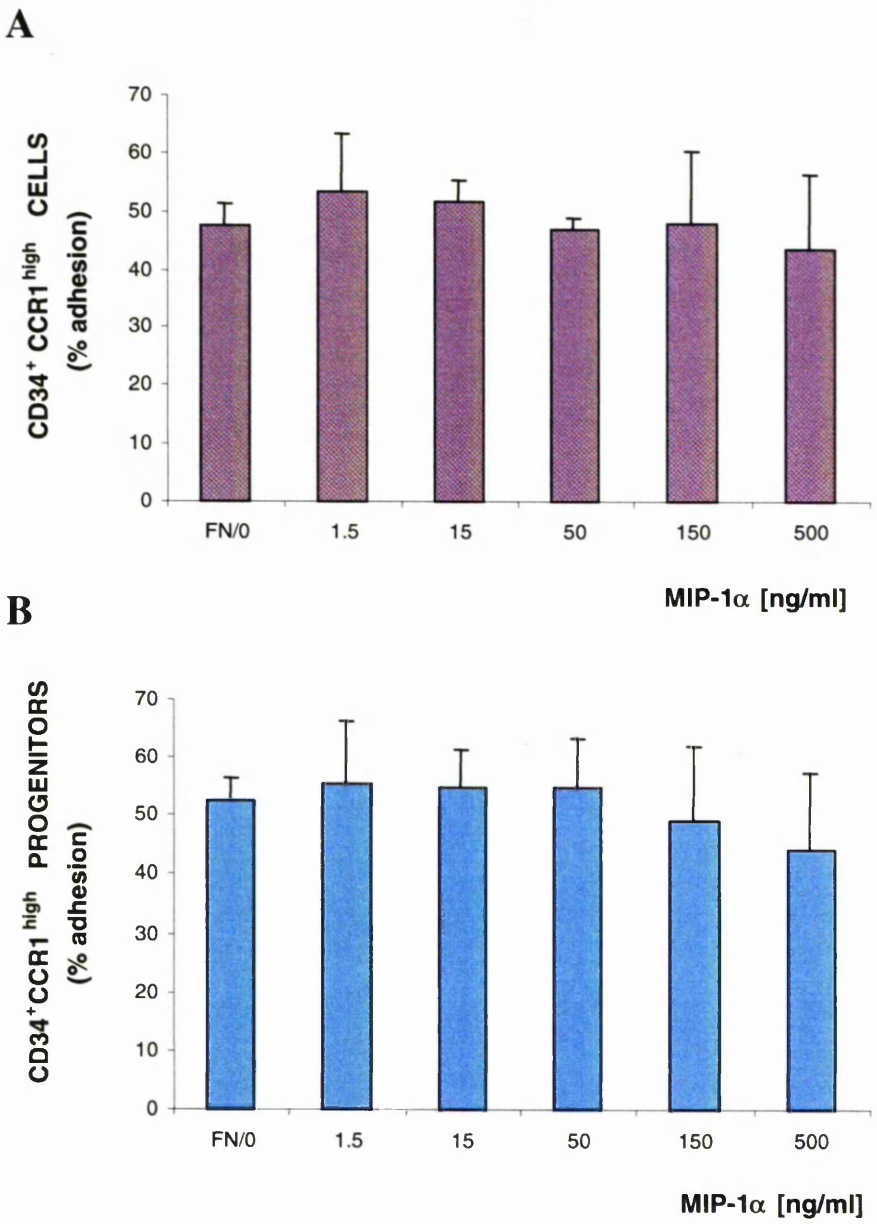


Figure 6.13. MIP-1α–mediated adhesion of CD34$^+$CCR1^{high} cells. CD34$^+$ cells from cord blood obtained after immunomagnetic separation (section 2.1.7.5) were labeled with anti-CCR1 antibody (section 2.1.7.6) and the CD34$^+$CCR1^{high} cells sorted by flow cytometry using the gating strategy depicted in Figure 5.14. CD34$^+$CCR1^{high} cells were plated in adhesion assay as described in section 2.1.17. After 1 hour incubation at 37°C in 5% CO_2 and O_2 in air, recovered adherent and non-adherent cells were enumerated using a haemocytometer (section 2.1.10) and plated in clonogenic assays as described in section 2.1.12.1. GM-CFC colonies were scored after 14 days. Adhesion is expressed as the mean percentage ± SEM or SD (where n=2) of bound cells/(unbound+bound cells) (**A**) and bound colonies/(unbound+bound colonies) (**B**) that adhered to fibronectin. The number of colonies of control cultures ranged from 137 to 339 (n=2-4).

evaluation of the MIP-1α effects on migration and adhesion of progenitors was performed only on $CD34^+$ cells expressing high levels of CCR1.

In the process of comparing the effects of MIP-1α in *in vitro* clonogenic assays on $CD34^+$ cells from cord blood and bone marrow, some interesting divergences were detected. The number of GM-CFC colonies and, to a much lesser extent, of erythroid progenitors from cord blood (Figure 6.1-A) increased in the presence of MIP-1α, whereas a significant dose-related suppressive effect was observed for GM-CFC and BFU-E progenitors derived from $CD34^+$ bone marrow cells (Figure 6.1-B). This is in agreement with previous findings (Bonnet *et al.*, 1995; Broxmeyer *et al.*, 1993b; Broxmeyer *et al.*, 1990; Chasty *et al.*, 1995; Mayani *et al.*, 1995; Su *et al.*, 1997; de Wynter *et al.*, 1998). Moreover, these differential effects were reproduced using the sorted $CD34^+CCR1^{high}$ (Figures 6.3, A and B), and $CD34^+CCR1^{-/low}$ (Figures 6.4, A and B) populations from cord blood and bone marrow, containing mostly GM-CFC and erythroid progenitors, respectively.

The *in vitro* effects of MIP-1α on proliferation of subsets of $CD34^+$ progenitors from other haemopoietic tissues such as foetal liver and mobilised peripheral blood have been recently assessed (de Wynter *et al.*, 1998; Durig *et al.*, 1999a; Keller *et al.*, 1994; Lu *et al.*, 1993a; Mayani *et al.*, 1995; Van Ranst *et al.*, 1996; Weekx *et al.*, 1998). These studies demonstrated that the effects of MIP-1α on foetal and cord blood cells were qualitatively different from those seen in bone marrow or peripheral blood. For instance, the MIP-1α suppressive effect observed for bone marrow (Broxmeyer *et al.*, 1990; Graham *et al.*, 1990) and mobilised peripheral blood (Durig *et al.*, 1999a) progenitors was not observed in cord blood (de Wynter *et al.*, 1998; Durand *et al.*, 1993) or foetal progenitors (Weekx *et al.*, 1998). Moreover, it has been shown that progenitor cells from different ontogenic sources have functional differences indicated by their cytokine requirements, growth factor responses, and spontaneous colony formation in clonogenic assays (Weekx *et al.*, 1998). Therefore, these studies suggest that the ability of a cell to respond to MIP-1α *in vitro* may depend not only on its ontogenic source, but also on a number of factors. These include the cell population under analysis, the cytokines to which the cell is exposed, and the assay conditions.

Several reports have proposed that the responses of haemopoietic progenitors to MIP-1α may differ qualitatively depending on the maturational stage of the progenitor cell population analysed (Broxmeyer *et al.*, 1990; Graham *et al.*, 1990; Keller *et al.*,

1994; Maze *et al.*, 1992). Throughout this work a relatively pure population of $CD34^+$ cells, as well as similar culture conditions were used for both cord blood and bone marrow cells. In addition, the *in vitro* growth of these cells was stimulated by EPO plus 5637 conditioned medium, known to contain a cocktail of cytokines (Coutinho *et al.*, 1993; Myers *et al.*, 1995), which stimulate both mature and more primitive haemopoietic cells. Therefore, it is possible that the differential responses of cord blood and bone marrow progenitors to MIP-1α are due to quantitative differences in the distribution of immature and more mature progenitors as suggested by others (Broxmeyer *et al.*, 1990), which could not be revealed by the assay conditions used.

On the other hand, it has been demonstrated that human $CD34^+$ cells from cord blood have an increased re-plating potential compared with $CD34^+$ cells from bone marrow (Lu *et al.*, 1993b). Evidence for a greater proliferative potential of haemopoietic stem/progenitor cells from foetal sources compared with adult bone marrow has also been documented (Micklem *et al.*, 1972; Lansdorp *et al.*, 1993; Lu *et al.*, 1993b; Rebel *et al.*, 1996). Moreover, it has been recently shown that the number of CFC/culture generated by foetal liver and cord blood LTC-ICs was 4- and 1.5-fold higher, respectively, compared to bone marrow after 6 weeks of culture in the presence of early acting cytokines (Eaves, 2000). These data, collectively, imply that the proliferative potential of primitive haemopoietic cells decreases during ontogeny as suggested by others (Keller *et al.*, 1994; Lansdorp, 1995; Lansdorp *et al.*, 1993; Weekx *et al.*, 1998; Zandstra *et al.*, 1998), or that the content of highly proliferative cells is greater in these tissues. These may influence the way in which cells of the same lineage, but from different haemopoietic sources, respond to similar stimuli.

Our results showed an increase in the number of GM-CFC colonies treated with MIP-1α only in cultures from cord blood (Figures 6.1-A and 6.3-A). These findings suggest that MIP-1α may be acting upon a sub-population within $CD34^+$ cells that has no clonogenic potential when directly cultured in clonogenic assay but, after exposure to the chemokine, acquires the ability to form colonies. This colony-forming ability is revealed through the increase in colony numbers. This sub-population can not be distinguished from the population that has the ability to grow as colonies when directly plated in clonogenic assays as the size and the morphology of the colonies were similar in all conditions tested. It has been suggested that some of the effects of MIP-1α on proliferation of haemopoietic progenitors may be related to its effect on the cell cycle

status of the cell population under analysis (Graham *et al.*, 1990; Lu *et al.*, 1993a; Pragnell *et al.*, 1988). Recent studies have shown that MIP-1α suppressed the number of bone marrow GM-CFC actively cycling whereas it stimulated cord blood GM-CFC into cycle (de Wynter *et al.*, 1998; Lu *et al.*, 1993b). If MIP-1α can influence the cell cycle of GM-CFC by triggering them into cycle, this response could be detected in clonogenic assays by an increase in the number of GM-CFC progenitors.

In contrast to its stimulatory effect on the GM-CFC progenitors from cord blood, MIP-1α has been shown to suppress erythroid progenitors from both cord blood and bone marrow (de Wynter *et al.*, 1998). The weak MIP-1α effects observed on BFU-E colony formation from cord blood (Figures 6.1-A and 6.4-A) in comparison with its significant inhibitory effects on erythroid progenitors from bone marrow (Figures 6.1-B and 6.4-B) may involve differences in receptor density and/or affinity, rendering cord blood cells less responsive to the effects of MIP-1α.

To gain additional insight into the biological role of MIP-1α in haemopoiesis, we investigated whether CCR1 could be the receptor mediating these effects by adding a 100-fold excess of the anti-CCR1 antibody to the clonogenic assay medium. Exposure of $CD34^+$ (Figure 6.5-A) and $CD34^+CCR1^{high}$ (Figure 6.5-B) cord blood cells to MIP-1α in the presence of an excess of anti-CCR1 antibody blocked the increase of GM-CFC colony formation. Moreover, it led to a simultaneous decrease in their numbers. The MIP-1α effects on the GM-CFC progenitors in the presence of an excess of anti-CCR1 antibody may be explained by two possible mechanisms. The continuous presence of the antibody may have acted as a competitive ligand, preventing MIP-1α binding to CCR1 or, alternatively, it may have downregulated CCR1 surface receptors, so that these receptors were no longer available for the cytokine to bind and exert its stimulatory effect. If this was true, then MIP-1α available in the culture could bind another, as yet unidentified MIP-1α receptor(s), and still exert an inhibitory effect. To better clarify this, it would be important to use this antibody on other $CD34^+$ populations, particularly from bone marrow, and observe the MIP-1α effects on GM-CFC progenitors generated after blocking CCR1.

The fact that a specific anti-CCR1 antibody could block the stimulatory effect of MIP-1α suggests that CCR1 is at least one of the receptors responsible for the stimulatory effect of MIP-1α on these cells. In support of this assumption, a recent report indicated that GM-CFC and M-CFC in CCR1-deficient (-/-) mice did not respond

to the stimulatory effects of MIP-1α (Broxmeyer *et al.*, 1999a). However, the multi-growth factor-stimulated myeloid progenitor cells from the CCR1 knockout mice were as sensitive to inhibition by MIP-1α as their wild-type counterparts (Broxmeyer *et al.*, 1999a). It is also of value to note that, according to the data obtained from the previous chapter (section 5.2.2), erythroid progenitors represent a selected population that express low levels of CCR1. A sligth decrease in the number of erythroid colonies was observed for $CD34^+$ cells (Figure 6.6-A), but not for $CD34^+CCR1^{-/low}$ sub-population (Figure 6.6-B), from cord blood after treatment with MIP-1α in the presence of an excess of anti-CCR1 antibody. Similar conditions applied to bone marrow erythroid progenitors, which were significantly inhibited by MIP-1α, have shown that the inhibitory effect of MIP-1α was only partially reversed (Su *et al.*, 1997). These findings collectively indicated that, in the absence of CCR1, the inhibitory effects of MIP-1α become more evident, suggesting that the inhibitory responses to MIP-1α does not occur through CCR1.

Apart from CCR1, the receptors CCR3 (Nibbs *et al.*, 1997a), CCR4 (Hoogewerf *et al.*, 1996), CCR5 (Power *et al.*, 1995; Raport *et al.*, 1996) and D6 (Nibbs *et al.*, 1997a; Nibbs *et al.*, 1997b) have been cloned as MIP-1α receptors. Of all MIP-1α receptors identified to date, only CCR1 and CCR5 have been shown to transduce signals in response to MIP-1α (Samson *et al.*, 1997). It is possible that one or more of these (or another as yet unidentified) receptors may be responsible for the MIP-1α inhibitory effects. As we have used a polyclonal anti-CCR1 antibody, another possibility that remains is a CCR1 sub-type(s) mediating the opposing effects of MIP-1α. The availability of neutralising monoclonal antibodies to CCR1 as well as to other MIP-1α receptors should facilitate a more detailed analysis of the MIP-1α mechanisms mediating haemopoietic progenitor growth.

MIP-1α activates several different intracellular effectors as reviewed in Chapter 1 (section 1.4.1). MIP-1α induces an increase in cytosolic Ca^{++} levels via pertussis toxin G-coupled receptors in haemopoietic cells (Durig *et al.*, 1999a; Gao *et al.*, 1993; Neote *et al.*, 1993; Sozzani *et al.*, 1993). Nevertheless, the MIP-1α signalling pathways in primary haemopoietic cells are not clear and have not yet been fully investigated. Therefore, differences in intracellular signalling events could also account for the differences in the response of myeloid progenitors from cord blood and bone marrow to MIP-1α.

SDF-1α was the first member of the chemokine family demonstrated to have chemotactic effects on $CD34^+$ cells and their progenitors (Aiuti *et al.*, 1997). This assay system has been useful for investigating and comparing migratory abilities of haemopoietic cells to other compounds. Although the migratory effect of MIP-1α was poor when compared with SDF-1α (Figures 6.8-A), the results showed that MIP-1α can attract $CD34^+$ cord blood cells (Figure 6.7-A), implying the presence of functional receptors. Moreover, we have demonstrated that GM-CFC and erythroid progenitors are among the $CD34^+$ cells that migrated towards MIP-1α (Figure 6.7-B). The ability of MIP-1α to mobilise marrow progenitors into circulation has been reported (McCourt *et al.*, 1994; Lord *et al.*, 1995), and CCR1 has been implicated in this activity since, in mice lacking functional CCR1, this *in vivo* mobilisation is absent (Broxmeyer *et al.*, 1999a). In an attempt to further explore this issue, selected $CD34^+$ cord blood cells expressing high levels of this receptor were tested for their migratory responses towards MIP-1α. $CD34^+CCR1^{high}$ cells, but not their progenitors, migrated efficiently towards MIP-1α (Figure 6.9, A and B, respectively). When the same cell populations were exposed to SDF-1α, a significant migration response occurred. These results suggest that CCR1 is involved in the migration of cord blood haemopoietic progenitors towards MIP-1α, but may be not the predominant receptor mediating this activity. This is further supported by the fact that chemotaxis towards MIP-1α of peripheral blood monocytes, which has been shown to express high levels of CCR1 (Figure 5.13-A) and reported to express a number of chemokine receptors capable of transducing signals by MIP-1α (Uguccioni *et al.*, 1995), was only partially reversed when these cells were exposed to a specific anti-CCR1 antibody (Su *et al.*, 1996).

Haemopoietic progenitor cells are mobilised from the bone marrow to the peripheral blood in response to radiation, chemotherapeutic agents or injected cytokines, and home to the bone marrow during transplantation. There is increasing evidence that the homing process requires specific adhesive interactions between adhesion molecule receptors and their ligands. Since MIP-1α affected progenitor cell proliferation and migration, we considered whether it could also be involved in haemopoietic cell adhesion. Our experiments showed that MIP-1α significantly enhanced adhesion of $CD34^+$ cells from cord blood to immobilised fibronectin (Figure 6.11-A). Moreover, GM-CFC progenitors and progenitors particularly of the erythroid lineage showed a partial response to MIP-1α in the adhesion assay (Figure 6.11-B), in agreement with

other reports (Peled *et al.*, 2000; Suehiro *et al.*, 1999; Suehiro *et al.*, 1999; Durig *et al.*, 1999a).

The mechanisms by which MIP-1α induces adhesion of haemopoietic progenitors to fibronectin are not known. Haemopoietic $CD34^+$ cells express α4β1 and α5β1 which are both receptors for fibronectin (Kerst *et al.*, 1993; Peled *et al.*, 2000; Rosemblatt *et al.*, 1991; Ryan *et al.*, 1991; Soligo *et al.*, 1990). MIP-1α is secreted endogenously by human bone marrow macrophages (de Wynter, 2000; Maltman *et al.*, 1993) and $CD34^+$ cells (Majka *et al.*, 1999). *In vitro*, MIP-1α transcripts have been detected in the adherent layer of LTBMC (Otsuka *et al.*, 1991). Recently, MIP-1α has been demonstrated in the adherent layer of LTBMC (de Wynter, 2000). Thus, MIP-1α seems to be a readily available 'resident' chemokine within the bone marrow microenvironment that could act in different haemopoietic processes simultaneously. Therefore, it is possible that MIP-1α may modulate the expression and/or interfere with the affinity of the integrins on progenitor cells. In this context, a growing number of studies indicate that bone marrow proteoglycans such as heparan sulphate (HS) can play a direct role in the molecular events that regulate cell adhesion, migration and proliferation by functioning either as receptors or as ligands for molecules that are required for these events to occur (Schofield *et al.*, 1999; Wight *et al.*, 1992). Recently, it has been shown that the sensitivity of CHO cells expressing the human CCR1 and CCR5 receptors to MIP-1α was enhanced by the presence of cell surface HS by increasing receptor affinity (Ali *et al.*, 2000). This suggests a possible mechanism for the sequestration of MIP-1α onto the cell surface, making it available for presentation to specific cell-signalling receptors, similar to that proposed for fibroblast growth factors (Yayon *et al.*, 1991). Local dynamic changes in MIP-1α concentration within stem cell niches could, therefore, anchor migrating primitive progenitors on site by inducing adhesion or release the cells by reversion to a migrational state. While maintaining adherence of haemopoietic progenitors to the stroma, MIP-1α could also play a role in proliferation by modulating their cell cycle status.

As MIP-1α significantly increased the adhesiveness of $CD34^+$ and progenitor cord blood cells to fibronectin (Figure 6.11), we investigated the role of CCR1 in mediating this activity. The analysis of the MIP-1α induced adhesion to fibronectin was hampered by technical difficulties associated with the use of the anti-CCR1 antibody. It was found that, in all doses tested, the extent of cell adhesion did not match that observed with the

unsorted cells or their progenitors when exposed to the same adhesion experiments (Figure 6.13, A and B, respectively).

It is important to note that the anti-CCR1 antibody promoted cell attachment also to the BSA-substrate. This event was not related to the possibility that the anti-CCR1 antibody itself had been adsorbed to the substrates as several cell washings were performed after the labelling and after the FACS sorting, eliminating unbound molecules. In addition, the presence of anti-CD34 molecules attached to the sorted cells did not interfere with cell adhesion as there was no difference in the cell adhesion profile to BSA or to fibronectin between the $CD34^+$ cells obtained directly from the Mini-MACS column and the FACS sorted $CD34^+$ cells (for which an additional anti-CD34 antibody was used). These findings indicated that the anti-CCR1 antibody may have bound to and subsequently activated the CCR1 receptors so that receptor occupancy resulted in enhanced non specific binding.

The anti-CCR1 antibody used for selecting the $CD34^+$ cells expressing high levels of CCR1 by flow cytometry did not interfere with the proliferation and migration assays performed as responses of the $CD34^+CCR1^{high}$ population to MIP-1α were observed. The results of these studies were inconclusive in defining a role for CCR1 in adhesion and further investigation is required. An alternative procedure to overcome this situation and to analyse the possible participation of CCR1 in MIP-1α-mediated adhesion to fibronectin would be to allow sorted cells to lose the expression of the antibody and then perform the adhesion assay.

In this work, we confirmed the ability of SDF-1α to attract and thus induce migration of haemopoietic progenitors. The potential of SDF-1α to influence haemopoietic cell growth was also tested, and in agreement with other recent published studies (Broxmeyer *et al.*, 1999b; Kim and Broxmeyer, 1999b; Lataillade *et al.*, 2000; Majka *et al.*, 2000a) no effect on proliferation of progenitors was observed. The effect of SDF-1α on proliferation of haemopoietic progenitor cells from sources other than bone marrow has been explored (Broxmeyer *et al.*, 1999b; Durig *et al.*, 1999a; Lataillade *et al.*, 2000) though the data are conflicting. SDF-1α has been reported to activate multiple signal transduction pathways that mediate cell growth, migration and transcriptional events (Ganju *et al.*, 1998a; Vila Coro *et al.*, 1999; Wang *et al.*, 2000). Therefore, the different responses of myeloid progenitors, in particular of erythroid lineage, from different ontogenic sources to SDF-1α may reflect particularities in

CXCR4 expression such as receptor density or in signal transduction as previously suggested for MIP-1α, and needs further investigation.

In line with a recently published reports (Peled *et al.*, 2000; Durig *et al.*, 1999a; Wang *et al.*, 1998), we have confirmed that SDF-1α induces adhesion of cord blood $CD34^+$ cells as well as myeloid progenitors to fibronectin (Figure 6.12, A and B, respectively). Taken together, these data suggest that SDF-1α acts not only as a chemoattractant, but also participates in modulating the growth and adhesion of haemopoietic progenitors to extracellular matrix components. How SDF-1α acts or discriminates between these activities is not known, and it is possible that, similarly to MIP-1α, in a steady-state haemopoiesis, SDF-1α produced by stromal cells (Tashiro *et al.*, 1993) may attract preferentially progenitor cells, allowing them to be maintained in close contact with the appropriate environment that support their development. In contrast, under stress circumstances or when a cytokine gradient is established, SDF-1α may be involved in the release of haemopoietic progenitors by suppressing their adhesiveness to extracellular matrix components. In both situations, modulation of the adhesion receptors would then be one important key of the process.

In summary, we have demonstrated that MIP-1α affected the proliferation of $CD34^+$ cells, inhibiting myeloid progenitors from bone marrow, while stimulating the GM-CFC population from cord blood. By using an anti-CCR1 antibody to block these effects, we provided the first evidence that CCR1 is at least one of the receptors that mediate the MIP-1α proliferation, but not inhibition, of $CD34^+$ progenitors. MIP-1α is a weaker chemoattractant for $CD34^+$ cells when compared with SDF-1α, but induces migration of progenitors. Moreover, both chemokines showed ability to induce adhesion of haemopoietic progenitors to immobilised fibronectin. These findings are likely to be relevant to the biological properties of these molecules, and provide more evidence that MIP-1α and SDF-1α have more comples roles in haemopoiesis that warrants further investigation.

Concluding remarks

Haemopoiesis is regulated *in vivo* by a controlled balance between stimulatory and inhibitory growth signals, many of which are generated within the bone marrow microenvironment by an interacting network of cytokines. The establishment of cell culture systems for the clonal development of haemopoietic cells and advances in DNA technology and protein purification have made it possible to discover those proteins that regulate cell viability, multiplication, and differentiation of different haemopoietic cell lineages. Therefore, much of the current understanding of the molecular basis of normal and abnormal blood cell development is based on *in vitro* studies using purified cytokines and a variety of cell culture systems.

The work reported in this thesis can be divided into two parts. In the first part (chapters 3 and 4), we investigated the potential role of the embryonic protein FGF3 on haemopoiesis based on previous studies with FGF3 knockout mouse (-/-) model, which showed marked haemopoietic defects (Cross *et al.*, 1997), and on the detection of FGF3 mRNA in FDCP-mix cells only under undifferentiated conditions. The knockout mouse model does not necessarily argue for an expression of FGF3 on haemopoietic cells, but for an effect on haemopoiesis which led to the present hypothesis and experiments. Because this protein is not available in a purified form, we used the FGF3 protein product purified from the conditioned medium after transfecting COS-7 cells with the cDNA of *Xenopus laevis,* and tested it in several cell culture systems using a variety of human and mouse haemopoietic cells.

Even though there was an indication that FGF3 may influence haemopoiesis according to the results obtained from our LTBMC studies and from the overexpression of the secreted form in FDCP-mix cells, a definite conclusion could not be drawn on such a small number of experiments performed and the wide heterogeneity of the results obtained, particularly in the population of normal controls. To further evaluate the FGF3 effects observed, these experiments would have to be extended using a purified secreted form of FGF3 after desalting procedures. The inclusion of heparin in the experiments would be also a priority. Studies focusing on the detection of receptors for FGF3 on haemopoietic progenitor cells and on stromal bone marrow cells could also be assessed. The use of other promoters and improvement of the FGF3 constructs that would result in better expression of the protein must also be pursued. The use of viral vector

constructs to drive the expression of FGF3 at acceptable levels is another possibility. Finally, if all these conditions are overcome, it would be interesting to test FGF3 on embryonic cell lines as these experiments would mimic early haemopoietic development, a time at which FGF3 is expressed.

The second part of this work was dedicated to study the effect of MIP-1α and SDF-1α, molecules that have already been shown to participate in several aspects of haemopoiesis. We focused on the effects of these chemokines on haemopoietic cells by (a) looking at the expression of their receptors on human peripheral blood, bone marrow and cord blood mononuclear and $CD34^+$ cells, which include progenitors; (b) studying their influence on *in vitro* proliferation of human GM-CFC and erythroid progenitor cells; (c) studying their participation in inducing migration and adhesion of cord blood progenitors, and finally (d) evaluating the functionality of the CCR1 receptor on MIP-1α-mediated effects, particularly on proliferation of haemopoietic progenitors. As demonstrated and discussed throughout the chapters 5 and 6, MIP-1α has shown to have a pleiotropic nature as a regulator of haemopoiesis. It has probably more than one function on haemopoietic cells, depending particularly on the concentration at which it is present and the target cells on which it acts. The participation of SDF-1α in triggering proliferation, migration and adhesiveness of haemopoietic progenitor has been demonstrated here and our results provide further baseline information of the roles of SDF-1α as a modulator of haemopoiesis.

The mechanisms by which MIP-1α and SDF-1α affect haemopoiesis are not well understood, but at least the initiation of these events must occur at the level of cell surface receptors. Little information is available regarding how the effects of these chemokines on haemopoietic progenitor cells are mediated at an intracellular level. Unfortunately, because of the rarity of haemopoietic stem/progenitor cells and the difficulty of isolating enough cells at high yield and purity, it is not possible to investigate most intracellular signalling mechanisms in these cells. We evaluated the expression of the chemokine receptors CCR1 and CCR5, and CXCR4 that bind MIP-1α and SDF-1α, respectively, on cells derived from different haemopoietic cell lineages (chapter 5). We found that CCR1 and CXCR4 are broadly expressed on HL-60, THP-1, TF-1, Jurkat, and K562 cells. However, it is not known whether these chemokines can induce biological responses on these cell lines. In assuming so, they would provide the framework for further studies to clarify the participation of these receptors on MIP-1α-

and SDF-1α-mediated effects. Of interest in this context is the fact that, although both cell lines were shown to express CCR1, HL60 cells express only the VLA-4 antigen while K562 cells express only VLA-5 (Hanenberg *et al.*, 1996). These differences in integrin expression can be explored to clarify whether CCR1 is involved in the mechanisms by which MIP-1α induces its effects, particularly in the adhesion of progenitor cells to fibronectin. The results would also be of value for understanding why normal and CML-Ph^+ progenitor cells exhibit different binding properties as preliminary experiments in our laboratory have shown that bone marrow and peripheral blood $CD34^+$ cells from CML patients express lower levels of CCR1 when compared to their normal counterparts (Dr. E. A. de Wynter, personal communication).

Bone marrow transplantation has been the major therapeutic modality in haematology, oncology and medical genetics. It depends upon the transfer of pluripotent stem cells from bone marrow, peripheral blood or cord blood that will lead to continuous generation of functionally normal progeny representing all blood and immune cell types (Mulligan, 1993). Retroviral vectors are currently the most effective vehicles to stably integrate new genes into the host cell genome, and have been studied extensively in attempts to genetically modify haemopoietic stem cells (Miller, 1992). Genetic transduction of haemopoietic stem cells using retrovirus has been hampered by low efficiency in gene transfer (Dunbar *et al.*, 1995; Kohn *et al.*, 1995). Inefficient transduction of these cells is likely due to the presence of a large number of cells in quiescent state (Miller *et al.*, 1990), a low density of receptors for the binding of retroviruses, and the inability to manipulate stem cells *in vitro* for prolonged periods of time without losing their repopulating ability. In this context, new approaches to gene transfer into haemopoietic cells have included methods to improve *ex vivo* manipulation of cells and/or modify virus/cell interactions. The use of cytokines such as G-CSF, SCF, Flt3, IL-3 and IL-6 alone or in combination has been shown to move quiescent progenitor cells into active cell cycling (Bodine *et al.*, 1991; Ikebuchi *et al.*, 1987). As such, they have contributed to improve transduction efficiency of *ex vivo* expanded $CD34^+$ cells using human stromal support layer (Dao *et al.*, 1997; Nolta *et al.*, 1992; Nolta and Kohn, 1990; Nolta *et al.*, 1995). Recently, fibronectin fragments have been shown to efficiently co-localise viral particles (Hanenberg *et al.*, 1996) and haemopoietic target cells, resulting in efficient transduction of murine and human LTC-IC (Moritz *et al.*, 1996; Moritz *et al.*, 1994; Moritz and Williams, 1994; Williams, 1999). Moreover, increased gene transfer efficiency into committed progenitor cells and

LTC-IC derived from cord blood *versus* adult bone marrow has been achieved using fibronectin as support instead stromal layers (Williams and Moritz, 1994). Recently, MIP-1α has been shown to improve the *ex vivo* expansion of cord blood LTC-ICs in combination with early acting cytokines (Capmany *et al.*, 1999). Our studies showed that MIP-1α stimulated the proliferation of haemopoietic progenitor cells from cord blood, possibly modulating their cell cycle status as suggested by others (de Wynter *et al.*, 1998; Lu *et al.*, 1993a). In addition, MIP-1α induced significantly their adhesion to fibronectin. Together, these data may favour the hypothesis that adding MIP-1α to gene therapy protocols using fibronectin coated dishes would enhance gene transfer into specific cell types necessary for sustaining multilineage haemopoiesis by exploiting unique ligand/receptor interactions.

The haemopoietic stem cell occupies a pivotal position in the hierarchy of the haemopoietic system and it is at this cellular level that all haemopoietic functions can be ultimately regulated. Much effort has, therefore, gone into characterizing regulators of stem cell proliferation with a view to enhancing our understanding of the regulation of this fundamental cell, and in addition to examining the potential clinical roles of such stem cell active factors. A large number of growth inhibitory molecules such as MIP-1α has been identified, but no clear picture has yet emerged concerning the relative physiological roles of them. Further studies are therefore necessary to elucidate the *in vivo* roles of these molecules. The redundancy in the cytokine system has been demonstrated many times not only in *in vitro* systems, but has been confirmed through knockout experiments that produce minor or mild changes in phenotypes. In some cases, these apparently trivial phenotypes can give clues to the basis of some human pathological conditions. In the future, more relevant insights into human diseases from this experimental approach can be expected. As a result of these insights we hope to see improvement in disease treatment and changes in clinical practice in the (not distant) future.

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